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Phenolic compounds and antioxidant activity of oat bran by various extraction methods

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PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF OAT BRAN BY VARIOUS EXTRACTION METHODS

A Thesis

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DEDICATION

First, I want to thank God for what he has given me. Then, thanks to my mom, Claire Holliday, I will never understand how much she sacrificed for me. I want to thank my brother and sister for pushing me to follow my dreams. Also, I want to recognize my teachers and friends throughout my life who have guided me through my different stages. Specifically, I want to thank Bill Koren, Michael Nolen, Douglas Harrison, George Charlet, George Kaslow, Gavin Estes and the faculty of the Food Science Department of Louisiana State University and the Chef John Folse Culinary Institute at Nicholls State University.

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ABSTRACT

Recent studies have suggested that the health promoting capabilities of oats are due to its antioxidants (tocopherols, tocotrienols, and sterols) found within the bran along with phenolic compounds, such as avenanthramides, *p*-hydroxybenoic acid and vanillic acid. Long-chain fatty acid oxidation is directly responsible for most off-flavors in food. Since oat bran is a good source of antioxidants, a concentrated extract could be used as a natural preservative, for foods rich in unsaturated long-chain fatty acids.

Three methods, traditional solvent (TSE), microwave-assisted solvent (MAS), and supercritical fluid treatment (SFT), were used to obtain the extracts. One extraction temperature in TSE, 60ºC, and two extraction temperatures in MAS, 60ºC and 100ºC, were tested. The DPPH (2, 2'-diphenyl-1-picrylhydrazyl) method demonstrated that the MAS-100ºC was the most efficient extraction in the group, thereby serving as MAS sample against the TSE and supercritical-treated samples.

For the treated samples, oat bran was exposed to supercritical CO_2 before extraction. Three different temperatures of CO_2 were tested, 25ºC, 50ºC, and 75ºC. The treated samples then underwent MAS-100ºC to gather extracts for analysis. The experimental results for the DPPH test favored the SFT-75ºC treatment at a 40µl concentration. Therefore, SFT-75ºC served as the treated sample in the final three experiments.

Antioxidant activity was further tested using two other methods: cholesterol oxidation and the DHA model. The total phenolic content was determined using Folin-Ciocalteau Method. The SFT-75ºC treatment showed statistically higher results for antioxidant activity in both the cholesterol oxidation and DHA oxidation experiments over the TSE-60ºC or MAS-100ºC. In terms of total phenolics, the SFT-75ºC treatment showed statistically higher results than TSE or MAS-100ºC in terms of catechin equivalency, but no statistical difference was seen among the

treatments when compared on the basis of total phenolics per gram of original oat bran sample. However, extraction techniques can be evaluated based on extract yield, which this research demonstrated would be SFE-75ºC.

In conclusion, the SFT-75ºC treatment was the optimal extraction based on antioxidant activity, catechin equivalency for total phenolics, and sample yield. This information could be used in the future development of food products as a natural antioxidant source.

CHAPTER 1. INTRODUCTION

Oxygen molecules combine with the lipid's molecular structure to cause lipid oxidation. This oxidation can occur in one of three ways: autoxidation, singlet and enzymatic oxidation. While all forms lead to lipid oxidation, they differentiate in how the free radicals bind to the lipid. Lipid oxidation in food is a negative feature because it can affect both the health benefits and flavor quality (Kanner et al. 1992). Lipid oxidation is known to produce harmful chemicals that cause the hardening of plaque in the body's arteries (McNeill 1998). As lipids are oxidized, they become rancid and produce new carbonyl compounds, which are detected as off-flavors.

Antioxidants are compounds known to slow or delay lipid oxidation. Preventative antioxidants can intercept free radical or singlet oxygen before any significant oxidation can occur. However, chain-breaking antioxidants retard or slow the oxidative processes after they begin.

As mentioned, lipid oxidation is believed to be the cause of most off-flavors in food products. Since oat bran naturally contains high levels of antioxidants the concentration of these antioxidants could be added to food products to lower the rate of lipid oxidation and potentially add additional health benefits. The applied use would be for product developers to use healthy but easily oxidized lipids when developing new food products and prevent oxidation by adding the antioxidant-rich oat bran extract.

The objective of this study is to identify the best method for the extraction of antioxidants from oat bran. The three methods of interest are traditional solvent extraction, microwave-assisted solvent extraction, and a supercritical pre-treatment. The objective for this research will be completed by determining and comparing the antioxidant activities and total phenolics of oat bran extracts from the extraction methods mentioned above.

The results expected from this research are to see an increase in antioxidant activity and total phenolics as extraction temperature and technology increase. By this, it is expected that the industry-used traditional solvent method will provide decent results but the microwave-assisted solvent method will provide better results because it more evenly heats the sample and allows for higher extraction temperatures and pressures. However, it is expected that the supercritical fluid pre-treatment will provide the best results because it starts by extracting in an oxygen-free environment, eliminating processing oxidation, and past research has shown it to be more selective in the compounds it extracts (Martinez 2004). It is also expected that the MAS will produce a higher yield because it can operate at higher temperatures.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Oats are defined a species of cereal grain. The seeds of this plant have played an important role throughout history as both animal feed and food source due to their high nutritional content (Riely 2003). It is one of the only cereals to be successfully grown in cooler northern climates, including Iceland, where many other cereal grains cannot grow. In fact, Russia, Canada, Poland, Austria, Finland, Germany, and the Ukraine are seven of the ten largest oat-producing countries. In 1995, as seen in the table below, these seven countries grew over 56% of the world's supply of oats according to the UN Food and Agriculture Organization.

Table 1: Top Oat Producers

Top Oat producers in 2005 (million metric tons)	
Russia	5.1
Canada	3.3
USA	1.7
Poland	1.3
Finland	1.2
Australia	1.1
Germany	1.0
Belarus	0.8
China	0.8
Ukraine	0.8
World Total	24.6

(FAOSTAT database 2006)

2.2 Oats

2.2.1 Oat History

Historical evidence may show oats to be one of the last cereal grains to be cultivated by man, around 3,000 years ago in Europe, but it could be found growing wild in ancient China as

long ago as 7,000 BC (Williams 2003). However, the modern oat (*Avena sativa*) is believed to be derived from the accidental crossbreeding of two wild grasses, the common wild oat (*A. fatua*) and the wild red oat (*A. sterilis*). Oats most likely made their way by merchant caravans or enemy raiding parties to northern Europe where they became the food of choice for the Scots and Norsemen. However, it was the Romans who gave them and other cultivated grain crops the term "cereal" (after the Roman goddess of agriculture Ceres) (Onstad 2004).

Oats became an American staple for the poor when they were brought to the "New World" in 1602 and planted along the Elizabeth Isles. The plant began to flourish and is now a major agricultural crop as seen in the figure below.

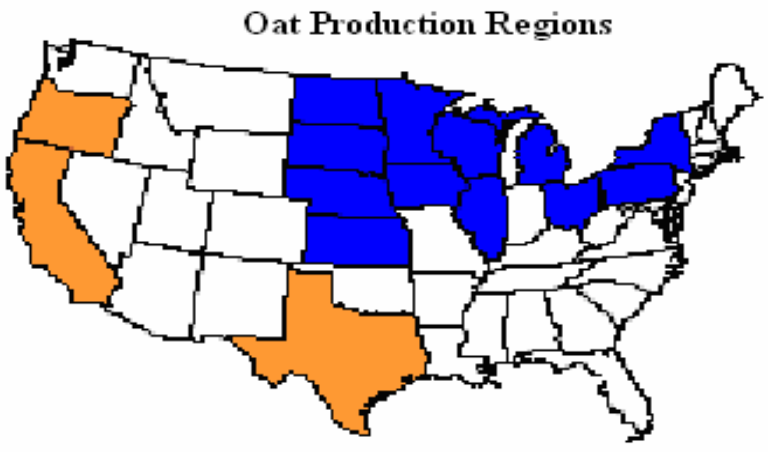


Figure 1: Two Major U.S. Oat Production Regions
(USDA Economic Research Service)

The major growing region is concentrated mainly from the Midwest to the Northeast, but some production comes from Texas, California, and Oregon.

However, with only 5% of the plant suitable for human consumption, it quickly began to undergo processing to increase its culinary uses, with the remaining going towards animal fodder (Onstad 2004).

