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Flavor and oxidative stability of walnuts partially defatted using supercritical carbon dioxide

by

Tammy Dawn Crowe

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

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ABSTRACT

During the past several years, walnuts have gained popularity because of their good taste, high n-3 fatty acid content, and reported hypocholesterolemic effects. Two negative components of walnut consumption are the relatively high fat content of this commodity (~ 70%), and the lack of oxidative stability caused by the high level of fat and its polyunsaturated nature. The objectives of this study were to 1) use supercritical carbon dioxide (SC-CO_2) extraction to decrease the total lipid content of walnuts, thus improving dietary fat content, and 2) determine the effects of SC-CO_2 lipid extraction on the oxidative stability, flavor and textural characteristics of the partially defatted walnuts initially after extraction and during storage. The relative lipid content of English walnut pieces was reduced by 25 and 40% using a pilot-scale SC-CO_2 extraction system. Full-fat, 25 and 40% partially defatted walnuts were stored at 25 °C and 40 °C for 8 weeks. Oxidative stabilities of walnut oils, a by-product walnut defatting, were also determined under accelerated conditions, and compared with a commercially prepared pressed walnut oil. Fatty acid profiles were not different for partially defatted and full-fat walnuts or SC-CO_2 and pressed walnut oils. Peroxide values and volatile compounds were significantly greater in full-fat walnuts than in partially defatted walnuts at both storage temperatures. Partially defatted walnuts were less astringent, and had less painty and rancid flavors, as judged by a trained sensory panel. Full-fat walnuts were determined by both sensory and instrumental texture profile analysis to have greater hardness than partially defatted walnuts. Consumer acceptance hedonic scores of 25%

reduced fat walnuts were not significantly different than full-fat walnut scores (6.5 and 7.0, respectively). The SC-CO_2 extracted oils were less stable during accelerated storage in the dark than was pressed walnut oil, as determined by peroxide value, volatile analysis and sensory methods. Photo-oxidative stability was greater in the SC-CO_2 extracted oils than pressed oil, probably because of the presence of chlorophyll in the pressed walnut oil. In general, reducing the relative fat contents of walnuts by 25 and 40% improved the oxidative stability while maintaining desirable flavor characteristics.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

During the past several years, nuts have gained popularity with consumers and nutrition professionals because of their good taste and health benefits. In particular, walnut consumption has been linked to the lowering of plasma cholesterol levels and blood pressure (Sabate *et al.* 1993). Two negative components of walnut consumption are the relatively high fat content of this commodity, and the lack of oxidative stability caused by the high level of fat and its polyunsaturated nature.

Supercritical CO_2 (SC-CO_2) extraction has emerged over the past decade as an ideal method for oil extraction in various food systems as it is relatively low-cost, non-flammable, non-toxic, and easily removed by depressurization (Goodrum *et al.* 1987). SC-CO_2 extraction is also unique, in that it allows controlled partial extraction from whole nut pieces, making it a viable technique for both nut production applications and the specialty oil industry. Further, SC-CO_2 is recognized as a potential technique for differential extraction of food lipid constituents. Indeed, fractionation of glycerides using SC-CO_2 has been demonstrated by the separation of alkyl esters derived from fish oil (Temelli *et al.* 1988), the removal of mono- and diglycerides from triglycerides (Nilsson *et al.* 1991), and the deacidification of olive oil (Brunetti *et al.* 1989). Several investigators have successfully partially defatted whole pecans and peanut kernels both to lower fat content and increase stability (Zhang *et al.* 1995; Chiou *et al.* 1996; Alexander

et al. 1997). Little attention has been paid, however, to triglyceride composition following partial defatting and oxidative stability of the extracted oil or nuts.

Oil extraction is generally associated with increased susceptibility to oxidation; therefore, it is necessary to assess the extent of oxidation in supercritically extracted oils and oil-bearing products. The primary goal of this study was to determine the influence of partial lipid extraction by SC-CO_2 on the flavor, composition and oxidative stability of walnut oil and walnut meat. It was hypothesized that SC-CO_2 extraction could be used to decrease the total lipid content of walnut, thus improving oxidative stability and dietary fat content, while still maintaining walnut flavor characteristics.

Dissertation Organization

This dissertation includes a General Introduction, which consists of an introduction to the study and a thesis organization section. This chapter is followed by a Literature Review encompassing topics related to supercritical fluid technology, lipid oxidation, lipid nutrition and background of the study. Five manuscripts to be submitted for publication in the *Journal of the American Oil Chemists' Society* are included (Chapters 3-7) in the thesis. These chapters include an introduction, followed by materials and methods, results and discussion, and figures and tables. The General Conclusions for the entire study are included in Chapter 8. References cited in thesis Chapters 1, 2, and 8 are included in the References section at the end of the dissertation. References cited in the manuscript chapters (Chapters 3-7) are included at the end of those sections.

CHAPTER 2. LITERATURE REVIEW

Supercritical Fluid Technology

General information

The supercritical state is achieved when the temperature and pressure of a substance are raised above its critical point, defined as the point at which an increase in the pressure will not convert the vapor into a solid or liquid state. In essence, a supercritical fluid behaves as a dense gas and is, therefore, capable of solvation. The solvent strength can be easily manipulated near the critical point by changing the temperature or pressure, thus altering the physiochemical properties of the substance, including density, diffusivity, dielectric constant and viscosity (McHugh and Krukonis 1986). The higher diffusion coefficients, and decreased viscosity and surface tensions of supercritical compared with liquid solvents, coupled with easily controllable solvation properties, make supercritical fluids a useful processing medium for extraction and separation techniques. Although there are a large number of compounds that have been utilized in supercritical applications, carbon dioxide (CO_2) is by far the most widely used (Sihvonen *et al.* 1999).

From the vantage point of pharmaceutical, nutraceutical and food applications, supercritical CO_2 performs well as a solvent and offers the advantages of being non-toxic, non-flammable, inexpensive and easily removed from the product. In addition, the critical temperature and pressure are relatively low (T_c = 31.1 °C, p_c = 72 bar), making CO_2 suitable for extracting thermally labile and non-polar bioactive compounds (Rivzi *et*

al. 1986). Unfortunately, because of the non-polar nature of CO_2, it cannot be used for dissolving polar molecules. The solubility of polar molecules can be increased by adding small quantities of co-solvents, such as ethanol, to the fluid. Co-solvent additions may decrease processing times, require milder processing conditions, and increase product yields. However, system thermodynamics are further complicated and capital costs are increased (Sihvonen *et al.* 1999).

