Effects of chemical, enzymatic and mechanical treatments on the phenolics and water holding capacity in wheat bran

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Abstract

Epidemiological studies have demonstrated that consumption of whole grain products is beneficial for human health due to its high dietary fiber content, and the consumption of fruits and vegetables can prevent chronic disease due to their high antioxidant activity. However, whole grain is also a good source of antioxidant, which has been underestimated by literature because most antioxidants in grains exist in their bound form, which cannot be detected using previous analysis methods. With the development of new approach to analyze the bound phenolics, scientists find the bound phenolics can take up almost as high as 70-80% of the total phenolics.

Wheat bran is a good source of dietary fiber and also phenolics. The phenolics in wheat bran are mainly ferulic acid, which is bound to arabinose, the side chain of arabinoxylans, through ester linkage. The bound phenolics reduce the bioactivity and bioaccesibility of wheat bran. Therefore, releasing them from the complex polysaccharides structure is beneficial for the functionality and bioactivity of wheat bran.

This objective of this study is to optimize the conditions to improve the bioavailability of free phenolics from wheat bran using combined physical, chemical, thermal, and enzymatic treatments. The treatments include alkaline treatment, enzymatic treatment, high shear mixing, and high-pressure homogenization. In this study, the optimal conditions of enzymatic treatment and combined alkaline and enzymatic treatments were evaluated. The phenolics acids content, flavonoids content, ferulic acid content and the water holding capacity were determined in processed wheat bran. Results showed that alkaline treatment was the most efficient in releasing bound phenolics in wheat bran due to its strong hydrolysis capacity of cleaving the ester linkage. Enzymatic

treatment was the most efficient in releasing flavonoids in wheat bran due to its effects on opening the polysaccharide structure. Mechanical treatments helped to reduce the particle size and opened the polysaccharide structure and improve the functionality and bioactivity of wheat bran as well.

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Literature review

Whole grain and health:

Whole grain has health benefits associated with reduced risk of chronic diseases such as cancer, diabetes, and cardiovascular disease[1] [2] [3] [4]. Therefore, Dietary Guidelines for Americans 2010 recommends consuming at least 3-ounce equivalents whole grain per day, but less than 5 percent of Americans makes it[5].

Jacobs et al. reviewed 40 case-control of cancers and colon ploys using meta-analysis and concluded the evidence that whole grain intake protected against various cancers[2]. Another review found whole-grain intake had positive effects on the insulin sensitivity thus prevented diabetes[6]. Liu also reviewed studies that proved the health beneficial of whole grain, and confirmed the positive results[4].

Four possible protective mechanisms are behind this: the large intestine model, the antioxidant model, the phytoestrogen model, and other potential mechanisms[7]. The large intestine model is related to the dietary fiber and resistant starch contained in whole grain. Since they are un-digestible in the GI tract, the microflora in large intestine will utilize them for fermentation and produces short-chain fatty acids and gases, which are considered of reducing the risk of cancer and lower serum cholesterol. The antioxidant model is related to the phenolics content in whole grain. It can balance the oxidative stress within human body and prevent the free radicals damage. The phytoestrogen model is related to lignans. They can influence the hormone metabolism that may have effects on the hormone-dependent cancer such as breast cancer. Since the whole grain food is a complex matrix containing hundreds of components that they are most likely to work together to perform their health benefits on human being.

However there are concerns about the antioxidant potential *in vivo*. Although many studies stated the high antioxidant capacity of whole grain *in vitro*, but few studies analyzed the antioxidant ability within the human body[8]. There are also concerns about the absorption and metabolism of antioxidants in human body. Do they really work to react with those generated free radicals or just be oxidized in the digestion process? More questions than answers is the current situation about the antioxidants beneficial effects on human being.

Nevertheless, whole grain is still a health food. USDA suggests the intake of at least 3-ounce equivalents whole grain per day, but most Americans fail to make the consumption due to the color, taste, and texture differences between the refined grain products and whole grain products. Therefore, whole grain products need to be modified and adjust to consumers' preferences to be widely accepted in public.

Wheat bran structure:

Wheat kernel is composed of three parts: endosperm, germ, and bran (Fig.1-1). The bran takes up about 14.5% of the kernel weight and contains of many layers to protect the kernel from harm including insects, bacteria, molds, and severe weather. Wheat bran is the by-product of fine wheat flour milling process, and used to feed animals in the past. Currently, with the public awareness of the benefits of consumption of whole grain, wheat bran is milled into the flour and also added as a resource of dietary fiber into food products.

The chemical compositions of wheat bran are protein, fat, crude fiber, cellulose, pentosans, vitamins, and minerals. Their contents vary with various wheat species[9]. However, the polysaccharides including arabinoxylans, cellulose, and lignin are the domain composition of wheat bran.

Arabinoxylan is a hemicellulose and contains two pentose sugars, arabinose and xylose. The main chain of arabinoxylans is the 1,4-linked xylose units with arabinose substituted on O-2 and/or O-3 position (Fig.1-2). Several studies extracted the arabinoxylans in wheat bran using acid, alkaline, enzymatic treatments and found the water-extractable arabinoxylans had a lower Arabinose/Xylose ratio than the water-unextractable one[10]. Mandalari et al. also detected ferulic acid and three forms of diferulic acid linked to arabinoxylans[10b].

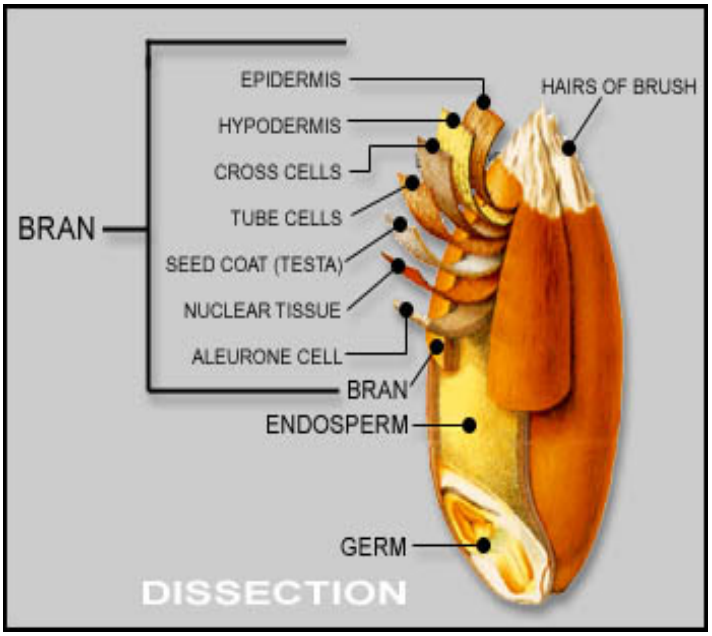


Figure 1-1 Wheat kernel dissection. (The New Zealand Institute for Crop & Food Research Limited 2002)

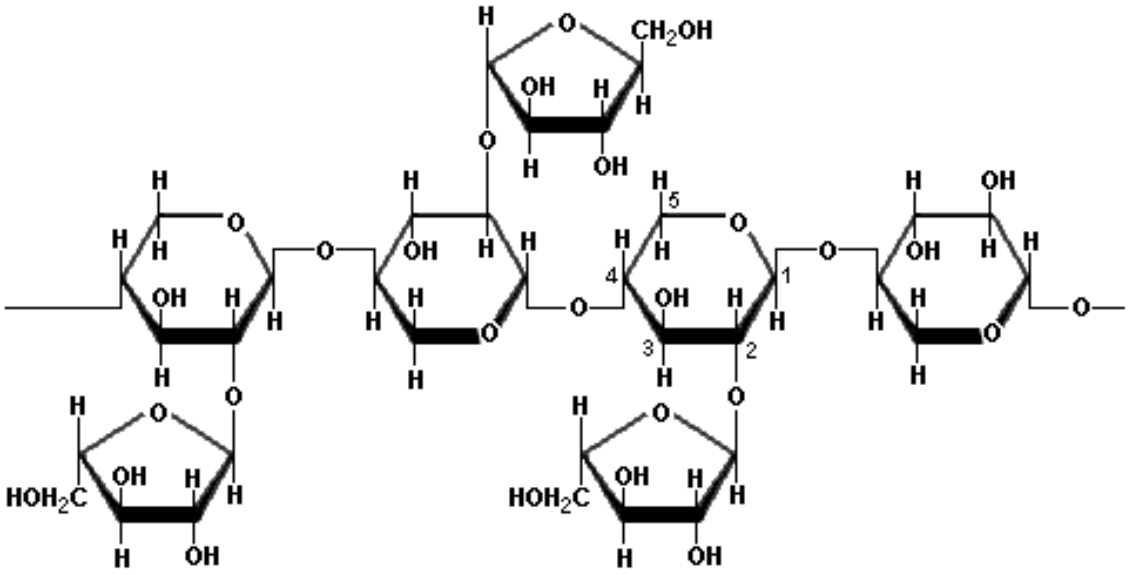


Figure 1-2 Structure of arabinoxylans
(http://www.scientificpsychic.com/fitness/carbohydrates2.html)

Phytochemicals in wheat bran:

"Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, whole grains, and other plant foods that have been associated with reduced risk of major chronic diseases"[11]. The content of phytochemicals in whole grain have been underestimated due to the analysis methods. Since the majority of phytochemicals in whole grain exists in their bound form, the commonly used extraction methods cannot efficiently extract them from the matrix. However, with the development of new knowledge about the bound phenolics and improved extraction technology, scientists have realized that whole grain is also a good source of antioxidants. In addition, whole grain contains unique phytochemicals that rarely found in fruit and vegetables such as ferulic acid[4].

The most important groups of phytochemials in wheat bran are phenolics, carotenoids, vitamin E compounds, and dietary fiber. The phenolics are discussed here. The most important phenolics in wheat bran are phenolics acids and flavonoids.

Flavonoids are a group of compounds that have a similar structure with flavones, with two aroma rings connected by a three-carbon structure[1]. They are the secondary metabolites of plant and cannot be synthesized in human and animals' body[12]. Feng et al. analyzed the flavonoids content in bran of four classes of wheat and found the variety due to the differences in species[13]. The durum wheat had the highest flavonoids content, followed by the white wheat, the hard red winter wheat, and the hard red spring wheat gave the lowest flavonoids content. McCallum and colleges detected the proanthocyanidins in wheat bran that may contribute to the color[14]. Adom et al. found the majority (79%) flavonoids were in the bran/germ fraction of wheat[15]. However, most of flavonoids existed in their bound form, linked to sugar, lignin, polysaccharides or protein within the wheat bran[4].

Phenolics acids are organic compounds with a phenolic ring and an organic carboxylic acid group. Phenolics acids can be categorized as hydroxybenzoic acid and hydroxycinnamic acid derivatives[4]. Common hydroxybenzoic acids derivatives are *p*-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acids. Common hydroxycinnamic acid derivatives are *p*-coumaric, caffeic, ferulic and sinapic acids.

The phenolics acids exist in wheat bran mostly in their bound form. They are either esterified to sugar, lignin, and protein, or ether to lignin. The bound form inhibits its absorption in rumen also in human body. Therefore it reduces the bioaccessibility of phenolics acids.

Extraction and measurement of phenolics:

Extraction methods for phenolics:

Phenolics in plant materials can be extracted through various solvents depending on the chemical nature such as structure and degree of polymerization of phenolics, and their existing form in the system[16]. Therefore, the optimal extraction conditions vary under different situations. The commonly used solvents to extract phenolics in plant materials are methanol, ethanol, acetone, water, ethyl acetate, and their combinations[17].

Several studies optimized the extraction of phenolics in different plant or food systems. Wettasinghe and Shahidi evaluated the antioxidants in evening primrose meal and found the optimal extraction condition was 56% acetone at 71ºC for 47 min[18]. Cacace and colleges used ethanol to extract anthocyanins from black currants and found the solvent to solid ratio is essential. The anthocyanins content increased with increasing ratio. The relationship between the anthocyanins content and the ethanol concentration was a bell-shaped curve with the top spot at 60% ethanol[19], which indicated the importance of the polymerization of anthocyanins during extraction. The extraction of phenolics compounds in wheat bran was optimized using response surface methodology[20]. They investigated three independent variables, namely solvent composition, extraction temperature, and time. The results showed the optimal conditions were 54% ethanol, 61ºC, 64min and 49% ethanol, 64ºC, 60min for whole grain and bran respectively.

Solvents were also used to extract free and bound phenolics. In Adom and Liu's study, they used 80% ethanol to extract the free phenolics in whole grain, whereas, ethyl acetate was used to extract the soluble-conjunct and bound phenolics[21]. In this study, a

similar extraction procedure was implemented with few modifications (see extraction part).

Loss of phenolics is found during the extraction due to the unstable characteristics of antioxidants. When adding with reducing agent, the detected phenolics content was significantly higher after extraction procedure[22], indicating the loss of phenolics during extraction. Therefore, the extraction condition is important especially the extraction temperature and time.

In this study, the extraction methods are modifications of Adom and Liu's[21], which is under ambient temperature and for no more than 1 hr to protect the phenolics in wheat bran.

Measurement of phenolics:

The most commonly used methods to detect total phenolics acids are Folin-Denis and Folin-Ciocalteu reagent. Both will generate a blue colored complex in the base condition when reacting with Folin-Denis or Folin-Ciocalteu reagent[16]. Folin-Ciocalteu reagent are more widely used currently for the analysis of total phenolics acids due to its greater sensitivity and reproducibility compared with Folin-Denis reagent[23]. The disadvantages of the spectrophotometric methods are: firstly, they fail to exclude the influence of protein and other similar structure compounds; secondly, the assay is the interference of reducing chemicals such as ascorbic acid[16].

The methods to separate and quantitate specific phenolics acids are chromatographic separations such as paper chromatography and thin-layer chromatography. The latter is still applied because it is fast, cheap and convenient[24].

HPLC and GC techniques are the primary approach to separate and analyze specific phenolics acid in the last twenty years. GC is less used than HPLC because of the

limited volatility of many phenolic acids even though suitable derivation are available[25]. Reverse phase-HPLC is commonly used to identify single phenolic acid. The column is normally composed of C_{18} stationary phase with length from 10 to 30mm, particle size from 3 to 5 μm, internal diameter from 2.1 to 5 mm. Gradient elution are more employed than isocratic elution. Mobile phase are composed of two solvents. Solvent A is the aqueous solvent with acids added in. Solvent B is the organic solvent. The acid added into solvent A are commonly acetic acid, but other acids such as sulfuric, trifluoroacetic, hydrochloric acids are also used. Solvent B are mostly methanol or acetonitrile, but ethyl acetate, butanol, propanol are employed as well. The run time range from 30 to 150 min. The flute rate varies from 0.15 to 1.8 mL/min, with the most common one 1 mL/min. The injection volume ranges from 10 to 20 μL. The column temperature is set from 20 to 45ºC. Phenolic acids are mostly detected by UV at wavelengths from 190 to 380 nm[24 26 25].

The measurement of flavonoids used $AlCl_3$ assay to generate a flavonoid-aluminum complex, the absorption of which can be read at 510nm[16]. The flavonoids can also be detected by HPLC with similar conditions as the HPLC analysis of phenolics acids[27].

However all the methods have their own limitation. Therefore, coupled methods are applied to find an optimum approach. The best methods are chromatography coupled with either mass or Fourier transform IR spectrometry[28].

In this study, the total phenolics acids are analyzed using Folin-Ciocalteu reagent, and the ferulic acid are analyzed using RP-HPLC technique.

Process technologies:

Physical and thermal processing:

Physical processes include grinding, high shear mixing, and high-pressure homogenization. The physical processes help to reduce the particle size of wheat bran and generate possible chemical structure changes of the dietary fiber in wheat bran. Antoine et al. studied the relationship between particle size and fragmentation, and found smaller particle size (through ball-milling) rapidly induces fractures in walls of cells in the aleurone layer[29]. Chau and colleges analyzed the effects of particle size on the functionality of a fruit insoluble fiber. They extracted the insoluble fiber from the pomace of carambola and micronized using three different micron technologies. Results showed that smaller particle samples contributed to better physicochemical properties such as water-holding capacity, swelling capacity, oil-holding capacity, cation-exchange capacity and glucose-adsorption capacity[30]. In Ferguson's study, larger particle size wheat bran could reduce the transit time in GI-tract in rats thus reduce the risk of colorectal cancer. It also increased the rate of excretion of faeces[31].

The most commonly used thermal process in food is cooking. Several scientists conducted research on the effects of heating on the antioxidant properties in whole grain. In Dietrych-Szostak's study, heating treatment drastically reduced the flavonoid content in buckwheat[32]. Li et al. also found the lost of total phenolics after heating of purple wheat bran, but it did not affect the ORAC and DPPH value[33]. However, heat treated sweet corn gave higher antioxidant activity due to the releasing of bound phenolics acids, even though 25% of vitamin C was lost during the heating process[34].

Extrusion-cooking is a technology using both thermal and physical treatments. Samples were treated at high temperature, under high pressure, under high shear forces,

and for a short time. Dietary fiber after extrusion-cooking were more soluble and easier for fermentation[35]. In wheat flour, the dietary fiber slight increased after extrusion-cooking due to the increase of non-digestible starch. The dietary fiber were more degraded[36]. Wang et al. found similar evidences when using twin-screw extrusion. In addition, they analyzed the baking quality of the extruded wheat bran, stating that the extruded wheat bran was an acceptable ingredient to be added into whole grain bread[37].

Biological processing:

Since phenolics are bound to arabinoxylans in wheat bran, reaction with certain enzymes can cleave the bound linkage and release the phenolics. The commonly used enzymes to release bound phenolics from wheat bran are xylanase, cellulase and ferulic acid esterase. Xylanase is a class of enzyme to cleave the linear β-1,4-xylan in to xylose. Cellulase is to hydrolysis the 1,4-β-D-glycodidic linkage. Ferulic acid esterase is a specific enzyme to cleave the ester linkage between ferulic acid and polysaccharides.

Several scientists studied the enzyme efficiency in releasing free phenolics in wheat bran. Maes et al. used two endoxylanases to solubilize water-unextractable arabinoxylans[38]. Szwajgier and Targonski also used five commercial enzyme preparations (Chlluclast, Viscozyme, Shearzyme, Cereflo, and Ultraflo) to treat brewer's spent grain to release free ferulic acids[39]. The use of ferulic acid esterase (FAE) had been proven to be very effective in releasing ferulic acid from wheat bran, but different kinds of ferulic acid esterases had different specificity and efficiency[40]. Faulds et al. treated wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger* together with a Trichoderma viride xylanase and released 95% total ferulic acid after 5 h incubation. They also showed that FAE could release ferulic acid on its own but the release was 24-

fold higher in the presence of xylanase. These might be because the prior work done by xylanase opened the structure of arabinoxylans, thus making ferulic acid esterase access to their active sites easier[41]. Another studies also showed the synergic effect of combined xylanase and ferulic acid esterase on releasing bound ferulic acid in wheat bran[42 43 44]. Faulds also prepared an enzyme from *Humicola insolens* with ferulic acid esterase activity that could release almost all of the ferulic acid[45].

Chemical processing:

Chemical treatments are another approach to release bound phenolics in plant cell walls besides the enzymatic treatment. In previous study, severe alkaline treatment was used to extract bound phenolics in wheat bran for the analysis purpose[46 26]. Adom and Liu analyzed the free, soluble-conjunct, and bound phenolics in wheat, corn and other uncooked whole grains using the alkali treatment to extract soluble-conjunct, and bound phenolics[21]. The commonly used chemical treatment is the alkaline treatment because several studies compared the efficiency of acid and alkaline treatment on releasing the bound phenolics in plant cell walls. Results showed that alkaline treatment is more efficient[47 48]. In Kim and his associates' study, the alkaline treatment (2M NaOH, 4h) could release more bound phenolics in wheat bran than the acid treatment (6M HCl, 1h, at 95ºC). In addition, the alkaline extracts had high antioxidant activity than the acid extracts. Verma et al. agreed with Kim's study, stating "the phenolics liberated by acid hydrolysis were significantly less than the phenolics liberated by alkaline hydrolysis". Alkaline and acid hydrolysis had different effects on releasing different phenolics acids. Alkaline hydrolysis was more efficient on releasing vanillic, cis-ferulic and sinapic acids. Whereas caffeic acid could only be found in the acidic-labile fraction, the p-coumaric acid could only be found in the alkali-labile fraction. However, both treatments had

similar effects on releasing trans-ferulic acid in wheat bran[48]. However, Arranz et al. published a paper one year later to demonstrate that acidic hydrolysis performed better in releasing polyphenols in cereals compared with the alkali hydrolysis. They explained it due to the differences in the acidic hydrolysis condition. Previous study used a mild acidic hydrolysis condition that could not release as much as bound phenolics possible. They also stated that their acidic hydrolysis could release the bound phenolics "that are trapped within the cores or bound to cell wall constituents", which is impossible to be done through alkali hydrolysis[49]. Other studies also developed combined alkali and acidic hydrolysis to release all the bound phenolics in grains[50].

Chapter 1: Enzymatic treatment of wheat bran in releasing bound phenolics acids in wheat bran

Introduction:

Since most phenolics are bound to polysaccharides in wheat bran reported by Liu etc. in 2002, stating that 75% of the total phenolics existed in their bound form[21], studies aiming to release the bound phenolics are necessary to enhance the bioactivity of wheat bran. Several studies examined the phenolic bound carbohydrate structure in wheat[51 52 53 54]. They found esterified ferulic acid, and diferulates bound to arabinose in arabinoxylans. Arabinoxylan is a hemicallulose with the main chain of 1,4-linked xylose units, substituted with 2,3 or 2,3-linked arabinose residues (Fig). Therefore, enzymes with specify to cleave the ester linkage or arabinoxylans are likely to release the bound phenolics in wheat bran to gain better bioactivity and bioaccessibility.

The commonly used enzymes to release bound phenolics from wheat bran are xylanase, cellulase and ferulic acid esterase. Xylanase is a class of enzyme to cleave the linear β-1,4-xylan in to xylose. Cellulase is to hydrolysis the 1,4-β-D-glycodidic linkage. Ferulic acid esterase is a specific enzyme to cleave the ester linkage between ferulic acid and polysaccharides. Several scientists studied the enzyme efficiency in releasing free phenolics in wheat bran. Maes et al. used two endoxylanases to solubilize water-unextractable arabinoxylans[38]. Szwajgier and Targonski also used five commercial enzyme preparations (Chlluclast, Viscozyme, Shearzyme, Cereflo, and Ultraflo) to treat brewer's spent grain to release free ferulic acids[39]. The use of ferulic acid esterase (FAE) had been proven to be very effective in releasing ferulic acid from wheat bran, but different kinds of ferulic acid esterases had different specificity and efficiency[40]. Faulds et al. treated wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger*

together with a Trichoderma viride xylanase and released 95% total ferulic acid after 5 h incubation. They also showed that FAE could release ferulic acid on its own but the release was 24-fold higher in the presence of xylanase. These might be because the prior work done by xylanase opened the structure of arabinoxylans, thus making ferulic acid esterase access to their active sites easier[41]. Another studies also showed the synergic effect of combined xylanase and ferulic acid esterase on releasing bound ferulic acid in wheat bran[42 43 44]. Faulds also prepared an enzyme from *Humicola insolens* with ferulic acid esterase activity that could release almost all of the ferulic acid[45].

In this study, xylanase, and cellulase were used to release bound phenolics and the optimal condition was developed and evaluated. The reason of not using ferulic acid esterase is that there has no commercialized ferulic acid esterase available at this moment. Our work is to release the majority bound phenolics in wheat bran using common enzymes coupled with other physical, chemical treatments.

Part 1 Enzymatic treatment

Materials:

King wheat bran is a light soft white winter wheat and was obtained from King Milling Company.

Enzymes:

Xylanase BX-AN and cellulase were purchased from Enzyme Development Company (New York, NY).

Methods:

The factorial experimental design was implement to determine the optimum condition for enzyme treatment. The variables and settings were listed in Table 1 below. 0.5000 g control wheat bran was blended with 25 mL distill water and certain amount of

enzyme to make slurry with 2% dry matter. The slurry was then stirred at 200 rpm at certain temperature for certain time (See table) using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried for further phenolics acids analysis.

Table 1 Factorial experimental design for the single enzyme treatment

Factors	Levels		
Enzyme	Xylanase	Cellulase	Xyl+Cel (1:1)
Enzyme Conc. (w/w)	0.3%	0.5%	1.0%
Time (h)	6	15	24
Temp. (°C)	30		50

Part 2: Extraction of free phenolics in enzymatic treated wheat bran

Materials:

Enzymatic treated wheat bran

Chemicals:

Ethanol was purchased from the University of Minnesota chemical store.

Reagent:

3L 80% ethanol solution: 2.4 L ethanol was measured using a 1 L measuring cylinder and mixed thoroughly with 0.6 L distilled water measured using a 1 L measuring cylinder.

Methods:

0.5000 g of each single enzymatic treated wheat bran samples (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight and added with 20 mL ethanol solution (80%) in 50 mL centrifuge tubes. All the samples were then stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY,

USA). After that, they were centrifuged at 2,000 g for 20 min. The supernatant was collected and poured into a new 50 mL centrifuge tube. The left residue was added with 20 mL ethanol solution (80%) to perform the extraction one more time. The supernatants were pooled (40 mL) and vacuum evaporated at 40ºC until no more than 10 mL liquid left. The final extracts were filled up to 10 mL using distilled water. The free antioxidant extracts were stored at -76ºC for further analysis. Duplicates of each sample were made and the standard deviations were calculated.

Part 3: Free Phenolics acids in enzyme treated wheat bran

Materials:

Free phenolics extracts of enzymatic treated wheat bran

Chemicals:

Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (Wilwaukee, WI, USA). Sodium carbonate was purchased from Fisher Chemicals (Pittsburgh, PA, USA).

Reagent:

200 mL 10% Sodium carbonate solution: 22.22 g sodium carbonate was weighed and added with 200 mL distilled water in a 500 mL beaker. Gently swirl the beaker until the solids were dissolved completely.

Methods:

0.2 mL of extracts were added with 0.2 mL Folin-Ciocalteu reagent in 12 mL tubes and stirred for 10 s before adding 0.8 mL sodium carbonate solution (10%) after 8 min. The mixture was then stirred and added with distilled water to a total volume of 5 mL. The absorbance value at 760nm was measured using a spectrophotometer after 90 min. Duplicates were made and the standard deviation was calculated.

Results:

The free phenolics acids contents were expressed as micrograms of gallic acid equivalent per 100 gram of wheat bran. The results were shown in Fig 2-1. As we can see, the free phenolics acids content of 1.0% xylanase treated wheat bran is the highest (30C 6h: 8.070 ± 0.675 mg gallic acid/100 g bran; 30C 15h: 9.710 ± 0.460 mg gallic acid/100 g bran; 30C 24h: 13.703 ± 0.994 mg gallic acid/100 g bran; 50C 6h: 15.016 ± 2.592 mg gallic acid/100 g bran; 50C 15h: 16.549 ± 0.287 mg gallic acid /100 g bran; 50C 24h: 17.622 ± 1.059 mg gallic acid/100 g bran), followed by 0.5% enzyme treated wheat bran (30C 6h: 7.388 mg gallic acid/100 g bran; 30C 15h: 6.735 ± 0.524 mg gallic acid/100 g bran; 30C 24h: 10.708 ± 1.523 mg gallic acid/100 g bran; 50C 6h: 9.009 ± 2.046 mg gallic acid/100 g bran; 50C 15h: 10.745 ± 1.014 mg gallic acid /100 g bran; 50C 24h: 15.065 ± 1.801 mg gallic acid/100 g bran). The 0.3% concentration xylanase treated wheat bran gave the lowest free phenolics acids (30C 6h: 3.994 ± 0.993 mg gallic acid/100 g bran; 30C 15h: 6.760 ± 0.404 mg gallic acid/100 g bran; 30C 24h: 6.816 ± 1.027 mg gallic acid/100 g bran; 50C 6h: 8.482 ± 1.127 mg gallic acid/100 g bran; 50C 15h: 9.300 ± 0.587 mg gallic acid /100 g bran; 50C 24h: 11.041 mg gallic acid/100 g bran).

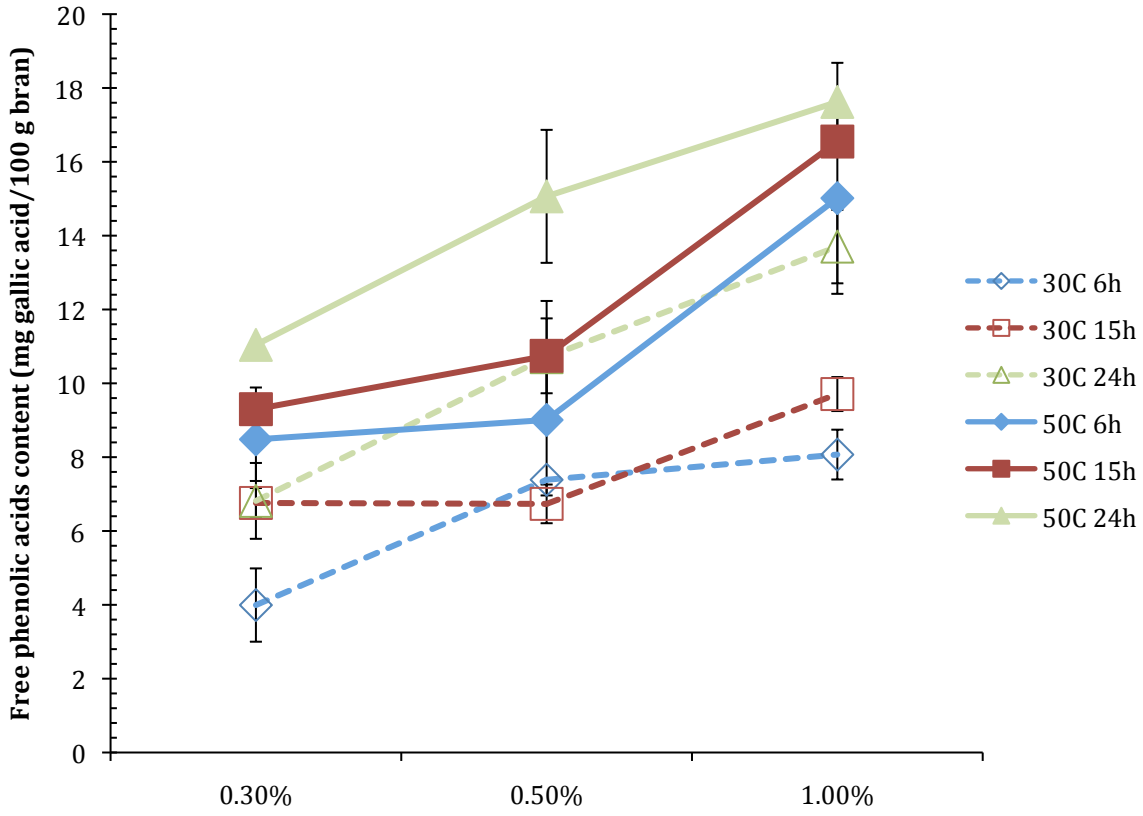


Figure 2-1 Free phenolics acids content in xylanase treated wheat bran.

Fig 2-2. Showed the free phenolics acids content in cellulase treated wheat bran. The 1.0% cellulase concentration treated wheat bran gave the highest phenolics acids content (30C 6h: 8.199 ± 1.581 mg gallic acid/100 g bran; 30C 15h: 6.144 mg gallic acid/100 g bran; 30C 24h: 9.285 ± 0.041 mg gallic acid/100 g bran; 50C 6h: 8.298 ± 0.726 mg gallic acid/100 g bran; 50C 15h: 12.967 ± 2.087 mg gallic acid /100 g bran; 50C 24h: 14.221 ± 2.483 mg gallic acid/100 g bran), which is similar to xylanase treated wheat bran, but the 30C 24h sample with 1.0% cellulase (9.285 ± 0.041 mg gallic acid/100 g bran) is lower than that with 0.3% cellulase (12.379 ± 1.071 mg gallic acid/100 g bran). The 0.3% and 0.5% cellulase treated wheat bran had similar free phenolics acids content (0.3%: 30C 6h: 7.743 ± 0.648 mg gallic acid/100 g bran; 30C

15h: 6.594 ± 1.240 mg gallic acid/100 g bran; 30C 24h: 12.379 ± 1.071 mg gallic acid/100 g bran; 50C 6h: 7.797 ± 0.462 mg gallic acid/100 g bran; 50C 15h: 8.982 ± 1.079 mg gallic acid /100 g bran; 50C 24h: 10.472 ± 1.654 mg gallic acid/100 g bran; 0.5%: 30C 6h: 7.270 ± 1.967 mg gallic acid/100 g bran; 30C 15h: 6.141 ± 0.069 mg gallic acid/100 g bran; 30C 24h: 8.535 ± 0.781 mg gallic acid/100 g bran; 50C 6h: 5.677 ± 1.203 mg gallic acid/100 g bran; 50C 15h: 7.796 ± 0.308 mg gallic acid /100 g bran; 50C 24h: 13.794 ± 0.730 mg gallic acid/100 g bran). However, most 0.5% cellulase treated wheat bran had a lower free phenolics acids content than those treated with 0.3% cellulase, expect the 50C 24h sample (13.794 ± 0.730 mg gallic acid/100 g bran). The 0.5% cellulase treated 50C 24h sample reached almost the same as that treated with 1.0% cellulase (14.221 ± 2.483 mg gallic acid/100 g bran).

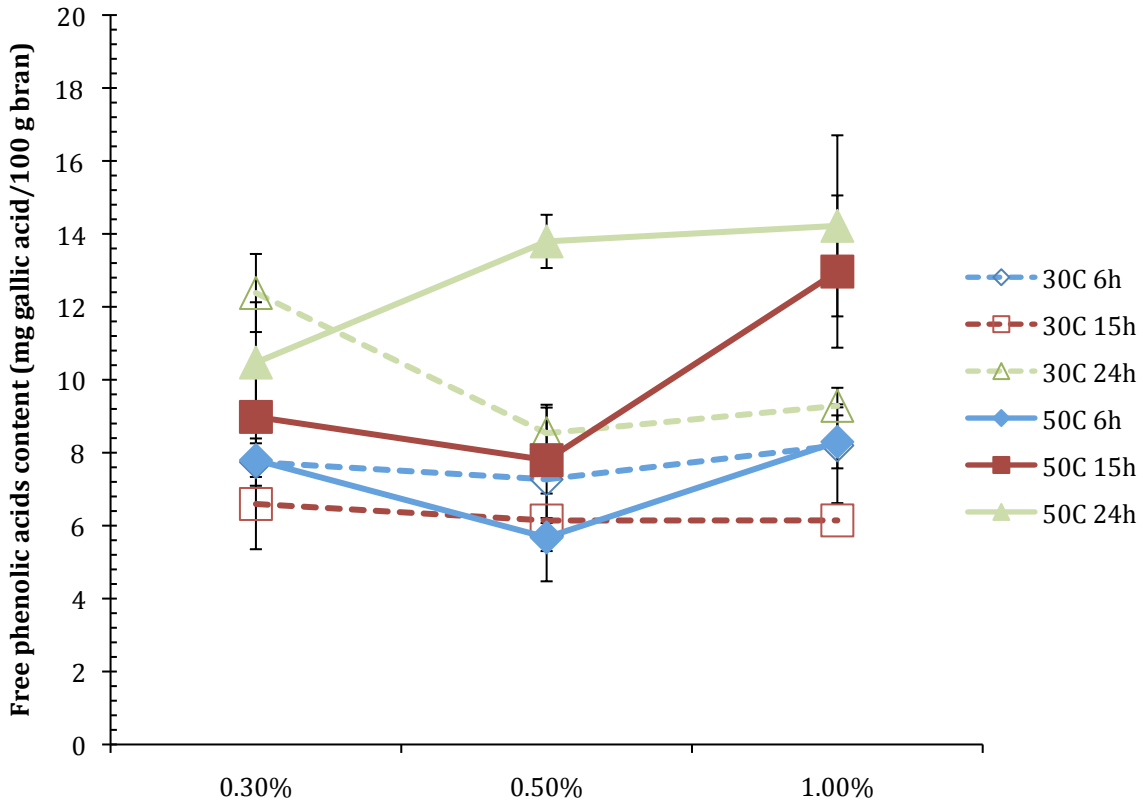


Figure 2-2 Free phenolics acids content in cellulase treated wheat bran.

The free phenolics acids content in combined xylanase and cellulase treated wheat bran had similar trend with the cellulase treated ones (Fig. 2-3). The 1.0% enzyme treated wheat bran gave the highest content (30C 6h: 8.950 ± 0.323 mg gallic acid/100 g bran; 30C 15h: 8.210 ± 0.789 mg gallic acid/100 g bran; 30C 24h: 9.413 ± 0.781 mg gallic acid/100 g bran; 50C 6h: 10.377 ± 0.803 mg gallic acid/100 g bran; 50C 15h: 12.998 ± 0.168 mg gallic acid /100 g bran; 50C 24h: 16.333 ± 0.863 mg gallic acid/100 g bran). The 0.3% and 0.5% enzyme treated gave similar free phenolics acids contents (0.3%: 30C 6h: 5.842 ± 1.928 mg gallic acid/100 g bran; 30C 15h: 5.903 ± 0.798 mg gallic acid/100 g bran; 30C 24h: 11.331 mg gallic acid/100 g bran; 50C 6h: 8.610 ± 1.333 mg

gallic acid/100 g bran; 50C 15h: 8.785 ± 0.000 mg gallic acid /100 g bran; 50C 24h: 13.043 ± 1.681 mg gallic acid/100 g bran; 0.5%: 30C 6h: 8.436 ± 0.428 mg gallic acid/100 g bran; 30C 15h: 7.598 ± 0.646 mg gallic acid/100 g bran; 30C 24h: 7.363 ± 0.259 mg gallic acid/100 g bran; 50C 6h: 8.103 ± 2.320 mg gallic acid/100 g bran; 50C 15h: 11.167 ± 0.943 mg gallic acid /100 g bran; 50C 24h: 13.170 ± 0.989 mg gallic acid/100 g bran). Some interesting samples are 0.3% 30C 24h (11.331 mg gallic acid/100 g bran, higher than 0.5% and 1.0% 30C 24h samples), and 0.3% 50C 6h (8.610 ± 1.333 mg gallic acid/100 g bran, higher than 0.5% 50C 6h sample).

