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Determination of antioxidant activity of polyphenol extract from grape seeds

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DETERMINATION OF ANTIOXIDANT ACTIVITY OF POLYPHENOL EXTRACT FROM GRAPE SEEDS

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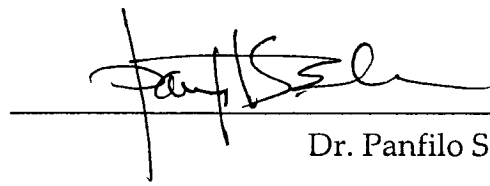

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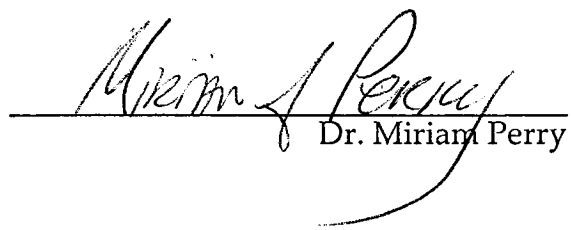

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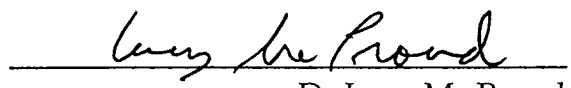

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ABSTRACT

DETERMINATION OF ANTIOXIDANT ACTIVITY OF POLYPHENOL EXTRACT FROM GRAPE SEEDS

by Jenab Shiyaji

The aim of the study was to determine the antioxidant property of grape seed polyphenol extract (GSPPE) and compare it with commercially available synthetic antioxidant butylated hydroxy toulene (BHT). Pure canola oil containing 0, 200, 500 ppm of GSPPE and BHT (200 ppm) were subjected to an accelerated oxidation test at 70°C with continuous aeration. Degree of oxidation was monitored at every 48 hours for 8 days using peroxide value (PV) and thiobarbituric acid (TBARS) analysis.

The total phenol content of grape seed extract was 43-48% gallic acid equivalent. Regression analyses for the three trials showed PV had a similar pattern of results unlike that of TBARS. This can be due to extreme heating conditions or/and interference of strong red color in spectrophotometric determination of TBARS. The GSPPE 500 ppm showed better antioxidant activity than GSPPE 200 ppm as measured by PV, but the activity was not prevalent throughout the storage period of 8 days. So based on PV, GSPPE 200 ppm and GSPPE 500 ppm are not effective antioxidants when compared with commercially used BHT.

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PREFACE

The following thesis is written in two publication styles. Chapters 1 and 3 are written according to the guidelines outlined in the Publication of the American Psychological Association, 5th edition, 2001. The second chapter is written according to Journal of Agricultural and Food Chemistry manuscript format.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Lipid oxidation is one of the major causes of food deterioration. It is of great economic importance to the food processing industry because the reaction leads to the development of rancid flavors and odors which can render edible oil and fat containing foods unacceptable or reduce their shelf life. Oxidative rancidity also reduces nutritional quality of food through the loss of essential fatty acids and fat soluble vitamins. Certain oxidative products are potentially toxic (Nawar, 1985).

Antioxidants are compounds that in small quantities delay the onset or greatly retard the oxidation of food that contain fat. Many types of compounds have been used as food antioxidants, both natural and artificial. Grape seed polyphenols are natural compounds having antioxidant properties (Negro et al., 2003; Jayprakasha et al., 2001).

Grapes (*Vitis vinifera*) are one of the world's largest fruit crops, with an annual production of approximately 58-61 million metric tons (FAO, 2002). The composition and properties of grapes have been widely investigated. It has been found that grapes contain high amounts of phenolic compounds (Bonilla et al., 1999; Guendez et al., 2005). Polyphenol in grapes have shown beneficial effects in human health such as the inhibition of oxidation of low-density lipoproteins, thereby decreasing the risk of heart diseases (Franklet et al., 1993). They also have anticarcinogenic properties (Bailey & Williams, 1994). Negro et al., (2003)

showed that grape seeds contain higher antioxidant activity than grape peel and grape marc. This is because grape seeds contain higher total phenolics than grape peel and marc.

Objective

The objective of the study was to determine the antioxidant property of grape seed polyphenolic extract (GSPPE) and compare it with the commercially used artificial antioxidant Butylated hydroxytoulene (BHT).

Significance of the Study

A lot of interest has been developed in recent years within the food industry in the use of naturally occurring antioxidants. There is increasing awareness and concern regarding the use of synthetic antioxidants like Butylated hydroxytoulene (BHT)/ butylated hydroxyanisole (BHA) since studies have provided evidence for their role as carcinogens (Madavi & Salunkhe, 1995). The use of these synthetic antioxidants is increasingly limited due to safety considerations. As a result there has been constant interest in the isolation, examination, and exploitation of natural antioxidants. Efforts have not only been focused on inexpensive plant sources but also on agricultural wastes rich in polyphenols such as canola meal and grape seeds. Moreover, of the many natural sources of antioxidants, seeds contain a variety of polyphenolic compounds that traditionally have shown to be effective in protecting the lipids within the seeds from oxidation (Murcia & Marlinez, 2001). In spite of the

richness of grape seeds in polyphenolic substances, mainly monomeric and oligomeric flavanols, studies on their use as a food lipid antioxidant are limited. Grape seed extract (GSE) has been shown to benefit inhibition of lipid oxidation in various lipid model systems (Jayprakasha et al., 2001; Negro et al., 2003). It would be of great economic significance to the grape juice and wine industry if grape seed extract is found to be as effective as synthetic antioxidants like BHT or BHA.

Review of Literature

Mechanism of Lipid Oxidation

The mechanism of fats/oils oxidation involves three phases: initiation, propogation, and termination as seen in Figure 1. (Nawar, 1985).

During initiation, the hydrogen atom (RH) adjacent to the double bond in the fatty acid is abstracted by exposure to light, heat, or metal catalysts to form a free radical. During the propogation step, the resultant free radical (R°) reacts with atmospheric oxygen to form an unstable peroxy free radical (ROO°), which may, in turn, abstract hydrogen from another unsaturated fatty acid to form hydroperoxide (ROOH) and a new free radical (R°). The resultant free radical (R°) reacts with oxygen, and the sequence of reaction is repeated. In the termination step, the chain reaction is terminated by the formation of non-radical products from the two free radicals. Also, this leads to the production of a

myriad of compounds of various molecular weights, flavor thresholds, and biological significance.

Initiation

RH---------- R° + H

Propogation

R° + O2---------- ROO°

ROO° + RH----------ROOH + R°

Termination

R° + R° ----------RR

R° + ROO° ---------- ROOR

ROO° + ROO°---------- ROOR + O2

Figure 1. Reaction of fats/oil oxidation.

