# IMPACT OF PH ON THE EXTRACTION OF DIFFERENT MUSTARD SEEDS AND THEIR APPLICATIONS

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## FORWARD

Chapter 2 and 3 of this thesis was prepared following a manuscript format. The manuscript I have been accepted by the Journal of Applied Food Research. Manuscript II has been accepted by the Journal of Food Science. Chapter 4 of this thesis has not yet been published.

**DEDICATION**

*I dedicate this document to my dearest parents, my older sister and my boyfriend for their support, trust, and encouragement and for always being there for my happiness and sadness throughout this journey.*

*And*

*In memory of my late supervisor, Dr. Usha Thiyam-Holländer.*

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## ABSTRACT

Canada was one of the world's top producers of mustard, in which the mustard yield in Canada varied between 50,000-286,700 tonnes in the last twenty years, according to the Canadian Grain Commission. Besides high proteins and oil, a large amount of phenolic compounds in mustard has initiated its ability in food production, nutraceuticals, as long as cosmetics. The most noticeable compounds that are found in mustard are sinapine, sinapic acid and its conversion to canolol. However, the production of mustard usually cannot fully exploit this oilseed nutrition and functional potential, especially its bioactivities. Therefore, recently, a desire for a more effective extraction method has been researched. In this study, a home-scale system with the application of temperatures, pressure and pH was designed to optimize the extraction of the sinapine, and sinapic acid concentration as long as the generation of canolol from Oriental, black and yellow mustard variety. Experiments proceeded with whole and crushed seeds using sautéing as a preheating treatment, followed by acidified-, neutralized- and alkaline-pressurized wet extraction. HPLC analysis, different antioxidant assays together with total phenolic content (TPC) and total flavonoid content (TFC) as long as anti-tyrosinase activity was used for the quantification of extraction efficiency. This extraction system was proved to be productive with the highest targeted mustard's major sinapates was obtained from yellow and black mustard. Moreover, strongest antioxidant and anti-tyrosinase activity was also observed for both yellow and black mustard extracts providing the *in-vitro* evidence of potential application of mustard as not only food ingredients but also in nutraceuticals and cosmetic production.

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# CHAPTER 1 Mustard: a potential oilseed literature review


## Abstract

Mustard has been a familiar oilseed since ancient times, contributing great value to global agriculture. Additionally, with a high concentration of phenolic content, especially sinapic acid and its derivatives, mustard has been studied lately as a potential preservative, and additive for the enhancement of food bioactivities as well as an excellent pharmaceutical. This study will discuss some of the mustard seed compositions including lipids, glucosinolates, and major mustard seed sinapates. Moreover, major sinapates in mustard are believed to be the main source of its bioactivities; therefore, their chemical, synthesis, stability, extraction, and measurement method are also covered in this review.



**Keywords:** Mustard, sinapates, sinapic acid, canolol, bioactivity, therapeutic effect, food application.

## 1. Introduction

Mustard *(Brassica alba, Brassica juncea, Brassica nigra*), belongs to *Brassicaceae* family that includes vegetables and seeds such as broccoli (*Brassica oleracea*), cauliflower (*Brassica oleracea* var. *botrytis*), cabbage (*Brassica oleracea* var. *capitata*), turnip (*Brassica rapa*) and canola (*Brassica napus*) (Nabavi & Silva, 2018). The different varieties of mustard include white or yellow mustard (*Sinapis alba*), Oriental, brown, or Indian mustard (*Brassica juncea*). Mustard gained increased attention due to their wide array of applications as edible oil, condiment, and the preservative (Edwards et al., 2007). It is a rich source of protein (18-24%), making it an excellent nutritional additive ingredient in the food industry.

According to Sanskrit records, mustard seeds have been used as condiments for more than 5,000 years by the Egyptians, Sumerians, Chinese and Romans. The word "mustard" comes from the Latin *mustum*, a Roman specialty condiment created by mixing fruits mixed, especially grapes juice, with ground mustard seed, for seasoning fish and meat (Thomas et al., 2012). The benefits from mustard were so valuable, that it became one of the most consumed oilseeds. In 2017, Nepal and Canada, were the world's top producers of mustard, with annual yields that accounted for 27.6% and 21% of total world production, respectively (Mitrović et al., 2020). In 2021, the total production of mustard in Western Canada was approximately 50 thousand tonnes, with Saskatchewan being the highest producer (76.3%) and followed by Alberta and Manitoba (Barthet, 2021).

The industry is primarily focused on oil production and, to a lesser extent, on protein and fiber, leaving behind a rich source of phenolic compounds in the mustard cake (Zamindar et al., 2017) Even though there are wide mustard varieties available around the world, the major ones in Canada are black mustard (*Brassica nigra*), Oriental mustard (*Brassica juncea*), and white/yellow

mustard (*B. hirta/ Sinapis alba*) (Barthet, 2021). They are quite distinguishable based on their phenotype, specifically their color. Another factor that makes each variety unique is their pungent aroma. Each mustard variety has a different level of pungent aromatics, ranging in order from the least to the most for yellow, Oriental and black mustards, respectively (Manohar et al., 2009). Compared to yellow mustard, brown and black mustard usually give more experience of heat in the nose and eyes (Thomas et al., 2012). The pungency of mustard results from an enzymatic reaction between water and ground seed (Thomas et al., 2012).

- **Oriental mustard**

Oriental mustard (*Brassica juncea*) is popularly known as Indian mustard or Chinese mustard, because of its origin (Thomas et al., 2012). It is grown in the orient temperate and subtropical climates, including United States, Canada, Mexico, Bangladesh, Central Africa, China, India, Japan, Nepal, and Pakistan, as well as in southern Russia north of the Caspian Sea (Kumar et al., 2011). *Brassica juncea* was reported to have both therapeutic and edible qualities due to its high content of polyphenolic and phenolic compounds (Dinis et al., 1994; Jahangir et al., 2009).

- **Black mustard**

Black mustard (*Brassica nigra*), an annual, aromatic weedy plant, is predominant in some parts of Europe, Western Asia, and North Africa, North and South America (Khaliq et al., 2017). According to Thomas et al. (2012), Canada was one of the most important countries in both mustard-growing and mustard condiment manufacturing and was the largest exporter of *B. nigra* during 2004-2008 (Alvarez & Boye, 2012). Black mustard seeds are widely used as a medicinal herb for specific diseases such as cardiovascular disease, diabetes, and related complications (Thomas et al., 2012). Not only the seeds of *Brassica nigra*'s, but also the leaves contain some critical minerals required by the human body (Saha et al., 2015).

- **Yellow mustard**

Yellow mustard (*B. hirta/Sinapis alba*) is a perennial crop of significant economic value (Marcone et al., 1997; Martinović et al., 2020). It is indigenous to North and South America, Northwestern Europe, China, Japan, Australia, New Zealand, the Middle East and India. Like the other two varieties, yellow mustard seeds are famous as condiments and spices, as well as providing an edible oil for salad dressing and emulsifiers for meat products (Ciubota-Rosie Camelia et al., 2009), and food preservatives (Rahman et al., 2018). Furthermore, research has demonstrated *S.alba*'s beneficial biological activities on human health, including chemo-protective (Zhu et al., 2012), antimicrobial (Boscaro et al., 2018), anticancer (Eskin et al., 2007), Its antioxidant activity includes its ability to enhance the natural antioxidant enzymes as superoxide dismutase, catalase, and glutathione peroxidase and their radical scavenging activity (Boscaro et al., 2018; Martinović et al., 2020; Terpinc et al., 2012).

## 2. Composition

### 2.1. Lipid

Despite having high-fat content, which varies from 23-47% depending on the mustard species, mustard oils are rarely used by the oil industry (Thomas et al., 2012). In Western countries, a recommended oil for a heart-healthy diet must contain large amounts (>80%) of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), and a low level (<20%) of saturated fatty acid (SAFs) (Arnett et al., 2019; Bowen et al., 2018; Sikand, 2021; Sikand & Severson, 2020). Consequently, mustard seeds oil's fatty acid profile is quite controversial, with PUFAs raging between 20-28%, SFAs 5-7%, and a notably large proportion of MUFAs 66-74%, which also includes a high level of erucic acid (Kaushik & Agnihotri, 2000; Wendlinger et al., 2014). According to Thomas et al., (2012), in fact, the erucic acid concentration

in mustard seed oil could reach 26.5-36.5% of total fatty acid content. The other components of mustard seed's fatty acid profile vary between varieties, including oleic acid (19.5-22%), and linoleic acid (9-15%) (Grygier, 2022).

The high erucic acid content of mustard may be more favorable in the polymer industry than in food consumption. The primary concern is the relationship between erucic acid and the progression of cardiac damage. This was highlighted by Charlton et al., (1975), who fed refined oil with three concentrations of erucic acid (1.6%, 4.3% and 22.3%) to male and female Sprague-Dawley rats for 112 days. At the end of the experiment, focal myocardial necrosis, myocardial lipidosis, and fibrosis were found to be proportional to the concentration of erucic acid, although a variable rate was observed in the different genders. Other studies by Kramer et al., (1979); Vogtmann et al., (1975) also confirmed similar results. This evidence has led to the banning of mustard oil in cooking by the Food and Drug Administration. On the other hand, Food Standards Australia and New Zealand just limit the daily intake of erucic acid to 2-5% of energy intake, which is equivalent to 7.5 mg/kg body weight/day. For the European Food Safety Authority, a lower daily intake (7 mg/kg body weight/day) was regulated.

In order to use mustard seed as food material, considerable research was conducted to develop a lower level of erucic acid mustard strain. Studies conducted in Canada by Getinet et al., (1994) obtained zero-erucic acid mustard through an interspecific cross between *B. carinnta* S-67 (brown seeded), Dodolla (yellow seeded) and *B. juncea* line Zem 2330. The fatty acid profile of the new seeds was promising, with a high concentration of oleic (28.3 ± 2.0%), linoleic (38.1 ± 2.9%), linolenic acid (22.9 ± 2.4%), and very low amount of erucic acid (0.1 ± 0.0%). However, genetically modified products are controversial due to the lack of scientific research on their long-term impact. Therefore, another approach was the introduction of microwave or preheat treatment

as an efficient method for reducing the erucic acid in mustard varieties. This method will be discussed in detail in Section 3.

### 2.2. Glucosinolates

Glucosinolates are a group of secondary metabolites found in *Brassicaceae* plants, including mustard seeds (Grygier, 2022; Tian & Deng, 2020). The main structure consists of a sulfonium sulfonate, β-D-glucose, with the side chain R of amino acid (Tian & Deng, 2020). There are 3 specific types of glucosinolates, aliphatic, aromatic and indole, structurally categorized based on the differences in their side chain R (Tian & Deng, 2020). Even though more than 100 different glucosinolates have been identified, *Brassicaceae* only has one-tenth of them, with even less recovered from mustard seeds (Wiseman, 2005). Nevertheless, glucosinolates are still important precursors in mustard varieties, responsible for their flavor and odor development (Augustine & Bisht, 2015; Liu et al., 2009). The nature and concentration of mustard's glucosinolates vary according to the varieties and distribution and include sinalbin (0.1-1.1%) in yellow mustard, sinigrin (0.8-0.9%) in Oriental mustard, and (0.4-0.9%) in black mustard, together with 2-hydroxy-oxazolidine, glucobrassicin, progoitrin, glucoerucin, gluconapine, glucoraphanin, glucoiberin, neoglucobrassicin and indole-3-methanol (Bell et al., 2017, 2018; Frazie et al., 2017).

Glucosinolates are themselves flavorless, but the scents and flavor come from thiocyanates, isothiocyanate, and nitriles, glucosinolate degradation products (Augustine & Bisht, 2015; Liu et al., 2009). These degradation processes occur via three main pathways: enzymatic, thermal and chemical (Bones & Rossiter, 2006). While glucosinolates are temperature resistant, chemical degradation can lead to health concerns sometimes. Enzymatic hydrolysis by myrosinase, however, is the most efficient process (Grygier, 2022). Nevertheless, for the enzymatic reaction to proceed the mustard seed's cell wall must be broken down by crushing, chopping or chewing

(Grygier, 2022). Myrosinase is mustard's intrinsic enzyme, and its concentration differs with each mustard variety. For example, the highest activity of 2.75 un/ml is in Oriental mustard, followed by 1.50 un/ml in black mustard, with the lowest activity of 0.63 un/ml in yellow mustard (Okunade et al., 2015). The myrosinase enzyme is thermally sensitive and can be readily inactivated when the temperature reaches 60ºC. However, the thermal threshold could be elevated when combined with pressure. According to Van Eylen et al., (2006), inactivation occurred at 300MPa and 70ºC. Okunade et al., (2015) also reported improved thermal and pressure tolerance for myrosinase in black and Oriental compared to yellow mustard.

Myrosinase generally hydrolyzes glucosinolates to glucose and unstable aglycones, which then form isothiocyanates, thiocyanates, epithionitriles, nitriles, and indoles (Grygier, 2022). However, there are also some glucosinolates that can generate distinctive compounds under special conditions. A study from Eylen et al., (2006) reported the production of allyl isothiocyanate (AITC) from sinigrin at a neutral pH in the presence of ferrous ions. AITC is not only responsible for the pungent flavor and odor of crushed mustard seed (Dai & Lim, 2015) but is also an effective antimicrobial agent, preventing the growth of bacteria, yeast, and mold in both liquid and gas phase (Peng et al., 2014). Another notable product is 4-hydroxybenzyl isothiocyanate, which is produced from sinalbin. This compound is responsible for the hot mouthfeel in yellow mustard and is reported to have sufficient bactericidal and bacteriostatic properties against gram negative organisms including *Salmonella enteritidis* and *Schizosaccharomyces pombe* (Bahmid et al., 2020; Dai & Lim, 2015).

### 2.3. Phenolic compounds

Over the last two decades, there has been a tremendous interest in endogenous phenolic compounds in plants and oilseeds due to their therapeutic potential and the ability of their bioactive

compounds to contribute to functional food products (Vuorela, 2005; Vuorela et al., 2004). Phenolic compounds usually contain one aromatic ring with one or more hydroxyl groups (Kougan et al., 2013). These groups help to categorize phenolic compounds as simple flavonoids and non-flavonoids (Roleira et al., 2018). Nonflavonoids are then sub-classified according to their carbon skeleton into simple phenolics, phenolic acid and derivatives, phenylacetic acid and derivatives, phenones, tannins and stilbenes (Cheynier, 2005; Laura et al., 2009). Phenolic acids and its derivatives are one of plants' most important secondary metabolites. They are located in roots, shells, leaves, and seeds and have protection mechanisms against environmental factors as well as insects (Lin et al., 2016). Phenolic acids are structured based on the binding between the carboxyl group and benzene ring and can be divided into benzoic acid derivatives and cinnamic acid derivatives (Laura et al., 2009). Benzoic acid derivatives mainly include vanillic acid, gallic acid, protocatechuic acid, and salicylic acid (Murkovic, 2003). On the other hand, cinnamic acid derivatives consist of sinapic acid, *p*-coumaric acid, ferulic acid, and caffeic acid (Macheix et al., 2018).

Mustard is a promising source of phenolic compounds, with the total phenolic content reaching 2.62-36.5mg/g dry weight (Dubie et al., 2013; Harbaum et al., 2008; Matthäus, 2002). The main phenolic compound in mustard is sinapine, which exists in both free and soluble bound forms (as esters or conjugates) (Nićiforović & Abramovič, 2014; Thiyam et al., 2006). Several phenolic compounds in mustard seed extract were identified as sinapine, sinapic acid, 4-vinylsyringol (canolol), sinapoyl esters, syringaldehyde (Nićiforović & Abramovič, 2014).

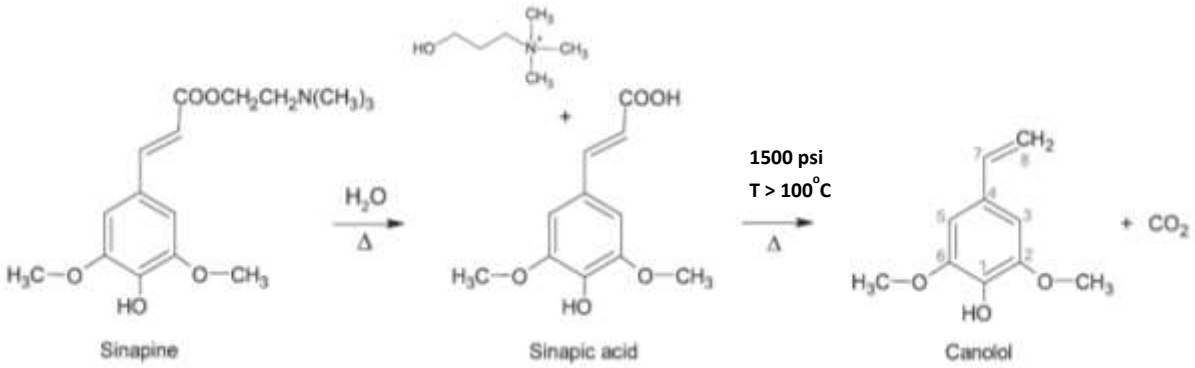


**Figure 1.1:** Formation of sinapine and canolol from sinapic acid

### 2.3.1. Sinapine and Sinapic Acid

#### 2.3.1.1. Chemical and Synthesis

Sinapic acid, a naturally occurring *p*-hydroxycinnamic acid and its derivatives constitute over 73% of phenolic acids (Thiyam et al., 2006). According to Naczk et al., (1998), free, esterified, and insoluble bound forms of sinapic acid account for 65-86, 71-97, and 7-32% of the phenolic acids**,** respectively. Sinapic acid is involved in both the shikimic acid pathway and phenylpropanoid pathway in plants, in which the latter pathway is the precursor for the formation of sinapic acid. On the other hand, the *p*-hydroxycinnamic acid derivatives can be obtained artificially through Knoevenagel-Doebner condensation of syringaldehyde and malonic acid (Flourat et al., 2021; Horbury et al., 2018). This phenolic compound has one hydroxyl group making it the major water-soluble antioxidant component in mustard meals (Das et al., 2009).

The main phenolic compound in the mustard seed is sinapine, a choline ester of sinapic acid. It accounts for about 70-90% of the total phenolic compounds present in seed, together with smaller amounts of minor derivatives (e.g., glucosidic ester, glucopyranosyl sinapate), and only a small amount of free sinapic acid (Engels et al., 2012; Hanses et al., 1995; R. Khattab et al., 2010; Vuorela et al., 2003). One of the earliest studies on sinapine in yellow mustard was reported by Clausen et al., (1982, 1983). They extracted sinapic acid extracted from yellow mustard seed and

let it react with bromocholine bromide to obtain pure sinapine. Clausen et al., (1982) reported that the highest amount of sinapine in yellow mustard seed was 33µmol/gram plant material. High-performance liquid chromatography analysis recorded around 62% (w/w) sinapine in mustard meal extracts compared to 16% (w/w) for sinapic acid (Das et al., 2009; Fang et al., 2008; Harbaum et al., 2008).

#### 2.3.1.2. Stability

Sinapic acid and its derivatives, like many phenolic compounds, are thermally sensitive. According to Shrestha et al., (2012), degradation of free sinapic acid (FSA) and total sinapic acid content after basic hydrolysis (TSAH) was observed after the preheat treatment of seed at 180$^{o}$C. For the Oriental variety, the FSA and TSAH before and after preheat treatment decreased from 46.92 ± 2.17 to 39.68 ± 1.23 µg/g of dry matter and from 6.72 ± 0.09 to 5.38 ± 0.06 mg/g of dry matter, respectively. A more significant trend was obtained in black and yellow mustard variety followed by Shrestha's study. The value from black mustard was 77.18 ± 4.11 to 26.50 ± 0.72 µg/g of dry matter and 5.63 ± 0.12 to 4.22 ± 0.21 mg/g of dry matter. Although the heat treatment of mustard varieties could significantly diminish the sinapic acid concentration, but it can also contribute a crucial function in the formation of canolol.

### 2.3.2. Canolol

#### 2.3.2.1. Chemical and Synthesis

Canolol, also known as 4-vinyl syringol, is formed from sinapic acid by loss of carbon dioxide following decarboxylation (Niu et al., 2013; Pudel et al., 2014). The formation of canolol from sinapic acid is presented in Figure 1. Since canolol is an unstable compound in the presence of oxygen, it appears to be converted into derivatives such as dimers, trimers, and oligomers (Harbaum-Piayda et al., 2010; Kraljić et al., 2015; Nandasiri et al., 2021a). Moreover, because of

its polarity, canolol can be complex with proteins and polysaccharides. Generally, extreme conditions of temperature and pressure are required to release canolol from these matrices (Guo et al., 2019; Nandasiri & Eskin, 2021). Previous researchers examined the effect of both heat and pressure treatment on the extraction of the major sinapates from mustard seed. A solvent-free dry heat pre-treatment method using a novel commercial RapidOxy® 100 system resulted in the maximum recovery of canolol at 160ºC for 10 minutes (Nandasiri et al., 2021b). This system established an optimum temperature and pressure for the generation of canolol, which enhanced its yield from mustard seed. Another study by Fadairo et al., (2021) applied air frying as a roasting pre-treatment method to enhance the yield of canolol and other lipophilic sinapates from mustard samples. Applying air circulation at pressurized temperature improved the solubility and mass transfer of phenolics by reducing the surface tension and viscosity of the extracting solvent.

#### 2.3.2.2. Stability

While stringent conditions are required for the formation of canolol, it is relatively unstable even at room temperature. Kraljić,et al., (2015) examined the shelf-life of canolol under three separate conditions including at freezer temperature of -20ºC, at fridge temperature of 4ºC, and at room temperature, which varied from 20 to 25ºC. The most favorable condition was -20ºC, after 5 months, but even under these optimum conditions there was still a 20% loss of canolol. The rate of degradation was far greater at 4ºC (50%) and room temperature, as no canolol could be detected.

### 2.3.3. Bioactivities of Mustard's Major Sinapates

#### 2.3.3.1. Antioxidant

Phenolic compounds have become very popular due to their bioavailability, bioactivity, and antioxidant effects. During human aging and exposure to environmental factors, the body's natural antioxidants become overwhelmed, reducing their ability to destroy free radicals (Lobo et al.,

2010). Consequently, there is a worldwide search for natural and safe antioxidants, and phenolic compounds from mustard seeds extract are a potential source. Research has been conducted to assess the efficacy of the antioxidant activity of sinapic and its derivatives. According to Kikuzaki et al., (2002), at a concentration of 20 μM, sinapic acid could scavenge 33.2% of DPPH radical. The scavenging effect of sinapic acid was then enhanced to 88.4% when the concentration increased to 0.5 μM (Nenadis & Tsimidou, 2002). Other studies by Hotta et al., (2002) and Jin et al., (2010) compared the DPPH radical scavenging properties of several hydroxycinnamic acids and showed the order of effectiveness ranged from caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid. When comparing the major sinapates from mustard seed, the antioxidant activity of sinapic acid was higher than sinapine and canolol (Harbaum-Piayda et al., 2010; Terpinc et al., 2011; Thiyam et al., 2006; Vuorela et al., 2005).

In addition to DPPH, the impact of sinapic acid and its derivatives on other free radicals was also investigated. Hydroxyl radicals are the most reactive free radicals capable of causing extensive damage to the human body compared to other reactive oxygen species (ROS) (Nordberg & Arnér, 2001). Thus, it became the most targeted ROS when considering antioxidant activity. Zou et al., (2002) reported that the ability of sinapic acid to quench hydroxyl radical of sinapic acid was $IC_{50}$ = 3.80 mM, which was effective than ascorbic acid ($IC_{50}$ = 4.56 mM) (Jalaludeen & Pari, 2011). The ability of sinapic acid to scavenge $O_2^{\cdot -}$, a free radical that causes oxidative stress in the human body leading to cell damage and mutagenesis (Nordberg & Arnér, 2001). Zou et al., (2002) found that the radical clearance activity of sinapic acid ($IC_{50}$ = 17.98 mM) was higher than well-known antioxidant Trolox ($IC_{50}$ = 7.24 mM). Unlike DPPH, the superoxide radical clearance activity was independent of decarboxylation, as the value from sinapic acid and canolol was insignificant (Terpinc et al., 2011). On the other hand, Galano et al., (2011) reported the efficacy

of canolol on the hydroperoxyl radical using the computational stimulation method. Besides, confirming the impact of canolol on hydroperoxyl radical, in which hydrogen atom transfer was the most favorable binding pathway between the radical and hydroxyl site of canolol, this study also showed that canolol was more sensitive in aqueous solution than in a nonpolar medium. The ratio between the two conditions was 32.6 times different (Galano et al., 2011).

#### 2.3.3.2. Anticarcinogenic and Anti-inflammatory

NF-κB plays an important role in signalling macrophages, in which the incorrect regulation of protein could link to cancer and inflammatory and autoimmune diseases (Shukla & Singh, 2011). The anticarcinogenic and anti-inflammatory properties of sinapic acid and its derivatives are due to its ability to inactivate nuclear factor-kappa B (NF-κB), thus, preventing the expression of proinflammatory mediators including nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, and interleukin-1β (Connelly et al., 2011; Hudson et al., 2000). A study by Cao et al., (2008) reported similar characteristics for canolol. Moreover, another pathway for the antiproliferative effect of sinapic acid against human breast cancer via T47D cell line was reported by Hudson et al., (2000) and Kampa et al., (2004). Sinapic acid was confirmed as an efficient time-dependent and dose-dependent phenolic compound in the suppression of colon and breast cancer cell activity (Nićiforović & Abramovič, 2014).

#### 2.3.3.3. Other Bioactivities

Sinapic acid and its derivatives have demonstrated a variety of health-promoting effects. Many in-vivo and in-vitro assays have reported their anticancer (Cao et al., 2008; Chen, 2016), antimutagenic (Kuwahara et al., 2004), neuroprotective (Ferreres et al., 2009; He et al., 2008), antimicrobial (Nowak et al., 1992), antihyperglycemic effects (Cherng et al., 2013). Canolol was shown to be a powerful antioxidant, antimutagenic and anticancer molecule (Siger et al., 2017).

Its antioxidant and antimutagenic properties were comparable to tocopherol, β-carotene, rutin, and quercetin (Kuwahara et al., 2004; Siger et al., 2017).

## 3. Technology for Extraction of Mustard

The mustard industry is primarily focused on oil production and, to a lesser extent, on protein and fibre concentration while ignoring the rich source of phenolic compounds in the mustard cake (Zamindar et al., 2017). Considerable research has been carried out to enhance the extraction of phenols from mustard seed by-products. However, there has been only limited success due to the flow characteristics of the biomass and the usage of large amounts of chemical solvents in the extraction. The incorporation of heat, pressure, and pH in the extraction process has significantly enhanced the yield of the major sinapates from mustard seeds. Other studies have pointed out that a mixture of organic solvents and water combined with heat treatment and pressure provides greater extraction efficiency (Li & Guo, 2016; Machado et al., 2015; Park et al., 2017; Szydlowska-Czerniak et al., 2015; Teh et al., 2015; Thiyam-Holländer et al., 2014; Wang et al., 2016).

### 3.1. Impact of preheat treatment.

Phenolic compounds in the plant membrane are present in both the free and bound forms requiring thermal and mechanical treatment to optimize their extraction. In addition, the most soluble phenolics are trapped in the plant cells' vacuoles, while insoluble-bound phenolics are located in the cell wall matrix, connecting covalently with macromolecules (Shahidi et al., 2016). The impact of heat may facilitate bioavailability by rupturing the cell walls and disrupting the intracellular bonds (Shahidi et al., 2016). Several types of pre-heat treatments were successfully applied, including roasting and microwave treatment.

#### 3.1.1. Roasting assistance

Vuorela, (2005) showed that roasting rapeseed promoted the decarboxylation of sinapic acid and the formation of canolol. Siger et al., (2018) showed the variability in antioxidant activity resulting from adjustments in pressing, roasting, moisture content, and their interactions. In the same study, they also reported a correlation between antioxidant activity and canolol content. An earlier study by Shrestha et al., (2012) reported that roasting was an effective method for extracting phenolics from different mustard seeds. They monitored the production of canolol from Oriental, black, and yellow mustard seeds, which increased after heat treatment but decreased slightly when overheated. No canolol was detected in the corresponding unheated seeds. The highest canolol levels were recorded in roasted Oriental mustard (135.56 ± 2.07 µg/g of dry matter), followed by black (143.00 ± 2.14 µg/g of dry matter) and yellow (75.96 ± 2.19 µg/g of dry matter) mustard seeds**.** Using roasting as a pre-treatment (Vaidya & Choe, 2010) increased the tocopherol content of mustard seed oil without detrimental effects on the free fatty acids and fatty acid composition. These changes were only observed when the roasting temperature was higher than 160ºC. A recent study by Fadairo et al., (2021) used air frying pre-treatment for the production of major sinapate**s** in the oil fraction of mustard. The increased recovery of canolol after roasting for 15 minutes at 170ºC was 1086.08 ± 12.62 µg canolol equivalent/100g lower grade yellow mustard oil. The highest sinapine content (415.64 ± 26.63 µg sinapine equivalent/100 g mustard powder) was recorded for Oriental mustard after air frying at 180ºC for 15 minutes.

### 3.1.2. Microwave Treatment

The canolol content of the seed, meal, and oil, was shown to be dependent on the temperature (Harbaum-Piayda et al., 2010; Nandasiri et al., 2019; Wroniak et al., 2016). Microwave-accelerated extraction is also an effective preheat treatment in which microwave energy potentiates the bioavailability of phenolics from binding to the plant matrix, enhancing

their antioxidant activity (Rodríguez-Bencomo et al., 2015). The increase in temperature vaporizes the free water within plant tissue, thus making phenolic compounds more available (Sparr Eskilsson & Björklund, 2000). According to Khattab et al., (2014), canolol production was more accessible when treated with microwave radiation under alkaline conditions. Rękas et al., (2017) reported a significant increase in canolol content after 10 minutes of exposure to microwave radiation. The concentration of canolol increased 63-fold from 28.66 ± 0.062 μg/g to 1807.63 ± 0.352 μg/g. A study by Verma et al., (2019) reported a positive effect of 2450 MHz microwave treatment on the total antioxidant activity of the Indian mustard variety. Besides enhancing the phenolic content performance, the microwave treatment also lowered the concentration of erucic acid and glucosinolates. The latter were considered for mustard's food application (Niu et al., 2013).

### 3.2. Impact of pH

The pH of the solvent extracts can greatly influence the electrostatic and stability of organic compounds. Theoretically, according to the pKa, the combinations will remain stable when a solvent reaches its equilibrium state. However, under acidic conditions, the targeted compounds can be more negatively charged or vice versa; when extracted with a more alkaline solution (Zhang et al., 2020). Chadni et al., (2021) showed that sinapic acid was a pH-dependent phenolic compound, in which the highest amount (13.22 ± 0.44 μmol/g of dry matter) was recovered from a buffered aqueous solution at pH 12. Galano et al., (2011) previously confirmed the structural transformation of sinapic acid under the influence of pH using the computational stimulation method. At neutral pH, sinapic acid mostly existed in an anionic form (carboxylate), which proved to be more active than the original.

According to Zhang et al., (2020), the amount of sinapine extracted from cold-pressed rapeseed meal under alkaline conditions slightly increased and then decreased, with pH 11.0 being optimum. Degradation was due to the electrostatic interaction between rapeseed protein and sinapine (Akbari & Wu, 2015). Two different pH treatments were recently applied, acid and alkaline hydrolysis. Acid hydrolysis is a convenient and simple method using 1%–5% hydrochloric acid in water/methanol to extract insoluble-bound phenolics. The main disadvantage of this method is that phenolic compounds are unstable at low pH, leading to their degradation. On the other hand, alkaline effectively hydrolyses both ether and ester bonds that are rarely broken by acid hydrolysis. However, this method requires a more complex procedure, including further extraction steps (Shahidi et al., 2016).

### 3.3. Enzyme-assisted extraction (EAE)

Besides physical influence, biochemical assistance also receives substantial attention due to its feasibility in phenolic extraction. Enzyme-assisted extraction (EAE) is one of these alternative methods that have an inherent ability to catalyze a reaction with exquisite specificity and their ability to function properly under mild processing conditions (Shahidi et al., 2016). Unlike temperature or pH pre-treatment, EAE requires moderate extraction conditions with a minimal number and concentration of substances involved and produces more potential as long as less deterioration of finished products (Cao et al., 2019). Enzymes act as phenolic releasing agents by digesting hemicellulose, cellulose, pectin, and proteins in plant cell wall matrix (Heemann et al., 2019). Thus, making both free and bound phenolic compounds become more purified and available for recovery. Cellulase, pectinase and proteases are the main enzymes in proceeding this process. Mushtaq et al., (2015) observed a significant improvement in pomegranate peel extract with the assistance of enzyme cellulases, pectinases and proteases.

On the other hand, some enzymes can work as phenolic compounds oxidation, which can potentially produce more beneficial derivatives. Phenol-oxidizing enzymes include polyphenol oxidase (tyrosinase, catecholase), laccases, and peroxidases (Peter & Wollenberger, 1997; Yahia, 2010). Polyphenol oxidases consist of copper in their active site and vary in size and substrate specifications (Yoruk & Marshall, 2003). Tyrosinase is one of the polyphenol oxidases that catalyze the oxidation of monophenols and diphenols without any additional co-factors. A study by Cao et al., (2019) investigated the enzymatic oxidation of sinapic acid catalyzed by horseradish peroxidase and tyrosinase. Although no canolol was detected, the result confirmed a potential oxidized effect of tyrosinase on sinapic acid, and sinapine.

### 3.4. Other potential extraction methods

According to Nandasiri & Eskin, (2022), pressure is also a prerequisite for the decarboxylation of sinapic acid during the generation of canolol. Accelerated Solvent Extraction (ASE) also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE) or high-pressure solvent extraction (HPSE) is a technique using high pressure and inert atmosphere over temperature range 35 – 200$^{o}$C (Nguyen et al., 2021). Not only facilitated the decarboxylation process, but pressure also contributed to breaking the analyte matrix bonds, thence enhancing the concentration of phenolic compounds released (Shahidi et al., 2016). Using high temperature (140-180$^{o}$C) and high pressure, Nandasiri et al., (2019) reported a better extraction yield in sinapic acid and its derivatives. Nandasiri et al., (2021b) further enhanced the yield of sinapic acid, sinapine and canolol using a modified RapidOxy® 100 compact system with a built-in pressurized heating chamber. This system is a solvent-free pre-treatment method for producing canolol from residual meals and resulted in the maximum recovery of canolol at 160$^{o}$C for 10 minutes (Cao et al., 2019; Nandasiri et al., 2021b).

Physical accelerators, including ultrasound, have also been employed in mustard's major sinapate extraction. The technique manipulates the passage of ultrasound ultrasonic waves into the sample-solvent mixture, materialization for the cavitation phenomenon, hence improving phenolic's extraction efficiency (Nandasiri et al., 2020). Szydlowska-Czerniak et al., (2015) practised using the ultrasound-assisted method in the extraction of yellow mustard seed cultivars, which resulted in the collection of considerable total phenolic content (27.16 mg/g of sinapic acid equivalent) and especially the limitation of erucic acid and glucosinolate concentration. Additionally, the application of the ultrasound-assisted method proved to be more efficient than the normal conventional method. With the same condition of temperature, solvent, and time (70% EtOH (v/v) at 80$^{o}$C for 3×30 min), the conventional and ultrasound-assisted methods had relatively equivalent phenolic content (8.81 ± 0.17 and 8.85 ± 0.38 mg/g sinapic acid equivalent, respectively); howbeit, the latter required less time and lower temperature.

## 4. Determination of phenolic compounds

Natural phenolics have been identified and quantified using a variety of methods which is presented in **Table 1**.

**Table 1.1*:* Quantification method for sinapic acid and its derivatives**

| Methods | Description | References |
|---|---|---|
| **Spectrophotometric methods** | Determination of total phenolic content by using Folin-Denis or Folin-Ciocalteu assays. The assay is based on determining the oxidation of phenolic compounds in the reaction with phosphomolybdate and phosphotungstate mixture, giving the blue color solution. Absorbance is measured with UV-vis spectrophotometer at wavelength 725-765 nm (Singleton & Rossi, 1965). The total phenolic content is calculated using sinapic acid standard. | (Koski et al., 2003; Naczk et al., 1992; Naczk & Shahidi, 1989; Thiyam et al., 2004; Xu & Diosady, 1997) |
| **Thin layer chromatography (TLC)** | Thin layer chromatography (TLC) is an affinity-based method between the mobile phase (organic solvent), and stationary phase (silica gel or aluminium oxide coated onto an inert plate surface, typically glass, plastic, or aluminium) used to separate compounds in a mixture. | (Amarowicz et al., 1995; Fenton et al., 1980; Krygier et al., 1982) |
| **Gas chromatography (GC)** | GC, known as vapour-phase chromatography (VPC), or gas-liquid partition chromatography (GLPC), is the process between a mobile phase (a mixture between gaseous or liquid samples and an inert gas such as helium, argon, nitrogen, or hydrogen) and stationary phase (Harvey, 2000). | (Kozlowska, Rotkiewicz, et al., 1983; Kozlowska, Zadernowski, et al., 1983) |
| **High-performance chromatography** | The most common method for the detection of sinapic acid and its derivatives is based on the separation between the mobile and stationary phases. Various phenolic compounds are separated using normal phase C18 or reversed phase (RP-C18) | (Cao et al., 2019; Fadairo et al., 2021; R. Khattab et al., 2010; Khattab et al., 2014; Kozlowska, Rotkiewicz, et al., 1983; Liang et al., 2018; Nandasiri et al., 2021a,b; |

| | | |
|---|---|---|
| | column in the presence of different polarity solvents. The pH of the mobile phase has to be kept at 2-4 and the detector can be varied depending on the phenolic compounds (Jahromi & Jahromi, 2019). | Nandasiri et al., 2019, 2020; Thiyam-Holländer et al., 2014) |
| **Mass Spectrometric** | Through differences in the ratio between the charges of atoms, molecules, and clusters and their respective masses, mass spectrometry (MS) is used to separate ionized particles, such as atoms, molecules, and clusters (Murayama et al., 2009). | (Asenstorfer et al., 2006; Cai et al., 1999) |
| **Nuclear Magnetic Resonance (NMR)** | NMR is based on the measurement of electromagnetic radiation when a weak oscillating magnetic field disturbs a strong constant magnetic field (Harvey, 2000). NMR has an application in the determination of phenolic structure (Jahromi & Jahromi, 2019). | (Asenstorfer et al., 2006; Cai et al., 1999; Niwa et al., 1999) |

## 5. Application of Mustard

### 5.1. Mustard in Food Application

Since ancient times, mustard has been used for flavoring and seasoning food. For instance, Indian mix sauces, chutneys, and pickles used oil-heated whole brown or black mustard seeds to enhance their flavor. While the Barbadians and other populations in the Caribbean use yellow or brown mustard with fruits and chilli peppers to improve their sauces, marinades and stews. Just by mixing mustard with salt and/or wine, and/or vinegar, a delicious sauce has been made and is ready to use (Thomas et al., 2012).

