ULTRASOUND-ASSISTED EXTRACTION OF RED RASPBERRY ANTHOCYANINS AND UTILIZATION OF MUSTARD MEAL-DERIVED PRODUCTS TO IMPROVE FOOD QUALITY AND SAFETY

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Amir Golmohamadi, submitted for the degree of Doctor of Philosophy with a major in Food Science and titled, "Ultrasound-assisted extraction of red raspberry anthocyanins and utilization of mustard meal-derived products to improve food quality and safety," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Power ultrasound (20-1000 kHz) potentially can improve the extraction of bioactive compounds. The objectives of this research were to study the effect of three frequencies of ultrasound (20, 490, and 986 kHz) on antioxidant activity (AOA), total phenolic content (TPC), and anthocyanin content (ACY) of red raspberry puree (RP), to extract thiocyanate (SCN^-) from mustard seed meal (*Sinapis alba*), and finally to generate and optimize hypothiocyanite ($OSCN^-$) production from mustard meal (MM) SCN^- in the lactoperoxidase system (LPSys). The RP was subjected to the three ultrasound frequencies for 10-30 minutes and aqueous solutions of MM were sonicated for 5-30 minutes at 20 kHz. The MM-SCN^- (or KSCN as control) was then mixed with lactoperoxidase enzyme (LPD) and exogenous H_2O_2 in a buffered solution (pH 6). Sustained production of H_2O_2 was achieved by using glucose and glucose oxidase (GOD). In the case of red raspberry puree, sonication at 20 and 490 kHz significantly ($P<0.05$) affected the AOA, ACY, and TPC of RP, while 986 kHz only increased TPC after 30 minutes ($P<0.05$). Sonication at 20 kHz for 10 minutes caused an increase ($P<0.05$) of AOA and ACY by 17.3 % and 12.6%. It was demonstrated that when limited to 10 minutes, 20 kHz was the most effective treatment for extraction of bioactive compounds in RP compared to 490 and 986 kHz. Therefore, 20 kHz was used to study the generation of SCN^- from mustard meal. Five and 30 minutes of sonication generated SCN^- concentrations that were equal to 24 h and 3 days of conventional extraction, respectively ($P<0.05$). Both of these protocols produced results that could also be achieved with 10 minutes of extraction at 70˚C. When exogenous H_2O_2 was used to activate the LPSYS, MM-SCN generated the same concentration of $OSCN^-$ as the KSCN-LPSys. The optimized LPSys at fixed LPD (3.92 IU/ml) contained 500 µM SCN^-, 32-64 mM glucose, and 342 IU/l GOD. This investigation

has shown that an increase in ultrasound power by itself cannot improve the- extraction. Mustard meal SCN^- would qualify as a generally recognized as safe material with potential applications in the food industry to inhibit the growth of food borne pathogens.

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DEDICATION

I dedicate my dissertation work to my wife *Reyhaneh* who brought love to my life, my little one *Ava*, whom presence created more love and grace for us, as well as my parents *Hossein* and *Kobra* for their prayers and support.

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CHAPTER 1: LITERATURE REVIEW

Along with physical activity, tobacco, and alcohol, the diet has a great impact on society's health and wellness. Today consumers are looking for a healthy diet that contains more natural and health improving compounds such as antioxidants and phenolic compounds. Consumer demand will affect food processing methods such as extraction and preservation techniques. The food research sector is trying to meet this demand by replacing conventional food processing methods such as solvent extraction with novel methods (for instance; ultrasound-assisted extraction) to reduce the harsh processing conditions caused by conventional methods and retain more bioactive compounds in the food.

In addition, by changing processing conditions, new food safety issues have emerged. Therefore in order to improve the safety and still follow the consumers' desire for natural compounds, some alternative preservatives are to be sought. One natural antimicrobial that is available in raw milk and the human body is the lactoperoxidase system, a system that can be applied in food products to reduce the risk of foodborne pathogens.

1.1. Bioactive compounds in plants and foods

Bioactive compounds are plant secondary metabolites exerting pharmacological or toxicological effects in human and animals. Nutrients such as minerals and vitamins do not fall under the category of bioactive compounds [1]. Production of bioactive compounds in plants seems to be the rule rather than the exception. Thus, the majority of even common food and feed plants are able to generate these. However, distinctive plant species contain elevated concentrations of more potent bioactive compounds [1].

1.2. Classification of bioactive compounds in plants

The frequent practice of classification of bioactive compounds is based on their chemical classes and biochemical pathways. Based on this method of classification and types of the plant materials that are used in this research, glycosides, flavonoids and proanthocyanins are presented here.

1.2.1. Glycosides

The glycosides consist of a variety of secondary metabolites attached to a monosaccharide, oligosaccharide, or to uronic acid (Figure 1-1). Glycosylation often happens at the last step of biosynthesis of natural compounds, effecting important changes in the natural molecules. Glycosylation contributes to production of incomparable variety and complexity of secondary metabolites.

The sugar or uronic acid part of the glycoside molecule is called glycone, and the other part is referred to as aglycone. Sugar acceptors are secondary metabolites, including phenolics, terpenoids, cyanohydrins (cyanogenic glycoside precursors), thiohydroximates (glucosinolate precursors), and alkaloids (such as betalains). The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins, and anthraquinone glycosides. In addition, flavonoids frequently occur as glycosides[1, 2] (Figure 1.1).

1.2.2. Flavonoids and proanthocyanins

Flavonoids are natural molecules with a central three-ring structure that constitute the largest group of plant phenolics (Figure **1-1** and 1-2). Various compounds are substituted on the ring C in the structure of these compounds. Thus, a variety of flavonoid classes such as flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins are created.

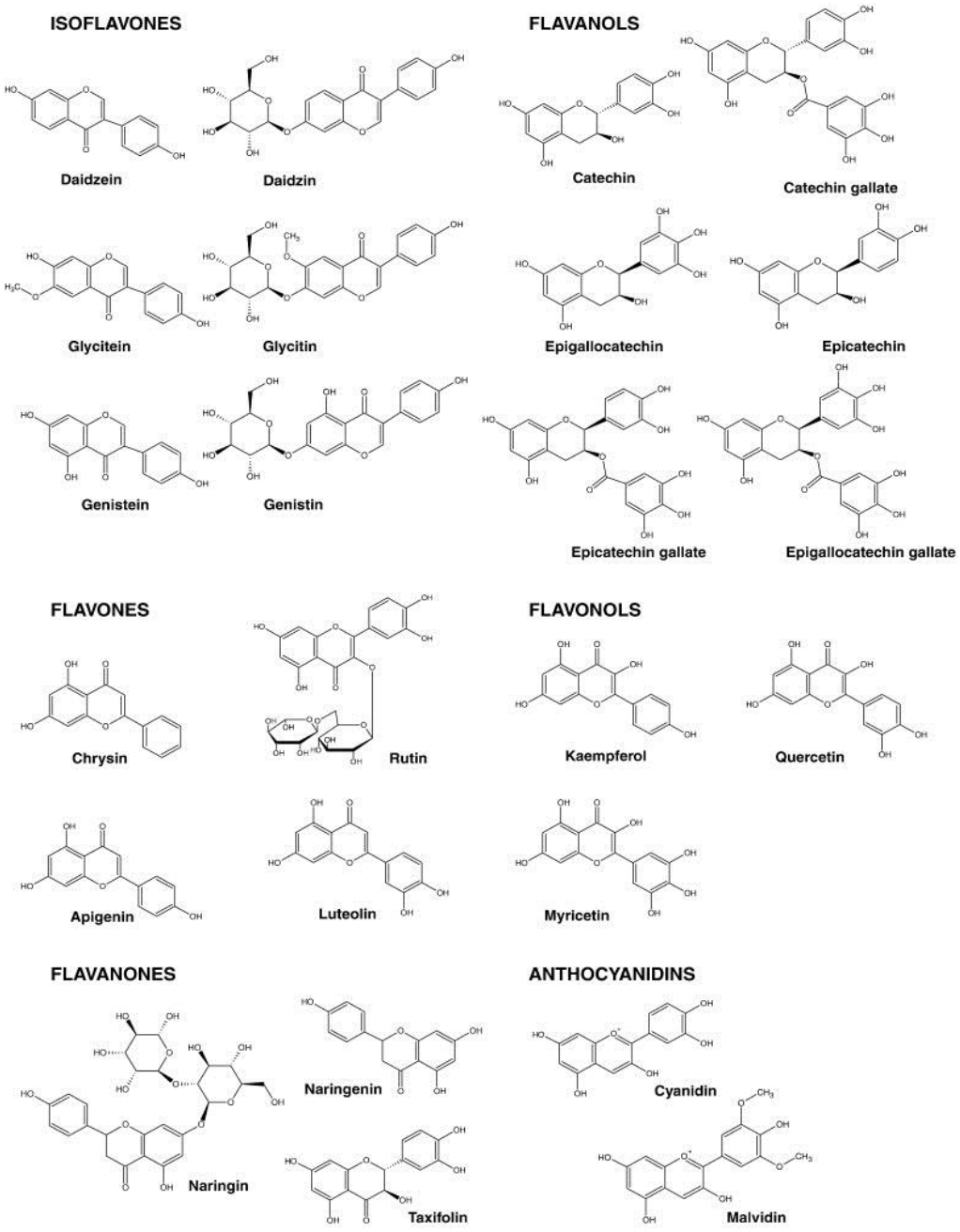

Figure 1-1. A few naturally occurring flavonoids[3]

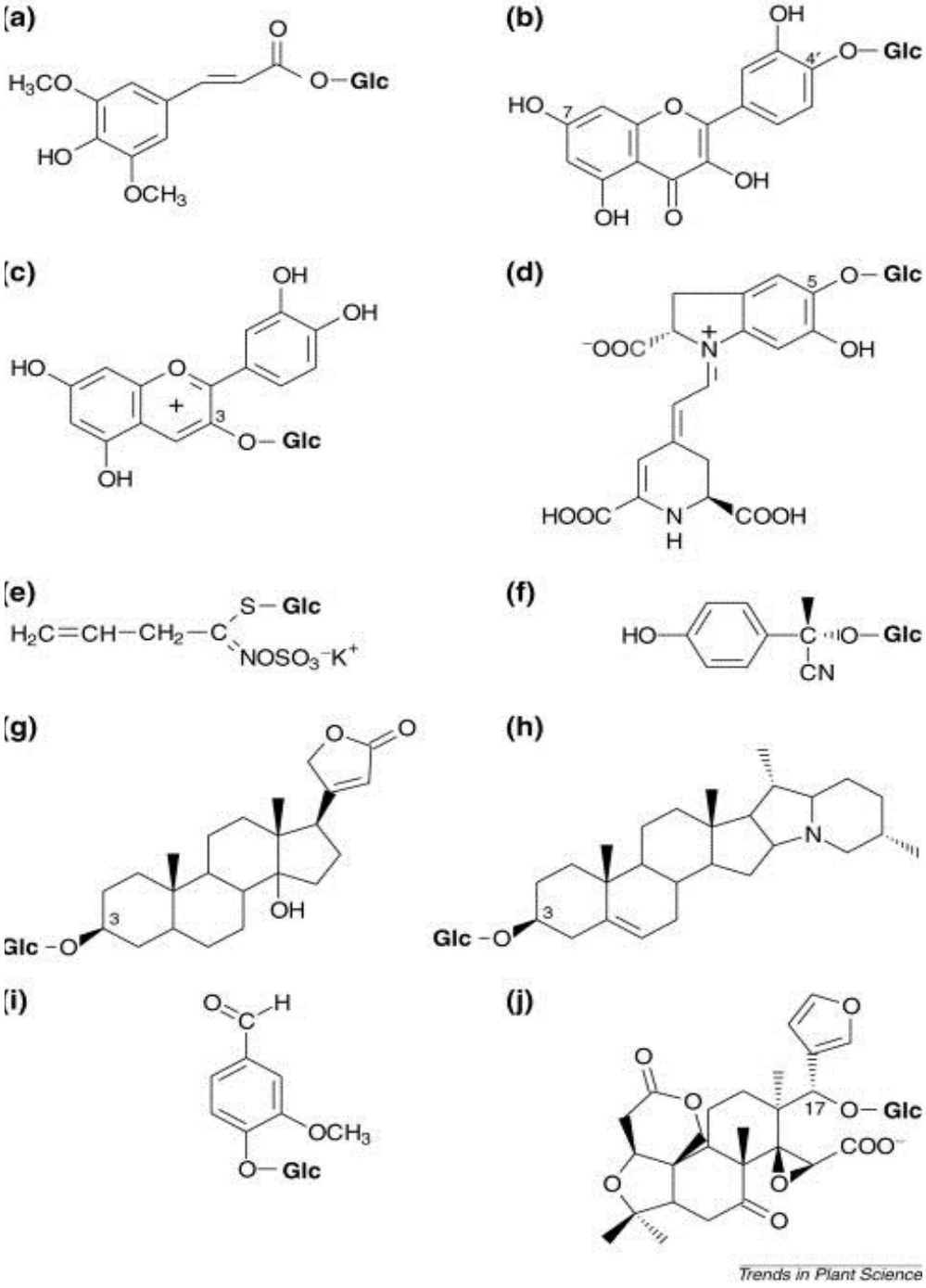

Figure 1-2. Glycosylated secondary plant products, (a) Hydroxycinnamic acids, for e.g. sinapoyl glucose (b) Flavonols, for e.g. quercetin 4′-O-glucoside. (c) Anthocyanins, for e.g. cyanidin 3-O-glucoside. (d) Betacyanins, for e.g. betanidin 5-O-glucoside. (e) Glucosinolates, for e.g. sinigrin. (f) Cyanogenic glucosides, for e.g. dhurrin. (g) Cardiac glycosides, for e.g. digitoxigenin glucoside. (h) Steroidal glycoalkaloids, for e.g. solanidine glucoside. (i) Glycosidically bound volatiles, for e.g. vanillin glucoside. (j) Triterpenoid glucosides, for e.g. limonoate 17-O-glucoside. Source: [2].

Flavonoids are referred to as proanthocyanidins. Flavonoids and proanthocyanidins are pigments that are created in a wide range of plant families[1, 4].

1.3. Extraction methods to extract bioactive compounds from plant materials

The history of essential oils extraction (mainly for medicinal use) started in countries such as old Persia, Egypt, and India. Interest in the traditional bioactive compounds from plants has been restored since more western scientists identified the clinical and biological benefits of this group of naturally occurring compounds [5]. Bioactivity and medicinal effectiveness of a plant extracts rely on a group of compounds that have synergistic effects; therefore, the extraction technique that is employed is a critical factor to target the specific range of compounds with more potent effects [5]. The major methods of extraction that are usually used in the food industry or the studies in this area are explained in the following review. The ultrasound extraction method is explained in detail.

1.3.1. Distillation

In distillation, plant materials are mixed with water and the mixture is heated or steam is introduced to the system. The resulting vapor is cooled by a cooling system and the extract is separated based on the difference between density of the water and extract.

This method is mainly used to extract essential oils from vegetal materials [6]. Combination of the distillation method with other novel methods such as 20 kHz ultrasound has been regarded as a pointless procedure. If an ultrasound unit is combined with this method, it is only to enhance the boiling points with little or no improvement in the yield [6].

1.3.2. Organic solvent extraction

Solvent extraction is a technique to extract the desired components by transferring that component from an aqueous or solid to an organic solvent.

To carry out the extraction in food-related studies, appropriate solvent should be selected. The factors that are important to select a solvent include:

1- Solubility of the target compound(s) in the solvent
2- Recovery ratio, which is the amount of solvent that is removed from the miscella (mixture of solid and solvents). The higher the recovery ratio the lower the residual of the solvent.
3- Interfacial tension and viscosity, the solvent should be able to wet the surface of the material and diffuse in it. The viscosity should be low enough so it can facilitate the flow of solvent through the pores and capillaries in the matrix.
4- The solvent should be safe, nontoxic with minimum flammability and as harmless to the environment as possible [7].

Solvent extraction and particularly solid liquid extraction of interest in this research rely on a multicomponent and multistage unsteady state mass transfer mechanism. The common and food grade extraction solvents in the food industry are water, ethanol, hexane, and carbon dioxide. The stages of the solvent extraction can be summarized in following steps created by [7]:

I. Solvent entry into the solid matrix ;
II. Solubilizing the target components ;
III. Carry the solutes to the surface of the solid matrix;

IV. Transport of the extracted solute from the exterior part of the matrix into the bulk solution;

V. Separation of the extract ;

The environmental regulations and new obligations of the food industry to improve the quality of the extracts as well as minimize residue reduction in consumption of substances that pose health risks to humans have opened new fields of research in the food industry. The aim of these new fields is to develop new technologies for the extraction of food products [8, 9]. An efficient extraction should maximize the target compound and cause minimal degradation using low cost raw materials and environmentally friendly technologies [10]. Among the novel extraction methods, supercritical fluid, microwave, and ultrasound assisted extraction have been investigated in several studies to perform the extraction process or improve the yield of conventional methods.

1.3.3. Supercritical fluid extraction

Supercritical fluid is an extraction technology that uses a fluid at a state above its critical point as the medium to extract target compounds. The temperature and pressure where above that the fluid cannot be liquefied regardless of the pressure applied is referred to as the critical point. The fluid above the critical point may gain a density close to the liquid state. Some solvents that are usually used as supercritical fluids are carbon dioxide, water, ethanol, ethane, and propane [11].

Supercritical carbon dioxide is an ideal solution for the extraction of bioactive compounds and thermally sensitive materials as the supercritical characterizations are 31.1˚C and 7.38 MPa. To design a supercritical fluid extraction system information such as

density, viscosity, diffusivity, heat capacity, and thermal conductivity are required. However the major disadvantage of this method is the high cost of the method due to the need for vessels capable of withstanding high pressures [11].

1.3.4. Microwave assisted extraction

All materials including food matrices possess inherit electromagnetic properties that govern their response to absorption or emission of electromagnetic radiation. Microwaves, radio waves, ultraviolet, and infrared are examples of electromagnetic radiation [12]. The electromagnetic properties of food and food ingredients are fundamental to microwave extraction. The magnetic field and electric field oscillate perpendicularly to each other in frequencies ranging from 0.3-300 GHz, which is the microwave frequency range. The efficiency of microwave energy is related to the ability of the sample to absorb microwave energy and dissipate heat to the surrounding molecules as is represented by tanδ [12].

$$\boldsymbol{tan\delta} = \frac{\boldsymbol{\varepsilon''}}{\boldsymbol{\varepsilon'}} = \frac{\boldsymbol{dielectric\ loss}}{\boldsymbol{dielectric\ constant}} \qquad (\mathbf{1})$$

Microwave-assisted extraction has some limitations such as the requirement for an additional step to remove solvent from the matrices, but more importantly that only polar materials can be used in this method. However this method, similar to ultrasound assisted extraction, is going to be one of the promising novel extraction methods that can be used in the food industry [13].

1.3.5. Ultrasound assisted extraction

Among the novel extraction methods, ultrasound extraction has been used in more investigations because the method itself is inexpensive and easy to perform. Also, the cavitation phenomenon has made it a good choice for cell disruption. Ultrasound not only causes damage to the cell wall, but it also increases the mass transfer into and out of the vegetal cell. Ultrasound increases the water uptake (swelling index) of the plant cell during sonication and increases the extraction because, in some cases, the cell walls will break and a washing out process is triggered [6]. During the 1950-1970s most of the research on ultrasound extraction was focused on determination of the frequency range in which maximum extraction yield would be obtained. Table 1-1 shows a summary of number of investigations conducted with this method.

1.3.5.1. Indirect sonication

In this case an ultrasonic bath (Figure 1-3) is used to do the extraction. A lab scale ultrasonic bath is a simple device that holds a small volume of water as the transmitting medium. This device is usually used as a complementary device for extraction of vitamins or some other compounds during lab experiments such as HPLC or GC quantification of food components. However, some researchers have used this device to conduct ultrasound extraction research [6].

1.3.5.2. Direct sonication

In this case, the system design is slightly different (Figure 1-4). A collimator, a plate type transducer, or a Langevin generator (horn type) is used to change the input frequency to ultrasound and emit it to the environment, which is usually a cylindrical chamber. The

power of sonication is reduced with increasing distance from transducer. In all types, shaking might be applied to introduce a uniform sonication [5, 6, 14].