The rate of oxidative reactions in oil is related to fatty acid composition and more specifically to the type and the amount of unsaturation in fatty acids. The relative rate of autoxidation of different unsaturated fatty acid esters were compared on the basis of oxygen absorption measurements. It was found that linoleate was 40 times more reactive than oleate, linolenate was 2.4 times more reactive than linoleate, and arachidonate was 2 times more reactive than linolenate (Frankel, 1998).

Food Antioxidants

Antioxidants have been used in foods for centuries. Antioxidants in food may be defined as substances which are capable of delaying, retarding, or preventing the development of rancidity or other flavor deterioration due to oxidation. Antioxidants delay the development of off-flavors by extending the induction period. The antioxidant property of an antioxidant varies with foods. The effectiveness of an antioxidant not only depends on the food but also on its processing and storage conditions. Factors such as legal standard, effectiveness, and cost determine the use of antioxidants.

Mechanism of Antioxidant Action

Antioxidants can be divided into two groups depending on their antioxidant activities (Gramza & Korczak, 2005).

1. Primary antioxidants or donors - they interrupt free radical chain reactions by donating hydrogen forming stable free radicals that don't further initiate or propogate the oxidation.
2. Secondary antioxidants or acceptors - which protect lipids by scavenging the oxygen or complexing with the metal ions or decomposing peroxides and non radical products or quenching singlet oxygen which results in the delay or inhibition of lipid oxidation.

Antioxidants can also be divided into natural or synthetic antioxidants. The most commonly used synthetic antioxidants are BHT, BHA, propyl

gallate (PG), and ascorbyl palmitate. Tertiary butylhydroquinone (TBHQ) is found to be more effective than BHT, BHA, or PG and more stable to heat processing. BHT is less soluble in fat than BHA, but both are more effective antioxidants in less unsaturated animal fats than polyunsaturated vegetable oil (Frankel, 1998). TBHQ is another effective antioxidant used in United States of America (USA) but not in Europe due to safety reasons. Though the synthetic antioxidants are used in fats and oils because of their effectiveness and cost, their safety has been questioned.

Federal Regulation of Antioxidants

In the United States, antioxidant use is subject to regulation under the Federal Food, Drug, and Cosmetic Act. Antioxidants for food products are also regulated under the Meat Inspection Act, the Poultry Inspection Act, and various state laws. The current approved levels for the use of BHA, BHT, and TBHQ in foods is 200 ppm, mixed or alone (Code of Federal Regulations, 2005).

Natural Antioxidants

Natural antioxidants may be obtained from plant or animal tissues, but the active compound may vary with the source. Most of the antioxidants are phenolic compounds and the most important groups of natural antioxidants are tocopherol, flavonoids, and phenolic acids. Flavonoids include flavones, flavonols, isoflavones, flavonones, and chalcones and they occur in all types of higher plant tissues. Phenolic acids include derivatives of benzoic acids, and

derivatives of cinnamic acids and are widely distributed in plant kingdoms (Pokorny et al., 2001).

Tocopherols are the most important natural antioxidants. They are present in all foods, at least in traces. The most important antioxidant of this group is alpha-tocopherol. Tocopherol works as an antioxidant by donating the hydrogen of the hydroxyl group to the lipid peroxy radical and stabilizes by forming stable peroxides. There are different forms of tocopherols and the hydrogen donating power of tocopherol in fats, oils, and lipoprotein is in the order of delta > beta = gamma > alpha (Pokerny et al., 2001). Unfortunately, tocopherols are much less effective than BHA or BHT (Kamal et al., 1996). Therefore, the search for natural antioxidants is highly desirable.

Various natural antioxidant extracts such as rosemary and sage have been exploited and proposed for their use as antioxidants. The antioxidant activity of rosemary and sage is due to phenolic diterpenes like carsonic and carnosal (Frankel, 1996). Significant research has also been focused on green tea extract for its use as a food antioxidant. The active ingredient present in green tea is catechin (Frankel, 1998). Studies have shown that grape seed extract is high in polyphenols, which have clear antioxidant activity (Jayprakasha et al., 2001; Negro et al., 2003). Grape seeds are also one of the industrial wastes of the wine and grape juice industry, and hence they can be used as one of the most potent and cost effective antioxidants.

Grapes

Production

Grape (*Vitis vinefera)* is one of the world's largest fruit crops, with an annual production of approximately 58-61 million metric tons. V. vinefera is called "old world grape" since most of its production occurs in Europe. This species of grape accounts for 90% of the world's grape production. Most of the production is used in making wine, but it is also the primary species used as table grapes and in the production of raisins.

The United States accounts for 11% of the world's production of grapes (USDA, 2002). In the United States, California leads with a grape production of 88% of the total. California accounts for 50% of the grapes used in wine production in the US (NASS, 2005).

Botanical Description

All Vitis are "lianas" or woody climbing vines. Grapes are cultivated on a trellis or fence. Leaves vary in size and shape depending on the variety of grapes grown. Buds of the grapes have multiple growing points or meristems. Grapes are small, round or oblong with up to four seeds. Their skin is thin and a source of anthocyanin compounds giving rise to red, blue, purple, and black colored grapes. Vinifera requires a Mediteranean climate: warm, rainless summer, low humidity, and mild winter temperatures.

Chemical Composition of Grapes

Grape composition varies with the climate, growing conditions, maturity of grape at harvest, and genetic strain or species (Fuleki et al., 1997; Negro et al., 2003). The composition and properties of grapes have been extensively investigated and it was found that grapes contain a large amount of phenolic compounds. Red grapes are found to be a richer source of polyphenolics than white grapes (Guendez et al., 2005). Various studies on phenolic content of different parts of grapes like grape marc, grape skin, and grape seeds have shown that grape seeds have the highest amount of phenolic compounds (Negro et al., 2003).

Grape Seeds

Grape seeds are a complex matrix which contain approximately 40% fiber, 16% oil, 11% proteins, and 7% complex phenols besides sugar, mineral salts etc (Kim et al., 2006). The active compound found in grape seed is proanthocyanidin (Negro et al., 2003). Grape seed proanthocyanidins refer to procyanidin mixtures extracted from grape (*Vitis vinifera*) seeds. Procyanidins are derivatives of the flavan-3-ol class of flavonoids. This class includes (+)-catechin, commonly referred to as catechin, and (-)-epicatechin, commonly referred to as epicatechin. Procyanidins are dimers and oligomers of catechin and epicatechin and their gallic acid esters. Both catechin and procyanidin exhibit their antioxidant activity via free radical scavenging (Silva et al., 1991).

The inhibitory potential of grape seed proanthocyanidin related to lipid peroxidation appears to increase with the degree of polymerization of the molecules. That is, grape seed proanthocyanidins with a greater number of catechin and epicatechin units appear to have more potent inhibitory activity than those with fewer catechin and epicatechin units (Gabetta et al., 2000).