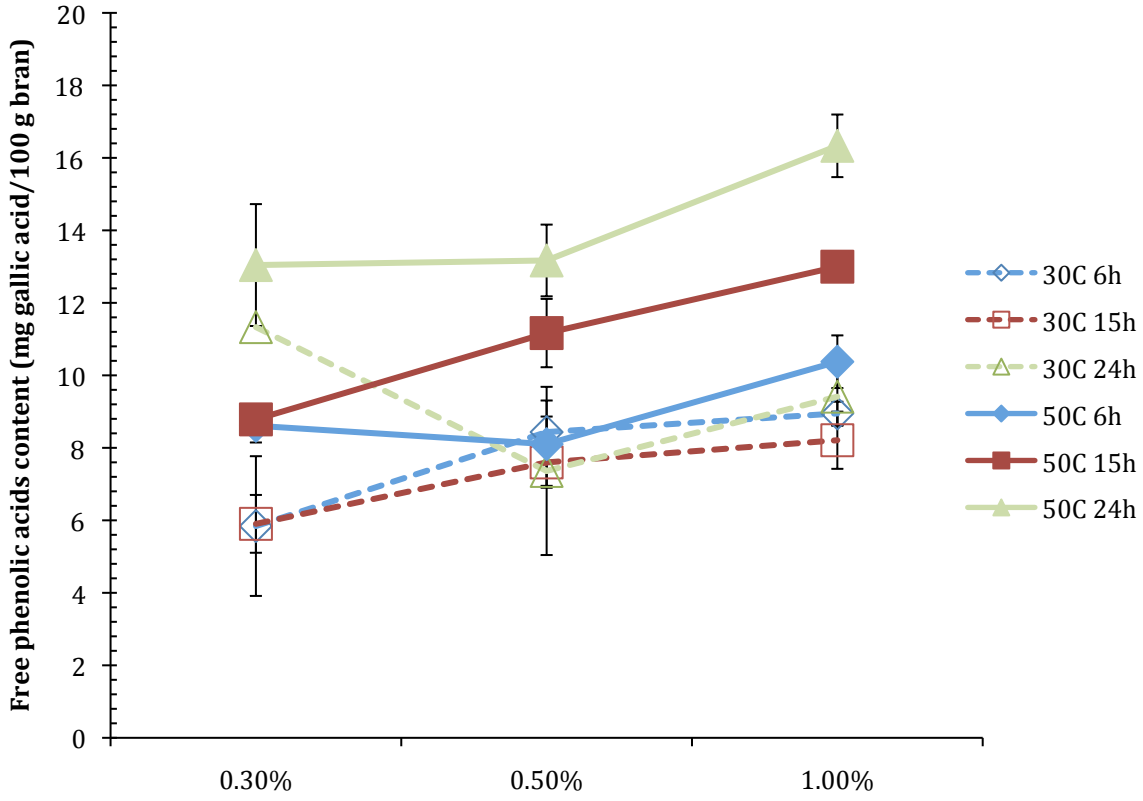


Figure 2-3 Free phenolics acids content in combined cellulase and xylanase treated wheat bran.

The effects of the single and combined enzymatic treatment on releasing free phenolics acids in wheat bran are not significantly different when the enzyme

concentration is 0.3% (Fig. 2-4). The cellulase and combined enzyme treated wheat bran appeared in similar trend (Cellulase 30C 6h: 7.743 ± 0.648 mg gallic acid/100 g bran; 30C 15h: 6.594 ± 1.240 mg gallic acid/100 g bran; 30C 24h: 12.379 ± 1.071 mg gallic acid/100 g bran; 50C 6h: 7.797 ± 0.462 mg gallic acid/100 g bran; 50C 15h: 8.982 ± 1.079 mg gallic acid /100 g bran; 50C 24h: 10.472 ± 1.654 mg gallic acid/100 g bran; Combined enzyme: 30C 6h: 5.842 ± 1.928 mg gallic acid/100 g bran; 30C 15h: 5.903 ± 0.798 mg gallic acid/100 g bran; 30C 24h: 11.331 mg gallic acid/100 g bran; 50C 6h: 8.610 ± 1.333 mg gallic acid/100 g bran; 50C 15h: 8.785 ± 0.000 mg gallic acid /100 g bran; 50C 24h: 13.043 ± 1.681 mg gallic acid/100 g bran), while the xylanase treated wheat bran gave a lower free phenolics acids content (30C 6h: 5.842 ± 1.928 mg gallic acid/100 g bran; 30C 15h: 5.903 ± 0.798 mg gallic acid/100 g bran; 30C 24h: 11.331 mg gallic acid/100 g bran; 50C 6h: 8.610 ± 1.333 mg gallic acid/100 g bran; 50C 15h: 8.785 ± 0.000 mg gallic acid /100 g bran; 50C 24h: 13.043 ± 1.681 mg gallic acid/100 g bran).

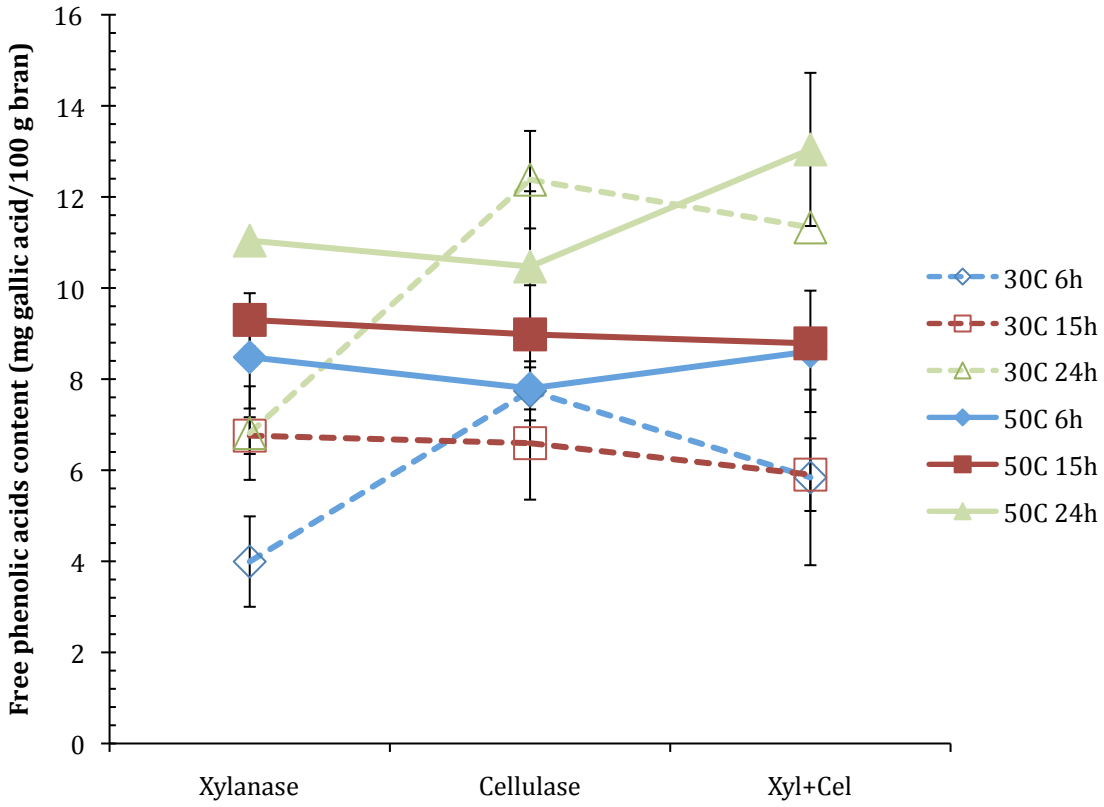


Figure 2-4 Free phenolics acids content in 0.3% enzyme treated wheat bran.

The 0.5% enzyme treated wheat bran gave a significant difference among the enzyme type levels (Fig. 2-5). The xylanase treated wheat bran had the highest free phenolics acids content (30C 6h: 7.388 mg gallic acid/100 g bran; 30C 15h: 6.735 ± 0.524 mg gallic acid/100 g bran; 30C 24h: 10.708 ± 1.523 mg gallic acid/100 g bran; 50C 6h: 9.009 ± 2.046 mg gallic acid/100 g bran; 50C 15h: 10.745 ± 1.014 mg gallic acid /100 g bran; 50C 24h: 15.065 ± 1.801 mg gallic acid/100 g bran). The cellulase gave the lowest free phenolics acids content (30C 6h: 7.270 ± 1.967 mg gallic acid/100 g bran; 30C 15h: 6.141 ± 0.069 mg gallic acid/100 g bran; 30C 24h: 8.535 ± 0.781 mg gallic acid/100 g bran; 50C 6h: 5.677 ± 1.203 mg gallic acid/100 g bran; 50C 15h: 7.796 ± 0.308 mg gallic acid /100 g bran; 50C 24h: 13.794 ± 0.730 mg gallic acid/100 g bran).

The free phenolics acids content of combined enzyme treated wheat bran lied in between (30C 6h: 8.436 ± 0.428 mg gallic acid/100 g bran; 30C 15h: 7.598 ± 0.646 mg gallic acid/100 g bran; 30C 24h: 7.363 ± 0.259 mg gallic acid/100 g bran; 50C 6h: 8.103 ± 2.320 mg gallic acid/100 g bran; 50C 15h: 11.167 ± 0.943 mg gallic acid /100 g bran; 50C 24h: 13.170 ± 0.989 mg gallic acid/100 g bran).

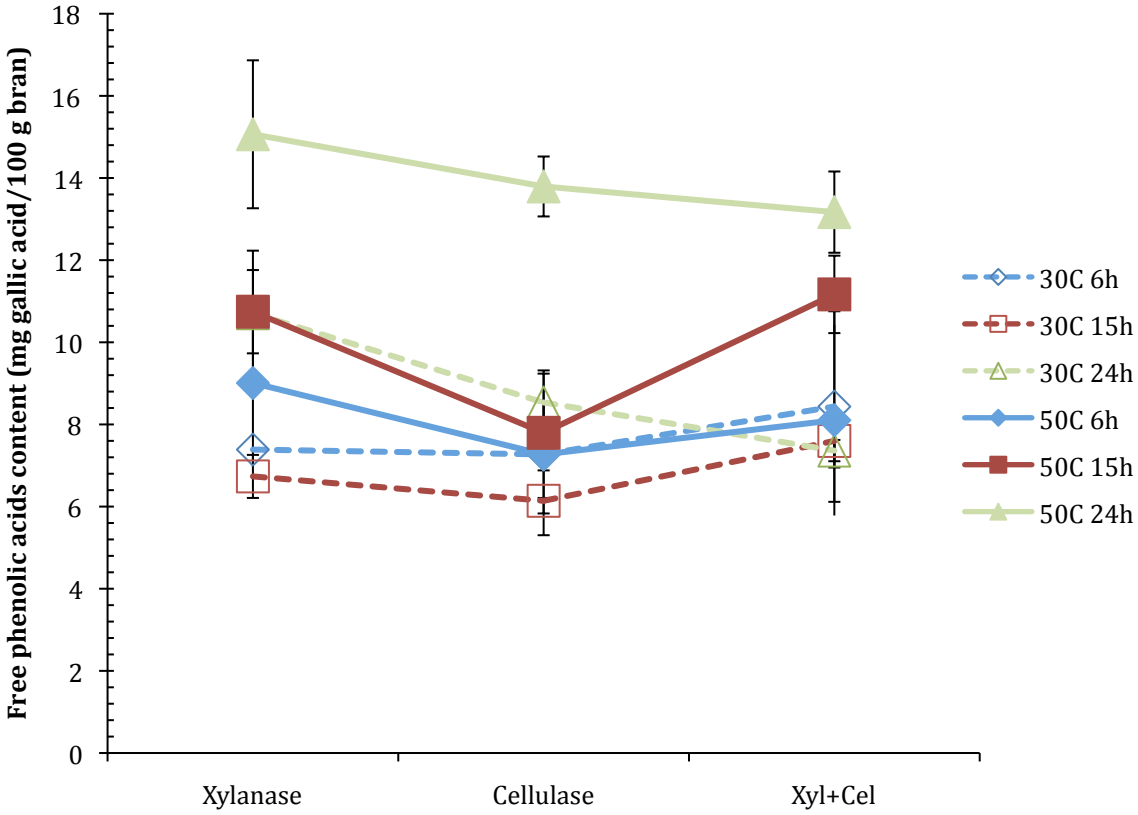


Figure 2-5 Free phenolics acids content in 0.5% enzyme treated wheat bran.

The free phenolics acids content in 1.0% treated wheat bran had the same trend with that in the 0.5% treated ones (Fig. 2-6). Xylanase is the most efficient enzyme to release bound phenolics acids in wheat bran (30C 6h: 8.070 ± 0.675 mg gallic acid/100 g bran; 30C 15h: 9.710 ± 0.460 mg gallic acid/100 g bran; 30C 24h: 13.703 ± 0.994 mg gallic acid/100 g bran; 50C 6h: 15.016 ± 2.592 mg gallic acid/100 g bran; 50C 15h:

16.549 ± 0.287 mg gallic acid /100 g bran; 50C 24h: 17.622 ± 1.059 mg gallic acid/100 g bran), followed by combined enzyme treated sample (30C 6h: 8.950 ± 0.323 mg gallic acid/100 g bran; 30C 15h: 8.210 ± 0.789 mg gallic acid/100 g bran; 30C 24h: 9.413 ± 0.781 mg gallic acid/100 g bran; 50C 6h: 10.377 ± 0.803 mg gallic acid/100 g bran; 50C 15h: 12.998 ± 0.168 mg gallic acid /100 g bran; 50C 24h: 16.333 ± 0.863 mg gallic acid/100 g bran). The lowest free phenolics acids content was the cellulase treated wheat bran (30C 6h: 8.199 ± 1.581 mg gallic acid/100 g bran; 30C 15h: 6.144 mg gallic acid/100 g bran; 30C 24h: 9.285 ± 0.041 mg gallic acid/100 g bran; 50C 6h: 8.298 ± 0.726 mg gallic acid/100 g bran; 50C 15h: 12.967 ± 2.087 mg gallic acid /100 g bran; 50C 24h: 14.221 ± 2.483 mg gallic acid/100 g bran).

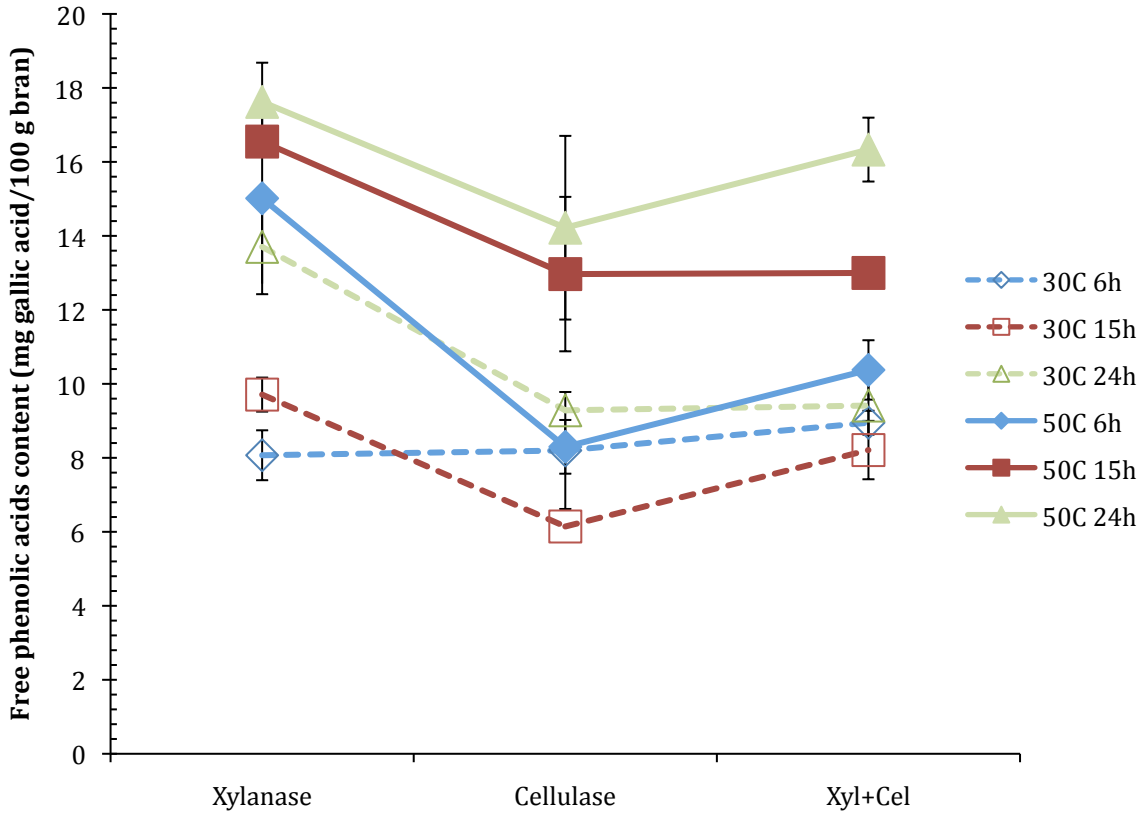


Figure 2-6 Free phenolics acids content in 1.0% enzyme treated wheat bran.

As we can see in Fig. 2-7, the longest enzyme treatment time (24h) gave the highest free phenolics acid contents in xylanase treated wheat bran (Xyl 0.3% 30C: 6.816 ± 1.027 mg gallic acid/100 g bran, Xyl 0.5% 30C: 10.708 ± 1.523 mg gallic acid/100 g bran, Xyl 1.0% 30C: 13.703 ± 0.994 mg gallic acid/100 g bran, Xyl 0.3% 50C: 11.041 mg gallic acid/100 g bran, Xyl 0.5% 50C: 15.065 ± 1.801 mg gallic acid/100 g bran, Xyl 1.0% 50C: 17.622 ± 1.059 mg gallic acid/100 g bran). The shortest treatment time (6h) contributed to the lowest free phenolics acids content (Xyl 0.3% 30C: 3.994 ± 0.993 mg gallic acid/100 g bran, Xyl 0.5% 30C: 7.388 mg gallic acid/100 g bran, Xyl 1.0% 30C: 8.070 ± 0.675 mg gallic acid/100 g bran, Xyl 0.3% 50C: 8.482 ± 1.127 mg gallic acid/100 g bran, Xyl 0.5% 50C: 9.009 ± 2.046 mg gallic acid/100 g bran, Xyl 1.0% 50C: 15.016 ± 2.592 mg gallic acid/100 g bran). The free phenolics acids content of the 15 hrs treatment time sample lied in between (Xyl 0.3% 30C: 6.760 ± 0.404 mg gallic acid/100 g bran, Xyl 0.5% 30C: 6.735 ± 0.524 mg gallic acid/100 g bran, Xyl 1.0% 30C: 9.710 ± 0.460 mg gallic acid/100 g bran, Xyl 0.3% 50C: 9.300 ± 0.587 mg gallic acid/100 g bran, Xyl 0.5% 50C: 10.745 ± 1.014 mg gallic acid/100 g bran, Xyl 1.0% 50C: 16.549 ± 0.287 mg gallic acid/100 g bran).

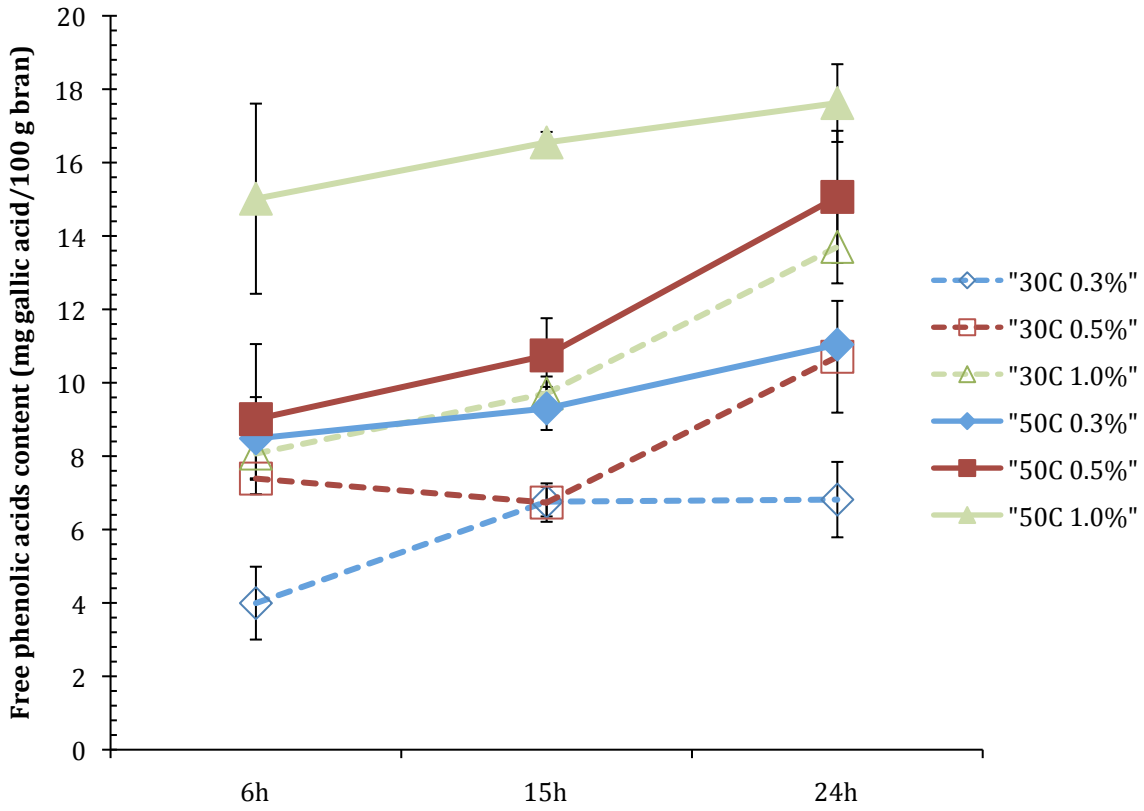


Figure 2-7 Free phenolics acids content in xylanase treated wheat bran.

The effect of treatment time on releasing free phenolics acids in cellulase treated wheat bran was significant (Fig. 2-8). Longer treatment time contributed to more free phenolics acids. The free phenolics acids content of the 24 h treated samples are Cel 0.3% 30C: 12.379 ± 1.071 mg gallic acid/100 g bran, Cel 0.5% 30C: 8.535 ± 0.781 mg gallic acid/100 g bran, Cel 1.0% 30C: 9.285 ± 0.041 mg gallic acid/100 g bran, Cel 0.3% 50C: 10.472 ± 1.654 mg gallic acid/100 g bran, Cel 0.5% 50C: 13.794 ± 0.730 mg gallic acid/100 g bran, Cel 1.0% 50C: 14.221 ± 2.483 mg gallic acid/100 g bran. The free phenolics acids of the 15 hrs treated samples are Cel 0.3% 30C: 6.594 ± 1.240 mg gallic acid/100 g bran, Cel 0.5% 30C: 6.141 ± 0.069 mg gallic acid/100 g bran, Cel 1.0% 30C:

6.144 mg gallic acid/100 g bran, Cel 0.3% 50C: 8.982 ± 1.079 mg gallic acid/100 g bran, Cel 0.5% 50C: 7.796 ± 0.308 mg gallic acid/100 g bran, Cel 1.0% 50C: 12.967 ± 2.087 mg gallic acid/100 g bran. The free phenolics acids content of the 6 hrs treated samples are Cel 0.3% 30C: 7.743 ± 0.648 mg gallic acid/100 g bran, Cel 0.5% 30C: 7.270 ± 1.967 mg gallic acid/100 g bran, Cel 1.0% 30C: 8.199 ± 1.581 mg gallic acid/100 g bran, Cel 0.3% 50C: 7.797 ± 0.462 mg gallic acid/100 g bran, Cel 0.5% 50C: 5.677 ± 1.203 mg gallic acid/100 g bran, Cel 1.0% 50C: 8.298 ± 0.726 mg gallic acid/100 g bran.

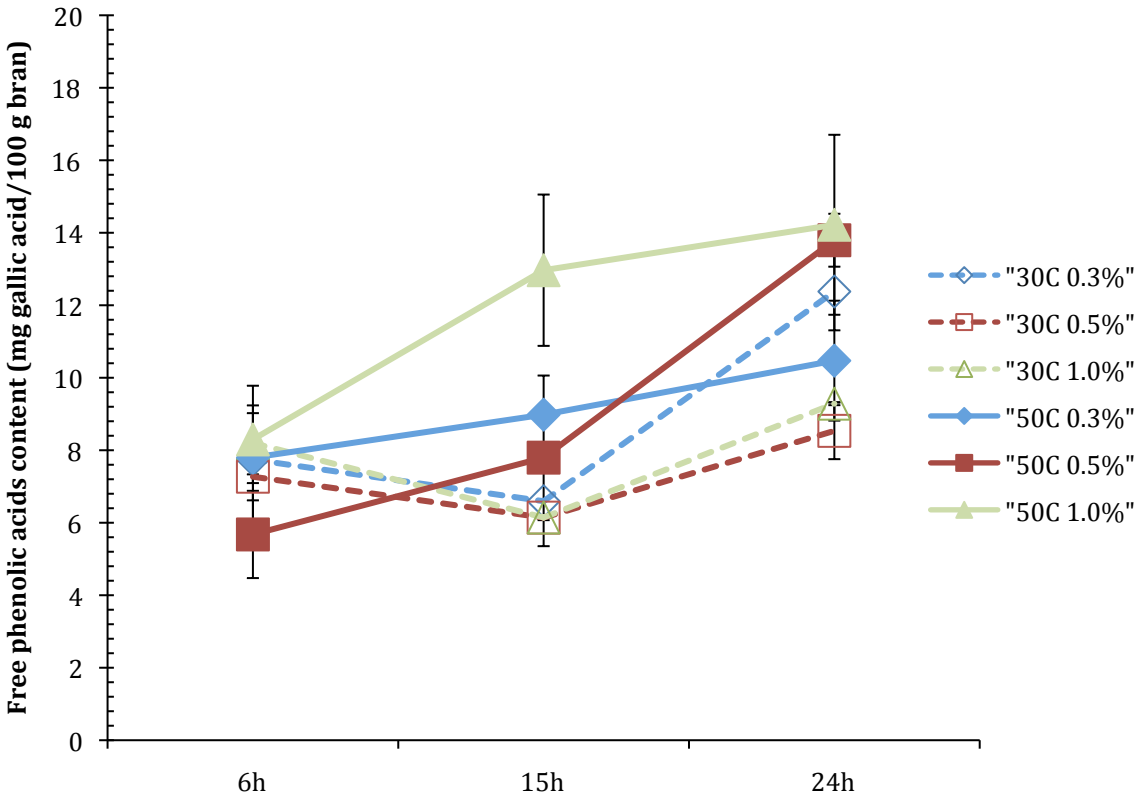


Figure 2-8 Free phenolics acids content in cellulase treated wheat bran.

The combined enzyme treated wheat bran had the same free phenolics content trend with single enzyme treated wheat bran when time is the factor (Fig. 2-9). Longer time gave more free phenolics acids content. The 24 h treated samples gave the highest

free phenolics acids content (Both 0.3% 30C: 11.331 mg gallic acid/100 g bran, Both 0.5% 30C: 7.363 ± 0.259 mg gallic acid/100 g bran, Both 1.0% 30C: 9.413 ± 0.781 mg gallic acid/100 g bran, Both 0.3% 50C: 13.043 ± 1.681 mg gallic acid/100 g bran, Both 0.5% 50C: 13.170 ± 0.989 mg gallic acid/100 g bran, Both 1.0% 50C: 16.333 ± 0.863 mg gallic acid/100 g bran), followed by the 15 h treated samples (Both 0.3% 30C: 5.903 ± 0.798 mg gallic acid/100 g bran, Both 0.5% 30C: 7.598 ± 0.646 mg gallic acid/100 g bran, Both 1.0% 30C: 8.210 ± 0.789 mg gallic acid/100 g bran, Both 0.3% 50C: 8.785 ± 0.000 mg gallic acid/100 g bran, Both 0.5% 50C: 11.167 ± 0.943 mg gallic acid/100 g bran, Both 1.0% 50C: 12.998 ± 0.168 mg gallic acid/100 g bran). The 6 h treated samples had the lowest free phenolics acids content (Both 0.3% 30C: 5.842 ± 1.928 mg gallic acid/100 g bran, Both 0.5% 30C: 8.436 ± 0.428 mg gallic acid/100 g bran, Both 1.0% 30C: 8.950 ± 0.323 mg gallic acid/100 g bran, Both 0.3% 50C: 8.610 ± 1.333 mg gallic acid/100 g bran, Both 0.5% 50C: 8.103 ± 2.320 mg gallic acid/100 g bran, Both 1.0% 50C: 10.377 ± 0.803 mg gallic acid/100 g bran).

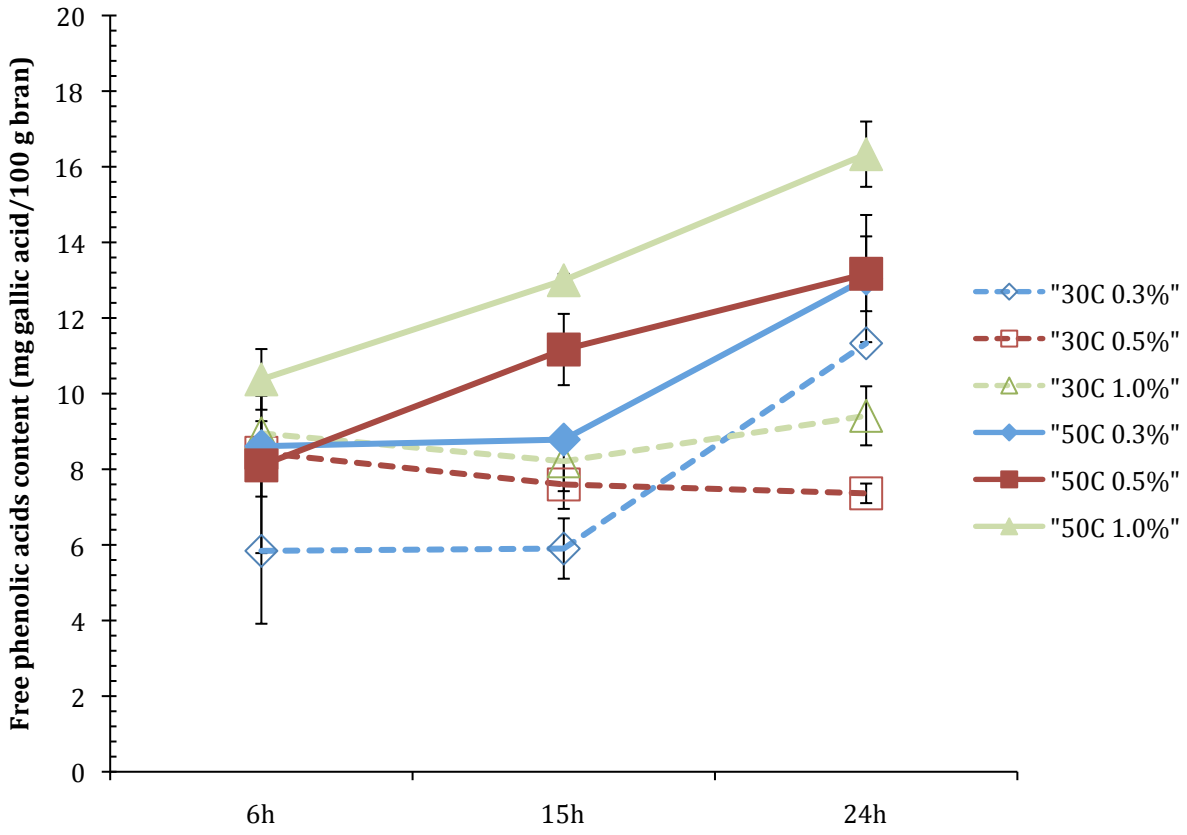


Figure 2-9 Free phenolics acids content in combined cellulase and xylanase treated wheat bran.

When the treatment time is six hours, the effects of treatment temperature on releasing free phenolics acids in enzymatic treated wheat bran are significant different, especially to the xylanase treated wheat bran, which indicated that xylanase may be more sensitive to temperature (Fig. 2-10). The free phenolics acids contents in 30ºC 6h treated samples are Xyl 0.3%: 3.994 ± 0.993 mg gallic acid/100 g bran, Xyl 0.5%: 7.388 mg gallic acid/100 g bran, Xyl 1.0%: 8.070 ± 0.675 mg gallic acid/100 g bran, Cel 0.3%: 7.743 ± 0.648 mg gallic acid/100 g bran, Cel 0.5%: 7.270 ± 1.967 mg gallic acid/100 g bran, Cel 1.0%: 8.199 ± 1.581 mg gallic acid/100 g bran, Both 0.3%: 5.842 ± 1.928 mg gallic acid/100 g bran, Both 0.5%: 8.436 ± 0.428 mg gallic acid/100 g bran, Both 1.0%:

8.950 ± 0.323 mg gallic acid/100 g bran. The 50ºC 6h samples gave higher free phenolics acids contents (Xyl 0.3%: 8.482 ± 1.127 mg gallic acid/100 g bran, Xyl 0.5%: 9.009 ± 2.046 mg gallic acid/100 g bran, Xyl 1.0%: 15.016 ± 2.592 mg gallic acid/100 g bran, Cel 0.3%: 7.797 ± 0.462 mg gallic acid/100 g bran, Cel 0.5%: 5.677 ± 1.203 mg gallic acid/100 g bran, Cel 1.0%: 8.298 ± 0.726 mg gallic acid/100 g bran, Both 0.3%: 8.610 ± 1.333 mg gallic acid/100 g bran, Both 0.5%: 8.103 ± 2.320 mg gallic acid/100 g bran, Both 1.0%: 10.377 ± 0.803 mg gallic acid/100 g bran).

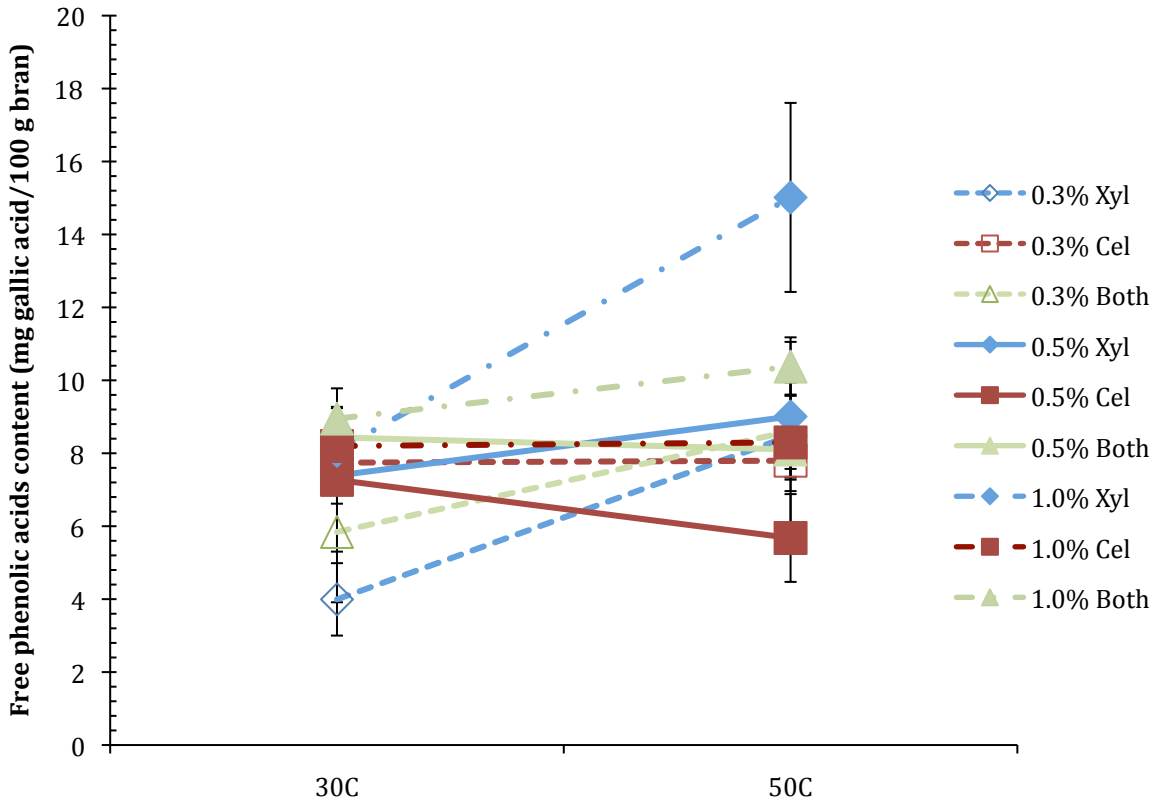


Figure 2-10 Free phenolics acids content in enzymatic treated wheat bran (6h).

The free phenolics acids in 15h 50ºC samples were all higher than that in 15h 30ºC ones, which indicated the treatment temperature was a main effect (Fig. 2-11). The free phenolics acids contents in 15h 50ºC samples are Xyl 0.3%: 9.300 ± 0.587 mg gallic

acid/100 g bran, Xyl 0.5%: 10.745 ± 1.014 mg gallic acid/100 g bran, Xyl 1.0%: 16.549 ± 0.287 mg gallic acid/100 g bran, Cel 0.3%: 8.982 ± 1.079 mg gallic acid/100 g bran, Cel 0.5%: 7.796 ± 0.308 mg gallic acid/100 g bran, Cel 1.0%: 12.967 ± 2.087 mg gallic acid/100 g bran, Both 0.3%: 8.785 ± 0.000 mg gallic acid/100 g bran, Both 0.5%: 11.167 ± 0.943 mg gallic acid/100 g bran, Both 1.0%: 12.998 ± 0.168 mg gallic acid/100 g bran. The 15h 30ºC samples gave lower free phenolics acids content (Xyl 0.3%: 6.760 ± 0.404 mg gallic acid/100 g bran, Xyl 0.5%: 6.735 ± 0.524 mg gallic acid/100 g bran, Xyl 1.0%: 9.710 ± 0.460 mg gallic acid/100 g bran, Cel 0.3%: 6.594 ± 1.240, Cel 0.5%: 6.141 ± 0.069 mg gallic acid/100 g bran, Cel 1.0%: 6.144 mg gallic acid/100 g bran, Both 0.3%: 5.903 ± 0.798 mg gallic acid/100 g bran, Both 0.5%: 7.598 ± 0.646 mg gallic acid/100 g bran, Both 1.0%: 8.210 ± 0.789 mg gallic acid/100 g bran).