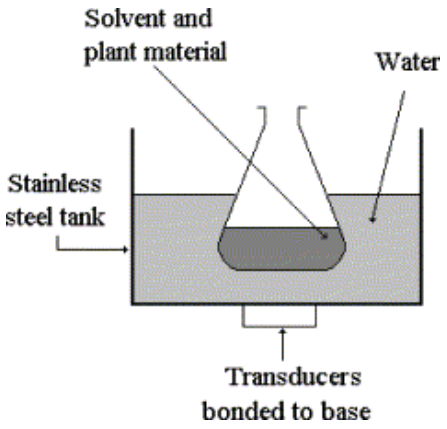

Figure 1-3. Experimental setup for indirect extraction using an ultrasonic cleaning bath [6]

1.4. Physical properties of sound

According to the Mason and Lorimer (2002), ultrasound is produced by the transducer and transmitted to the medium with total pressure P (Pa) given by:

$$P = P_a \sin 2\pi ft \qquad (2)$$

Where;

P_a = Applied acoustic pressure

f = Frequency of the sound

t = sonication time

Table 1-1. Examples of research on application of ultrasound in food and pharmaceutical studies

Product	Ultr. specifics	Solvent	Performance	Reference
Almond oils	Batch, 20 kHz	Supercritical Co_2	30% yield increase in yield, time was reduced	Riera et al. (2004)
Lycopene	US bath , 40 kHz	Ethyl acetate	90% total lycopene in 20 min	Liangfu & Zelong, (2008)
Raspberry (anthocyanins)	Batch, 22 kHz	1.5 M HCl-95% EtOH (15:85)	3.3 min US compared to 53 min conventional	Chen et al. (2007)
Citrus peel (phenolics)	US bath, 60 kHz	15 ˚C and 40˚C 80% methanol/ 1h	Higher extraction compared to maceration, lower temperature better	Ma et al. (2009)
Cinchona bark (medicinal drug)	2400 kHz	Water	No yield increase (too high freq.)	Schultz & Arzneim (1954)
Digitalis leaves	800 kHz	Water	Similar or better yield	Suss, 1972

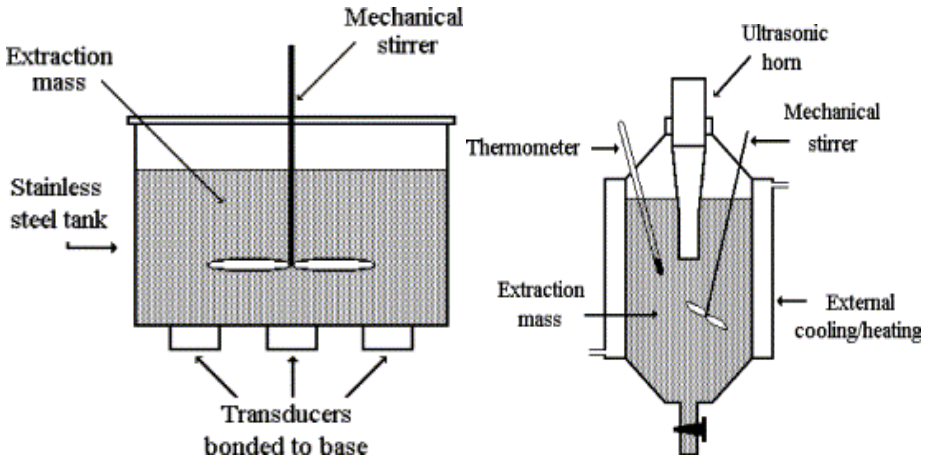

Figure 1-4. Lab scale experimental setups for direct application of ultrasound in media [6].

This pressure will be imposed to the medium and equals the sum of hydrostatic and acoustic pressure. Therefore, the total pressure, P, at a time t, is given by:

$$P = P_h + P_a \qquad (3)$$

P_a = applied acoustic pressure

P_h = hydrostatic pressure

The hydrostatic pressure in small batch systems in laboratory scale can be neglected. Therefore, according to the equation 1, time and frequency are major factors in this equation in laboratory ultrasound experiments [15]. Ultrasonic power can be measured by measuring the rate of heat energy production over time during the sonication process.

Ultrasonic power usually produces some energy that is considered mechanical energy including friction and heat production that cause a temperature increase in the medium.

Therefore, measuring the temperature increase as a function of time leads to the estimation of the acoustic power in watts. According to Jambrak *et. al.* (2009) [16];

$$P = m.Cp.\left(\frac{dT}{dt}\right) \quad (3)$$

Where;

m: mass of the sonicated liquid (kg)

Cp: Specific heat at a constant pressure (J/kg °C)

dT/dt: is the slope of the initial region of the heat over time curve

P: can be expressed as W/cm^2 or W/cm^3 of the liquid subjected to sonication.

Another factor that will impact the ultrasound effect on systems is the intensity of the ultrasound as controlled by the depth to which the sonotrode is immersed into the medium or the device setting. The intensity of the sound decreases as the distance between the traveling sound wave and the source of sound increases [17].

1.5. Ultrasound-assisted extraction (UAE):

General results from the ultrasonic-assisted extraction have shown that ultrasonication not only increased the extraction rate and yield but also considerably decreased the extraction time. Mass transfer rate of the cell content from inside to the outside of the cell is increased. Also mass transfer of the solvent into plant cells is increased especially during the initial minutes [10].

Usually during this process the temperature of the medium increases. Increase in temperature and extraction yield is due to production of air bubbles during the compression and rarefaction cycles. The asymmetric collapse of bubbles close to the surfaces of cell walls as well as the micro jets, damage cellular materials as the result of ultrasound travel within the fluid. Part of the energy can be absorbed by the medium and changed to heat so an increase in temperature is observed. However the temperature increase is usually lower than 70°C [18] as long as a cooling system is available during sonication.

The ultrasound extraction yield is affected by several factors. Parameters such as *frequency* and *power intensity* (W/cm^2), *material's structure, type and amount of extracted substance, physical properties of the solvent* (vapor pressure, material viscosity, surface tension, etc) have substantial influence on the extraction yield [18, 19].

In lab scale sonication, sample size range, extraction time, and solvent volume in numerous studies have been between 1-30 g, 10-60 minutes, and 50-200 ml, respectively [9]. The lab scale UAE has been carried out in several studies and the investigations in this area recently have been focused on the optimization of UAE.

1.6. Extraction of antioxidants and bioactive compounds from fruits and berries with ultrasound-assisted extraction

Today consumers are aware of the direct relation of the health and diet. Gutteridge and Halliwell (2010) defined antioxidants as "any substance that delays, prevents or removes oxidative damage to a target molecule". Antioxidants could be either complex molecules such as anthocyanins or simpler forms as uric acid and ascorbic acid [20].

The use of antioxidants to eliminate or obstruct oxidative damage can be traced in ancient Egypt. Egyptians used plant extracts rich in polyphenols to preserve their corpses [20]. Naturally occurring antioxidants in food, namely fruits, vegetables, and grains, assist the human body to maintain health and delay disease onset. The in vivo contribution of antioxidants to these effects is uncertain. Adequate amounts of antioxidants are beneficial, however high-doses of antioxidant supplements do not result in additional positive health effects [20].

Berries such as cranberry, raspberry, and black currant are considered as important sources of antioxidants amongst fruits and vegetables, because they contain a considerable amount of anthocyanins, which are effective antioxidants[21, 22]. Raspberries are unique among the berry fruits because of a nutritional profile of low calories, fat and saturated fats, high fiber, several essential micronutrients, and a phytochemical composition that includes flavonoids, phenolic acids, lignans, and tannins. These compounds play a significant role in reducing the damaging effects of oxidative stress on cells and the risk of chronic diseases. Among the polyphenolic compounds, raspberries carry significant amounts of elagitanins and anthocyanins, which contribute to their appealing color and ability to promote human health [23]. Xylitol, a low-calorie sugar substitute with immense applications in human health products, is present in strawberries and red raspberries. The level of this type of sugar in raspberries (*Rubus idueus*) is approximated to 400 µg per 1.0 g of fresh weight. This amount in other berries ranged from 7.5–280 pg per 1.0g of fresh weight [24]. In addition, the agronomic characteristics such as higher fruit weight and pest and disease resistance of selected genotypes [25], high yield, and long harvesting period [26] could make this type of

berry a good selection for the agro-industry. Ultrasound can be applied to improve the extraction of anthocyanins from red raspberry and reduce the extraction time.

1.7. Ultrasound technology pitfalls and limitations

Ultrasound is a novel technology with several applications in science and engineering. One of the applications of ultrasound is to extract herbal, oil, and bioactive compounds from plant materials. Several studies have demonstrated that ultrasound improves the extraction rate and yield, however the majority of the studies have been investigated at laboratory scales. Scale up of the ultrasound extraction process has faced numerous problems such as difficulty to control the temperature and power input. In addition, to create an ultrasound reactor with dual or multiple frequencies capability, more than one transducer, each with its own generator is needed. The design and operation of this system becomes considerably complicated and lacks economic feasibility [27]. Moreover, a large amount of radicals are produced due to the cavitation process and at the contact of the transducer to the fluid especially when the power intensity is high. Likelihood of the oxidation of fats, and denaturation of proteins and enzymes is significantly large [28]. Thus, several steps are needed to remedy the disadvantages, scale up the process, and reduce the pitfalls of this novel procedure.

1.8. Extraction of potential antimicrobial compounds from mustard meal:

Mustard is a member of the cruciferous plants that include various cabbages, broccoli, cauliflower, and brussel sprouts [29]. Cruciferous plants contain compounds called glucosinolates (GLS) (figure 1-5) that play an important role in resistance to fungi, nematodes, or other plant pests. Glucosinolates are so diverse that so far 120 structurally

different glucosinolates have been reported [29, 30]. Glucosinolates are highly polar and water soluble and mostly concentrated in the seed. They are responsible for the pungent flavor of plants in the family Brassicaceae [31, 32]. Spiciness of rapeseed and mustard is caused by a group of compounds known as isothiocyanates. A variety of substituents including aliphatic, aromatic or heterocyclic groups [33] are seen in the side chain of glucosinolate including allyl (sinigrin), benzyl (sinalbin) and indol (Botti et al., 1995). Naturally occurring GLSs are (Z) N hydroximinosulfate esters with a sulfur-linked ß-D-glucopyranose moiety and an amino acid-derived side chain.

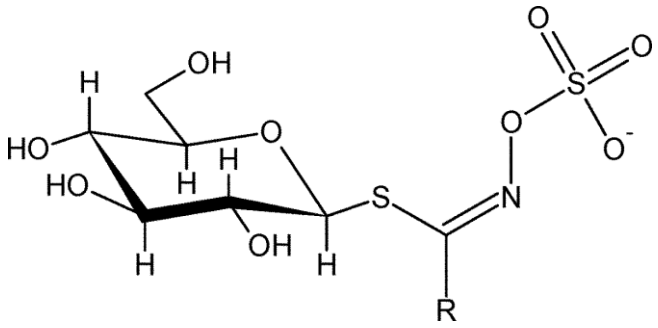

Figure 1-5. Glucosinolates structure [33]

Glucosinolates that are present in the plant cells are hydrolyzed as soon as water is introduced to the system as catalyzed by enzyme myrosinase. After hydrolyzing, they produce a wide range of products and the concentration of GLSs is reduced significantly[31]. Hydrolysis of sinigrin produces allyl isothiocyanate (AITC), allyl thiocyanate, 1-cyano-2, 3 epithiopropane and allyl cyanide (ACN^-). The product formed from any type of glucosinolates can be controlled by controlling the pH, ferrous ion concentration, and application of different coenzymes and protein specifiers [30, 34]. Major hydrolysis products

are stable at pH 6-7. Glucosinolate content is determined by the HPLC method (ISO 9167-1:1992-(E)).

1.9. The role of myrosinase in glucosinolate hydrolysis:

Myrosinase is an endogenous enzyme thioglucoside glucohydrolase (E.C. 3.2.1.147) that hydrolyses glucosinolates. In *Brassica* plants, myrosinase is a part of the defense system along with glucosinolates that forms the glucosinolates-myrosinase defensive system against herbivores and insects [35].

The myrosinase enzyme is a glucopeptide containing a large number of carbohydrates, and in particular mannose residues with a range of thiol groups, disulfide, and salt bridges. A zinc atom is presented at the interface of the myrosinase subunits[36].

Myrosinase has been isolated from some plant sources including *Lepidium sativum L.* (light ground cress), *Sinapis alba* (white mustard) and *Brassica napus* (rapeseed). The molecular weight of mustard and rapeseed enzymes is between 120K and 150K (Botti *et al*, 1995).

Hydrolysis of glucosinolates in the presence of water and myrosinase usually happens when myrosinase becomes active due to cell damage as a result of physical injury of plants or food processing (crushing, sieving, etc.) [37]. After tissue damage, myrosinase hydrolyzes the thioglucosidic bond (figure 1-6) thereby glucose and an unstable aglycone (thiohydroxamate-o-sulfonate) are produced [33]. The latter product spontaneously rearranges into different products including isothiocyanates (ITCs), nitriles, elemental sulfur, thiocyanate, epithio nitriles, oxazolidinie-2-thiones, or indolyl compounds. The

chemical structure of the side chains is a key factor for types of hydrolysis compounds formed [33].

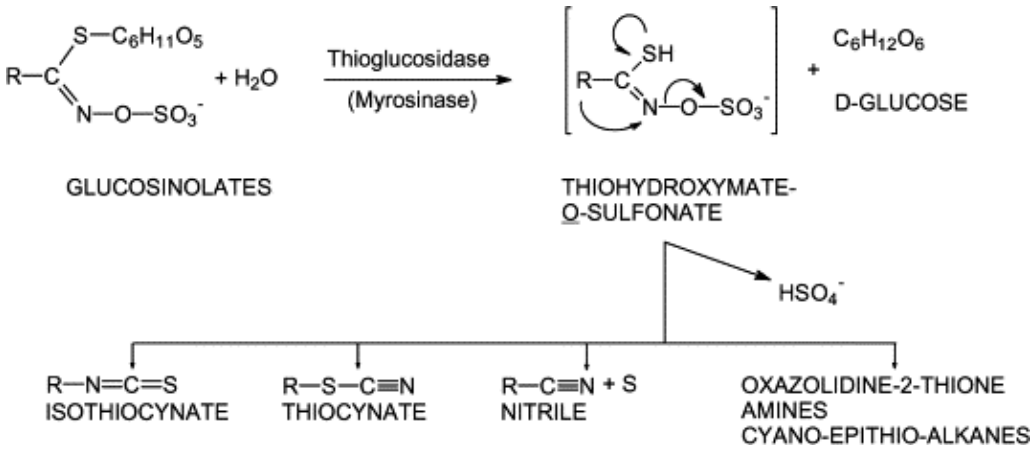

Figure 1-6. Glucosinolates and myrosinase in the intact plant are stored separately. Upon tissue damage, the two components come into contact and myrosinase catalyzes the hydrolysis of the glucosinolates (adapted from [38])

All glucosinolates are hydrolyzed at the same rate regardless of side chain [37]. Ascorbic acid is a specific activator of myrosinase which 1mM of ascorbic acid can increase the myrosinase activity by 25 fold [37].

Denaturation of myrosinase may happen with heating, ethanolic extraction, even microwave treatment, or in the presence of some organic solvents [39]. For example, the crushed seeds of rapeseed are heated 90-110°C for 20-25 minutes to inactivate myrosinase [40]. Myrosinase extracted from *S. alba* L. is stable until 60°C for 10 minutes in a buffer system; however beyond this temperature inactivation becomes significant. Thermal treatment at 75°C for 10 minutes causes a 90% drop in the enzyme activity [34]. Generally myrosinase is a very heat stable enzyme and it is hard to completely remove from mustard meal. The half-

life of myrosinase at 37°C was 40 h with 20% methanol and 8 hours with 40 % methanol [37]. The final product of myrosinase in *Sinapis alba* is the thiocyanate ion (SCN^-), which can be used in lactoperoxidase system (a well-known antibacterial system in raw milk).

1.10. Application of glucosinolate hydrolysis products as antimicrobials in food

Glucosinolates are naturally present in mustard flour, which contain the myrosinase enzyme, and could serve as a source of allyl and other isothiocyanates. A study in 2005 was conducted to determine the effect of glucosinolate products on the *Escherichia coli* O157:H7 in ground beef patties. This study tested if the concentration of glucosinolate products was sufficient to kill *E.coli* O157:H7 inoculated in ground beef at three different levels during refrigerated storage of the meat under nitrogen. The total number of microorganisms showed larger reductions at higher concentrations of mustard flour. However, sensory evaluation results showed that the difference between mustard meal-added patties and control patties was obvious to panelists [41]. Therefore, most studies on the application of mustard flour or meal have been focused on incorporation of the meal into the packaging.

Lee et al. (2012) studied the antimicrobial effects of defatted mustard meal on *Listeria monocytogenes*. Mustard meal was added to the film solution at pH 5.5 and pH 2 in the presence or absence of myrosinase enzyme to study the effect of pH, SCN^- concentration, and their synergistic effect on *Listeria monocytogenes* growth. Similar to other researchers, the growth of the microorganism was inhibited by SCN^-. There was a direct correlation between the time and concentration of SCN^-, and the antibacterial effect was more effective after 24 hours. The antimicrobial effect of DMM film was higher when the pH of the film

solution was lower. The inhibition of *Listeria monocytogenes* was more effective when the coating was created before inoculation[42].

Although AIT from plant sources has been confirmed to have significant antimicrobial activity against other pathogenic bacteria, since the odor is not acceptable, it has infrequently been employed in food matrices as an antimicrobial agent [41].

1.11. Application of lactoperoxidase system to improve food safety

Food safety is always associated with pathogenic microorganisms and foodborne pathogens. Food borne disease outbreaks are a major concern of the food industry and regulatory agencies. Annually, food born bacteria cause significant numbers of foodborne illnesses in the USA [43]. The Center for Disease Control and prevention (CDC) data confirms that each year foodborne diseases make roughly 1 in 6 Americans (or 48 million people) sick. Among them, 128,000 hospitalizations and 3,000 deaths are recorded. *Salmonella* (nontyphoidal), *Toxoplasma qondii*, *Listeria monocytogenes*, *Norovirus,* and *Campylobacter* Spp. are the top five pathogens that cause death (Table 1.2). Even food companies with sophisticated production lines and well established automatic unit processes are required to launch numerous preventive strategies such as Hazard Analysis Critical Control Points (HACCP) and Good Agriculture Practice (GAP) to minimize the risk of foodborne pathogens. The introduction of preservatives into the final product during processing is another potential preventive strategy.

Table 1-2. Top five pathogens contributing to nationally acquired foodborne diseases resulting in death (CDC, 2012)

Pathogen	**Estimated number of deaths**	**%**
Salmonella, nontyphoidal	378	28
Toxoplasma gondii	327	24
Listeria monocytogenes	255	19
Norovirus	149	11
Campylobacter spp.	76	6
Subtotal		88

Today, consumers demand processed foods with properties close to the original unprocessed food. Food additives and preservatives are normal ingredients in food to retard spoilage and prevent microbial growth in the system. To address consumers' needs, increase safety, and improve shelf life researchers are looking for methods to replace chemical preservatives with natural or so called bio-preservatives. The "lactoperoxidase system" is a natural antimicrobial and preventive mechanism found in raw milk that has potential applications in the food industry.

The lactoperoxidase system (LPSys) is a three component system that includes thiocyanate ion (SCN^-), hydrogen peroxide (H_2O_2), and the lactoperoxidase enzyme. It is an antimicrobial system available naturally in raw milk that is able to prevent growth of pathogenic microorganisms within the first 24 hours of unrefrigerated milk storage.

Lactoperoxidase is a promising natural antimicrobial system that can be employed in food products to improve the safety of the product. To create the LPSys, each component can be used separately. Hydrogen peroxide can be added exogenously or created by the glucose oxidase enzyme endogenously, lactoperoxidase enzyme is available commercially,

and so far non-food grade potassium thiocyanate (KSCN) has been used as the source of SCN^-. Therefore, if food grade SCN^- is available, a food grade LPSys can be used as a natural antimicrobial. *Brassicaceae* meal extracts contain glucosinolates that can be hydrolyzed by an endogenous enzyme called myrosinase and produce thiocyanate (SCN^-) as one of the several resulting hydrolysis products. Therefore, the combination of the extract and lactoperoxidase with hydrogen peroxide can be used as a natural preservative.