Mustard is a rich source of antioxidants and glucosinolates, which elicit many benefits as potential additives. Traditional preservatives in meat-based products are nitrates and nitrites, which give the meat a smoky flavor, pinkish color, and protection from harmful bacteria (Gassara et al., 2016). However, their presence can have detrimental effects on human health and the environment. An alternative to nitrates and nitrites as food preservatives was studied by Çağlar et al., (2018), who adding ground mustard into meatballs. They found that mustard significantly improved the shelf life by delaying lipid peroxidation as well as inhibiting the growth of microorganisms. Moreover, the addition of mustard also embellished the sensory properties of meatballs, with white or yellow mustard being more favourable than black and brown mustard. Earlier studies by Karwowska et al. (2014, 2015) and Wójciak et al. ( 2014) also confirmed the efficacy of mustard as a natural additive. The combination of autoclaved mustard seed and acid whey in the organic fermented sausage was found to prolong the product's shelf life by up to 90 days. Additionally, not only was the quality preserved, but the increase in antioxidant capacity and a decrease in oxidation-reduction potential were also observed. This could be explained by the

high-temperature pressurized extraction of the autoclave, which significantly improve the phenolic content in the additive mixture (David et al., 2013; Karwowska et al., 2014).

In addition to its effectiveness as a potential additive, mustard seed extract was also applied as an antimicrobial film on bologna sausages and successfully reduced the levels of viable *Listeria monocytogenes* in the samples (Lara-Lledó et al., 2012). Hendrix et al. (2012) applied defatted mustard seed by-products in food-based biopolymer film by mixing the meal with 0.6% glycerol. The high protein content of mustard also made it a suitable material for food-grade vegetable protein and bread making (Marnoch & Diosady, 2006; Păucean et al., 2018).

### 5.2. Mustard in Health Promotion and Disease Prevention

Mustard was used by ancient Greeks as an antidote against scorpion and snake bites. In addition, different varieties are used for blood flow stimulation, cold feet vapour warming, muscle relaxation, and even treatment of rheumatism and arthritis diseases. Aside from that, mustard's high sulphur concentration made it a highly effective treatment for skin ailments (Thomas et al., 2012).

Not only do phenolic compounds in the mustard extract provide health benefits, but also the high glucosinolates and their degradation products also have health benefits. **Table 2** summarises the potential therapeutic effects of mustard on human health.

**Table 1.2: potential therapeutic effects of mustard on human health.**

| Therapeutic Effect | Mustards | Targeted | References |
|---|---|---|---|
| Anticancer | Sinigrin | Inhibit the proliferation of liver cancer cell by through up-regulation of p53 and down-regulation of Bcl-2 family members and caspases | (Jie et al., 2014) |
| | Sinigrin | Inhibit the occurrence of tongue cancer in male ACI/N rats | Tanaka et al., (1992) |
| | Glucosinolates | Protect against four different cancer cells SNU-251, SNU-354, SNU-C4, and MCF-7 | Kim et al., (2011) |
| | Allyl isothiocyanate, benzyl isothiocyanate | Inhibit the activity of lung cancer cells | Jeong et al., (2017); Tripathi et al., (2015) |
| | Sulforaphane | Inhibit the activity of lung cancer cells, colon cancer cells and esophageal adenocarcinoma cells. | Pappa et al., (2006); Qazi et al., (2010); Tripathi et al., (2015) |
| | Indole-3-methanol | Inhibit colon cancer cells, breast cancer cells and tongue cancer in male ACI/N rats. | Hajra et al., (2018); Martín-Ruiz et al., (2018); Pappa et al., (2006); Tanaka et al., (1992) |
| | *Brassica juncea*'s extract | Inhibit the proliferation of the activity of breast cancer cells (MCF-7, MDA- MB-231), colon cancer (HCT116), prostate cancer (PC-3), cervical cancer (HeLa), and lung cancer (A-549) cells. | Bassan et al., (2018) |

| | | | |
|---|---|---|---|
| | *Sinapis Alba* | Suppress 1,2-Dimethylhydrazine- induced immuno-imbalance and colonic carcinogenesis in rats | Zhu et al., (2012) |
| | Canolol | Inhibit the $H_2O_2$-stimulated cell death of HepG2 cells→ Suppress human liver cancer cell | Xia et al., (2019) |
| | Canolol | Inhibit gastric tumors initiation and progression through COX-2/PGE2 pathway in K19-C2mE transgenic mice | Cao et al., (2015) |
| | Sinapic acid | Inhibit tumorigenic colon cells, breast cancer cells | Hudson et al., (2000) |
| | Sinapic acid | Inhibit the proliferation of human breast cancer T47D cell line | Kampa et al., (2004) |
| Anti-hyperglycemia | Phenolic compounds and glucosinolates | Inhibit α-amylase and α-glucosidase (antihyperglycemic potential) | Jo et al., (2018) |
| Neuroprotective | Sinapine | Inhibit acetylcholine (ACh) esterase activity | Ferreres et al., (2009); He et al., (2008) |

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## CHAPTER 2

# Optimization of pH for Extracting Sinapates from Mustard Varieties using Green Technology

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**Abstract**

Mustard seeds contain high levels of phenolic compounds, especially sinapine, compared to many other oilseeds. This study investigated the effectiveness of an innovative green pre-treatment for extracting sinapates, particularly canolol. Three mustard varieties, Oriental, black, and yellow, were pretreated by sautéing and adjusting the pH of the extract to acid, neutral, and alkaline conditions using safe household products, vinegar and sodium bicarbonate. The treated whole and crushed seeds were then extracted by pressurized heating at 115oC and 10.2 psi. and the phenolics measured by HPLC-DAD. Statistical analysis showed an interesting trend in which pH affected the mustard endogenous phenolic profile with the neutral pH proving superior to either acid or alkaline treatment. Of the varieties examined, both yellow and black mustard yielded competitive amounts of sinapine, sinapic acid, and canolol. Sinapic acid and canolol, however, were significantly ($p<0.05$) higher in crushed black mustard seeds accounting for 2.7+ 0.08 and 2.61+ 0.35 mg/g DW, respectively. Crushed yellow mustard seeds had the highest level of sinapine at 385+ 49.7 mg/g DW. The effectiveness of the treatment was attributed to the electrostatic interaction between the solvents and extractants, using water in combination with the preheat treatment and pressure. It is evident that the alternative green extraction method described maximized the extraction of phenolic compounds from Oriental, black, and yellow mustard seeds.

**Keywords:** mustard seeds, sinapates, green technology, pH, pressurized extraction

## 1. Introduction

Mustard has gained increased attention due to its wide array of applications as an edible oil, condiment, preservative, and medicinal supplement (Edwards *et al.* 2007). Its rich protein content (18-24%) makes it an excellent nutritional additive ingredient in the food industry (Abul-Fadl *et al.* 2011). For example, Oriental, black and yellow mustards are used in Indian and Caribbean traditional sauces as flavour enhancers (Edwards *et al.* 2007; Thomas *et al.* 2012; Tian & Deng, 2020). Gokhalet *et al.* (2004) and Monsalve *et al.* (2001), both reported the presence of mustard as an ingredient in sauce (barbecue, curry, condiment, ketchup), mayonnaise, wine, vinegar, and commercial oil products. According to Thomas *et al*. (2012), Canada is one of the most important countries in both mustard-growing and mustard condiment manufacturing. In fact, Canada occupied 21% of the world's mustard yield (Lietzow, 2021). In 2021, the total production of mustard in Western Canada was approximately 50, 000 tonnes, with Saskatchewan being the highest producer (76.3%) followed by Alberta at 23.7% (Barthet, 2021). Even though there are a wide array of mustard varieties available around the world, the major ones in Canada are black (*Brassica nigra*), Oriental (*Brassica juncea*), and white/yellow (*B. hirta/ Sinapis alba*) (Barthet, 2021).

Over the past two decades, there has been a tremendous interest in endogenous compounds in oilseeds due to their therapeutic potential and ability to contribute bioactive phenolic compounds for functional food products (Kozlowska, *et al.* 1983a, b; Lietzow, 2021; Manohar *et al.* 2009; Thiyam-Holländer *et al.* 2014; Vuorela, 2005; Xu & Diosady, 1997). Mustard with an abundance of phenolic compounds meets the criteria as a functional food. The main phenolic compound in mustard is sinapine, which is present in both free and soluble bound forms (as esters or conjugates) (Nićiforović & Abramovič, 2014; Thiyam *et al.* 2006). The other sinapate derivative is sinapic acid, which accounts for over 73% of the total phenolic acids (Thiyam *et al.* 2006). High-

performance liquid chromatography (HPLC) analysis showed that sinapine accounted for 62% (w/w) in mustard meal extracts compared to around 16% (w/w) for sinapic acid (Das *et al.* 2009; Fang *et al.* 2008; Harbaum *et al.* 2008). A novel phenolic compound identified in mustard extracts is 2,6-dimethoxy-4-vinyl phenol or canolol. a very promising bioactive (Nandasiri & Eskin, 2022). It is formed from sinapic acid by decarboxylation, initiated by extreme pressure and temperature (Nandasiri *et al.* 2021 a,b,c; Nandasiri & Eskin, 2021,2022). The antioxidant activity of canolol was reported by Koski et al. (2003) to be comparable to α-tocopherol.

Recent research has focused on optimizing the conditions for the extraction of mustard's major sinapates. A study by Shahidi & Yeo, (2016), showed that heat facilitates their bioavailability by rupturing the cell walls and disrupting the intracellular bonds. Previous work by Shrestha *et al.* (2012) significantly increased the canolol content from *Brassica nigra*, *Brassica juncea*, and *Sinapis alba*' by roasting the seeds. No canolol was detected in the unheated seeds, but high amounts were measured in roasted Oriental (135.56 ± 2.07 µg/g of $_{DM}$), black (143.00 ± 2.14 µg/g of $_{DM}$) and yellow (75.96 ± 2.19 µg/g of $_{DM}$) mustard seeds. According to Nandasiri & Eskin, (2022), pressure is also a prerequisite for the formation of canolol by decarboxylation of sinapic acid. Nandasiri *et al.* (2021b) adapted the RapidOxy® 100, a compact system with built-in pressurized heating chambers, as a solvent-free pre-treatment for producing canolol from residual meals. They maximumized the recovery of canolol (453.40 ± 17.66 µg/g dry weight) after heating under pressure at 160$^{o}$C for 10 minutes. Although this research was not carried out on mustard, the study established a promising application of pressure and temperature for the extraction of phenolic compounds from oilseeds.

While the application of extreme heat and pressure was effective for the production of canolol**,** it was expensive, time-consuming, and unsustainable. To overcome this problem, efforts were made to incorporate a more environmentally friendly technology method. These not only

minimize losses in nutritional value but also reduce the amount of energy and solvents used. Recently, several studies established the significant impact that pH had on the concentration, stability, and bioactivity of plant phenolic extract**s** (Friedman & Jürgens, 2000; Librán *et al.* 2013; Roselló-Soto *et al.* 2019). This novel approach of adjusting the pH of the solvent extractants affects the electrostatic properties and stability of the organic compounds. Theoretically, following the pKa formula, the combinations will remain stable when a solvent reaches its equilibrium state. However, under acidic conditions, the targeted compounds will be more negatively charged and positively charged when extracted with a more alkaline extractant (Zhang *et al.* 2020).

In this study, a home-scale extraction system that included the application of temperature and pressure with the adjustment of pH was developed to extract major sinapates and other thermo-generative sinapates from the different mustard varieties. The three different pH conditions selected were acidic (2.59), neutral (5.94) and alkaline (7.78) to establish their potential for extracting targeted phenolic compounds.

## 2. Material and Methods

### 2.1. Raw Materials

GS Dunn. Ltd provided Oriental mustard seed. (Montreal, QC). Commercial black mustard seeds were purchased from Gill's supermarket (Winnipeg, MB, Canada), which was imported by PTIFOODS© (Mississauga, ON, Canada). Yellow mustard seeds were purchased from Real Canadian Superstore (Winnipeg, MB, Canada), which was imported by Loblaw Inc®/™ (Toronto, ON, Canada). In order to prolong the shelf life of samples, all the seeds were packed, and sealed in Ziploc bags (Ziploc®, SC Johnson & Son Inc., Racine, WI, USA) and kept in the fridge at 4ºC until further analysis. Whole seed and crushed seed orientations were used in the current study. The crushed seeds were prepared by grinding the whole seeds with a pestle and mortar for 10 minutes.

### 2.2. Chemicals

Vinegar (No Name®, CA), ARM & HAMMER™ Baking Soda Box was purchased from Real Canadian Superstore (Winnipeg, Manitoba, Canada). Methanol, (Optima™ LC/MS Grade) ethanol and formic acid were purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). Sinapic acid (purity >98%) was purchased from Fisher Scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity >97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China). Canolol was synthesized in the lab (purity>97%), and its purity was confirmed by HPLC.

### 2.3. Extraction Process

#### 2.3.1. Description

The extraction process is presented as a flow chart (**Figure 1**). In summary, whole and crushed mustard seeds underwent three main processes: pre-heat treatment, extraction, and analysis steps. Pre-heat treatment involved sautéing for 0, 2, 8, and 10 minutes. After cooling to ambient temperature, the samples were then adjusted to acid, neutral, or alkaline conditions and then subjected to pressurized wet extraction (115$^{o}$C at 10.2 psi) for 3 minutes. The extractants were then analyzed using High-Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD). Data were analyzed by ANOVA to determine the optimum condition**s** for the extraction of major sinapates in selected mustard varieties.

**Processing Condition**

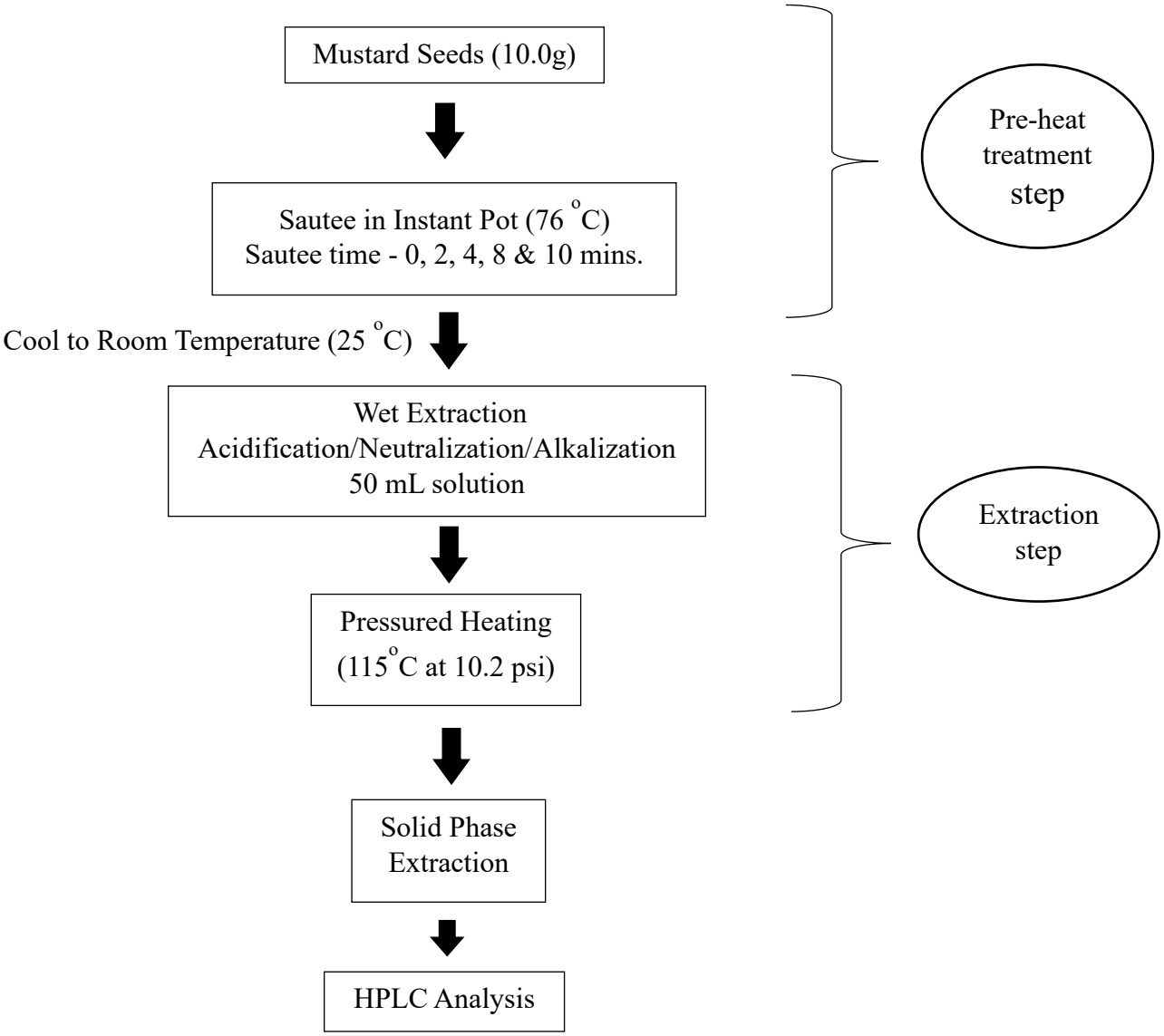


**Figure 2.1 Procedure for pressurized wet extraction of Mustard seed**

### 2.3.2. Optimization

#### 2.3.2.1. Pre-heat treatment

Samples (each treatment contained 5 batches of replicates) were sautéed in an instant pot (Instant Pot Duo Mini 3 Qt Model number: IPDUOMINI 3Qt, Instant Brands Inc, Downers Grove, IL, USA) at a constant temperature of 76$^{o}$C. Initially, the impact of the sauté process on the change in phenolic compounds was examined using Oriental mustard seeds at 0, 2, and 8 minutes. Preliminary results showed that there was an increase in sinapine, sinapic acid, and canolol over pre-treatment time. Thus, the experiment was then continued for 10 minutes of sautéed time. Under these conditions, despite the enhancement in sinapine, sinapic acid, and canolol, the compounds started to degrade as summarized in **Table S1** (Supplement Information). Hence, the time parameters were limited to 0, 2, & 10 minutes in further studies. After each pre-heat treatment, sautéing samples (5-10g) were kept in the desiccator to cool down to ambient temperature (21$^{o}$C).

#### 2.3.2.2. pH value for the used solvent

The highlight of this project was using environmentally friendly and edible solvents or ingredients for the extraction of mustard phenolics. The different pH values were adjusted using common household solvents or ingredients. For the acidic treatment, acetic acid in the form of household vinegar (non-fruit type) was used while deionized water was used for the neutral treatment. Finally, sodium bicarbonate or household baking soda was used as an environmentally friendly and edible ingredient for the alkali treatment. The pH values were measured using Fisherbrand™ accumet™ AB150 pH Benchtop Meters™, Ottawa, ON, Canada, before and after each extraction was carried out. The initial pH values recorded for the acidic, neutral, and buffer treatments were 2.59, 5.94 and 7.78, respectively (**Table 1**).

#### 2.3.2.3. Optimization of the pressurized heat treatment

Wet extraction of the mustard seeds was carried out using the three types of solvents responsible for different pH conditions. For the acidic conditions, different concentrations of **c**ommercial vinegar (No Name®, CA containing 5% (v/v) of acetic acid) were used, which was presented in **Table S1** (Supplement Information) The extraction was conducted using pressurized heating (Instant Pot Duo Mini 3 Qt Model number: IPDUOMINI 3Qt, Downers Grove, IL, USA) at 115$^{o}$C at 10.2 psi. The extractants were further analyzed using the HPLC-DAD (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States) to determine the optimal pH condition for maximum yield of sinapates. The HPLC analysis of the preliminary data showed that the solution of 3% acetic acid (v/v) produced optimum results.

Similar to acid treatment, deionized water was used as the neutral treatment for the pressurized cooking of the mustard samples. For the alkali treatment, at first, the same ratio between sodium bicarbonate and water 3:2 (w/v) was used for evaluation. However, according to Haynes, (2011), in order to dissolve this concentration, the temperature must reach 60$^{o}$C, raising concerns for its stability and shelf-life. On the other hand, at 20$^{o}$C, the solubility of sodium bicarbonate was approximately 10 g/L. Consequently, a lower concentration (10% w/v) was used.

### 2.3.3. Solid Phase Extraction (SPE) Method for Mustard Seed Samples

Solid phase **e**xtraction of the obtained extracts was conducted following the method described by (Nandasiri *et al.* 2021b**)** with some minor adjustments. Samples were prepared using a $C_{18}$, 200mg 3mL (Agilent Technology Bond Elute, Santa Clara, CA, U.S.A) SPE column tube. The extraction was performed using a vacuum filtration unit (Agilent Vac Elut 12 and 20 Position, Agilent Technologies Canada Inc., Mississauga, ON, CA). Prior to each extraction, the column was conditioned using 3 mL of absolute methanol and 6 mL of distilled water. Afterwards, 3 mL of the sample was loaded with the excess sample, and the water-soluble materials, including the

sugars, were washed away using another 6 mL of de-ionized water. The concentrated extract was finally eluted with 1mL of acidified (1% v/v formic acid) methanol. Each extract was further diluted (1:1) using acidified methanol and filtered using a 0.2µm syringe filter (Agilent Technologies Canada Inc., Mississauga, ON, CA) prior to the HPLC-DAD analysis.

### 2.4. Physicochemical analysis of selected mustard varieties

#### 2.4.1. Moisture Content

The moisture content of each mustard sample was determined using a rapid method based on the AOAC method. The moisture meter (IR-35 Infrared Moisture Analyzer, Denver instrument, Arvada, CO, USA) was used. In brief, samples were kept at 130$^{o}$C for 4 minutes, and the moisture content was determined on the dry weight basis (n=5).

#### 2.4.2. Determination of the oil content of the Mustard samples

The oil content of the samples was determined using Soxtec 2050 (Foss-Tecator, Foss North America, Eden Prairie, MN, United States) as described by Khattab *et al.* (2014) with slight modifications. In brief, 16 grams of sample (n=5) was measured in a thimble and put in an extraction cup. Each sample was extracted two times to maximize the extractability of the oil. After each extraction, the residual oil-containing cups were left overnight in the fume hood for 24 hours for solvent evaporation. The percentage oil was calculated based on the weight.

### 2.5. HPLC analysis of sinapic acid derivatives

The major sinapates in Oriental, black, and yellow, seeds were tested qualitatively and quantitatively using the HPLC-DAD (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States) method as described by Nandasiri *et al.* (2019) and modified by Fadairo *et al.* (2021). The separation was carried out on a KinetexR® Biphenyl $C_{18}$ 100 Å RP column (2.6 µm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States), with a 0.4 mL/min flow rate and a 10-µL injection

volume. The mobile phase consisted with formic acid (0.1%, v/v) in water as solvent A, and formic acid (0.1%, v/v) in methanol as solvent B.

### 2.6. Statistical Analyses

Results were presented as a mean ± standard deviation for all experiments conducted with five replicates. Examination of residuals confirmed normally distributed data and constant variance (Pallant, 2020). In order to obtain normalized data, the raw data points were tested using the normality test, and the necessary transformations were made to the raw data. As part of the current study, square root and logarithm transformations were used to transform normalized data (Pallant, 2020). The impact of variety (Oriental, black, yellow), orientation of the seeds (whole and crushed), time of pre-heat treatment (0, 2 and 10 minutes of sauté), pH (acid, neutral and alkaline condition) and their combinations were inspected on mustard's major sinapates concentration with 95% confidence level. Therefore, each factor/their combinations were considered as a treatment. With the application of factorial design in statistical analysis, the treatments were introduced as single-factor, two-way (variety*orientations, variety*time, variety*pH, orientation*time, orientation*pH, time*pH), three-way (variety*orientation*time, variety*time*pH, orientation*time*pH), and four-way interactions (variety*orientation*time*pH). Additionally, in order to obtain higher statistical power for factorial analysis for each test, the insignificant factors/treatments were eliminated from the system. Multiple mean comparisons of sinapine, sinapic acid, and canolol were conducted using Tukey's post-hoc analysis. Analyses of the data were performed using a general linear multiple regression model with a two-way analysis of variance. Multiple mean comparisons were achieved with the Tukey's test, with 5% statistical differences ($P > 0.05$) considered statistically significant (Pallant, 2020). SPSS statistical software version 26 (IBM, New York, NY, United States) was used to analyze the data.

## 3. Result and Discussion

### 3.1. Optimization of the operating conditions of the extraction process

Statistical analysis results for the selected mustard varieties' major sinapates are shown in **Table 1**. The impact of variety (Oriental, black, yellow), orientation of the seeds (whole and crushed), time of pre-heat treatment (0,2 and 10 minutes of sauté), pH (acid, neutral and alkaline condition) and their combinations were inspected on mustard's major sinapates concentration with 95% confidence level, as summarized in Tables 2a, b, and c.

According to the mean sum of squares in **Table 1**, orientation (whole or crushed seeds) was the only factor that did not significantly affect the sinapic acid and canolol content in the three mustard varieties. Furthermore, the interaction effects of the parameters clarified two-way ANOVA test including orientation*time, orientation*pH, pH*time, variety*pH significantly affected the major phenolic compounds of the selected mustard. The only factor that had negative results was the impact of variety*time on the canolol concentration ($p = 0.056$). All three-way interactions and all four-way interactions were also significantly different. Furthermore, all the results obtained from the HPLC-Analysis of sinapine, sinapic acid and canolol had high coefficients of variance: $R^2 = 0.997, 0.940, 0.983$, respectively. From **Table 2**, variety, pH, and time positively affected the mustard's phenolic changes, except for sinapine content.

**Table 2.1: Statistical Analysis of Major Phenolic Changes in Selected Mustard Varieties**

| Source | Sum of Squares | df | Mean Square | Sig. |
|---|---|---|---|---|
| Sinapine | | | | |
| Corrected Model | 393.43[a] | 53 | 7.42 | 0.00 |
| Intercept | 4768.64 | 1 | 4768.65 | 0.00 |
| Orientation | 12.02 | 2 | 6.01 | 0.00 |
| pH | 345.27 | 2 | 172.64 | 0.00 |
| Time | 0.34 | 2 | 0.17 | 0.00 |
| Orientation * Time | 4.34 | 2 | 2.17 | 0.00 |
| Orientation * pH | 7.58 | 2 | 3.79 | 0.00 |
| Variety * Time | 0.73 | 4 | 0.18 | 0.00 |
| pH * Time | 0.21 | 4 | 0.05 | 0.00 |
| Variety * pH | 8.22 | 4 | 2.06 | 0.00 |
| Variety * Orientation * Time | 0.29 | 4 | 0.07 | 0.00 |
| Orientation * pH * Time | 0.77 | 4 | 0.19 | 0.00 |
| Variety * Orientation * pH | 4.06 | 4 | 1.02 | 0.00 |
| Variety * pH * Time | 2.24 | 8 | 0.28 | 0.00 |
| Variety * Orientation * pH * Time | .40 | 8 | 0.05 | 0.00 |
| Error | 1.09 | 414 | 0.00 | |
| Total | 5348.13 | 468 | 7.42 | 0.00 |
| Corrected Total | 394.53 | 467 | 4768.65 | 0.00 |
| Sinapic acid | | | | |
| Corrected Model | 1566061.65[a] | 53 | 29548.33 | 0.00 |
| Intercept | 8412807.08 | 1 | 8412807.08 | 0.00 |
| Variety | 41679.75 | 2 | 20839.88 | 0.00 |
| Orientation | 260471.46 | 1 | 260471.46 | 0.00 |
| pH | 315737.82 | 2 | 157868.91 | 0.00 |
| Time | 39429.15 | 2 | 19714.58 | 0.00 |
| Orientation * Time | 17875.83 | 2 | 8937.92 | 0.00 |
| Variety * Orientation | 33010.04 | 2 | 16505.02 | 0.00 |
| Orientation * pH | 71689.84 | 2 | 35844.92 | 0.00 |
| Variety * Time | 29950.57 | 4 | 7487.64 | 0.00 |
| pH * Time | 340663.16 | 4 | 85165.79 | 0.00 |
| Variety * pH | 31218.51 | 4 | 7804.63 | 0.00 |
| Variety * Orientation * Time | 48628.12 | 4 | 12157.03 | 0.00 |
| Orientation * pH * Time | 41542.71 | 4 | 10385.68 | 0.00 |
| Variety * Orientation * pH | 24073.67 | 4 | 6018.42 | 0.00 |
| Variety * pH * Time | 147984.45 | 8 | 18498.06 | 0.00 |
| Variety * Orientation * pH * Time | 122826.84 | 8 | 15353.35 | 0.00 |

| | | | | |
|---|---|---|---|---|
| Error | 100395.76 | 409 | 245.47 | |
| Total | 10087032.20 | 463 | | |
| Corrected Total | 1666457.41 | 462 | | |
| Canolol | | | | |
| Corrected Model | 69.78[a] | 49 | 1.42 | 0.00 |
| Intercept | 1665.77 | 1 | 1665.77 | 0.00 |
| Variety | 6.04 | 2 | 3.02 | 0.00 |
| pH | 11.76 | 2 | 5.88 | 0.00 |
| Time | 10.72 | 2 | 5.36 | 0.00 |
| Orientation * Time | 0.17 | 2 | 0.08 | 0.00 |
| Variety * Orientation | 9.19 | 2 | 4.59 | 0.00 |
| Orientation * pH | 3.18 | 2 | 1.59 | 0.00 |
| pH * Time | 0.86 | 4 | 0.22 | 0.00 |
| Variety * pH | 9.624 | 4 | 2.41 | 0.00 |
| Variety * Orientation * Time | 0.17 | 4 | 0.04 | 0.00 |
| Orientation * pH * Time | 0.77 | 4 | 0.19 | 0.00 |
| Variety * Orientation * pH | 1.44 | 4 | 0.36 | 0.00 |
| Variety * pH * Time | 3.18 | 7 | 0.46 | 0.00 |
| Variety * Orientation * pH * Time | 0.32 | 5 | 0.06 | 0.00 |
| Error | 1.20 | 388 | 0.00 | |
| Total | 2038.63 | 438 | | |
| Corrected Total | 70.99 | 437 | | |

**Table 2.2**: **Multiple comparisons for Main Factors from transformed variables.**

| | | | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| **a.** Sinapine | | | | | | | |
| Variety | OM | BM | 0.14* | 0.01 | 0.00 | 0.12 | 0.15 |
| | | YM | -.015* | 0.01 | 0.00 | -0.16 | -0.13 |
| | BM | OM | -0.17* | 0.01 | 0.00 | -0.15 | -0.12 |
| | | YM | -0.29* | 0.01 | 0.00 | -0.30 | -0.27 |
| | YM | OM | 0.15* | 0.01 | 0.00 | 0.13 | 0.16 |
| | | BM | 0.29* | 0.01 | 0.00 | 0.27 | 0.30 |
| pH | 2.59 | 5.94 | -0.60* | 0.01 | 0.00 | -0.61 | -0.59 |
| | | 7.78 | 1.45* | 0.01 | 0.00 | 1.43 | 1.46 |
| | 5.94 | 2.59 | 0.60* | 0.01 | 0.00 | 0.59 | 0.61 |
| | | 7.78 | 2.05* | 0.01 | 0.00 | 2.03 | 2.06 |
| | 7.78 | 2.59 | -1.45* | 0.01 | 0.00 | -1.46 | -1.43 |
| | | 5.94 | -2.05* | 0.01 | 0.00 | -2.06 | -2.03 |
| **b.** Sinapic Acid | | | | | | | |
| Variety | OM | BM | -19.66* | 1.77 | .000 | -23.8207 | -15.5014 |
| | | YM | 2.19 | 1.79 | .442 | -2.0318 | 6.4115 |
| | BM | OM | 19.66* | 1.77 | .000 | 15.5014 | 23.8207 |
| | | YM | 21.85* | 1.79 | .000 | 17.6423 | 26.0594 |
| | YM | OM | -2.19 | 1.79 | .442 | -6.4115 | 2.0318 |
| | | BM | -21.85* | 1.79 | .000 | -26.0594 | -17.6423 |
| pH | 2.59 | 5.94 | -60.71* | 1.82 | 0.00 | -65.00 | -56.43 |
| | | 7.78 | -19.54* | 1.76 | 0.00 | -23.68 | -15.39 |
| | 5.94 | 2.59 | 60.71* | 1.82 | 0.00 | 56.43 | 65.00 |
| | | 7.78 | 41.18* | 1.78 | 0.00 | 37.00 | 45.35 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7.78 | 2.59 | 19.54* | 1.76 | 0.00 | 15.39 | 23.68 |
| | | 5.94 | -41.18* | 1.78 | 0.00 | -45.35 | -37.00 |
| **c.** Canolol | | | | | | | |
| Variety | OM | BM | -0.06* | 0.01 | 0.00 | -0.07 | -0.05 |
| | | YM | -0.48* | 0.01 | 0.00 | -0.49 | -0.46 |
| | BM | OM | 0.06* | 0.01 | 0.00 | 0.05 | 0.07 |
| | | YM | -0.42* | 0.01 | 0.00 | -0.43 | -0.40 |
| | YM | OM | 0.48* | 0.01 | 0.00 | 0.46 | 0.49 |
| | | BM | 0.42* | 0.01 | 0.00 | 0.40 | 0.43 |
| pH | 2.59 | 5.94 | -0.13* | 0.01 | 0.00 | -0.14 | -0.11 |
| | | 7.78 | -0.40* | 0.01 | 0.00 | -0.41 | -0.38 |
| | 5.94 | 2.59 | 0.13* | 0.01 | 0.00 | 0.11 | 0.14 |
| | | 7.78 | -0.27* | 0.01 | 0.00 | -0.28 | -0.25 |
| | 7.78 | 2.59 | 0.40* | 0.01 | 0.00 | 0.38 | 0.41 |
| | | 5.94 | 0.27* | 0.01 | 0.00 | 0.25 | 0.28 |
| Time | 0 | 2 | -0.28* | 0.01 | 0.00 | -0.30 | -0.27 |
| | | 10 | -0.40* | 0.01 | 0.00 | -0.41 | -0.38 |
| | 2 | 0 | 0.28* | 0.01 | 0.00 | 0.27 | 0.30 |
| | | 10 | -0.11* | 0.01 | 0.00 | -0.13 | -0.10 |
| | 10 | 0 | 0.40* | 0.01 | 0.00 | 0.38 | 0.41 |
| | | 2 | 0.11* | 0.01 | 0.00 | 0.10 | 0.13 |

OM: Oriental Mustad; BM: Black Mustard; YM: yellow mustard; Std Error: standard error; Sig: level of significant

### 3.2. Physicochemical analysis of selected mustard varieties

Physicochemical characterization, including moisture, oil, and changes in pH of the extracts, was recorded for all the mustard varieties (**Table 3**). pH was the most notable for its effect on the major sinapates of the mustard seeds. A study conducted by Chadni *et al.* (2021) applied 3 types of pH (0.1 M carbonate-bicarbonate buffer (pH = 12), acetic acid solution (pH = 2), and distilled water (pH = 4.5) and recovered a considerable amount of mustard's sinapine (15.75 ± 0.54 mg/g $_{DM}$) at pH =2, and sinapic acid (13.22 ± 0.44 mg/g $_{DM}$) at pH = 12. Although no canolol concentration was reported, this study provided compelling evidence for the effect of pH on the extraction process. Consequently, in this study, we examined the impact of acidic, neutral, and alkaline conditions. For the acid treatment, the recorded initial pH was 2.59 but after wet-pressurized extraction, the pH ranged between 3.30 - 3.62 depending on the variety, orientation, and treatment time (**Table 3**). For the neutral treatment, the initial pH was recorded as 5.94 and decreased to between 3.94 - 5.24 after wet-pressurized extraction (**Table 1**). Finally, for the alkaline treatment, the initial pH was 7.78, which increased to between 8.90 - 9.30 after wet-pressurized extraction (**Table 3**). Changing the sodium bicarbonate concentration (15% to 10%) did not affect the pH value of the solvent. Furthermore, using a lower concentration of food-grade sodium carbonate proved to be a more favorable method of pre-treatment for extracting phenolic derivatives, including canolol.