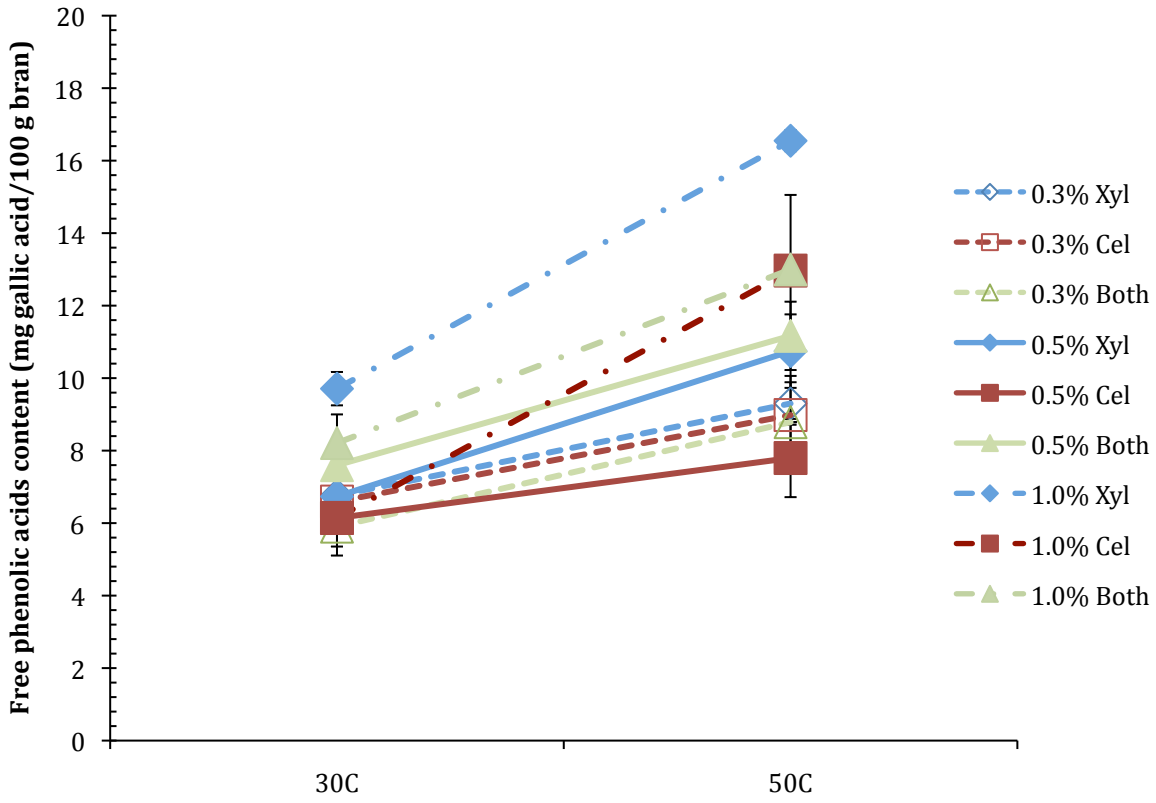


Figure 2-11 Free phenolics acids content in enzymatic treated wheat bran (15h).

The free phenolics acids content in 24 h treated samples had the same trend with that in 6 h and 15 h treated samples (Fig. 2-12). Even though the free phenolics acids content in Cel 0.3% 30ºC sample (12.379 ± 1.071 mg gallic acid/100 g bran) was higher than that in Cel 0.3% 50ºC one (10.472 ± 1.654 mg gallic acid/100 g bran), the 50ºC treated samples gave higher free phenolics acids content (Xyl 0.3%: 11.041 mg gallic acid/100 g bran, Xyl 0.5%: 15.065 ± 1.801 mg gallic acid/100 g bran, Xyl 1.0%: 17.622 ± 1.059 mg gallic acid/100 g bran, Cel 0.3%: 10.472 ± 1.654 mg gallic acid/100 g bran, Cel 0.5%: 13.794 ± 0.730 mg gallic acid/100 g bran, Cel 1.0%: 14.221 ± 2.483 mg gallic acid/100 g bran, Both 0.3%: 13.043 ± 1.681 mg gallic acid/100 g bran, Both 0.5%: 13.170 ± 0.989 mg gallic acid/100 g bran, Both 1.0%: 16.333 ± 0.863 mg gallic acid/100 g bran). The 30ºC treated samples had lower free phenolics acids content (Xyl 0.3%: 6.816 ± 1.027 mg gallic acid/100 g bran, Xyl 0.5%: 10.708 ± 1.523 mg gallic acid/100 g bran, Xyl 1.0%: 13.703 ± 0.994 mg gallic acid/100 g bran, Cel 0.3%: 12.379 ± 1.071 mg gallic acid/100 g bran, Cel 0.5%: 8.535 ± 0.781 mg gallic acid/100 g bran, Cel 1.0%: 9.285 ± 0.041 mg gallic acid/100 g bran, Both 0.3%: 11.331 mg gallic acid/100 g bran, Both 0.5%: 7.363 ± 0.259 mg gallic acid/100 g bran, Both 1.0%: 9.413 ± 0.781 mg gallic acid/100 g bran).

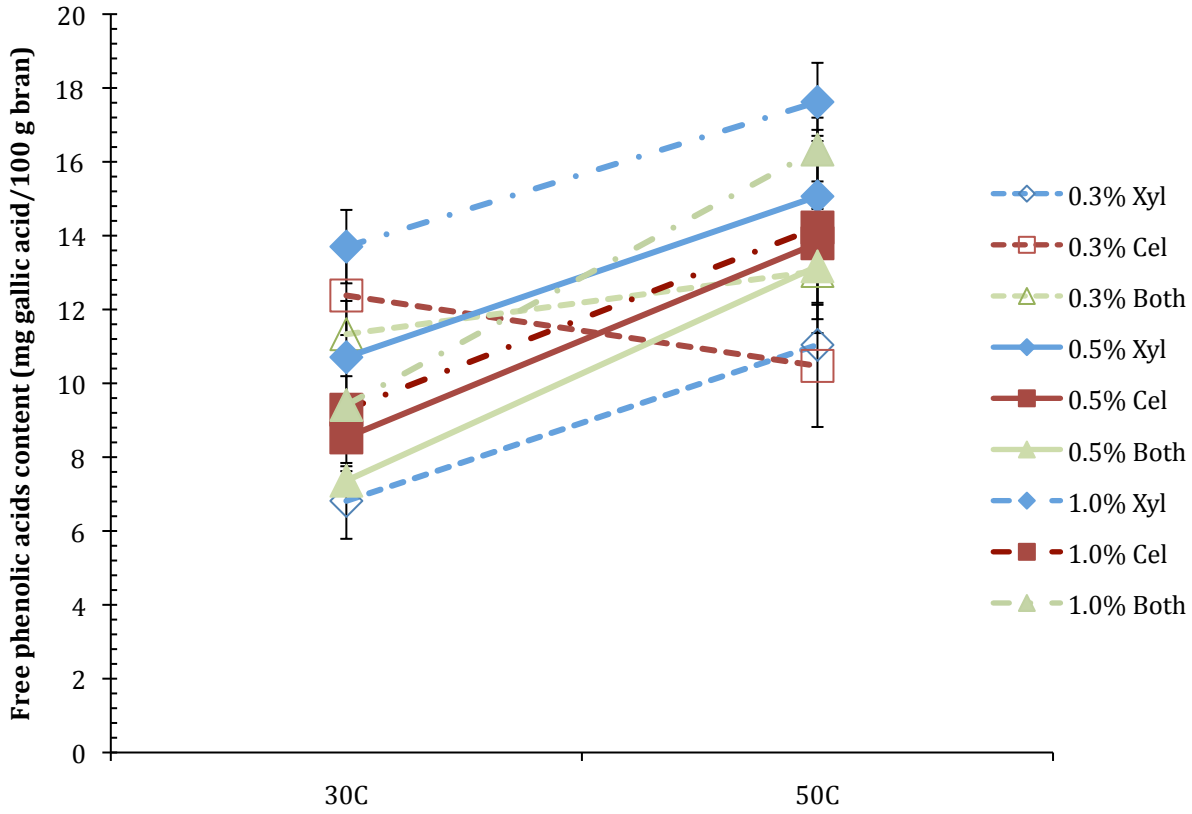


Figure 2-12 Free phenolics acids content in enzymatic treated wheat bran (24h).

Discussion:

The multilevel factorial design was designed with four factors (Enzyme, Enzyme Conc., Soaking time, Soaking temperature), two replicates. The numbers of levels were three, three, two, and three respectively. The ANOVA test (shown below in table 2) demonstrated whether the factors were significant or not. As we can see, all the single factors were significant with P value of almost 0.000 (Enzyme: 0.003, Enzyme Concentration: 0.000, Soaking Temperature: 0.000, Soaking Time: 0.000). Several factor interactions failed to show significance (Enzyme*Soaking Time: 0.088, Enzyme*Soaking time: 0.450, Enzyme Conc. Soaking time: 0.813, Enzyme*Enzyme Conc.*Soaking Time:

0.408, Enzyme*Enzyme Conc.*Soaking Temp.:0.130), while others showed their significance (Enzyme*Enzyme Conc.: 0.000, Enzyme Conc. Soaking time: 0.001, Soaking Time*Soaking Temp.:0.001, Enzyme Conc.*Soaking Time*Soaking Temp.: 0.047, Enzyme*Enzyme Conc.*Soaking Time*Soaking Temp.: 0.002).

Table 2 ANOVA test for the single enzymatic treated wheat bran

Term	P value
Enzyme	**0.003**
Enzyme Concentration	**0.000**
Soaking time	**0.000**
Soaking temperature	**0.000**
Enzyme*Enzyme Conc.	**0.000**
Enzyme*Soaking time	0.088
Enzyme*Soaking Temp.	0.450
Enzyme Conc.*Soaking Time	**0.001**
Enzyme Conc.*Soaking Temp.	0.813
Soaking Time*Soaking Temp.	**0.001**
Enzyme*Enzyme Conc.*Soaking Time	0.408
Enzyme*Enzyme Conc.*Soaking Temp.	0.130
Enzyme Conc.*Soaking Time*Soaking Temp.	**0.047**
Enzyme*Enzyme Conc.*Soaking Time*Soaking Temp.	**0.002**

The effect of enzyme on releasing free phenolics acids in wheat bran is significant. Fig. 2-13 showed the differences among the three levels. The xylanase is the most efficient enzyme to release free phenolics acids in wheat bran, followed by the combined enzyme. The cellulase is the least efficient in releasing phenolics acids in wheat bran. This is due to the enzyme specificity. Xylanase is specific for breaking down the linear polysaccharides beta-1,4-xylan into xylose. Cellulase is less specific and could work only for the hydrolysis of cellulose. In the plant cell wall of wheat bran, the main polysaccharides are arabinoxylans with backbone of 1,4-linked xylose. Therefore, xylanase can chop down the backbone and release the bound phenolics acids, which is more specific and efficient compared to cellulase. The combined enzyme treatment failed to show the synergic effect also due to the enzyme specificity.

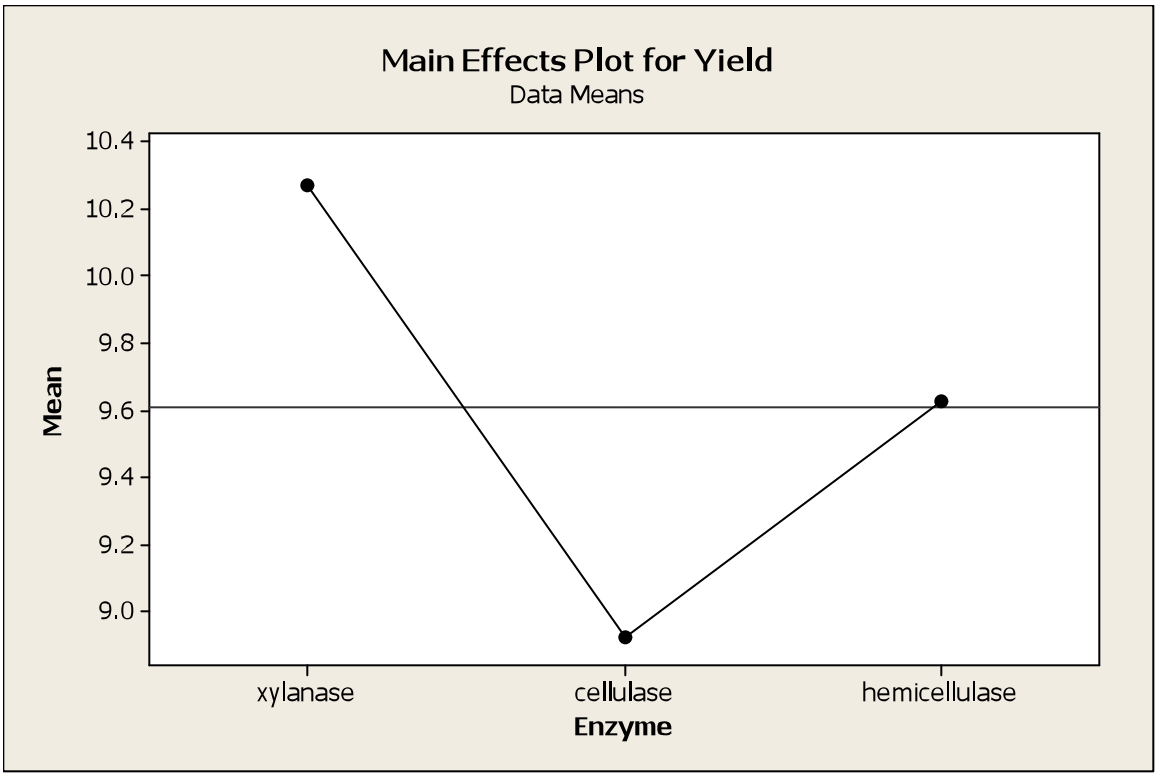


Figure 2-13 Enzyme type effect plot for yield.

The free phenolics acids contents increased with increasing enzyme concentration (Fig. 2-14). The mean free phenolics acids content of 0.3% enzyme treated wheat bran is approximately 8.2 mg gallic acid/100 g bran. The 0.5% enzyme treated wheat bran raised it to around 9.0 mg gallic acid/100 g bran. The 1.0% enzyme treatment further increased the free phenolics acids content to more than 11 mg gallic acid/100 g bran. High concentration could provide more opportunities to cleave the main chain of arabinoxylans, thus free more bound phenolics acids. In consequence, the optimal condition of enzymatic treatment is to select the 1.0% enzyme concentration.

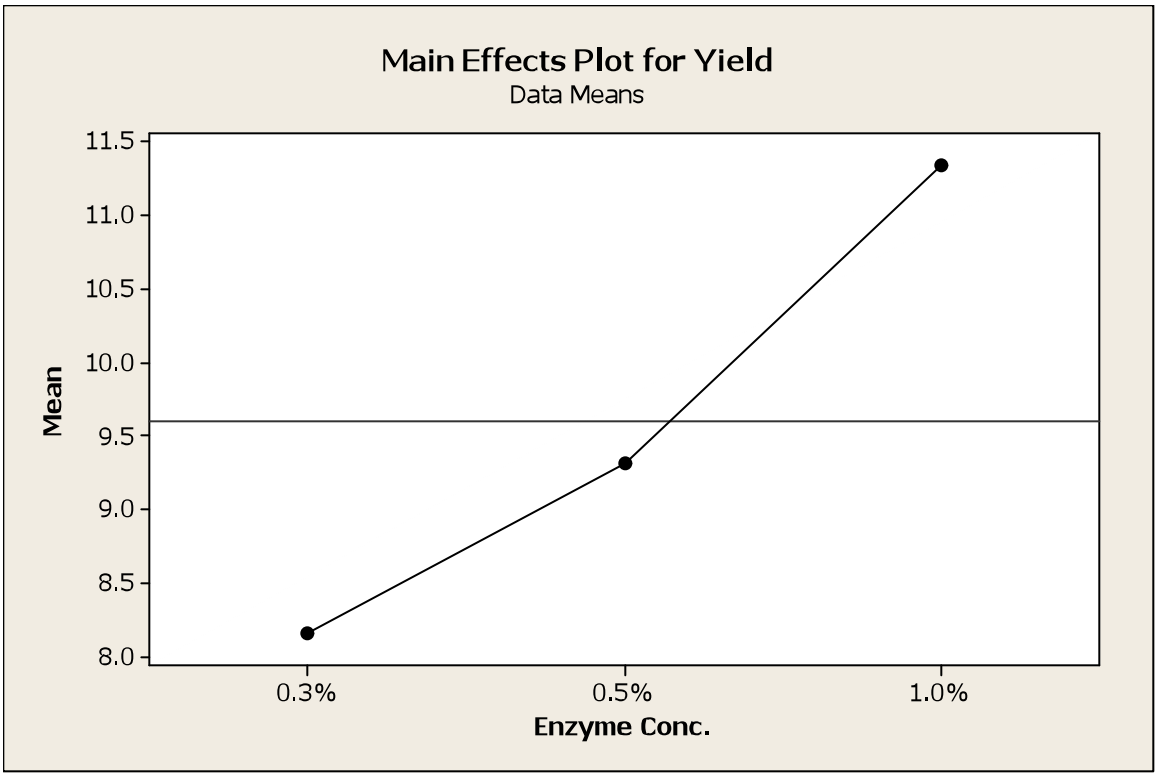


Figure 2-14 Enzyme concentration effect plot for yield.

The effect of soaking temperature on releasing free phenolics acids in wheat bran is significant as well (Fig. 2-15). This is because enzyme is sensitive to temperature. Their performance is closely related to soaking temperature. When the temperature is too low, the enzyme cannot be active. While, when the temperature is too high, the enzyme may be denatured and lose their functionality due to their protein characteristics. Therefore, temperature is essential in enzyme performance. In this case, 50ºC is much better than 30ºC in releasing bound phenolics acids in wheat bran.

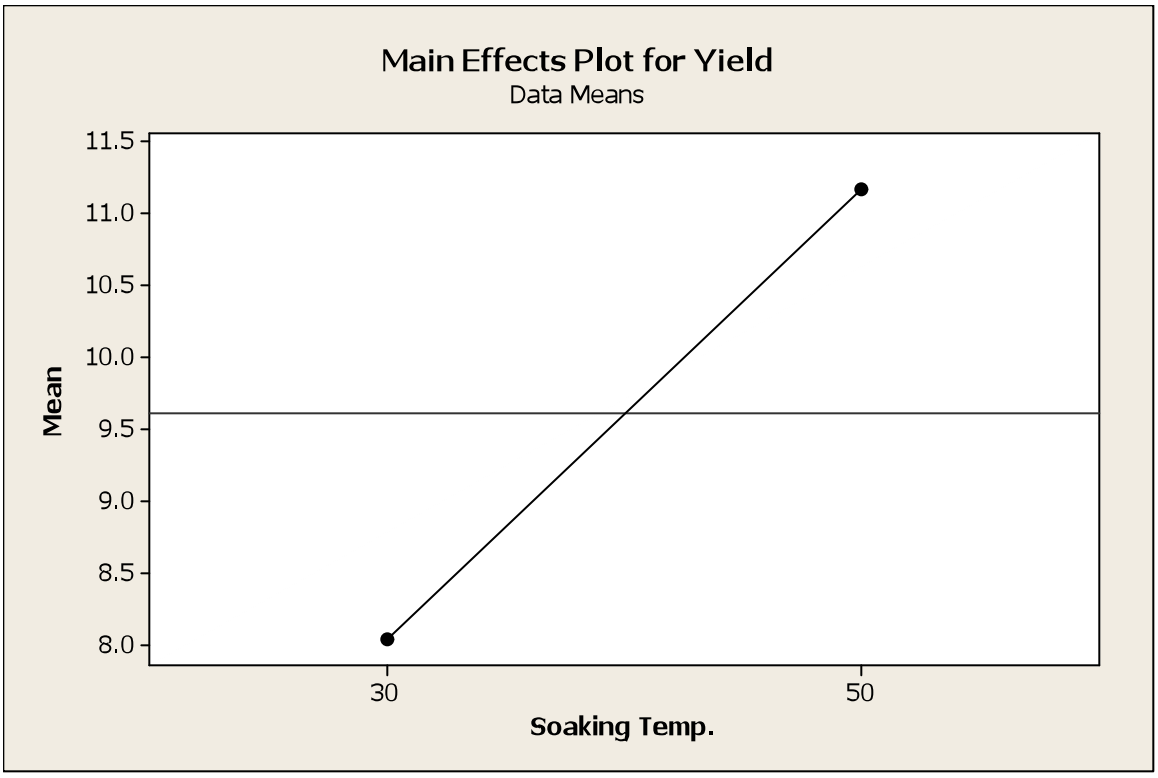


Figure 2-15 Soaking temperature effect plot for yield.

Longer soaking time contributes to more phenolics acids content (Fig. 2-16). The mean free phenolics acids content of 6 h treated wheat bran were around 8.2 mg gallic acid/100 g bran, which increased to more than 9.0 mg gallic acid/100 g bran when increasing the treatment time to 15 h. The 24 h treated wheat bran gave a much higher free phenolics acids content (around 11.7 mg gallic acid/100 g bran). It indicated that time is important in enzyme performance as well. Longer time gave enzyme enough time to find their specific binding sites and work on cleaving them.

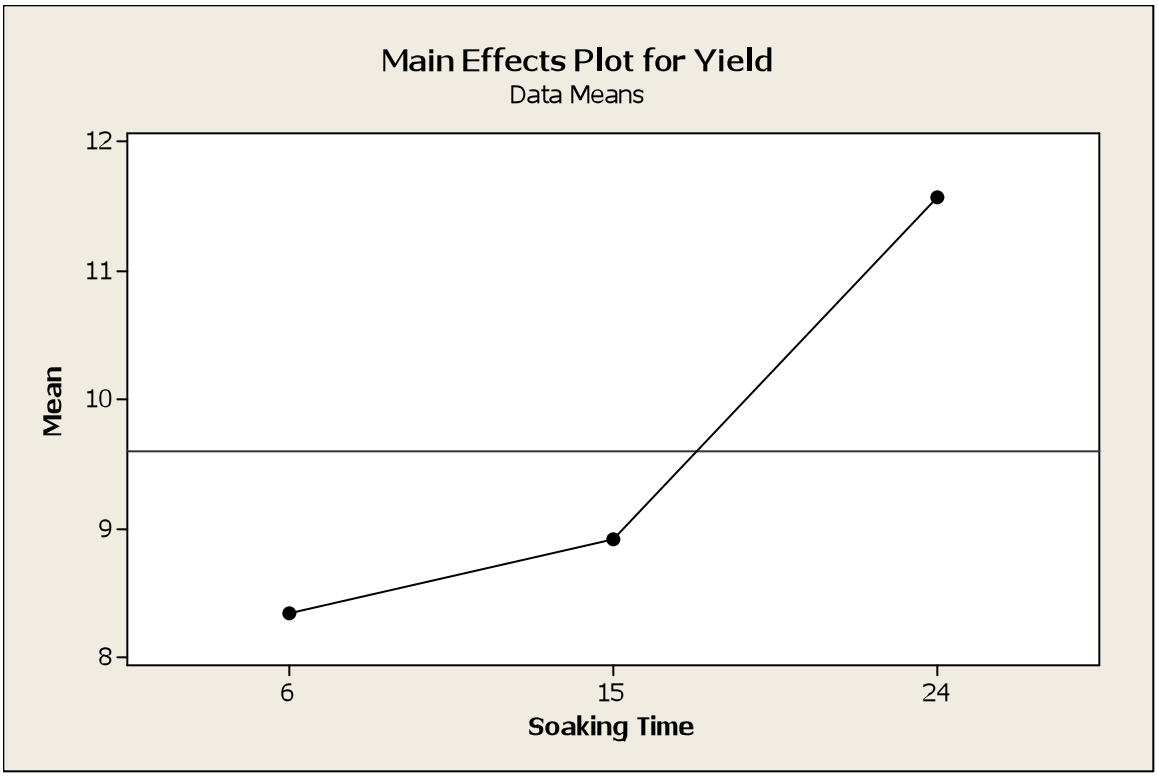


Figure 2-16 Soaking time effect plot for yield.

To sum up, the optimal enzyme treatment condition should be:

Enzyme	Enzyme Conc.	Soaking Time	Soaking Temp.
Xylanase	1.0%	24h	50ºC

Chapter 2: Combined alkaline and enzymatic treatment in releasing bound phenolics acids in wheat bran

Introduction:

Chemical treatments are another approach to release bound phenolics in plant cell walls besides the enzymatic treatment. In previous study, severe alkaline treatment was used to extract bound phenolics in wheat bran for the analysis purpose[46 26]. Adom and Liu analyzed the free, soluble-conjunct, and bound phenolics in wheat, corn and other uncooked whole grains using the alkali treatment to extract soluble-conjunct, and bound phenolics[21]. The commonly used chemical treatment is the alkaline treatment because several studies compared the efficiency of acid and alkaline treatment on releasing the bound phenolics in plant cell walls. Results showed that alkaline treatment is more efficient[47 48]. In Kim and his associates' study, the alkaline treatment (2M NaOH, 4h) could release more bound phenolics in wheat bran than the acid treatment (6M HCl, 1h, at 95ºC). In addition, the alkaline extracts had high antioxidant activity than the acid extracts. Verma et al. agreed with Kim's study, stating that "the phenolics liberated by acid hydrolysis were significantly less than the phenolics liberated by alkaline hydrolysis". Alkaline and acid hydrolysis had different effects on releasing different phenolics acids. Alkaline hydrolysis was more efficient on releasing vanillic, cis-ferulic and sinapic acids. Whereas caffeic acid could only be found in the acidic-labile fraction, the p-coumaric acid could only be found in the alkali-labile fraction. However, both treatments had similar effects on releasing trans-ferulic acid in wheat bran[48]. However, Arranz et al. published a paper one year later to demonstrate that acidic hydrolysis performed better in releasing polyphenols in cereals compared with the alkali hydrolysis. They explained it due to the differences in the acidic hydrolysis condition. Previous study

used a mild acidic hydrolysis condition that could not release as much as bound phenolics possible. They also stated that their acidic hydrolysis could release the bound phenolics "that are trapped within the cores or bound to cell wall constituents", which is impossible to be done through alkali hydrolysis[49]. Other studies also developed combined alkali and acidic hydrolysis to release all the bound phenolics in grains[50].

However, the concentrations of either alkali or acidic solution are still high and it consumes a great amount of solvent. Therefore, this study aims to lower the consumption of solvent through combining enzymatic and other treatments to release the bound phenolics in wheat bran. This study implemented a factorial design to optimize the alkali hydrolysis condition.

Part 1: Combined enzymatic and alkaline treatments

Materials:

King wheat bran is a light soft white winter wheat and was obtained from King Milling Company.

Enzymes:

Xylanase BX-AN was purchased from Enzyme Development Company (New York, NY).

Chemicals:

Sodium hydroxide was purchased from Fisher Chemicals (Pittsburgh, PA, USA)

Reagents:

500 mL 0.03N NaOH solution: 0.60 g sodium hydroxide (white plates) was weight and firstly dissolved in approximately 80 mL distilled water in 100 mL beaker. The solution was then transferred into a 500 mL volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and

all the wash water were transferred into the 500 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 500 mL volumetric flask. Then added approximately 300 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

500 mL 0.05N NaOH solution: 1.00 g sodium hydroxide (white plates) was weight and firstly dissolved in approximately 80 mL distilled water in 100 mL beaker. The solution was then transferred into a 500 mL volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 500 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 500 mL volumetric flask. Then added approximately 300 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

500 mL 0.1N NaOH solution: 2.00 g sodium hydroxide (white plates) was weight and firstly dissolved in approximately 80 mL distilled water in 100 mL beaker. The solution was then transferred into a 500 mL volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 500 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 500 mL volumetric flask. Then added approximately 300 mL distilled water and swirled the

flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

Methods:

The factorial experimental design was implement to determine the optimum condition for combined enzymatic and alkaline treatment. The variables and settings were listed in Table 3 below. 0.5000 g control wheat bran was blended with 25 mL distill water and 0.0500 g xylanase BX-AN (1.0% w/w) to make a 2% dry matter slurry. The slurry was then stirred at 200 rpm at 50ºC for 24 h using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried. The dried powders were then added with 25 mL sodium hydroxide solution and stirred at 200 rpm at certain temperature for certain time using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried for further phenolics analysis.

Table 3 Factorial experimental design for the combined enzymatic and alkaline treatment

Factors	Levels		
Enzyme		Xylanse	
Enzyme Conc.		1.0%	
Enzymatic Treatment Time (h)		24	
Enzymatic Treatment Temp. (ºC)		50	
NaOH Conc. (N)	0.03	0.05	0.10
NaOH treatment time (h)	6	15	24
NaOH treatment temp.	40		60

(ºC)

Part 2: Extraction free phenolics in combined enzymatic and alkaline treated wheat bran.

Materials:

Combined enzymatic and alkaline treated wheat bran

Chemicals:

Ethanol was purchased from the University of Minnesota chemical store.

Reagent:

2L 80% ethanol solution: 1.6 L ethanol was measured using a 1 L measuring cylinder and mixed thoroughly with 0.4 L distilled water measured using a 500 mL measuring cylinder.

Methods:

0.5000 g of each combined enzymatic and alkaline treated wheat bran samples (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight and added with 20 mL ethanol solution (80%) in 50 mL centrifuge tubes. All the samples were then stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, they were centrifuged at 2,000 g for 20 min. The supernatant was collected and poured into a new 50 mL centrifuge tube. The left residue was added with 20 mL ethanol solution (80%) to perform the extraction one more time. The supernatants were pooled (40 mL) and vacuum evaporated at 40ºC until no more than 10 mL liquid left. The final extracts were filled up to 10 mL using distilled water. The free antioxidant extracts were stored at -76ºC for further analysis. Duplicates of each sample were made and the standard deviations were calculated.

Part 3: Free Phenolics acids in combined enzymatic and alkaline treated wheat bran

Materials:

Free Phenolics extracts of combined enzymatic and alkaline treated wheat bran

Chemicals:

Folin-Ciocalteu reagent was purchased from Sigma-Aldrich (Wilwaukee, WI, USA). Sodium carbonate was purchased from Fisher Chemicals (Pittsburgh, PA, USA).

Reagent:

200 mL 10% Sodium carbonate solution: 22.22 g sodium carbonate was weighed and added with 200 mL distilled water in a 500 mL beaker. Gently swirled the beaker until the solids were dissolved completely.

Methods:

0.2 mL of extracts were added with 0.2 mL Folin-Ciocalteu reagent in 12 mL tubes and stirred for 10 s before adding 0.8 mL sodium carbonate solution (10%) after 8 min. The mixture was then stirred and added with distilled water to a total volume of 5 mL. The absorbance value at 760nm was measured using a spectrophotometer after 90 min. Duplicates were made and the standard deviation was calculated.

Results:

The alkaline treatment is a main effect on releasing free phenolics acids in wheat bran. The free phenolics acids contents increased with increasing sodium hydroxide concentration (Fig. 3-1). The 0.1N sodium hydroxide treated wheat bran gave the highest free phenolics acids content (6h 40C: 36.475 ± 4.853 mg gallic acid/100 g bran, 15h 40C: 32.470 ± 0.684 mg gallic acid/100 g bran, 24h 40C: 32.822 ± 0.293 mg gallic acid/100 g bran, 6h 60C: 36.434 ± 1.974 mg gallic acid/100 g bran, 15h 60C: 32.074 ± 2.436 mg

gallic acid/100 g bran, 24h 60C: 30.493 ± 2.312 mg gallic acid/100 g bran), followed by the 0.05N sodium hydroxide treated wheat bran (6h 40C: 22.221 ± 0.053 mg gallic acid/100 g bran, 15h 40C: 25.714 ± 4.496 mg gallic acid/100 g bran, 24h 40C: 24.173 ± 1.323 mg gallic acid/100 g bran, 6h 60C: 29.999 ± 0.319 mg gallic acid/100 g bran, 15h 60C: 31.742 ± 5.280 mg gallic acid/100 g bran, 24h 60C: 33.486 ± 4.504 mg gallic acid/100 g bran). While, the 0.03N sodium hydroxide treated wheat bran had the lowest free phenolics acids content (6h 40C: 17.731 ± 0.394 mg gallic acid/100 g bran, 15h 40C: 17.778 ± 0.890 mg gallic acid/100 g bran, 24h 40C: 18.353 ± 0.207 mg gallic acid/100 g bran, 6h 60C: 16.549 ± 2.686 mg gallic acid/100 g bran, 15h 60C: 17.599 ± 0.260 mg gallic acid/100 g bran, 24h 60C: 15.071 ± 0.713 mg gallic acid/100 g bran). However, when the treatment time and temperature was long and high enough, in this case, the time was 24h and the temperature reached to 60C, the free phenolics acids content was not significantly different between the 0.1N and 0.05N sodium hydroxide treated wheat bran (0.1N 24h 60C: 30.493 ± 2.312 mg gallic acid/100 g bran, 0.05N 24h 60C: 33.486 ± 4.504 mg gallic acid/100 g bran), which indicated the coeffects of three factors were not significant different that has been approved by the ANOVA test giving a P value of 0.765 (see table 4).

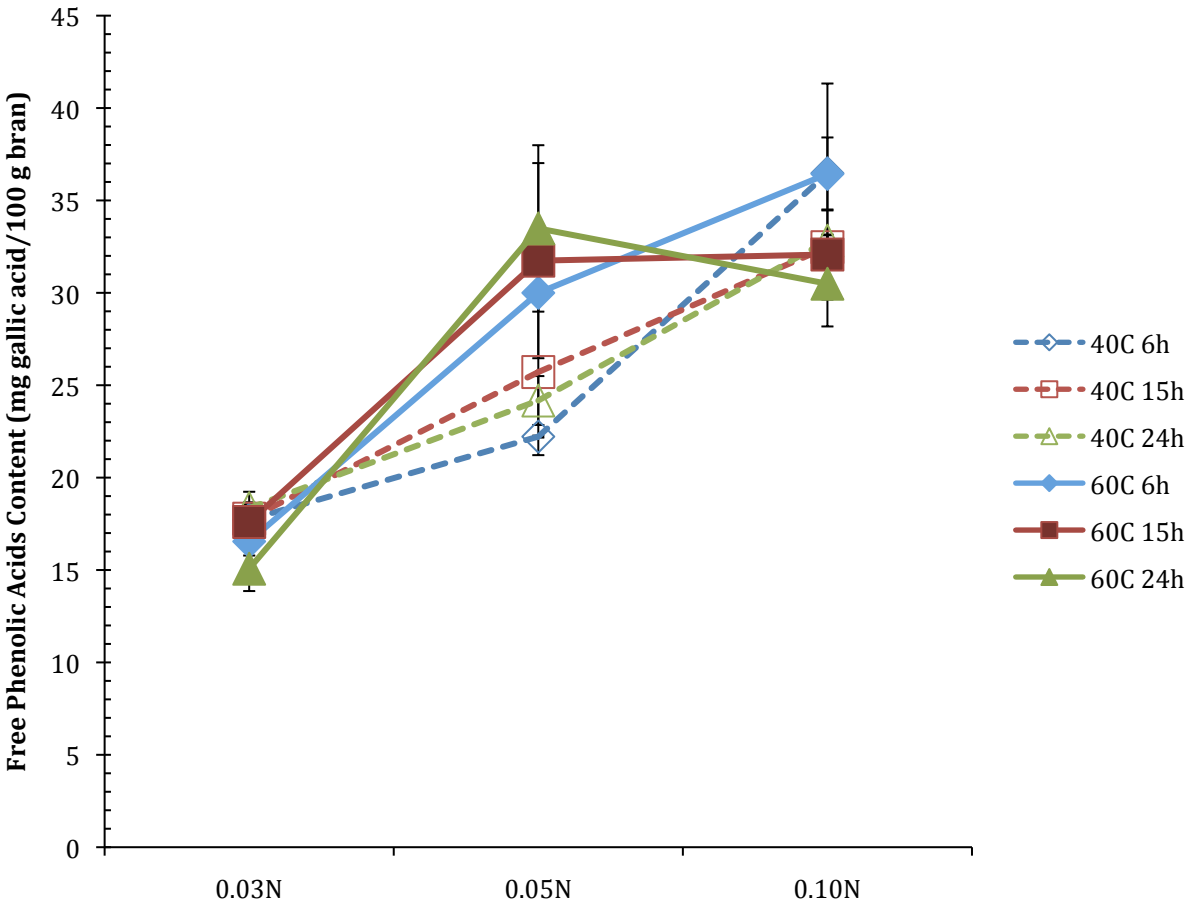


Figure 3-1 Free phenolics acids content in combined enzymatic and alkaline treated wheat bran with alkaline concentration as the variable.

The free phenolics acids content in wheat bran treated for different time period were similar in both values and trends (Fig. 3-2). They all showed an increase trend with increasing sodium hydroxide concentration and temperature, and gave similar free phenolics acids content (6h: 0.03N 40C: 17.731 ± 0.394 mg gallic acid/100 g bran, 0.03N 60C: 16.549 ± 2.686 mg gallic acid/100 g bran, 0.05N 40C: 22.221 ± 0.053 mg gallic acid/100 g bran, 0.05N 60C: 29.999 ± 0.319 mg gallic acid/100 g bran, 0.1N 40C: 36.475 ± 4.853 mg gallic acid/100 g bran, 0.1N 60C: 36.434 ± 1.974 mg gallic acid/100 g bran; 15h: 0.03N 40C: 17.778 ± 0.890 mg gallic acid/100 g bran, 0.03N 60C: 17.599 ± 0.260 mg gallic acid/100 g bran, 0.05N 40C: 25.714 ± 4.496 mg gallic acid/100 g bran, 0.05N

60C: 31.742 ± 5.280 mg gallic acid/100 g bran, 0.1N 40C: 32.470 ± 0.684 mg gallic acid/100 g bran, 0.1N 60C: 32.074 ± 2.436 mg gallic acid/100 g bran; 24h: 0.03N 40C: 18.353 ± 0.207 mg gallic acid/100 g bran, 0.03N 60C: 15.071 ± 0.713 mg gallic acid/100 g bran, 0.05N 40C: 24.173 ± 1.323 mg gallic acid/100 g bran, 0.05N 60C: 33.486 ± 4.504 mg gallic acid/100 g bran, 0.1N 40C: 32.822 ± 0.293 mg gallic acid/100 g bran, 0.1N 60C: 30.493 ± 2.312 mg gallic acid/100 g bran).