The solvating ability of a supercritical fluid is considerably more dependent on the temperature and pressure of the fluid than on the solvating ability of a liquid. At low pressure (e.g. 100 bar), the solubility of a compound actually falls as the temperature rises (this is the opposite effect found with liquids such as water at atmospheric pressure). At higher pressures, the solubility increases with temperature at higher pressure (e.g. 150 bar). The difference in solubility as a function of temperature at various pressures is primarily due to the change in density of the solvent. At low pressure, an increase in temperature leads to a significant decrease in density. As the pressure increases, the change in temperature leads to a much smaller change in the density. Generally, the density of a supercritical fluid approaches that of a liquid at pressures that are 1.7 to 2 times that of the critical pressure (Lira 1996). In supercritical fluid extraction, the extraction conditions and solubility are frequently defined in terms of the density, which is closely related to the solvent strength. There are several factors that make a supercritical fluid a more effective solvent than liquid including (Eggers 1996):

Rate of mass transfer: The greater rate of mass transfer of a supercritical fluid allows for a significant increase in the rate of transfer of the compound that is extracted from the solid phase into the liquid.

Solvent diffusivity: The greater solvent diffusivity of a supercritical fluid provides for more rapid removal of the extracted compound from the intermediate point of extraction. In addition, it allows for more rapid replenishment of fresh solvent for extraction of the analyte from the sample.

Solvent viscosity: The low viscosity of a supercritical fluid makes it easier to flow through the pores of a sample matrix.

Surface tension of the solvent: The lower surface tension of a supercritical fluid leads to greater ease of penetration into the small crevices of the sample.

Lower temperature requirement: Supercritical fluids can frequently extract the compounds of interest at temperatures lower than needed by a liquid (e.g. using a refluxing solvent and a Soxhlet extractor). This feature is especially important if the compounds of interest are thermally labile.

Solvent strength: The solvent strength of a supercritical fluid can be easily controlled. The solvent strength of a supercritical fluid is dependent on the temperature and pressure and can thus be changed. At a constant temperature, lower pressure favors the extraction of non-polar compounds and higher pressure favors the extraction of more polar compounds.

Although numerous types of commercial and customized supercritical extraction apparatuses are in use, most are based on two basic operational types, either static or dynamic extraction.

Static extraction occurs when a quantity of supercritical fluid is allowed to enter the vessel containing the sample and is left in the vessel for a specific period of time. This mode of operation is most commonly used when the compound of interest is not especially soluble in the fluid because of poor kinetics and/or if the matrix does not provide many readily accessible pores (Eggers 1996).

With dynamic extraction, the supercritical fluid is pumped through the vessel at a fixed flow rate. This replenishes the supply of fresh solvent and is used when the analytes are not very soluble and the matrix is easily penetrated by the supercritical fluid. Dynamic extraction also reduces solubility saturation.

Several techniques using either static or dynamic extraction can be used to achieve the desired extracts. In the case of fractionation, multistage separation may be used. Supercritical extraction of a multicomponent material over a range of operational conditions creates selectivity in the process. A stepwise increase in solvent power of the compressed gas results in portions of extracts with varying composition. In the vicinity of the critical point the gradient in density is steep, and small changes in pressure and temperature cause wide-range variation in the solvent power (Brogle 1982). The extraction is begun near the critical point and is advanced to higher pressures until the solubility of the least soluble analyte is reached. Sufficient difference in CO_2 solubility

of the substances to be fractionated is required for them to be extracted separately (Stahl and Gerard 1985).

Countercurrent extraction is useful for fractioning closely related compounds. By attaching the extraction system to a column, where countercurrent rectification occurs (i.e. internal refluxing), improved separation can be achieved (Zosel 1978). The dense gas is passed through the column, in which a gradient of increasing temperature causes a countercurrent as the dissolved substances flow backward against the rising loaded gas phase. The most soluble component is collected in the separation vessel and subsequently separated by lowering the pressure isothermally. The least soluble components are concentrated in the extract. Because of the high number of theoretical plates in comparison to single extraction, the separation of substances with similar solubility in dense CO_2 can be achieved. Zosel (1978) separated triglycerides of cod liver oil into 50 fractions with increasing molecular weight and degree of saturation. Gerard (1984) presented a continuously operating countercurrent fractionation system for the deterpenation of citrus peel oils. An analytical scale apparatus has been constructed for simulation of the continuous deodorization process using supercritical CO_2 (Bitner *et al.*, 1986).

The use of an entrainer, a substance of intermediate volatility or a co-solvent, can enhance the efficiency of the separation (Stahl *et al.* 1988). Entrainers are thought to aid extraction according to two principles: (1) increasing the solvent strength of the dense gas; and (2) competing with the adsorbed compounds for active sites in the matrix (Hills *et al.*, 1991; Pawilszyn 1993). Francis (1955) suggested that almost any liquid augments

the mutual solubilities of another dissimilar liquid with carbon dioxide. The entrainer may also increase the difference in solubility between analytes to that selectivity increases through special interactions of the extraction phase with the substrate (Lira 1996; Stahl *et al.* 1988). An appropriately selected entrainer can decrease the required temperature and pressure, increase yield, decrease the extraction time, and even increase the degree of fractionation (Bott 1980; Peter and Brunner 1978).

Preferential extraction of lipids

The separation of polyunsaturated fatty acids (PUFA) by supercritical fluid extraction (SFE) is dependent on the molecular size of the components involved rather than the degree of unsaturation; therefore, a prior concentration step is needed to achieve a high concentration of PUFA in the final product (Mishra *et al.* 1993). Oils to be used for ω-3 concentration by SFE require preparation steps for extraction, hydrolysis, and esterification by conventional methods (Nilsson *et al.* 1989). SFE has been effectively used to refine fish oils and remove cholesterol, polychlorinated biphenyls, vitamin E, and other components (Rivzi *et al.* 1986). Several research groups have reported the fractionation of mixtures of mono-, di- and triacylglycerols using various fluids (Peter and Brunner 1978; Brunner and Peter 1982). Stout and Spinelli (1987) demonstrated that fish oil esters could be fractionated by SFE to produce an oil with 60-65% docasahexanoic acid (DHA). The fractionation of free fatty acids using SFE also has been reported (Rivzi *et al.* 1986). Both of these studies showed a low recovery of ω-3-PUFA during the process, the reasons for which remain speculative. But, use of

extremely high pressures and high capital costs might limit widespread use of this method for production of concentrated ω-3-PUFA to the larger processing companies. Recently, the use of propane has gained more interest in extraction technology especially in the nutraceutical industry (Lira 1996). Several studies have examined the use of supercritical conditions, using propane as a solvent, to promote the hydrogenation of fatty acid methyl esters to fatty alcohols, which are widely used as surfactants, lubricants, or additives in many industrial products. The current manufacturing process for fatty alcohols can result in the formation of trans-fatty acids, and requires severe conditions for hydrogenation of the carboxyl group. The supercritical process was several orders of magnitude more time efficient than commonly used techniques and resulted in less by-product formation (van de Hark 1999).

Fractionation of fat from cocoa beans to produce cocoa oil and cocoa butter with a strong cocoa flavor was reported (Vitzthum 1975). De Hann *et al.* (1990) concentrated milk fat flavor 20 to 50-fold with supercritical CO2 extraction from anhydrous milk fat. Supercritical CO_2 concentrated volatile flavor compounds up to 30-fold when applied to the isolation of the flavor fraction from heated beef (Um 1992) and pork fat (Bailey 1993).