In wine and grapes, proanthocyanidins play an important role in organoleptic features such as color, astringency and bitterness, mouthfeel, and ageing characteristics.

Antioxidative activity of grape seed extract has been confirmed by beta-carotene linoleate and linoleic acid peroxidation methods (Jayaprakasha et al., 2001) as well as by diphenyl picryl hydrazyl (DPPH) and phosphomolybdenum complex methods (Jayaprakasha, Selvi, & Sakariah, 2003). Studies have also shown that GSPPE has antimicrobial activity (Baydar et al., 2004), suppresses peroxidation and reduces hypoxic ischemic injury in rats (Feng et al., 2005), shows antithrombotic effect (Sano et al., 2005), is anticarcinogenic, and shows antiulcer activity in rats (Yilmez et al., 2004).

These health benefits have led to the formulation of dietary supplements of grape seeds extracts. These are sold commercially in the form of tablets under various brand names, though the legal regulations of the products are still under consideration (Monagas et al., 2005).

Several in vitro studies have been done to test the efficacy of GSPPE for their use as food lipid antioxidants. Studies done to measure the antioxidant activity of GSPPE in various meat products such as cooked beef (Ahn et al., 2002), turkey meat (Lau et al., 2003; Meilinki, 2005), and in raw and cooked pork (Carpenter et al., 2007) found that GSPPE at 400-10,000 ppm was very effective in inhibiting the lipid oxidation in meat products. In another study done using GSPPE at 2000 ppm in refined poppy seed oil tested under accelerated conditions, it was seen that GSPPE was more active than BHT and BHA (Baydar et al., 2006). However GSPPE at 200 ppm in sunflower oil under accelerated conditions had no effect on primary oxidation products and showed prooxidant activity on secondary antioxidant products.

Accelerated Test for Lipid Oxidation

Accelerated Stability Test

The accelerated test for lipid oxidation is carried out to estimate the stability or susceptibility of a fat to oxidation. The test is carried out by subjecting the sample to an accelerated oxidation test under standardized conditions and a suitable end point is chosen to determine signs of oxidative deterioration. For this, several parameters such as temperature, oxygen pressure, metal catalyst, or/and shaking are manipulated to measure rancidity or stability of oil, or efficacy of antioxidant (Frankel, E. N., 1993). Frankel, (1993) have

reviewed the various conventional stability tests which show the oxidation test based on the severity of the oxidation condition used, as shown in Table 1.

Table 1 *Table showing the Standard Accelerated Stability Test*

Test	Conditions
Ambient temperature	Room temperature, atmospheric pressure
Light	Room temperature, atmospheric pressure
Metal catalyst	Room temperature, atmospheric pressure
Weight gain method	30-80°C, atmospheric pressure
Schaal oven	60-70°C, atmospheric pressure
Oxygen uptake	80-100°C, atmospheric pressure
Oxygen bomb (ASTM)[1]	99°C, 65-115 psi oxygen
Active oxygen (AOM)	98°C, air bubbling
Rancimat	100-140°C

[1] ASTM, American society for testing materials.

Decker et al., (2005) has developed model systems for the evaluation of antioxidants in different food systems. In bulk oil, the autoxidation by oven storage test under accelerated conditions in dark at 60°C and in oil-in-water emulsions, oxidation conducted at temperature less than 60°C. However the model systems are not inclusive of all the possible methods that measure lipid oxidation.

Lipid Oxidation Assessment

Various chemical methods, sensory tests, and instrumental techniques are often used to measure the oxidation of lipids (Wanasundara et al., 1994). The method selected for assessing the oil quality depends on various factors such as the accuracy of the measurement desired, resources available for carrying out the test, and the time allowed or available.

A classic method used for the quantification of hydroperoxide is the determination of peroxide value (PV). Hydroperoxide is the primary product of lipid oxidation, so PV is often used as an indicator for initial stage of lipid oxidation (Gray, 1978; Baydar et al., 2007). Although determination of PV is common, the usefulness of this measure is generally limited to the initial stages of lipid oxidation since peroxides decompose rapidly during storage and upon heating.

Measuring conjugate dienes and conjugate trienes are quick physical methods used to assess the oxidative stability of oil. Studies have shown that the PV and conjugate diene methods of edible oil correlate well during their oxidation. So the conjugate diene method may be used as an index of stability of lipids in place of or in addition to, peroxide value.

Decomposition of the primary products forms secondary oxidative products. A classic method used to measure the secondary oxidative products is the 2-thiobarbituric acid (TBA) test (Gray, 1978). TBA measures malonaldehyde and other TBA-reactive substances, hence it is referred to as the thiobarbituric acid reactive substances (TBARS) method (Frankel, 1998). TBARS is the most widely used method to determine the extent of lipid oxidation. The other method is p-Anisidine value that measures secondary products such as carbonyl compounds.

Although chemical and physical methods are used to assess the oxidative stability, sensory evaluation is the ultimate method to determine the flavor, odor, and color quality of vegetable oil.

Extraction of the Phenolics

Extraction of plant phenols is carried out usually by the solvent extraction method using various solvents at different concentations (Pekic et al., 1998). Grape seed phenolics mainly proanthocyanidins viz. catechins and epicatechins are well soluble in ethyl acetate. Water as a solvent has shown to increase the permeability of grape seeds, as a result increasing the phenol content. A recent study has shown that ethyl acetate:methanol:water (60:30:10) yielded higher phenolic extract than acetone:water:acetic acid (90:9.5:0.5) (Baydar et al., 2004).

Total phenol analyses. There have been several methods proposed for estimating phenols. Of these methods only two had survived: the Neubauer-Lawenthal method and Folin-Denis method. The first one was for some time the official method but was later replaced by the Folin-Denis method. More recently Folin-Ciocalteu reagent replaced the Folin-Denis reagent method. The difference is that Folin-Ciocalteu reagent contains a higher percentage of molybdate in the complex and is more easily reduced. The lithium present, prevents precipitation, which causes problems using the Folin-Denis reagent. However total phenol

assay does not identify a particular phenolic compound that is responsible for the antioxidant activity in the sample.

Oxidation Test Substrate or Medium

The antioxidant behavior in lipids and lipid containing foods also depends on the substrate used, since the rate of lipid oxidation is dependent on the type and degree of lipid unsaturation, besides the other test conditions like temperature, pressure, metal, etc. (Pokerny, 2001).

Canola Oil as a Medium

Definition. Canola is a rapeseed cultivar that contains less than 2% erucic acid in its oil and 30 micromoles/ g of glucosinolates in defatted meal. In 1988, the United States Food and Drug Administration (FDA) granted the GRAS (Generally Recognized as Safe) status to canola oil. Canola oil is being widely used as salad and cooking oil, tablespread, and shortening.