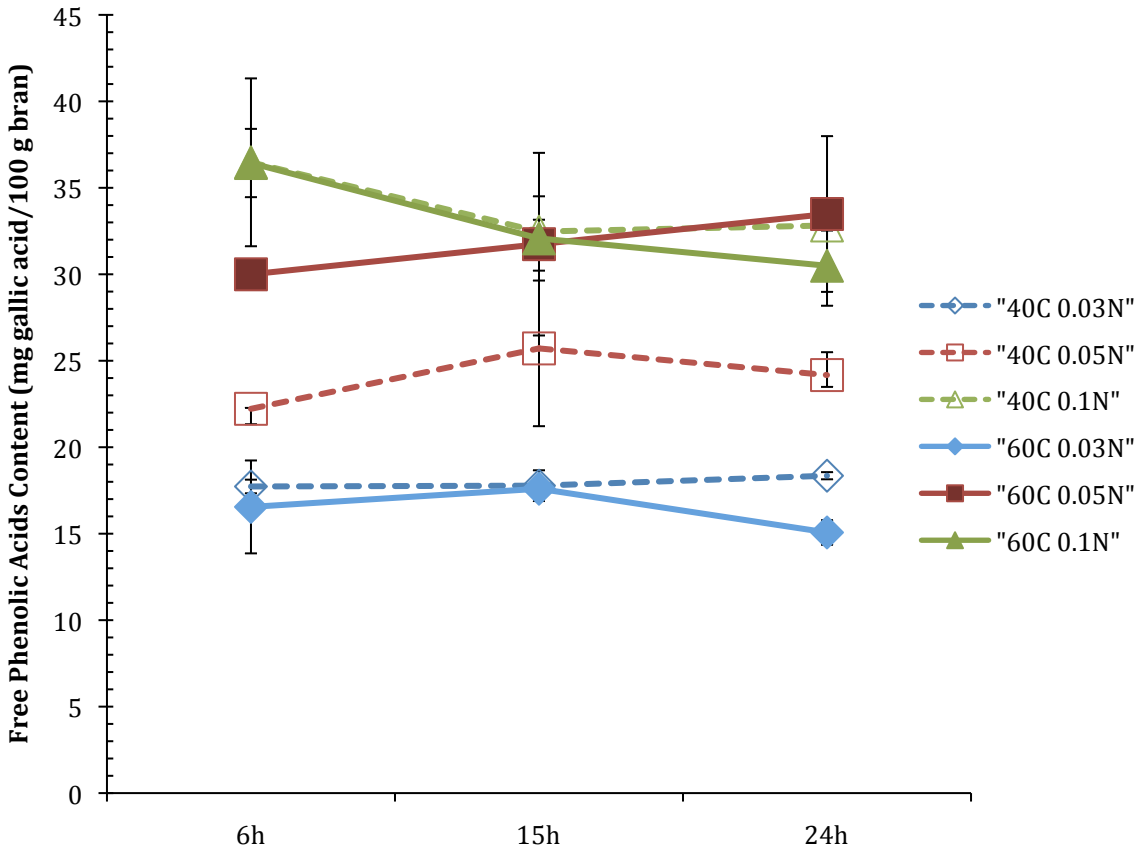


Figure 3-2 Free phenolics acids content in combined enzymatic and alkaline treated wheat bran with alkaline treatment time as the variable.

The temperature differences did not cause significant differences in free phenolics acids contents in wheat bran as well, even though at several points, the 60ºC treated wheat bran gave higher free phenolics acids content than the 40ºC treated ones (Fig. 3-3).

The overall trend and value of both temperature treated wheat bran were similar. The free phenolics acids contents in 40ºC treated wheat bran were 0.03 6h: 17.731 ± 0.394 mg gallic acid/100 g bran, 0.03 15h: 17.778 ± 0.890 mg gallic acid/100 g bran, 0.03 24h: 18.353 ± 0.207 mg gallic acid/100 g bran, 0.05 6h: 22.221 ± 0.053 mg gallic acid/100 g bran, 0.05 15h: 25.714 ± 4.496 mg gallic acid/100 g bran, 0.05 24h: 24.173 ± 1.323 mg gallic acid/100 g bran, 0.1 6h: 36.475 ± 4.853 mg gallic acid/100 g bran, 0.1 15h: 32.470 ± 0.684 mg gallic acid/100 g bran, 0.1 24h: 32.822 ± 0.293 mg gallic acid/100 g bran. The free phenolics acids contents in 60ºC treated wheat bran were 0.03 6h: 16.549 ± 2.686 mg gallic acid/100 g bran, 0.03 15h: 17.599 ± 0.260 mg gallic acid/100 g bran, 0.03 24h: 15.071 ± 0.713 mg gallic acid/100 g bran, 0.05 6h: 29.999 ± 0.319 mg gallic acid/100 g bran, 0.05 15h: 31.742 ± 5.280 mg gallic acid/100 g bran, 0.05 24h: 33.486 ± 4.504 mg gallic acid/100 g bran, 0.1 6h: 36.434 ± 1.974 mg gallic acid/100 g bran, 0.1 15h: 32.074 ± 2.436 mg gallic acid/100 g bran, 0.1 24h: 30.493 ± 2.312 mg gallic acid/100 g bran.

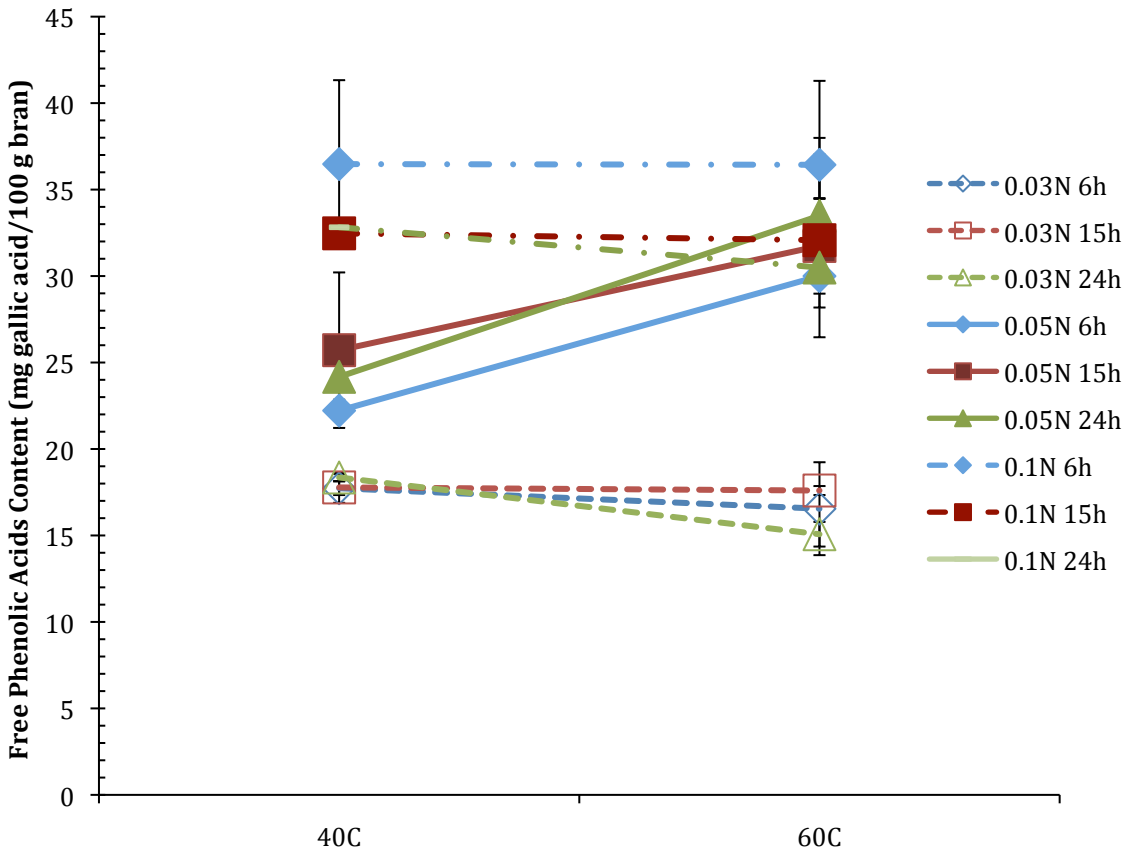


Figure 3-3 Free phenolics acids content in combined enzymatic and alkaline treated wheat bran with alkaline treatment temperature as the variable.

Discussion:

The multilevel factorial design was designed with three factors (Alkaline Conc., Soaking time, Soaking temperature), two replicates. The numbers of levels were three, three, and two respectively. The ANOVA test (Table 4) showed that only the NaOH Conc. and the NaOH Conc.*Temp gave significant differences (P=0.000 for both). The time and temperature failed to show the significance. This is due to the previous enzymatic treatment. The prior enzymatic treatment helped to open the polysaccharides structure and make alkaline hydrolysis easier. The shorter treatment time and lower treatment temperature saved time and energy.

Table 4 ANOVA test of combined enzymatic and alkaline treated wheat bran.

Term	P
NaOH Conc.	**0.000**
Time	0.729
Temperature	0.056
NaOH Conc.*Time	0.063
NaOH Conc.*Temp.	**0.000**
Time*Temp.	0.901
NaOH Conc.*Time*Temp	0.765

Fig. 3-4 showed the effect of sodium hydroxide concentration on releasing free phenolics acids in wheat bran. As we can see, the free phenolics acids content increased with increasing sodium hydroxide concentration. The increasing rate from 0.03N to 0.05N was higher than that from 0.05N to 0.1N. However, the 0.1N treated wheat bran gave the highest free phenolics acids. The mean value was more than 33 mg gallic acids/100 g bran.

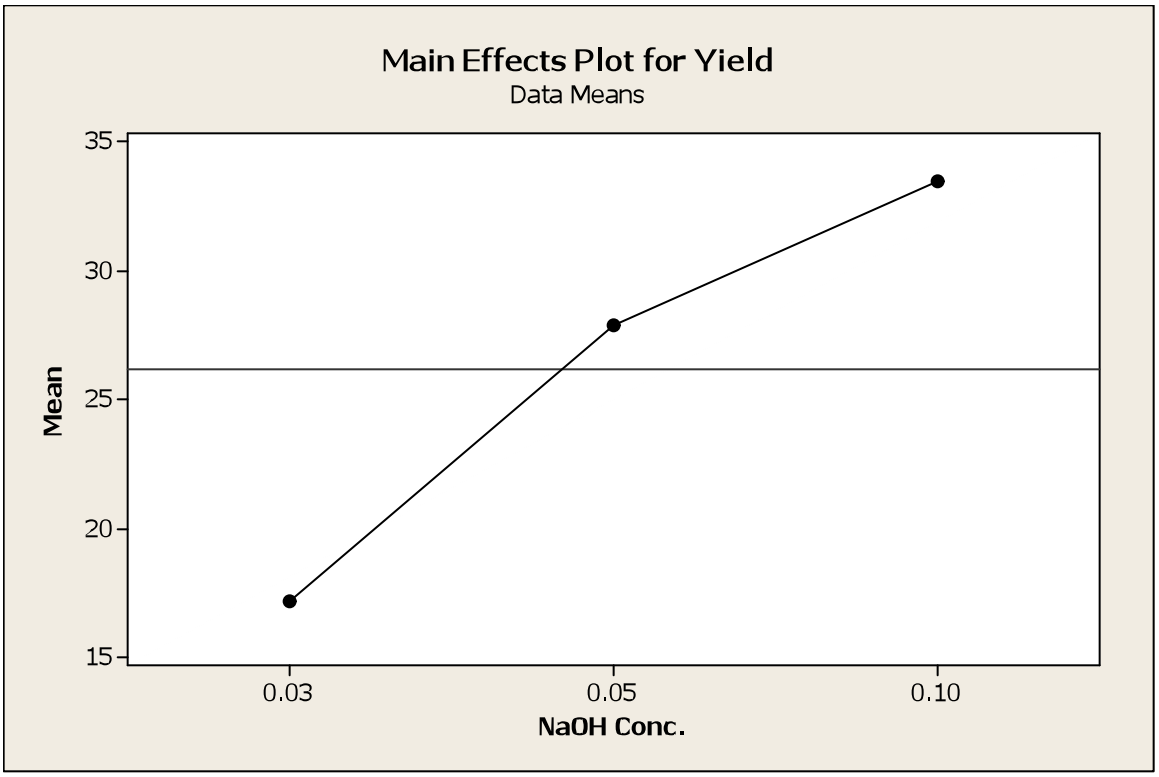


Figure 3-4 NaOH concentration effect plot for yield.

Fig 3-5 showed that the free phenolics acids contents in wheat bran decreased with increasing treatment time. This is interesting because we assumed that longer time gave more opportunities for the alkaline to hydrolyze the bound phenolics acids. The reasons may be, first, longer time also contributed to phenolics acids lost due to the unstable characters of antioxidants; second, the 6h treated wheat bran gave around 26.58 mg gallic acid/100 g bran phenolics acid content, while, the 24h treated wheat bran gave around 25.75 mg gallic acid/100 g bran phenolics acid content. The differences between them were only 0.8 mg gallic acid/100 g bran, thus it was not a significant decline trend. Therefore, the time differences did not have significant effect on releasing free phenolics acids in wheat bran (P=0.729).

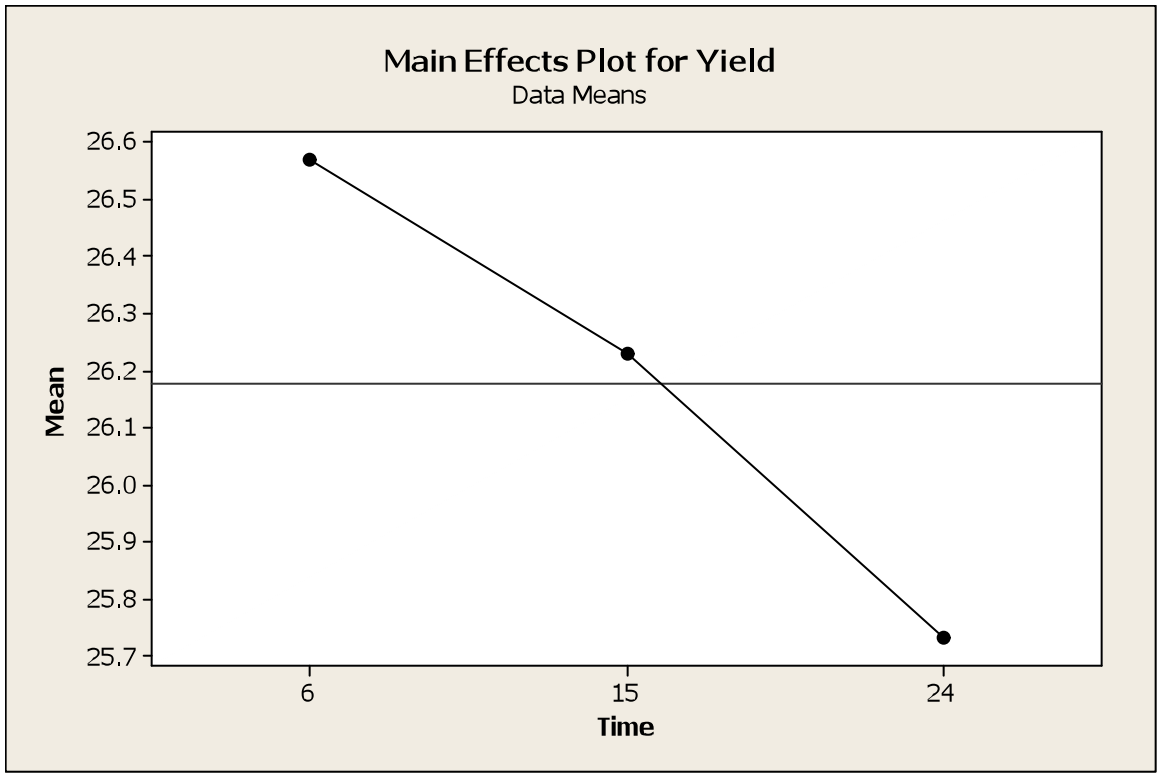


Figure 3-5 Time effect plot for yield.

The free phenolics acids content in wheat bran increased with increasing treatment temperature (Fig. 3-6). The mean free phenolics acids content of 40ºC treated wheat bran was about 25.2 mg gallic acid/100 g bran, while that of 60ºC treated wheat bran was more than 27.2 mg gallic acid/100 g bran. However, the differences were still not huge enough to cause significant differences according to the ANOVA test (P=0.056).

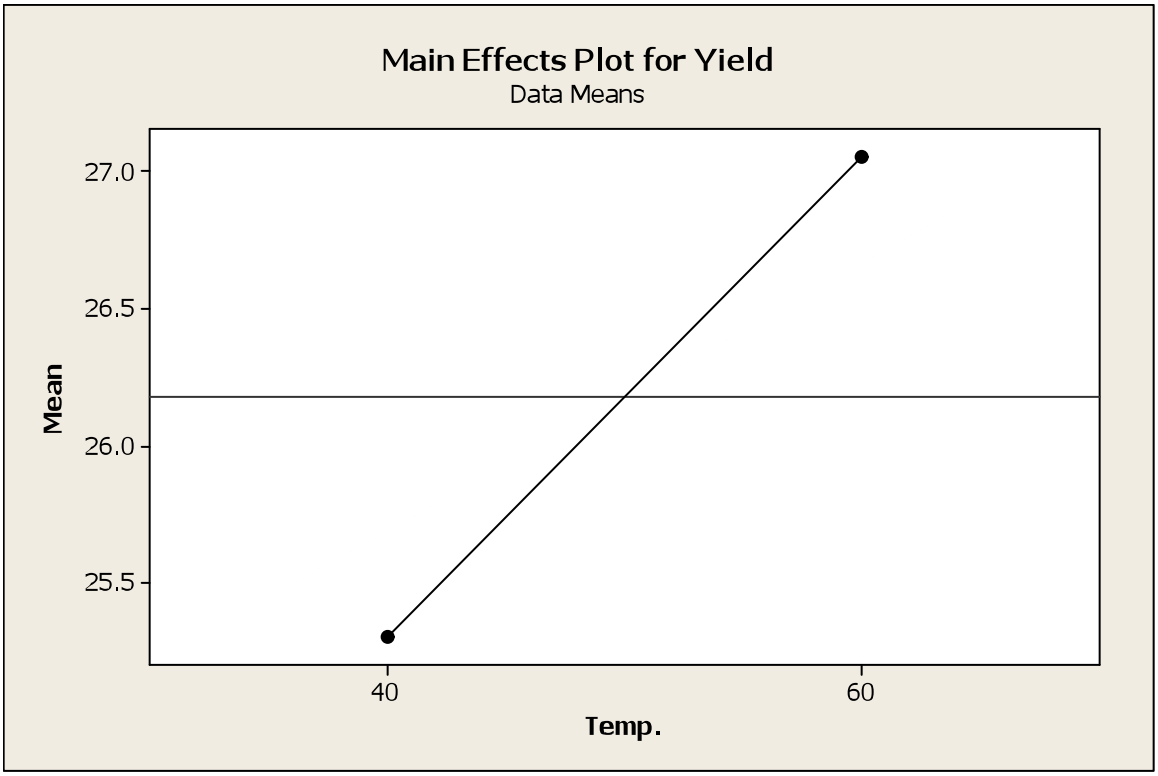


Figure 3-6 Temperature effect plot for yield.

In summary, the optimal condition for combined enzymatic and alkaline treatment is listed below (table 5):

Table 5 The optimal condition for combined enzymatic and alkaline treatment.

Enzyme	Xylanase
Enzyme Conc.	0.1%
Enzyme treatment time	24h
Enzyme treatment temperature	50ºC
Alkaline Conc.	0.1N

Alkaline treatment time	6h
Alkaline treatment temperature	40ºC

Chapter 3: The multiple treatments of wheat bran

Materials and Methods

Materials:

King wheat bran is a light soft white winter wheat and was obtained from King Milling Company.

Chemicals:

Sodium Hydroxide (NaOH) was purchased from Fisher Chemicals (Pittsburgh, PA, USA)

Xylanase BX-AN was purchased from Enzyme Development Company (New York, NY).

Reagents:

2 L 0.1N NaOH solution: 8.00 g sodium hydroxide (white plates) was weight and firstly dissolved in approximately 200 mL distilled water in 500 mL beaker. The solution was then transferred into a 2 L volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 2 L volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 2 L volumetric flask. Then added approximately 1,500 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

190 mL Xylanase solution: 0.1000 g xylanase (white powder) was weight and added with 190 mL distilled water until thoroughly dissolved in water before use.

Process equipments:

Rotor beater mill (Retsch GmbH, Model SR 300 Haan, Germany), Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA), M-110Y laboratory Microfluidizer Processor (Microfluidics, Newton, MA, USA) equipped with 200µm and 100µm chambers. Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NJ, USA), VirTis Freezemobile 25EL freeze-dryer (Gardiner, NY, USA), Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA).

Processes:

Control (labeled as "C"): King wheat bran was milled into powders with particle size no greater than 0.5mm using the Retsch GmbH SR300 rotor beater mill (Haan, Germany).

Alkaline treated wheat bran (labeled as "A"): 10.00 g control wheat bran was added with 190 mL NaOH (0.1N) to make a slurry with 5% dry matter. The slurry was then stirred at 200 rpm and incubated at 60°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Alkaline and high mixer treated wheat bran (labeled as "AH"): 10.00 g control wheat bran was added with 190 mL NaOH (0.1N) to make a slurry with 5% dry matter. The slurry was then stirred at 200 rpm and incubated at 60°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The

diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA) at level 6 for 5 min. The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Alkaline, high shear mixed, and high pressure homogenized wheat bran (labeled as "AHH"): 10.00 g control wheat bran was added with 190 mL NaOH (0.1N) to make a slurry with 5% dry matter. The slurry was then stirred at 200 rpm and incubated at 60°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA) at level 6 for 5 min, then was high-pressure homogenized using M-110Y Laboratory Microfluidizer Processor (Microfluidics, Newton, MA, USA). The 2% dry matter slurry was fed into the microfluidizer at 2.3k psi first through the 200 um chamber, then the 100 um chamber. The whole process was done three times. The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic treated wheat bran (labeled as "E"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000 g xylanase, which is 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). The viscosity of the slurry was measured at this point using Rapid

Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic and high shear mixed wheat bran (labeled as "EH"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000 g xylanase, which is 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA) at level 6 for 5 min. The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic, high shear mixed, and high pressure homogenized wheat bran (labeled as "EHH"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000 g xylanase, which was 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA) at level 6 for 5 min, then was high-pressure homogenized using M-110Y Laboratory Microfluidizer Processor (Microfluidics, Newton, MA, USA). The 2% dry matter slurry was fed into the microfluidizer at 2.3k psi first through the 200 μm chamber, then the 100 μm chamber. The whole process was done three times. The

viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic and alkaline treated wheat bran (labeled as "EA"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000g xylanase, which was 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried and crushed into powders in a mortar. The powders was added with 190 mL NaOH solution (0.1N), and then stirred at 200 rpm and incubated at 40°C for 6 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic, alkaline, and high shear mixed wheat bran (labeled as "EAH"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000g xylanase, which was 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried and crushed into powders in a mortar. The powders was added with 190 mL NaOH solution (0.1N), and then stirred at 200 rpm and incubated at 40°C for 6 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works,

Wilmington, NC, USA) at level 6 for 5 min. The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Enzymatic, alkaline, high shear mixed, and high pressure homogenized wheat bran (labeled as "EAHH"): 10.00 g control wheat bran was added with 190 mL xylanase solution (0.1000 g xylanase, which is 1% of the weight of wheat bran) to make a 5% dry matter slurry. The slurry was then stirred at 200 rpm at 50°C for 24 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was freeze-dried and crushed into powders in a mortar. The powders was added with 190 mL NaOH solution (0.1N), and then stirred at 200 rpm and incubated at 40°C for 6 hrs using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, the slurry was added with water to 500 mL to make a 2% dry mass liquid mixture. The diluted slurry was high-shear mixed by Ultra-Turrax T25 Basic high shear mixer (IKA-Works, Wilmington, NC, USA) at level 6 for 5 min, then was high-pressure homogenized using M-110Y Laboratory Microfluidizer Processor (Microfluidics, Newton, MA, USA). The 2% dry matter slurry was fed into the microfluidizer at 2.3k psi first through the 200 μm chamber, then the 100 μm chamber. The whole process was done three times. The viscosity of the slurry was measured at this point using Rapid Visco Analyzer (RVA, Newport Scientific, Springfiled, IL, USA). After that, the slurry was freeze-dried for further analysis.

Chapter 4: The phenolics in multiple treated wheat bran

Extraction of free, soluble-conjunct, and bound antioxidants in processed wheat bran samples (for phenolics analysis)

Materials:

Processed wheat bran: C, A, AH, AHH, E, EH, EHH, EA, EAH, EAHH.

Chemicals:

Sodium hydroxide, and hydrochloride were purchased from Fisher Chemicals (Pittsburgh, PA, USA).

Ethanol was purchased from the University of Minnesota chemical store.

Ester Ethyl, and hexane were purchased from Sigma-Aldrich (Wilwaukee, WI, USA).

Reagents:

1L 2N sodium hydroxide solution: 80.00 g sodium hydroxide (white plates) was weighed and firstly dissolved in approximately 400 mL distilled water in 500 mL beaker. The solution was then transferred into a 1 L volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 1 L volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 2 L volumetric flask. Then added approximately 300 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

2L 80% ethanol solution: 1.6 L ethanol was measured using a 1 L measuring cylinder and mixed thoroughly with 400 mL distilled water measured using a 500 mL measuring cylinder.

Extraction of free antioxidants in processed wheat bran:

0.5000 g of each processed wheat bran samples (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight and added with 20 mL ethanol solution (80%) in 50 mL centrifuge tubes. All the samples were then stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, they were centrifuged at 2,000 g for 20 min. The supernatant was collected and poured into a new 50 mL centrifuge tube. The left residue was added with 20 mL ethanol solution (80%) to perform the extraction one more time. The supernatants were pooled (40 mL) and vacuum evaporated at 40ºC until no more than 10 mL liquid left. The final extracts were filled up to 10 mL using distilled water. The free antioxidant extracts were stored at -76ºC for further analysis. Triplates of each sample were made and the standard deviations were calculated.

Extraction of soluble-conjunct antioxidants in processed wheat bran:

0.5000 g of each processed wheat bran samples (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight and added with 20 mL ethanol solution (80%) in 50 mL centrifuge tubes. All the samples were then stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, they were centrifuged at 2,000 g for 20 min. The supernatant was collected and poured into a new 50 mL centrifuge tube. The left residue was added with 20 mL ethanol solution (80%) to perform the extraction one more time. The supernatants were pooled

(40 mL) and vacuum evaporated at 40ºC until no more than 10 mL liquid left. The final extracts were filled up to 10 mL using distilled water. 0.5 mL of the free antioxidant extracts were added with 10 mL NaOH (2N), and stirred at 200 rpm for 1 hr under nitrogen gas using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). The alkaline treated solution was adjusted the pH to approximately two using HCl (6N). After that, the solution was extracted with ester ethyl four times using 10 mL ester ethyl each time. The oil fraction was collected and evaporated at ambient temperature to dryness, then refilled with 10 mL distilled water. The soluble-conjunct antioxidant extracts were stored at -76ºC for further analysis. Triplicates of each sample were made and the standard deviations were calculated.

Extraction of bound antioxidants in processed wheat bran:

0.5000 g of each processed wheat bran samples (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight and added with 20 mL ethanol solution (80%) in 50 mL centrifuge tubes. All the samples were then stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After that, they were centrifuged at 2,000 g for 20 min. The supernatant was discarded. The left residue was added with 20 mL ethanol solution (80%) to perform the extraction one more time. The final left residue was added with 20 mL hexane to remove the lipids, and stirred at 200 rpm for 30 min using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). After centrifuged at 2,000g for 20 min, the supernatant was discarded. The left residue was added with 10 mL NaOH (2N), and stirred at 200 rpm for 1 hr under nitrogen gas using the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NY, USA). The alkaline treated solution was adjusted the pH to approximately two using HCl (6N). After that, the solution was extracted with ester ethyl

four times using 10 mL ester ethyl each time. The oil fraction was collected and evaporated at ambient temperature to dryness, then refilled with 10 mL distilled water. The bound antioxidant extracts were stored at -76ºC for further analysis. Triplicates of each sample were made and the standard deviations were calculated.

Analysis of phenlics in multiple treated wheat bran

Phenolics acids

Part 1: Introduction

Phenolics acids (Fig. 5-1) are organic compounds with a phenolic ring and an organic carboxylic acid function. Phenolics acids can be categorized as hydroxybenzoic acid and hydroxycinnamic acid derivatives[4] (Fig. 5-2). Common hydroxybenzoic acids derivatives are *p*-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acids. Common hydroxycinnamic acid derivatives are *p*-coumaric, caffeic, ferulic and sinapic acids.

Ferulic, syringic, *p*-hydroxybenzoic, vanillic, and coumatic acids were found in wheat bran[69]. Among those, ferulic acid is the predomain phenolics acids. The phenolics acids contents vary with various wheat species[1].

Phenolics acids are bioactive compounds that can be beneficial to human health. They can perform as antioxidants to balance the oxidative stress in human body and prevent the free radical to attack DNA. Epidemiological studies showed that consumption of whole grain can reduce the risk of several chronic disease such as cardiovascular disease, type 2 diabetes and cancer[4]. The reason may partially due to the antioxidant ability of phenolics acids contained in whole grain.

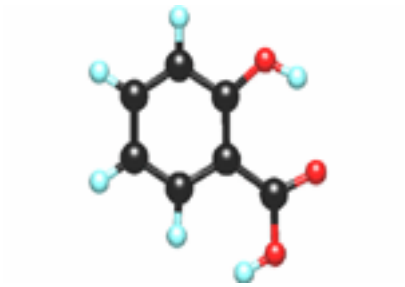
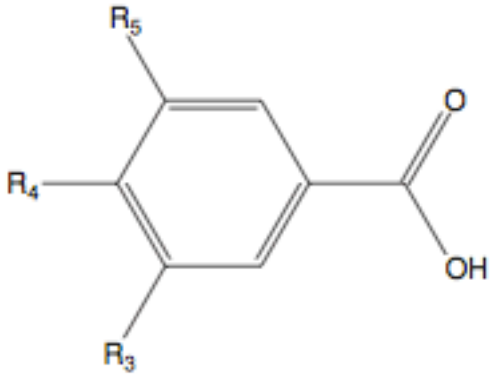


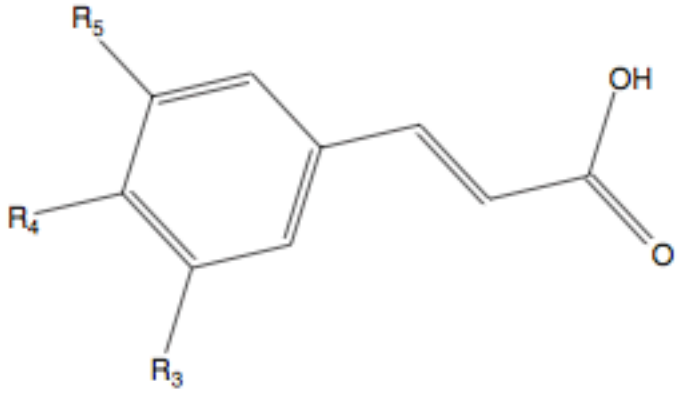
Figure 5-1 Structure of phenolics acids
(http://www.rowett.ac.uk/divisions/ghp/g_duthie.html)

Benzoic Acid Derivatives



	R_3	R_4	R_5
p-hydroxybenzoic acid	H	OH	H
gallic acid	OH	OH	OH
syringic acid	OCH_3	OH	OCH_3

Cinnamic Acid Derivatives



	R_3	R_4	R_5
p-coumaric acid	H	OH	H
ferulic acid	OCH_3	OH	H
sinapic acid	OCH_3	OH	OCH_3

Figure 5-2 Structures of benzoic acid and cinnamic acid derivatives.

The phenolics acids exist in wheat bran mostly in their bound form. They are either esterified to sugar, lignin, and protein, or ether to lignin. The bound form inhibits its absorption in rumen also in human body. Therefore it reduces the bioaccessibility of phenolics acids. For better accessibility, several treatments were carried out to release the bound phenolics acids in wheat bran.

Part 2: The standard curve of phenolics acids

Chemicals:

Folin-Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich (Wilwaukee, WI, USA). Sodium carbonate was purchased from Fisher Chemicals (Pittsburgh, PA, USA).

Reagent:

Storage gallic acid solution: 0.020 g gallic acid was weighed and firstly dissolved in approximately 40 mL distilled water in a 50 mL beaker. The solution was then transferred into a 100 mL volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 100 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 100 mL volumetric flask. Then added approximately 30 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution. This was the store solution and stored at refrigerator covered with aluminum foil before use.

Standard curve gallic acid solutions: The store solution was diluted by pipetting 2 mL store solution and adding with 6 mL distilled water to make a 50 mg/L gallic acid

solution. After that, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mL of the 50 mg/L gallic acid solution were pipette and filled up to a total of 1mL using distilled water to make a 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mg/L gallic acid solutions respectively.

200 mL 10% Sodium carbonate solution: 22.22 g sodium carbonate was weighed and added with 200 mL distilled water in a 500 mL beaker. Gently swirled the beaker until the solids were dissolved completely.

Methods:

0.2 mL of standard gallic acid solution were added with 0.2 mL Folin-Ciocalteu reagent in 12 mL tubes and stirred for 10 s before adding 0.8 mL sodium carbonate solution (10%) after 8 min. The mixture was then stirred and added with distilled water to a total volume of 5 mL. The absorbance value at 760nm was measured using a spectrophotometer after 90 min. Triplets were made and the standard deviations were calculated.

Results:

The standard curve was calculated and drawn using Microsoft office 2008 for Mac (home and student edition) Excel software. Below is the figure of the standard curve (Fig. 5-3) with 11 spots. Table 6 showed the absorbance values of each gallic acid solution.

Table 6 absorption value of gallic solution at 760 nm

Gallic acid concentration (mg/L)	1	2	3	Average	Standard deviation
0	0.000	0.005	0.000	0.002	0.003
5	0.261	0.264	0.263	0.263	0.002
10	0.488	0.497	0.498	0.494	0.006
15	0.711	0.719	0.704	0.711	0.008

20	0.942	0.922	0.900	0.921	0.021
25	1.132	1.120	1.113	1.112	0.010
30	1.315	1.284	1.359	1.319	0.038
35	1.448	1.368	1.514	1.443	0.073
40	1.660	1.566	1.650	1.625	0.052
45	1.669	1.931	1.802	1.801	0.131
50	1.749	1.971	2.088	1.936	0.172

As we can see, the absorbance value increased proportionally when the concentration of gallic acid increased.

The equation of the standard curve is

$Y = 0.0383X + 0.0998$ $(R2=0.9924)$

Calculations of phenolics content in processed wheat bran:

Set A as the absorbance value,

Then, the gallic acid concentration in 5 mL mixture is:

$X = (A - 0.0998)/0.0383$ mg/L

Then, the phenolics content in processed wheat bran is:

C = X*0.010 L/wheat bran weight g*100 mg/100g bran

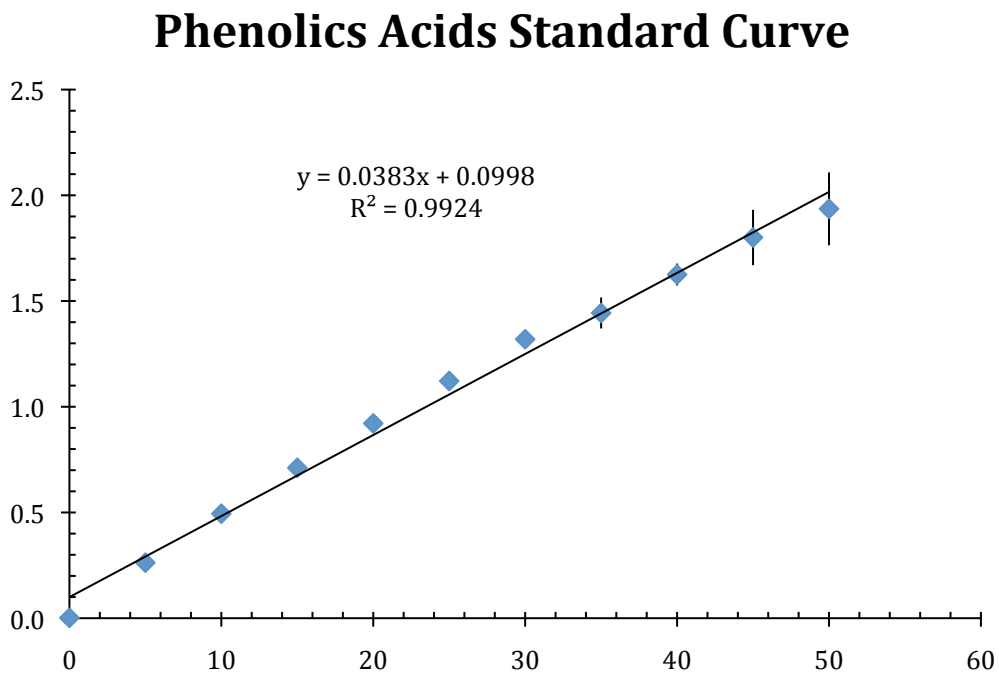


Figure 5-3 Phenolics acids standard curve.

However, the soluble-conjunct phenolics content in processed wheat bran and the bound phenolics content in several processed wheat bran were all very little. The absorbance values were almost zero, which turned to be a problem that the phenolics content would be below zero when using the equation above. Therefore, the standard curve was reconstructed by narrowing the concentration range from 0 to 15 mg/L (Fig. 5-4)

The equation of the standard curve is

$Y = 0.0493X + 0.0066$ (R2=0.99882)

Calculations of phenolics content in processed wheat bran:

Set A as the absorbance value,

Then, the gallic acid concentration in 2.06 mL mixture is:

$X = (A - 0.0066)/0.0493$ mg/L

Then, the phenolics content in processed wheat bran is:

C = X*0.01 L/wheat bran weight g*100 mg/100g bran

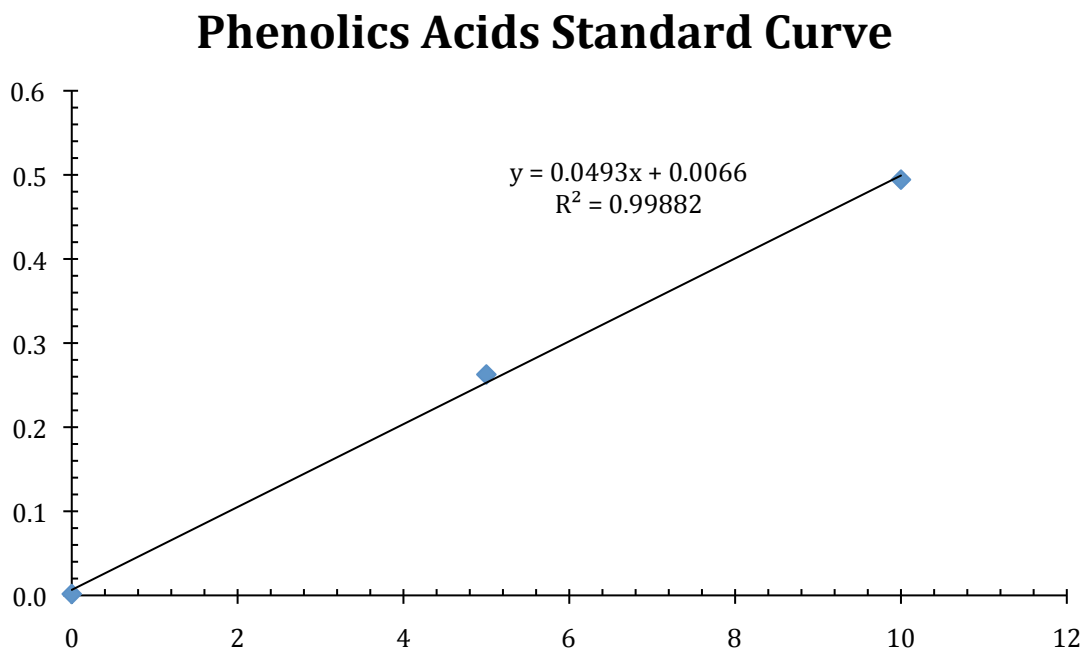


Figure 5-4 Phenolics acid standard curve complimentary.