The extractability of aroma and flavor compounds with carbon dioxide is high (Kerrola 1995). The major market implication of this feature is in the decaffeination of coffee and tea. Hop extraction is another commercial application of supercritical CO_2 extraction. Flavor-containing liquids, such as fruit and vegetable juices, may be extracted

with CO_2 to obtain flavor concentrates. Shultz *et al.* (1970) concentrated flavors of vegetable materials including tomatoes, carrots, celery, and watercress.

The use of essential oils and oleoresins as an alternative to crude spices has increased, and spice extracts of various compositions can be obtained using supercritical extraction (Kerrola 1995). The advantages of this process include selective isolation and avoidance of thermally induced reactions by using low operational temperatures. In addition, the exclusion of air by chemically inert CO_2 hinders various oxidation reactions. Essential oils were removed from spices primarily with liquid CO_2, under mild operating conditions, followed by isolation of the less volatile flavor compounds with supercritical CO_2 with ~ 62% oil removal in fennel. Capsicum and carotenoid color fractions from paprika were successfully extracted by consecutive extractions with supercritical CO_2. The CO_2-extracted essential oil fractions were evaluated by an expert panel as representing higher quality than steam distillates.

Supercritical techniques are not widely used in the industry at this time. The diversity of patents and articles in the late 1990's, however, indicates that there is much interest in utilizing of supercritical techniques in a wide variety of applications. According to Perrutt (1999), the four aspects to consider when trying to foresee the future of supercritical technology are:

1. Regulatory issues
2. Quality considerations
3. Innovative products
4. Innovative processes

In recent years, growing environmental and occupational concerns regarding the use of organic solvents has prompted regulatory agency action. In particular, disposal regulations and emissions requirements have become stricter, and many organic solvents have already been banned in food processing. These regulations have resulted in increased processing costs, and in some cases, lack of a suitable food-grade solvent. Thus, there is pressure to develop and optimize alternative, environmentally safe processes. In addition, the food industry's strong emphasis on value-added products such as food additives, functional foods, and nutraceuticals has created another lucrative niche for supercritical processing. Supercritical fluid technologies can also be applied in the development of new, innovative products. One of the very promising areas of research is microencapsulation of drug molecules, which is used for controlled drug release in the human body. Although the use of supercritical technology in the formation of homogeneous microparticles has not yet been widely used in food applications, these substances could be a useful tool in processing of nutraceuticals and functional food ingredients in the future. Currently used microparticle formation techniques, such as recrystallization from liquid antisolvents, spray drying, milling, grinding, and lyophilization cannot be used to produce certain pharmaceutical, dye, or explosive particles. The gas anti-solvent process has already been applied to the separation of β-carotene (Chang *et al.* 1991).

Lipid Oxidation in Foods

Autoxidation

Autoxidation is a radical chain reaction in which a neutral reactive intermediate with an odd electron is formed (initiation). These free radical species are highly reactive with oxygen and rapidly converted to peroxides (propagation – fast step). In the rate determining step of this reaction, the peroxy radical is reduced to form a hydroperoxide (propagation – rate determining), thus creating a fresh alkyl radical. The breakdown of hydroperoxides generates additional free radicals, further propagating the chain reaction. This autocatalytic chain reaction continues until two radicals form a stable dimer.

The hydroperoxides formed during autoxidation vary with fatty acid composition. The free electron is delocalized over adjacent carbon atoms, allowing for oxygen attachment at multiple positions (Frankel 1985). Theoretically, this mechanism should result in a mixture of hydroperoxides, with -OOH groups present in equal amounts for each position. However, greater amounts of 8- and 11-hydroperoxides, 9- and 13-hydroperoxides, and 9- and 16-hydroperoxides were measured in oleate, linoleate and linolenate, respectively, probably because of kinetic and thermodynamic factors responsible for the steriochemistry of the allylic carbon and/or peroxyl radical intermediates (Frankel 1998).

Photooxidation

Photo, or singlet oxygen oxidation, occurs when the ground state of molecular oxygen (triplet oxygen $^3O_2$) is converted to excited state singlet oxygen ($^1O_2$). Singlet

oxygen can be generated in a variety of ways. In foods, creation of singlet oxygen by photosensitization is probably the most important. Several substances commonly found in fat-containing foods can act as sensitizers to produce an alkyl radical as in autoxidation (Type I photosensitized oxidation) or to produce singlet oxygen (Type II photosensitived oxidation). These sensitizing agents include natural pigments, such as chlorophyll and heme compounds (Min *et al.* 1989). Singlet oxygen reacts much faster than triplet oxygen with moieties of high electron density, such as carbon-carbon double bonds. The formation of hydroperoxides by singlet oxygen proceeds via mechanisms different from those of autoxidation. Oxygen may combine directly with the double bond, which then shifts to form an allylic hydroperoxide in the trans configuration. Accordingly, oleate produces 9- and 10-hydroperoxides, linoleate produces 9-,10-,12-, and 13-hydroperoxides, and linolenate produces 9-,10-,12-,13-,15-, and 16- hydroperoxides. These hydroperoxides can then cleave to initiate conventional free radical chain reactions (Frankel 1998).

Photooxidation differs from autoxidation in several important ways:

1. It involves reaction with singlet oxygen produced from triplet oxygen by light and a sensitizer.
2. It is an ene reaction (i.e. oxygen is inserted directly at either side of the double bond) and not a radical chain process.
3. It displays no induction period.
4. It is unaffected by antioxidants normally used to inhibit autoxidation, but is inhibited by singlet oxygen quenchers (e.g. β-carotene).

5. It is confined to olefinic carbon atoms, and accompanied by double bond migration with change of configuration from *cis* to *trans*.
6. It gives products that are similar in type, but not identical in structure to those obtained by autoxidation.
7. It proceeds much faster than autoxidation.

Factors influencing oxidation

The free radical and hydroperoxide reactions have high activation energies; therefore, if a peroxide is to form, some factor must assist in the process. Lipid oxidation in food systems is much more complex than in pure systems, e.g. bulk oils or isolated fats and oils. A wide variety of components contained in the food matrix may influence the rate of oxidation, particularly compounds such as metals, antioxidants, prooxidants, pigments and enzymes. The nature of the lipid moeities, and the way in which they are dispersed within the food system are important variables.

Lipid structure: The relationship between lipid structure, primarily degree of unsaturation, and the rate of oxidation has been the subject of much research. In autoxidation, where the removal of hydrogen to form the free radical is influenced by the presence of allylic methylene groups, the methylene interrupted diene system (e.g. linoleic and linolenic acid) is significantly more reactive than the isolated double bond of a monoene (e.g. oleic acid; Frankel 1985). Various relative rates of oxidation for individual unsatured fatty acids have been reported (Table 1). Oxidation rates for photosensitized purifed forms of oleic, linoleic and linolenic acids were 1:1.7:2.3, much

Table 1. Relative rates of autoxidation of oleate, linoleate and linolenate.