Fatty acid composition. Canola oil has a healthy fatty acid profile because of its low level of saturates and high level of unsaturates. Canola oil has a high content (8-12%) of alpha linolenic acid (C18:3 w3) as compared to soybean oil (8.0%), sunflower oil (0.2%), and olive oil (0.8%). However the content of linoleic fatty acid (18:2 w6) is low (22-25%) compared to soybean oil (57-60%). The ratio of linolenic to linoleic acid is 1:2; which is considered to be nutritionally favorable.

Stability of canola oil. The high content of unsaturated fatty acids in canola oil influences the stability and quality of the oil. The rate of oxidation increases with the increase in unsaturation of fatty acids. Linolenic fatty acid oxidizes twice as fast as linoleic acid, and 25 times faster than oleic acid. Oxidation of oil results in development of off flavor and odor is a major concern for canola oil during storage and heating.

CHAPTER 2

JOURNAL ARTICLE

Author's Title Page

Determination of Antioxidant Activity of Polyphenols from Grape Seeds

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ABSTRACT

The aim of the study was to determine the antioxidant property of grape seed polyphenol extract (GSPPE) and compare it with commercially available synthetic antioxidant butylated hydroxy toulene (BHT). Pure canola oil containing 0, 200, 500 ppm of GSPPE and BHT (200 ppm) was subjected to an accelerated oxidation test at 70°C with continuous aeration. Degree of oxidation was monitored at every 48 hours for 8 days using peroxide value (PV) and thiobarbituric acid (TBARS) analysis.

The total phenol content of grape seed extract was 43-48% gallic acid equivalent. Regression analyses for the three trials showed PV had a similar pattern of results unlike that of TBARS. This can be due to extreme heating conditions or/and interference of strong red color in spectrophotometric determination of TBARS. The GSPPE 500 ppm showed better antioxidant activity than GSPPE 200 ppm as measured by PV, but the activity was not prevalent throughout the storage period of 8 days. So based on PV, GSPPE 200 ppm and GSPPE 500 ppm are not effective antioxidants when compared with commercially used BHT.

KEYWORDS: Grape seed extract; antioxidant; polyphenols; canola oil

INTRODUCTION

Autoxidation is one of the main routes of spoilage in fats and oils, this may lead to oxidative rancidity via free radical mechanism *(15)*. The rate of oxidation reaction is related to fatty acid composition and more specifically to the type and amount of unsaturation. Vegetable oil contains substantial amounts of unsaturated fatty acids, which may undergo rapid oxidation leading to off flavor and off odor.

Antioxidants are the substances that are added to fats and oils to retard oxidation of unsaturated fatty acids and thus decrease the development of rancidity *(15)*. Phenolic substances have shown clear antioxidant activity by inhibiting oxidative reaction generally by donating hydrogen and forming relatively stable free radical products *(13)*. Commonly used synthetic antioxidants are butylated hydroxy toulene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroquinone (TBHQ). There is increasing awareness concerning the use of these synthetic antioxidants since they have been shown as promoters of carcinogenesis. Hence there is constant search for the identification of naturally available antioxidants.

The most common sources of naturally occurring phenolics are plants, but the phenolics may vary with the source viz. different parts of a plant like seeds, stems, barks, or leaves and also with different plants. In recent years, there has

been focus on inexpensive plant sources such as industrial waste, especially that containing residual phenols from the plant raw materials used.

Grape (*Vitis vinefera)* is one of the world's largest fruit crops, with an annual production of approximately 58-61 million metric tons (FAO, 2002). Studies on polyphenol content in various parts of the grape have shown that polyphenol content from grape seeds is higher than those from grape skin and pomace *(12, 13).* The main phenolic compound present in grape seed is proanthocyanidin, which acts as a free radical scavenger. Negro et al. *(12)* and Jayprakasha et al. *(9)* have shown clear antioxidant activity of grape seed polyphenol extract (GSPPE) in various lipid model systems. Thus in spite of the richness of grape seed in polyphenols, studies on their use as food lipid antioxidant is limited. Also it would be of great economic significance to grape and wine industries if GSPPE has antioxidant activity comparable to BHT. It is possible that under accelerated oxidation test conditions the antioxidant activity of polyphenols of grape seeds may have activity comparable to BHT. The results of the study will not only contribute to the knowledge of antioxidant activity of grape seeds, but also open future research prospects for their use as natural food lipid antioxidants. For these reasons, the current study was undertaken to measure the antioxidant properties of polyphenols from grape seeds and compare it with BHT in canola oil.

MATERIALS AND METHODS

Materials. Red grapes (variety Red Globe) and pure canola oil (Wesson brand) with no added antioxidants were purchased from a local grocery store. BHT was purchased from Sigma chemicals. All the reagents used were of analytical grade.

Preparation of Grape Seed Extracts (GSE). GSE was prepared according to the method described by Baydar et al. *(3)*. Grape seeds were manually separated from the berries and were dried at 70°C for 24 hours. The dried grape seeds were ground to fine powder with a grinder. Then the powdered grape seeds (90 g) were extracted in a Soxhlet extractor (Precision Soxhlet) with hexane at 60°C for six hours to remove fatty materials from seeds. The defatted grape seed powder was reextracted in the Soxhlet apparatus (Precision Soxhlet) for eight hours with 200 ml of ethyl acetate: methanol: water (60:30:10, v/v/v) at 60°C. The extract obtained was concentrated by using rotary evaporation (Rotavapor, Buchi laboratory-Technik Ag) under vacuum at 70°C to get a concentrated extract. This extract was dried in a vacuum oven to get a dried crude extract. This extract was stored under refrigeration until use.

Total Phenol Analysis. Analysis of total phenols was performed according to the method of Singleton and Rossi *(16)* using gallic acid as the standard solution. The principle of the method is based on the observation that the phenolics present in the sample are converted to phenolate ions in presence

of an oxidizing agent i.e Folin-Ciacalteau, and an alkali i.e. Sodium carbonate to give a blue color complex, which is read colorimetrically at 765 nm and compared with gallic acid standards.

Procedure. Estimation was carried out in duplicates. Samples (0.2 ml) were mixed with 1.0 ml of tenfold diluted Folin-Ciocalteau reagent and 0.8 ml of 7.5% sodium carbonate solution. After standing for 30 minutes at room temperature the absorbance was measured at 765 nm.

To determine the concentration of total phenols in our sample, a standard curve was plotted based on the obtained absorbance of several known concentrations of the standard solutions of total phenols (0-500 mg/L). Then the rest of the procedure was performed as described previously. Total phenolics were calculated as per gram of the extract. Commercial standard BHT was used at 200 ppm.