One possible reason people have been slow to embrace the oat is because of how quickly they go rancid, due to both the presence of natural fats and a fat-dissolving enzyme present in the

grain (Williams 2003). One important note is that oat groats have the highest lipid content among the common cereal grains (Liukkonen 1992). Short processing times and steam treatments are typical pretreatments in industry to eliminate undesired enzyme activities (Knehr 1998).

The oat can be broken down into three distinct parts, which can be seen in Figure 2 below, adapted from the picture found in Fermented Cereals: A Global Perspective by the Food and Agriculture Organization of the United Nations.

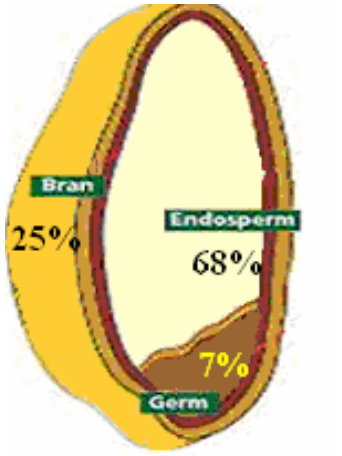


Figure 2: Breakdown of an Oat Groat (Entire Seed Kernel)

(Marquart et al. 2005)

In culinary terms, the term "oats" would be more accurately labeled as "oat products" since whole oats can be, and normally are, processed into many different forms (from least to most processed): whole oat grain or groats, steel-cut oats, rolled oats or oatmeal, and instant oats (Knehr 1998). But besides different processing forms, there are also a variety of raw oat products, such as whole oat groats, oat flour, oat germ, and oat bran. These varieties, as compared with the processed varieties, have a greater variation because the actual oat is broken down into its different forms.

Today, a majority of the world's oat production is still used as animal fodder, with the last know estimate of less than 5% of the world's crop being grown for human consumption (Eborn 2001). But due to increased knowledge about the many nutritional benefits of oats, consumption has increased around the world. Also, another growing segment for oats is in cosmetics due to the compounds in oat extract that have natural skin conditioners and anti-inflammatory properties (Williams 2003).

2.2.2 Oat Bran Health Aspects

It has been well known for many centuries that oats offer numerous health benefits, but science has only recently proven that it is partly due to the insoluble fiber they contain that helps in adding bulk to the body's waste material, which also aids in moving foods through the intestines (Gibson et al. 2002) (Anderson et al. 1994). More importantly, oats are high in soluble fiber, specifically β-glucan, which reduces blood cholesterol levels by increasing the excretion of bile in the body (Jenkins et al. 2002). This information led the United States Food and Drug Administration (FDA) to endorse health claims for oat and oat products in 1997 by stating that "soluble fiber from foods such as oat bran, rolled oats, or oatmeal and whole oat flour, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (21CFR101.81 2005). In fact, the FDA is currently pushing a diet based on a higher consumption of whole grains in its new Food Guide Pyramid in order to help lower the rate of heart disease and obesity in the United States (Jenkins et al. 2002).

Celiac disease, from Greek "koiliakos," is a disease often associated with ingestion of a group of proteins labeled prolamines or, commonly, gluten. Oats, however, lack many of the prolamines found in other grains such as wheat, rye and barley, making it a possible food source for some celiacs. Conversely, oats do contain avenin, which is a prolamine toxic to the intestinal submucosa that can trigger a reaction in some celiacs (Thompson 2003). In Finland and Sweden,

however, both countries have developed “pure oat” products, which can serve as part of a gluten-free diet.

With this knowledge at hand, it is no wonder the large food conglomerates are using their time and resources to start adding oats and oat products to the many products they produce, not only as a bulking agent but also for the health benefits (Stevenson et al. 2005) (Heasman et al. pg 3).

Table 2: Nutritional Breakdown for Oats per 100g

Carbohydrates	66 g
- Dietary fiber	11 g
Fat	7 g
Protein	17 g
Pantothenic acid (B5)	1.3 mg
Folate (Vit. B9)	56 µg
Iron	5 mg
Magnesium	177 mg
β-glucan (soluble fiber)	4 g

(USDA National Nutrient Database for Standard Reference, Release 19 NDB No: 20033)

While the consumption of whole grains should be encouraged, the bran and germ supply the majority of biologically active compounds found in the grain. These compounds include, but are not limited to, amino acids, B vitamins (such as thiamin, riboflavin, niacin, and pantothenic acid), and many minerals (calcium, magnesium, phosphorus, potassium, sodium, and iron) (Marquart et al. 2005). Even though oat bran only makes up 25-41% of the groat weight, it seems to contain most of the healthy compounds in oats, which can be seen in the table above with data calculated from the USDA Nutrient Database for Standard Reference Release 193 USDA-ARS Nutrient Data Laboratory (Chen et al. 2002).

2.2.3 Antioxidants in Oat Bran

Besides being high in both soluble and insoluble fiber and a good source of essential fatty acids, protein, minerals and vitamins, the bran contains most of the antioxidants found in the oat groat kernel (Peterson 2001). Antioxidants function in helping maintain the stability of processed oat products and can stabilize oils and fats against rancidity (Peterson 2001). The types of antioxidants oat bran contains include: tocopherols, tocotrienols, sterols, avenanthramides (unique to oats), *p*-hydroxybenoic acid, and vanillic acid. Similar to actions in other foods, simple phenolic acids and polyphenolic compounds from oats may serve as potent antioxidants by scavenging reactive oxygen and nitrogen species and/or by chelating transition minerals both in plants and in those animals that consume them (Chen et al. 2004). Research has shown that most phenolic compounds are located in the bran layer and that it has a higher antioxidant activity than whole oats and oat flour (Chen et al. 2002) (Handelman et al. 1999). Therefore, consumption of oats with oat bran could be a significant dietary source for these compounds (Chen et al. 2004).

While it is known that heating the oats destroys enzymes responsible for rancidity, no evidence has proven lipid oxidation is prevented. However, the antioxidant capacity of oat phenolics was demonstrated via *in vitro* studies (Chen et al. 2004). Even though oat bran contains high levels of the previously mentioned antioxidants, the lipids in the oats and oat bran still seem to oxidize rather quickly. Oats are inherently unstable as soon as they are ground or flaked, due to their relatively high oil concentration and the presence of lipase activity on the surface of the ungerminated groats (Peterson 2001). Past research has shown that the tocopherols were stable in unprocessed oats for over seven months of storage at room temperature, but were significantly degraded within one to two months in all processed oat products, including dried groats (Peterson 2001). One idea is that the antioxidants are bound

inside the oat bran's food matrix. These oat components have been added to food and beverage products to preserve quality and are associated with flavor, color, and/or aroma (Handelman et al. 1999). With proper extraction and concentration techniques, these antioxidants can be reapplied to the oats or oat bran or a variety of other food products to further or potentially better prevent lipid oxidation. Antioxidant phytochemicals may also play an important role in human health by scavenging reactive oxygen and nitrogen species and modulating several enzyme systems such as lipoxygenases (Handelman et al. 1999).

2.3 Lipid Oxidation

2.3.1 Basic Lipid Oxidation

Oxidation arises when oxygen binds to a molecule's structure through free radical chain reactions as seen in Figure 3 below. Even though a large assortment of organic molecules are susceptible to oxidation, lipids have been a major focus because of their unique oxidative damage (Kanner 1992).

At the nutritional level, the oxidation of fats and lipids is the major chemical factor in the deterioration of food quality through the breakdown of pleasant flavors and aromas, along with a loss in the nutritional and safety qualities (Erikson 1982).

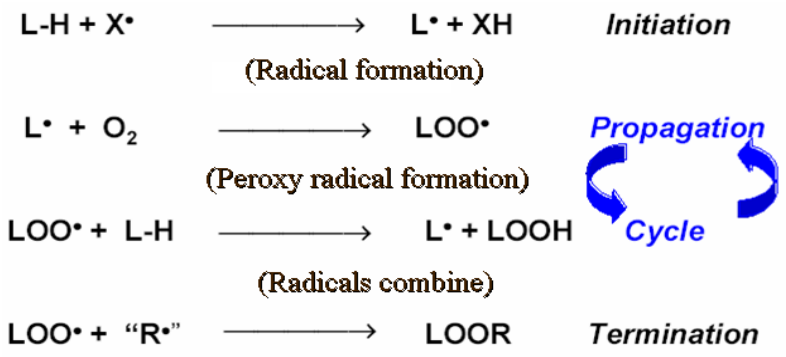


Figure 3: Steps in Basic Lipid Oxidation
(Ohshima 2005)

Recently, biological and nutritional aspects have merged to study how diets with an intake of a large concentration of oxidized lipids have resulted in increased amounts of

carcinogenesis, premature aging, and other diseases (Bruce et al. 2000). Oxidation of lipids can occur in foods with a high percent of fat and lipids, such as cream, cheese, meat products, oils, and nuts, but also in foods containing minor amounts of lipids, such as dried fruit and vegetable products, bread, or pasta. This process occurs in three ways, autoxidation, singlet oxidation, and enzymatic oxidation.