	18:1	18:2	18:3
Gunstone and Hilditch (1945)	1	12	25
Gunstone (1984)	1	27	77
Holman and Elmer (1947)	1	41	98
Fatemi and Hammond (1980)	1	10	20

closer to the number of double bonds present in these fatty acids (Terao and Matsushita 1980).

Fatty acid position in triacylglycerols: The relationship between oxidative stability and relative positional distribution of fatty acids in triacylglycerol molecules is controversial. Several investigators have observed increased oxidation rates of oils after randomization (Hoffman *et al.* 1973), particularly when the unsaturated fatty acids were located in the sn-2 position (Raghuveer and Hammond 1967). Working with synthesized triacylglycerols, Hoffman *et al.* (1973) proposed that molecular symmetry of triacylglycerol molecules may influence oxidative stability. They concluded that oxidative stability was lower if the same fatty acid occupied the 1- and 2-positions, e.g.,

OOL, as opposed to the 1- and 3-positions, e.g. OLO. However, Fatemi and Hammond (1980) investigated the affect of asymmetry using randomized olive and soybean oils and calculated that any possible impact on oxidative stability would be minimal. Neff *et al.* (1994) found that the oxidative stability of canola oil was positively correlated with the amount of linolenic acid on nonspecific locations on glycerol, the amount of linoleic acid in the sn-2 position and with the sn-oleoyllinoleoyllinolenoyl glycerol content.

Free fatty acids: In general, free fatty acids oxidize faster than their glycerol esters. These products are generally the result of lipolytic rancidity in which enzyme action, heat or moisture causes the hydrolysis of ester bonds, thus liberating fatty acids from the glycerol backbone. The presence of relatively large amounts of free fatty acids in commercial oil can also enhance pick-up of trace metals from equipment or storage tanks. Even small amounts of free fatty acids in some foods, e.g. nuts, contribute to off-flavor and unacceptability of the product, and may have a profound impact on the rate of oxidation (Frankel 1998).

Oxygen concentration: At very low oxygen pressure, the rate of oxidation is approximately proportional to oxygen pressure. If the supply of oxygen is unlimited, the rate of oxidation is independent of oxygen pressure. The availability of oxygen clearly plays a critical role in determining competitive oxidative pathways (e.g. peroxidation versus homolytic cleavage or polymerization). Oil oxidation occurs at the oxygen interface. The rate of oxidation increases proportionally to the surface area of the liquid exposed to air. In oil-and-water emulsions, oxygen must gain access to the lipid by diffusion into the aqueous phase and passage through the oil-water interface. The rate of

concentration of emulsifier, size of oil droplets, surface area of interface, viscosity of the aqueous phase, composition and porosity of the aqueous matrix, and pH (Frankel 1998).

Temperature: Rates of reaction increase with increases in temperature. As the temperature increases, however, the increase in rate with increasing oxygen concentration becomes less evident, because oxygen becomes less soluble (Nawar 1985).

Water activity: In dried foods with very low moisture content [water activity (a_w) < 0.1], oxidation proceeds very rapidly. Increasing a_w to ~ 0.3 retards lipid oxidation, reportedly by reducing metal catalysts, quenching free radicals, promoting nonenzymatic browning, and impeding oxygen accessibility. At higher a_w (0.55-085), the rate of oxidation increases again, presumably because of increased mobilization of the catalysts (Nawar 1985).

Prooxidants: Enzymes can catalytically promote oxidation. Transition metal ions, particularly copper and iron, are also major catalysts for oxidation. At very low concentrations, < 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (Min 1989). Such metal ions, either in free or bound forms, occur naturally in plant and animal tissues, membranes, and enzymes. Prooxidants are also introduced into food by contact with metallic equipment used in processing and storage. The roles of natural and synthetic pigments in the initiation of photooxidation by generation of singlet oxidation were discussed previously.

Antioxidants: Antioxidants may occur naturally or be synthesized, and their presence can delay the onset or slow the rate of oxidation. The main antioxidants used in food are mono- or polyhydric phenols with ring substitutions, and include tocopherols, rosemary

extracts, buylated hydroxytoluene and tert-butylhydroxyquinone (Frankel 1998). They either inhibit the formation of free radicals at the initiation step or interrupt the propagation of the free radical chain reaction. Many food antioxidants, particularly synthetic antioxidants, are chain-breaking inhibitors of lipid peroxidation. For maximum efficiency, primary antioxidants are used in combination with other phenolic antioxidants or with metal-sequestering agents. Singlet oxygen quenchers, such as β-carotene, are important inhibitors of photooxidation.

Interactions in foods: The interactions of the oxidizing lipid itself or its oxidation products with other components greatly influence oxidative and antioxidative processes. Lipids usually exist as mixtures (e.g. triacylglycerols of different fatty acid constituents, phospholipids, cholesterol). In foods, both lipid and non-lipid components (e.g. protein, carbohydrate, water) often exist with close proximity, or in some bound form. The mobility of the food constituents and their breakdown products depend to a great extent on the physical structures of the food.

Under oxidative conditions, some very complex interactions will take place. Free amino groups (e.g. in amino acids, primary amines, or phosphatidylethanolamine) with aldehydes produced by either oxidation of the lipids or decomposition of the carbohydrate or protein constituents have long been recognized as important (Frankel 1998). Their reaction products affect color, texture and flavor, and some may have prooxidative or antioxidative properties.

The effects of different oxidizable components in a mixture on oxidation of each other are much less clear. Some researchers have suggested that oxidizable substrates

can inhibit and or accelerate the oxidation of another component in a mixture depending on the interplay of a number of parameters, including the nature of the substrate and their concentrations, temperature, pH and physical state (Nawar 1996). These interactions, although complicated, are important to understanding food deteriorative processes which impact quality and acceptability.

Indicators of oxidation

No single test can measure all oxidative events, and no single test is universally applicable to all foods. Obviously, a more reliable evaluation can be obtained by using a combination of tests. A ranking of lipid oxidation tests is listed in Table 2, with the procedures ranked in decreasing order of usefulness in predicting the oxidative stability of a food product (Frankel 1998). Some of the commonly used methods are discussed.

Sensory evaluation: Sensory evaluation is, perhaps, the most important indicator of oxidation, because it measures how the consumer views a product. Many lipid oxidation products have distinctive flavor characteristics that contribute to the overall perception of objectionable changes in an oxidized food. The testing of oxidative off-flavors can be conducted by trained taste panels (Warner 1988). Unfortunately, depending on the type of food, the stage of oxidation (e.g. early, late), and conditions contributing to oxidation, such off-flavors vary not only in their intensity, but also in their character. Terms such as rancid, stale, beany, metallic, cardboard, green, fishy, etc., are often used to describe flavors attributed to oxidation in foods.

Table 2. Ranking of Lipid Oxidation Tests (Frankel 1998).