Experimental Design. To determine the antioxidant property of the GSPPE, a total of three trials were carried out involving accelerated lipid oxidation of canola oil as shown in **Figure 2**. The accelerated test was carried out in a 250 ml conical flask using 200 ml 100% pure canola oil. The flasks were kept in a drying oven (American drying oven, American Scientific products). The temperature of the oven was maintained at 70°C with constant aeration and the rate of oxidation was controlled by connecting to a vacuum and adjusting it to 175-180 bubbles/minute. Continuous aeration and heating were maintained for

a period of eight days. Thirty ml oil samples from each flask were withdrawn at an interval of every 48 hours and analyzed for peroxide values, and TBARS analysis. No fresh oil was added to replace the oil removed during sampling. Three replicate trials were run at different times.

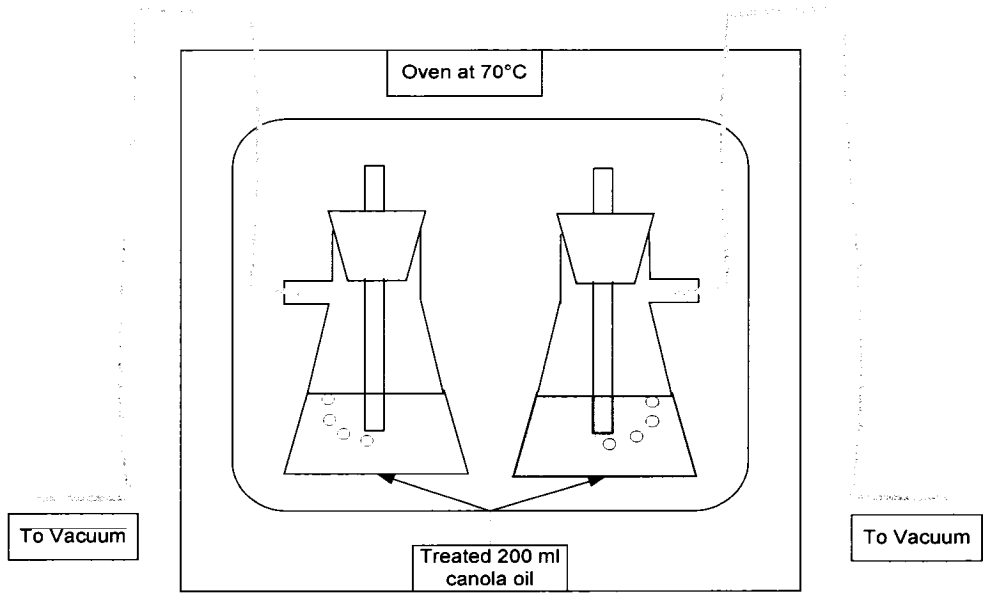

Figure 2. Schematic diagram for accelerated oxidation test.

The oxidation of canola oil was studied using three antioxidant treatments. These were: pure canola oil treated with 200 ppm of GSPPE, pure canola oil treated with 500 ppm of GSPPE, and pure canola oil treated with 200 ppm of BHT. Pure canola oil with no added antioxidant was used as control. The additives were dissolved in minimum amount of ethanol and then added to the oil. In the control sample with no added antioxidant, the same amount of ethanol was added.

Evaluation of Lipid Oxidation in Canola Oil. The evaluation of lipid oxidation was carried out by measuring peroxide value (PV) and TBARS at every 48 hours interval for a period of eight days.

Peroxide Value Determination. Peroxide value is expressed as milliequivalent of oxygen per kg of oil. PV was measured according to American Oil Chemist Society method 965.33 *(2)*. The principle is based on the reduction of the hydroperoxide group (ROOH) with iodide (I-). The amount of iodine (I_2) released is proportional to the concentration of peroxide present. Released I_2 is assessed by titration against a standardized solution of sodium thiosulfate ($Na_2S_2O_3$) using a starch indicator.

For the peroxide value estimation, thirty ml of acetic acid-$CHCl_3$ solution was added to 5 ml of the sample. Flask was swirled until the sample dissolved in the solution. Then 0.5 ml saturated potassium iodide (KI) was added. The solution was made to stand and occasionally shaken for one minute. It was then titrated against sodium thiosulphate solution until the blue color disappeared. Starch solution was used as an indicator.

Thiobarbituric Acid Reactive Substances (TBARS) Determination. The TBARS values of the oil samples were expressed as micrograms of malonaldehyde (MDA) per gram of oil. TBARS test was carried out by the method outlined by Botsoglou et al. *(5)*. This method is based on spectrophotometric quantification

of the pink colored complex formed after the reaction of MDA with two molecules of TBARS. The results were the mean of duplicate measurements.

For the TBARS estimation, eight ml of extracting solution that is 5% Trichloroaceitic acid (TCA) was added to 2 ml of the sample. To ensure no oxidation proceeds, further 0.8% BHT was added. The solution was than centrifuged and the top hexane layer was discarded. The bottom aqueous layer was brought up to 10 ml with 5% TCA, 2.5 ml aliquot was pipetted, to which a volume of 1.5 ml 0.8% thiobarbituric acid (TBA) was added. It was then incubated for 30 minutes at 70°C and cooled under tap water. Absorbance was read at 521.5 nm spectrophotometrically against a reagent blank sample.

To determine the TBARS value in our samples, a standard curve was plotted based on the obtained absorbance of several known concentrations of standard solutions of MDA (0.15-2.39 microgram/ml). Five percent of TCA was added to the standards to bring the volume up to 2.5 ml. Then 1.5 ml 0.8% TBA was added, mixed, and kept aside. It was read spectrophotometrically at 521.5 nm. From the standard curve, MDA (microgram of MDA/g of oil) concentration was calculated, and TBARS was determined.

Statistical Analysis. Microsoft Excel 11.0 was used for the statistical analysis in this study. Two-way analysis of variance (ANOVA) was performed to determine if there were significant differences in the PV and TBARS values between different storage times and different antioxidant treatments at 95%

confidence level. Least Significant Difference (LSD) was calculated to compare the means when analysis of variance indicated a significant difference at $p< 0.05$. Using t-statistics, commonly used in biostatistics when comparing two treatments, hypothesis testing on equality of the slopes of the regression was performed at 95% confidence interval, as shown in Appendix *(17)*.

RESULTS AND DISCUSSION

Total Phenolics of Grape Seed Extract. The total phenolics of grape seed extract were determined using the method proposed by Singleton and Rossi *(16)*. **Table 2** shows the polyphenol content of fresh grape seed as gallic acid equivalents (GAE) from Red Globe variety red grape. Another study found that Negro Amaro red grape variety had 8.58% GAE on dry basis *(12)*. A study by Guedez et al. *(8)* showed that polyphenol content in several varieties of red grapes seeds was between 44 -1083 mg/100g grape seed. The differences in the polyphenol content may be due to genetic potential of individual species for polyphenol synthesis, maturation, irrigation, and/or climatic conditions *(8)*.