**Table 2.3: Changes in Oil Content, Moisture Content, and pH of selected variety of Mustard**

| | | | | Whole seed | | | Crushed seed | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Oriental | Black | Yellow | Black | Oriental | Yellow |
| Oil Content | | | | 0.64% | 0.34% | 1.10% | 12.93% | 14.26% | 6.23% |
| Moisture Content | | | | 96.27% | 95.82% | 96.61% | 94.22% | 93.9% | 92.68% |
| pH | AT | Before | | 2.59 | | | | | |
| | | After | 0 min | 3.62 ± 0.01 | 3.45 ± 0.02 | 3.51 ± 0.04 | 3.62 ± 0.02 | 3.55 ± 0.01 | 3.46 ± 0.02 |
| | | | 2 min | 3.38 ± 0.02 | 3.42 ± 0.05 | 3.47 ± 0.03 | 3.42 ± 0.01 | 3.54 ± 0.02 | 3.48 ± 0.06 |
| | | | 10 min | 3.29 ± 0.01 | 3.34 ± 0.05 | 3.33 ± 0.07 | 3.28 ± 0.04 | 3.37 ± 0.02 | 3.4 ± 0.05 |
| | NT | Before | | 5.94 | | | | | |
| | | After | 0 min | 5.24 ± 0.06 | 5.39 ± 0.08 | 5.78 ± 0.17 | 5.2 ± 0.06 | 5.33 ± 0.03 | 4.84 ± 0.05 |
| | | | 2 min | 4.23 ± 0.04 | 4.86 ± 0.1 | 4.7 ± 0.27 | 4.68 ± 0.05 | 4.99 ± 0.06 | 5.01 ± 0.01 |
| | | | 10 min | 3.94 ± 0.07 | 4.51 ± 0.02 | 4.35 ± 0.05 | 4.26 ± 0.05 | 4.53 ± 0.09 | 4.54 ± 0.06 |
| | BT | Before | | 7.78 | | | | | |
| | | After | 0 min | 8.94 ± 0.02 | 9.14 ± 0.02 | 9.02 ± 0.03 | 9.23 ± 0.02 | 9.34 ± 0.03 | 9.28 ± 0.03 |
| | | | 2 min | 9.03 ± 0.03 | 9.05 ± 0.01 | 9.04 ± 0.03 | 9.11 ± 0.03 | 9.05 ± 0.03 | 9.02 ± 0.03 |
| | | | 10 min | 9.1 ± 0.01 | 9.14 ± 0.01 | 9.02 ± 0.03 | 9.06 ± 0.01 | 9.02 ± 0.02 | 9.04 ± 0.02 |

0 min:0-minute sautee time with pressurized cooking; 2 min: 2-minute sautee time with pressurized cooking; 10 min:10-minute sautee time with pressurized cooking; AT: acid treatment; NT: neutral treatment; BT: buffer treatment; min: minutes

### 3.3. Mustard Extraction and HPLC Analysis

#### 3.3.1. Sinapine Content

Sinapine the choline ester of sinapic acid, was detected at a retention time of 12 minutes based on the HPLC analysis (320 nm) (**Figure S1**)**.** It is the most abundant phenolic compound present in mustard; therefore, the concentration of sinapine was expected to be the highest among the three targeted phenolic compounds. According to **Table 4a**, the highest amount of sinapine was observed for both whole and crushed yellow mustard seeds. Statistical analysis indicated that there was no significant difference between the two different orientations. **Table 4a** also indicated that the highest values in whole and crushed yellow mustard seeds were 123.48 ± 90.14 mg/g DW and 384.99 ± 49.47 mg/g DW, respectively. Besides orientation, sauté time also had no significant ($p > 0.05$) effect when comparing the samples. However, differences between the varieties were notable, with the highest levels of sinapine recorded with the neutral treatment of yellow mustard (**Table 4a**). Both acid and buffer treatment had similar trends but at a much lower amount. For acid treatment, the sinapine concentration of whole and crushed yellow mustard was 16.86 ± 2.51 mg/g DW, respectively. Meanwhile**,** for the buffer treatment, the concentration from the whole and crushed seeds were reduced to 0.19 ± 0.01 and 0.11 ± 0.01 mg/g DW. The difference between the three pHs was significant ($p < 0.05$), with the neutral treatment having the largest impact on the extraction of phenolic derivatives from the mustard samples.

Dubie *et al*. (2013) conducted their extraction using the more conventional method of combining ethanol (70%) and temperature (80$^{o}$C) on the *Brassica juncea*'s meal, and obtained a much lower sinapine concentration of 8.81 ± 0.17 mg SAE/g. A similar sinapine concentration (8.8mg/g) was reported by Reungoat *et al*. (2020) by reducing the temperature to 75 $^{o}$C. A study by Matthäus, (2002) also investigated the impact of the conventional method using 70% methanol, 70% acetone, water, and ethyl acetate as the extractant solvents on *S. alba*'s sinapine content. The

highest sinapine concentration of 122.1 mg/g was obtained with 70% ethanol, equivalent to the whole seed sample used in this study but three times lower than the corresponding crushed seed. Moreover, when comparing water as an extractant solvent, the sinapine content was 32.5 mg/g extract. Using a combination of temperature and pressure in this study, the value was twelve-time greater than the concentration reported by Matthäus, (2002). Another study by Thiyam-Holländer *et al*. (2014) also applied the conventional extraction method to ground yellow and Oriental mustard but reported much lower sinapine concentrations, 7.81 ± 0.01 and 21.34 ± 0.01 mg/g, DW, respectively. It was evident, that the combination of temperature, pressure and pH significantly affected the major sinapates of selected mustard varieties.

**Table 2.4** :**Sinapine and Sinapic Acid Content of Selected Mustard Varieties**

| | | Whole seed | | | Crushed seed | | |
|---|---|---|---|---|---|---|---|
| | | Oriental | Black | Yellow | Black | Oriental | Yellow |
| a. | Sinapine (mg/g DW) | | | | | | |
| AT | 0 min | 23.47 ± 3.4 | 1.99 ± 0.37 | 19.94 ± 2.82 | 3.21 ± 0.24 | 3.64 ± 0.24 | 122.29 ± 15.07 |
| | 2 min | 16.4 ± 2.47 | 2.99 ± 0.17 | 16.86 ± 2.51 | 3.71 ± 0.55 | 21.7 ± 2.31 | 139.14 ± 11.88 |
| | 10 min | 17.67 ± 2.93 | 2.84 ± 0.15 | 16.37 ± 1.42 | 17.51 ± 3.11 | 21.34 ± 3.06 | 115.26 ± 15.94 |
| NT | 0 min | 36.83 ± 2.24 | 7.79 ± 0.58 | 75.57 ± 1.49 | 205.34 ± 21.75 | 176.13 ± 15.1 | 228.56 ± 6.72 |
| | 2 min | 33.32 ± 3.46 | 11.05 ± 1.7 | 123.48 ± 9.01 | 180.59 ± 26.66 | 172.5 ± 20.19 | 384.99 ± 49.47 |
| | 10 min | 29.59 ± 3.6 | 14.51 ± 0.84 | 97.9 ± 8.56 | 192.4 ± 19.13 | 197.7 ± 14.46 | 164.24 ± 11.97 |
| BT | 0 min | 0.13 ± 0.02 | 0.15 ± 0.03 | 0.07 ± 0.01 | 0.13 ± 0.02 | 0.08 ± 0.01 | 0.11 ± 0.01 |
| | 2 min | 0.14 ± 0.02 | 0.21 ± 0.02 | 0.19 ± 0.01 | 0.08 ± 0.01 | 0.09 ± 0.01 | 0.11 ± 0.02 |
| | 10 min | 0.14 ± 0.02 | 0.16 ± 0.02 | 0.43 ± 0.04 | 0.06 ± 0.01 | 0.08 ± 0.01 | 0.17 ± 0.02 |
| b. | Sinapic acid (mg/g DW) | | | | | | |
| AT | 0 min | 0.23 ± 0.02 | 0.47 ± 0.04 | 0.16 ± 0.03 | 0.29 ± 0.03 | 0.27 ± 0.02 | 1.44 ± 0.08 |
| | 2 min | 0.15 ± 0.02 | 0.37 ± 0.03 | 0.17 ± 0.02 | 0.29 ± 0.06 | 1.31 ± 0.2 | 0.95 ± 0.14 |
| | 10 min | 0.2 ± 0.03 | 0.18 ± 0.02 | 0.16 ± 0.02 | 0.82 ± 0.09 | 1.14 ± 0.15 | 0.72 ± 0.22 |
| NT | 0 min | 0.52 ± 0.02 | 0.57 ± 0.05 | 0.84 ± 0.14 | 2.41 ± 0.18 | 2.71 ± 0.08 | 1.95 ± 0.34 |
| | 2 min | 0.32 ± 0.02 | 0.24 ± 0.03 | 0.82 ± 0.18 | 1.37 ± 0.19 | 1.84 ± 0.19 | 1.71 ± 0.13 |
| | 10 min | 0.15 ± 0.01 | 0.18 ± 0.01 | 1.41 ± 0.04 | 1.52 ± 0.25 | 1.64 ± 0.14 | 1.33 ± 0.11 |
| BT | 0 min | 0.08 ± 0 | 0.04 ± 0 | 0.06 ± 0.01 | 0.13 ± 0.01 | 0.12 ± 0.01 | 0.17 ± 0.02 |
| | 2 min | 0.11 ± 0.02 | 0.12 ± 0.01 | 0.06 ± 0.01 | 0.16 ± 0.02 | 0.14 ± 0.01 | 0.19 ± 0.03 |
| | 10 min | 0.07 ± 0.01 | 0.17 ± 0.02 | 0.17 ± 0.02 | 0.17 ± 0.02 | 0.22 ± 0.03 | 0.15 ± 0.02 |

0 min:0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking); AT-acid treatment; NT- neutral treatment; BT: buffer treatment; min: minutes; mg: milligram; g: gram; DW: dry weight

### 3.3.2. Sinapic Acid Content

Sinapic acid accounts for over 73% of the free phenolic acids and exhibits higher free-radical activity than either sinapoyl glucose or sinapine (Thiyam *et al.* 2006). Sinapic acid was identified at a wavelength of 320 nm (Khattab *et al.* 2010). **Table 4b** reports the sinapic acid concentration in Oriental, black, and yellow mustard. The sinapine and sinapic acid content showed a distinctive trend between the varieties. For the whole mustard seeds, the highest value of sinapic acid was observed in yellow mustard at 1.41 ± 0.04 mg/g DW. However, for the crushed mustard seeds, black mustard had the highest amount of sinapic acid, with 2.71 ± 0.08 mg/g DW.

Moreover, sauté time also had a significant effect ($p < 0.05$) on the mustard extract. In the case of the whole seed extract from black and Oriental mustard, the concentration of sinapic acid decreased with an increase in sauté time (**Table 4b**). On the other hand, there was a slight increase for yellow mustard when the sauté time was prolonged (**Table 4b**). This was attributed to the protection by the mucilage in the yellow mustard seed coat (Cui *et al*. 2006). However, Aguilar & Ziegler, (1990) established that the longer the exposure of the seeds to heat, the lower amount of mucilage was extracted**,** potentially increasing the sinapic acid yield. Prior to preheat treatment, the sinapic acid concentration of yellow mustard was 0.84 ± 0.1 mg/g DW, which increased to 1.41 ± 0.01 mg/g DW after 10 minutes of preheat treatment. However, the changes were much more distinctive with the crushed mustard seeds as the sinapic acid concentration generally reached its peak when no sauté time was applied, accounting for 2.41 ± 0.01, 2.71 ± 0.08, 1.95 ± 0.3 mg/g DW for Oriental, black, and yellow mustard, respectively. The levels of sinapic acid started to decline when the temperature was applied, with yellow mustard having the lowest (1.33 ± 0.1 mg/g DW). An exception to this was the acid-treated black mustard crushed seed extract in which sinapic acid reached its highest level of 1.31 ± 0.2 mg/g DW after a 2-minute sauté time. The pH value proved to be the critical factor in this study, as, in the case of sinapic acid, the solvent's electrostatic

properties had a significant impact on the targeted samples. With both whole and crushed mustard seeds, the neutral pH **was** the most effective treatment followed by acid pH, with alkaline pH being the least effective treatment (**Table 4b**).

Smyk & Drabent, (1989) confirmed the structured transformation of sinapic acid under the influence of pH. (1.9 - 11.5) using spectrophotometry and spectrofluorimetry. Generally, sinapic acid could exist under three ionic forms ( **Figure 2 I, II, III**). According to Smyk and Drabent's study, sinapic acid retained its neutral form (**I**) when the solution had pH ≤ 2. In a solution of 2 < pH ≤ 6, the ionic form (**II**) coexisted with the neutral form, howbeit, with a pH lower than 5, the original structure of sinapic acid was the most excited form. On the other hand, at pH > 6, form (**III)** was found to be in equilibrium with form (**II**). Later studies by Beltrán *et al.* (2003) and Erdemgil *et al.* 2007 also certified this theory by using potentiometric, spectrophotometric, and liquid chromatographic (LC) measurements. Therefore, the dissociation of sinapic acid under different pH could significantly affect its extracted concentration. On the other hand, each of these ionic forms of sinapic acid can be detected at different wavelengths with the neutral form at 320nm while forms (**II**) and (**III**) came at ≈ 310 and 365 nm, respectively (Smyk & Drabent, 1989). Therefore, when considering 320 nm, only the original and anionic form of sinapic acid was detected. In our study, because the pH value of both acid and neutral was lower than 6, sinapic acid existed mostly in the form (**I**) and (**II**). Meanwhile, for buffer treatment, the pH of the solution varied from 8.90 – 9.30 with form (**III**) being the main one together with a smaller amount of form (**II**). This could explain the variation in sinapic acid concentration at the different pH treatments, and why buffer treatment produced the lowest level of this phenolic.

Chadni *et al.* (2021) examined the effect of pH on the sinapic acid from mustard seed meal using different methanol concentrations while adjusting the pH with acetic acid, sodium carbonate, sodium hydroxide, and potassium hydroxide. They reported the highest sinapic acid content with

buffer condition at pH 12, which was 13.22 ± 0.44 µmol/ $g_{DM}$ ≈ 2 924 ± 98.65 µg/g. A different value was recorded in our study using the same treatment, in which the highest value was 169.56 ± 24.94 mg/g DW after 10 minutes of preheat treatment. However, this may be attributed to the higher heat and pressure and the milder pH conditions applied in the current study. Shrestha *et al.* (2012) also employed a pre-heat treatment of black mustard (*Brassica nigra*), Oriental mustard (*Brasssica juncea*), and yellow mustard (*Sinapis alba*) seed, using methanol/water/acetic acid (70:30:0.2, v/v/v) as extractant solvent. The amount of sinapic acid from roasted seed and roasted powder of black, Oriental, and yellow mustard was 56.90 ± 1.52, 58.52 ± 0.92, 26.50 ± 0.72, 35.97 ± 1.25, 29.58 ± 1.46, 23.10 ± 0.64 µg/g DW, respectively. In our study, however, the highest sinapic acid concentration obtained from whole and crushed seeds of Oriental, black and yellow was 523.96 ± 19.71, 2408.76 ± 184.64, 573.22 ± 48.01, 2712.24 ± 84.59, 1409.5 ± 41.74, and 1953.94 ± 338.86 µg/g DW, respectively. Using an air-frying pre-heat treatment on Oriental mustard powder, Fadairo *et al.* (2021) obtained a sinapic acid concentration of 415.64 ± 26.63 µg/100 g ≈ 0.004 mg/g**,** when roasting the seeds at 180$^{o}$C for 15 minutes. Meanwhile, with the assistance of pressure, our study increased the concentration to 2.41 ± 0.18, 1.37 ± 0.19, 1.52 ± 0.25 mg/g, with respect to 0, 2, and 10 minutes of preheat treatment. Consequently, pre-heat treatment with pressurized wet extraction proved to be the optimum condition for extracting sinapic acid from all mustard varieties examined.

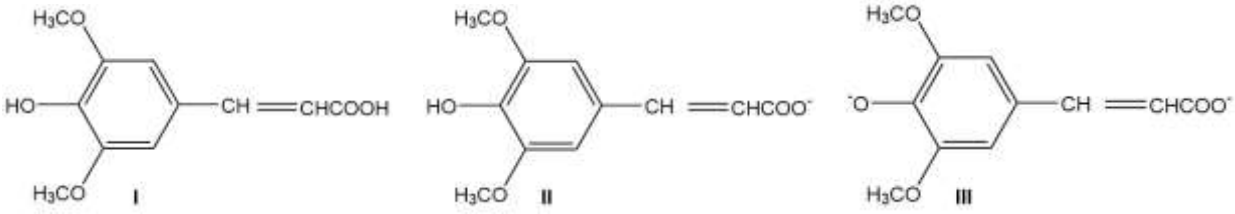


**Figure 2.2 The ionic form of sinapic acid**

### 3.3.3. Canolol Content

Because of its polarity, sinapic acid usually exists complex with proteins and polysaccharides, thereby obstructing decarboxylation (Nićiforović & Abramovič, 2014). Consequently, extreme temperature and pressure conditions are required to release both free and bound sinapic acid from these matrices to facilitate the formation of canolol (Guo *et al.* 2019; Nandasiri & Eskin, 2021). The canolol levels reported in this study showed very promising results. Similar to sinapic acid, the highest canolol values were obtained from different mustard seeds that were processed with no significant differences between the whole and crushed seeds. Of the whole seeds, yellow mustard had the highest canolol value of 2.32 ± 0.13 mg/g DW after 2 minutes of sauté time and neutral treatment. On the other hand, crushed black mustard attained a higher canlolol content (2.61± 0.35 mg/g DW) after 10 minutes of pre-heat treatment followed by neutral conditions for pressurized wet extraction. In the case of acid treatment**,** no canolol was detected in either whole or crushed (orientation) yellow mustard seed prior to pre-heat treatment.

This was consistent with a study by Cools & Terry, (2018), who explained that thermal degradation was effective for breaking down glucosinolates. As a result, more endogenous phenolic compounds were liberated from the matrix facilitating the production of canolol. The highest canolol content from the mustard extracts was also associated with the neutral treatment for both whole and crushed mustard seeds (orientations). While there were no significant differences between the whole and crushed seeds, some improvements in extraction were observed, especially following neutral treatment. According to **Figure 3** and **Figure 4**, the neutral

pH treatment increased the recovery of canolol from Oriental, black, and yellow crushed mustard seeds which further increased with the longer pre-heat treatment time. Therefore, such modifications can significantly enhance the canolol content of the mustard extracts.

Shrestha *et al.* (2012) also showed that using roasting as a pre-heat treatment of selected mustard varieties (Oriental, black and yellow), significantly increased the canolol content. While canolol was undetected in the unroasted samples, its concentration increased to 135.56 ± 2.07, 143.00 ± 2.14 and 75.96 ± 2.19 µg/g DW, respectively, when roasted at 180ºC. Meanwhile, in our study, with the assistance of pressure, canolol content was increased to 605.15 ± 86.23, 173.44 ± 13.08, 621.61 ± 70.01 µg/g DW under 10 minutes of sauté time and acid treatment. On the other hand, this study also noticed a significant decrease in canolol when the orientation changed from whole to crushed seeds**.** The canolol concentration of the latter was 118.97 ± 2.83, 95.50 ± 5.86, and 56.67 ± 0.97 µg/g DW, respectively. The use of lower pressure and lower temperature (115ºC) in our study, not only preserved canolol but also enhanced its concentration to 1241.88 ± 116.37, 731.30 ± 98.80, and 1209.27 ± 86.99 µg/g DW under similar treatments. A study by Fadairo *et al.* (2021) manipulated air frying pre-treatment as home-scale seed roasting for the production of major sinapates in the oil fraction of mustard samples. The canolol content after roasting for 15 minutes at 170ºC was 1086.08 ± 12.62 µg CAE/100g for the low-grade yellow mustard oil. When the roasting temperature was increased to 180ºC, the canolol concentration of this sample then decreased to 1055.49 ± 12.62 µCAE/ 100g. On the other hand, for Oriental mustard, the canolol content increased from 380.68 ± 49.59 to 513.96 ± 48.63 µg CAE/100g when the temperature changed from 170 to 180ºC. Our study observed a similar trend with much more Oriental mustard. The longer the preheat treatment time, the higher the canolol content, with the concentration, improved from 869.90 ± 80.80 to 2222.10 ± 289.00 µg/g DW. The combination of pressure, temperature and pH can potentially affect the generation and preservation of canolol in selected

mustard samples. Consequently, this home-scale processing was not only a cost-saving green technology but also an effective method for the extraction of phenolic compounds from mustard seed.

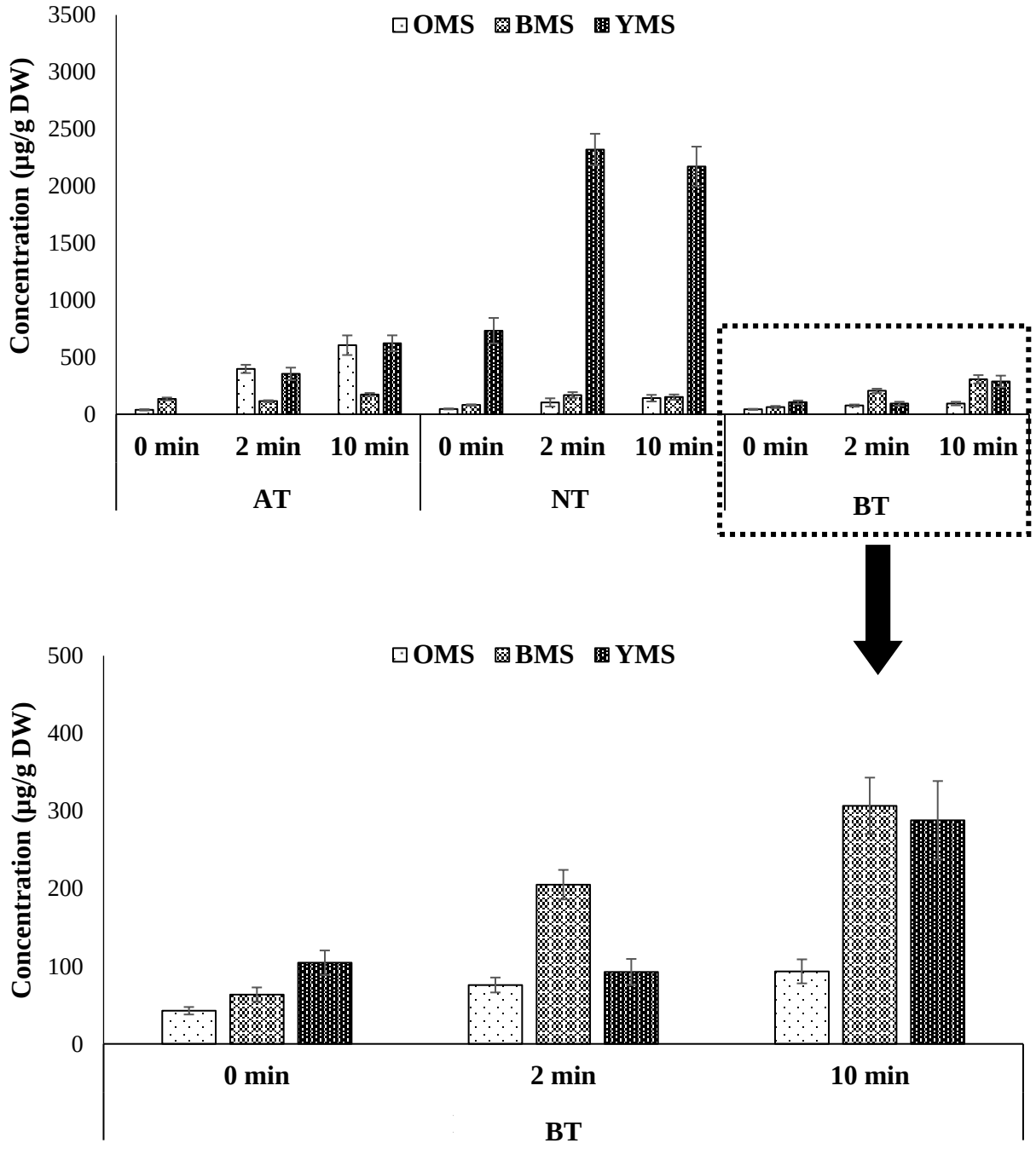


**Figure 2.3: Canolol Content of Selected Varieties of Mustard Seed with three pH treatment**

OMS: Oriental Mustad Seed; BMS: Black Mustard Seed; YMS: Yellow Mustard Seed (n=5)
AT: Acid Treatment; NT: Neutral Treatment; BT: Buffer Treatment, min; minutes µg; microgram, g; gram, DW; dry weight

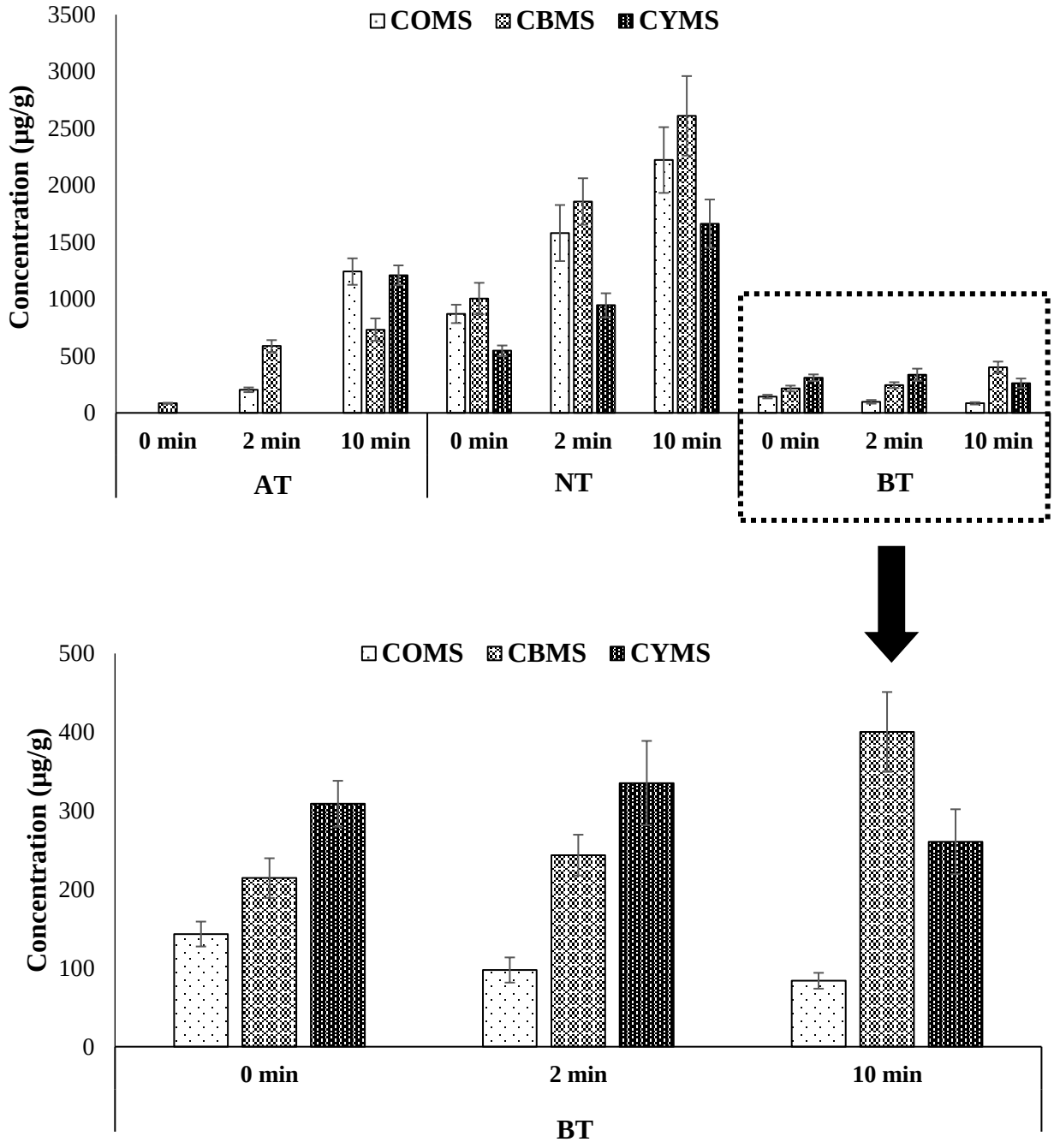


**Figure 2.4: Canolol content of selected varieties of crushed mustard seed with three pH treatment**

COMS: crushed oriental mustard seed; CBMS: crushed black mustard seed; YMS: crushed yellow mustard seed (n=5), AT: acid treatment; NT: neutral treatment; BT: buffer treatment, min; minutes µg; microgram, g; gram, DW; dry weight

### 3.3.4. Other sinapates

Besides the major sinapates, other thermo-generative compounds were also detected in the mustard extracts, with their values presented in **Table 5**. Four compounds with retention times of 19, 35, 39, and 40.5 minutes were recorded. An unknown compound 1 generated at a retention time of 14 minutes, belonged to Oriental mustard (whole and crushed) under both acid and neutral treatment and black mustard (whole and crushed) after acid treatment. The variation of this compound depended on the variety, pH of solvent, and time of pre-heat treatment. The highest concentration of this compound, 506.65 ± 51.56 CAE µg/g DM**,** was found in crushed Oriental mustard seed prior to pre-heat treatment but decreased with a longer pre-heat treatment time to 403.66 ± 48.94 µg CAE/g DM being the lowest after sautéing for 10 minutes. The same trend was observed with Oriental mustard seed after acid treatment with the value reaching 236.36 ± 44.72 µg CAE/g DW prior to sautéing but decreased to 139.8 ± 11.84 µg CAE/g DW after sautéing for 10 minutes. Meanwhile, for the rest of the samples, this compound had the highest concentration after 2 minutes of sauté time at 250.56 ± 25.75 for crushed Oriental mustard seed), 218.21 ± 86.13 (black mustard seed), and 272.06 ± 34.02 µg (crushed black mustard seed) µg CAE/g DW) but then decreased (140.11 ± 17.89, 207.88 ± 18.68, and 257.68 ± 45.65 µg CAE/g DW, respectively). Oriental mustard seed, after neutral treatment, was the only sample that did not follow these trends, as the concentration was stable at 0 and 2 minutes of pre-heat treatment (218.48 ± 18.02 and 218.24 ± 17.05 µg CAE/g DW and increased to 251.51 ± 18.4 µg CAE/g DW when pre-heated for 10 minutes.

The thermo-generative compounds observed at a retention time of 35 minutes (Unknown compounds 2, 3, and 4) were believed to be canolol derivatives. These compounds were thermally sensitive since the longer they were exposed to heat, the lower was their concentration. The highest levels observed for unknown compounds 2, 3, and 4 were 490.07 ± 40.27, 1115.75 ± 54.75, and

459.93 ± 22.56 µg CAE/g DW, respectively. Notably, the concentration of these canolol derivatives was higher than canolol itself (308.92 ± 29.21 µg/g) under the same extraction condition.

The thermo-generative compounds all had the same wavelength as canolol, but different retention times, as they came after canolol (31.6 minutes) at 35, 39 and 40.5 minutes. Such compounds are likely to be canolol derivatives. Canolol appeared to be more stable than these compounds as its concentration did not vary as much with the different pre-heat treatment times. Since canolol is unstable in the presence of oxygen, its conversion into canolol derivatives, including dimers, trimers, and oligomers, is not unexpected. (Harbaum-Piayda *et al.* 2010; Kraljić *et al.* 2015; Nandasiri, *et al.* 2021a,b,c).

**Table 2.5: Impact of pressurized cooking on other thermo-generative sinapates**

| Substrate | Time | RT 14 (µg CE/g DW) | RT 35 (µg CE/g DW) | RT 39 (µg CE/g DW) | RT 40.5 (µg CE/g DW) |
|---|---|---|---|---|---|
| OMS (AT) | 0 min | 236.36 ± 44.72[a] | nd | nd | nd |
| | 2 min | 199.22 ± 25.81[a] | nd | nd | nd |
| | 10 min | 139.8 ± 11.84[b] | nd | nd | nd |
| COMS (AT) | 0 min | 194.16 ± 12.99[a] | nd | nd | nd |
| | 2 min | 250.56 ± 25.75[a] | nd | nd | nd |
| | 10 min | 140.11 ± 17.89[b] | nd | nd | nd |
| OMS (NT) | 0 min | 218.48 ± 18.02[a] | nd | nd | nd |
| | 2 min | 218.24 ± 17.05[a] | nd | nd | nd |
| | 10 min | 251.51 ± 18.4[a] | nd | nd | nd |
| COMS (NT) | 0 min | 506.65 ± 51.56[c] | nd | nd | nd |
| | 2 min | 454.1 ± 112.51[c] | nd | nd | nd |
| | 10 min | 403.66 ± 48.94[d] | nd | nd | nd |
| BMS (AT) | 0 min | 187.16 ± 39.77[a] | nd | nd | nd |
| | 2 min | 218.21 ± 86.13[a] | nd | nd | nd |
| | 10 min | 207.88 ± 18.68[a] | nd | nd | nd |
| CBMS (AT) | 0 min | 132.15 ± 27.06[b] | nd | nd | nd |
| | 2 min | 272.06 ± 34.02[a] | nd | nd | nd |
| | 10 min | 257.68 ± 45.65[a] | nd | nd | nd |
| CYMS (AT) | 0 min | nd | 490.07 ± 40.27[a] | 1115.75 ± 54.75[a] | 459.93 ± 22.56[a] |
| | 2 min | nd | 283.95 ± 43.22[b] | 414.64 ± 57.98[b] | 163.35 ± 23.31[b] |
| | 10 min | nd | 156.6 ± 22.36[c] | 140.94 ± 22.86[c] | 57.73 ± 8.72[c] |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$); AT-acid treatment; NT- neutral treatment; nd: not detected; 0 min:0-minute sautee time with pressurized cooking; 2 min: 2-minute sautee time with pressurized cooking; 10 min:10-minute sautee time with pressurized cooking; RT:

retention time; CE: canolol equivalent; OMS: Oriental Mustad Seed; BMS: Black Mustard Seed; COMS: Crushed Oriental Mustad Seed; CBMS: Crushed Black Mustard Seed; CYMS: Crushed Yellow Mustard Seed; µg: micro gram; g: gram; DW: dry weight; RT: retention time

## 4. Conclusion

The processing conditions were strictly followed as outlined in Figure 1**.** However, there were some problems with the absorption of the crushed seed during the treatment since it appeared to absorb solvent better than the whole seed. Consequently, few extractants were left for HPLC analysis and antioxidant assays. Solvent was then added to increase the weight of the mustard sample using the same ratio. Pre-heat treatment was then applied using 0, 2, 10 minutes of sauté treatment and pressurized cooking with the different pH solutions.

Optimization was carried out to establish the best extraction condition for sinapine, sinapic acid, with a primary focus on canolol content. The first factor tested was the time of pre-treatment. Three different time conditions were applied using the same ratio of solvent for comparison. The results showed that the 8- and 10-minute treatments generated relatively similar amounts of phenolic compounds. The 10-minute treatment, however, produced slightly higher levels of phenolic compounds in all tested conditions. Therefore, the application of an 8-minute treatment proved unnecessary. The next factor examined was the ratio of solvent. Different proportions of vinegar and water were tested with the most optimal ratio established as 3:2(v/v).

Both yellow and black mustard had competitive amounts of sinapine, sinapic acid, and canolol. For sinapine content, however, yellow mustard had the highest amount in both the whole and crushed seeds. While crushed black mustard seeds were slightly high in both sinapic acid and canolol content. Acid and buffer treatment proved to be far less effective than the neutral treatment. Overall, the amount of targeted phenolics increased with longer pre-heat treatment.

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## 6. Author Contributions

TN and RN designed the study. TN performed the experiments under the guidance of both RN and ME, interpreted the results and drafted the manuscript. ME, and RN proofread the manuscript. Funding was acquiesced by UTH and ME.