2.3.2 Autoxidation

In his book "Food Chemistry," Fennema states that it is generally agreed that autoxidation is the main reaction involved in oxidative deterioration of lipids (Fennema 255). In autoxidation, a di-radical is formed during the initiation stage when a carbon-hydrogen bond is broken. The O_2 molecule splits and a single oxygen atom binds with either the carbon or the hydrogen. This produces two radicals, which go on to break up other bonds. The molecular structure of the lipid is changed and new compounds are created.

2.3.3 Singlet Oxidation

In singlet oxidation, however, no di-radical is formed due to oxygen directly attaching to the double bond and not to the individual hydrogen or carbon. A more satisfactory explanation is that singlet oxygen is responsible for the initiation of oxidative deterioration of lipids (Fennema 259). The major differentiation between the two forms of oxidation is that two peroxides are formed with autoxidation and four peroxides are formed under singlet oxidation. Therefore, singlet oxidation occurs 1400 times faster than its counterpart, autoxidation (Min et al. 2002).

2.3.4 Enzymatic Oxidation

Different enzymes are responsible for the hydrolysis of neutral triacylglycerols and polar phospho- and glycophospholipids. Cereal seeds contain apparently only 1 or up to 3 different isoenzymes of lipases acting on storage lipids (Futterman 2002). During various processes,

cereal lipids may also be exposed to microbial lipase, and the role of microbial enzymes in cereal lipid reactions is a controversial subject (O'Connor et al. 1992). However, the heat treatment of oats during processing to inactivate the enzymes responsible for oxidation unfortunately leads to increased autoxidation and singlet oxidation (Lehtinen et al. 2002).

2.3.5 Rancidity Due To Lipid Oxidation in Oats

During processing and/or storage, two distinct reactions of oat lipids take place. In the first reaction, hydrolytic deterioration occurs when triacylglycerols or phospholipids are converted to free fatty acids. In the second reaction, oxidative deterioration occurs when polyunsaturated fatty acids are converted to hydroperoxides and then further to secondary oxidation products (Heinio et al. 2002). It should be noted though that oat is exceptionally high in lipase, catalyzing the hydrolytic deterioration, whereas the lipoxygenase activity, catalyzing the oxidative deterioration, is low (Heinio 2003).

2.3.6 Negative Aspects of Lipid Oxidation

Lipid oxidation in the body is directly related to cholesterol oxidation and hardening of plaque on the artery walls, two leading contributors to coronary heart disease. Fat oxidation during storage can lead to the loss of nutritive value and generation of toxic products such as lipid peroxides (Rufián-Henares et al. 2005). Lipid oxidation is also a key player in the production of off-flavors in foods. As lipids oxidize, they become rancid and produce new carbonyl compounds, such as aldehydes, ketones, and acids, which are perceived as causing rancid flavors and can be poisonous at high levels, along with hydrocarbons, alcohols, and epoxides.

2.3.7 Preventing Lipid Oxidation

Preventing lipid oxidation is important for two main reasons stated earlier: health and flavor. This can be achieved by completely sealing off the fat or lipid from any oxygen source,

but that is highly inefficient. Phenolic compounds, including antioxidants, have been shown in previous studies to exhibit antioxidant and anti-inflammatory properties (Vuorela et al. 2005). This is the method that the food industry is beginning to use more and more frequently (Morris 2002). Antioxidants are compounds that inhibit or retard oxidation by reacting with and neutralizing the free radicals or the chemicals that release these free radicals. There are two main types of antioxidants, preventative and chain-breaking, and each works differently depending on what stage of oxidation is present. Preventative antioxidants are antioxidants that, during the initiation stage, intercept oxidizing compounds before any oxidation can occur. On the other hand, chain-breaking antioxidants are antioxidants that interrupt the propagation stage by slowing or stopping oxidative processes after they begin, by intercepting the chain-carrying radicals (Pokorny et al. pg. 253).

2.4 Oxidation of Oat Bran and Its Effects

The fat content of oats is relatively high at around 5-7% (Ekstrand et al. 1993). This is possibly the most important reason contributing to why oats have such a pronounced and easily detectable flavor and aroma and possibly why they oxidize so quickly.

Oats contain a variety of antioxidants, including tocopherols and phenolic acids, and therefore are theoretically fairly stable towards nonenzymatic oxidation. However, the lipid content of oats is roughly five times higher than wheat, the standard for cereal grains in the United States, and the lipolytic enzymes are 10-15 times more active than those found in wheat (1. Molteberg et al. 1996) (Matlashewsky et al. 1982). These high levels of unsaturated free fatty acids and the presence of lipoxygenases favor lipid oxidation (2. Molteberg et al. 1996). The problem with this is that most commercially processed oats for human consumption are stabilized by heat-treatment to inactivate the lipolytic enzymes and develop the characteristic,

pleasant flavor that is associated with high quality commercial oat products (2. Molteberg et al. 1996).

The heat-treatment for the stability of the processed oats actually denatures some of the antioxidants during the processing since many are heat-lable (Heinio et al. 2002). In addition, heat is commonly known to promote oxidation (Fast 1990). The loss of antioxidants and the added heat lead to oxidation and the formation of hexanal. As expected, hexanal is the major volatile produced by lipid oxidation. Past research has demonstrated that a rancid odor begins to occur in ready-to-eat oat cereals when the hexanal concentration reaches around 5-10 mg/kg (Sjövall et al. 1997).

However, hexanal accumulates only partly in food products because it may evaporate or be quenched into non-volatile compounds (Zhou et al. 1999). Oat is a source of many quenching agents: vitamin E, phytic acid, sterols, and phenolic compounds such as avenanthramides, and flavenoids (Peterson 2001). These compounds can be extracted into antioxidant rich oil-based solutions and used to inhibit off-flavor production. These oils can be obtained by either crushing the seeds with a press or extracting the oils with chemical solvents (Moreau et al. 2003).

2.5 Solvents and Solvent Extraction

A solvent is usually a liquid that dissolves a solid, liquid, or gaseous substance (the solute), resulting in the formation of a solution. Solvents are used to extract soluble compounds from a mixture. As a solvent dissolves a compound, it will create various weak chemical interactions with the solute in order to solubilize it. The most common of these interactions, in increasing strength, are the Van der Waals interactions consisting of induced dipole interactions, the dipole-dipole interactions, and the hydrogen-bond interactions (Miller et al. 1981).

Solvents and solutes can be broadly classified as polar (hydrophilic) or non-polar (lipophilic). It is this polarity of a solvent that determines what type of compounds it is able to

dissolve and with what other solvents or liquid compounds it is miscible. Relatively more polar solvents dissolve polar compounds best and similarly, non-polar solvents dissolve non-polar compounds best.

For determination of which solvents to use in this experiment, previous research was consulted. Past research has shown that hexane efficiently extracts all of the major lipid components from cereal grains; however, hexane is believed to cause health implications when used in food-grade lipid extractions. In general, for oats, past research has shown that increasing solvent polarity resulted in increased yields of polar lipids (Moreau et al. 2003). Hexane is a non-polar solvent with a polarity of 20 and a density of 0.655 g/ml, while methanol is a polar solvent with a polarity of 33 and a density of 0.791 g/ml (Asano et al. 1984). Therefore, polar methanol will be used for all chemical extractions. This solvent's low boiling point, 64ºC, will allow it to evaporate easily or be removed by distillation from extract, thereby leaving the extract behind for identification and analysis (Asano et al. 1984).

2.6 Extraction Techniques

The challenges of extracting substances for analysis from food matrix systems often lead to techniques that are wasteful of analytical time and reagents. Methods are chosen because they can be put into practice immediately or because they do the job well. Therefore, it is not surprising that there is a great interest in improving extraction methods (Brühl et al. 1999).

Large-scale operations follow the traditional solvent extraction (TSE) or Soxhlet Method for removing the oil from cracked seeds at low temperatures with either a toxic or nontoxic fat solvent, depending on the end result of the oil, by percolating it through or steeping the seeds. After all the oil is extracted, the solvent is distilled from the oil and recovered for reuse (Potter et al. 1998). Solvent extraction generally obtains more oil from seeds than is possible by pressing alone because it provides a soaking effect for the sample. It should be noted that the Soxhlet

Method is often considered the standard method by which the others are evaluated (Nielson 2003).