Part 3: The methodology of anaylysis of phenolics acids in wheat bran extracts

Materials:

Free antioxidant extracts, Soluble-conjunct extracts, and bound extracts.

Methods:

0.2 mL of extracts were added with 0.2 mL Folin-Ciocalteu reagent in 12 mL tubes and stirred for 10 s before adding 0.8 mL sodium carbonate solution (10%) after 8 min. The mixture was then stirred and added with distilled water to a total volume of 5 mL. The absorbance value at 760nm was measured using a spectrophotometer after 90 min. Triplets were made and the standard deviation was calculated.

Part 4: Results and Discussion

Results:

The phenolics acids content in processed wheat bran was expressed as microgram gallic acid equivalent per 100 grams of wheat bran. The results of free phenolics acids content in processed wheat bran were shown in fig. 5-5. As we can see, all treatments had an improving effects on free phenolics acids content compared to control wheat bran. The only alkaline treated and combined alkaline and enzymatic treated wheat bran gave similar free phenolics acids content (A: 45.817 ± 1.469 mg gallic acid/100 g bran, AH: 43.077 ± 4.038 mg gallic acid/100 g bran, AHH: 42.202 ± 9.140 mg gallic acid/100 g bran, EA: 44.836 ± 10.901 mg gallic acid/100 g bran, EAH: 45.909 ± 7.930 mg gallic acid/100 g bran, EAHH: 41.168 ± 5.723 mg gallic acid/100 g bran). The only enzymatic treatment released less bound phenolics compared with the two above (E: 25.409 ± 2.093 mg gallic acid/100 g bran, EH: 30.254 ± 4.218 mg gallic acid/100 g bran, EHH: 30.997 ± 4.795 mg gallic acid/100 g bran).

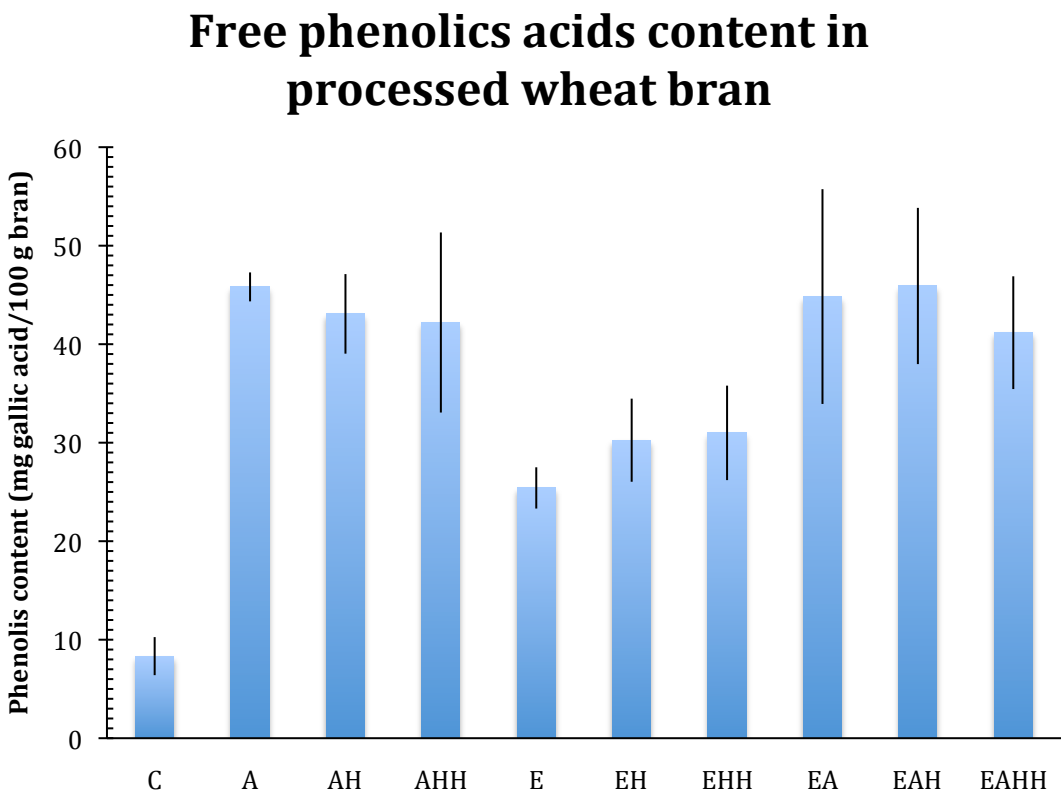


Figure 5-5 Free phenolics acids content in processed wheat bran.

The soluble-conjunct phenolics acids content were almost none, which is the same as the soluble-conjunct flavonoids content (Fig. 5-6).

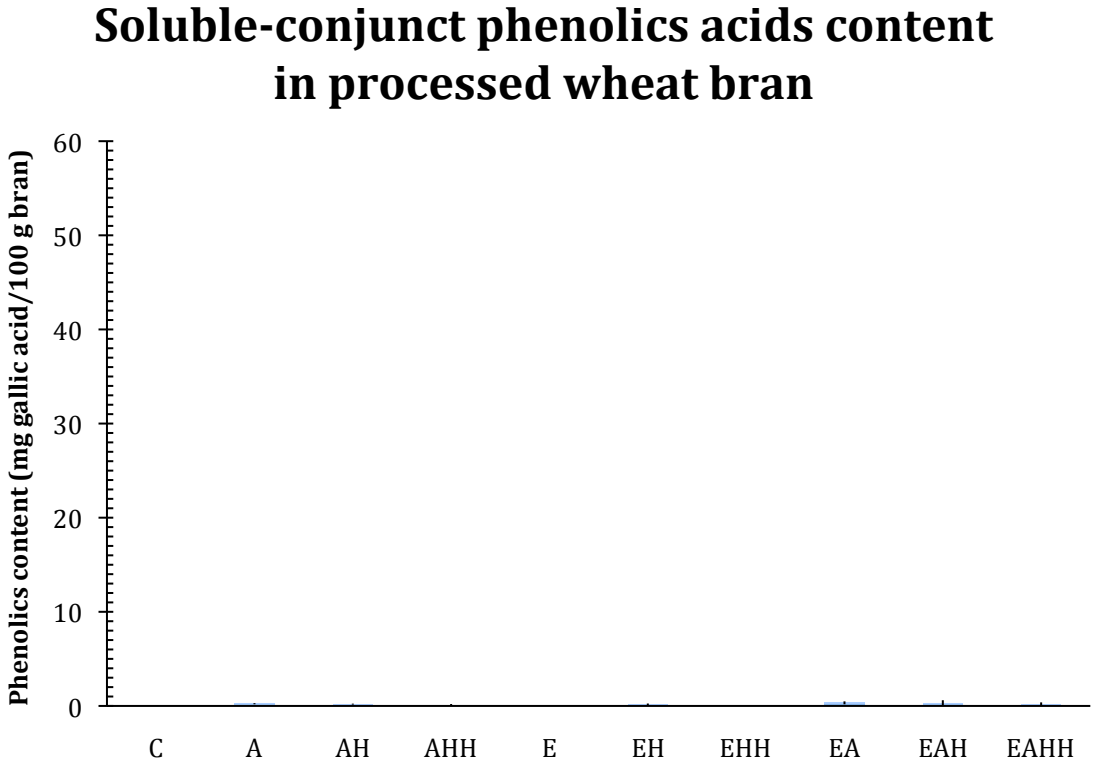


Figure 5-6 Soluble-conjunct phenolics acids content in processed wheat bran.

The only alkaline treated wheat bran did not contain any bound phenolics acids, which indicated the alkaline treatment released almost all bound phenolics acids (Fig. 5-7) (A: 0.000 ± 0.000 mg gallic acid/100 g bran, AH: 0.000 ± 0.000 mg gallic acid/100 g bran, AHH: 0.000 ± 0.000 mg gallic acid/100 g bran). The only enzymatic treated wheat bran still contained a small amount of bound phenolics acids (E: 13.087 ± 1.102 mg gallic acid/100 g bran, EH: 11.813 ± 2.319 mg gallic acid/100 g bran, EHH: 14.368 ± 1.767 mg gallic acid/100 g bran). The both enzymatic and alkali treated wheat bran also contained bound phenolics acids (EA: 1.931 ± 0.325 mg gallic acid/100 g bran, EAH: 1.738 ± 0.868 mg gallic acid/100 g bran, EAHH: 2.695 ± 0.989 mg gallic acid/100 g bran).

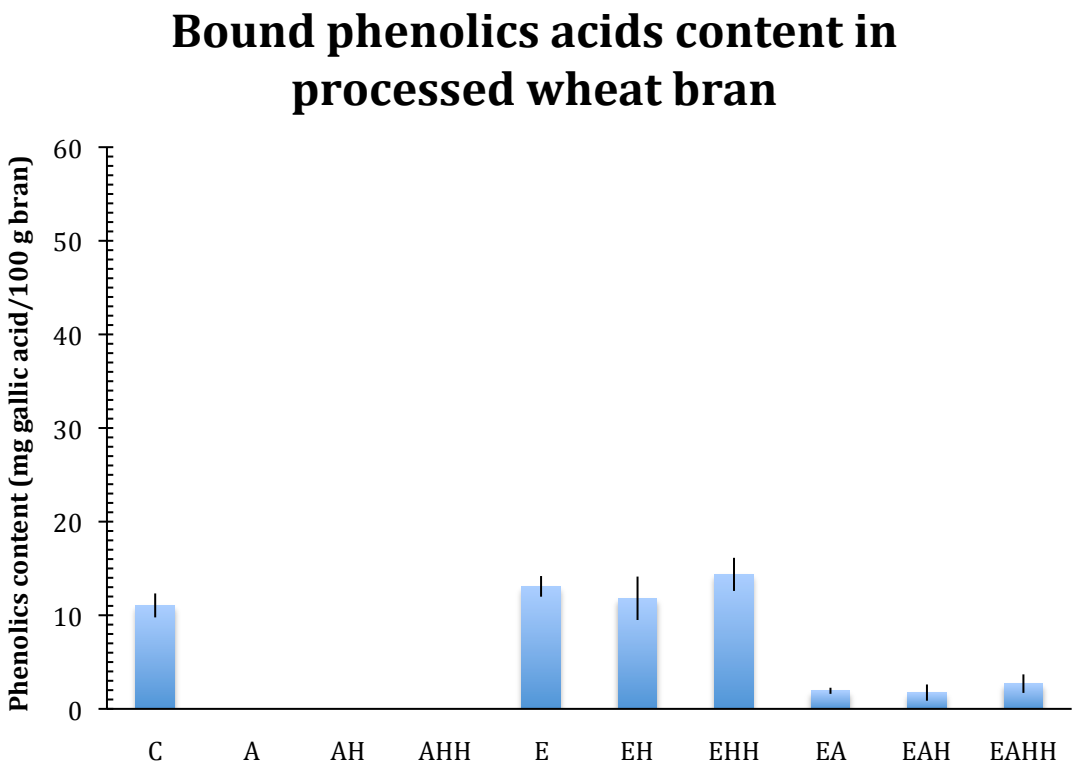


Figure 5-7 Bound phenolics acids content in processed wheat bran.

Discussion:

The alkaline treatment is more efficient than the enzymatic treatment on releasing bound phenolics acids in wheat bran (Fig. 5-8). After soaked in 0.1N sodium hydroxide for 24h at 60ºC, the bound phenolics acids in wheat bran existed almost all in their free form. This is due to the completely hydrolysis of ferulic acid ester linkage. As stated before, the bound phenolics acids are mostly ferulic acid, which is attached to arabinose through ester linkage on the arabinoxylans. Di, and tri-ferulic were also found in the complex matrix to contribute to the structure. Alkaline treatment can break the ester linkage thus free the bound ferulic acid and also open the fiber structure in some extent.

Ferulic acid esterase can degrade the ester linkage as well, but since it is not commercially available, it was not applied in this study. Xylanase is a class of enzyme to

degrade xylans into xylose. Since ferulic acids were bound to arabinoxylans, the degradation of xylans may indirectly help to release bound ferulic acids, which has been approved according to the results. Data showed that enzymatic treatment did release approximately half bound phenolics acids, but still left the other half in their original form.

Combined alkaline and enzymatic treatment do not have the synergic effect because alkaline treatment alone is efficient enough to release all the bound phenolics acids. However, combined treatment seems not work better than single alkaline treatment, which left a small amount of bound phenolics acids still attached to arabinoxylans. The reason may be the difference of alkaline treatment conditions. The single alkaline treatment is carried out using 0.1N sodium hydroxide for 24h at 60ºC, whereas the combined alkaline treatment is using 0.1N sodium hydroxide for 6h at 40ºC. The shorter time and lower temperature may have effects on alkali hydrolysis efficiency.

Mechanical processes including high shear mixing and high pressure homogenization seems not have positive effects on releasing bound phenolics acids in wheat bran. Although a slight increase can be detected when comparing the only enzymatic treated wheat bran, the standard deviation is too large to show any significant difference. The situation is the same when comparing the combined alkaline and enzymatic treatment. The mechanical processes help to open the structure, reduce the particle size, and increase the viscosity. They have an improving effect on physical characteristics of wheat bran. However, since the previous alkaline and enzymatic treatment are so efficient that further open structure through mechanical processes cannot free any more bound phenolics acids.

In summary, the alkaline treatment is the most efficient in releasing bound phenolics acids in wheat bran. Enzymatic treatment can free phenolics acids as well but with a lower efficiency. Mechanical processes fail to show any effects in releasing bound phenolics acids.

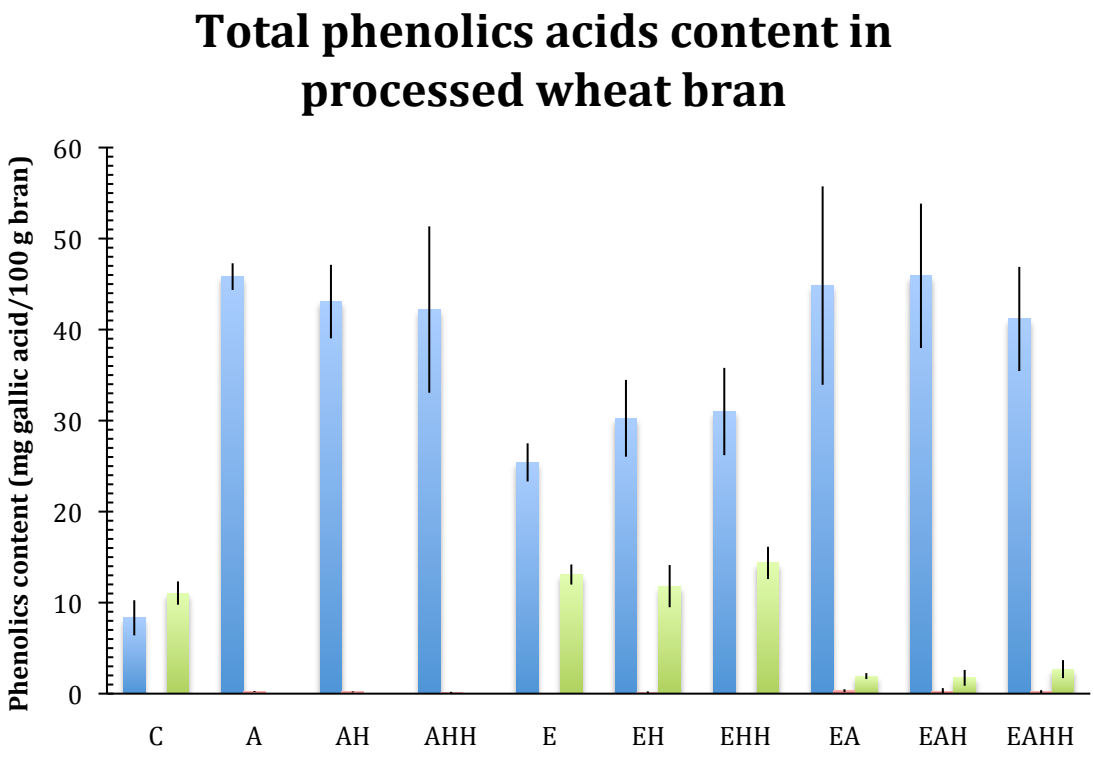


Figure 5-8 The total phenolics acids content in processed wheat bran.

Flavonoids

Part 1: Introduction

Flavonoids are a group of compounds that have a similar structure with flavones, with two aroma rings connected by a three-carbon structure[1]. They are the secondary metabolites of plant and cannot be synthesized in human and animals' body[12]. Therefore, flavonoids are obtained through our daily diet. Fruits, vegetables, tea and wines are considered good resource of flavonoids[12].

Flavonoids are also contained in whole grains. Feng et al. analyzed the flavonoids content in bran of four classes of wheat and found the variety due to the differences in species[13]. The durum wheat had the highest flavonoids content, followed by the white wheat, the hard red winter wheat, and the hard red spring wheat gave the lowest flavonoids content. McCallum and colleges detected the proanthocyanidins in wheat bran that may contribute to the color[14]. Adom et al. found the majority (79%) flavonoids were in the bran/germ fraction of wheat[15]. However, most of flavonoids existed in their bound form, linked to sugar, lignin, polysaccharides or protein within the wheat bran[4].

Flavonoids have several health benefits due to their antioxidant characteristics. They have been associated with reducing risk of several disease including cancer, and cardiovascular disease[64]. They help to maintain a balanced oxidative stress in human body and prevent the damage that free radicals can cause on DNA. It may also due to their other biological activities including antiallergic, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic activities[12].

Anticancer effects of flavonoids were found in several studies. Knekt et al. reported an inverse association between the flavonoids intake and occurrence of cancer[65]. The different anticancer effects in different wheat class were also found due to the content differences in variety[66]. Ferguson and colleges also stated the anticancer effect of wheat bran was not only because of the dietary fiber, but also mostly due to the phytochemicals and their synergic effects[67].

However, flavonoids existed in foods are considered non-digestive because of their bound form. Only the free form flavonoids can pass through the gut wall and perform their biological activities. Therefore, in this study, several treatments were

implemented to try to release the bound flavonoids in wheat bran and increase the bioactivity and also bioaccessibility of wheat bran.

Part 2: The standard curve of flavonoids

Chemicals:

Catechin and Aluminum chloride were purchased from Sigma-Aldrich (Wilwaukee, WI, USA). Sodium hydroxide and sodium nitrite were purchased from Fisher Chemicals (Pittsburgh, PA, USA).

Reagents:

Storage Catechin solution: 0.005 g catechin was weighed and firstly dissolved in approximately 8 mL distilled water in a 10 mL volumetric flask using a clean glass rod, and swirl the flask gently until the solids were thoroughly dissolved. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution. This was the store solution and stored at refrigerator covered with aluminum foil before use.

Standard curve catechin solutions: The store solution was diluted ten times by pipetting 1 mL store solution and adding with 9 mL distilled water to make a 0.05 mg/mL catechin solution. After that, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mL of the 0.05 mg/mL catechin were pipette and filled up to a total of 10 mL using distilled water to make a 0.000, 0.005, 0.010, 0.015, 0.020, 0.025, 0.030, 0.035, 0.040, 0.045, 0.050 mg/mL catechin solutions respectively.

100 mL 1N Sodium hydroxide: 4.00 g sodium hydroxide was weighed and firstly dissolved in approximately 40 mL distilled water in a 50 mL beaker. The solution was then transferred into a 100 mL volumetric flask using a clean glass rod. After that, the

beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 100 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 100 mL volumetric flask. Then added approximately 30 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to homogenize the solution.

40 mL Sodium nitrite (1:20, w:w): 2.00 g sodium nitrite was weighed and added with 40 mL distilled water. The solids were dissolved thoroughly before use.

100 mL Aluminum Chloride (1: 10, w:w): 10.00 g Aluminum chloride was weighed and added with 100 mL distilled water. The solids were dissolved thoroughly before use.

Methods:

1 mL of the above made catechin solutions were pipette into 12 mL tubes. Solutions were added with 0.06 mL sodium nitrite solution (1: 20, w:w) and stirred for 10 s. 0.6 mL aluminum chloride (1:10, w:w) were added after 5 min and stirred for 10 s. 0.4 mL sodium hydroxide (1N) were added after 6 min and stirred for 10 s. The solution with catechin in it turned to a yellow orange color at this point. The mixture was then measured at 510 nm using spectrophotometer equipment immediately. Triplicates were made and standard deviations were calculated.

Results:

The standard curve was calculated and drawn using Microsoft office 2008 for Mac (home and student edition) Excel software. Below is the figure of the standard curve (Fig. 5-9) with 11 spots. Table 7 showed the absorbance values of each catechin solution.

Table 7 absorption value of catechin solution at 510 nm

Catechin concentration (mg/mL)	1	2	3	Average	Standard deviation
0.000	0.000	0.001	0.000	0.000	0.001
0.005	0.033	0.032	0.032	0.032	0.001
0.010	0.068	0.067	0.066	0.067	0.001
0.015	0.104	0.103	0.104	0.104	0.001
0.020	0.143	0.141	0.141	0.142	0.001
0.025	0.177	0.178	0.179	0.178	0.001
0.030	0.216	0.218	0.216	0.217	0.001
0.035	0.247	0.248	0.245	0.247	0.002
0.040	0.283	0.282	0.280	0.282	0.002
0.045	0.307	0.303	0.300	0.303	0.004
0.050	0.313	0.308	0.307	0.309	0.003

As we can see, the absorbance value increased proportionally when the concentration of catechin increased at the beginning. However, when the concentration of catechin reached more than 0.04 mg/mL, the absorbance value began to increase slowly and had the trend of keeping constant around 0.300 mg/mL. However, since the absorbance value of the samples were all lower than that of 0.03 mg/mL catechin solution, the standard curve (Fig. 5-10) was reconstructed by deleting the high catechin concentration spots, which turned to be a straight line with a high R2 value (R^2=0.99943). It was used to calculate the flavonoids content in processed wheat bran.

The equation of the standard curve is

Y = 7.14X – 0.0019 (R2=0.99943)

Calculations of flavonoids content in processed wheat bran:

Set A as the absorbance value,

Then, the catechin concentration in 2.06 mL mixture is:

X = (A + 0.0019)/7.14 mg/mL

Then, the flavonoids content in processed wheat bran is:

C = X*10 mL/wheat bran weight g*100 mg/100g bran

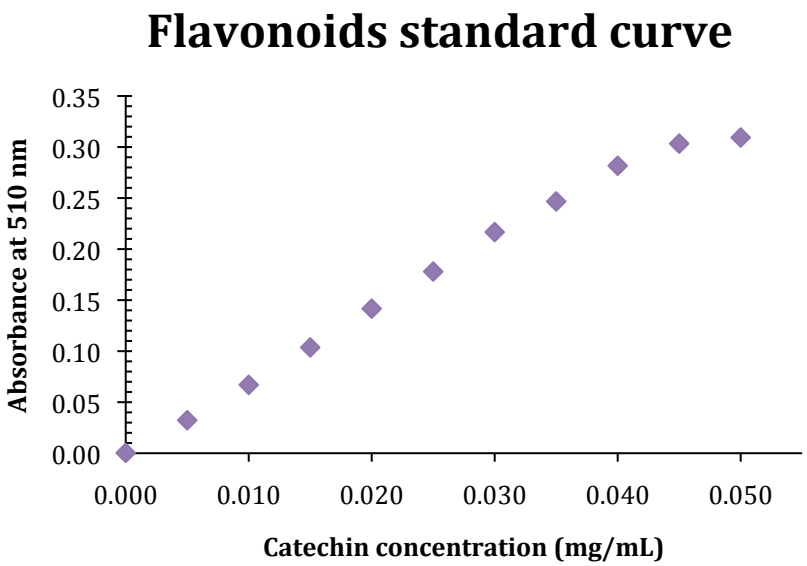


Figure 5-9 Flavonoids standard curve.

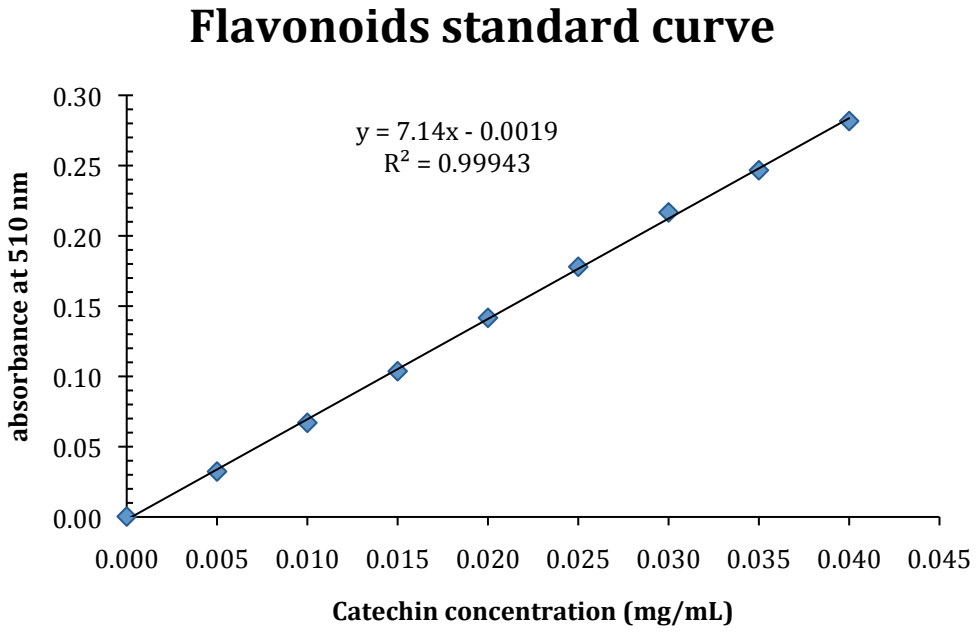


Figure 5-10 Reconstructed flavonoids standard curve.

Part 3: The methodology of anaylysis of flavonoids in wheat bran extracts

Materials:

Free antioxidant extracts, Soluble-conjunct extracts, and bound extracts.

Methods:

1 mL of extracts (free, soluble-conjunct, and bound extracts) were pipette into 12 mL tubes. Extracts were added with 0.06 mL sodium nitrite solution (1: 20, w:w) and stirred for 10 s. 0.6 mL aluminum chloride (1:10, w:w) were added after 5 min and stirred for 10 s. 0.4 mL sodium hydroxide (1N) were added after 6 min and stirred for 10 s. The solution with flavonoids content turned to a yellow orange color at this point. The mixture was then measured at 510 nm using spectrophotometer equipment immediately.

Part 4: Results and Discussion

Results:

The free flavonoids contents in processed wheat bran were expressed as microgram of catechin equivalent per 100 grams of wheat bran (Fig. 5-11). The enzymatic treated wheat bran (E) had the highest free flavonoids content (59.723 ± 9.060 mg/100 g bran), followed by enzymatic and high shear mixed wheat bran (EH) (31.693 ± 4.750 mg/100 g bran). The control wheat bran (C) had the lowest free flavonoids content (5.154 ± 0.593 mg/100 g bran). Other samples had similar free flavonoids content around 20 mg/ 100 g bran in the middle. Among which, the alkaline, high shear mixing, and high pressure homogenized wheat bran (AHH) had higher free flavonoids content (28.566 mg/100 g bran) compared to other alkaline treated wheat bran, and the alkaline treated

wheat bran (A) and the alkaline and high shear mixed wheat bran (AH) had lower flavonoids content (18.632 ± 4.376, 18.815 ± 7.515 mg/100 g bran respectively).

The soluble-conjunct flavonoids contents in processed wheat bran were expressed as microgram of catechin equivalent per 100 grams of wheat bran (Fig. 5-12). The soluble-conjunct flavonoids content in all samples were similar and had no significant differences. All were approximately 2 mg/100 g bran with standard deviation ranging from 0.170 to 1.684.

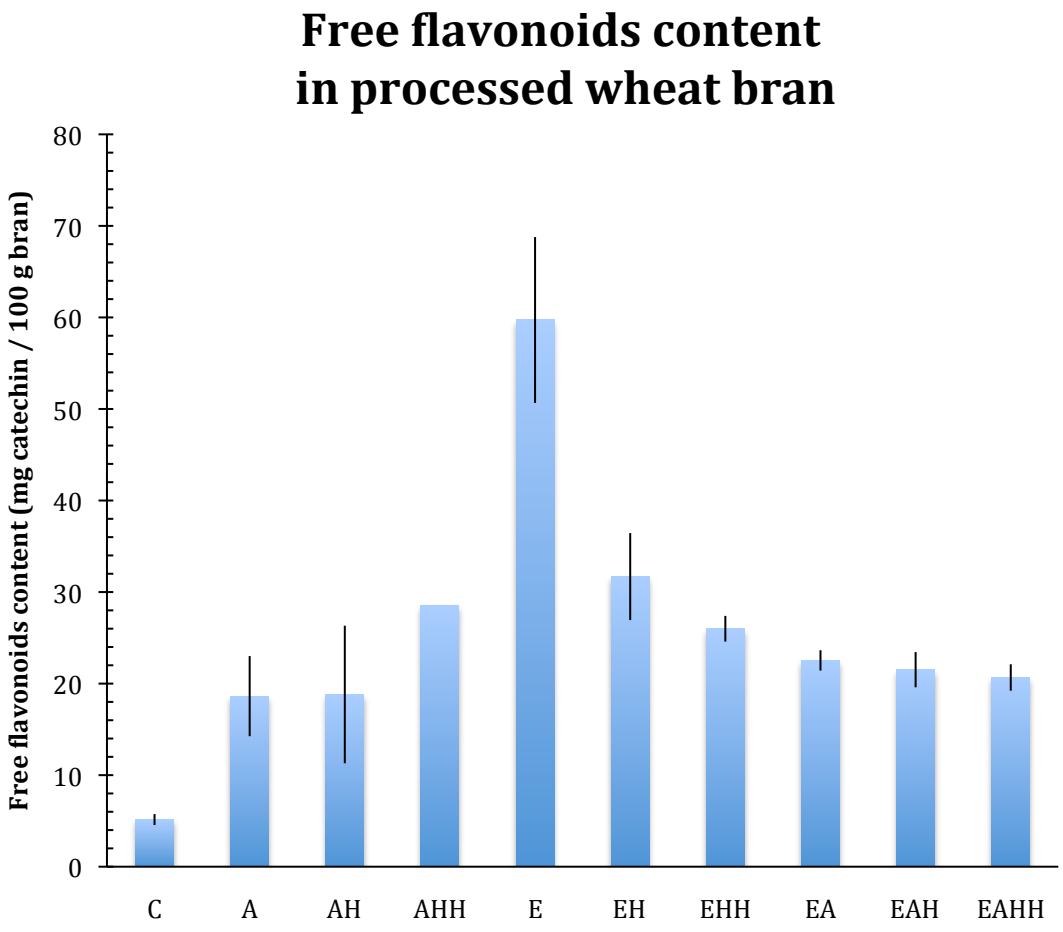


Figure 5-11 Free flavonoids content in processes wheat bran.

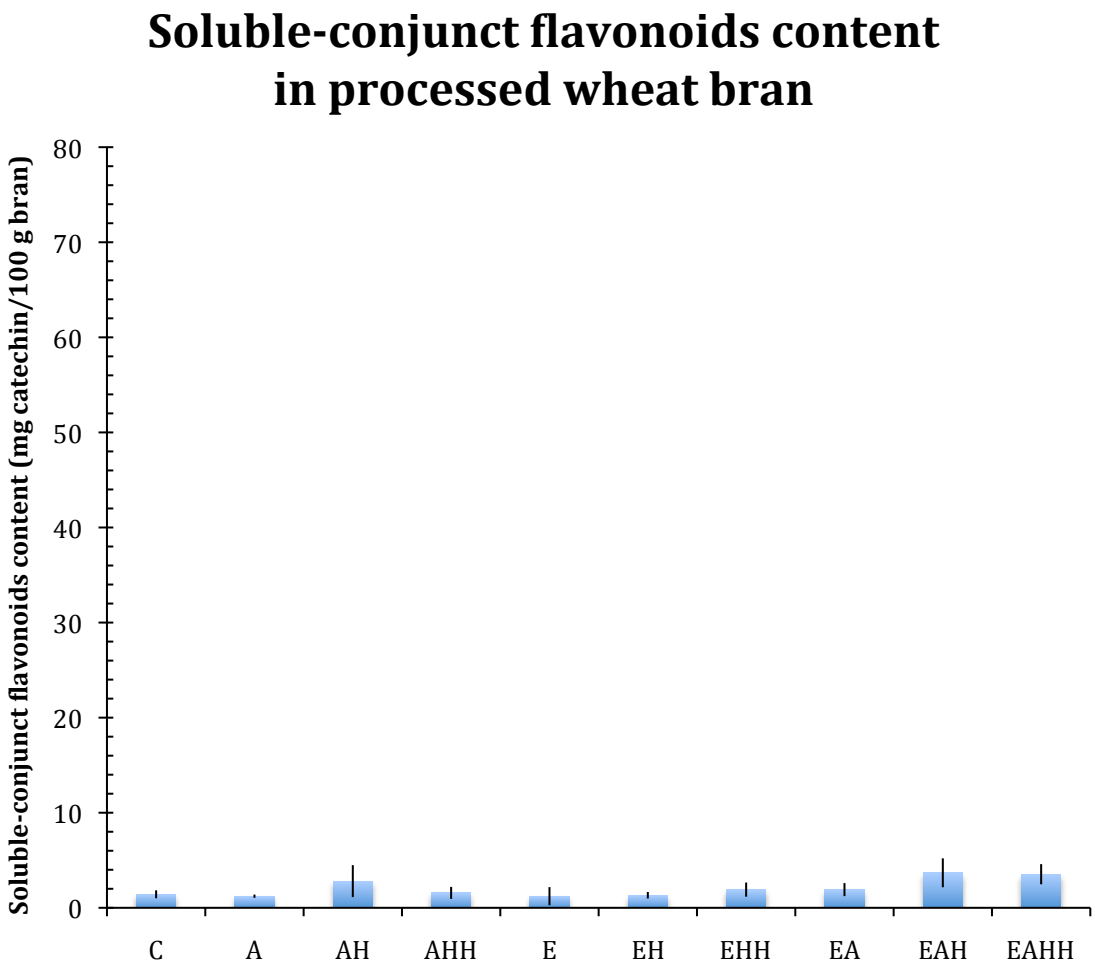


Figure 5-12 Soluble-conjunct flavonoids content in processed wheat bran.

The bound flavonoids contents in processed wheat bran were expressed as microgram of catechin equivalent per 100 grams of wheat bran (Fig. 5-13). The enzymatic, no alkaline treated wheat bran had higher flavonoids contents (E: 34.037 ± 12.199 mg/100 g bran, EH: 37.763 ± 11.247 mg/100 g bran, EHH: 37.448 ± 9.221 mg/100 g bran, respectively) but with larger standard deviation. The alkaline, no enzymatic treated wheat bran had lower flavonoids contents (A: 15.444 ± 3.956 mg/100 g bran, AH: 16.042 ± 1.747 mg/100 g bran, AHH: 14.767 ± 1.527 mg/100 g bran, respectively) but with smaller standard deviation. The flavonoids contents of enzymatic

and alkaline treated wheat bran are in the middle (EA: 20.169 ± 4.512 mg/100 g bran, EAH: 24.334 ± 4.745 mg/100 g bran, EAHH: 25.990 ± 2.074 mg/100 g bran) also with smaller standard deviation.

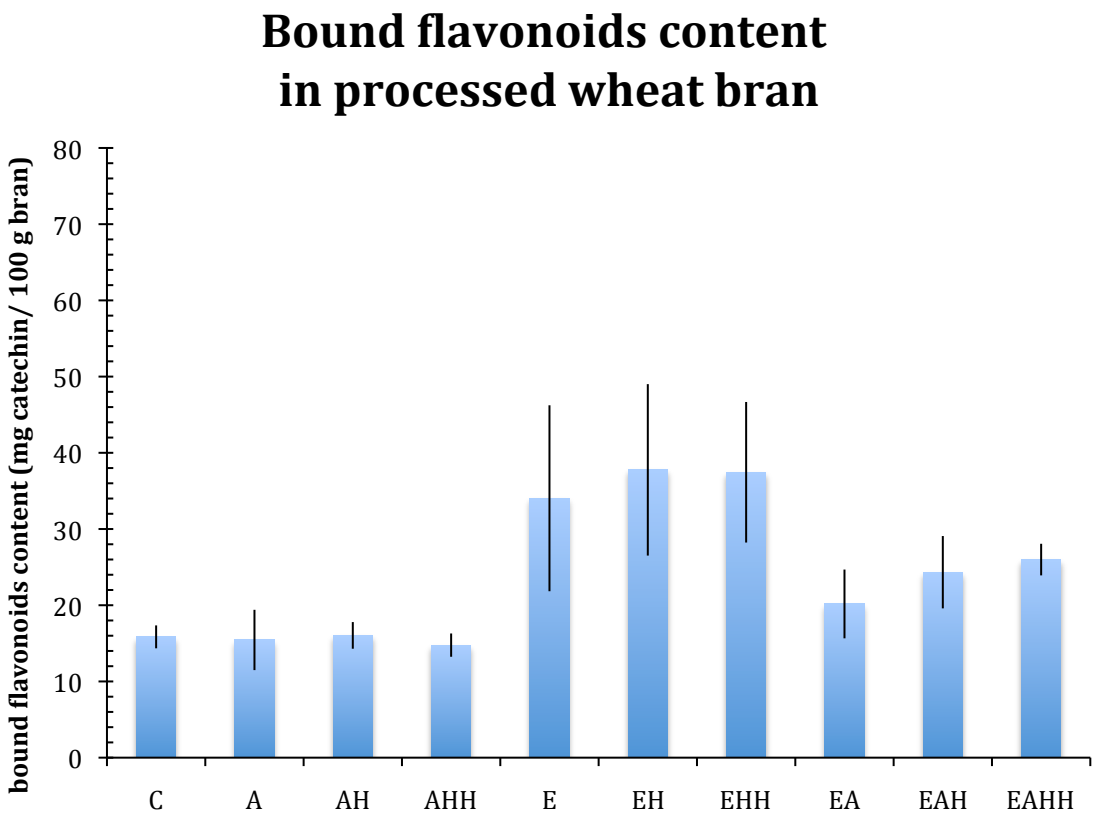


Figure 5-13 Bound flavonoids content in processed wheat bran.