Table 2. Polyphenolic Content of Grape and Grape Seed Extract

Sample	Percent Polyphenol content (Gallic acid equivalent)
Grape Seed (wet basis)	2.3-2.5
Grape Seed (dry basis)	4.0-4.5
Grape seed extract	43-48

The phenolic content of grape seed extract for all the three trials was different, ranging from 43-48% GAE. Based on the phenolic content, the appropriate weights of crude grape seed phenolic extract were used to bring the final concentration to 200 ppm and 500 ppm of polyphenols. The total phenolics in grape seed extract obtained by Baydar et al. *(3)* and Shaker (2005) were 62.7% GAE and 69.2% catechin equivalents (CE) respectively. This may be due to differences in the individual species of grapes *(8)* and different extraction procedures.

Oxidation of Canola Oil. The addition of natural and synthetic antioxidants to canola oil affected the Peroxide Value and TBARS values to different degrees during accelerated oxidation at 70°C for 8 days. Peroxide Value (PV) measures primary products of lipid oxidation and TBARS Value measures the formation of secondary oxidation products, mainly malonaldehyde, which may contribute off-flavor to oxidized oil.

Peroxide Value. As shown in **Figure 3** and **Table 3**, as the oxidation time increases from day 0 to day 8, the PV also increases for all the treated and control samples. As seen from **Figure 3**, BHT had the lowest PV (expressed as meq of O_2/kg) throughout the accelerated oxidation condition followed by the GSPPE-500 ppm, GSPPE-200 ppm, and the control sample.

Table 3. Peroxide Values (milliequivalent of oxygen per kg of oil) of the Canola Oil treated with BHT and GSPPE[1]

Days	Treatment			
	Control	BHT	GSPPE-200 ppm	GSPPE-500 ppm
0	x12.42[a] ±1.09	x5.25[a] ±2.19	x9.50[a] ±0.28	x3.35[a] ±3.46
2	x33.48[a] ±11.14	x15.60[a] ±7.49	x22.40[a] ±23.72	x19.90[a] ±20.22
4	y186.60[a] ±99.01	x40.40[b] ±15.36	y90.40[c] ±68.22	x38.50[b] ±4.81
6	y179.80[a] ±28.11	y100.90[b] ±100.06	z202.20[a] ±93.74	y164.10[a] ±71.55
8	y207.00[a] ±12.55	y120.60[b] ±98.86	z192.40[a] ±29.20	y180.90[a] ±64.07

[1] Values are the average of three trials. abc - mean values (± SD) within the same row bearing different superscripts are significantly different at $p<0.05$. xyz - mean values (± SD) within the same column bearing different superscripts are significantly different at $p<0.05$.

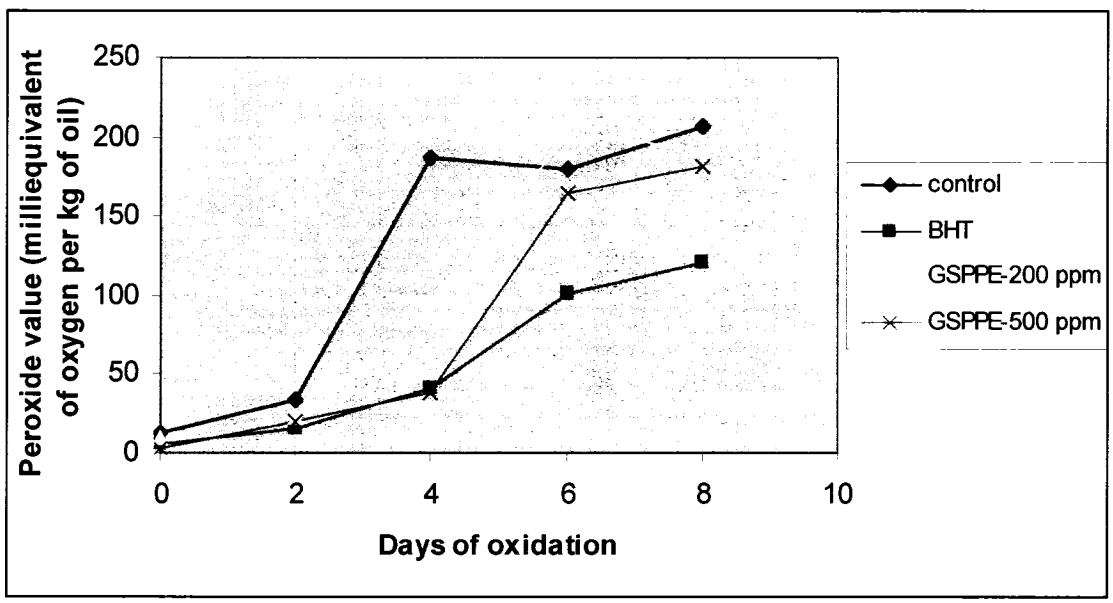

Figure 3. Peroxide Values (milliequivalent of oxygen per kg of oil) of canola oil from day 0 to day 8.

Analysis of variance of the data showed that there were significant differences in the PV of the canola oil samples, between different oxidation times ($p<0.05$) and different treatments ($p<0.05$). Based on Least Significant Difference (LSD) value, there was no significant difference in the PV between BHT and GSPPE 500 ppm treated sample up to day 4. However GSPPE 200 ppm and control sample were significantly different from the BHT after day 2. Also GSPPE 500 ppm showed significant difference from the control sample as well as GSPPE 200 ppm sample after day 2. Throughout the experiment, the PV of GSPPE 200 ppm treated sample was significantly different from the BHT treated sample. It can be said that GSPPE 200 ppm is not effective in lowering the primary oxidation products. Similar result was also found by Shaker, (2005) GSPPE at 200 ppm did not have any effect on the conjugated diene hydroperoxide in sunflower oil, when the oil was subjected to accelerated conditions at 60°C.

Table 3 shows the mean of three trials for PV. The values show that standard deviation is very high. Hence it is important to analyze individual trials to determine the consistency among the trials, and to determine the relationship between the treatments within each trial. Based on **Table 4** for the regression analyses, trial 1 and trial 3 showed almost similar patterns of PV throughout the experiment.

Table 4. Table showing the calculated t-value from Regression Analyses of Peroxide Value (PV) for each trial

Treatments	Trials		
	Trial 1	Trial 2	Trial 3
Control vs BHT	2.48*	1.49	5.56*
Control vs GSPPE 200 ppm	0.05	4.01*	0.37
Control vs GSPPE 500 ppm	0.16	4.63*	2.46*
BHT vs GSPPE 200 ppm	3.85*	2.99*	5.54*
BHT vs GSPPE 500 ppm	2.45*	3.64*	7.7*
GSPPE-200 vs GSPPE-500	0.31	0.01	2.18

* Significant difference between two slopes at $p<0.05$.

Regression analyses show the overall difference between the treatments from day 0 to day 8. The BHT treated sample was significantly different from the GSPPE 500 ppm, GSPPE 200 ppm, and the control samples. Also, no significant difference was seen between the GSPPE 500 ppm and GSPPE 200 ppm samples.