Microwave-assisted solvent extraction (MAS) is becoming more widely used for sample preparation in order to replace other extraction methods, specifically, TSE. In MAS, a sample in solvent is encased and subjected to pressure and convection heat rather than conduction heat (like a water bath) alone. In addition, the microwaves rapidly increase the heating of the sample, allowing the extraction to start earlier. This method also allows for pressure and rate of temperature increase to be controlled, allowing for a more controlled extraction.

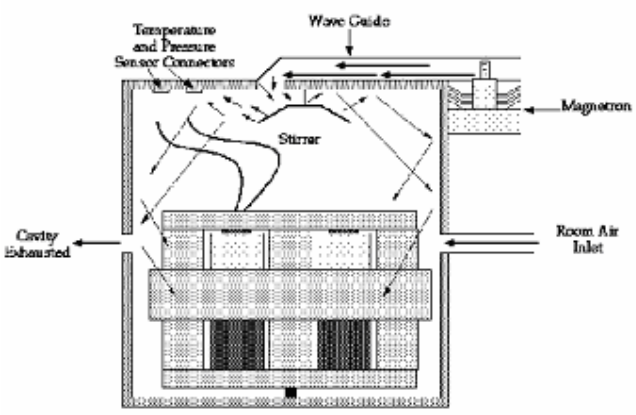


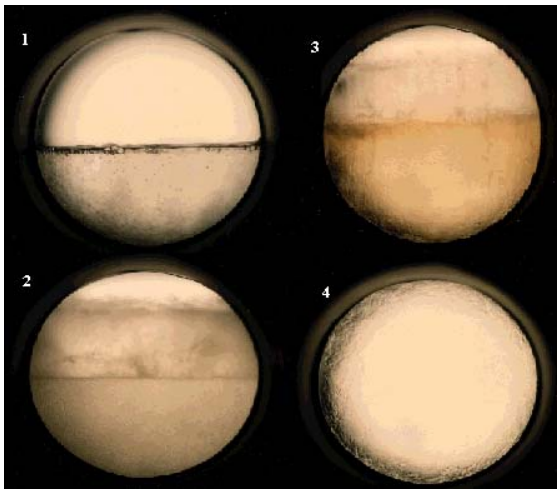
Figure 4: Microwave-assisted Extraction Process
(Milestone Microwave Laboratory Systems)

This extraction method has numerous advantages over the traditional extraction method. For example, MAS considerably reduces extraction time and/or enhances the efficiency of the extraction. Plus, since it is not tedious and not restricted in solvent selectivity, no concentration/evaporation step is required, and the possibility of contaminating the sample with solvent impurities is much lower. These problems can also be successfully avoided by using supercritical fluid extraction (SFE) (Csiktusnadi Kiss et al. 2000).

Concern over solvent residues in extracted products has catalyzed a search for alternative processing methods such as the use of extraction with supercritical fluids. SFE is a relatively

new concept. Supercritical fluids are produced by heating a gas above its critical temperature or compressing a liquid above its critical pressure (Perrut 2000). The main advantages of using supercritical fluids for extractions are that they are inexpensive, contaminant-free, and less costly to dispose of safely than organic solvents. The properties of supercritical fluids also provide some advantages for analytical extractions. Supercritical fluids can have solvating powers similar to organic solvents, but with higher diffusivities, lower viscosity, and lower surface tension (Perrut 2000).

Carbon dioxide is the most utilized gas because of its nontoxicity, low reactivity, moderate critical temperature and pressure, availability, low cost, and nonflammability. Under these conditions, the supercritical fluid offers properties of both liquids and gases. Once a gas reaches its supercritical fluid state, no distinct phase can be determined.



1) Here the separate phases of carbon dioxide with the meniscus are easily observed.
2) An increase in temperature causes the meniscus to begin to diminish.
3) Increasing the temperature further causes the two densities to become more similar. The meniscus is less easily observed but still evident.
4) Once the critical temperature and pressure have been reached, the two distinct phases are no longer visible.

Figure 5: Different States Leading To Supercritical Fluid

As a supercritical fluid, carbon dioxide (CO_2) has unique physicochiemical properties, such as high density and low viscosity that make it suitable as an extraction solvent. The solvent power of supercritical carbon dioxide (SC-$C0_2$) depends on the density. This can be adjusted conveniently by changing the pressure and temperature, as seen in Figure 6 below, which was adapted from a presentation found on the University of Leeds Cleaner Synthesis Group website.

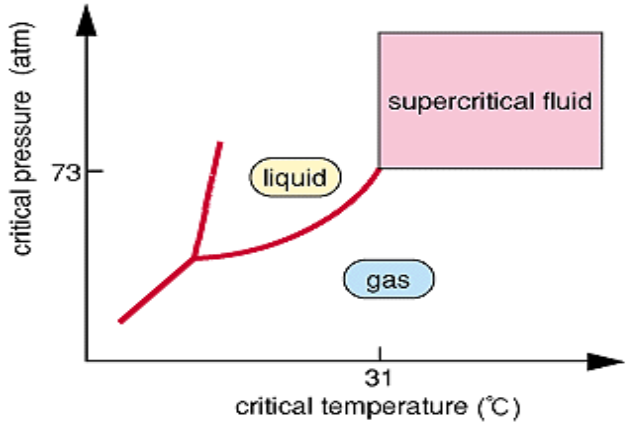


Figure 6: Phase Diagram of Supercritical CO_2

Increasing pressure of the SC-CO_2 raises its density and allows a closer interaction of the fluid molecules with those of the compounds to be extracted. The increase of temperature raises the vapor pressure of the components to be extracted and therefore improves their extractability (Reverchon 1997). The use of CO_2 as a supercritical solvent permits the processing of thermo-sensitive material (Reverchon et al. 1993). However, SC-CO_2 has been shown to be a poor solvent for polar compounds. The supercritical extraction method has been shown to be faster, more productive, and more environmentally friendly than the classical methods. The initial high investment cost involved in supercritical fluid technology can therefore be paid off through the benefits gained for the end products (Perrut 2000).

2.7 Analysis Methods

The DPPH (2,2'-diphenyl-1-picrylhydrazyl) method provides information on the reactivity of compounds with a stable free radical. Because of the odd electron, DPPH shows a

strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorisation is proportional to the number of electrons taken up (Miller et al. 2000).

In the Folin-Ciocalteau method, monohydric phenols, polyphenols, flavonoids, tannins, and ascorbic acid are oxidized by a mixture of two strong inorganic oxidants, phosphotungstic and phosphomolybendic acids, found in the Folin-Ciocalteau reagent. This results in the production of a complex molybdenum-tungsten blue colored solution. The phenols oxidize rapidly in the alkaline solution, which first converts them to phenolate ions.

Cholesterol is a molecule with an unsaturated bond at position Δ 5-6 of the sterol nucleus; therefore it is prone to oxidation (Maerker, 1987). The molecule undergoes autoxidation by a free-radical mechanism leading to the formation of hydroperoxides and then to a number of oxidation products. It has been estimated that approximately 1% of the cholesterol consumed in a mixed Western diet is oxidized cholesterol (oxysterol) (van de Bovenkamp et al. 1988). In light of the potentially dangerous effects of oxysterol for human health, efforts to prevent or to reduce oxysterol consumption are now currently being made. Since cholesterol oxidation proceeds via a free radical mechanism, similar to polyunsaturated fatty acid oxidation (Kubow 1993), antioxidants used to inhibit general fat and oil oxidation are also able to prevent or retard cholesterol oxidation. Analytical procedures for determining the extent of cholesterol oxidation in food, especially in heated and/or stored foods with high cholesterol content, include capillary gas-chromatography, GC-mass spectrometry, and high performance liquid chromatography (HPLC) (Park et al. 1985). HPLC greatly simplifies the quantification procedure and methods have been applied to the separation of oxysterols by several researchers (Park et al. 1985).

DHA is one of the most unsaturated fatty acids found in large quantities in the bodies of animals. Therefore, it is likely to be more vulnerable to lipid peroxidation than most other fats

(Best 1997). Since DHA oxidizes quickly, it can serve as an excellent model for evaluating the efficiency of antioxidants in reducing oxidation.