Method	Sensitivity	Precision	Information
Sensory	High	Low	High
Volatiles by GC[a]	High	Low	High
UV[b] absorption	High	High	Low
Carbonyls	Low	High	Low
Anisidine value	Low	High	Low
Peroxide value	Low	High	Low
Oxygen uptake	Low	High	Low
TBA[c]	Low	High	Low
Volatile acids[d]	Low	Low	Low

[a]Gas chromatography

[b]Ultraviolet

[c]Thiobarbituric acid

[d]Rancimat

Peroxide value (PV): The PV is one of the most widely used tests in lipid oxidation. Peroxides are primary oxidation products, which form quickly following free radical initiation. Standard methods for measurement of peroxides are based primarily on their ability to liberate iodine from potassium iodide or to oxidize ferrous to ferric ions. PVs are usually expressed in terms of milliequivalents of oxygen per kilogram of fat. Although the PV is useful for monitoring oxidation at the early stages, it can be misleading because its accuracy may vary with the procedure used and the storage history and nature of the food product. As oxidation progresses, the PV reaches a peak and then declines. If the food is subjected to elevated temperatures, for example during frying, peroxides will decompose and no longer serve as an effective indicator of oxidation. Accordingly, a low PV does not always indicate a low level of oxidation, particularly as PV levels vary widely depending on the food system. In addition, peroxides themselves are odorless, tasteless compounds and, therefore, may not be indicative of flavor changes in a food product at a single point in time. When used to identify changes over time, under known storage conditions, the PV can provide excellent data regarding the oxidative stability of a product.

Volatile analysis: Gas chromatographic analysis of volatile oxidation products can be used to detect low levels of oxidation in oil- and lipid-containing foods, and provides useful data regarding the flavor impact of oxidation. Hydroperoxides break down to form short-chain compounds, including aldehydes, ketones, alcohols, acids, esters, lactones, ethers and hydrocarbons, which contribute to odor and flavor (Frankel 1991). Of these compounds, aldehydes are probably the most significant with regard to flavor.

Correlation of specific volatile components with perceived human sensory panel flavors or odors can be used to determine their contribution to both desirable and undesirable flavors. Quantitative analysis of volatile compounds can be achieved by several methods, including direct injection, dynamic headspace and static headspace.

For direct injection, the sample is added directly to the GC injector port packed with glass wool, or in the case of solid samples, the sample may be packed in a tube which can be inserted into the injection port. The advantages of this method include detection of both low- and high-molecular weight volatile compounds. In addition, this method can be more rapid and inexpensive than dynamic headspace methods. Unfortunately, the elevated desorption temperatures required cause thermal decomposition of hydroperoxides (Snyder *et al.* 1988).

Dynamic headspace sampling, otherwise known as "purge and trap," involves purging a sample using an inert gas such as nitrogen or helium and collecting the volatile compounds onto an adsorbent such as Tenax. The volatile compounds are then thermally desorbed into the GC inlet. The advantages of this method are low temperatures required for the collection of volatile compounds and concentration of compounds onto the adsorbent. This method is also time-consuming, labor-intensive, and prone to methodological problems (Zhang *et al.* 1994; Song *et al.* 1997).

Static headspace analysis involves equilibration of a sample with the headspace in a closed system, such as a sealed vial. Following equilibration, an aliquot of headspace is extracted and injected into the GC inlet for analysis. This method is simple, rapid and inexpensive but lacks sensitivity compared with other methods because of volatile

compound dilution by the headspace (Snyder *et al.* 1988; Zhang *et al.* 1994). In addition, static headspace analysis is generally only effective for compounds with low boiling points and/or high vapor pressures (Snyder *et al.* 1985; Chin *et al.* 1996). Snyder *et al.* (1988) reported higher relative proportions of acrolein, propanal and pentanal than dynamic headspace or direct injection in samples from oxidized soybean oils.

Solid phase microextraction is a relatively new technique for the analysis of volatile compounds (Arthur and Pawliszyn 1990). This technique utilizes a fused silica fiber with a polymeric coating to adsorb volatile compounds. Following exposure of the fiber to the headspace above the sample, or to the sample itself, the fiber is retracted into a protective needle attachment. The needle is inserted into the GC port, the fiber is pushed out of the needle and thermally desorbed onto the GC column. Similar to static headspace analysis, the SPME method is rapid, easy-to-use, and relatively inexpensive. Unlike traditional static headspace, however, SPME allows concentration of volatile compounds, thereby increasing sensitivity (Zhang *et al.* 1994).

Thiobarbituric acid (TBA) test: Oxidation products of unsaturated systems produce a color reaction with TBA that can be measured spectrophotometrically. It is believed that the chromogen arises from condensation of 2 moles of TBA with 1 mole of malondialdehyde (MDA), a hydroperoxide decomposition product. The test can be conducted directly on the food, however, there is much controversy regarding appropriate extraction procedures and a wide variation in results depending on the method and solvent used. Malondialdehyde itself is not always present in oxidized samples, and many other compounds have reported to produce the characteristic pigment upon reaction

with TBA. Various oxidized lipid products, such as 2-alkenals, 2,4,-alkadienals, and 4-hydroxyalkenals can also react in this procedure (Kosugi et al 1987, Esterbauer et al 1991, Raharjo et al 1993). Other nonlipid materials such as sugars, amino acids, urea, biliverdin, glyoxal and furfuraldehyde may also react with TBA to form complexes that absorb at 530-535 nm, the same wavelength range in which the TBA-MDA adduct is measured (Kosugi et al 1987; Hoyland and Taylor 1991).

Such interference may arise not only from other products of lipid oxidation, but also from nonlipid food components. In addition, direct gas chromatographic analyses of malondialdehyde does not correlate with TBA values (Frankel 1991). The amount of TBA-reactive substances (TBARS) varies depending on the balance between the rates of their formation and the rates of decomposition. Consequently, although this test may be applicable for comparison of a single material at different stages of oxidation, or of samples with known storage conditions (e.g. shelf-life studies), it is not always useful for evaluating foods of unknown history (Nawar 1985).

Conjugated dienes: Double bond shifts during the initial stages of oxidation result in diene and triene conjugation which show typical absorbance bands at 234 nm and 268 nm, respectively. This test measures only primary oxidation products, and is therefore limited to the early stages of oxidation (AOCS 1989).

Carbonyls and anisidine value: These tests provide information regarding secondary oxidation products, including many of the same compounds measured using gas chromatography, but they do not differentiate between volatile and non-volatile carbonyls. The p-anisidine value is a spectrophotometric method that measures primarily

2-alkenals and 2,4-dienals. Both of these methods are often correlated with flavor scores, although the flavor significance of carbonyls in general has not been established (Frankel 1998).