1.12. Lactoperoxidase system

There are primarily three components available in LPSys: lactoperoxidase enzyme (LPD), hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) (Figure 1-7 and 1-8). Hydrogen peroxide may be produced by a number of microorganisms such as *Lactobacilli, Streptococci,* and *Lactococci* by oxidation of ascorbic acid or oxidation of glucose, or added exogenously. Thiocyanate is naturally available in some biological systems and raw milk that is part of the diet. The lactoperoxidase system is available in raw milk and the human body. Lactoperoxidase is secreted from salivary, mammary, and other mucosal glands in the human body and functions as a natural antibacterial agent by creating the lactoperoxidase system. Thus, it plays an important role in the human immune defense system [43].

The LPD catalyzes peroxidation of H_2O_2 in the presence of SCN^- as the two electron donor compound I (Figure 1-7) [44] and LPSys is activated. After activation usually several intermediate oxidation products are created, of which hypothiocyanite ($OSCN^-$) is the major compound. $OSCN^-$ is in equilibrium with hypothiocyanous acid (HOSCN) at pHs between 5 and 7.

The antimicrobial potential of the lactoperoxidase system is that it is GRAS (Generally Recognized as Safe) having been extensively investigated (Elliot et *al.,* 2004). The LPSys inhibits the growth of both Gram negative (e.g. *E.coli* O157:H7) and Gram positive bacteria (e.g. *L.monocytogenes,* and *S.aureus*).

The lactoperoxidase system can inhibit or reduce the number of microorganisms. The microorganisms include a wide range of fungi, viruses, and bacteria. The intermediate oxidation products of thiocyanate oxidize the essential sulfhydryl groups in proteins and impair transport systems, membrane stability, and enzyme metabolism of microorganisms [43, 45, 46]. The inhibitory effects of the lactoperoxidase system at the molecular level depend on the type of electron donor, test media, temperature, and pH. The system may impose an oxidative killing, block or interfere with glycolytic pathways, and cause cytopathic effects [46].

The lactoperoxidase system has a bacteriostatic effect on the Gram positive, catalase negative bacteria such as *Streptococci* and *Lactobacilli*. However, Gram negative and catalase positive bacteria (e.g. *Pseudomonas, coliforms, Salmonellae* and *Shigellae*) are inhibited and may be killed by the LPSys. Bactericidal activity of LPSys has been shown against *Campylobacter jejuni, Salmonella spp, Escherichia coli,* and *Pseudomonas spp* [47].

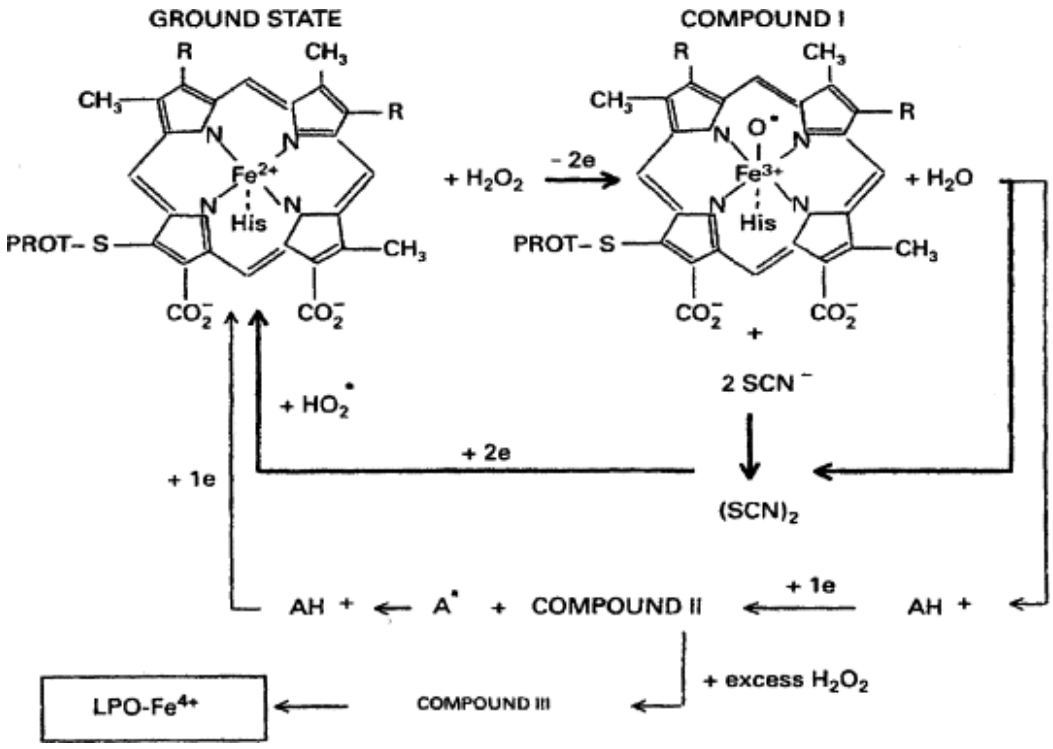

Figure 1-7. Pathways in the lactoperoxidase catalyzed system. The normal product of the peroxidatic cycle creates compound I. If the two electron donor is not sufficient the compound II is formed. Excessive hydrogen peroxide remains in the system and results in the formation of compound III [48, 49]; courtesy of Elsevier Science publishers.

The decay rate depends on the medium conditions (pH, temperature, cell density, and incubation time) given that hydrogen peroxide is introduced exogenously[46, 50, 51]. Highest concentration of allyl cyanide (ACN-) was recorded at 20°C rather than 60°C [39].

$OSCN^- + R\text{-}SH \rightarrow R\text{-}S\text{-}SCN + OH^-$ (Pruitt et al., 1982)

It is interesting to note that only a portion of bacterial sulfhydryl bonds are damaged by $OSCN^-$ and an excessive amount of $OSCN^-$ does not oxidize more sulfhydryl bonds (Pruitt et al., 1982). The stability of the $OSCN^-$ is impacted by several factors such as pH, light, metal ions, glycerol, and ammonium sulfate [44, 46].

The potential bacterial inhibition effect of LPSys in food systems has been investigated by Elliot *et al.* (2004) and Kennedy *et al.* (2000) in beef cubes and ground beef, respectively. Application of the LPSys on beef cubes significantly reduced the number of microorganisms including *S. aureus, S.typhomurium, L.monocytogenes, E.coli* O157:H7, *P.aeruginosa, and Y. enterocolitica* after 7 days incubation at 12°C. At this temperature, the greatest inhibition was recorded for *Y. enterocolitica* with a 4 log reduction. All other bacteria in this study except *S. aureus* showed a 2 log reduction compared to the control sample. Likewise, growth of most pathogens in beef cubes was inhibited using a combination of the LPSys and a chilling regime (12 to -1°C) (Elliot *et al.,* 2004). Formerly, it was similarly shown that lactoperoxidase activation is significantly affected by temperature, length of incubation, and the lactoperoxidase concentration as well as the level of thiocyanate. An activated LPSys was found to be more stable at 4°C than at 8°C [47].

1.13. Antimicrobial properties of the mustard meal

There are various species of mustard available under the family of *Brassicaceae*. The meal extracts from some members of this family can inhibit several important pathogens especially bacterial pathogens (Hashmi *et al.*, 2011). As an example, benzyl isothiocyanate (BITC) is applied to treat infections of urinary and respiratory system [52]. Taking the structure of the bacterial cell wall into account, Gram negative bacteria are less sensitive than Gram positive bacteria to isothiocyanates [52].

$$H_2O_2 + 2\,SCN^- + 2H^+ \xrightarrow{Lactoperoxidase} 2H_2O + (SCN)_2$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^-$$

$$\mathbf{HOSCN} \underset{pH\,5-7}{\Longleftrightarrow} \mathbf{H^+ + OSCN^-}$$

Or

$$H_2O_2 + \mathbf{SCN^-} \xrightarrow{Lactoperoxidase} \boldsymbol{OSCN^-} + H_2O$$

Figure 1-8. Lactoperoxidase system. $OSCN^-$ is the major product of the lactoperoxidase-thiocyanate peroxide system [53]

Isothiocyanates, benzyl isothiocyanate, and 2-phenylethylisothiocyanate inhibited bacterial growth (Gram positive and Gram negative bacteria) more than traditional antibiotics such as gentamycin or streptomycin. *E.coli* growth was strongly inhibited by BITC and allyl isothiocyanates (AITC) and both had strong antibacterial activity against *P.aeruginosa* [45]. Allyl isothiocyanate may damage the cell membrane, reduce the level of ATP, or alter the internal structure of bacteria. Allyl isothiocyanate might be more active in acid foods and has a minimum inhibitory concentration of 25 µl/l [32]. Aromatic isothiocyanates have higher antibacterial effect than aliphatic isothiocyanates [45], however, this effect was more evident on Gram positive bacteria.

Tenore *et al.* (2012) treated 12 individual bacteria strains, two types of yeast, and four types of molds with a *Brassica rapa* L. var rapa meal extract (RCN meal extract). The bacteria were known food borne Gram positive and Gram negative pathogens. The minimum inhibitory concentration method was used to monitor the growth of microorganisms. Comparable to the previous reports, Gram positives were the most sensitive bacteria to the

RCN meal extract. The most sensitive among Gram negative bacteria were *Y. enterocolitica* and *E. cloaceae*. The inhibition on yeasts was more than molds[54].

In addition to GLS products, phenolic compounds that are presented in different mustard meals induce antimicrobial effects, but such effects are less pronounced. Saavedray et al. (2010) compared the antibacterial activity of a group of common dietary phytochemicals including phenolics such as tyrosol, gallic acid, caffiec acid, chlorogenic acid, glucosinolate products (allyl isothiocyanate, benzyl isothiocyanate and 2 phenylethylisothiocyanate) against *Escherichia coli, Pseudomonas aeroginosa* , *Listeria monocytogenes and Stophilococus aureus* . They also studied the synergic effects of a dual combination of streptomycin with either glucosinolates products or phenolic compounds and the effects were compared with single antibiotics (ciprofloxacin, gentamicin, and streptomycin) on the growth of those bacteria, Glucosinolates significantly improved antimicrobial activity, but the phenolics showed much lower antibacterial potential compared to the reference antibiotics. Therefore, it is assumed that if mustard meal extract can be used as a source of thiocyanate in the lactoperoxidase system, no noteworthy additional antimicrobial effect will be induced by phenolics and antioxidants in the mustard meal extract.

1.14. Mustard meal lactoperoxidase system pitfalls and limitations

Fractionation and industrial scale purification of lactoperoxidase from whey, whey proteins concentrate, or milk has been developed by Burling (1989) and Uchida (1996). Cation exchange resins have been used to obtain purified lactoperoxidase on a large scale [55]. Hydrogen peroxide can be added exogenously or may be produced by adding glucose

and glucose oxidase into the system. By supplementing with thiocyanate from a mustard meal extract, the lactoperoxidase system can be activated.

Hypothiocyanite ($OSCN^-$) is the intermediate product of peroxidase-catalyzed oxidation of thiocyanate (SCN^-) and is the major product of this oxidation system in a pH range of 6-8 [56]. Maximum concentration and accumulation of $HOSCN/OSCN^-$ is limited by the combination of SCN^- and H_2O_2 concentrations in the system. Excessive hydrogen peroxide at pH levels higher than 6.5 with equimolar initial concentrations of SCN^- and H_2O_2- accelerated hypothiocyanite decay rate [56]. In addition, Hoogendoom et al. (1977) postulated that after reaching the maximum concentration, one molecule of $OSCN^-$ deteriorates another three molecules of hypothiocyanite, which ultimately produce CO_2, NH_4^+ and SO_4^{2-} [57].

As was described in the literature, the stability of hypothiocyanite can be affected by several factors including, pH, thiocyanate, lactoperoxidase, and even $OSCN^-$ itself. Thereby, when the lactoperoxidase system with mustard meal-sourced SCN^- (which is a mixture of plant components, polyphenols, sulfur compounds, and several other limiting compounds) is activated, the stability of the $OSCN^-$ is negatively affected by several unknown molecules even though one might claim that there may be other synergic factors in the mustard meal extract that can improve the antimicrobial effect of the mustard meal lactoperoxidase system.

1.15. Hypothesis

The following hypothesis will be tested that:

1) Application of higher ultrasound frequencies will increase the recovery of bioactive compounds in red raspberry puree.

2) Ultrasound can improve the production of thiocyanate from mustard meal.
3) Mustard meal extract thiocyanate can activate the lactoperoxidase system.

1.16. Objectives

1) To investigate the effect of three frequencies of ultrasound (20, 490, and 986 kHz) on antioxidant activity, total phenolics content, and anthocyanin content of red raspberry puree.

2) To investigate the effect of 20 kHz ultrasound on increasing the rate of SCN^- production in a 20:1 solvent (water) to meal (*Sinapis alba*) ratio.

3) To study hypothiocyanite ion ($OSCN^-$) production and decay pattern in a mustard meal (*Sinapis alba*)-derived lactoperoxidase system over 0-24 hours.

CHAPTER 2: EFFECT OF ULTRASOUND FREQUENCY ON ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND ANTHOCYANIN CONTENT OF RED RASPBERRY PUREE [1]

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2.1. Abstract

Ultrasound in the 20 to 1000 kHz range show unique propagation characteristics in fluid media and possesses energy that can break down fruit matrices to facilitate the extraction of valuable bioactive compounds. Red raspberries (such as Herritage, Kiwigold, Goldie, and Anne) contain significant amounts of specific antioxidants (varies based on methodology), including ellagitannins and anthocyanins that are important for human health. The objective of this study was to investigate the efficacy of ultrasound extraction including the effects of ultrasound frequencies associated with cavitation (20 kHz) and microstreaming (490 and 986 kHz) on total antioxidant activity (AOA), total phenolics content (TPC), and total monomeric anthocyanin content (ACY) of red raspberry puree (RP) prepared from crushed berries. The pureed fruit was subjected to high-intensity (20 kHz) and higher frequency-low intensity (490 and 986 kHz) ultrasound for 30 minutes at room temperature. The temperature of treated purees increased to a maximum of 56°C with 986 kHz on ice bath. Sonication at 20 and 490 kHz significantly ($P<0.05$) affected the

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AOA, ACY, and TPC of red raspberry puree, while 986 kHz had no significant effect on ACY and AOA ($P<0.05$) compared to the untreated control. At 20 kHz, AOA and ACY increased by 17.3 % and 12.6% after 10 minutes. Treatment at 20 kHz ultrasound treatment, when limited to 10 minutes, was the most effective for extraction of bioactive compounds in red raspberry compared to 490 and 986 kHz. In all cases, ultrasound treatment had significant and positive effect on at least one of the measured parameters up to 30 minutes. Sonication beyond 10 minutes (and up to 30 minutes) using 20 kHz either produced no change or caused a drop in AOA and ACY extraction ability.. However, for 986 and 20 kHz, TPC, increased by 10% and 9.5% respectively after 30 minute ($P<0.05$) compared to the control.

Keywords: Ultrasound, Extraction, Antioxidant activity, Anthocyanin content.

2.2. Introduction

Ultrasound is widely used in the areas of science and engineering because it is a technique with multiple capabilities for cellular tissue disruption that makes it suitable for different industrial applications, including the food industry. Sound frequencies equal to or higher than 20 kHz are defined as ultrasound [15]. Power ultrasound frequencies range from 20 kHz to 1MHz, and are considered so because of ability to deliver high energy uniformly throughout a fluid or semisolid medium and under conditions in which tissues of different acoustic impedance can be selectively targeted. Within the frequency range specified above, ultrasound waves can generate cavitation and microstreaming (microscopic fluid movement) effects that cause changes (eg. improve extraction, disrupt cells, etc.) in a liquid medium [58]. Cavitation is the phenomenon associated with production, growth and collapse of bubbles during the compression and rarefaction cycles in sound propagations. This

phenomenon creates eddy currents in the fluid near the vibrating bubbles and the eddy currents consecutively apply a distortion and rotational motion on nearby cells or particles.

The application of ultrasound in the 20-1000 kHz range is divided into two bands, namely, (1) low power ultrasound having low amplitude and high frequency (100-1000 kHz), (2) high power ultrasound generated at high amplitude and low frequency (20 - 100 kHz). This second type is used in food processing, welding, and cleaning processes because cavitation bubbles are more difficult to form at the higher ultrasonic frequencies [15, 59]. The mechanism of ultrasound assisted extraction (UAE) of bioactive compounds in plants involves breaking the cell walls, diffusion of the solvent through the cell matrix and washing out of the cell contents. Ultrasound is beneficial in extraction because it damages the plant cell wall and increases the solvent uptake by the cell during sonication thereby increasing the extraction yield [60]. Usually during this process the tissue temperature increases due to cavitation, asymmetric collapse of bubbles close to the surfaces of cell walls, and the production of micro jets. These episodes produce energy that is partly absorbed by the medium and dissipated as heat, resulting in temperature increase. However, the temperature remains low (less than 70°C) when the reactor is equipped with a cooling system [18].

Ultrasonic extraction of various food components has been reported mainly for 20-40 kHz; and the sample size, extraction time and solvent volume generally used by many researchers for solid-liquid extraction is between 1-30 g, 10-60 min, and 50-200 ml, respectively [9].For example, Tiwari *et al.* [61] applied a 1500 W ultrasonic processor at a constant frequency of 20 kHz to investigate the effect of ultrasound on the degradation of anthocyanin and ascorbic acid in strawberry juice.

Current research suggests that a diet rich in bioactive compounds (such as those provided by antioxidants found in fruits and vegetables) can play a role in protecting the body from degenerative diseases[62, 63]. Therefore, it is recommended to include a daily intake of fruits and vegetables, although according to USDA, most Americans do not consume the quantities and varieties that are recommended. Fruit juices are more convenient and often less expensive than whole fruits [64]. In 2010, the juice retail market in the U.S. was valued at $ 16.2 billion and Americans consumed an average of 30.3 liters per person [65]. Efficient extraction of bioactive compounds for preparation of juices and other products, while not correlated with the absorption and effectiveness of the antioxidants *in vivo,* can provide options for increased nutritional or food functionality, and more efficient delivery of nutritent that may not be possible with the consumption of whole fruits and vegetables [66]. Enhanced recovery of anthocyanins from fruit or vegetable matrices is also of interest to food processors because of their use as colorants, additives to promote nutritional value of other foods, or even as nutritional supplements.

Many berries contain polyphenols, anthocyanins and ascorbic acid [67, 68] in varying quantities; and cranberry, raspberry, and black currant are considered important sources of anthocyanins [21, 22]. Raspberries are unique among the berry fruits because of a nutritional profile of low calories, high fiber, and phytochemical composition that include flavonoids, phenolic acids, lignans, and tannins. Among the phenolic compounds, raspberries carry significant amounts of ellagitannins and anthocyanins which contribute to their appealing color and health-promoting properties [23]. Xylitol, a low-calorie sugar substitute with immense applications in human health products, is found in red raspberries and strawberries. The level of xylitol in raspberries (*Rubus idaeus*) per gram of fresh weight is approximately 400 µg compared to 7.5–280 pg in other berries [24].

The Pacific Northwest region of the United States is a major producer of red raspberry, especially Western Washington and Oregon where there is large scale commercial production and processing [69]. Processed red raspberry in the State of Washington alone accounts for nearly 95% of the U.S. production (about 30 million kg/year) [70]. Because of this economic importance, it is important to not only increase fresh fruit production, but to investigate alternative processing techniques to improve juice extraction and anthocyanin recovery. In conventional juice extraction, a mash of red raspberry fruit is treated with enzymes (pectinase) to increase the free-run juice, shorten the pressing time, and minimize energy consumption [71]. The juice yield is improved by about 20% when the mash is treated with enzymes[72]. Ultrasound assisted extraction (UAE) may be a treatment step that can enhance extraction yield and increase the antioxidant capacity of extracts [73] used either in conjunction with enzyme treatment or in place of it.