CHAPTER 3. SOLVENT EXTRACTION AND EXTRACT ANALYSIS

3.1 Materials

Oat grain (*Avena sativa* L.) was a gift from the Bell Institute of Health and Nutrition of General Mills Company (Minneapolis, MN). It was stored at 4ºC before use. DPPH (2,2'–Diphenyl-l-picryhydrazyl), heptadecanoic acid (C17:0), DHA and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO). BCI_3-methanol and 2,2-dimethoxypropane were from Supelco (Bellefonte, PA). Methanol, acetone and hexane were HPLC grade and purchased from Fisher Scientific (Springfield, NJ). All data was analyzed using the Statistical Analysis Software System, Version 9.1 (SAS® Institute, Cary, NC).

3.2 Methods

3.2.1 Extraction Methods

Methanol was used to extract the lipids from the oat bran sample for both the traditional solvent extraction and the microwave-assisted solvent extraction using a 2:1 ratio of solvent to solute based on volume, and carbon dioxide served as the solvent during the supercritical fluid treatment.

Each extraction was run with six replications. After the extraction process for traditional solvent and microwave-assisted solvent, the extract and solvent with dissolved solids were collected in centrifuge tubes and spun at 4000rpms for fifteen minutes under a constant 20°C. After removing the tubes, the supernatant was measured and frozen until further experimentation was to be conducted.

3.2.1.1 Traditional Solvent Extraction (TSE)

For TSE, five replications of ten grams of oat bran were combined with 40 mL of methanol in a 150 mL flask and swirled to evenly mix the oat bran with the methanol. The mixture was then placed in a 60°C water bath for twenty-five minutes. After running, the extract was gathered as specified.

3.2.1.2 Microwave-Assisted Solvent Extraction (MAS)

For the MAS, an *Ethos EX Labstation* from Milestone Microwave Laboratory System (Shelton, CT) was used. Ten grams of oat bran were combined with 40 mL of methanol in special Teflon cups with the addition of a magnetic stirrer to prevent hot spots from forming. These cups were then inserted into thermal-protective sleeves and covered with Teflon lids. The lids were locked into place in heavy plastic canisters and connected to the turntable in the microwave oven. When all the canisters were in place, a thermal probe was inserted into one canister to control measure the internal temperature and pressure. Since MAS has a larger variable temperature range, two extraction temperatures were utilized, 60°C to compare to the TSE and 100°C. Once the sample was in the oven, the computer was set for 6.0 psi and 800 watts. The heating program was scheduled to increase the sample's internal temperature according to the set program, depending on which temperature was being tested, over 5 minutes and then held at that specific temperature for 20 minutes. Upon completion of the extraction, the samples sat for 20 minutes to cool and allow the pressure to lessen before opening so that no extract would be lost if the solvent was misted into the air. Once the canisters were unlocked and cups removed and uncapped, the liquid extract was gathered as specified earlier.

3.2.1.3 Supercritical Fluid Treatment (SFT)

For supercritical fluid extraction, 250 grams of oat bran was passed through a series of four sieves in order to remove the particles smaller than 40-mesh. Once removed, 18 containers were filed with 10 grams of the remaining oat bran, 79% of the starting amount. This step prevented the chance of losing the sample when spun in the autoclave, 1L bolted 3-16 stainless steel with a 3-inch diameter and 9-inches deep, with a Modular Temperature Controller for Process model 93102199-1 to regulate the internal temperature. One at a time, each sample was placed into the mesh basket, inserted onto the spindle of the motor, locked into place, and capped

with a bladed nut. The spindle with basket was then inserted into the well of an autoclave and bolted down to prevent gas leaks. Using a Ruska Gear Pump, liquid CO_2 was released into autoclave until 950 psi was achieved. The CO_2 in the autoclave was heated to one of the desired temperatures, 25°C, 50°C, or 75°C, regulated by a thermal probe. The motor was run at 250rmp to ensure that the CO_2 passed through the mesh basket and over the sample as it continuously spun in the well. After the extraction process, the CO_2 was allowed to exit the well into a fume hood while maintaining the desired temperature to ensure full vaporization. The oat bran was collected and then frozen until further extraction. The equipment and process can be seen in Figure 7.

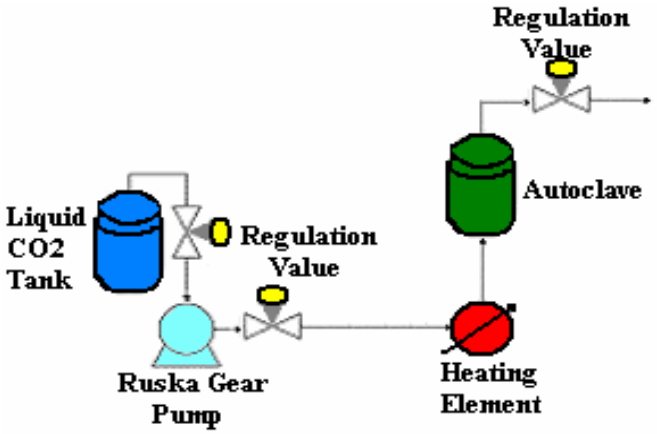


Figure 7: SFT Process

In order to collect an extract to test for antioxidants and phenolics, the bran went through MAS after the supercritical fluid treatment. This double extraction allowed for the determination of what compounds were extracted from the oat bran during the SFT and which compounds remained.

3.2.2 Extract Analysis

To determine which method is most efficient in the extraction of phenolic compounds and antioxidants, several analyses can be performed. For total phenolic compounds, each extract will be analyzed using the Folin-Ciocalteau Method. The antioxidant activity will be evaluated

using three different tests: the DPPH (2,2'-diphenyl-1-picrylhydrazyl) method, cholesterol oxidation, and the DHA (docosahexaenoic acid) model. The relationship between phenolic content and antioxidant activities was examined.

3.2.2.1 DPPH Method

The oat extract solution for the DPPH test was prepared by dissolving 0.2 g of the thawed extract in 10 ml methanol. Two ml of a DPPH solution with a concentration of 0.025 g of DPPH in 1000 ml of methanol was mixed with 40, 80, 120 µL of the extract solution in a cuvette. After a 30-minute incubation at room temperature, the reaction solution was examined at 515 nm by using a spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

$$\text{Inhibition } \% = [(Abs_{t0} - Abs_{t30min})/Abst0] \times 100$$

Abs_{t0min} was the absorbance of DPPH at time zero. Abs_{t30min} was the absorbance of DPPH after the 30 minutes of incubation.

The inhibition percentage determined from the absorbance of DPPH was compared between each concentration of the oat extract solution added.

3.2.2.2 Total Phenolics/Folin-Ciocalteau Method

The Folin-Ciocalteau reagent was diluted by a factor of ten with deionized water. The oat extract solution for the DPPH test was prepared by dissolving 0.2 g of the thawed extract in 10 ml methanol. Forty, eighty, and one hundred and twenty micro liters of extract solution was mixed with 0.75 ml of the diluted Folin-Ciocalteau reagent and incubated at room temperature for 5 minutes. Then 0.75 ml of 60-g/L sodium bicarbonate solution was added, and the mixture was incubated at room temperature for 90 minutes. Using a spectrophotometer set at 750nm, the absorbance of the solution was determined after first zeroing with deionized water. Catechin

was used to create a standard curve and the total phenolics content of the sample being expressed in terms of catechin equivalent.

3.2.2.3 DHA Oxidation

One milliliter of DHA (0.1 mg/mL of hexane) was added in each test tube (13x100mm). Each oat extract for this test was prepared by dissolving 0.2 g of the extract in 10 mL of methanol. For each oat extract treatment group, 50, 250, and 500 μL of the prepared oat extract solution was added to the DHA tube and vortexed for 30 seconds. The DHA test tube without any oat extract solution added served as the control. Then, the solvent in the test tubes was evaporated under vacuum in a centrifuge evaporator for 30 minutes at 60°C. After evaporating, the tubes were immersed in a 150°C sand bath for 15 minutes. One-milliliter of heptadecanoic acid (C17:0) (0.1 mg/mL) as an internal standard for the DHA analysis was added in each tube after the test tube was cooled.

The test tube was capped and incubated at 60°C in a water bath for 10 minutes after adding 2 mL BCI_3-methanol and 1 mL 2,2-dimethoxypropane to perform fatty acid ester derivatization. Then, a 1 mL hexane layer was transferred to a GC vial after being passed through a filter compacted with anhydrous sodium sulfate.

A gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA) with an FID detector was used to determine DHA concentration. Helium was used as a carrier gas and at column flow rate 1.2 mL/minute. The injection volume was 5 μL and the split ratio was 1:100. The injector and detector temperature were 250 and 270°C. the column was a Supelco SP 2380 (30m x 0.25mm) (Bellfonte, PA). The oven temperature program was holding at 50°C for 3 minutes and then increasing standard as a reference. The percentage of remaining DHA in each tube was obtained by its determined concentration to its original added concentration.

3.2.2.4 Cholesterol Oxidation

One-milliliter cholesterol solution (0.1 mg/mL) in hexane was added in each test tube (13x100 mm). Similar to the DHA oxidation procedure, the oat extract treatment volumes of 50, 250, and 500 μL of 0.02g of extract dissolved in 10mL of methanol was added to the tube containing cholesterol and vortexed for 30 seconds. Two tubes without any oat extract solution served as the control. Then, the solvent in the test tubes was evaporated using a vacuum centrifuge evaporator for 10 minutes at 60°C. After evaporating, the tubes were immersed in a 150°C sand bath for 15 minutes. After the tube was cooled, 1 ml of methanol was added to each tube and vortexed for 30 seconds. This methanol solution was then transferred to a HPLC vial.

An HPLC system was used to determine the cholesterol concentration. The HPLC included a Waters 2690 System and a 960 PDA detector (Milford, MA), and a 25 cm x 4.6 mm diameter 5-μm C18 Discovery column (Supelco, Bellefonte, PA). The mobile phase was acetone: methanol (10:90) with a flow rate at 0.8 mL/minutes. The HPLC was controlled by Waters Millennium chromatography software. The wavelength for quantifying cholesterol was selected at 215 nm. The percentage of remaining cholesterol in each tube was obtained by comparing its determined concentration to its original added concentration.

3.3 Statistical Analysis Method

Five replications of each experiment were performed with the data's average serving as the reported value. All data was analyzed with a confidence level of 95% ($\alpha = 0.05$).

The analysis of variance (ANOVA) is a statistical method for determining which of the various effects, operating at the same time, are important and have an influence on the results (Piggot 1996). Therefore, it is "a method of multiple-comparison for pairwise comparisons of k means and for the simultaneous estimation of differences between the means by confidence intervals" (Gacula et al. 1987). Generally, the analysis of variance is used when studying the

effects of qualitative factors on quantitative measurements. The basic idea for ANOVA is that it measures the total variation of a data set and expresses it as a sum of terms (Freund et al. 1999). The assumptions behind ANOVA are that it has normally distributed variables, variance equality and independence of the errors.

Analysis of variance was used in this study to determine if there was any significant difference between the means and standard deviations for each of the three oat bran treatments within a given concentration in each experiment. If a significant difference was perceived, it was determined where those differences lie.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Temperature Increase Rate of Different Extract Treatments

One of the most important differences between the three extraction methods is their ability to quickly raise the temperature of the sample to the desired extraction temperature. The graphs below demonstrate that MAS was the quickest method for bringing the sample up to the desired temperature when compared to TSE and SFT, except for the SFT-25°C, which was already at room temperature.

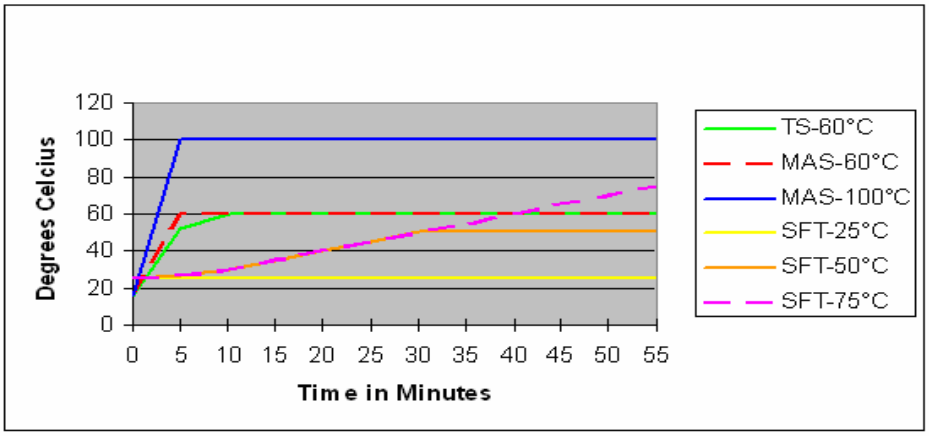


Figure 8: Comparison of Extraction Temperatures

The difference in the time it took to bring the samples up to their desired temperatures is one example of the unique properties of each extraction method. Since it is known that certain antioxidants in oat bran are heat-libel, the slower the sample is brought up to temperature the less likely that these antioxidants will be damaged or destroyed. However, future research would be needed to test that hypothesis.

4.2 Extract Yields From Different Extraction Conditions

Even though each sample was extracted using 40mL of methanol, the different extraction methods gave different final extract yields. The average total is the ml of extract plus methanol collected from the extraction. The average yield is the grams of pure extract per gram of oat

bran. The concentration in mg/ml is the amount of pure extract in 1ml of the extract plus methanol solution. All the values can be seen in the table below.

Table 3: Extract Volumes and Yield

Extractions:	TSE	MAS (60°C)	MAS (100°C)	SFT (25°C)	SFT (50°C)	SFT (75°C)	STD. DEV
Avg. Totals:	(a)16.4	(b) 18.6	(c) 19.7	(d) 14.9	(a) 16.7	(c) 20.3	2.1
Avg. Yield (g)	1.4	1.8	1.7	1.3	1.4	1.7	0.2
Concentration (mg/ml)	23.38	30.18	41.23	79.80	107.63	130.05	44.0

As seen in the table above, the average totals with methanol increased as the temperature increased. It is worthy to note that SFT-25°C offered statistically the lowest volume of extract, with TSE and SFT-50°C offering statically not different volumes, followed by MAS-60°C, and finishing with statistically not different volumes from MAS-100°C and SFT-75°C.

In terms of grams of overall yield from 10 grams of oat bran, SFT-75°C and MAS-100°C provided the same yield, which is higher than TSE. In terms of concentration, SFT-75°C was higher than every other sample analyzed. Meanwhile, SFT had overall a higher concentration than MAS and TSE. This information must be kept in mind when looking at the gathered results but will be explained in further detail later in this chapter.

4.3 Antioxidative Capabilities and Total Phenolic Contents in Different Extracts

When reviewing the data generated from the DPPH experiment for TSE and MAS, which can be seen in Figure 9 below, the extraction with the highest antioxidant activity was MAS-100ºC. Antioxidant activity was determined by using the DPPH inhibition percent formula provided earlier. As the inhibition percent increases, the antioxidants are more abundant and more active. Past research has shown that an increase in the temperature used during extraction as a tendency to increase antioxidant activity (Brand-Williams et al. 1995). According to research conducted at General Mills, Inc., the antioxidant activity is quite low for many

compounds but cereals have remarkably high antioxidant content (Miller et al. 2000). This information was used in selecting MAS-100ºC as the extraction method for the supercritical fluid treated samples.

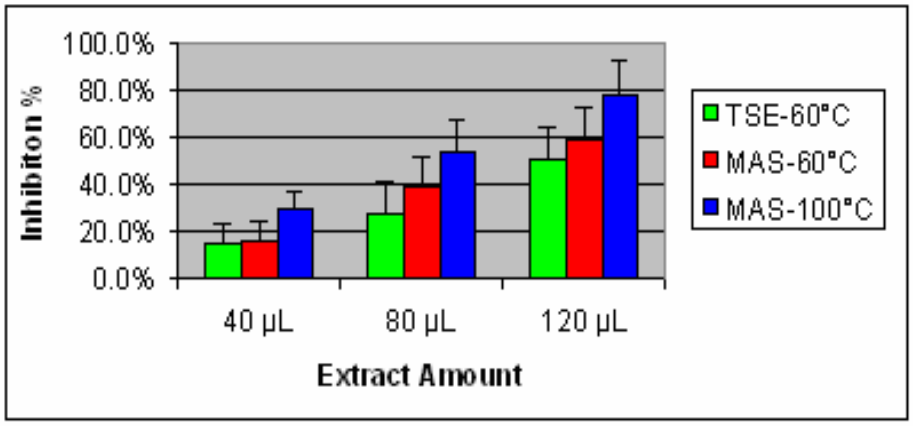


Figure 9: Measurement of Antioxidant Activity Using DPPH for TSE and MAS

MAS offered a higher antioxidant activity for both treatments when compared to the TSE. This is due to MAS building up of pressure during the extraction process for an increase in antioxidants to be drawn from the oat bran matrix. Increasing pressure leads to higher boiling points allowing for a higher temperature extraction. Because methanol has a boiling point around 64ºC, it would be impossible to reach the 100ºC temperature used for one of the MAS treatments. The pressure-aided extraction can be further supported in this experiment with the MAS-100ºC offering the highest antioxidant activity.