Accelerated tests: The oxidative stability instrument (OSI), Rancimat, Schall oven test, or active oxygen method (AOM) are examples of accelerated tests designed to predict the resistance of an oil to oxidative rancidity. The Schall oven test provides the most reliable results because relatively mild temperatures (50-60 °C) are used to promote oxidation. Most of these methods involve storage at high temperatures (> 100 °C) until a certain PV is reached. The Rancimat an OSI measure the conductivity of low-molecular weight fatty acids, which are tertiary products of lipid autoxidation. Oxidation mechanisms are altered at elevated temperatures and the testing conditions may lead to polymerization, cyclization, rapid breakdown of peroxides, and production of thermal decomposition products (Frankel 1998). Although these methods provide quick results, the PV endpoints are often far too high to be of any practical flavor significance. Accelerated methods are widely used, and offer value in evaluating the relative oxidative stability of similar-type samples.

Lipid Nutrition

Polyunsatuated fatty acids

Large volumes of research regarding the health implications of lipids exist. Lipid intake has been implicated in cardiovascular disease (CVD), obesity, cancer, stroke, hypertension and immune disorders (Simopoulos 1999). The purpose of this review is to

examine the potential positive and negative health implications of n-6 and n-3 fatty acids, particularly linoleic and linolenic acid.

Linoleic (18:2 n-6) and linolenic acid (18:3 n-3) are considered to be essential fatty acids because they are required for important bodily functions such as growth, skin and hair qualities, and since they cannot be synthesized in the body they must be supplied by the diet. It has been proposed that the ratio of linoleic to linolenic acid currently consumed in Western diets (~ 20-30:1) is far too high, and that these excess levels of linoleic acid give rise to increased levels of arachidonic acid (AA, 22:4 n-6) and may lead to hypersensitivity, depression of the immune system, and inflammatory diseases (Simopoulos).

Linolenic acid may be converted to eicosapentanoic acid (EPA, 20:5 n-3) and docosahexanoic acid (DHA, 22:6 n-3). EPA and DHA are the fatty acids found in fish oils. It has been well established that n-3 fatty acids lower plasma triglyceride levels, however, when fish oil is fed with a constant saturated fat intake, low-density lipoprotein cholesterol (LDL) increases or does not change, and levels of high-density lipoprotein cholesterol (HDL) increases slightly (Harris 1989).

The mechanism by which long chain, n-3 PUFAs reduce very low density lipoprotein (VLDL) is only partially understood. It appears that these compounds suppress both triglyceride synthesis in the liver and gut and the assembly of nascent VLDL (Yeo and Holub 1990). It is not known how these fatty acids interfere with synthesis but it is assumed that they may specifically inhibit one or more of the enzymes that lead to the formation of acyl CoAs that are needed to activate fatty acids for further

metabolic utilization. Perhaps they act on the 1,2-diacylglycerol acyltransferases (Rustan 1988). Rustan *et al.* (1989) found that incorporation of oleyl-CoA into triglycerides was inhibited by EPA in rat hepatocytes. This group also reported that EPA CoA decreased the incorporation of oleate into cholesterol ester, specifically into cholesterol esters secreted into VLDL molecules.

Eicosanoids are cellular activity regulation compounds formed from 20 carbon PUFA. The fatty acid composition of the diet regulates membrane phospholipid fatty acid profiles and, in turn, modulates eicosanoid synthesis. The replacement of n-3 PUFA is due to competition for the delta-6 desaturase enzymes. As the amount of n-3 PUFA increases in diets, there are associated decreases in n-6 metabolites, and lower levels of AA available as substrate (Peck 1994). Dietary PUFA manipulations are highly significant because many diseases are associated with the overproduction of eicosanoids from AA, derived from linoleic acid. Interestingly, the AA for prostaglandins in humans is not quickly influenced by dietary linoleic acid and AA because of the large pool size of AA and low conversion to linoleic acid to AA. In contrast, the n-3 PUFA pool(s), which are considerably smaller, is immediately influenced by n-3 PUFA supplementation (Hamazaki 1989). There is growing evidence to indicate that although the average intake of AA in the diet (~ 100 mg/day) is ~ 100 times lower than linoleic acid, its consumption may have physiological relevance (Mann *et al.* 1995). Since AA bypasses some of the normal regulatory rate-limiting enzymatic steps that control membrane AA content, it bypasses the attenuation effects of n-3 PUFA (German *et al.* 1995).

North Americans consume greater than 10 times the amount of n-6 PUFA minimally required (Simopoulos 1991) and this excess consumption is creating an imbalance in long-chain fatty acid metabolism. This issue is of particular importance in view of the relative n-3 PUFA deficiency syndrome seen in Japan, where an increase in the n-6/n-3 ratio from 2.8 to 4.0 during the last 40 years is associated with the rising incidence of cancer of the colon, breast, prostate and pancreas (Okuyama *et al.* 1996). In addition, it has been suggested that neurodegenerative disorders, such as Parkinson's and Alzheimer's disease may be related to membrane loss of PUFA, particularly DHA (Youdim *et al.* 2000). These authors suggest that optimizing the n-6/n-3 ratio may help delay the onset, or reduce the insult of neurodegenerative disorders.

Several investigators have argued that more attention must be paid to promoting the consumption of n-3 PUFA-rich foods while reducing the intake of n-6 PUFA (Okuyama *et al.* 1996; Simopoulos 1999), but exactly how much n-3 PUFA to include in the diet is a question of much controversy. It has been shown that when rats were fed graded amounts of n-3 fatty acids in the presence of a constant amount of linoleic acid, the levels of AA in tissue lipids and the formation of eicosanoids derived from AA were suppressed in a dose-dependent fashion. However, if rats were fed graded amounts of the n-3 fatty acids with constant ratios of n-3/n-6 fatty acids (0.62 for linolenate and 0.3 for menhaden oil groups) with concomitant increases in n-6 fatty acid, then there was no dose-dependent response within the groups fed different levels of the same dietary fat at a constant n-3/n-6 ratio (Boudreau *et al.* 1991). These results indicate that the ratio of

n-3/n-6 fatty acids in the diet, rather than the absolute amount of n-3 fatty acids, is the determining factor in inhibiting eicosanoid biosynthesis from AA.

Nutritional benefits of walnuts

Walnuts are high in fat (~70%), which is largely polyunsaturated (~ 63%). Walnuts also contain significant quantities of dietary fiber (4.8%), vitamin E (> 300 ppm), and are rich in arginine (2.1% w/w), the dietary precursor of nitric oxide (Fraser 1999). Several studies (Table 3) have evaluated the effect of walnut consumption on serum cholesterol levels (Sabate *et al.* 1993; Abbey *et al.* 1994; Chisholm *et al.* 1998; Lavedrine *et al.* 1999; Iwamoto *et al.* 2000; Zambon *et al.* 2000), and have demonstrated reductions in low-density lipoprotein (LDL) cholesterol. In addition, four large, nutritional epidemiological, cohort studies have reported that frequent consumption of nuts is associated with a 30-50% decreased risk of coronary heart disease (Fraser *et al.* 1992; Kushi *et al.* 1996; Hu *et al.* 1998; Albert *et al.* 1998).