Small scale UAE has been practiced since the 1950s [9], and most recently Chen and others [74] reported results of a laboratory scale extraction of red raspberry anthocyanins using ultrasound. However, optimization of the process to determine suitable frequencies and strategies for scale up are still poorly understood. The extent of cell disruption, juice yield and antioxidant activity as affected by ultrasound frequency, power intensity, and material structure [18, 19] need further investigation. The effect of frequency on sonochemical reactions has been investigated using an interesting ultrasound system with a single transducer that was capable of generating 0-5 Watts of ultrasound power at multiple ultrasound frequencies (20, 40, 150, 200, 300 and 450 kHz) [75]. In a different study [76], the degradation of alachlor (a herbicide) residues in water at 20 kHz and 300 kHz was more rapid at 300 kHz than at 20 kHz under similar acoustic energy input.

In the case of UAE, no published literature was found where the effect of high frequency ultrasound (> 50 kHz) on antioxidant capacity and recovery of anthocyanins from fruits was investigated. Many studies are available on the application of 20 kHz-40 kHz ultrasound for cell disruption or extraction [9], but just like in sonochemistry, most of those studies are based on the availability of commercial ultrasound generators which are designed to function at lower frequencies (<100 kHz). There is a need to evaluate other frequencies as a step toward optimization and scale up ultrasound assisted extraction. Therefore, the objective of this study was to investigate the effect of three ultrasound frequencies (20, 490 and 986 kHz) on antioxidant activity, total phenolics content and anthocyanin content of red raspberry puree as a berry product with important health-promoting attributes. The effect of higher frequencies to increase or decrease the recovery of anthocyanins in red raspberry puree was tested.

2.3. Materials and methods

2.3.1. Materials

Reagent grade L-ascorbic acid powder (99.5% purity) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical were obtained from Sigma Co. (St. Louis, MO). A kit (ACW kit) for the analysis of water soluble antioxidants was purchased from Analytik-Jena AG, Jena, Germany. Ascorbic acid solution (AAS), used as model to determine antioxidant activity assays and ultrasound intensity in the fluid, was prepared by dissolving 27 mg of L-ascorbic acid in 100 ml distilled water. Red raspberries were purchased from a local grocery store, crushed, blended and mixed thoroughly to obtain purees (with 10.9% solid content) with fine and smooth texture.

2.3.2. Sonication

For the sonication treatments, 150 ml samples of either ascorbic acid solution or red raspberry puree were used. Three different ultrasound frequencies (20, 490 and 986 kHz) were applied for treatment of ascorbic acid solution and red raspberry puree in batch systems. During those treatments, the reactor vessel jacket was filled with ice water to keep the temperature as low as possible. Samples were collected at 10-minute intervals from start up to 30 minutes.

2.3.2.1. Ultrasound treatment at 20 kHz

A 400W capacity batch sonication system (Branson Sonifier, S-450A, Danbury, CT) with a 7 cm vibrating titanium tip was used for treatments with the probe immersed half way into the liquid. The mashed raspberries were sonicated through continuous pulsation at 50% output power setting. A 500 ml glass beaker (15 cm inside diameter) was put inside another plastic beaker (25 cm inside diameter) containing a mixture of ice and water. A magnetic stirrer was placed at the bottom of the plastic beaker for continuous mixing of the cold water for effective cooling (Figure **2-1**). Samples were subjected to sonication for 30 minutes, and at each time interval, a 5 ml sample was taken. Each fresh sample was maintained in an ice chest during the experiment, centrifuged at 1880 g for 20 minutes, and the clear supernatant was collected and kept frozen at -20 °C for analysis afterwards. Preliminary experiments were carried out to secure uniform sampling and mass balance among samples.

2.3.2.2. Ultrasound treatment at 490 and 986 kHz

The higher ultrasound frequencies (490 and 986 kHz) were produced by a custom-made ultrasound generator (Figure **2-2**) equipped with a parabolic stainless steel horn collimator

designed to focus sound waves from a 3.2 mm thick and 35mm diameter piezoelectric transducer (APC-841, American Piezo Ceramics, Mackeyville, PA). The collimator was equipped with a copper backing that provided the electrical input and base for the transducer. The collimator itself was connected to a stainless steel reaction chamber (27.0 ± 0.1 cm in length and 7.5 ± 0.0 cm in diameter) which was mounted inside concentric cylindrical cooling chamber [77]. The head space was purged by argon gas (also bubbled through the liquid) to minimize the potential effect of oxidation of berry antioxidants. The sonication time, sampling procedure, and sample preparation was similar to that at 20 kHz as described above.

2.3.3. Ultrasound power measurement

The acoustic energy supplied by the transducers and delivered into the liquid is eventually dissipated as heat resulting in a moderate temperature increase (~ 50°C). Therefore, knowing the physical properties of liquid and by measuring the temperature change as a function of time, an estimation of the acoustic power (W) should be obtained. According to Jambrak et al. [16], this rate of acoustic energy dissipation rate (P) (which may be calculated per unit volume of liquid) is given by:

$$P = m.C_p.\left(\frac{dT}{dt}\right) \qquad (1)$$

Where m: mass of the sonicated liquid (kg); C_p: Specific heat capacity at a constant pressure (J/kg °C). Since water forms 90% of the RP, value of C_p was equal to that of water (4180 J/kg °C); dT/dt is the rate of temperature increase, obtained from the slope of the initial region of the heating curve.

In order to monitor temperature within the puree, thermocouples were inserted inside three narrow (2 mm internal diameter) glass tubes with the sensing tips protruding slightly

out of the tubes. The tubes, positioned vertically with 1.5 cm spacing, were then inserted half way in the ascorbic acid solution or red raspberry puree to measure temperature at the center, near the wall of the reactor and at a midpoint location between the wall and the center. One minute before the end of sonication, the material being treated was mixed to obtain the final uniform temperature.

2.3.4. Determination of antioxidant activity

2.3.4.1. Photochemiluminescence (PCL) method

This method combines the fast photochemical excitation of radical generation with sensitive luminometeric detection. The advantage is that it is highly sensitive, involves few preparation steps, and the measurement time is reduced to a few minutes per measurement and [78]. Photochem® (Analytik Jena AG, Jena, Germany) ACW kit was used to measure the hydrophilic antioxidant activity with ascorbic acid as reference. The standard curve was plotted from zero to 3 nmol/L and concentration of every sample for analysis was adjusted to be within the linear region of this curve.

2.3.4.2. DPPH decoloration method

The DPPH decoloration method is an established procedure to measure the radical scavenging capacity of a compound [79]. Briefly, 2.5 mg of DPPH powder was dissolved in 100 ml of methanol. The solution was mixed completely, capped and covered with aluminum foil to protect it from light. The DPPH solution was prepared fresh daily. A UV/Visible spectrophotometer with "UV Winlab" software (Perkin Elmer PTP6., San Jose, CA) was used to measure the antioxidant activity of ascorbic acid solution by monitoring the absorbance at 517 nm using methanol as blank. The percentage of DPPH quenched was calculated each minute as follows:

$$\%\ \text{DPPH quenched} = 1 - \left(\frac{\text{Abs(sample)}}{\text{Abs(control)}}\right) \qquad (2)$$

This DPPH method was used only for the ascorbic acid solution in order to validate the PCL method for determining the antioxidant activity of red raspberry puree. Control was dionized water and DPPH solution.

2.3.5. Determination of total monomeric anthocyanins

The red raspberry total monomeric anthocyanin (ACY) content was determined by the pH differential method - a spectrophotometric method that involves measurement of absorbance at pH 1.0 and 4.5. The absorbance of the extract was measured at 520 and 700 nm using model PTP6 spectrophotometer (Perkin Elmer, San Jose, CA). Total anthocyanins content (mg/L) was expressed as cyanidin-3-glucoside (MW: 449.2) according to this equation [80]:

$$\text{Anthocyanin content}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{A}\times\text{MW}\times\text{DF}\times 1000}{\varepsilon\times\text{L}} \qquad (3)$$

Where $A = (A_{520nm,\ pH\ 1.0} - A_{700nm,\ pH\ 1.0}) - (A_{520nm,\ pH\ 4.5} - A_{700nm,\ pH\ 4.5})$, or the difference between the absorbance readings at 520 nm measured at pH 1.0 and 4.5 with each reading corrected for haze at 700 nm; MW: molecular weight of cyanidin-3-glucoside = 449.2, g/mol; DF: Dilution factor; ε: molar absorptivity of cyanidin-3-glucoside = 26,900; and L: cell path length (1cm).

2.3.6. Total phenolics content of red raspberry puree

The total phenolics content (TPC) of sonicated red raspberry was determined according to the Folin-Ciocalteu method[81]. Briefly, 20 µl of diluted sample was mixed with 1580 µl of DI water and 100 µl of 2 N Folin-Ciocalteu's reagent. The mixture was left to stand for 3

minutes at room temperature before adding 300 µl sodium carbonate solutions (20% w/v). The cuvettes were incubated at 40°C for 30 min then the absorbance was read at 765nm using dionized water as blank. The TPC was reported as mg Gallic acid equivalent per liter (mg/L). The standard curve was plotted within 50-500 mg/L of Gallic acid in DI water. The stock solution (0.5 g/100 ml) was prepared in 10% aqueous ethanol.

2.3.7. Statistical analysis

All experiments were repeated and data reported represents three measurements from three independent ultrasound treatments or chemical tests. The effect of ultrasound on each of the tested parameters was determined by analysis of variance (ANOVA) using SAS statistical analysis program version 9.0 (SAS, Cary, NC). The significance level (p-value of 0.05) was used to assess whether the individual treatments presented statistically different results. The difference among group means was tested with Duncan's test.

2.4. Results and discussion:

2.4.1. Effect of ultrasound on the antioxidant activity of ascorbic acid:

The DPPH and PCL analysis showed that the antioxidant activity of the ascorbic acid solution did not change significantly ($p<0.05$) when low frequency high intensity ultrasound (20 kHz) or medium intensity ultrasound frequency (490 kHz) was used. However, the PCL analysis showed that there was significant drop in antioxidant activity of ascorbic acid solution after 30 minutes of sonication at the highest frequency (986 kHz) investigated (Figure **2-3**). This apparent drop was not observed with the DPPH assay (data not shown) since it was not as sensitive as PCL method. At 986 kHz, the PCL assay showed a 7.5% decrease in antioxidant activity after 10 minutes and then another drop (47.6%) was recorded after 30 minutes. There was a good correlation between the results of antioxidant

activity measured with DPPH and PCL methods except for the data obtained at 986 kHz. Because of this observed correlation, the PCL method was chosen to analyze the raspberry puree as it was possible to measure changes in antioxidant activity down to concentrations as low as 0.5 nM of ascorbic acid equivalents.

Ultrasound is reported to have a minimal effect on the quality of fruit juices that contain heat labile vitamins [82]. In one study, the ascorbic acid content of orange juice treated with ultrasound decreased by only 5% when a maximum acoustic energy density of 0.81 W/cm^3 was used [83]. However, ascorbic acid degradation is greatly affected by temperature and the loss is more rapid at higher temperatures. The temperature used in this study did not exceed 34°C at the two lower frequencies (20 and 490 kHz), so the effect of temperature on extraction was eliminated and any change in antioxidant activity could only be attributed to ultrasound However, the effect may. In the case of 986 kHz, the observed drop was probably due to the synergic effect of the ultrasound and temperature increase. Vikram et al. [84] found that with conventional heating of orange juice at 50°C and 75°C, it took 65.7 and 27.0 minutes respectively, for ascorbic acid concentration to decrease to one tenth of its initial value. Ascorbic acid degradation during ultrasound treatment has been attributed mainly to free radical production and extreme physical conditions (due to cavitation) experienced under power ultrasound treatments [85].

2.4.2. Effect of ultrasound on product temperature

Table **2-1** shows changes in temperature of ascorbic acid solution and red raspberry puree during sonication at the three tested frequencies. The results showed that with ascorbic acid solution, only a 9.3 °C temperature increase was recorded after 30 minutes of sonication using the 20 kHz system (with cooling). However, under the same setting, a significant increase (48 °C) in temperature was observed without cooling. This depression

of temperature increase showed that the designed cooling system was very efficient during the treatment of ascorbic acid solution. The power intensity comparison of three tested frequencies in ascorbic acid solution showed that within the first 100 seconds, 986 kHz created almost two orders of magnitude higher acoustic energy than 20 kHz and 3.5 times higher than 490 kHz. After 10 minutes the increase in medium temperature was much lower and the acoustic power delivered almost leveled off (Figure **2-5**). A similar pattern was observed with red raspberry puree (data not shown).

The temperature increased by about 44.6 °C (at an average rate of 0.7 °C/min) during a 30minute sonication of red raspberry puree at 20 kHz. Sonication of ascorbic acid solution at 986 kHz (with the ice bath cooling) resulted in a final temperature very close to 50°C after 30 minutes. The same experiment, without cooling, resulted in a final temperature of 83.3°C. The maximum temperature of the puree was recorded close to the sonotrode or transducer. Temperature increases were higher within the first 10 minutes (contributing to almost 80-85% of total increase) than in the next 20 minutes when the change was more gradual.

High temperatures coupled with exposure to molecular oxygen may degrade certain groups of bioactive compounds including anthocyanins which are the major group in red raspberry. However, the degradation of anthocyanins increases with processing time and temperature and the effect of the latter was particularly true above 60°C. The half-life of cynidin-3-glucoside at 60°C and 90°C was reported as 16.7 and 2.9 h, respectively [86]. Now, the highest temperature of red raspberry puree after sonication for 30 minutes at 986 kHz was 55.4°C, suggesting that any effects on ACY content would be minimal.

It is known that elevated temperatures enhance extraction efficiency as the diffusion coefficient is increased and solubility of the target compounds into the solvent is boosted.

Additionally, cell wall structure also gets weakened, thus facilitating the extraction of more from cellular compounds[87, 88]. Therefore, an increase in temperature may not always lead to undesirable effects, but a balance must be struck between extraction yield and possible loss of antioxidant activity. Blueberry anthocyanins are shown to be almost stable in the juice up to 60°C; and after 120 minutes of heating, only 10% loss in total anthocyanin content was observed [89]. The anthocyanins of blackberry juice and blood orange juice showed a negligible drop (< 1%) at temperatures below 70°C, but the drop increased significantly at temperatures above 70°C [90].

2.4.3. Change in total monomeric anthocyanin content

Total monomeric anthocyanin (ACY) content of red raspberry puree increased by 12.6% at 20 kHz and by 6.7% at 490 kHz after 10 minutes sonication ($p < 0.05$) (Table 2-2). However, at 20 kHz, no significant increase was recorded after 20 minutes. At 986 kHz no significant change in ACY content was observed. Holtung *et al*. [91] also found no significant change in TPC and ACY content after black currant press residue was sonicated at 20, 40, 55 and 80°C for 15 and 30 minutes. Tiwari *et al.* [61] similarly observed only a slight increase (<1%) in the anthocyanin content of strawberry juice sonicated for less than five minutes at 20 kHz and lower amplitudes (<60% of full scale). Chen et al. [74] suggested keeping the temperature below 40°C to optimize the extraction of anthocyanins from red raspberry. In our case, the temperature of red raspberry puree reached 35°C within the first 10 minutes of sonication, and after 20 minutes it was close to 40°C (which was within the optimal temperature range). This increase in temperature and power input improved the mass transfer, resulting in more extraction. Considering the proportion of the power input within the first 10 minutes, it is realized that the power level increased by 80-85%, and this had a positive effect on the extraction rate. The increase in extractable anthocyanin pigments

within 10 minutes of sonication may be due to the large power input and cavitation effects that cause significant cell disruption. Thereafter, other factors such as solvent type may play a greater role.

Chen et al. [74] showed that at temperatures below 40°C, the optimum extraction time was 3.3 minutes with 400 W power input. This processing condition extracted 34.5 mg of anthocyanin (cyanidin-3-glucoside equivalent) per 100 g of fresh fruit which was similar to the yield after 53 minutes of conventional extraction at 71°C. The improvement in extraction time was attributed to cavitation which increases the number of damaged cells and/or mass transfer from cell matrix to solvent. The power input for 20 kHz and 986 kHz was close, but the 986 kHz had minimal effect on the puree because, as the sonication frequency approached 1 MHz, the cavitation was not sufficient to disrupt the cell walls. Therefore, no noticeable extraction occurred and hence the level of anthocyanins remained constant.

2.4.4. Effect of sonication on total phenolic content of red raspberry puree

There was no significant change in TPC of red raspberry puree at all the ultrasound frequencies ($p<0.05$) investigated except for sonication at 986 kHz for 30 minutes where there was 10.9% increase (Table 2-2). This increase can be attributed to the greater overall temperature increase at this frequency. Viljanen et al. [92] reported that two major phenolics compounds could be found in red raspberry, with elagitanins and anthocyanins accounting for 51% and 31% of total phenolics, respectively. Our results showed that none of the three ultrasound frequencies had any negative effect on the total phenolics content of the red raspberry puree. No significant increase in total phenolics content was observed after 10 minutes of sonication using 20 and 490 kHz. However, the recovery of anthocyanins increased. This suggests that anthocyanins may not have contributed much to the observed total phenolics content of the red raspberry puree.

The increase in total phenolics at 986 kHz could be explained by temperature increase which can increase the transfer of the red raspberry phenolic compounds from the puree to the extraction solvent. This suggests that this approach could be used to extract the same compounds from pulp, seeds or both. It is probable that the synergic effect of temperature and sonication caused the increase in total phenolics content. In a study with black currant press-residue [91], the effect of extraction time on total phenolics content and total monomeric anthocyanins content was not dependent on extraction temperature when the extraction medium was kept at lower temperatures (~ 20°C). However, at higher temperatures (40, 55 and 80°C), longer extraction time increased the extraction of total phenolics and total monomeric anthocyanins.

2.4.5. Total antioxidant activity of red raspberry puree

The total antioxidant activity (AOA) of red raspberry puree significantly increased after 10 minutes ($p<0.05$) of treatment at 20 kHz and 490 kHz. For both frequencies, no significant ($p<0.05$) change was observed after 10 minutes except for the 20 kHz treatment where the antioxidant activity decreased by 6.6% but remained higher than that of the control after 30 minutes. Treatments at 986 kHz showed no significant change in antioxidant activity. These results revealed that power ultrasound had no deteriorative effect on antioxidants in red raspberry puree. However, results from frequencies tested did not support the hypothesis that increase in frequency can lead to an increase in the antioxidant activity of the extracts. In addition, it can be inferred that the major contributor to antioxidant activity are anthocyanins, as the trend in antioxidant activity paralleled that of anthocyanin content. This agrees with the findings of Liu et al. [93] which showed that anthocyanins have major contribution to berry color, and that darker colored raspberry

cultivars (e.g. Heritage) have higher antioxidant activity than lighter colored cultivars (e.g. Anne).

2.5. Conclusion

Results of treatments using three ultrasound frequencies (20, 490 and 986 kHz) showed that none of the frequencies had negative impact on recovery major bioactive compounds in red raspberry puree. In addition, high intensity (20 kHz) and the higher frequency (490 kHz) ultrasound can increase the antioxidant activity and total monomeric anthocyanin content of red raspberry puree due to better extraction. Ultrasound treatment at 490 kHz had less effect compared to 20 kHz while treatment at 986 kHz had no significant effect on antioxidant activity and total monomeric anthocyanin content. The greatest positive impact was the increase in antioxidant activity and total monomeric anthocyanin content by 17.3% and 12.6%, respectively when 20 kHz was applied for 10 minutes. No decrease in the antioxidant activity of ascorbic acid was observed at 20 kHz. The positive effects of ultrasound assisted extraction decreased at higher frequencies regardless of power input. The results also showed that ultrasound extraction at the three tested frequencies was most efficient up to10 minutes. Treatment temperature and 986 kHz had a synergic effect on the loss of vitamin C after 30 minutes of sonication although the antioxidant activity and total phenolic content of red raspberry puree did not change at this higher frequency at level of total power exposure. As frequency increased, there was less cavitation and the investigated bioactive compounds in red raspberry puree were less affected. Sonication at different amplitudes and power intensities need to be optimized for achieving maximum extraction should be investigated to define parameters that would be suitable for treatment of similar fruit purees.