PV measures the primary product of lipid oxidation. Based on **Table 3** GSPPE at 500 ppm shows lower PV than the control sample. This shows that GSPPE has antioxidant activity and delays or slows the rate of oxidation up to day 4 comparable to BHT. However, due to stressful conditions of oxidation and rapid decomposition of peroxides upon heating, there was significant difference between the BHT and the GSPPE 500 ppm samples overall (day 0 to day 8).

TBARS Value. The results of the TBARS test of the canola oil sample showed that for all the three treatments, the TBARS values (expressed as microgram of MDA per gram of oil) increased as time progressed from 0 to 6 days (**Table 5**), and the value declined after that. The MDA value was highest

for the GSPPE 200 ppm treated oil sample and lowest for the BHT treated canola oil sample during the entire storage period of 8 days. As shown in **Figure 4,** increase in TBARS values was slow up to 4 days and than the values rose very fast. This may be due to the fact that TBARS values measures the secondary oxidative product, malonaldehyde. From the **Figure 4,** the BHT treated canola oil sample had the lowest TBARS values followed by the control sample, and grape seed extract samples.

Table 5. TBARS Values (microgram of malonaldehyde /g of oil) of the Canola Oil treated with BHT and GSPPE[1]

Days	Treatment			
	Control	BHT	GSPPE-200 ppm	GSPPE-500 ppm
0	x0.38^{a} ±0.44	x0.29^{a} ±0.28	x0.72^{bc} ±0.26	x0.45^{ac} ±0.12
2	xy0.60^{a} ±0.00	x0.37^{a} ±0.10	x0.95^{b} ±0.48	x0.36^{a} ±0.01
4	y0.71^{a} ±0.51	x0.31^{b} ±0.22	x0.98^{ac} ±0.56	y1.31^{c} ±1.46
6	z1.60^{a} ±0.89	y1.53^{a} ±1.85	y2.20^{c} ±1.31	z1.77^{a} ±0.91
8	z1.86^{a} ±0.93	z1.08^{b} ±0.85	y1.90^{a} ±0.53	z1.73^{a} ±1.24

[1] Values are the average of three trials. abc - mean values (± SD) within the same row bearing different superscripts are significantly different at $p<0.05$.xyz – mean values (± SD) within the same column bearing different superscripts are significantly different at $p<0.05$.

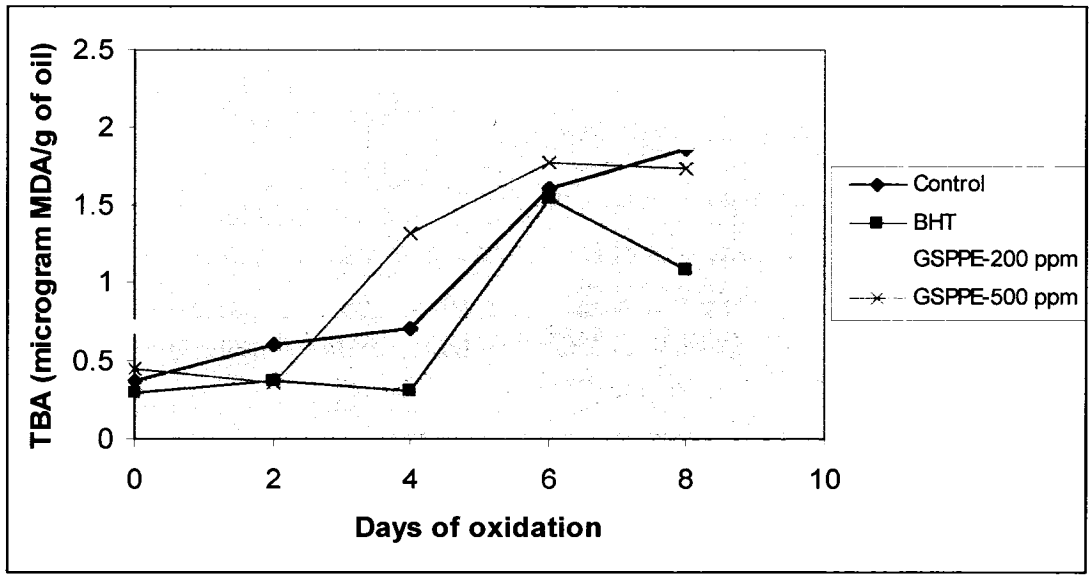

Figure 4. TBARS Values (microgram of MDA/gram of oil) of canola oil from day 0 to day 8.

Analysis of variance data shows that at $p<0.05$, there exists a significant difference between the treatments and between the oxidation times. Based on LSD value, no significant difference was found in the TBARS values for all the treated samples and control samples from day 0 to day 2 as well as between day 6 and day 8. Also, LSD value showed that there was a significant difference between the GSPPE 200 ppm and BHT throughout the storage time. This shows that GSPPE 200 ppm had no effect on TBARS values.

Table 6 shows the mean of three trials for TBARS. The values show that standard deviation is very high. It is therefore important to analyze individual trials to determine the consistency among the trials, and to determine the relationship between the treatments within each trial.

Table 6. Table Showing the calculated t-value from Regression Analysis of TBARS for each trial

Treatments	Trials		
	Trial 1	Trial 2	Trial 3
Control vs BHT	0.48	12.5	0.65
Control vs GSPPE 200 ppm	1.78	20.25*	1.61
Control vs GSPPE 500 ppm	3.88*	20.25*	1.41
BHT vs GSPPE 200 ppm	1.375	0.81	1.29
BHT vs GSPPE 500 ppm	3.14*	0.80	1.42
GSPPE 200 ppm vs GSPPE 500 ppm	1.09	0.02	0.90

* Significant difference between two slopes at $p<0.05$.

Based on the **Table 6** for the regression analyses for TBARS value, no consistency or similarity was observed in the results between the three trials. The possible reasons might be extreme heating conditions (70°C) since the mechanism and kinetics of oxidation are not the same as those at lower temperatures (less than 60°C). Also temperatures above 60°C can cause rapid decomposition of hydroperoxides, decomposition or volatility of antioxidants, and depletion of oxygen as solubility of oxygen decreases with increasing temperature *(7)*. Besides, GSPPE had a strong red color, which might have also affected or interfered with the results of the spectophotomeric determination of TBARS test.