A serious limitation of each of these studies was the failure to provide a washout period between the experimental and reference diets. Despite utilization of a randomized crossover design, the effects of the preceding diet cannot be overlooked. This issue was addressed by Sabate *et al.* (1993) and Iwamoto *et al.* (2000), with no significant influence of dietary sequence reported in these studies.

Table 3 (Continued). Summary of studies that evaluated the nutritional benefits of walnuts.

Source	Subject Profile	Study Type	Results	Limitations
Abbey *et al.* (1994)	16 normolipidemic males	Dietary intervention using 36% fat (reference diet) and 36% fat diet supplemented with peanuts (50g/day), almonds (84g/day), or walnuts (68g/day) for three weeks.	Total and LDL cholesterol were reduced by 5 and 9% respectively for the walnut supplemented vs. reference diets.	Reference diet significantly higher in saturated fat.
Zambon *et al.* (2000)	49 hypercholesterolemic males and females	Dietary intervention using Mediterranean diet (reference) and a similar diet with walnuts replacing 35% energy frommonounsaturated fat (experimental diet.	Reference diet serum cholesterol, LDL, and apo A were reduced by 4.1, 5.9, and 6.2% respectively vs. experimental diet. Both diets significantly lowered cholesterol from baseline.	
Iwamoto *et al.* (2000)	40 males and females	Dietary intervention using an average Japanese diet (reference) and a similar diet with 12.5% of energy (offset by decreasing fatty foods, meat, and visible fat) derived from walnuts (43-57g/day; experimental diet).	Reference diet had lower serum cholesterol, LDL, LDL/HDL ratio, and apo B than men and women on the experimental diet.	

Table 3. Summary of studies that evaluated the nutritional benefits of walnuts.

Source	Subject Profile	Study Type	Results	Limitations
Sabate *et al.* (1993)	18 normolipidemic males	Dietary intervention using National Cholesterol Education Program Step 1 diet (reference diet) and NCEP Step 1 diet with 20% of calories replaced with walnuts (experimental diet) for four weeks. 30% fat in both diets.	Serum toal, LDL, HDL cholesterol reduced by 12.4, 16.3, and 4.9% respectively.	Similar fatty acid compositions of both diets; ~3% greater sat fats, 1.2% greater mono and 7% less PUFA in reference diet. Cholesterol: 237mg/day reference diet, 125mg/day in walnut intervention.
Lavedrine *et al.* (1999)	793 males and females	Cross-sectional survey (1-year dietary recall); Measured serum total, LDL, and HDL cholesterol, and apo A1 and B.	High HDL and apo A1 positively associated with walnut consumption.	Dietary recall data; non-representative population.
Chisholm *et al.* (1998)	21 hyperlipidemic males	Dietary intervention using 30% (low-fat reference diet) and walnuts as 20% of total energy and 55% of total fat (experimental diet) for four weeks.	Total and LDL cholesterol and apo B were reduced on the experimental diet compared with the baseline and reference diet. HDL cholesterol increased from baseline for both diets.	Total fat intake was not well-controlled with 8% increase in total fat (mostly PUFA) in the walnut diet. Cholesterol was 320mg/dL on the low-fat vs. 230 mg/dL on the walnut diet.

Iwamoto *et al.* (2000) fed all meals to subjects at the nutrition research facility, and randomly selected 15 different days in which to analyze diets for macronutrient composition and fatty acids. Surprisingly, despite the link between walnut fatty acid composition and the observed health benefits reported by all studies, no study examined the fatty acid composition of walnuts individually. Fatty acid compositions and total fat content of walnuts vary widely with cultivar and growing season (Savage *et al.* 1999). Data from our laboratory showed up to a 10% decrease in total fat of California Walnuts from different growing seasons (unpublished data). If specific fatty acids are responsible for the reported health benefits of walnuts, accurate measurement of dietary contents is necessary. The study by Iwamoto *et al.* (2000) was the only feeding trial where dietary intakes of cholesterol on both the reference and experimental diets were similar. Failure to control cholesterol intake on the reference diet has been a limitation with other studies (Sabate *et al.* 1993; Chisholm *et al.* 1998; Zambon *et al.* 2000). This discrepancy is particularly important in lieu of the fact that cholesterol is the parameter being measured.

Although dietary fiber intakes were estimated for most studies (Sabate *et al.* 1993; Abbey *et al.* 1994; Chisholm *et al.* 1998; Iwamoto *et al.* 2000; Zambon *et al.* 2000), the potential role of dietary antioxidants has been largely overlooked. Indeed, Zambon *et al.* (2000) was the first study to report Vitamin E and α-tocopherol levels of the diets, although these values were calculated from food tables rather than measured. The Nutrition Data System (Nutrition Coordinating Center, University of Minnesota. Version 2.5, 1993) reports high levels of vitamin E (expressed as alpha-tocopherol equivalents) for almonds (16.1 mg), walnuts (3.1 mg), filberts (21.9 mg), pecans (2.7 mg), pistachios

(4.5 mg) and peanuts (6.3 mg). However, these values may vary widely with geographic origin of the nuts and no values are reported for specific tocopherol isomers, which may significantly influence antioxidant activity. In a cohort study of 34,000 Seventh-Day Adventists, Fraser *et al.* (1992) reported a 50% risk reduction of a coronary event in individuals who consumed nuts frequently versus those who consumed nuts rarely. The Iowa Women's Study (n = 34,000) also reported a 40% risk reduction of coronary heart disease with nut consumption of greater than one time per week (Kushi *et al.* 1996). Adjustment for the vitamin E content of the nuts reduced the risk to 28%.

Despite the experimental limitations of many studies that have examined the health benefits of walnuts, there seems to be a clear link between walnut consumption and hypocholesterolemic effects among several different groups. What has yet to be elucidated is a clear mechanism for these effects, including both the identification and action of the walnut components responsible.

It has been suggested that the relatively high n-3 (α-linolenic acid) polyunsaturated fatty acid content of walnuts may explain the cardioprotective benefit of nuts. In both plasma and membrane phospholipids, especially in platelets and endothelial cells, longer-chain n-3 polyunsaturated fatty acids competitively displace arachidonic acid, reducing the ratio of n-6/n-3 polyunsaturated fatty acids. The shift to a higher concentration of n-3 polyunsaturated fatty acids has a host of cardioprotective benefits, including a substantial modification of the aggregatory, vasoactive, and thrombogenic potential of platelets. Also, since the uptake of oxidized LDL by specific receptors on macrophages is very avid and is not down-regulated by internal macrophage cholesterol

content, it leads to the accumulation of lipid-laden foam cells within the arterial wall (Fisher *et al.* 1990). Higher plasma levels of n-3 polyunsaturated fatty acids reduce oxidation, and hence the atherogenicity, of LDL cholesterol.