2.6. Acknowledgements

This work has been funded by the USDA NIFA Grant # 2009-35503-05207. The authors would like to thank Dr. Farhad Foroudi for his valuable help with statistical analysis of the data.

2.7. Figures

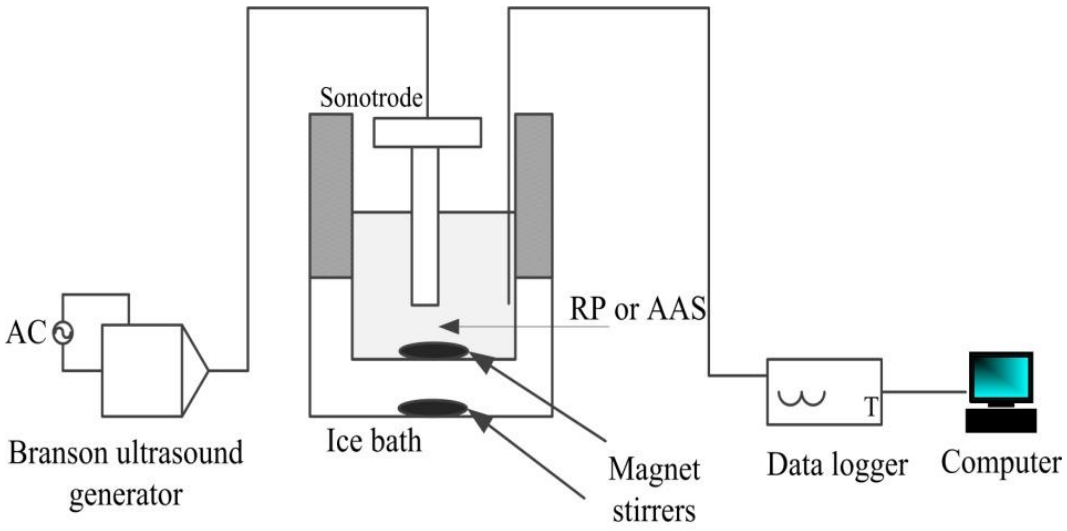

Figure 2-1. Schematic of a 20 kHz ultrasound reactor for treatment of red raspberry puree (RP) and ascorbic acid solution (AAS).

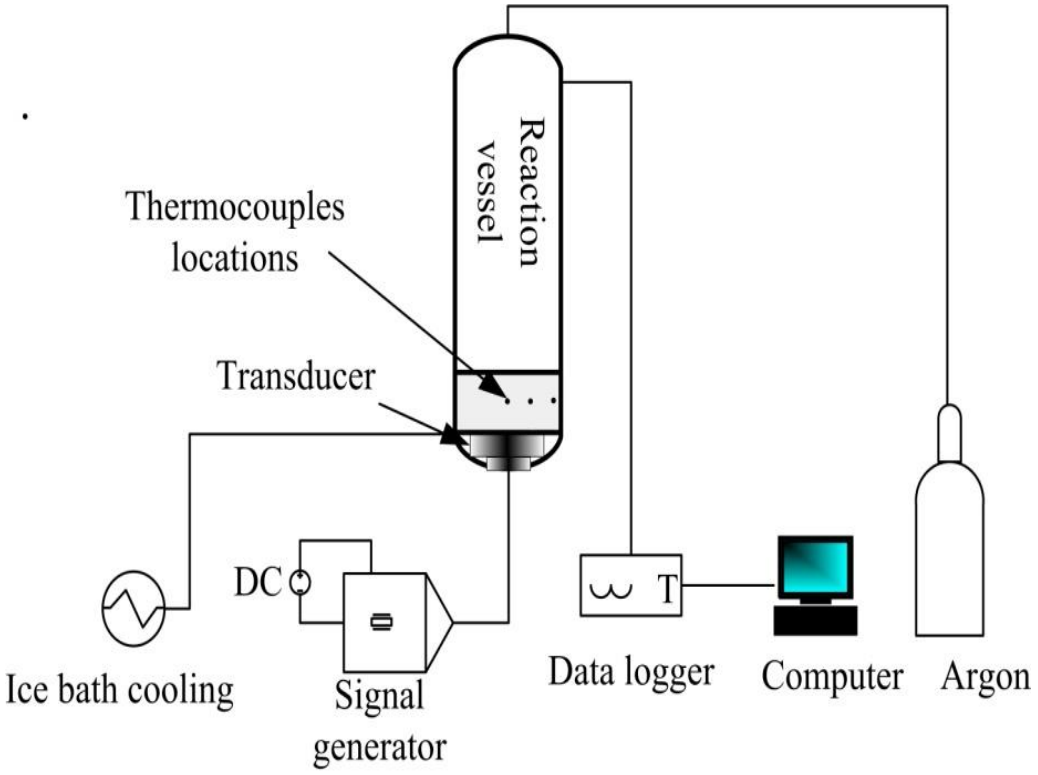

Figure 2-2. Schematic of 490 and 986 kHz ultrasound reactor and a provision for sample purging with argon gas.

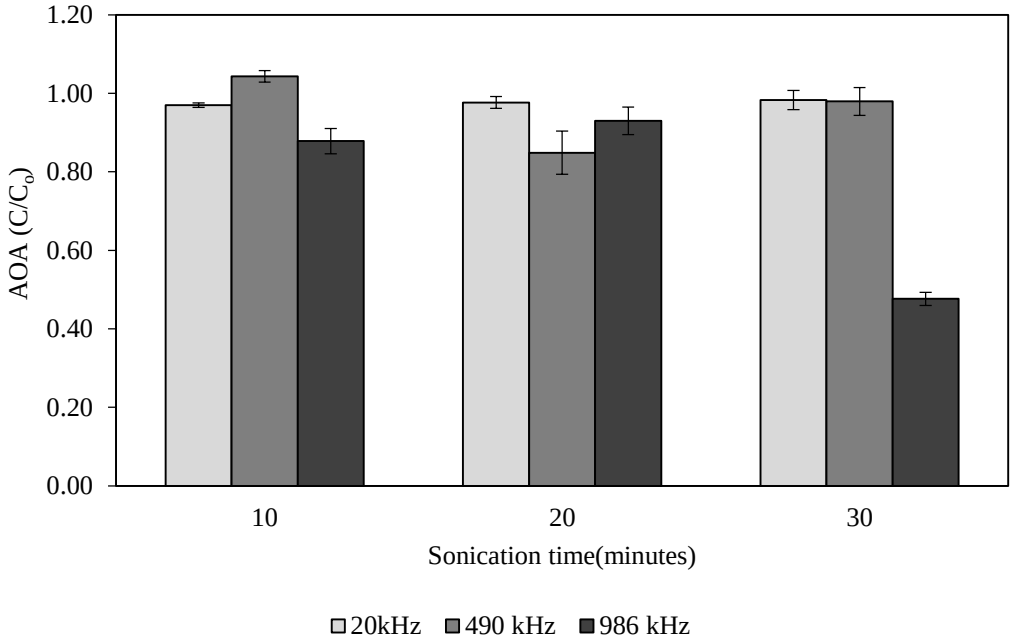

Figure 2-3. Change in antioxidant activity (AOA) of ascorbic acid solution measured with PCL assay after 30 minutes sonication at 986 kHz, 490 kHz, and 20 kHz. Each column represents mean of three individual experiments at $P<0.05$ and error bars are standard error). No significant difference between the time points at each treatments except for 30 minutes sonication at 986 kHz.

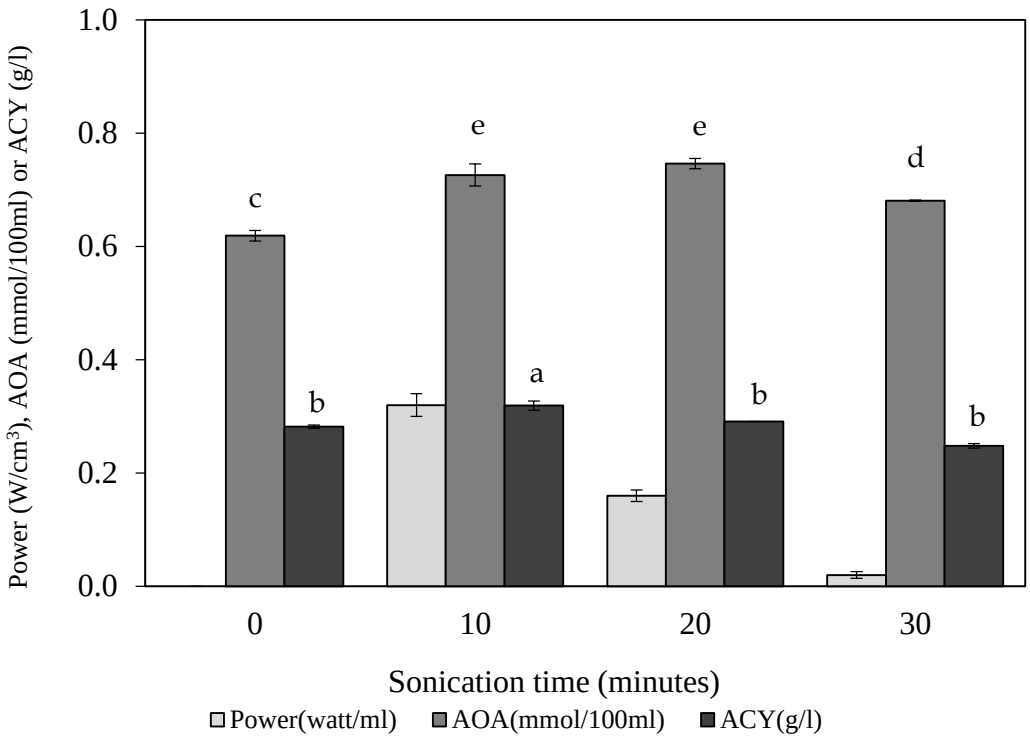

Figure 2-4. The relation between change in power (P, W/cm^3), antioxidant activity (AOA, mmol/100ml) and anthocyanins content (ACY, g/l) at 20 kHz. Significant increase ($p<0.05$) was observed between the AOA and ACY of samples at 10 minutes compared to the base. Significant decrease ($p<0.05$) in AOA and ACY was recorded after 30 minutes sonication. Each column is the mean of three individual experiments and error bars show the standard error.

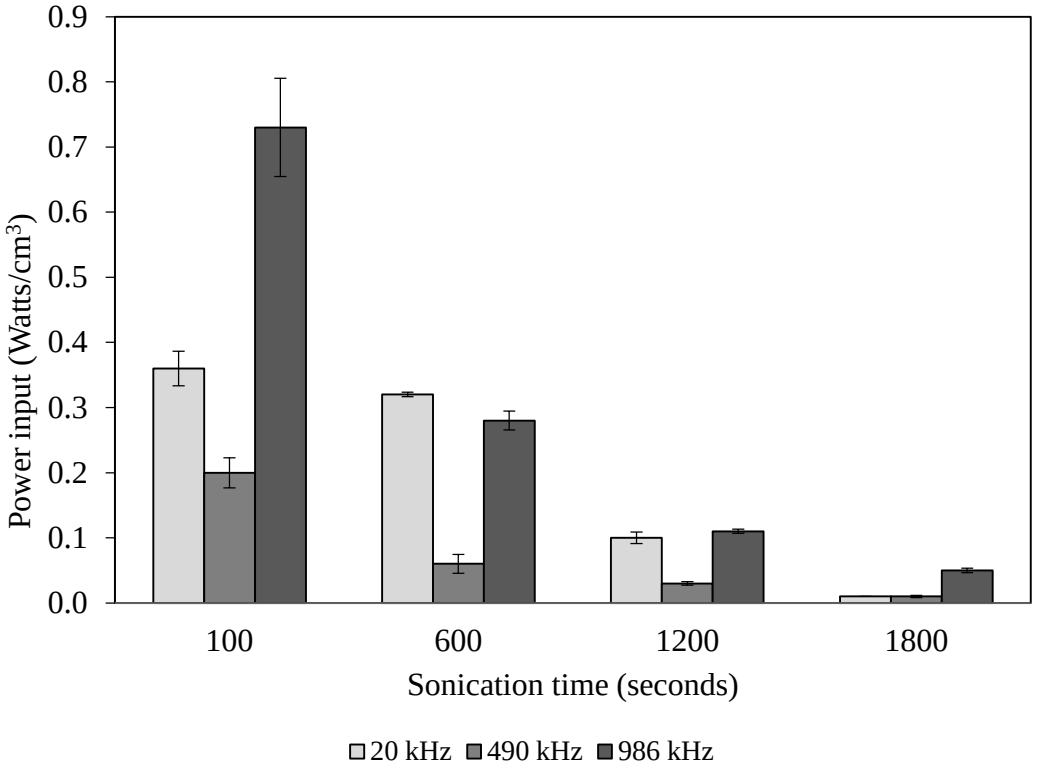

Figure 2-5. Change in the power (W/cm^3) during sonication of ascorbic acid solution at 20 kHz, 490 kHz and 986 kHz. Each column is the mean of three individual experiments and error bars show the standard error. Significant difference was observed between time points ($p<0.05$).

2.8. Tables

Table 2-1. Temperature (°C) of red raspberry puree and ascorbic acid solution during ultrasound treatment in a reactor with ice bath cooling

Samples*	20 kHz	490 kHz	986 kHz
A1	24.5±0.1	20.6±0.5	19.1±0.5
A2	33.8±0.5	21.0±0.7	48.0±0.1
J1	23.6±0.7	17.0±0.9	25.5±0.3
J2	44.6±0.6	26.2±0.6	56.5±0.2

* A1: Ascorbic acid solution before treatment; A2: Ascorbic acid solution after treatment (30 minutes);

J1: Red raspberry juice before treatment; and J2: Red raspberry juice after treatment (30 minutes). Each value is the mean of three measurements.

Table 2-2. Effect of ultrasound frequency and time on total antioxidant activity (AOA), total phenolic content (TPC) and monomeric anthocyanin content (ACY) of red raspberry puree [a].

f (kHz)	Total antioxidant activity (μmol/l)				Total phenolic content (mg/l)				Monomeric anthocyanin content (mg/l)			
	0	10	20	30	0	10	20	30	0	10	20	30
20	6189±94^{c}	7260±195^{a}	7464±89^{a}	6808±12^{b}	1454±29^{b}	1529±28^{ab}	1594±57^{a}	1628±30^{a}	282±3^{b}	317±8^{a}	291±3^{b}	248±8^{b}
490	3388±58^{b}	3656±84^{ab}	3884±64^{a}	3758±131^{a}	1161±35^{a}	1130±28^{a}	1151±24^{a}	1097±6^{a}	319±3^{b}	341±4^{a}	341±0^{a}	328±4^{b}
986	3412±61^{a}	3547±71^{a}	3522±102^{a}	3406±63^{a}	1107±47^{b}	1103±14^{b}	1086±17^{b}	1228±38^{a}	305±10^{a}	315±2^{ab}	293±10^{ab}	289±10^{b}

[a] Mean±standard error of three measurements. Means in each row with different letters are significantly different ($p<0.05$)

CHAPTER 3: UTILIZATION OF MUSTARD MEAL (*Sinapis alba*) EXTRACT TO ACTIVATE AND OPTIMIZE THE LACTOPEROXIDASE SYSTEM AND ITS POTENTIAL TO IMPROVE FOOD SAFETY

Amir Golmohamadi, Matthew Morra, Caleb Nindo

3.1. Abstract

Mustard meal (*Sinapis alba*), a by-product of biofuel industry, contains glucosinolates that can be hydrolyzed to thiocyanate (SCN^-) and generate hypothiocyanite ($OSCN^-$), an antimicrobial agent, in lactoperoxidase system (LPSys). The objective of this study was to extract SCN^- from mustard meal (MM-SCN) using and to compare $OSCN^-$ production from MM-SCN and KSCN. Aqueous solution (1:20) was extracted by 3-4 days shaking, 20 kHz ultrasound and in 70°C water bath. To develop LPSys, a fixed concentration of lactoperoxidase (3.92 IU/ml) with exogenous and endogenous H_2O_2 was used. The $OSCN^-$ production and change in its concentration at room temperature was traced over several hours. Ultrasound did not improve the generation of SCN^- and extraction for 10 minutes at 70˚C was similar to shaking for 3 days. When exogenous H_2O_2 was used, MM- SCN generated the same concentration of $OSCN^-$ as KSCN-LPSys. In MM-LPSys, a sustained production of $OSCN^-$ was achieved for 4-6 hours when H_2O_2 generated endogenously. The optimum levels of SCN^-, glucose and GOD were determined 500 µM, 32-64 mM and342 U/l respectively. The resulting LPSys is a GRAS material that has potential applications in the food industry to inhibit the growth of both gram negative and gram positive bacteria.

3.2. Introduction

The lactoperoxidase system (LPSys) is a naturally occurring antimicrobial multi-component system [43] inherently available in raw milk and human body fluids such as saliva. There are three primary components in the LPSys: lactoperoxidase enzyme (LP), hydrogen peroxide (H_2O_2), and thiocyanate (SCN^-). Hydrogen peroxide is produced by a number of microorganisms such as *Lactobacilli, Streptococci*, and *Lactococcus* through oxidation of ascorbic acid or oxidation of glucose by glucose oxidase [44]. Lactoperoxidase catalyzes oxidation of SCN^- by H_2O_2 to produce hypothiocyanite ($OSCN^-$) [94], which oxidizes essential sulfhydryl groups in proteins and impairs transport systems, membrane stability, and enzyme metabolism [43, 45]. The reactions inhibit growth of both Gram negative (e.g., *E. coli* O157:H7) and Gram positive bacteria (e.g., *L. monocytogenes*), thus making the LPSys potentially useful in improving food safety [43].

Although investigators have demonstrated the effect of the LPSys in milk [46, 49, 95], application in other food systems is limited. For example, Elliot *et al.* (2004) reported that application of the LPSys in beef cubes significantly reduced the number of microorganisms including *L. monocytogenes* after 7 days of incubation at 12°C [43]. Kennedy *et al.* (2000) showed that *E. coli* O157:H7, *L .monocytogenes*, and *S. aureus* were inhibited by the LPSys in ground beef [96]. In other food products, the LPSys inhibited S*almonella enteritidis* in tomato juice, carrot juice, milk, liquid whole egg, and chicken skin extract[97]. In almost all the LPSys studies an existing concentration of SCN^- was used or the ion was added from non-food-grade potassium or sodium thiocyanate (KSCN or NaSCN). Therefore, even though the LPSys might be Generally Recognized as Safe (GRAS), it may not be applied in vitro except in raw milk that inherently contains SCN^- to keep the LPSys active.

Provision of a natural source of food-grade SCN^- would facilitate integration of the LPSys into a broader range of food products. *Sinapis alba* (white or yellow mustard) contains a glucosinolate called sinalbin that constitutes over 90% of the total glucosinolate pool. Enzymatic hydrolysis of this glucosinolate by the enzyme myrosinase (EC 3.2.1.147) produces an unstable isothiocyanate that non-enzymatically undergoes conversion to SCN^- [37, 98], thus, supplying substrate for the LPSys that can be substituted for KSCN or NaSCN.

Glucosinolate concentrations are highest in the seed of *S. alba* and correspondingly in the seed meal after oil is removed. Although *S. alba* seed can be crushed to produce an oil feedstock for biodiesel with improved cold-flow properties compared to soybean biodiesel [99], relatively low economic returns limit *S. alba* oil availability. One way to increase economic viability of biodiesel production from *S. alba* seed is to develop value-added products from the seed meal remaining after oil removal. *Brassicaceae* seed meals are most often used for animal feed, however higher value products are required to increase economic returns and expand oilseed acreages [100].

In a recent study, defatted *S. alba* meal was used to develop an edible film and to test the film's effect on *Listeria monocytogenes* [42]. An antimicrobial effect beyond that of control films in which myrosinase was inactivated was observed both in assays involving disks of the film on *L. monocytogenes*-inoculated agar plates and when the films were coated on inoculated salmon. Thiocyanate production in the films was quantified, but other components of the LPSys were not included. To our knowledge, there are no reported studies that have applied SCN^- sourced from mustard meal in the LPSys.