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**Supplement Information**

**Table S 2.1:** Vinegar Concentration used for the optimization of the pressurized heat treatment.

| | **Acetic Concentration (vinegar: water) (v/v)** | | |
|---|---|---|---|
| **Commercial Vinegar** | 5% | 5% | 5% |
| **1st dilution** | 5% | 2.5% | 1.25% |
| **2nd dilution (ratio 3:2 v/v)** | 3% | 1.5% | 0.75% |

**Table S 2.2:** Phenolic changes under different sauté time from Oriental mustard sample

| | **Sinapine** (µg/g) | **Sinapic acid** (µg/g) | **Canolol**(µg/g) |
|---|---|---|---|
| **0 mins** | 14144.15 ± 988.40 | 91.48 ± 5.41 | nd |
| **2 mins** | 11978.43 ± 1083.27 | 148.98 ± 14.07 | 331.10 ± 23.39 |
| **8 mins** | 7920.43 ± 802.23 | 122.73 ± 8.35 | 374.41 ± 14.55 |
| **10 mins** | 10401.16 ± 762.40 | 146.59 ± 18.17 | 431.64 ± 37.43 |

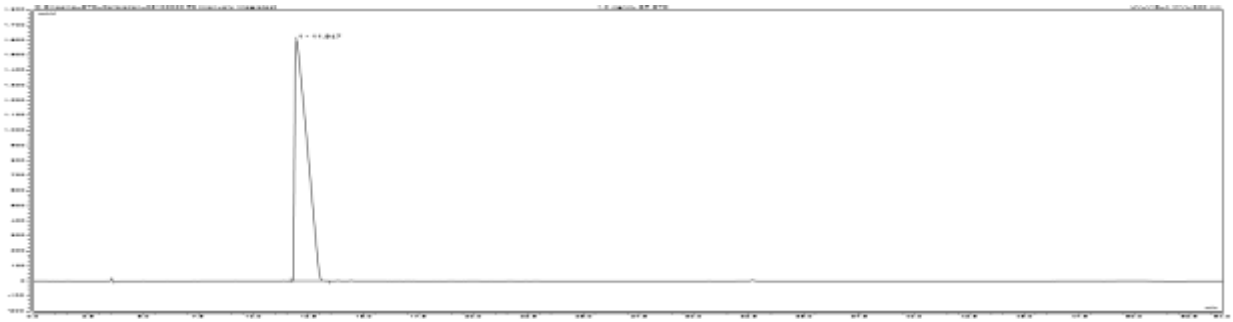

**Figure S 2.1 HPLC-Chromatogram of Sinapine Standard (320nm)**

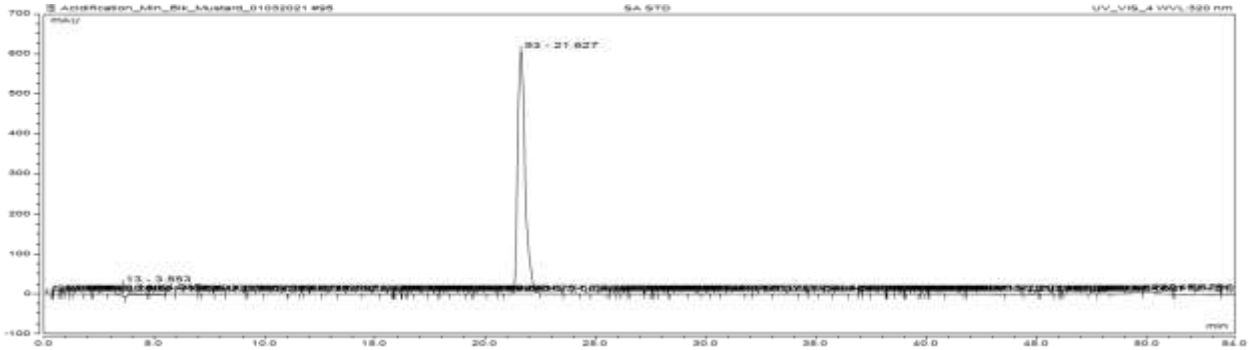

**Figure S 2.2: HPLC-Chromatogram of Sinapic Acid Standard (320nm)**

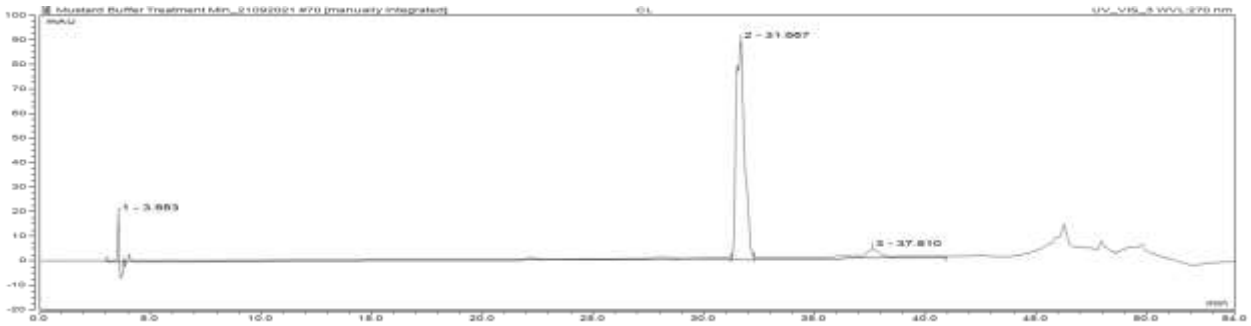


**Figure S 2.3** :**HPLC-Chromatogram of Canolol Standard (270nm)**

## CHAPTER 3

# The Effect of pH on the Phenolic Content and Antioxidant Properties of Three Different Mustard Extracts

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**Abstract**

Mustard seeds are cultivated worldwide due to their substantial agronomic value of their high protein, oil, and phenolic content. The latter bioactive compounds give mustard seeds various applications in the food and pharmaceutical industries, as antimicrobial, antioxidant, and chemoprotective agents. By modifying the pretreatment and extraction conditions, a significant improvement in the quantity and quality of these crucial compounds was obtained. Based on the electrostatic interactions between the solvents and the extracts, an alternative green extraction procedure was used on three varieties of mustard seeds (Oriental, Black, and Yellow). Preliminary results demonstrated an interesting trend in which the isoelectric pH value affected the antioxidant activity of the extracts. A number of different antioxidant assays together with total phenolic content (TPC) and total flavonoid content (TFC) were conducted on the three different mustard seeds as affected by different combinations of times and pHs. With the exception of metal ion chelation assay, the other antioxidant methods, including ferric reducing/ antioxidant power assay, DPPH free radical-scavenging assay and ABTS•+ Scavenging Assay, significantly ($p<0.05$) increased with the pretreatment time for all three pH levels studied. Interestingly, the total phenolic content significantly increased ($p<0.05$) with the lower pH level treatments. The highest total phenolic content (2040.32 ± 360.12 mg/g dry weight basis) was obtained from yellow mustard seed under neutral treatment. Conversely, TFC showed no significant differences among the different pretreatment time conditions closer to the neutral pH.

**Keywords**: Mustard, pH, TPC, TFC, antioxidant activity

**Practical Application:** The usage of food-based solvents with the assistance of a home-scale pressurized wet extraction model represents a green technology which can contribute to a wide variety of applications. This method significantly improved the phenolic content, flavonoid content and antioxidant-potential of the mustard extracts, thus making water the most promising extracted solvent.

## 1. Introduction

Mustard, a member of the *Brassicaceae* family, has gained increased attention due to the wide array of applications as an edible oil, condiment, preservative, and use as medicinal supplements (Edwards et al., 2007). The ranking of the top five canola-/rapeseed-/mustard-producing countries has remained similar for the last 30 years, with Canada overtaking China in 2011 (Warwick et al., 2009). According to Thomas et al. (2012), Canada was one of the most important countries in both mustard-growing and mustard condiment manufacturing, especially the highest exporter of *B. nigra* during 2004-2008 (Alvarez & Boye, 2012). While there are many mustard varieties available around the world, the major ones are black (*Brassica nigra*), Oriental (*Brassica juncea*), and white/yellow (*B. hirta/Sinapis alba*). These mustard varieties are not only famous as condiments and spices, but also for their therapeutic qualities due to their high content of polyphenolic and phenolic compounds (Boscaro et al., 2018; Dinis et al., 1994; Jahangir et al., 2009; Martinović et al., 2019; Terpinc et al., 2012; Thomas et al., 2012; Yang et al., 2013). Mustards are high in oil (23.4-43.4%) and protein content (22.8-37.4%), which varies between the different mustard varieties. Yellow mustard has the highest protein (26.9-37.4%) and the lowest oil content (23.4-33.7%), while Oriental mustard has the lowest oil (22.8-30.9%) and the highest protein content (34.8-43.4 %) (Siemens & Barthet, 2019). Black mustard, on the other hand, was reported to have the same constituents as the Oriental variety (Thomas et al., 2012). The three main varieties are also distinguishable by their color and seed phenotype. Another factor that makes thee mustard varieties unique are their pungent aromas. Different mustards vary in their levels of pungent aromatics ranging from least to most in following order, yellow> Oriental> black (Manohar et al., 2009).

Mustard is a functional food with a rich source of safe and effective natural antioxidants. The total phenolic content of mustard seeds could reach 2.62-36.5mg/g dry weight (Dubie et al., 2013; Harbaum et al., 2008; Matthäus, 2002). The main phenolic compound in mustard seeds is sinapic acid and its derivatives (Thiyam et al., 2006). Moreover, sinapic acid and its derivatives have demonstrated a variety of health-promoting effects. A number of *in-vivo* and *in-vitro* assays have reported their antimicrobial, anti-inflammatory, antioxidant, anti-thrombotic, and anti-carcinogenic effects (Chen, 2016; Nićiforović & Abramovič, 2014), especially 2,6-dimethoxy-4-vinylphenol or 4-vinyl syringol of canolol (Nandasiri & Eskin, 2022).

Extraction of phenolic compounds from oilseeds, including mustard, has been carried out for many years using a wide variety of methods in which canolol has gained considerable attention lately. Canolol is a thermally generated phenolic compound that is not naturally present (Fadairo et al., 2022; Nandasiri et al., 2023 a,b). However, with the application of thermal treatments canolol is formed via decarboxylation of sinapic acid (Nandasiri et al., 2023a, b).. Prolonged thermal treatments have been reported to have detrimental effects on the concentration of canolol as it undergoes dimerization (Nandasiri et al., 2023 a, b; Nandasiri & Eskin, 2022). The decrease in canolol when extracted at 180 $^{o}$C for 20 minutes could be due to its conversion to other forms (Aachary & Thiyam-Hollander, 2012; Fadairo et al., 2021; Nandasiri et al., 2019; Nandasiri, et al., 2021a, b; Nandasiri & Eskin, 2021, 2022). Consequently, milder temperatures are required such as provided by pressurized temperature processing (Fadairo et al., 2021; Nandasiri et al., 2019; Nandasiri, Imran, et al., 2021)**.**

Researchers have examined the effect of both heat and pressure treatment on the extraction of the major sinapates from mustard seed. A solvent-free dry heat pre-treatment method, using a novel commercial RapidOxy® 100 system, resulted in the maximum recovery of canolol at 160$^{o}$C

for 10 minutes (Nandasiri et al., 2021a). This system established an optimum temperature and pressure for the generation of canolol, which enhanced its yield from mustard seed. Another study by Fadairo et al., (2021) applied air frying as a roasting pretreatment condition to increase the canolol and other lipophilic sinapates content from mustard samples. This method applied forced air circulation at high temperatures to produce convection effects leading to a more efficient heat transfer. Another approach is to examine the effect pH of the extracted solvent as it can change the isoelectric status of the solvent. This can affect the targeted phenolic compounds differently and could facilitate an increase in antioxidant activity.

In this study, three mustard varieties were extracted using three types of food-based solvents to establish acid, neutral and buffer conditions followed by a pressurized wet extraction to provide a combination of both temperature and pressure. The extracts were then evaluated to assess the impact of the changes in pH on their phenolic content and antioxidant activity.

## 2. Material and Method

### 2.1.Materials

GS Dunn. Ltd (Montreal, QC, Canada), provided Oriental Mustard seed. Commercial black mustard seeds were purchased from Gill's supermarket (Winnipeg, MB, Canada), which was imported by PTIFOODS© (Mississauga, ON, Canada). Yellow mustard seeds were purchased from Real Canadian Superstore (Winnipeg, MB, Canada), which was imported by Loblaw Inc®/™ (Toronto, ON, Canada). In order to prolong the shelf life of samples, all the seeds were packed, sealed, and kept under refrigeration conditions at 4 $^{o}$C. Experiments were conducted on both the whole seeds and ground seeds. The latter was prepared fresh every day using a mortar and pestle.

### 2.2.Chemicals

Formic Acid was purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). No Name brand Vinegar, (ARM & HAMMER™). Baking soda (Arm and Hammer brand) was purchased from Real Canadian Superstore (Winnipeg, Manitoba, Canada). Methanol, ethanol (Optima™ LC/MS Grade) was also purchased from Fisher Scientific Canada Ltd.

Folin–Ciocalteu's (FC) reagent, iron (II) chloride hexahydrate (98%), iron (III) chloride hexahydrate (97%), iron (II) sulphate heptahydrate (99%), glacial acetic acid (99.8%), hydrogen chloride (HCl, 99%), sodium acetate, 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ >98%), sinapic acid (>97%), and 2,2- diphenyl-1-picrylhydrazyl (DPPH, 97%), 3-ethylbenzothiazoline-6-sulphonic acid, potassium persulphate, potassium buffer saline were all purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). Quercetin hydrate (>95%) and 2-amino-ethyl-diphenyl borate (98%) were purchased from Acros (Mississauga, ON, Canada).

## 2.3.Methods

### 2.3.1. Sample preparation

The extraction process was presented as a flow chart shown in **Figure 1**. Briefly, 10.0 grams of mustard were first sautéd in an instant pot (Instant Pot Duo Mini 3 Qt Model number: IPDUOMINI 3Qt, Instant Brands Inc.) at a constant temperature of 76$^{o}$C for 0, 2 & 10 minutes. The selection of preheat treatment time was based on the changes of major sinapates in the mustard extract. Preliminary studies showed an increase in the indigenous phenolic content with the treatment times from 0 min to 8 min (data not shown). Prior to preheat treatment, mustard varieties had the highest sinapine concentration. After 2 minutes, the sinapine concentration decreased, leading to an increase in sinapic acid. Meanwhile, canolol had the highest recovery at 10 minutes of preheat treatment. Hence, we used 0, 2, and 10 minutes. The samples were then cooled at ambient temperature in a desiccator. After 10 minutes, the sautéd seeds were treated by wet

extraction under pressurized heating (115 $^{o}$C at 10.2psi) with 3 types of solvents. The conditions for each treatment were presented in **Table S1** (Supplement information). For the acid treatment, acetic acid was added to water in a ratio of 3:2 (v/v). The concentration between two aqueous solutions was based on which ratio yielded the highest sinapate concentration. The pH value was measured before and after the extraction was carried out. The initial pH value was 2.59, but after extraction, it ranged from 3.3-3.62 depending on the variety, orientation (whole and crushed), and treatment time. For neutral treatment, deionized water was used, and the measuring step was carried out similarly as described above. The initial pH value was 5.94 but was reduced to 3.94-5.24 depending on the application. For the basic pH, the initial concentration was 1.5:10 w/v. However, because of the low solubility of sodium bicarbonate in water (≈10 g/L at 20 $^{o}$C), a lower concentration of sodium bicarbonate (1:10 w/v) was used as an environmentally friendly and consumable solvent for the buffer treatment. The initial pH value started from 7.78 but increased to 8.9-9.3. Thereafter, the extracts were stored at -20$^{o}$C until analyzed.

**Processing Condition**

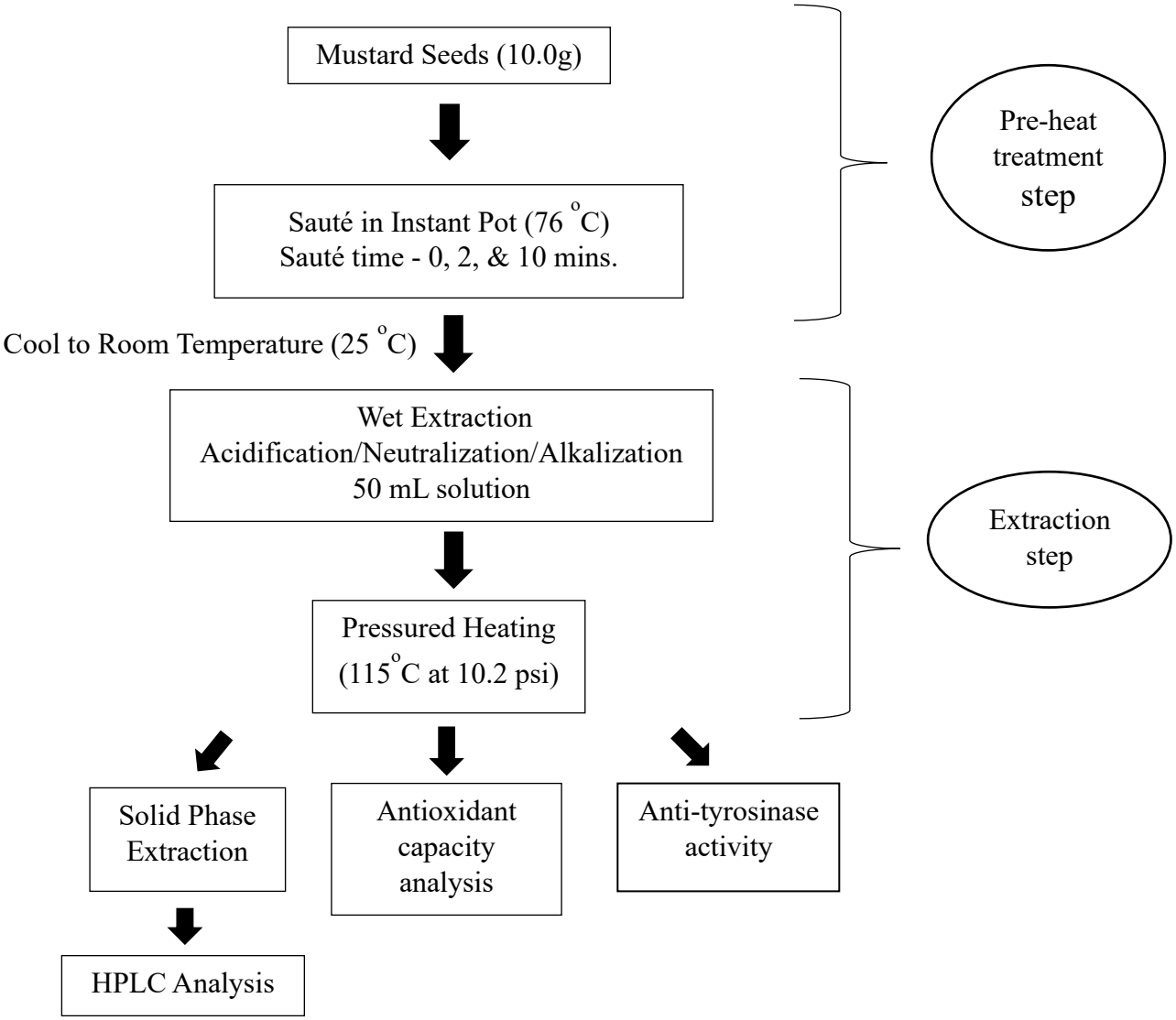



**Figure 3.1: Procedure for pressurized wet extraction of Mustard seed**

### 2.3.2. Total phenolic content determination

The Total Phenolic Content (TPC) of the extracts was estimated using the Folin- Ciocalteu method described by Thiyam et al., (2006) with slight modification. First, samples were diluted with distilled water using 1:100 (v/v) ratio. The diluted extractant was mixed with 0.5mL Folin-Ciocalteu's reagent and 1.0 mL 19% (v/v) Sodium Carbonate. Distilled water was then added to make 10.0mL total volume, and then vortexed. The reaction was conducted in the dark for 60 minutes with intermittent vortexing after 30 minutes. Absorbance was measured using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A) at 750 nm. Methanol was substituted as blank, and a calibration curve was prepared using (1.0 mM) sinapic acid solution.

### 2.3.3. Total flavonoid content determination

The total flavonoid content (TFC) of the mustard extracts was determined by the aluminum chloride colorimetric method described by Zhishen et al., (1999) with slight modifications. A 0.5 mL aliquot of sample was diluted with distilled water in a ratio of 1:4 (v/v). The diluted sample was then mixed with 0.15mL of sodium nitrite 5% (w/v), and the reaction mixture was held at room temperature for 6 minutes. After that, 0.3mL of aluminium chloride 10% (w/v) was added and kept for an additional 5 minutes. After 5 minutes, 1.0 mL of NaOH (1 M) was mixed with the previous solution by a Vortex mixer (VWR™ Analog Vortex Mixer). The absorbance was measured at 510 nm with the total flavonoid content expressed as milligrams of quercetin per gram of mustard seed. The standard curve was presented in **Figure S2**.

### 2.3.4. DPPH free radical–scavenging activity

The DPPH radical scavenging activity of the extracted solution was measured using the DPPH assay described by Thiyam et al., (2006), with some modifications. Briefly, 10 µL of mustard seed extracts were mixed with 2.98 mL of prepared DPPH solution. The solution was then measured using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A) at 516 nm after 10 minutes. The free radical scavenging activity was measured using the following equation:

$$\text{Scavenging Effect (\%)} = \frac{(A_c - A_s) \times 100}{A_c}$$

Where $A_c$ is the absorbance of solvent control

$A_s$ is the absorbance of the sample.

### 2.3.5. $ABTS^{\bullet+}$ Scavenging Assay

The ABTS assay was performed using the method described by Sen & Sarkar (2013). Extracts (20 µL) were diluted with water in a dilution ratio of 1:20 and then mixed with 2980 µL $ABTS^{\bullet+}$ solution for detection at 0 minutes and 6 minutes of the reaction. The $ABTS^{\bullet+}$ solution was prepared by mixing ABTS (7 mM) and potassium persulfate (2.45 mM) in potassium buffer saline (pH 7.4) in 1:1 (v/v) ratio. This solution was then kept for countering in dark conditions for 14-16 hours; until the targeted radical was formed. In order to dilute the stock of ABTS radical solution, phosphate buffer saline was made and adjusted to pH 7.4. The $ABTS^{\bullet+}$ solution was then mixed with phosphate buffer saline to an absorbance of 0.7 ± 0.01 (Ozgen et al., 2006), using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A). According to Lyasov et al. (2020), at the wavelength of 734nm.

The radical scavenging effect is calculated using the equation:

$$\text{Radical scavenging activity (\%)} = \frac{A_c - A_{(t_0 - t_{10})}}{A_c} \times 100$$

Where $A_c$ is the absorbance of blank

$A_s$ is the absorbance of the sample at 0 min subtracted by absorbance of the sample after 10 minutes.

### 2.3.6. Ferrous-ion-chelating activity assay antioxidant capacity

The metal chelating activity assay for assessing antioxidant capacity was determined in this study. This analysis was followed according to that described by Dinis et al., (1994) with some modifications. The dilution factor was 1:1 (v/v) for acid and neutral treatments and was diluted with deionized water. With respect to the buffer treatment, due to the high color intensity, the dilution was further increased to 1:100 (v/v). After dilution, 1.0 mL of the sample was mixed with 0.05 mL of 2.0 mM ferrous chloride solution and 0.2 mL of 5.0 mM ferrozine solution. These solutions were freshly prepared every day. Deionized water was then added to dilute the solution to 4.0 mL of total volume. The mixture was then kept at room temperature for 10 minutes and the absorbance was measured using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A) at 562 nm wavelength. The ferrous-ion-chelating activity was calculated using disodium EDTA salt. The ferrous-ion-chelating activity was calculated using Disodium EDTA salt. **Figure S3** was used for calculation.

### 2.3.7. Ferric reducing/antioxidant power assay (FRAP assay)

FRAP assay is a colorimetric method evaluating the reduction of $Fe^{3+}$-tripyridyltriazine complex ($Fe^{3+}$-TPTZ) to the ferrous form ($Fe^{2+}$-TPTZ). The method used is that described by Benzie & Strain, (1996) with a few modifications The FRAP reagent was prepared by mixing

acetate buffer (300mM, pH = 3.6), TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) solution with a 20 mM ferric chloride solution in a ratio of 10:1:1 and kept at 37 $^{o}$C until used. The prepared solution was a combination of 100 µL extracted sample, 900 µL Distilled water, and 2.0 mL FRAP reagent. The mixture was then left to react in the dark for 30 minutes and the absorbance was measured using the UV-Visible Spectrometer FL6500 (Perkin Elmer Inc., Shelton, Connecticut, U.S.A) at 593 nm wavelength. Deionized water was used as the blank with Trolox as the standard solution. The standard curve is presented in **Figure S4**.

### 2.3.8. High-Performance Liquid Chromatography - Diode Array Detection (HPLC-DAD) analysis of sinapic acid derivatives

Major sinapates of different mustard seeds, including Oriental, black, and **y**ellow, were tested qualitatively and quantitatively using the HPLC-DAD (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States) method described by Nandasiri et al., (2019) as modified by Fadairo et al., (2021) The separation was carried out on a KinetexR® Biphenyl C18 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States), with a 0.4 mL/min flow rate and a 10-µL injection volume.

### 2.3.9. Statistical Analysis

The results are presented as a mean ± standard deviation for all experiments conducted with five replicates. Examination of residuals confirmed normally distributed data and constant variance (Pallant, 2020). To obtain normalized data, the raw data points were tested using the normality test, and the necessary transformations were made to the raw data. As part of the current study, square root and logarithm transformations were used to transform normalized data (Pallant, 2020). A factorial design was used for the study with a variety, orientations (whole and crushed),

preheat treatment time, and pH as independent factors. Analyses of the data were performed using a general linear multiple regression model with a two-way analysis of variance. Multiple mean comparisons were achieved with Tukey's test, with 5% statistically significant differences (P * 0.05) considered statistically significant (Pallant, 2020). SPSS statistical software version 26 (IBM, New York, NY, United States) was used to analyze the data.

## 3. Result and Discussion

### 3.1.Statistical result

A multifactor comparison of the total phenolic content, total flavonoid content, and four different antioxidant assays were conducted using Tukey's *post-hoc* analysis. The statistics were presented in **Tables 1** and **2**. According to the mean sum of squares results, variety, orientations of the seed (whole and crushed), preheat treatment, time, and pH had a positive effect on the tested assays except for the DPPH and metal chelation ion assays. In order to obtain higher statistical power for factorial analysis for each test, the insignificant factors were eliminated from the system. For the DPPH scavenging effect, those factors that did not affect the mustard extracts were variety and orientation with $p = 0.262$ and $p= 0.281$, respectively. For the metal ion chelation assay, those factors were variety and time with $p = 0.204$, and 0.745, respectively. Furthermore, the interaction effects of the parameters using the two-way ANOVA test, orientation*time, orientation*pH, pH*time, and variety*pH, were different between each test. All interactions had a significant effect on total phenolic content and FRAP value ($p < 0.05$). However, for total flavonoid content, the comparison between variety*time, and variety*orientation with $p = 0.508$, 0.645 showed no significance. For the metal chelation assay, a similar comparison included orientation*time, variety*time and pH*time, while for the ABTS assay, it was variety*orientation, and for the DPPH assay, orientation. All three-way interactions and four-way interactions were also significantly

different, except for the DPPH assay. Accordingly, for most of the tests, when orientation was combined with other factors, there was no significance. Moreover, DPPH was not considered as a good model for this study. All the results obtained from total phenolic content, total flavonoid content, with FRAP, metal ion chelation, ABTS scavenging effect, and DPPH assays, however, had high coefficients of variances: $R^2$ = 0.989, 0.974, 0.995, 1.000, 0.980, 0.951, respectively.

**Table 3.1: Multiple comparisons for Main Factors from Total Phenolic Content, Total Flavonoid Content, and Antioxidant Assay value variables**

| | | | Mean Difference | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| | | **a.** Total Phenolic Content | | | | | |
| Variety | Oriental Mustard | Black mustard | -2.17* | 0.15 | 0.00 | -2.52 | -1.82 |
| | | Yellow Mustard | -12.88* | 0.15 | 0.00 | -13.23 | -12.53 |
| | Black Mustard | Oriental Mustard | 2.17* | 0.15 | 0.00 | 1.80 | 2.52 |
| | | Yellow Mustard | -10.71* | 0.15 | 0.00 | -11.06 | -10.36 |
| | Yellow Mustard | Black Mustard | 12.88* | 0.15 | 0.00 | 12.53 | 13.23 |
| | | Oriental Mustard | 10.71* | 0.15 | 0.00 | 10.36 | 11.06 |
| pH | 2.59 | 5.94 | 0.47* | 0.16 | 0.01 | 0.08 | 0.85 |
| | | 7.78 | 4.70* | 0.15 | 0.00 | 4.35 | 5.05 |
| | 5.94 | 2.59 | -0.47* | 0.16 | 0.01 | -0.85 | -0.08 |
| | | 7.78 | 4.23* | 0.15 | 0.00 | 3.89 | 4.58 |
| | 7.78 | 2.59 | -4.70* | 0.15 | 0.00 | -5.05 | -4.35 |
| | | 5.94 | -4.23* | 0.15 | 0.00 | -4.58 | -3.89 |
| Time | 0 | 2 | -3.33* | 0.15 | 0.00 | -3.69 | -2.99 |
| | | 10 | -6.05* | 0.15 | 0.00 | -6.40 | -5.70 |
| | 2 | 0 | 3.33* | 0.15 | 0.00 | 2.99 | 3.69 |
| | | 10 | -2.71* | 0.15 | 0.00 | -3.06 | -2.36 |
| | 10 | 0 | 6.05* | 0.15 | 0.00 | 5.70 | 6.40 |
| | | 2 | 2.71* | 0.15 | 0.00 | 2.36 | 3.06 |
| | | **b.** Total Flavonoid Content | | | | | |
| Variety | Oriental Mustard | Black mustard | -0.13* | 0.01 | 0.00 | -0.14 | -0.11 |
| | | Yellow Mustard | -0.14* | 0.01 | 0.00 | -0.16 | -0.12 |
| | Black Mustard | Oriental Mustard | 0.13* | 0.01 | 0.00 | 0.11 | 0.14 |
| | | Yellow Mustard | -0.01* | 0.01 | 0.37 | -0.03 | 0.01 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Yellow Mustard | Black Mustard | .014* | 0.01 | 0.00 | 0.12 | 0.16 |
| | | Oriental Mustard | 0.01* | 0.01 | 0.37 | -0.01 | 0.03 |
| pH | 2.59 | 5.94 | -0.18* | 0.01 | 0.00 | -0.20 | -0.17 |
| | | 7.78 | -0.59* | 0.01 | 0.00 | -0.61 | -0.57 |
| | 5.94 | 2.59 | 0.18* | 0.01 | 0.00 | 0.17 | 0.20 |
| | | 7.78 | -0.41* | 0.01 | 0.00 | -0.43 | -0.39 |
| | 7.78 | 2.59 | 0.59* | 0.01 | 0.00 | 0.57 | 0.61 |
| | | 5.94 | 0.41* | 0.01 | 0.00 | 0.39 | 0.43 |
| Time | 0 | 2 | -0.11* | 0.01 | 0.00 | -0.13 | -0.09 |
| | | 10 | -0.23* | 0.01 | 0.00 | -0.25 | -0.21 |
| | 2 | 0 | 0.11* | 0.01 | 0.00 | 0.09 | 0.13 |
| | | 10 | -0.12* | 0.01 | 0.00 | -0.14 | -0.11 |
| | 10 | 0 | 0.23* | 0.01 | 0.00 | 0.21 | 0.25 |
| | | 2 | 0.12* | 0.01 | 0.00 | 0.11 | 0.14 |
| | | | **c.** FRAP Value | | | | |
| Variety | Oriental Mustard | Black mustard | 0.04* | 0.00 | 0.00 | 0.03 | 0.05 |
| | | Yellow Mustard | 0.10* | 0.00 | 0.00 | 0.09 | 0.11 |
| | Black Mustard | Oriental Mustard | -0.04* | 0.00 | 0.00 | -0.05 | -0.03 |
| | | Yellow Mustard | 0.06* | 0.00 | 0.00 | 0.05 | 0.07 |
| | Yellow Mustard | Black Mustard | -.010* | 0.00 | 0.00 | -0.11 | -0.09 |
| | | Oriental Mustard | -0.06* | 0.00 | 0.00 | -0.07 | -0.05 |
| pH | 2.59 | 5.94 | 0.20* | 0.00 | 0.00 | 0.19 | 0.21 |
| | | 7.78 | -0.57* | 0.00 | 0.00 | -0.58 | -0.56 |
| | 5.94 | 2.59 | -0.20* | 0.00 | 0.00 | -0.21 | -0.19 |
| | | 7.78 | -0.77* | 0.00 | 0.00 | -0.78 | -0.76 |
| | 7.78 | 2.59 | 0.57* | 0.00 | 0.00 | 0.56 | 0.58 |
| | | 5.94 | 0.77* | 0.00 | 0.00 | 0.76 | 0.78 |
| Time | 0 | 2 | -0.15* | 0.00 | 0.00 | -0.16 | -0.15 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10 | -0.23* | 0.00 | 0.00 | -0.23 | -0.22 |
| | 2 | 0 | 0.15* | 0.00 | 0.00 | 0.15 | 0.16 |
| | | 10 | -0.07* | 0.00 | 0.00 | -0.08 | -0.06 |
| | 10 | 0 | 0.23* | 0.00 | 0.00 | 0.22 | 0.23 |
| | | 2 | 0.07* | 0.00 | 0.00 | 0.06 | 0.08 |
| | | | **d.** Metal Ion Chelation | | | | |
| pH | 2.59 | 5.94 | 0.20* | 0.00 | 0.00 | 0.19 | 0.20 |
| | | 7.78 | -0.44* | 0.00 | 0.00 | -0.45 | -0.43 |
| | 5.94 | 2.59 | -0.20* | 0.00 | 0.00 | -0.20 | -0.19 |
| | | 7.78 | -0.64* | 0.00 | 0.00 | -0.64 | -0.63 |
| | 7.78 | 2.59 | 0.44* | 0.00 | 0.00 | 0.43 | 0.45 |
| | | 5.94 | 0.64* | 0.00 | 0.00 | 0.63 | 0.64 |
| | | | **e.** ABTS scavenging effect | | | | |
| Variety | Oriental Mustard | Black mustard | 0.02* | 0.01 | 0.00 | 0.00 | 0.03 |
| | | Yellow Mustard | -0.26* | 0.01 | 0.00 | -0.27 | -0.25 |
| | Black Mustard | Oriental Mustard | -0.02* | 0.01 | 0.00 | -0.03 | 0.00 |
| | | Yellow Mustard | -0.28* | 0.01 | 0.00 | -0.29 | -0.26 |
| | Yellow Mustard | Black Mustard | 0.26* | 0.01 | 0.00 | 0.25 | 0.27 |
| | | Oriental Mustard | 0.28* | 0.01 | 0.00 | 0.26 | 0.29 |
| pH | 2.59 | 5.94 | 0.05* | 0.01 | 0.00 | 0.04 | 0.06 |
| | | 7.78 | -0.10* | 0.01 | 0.00 | -0.11 | -0.09 |
| | 5.94 | 2.59 | -0.05* | 0.01 | 0.00 | -0.06 | -0.04 |
| | | 7.78 | -0.15* | 0.01 | 0.00 | -0.16 | -0.13 |
| | 7.78 | 2.59 | 0.10* | 0.01 | 0.00 | 0.09 | 0.11 |
| | | 5.94 | 0.15* | 0.01 | 0.00 | 0.13 | 0.16 |
| Time | 0 | 2 | -0.11* | 0.01 | 0.00 | -0.12 | -0.10 |
| | | 10 | -.013* | 0.01 | 0.00 | -0.14 | -0.12 |
| | 2 | 0 | 0.11* | 0.01 | 0.00 | 0.10 | 0.12 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10 | -0.02* | 0.01 | 0.00 | -0.03 | -0.01 |
| | 10 | 0 | 0.13* | 0.01 | 0.00 | 0.12 | 0.14 |
| | | 2 | 0.02* | 0.01 | 0.00 | 0.01 | 0.03 |
| | | | **f.** DPPH scavenging effect | | | | |
| pH | 2.59 | 5.94 | 3.34* | 1.00 | 0.00 | 0.98 | 5.69 |
| | | 7.78 | 52.77* | 1.06 | 0.00 | 50.27 | 55.27 |
| | 5.94 | 2.59 | -3.348* | 1.00 | 0.00 | -5.69 | -0.98 |
| | | 7.78 | 49.43* | 1.05 | 0.00 | 46.96 | 51.91 |
| | 7.78 | 2.59 | -52.77* | 1.06 | 0.00 | -55.27 | -50.27 |
| | | 5.94 | -49.43* | 1.05 | 0.00 | -51.91 | -46.96 |
| Time | 0 | 2 | -11.95* | 1.03 | 0.00 | -14.38 | -9.52 |
| | | 10 | -13.45* | 1.03 | 0.00 | -15.89 | -11.00 |
| | 2 | 0 | 11.95* | 1.03 | 0.00 | 9.52 | 14.38 |
| | | 10 | -1.50 | 1.03 | 0.31 | -3.92 | 0.93 |
| | 10 | 0 | 13.45* | 1.03 | 0.00 | 11.00 | 15.89 |
| | | 2 | 1.50 | 1.03 | 0.31 | -0.93 | 3.92 |