Discussion:

The result of free flavonoids contents in processed wheat bran indicated that the enzymatic treatment had significant effects on releasing flavonoids in wheat bran (Fig. 5-14). The xylanase can cleave the backbone of arabinoxylan and chop the long main chain into short pieces, therefore, release the pigments trapped within the polysaccharides structure. The alkaline treatment did not release much flavonoids in wheat bran compared to enzymatic treatments. Because the alkaline treatment is a hydrolysis reaction which

cleaves the ester linkage between ferulic acid and arabinoxylan but does not have much effects on the main chain changes. Therefore, the trapped flavonoids within the polysaccharides structure could not be freed. The mechanical treatments such as high shear mixer and high pressure homogenization had rarely no effects on releasing flavonoids in wheat bran, on the contrary, they caused several flavonoids loss due to the unstable character of flavonoids. The flavonoids have high antioxidant activity both *in vivo* and *in vitro*. *In vitro*, the flavonoids have stronger antioxidant activity than those of vitamin C and E[68]. Therefore, during the mechanical processes, the samples were exposed to air (oxygen) and maybe heat generated by shearing and high pressure pumping. The flavonoids loss caused by mechanical processes after enzymatic treated wheat bran is larger than those after alkaline and both enzymatic and alkaline treated wheat bran. This may be because the alkaline treatment could cause flavonoids loss as well due to samples exposing to oxygen and high temperature (60ºC). However, all the treatments and processes contribute to release flavonoids trapped within the polysaccharides structure.

The result of soluble-conjunct flavonoids contents in processed wheat bran showed rarely flavonoids contained in those samples, indicating that flavonoids are mostly in the free form in the ethanol extracted samples.

The result of bound flavonoids contents in processed wheat bran indicated all the treatments failed to release all the trapped flavonoids, leaving around 40 mg/100 g bran bound flavonoids content. The enzymatic, no alkaline treated wheat bran had higher bound flavonoids content, while the alkaline, no enzymatic treated wheat bran contained less flavonoids, but they did not have significant differences considering the variation.

This also indicated that not all the flavonoids being released after the 2N NaOH incubation, because alkaline treatment is only aimed to release all the phenolics acids, especially attached ferulic acid, but not to chop down polysaccharides and free the trapped flavonoids within it. That is why the total amounts combined free, soluble-conjunct, and bound flavonoids were different among all the processed wheat bran. Another reason may be due to the wheat bran color since flavonoids are a large class of plant pigments. Enzymatic, alkaline and mechanical treatments could contribute to color changes of wheat bran, which influenced the color of the extracted bound solution. Therefore, it is likely to have effects on the results considering that the method to measure the flavonoids content is a spectrophotometric approach, which measures the absorbance at a certain wavelength mostly depending on the color of the solution.

In conclusion, alkaline, enzymatic, and mechanical treatments help to release trapped flavonoids in wheat bran. Enzymatic treatment is the most significant approach, followed by alkaline treatment. Both alkaline treatment and mechanical processes cause loss of freed flavonoids due to exposing to air, light, and heat.

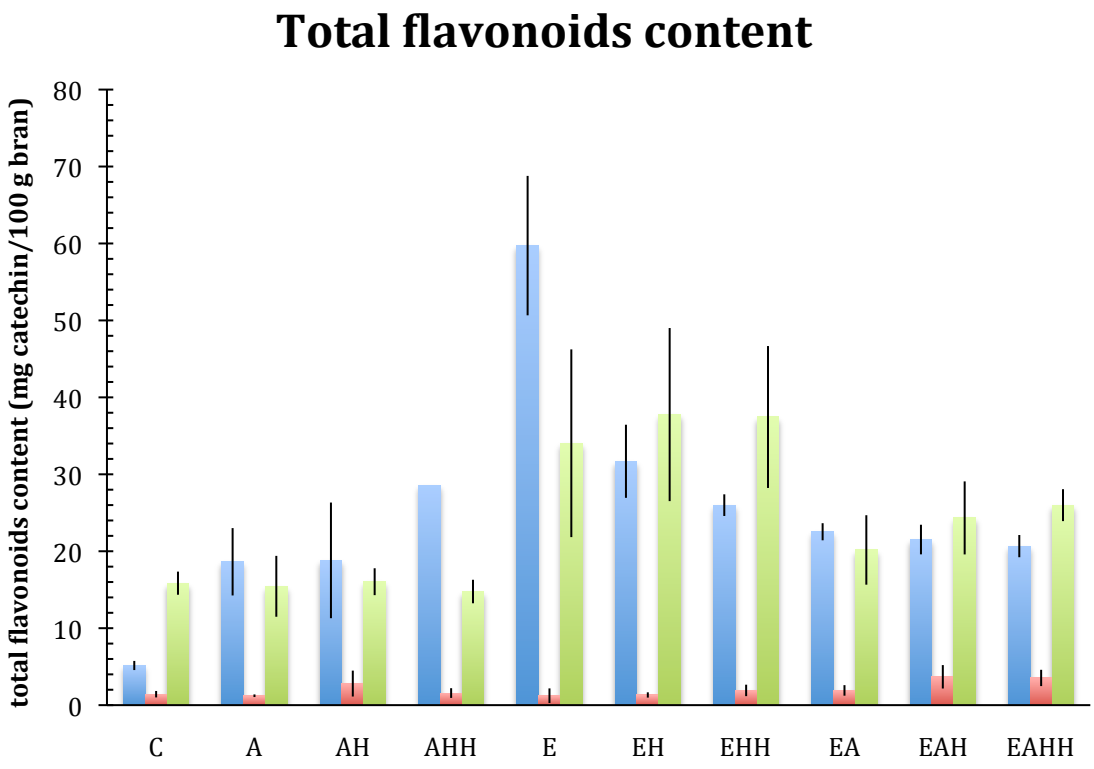


Figure 5-14 The total flavonoids content in processed wheat bran.

Ferulic acid

Part 1: Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) belongs to hydroxycinnamic acid derivatives. It is the main phenolic acid existed in plant cell walls[70] (Fig. 5-15). Ferulic acid is synthesized through the metabolism of phenylalanine and tyrosine, which is also the pathway to synthesis diverse secondary metabolites including lignin[71 72 73].

Ferulic acid occurs in various fruits, vegetables, and whole grains including berries, peas, tomatoes, asparagus, oat, barley, and wheat[73], but it mostly exists abundant in whole grains in both free form and bound form, with a free to bound ratio of 0.1:100[21]. In wheat bran, ferulic acid was attached to the polysaccharides such as arabinoxylans, and lignin through ester and ether linkages[74 70]. F-Ara-(1→3)-Xyl and F-Ara was found

in wheat bran (*Triticum aestivum*). (F-Ara)-(1→3)-Xyl-(1→4)-Xyl, Xyl-(1→2)-(F-Ara), and Gal-(1→4)-Xyl-(1→2)-(F-Ara) were also found in barley and maize bran[75]. Ferulic acid can also form dimer or trimer in wheat bran cell wall to cause cross-linking of arabinoxylans, which not only strengths the cell wall, but also prevents microorganism's infection[54 75] (Fig. 5-16).

Ferulic acid, as a bioactive compound, has several pharmaceutical functions such as antioxidant activity, cholesterol-lowering activity, prevention against thrombosis and atherosclerosis, antimicrobial and anti-inflammatory activity, and anti-cancer effect[76]. The mechanisms are various and have not been clearly understood so far, but it mainly contributes to the antioxidant activity of ferulic acid and its ability of inhibition and prevention of active enzymes.

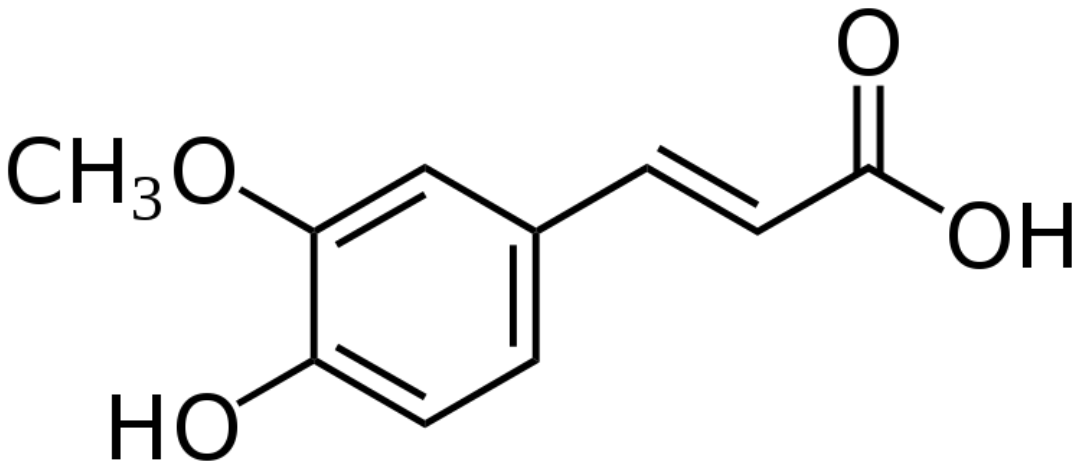


Figure 5-15 Structure of ferulic acid.
(http://en.wikipedia.org/wiki/File:Ferulic_acid_acsv.svg)

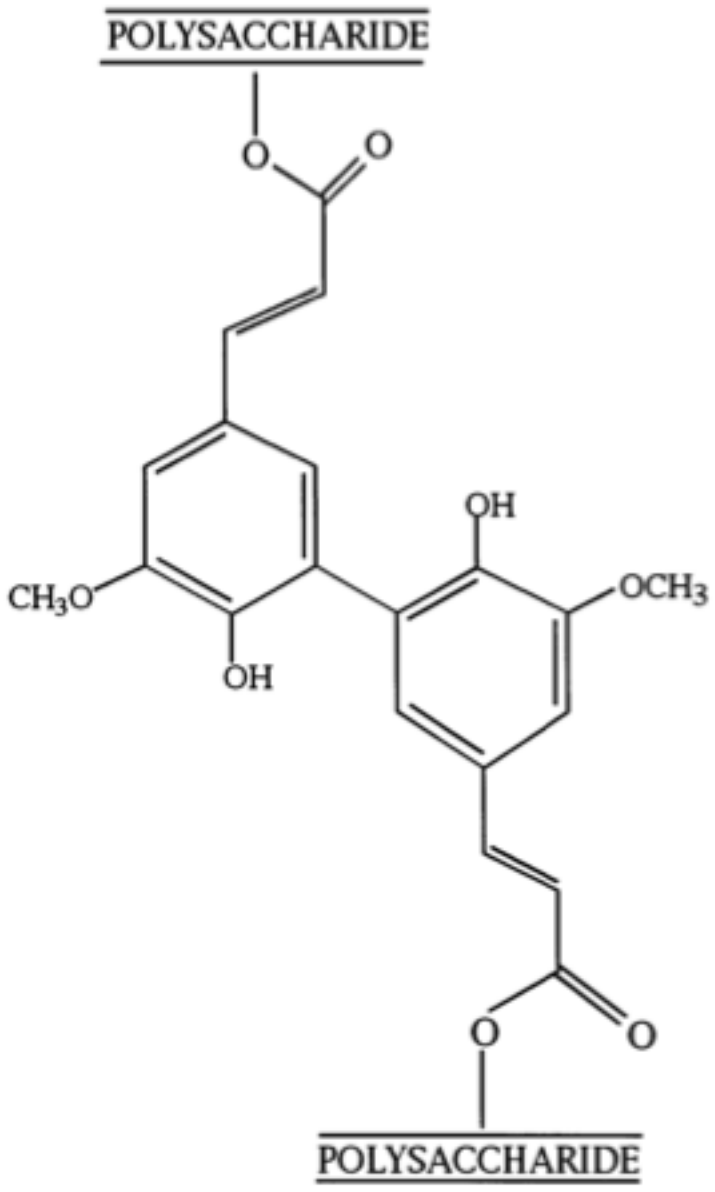


Figure 5-16 Structure of diferulate cross-link between polysaccharide.

Ferulic acid can be absorbed in stomach, jejunum, and ileum[71]. Studies investigated the absorption of ferulic acid in rats, and stating that ferulic acid could be recovered as conjugated forms in plasma and bile secretion and the cereal complex matrixes prevented the absorption of ferulic acid, thus lower its bioavailability[77]. Ferulic acid can be metabolized into a number of ferulic acid conjugates. Zhou et al. analyzed the

metabolites in urine, feces, and bile in rats, and found they are all FA-glucuronide conjunctions, among which ferulic acid conjugated with two glucuronic acids was the domain components[78].

The bioactivity of ferulic acid mostly depends on its form. Soluble ferulic acid conjunctions are considered more active than the bound ones[71]. However, in wheat bran, almost all ferulic acid are in their bound form, therefore releasing them can improve the bioactivity of wheat bran. In this study, alkaline, enzymatic, and mechanical treatments were applied to improve the free ferulic acid content in wheat bran.

Part 2: The standard curve of ferulic acid

Chemicals:

Ferulic acid, triflouroacetic acid, and methanol were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA).

Reagents:

1L pH=2, triflouroacetic acid (TFA) solution: 1 L Milli-Q water was added with triflouroacetic acid to adjust pH to 2 in 2 L beaker.

Storage ferulic acid solution: 0.1000 g ferulic acid was weighed and firstly dissolved in approximately 20 mL distilled water in a 25 mL beaker. The solution was then transferred into a 50 mL volumetric flask using a clean glass rod. After that, the beaker was washed using a small amount of distilled water four times and all the wash water were transferred into the 100 mL volumetric flask as well. The glass rod then was washed by distilled water too and the wash water was pooled into the 100 mL volumetric flask. Then added approximately 15 mL distilled water and swirled the flask gently. Then added more distilled water until the bottom of the meniscus touches the graduation line. The stopper was then placed in the neck and the whole flask inverted repeatedly to

homogenize the solution. This was the store solution and stored at refrigerator covered with aluminum foil before use.

Standard curve ferulic acid solutions: The store solution was diluted by pipetting 1 mL store solution and adding with 39 mL distilled water to make a 0.05 g/L ferulic acid solution. After that, 0, 1, 2, 3, 4, 5 mL of the 0.05 g/L ferulic acid solution were pipette and filled up to a total of 5 mL using distilled water to make a 0, 0.01, 0.02, 0.03, 0.04, 0.05 g/L ferulic acid solutions respectively.

Methods:

Free ferulic acid was quantified by HPLC analysis. Ferulic acid solutions prepared above were passed through 45μm filter and 10μL were injected into a Varian Polaris HPLC system (Varian Inc. Palo Alto, CA, USA) equipped with a Luna 5u Phenyl-Hexyl column (3.0 μm particle size, 250*4.6mm ID, Phenomenex, Torrance, CA) and coupled online with a Varian Prostar 400 AutoSampler and Varian Prostar 325 UV-Vis detector. Elution was carried out with solvent A (Milli-Q water + trifluroacetic acid (TFA), pH=2) and solvent B (methanol) following this gradient: 0-25min, from 95%A, 5%B to 70%A, 30%B, linear gradient; 25-35min, from 70%A, 30%B to 50%A, 50%B, linear gradient; 35-45min, from 50%A, 50%B to 0%A, 100%B, linear gradient; 45-55min, 0%A, 100%B; 55-60min, from 0%A, 100%B to 95%A, 5%B, linear gradient; 60-70min, 95%A, 5%B. These conditions were applied at a constant temperature of 30°C and at a flow rate of 1mL min^{-1}. Ferulic acid was detected at 280nm.

Results:

The standard curve was calculated and drawn using Microsoft office 2008 for Mac (home and student edition) Excel software. Below is the figure of the standard curve (Fig. 5-17). Table 8 showed the peak area of each ferulic acid solution.

Table 8 Peak area of each ferulic acid solution.

Ferulic acid concentration (g/L)	1	2	Average	Standard Deviation
0.00	0	0	0	0
0.01	2614848	2689957	2652403	53110.08
0.02	5285381	5272743	5279062	8936.416
0.03	7698182	7989358	7843770	205892.5
0.04	10449541	10328781	10389161	85390.21
0.05	13022892	13173514	13098203	106505.8

As we can see, the peak area increased proportionally when the concentration of ferulic acid increased.

The equation of the standard curve is

$Y = 3E+08X + 24767$ $(R2=0.99993)$

Calculations of ferulic acid content in processed wheat bran:

Set A as the peak area,

Then, the ferulic acid concentration in 10 mL mixture is:

$X = (A - 24767)/3E+08$ g/L

Then, the phenolics content in processed wheat bran is:

$C = X*1000*0.010$ L/wheat bran weight g*100 mg/100g bran

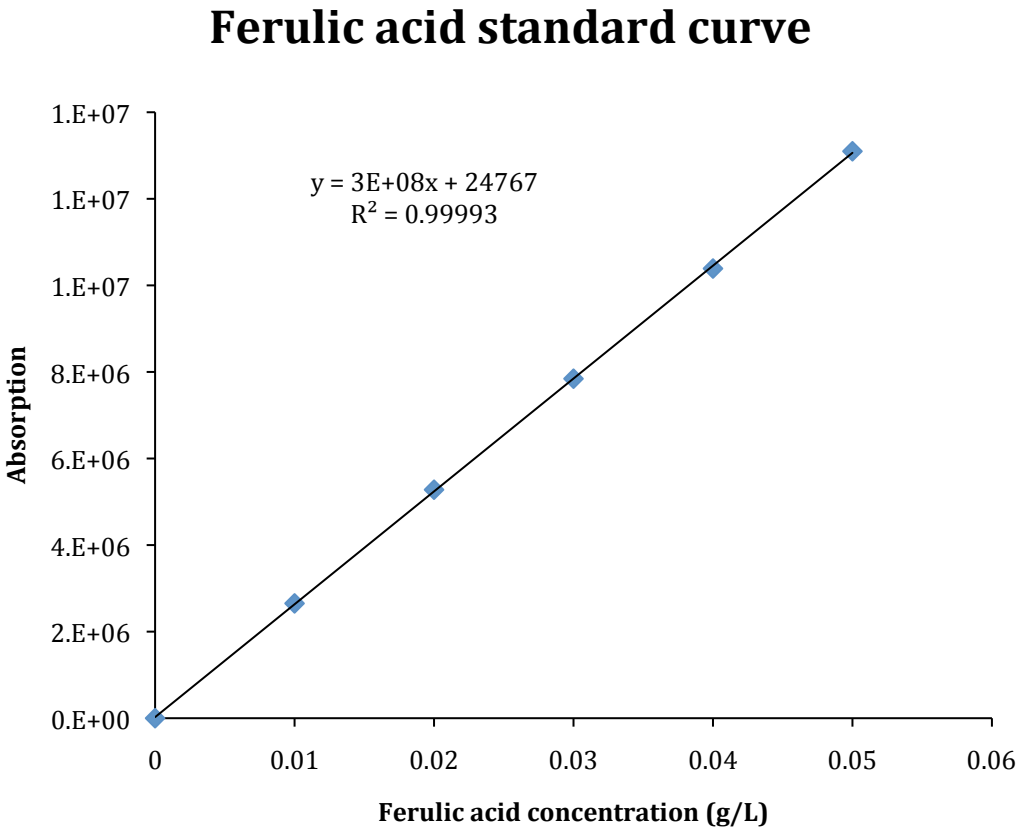


Figure 5-17 The ferulic acid standard curve

Part 3: The methodology of anaylysis of ferulic acid in wheat bran extracts

Materials:

Extracts of free phenolics in processed wheat bran (C, A, AH, AHH, E, EH, EHH, EA, EAH, EAHH)

Methods:

Free ferulic acid was quantified by RP-HPLC analysis. Extracts were passed through 45µm filter and 10µL were injected into a Varian Polaris HPLC system (Varian Inc. Palo Alto, CA, USA) equipped with a Luna 5u Phenyl-Hexyl column (3.0 µm particle size, 250*4.6mm ID, Phenomenex, Torrance, CA) and coupled online with a Varian Prostar 400 AutoSampler and Varian Prostar 325 UV-Vis detector. Elution was

carried out with solvent A (Milli-Q water + trifluroacetic acid (TFA), pH=2) and solvent B (methanol) following this gradient: 0-25min, from 95%A, 5%B to 70%A, 30%B, linear gradient; 25-35min, from 70%A, 30%B to 50%A, 50%B, linear gradient; 35-45min, from 50%A, 50%B to 0%A, 100%B, linear gradient; 45-55min, 0%A, 100%B; 55-60min, from 0%A, 100%B to 95%A, 5%B, linear gradient; 60-70min, 95%A, 5%B. These conditions were applied at a constant temperature of 30°C and at a flow rate of 1mL min-1. Ferulic acid was detected at 280nm. The free ferulic acid concentration in samples was extrapolated from the pure ferulic acid standard curve.

Part 4: Results and Discussion

Results:

The free ferulic acid contents in each sample were expressed as micro grams of ferulic acid per 100 grams of wheat bran. Table 9 showed the ferulic acid content in alkaline treated and both alkaline and enzymatic treated samples. The free ferulic acid in control and enzymatic treated samples were either not detected or hard to determine, because several peaks were eluted during the ferulic acid retention time and it was hard to identify the ferulic acid peak (Shown in appendix).

As we can see in table 9, alkaline treated samples gave higher free ferulic acid content (A: 308.602 ± 20.352 mg/100g bran, AH: 236.105 ± 43.007 mg/100g bran, AHH: 336.715 ± 52.582 mg/100g bran). The AH sample contained less free ferulic acid compared with A and AHH samples. The both alkaline and enzymatic treated bran had around one third free ferulic acid content of that contained in the alkaline treated ones (EA: 109.408 ± 9.011 mg/100g bran, EAH: 114.406 ± 18.664 mg/100g bran, EAHH: 101.324 ± 27.379 mg/100g bran).

Table 9 The free ferulic acid content in processed wheat bran.

Sample	Ferulic acid content (mg/100g bran)	SD
A	308.602	20.352
AH	236.105	43.007
AHH	336.715	52.582
EA	109.408	9.011
EAH	114.406	18.664
EAHH	101.324	27.379

Discussion:

The alkaline treatment is efficient in releasing esterified ferulic acid from wheat bran due to its hydrolysis effects. The sodium hydroxide solution can help hydrolyze the ester linkage and free the bound ferulic acid. That is why several studies used mild or severe alkaline treatment as the pre-process for the bound phenolics analysis in various food systems[16, 21, 26, 46]. The alkaline treatment conditions they normally used were 1-4N sodium hydroxide and for 1-24 hours. In this study, the concentration of sodium hydroxide is only 0.1N and can also release almost all bound ferulic acid, which reduce the consumption of chemicals. The alkaline treatment also releases gallic acid compared with the control wheat bran. The peak area of alkaline treated wheat bran is more than ten times higher than that of control samples.

The enzymatic treatment seems rarely releasing bound ferulic acid from wheat bran according to the HPLC results. Even though several peaks were eluted during the ferulic acid retention time, but their peak area is small and the ferulic acid peak cannot be identified. The enzymatic treatment also helps to release gallic acid, but not as high as the alkaline treatment. It releases cinnamic acid, which is not detected in only alkaline treated

wheat bran. However, it cannot be quantitated due to unavailable standard of cinnamic acid, because cinnamic acid is not the domain phenolics in wheat bran.

The combined enzymatic and alkaline treated wheat bran contain both free ferulic acid and cinnamic acid. However, the amount of free ferulic acid is less than the alkaline only treated samples. The reason is that the alkaline treatment condition is different. The alkaline only treated wheat bran samples are soaked in 0.1N sodium hydroxide at 60ºC for 24h, while the combined enzymatic and alkaline treated wheat bran are soaked in 0.1N sodium hydroxide at 40ºC for 6h. It also indicates that temperature and time are main effects in releasing bound ferulic acid in wheat bran. However, they are not significant effects in releasing bound phenolics in wheat bran according to the total phenolics content results, indicating that several phenolics acids are contained in wheat bran besides ferulic acid, and various treatments aim at releasing different phenolics acids. In the total phenolics acids content test, each single phenolics acid cannot be evaluated, whereas in the HPLC test, each phenolics acid represents a peak and elutes at certain retention time. Therefore, it is easy to identify the various effects of various treatments.

The mechanical processes do not help to increase the free ferulic acid in wheat bran but they do not give rise to significant lost of free ferulic acid also. Since the mechanical processes help to improve the functionality of wheat bran such as the viscosity and particle size, the no effects on antioxidants may be good news.

The free ferulic acid results are not present as our expected. According to the total phenolic acids content, we assume that the ferulic acid content should have the same trend. However, the enzymatic treatment did not release much bound ferulic acid, while,

the temperature and time of alkaline treatment are the main effects in releasing esterified ferulic acid. The unexpected results showed the different working mechanism of enzyme and alkaline. Xylanase aims to degrade the main chain of arabinoxylans, which frees the trapped phenolics within the wheat bran structure. The alkaline works to cleave the ester link and free the bound ferulic acid.

In conclusion, the alkaline treatment is the most efficient approach to release bound ferulic acid in wheat bran. The enzymatic treatment helps to release other phenolics acids such as cinnamic acid. The mechanical processes have no effects on improving antioxidant in wheat bran.

Chapter 5: Water Holding Capacity (WHC) of multiple treated wheat bran

Introduction:

Water holding capacity (WHC) is an ability of a matrix to retain inherent water. Dietary fiber has a WHC, which is associated with chemical composition and fiber structure[55]. The chemical composition determined the amount of bound water whereas the fiber structure determined the amount of trapped water. Roberson examined factors that may affect the WHC of dietary fiber and stated that "WHC is more a function of fiber structure than chemical composition". That is why the various WHC held by various cereal, vegetable, and fruit fiber[55]. Vegetable and fruit fiber have a higher WHC compared with cereal fiber. Chen compared apple fiber, wheat and oat bran and concluded a much higher WHC of apple fiber due to the different fiber structure, the higher fiber content, and the smaller particle size of apple fiber[56]. As early as 1978, Scientists in Oxford found coarse bran positively affected the stool weight and gave a greater stool weight than fine bran, which was partially related to WHC[57]. When analyzing the effects of particle size on WHC, Cadden found that the WHC of wheat bran decreased with reduced particle size because of the lost of insoluble fiber. However, the WHC of oat bran was not affected with reduced particle size[58]. This confirmed that previous study carried out by Robertson stating the importance of fiber structure on WHC. However, the WHC of wheat bran was strictly associated with the insoluble fiber content and particle size distribution[59].

Several researches studied the effects of thermal and enzyme treatment on WHC of dietary fiber. Thermal treatments including baking and extrusion-cooking were implemented in Camire's research. The results showed the baking process had no effect

on WHC in corn meal, oat meal and potato peels, whereas the extrusion process increased the WHC of corn meal, and oat meal[60]. Rouzu et al. studied the enzyme effects on WHC of wheat flour and found no significant effect[61].

Pentosans is an important component in wheat and flour and contributes to dough and baking performance. It can absorb a large amount of water and have significant influence on the rheological properties of dough[62]. The water-soluble pentosans had a positive effect in dough, however, the water-insoluble pentosans had a higher WHC[63].

Since many factors could affect the WHC of wheat bran, the WHC of different treated wheat bran was analyzed in this study. The enzymatic, alkali treatment, and mechanical processes could change the fiber structure and thus cause changes in WHC. This study is to evaluate the effects of different treatment on WHC of wheat bran.

Materials and Methods:

Materials:

The processed wheat bran: C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH.

Methods:

0.1000 g processed wheat bran (C, A, AH, AHH, E, EH, EHH, EA, EAH, and EAHH) were weight in 50 mL centrifuge tubes. The centrifuge tubes were capped and weighed (labeled as W1), and the weights were recorded. All the samples were then added with 20 mL distilled water and shaken vigorously. The mixture was rested for 24 hrs and then was centrifuged at 2,000 g for 2 hrs. The supernatant was discarded and the centrifuge tubes were drained for 20 mins by leaning downward at a 45-degree angle. The left water drops on the inner and out walls of centrifuge tubes were wiped dry. After that, the centrifuge tubes with caps on were weighed (labeled as W2). Triplet samples

were made and the standard deviations were calculated. The water holding capacity was calculated using the equation below:

WHC= (W2-W1)/processed wheat bran dry weight

The water holding capacity was expressed as the gain water weight per gram of the processed wheat bran dry weight (g water/g wheat bran). The calculation was done by Microsoft Office 2008 for Mac (home and student edition) Excel software.

Results:

The water holding capacity (WHC) was expressed as gram of water per gram of bran. The results were shown in fig. 6-1. As we can see, the alkaline, high shear mixing and high-pressure homogenized wheat bran gave the highest WHC (6.091 ± 0.236 g water/g bran), followed by alkaline, high shear mixed wheat bran (5.776 g water/g bran). The WHC of control wheat bran was 3.747 ± 0.163 g water/g bran. The alkaline, no enzymatic treated wheat bran gave higher WHC than control (A: 4.647 ± 0.149 g water/g bran; AH: 5.776 g water/g bran; AHH: 6.091 ± 0.236 g water/g bran). The enzymatic, no alkaline treated wheat bran had lower WHC compared to the control wheat bran (E: 3.443 ± 0.478 g water/g bran; EH: 2.814 ± 0.130 g water/g bran; EHH: 2.481 ± 0.890 g water/g bran), with a decreasing trend from E to EHH. The alkaline, enzymatic treated wheat bran gave lower WHC as well (EA: 2.984 ± 0.529 g water/g bran, EAH: 2.982 ± 0.185 g water/g bran), but EAHH had a higher WHC (4.200 ± 0.159 g water/g bran), which was interesting.

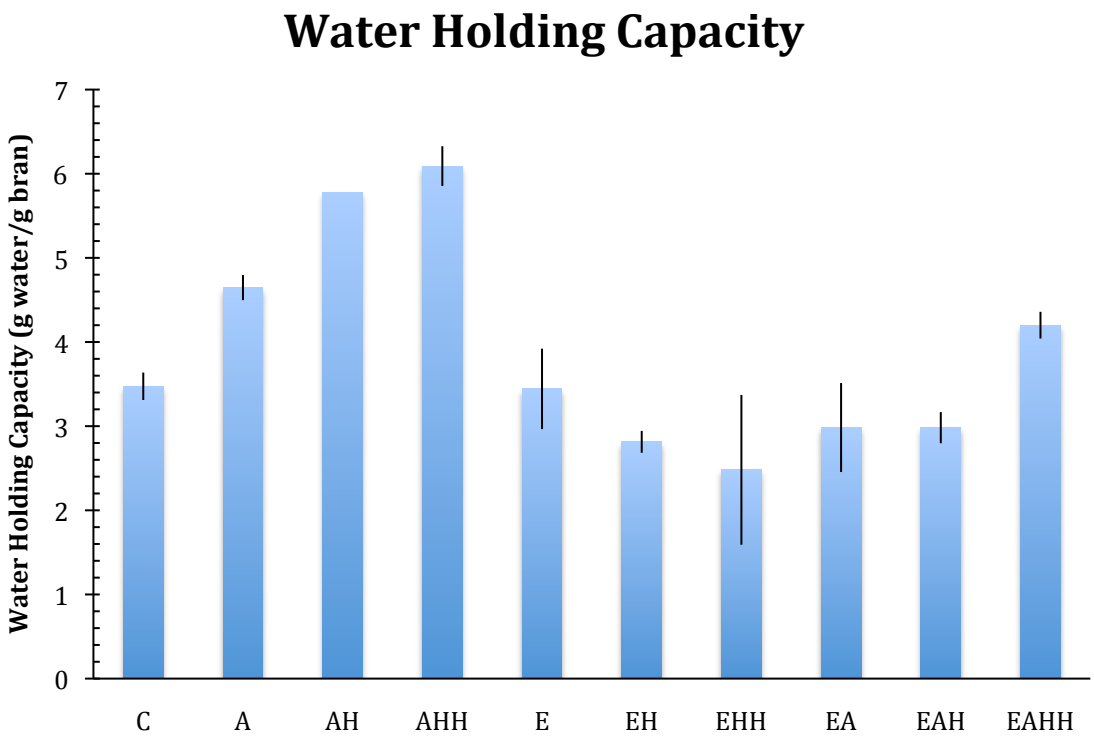


Figure 6-1 WHC of processed wheat bran

Discussion:

The alkaline, no enzymatic treated wheat bran significantly increased the water holding capacity compared to the control wheat bran (Fig. 6-2). The single alkaline treatment increased the WHC from 3,474 ± 0.163 to 4.647 ± 0.149 gram of water per gram of bran. The high shear mixture further raised it up to 5.776 gram of water per gram of bran. It finally reached to 6.091 ± 0.236 gram of water per gram of bran after high-pressure homogenization. All three treatments have positive effects on WHC of wheat bran. The increase in WHC caused by alkaline treatment may due to the increase of water binding sites after ester linkages being hydrolyzed in sodium hydroxide solution. The main effect of alkaline treatment is to cleave the ester linkage between ferulic acid and arabinoxylans, leaving un-bond active sites to likely bind water. In addition, ferulic acid

plays an important role in holding wheat bran structure through cross-linking arabinoxylans. Therefore, by releasing bond ferulic acid, it also frees the complex cross-linked structure, thus generating new binding sites to absorb water. The high shear mixture helped to reduce the particle sizes and was likely to open the structure, which turned to increase the water binding sites as well. However, the mechanism is different from the alkaline treatment although they all resulted the increase of water binding sites. The alkaline treatment (chemical treatment) was more efficient because it was more specific. While the high shear mixture (mechanical treatment) was not as significant as alkaline treatment. However, the combined chemical and mechanical treatments gave higher WHC than single treatment (A: 4,647 ± 0.149 g water/g bran, AH: 5.776 g water/g bran). The high-pressure homogenization also helped to reduce the particle sizes and opened the structure but failed to significantly increase the WHC when comparing AH and AHH (AH: 5.776 g water/g bran, AHH: 6.091 ± 0.236 g water/g bran). It may due to the previous high shear mixing process has already had the same effects even though the high pressure homogenization reduced the particle size a lot and increased the surface area.

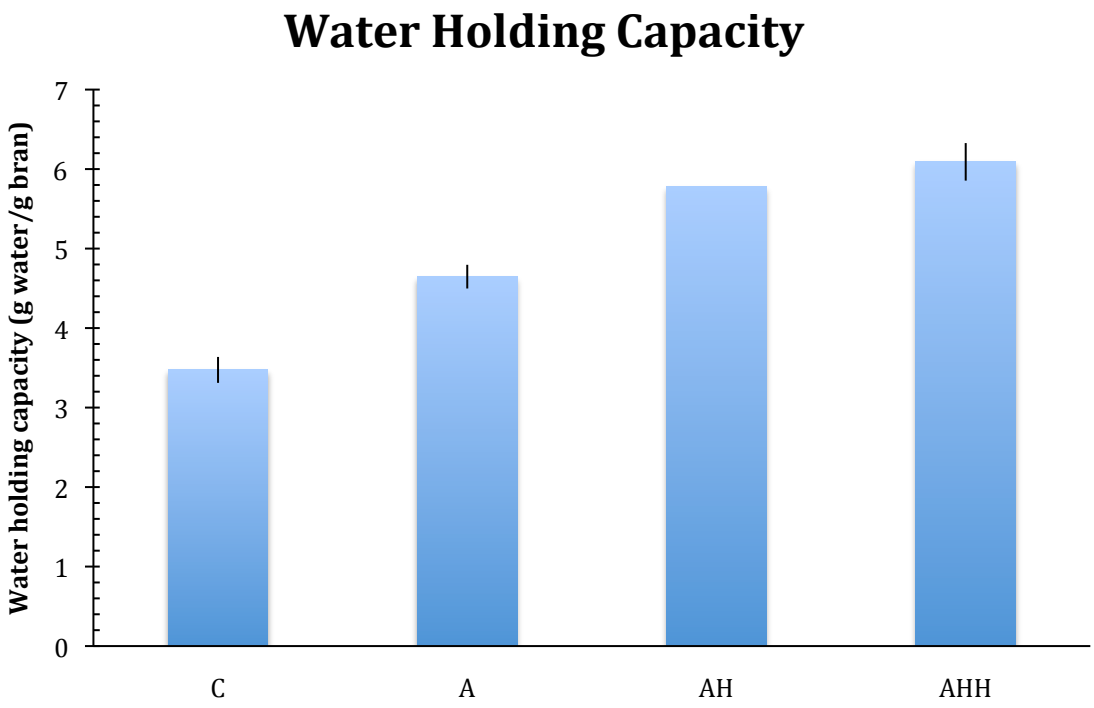


Figure 6-2 WHC of alkaline, no enzymatic treated wheat bran.

The enzymatic, no alkaline treated wheat bran had negative effects on WHC of wheat bran (Fig. 6-3). The single enzymatic treatment gave similar WHC compared to the control wheat bran (C: 3.474 ± 0.163 g water/g bran, E: 3.443 ± 0.478 g water/g bran). The high shear mixture then decreased it to 2.814 ± 0.130 gram of water per gram of bran. It was finally down to 2.481 ± 0.890 g water/g bran after the high pressure homogenization. The decreasing trend is very interesting and may due to the prior enzymatic treatment. The enzymatic treatment is to cleave the xylose backbone of arabinoxylans. It chops down the backbone and generates shorter and smaller polysaccharides, which are more likely to able to solve in water. In baking industry, pentosans have significant effects on dough rheology and bread quality due to its high affinity for water[61]. The water-insoluble pensotans have higher WHC than the water-soluble one[63]. In this case, the pensotans is arabinoxylans. Since enzymatic treatment

contributed to more water-soluble arabinoxylans, resulting in a decrease in WHC of wheat bran. The further high shear mixture and high-pressure homogenization processes also helped to reduce particle size and further generate more water-soluble arabinoxylans. The alkaline treated wheat bran may contain less water-soluble arabinoxylans compared with the enzymatic treated wheat bran due to their different specific cutting sites, therefore the alkaline treated wheat bran gave higher WHC, while enzymatic treated wheat bran had lower WHC.