Our objective was to determine whether *S. alba* seed meal extracts can be used as a source of SCN^- for the LPSys to produce $OSCN^-$. Associated with this objective is the need to 1) develop efficient extraction procedures for SCN^- using conventional or ultrasound-assisted processes and 2) determine if additional compounds in the seed meal extracts affect $OSCN^-$ production rates. By developing methods to utilize SCN^- produced in *S. alba* meal, multiple benefits result including the provision of a natural source of SCN^- for the LPSys, development of a value-added product from residual meals produced after oil is removed for biodiesel, and increased palatability of the extracted seed meal as an animal feed.

3.3. Materials and methods

3.3.1. Mustard meal and chemicals

All experiments were carried out using meal remaining after cold pressing *S. alba* "IdaGold" seed using a mechanical seed crusher at the University of Idaho (Moscow, ID) [30]. Mustard meal (*S. alba* with ~ 10% oil content) was used as the source of extract for mustard meal SCN^- (MM-SCN^-). Potassium thiocyanate (KSCN, 98.5%), 5, 5′dithiobis (2-nitrobenzoic acid, Nbs_2 or DNTB), diethylenetriaminepentaacetic acid (DETAPAC), sodium phosphate monobasic monohydrate, sodium phosphate dibasic hepta hydrate, and lactoperoxidase were supplied by Sigma (St. Louis, MO). Hydrogen peroxide, concentrated nitric acid, and ferric nitrate ($Fe(NO_3)_3.9H_2O$) were purchased from Macron Chemicals (Center Valley, PA).

3.3.2. Equipment setup

Ultrasound treatment was performed by using a 400W-capacity batch sonication system (Branson Sonifier, S-450A, Danbury, CT) operated at 20 kHz and 35% of full scale amplitude with a 7-cm vibrating titanium tip. A UV/Visible spectrophotometer with "UV Winlab"

software (Perkin Elmer PTP6., San Jose, CA) was used to measure SCN^- , $OSCN^-$, and LP activity.

3.3.3. Thiocyanate production

Two sets of experiments were designed to extract SCN^- from mustard meal. The first involved conventional solvent extraction, whereas ultrasound was used in the second method in an attempt to improve extraction efficiency.

3.3.3.1. Conventional extraction

Fifteen grams of mustard meal were ground for 30 sec using a Scienceware micromill (Bel-art products, Pequannock, NJ). Residual oil in a portion of the meal was extracted using the method of Borek and Morra (2005). Two grams of both the oil-containing meal (MM) and the defatted (DMM) meals were added to separate 40-ml aliquots of deionized water. Mustard meal solutions were placed on an orbital shaker (VWR-S-500, San Francisco, CA) and the speed was set at 100 rpm for 3 d. Samples were centrifuged at 2000 g for 20 min or until we obtained clear supernatants that were then decanted into glass vials for the measurement of SCN^- using methods described below.

3.3.3.2. Ultrasound-assisted SCN^- production

Two grams mustard meal (MM) were added to 40 ml of deionized water in a beaker. The solution was then sonicated for 5, 10, 15, and 30 min with cooling using an ice bath or for the same intervals with no cooling. The temperature change was recorded during the sonication period for each run [101]. After sonication, samples were placed on an orbital shaker for 4 d. Thiocyanate concentrations in the centrifuged solutions were quantified

immediately after sonication (see below) and at 1-d intervals. The concentration of SCN^- after 30 min of shaking at room temperature was reported for the control at day 0.

3.3.4. Production of SCN^- in different temperatures

Two grams of mustard meal were added to 40 ml of deionized water and incubated in a shaking water bath at 30, 60, 70, and 90°C for 30 min. Samples were removed at 10-min intervals, centrifuged, and SCN^- concentrations measured.

3.3.5. Measurement of lactoperoxidase enzyme activity

The method of Pruitt and Kamau (1994) as detailed by Campbell et al. (2012) was used[102, 103] to measure LP activity. Potassium phosphate buffer (0.1 M) adjusted to pH 6.0 served as a solution to dissolve LP and to make the ABTS solution (1 mM).

Three milligrams of lyophilized LP were dissolved in 10 ml of potassium phosphate buffer. To measure the activity, dilutions were made to keep the absorbance within the linear range of the spectrophotometer. Two milliliters of the ABTS solution were added to a disposable cuvette with a 1-cm path length and 0.1 ml of the LP solution was added. The reaction was started by adding 1 ml of 3.2 mM H_2O_2 to the solution. The reaction was monitored for 10 min, absorbance recorded at 412 nm, and results expressed as µmol of oxidized ABTS per minute (IU).

3.3.6. Lactoperoxidase system

A method by Adolphe and others [104] for optimization of the LPSys was followed using SCN^- supplied from mustard meal extracts or KSCN. This comparison was performed to determine if additional compounds in mustard meal extracts affect the behavior the SCN^- in the LPSys. The protocol involved adding 250 µM H_2O_2 to 3.92 IU/ml LP in a final volume of

50 ml of solution buffered at pH 6.0 (sodium phosphate buffer). The concentration of SCN^- was varied from 125 to 1000 μM and $OSCN^-$ and SCN^- concentrations measured at 0, 20, 40, 60, and 120 min. The concentration of SCN^- that generated the greatest amount of $OSCN^-$ was then used in assays with concentrations of H_2O_2 ranging from 500-1500 μM. To create sustained production of H_2O_2, exogenous H_2O_2 was generated in-situ using glucose oxidase and glucose. Concentrations of glucose were varied from 0 to 64 mM with a fixed glucose oxidase activity of 85.5 IU/l. The glucose concentration that maximized $OSCN^-$ production was determined and finally the level of glucose oxidase (GOD) was varied 1X, 2X, and 4X to further increase $OSCN^-$ production. The system capable of producing the highest concentration of $OSCN^-$ was regarded as optimum. All of the experiments were repeated with MM-SCN and KSCN for comparison purposes.

3.3.7. Measurement of thiocyanate (SCN-) concentration

Ion chromatography (IC) was used to verify accuracy of the more routinely used colorimetric method.

3.3.7.1. Ion chromatography

To quantify SCN^- concentration using IC, the samples were filtered using 0.45-μm filters (Millipore, MA, USA) followed by 0.1-μm syringe filters (Millipore, MA, USA), and diluted as required. Concentrations of SCN^- were quantified based on an external calibration curve. Ion chromatographic analysis was performed using a Dionex Ion Analyzer equipped with a GP40 gradient pump, ED40 electrochemical detector, and an AS40 auto sampler. Chromatographic separation was achieved on a Dionex Ion-Pac AS11 (250 mm×4mm) column using 100 mM NaOH as a mobile phase at a flow rate 0.5 ml/min. The overall run time was 10 min with SCN^- elution occurring at 5.3 min. The injection volume was 20 μl. The

detector stabilizer temperature was set at 30 °C with temperature compensation of 1.7%/°C. Anion suppressor current was set to 300 mA.

3.3.7.2. Spectrophotometric method

Routine analysis of SCN^- was performed according to the method of Pruitt and coworkers [105] using spectrophotometric analysis of an Fe-SCN complex at 460 nm. A stock solution of ferrous nitrite was prepared by dissolving 10 g of ferric nitrite ($Fe(NO_3)_3.9H_2O$) in 20 ml of concentrated nitric acid and diluted with water to a final volume of 200 ml. To measure SCN^- concentration, 0.5 ml of the LPSys was added to 1 ml ferric nitrite stock solution and the absorbance was recorded with ferrous nitrite as a reference. The sample absorbance was determined by comparison with an external standard curve obtained using KSCN concentrations ranging from 0.0625 to 1 mM.

3.3.8. Hypothiocyanite ($OSCN^-$) production

The production $OSCN^-$ was measured according to the methods of Thomas et al., [106] and Bosch et al., [107] as modified by Min et al. [108]. Twenty milligrams of Nbs_2 were dissolved in 50 ml of ice-cold phosphate buffer (pH 7.2) containing 0.1 mM DETAPAC. The mixture was reduced with 1 ml of a freshly prepared solution of 42 µl 2-mercaptoethanol in 20 ml water to form a 0.6 mM Nbs solution. An aliquot of the LPSys (0.5 ml) was added to the cuvette containing 0.5 ml of cold buffered sodium chloride (pH 7.2), followed by introduction of 125 µl of the 0.6 mM Nbs solution. The absorbance at 409 nm was recorded using deionized water as a blank. The $OSCN^-$ concentration was calculated using equation 1:

$$[OSCN^-] = \frac{(A_S - A_C) \times R_{f/s}}{0.01405 \times 2} \qquad (1)$$

Where:

A_s: absorbance of the sample at 409 nm (A_{409})

A_c: absorbance for control at 409 nm

$R_{f/s}$: the ratio of final and initial volumes (2 in this case)

0.01405: extinction coefficient of ABTS at 409 nm (μM/cm)

For the mustard meal-LPSys, the absorbance of the aqueous solution of LP and mustard meal extract in phosphate buffer (pH 6) before adding or generating the H_2O_2 was added to the value of A_c.

3.3.9. Statistical analysis

All experiments were repeated three times and data reported represent the mean of three measurements or chemical tests. The treatment effect on each of the tested parameters was determined by analysis of variance (ANOVA) using SAS version 9.0 (SAS, Cary, NC). A significance level (p-value) of ≤ 0.05 was used to assess whether the individual treatments represented statistically different results. The differences among group means were tested with the Least Square Mean test.

3.4. Results

3.4.1. Extraction of SCN^-

3.4.1.1. Thiocyanate production from regular and defatted mustard meal

Results from conventional extraction (using water and shaking for 3 d) of SCN^- from MM and DMM showed that removing residual oil from mustard meal had no effect ($p<0.05$) on the concentration of SCN^- extracted (DMM: 152±1.6 μM/g and MM: 155±4.2 μM/g).

Therefore, mustard meal with an oil content of approximately 10% by weight was used as the SCN^- source in subsequent experiments.

3.4.1.2. Ultrasound-assisted SCN^- production

Maximum SCN^- concentrations (190 μmol g^{-1} meal) were achieved by 30 min of sonication, with no additional SCN^- being produced during 4 additional days of incubation (Figure **3-1**). In contrast, both no sonication and 5-min sonication treatments showed increased SCN^- production with incubation time, reaching a maximum for 5 min of sonication in 1 d and no sonication in 2 d. The SCN^- maxima for these two treatments were similar at approximately 150 μmol g^{-1} meal.

The potential for sonication to improve SCN^- yield was further tested at additional time intervals, with and without cooling. Increases in sonication time from 5 to 30 min increased SCN^- production in the non-cooled treatments (Figure **3-2**). Cooled treatments displayed lower SCN^- yields as compared to the respective non-cooled treatments. The temperature profile of sonication reached a maximum of 45°C in the cooled system and 96°C in the non-cooled system (Figure 3-3). These results confirmed that heat generated during sonication increased SCN^- yields.

This increase in temperature generated by sonication could potentially increase SCN^- yield either by improving extraction efficiency or accelerating myrosinase activity, or through a combination of both mechanisms. Yields of SCN^- determined at increasing temperatures from 30 to 90°C showed that myrosinase was active up to 70°C and that maximum SCN^- concentration reached approximately 145 μmol g^{-1} meal (Table **3-1**).

The optimal temperature of *S. alba* myrosinase activity in the presence of 3.38 mM ascorbic acid and 0.15 mM magnesium chloride in Bis-Tris buffer solution (pH 6.5) was measured by Van Eylen et al. [34]. It was shown that myrosinase activity increased up to 60˚C and the enzyme experienced deactivation after this temperature. Stability of myrosinase up to 70 ˚C in systems containing *S. alba* meal possibly results from increased complexity afforded by the inclusion of mustard meal as compared to the buffered solution used by Van Eylen et al. [34].

These data indicate that a large fraction of increased SCN^- yield observed with sonication likely results from the effect of increased temperature on enzymatically catalyzed glucosinolate hydrolysis. However, the fact that SCN^- yields with sonication were 190 μmol g^{-1} meal (Figure **3-1**) as compared to 145 μmol g^{-1} meal without sonication (Table **3-1**) suggests that mixing as occurs with sonication further increases SCN^- yields either by enhancing enzymatic activity or improving SCN^- extraction efficiency from the meal-water slurry. Ultimately, incubation with shaking at 70°C was chosen as the method for production of SCN^-. Although SCN^- yields were greater with sonication, water bath incubation was a more convenient method to achieve the desired concentration (16-18 mM SCN^- to create a concentrated stock solution of MM-SCN).

3.4.2. Hypothiocyanite production and optimization of the mustard meal lactoperoxidase system

Thiocyanate generated by shaking water-meal mixtures at 70°C for 10 min was used as one of the components to create an LPSys in which H_2O_2 (250 μM final concentration) was added exogenously. At the range of SCN^- concentrations tested in this experiment, the highest $OSCN^-$ concentration (82.7 μM) was achieved with 500 μM SCN^- ($p<0.05$) and no readily explainable trend was observed (Figure **3-4**). For comparison purposes, KSCN was

used in place of the seed meal extract in the LPSys to yield maximum $OSCN^-$ concentrations of 71.5 μM for all treatments except in the case of 125 μM SCN^- (Figure **3-5**). In addition to a lower maximum as compared to MM-derived SCN^- treatments, the data for KSCN exhibited a more consistent trend with all KSCN concentration ≥ 500 μM showing similar $OSCN^-$ concentrations at each time point (Figure **3-5**).

Differences in $OSCN^-$ production between the two SCN^- sources occurs as result of the inclusion of a variety of compounds in the mustard meal extract in addition to SCN^-. These constituents may have effects on both the production and stability of $OSCN^-$ by 1) protecting LP against inactivation, 2) providing additional substrate for OSCN- production, and 3) supplying antioxidants that aid in the preservation of $OSCN^-$. Our data indicate that mustard meal extracts containing 500 μM SCN^- allow for maximum $OSCN^-$ and that other constituents in the extract have no or a slight positive effect on such production.

In an attempt to generate additional $OSCN^-$, higher concentrations of H_2O_2 were tested at fixed concentrations of LP and SCN^- (500 μM). When additional H_2O_2 was provided, $OSCN^-$ production decreased at the initial 10-min measurement in both the MM (Figure **3-6**) and KSCN systems (Figure **3-7**).

Our data are in agreement with previously reported results showing that in an LPSys with 50-100 μg of LP and 2-5 mM of SCN^-, maximum concentration of $OSCN^-$ was achieved when equimolar concentrations of H_2O_2 and SCN^- were used [109]. This is most likely caused by the enhanced decay rate of $OSCN^-$ as caused by elevated H_2O_2 concentrations [56].

An LPSys can thus be generated with MM extracts containing SCN^- , but rapidly generated $OSCN^-$ decays quickly when H_2O_2 is supplied exogenously. Our results are in agreement with Pruitt et al. (1986) who used an LPSys containing KSCN (1 mM) and H_2O_2

(1mM) at 37˚C to study the rate of SCN^- and H_2O_2 consumption and $OSCN^-$ production. They recorded a rapid reduction in SCN-concentration and production of HOSCN/$OSCN^-$ within the first minute of reaction initiation. After the first minute, there was little change in the concentration of HOSCN/ $OSCN^-$ throughout the time period of the experiment (2 min) [110]. This suggests that the LPSys reaches equilibrium within the first few minutes and that $OSCN^-$ decomposition occurs thereafter. The decomposition of HOSCN/$OSCN^-$ may result from its interaction with SCN^- [111] or through self-decomposition in which one molecule of $OSCN^-$ destroys three molecules of $OSCN^-$, ultimately producing CO_2, NH_4^+, and SO_4^{2-} [57].

In an attempt to promote longer-term production of $OSCN^-$ beyond the initial flush provided through the use of H_2O_2, glucose and glucose oxidase where substituted into the LPSys. Initially, we attempted to determine if glucose concentrations in the MM extract were sufficient to sustain OSCN- generation given that glucose is produced in an equimolar amount to SCN^- during the glucosinolate hydrolysis reaction.

Systems supplemented with 0 to 4 mM glucose showed more sustained production of $OSCN^-$ during the 8-h test period, with the 4 mM glucose treatment yielding the highest average concentrations (Figure **3-8**). The 0 glucose treatment yielded the lowest average $OSCN^-$ concentration indicating that supplemental glucose is required to maximize $OSCN^-$ production. Similar systems in which KSCN was used instead of MM-derived SCN^- showed maximum OSCN concentrations in the 4 mM glucose treatment and maximums nearly double those produced with MM extracts (Figure 3-9).

The data indicated that the reaction remained glucose limited, so even higher concentrations of glucose (8 to 64 mM) were tested to determine if additional $OSCN^-$ could

be produced in the LPSys with MM extracts. Although only a small increase in the maximum concentration of $OSCN^-$ was observed, sustained $OSCN^-$ production was achieved in the 32 and 64-mM treatments for approximately 9 h (Figure **3-10**). Unlike the MM-LPSys, the KSCN system showed sustained $OSCN^-$ production for 8 h with only 8 mM glucose (Figure **3-11**). The KSCN-LPSys was a pure system with production and degradation of $OSCN^-$ being governed only by LPSys components. In contrast, higher glucose concentrations are required with MM extracts, because the extract contains a variety of compounds such as sinapine [112] that potentially interfere with reaction kinetics.

Even with increased glucose, $OSCN^-$ concentrations of approximately 15 μM (Figure 3-10) were still far below the concentrations (82.7 μM) that were obtained using exogenous H_2O_2 and MM extracts (Figure **3-4**). In order to increase H_2O_2 production, the highest concentration of glucose (64 mM) was chosen and concentrations of GOD were increased by 2 and 4 times. Doubling the concentration of GOD doubled the concentration of $OSCN^-$ in the MM-LPSys, generating a maximum of approximately 20 μM $OSCN^-$ (Figure **3-12**). With 4 times the GOD concentration, $OSCN^-$ was proportionally increased, reaching a maximum of approximately 40 μM. Similar to previous comparisons between MM extracts and KSCN, higher $OSCN^-$ concentration of 60 μM were obtained with KSCN extracts as a result of fewer interfering constituents. However, in all LPSys it was observed that higher maximum $OSCN^-$ concentrations resulted in a greater rate of OSN^- disappearance with time (Figure **3-12**).

Thus, at a fixed level of LP (4.92 IU/ml) the optimized concentrations of components in the mustard meal derived LPSys were 32-64 mM glucose, 342 IU/l GOD, and 500 μM SCN^-. Even though the LPSys has been extensively studied, there are few reports in which the

system has been optimized. In one of the latest studies, the components of the LPSys were optimized using response surface methodology and optimal concentration for a fixed concentration of SCN^- (500 µM) at 25°C was suggested to be 85.5 IU/l for GOD, 8 mM for glucose, and 3927 IU/l LP [104]. However, optimization was not based on the product of the system ($OSCN^-$), but on residual SCN^- concentration. Similar to Adolphe et al. (2006), $OSCN^-$ was not measured in most other chemical and microbial studies[104, 113-115] and the activity of GOD was not reported. Perhaps the most significant difference and a primary accomplishment of our research is the establishment of MM SCN^- as a substitute for non-food-grade KSCN.

3.5. Conclusion

White mustard meal (*Sinapis alba*) SCN^- was successfully extracted from by-products of biofuel processing using conventional and ultrasound-assisted methods. The initial results for sonication without cooling showed that ultrasound increased the extraction of SCN^- from mustard meal. However, the effect of ultrasound was minimal when system was cooled. The combined effect of heating and sonication became more visible after 15 min.

Ultrasound improved the production of SCN^- and did not inactivate myrosinase. For the first time, in this study an LPSys was created and optimized with potentially GRAS components. The optimization was based on the antimicrobial product $OSCN^-$ as generated using SCN^- derived from MM. It has been shown that 5µM $OSCN^-$ is sufficient to kill *E.coli* and reduce the bacterial population by 10 fold within 2 hours of incubation [116]. Likewise, *Salmonella enteritidis* in carrot juice (pH 6.5) was reduced by 3.7 log units after 4 h incubation with 125 µM $OSCN^-$ [97]. Previous studies have uniformly reported a bacteriostatic effect of $OSCN^-$ on *Listeria monocytogenes* [104, 114, 117]. The optimized LPSys in this study may have

bacteriostatic effect on *Listeria monocytogenes* in liquid foods or on *E.coli* in several food matrices such as fresh produce. Potential future studies to improve this system are:

1- Study the effect of additional GOD or LP after 4-5 hours to the system and determine if the $OSCN^-$ levels can be returned to the maximum or close to the maximum level.
2- Study the effect of *Sinapis alba* antioxidants on the preservation of the $OSCN^-$ or the LPSys in general.
3- Study the more developed system on microorganisms to see if antimicrobial effects are observed.