**Table 3.2: Statistical Analysis of Antioxidants Assay value from Selected Varieties**

| Source | Sum of Squares | df | Mean Square | Sig. |
|---|---|---|---|---|
| a. Total Phenolic Content | | | | |
| Corrected Model | 27044.48[a] | 53.00 | 510.27 | 0.00 |
| Intercept | 108070.45 | 1.00 | 108070.45 | 0.00 |
| Variety | 12075.60 | 2.00 | 6037.80 | 0.00 |
| Orientation | 5701.86 | 1.00 | 5701.86 | 0.00 |
| pH | 1537.97 | 2.00 | 768.99 | 0.00 |
| Time | 1497.80 | 2.00 | 748.90 | 0.00 |
| Orientation * Time | 462.69 | 2.00 | 231.34 | 0.00 |
| Variety * Orientation | 1470.65 | 2.00 | 735.33 | 0.00 |
| Orientation * pH | 259.52 | 2.00 | 129.76 | 0.00 |
| Variety * Time | 72.48 | 4.00 | 18.12 | 0.00 |
| pH * Time | 96.10 | 4.00 | 24.03 | 0.00 |
| Variety * pH | 3162.31 | 4.00 | 790.58 | 0.00 |
| Variety * Orientation * Time | 483.64 | 4.00 | 120.91 | 0.00 |
| Orientation * pH * Time | 128.04 | 4.00 | 32.01 | 0.00 |
| Variety * Orientation * pH | 1046.92 | 4.00 | 261.73 | 0.00 |
| Variety * pH * Time | 84.74 | 8.00 | 10.59 | 0.00 |
| Variety * Orientation * pH * Time | 82.91 | 8.00 | 10.36 | 0.00 |
| Error | 309.70 | 264.00 | 1.17 | |
| Total | 136263.09 | 318.00 | | |
| Corrected Total | 27354.18 | 317.00 | | |
| b. Total Flavonoid Content | | | | |
| Corrected Model | 36.29[a] | 45.00 | 0.81 | 0.00 |
| Intercept | 692.5 | 1.00 | 692.5 | 0.00 |
| Variety | 1.14 | 2.00 | 0.57 | 0.00 |
| pH | 9.87 | 1.00 | 9.87 | 0.00 |
| Time | 14.34 | 2.00 | 7.17 | 0.00 |
| Orientation * Time | 2.41 | 2.00 | 1.21 | 0.00 |
| Orientation * pH | 0.53 | 2.00 | 0.27 | 0.00 |
| pH * Time | 0.27 | 2.00 | 0.14 | 0.00 |
| Variety * pH | 2.49 | 4.00 | 0.62 | 0.00 |
| Variety * Orientation * Time | 2.37 | 4.00 | 0.59 | 0.00 |
| Orientation * pH * Time | 0.5 | 4.00 | 0.13 | 0.00 |
| Variety * Orientation * pH | 1.87 | 4.00 | 0.47 | 0.00 |
| Variety * pH * Time | 0.71 | 4.00 | 0.18 | 0.00 |
| Variety * Orientation * pH * Time | 0.5 | 8.00 | 0.06 | 0.00 |

| | | | | |
|---|---|---|---|---|
| Error | 0.96 | 201.00 | 0.01 | |
| Total | 723.53 | 247.00 | | |
| Corrected Total | 37.25 | 246.00 | | |
| c. FRAP Assay | | | | |
| Corrected Model | 58.94[a] | 53.00 | 1.11 | 0.00 |
| Intercept | 397.85 | 1.00 | 397.85 | 0.00 |
| Variety | 0.63 | 2.00 | 0.32 | 0.00 |
| Orientation | 5.00 | 1.00 | 5.00 | 0.00 |
| pH | 30.37 | 2.00 | 15.19 | 0.00 |
| Time | 5.73 | 2.00 | 2.86 | 0.00 |
| Orientation * Time | 4.81 | 2.00 | 2.40 | 0.00 |
| Variety * Orientation | 1.73 | 2.00 | 0.87 | 0.00 |
| Orientation * pH | 8.04 | 2.00 | 4.02 | 0.00 |
| Variety * Time | 0.76 | 4.00 | 0.19 | 0.00 |
| pH * Time | 2.81 | 4.00 | 0.70 | 0.00 |
| Variety * pH | 1.15 | 4.00 | 0.29 | 0.00 |
| Variety * Orientation * Time | 0.42 | 4.00 | 0.11 | 0.00 |
| Orientation * pH * Time | 2.23 | 4.00 | 0.56 | 0.00 |
| Variety * Orientation * pH | 0.86 | 4.00 | 0.21 | 0.00 |
| Variety * pH * Time | 0.42 | 8.00 | 0.05 | 0.00 |
| Variety * Orientation * pH * Time | 0.37 | 8.00 | 0.05 | 0.00 |
| Error | 0.31 | 370.00 | 0.00 | |
| Total | 439.62 | 424.00 | | |
| Corrected Total | 59.26 | 423.00 | | |
| d. Metal Ion Chelation | | | | |
| Corrected Model | 10023358.75[a] | 53.00 | 189119.98 | 0.00 |
| Intercept | 5945175.44 | 1.00 | 5945175.44 | 0.00 |
| Orientation | 4458.88 | 1.00 | 4458.88 | 0.00 |
| pH | 9998967.77 | 2.00 | 4999483.88 | 0.00 |
| Variety * Orientation | 230.81 | 2.00 | 115.41 | 0.00 |
| Orientation * pH | 491.30 | 2.00 | 245.65 | 0.00 |
| Variety * pH | 2670.29 | 4.00 | 667.57 | 0.00 |
| Variety * Orientation * Time | 151.84 | 4.00 | 37.96 | 0.00 |
| Orientation * pH * Time | 230.78 | 4.00 | 57.70 | 0.00 |
| Variety * Orientation * pH | 1044.53 | 4.00 | 261.13 | 0.00 |
| Variety * pH * Time | 755.76 | 8.00 | 94.47 | 0.00 |
| Variety * Orientation * pH * Time | 1044.69 | 8.00 | 130.59 | 0.00 |
| Error | 731.61 | 193.00 | 3.79 | |
| Total | 14703434.69 | 247.00 | | |

| | | | | |
|---|---|---|---|---|
| Corrected Total | 10024090.36 | 246.00 | | |
| e. ABTS Scavenging Effect | | | | |
| Corrected Model | 10.80[a] | 53.00 | 0.20 | 0.00 |
| Intercept | 739.80 | 1.00 | 739.80 | 0.00 |
| Variety | 3.58 | 2.00 | 1.79 | 0.00 |
| Orientation | 0.51 | 1.00 | 0.51 | 0.00 |
| pH | 0.89 | 2.00 | 0.45 | 0.00 |
| Time | 0.89 | 2.00 | 0.44 | 0.00 |
| Orientation * Time | 1.24 | 2.00 | 0.62 | 0.00 |
| Orientation * pH | 1.30 | 2.00 | 0.65 | 0.00 |
| Variety * Time | 0.10 | 4.00 | 0.03 | 0.00 |
| pH * Time | 0.33 | 4.00 | 0.08 | 0.00 |
| Variety * pH | 0.99 | 4.00 | 0.25 | 0.00 |
| Variety * Orientation * Time | 0.12 | 4.00 | 0.03 | 0.00 |
| Orientation * pH * Time | 0.32 | 4.00 | 0.08 | 0.00 |
| Variety * Orientation * pH | 0.21 | 4.00 | 0.05 | 0.00 |
| Variety * pH * Time | 0.06 | 8.00 | 0.01 | 0.00 |
| Variety * Orientation * pH * Time | 0.11 | 8.00 | 0.05 | 0.00 |
| Error | .22 | 194.00 | 0.01 | 0.00 |
| Total | 756.45 | 248.00 | 0.01 | 0.00 |
| Corrected Total | 11.02 | 247.00 | 0.00 | 0.00 |
| f. DPPH Scavenging Effect | | | | |
| Corrected Model | 164427.34[a] | 53.00 | 3102.40 | 0.00 |
| Intercept | 389616.06 | 1.00 | 389616.06 | 0.00 |
| pH | 130777.87 | 2.00 | 65388.93 | 0.00 |
| Time | 7588.13 | 2.00 | 3794.06 | 0.00 |
| Variety * Orientation | 3117.87 | 2.00 | 1558.93 | 0.00 |
| Orientation * pH | 1250.58 | 2.00 | 625.29 | 0.00 |
| Variety * Time | 781.22 | 4.00 | 195.30 | 0.00 |
| pH * Time | 4099.28 | 4.00 | 1024.82 | 0.00 |
| Variety * pH | 2325.37 | 4.00 | 581.34 | 0.00 |
| Orientation * pH * Time | 558.42 | 6.00 | 93.07 | 0.05 |
| Variety * Orientation * pH | 8337.01 | 4.00 | 2084.25 | 0.00 |
| Variety * pH * Time | 873.76 | 8.00 | 109.22 | 0.01 |
| Variety * Orientation * pH * Time | 616.38 | 12.00 | 51.37 | 0.30 |
| Error | 8424.51 | 193.00 | 43.65 | |
| Total | 618420.80 | 247.00 | | |
| Corrected Total | 172851.86 | 246.00 | | |

### 3.2. Total Phenolic Content

Phenolic compounds are complex plant derivatives contributing to plant foods' flavor, color, and antioxidants (Cai et al., 2004). The assay for determining total phenolic content in food products was based on the oxidation of phenolic compounds in the reaction with phosphomolybdate and phosphotungstate mixture. The total phenolic content in whole and crushed yellow mustard seeds had the highest phenolic content, which was 2040.32 ± 360.12 and 1692.96 ± 29.78 mg/g DW, respectively. According to **Table 1a**, the whole and crush ed seeds produced a relatively high amount of total phenolic content, particularly during neutral treatment. However, except for yellow mustard seed, less phenolic content was found in whole seed than in crushed seed. This can be explained by the fact that crushed seed provided more surface area with the extracting solvent than the whole seed.

Moreover, some of the plant cell walls were broken in the crushed seed compared to the whole seed, enabling the phenolics to exude out and not be trapped inside the seed. With the exception of yellow mustard seed, the variation in phenolic content between the pre-heat treatment time was very limited in the other mustard varieties. However, the extraction of phenols increased over time. Of the different pH conditions examined, the highest phenolic content was obtained under neutral conditions compared to far less under acidic conditions, with the lowest amount under alkaline buffer conditions (**Table 1a).** The phenolic content of yellow mustard seed reached its peak, 2040.32 ± 360.12 mg/g and 1692.96 ± 29.78 mg/g dry weight basis, when extracted with modified water. The data above indicates the results obtained from whole and crushed seeds, respectively. According to the statistical results, all the applied factors and their combinations significantly affected the extraction of phenolic compounds from the different mustard varieties examined.

Without the use of temperature and pressure, a much lower amount of phenolic content of 36.5 mg/g and 125 mg/g were reported by both Matthäus, (2002) and Lee et al., (2015) for yellow and black mustard, respectively. Of all the extraction solvents used, water proved to be the most effective. This was consistent with the study by Matthäus, (2002), who reported that the total phenolic content of yellow mustard extracted with methanol, acetone, water and ethyl acetate was 17.6, 17.6, 36.5, 9.2 mg/g, respectively. A later study by Lee et al. (2015) also investigated the extraction of phenolic compounds from black mustard seed using dichloromethane, ethanol, methanol, hexane and ethanol-acetone mixture as extractants. The highest amount was also obtained using water which yielded 125 mg/g fresh weight of powder. The effect of solvent pH on phenolics was investigated with Henna leaves (*Lawsonia inermis*) by Uma et al., (2010). In their research, the leaves were extracted with different concentrations of acetone, representing different solvent polarities. Optimum conditions were reported at an acetone concentration of 48.07% with a maximum yield of total phenolics of 72.03 mg/g DW. A parallel study by Chen et al., (2008) reported that the highest phenolic content ≈200 µg/g fresh weight, was obtained from various yam cultivars was the pH was around 5.

### 3.3.Total flavonoid content

Flavonoids belong to a group of natural substances and occupy almost half of the existence of phenolic compounds in plants (Ignat et al., 2011; Pérez-Gregorio et al., 2014). They have been well-known for their health benefits, including antioxidants, anti-inflammatory, antimicrobial, and anti-aging activity (Quecan et al., 2019; Sharma et al., 2018; Tiwari & Cummins, 2013) through their protective effects against oxidizing agents and free radicals (Assefa et al., 2018; Russo et al., 2012; Wang et al., 2018). The total flavonoid content (TFC) showed a different trend compared to phenolic content **(Table 1b)**. Under acid and neutral treatment, the flavonoid content did not seem

to vary much, especially for the whole mustard seeds. However, yellow mustard seeds were higher in flavonoid content than the other mustard varieties examined, reaching 39.2 ± 1.28 mg/g and 98.47 ± 9.51 mg/g dry weight basis for whole and crushed seeds, respectively. On the other hand, under buffer conditions, a much higher amount of flavonoid was observed, especially in black mustard seed. The amount reached 275.80 ± 16.63 mg/g QE, which was five-fold higher compared to the corresponding acid and neutral treatments. Some research had been carried out on the effect of pH on the variation of flavonoid antioxidant activity. Using a computer-based method for examining the deprotonation energies, Lemańska et al., (2001) found that increased pH significantly improved the antioxidant activity of the flavonoids, including quercetin. This study confirmed the influence of protonation on antioxidant activity by lowering the ionization potential (IP) rather than bonding dissociation energies (BDE), therefore, providing a more accessible electron donation for flavonoid compounds containing C3-OH, C5-OH and C4=O. Normally, for 3- and 5- hydroflavone, the strong hydrogen bond with oxygen atoms from C4=O group may inhibit their deprotonationation and radical scavenging activity. However, when increasing pH, the radical cations shift from C3- and/or C5- hydroxyl group to C4= carbonyl group, resulting in more stable cations. This would decrease the calculated IP, and promote electron donation for flavonoid molecules containing C3-OH, C5-OH and C4=O, thus favorable for antioxidants action (Lemańska et al., 2001). In his research, Matthäus, (2002) also investigated the effect of solvent on the total flavonoid content of yellow mustard extracts. However, without the pressure and temperature, the amount of flavonoid obtained (0.07mg/g) was much lower when compared with no pressure and temperature application.

Moreover, the orientation of the seed definitely affected the amount of flavonoids extracted. For instance, the amount of flavonoid recovered from the whole seed is significantly

lower than the crushed one. The impact of the buffer treatment was particularly evident for the Oriental mustard variety. However, in the case of black mustard, the flavonoid content of crushed seed showed little variation between the pretreatment times. A sharp increase in flavonoid content for the whole seeds was observed, however, when the time for heat exposure was increased. The level of flavonoids at 10 minutes sauté time was even higher than in the seed crushed under the same condition. For acid and neutral treatment, preheating time did not significantly affect the samples. The same trend was evident for all the crushed seed varieties, but not for the whole seeds. The flavonoid content increased with increased sautéing time. With the exception of the combination of variety*time and variety*orientation, all the other applied factors significantly increased the extraction of the total flavonoids.

**Table 3.3: Total Phenolic Content and Total Flavonoid Content of Selected Mustard Varieties**

| | | Whole seed | | | Crushed seed | | |
|---|---|---|---|---|---|---|---|
| | | Oriental | Black | Yellow | Black | Oriental | Yellow |
| | | a. Total Phenolic Content (mg/g DW) | | | | | |
| Acid Treatment | 0 min | 215.52 ± 35.86 | 62.97 ± 18.77 | 342.42 ± 54 | 98.14 ± 18.67 | 50.19 ± 14.42 | 332.51 ± 43.71 |
| | 2 min | 296.09 ± 31 | 184.99 ± 9.64 | 611.55 ± 27.71 | 161.74 ± 28.9 | 153.38 ± 63.03 | 572.03 ± 64.76 |
| | 10 min | 255.47 ± 24.68 | 255.15 ± 40.39 | 887.34 ± 33.8 | 257.58 ± 133.77 | 149.26 ± 22.54 | 835.41 ± 52.14 |
| Neutral Treatment | 0 min | 95.74 ± 23.46 | 163.71 ± 12.36 | 2040.32 ± 360.12 | 192.15 ± 49.92 | 307.68 ± 45.93 | 1498.88 ± 66.09 |
| | 2 min | 172.69 ± 29.27 | 307.99 ± 15.61 | 1318.04 ± 924.34 | 353.41 ± 62.79 | 300.42 ± 22.19 | 1692.96 ± 29.78 |
| | 10 min | 348.49 ± 247.52 | 434.31 ± 19.77 | 1603.08 ± 136.65 | 291.21 ± 48.56 | 476.85 ± 80.01 | 1414.23 ± 120.53 |
| Buffer Treatment | 0 min | 11.62 ± 0.9 | 39.5 ± 3.27 | 42.32 ± 6.06 | 301.95 ± 14.33 | 404.04 ± 42.83 | 583.73 ± 41.33 |
| | 2 min | 36.73 ± 3.9 | 135.94 ± 15.23 | 133.43 ± 13.77 | 322.44 ± 19.67 | 434.8 ± 34.96 | 660.07 ± 43.87 |
| | 10 min | 97.16 ± 12.36 | 319.36 ± 19.3 | 541.9 ± 46.8 | 342.51 ± 10.92 | 560.96 ± 26.56 | 602.89 ± 34.42 |
| | | b. Total Flavonoid Content (mg/g DW) | | | | | |
| Acid Treatment | 0 min | 19.7 ± 2.46 | 11.58 ± 3.7 | 13.59 ± 1.34 | 21.98 ± 2.48 | 51.25 ± 6.94 | 51.25 ± 6.94 |
| | 2 min | 22.55 ± 1.46 | 20.17 ± 1.14 | 19.46 ± 3.21 | 68.74 ± 8.48 | 47.28 ± 3.34 | 47.28 ± 3.34 |
| | 10 min | 20.55 ± 3.11 | 23.61 ± 1.32 | 68.85 ± 2.8 | 53.05 ± 7.57 | 49.69 ± 3.53 | 49.69 ± 3.53 |
| Neutral Treatment | 0 min | 20.67 ± 2.33 | 30.04 ± 1.64 | 67.45 ± 8.2 | 56.59 ± 4.37 | 98.47 ± 9.51 | 98.47 ± 9.51 |
| | 2 min | 17.83 ± 1.2 | 18.94 ± 2.66 | 56.88 ± 9.87 | 48.32 ± 6.45 | 97.23 ± 7.78 | 97.23 ± 7.78 |
| | 10 min | 17.14 ± 2.31 | 17.05 ± 1.66 | 54.26 ± 5.21 | 54.14 ± 5.2 | 92.69 ± 9.21 | 92.69 ± 9.21 |
| Buffer Treatment | 0 min | 13.4 ± 2.04 | 45.28 ± 5.96 | 138.38 ± 10.25 | 258.97 ± 8.41 | 121.93 ± 7.59 | 121.93 ± 7.59 |
| | 2 min | 45.71 ± 5.2 | 121.28 ± 10.83 | 145.35 ± 9.3 | 263.1 ± 10.28 | 134.93 ± 3.91 | 134.93 ± 3.91 |
| | 10 min | 91.5 ± 12.43 | 314.52 ± 23.57 | 180.33 ± 6.53 | 275.8 ± 16.63 | 164.91 ± 2.17 | 164.91 ± 2.17 |

0 min: 0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking.

### 3.4.Ferric Reducing/Antioxidant Power Assay (FRAP assay)

Parallel with the total flavonoid content, the FRAP assay also showed a positive increase with buffer treatment **(Table 2a)**. Treatment under the first two pH conditions showed only a modest difference for each factor examined, whereas acid treatment was slightly higher than under neutral conditions. A significantly higher value was observed in the extracts under buffer extraction conditions. Black mustard seed extracts obtained after 10 minutes of sauté time and alkaline wet extraction had the highest FRAP value of 92.77 ± 1.38 mg/g TE. For both Oriental mustards, the FRAP value increased with the increase in time, but the variation with crushed mustard was not as distinct as whole seed counterpart. The value for yellow mustard was highest with the whole seed, resulting in 1.45 ± 0.05 mg/g TE, 9.79 ± 0.38 mg/g TE, 71.21 ± 0.87 mg/g TE, with increasing sauté time of 0, 2, and 10 minutes, respectively. A similar trend was also observed for Oriental and black mustard whole seeds. Besides the pH, preheat treatment also had a significant impact on the selected mustard varieties. While this trend was relatively flat for the acid and neutral treatment, it intensified with the buffer treatment. The data showed that with the increase in time, the ability to reduce ferric (III) to ferrous (II) ions also increased. All the factors, including variety, orientation, time, pH and their combinations significantly impacted the dependent variables, particularly the FRAP value.

A study by Apenten (2015) evaluated different pH conditions on the FRAP assay. They showed that changes in pH caused a variation in antioxidant activity, in which the lower pH conditions lowered the redox potential. In this study, the FRAP value of ascorbic acid and gallic acid under pH=4 condition is 1.8 and 3.0, while an increase in pH can double the FRAP value to 3.4 and 7.02, respectively. According to Nernst equation $Eh = E^o \pm (0.059/n)$ pH, redox potential is pH-dependent, in which the redox potential changes by 59 mV for each unit pH change with a

one-electron reduction (n=1). $E^o$ is the standard redox potential when pH = 0, and Eh is the redox potential at any pH (Schafer & Buettner, 2001). Therefore, protonation at low pH also increases the Eh and decreases the antioxidant activity (Apenten, 2005).

### 3.5.Ferrous-ion-chelating activity assay (Metal Ion Chelation assay)

The metal chelation ion value for the acid and neutral treatments was much lower than the buffer treatment. The data belonging to these treatments were presented in **Table 2b**. Ferrous ion chelating activity under different pH conditions was examined by Hrdina & Macakova, (2011). These researchers claimed that, at a pH between 6.8-7.5, many phenolic compounds could be considered potent ion chelators (the ability to chelate $Fe^{2+}$ could approximately reach 100%) as long as they form stable complexes with iron. Phenolic compounds with 3-hydroxy-4-keto groups can form a complex with one iron atom under both acidic and neutral conditions. Those phenols with a catechol B ring, however, are unable to chelate iron under acidic conditions. Under neutral conditions, phenols with 2 catecholic B rings can bind to one iron atom and form a complex. At pH 5.5 and lower, very few phenolic compounds showed the same potential, which explains the results in this assay. According to the statistical analysis, differentiation between time, variety, and orientation were not significantly different. In addition to the insignificant effect of variety and time, the combination between orientation*time, variety*time, and pH*time had no major impact on the mustard. Consequently, these factors were excluded from the model. In conclusion, the pH value was the only factor relevant for consideration for this type of antioxidant assay. As a consequence of the electrostatic effect on its mechanism, the metal ion chelation assay proved to be an unsuitable evaluation method.

**Table 3.4: FRAP value and Metal Ion Chelation value of Selected Mustard Varieties**

| | | Whole seed | | | Crushed seed | | |
|---|---|---|---|---|---|---|---|
| | | Oriental | Black | Yellow | Black | Oriental | Yellow |
| | | a. FRAP value (mg/g DW) | | | | | |
| Acid Treatment | 0 min | 9.86 ± 0.38 | 5.82 ± 0.29 | 6.1 ± 0.79 | 9.21 ± 0.38 | 9.75 ± 0.72 | 9.75 ± 0.72 |
| | 2 min | 14.16 ± 0.3 | 8.84 ± 0.23 | 8.49 ± 0.29 | 7.66 ± 0.47 | 9.24 ± 0.17 | 9.24 ± 0.17 |
| | 10 min | 11.45 ± 0.38 | 9.5 ± 0.91 | 6.89 ± 0.49 | 6.59 ± 0.47 | 9.72 ± 0.62 | 9.72 ± 0.62 |
| Neutral Treatment | 0 min | 6.74 ± 0.34 | 3.87 ± 0.29 | 5.75 ± 0.48 | 5.66 ± 0.43 | 5.57 ± 0.28 | 5.57 ± 0.28 |
| | 2 min | 9.59 ± 0.83 | 6.81 ± 0.29 | 4.68 ± 0.3 | 4.7 ± 0.38 | 6.83 ± 0.26 | 6.83 ± 0.26 |
| | 10 min | 9.76 ± 0.58 | 7.79 ± 0.18 | 5.49 ± 0.28 | 6.06 ± 0.3 | 7.61 ± 0.21 | 7.61 ± 0.21 |
| Buffer Treatment | 0 min | 4.78 ± 0.41 | 4.17 ± 0.28 | 72.09 ± 1.64 | 75.88 ± 1.06 | 56.89 ± 0.7 | 56.89 ± 0.7 |
| | 2 min | 13.16 ± 1.47 | 33.02 ± 1.61 | 64.76 ± 1.13 | 83.81 ± 5.68 | 71.85 ± 0.84 | 71.85 ± 0.84 |
| | 10 min | 38.71 ± 1.34 | 58.58 ± 2.28 | 68.78 ± 0.54 | 92.77 ± 1.38 | 77.19 ± 1.14 | 77.19 ± 1.14 |
| | | b. Metal Ion Chelation value | | | | | |
| Acid Treatment | 0 min | 2.6 ± 0.42 | 5.12 ± 0.25 | 3.59 ± 0.41 | 4.7 ± 0.26 | 3.5 ± 0.29 | 11.62 ± 0.53 |
| | 2 min | 1.58 ± 0.17 | 4.29 ± 0.14 | 2.69 ± 0.31 | 2.72 ± 0.35 | 10.66 ± 3.29 | 14.22 ± 0.44 |
| | 10 min | 2.07 ± 0.33 | 2.99 ± 0.24 | 2.45 ± 0.27 | 6.54 ± 0.94 | 11.87 ± 1.82 | 7.02 ± 1.17 |
| Neutral Treatment | 0 min | 4.8 ± 0.33 | 6.18 ± 0.34 | 12.95 ± 0.42 | 14.28 ± 1.07 | 15.22 ± 1.93 | 12.5 ± 0.76 |
| | 2 min | 3.28 ± 0.27 | 4.86 ± 0.54 | 11.49 ± 0.22 | 13.95 ± 1.92 | 16.85 ± 1.22 | 14.59 ± 0.34 |
| | 10 min | 3.18 ± 0.46 | 5.39 ± 0.64 | 10.63 ± 0.49 | 16.17 ± 0.91 | 20.32 ± 1.51 | 14.93 ± 0.37 |
| Buffer Treatment | 0 min | 457.52 ± 4.87 | 440.81 ± 0.72 | 432.65 ± 1.24 | 455.49 ± 1.44 | 464.93 ± 1.15 | 454.21 ± 1.19 |
| | 2 min | 457.07 ± 3.23 | 446.23 ± 3.82 | 443.25 ± 9.1 | 461.79 ± 2.54 | 459.58 ± 1.35 | 447.7 ± 0.9 |
| | 10 min | 457.71 ± 6.34 | 440.41 ± 2.38 | 433.19 ± 0.99 | 466.32 ± 1.62 | 445.06 ± 1.04 | 463.07 ± 1.27 |

0 min: 0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking

### 3.6.ABTS$^{\bullet+}$ and DPPH Scavenging Assay

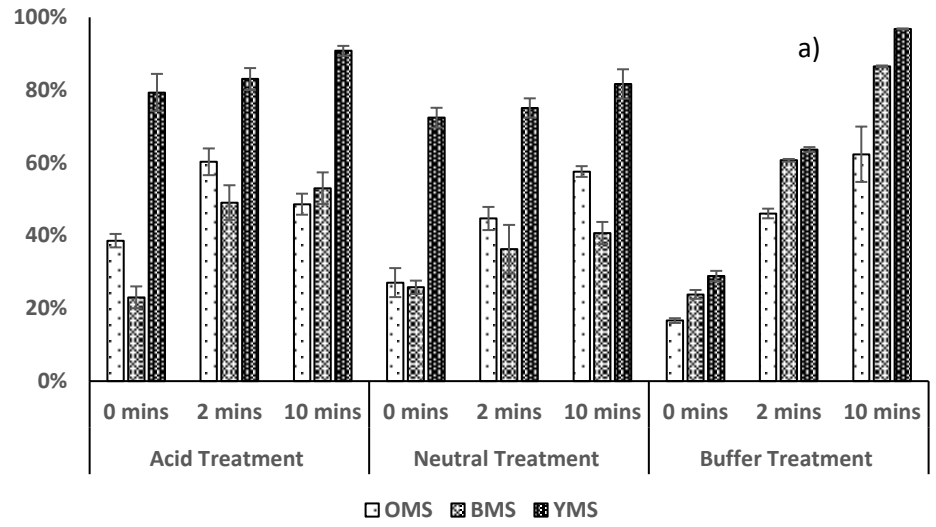
This method was based on the principle of a colorimetric method to evaluate the decay of ABTS•+ in the presence of an antioxidant agent. In plant foods containing hydrophilic, lipophilic, and high-pigmented antioxidant compounds, the ABTS assay is superior to DPPH. Dudonné et al., (2009) and Samaniego Sánchez et al., (2007) established a correlation between ABTS assays, and total phenolic content is $R^2$ = 0.966, 0.9905 while for DPPH it was lower at 0.939, 0.7387 when testing with oil and plant extract. Solvents have also been shown to influence antioxidant tests but in different ways. ABTS and DPPH assays are the most widely used methods for determining the antioxidant activity of phenolic compounds. ABTS values increase with increasing polarity of the solvent (Pérez-Jiménez & Saura-Calixto, 2006), while the use of solvents with high polarity gives lower DPPH values (Molyneux, 2004).

Yellow mustard seeds exhibited outstanding scavenging effects compared to other varieties, with slightly higher values than the crushed seed. This is evident by the scavenging activity of whole and crushed yellow mustard seeds being 96.81 ± 0.13% and 98.45 ± 0.19%, respectively. The variation between the samples was more clearly observed with the whole seeds than with the crushed seeds. Although there was a slight difference between the two orientations, they were insignificant. Based on statistical analysis, the examined factors and their combinations on the ABTS scavenging effect of mustard extracts were significant, except for orientation and variety. ABTS scavenging effect significantly increased ($p<0.05$) over time, although this trend was more evident with the whole seed than the crushed seed. Oriental mustard and Black mustard seeds did not demonstrate greater than 60% scavenging activities **(Figure 2)**. On the other hand, alkaline conditions impact the ability of radical clearance by the mustard extracts in a different way than other solvents. Under the buffer conditions, the crushed seed extracts demonstrated a

better scavenging ability compared to the whole seeds. With all the tested varieties and preheat treatment times, the ABTS scavenging effect was close to reaching 100%. Meanwhile, for the whole seed, there was a time-dependent trend, in which the longer the pre-treatment time, the higher the antioxidant activity. This ability increased with the whole seeds ranging in decreasing order from Oriental > Black > Yellow mustard seed. For the pH treatments, there were slight changes between orientations with the radical clearance activity of whole seed extracts increasing with pre-treatment time while a slight decrease was observed for the crushed seed extracts.

DPPH assay, on the other hand, is a colorimetric method based on measuring the scavenging capacity of antioxidants towards DPPH• radicals. Unlike the ABTS, DPPH had more promising results with acidic and neutral conditions compared to the buffer treatment **(Figure 3)**. The highest scavenging ability was observed for yellow mustard seeds when subjected to wet, acidic extraction, reaching 87.58% in value. Acidic conditions proved more favorable for antioxidant activity, as the lowest result was 29.98 ± 1.85%, and the highest was 87.58 ± 1.83%. Meanwhile, the value under neutral conditions was 17.05 ± 4.30% and 73.93 ± 1.65%, respectively. Based on statistical analysis, the differentiation between the variety and orientation were not significantly different. Besides the lack of significance for variety and orientation, the combination between orientation*time, variety*orientation*time and variety*orientation*time*pH also had no compelling effect on the mustard. Under acidic conditions, however, the whole seeds had the highest DPPH scavenging effect, which was most pronounced in yellow mustard. This trend was not evident with the latter orientation. The highest effect obtained from this orientation was under neutral treatments. In this treatment, crushed yellow mustard seeds significantly increased the free radical clearance activity compared to the whole seeds. In sharp contrast, alkaline pH wet extraction appeared to have a detrimental impact

on the scavenging activity of the bioactive compounds present in the mustard extract. Under this treatment, the scavenging ability of mustard could not reach 10%. Bioactive compounds can react with radicals in four main pathways: Proton Coupled-Electron Transfer (PC-ET), Electron Transfer-Proton Transfer (ET-PT), Sequential Proton Loss Electron Transfer (SPLET), and Adduct Formation (AF) (Litwinienko & Mulder, 2009; Zhang & Ji, 2006). The reaction between antioxidants, especially phenolic compounds, and DPPH radicals followed PC-ET and SPLET mechanisms. PC-ET was slower and more favorable with non-polar, low dielectric constant, and low basicity solvents. Meanwhile, the SPLET mechanism was faster and followed the solvents with dielectric constants that were highly basic by encouraging ionization (Friaa & Brault, 2006; Litwinienko & Mulder, 2009) and was dominant in methanol with a dielectric constant $\varepsilon = 33$. Another aspect of hydrogen ion donation activity was reviewed in Dawidowicz & Olszowy, (2012), in which increased hydrogen ions led to PC-ET concentration. Regarding this study, acid treatment used an aqueous solution of acetic acid, which existed in the form of $COO^-$ and $H^+$. Besides, the application of methanol as a delivery system for the DPPH radical, the acid treatment reacted with both mechanisms with a greater ability to scavenge radicals, while a reduced ability was evident following modified water and buffer treatment.



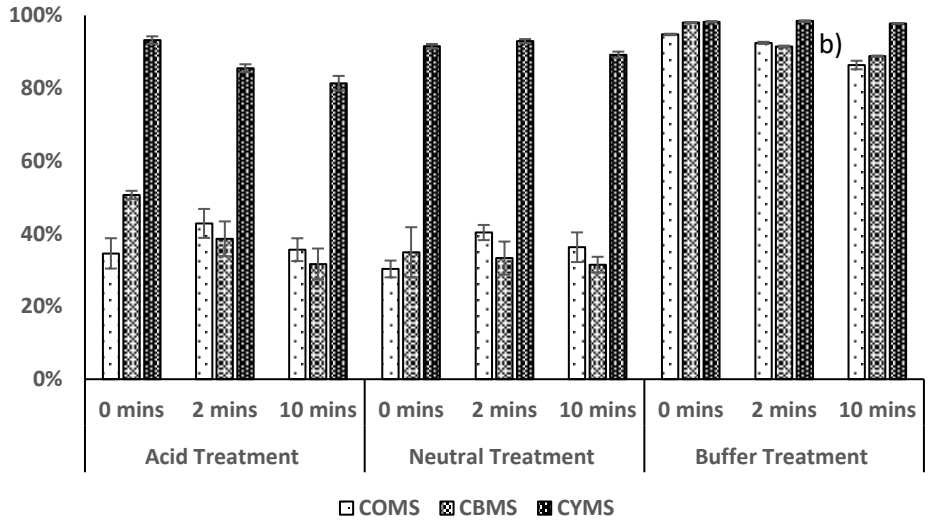




**Figure 3.2: a)** ABTS radical scavenging of Selected Varieties of Mustard Seed with three pH treatments; OMS: Oriental Mustad Seed; BMS: Black Mustard Seed; YMS: Yellow Mustard Seed

**b)** ABTS radical scavenging of Selected Varieties of Mustard Seed with three pH treatments;COMS: Crushed Oriental Mustard Seed; CBMS: Crushed Black Mustard Seed; CYMS: Crushed Yellow Mustard Seed

0 min: 0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking

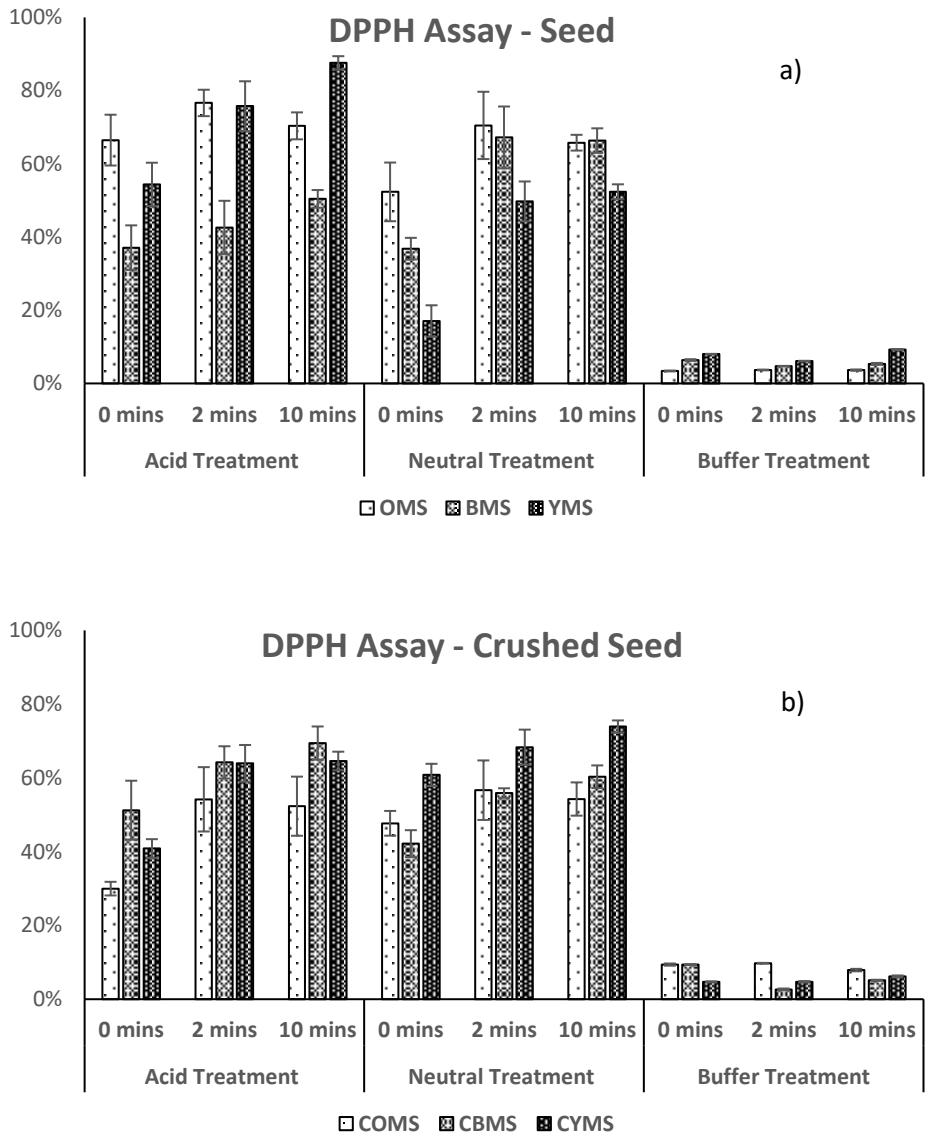



**Figure 3.3: a)** DPPH radical scavenging value of Selected Varieties of Mustard Seed with three pH treatments; OMS: Oriental Mustad Seed; BMS: Black Mustard Seed; YMS: Yellow Mustard Seed

**b)** DPPH radical scavenging value of Selected Varieties of Crushed Mustard Seed with three pH treatments; COMS: Crushed Oriental Mustard Seed; CBMS: Crushed Black Mustard Seed; CYMS: Crushed Yellow Mustard Seed

0 min: 0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking

### 3.7.HPLC-DAD Analysis

The conditions that gave the highest total phenolic content (2-minute sauté time with pressurized cooking) were designated for HPLC Analysis. The results from this experiment were presented in **Table 5**. The HPLC chromatography of these samples was presented in **Figure S5** and **S6** (Supplement Information). According to **Table 5,** Yellow Mustard seed had the significantly highest amount of sinapine, sinapic acid, and canolol among the whole seed**s**, 123476.2 ± 22014.60; 817.8 ± 183.83; and 2320.72 ± 137.83 µg/g DW, respectively, which was equivalent to the total phenolic results. However, the corresponding crushed seed demonstrated a more varied sinapic acid and canolol content. With respect to the crushed samples, Yellow mustard still prevailed in sinapine content (384992.79 ± 49470.66 µg/g DW) ($p<0.05$). However, because of the cell wall breaking, more sinapine was recovered from Oriental and black varieties. In the case of Oriental mustard, the sinapine concentration was 5.4 times higher in the crushed seeds compared to the whole seeds yielding 33319.36 ± 3458.95 and 180590 ± 26661.42 µg/g DW, respectively. The same trend was observed for black mustard, with the sinapine content increasing from 11051.51 ± 1704.21 to 172502.00 ± 20191.37 µg/g DW. On the other hand, for canolol, yellow mustard appeared to produce significantly ($p<0.05$) less sinapic acid decarboxylation in both the whole and crushed seeds compared to the other varieties. The canolol concentration for this variety decreased from 2320.72 ± 137.83 to 945.8 ± 105.51 µg/g DW. This was attributed to canolol being a thermal-sensitive compound. On the other hand, the cell wall of black and Oriental mustards appeared to preserve canolol, and breaking it facilitated decarboxylation. As a result, the canolol content of Oriental and black mustards increased from 229.19 ± 36.06 and 168.87 ± 24.87 µg/g DW in the whole seed to 1580.53 ± 246.35 and 1857.62 ± 204.43 µg/g DW in the crushed seeds. Both yellow and black mustards had a comparative level of sinapic acid. The amount of

sinapic acid detected in all three mustard varieties increased when the whole seeds were crushed. The concentration of sinapic acid from whole and crushed Oriental, black and yellow mustard was 317. 04 ± 21.76, 1370.88 ± 187.69, 235.09 ± 29.17, 1838.97 ± 185.8, and 817.8 ±183.83, 1705.85 ± 129.6 µg/g DW, respectively. Shrestha et al., (2012) also examined black mustard (*Brassica nigra*), Oriental mustard (*Brassica juncea*), and yellow mustard (*Sinapis alba*) seeds using different preheat treatments and water as extract solvents. The amounts of sinapic acid from roasted seed and roasted powder of black, Oriental, and yellow mustard were 56.90 ± 1.52, 58.52 ± 0.92, 26.50 ± 0.72, 35.97 ± 1.25, 29.58 ± 1.46, 23.10 ± 0.64 µg/g of dry weight, respectively. Another study on mustard seed by Fadairo et al., (2021) showed that the highest canolol content obtained was 1086.08 ± 12.62 (µg CE/100 g) after roasting at 170ºC for 10 minutes.