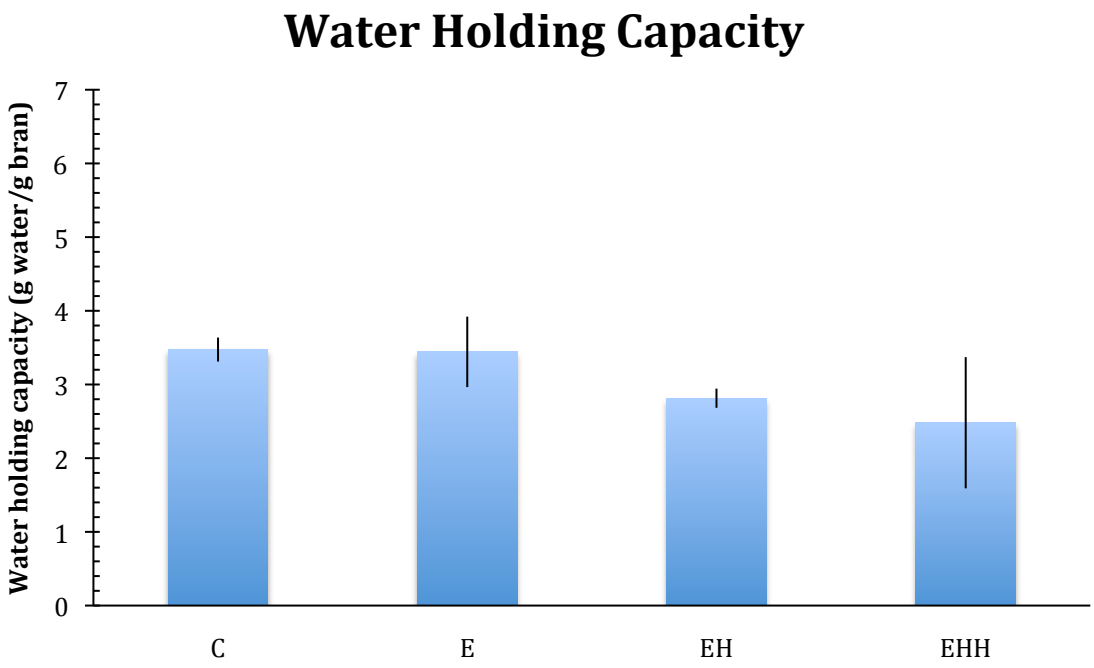


Figure 6-3 WHC of enzymatic, no alkaline treated wheat bran.

The alkaline and enzymatic treated wheat bran gave similar WHC compared to the control wheat bran (Fig. 6-4). The only alkaline and enzymatic treated wheat bran gave a lower WHC (2.984 ± 0.529 g water/g bran). The high shear mixture did not have any effects on WHC in this case (2.982 ± 0.185 g water/g bran). The high-pressure homogenization increased the WHC to 4.200 ± 0.159 g water/g bran, which is also

interesting and hard to explain due to various factors. Those factors worked together and generated a complex situation with no significant trend here. The lower WHC of EA may due to the domain of the enzymatic effects of producing more water-soluble arabinoxylans. The high shear mixing did not change the situation, and gave a similar WHC of EAH. However, the high-pressure homogenization seems to produce more water binding sites through re-construction of the pensotans structure and finally gave a higher WHC of EAHH.

Several factors have effects on WHC of wheat bran. The alkaline treatment hydrolyzes the ester linkage, thus generating new water binding sites. The enzymatic treatment chops down the main chain and producing water-soluble arabinoxylans. The mechanical processes also generate new water binding sites but through physical re-construction of structure. The alkaline and mechanical treatment help increase the WHC, while the enzymatic treatment has negative effects on WHC in wheat bran. The treatments worked together, resulting in different effects on WHC.

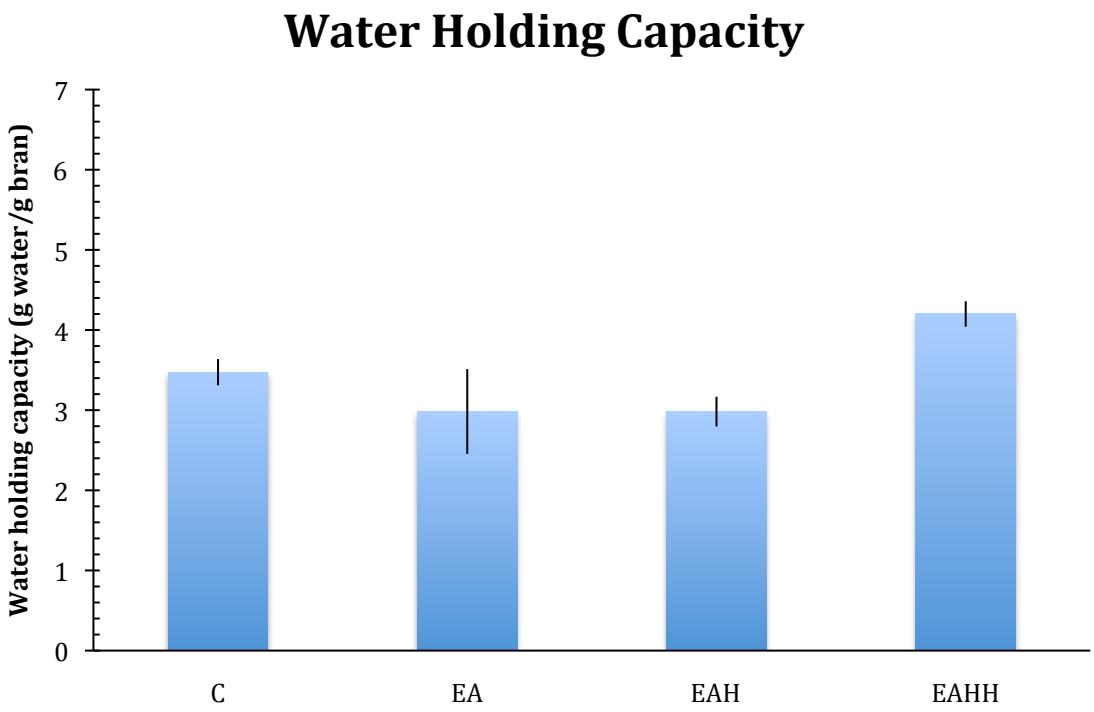


Figure 6-4 WHC of alkaline and enzymatic treated wheat bran.

Summary

This study evaluated multiple treatments' effects on the phenolics and water holding capacity in wheat bran. The alkaline treatment is efficient in releasing bound phenolics acids, especially ferulic acid from wheat bran. It also helps to increase the water holding capacity by creating more water binding sites. The enzymatic treatment is most efficient in releaing bound flavonoids in wheat bran. It also can release bound phenolics acids, but rarely release ferulic acid. It does not improve the water holding capacity of wheat bran. The main reason of this may be that it produces more soluble dietary fiber with smaller molecular size compared to the insoluble dietary fiber. The mechanical treatments do not have significant positive effects on the phenolics in wheat bran, but they do not have negtive effects also. It may be a good thing, because the

mechanical treatments are more contributing to improve the physical properties of wheat bran including viscosity, particle size, and water holding capacity. Therefore, as long as they do not have influnce on the phenolics content in wheat bran, they are still necessary and essencial in the wheat bran treatments.

In summary, the treated wheat bran have a higher potencial to be a functional ingredient in food products. The released phenolics will benefit consumers' health and the improved physical properties will produce whole grain products with better quality, wider consumption, and easier for acceptance by consumers.

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Appendix: HPLC images

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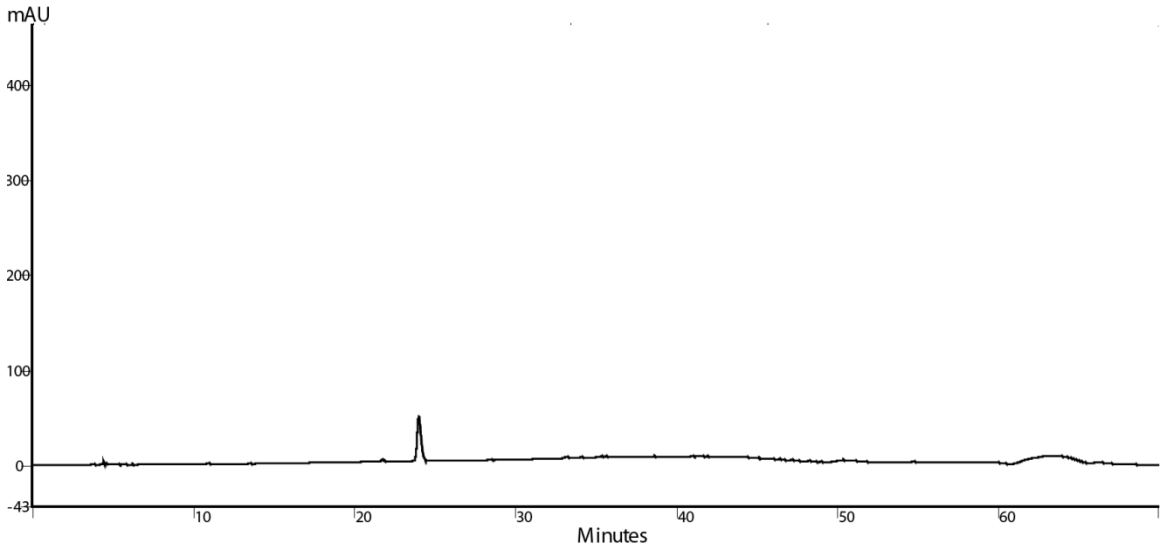

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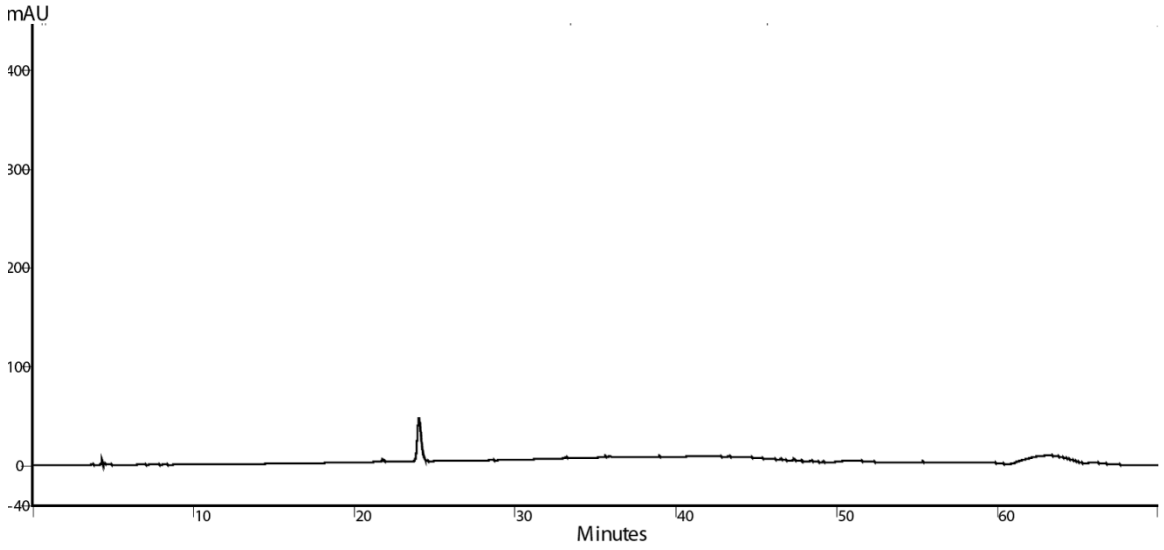

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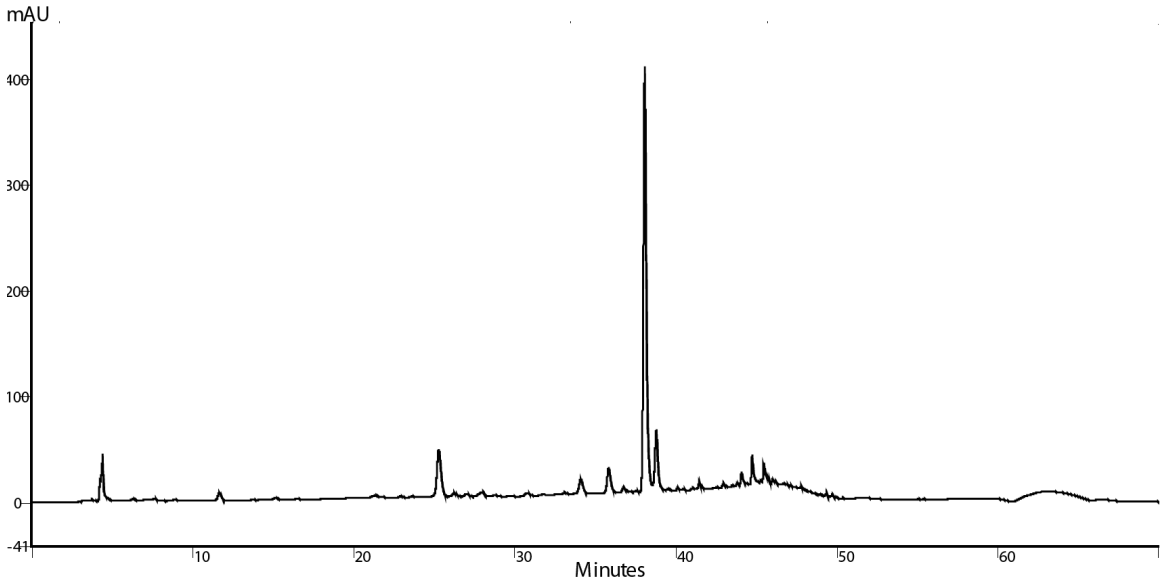
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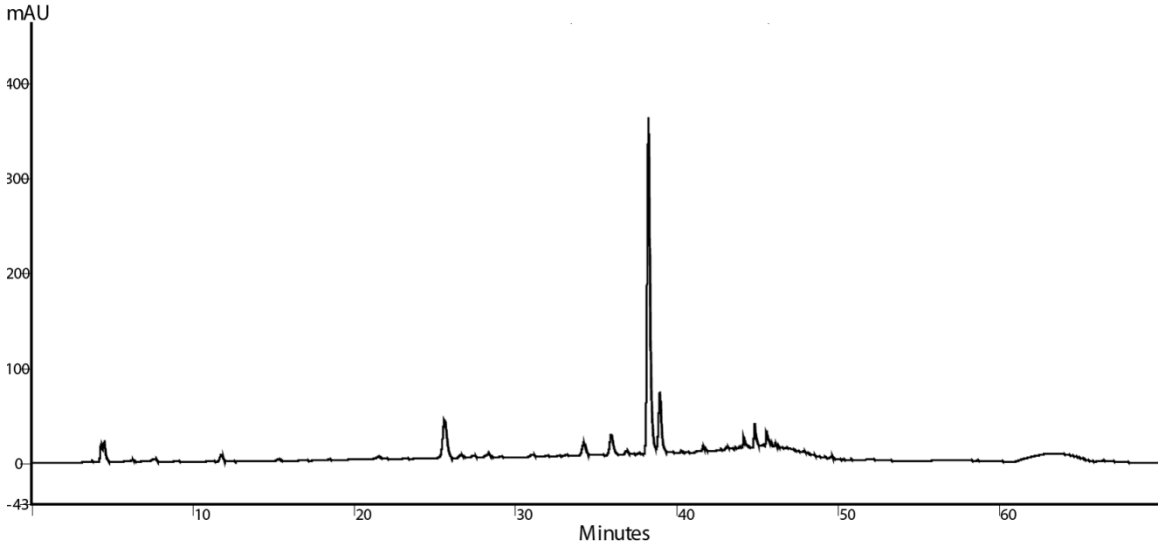
A2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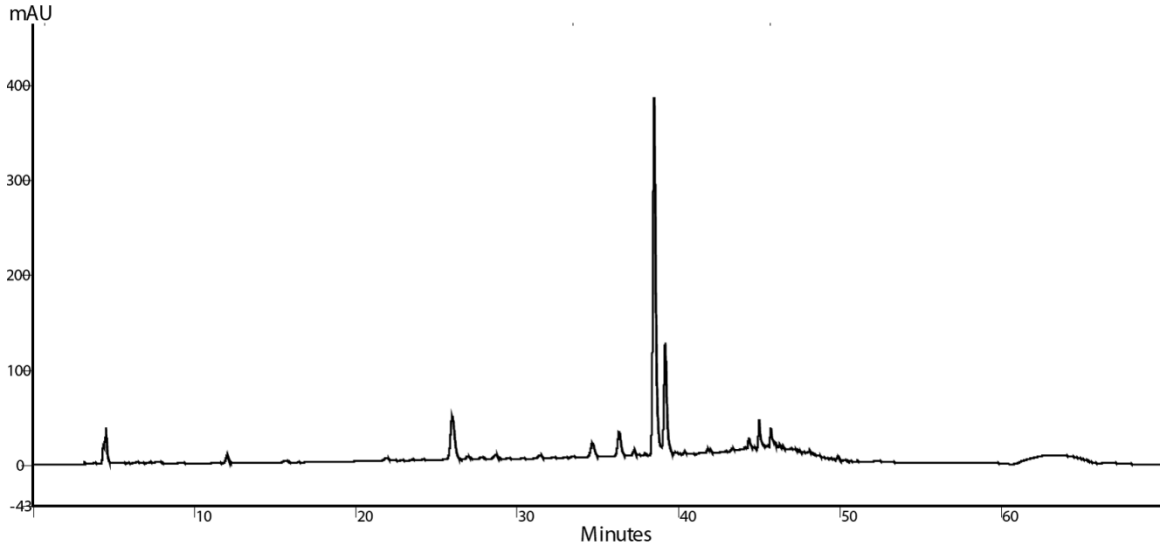
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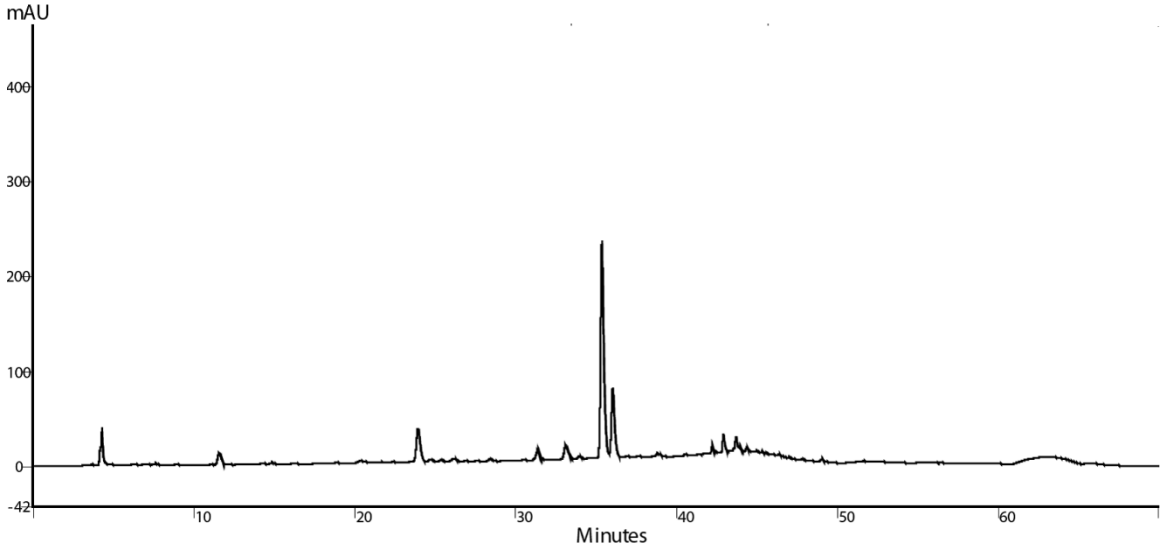
Minutes

AH1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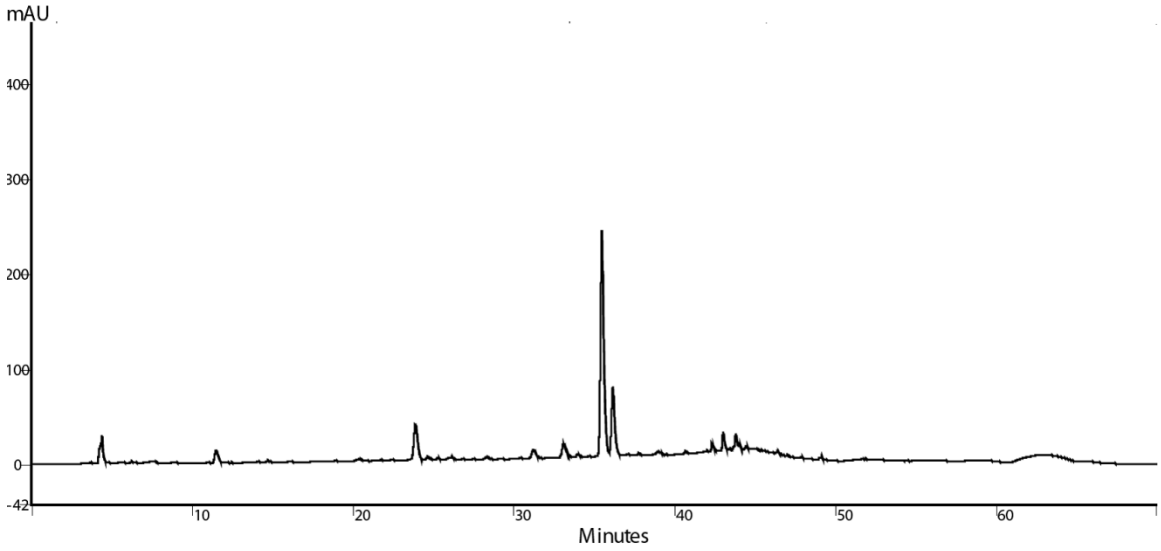
AH2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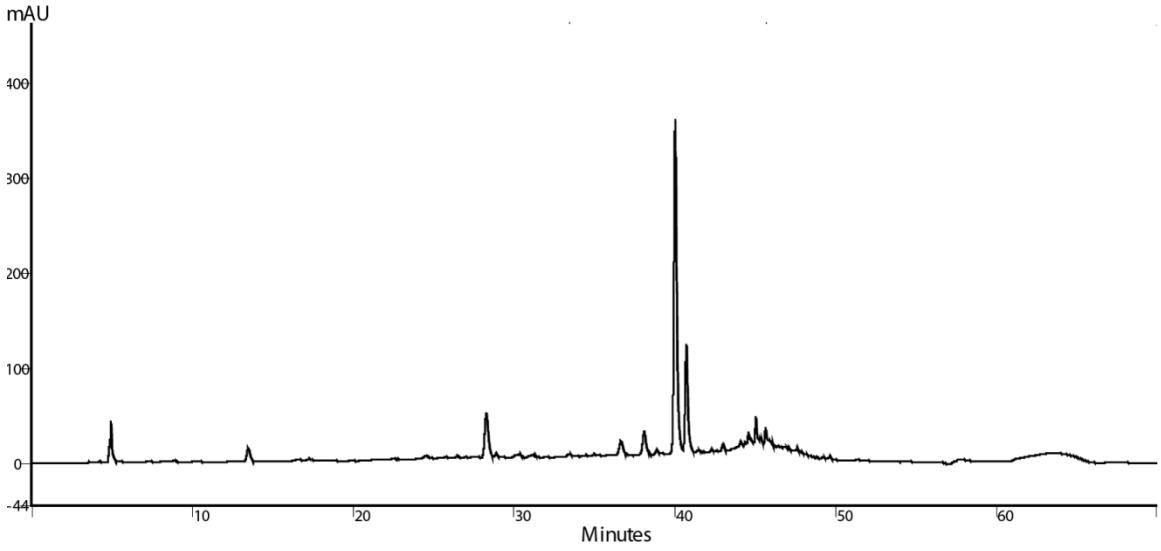
60
Minutes

AH3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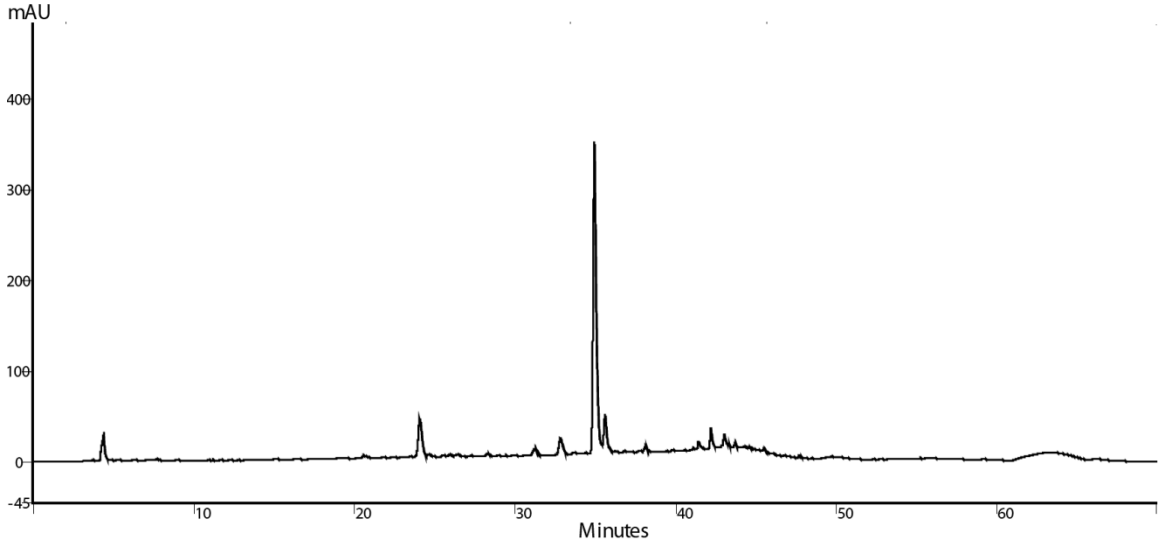
Minutes

AHH2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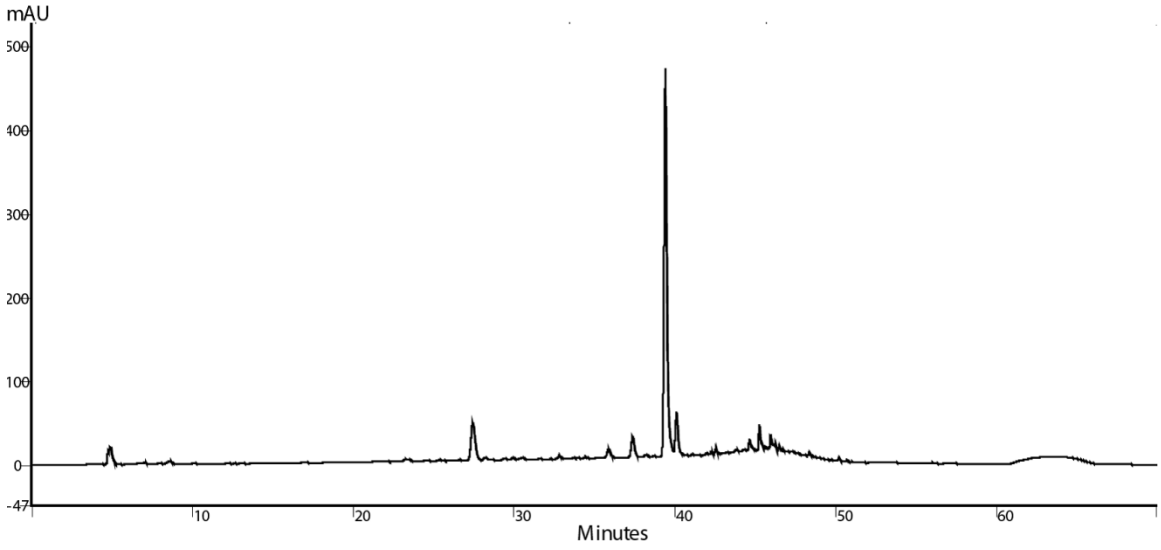
Minutes

AHH3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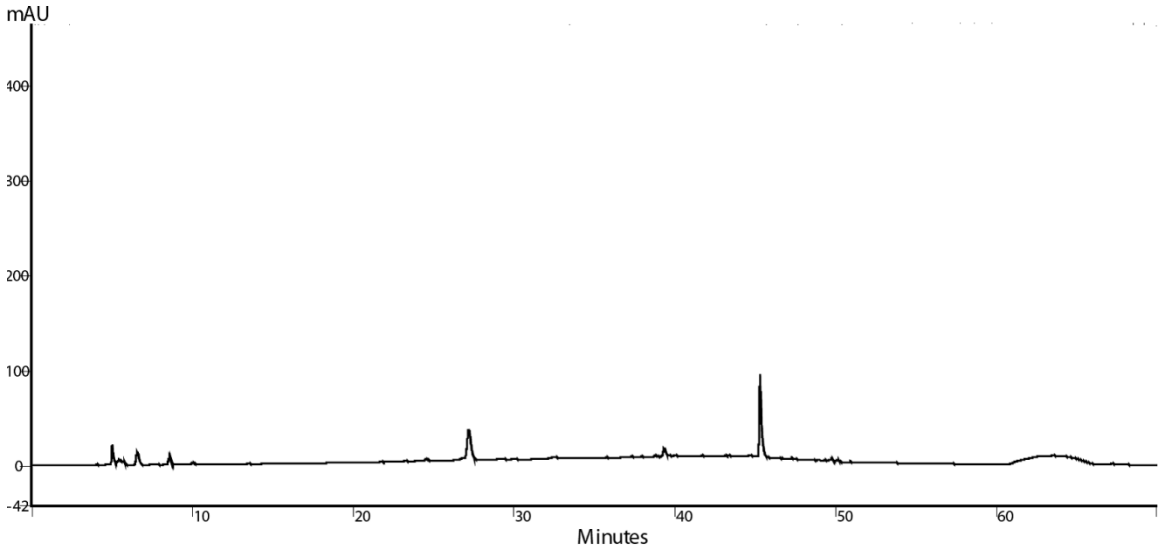
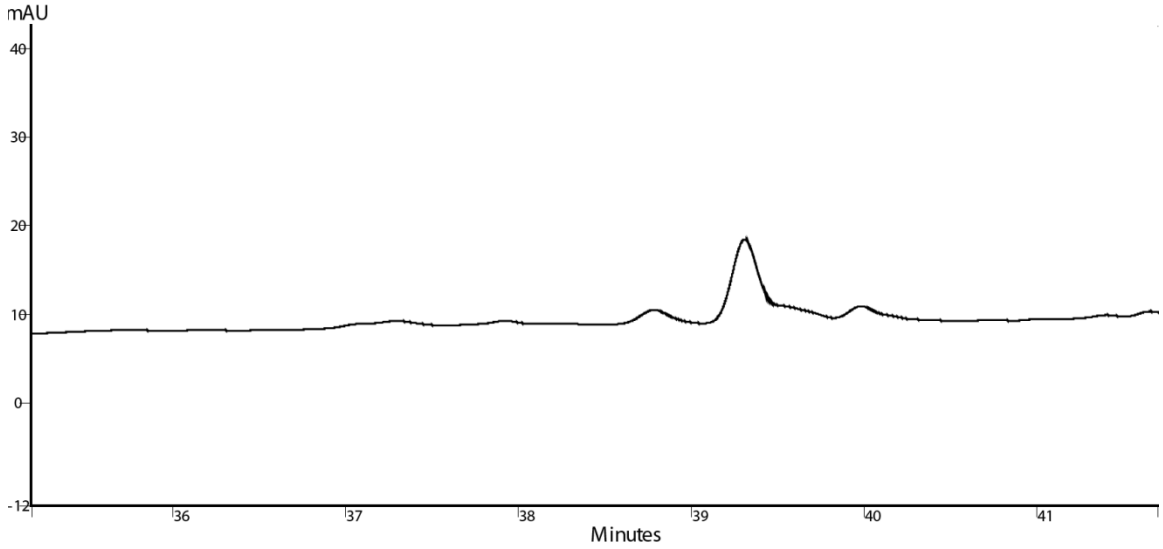
Minutes

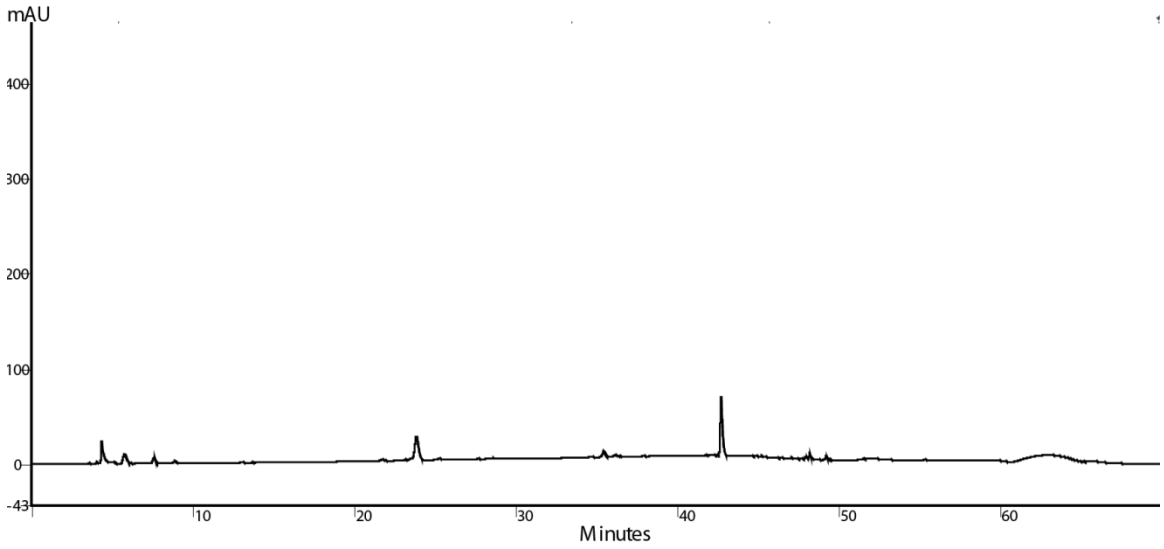
E1



E1-amplify (30-40mins)



E2



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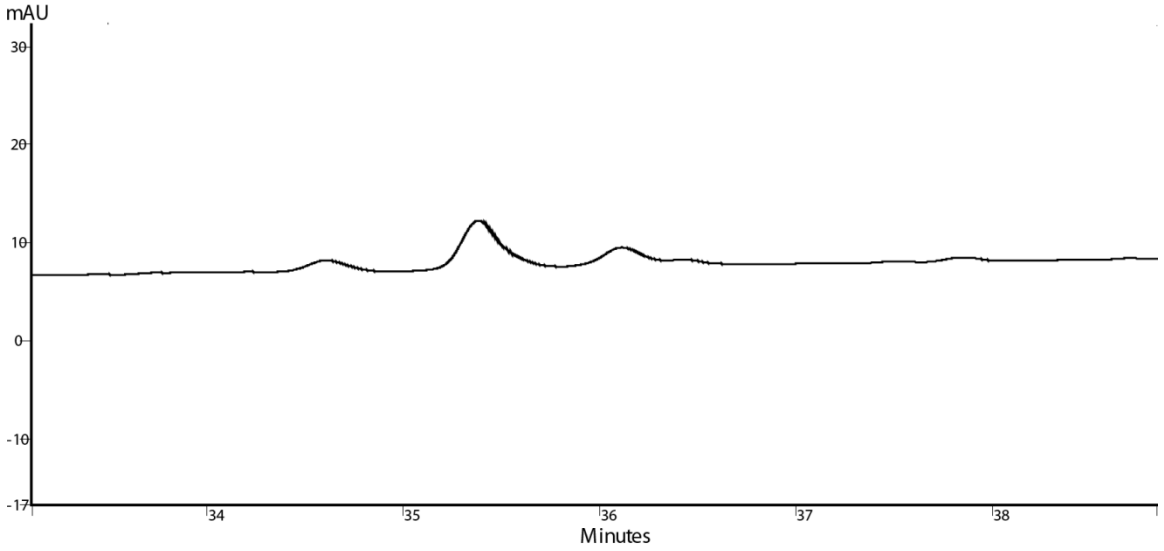
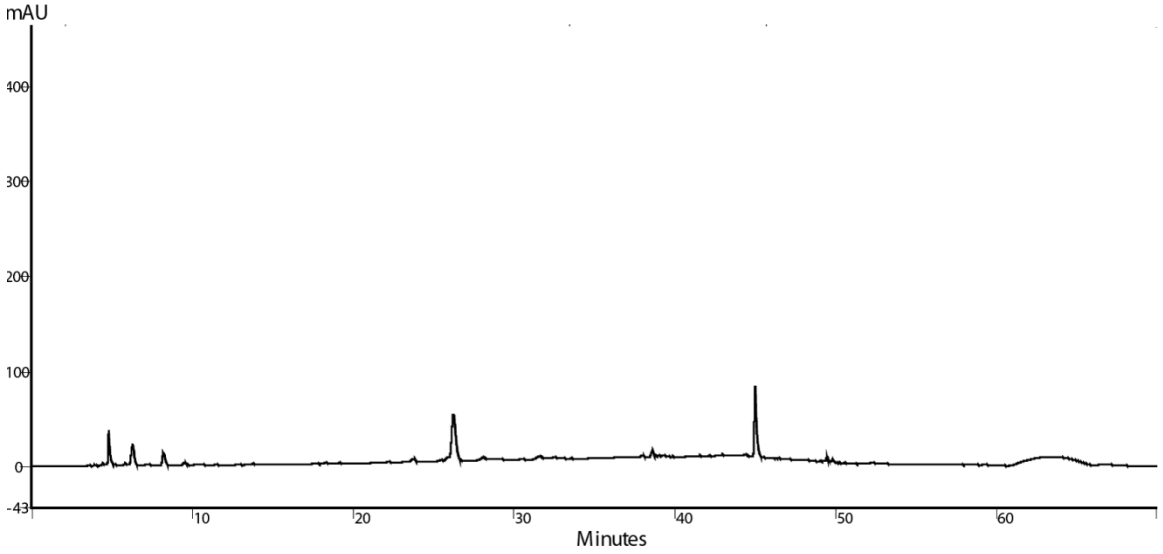