Figure 10 below shows in graphic form how the SFT samples ranked against MAS-100ºC. The extraction has been statistically proven to offer higher antioxidant activity in past research than liquid solvent (Tena et al. 1997). Surprisingly, however, it showed higher results in every extraction volume. This is potentially a result of the supercritical fluid treatment removing lipids and concentrating the antioxidants in the remaining extract (Csiktusnadi Kiss et al. 2000).

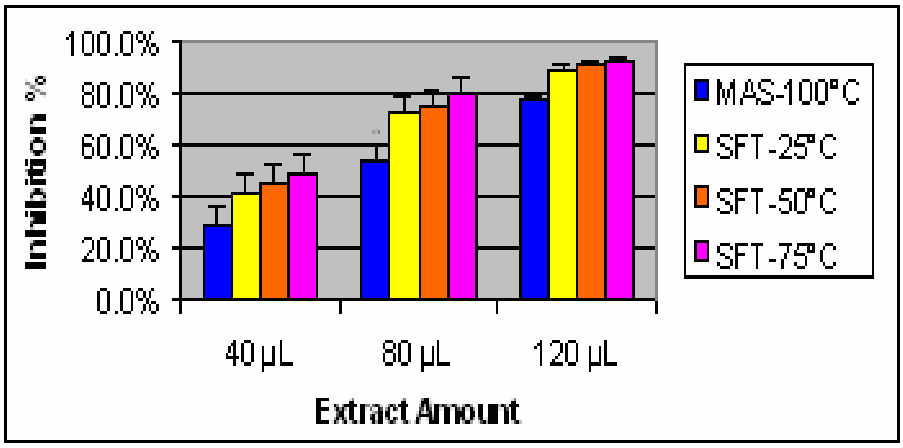


Figure 10: Measurement of Antioxidant Activity Using DPPH for MAS and SFT

Due to the results generated from the DPPH method, it was decided that the remaining analyses would evaluate only the TSE, MAS-100ºC, and SFT-75ºC treatments.

It was therefore deemed necessary to compare the DPPH inhibition results for TSE, MAS-100ºC, and SFT-75ºC. Auerbach et al. published results in the Journal of the Science of Food and Agriculture stating that the antioxidant activity of oats was 70% inhibition in methanol (Auerbach et al. 1999). However, the TSE and MAS-100ºC were less than 70% but the SFT-75ºC showed to be well over the 70% inhibition.

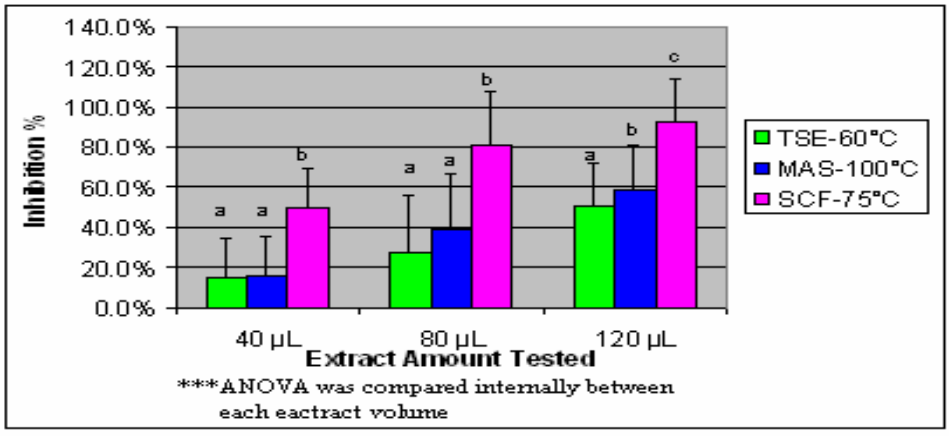


Figure 11: Results of DPPH Inhibition for TSE, MAS-100ºC, and SFT-75ºC

The results showed that the SFT-75ºC was the only statistically different sample for the 40 and 80µl volumes, but when the extract volume analyzed was increased to 120µl, each

extraction became statistically different. Once again, the possibilities for these results are the removal of lipids and other non-phenolic substances during the supercritical treatment prior to the microwave-assisted extraction and the increased efficiency when increasing antioxidant concentration.

For the total phenolics or the Folin-Ciocalteau method, TSE showed to be significantly different from MAS-100ºC, which was statically different from SFT-75ºC in terms of catechin equivalency, with the values for each being seen in Figure 12 below. It is not surprising that all three extractions would be statistically different since new technology may only increase purity or yield by removing antioxidant impurities (Auerbach 1999). The reason for this is increased antioxidant activity for MAS-100ºC and SFT-75ºC, which leads to a higher phenolic content since antioxidants are a type of phenolic (Kahkonan et al. 1999).

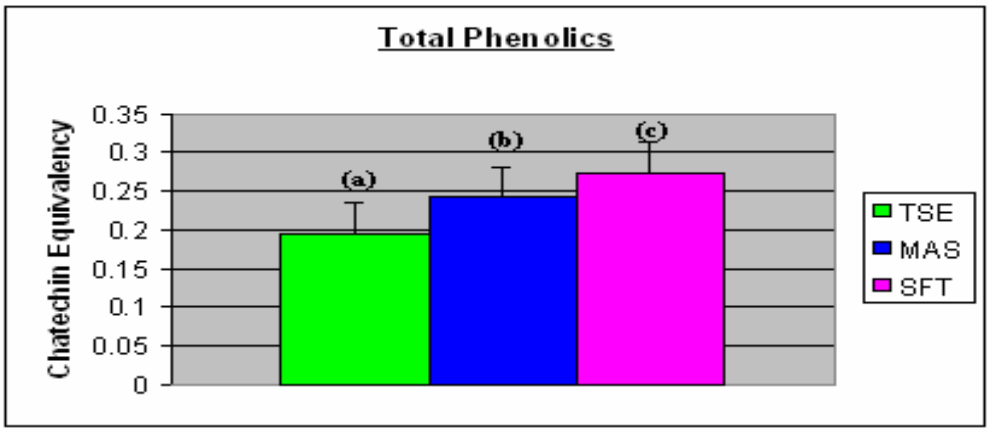


Figure 12: Catechin Equivalency for Total Phenolics

Emmons et al. published in their 1999 publication "Antioxidant Activity and Phenolic Contents of Oat Groats and Hulls" in Cereal Chemistry that data showed the catechin equivalency for phenolic content of oat from methanol extractions to be between 9.8-44.4 mg/kg. The data from this experiment fits this profile with TSE, MAS-100ºC, and SFT-75ºC ranging from 20.0 to 27.0 mg/kg of oat bran. Past research conducted at Louisiana State University

showed a 26.7µg of catechin equivalency per gram (Sun et al. 2006). This research showed a 20.0 to 27.0µg of catechin equivalency per gram, so some variation in samples does occur.

However, in terms of total phenolics per gram of extract, none of the extractions were significantly different from each other.

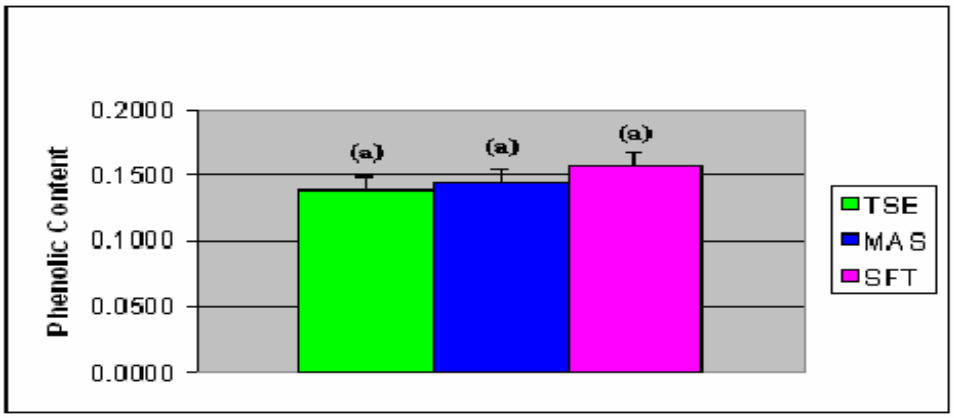


Figure 13: Total Phenolics per Gram of Extract

4.4 Inhibition of the Extracts on Cholesterol and Long-Chain Fatty Acid Oxidation

Cholesterol oxidation analysis using the three extract samples demonstrated similar results to the total phenolics analysis. Statistically each extraction proved to offer different levels of cholesterol oxidation with the results seen in Figure 14 below.