**Table 3.5: Major sinapates content of Selected Mustard Varieties under 2-minute of sauted time and neutral treated**

| | Neutral treatment – 2-minute sauted time | | |
|---|---|---|---|
| | Sinapine (µg/g) | Sinapic Acid (µg/g) | Canolol (µg/g) |
| OMS | 33319.36 ± 3458.95[a] | 317.04 ± 21.76[a] | 229.19 ± 36.06[a] |
| BMS | 11051.51 ± 1704.21[a] | 235.09 ± 29.17[a] | 168.87 ± 24.87[a] |
| YMS | 123476.2 ± 22014.6[b] | 817.8 ± 183.83[b] | 2320.72 ± 137.83[b] |
| COMS | 180590.24 ± 26661.42[c] | 1370.88 ± 187.69[c] | 1580.53 ± 246.35[c] |
| CBMS | 172502. ± 20191.37[c] | 1838.97 ± 185.8[d] | 1857.62 ± 204.43[d] |
| CYMS | 384992.79 ± 49470.66[d] | 1705.85 ± 129.6[d] | 945.8 ± 105.51[e] |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
OMS: Oriental Mustad Seed; BMS: Black Mustard Seed; YMS: Yellow Mustard Seed; COMS: Crushed Oriental Mustard Seed; CBMS: Crushed Black Mustard Seed; CYMS: Crushed Yellow Mustard Seed

**k**

## 4. Conclusion

Yellow mustard seeds treated with a water-based medium under neutral conditions yielded the largest amount of total phenols compared to the other mustard varieties. These phenolics are effective antioxidants with variable responses to the different antioxidant assays, particularly those methods based on radical scavenging. The metal chelation ion and FRAP assays can be altered by pH, as the main principle of these methods is based on ion interactions. The highest FRAP and metal ion chelating activities were observed for yellow mustard using an alkaline wet extraction method. Black mustard seeds had the highest results for total flavonoid content compared to the other mustard varieties, particularly when exposed to buffer conditions. The orientation and sauté time also affected the antioxidant activity of the mustard extract. In the case of whole mustard seeds, most of the samples exhibited increased antioxidant activity with longer temperature exposure. There are no plant cell walls to break with the crushed seeds, so the temperature could easily interact with mustard phenolic. The longer the exposure, however, the more phenols were

degraded. Consequently, the sauté*time factors were not necessary for producing phenols from crushed seed extracts.

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## 6. Author Contributions

TN and RN designed the study. TN performed the experiments under the guidance of both RN and ME, interpreted the results and drafted the manuscript. ME, RN, and OF proof-read the manuscript. Funding was acquiesced by UTH.

## Conflicts of Interest

There is no conflicts of interest.

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**Supplemental Information**

**Table S 3.1: Changes in Oil Content, Moisture Content, and pH of selected variety of Mustard**

| | | | | Whole seed | | | Crushed seed | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Oriental | Black | Yellow | Black | Oriental | Yellow |
| Oil Content | | | | 0.64% | 0.34% | 1.10% | 12.93% | 14.26% | 6.23% |
| Moisture Content | | | | 96.27% | 95.82% | 96.61% | 94.22% | 93.9% | 92.68% |
| pH | Acid Treatment | Before | | 2.59 | | | | | |
| | | After | 0 min | 3.62 ± 0.01 | 3.45 ± 0.02 | 3.51 ± 0.04 | 3.62 ± 0.02 | 3.55 ± 0.01 | 3.46 ± 0.02 |
| | | | 2 min | 3.38 ± 0.02 | 3.42 ± 0.05 | 3.47 ± 0.03 | 3.42 ± 0.01 | 3.54 ± 0.02 | 3.48 ± 0.06 |
| | | | 10 min | 3.29 ± 0.01 | 3.34 ± 0.05 | 3.33 ± 0.07 | 3.28 ± 0.04 | 3.37 ± 0.02 | 3.4 ± 0.05 |
| | Neutral Treatment | Before | | 5.94 | | | | | |
| | | After | 0 min | 5.24 ± 0.06 | 5.39 ± 0.08 | 5.78 ± 0.17 | 5.2 ± 0.06 | 5.33 ± 0.03 | 4.84 ± 0.05 |
| | | | 2 min | 4.23 ± 0.04 | 4.86 ± 0.1 | 4.7 ± 0.27 | 4.68 ± 0.05 | 4.99 ± 0.06 | 5.01 ± 0.01 |
| | | | 10 min | 3.94 ± 0.07 | 4.51 ± 0.02 | 4.35 ± 0.05 | 4.26 ± 0.05 | 4.53 ± 0.09 | 4.54 ± 0.06 |
| | Buffer Treatment | Before | | 7.78 | | | | | |
| | | After | 0 min | 8.94 ± 0.02 | 9.14 ± 0.02 | 9.02 ± 0.03 | 9.23 ± 0.02 | 9.34 ± 0.03 | 9.28 ± 0.03 |
| | | | 2 min | 9.03 ± 0.03 | 9.05 ± 0.01 | 9.04 ± 0.03 | 9.11 ± 0.03 | 9.05 ± 0.03 | 9.02 ± 0.03 |
| | | | 10 min | 9.1 ± 0.01 | 9.14 ± 0.01 | 9.02 ± 0.03 | 9.06 ± 0.01 | 9.02 ± 0.02 | 9.04 ± 0.02 |

0 minute:0-minute sauté time with pressurized cooking; 2 min: 2-minute sauté time with pressurized cooking; 10 min:10-minute sauté time with pressurized cooking

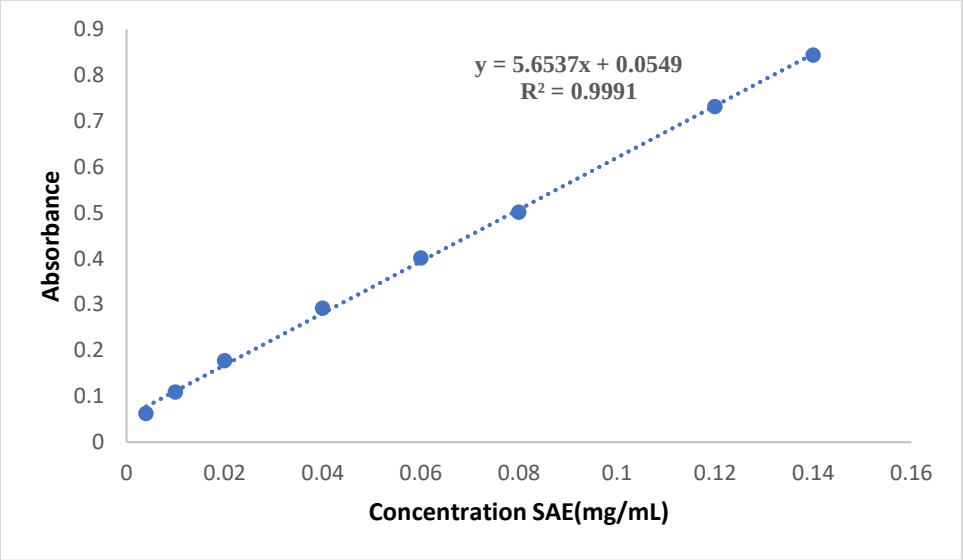


**Figure S 3.1:** Total Phenolic Content Standard Curve uisng Sinapic Acid Solution (1mM) as Standard

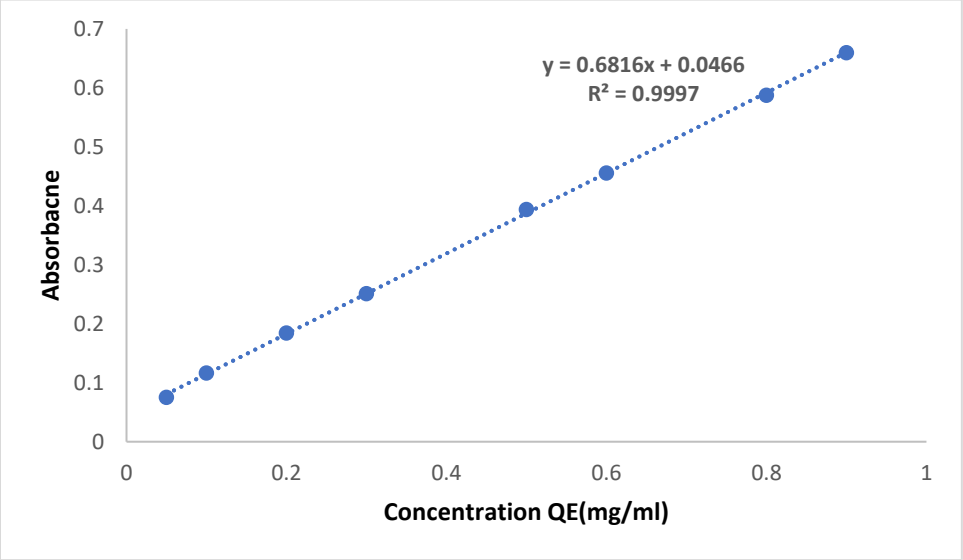


**Figure S 3.2:** Total Flavonoid Content Standard Curve uisng Quercetin solution (1mM) as Standard

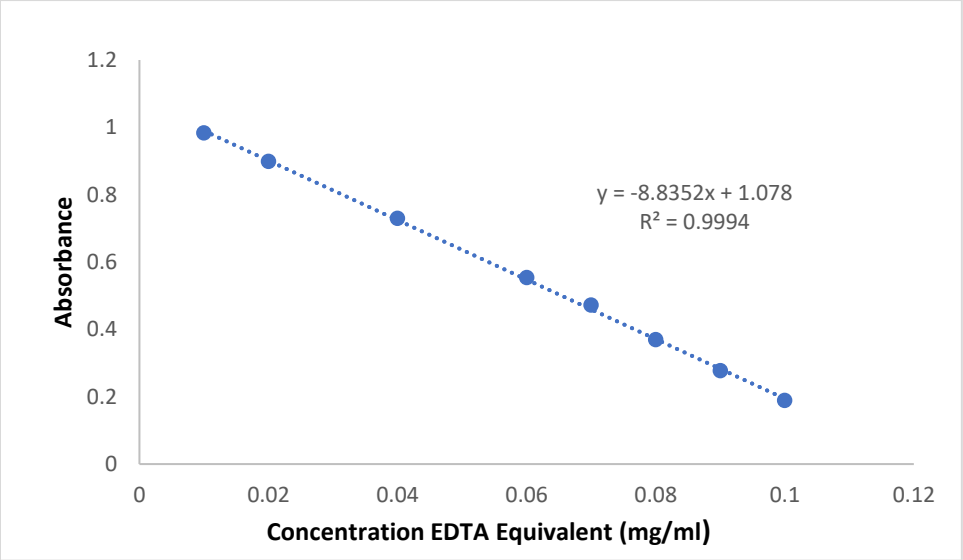


**Figure S 3.3:** Ferrous-ion-chelating activity assay standard curve using $Na_2EDTA$ as standard

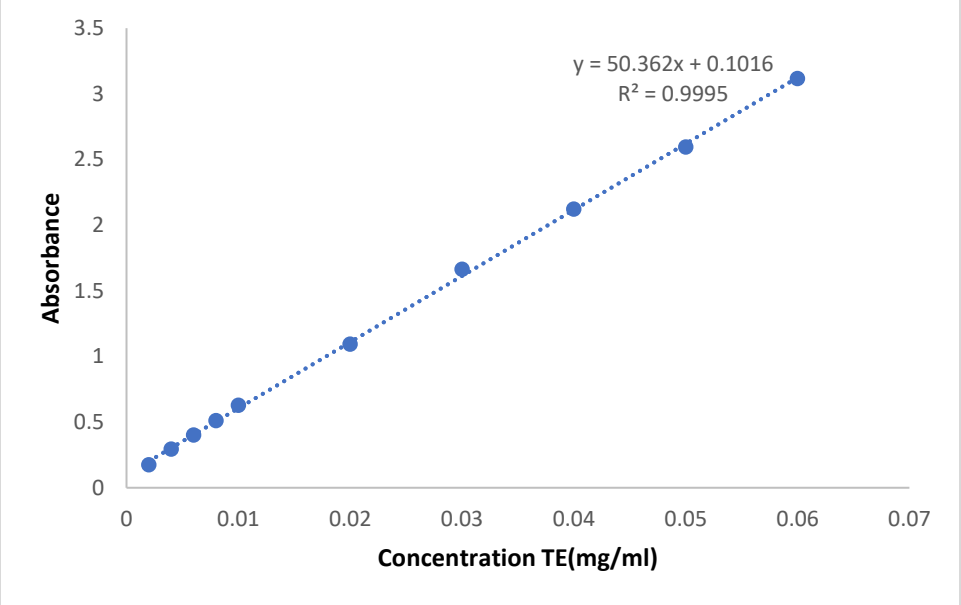


**Figure S 3.4:** FRAP assay standard curve using Trolox as standard**.**

**Figure S 3.5:** HPLC Chromatography of major sinapates content from Selected Mustard Varieties under 2-minute of sauted time and neutral treated. (Oriental: black line; Black: blue line; Yellow: pink line)

**Figure S 3.6:** HPLC Chromatography of major sinapates content from Selected Mustard Varieties (Crushed Seed) under 2-minute of sauted time and neutral treated. (Oriental: black line; Black: blue line; Yellow: pink line**)**

# CHAPTER 4

## Mustard's Sinapates: Potential Tyrosinase Inhibitors

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**Abstract**

Melanin pigmentation protects the skin from harmful effects including ultraviolet radiation. In general, two types of melanin pigments are produced in the skin, eumelanin and pheomelanin. While eumelanin is generally photo-protective, pheomelanin is considered photo-toxic and capable of producing melanoma, a deadly type of skin cancer. It was reported that melanogenesis could be prevented by inhibiting tyrosinase activity. Research has been carried out for anti-tyrosinase compounds, and sinapates have been reported as promising candidates due to their excellent metal chelators activity. In the current study, the sinapine content in selected extracts obtained from untreated whole yellow mustard seeds (YMS), crushed yellow mustard seeds (CYMS) and crushed black mustard seeds (CBMS), were compared to extracts obtained from treated whole yellow mustard seeds sautéed for 2 (YMS-N2-H) and 10 minutes (YMS-N10-H), crushed yellow mustard seed sautéed for 2 minutes (CYMS-N2-H), and crushed black sautéed for 10 minutes (CBMS-N10-H). The highest sinapine, sinapic acid, and canolol levels were detected in the CBMS extract accounted for 102.59 ± 4.85, 7.86 ± 0.83, 2.98 ± 0.56 µg/g DM, respectively. CYMS followed by sinapine, sinapic acid, and canolol, accounting for 57.69 ± 1.35, 14.14 ± 0.17, 5.76 ± 0.45 µg/g DM, respectively. The strongest anti-tyrosinase activity was also observed for both CBMS and CYMS extracts providing the *in-vitro* evidence towards using sinapates as possible tyrosinase inhibitors, suggesting the suitability of sinapates towards treating skin cancer and application towards the cosmetic industry.

**Keywords**: sinapine, sinapic acid, canolol, mustard, ani-tyrosinase, melanoma

## 1. Introduction

Skin is the most significant part of the human body, which protects internal organs from harmful environmental factors. Skin structure can be divided into three main regions: the epidermis, the dermis, and the subcutaneous tissue. The epidermal layer is mainly responsible for a barrier in preventing water loss and entering destructive components such as chemicals, microorganisms, and especially sunlight (Gragnani et al., 2014). Ultraviolet (UV) A makes up most of solar radiation (approximately 95%), which causes damage by generating reactive oxygen species (ROS). Meanwhile, the other 5% is UVB, which is more destructive radiation with the alteration of nucleotide structure and DNA damage properties (Nasti & Timares, 2015).

Skin pigmentation is one of the body's protection pathways against UV radiation damage. Generally, a compound named melatonin is generated as a natural sunscreen to absorb the harmful UV radiation and intercept its further destruction by causing skin coloration (Kadekaro et al., 2003). Usually, melanogenesis produces two major pigments, including eumelanin and pheomelanin, with the catalyzation of the enzyme tyrosinase (Morgan et al., 2013). Eumelanin is a product of the serial enzymatically catalyzed and chemical reaction of 3,4-dihydroxyphenylalanine (DOPA) and DOPA quinone, which gives a dark brownish-black color to the skin (Ito & Wakamatsu, 2003). On the other hand, pheomelanin is yellow to reddish-brown pigmented melanin derivative with the incorporation of cysteine during bio-aggregation (Nasti & Timares, 2015). However, pheomelanin is also known to produce considerable pathological consequences causing skin cancer. The production of pheomelanin depends on glutathione (Nasti & Timares, 2015) and a shortage of this enzymatic antioxidant potentially leads to oxidative stress, DNA damage, and carcinogenesis (Galván et al., 2012; Galván & Møller, 2011; Morgan et al., 2013). Moreover, the retention of sulfur in pheomelanin's aromatic ring reduces its ionization

potential, thus becoming unstable and vulnerable to free radical disturbance (Ancans et al., 2001; Ranadive et al., 1986; Thody et al., 1991). On the other hand, according to Pavel et al., (2004), a higher pheomelanin content was found in melanoma cells than in normal melanocytes from the same patients. This study further demonstrated a positive correlation between the generation of pheomelanin and melanoma (Schadendorf et al., 2015). Additionally, the inactivation of the melanogenesis-catalyzed enzyme tyrosinase appeared to intercept melanoma development in mice (Morgan et al., 2013). Therefore, the inhibition of tyrosinase enzyme might become a key in the prevention of pigmentation as well as skin cancer.

Tyrosinase is a multifunctional copper-containing enzyme involved in the first two steps of melanogenesis (Sánchez-Ferrer et al., 1995). The over-excessive melanin synthesis might have a detrimental effect on human skin, including melanoma. To limit the activity of this enzyme, several natural and synthetic sources have been examined. The use of thiol-containing compounds has soon been introduced to the food industry. However, this method has many constraints, including off-flavour, and allergies leading to its application in food industries by U.S. Food and Drug Administration (Loizzo et al., 2012). Melanogenesis inhibition was carried out with the use of another method which includes ascorbic acid and citric acid. These compounds reversed the transformation of DOPA to *o*-dopaquinone, thus, refraining the melanin generation (Chang, 2009). Nevertheless, the ascorbic acid compound was less efficient and sustainable than the sulfiting agents (Santerre et al., 1988). Therefore, research is still proceeding in the current area of study. In recent times the application of phenolic compounds, including caffeic acid as an alternative enzyme substrate as tyrosinase inhibitors, has recently gained much attentin. The good affection between phenolic compounds and the enzyme tyrosinase can reduce the formation of dopachrome,

an intermediate in eumelanin and pheomelanin synthesis which will facilitate the inhibitory activity (Chang, 2009).

Mustard, a rich source of both phenolic and flavonoid content, could be considered as a potential candidate for skin cancer prevention and further application in cosmetics. Sinapine is reported to be the major phenolic compound in mustard seed, which is accounted for 70-90% of the total phenolic compound present, together with smaller amounts sinapic acid and canolol (Aachary & Thiyam-Hollander, 2012; R. Khattab et al., 2010; Nandasiri et al., 2019; Nandasiri, et al., 2021a,b; Nandasiri & Eskin, 2021, 2022). Our research has found that the optimization of the extraction condition of mustard seed derived an abundant amount of mustard phenolic compounds including sinapine and sinapic acid. On the other hand, *Agaricus bisporus* mushroom tyrosinase, which has an acceptable level of mimicking human tyrosinase, is a major and cost-efficient source of tyrosinase (Zolghadri et al., 2019 for further application in the skin care and cosmetic industry.

## 2. Material and Method

### 2.1. Material and Chemicals

GS Dunn. Ltd provided Oriental mustard seed. (Montreal, QC), while black and **y**ellow mustard seeds were purchased from Real Canadian Superstore (Winnipeg, MB) and Gill's supermarket (Winnipeg, Canada), respectively. Commercial black mustard seeds were purchased from Gill's supermarket (Winnipeg, MB, Canada), which was imported by PTIFOODS© (Mississauga, ON, Canada). Yellow mustard seeds were purchased from Real Canadian Superstore (Winnipeg, MB, Canada), which was imported by Loblaw Inc®/™ (Toronto, ON, Canada). In order to prolong the shelf life of samples, all the seeds were packed, sealed in Ziploc bags (Ziploc®, SC Johnson & Son Inc., Racine, WI, USA) and kept in the fridge at 4$^{o}$C until further analysis.

Seeds were grounded using the motor and pestle for 10 minutes to obtain the crushed seeds and whole seeds were used as it is for the current study.

Formic Acid, Sodium Phosphate Dibasic Dihydrate, and Citric Acid were purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). No Name Vinegar, ARM & HAMMER™ Baking Soda Box was purchased from Real Canadian Superstore (Winnipeg, Manitoba, Canada). Methanol, and ethanol (Optima™ LC/MS Grade) was also purchased from Fisher Scientific Canada Ltd, and Sinapic acid and mushroom tyrosinase 25KU were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sinapic acid (purity >98%) was purchased from Fisher Scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity >97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China). Canolol was synthesized in the lab (purity>97%), and its purity was confirmed via HPLC.

## 2.2. Methods

### 2.2.1. Sample preparations

The experiment was designated based on the reaction between tyrosinase enzyme and raw (non-extracted), extracted mustard, and residue after extraction samples (n=4). In this study, only the samples with the highest concentration of major sinapates content (sinapine, sinapic acid, and canolol) were selected for further analysis. Therefore, each selected sample was examined as an individual experiment, and no comparison of the enzymatic activity was further carried out between them. For the enzyme reaction of non-extracted mustard seed, the samples were raw yellow mustard seed (YMS), crushed yellow mustard seed (CYMS), and crushed black mustard seed (CBMS). The extracted samples were the yellow mustard seed with preheat treatment time of 2 and 10 minutes (YMS-N2, YMS-N10), crushed yellow mustard seed with preheat treatment time of 2 minutes (CYMS-N2), and crushed black mustard seed with preheat treatment time of 0

and 10 minutes (CBMS-N0, CBMS-N10). On the other hand, mustard residues after the extraction process were separated from phenolic extracts and underwent alkaline hydrolyzation to isolate the bound phenolic compounds. The tyrosinase enzyme reaction was then carried out using alkaline extracted mustard residues including yellow mustard seed with preheat treatment time of 2- and 10-minute residues (YMS-N2-H, YMS-N10-H), crushed yellow mustard seed with preheat treatment time of 2 minutes residues (CYMS-N2-H), and crushed black mustard seed with preheat treatment time of 0 and 10 minutes residues (CBMS-N0-H, CBMS-N10-H).

### 2.2.2. Enzyme reaction with pure Sinapic Acid

The effect of pure sinapic acid on the tyrosinase enzyme was carried out based on the previous study by Cao et al. (2019) with some modifications. Mushroom tyrosinase enzymes were diluted to 40.3U using phosphate citrate buffer (pH=6). The buffer was then used as a diluted solution for the mixture between 40µL of 40.3U tyrosinase and 1mL of 5 mM sinapic acid. The measurement was then carried out at 0, 10, 20, 30, 40, 50, and 60 minutes after incubation with gentle shaking in 25 $^{o}$C water bath. The spectrophotometric analysis was accomplished using Evolution™ One/One Plus UV-Vis Spectrophotometers (Thermo Scientific™, Ottawa, ON, Canada) at wavelengths ranging from 220 to 500 nm with 1200nm/min of speed.

### 2.2.3. Enzyme Reaction with Raw (Non-extracted) Mustard Seed

Both raw whole and crushed mustard seeds were used in this study. The experiment was performed using the procedure from Cao et al., (2019) with some modifications. First, 2.5 grams of mustard samples were weighed in centrifuge tubes and mixed with 500 µL of 40.3U Tyrosinase enzyme. Phosphate citrate (0.1M) was then added up to reach a volume of 25 mL. Incubation conditions remained the same in Section 2.3.2 (25$^{o}$C water bath, gentle shaking) with different time intervals (0, 30, 60, 90, 120 minutes). The solution was then centrifuged at 2000g at 37$^{o}$C for

20 minutes (Sorvall Biofuge Primo R Refrigerated Centrifuge, SO-BPRC, Thermo Scientific™, Ottawa, ON, Canada). The reaction mixture was then spectrophotometric analysed by Evolution™ One/One Plus UV-Vis Spectrophotometers (Thermo Scientific™, Ottawa, ON, Canada) to confirm the existence of the targeted phenolic compounds, followed by the HPLC analysis **Figure S1**.

### 2.2.4. Enzyme reaction with Extracted Mustard Samples

Generally, 10.0 grams of mustard were first sautéed in an instant pot (Instant Pot Duo Mini 3 Qt Model number: IPDUOMINI 3Qt, Instant Brands Inc, Downers Grove, IL, USA) at a constant temperature of 76$^{o}$C for 0, 2 & 10 minutes. The samples were then cooled at ambient temperature in a desiccator. After 10 minutes, the sautéed seeds were treated by wet extraction under pressurized heating (115$^{o}$C at 10.2 psi) in the presence of water. Thereafter, the extracts and mustard flesh were stored at -80$^{o}$C until analyzed. The experiment was then performed using 1 mL of the extracted solution, mixed with 500 µL of 40.3U tyrosinase enzyme and added up to 25 mL by using phosphate citrate buffer. The measurement was then carried out at 0, 0.5, 1, 1.5, 2, 18 and 24 hours after incubation with gentle shaking at 25 $^{o}$C water bath. The solution was centrifuged at 2000g at 37$^{o}$C for 20 minutes (Sorvall Biofuge Primo R Refrigerated Centrifuge, SO-BPRC, Thermo Scientific™, Ottawa, ON, Canada). The reaction mixture was analysed using the Evolution™ One/One Plus UV-Vis Spectrophotometers (Thermo Scientific™, Ottawa, ON, Canada) to confirm the presence of targeted compounds.

### 2.2.5. Enzyme reaction with Alkaline treated Mustard Residue

After the extraction steps (Section 2.3.4), the residues were collected. The experiment was then performed as per the procedure of Cao et al. (2019) with slight modifications **(Figure S2)**. Each residue was weighed exactly 1 gram and mixed with 20 mL 4M Sodium Hydroxide. The mixture was then covered with foil and left for hydrolysis under 250 rpm, continually stirring

(Khattab et al., 2014). After 4 hours, the supernatant was collected using Sorvall Biofuge Primo R Refrigerated Centrifuge, SO-BPRC (Thermo Scientific™, Ottawa, ON, Canada) at 2500g for 10 minutes at 5°C. The solution was then neutralized to pH=7 using 6M Acid Hydrochloric solution. Further steps were accomplished using the same procedure as Section 2.3.4 with different enzyme incubation times (0, 0.5, 1, 1.5, 2, and 18 hours). Both spectrometrical and HPLC analyses were used for the identification of the targeted compounds.

### 2.2.6. Anti-tyrosinase activity

The anti-tyrosinase activity was performed according to (Mayr et al., 2019) with some modifications. Spectrophotometric assays were carried out with Evolution™ One/One Plus UV-Vis Spectrophotometers (Thermo Scientific™, Ottawa, ON, Canada) at wavelengths ranging from 220 to 500 nm with 1200nm/min of speed. Two wavelengths, 320, and 270 nm, were selected primarily to understand the presence of sinapine, sinapic acid and canolol respectively. The anti-tyrosinase activity was expressed on the basis of changes of absorbance in measurement time.

### 2.2.7. High-Performance Liquid Chromatography - Diode Array Detection (HPLC-DAD) analysis of sinapic acid derivatives

Major sinapates of different enzymatic hydrolysis samples, were tested qualitatively and quantitatively using the HPLC-DAD (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States) method described by Nandasiri et al., (2019) as modified by Fadairo et al., (2021) The separation was carried out on a KinetexR® Biphenyl C18 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States), with a 0.4 mL/min flow rate and a 10-µL injection volume. Before going through HPLC analysis, samples were filtered using 0.45 µm syringe filter.

### 2.2.8. Statistical analysis

All the enzyme reactions and analyses operated in duplicates. The results were reported as mean ± standard deviation. Mean comparisons were achieved with Tukey's test, with 5% statistically significant differences (P >0.05) considered statistically significant. SPSS statistical software version 26 (IBM, New York, NY, United States) was used to analyze the data.

## 3. Result and Discussion

### 3.1. Enzyme reaction with pure Sinapic Acid

Pure sinapic acid was used as the standard to confirm the oxidation effect of tyrosinase. According to **Figure S3**, the trendline reached its maximum absorbance at around 310-320nm, which was the absorbance wavelength of sinapic acid (Ayyappan & Usha, 2013). The absorbance reading was reported its highest at 0 minutes of incubation and then started to decrease slightly. This trend suggested the disappearance of sinapic acid during the time of incubation; therefore, it certified the hypothesis that sinapic acid could perform as a tyrosinase substrate, and inhibit melanogenesis, a serial chemical and biological reaction causing skin cancer. Additionally, besides sinapic acid, there was also a smaller peak detected at 220-240 nm, advised to be an oxidized product of sinapic acid.

### 3.2. Enzyme reaction with Raw Mustard Seed

**Figure S4a** and **Table 1** were the value obtained from the spectrophotometric and HPLC analysis of raw YMS, respectively. In this experiment, the spectrophotometric reading was accumulated every half an hour. According to **Figure S4a,** the highest absorbance was obtained at the 120 minutes of incubation at wavelength 300nm. Meanwhile, sinapic acid and its derivatives, which were recognized at a wavelength of around 320nm, despite the growth with time, the recovered value was not phenomenal. Moreover, after 2 hours of incubation, another promising

compound was observed at wavelengths 255nm and 270nm. Accordingly, the sinapine content obtained from HPLC analysis was not considerable. The highest sinapine concentration obtained from YMS was 0.58 ± 0.01 µg/g DW after 30 minutes of incubation with tyrosinase enzyme. Additionally, no significance was detected between each incubation time with no sinapic acid and canolol getting recovered. This deficiency also affected the anti-tyrosinase activity of YMS **(Figure 1)**. Despite the slight increase in absorbance after 2 hours of tyrosinase incubation, the obtained values from both wavelengths 270 and 320nm were still very low (0,02 ± 0.00 and 0.25 ± 0.00, respectively), suggesting YMS is not very effective against tyrosinase activity.

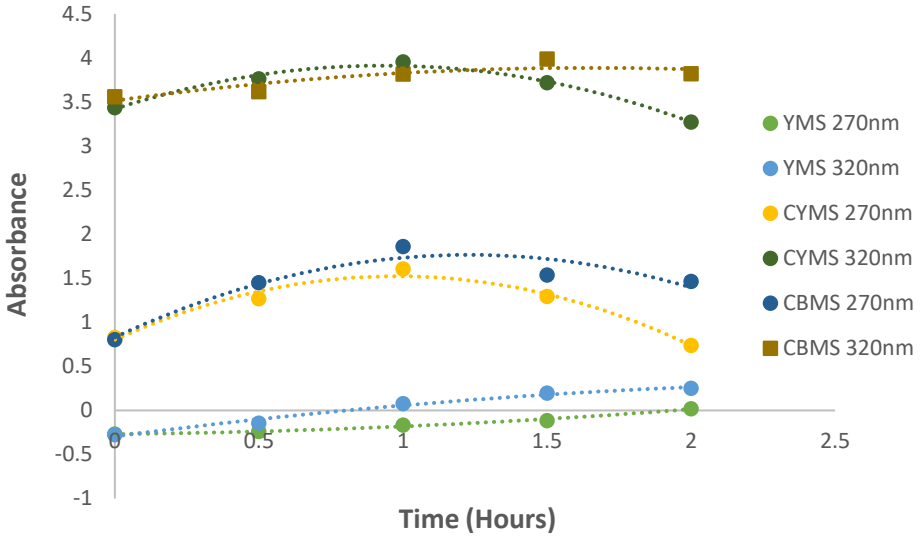


**Figure 4.1:** Anti-tyrosinase activity of raw mustard sample

**Table 4.1:** Phenolic changes of YMS**.**

| | **Sinapine (µg/g)** | **Sinapic Acid (µg/g)** | **Canolol(µg/g)** |
|---|---|---|---|
| | **Yellow Mustard Seed** | | |
| **0 min** | $0.46 \pm 0.01^{a}$ | nd | nd |
| **30 min** | $0.58 \pm 0.01^{b}$ | nd | nd |
| **60 min** | $0.45 \pm 0.03^{a}$ | nd | nd |
| **90 min** | $0.57 \pm 0.07^{b}$ | nd | nd |
| **120 min** | nd | nd | nd |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 min**: 0 minutes of incubation, **30 min**: 30 minutes of incubation, **60 min**: 60 minutes of incubation, **90 min**: 90 minutes of incubation, **120 min**: 120 minutes of incubation

The sinapine, sinapic acid, and canolol concentrations of CYMS were presented in **Table 2**. Generally, the concentrations of mustard's major sinapates were higher than the YMS, despite having the same trend of UV-scanning (**Figure S4b**). The difference in orientation might take responsibility for the variation. The highest absorbance obtained from this sample was detected after 60 minutes of tyrosinase incubation and started to decline afterwards. Like the YMS, the absorbance peaked at 320nm; however, the wavelength prolonged from 260nm to 400nm. In **Table 2**, crushing the seed contributed a better effect on the major sinapates content. The highest sinapine content was detected under 0 minutes of incubation time, which was 57.69 ± 1.35 µg/g DM. Sinapine content showed an inverse relationship with time, in which lower sinapine content was detected under longer incubation times. On the contrary, sinapic acid and canolol recovered the highest content was penetrated at 30 minutes (14.14 ± 0.17 µg/g) and 60-90 minutes (4.96 ± 0.57; 5.76 ± 0.45 µg/g) of incubation, respectively. Accordingly, **Figure 1** guaranteed the anti-tyrosinase activity of CYMS after 2 hours of enzyme incubation. Sinapic acid and canolol compounds performed the most productive anti-tyrosinase activity after 1 hour of incubation, in which the absorbances were 3.95 ± 0.00 and 1.60 ± 0.00, respectively. The absorbance decreased afterwards, suggesting efficient reactions between mustard's major sinapates and mushroom tyrosinase enzyme.

**Table 4.2:** Phenolic changes of CYMS.

| | Sinapine (µg/g) | Sinapic Acid (µg/g) | Canolol(µg/g) |
|---|---|---|---|
| **Crushed Yellow Mustard Seed** | | | |
| **0 min** | $57.69 \pm 1.35^{a}$ | $8.65 \pm 0.42^{a}$ | $3.67 \pm 0.41^{a}$ |
| **30 min** | $30.71 \pm 1.51^{b}$ | $14.14 \pm 0.17^{b}$ | $3.87 \pm 0.12^{a}$ |
| **60 min** | $5.83 \pm 0.13^{c}$ | nd | $4.96 \pm 0.57^{b}$ |
| **90 min** | $3.22 \pm 0.63^{d}$ | nd | $5.76 \pm 0.45^{b}$ |
| **120 min** | $3.65 \pm 0.5^{d}$ | nd | $1.85 \pm 0.27^{c}$ |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 min**: 0 minutes of incubation, **30 min**: 30 minutes of incubation, **60 min**: 60 minutes of incubation, **90 min**: 90 minutes of incubation, **120 min**: 120 minutes of incubation

**Table 4.3:** Phenolic changes of CBMS**.**

| | Sinapine (µg/g) | Sinapic Acid (µg/g) | Canolol(µg/g) |
|---|---|---|---|
| **Crushed Black Mustard Seed** | | | |
| **0 min** | $53.41 \pm 0.68^{a}$ | $2.15 \pm 0.52^{a}$ | $2.06 \pm 0.2^{a}$ |
| **30 min** | $58.62 \pm 3.94^{ab}$ | $3.43 \pm 0.72^{ab}$ | $2.98 \pm 0.56^{a}$ |
| **60 min** | $69.73 \pm 8.51^{b}$ | $5.07 \pm 1.15^{b}$ | $2.24 \pm 0.02^{b}$ |
| **90 min** | $98.68 \pm 5.69^{c}$ | $4.1 \pm 1.38^{ab}$ | $2.04 \pm 0.19^{b}$ |
| **120 min** | $102.59 \pm 4.85^{c}$ | $7.86 \pm 0.83^{c}$ | $1.39 \pm 0.18^{c}$ |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 min**: 0 minutes of incubation, **30 min**: 30 minutes of incubation, **60 min**: 60 minutes of incubation, **90 min**: 90 minutes of incubation, **120 min**: 120 minutes of incubation

The interaction between mustard seeds and tyrosinase enzyme varied with different varieties. For CBMS, the absorbance escalated with the increased incubation time **(Figure S4c)**. The anti-tyrosinase activity of CBMS was different from YMS and CYMS, in which CBMS demonstrated a better inhibitory activity. At 270nm, the reading value peaked after 1 hour of enzyme incubation (1.53 ± 0.00) and slightly decreased afterwards **(Figure 1)**. On the other hand, at 320nm, the highest absorbance was detected after 1.5 hours of incubation. Despite the difference, both sinapic acid and canolol can be considered as phenolic compounds with anti-tyrosinase activity. On the other hand, the HPLC analysis presented a more complex result in CBMS major sinapates profile (**Table 3**). The oxidation of tyrosinase cannot suppress the development of sinapine concentration, in which the highest sinapine content (98.68 ± 5.69; 102.59 ± 4.85 µg/g) was recovered after 90-120 minutes of incubation time. The same trend was observed with sinapic acid concentration (7.86 ± 0.83 µg/g). On the contrary, canolol concentration decreased with the increase in incubation time. At 0 minute of incubation, the concentration was 2.06 ± 0.2 µg/g, then decreased to 1.39 ± 0.18 µg/g after 2 hours of incubation. No significance was found between the incubation time from 0 to 90 minutes. This suggested that among all the major sinapates of BMS, canolol was the first compound that had been oxidized. This trend was also observed in **Figure 1**. The anti-tyrosinae activity of canolol reached its maximum after 1 hour of incubation and declined afterward. However, different from CYMS, the decrease rate was slower. For the compounds that was found in 320nm, CYMS and CBMS had quite similar performance, but lesser degradation at the end of the experiment. Interestingly. CBMS demonstrating the better tyrosinase inhibitory activity then YMS and CYMS. Additionally, canolol (270nm) was more efficient in tyrosinase inhibitory than sinapine and sinapic acid (320nm).