3.6. Tables

Table 3-1. Change in the production of SCN^- (µmol/g meal) with time and temperature for 2g mustard meal/40ml in water.

Temp (◦C)	Time (min)		
	10	20	30
30	17±1.4	31±1.9	40.4±2.4
60	106.8±5.9	130.6±2.1	145.8±4.6
70	132.8±1.9	140.3±2.1	144.8±2.1
90	16.8±2.6	32.7±3.1	47.2±2.6

Values are mean of triplicates ± standard error.

3.7. Figures

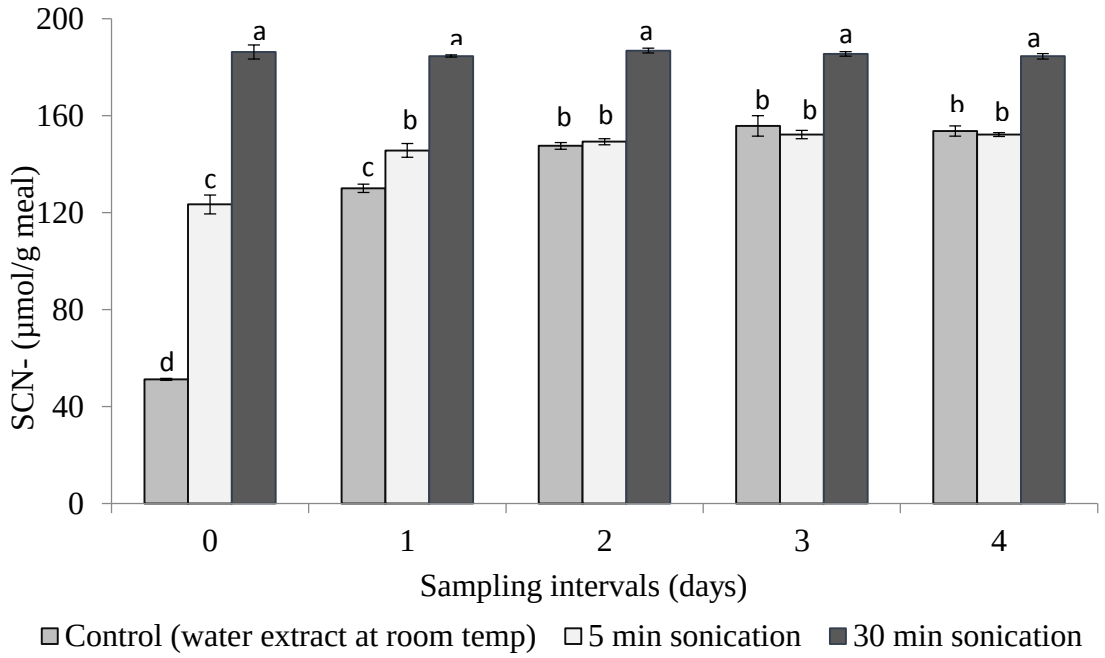

Figure 3-1. Comparison of conventional and ultrasound-assisted extraction of SCN^- from *S. alba* seed meal. A single sonication treatment for 0, 5, or 30 min was followed by incubation of the samples for 4 d. Error bars represent the standard errors of three replicates. Means which share a letter are not significantly ($p<0.05$) different.

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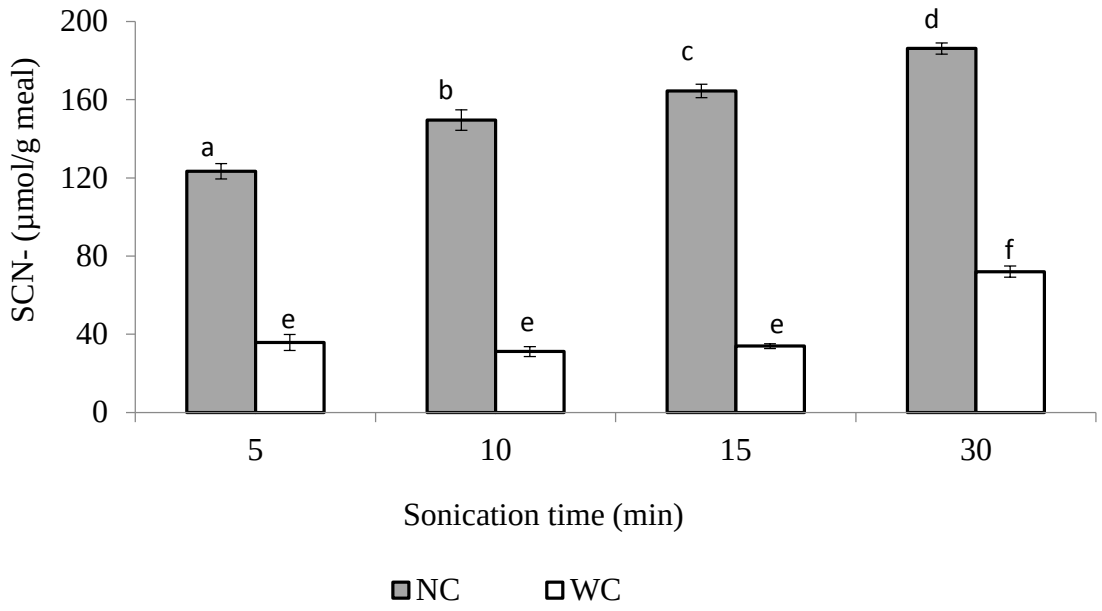

Figure 3-2. Change in the ultrasound assisted production of SCN^- with sonication time, no-cooling (NC), and with-cooling (WC) Error bars represent standard error of three replicates. Means which share a letter are not significantly ($p<0.05$) different.

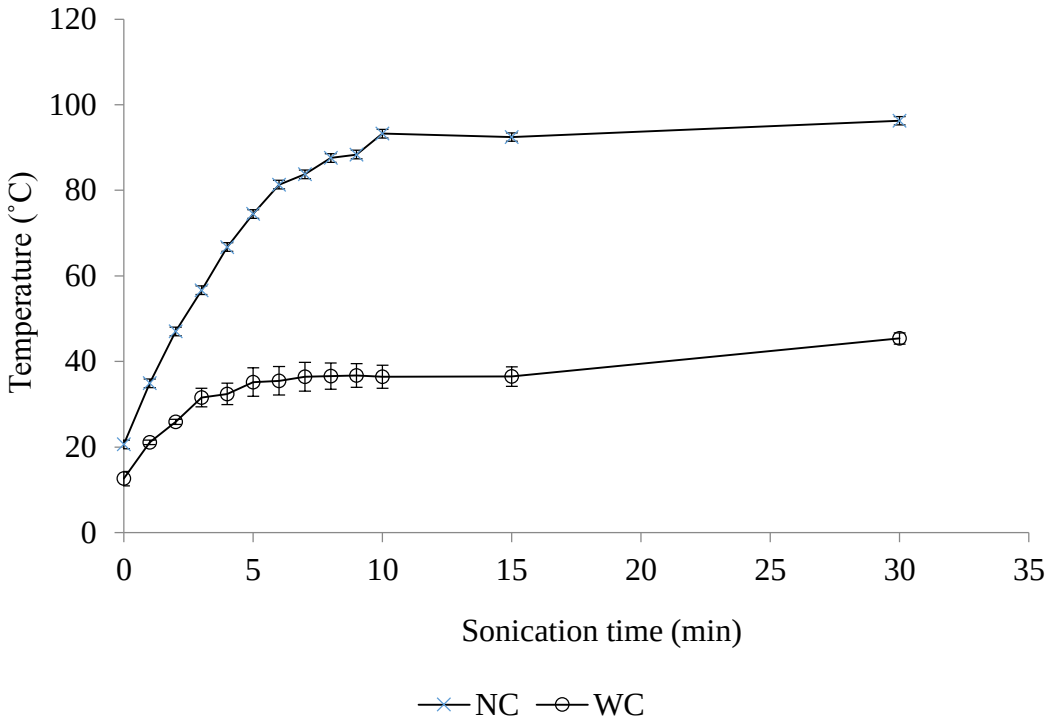

Figure 3-3. Temperature profile during the no-cooling sonication (NC) and with cooling (WC) sonications of ultrasound assisted extraction of SCN^-.

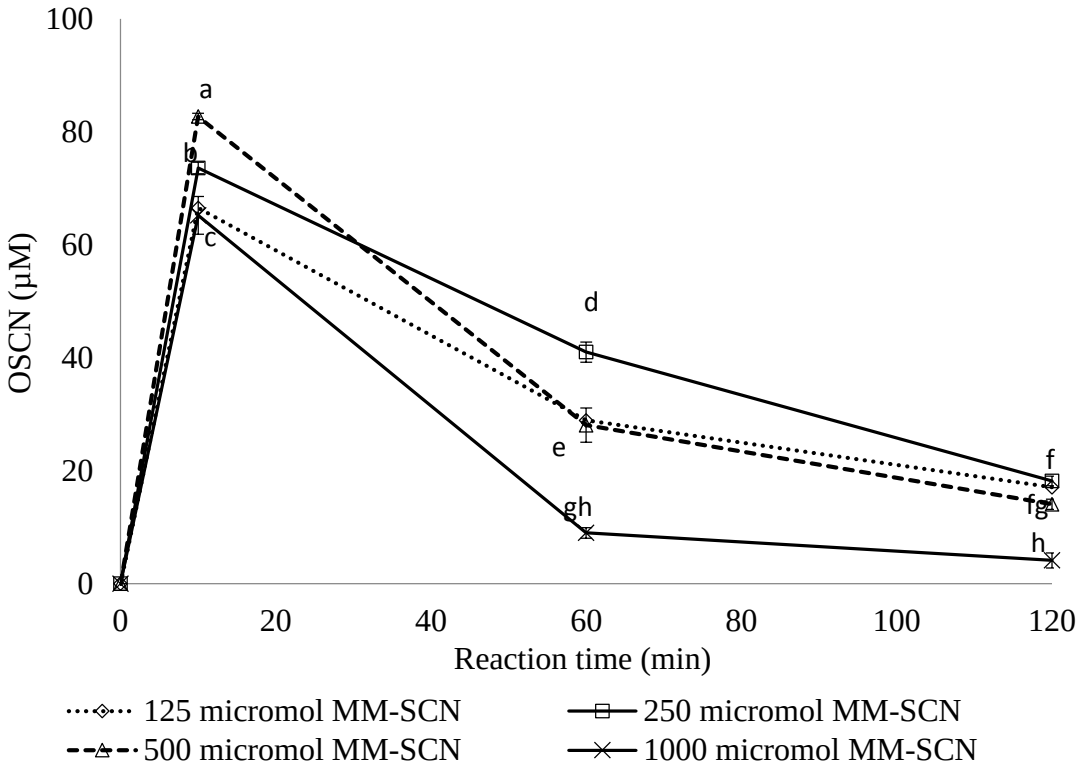

Figure 3-4. Change in mustard meal LPSys hypothiocyanite concentration during a 2 h incubation at room temperature with 250 µM exogenous H_2O_2. Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different.

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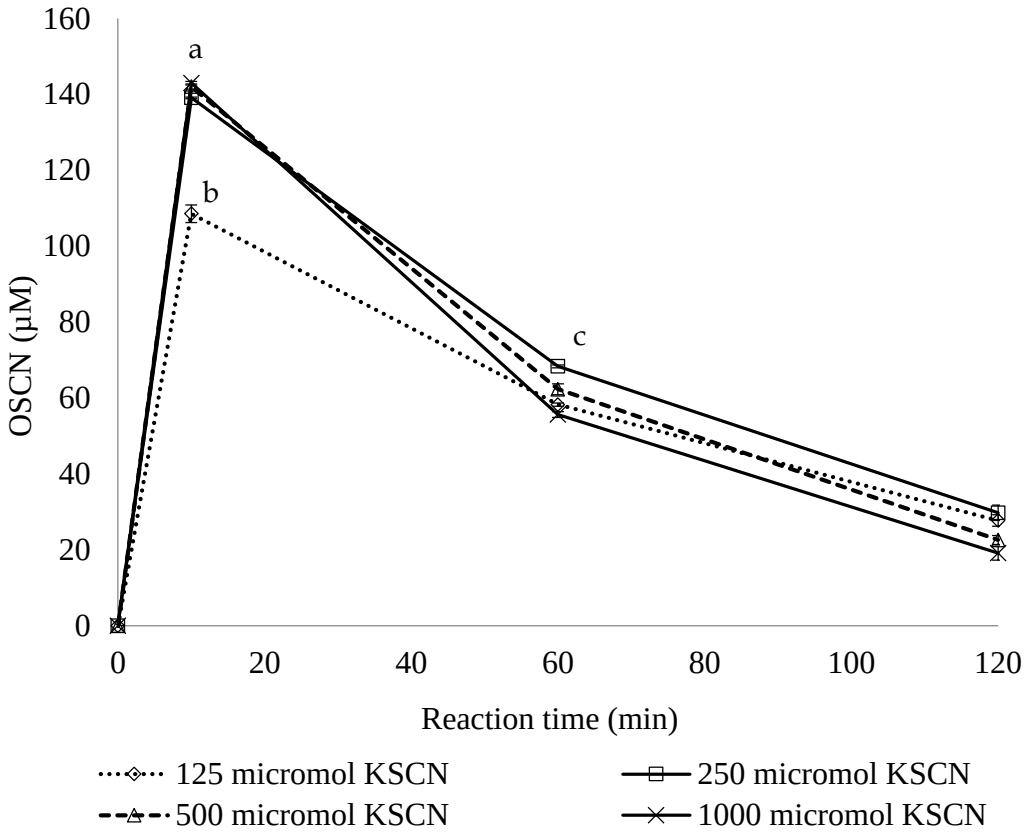

Figure 3-5. Change in KSCN- LPSys hypothiocyanite concentration during a 2 h incubation at room temperature with 250 µM exogenous H2O2. Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different.

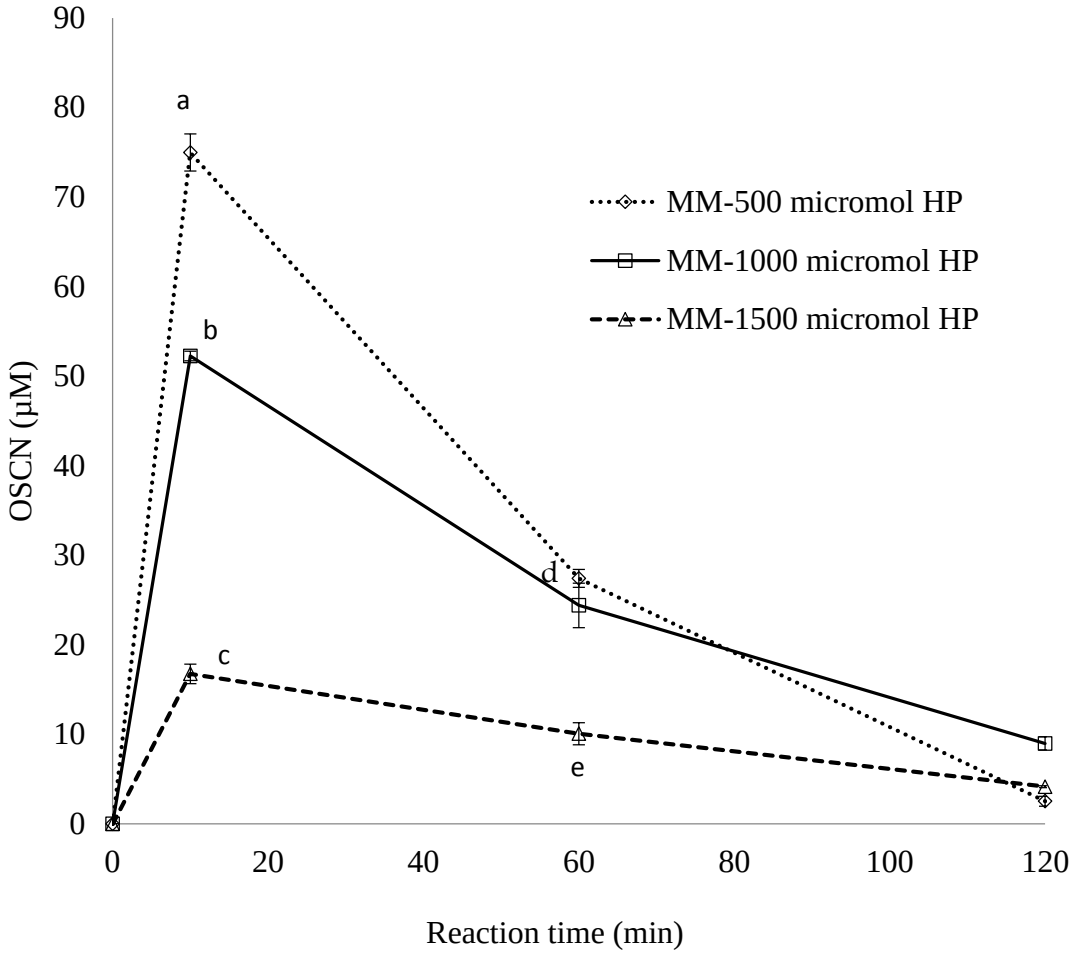

Figure 3-6. Change in mustard meal LPSys hypothiocyanite concentration during a 2-h incubation at room temperature with 500 µM SCN^- and variable concentrations of exogenous H_2O_2. Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different.

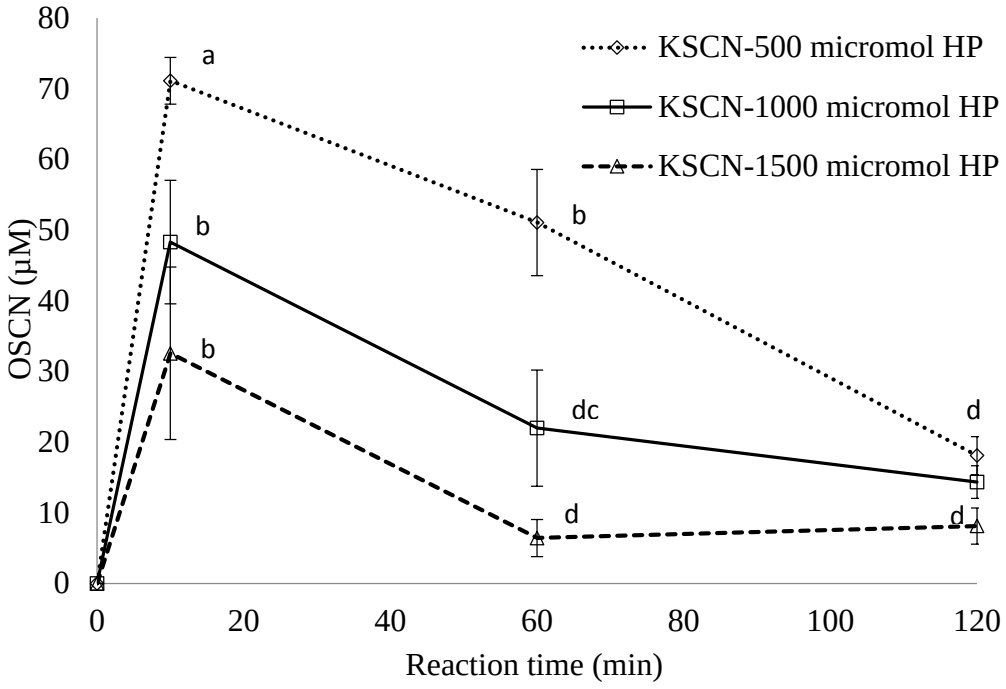

Figure 3-7. Change in KSCN- LPSys hypothiocyanite concentration during a 2-h incubation at room temperature with 500 µM SCN^- and variable concentrations of exogenous H_2O_2. Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different.