The results from this study demonstrated a statistically different percent of oxidized cholesterol from each treatment. The control sample had almost 50% of the original level of cholesterol oxidized while the oat extracts prevented an additional 10-28% in cholesterol oxidation. Similarly, Sun et al. used the cholesterol oxidation model to test the antioxidant activity in oat extracts with results showing a positively statistically different cholesterol oxidation percent compared to the control. The results from Sun et al. show methanol to prevent 75% of the cholesterol from oxidizing, while data from this research prevented 70% from oxidizing using the methanol extraction (Sun et al. 2006). Meanwhile, the extract representing the supercritical treatment followed by methanol extraction was able to inhibit 81% of the

cholesterol from oxidizing, which would be statistically different from the optimal results from Sun et al.

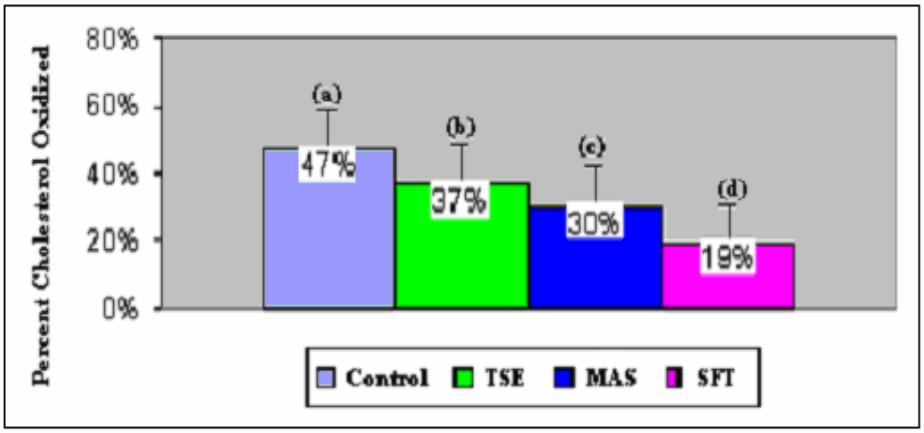


Figure 14: Percent Cholesterol Oxidized

However, Xu et al. published research showing that α-tocopherol and α-tocotrienol were not statistically different from a control sample when testing cholesterol oxidation. Although oats are known to contain α-tocopherol and α-tocotrienol, the benefits for the prevention of cholesterol oxidation might come from the avenanthramides and avenalumic acids, which are only found in oats (Miller et al. 2000).

Again, DHA showed that the results from TSE, MAS-10075 ºC, and SFT-75 ºC were statistically different. This again shows that the extracts gathered using the non-traditional methods increased the prevention of oxidation.

Past research conducted at Louisiana State University demonstrated that antioxidants found in soy gums have shown to reduce DHA oxidation more than antioxidants found in soy oils. The reasoning behind the results was the increase in tocopherols found trapped in the gum based on oils matrix, pH, temperature, and oxygen pressure at time of extraction (Yuan pg. 44-45). The results from this research demonstrate that the tocopherols can be extracted into a lipid-based extract under the right conditions. If this data was compared to the results from Sun et al., both

had oat extracts that were statistically different from the control, showing greater inhibition capacity.

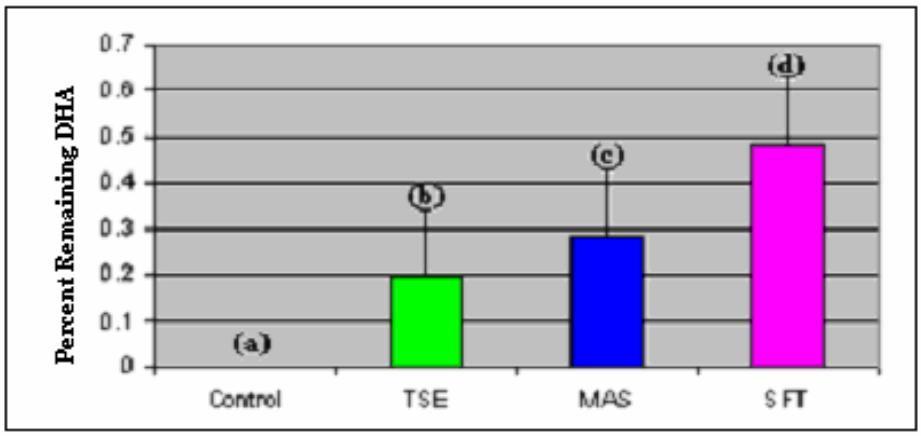


Figure 15: Un-reacted DHA Determined By GC-FID

However, Sun et al. was able to prevent 80% of the DHA from oxidizing, while the optimal sample, SFT-75ºC, from this research was able to prevent only around 50% DHA oxidation (Sun et al. 2006). This discrepancy difference might be due to the increased amount of solvent used in the research conducted by Sun et al.

CHAPTER 5. SUMMARY AND CONCLUSION

Oats have long been known for their nutritional benefits, but science has recently been showing how beneficial they are for other reasons: heath and food stability. Oats contain high levels of antioxidants. These antioxidants when concentrated in the outer layers of foods can serve a duel purpose: they can prevent oxidation of healthy oils in foods, thereby allowing better compounds to enter the body such as oat and oat bran, which in turn aids in preventing disease such as hardening of plaque, oxidized cholesterol, in the heart.

Three different sample treatments tested TSE, MAS, and SFT using four different analysis methods to determine which method was best suited for the extraction of phenolics and antioxidants from oat bran.

The results from the extract analyses demonstrated how the extract from the supercritical fluid treatment at 75ºC performed better at preventing oxidation of DHA and cholesterol, oxygen scavenging in the DPPH analysis, and offered a higher catechin equivalency value in total phenolics. However, when examined in terms of concentration, all extractions showed statistically no difference in terms of total phenolics per gram of sample.

The research shows that high quality extracts of these antioxidants can be acquired. If these extracts can be used in foods, then the potential health problems or lipid stability problems might be eliminated, leading to the development and creation of new food products on the market that are both healthy and good tasting.

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APPENDIX: STATISTICAL ANALYSIS FOR RESEARCH DATA

1. ANOVA Tables

Extraction Volumes

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	20.3333	3	SFT-75
A	19.5333	3	MAS-100
B	18.2667	3	MAS-60
C	16.6333	3	SFT-50
C	16.4667	3	TSE
D	15.0333	3	SFT-25

Percent Cholesterol Oxidized

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	57.000	4	TSE
B	27.750	4	MAS-100
C	19.250	4	SFT-75

DHA Oxidation

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.482000	4	SFT-75
B	0.282750	4	MAS-100
C	0.192000	4	TSE

Catechin Equivalency

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.270499	3	SFT75
B	0.241666	3	MAS100
C	0.197290	3	TSE

Total Phenolics per Gram of Extract

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.151913	3	SFT-75
A	0.149761	3	MAS-100
A	0.145684	3	TSE

DPPH Analysis

40µL

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.49867	3	SFT-75
B	0.26200	3	MAS-100
B	0.21033	3	TSE

80µL

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.84067	3	SFT-75
B	0.49400	3	MAS-100
B	0.37100	3	TSE

120µL

Means with the same letter are not significantly different.			
Duncan Grouping	**Mean**	**N**	**Treatment**
A	0.92067	3	SFT-75
B	0.73667	3	MAS-100
C	0.55700	3	TSE

2. SAS Coding For Running ANOVA

SAS Coding

```
title1 'Thesis data';
   data one;
      input Treatment $ Phenolics @@;
      datalines;
 (DATA)
   ;
     ods html;
   ods graphics on;

   proc anova data = one;
      class Treatment;
      model Phenolics = Treatment;
        means Treatment / lsd tukey cldiff;
           means Treatment / duncan waller;
               means Treatment/tukey lines;
run;

   ods graphics off;
   ods html close;
```

VITA

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