According to Shahidi et al., (2016), phenolic compounds were usually observed in free, soluble and insoluble-bound forms. Most of these secondary metabolite compounds covalently bind to the vacuoles of the plant cell walls; therefore, the disruption of the cell wall matrix was necessary for the release of phenolic compounds. In the experiment with the YMS, because of the lack of physical interaction, trivial amounts of sinapic acid and its derivatives were recovered from the oxidized tyrosinase enzyme. The crushing steps reduced the impact of mucilage, which was also an active barrier of yellow mustard seed (Cui et al., 1993). Therefore, more phenolic compounds were released from CYMS and CBMS; hence, better anti-tyrosinase activity was observed.

### 3.3. Enzyme reaction with Extracted Mustard Samples

Extracted samples, on the other hand, were slow anti-tyrosinase inhibitory. **Figures S5, S6,** and **S7** were scanned spectrums of extracted samples when reacting with 40.3U mushroom tyrosinase. **Figures S5a** and **b** presented results from YMS with different times of preheat treatment (YMS-N2 and YMS-N10). Generally, the reaction was stable for the first two hours of enzyme incubation and slightly decreased after 18 hours of incubation. Both samples were completely degraded after 24 hours of incubation, yet YMS-N10 presented a better inhibitory activity than YMS-N2. In **Figure 2**, we obtained the enzymatic reaction equation of YMS-N2 and YMS-N10 at 270 and 320 nm ($y = -0.0019x^2 + 0.0255x + 0.2845$, $y = -0.0149x^2 + 0.2447x + 1.7239$, $y = -0.0013x^2 + 0.0051x + 0.5092$, and $y = -0.0095x^2 + 0.133x + 1.7431$ with $R^2$ = 0.8722, 0.7342, 0.8079, and 0.9889, respectively). Therefore, we were able to calculate the time and maximum absorbance of these samples. Canolol of YMS-N2 and YMS-N10 had the highest anti-tyrosinase activity (absorbance 0.37 and 0.51) at approximately 6.7 and 1.96 hours after incubation, respectively. For sinapic acid, the maximum absorbances were 2.73 and 2.2 after 8.2 and 7 hours of incubation, confirming the better anti-tyrosinase activity of canolol.

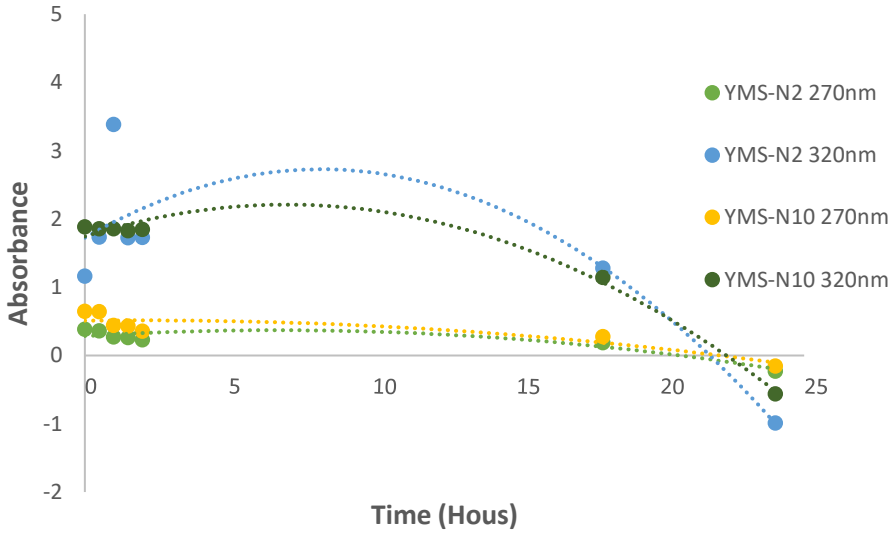


**Figure 4.2:** Anti-tyrosinase activity of YMS-N2 and YMS-N10

For the crushed samples, both CYMS-N2, BMS-N0 and CBMS-N10, higher amounts of mustard's sinapate were observed. For these samples, the scanned spectrums were also stable after the first two hours of enzyme incubation **(Figure S6** and **Figure S7)**. However, the increase in absorbances was detected after 18 hours of enzyme incubation. After 24 hours of tyrosinase incubation, all the phenolic compounds from these mustard samples were completely degraded. **Figure 3** and **Figure 4** gave us a more precise picture of how these samples might react with the mushroom tyrosinase enzyme. For CYMS-N2, the enzymatic reaction equations were $y = -0.0106x^2 + 0.2422x + 0.3086$ and $y = -0.0227x^2 + 0.4487x + 2.7247$ ($R^2$ = 0.9586 and 0.9844) for wavelengths 270 and 320 nm, respectively. As a result, canolol achieved the highest anti-tyrosinase activity (absorbance 1.69) after 11.4 hours of enzyme incubation. Meanwhile, for sinapic acid, the reaction was faster when the maximum absorbance (4.94) was reached after 9.88 hours of enzyme incubation. For CBMS-N0 and CBMS-N10, the enzymatic reaction equations at 270 and 320 nm were $y = -0.0039x^2 + 0.0744x + 0.3422$, $y = -0.0184x^2 + 0.3642x + 1.7658$, $y = -0.0055x^2 + 0.1069x + 0.4517$, and $y = -0.0191x^2 + 0.3799x + 1.8827$ ($R^2$ = 0.8979, 0.9307, 0.8226, and 0.9286), respectively. Accordingly, the maximum absorbances of canolol from CBMS-N0 and CBMS-N10 were 0.7 and 0.97 after 9.54 and 9.7 hours of enzyme incubation, respectively. The time sinapic acid took to reach maximum absorbance (3.57 and 3.77) was 9.9 hours for both CBMS-N0 and CBMS-N10.

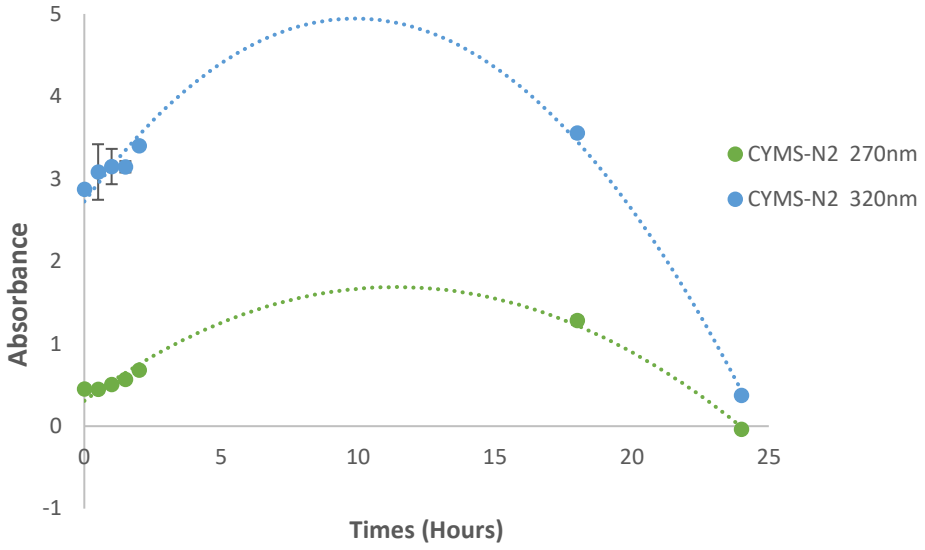


**Figure 4.3:** Anti-tyrosinase activity of CYMS-N2

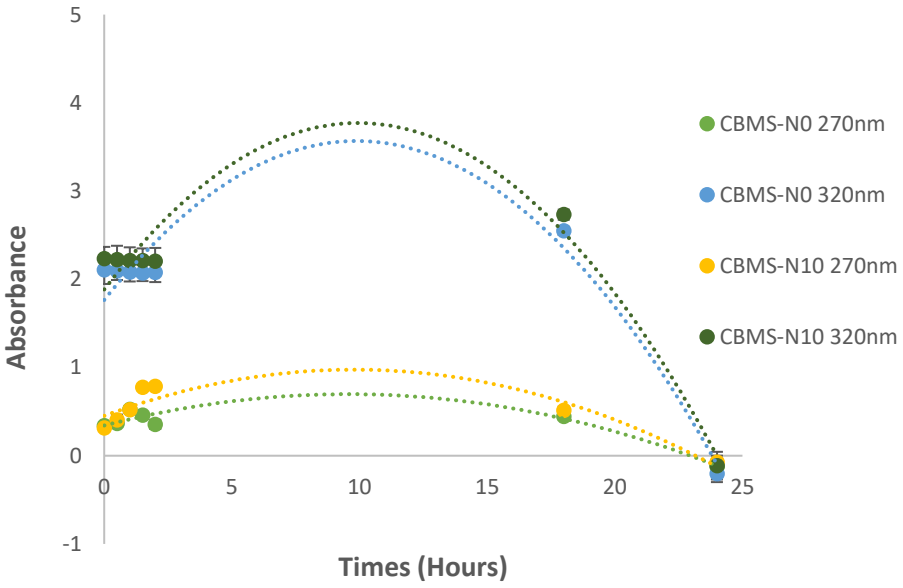


**Figure 4.4:** Anti-tyrosinase activity of CBMS-N0 and CBMS-N10

### 3.4. Enzyme reaction with Alkaline-Treated Mustard Seed

After undergoing preheat treatment and pressurized cooking, the majority of sinapine, sinapic acid, and canolol concentrations had already been removed from the mustard seed, leaving only bound phenolic compounds. Therefore, hydrolyzation was performed for the breaking of covalently bound phenolic acids. The alkaline-treated samples were then neutralized (pH = 7) before going through a tyrosinase enzymatic reaction. According to **Figure S8 a** and **b**, despite the difference in pre-treatment time, the YMS-N2-H and YMS-N10-H expressed a quite similar trend, in which the highest spectrophotometric absorbance was observed after 18 hours of enzyme incubation. However, at this time, the highest absorbance belonged to wavelength 290nm, suggesting a different compound was formed other than mustard's major sinapates. A clearer trend was observed when considering selected wavelengths 270 and 320 nm **(Figure 5)**. At 270 nm, no canolol was detected after 2.36 and 3.2 hours of enzyme incubation for YMS-N2-H and -N10-H, respectively. However, after 12 hours, the absorbance of both samples started to increase, establishing the generation of new compounds in both targeted wavelengths. The same trend was performed with wavelength 320nm, in which after 7.2 and 4.7 hours of enzyme incubation, the absorbance reached 0 for both YMS-N2-H and YMS-N10-H. After pressurized wet extraction and hydrolyzation, there was little sinapine, sinapic acid, and no canolol was recovered from the yellow mustard seed samples. The highest sinapine (1 ± 0.12 µg/g DW) and sinapic acid (4.52 ± 0.6 µg/g DW) concentrations were collected from YMS-N2-H after 0.5 and 1 hour of incubation, respectively **(Table 4)**. For YMS-N10-H, sinapine and sinapic acid reached their highest concentration (1.3 ± 0.12 and 0.95 ± 0.03 µg/g DW, respectively) after 2 hours of tyrosinase incubation. However, no significant difference was observed between each time interval for both alkaline-treated yellow mustard samples.

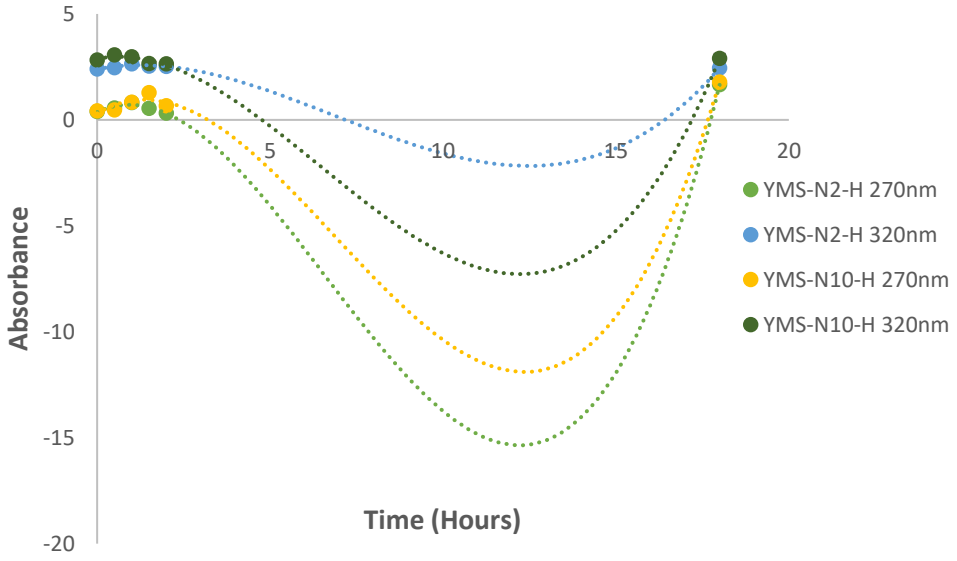


**Figure 4.5:** Anti-tyrosinase activity of YMS-N2-H and YMS-N10-H

**Table 4.4:** Phenolic changes of YMS-N2-H and YMS-N10-H

| | YMS-N2-H | | | YMS-N10-H | | |
|---|---|---|---|---|---|---|
| | **Sinapine (µg/g)** | **Sinapic Acid (µg/g)** | **Canolol (µg/g)** | **Sinapine (µg/g)** | **Sinapic Acid (µg/g)** | **Canolol (µg/g)** |
| **0 hr** | $0.98 \pm 0.07^{a}$ | $4.32 \pm 0.35^{a}$ | nd | $1.23 \pm 0.17^{a}$ | $0.94 \pm 0.05^{a}$ | nd |
| **0.5 hrs** | $1 \pm 0.12^{a}$ | $4.37 \pm 0.25^{a}$ | nd | $1.18 \pm 0.17^{a}$ | $0.87 \pm 0.03^{a}$ | nd |
| **1 hr** | $0.96 \pm 0.08^{a}$ | $4.52 \pm 0.6^{a}$ | nd | $1.28 \pm 0.11^{a}$ | $0.95 \pm 0.03^{a}$ | nd |
| **1.5 hrs** | $0.99 \pm 0.07^{a}$ | $4.48 \pm 0.33^{a}$ | nd | $1.28 \pm 0.17^{a}$ | $0.9 \pm 0.01^{a}$ | nd |
| **2 hrs** | $0.95 \pm 0.05^{a}$ | $4.37 \pm 0.35^{a}$ | nd | $1.3 \pm 0.12^{a}$ | $0.94 \pm 0.06^{a}$ | nd |
| **18 hrs** | $0.96 \pm 0.07^{a}$ | $4.38 \pm 0.38^{a}$ | nd | $0.5 \pm 0.04^{b}$ | $0.92 \pm 0.03^{a}$ | nd |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 hr**: 0 hour of incubation, **0.5 hrs**: 30 minutes of incubation, **1 hr**: 60 minutes of incubation, **1.5 hrs**: 90 minutes of incubation, **2 hrs**: 120 minutes of incubation, **18 hrs**: 18 hours of incubation

The enzyme reaction was different with the crushed seed sample (**Figure S9**, **S10**). The scanned spectrum of CYMS-N2-H, CBMS-N0-H and CBMS-N10-H established the same trend that reached the highest absorbance after 1 hour of tyrosinase incubation. However, there were some differences in compounds and absorbance. For CYMS-N2-H, besides the peak at 300-320nm, there was another compounds that were found at 230 and 270nm. On the other hand, for CBMS-N0-H, the highest absorbance was found at 210 nm, then 320nm, and finally 230 and 250nm. Meanwhile, for CBMS-N10-H, compound at 320nm had the highest absorbance. Two other compounds at 245nm and 270nm was also recorded. Like yellow mustard samples, after 18 hours of enzyme incubation, a new set of phenolic compounds was discovered (**Figures 6** and **7**). For CYMS-N2-H sample, after 2.5 hours of incubation, no absorbance was obtained from both selected wavelengths. However, after 17.5 hours of incubation, the absorbance started to raise again. Same trend was observed from CBMS-N0-H and CBMS-N10-H, but different in timeline that the absorbance reached 0. CYMS-N2-H, no canolol and sinapic acid were detected after 2.5 and 2.1 hours of enzyme incubation. For CBMS-N0-H and CBMS-N10-H, at wavelength 270 and 320 nm, the timelines were 2.17, 3.1, 2.1, and 3.7 hours after the enzyme reaction began, respectively. Additionally, after 12 hours of enzyme incubation, the absorbance started to increase, demonstrating the production of a new compound.

The HPLC results from these samples were also not promising since no significant difference was observed ($p>0.05$) **(Table 5** and **Table 6)**. No canolol was detected from these samples. **Table 5** was the phenolic changes of CYMS-N2-H sample. From this sample, small amount of sinapine and sinapic acid was detected, and both compounds had their concentration decreased with an increase of time. From 0-1.5 hours of enzyme incubation, the concentration of sinapine was quite stable, then decreased to $0.65 \pm 0.19$ µg/g when reached 2-hour and $0.49 \pm 0.03$

µg/g after 18-hour. Similarly, the concentration of sinapic acid was 1.16 ± 0.12 at initial, then declined to 0.63 ± 0.17 µg/g after 18 hours of enzyme incubation. On the other hand,CBMS-N0-H and CBMS-N10-H has lower sinapine and sinapic acid concentration than CYMS-N2-H. The highest sinapine concentration for both samples were 0.53 ± 0.02 and 0.82 ± 0.11 µg/g after 1 and 1.5 hours of incubation, respectively. On the other hand, no sinapic acid was obtained from CBMS-N10-H and little was found for CBMS-N0-H. After 1.5 hours of incubation, no sinapic acid was detected from CBMS-N0-H sample.

According to Vuorela et al., (2003), alkaline hydrolysis can damage an estimated 20% of oilseed phenolic compounds. This could explain the reduction of major sinapates with the introduction of the alkaline hydrolysis. Despite that, the HPLC results established that there were still enough compounds left to constrain the participation of tyrosinase in the depigmentation process. The lack of canolol in hydrolyzation samples promoted two possible pathways. The first one could be established as the pressurized wet extraction and alkaline hydrolyzation had already eliminated all the canolol in these samples. The second one could be canolol was the first compound that had been oxidized by the tyrosinase enzyme confirming the potent anti-tyrosinase activity of canolol.

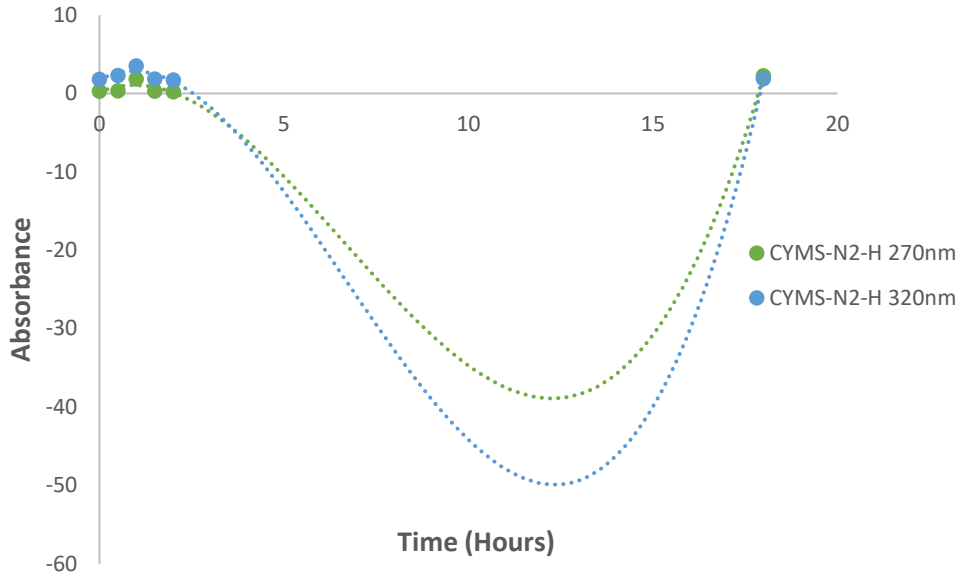


**Figure 4.6:** Anti-tyrosinase activity of CYMS-N2-H

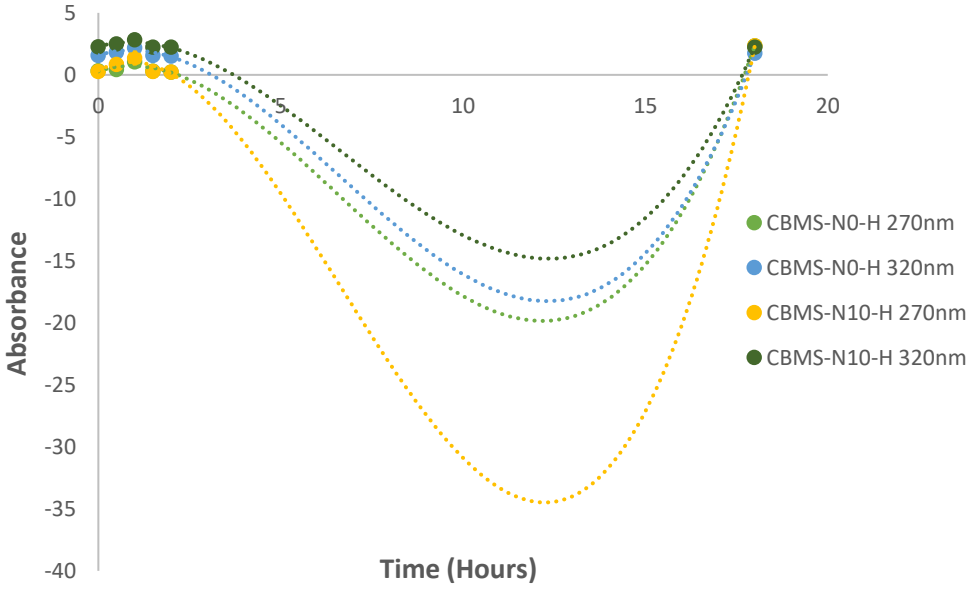


**Figure 4.7:** Anti-tyrosinase activity of CBMS-N0-H and CBMS-N10-H

**Table 4.5:** Phenolic changes of CYMS-N2-H

| | Sinapine (µg/g) | Sinapic Acid (µg/g) | Canolol(µg/g) |
|---|---|---|---|
| | **Crushed Yellow Mustard Seed** | | |
| **0 hr** | $0.89 \pm 0.13^{a}$ | $1.16 \pm 0.12^{a}$ | nd |
| **0.5 hrs** | $0.85 \pm 0.07^{a}$ | $1.15 \pm 0.01^{a}$ | nd |
| **1 hr** | $0.86 \pm 0.12^{a}$ | $1.16 \pm 0.16^{a}$ | nd |
| **1.5 hrs** | $0.89 \pm 0.08^{a}$ | $1.01 \pm 0.15^{a}$ | nd |
| **2 hrs** | $0.65 \pm 0.19^{a}$ | $0.88 \pm 0.17^{a}$ | nd |
| **18 hrs** | $0.49 \pm 0.03^{a}$ | $0.63 \pm 0.17^{a}$ | nd |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 min**: 0 minutes of incubation, **30 min**: 30 minutes of incubation, **60 min**: 60 minutes of incubation, **90 min**: 90 minutes of incubation, **120 min**: 120 minutes of incubation

**Table 4.6:** Phenolic changes of CBMS-N0-H and CBMS-N10-H

| | CBMS-N0-H | | | CBMS-N10-H | | |
|---|---|---|---|---|---|---|
| | **Sinapine (µg/g)** | **Sinapic Acid (µg/g)** | **Canolol (µg/g)** | **Sinapine (µg/g)** | **Sinapic Acid (µg/g)** | **Canolol (µg/g)** |
| **0 hr** | $0.5 \pm 0.0^{a}$ | $0.49 \pm 0.02^{a}$ | nd | $0.61 \pm 0.08^{a}$ | nd | nd |
| **0.5 hrs** | $0.52 \pm 0.03^{a}$ | $0.49 \pm 0.04^{a}$ | nd | $0.53 \pm 0.0^{a}$ | nd | nd |
| **1 hr** | $0.53 \pm 0.02^{a}$ | $0.5 \pm 0.02^{a}$ | nd | $0.6 \pm 0.05^{a}$ | nd | nd |
| **1.5 hrs** | $0.48 \pm 0.05^{a}$ | nd | nd | $0.82 \pm 0.11^{b}$ | nd | nd |
| **2 hrs** | nd | nd | nd | $0.69 \pm 0.08^{ab}$ | nd | nd |
| **18 hrs** | nd | nd | nd | $0.61 \pm 0.08^{a}$ | nd | nd |

Note: Values with different superscripts in the same column are significantly different ($p<0.05$)
nd: Not detected
**0 hr**: 0 hour of incubation, **0.5 hrs**: 30 minutes of incubation, **1 hr**: 60 minutes of incubation, **1.5 hrs**: 90 minutes of incubation, **2 hrs**: 120 minutes of incubation, **18 hrs**: 18 hours of incubation

### 3.5. Other sinapates

Besides major sinapates, the alkaline-treated mustard samples promoted the formation of an unknown compound, which recovered at wavelength 320nm and a retention time of 29.9 minutes. Generally, this compound appeared to decrease with the increase in incubation time; however, its concentration varied between the mustard varieties. The highest concentration was recovered from YMS-N2-H after 0.5 hours of enzyme incubation (46.01 ± 3.14 µg/g DW). Moreover, this sample also performed the least variation between the incubation time. For YMS-N2-H, the concentration of this compound was 42.68 ± 1.54 µg/g DW at the beginning of the incubation process, reaching the highest concentration (46.01 ± 3.14 µg/g DW) at 0.5 hours and started to decline. After 18 hours of incubation, the concentration was still stable at 39.28 ± 0.46 µg/g DW. CYMS-N2-H and CBMS-N10-H also obtained the lowest amount (14.48 ± 3.72, and 16.11 ± 0.17 µg/g DW, respectively), suggesting the dependence of this compound on the preheat treatment. On the other hand, the most distinguishable phenolic changes were observed from CBMS-N10-H. The concentration sharply declined from 29.94 ± 1.02 µg/g DW to 15.95 ± 4.05 µg/g DW after 2 hours of tyrosinase incubation. For YMS-N10-H, no variation was detected between 0, 0.5 and 1 hour of enzyme incubation, 35.98 ± 0.42, 33.43 ± 2.35, and 35.30 ± 0.76 µg/g DW, respectively. The concentration was then gradually decreased to 32.20 ± 1.26 µg/g DW after 1.5 hours of enzyme incubation and declined to 24.99 ± 0.66 and 23.83 ± 2.75 µg/g DW at the last two intervals. This unknown compound was only detected with the presence of tyrosinase, suggesting this was the by-product of the enzyme oxidation process.

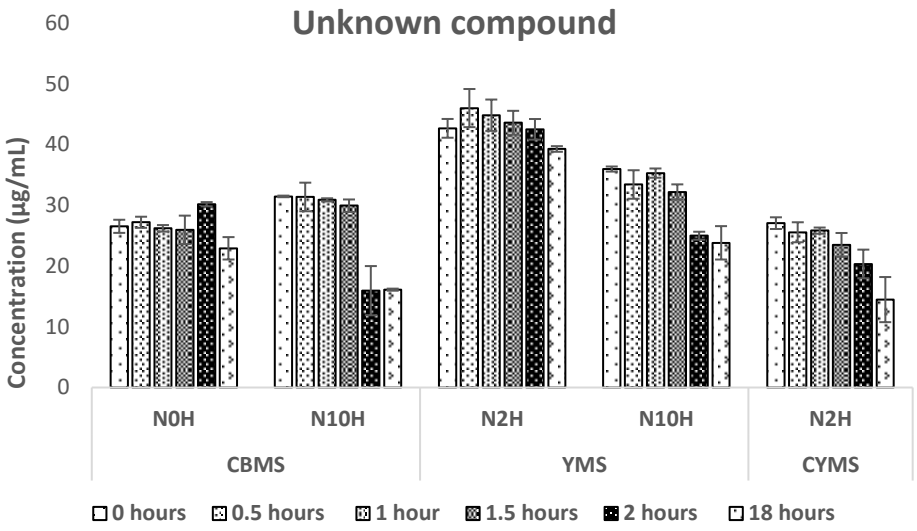


**Figure 4.8:** Unknown compound from treated samples

### 3.6. Discussion

Tyrosinase was a copper-containing enzyme that normally has two active copper binding sites (Ramsden & Riley, 2014). Depending on the binding bridge between the metallic domain, enzyme tyrosinase are categorized into *met*-tyrosinase (hydroxide bond), *oxy*-tyrosinase (peroxide bond) and *deoxy*-tyrosinase (co-ordination arrangement). According to Zolghadri et al., (2019), there were two mechanisms for inhibiting phenolic compounds on the tyrosinase mechanism. The first one was detailly described by Muñoz-Muñoz et al., (2010). The reactive compounds were called suicide activators or mechanism-based inhibitors, which changed tyrosinase's tertiary and quaternary structure. The second mechanism was “true inhibitors”, which consisted of four main types: competitive, uncompetitive, competitive/uncompetitive (mixed type), and non-competitive. A competitive inhibitor had the ability to combine free enzymes and prevent the creation of an enzyme-substrate complex. As tyrosinase was a metalloenzyme, copper chelator compounds including phenolic acid, aromatic acids, and polyphenol compounds can perform as competitive inhibitors. On the other hand, the uncompetitive inhibitor can only bind to the enzyme-substrate complex. Non-competitive and uncompetitive ones performed their activity on both free enzymes and an enzyme-substrate complex but with different equilibrium rates (Zolghadri et al., 2019).

Although there was much research on phenolic acid as an anti-tyrosinase compound, the mechanism of how sinapic acid and its derivatives on prevention of depigmentation have not been accomplished. However, some studies on the anti-tyrosinase activity of cinnamic acid derivatives can be used as references. The structure of sinapic acid consisted of two methoxy groups (C3 and C5) and one hydroxy group (C4), hence, the cinnamic acid, 3-methoxycinnamic acid, and p-coumaric acid were considered. A study by Garcia-Jimenez et al., (2018) confirmed the competitive tyrosinase inhibition effects of cinnamic acid and 3-methoxycinnamic acid. The

presence of methoxy group in the latter increased the electron density of the benzene ring by electron-donating effect, thus making 3-methoxycinnamic acid had a better affinity to di-nuclear copper active site of tyrosinase than cinnamic acid. In contrast, (Garcia-Jimenez et al., (2017) showed that *p*-coumaric acid (4 -hydroxycinnamic acid) was the tyrosinase substrate for forming *o*-caffeoquinone. However, using molecular docking, the study also established that the distance between the hydroxyl group (C4) was close to the oxygen atoms of peroxide ions (*oxy*-tyrosinase (2.9 and 3.0 Å), hence the hydrogen-bond interaction between p-coumaric acid and tyrosinase was possible. Indeed, with just one more functional group, 3,4 – hydroxycinnamic acid was verified to be a suicide substrate of tyrosinase (Garcia-Jimenez, Teruel-Puche, et al., 2018; García-Molina et al., 2005). Therefore, sinapic acid and its derivatives were potential tyrosinase inhibitors, and their structures suggested both suicide substrate and "true inhibitor".

## 4. Conclusion

The experiment proposed that major sinapates of mustard have a positive impact on the inhibition of mushroom tyrosinase. The most promising results were obtained from raw crushed yellow and black mustard seeds. The highest sinapine, sinapic acid and canolol content were also recovered from these samples, suggesting that the phenolic compound found in these samples prevailed the anti-tyrosinase activity, and continued to release. On the other hand, the samples that suffered from pressurized wet extraction and hydrolyzation acquired limited mustard phenolic compounds because of exhaustion during multiple processes. Moreover, an unknown compound at a retention time of 29.9 minutes of the alkaline-treated samples could also be a promising discovery for the application of enzyme extraction in different mustard varieties. Even though the mechanism behind the enzyme reaction between sinapic acid derivatives and tyrosinase was not yet clarified, their chemical structures potentially allowed them to inhibit tyrosinase activity in

both suicide substrate and “true inhibitor” mechanism. As a result, mustard can be considered a potential material for the inhibition of tyrosinase enzyme.

## 5. Acknowledgement

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## 6. Author Contributions

TN and RN designed the study. TN performed the experiments under the guidance of both RN and ME, interpreted the results and drafted the manuscript. ME, RN, and OF proof-read the manuscript. Funding was acquiesced by UTH.

## Conflicts of Interest

There is no conflicts of interest.