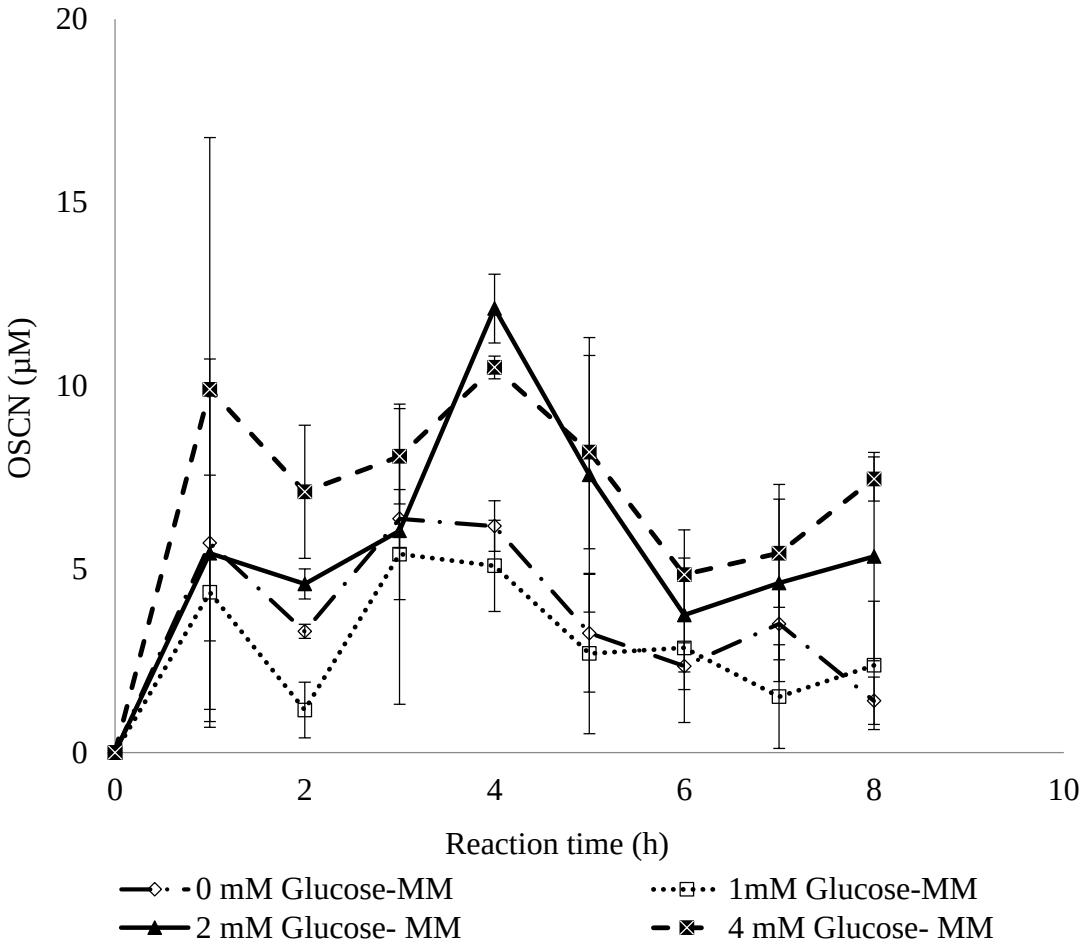

Figure 3-8. Change in mustard meal LPSys hypothiocyanite concentration during an 8-h incubation at room temperature with 500 µM SCN^-, 85.5 U/l GOD, and variable glucose concentrations (0-4 mM). Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different.

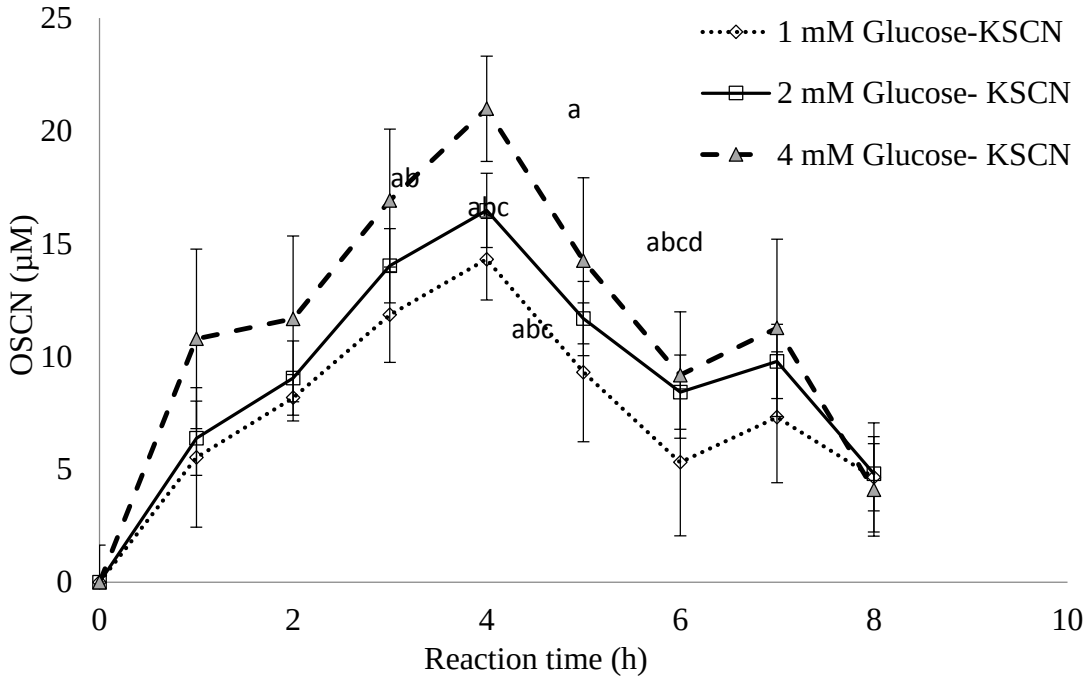

Figure 3-9. Change in KSCN-LPSys hypothiocyanite concentration during an 8-h incubation at room temperature with 500 µM SCN-, 85.5 U/l GOD and variable glucose concentrations (1-4 mM). Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different. No statistics symbol means a significant difference.

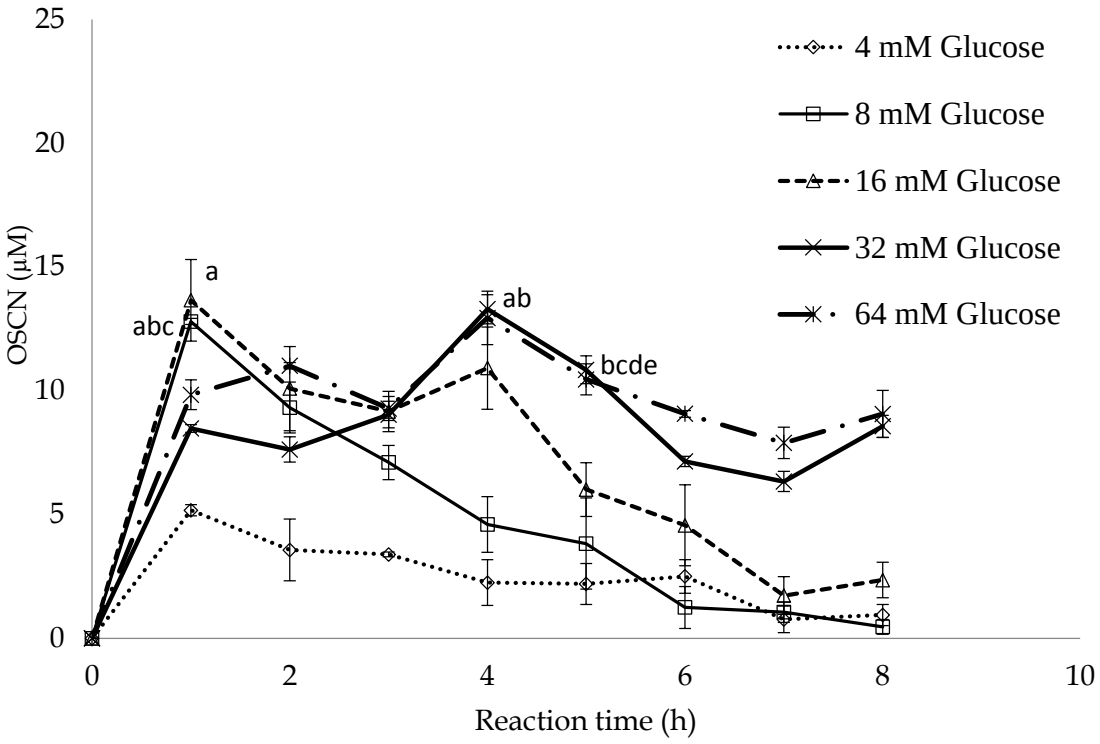

Figure 3-10. Change in mustard meal LPSys hypothiocyanite concentration during an 8-h incubation at room temperature with 500 µM SCN-, 85.5 U/l GOD and variable glucose concentrations (4-64 mM). Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly different ($p<0.05$). No statistics symbol means a significant difference.

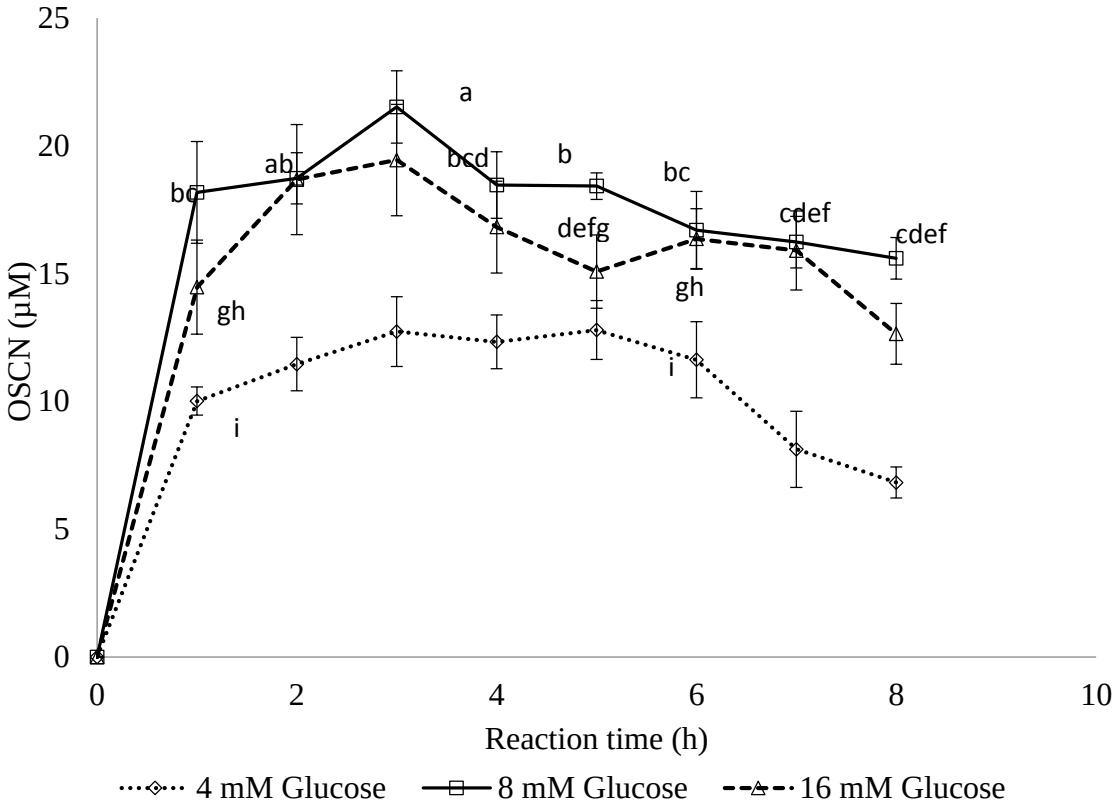

Figure 3-11. Change in KSCN-LPSys hypothiocyanite concentration during an 8-h incubation at room temperature with 500 µM SCN-, 85.5 U/l GOD and variable glucose concentrations (4-16 mM). Error bars represent standard error of the mean for three replicates. Means which share a letter are not significantly ($p<0.05$) different. No statistics symbol means a significant difference.

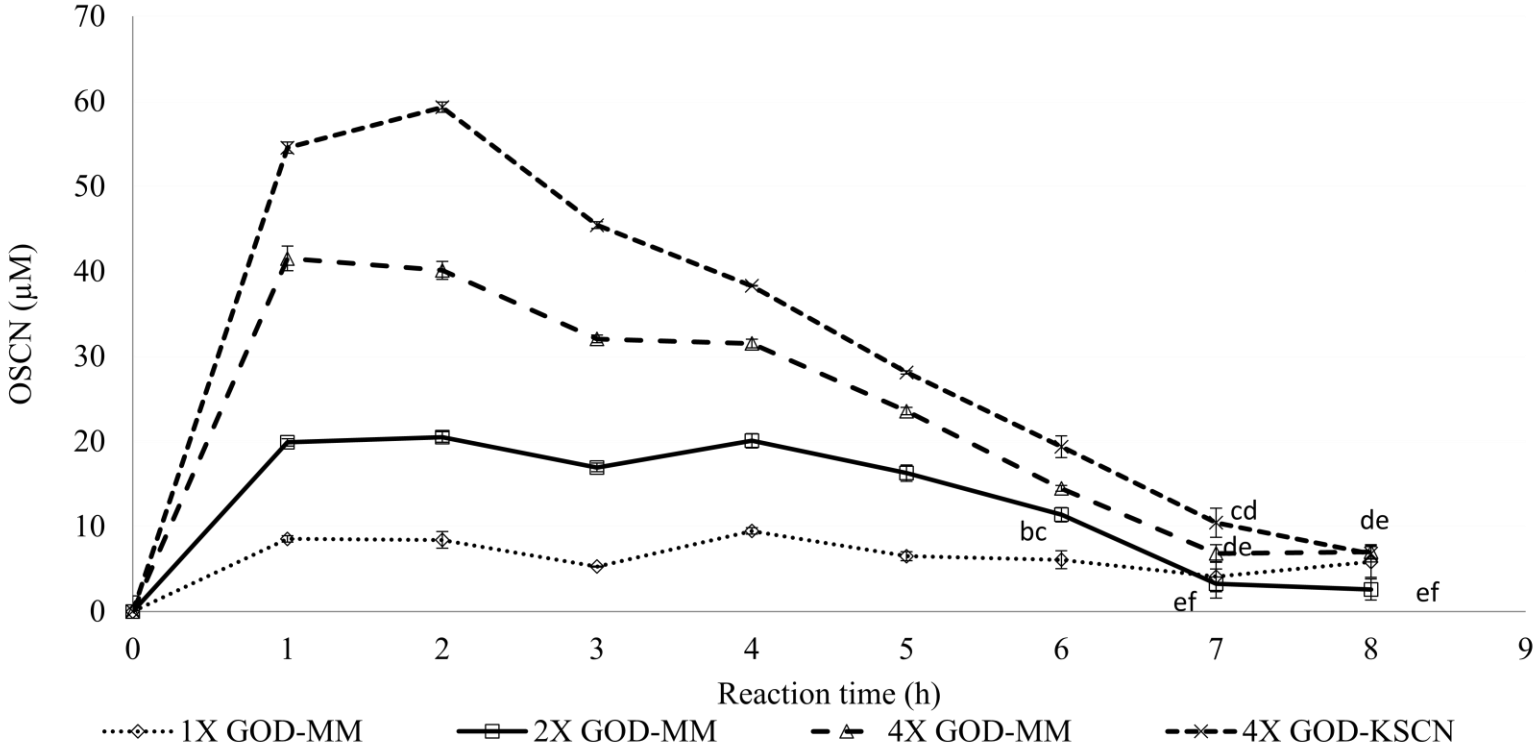

Figure 3-12. Change in the OSCN- concentration of MM-LPSys with 500 μM SCN, 64 mM Glucose, 3.92 U/ml LP and variable concentrations of glucose oxidase (GOD) at room temperature. Means which share a letter are not significantly ($p<0.05$) different. No statistics symbol means a significant difference.

CHAPTER 4: OVERALL CONCLUSION AND FUTURE REMARKS

This study was designed to explore the application ultrasound as an emerging and novel technology for the extraction of bioactive compounds from red raspberry and mustard meal (*Sinapis alba*). Effects of three different ultrasound frequencies (20, 490, and 980 kHz) on the extraction of anthocyanins, antioxidants, and total phenolic content of red raspberry puree were examined. In addition, we determined whether ultrasound can be applied to improve the generation of thiocyanate (SCN^-) from mustard meal and if the extract can be used in the lactoperoxidase system to generate hypothiocyanite ($OSCN^-$), an antimicrobial agent. The general pool of literature on ultrasound has been focused on one ultrasound frequency, presumably due to the availability of commercial and bench top ultrasound generator units. In addition, to our knowledge all of the studies on the lactoperoxidase system have used non-organic source of SCN^- (KSCN or NaSCN). Thus, this study was conducted to answer the following questions:

1- Do higher ultrasound frequencies act similar or better than the 20 kHz power ultrasound?
2- Can power ultrasound (20 kHz) be used to improve myrosinase-catalyzed hydrolysis of the glucosinolates to SCN^- in mustard meal (MM-SCN)?
3- Will it be possible to apply MM-SCN in the lactoperoxidase system (LPSys) instead of using KSCN and how it will behave differently from KSCN? How might the LPSys components can be optimized?

To answer these questions we hypothesized that:

a- Higher power ultrasound frequencies will improve the extraction rate. This hypothesis was tested on red raspberry puree.

b- The ultrasound can increase the rate of glucosinolate hydrolysis catalyzed by myrosinase. This was tested on an aqueous solution of mustard meal (20:1) after the best ultrasound frequency (under our test condition) was determind.

c- Thiocyanate from mustard meal can activate the lactoperoxidase system.

The results of this research revealed that among the three ultrasound frequencies (20, 490, and 980 kHz), the lowest frequency was the most appropriate for extraction of anthocyanins and antioxidants from red raspberry puree. The synergistic effect of heat and ultrasound improved the production of SCN^- after 30 minutes of sonication at 20 kHz. Heat generated during the sonication had a substantial contribution to SCN^- generation from mustard meal. When the effect of heat was determined, it was concluded that 10 minutes shaking of mustard meal in aqueous solution at 70°C generated sufficient SCN^- ; therefore, this method was chosen to extract SCN^-.

The extract of SCN^- was tested to determine if it could activate the lactoperoxidase system (SCN^-, H_2O_2, and lactoperoxidase enzyme). The results of this experiment showed that mustard meal SCN^- can be used as a source to create a lactoperoxidase system with potential as GRAS antimicrobial. In subsequent research, a sustained production of H_2O_2 and subsequently $OSCN^-$ was achieved using glucose and glucose oxidase. The optimized lactoperoxidase system that produced 41.5 μM of $OSCN^-$at a fixed lactoperoxidase activity (3.92 IU/ml), contained 500 μM SCN^-, 32-64 mM glucose, and 342 IU/l glucose oxidase. This suggests that mustard meal, a by-product of the biofuel industry, can gain value by creating a potentially GRAS antimicrobial system.

The results of this study can be used for future research and developments of ultrasound-assisted extraction of bioactive compounds from red raspberry and further optimization of the lactoperoxidase system. The followings are potential future objectives to improve the results of this study:

Comparing the effect of different amplitude levels of 20 kHz ultrasound on the extraction yield of bioactive compounds in red raspberry puree within 10 minutes. Subsequently investigate the effect of the best ultrasound conditions on degradation kinetics of elagitanin, a strong anticarcinogenic compound in red raspberry puree.

Investigating the highest ultrasound amplitude level at 20 kHz on the extraction of SCN^- from mustard meal in different meal to solvent ratios. Compared to the results of this study, a better synergistic effect of ultrasound and heat might be seen at specific meal to solvent ratios and shorter times.

Studying and comparing the effect of two developed lactoperoxidase systems in this study on number of pathogenic microorganism in fresh produce, food industry, and carcass surfaces. This study will give a better understanding of the effect of this potential antimicrobial on system economics. The relative costly components of this system are the enzymes (LP and GOD). If any of the enzymes can be removed or replaced by a more economically feasible but effective source, the system will be competitive to the other conventional antiseptic methods such as chlorine.

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