EH1



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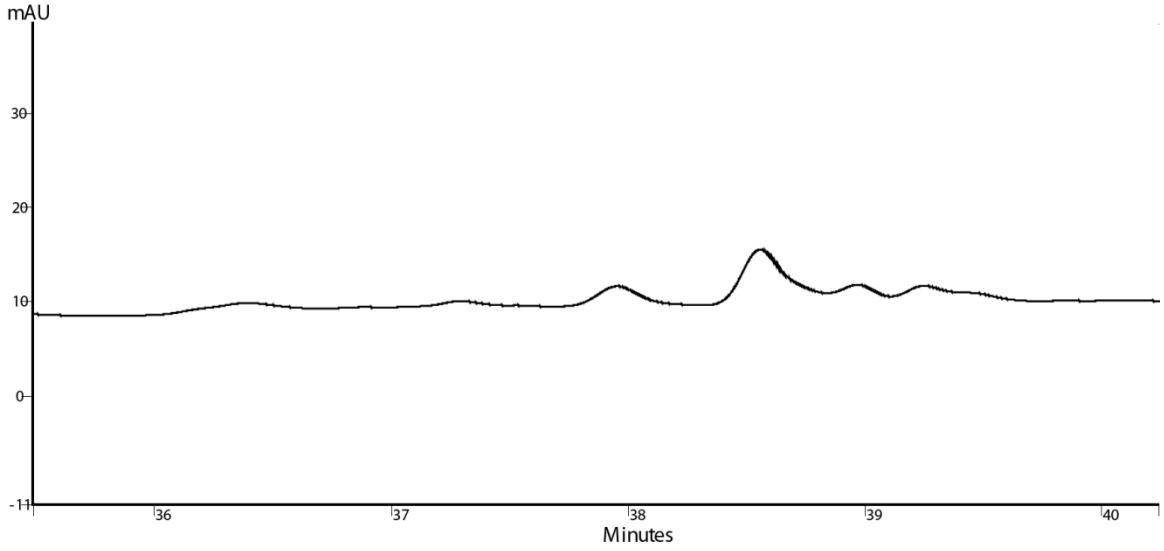

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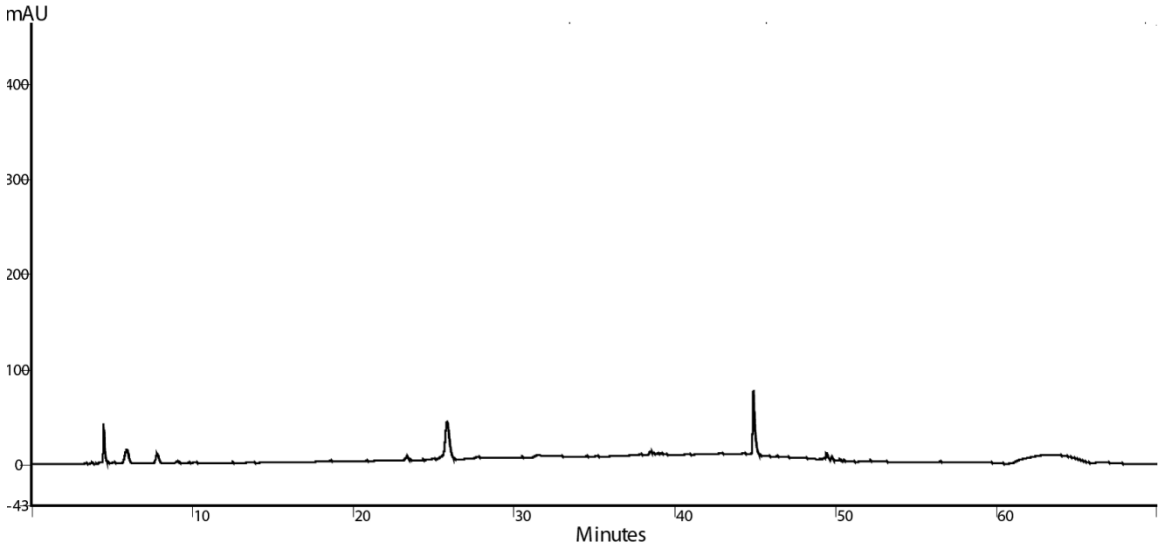

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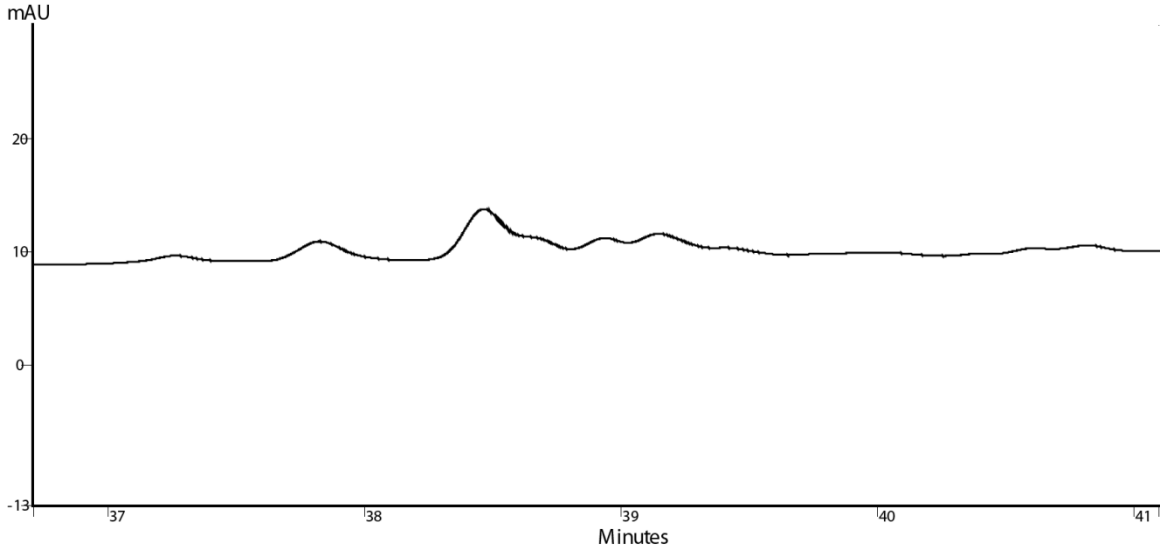

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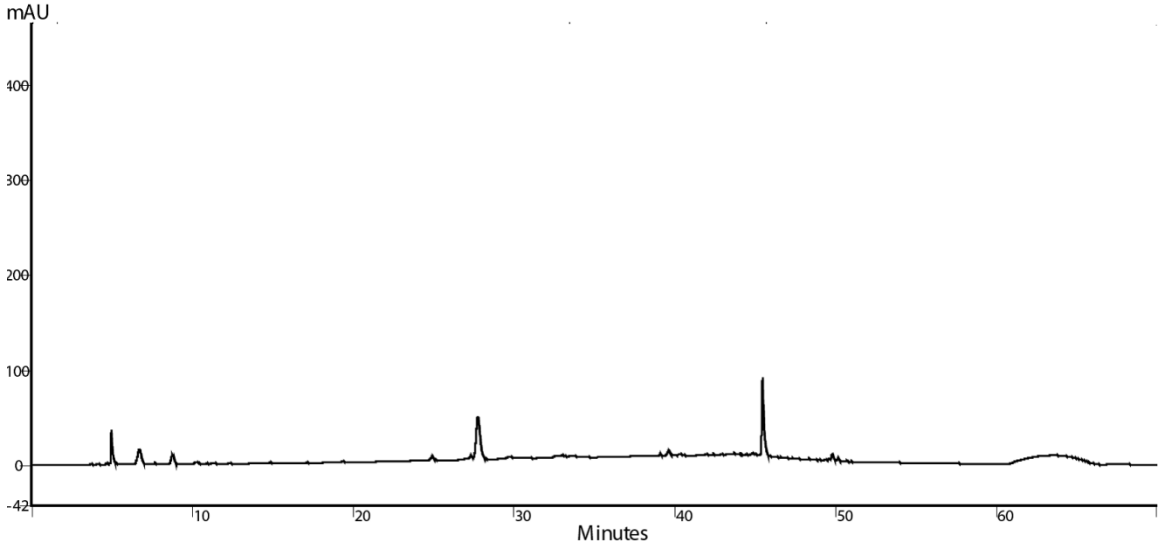

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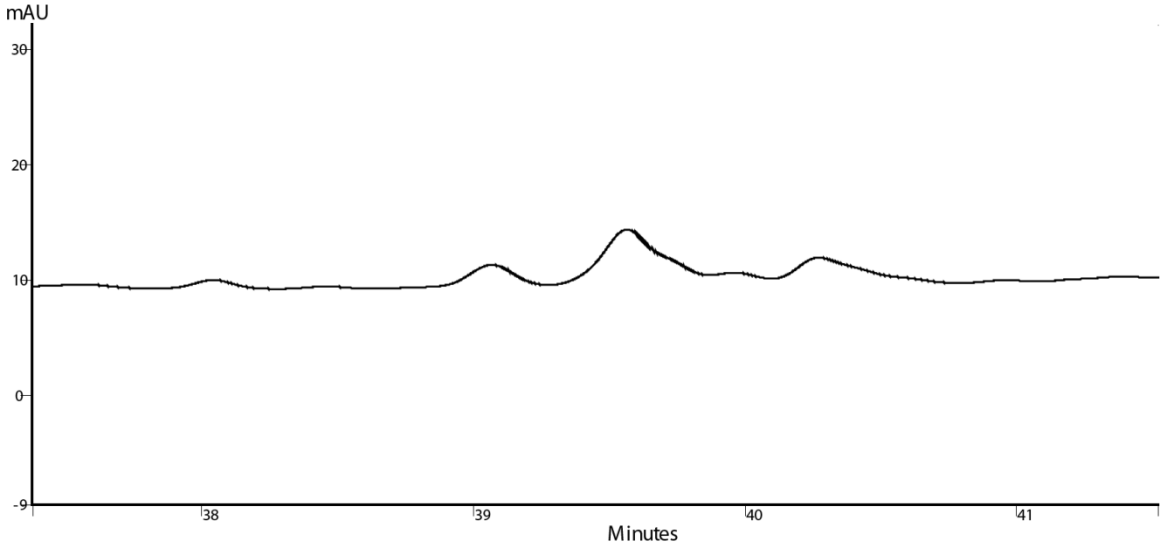

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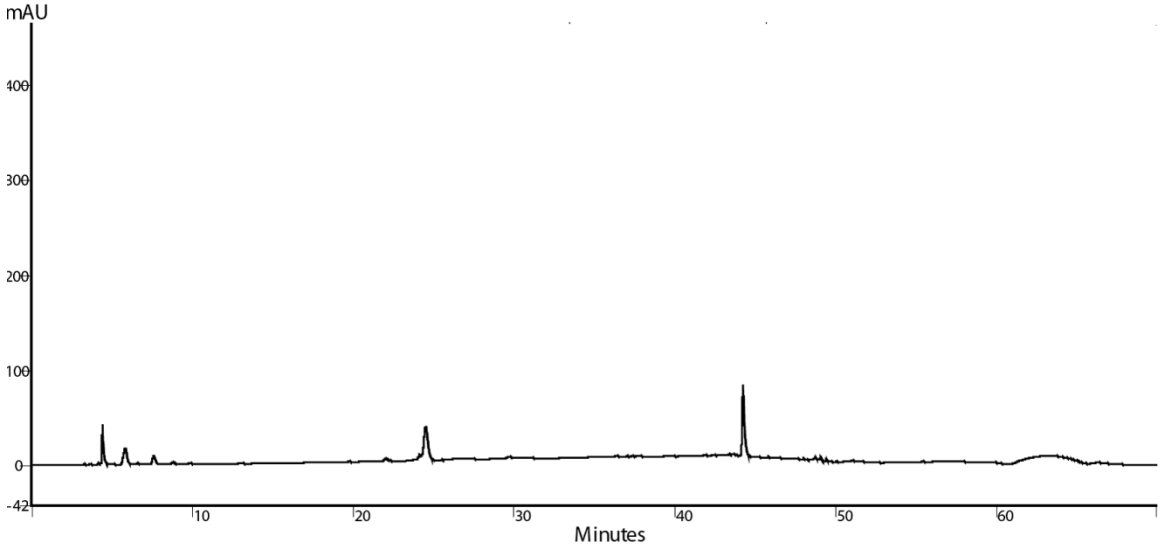

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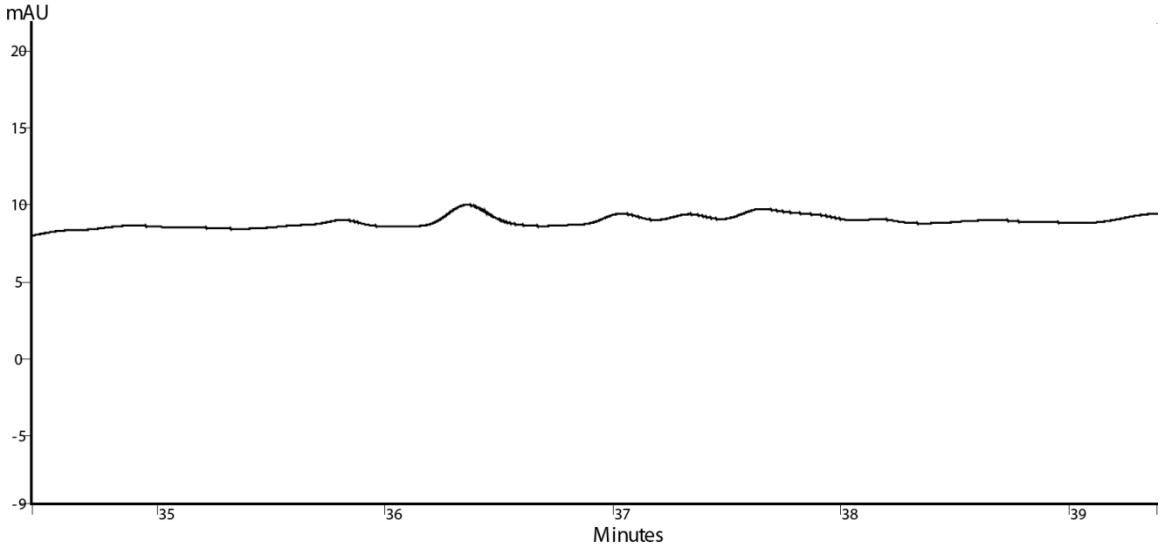

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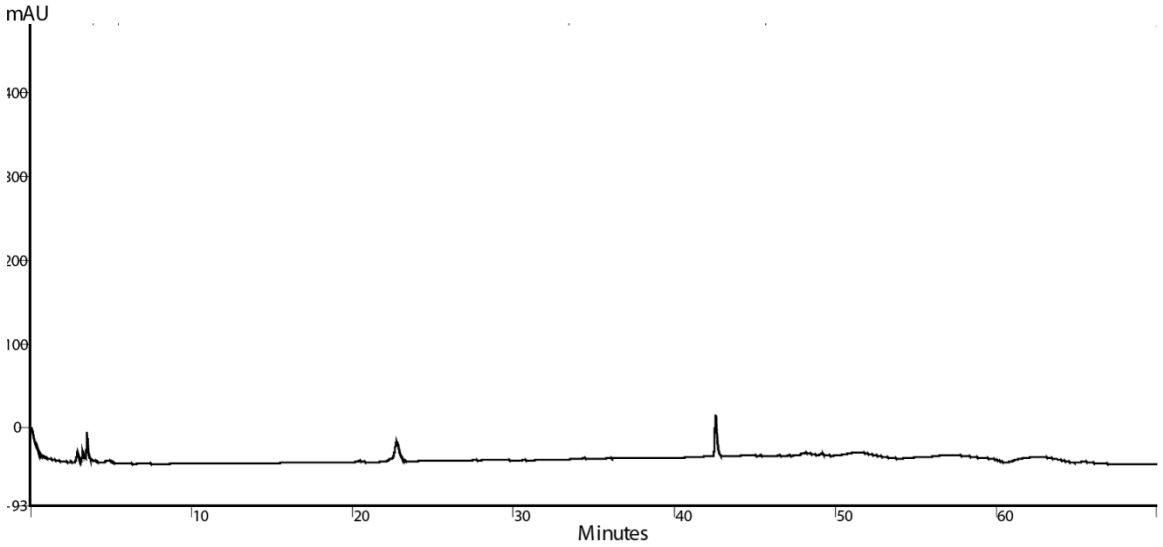

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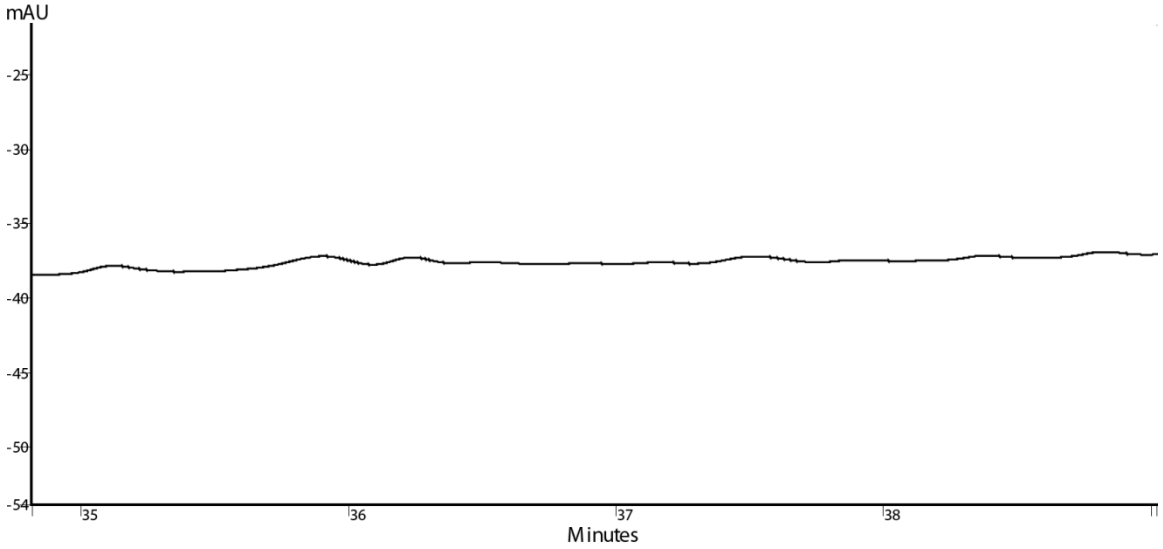

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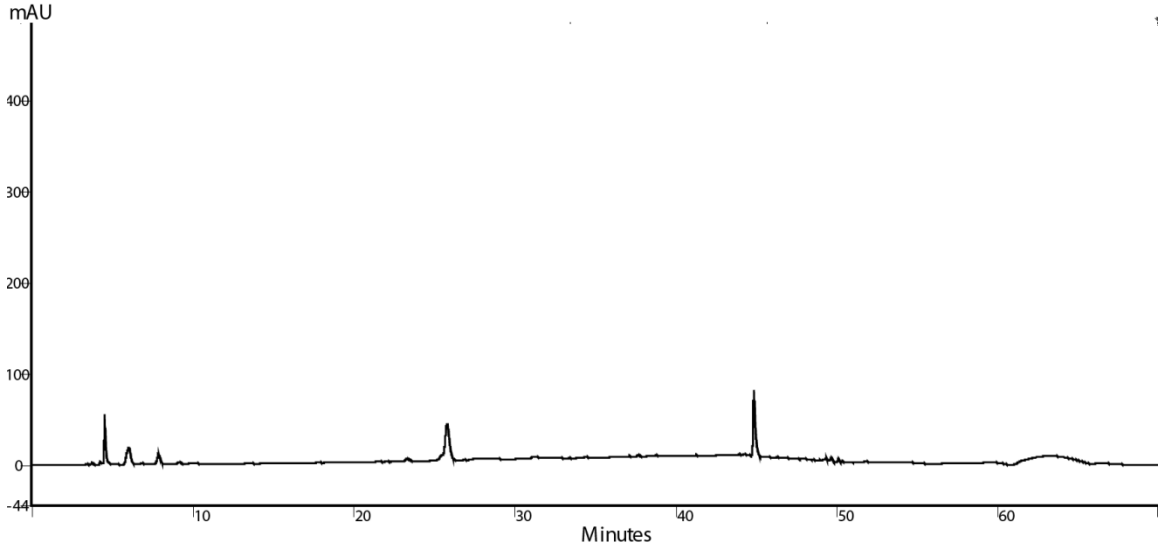

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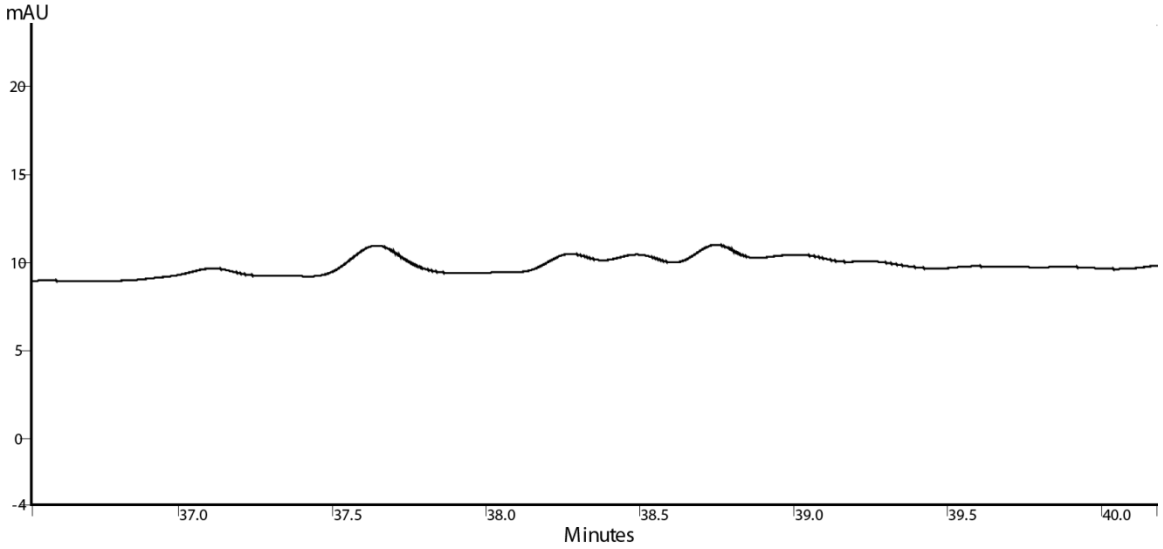

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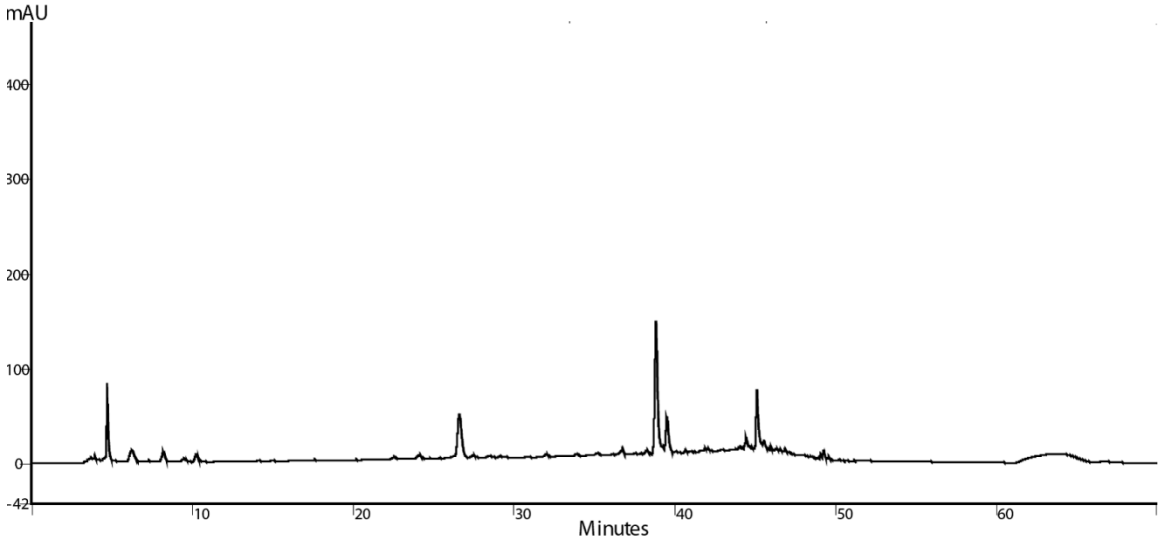
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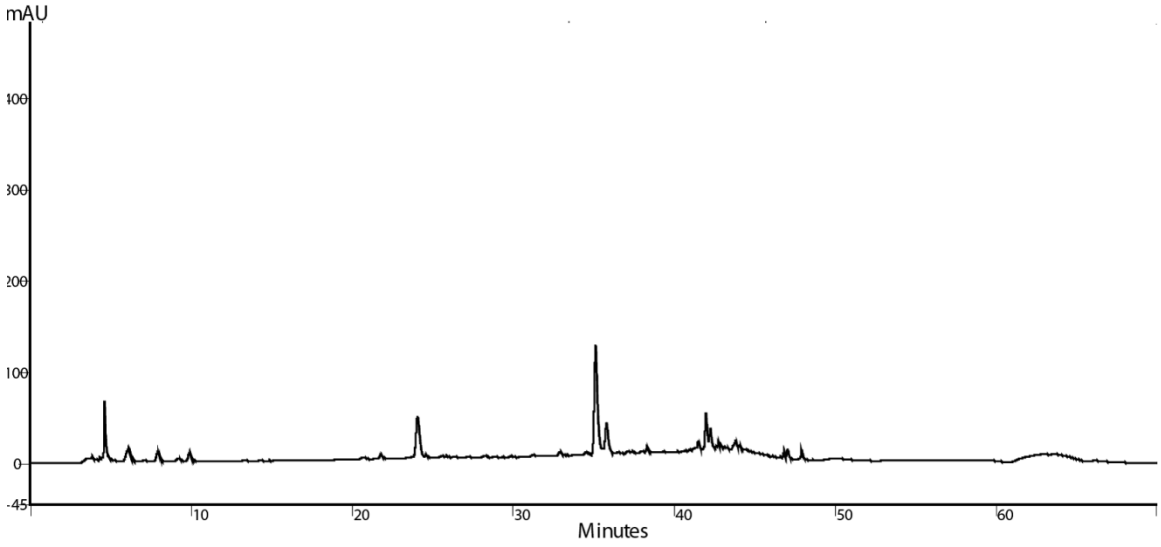
Minutes

EA2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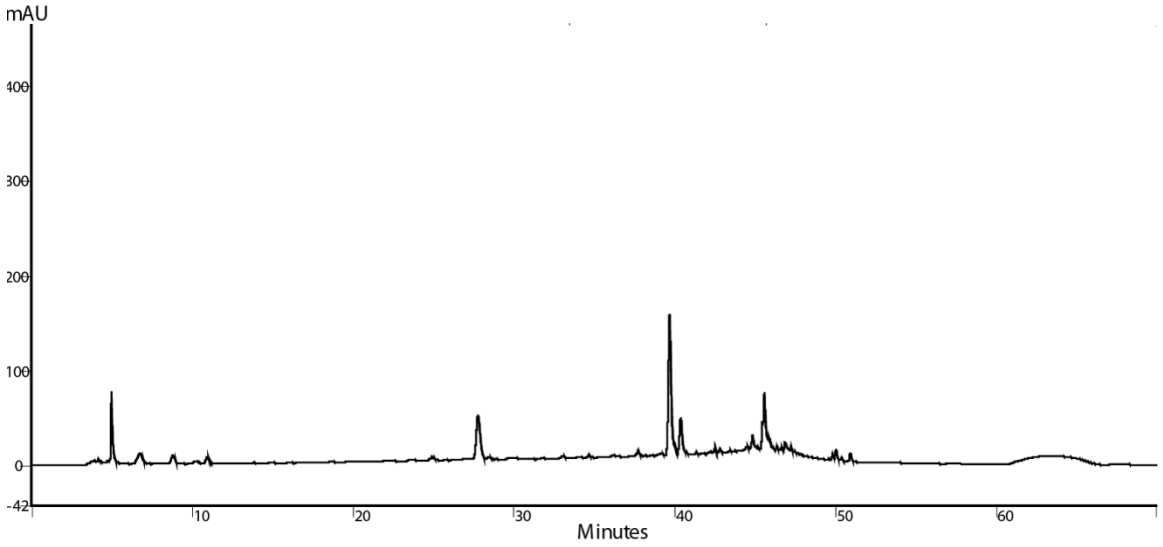
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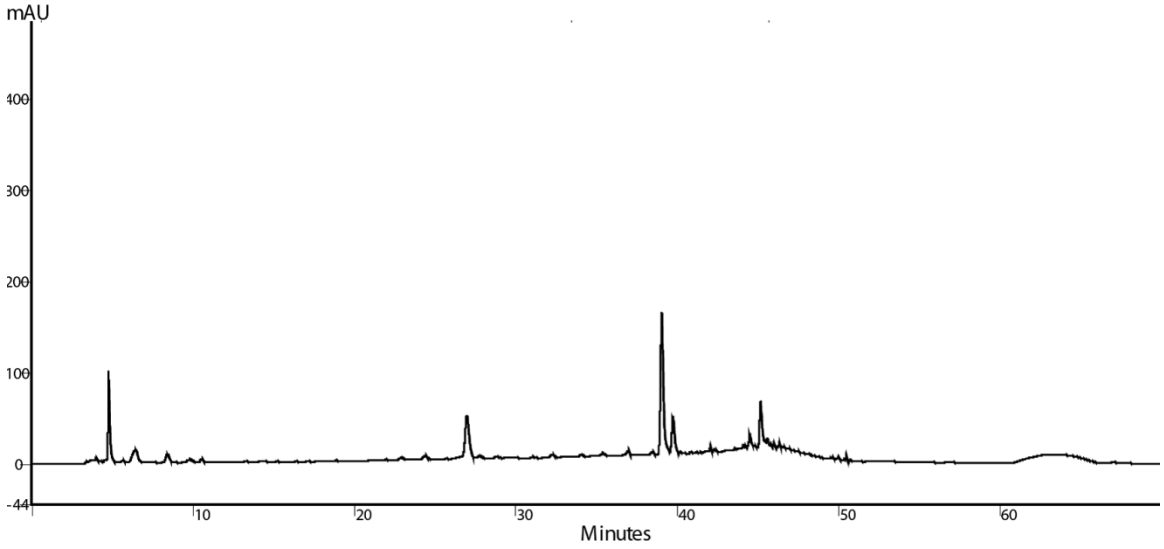
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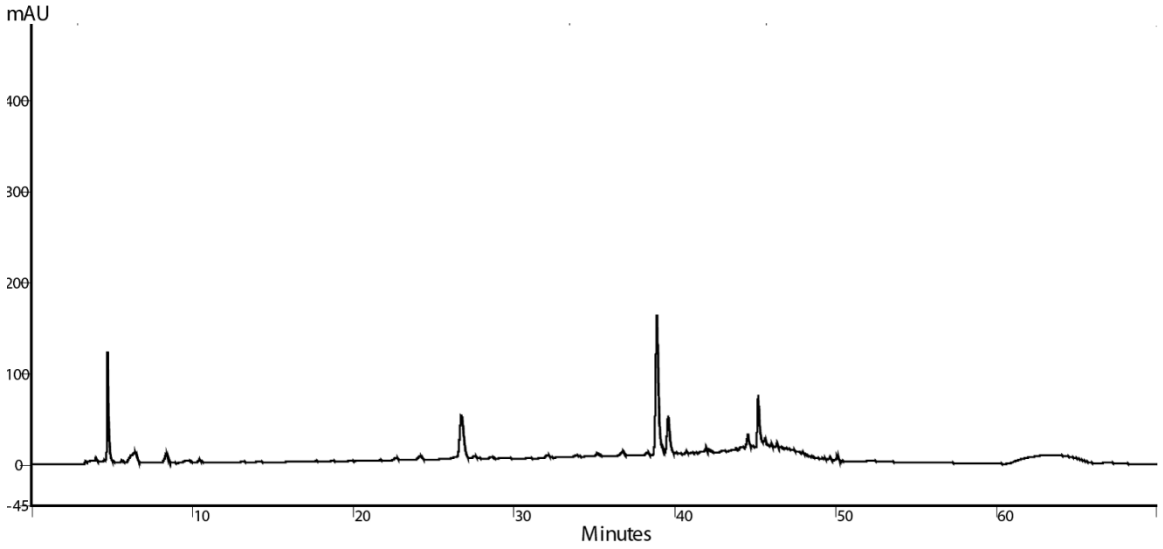
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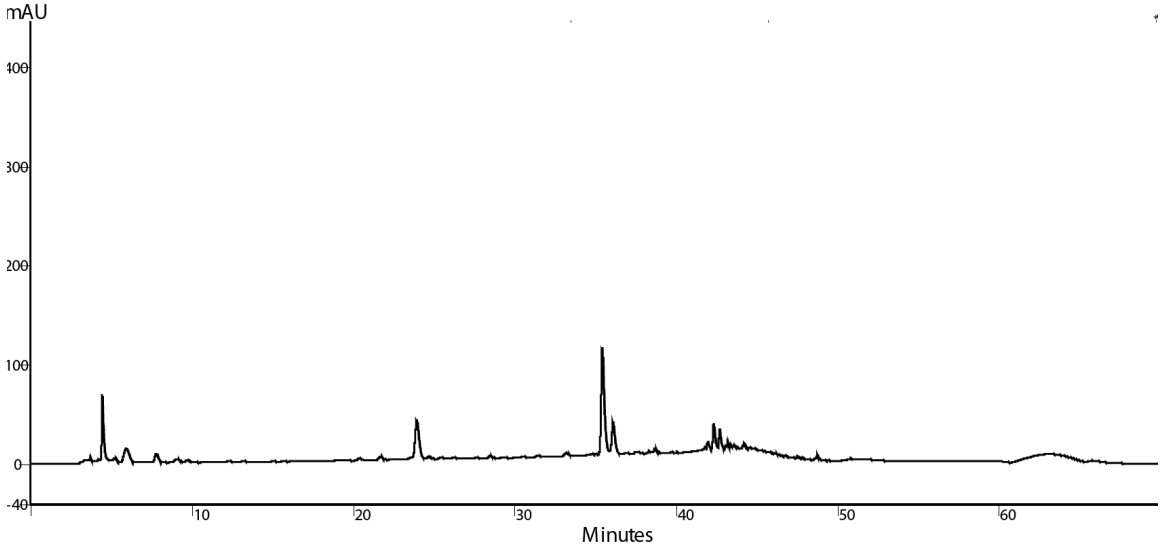
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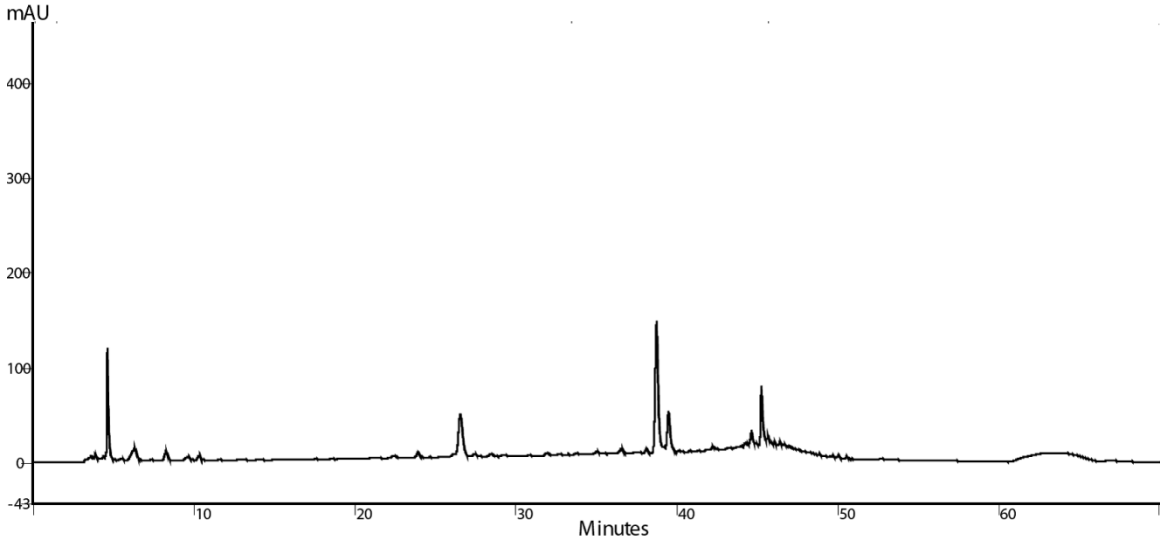
Minutes

EAHH1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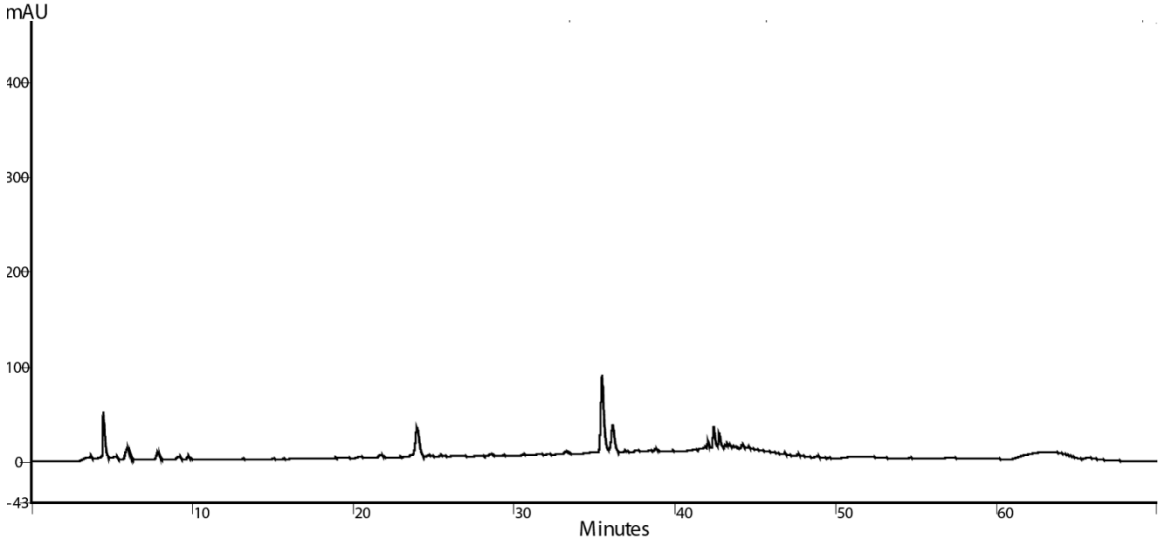
Minutes

EAHH2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EAHH3