Based on the results of the study it was observed that over the three trials PV showed similar pattern of results unlike that of TBARS values. The GSPPE at 500 ppm showed better antioxidant activity than GSPPE at 200 ppm as measured by PV, but the activity was not prevalent throughout the storage period of 8 days

when compared to BHT. So based on PV, GSPPE 200 ppm and GSPPE 500 ppm are not effective antioxidants when compared with commercially used BHT. Shaker, (2005) reported that GSPPE at 200 ppm in sunflower oil showed no effect on primary oxidation products measured by conjugate diene hydroperoxides (CD). It also found pro-oxidant activity for the secondary lipid oxidation products, measured by static headspace gas chromatography (SHGC) and Proton transfer reaction-mass spectrometry (PTR-MS) during the seven day storage at 60°C. The author's explanation for proxidant activity of GSPPE was that the sunflower oil has naturally present antioxidants and hence it did not require any added antioxidants for the stability of the oil. The result of the present study did not find any apparent antioxidant activity of GSPPE 200 ppm, based on PV and TBARS values.

However, according to the study done by Baydar et al. *(4)*, GSE at 2000 ppm in refined poppy seed oil tested at 70°C in the dark for eight weeks where oxidation was measured by PV value found that GSPPE was more active than BHT and BHA. The explanation for the difference in the antioxidant activity of GSPPE in the current study might be due to the fact that the concentrations used in the current study were GSPPE 200 ppm and 500 ppm which were very low compared to 2000 ppm of GSPPE in the above mentioned study.

As shown by various studies done in different lipid model systems such as antiradical activity measured by beta-carotene bleaching test and diphenyl

picryl hydrazyl (DPPH) method for GSPPE concentration at 25-160 ppm, the activity of GSPPE was concentration dependent and comparable to that of BHT and BHA *(9, 12)*. In all of the above mentioned studies the antioxidant activity of GSPPE was attributed to its hydrogen donating ability and thus is a primary antioxidant that reacts with free radicals forming stable free radical which do not further initiate or propagate the oxidation.

Studies done to determine the antioxidant activity in various meat products such as cooked beef *(1)*, turkey meat *(10, 11)*, and in raw and cooked pork *(6)* found that GSPPE was very effective in inhibiting the lipid oxidation in meat products. In these studies, commercially available grape seed extract was used, which has a high content of polyphenolics. Difference in the antioxidant activity in these studies and the current study may be due to the use of higher polyphenolic concentration (400 -10000 ppm) of grape seed extract in these meat products, as well as different storage times and temperatures. The difference in the meat product studies may be also due to the use of actual commercial situations unlike the accelerated conditions used in the current study.

Above discussion shows that antioxidant activity of an antioxidants is dependent on its concentration used, type of substrate, condition used such as light and temperature, as well as method of extraction for polyphenolics extracts.

CONCLUSION

Polyphenol extract of grape seeds showed no apparent antioxidant activity compared to that of BHT in canola oil during an eight day storage period under accelerated condition, as measured by PV and TBARS values of the canola oil sample. GSPPE at 500 ppm did lower the PV from day 0 to day 4 and it was comparable to the BHT. However, during the entire storage period of eight days, the activity of GSPPE 500 ppm was not comparable to the BHT. The results of the study also showed that over the three trials PV showed similar pattern of the results unlike that of TBARS.

Based on the results of the current study, GSPPE may be investigated to test its efficacy at higher concentrations (> 500 ppm), accelerated conditions with the temperature less than or equal to 60°C, and with more frequent sample analyses for oxidation.

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CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

Autoxidation is considered to be the main route of spoilage for edible oils and its progression leads to oxidative rancidity via a free radical mechanism. Vegetable oils contain substantial amounts of unsaturated fatty acids which undergo rapid oxidation and produce off flavors and off odors. Antioxidants are therefore added to fats and oils to retard oxidation of unsaturated fatty acids which in turn increase the shelf life of the fats/oils. Due to toxicological issues, use of synthetic antioxidants such as BHT/BHA is reduced so there is constant interest in the isolation, examination, and exploitation of natural antioxidants. Phenolic substances have shown to possess strong antioxidant properties. So the focus is not only on inexpensive plant sources but also on the agricultural waste rich in polyphenols. The aim of this study was to determine the antioxidant properties of polyphenol from grape seed extract and to compare it with that of BHT. The antioxidant property of GSPPE (GSPPE 200 ppm and 500 ppm) and BHT (200 ppm) was evaluated by measuring the PV, and TBARS values using accelerated oxidation carried out at 70°C with continuous aeration for 8 days. A Canola oil sample without addition of antioxidants was used as a control. The sample was collected every 48 hours for analyses.

The results of the study indicated that during the eight days of storage period under accelerated conditions, GSPPE for the tested concentrations does not possess the apparent antioxidant activity comparable to the BHT. The

GSPPE at 500 ppm showed better antioxidant activity than GSPPE at 200 ppm as measured by PV but the activity was not prevalent throughout the storage period of 8 days.

Recommendations

In this study, the antioxidant activity of GSPPE was determined in canola oil under accelerated oxidation conditions in order to test the efficacy in a pure state. Based on the results it is recommended that the efficacy also be tested at ambient temperature or at temperatures less than 60°C, since accelerated conditions used in the current study are more extreme conditions. Also various higher concentrations (greater than 500 ppm) of GSPPE may be tested for the most effective level.

Further modification is needed in the extraction of polyphenols from grape seed since it will not only increase the polyphenol content i.e. a purer extract, but will also affect the solubility in oil. Comparison should also be made using commercially available grape seed polyphenolic tablets since they contain higher phenolics. Since lipid oxidation under accelerated conditions is faster, more frequent sample analyses for oxidation should be carried out.

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Appendix

Regression Analyses

Regression analyses for comparing two slopes using t-test

Test the hypothesis that the slopes are equal for different treatments:

H_0: $\beta_1 = \beta_2$ and H_1: $\beta_1 <> \beta_2$ (significance level $\alpha = 0.05$)

Test statistics:

Xi =Independent variable, Yi= Dependent variable

$\Sigma x^2 = \Sigma Xi^2 - (\Sigma Xi)^2/n$ and $\Sigma y^2 = \Sigma Yi^2 - (\Sigma Yi)^2/n$

$\Sigma xy = \Sigma XiYi - (\Sigma Xi \Sigma Yi/n)$

$B = \Sigma xy / \Sigma x^2$

Residual SS = $\Sigma y^2 - (\Sigma xy)^2 / \Sigma x^2$

Residual DF = n-2

$(Syx)^2p$ = (Residual SS)$_1$ + (Residual SS)$_2$/ (Residual DF) $_1$ + (Residual DF)$_2$

$Sb1\text{-}b_2 = \sqrt{ ((Syx)^2p / (\Sigma x^2)_1 + (Syx)^2p / (\Sigma x^2)_2)}$

Test statistic $t_0 = (b_1\text{-}b_2)/ Sb_1\text{-}b_2$

Degree of Freedom = $(n_1 - 2) + (n_2 -2) = n_1 + n_2 -4$

Rejection region: Reject H_0 if $t_0 > t\alpha/_2, n_1+n_2-4$ or $t_0 < t\alpha/_2, n_1+n_2-4$