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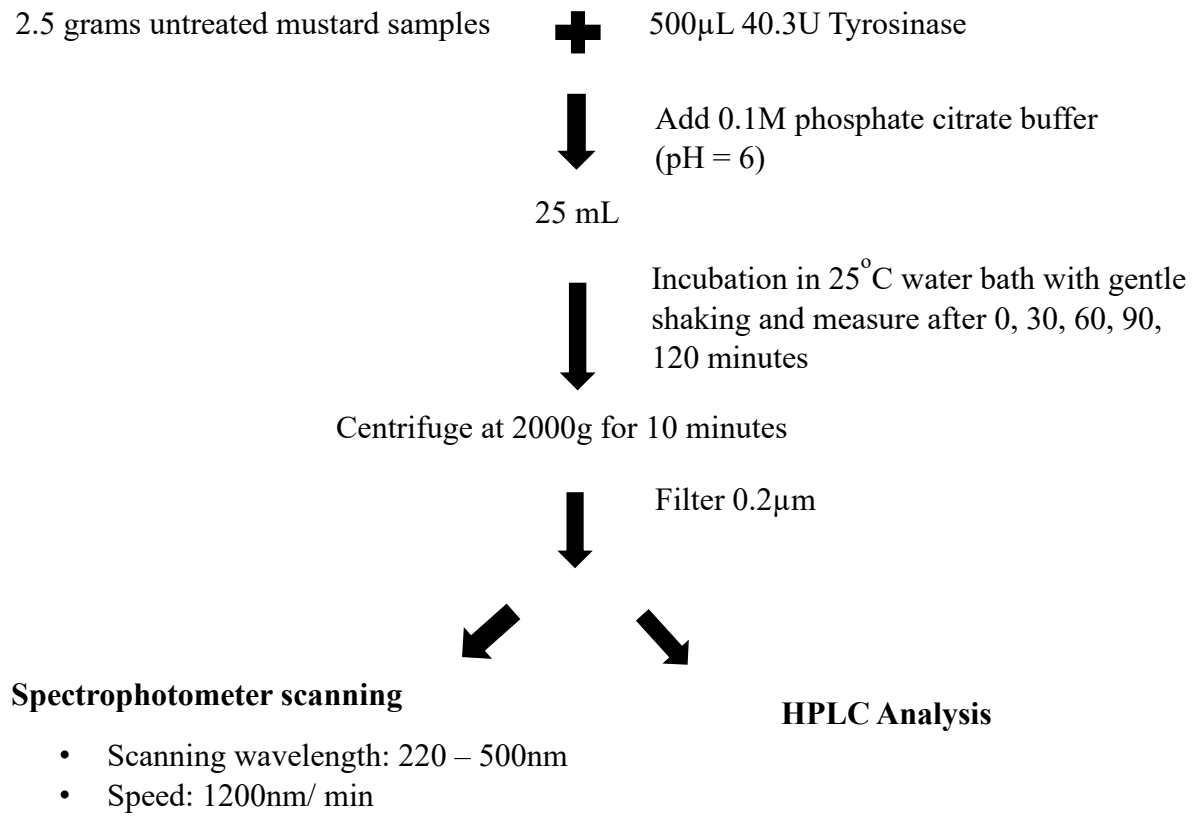
**Supplement Information**

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- Speed: 1200nm/ min

**Figure S 4.1:** Preparation for the Enzymatic reaction of Untreated Mustard Seed

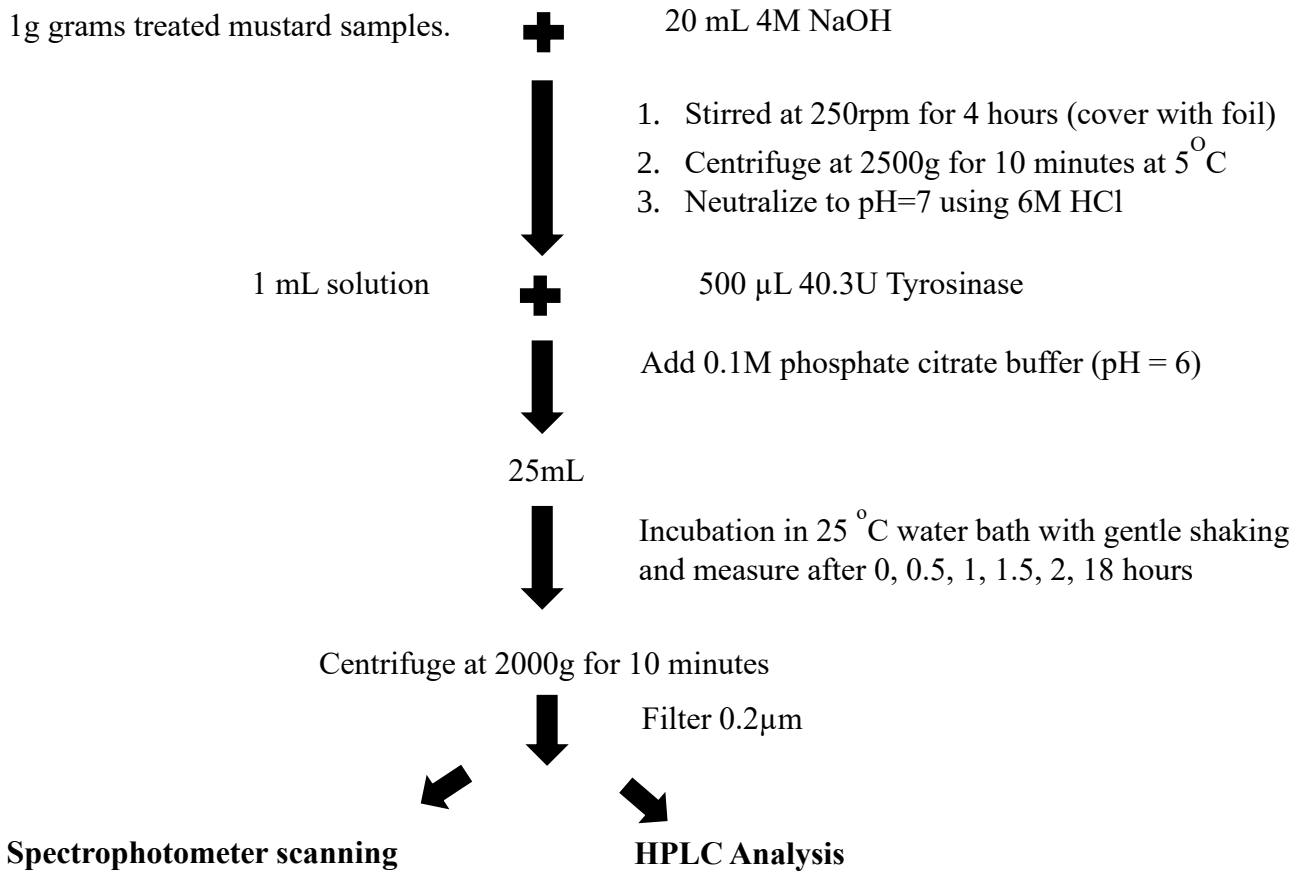


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**Figure S 4.2:** Preparation for the Enzymatic reaction of Treated Mustard Seed

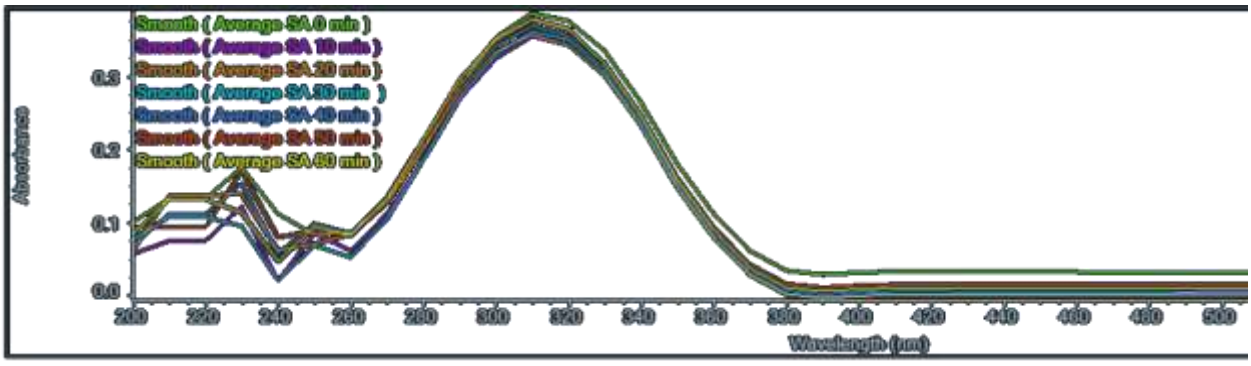



**Figure S 4.3:** The absorption spectra of pure sinapic acid with 40.3U Tyrosinase

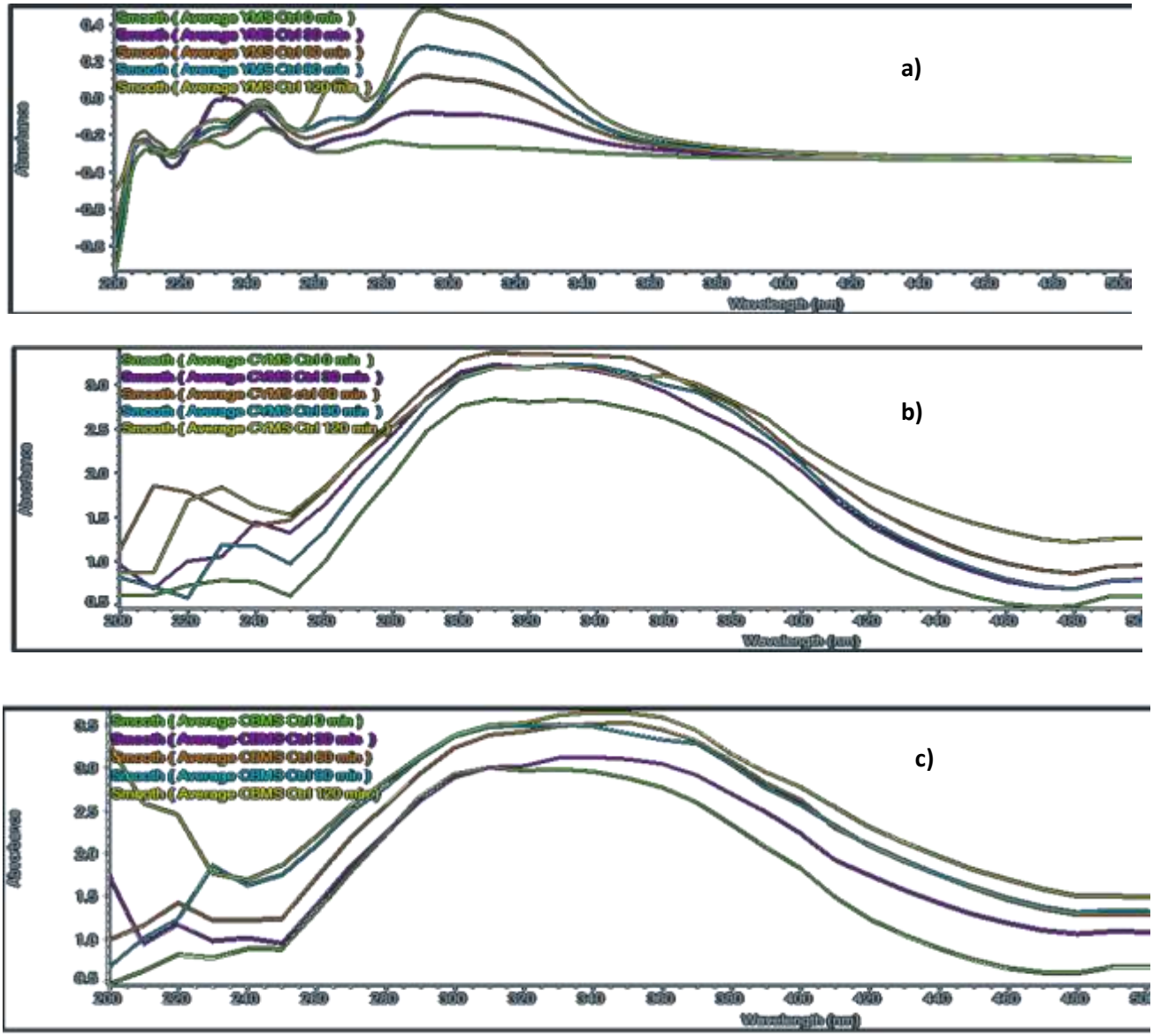



**Figure S 4.4:** The absorption spectra of raw a) YMS, b) CYMS, and c) CBMS with 40.3U Tyrosinase.

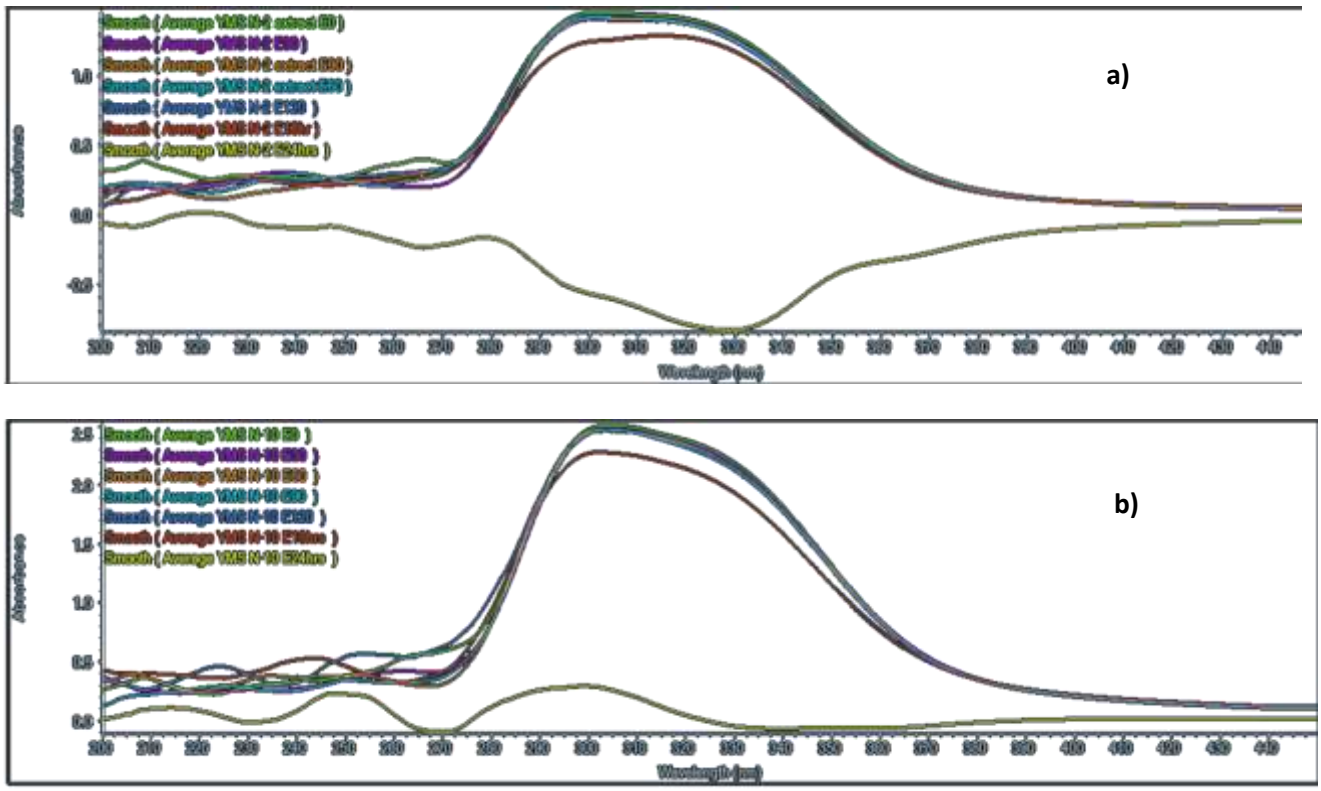



**Figure S 4.5:** The absorption spectra of a) YMS-N2, b) YMS-N10 with 40.3U Tyrosinase.

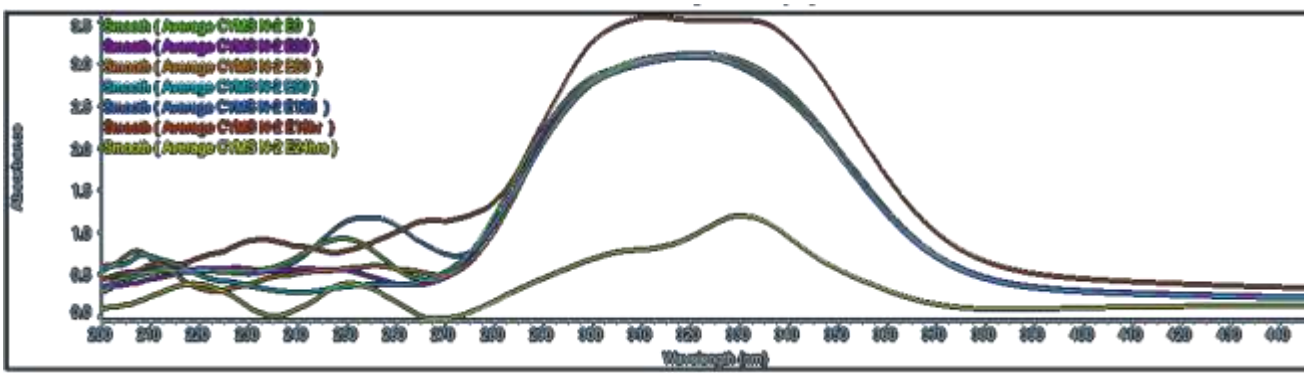


**Figure S 4.6: The absorption spectra of CYMS-N2 with 40.3U Tyrosinase.**

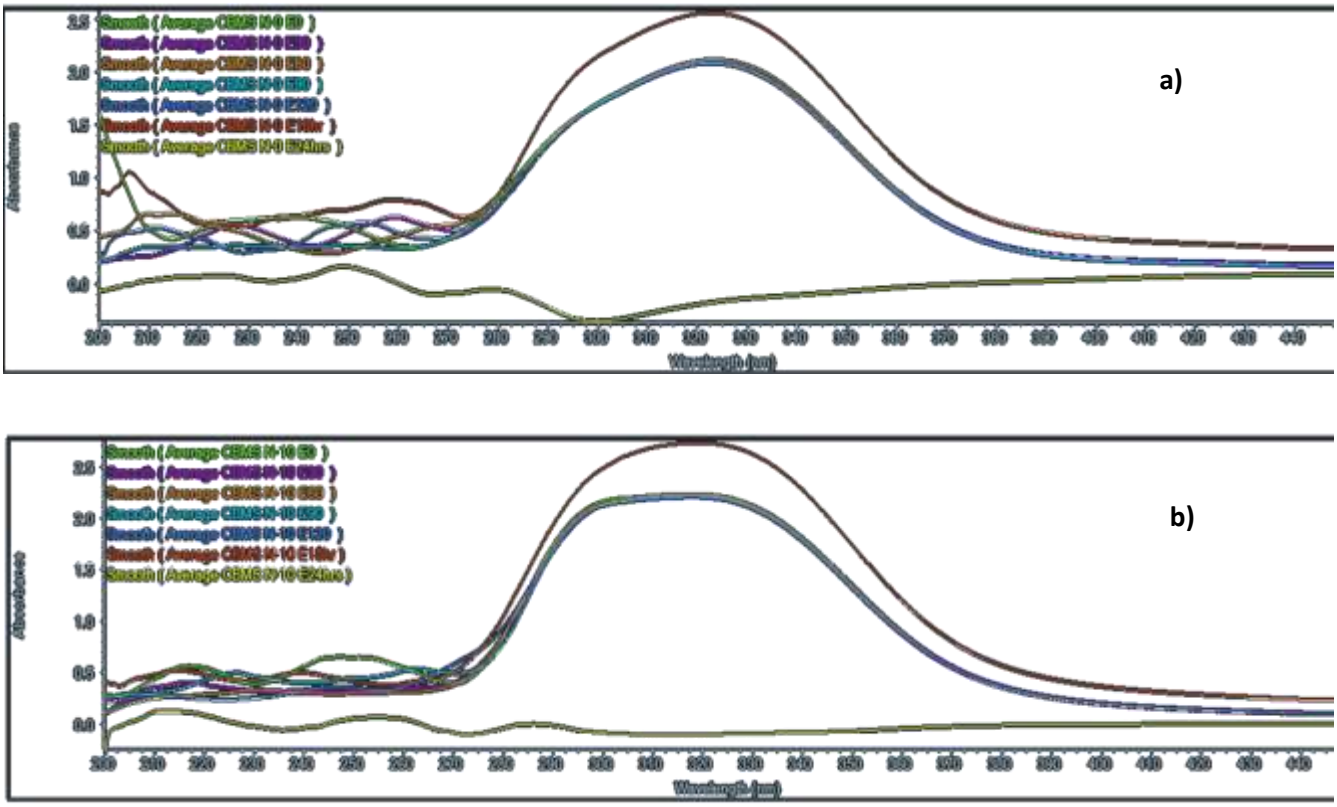



**Figure S 4.7:** The absorption spectra of a) CBMS-N0 b) CBMS-N10 with 40.3U Tyrosinase.

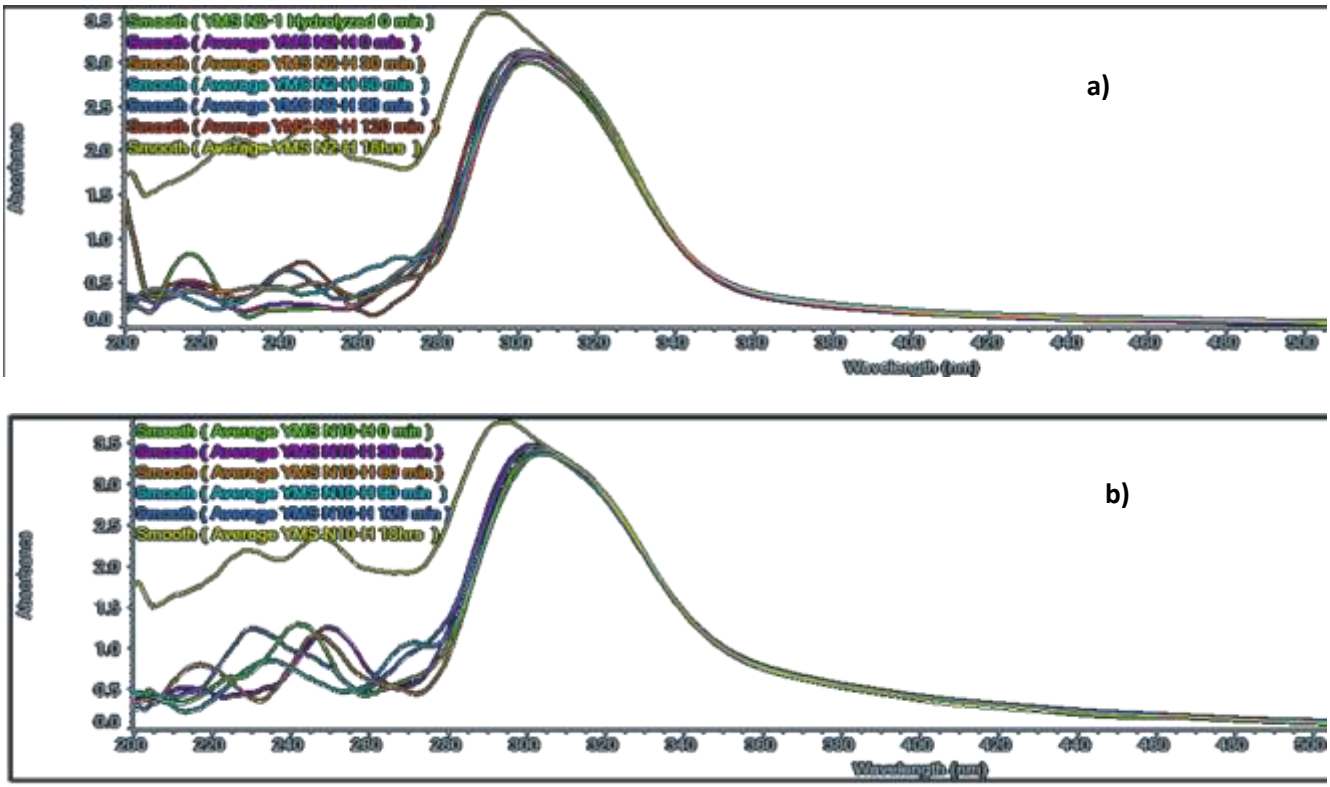



**Figure S 4.8**: The absorption spectra of a) YMS-N2-H b) YMS-N10-H with 40.3U Tyrosinase.

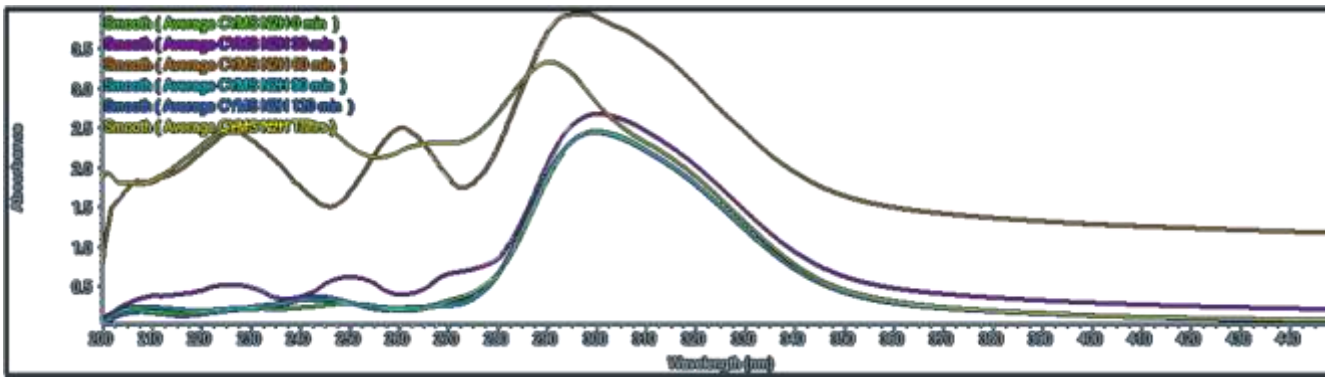



**Figure S 4.9:** The absorption spectra of CYMS-N2-H with 40.3U Tyrosinase.

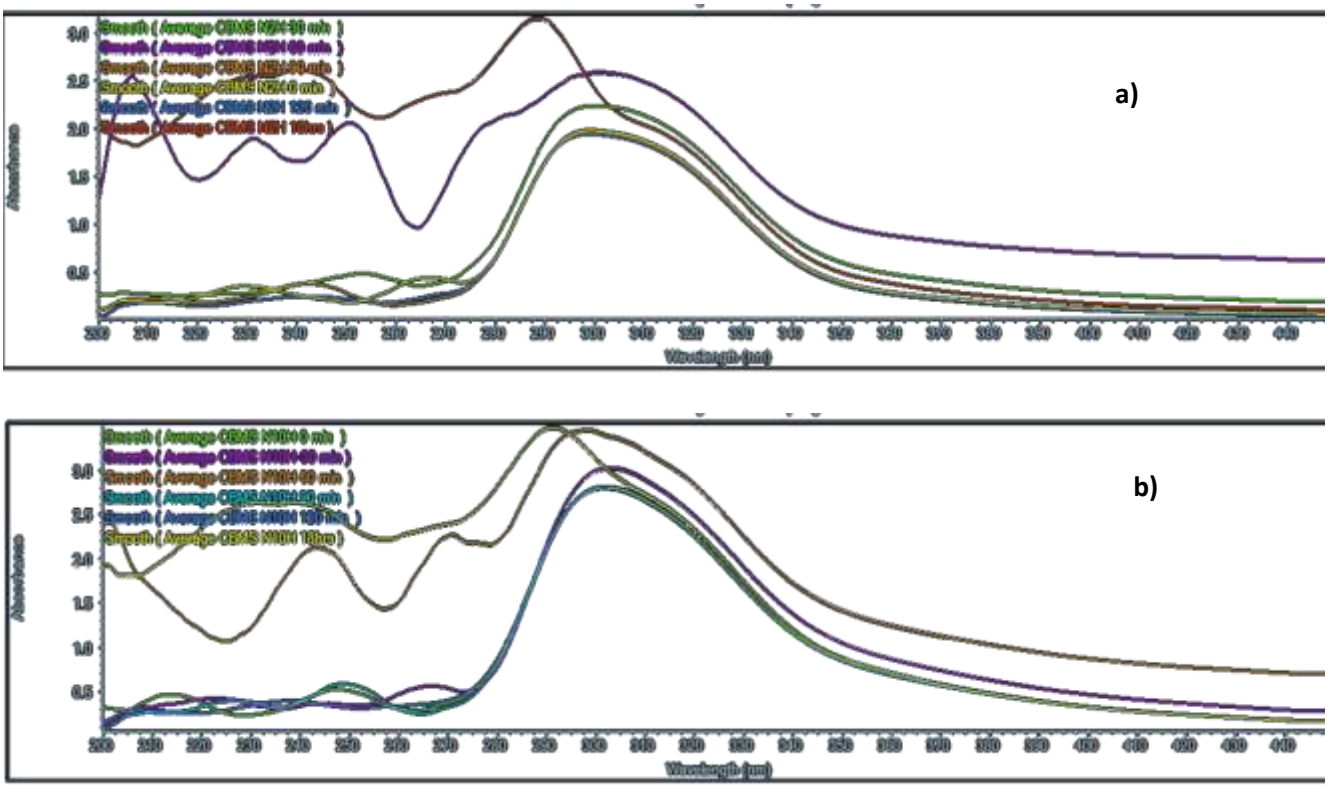



**Figure S 4.10:** The absorption spectra of a) CBMS-N0-H, b) CBMS-N10-H with 40.3U Tyrosinase.

# CHAPTER 5

# CONCLUSION

Mustard seed is cultivated worldwide for its agronomic value due to its high protein, oil and phenolic content. Mustard is a rich source of phenolic compounds, especially sinapic acid and its derivatives. Therefore, in this thesis, a home-scale extraction system was designated for the optimization of the extraction of mustard's major sinapates. The impact of three varieties (Oriental, black, yellow), two orientations of the seeds (whole and crushed), three of pre-heat treatment times (0,2 and 10 minutes of sauté), and three pH conditions (acid, neutral and alkaline) using food-based solvent (vinegar, water and sodium bicarbonate solution, respectively) with the assistance of pressure (10.2 psi) and temperature (115 $^{o}$C) were examined their effect on the extraction of sinapine, sinapic acid and generation of canolol. Both black and yellow mustard had competitive amounts of sinapine, sinapic acid and canolol and Oriental has the lowest. The impact of preheat treatment time varied between the samples. Generally, for most whole seed samples, the amounts of targeted phenolic compounds increased with the increase of time. Meanwhile, for most crushed seed samples, opposite trend was observed due to the larger surface was exposure to heat. An obvious difference was determined between the samples when considering pH, in which neutral treatment prevailed acid and alkaline one, suggesting water was the most productive solvent. Orientations of seed (whole and crushed), on the other hand, has least impact on the mustard samples. Overall, this system was proved to be effective for the extraction of mustard phenolic compounds.

The same factors were investigated in their impact on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of extracted mustard samples. Yellow mustard seeds treated with a water-based medium under neutral conditions yielded the largest amount of

total phenols compared to the other mustard varieties. The metal chelation ion and FRAP assays can be altered by pH, as the main principle of these methods is based on ion interactions. The highest FRAP and metal ion chelating activities were observed for yellow mustard using an alkaline wet extraction method. Black mustard seeds had the highest results for total flavonoid content compared to the other mustard varieties, particularly when exposed to buffer conditions. The effect of orientation and sauté time on antioxidant activity of mustard extract was similar to their effect on mustard's major sinaptes.

Melanin pigmentation protects the skin from the harmful effects of ultraviolet radiation. Two types of melanin pigments are produced in the skin, eumelanin and pheomelanin. While eumelanin is generally photoprotective, pheomelanin is considered phototoxic and capable of producing melanoma, a deadly skin cancer. Consequently, it is beneficial to prevent melanogenesis by inhibiting tyrosinase activity. In this study, only the samples with the highest concentration of major sinapates content (sinapine, sinapic acid, and canolol) were selected for further analysis. Therefore, each selected sample was examined as an individual experiment, and no comparison of the enzymatic activity was further carried out between them. For the enzyme reaction of non-extracted mustard seed, the samples were raw yellow mustard seed (YMS), crushed yellow mustard seed (CYMS), and crushed black mustard seed (CBMS). The extracted samples were the yellow mustard seed with preheat treatment time of 2 and 10 minutes (YMS-N2, YMS-N10), crushed yellow mustard seed with preheat treatment time of 2 minutes (CYMS-N2), and crushed black mustard seed with preheat treatment time of 0 and 10 minutes (CBMS-N0, CBMS-N10). On the other hand, mustard residues after the extraction process were separated from phenolic extracts, and underwent alkaline hydrolyzation to isolate the bound phenolic compounds. The tyrosinase enzyme reaction was then carried out using alkaline extracted mustard residues including yellow

mustard seed with preheat treatment time of 2- and 10-minute residues (YMS-N2-H, YMS-N10-H), crushed yellow mustard seed with preheat treatment time of 2 minutes residues (CYMS-N2-H), and crushed black mustard seed with preheat treatment time of 0 and 10 minutes residues (CBMS-N0-H, CBMS-N10-H). The highest sinapine, sinapic acid and canolol levels were detected in the CBMS and CYMS extracted samples. The strongest anti-tyrosinase activity was also observed for both samples above, suggesting their potential application in the cosmetic industry.

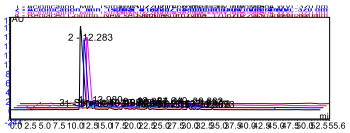
## FUTURE PROSPECTS

This study developed a home-scale system with the assistance of temperatures, pressure and pH. Despite, interesting results were obtained proving the efficient of this system, some of the limitations can also be improved.

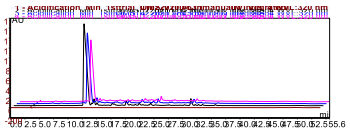
1. While the application of pH was confirmed to be effective, the mechanism behind the reactions between solvents and mustard's phenolic compounds was remain unexplainable, especially for sinapine and canolol compounds. Further research on structure transformation is necessary better understanding the impact of pressurized heat processing and pH on the mustard seed.
2. Several thermal-generative phenolic compounds were detected during the extraction of mustard samples. Because of time limitations, the identification and quantifications of these compounds was not possible. Further development including Nuclear Magnetic Resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) could be carried out.
3. *In vitro* chemicals study confirms the effect of this home-scale system on the extraction of phenolic compounds of mustard seed as long as its antioxidant and anti-tyrosinase activity. Further development such as *in vitro* cell-based assays and further *in-vivo* animal based model is needed to confirm the benefits and eliminate the drawbacks before putting to use.
4. Further statistical modeling is required for the development of this method to pilot-scale to establish feasibility for industrial scale.

**Appendices**

**Appendix 1: HPLC chromatogram of Oriental mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

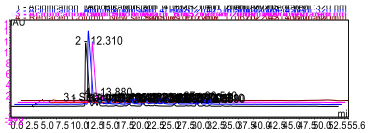


A-HPLC chromatogram of Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

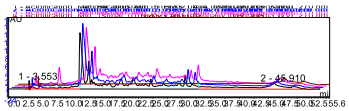


B-HPLC chromatogram of Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 2: HPLC chromatogram of black mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

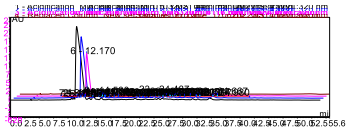


A-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

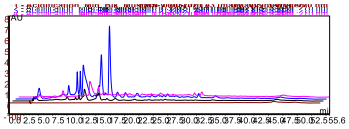


B-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 3: HPLC chromatogram of yellow mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

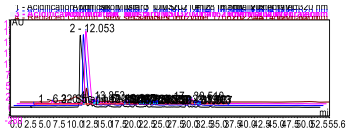


A-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

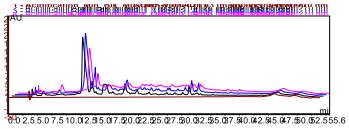


B-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 4: HPLC chromatogram of crushed Oriental mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

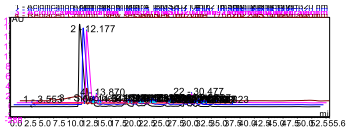



A-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

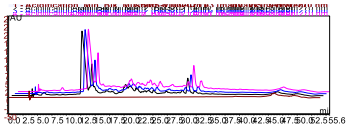



B-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 5: HPLC chromatogram of crushed black mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

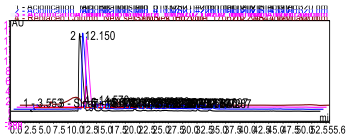


A-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

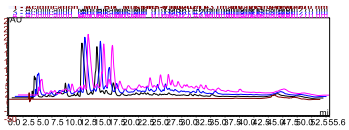


B-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 6: HPLC chromatogram of crushed yellow mustard seed under vinegar pressurized wet extraction (A-320nm, B-270nm)**

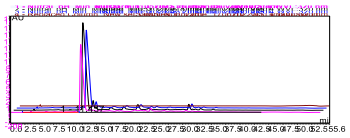



A-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

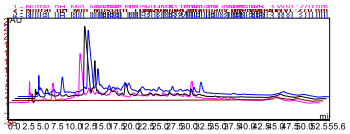



B-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and vinegar pressurized wet extraction.

**Appendix 7: HPLC chromatogram of Oriental mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

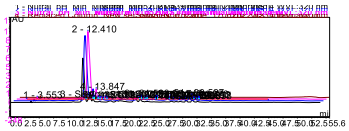


A-HPLC chromatogram of Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

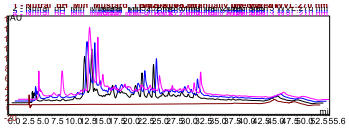


B-HPLC chromatogram of Oreintal mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 8: HPLC chromatogram of black mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

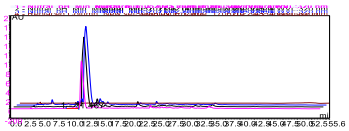



A-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

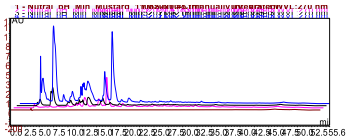



B-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 9: HPLC chromatogram of yellow mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

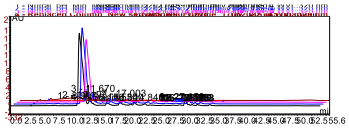


A-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

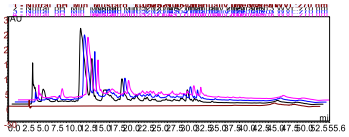


B-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 10: HPLC chromatogram of crushed Oriental mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

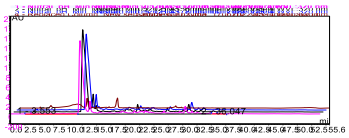


A-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

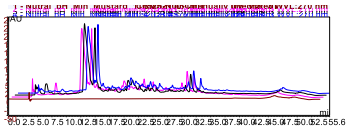


B-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 11: HPLC chromatogram of crushed black mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

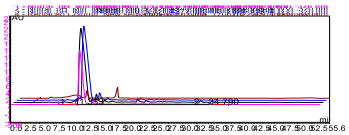


A-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

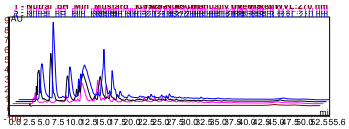


B-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 12: HPLC chromatogram of crushed yellow mustard seed under neutral pressurized wet extraction (A-320nm, B-270nm)**

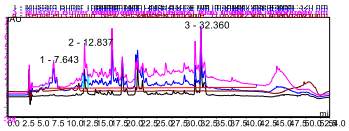


A-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

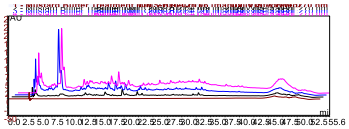


B-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and neutral pressurized wet extraction.

**Appendix 13: HPLC chromatogram of Oriental mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**

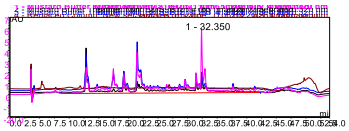



A-HPLC chromatogram of Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

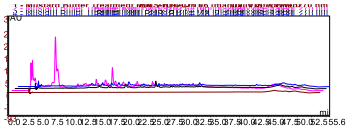


B-HPLC chromatogram of Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

**Appendix 14: HPLC chromatogram of black mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**

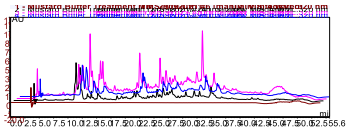



A-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

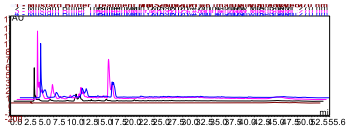



B-HPLC chromatogram of black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

**Appendix 15: HPLC chromatogram of yellow mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**

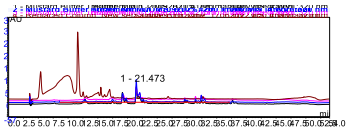


A-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

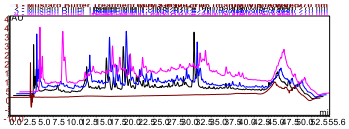


B-HPLC chromatogram of yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

**Appendix 16: HPLC chromatogram of crushed Oriental mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**

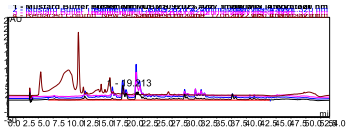



A-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

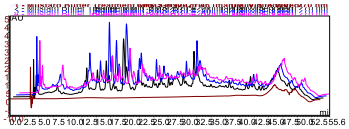


B-HPLC chromatogram of crushed Oriental mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

**Appendix 17: HPLC chromatogram of crushed black mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**

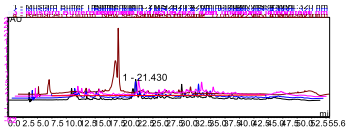


A-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

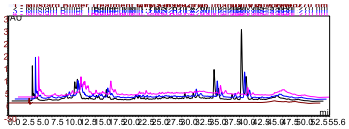


B-HPLC chromatogram of crushed black mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.

**Appendix 18: HPLC chromatogram of crushed yellow mustard seed under alkaline pressurized wet extraction (A-320nm, B-270nm)**




A-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.



B-HPLC chromatogram of crushed yellow mustard seed under 0 minutes (black),2 minutes (blue), 10 minutes (pink) of preheat treatment and alkaline pressurized wet extraction.