Present study

In recent years, methods for the production and development of nutritionally functional lipids and lipid foods have been heavily investigated. Supercritical fluid extraction (SFE) is a viable method for fat reduction in foods. We chose to apply this processing technology to a nutritionally desirable product, walnuts, which are high in PUFA, a good source of n-3 fatty acids, high in antioxidants and has been linked to hypocholesterolemic and antihypertensive effects (Sabate *et al.* 1993; Abbey *et al.* 1994; Chisholm *et al.* 1998; Iwamoto *et al.* 2000; Zambon *et al.* 2000). Further, by using CO_2 as an extraction solvent, the resulting reduced-fat walnut is also organic.

The primary objective of this study was to determine the effects of SFE on the oxidative and flavor stability of the modified walnut product. A secondary objective of this study was to reduce the overall fat content, thus resulting in a lower-fat product. The following are benefits of the project:

1. Determine the influence of SC-CO_2 extraction using an inexpensive CO_2 on the oxidative stability of an extracted product.
2. Determine the feasibility of partially defatting walnut pieces using SC-CO_2.
3. Determine the defatting thresholds for walnut pieces using SC-CO_2.
4. Determine the flavor and textural attributes of SC-CO_2 extracted walnuts.

5. Develop a reduced-fat walnut product.
6. Determine the oxidative stability of the extracted walnut oil co-product from SC-CO_2 defatting.
7. Develop a walnut product with increased oxidative stability.
8. Develop a walnut product less prone to oxidative instability in intermediate moisture food applications, such as confections.

In order to achieve these objectives, several other investigations were performed. To determine extraction conditions appropriate for removing oil from oxidized walnuts for analyses, we examined the influence of several different extraction methods on the oxidative products in oxidized and unoxidized walnuts. Development of a micro-titration method for measurement of peroxides was desirable because only a small amount of sample was available for testing. In addition, many portions of the study required that a small number of samples be tested on a single day, making it inefficient to save samples for testing using other micro-methods. Finally, methodology to measure volatile oxidation products was developed using solid-phase microextraction.

CHAPTER 3. ADAPTATION OF THE AOCS OFFICIAL METHOD FOR MEASURING HYDROPEROXIDES FROM SMALL-SCALE OIL SAMPLES

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ABSTRACT:

An adaptation of the AOCS Official Method Cd 8-53 for determining peroxides in fats and oils using a 0.5-g sample is described. Comparisons of the Official and small-scale methods were performed on soybean oil samples spiked with *t*-butyl hydroperoxide, and autoxidized soybean oil samples. A linear relationship between the Official and small-scale method was obtained with an R^2 of 0.998. The small-scale method is sensitive, precise, suitable for small sample sizes, and uses only about 10% of the chemicals necessary for the Official Method.

INTRODUCTION:

The AOCS Official Method Cd 8-53 for determining peroxide value (PV) is an effective, easy-to-use method, which requires equipment and glassware readily available in most laboratories. One negative aspect of this method is the need for a relatively large sample size of ~ 5.0 g (1). In some research studies, oil quantities may be limited, either by physical space during storage studies, or by sample availability, as in the case of experimental or exotic oilseed crops. Thus, the AOCS procedure is not a viable method in these situations. Several methods suitable for use with small sample sizes have been

proposed (2-5), however, these methods are often too complex for evaluation of small numbers of samples and can require special reagents or equipment. The use of smaller sample sizes with the AOCS Official Method would result in less chemical waste and reduced exposure to potentially toxic solvents, such as chloroform. The objective of this study was to modify and evaluate the AOCS Official Method Cd 8-53 of PV determination for use with 0.5 g-oil samples, a sample size of about 10% of the weight recommended in the AOCS Official Method.

EXPERIMENTAL PROCEDURES:

Refined, bleached and deodorized (RBD) soybean oil was obtained from Archer Daniels Midland Company (Decatur, IL). Three sets of samples were evaluated, stored soybean oil samples (Oil Set I), standard soybean oil samples spiked with different amounts of *tert*-butyl hydroperoxide (TBHP; Oil Set II), and a variety of oils from several sources oxidized to various levels (Oil Set III). The PV analyses by the Official and small-scale methods were performed in triplicate. The entire experiment was replicated three times for Oil Sets I and II.

Stored soybean oil (Oil Set I).

To simulate oxidative stability testing conditions, RBD soybean oil was subjected to accelerated storage conditions. The oil was stored in a 60 °C oven in the dark for 14 days in 100-mL beakers containing 50 mL of oil (surface area to volume ratio 0.03 mm^2/mL) and loosely covered with plastic wrap. The PV were measured every two days.

Standard RBD soybean oil spiked with TBHP (Oil Set II).

The TBHP solution (5.5 M in isooctane; 95% TBHP, 5% H_2O, and 5% butanol) was obtained from Aldrich Chemicals (Milwaukee, WI). Various amounts of the TBHP solution were added to the RBD soybean oil, which had been sparged at 50 °C with helium for 16 h to destroy any existing hydroperoxides, to produce PV ranging from 0 to 100 meq/Kg oil.

Oil from various sources (Oil Set III).

To evaluate the small-scale PV method in a variety of samples, several oils purchased at a local grocery store (soybean, corn, olive, and sunflower oils) or extracted in our laboratory using supercritical carbon dioxide extraction (walnut oils 1 and 2) were analyzed for PV in triplicate. These oils were allowed to oxidize at room temperature for periods of between two weeks and two years.

AOCS Official Method Cd 8-53.

The AOCS iodometric procedure Cd 8-53 (1) was performed without modification using oil samples of approximately 5.0 g. A 0.1 N solution of sodium thiosufate ($Na_2S_2O_3$) was prepared and standardized according to the AOAC Method #942.27 (6).

Small-scale method.

A 0.001 N sodium thiosulfate solution was prepared by diluting the 0.1 N solution

100 times with boiled Milli Q-water. The 0.001 N solution was standardized according the AOAC Method #942.27 (6). The AOCS iodometric procedure Cd 8-53 (1) was performed using a 0.5 g sample, with all reagents at 10% of the amounts recommended for the standard procedure. Oil samples were titrated using a 0.001 N sodium thiosulfate solution into 50-mL beakers.

Statistical analysis.

Data were analyzed by analysis of variance (ANOVA) using the General Linear Model procedure of SAS (7). Coefficients of determination (R^2) and coefficients of variation were determined to evaluate the suitability of the method. Significance was established at $P < 0.05$.

RESULTS AND DISCUSSION:

Oil Set I.

Table 1 shows the PV determined by the AOCS Official Method and by the small-scale method. There was no significant ($P < 0.05$) difference in the overall means for the PV determinations by the two methods in samples oxidized at 60 °C in the dark with PV levels of 0 to 100 meq/kg of oil. The percentage difference between the two methods was greatest at the lowest PV level. Subjective determination of the end-point using this procedure was difficult, particularly in samples with low PV, so a 50-mL beaker was used for the small-scale procedure in place of the Erlenmeyer flask recommended in the Official Method. The greater depth of field of the beaker allowed easier visualization of

easier visualization of the colorimetric end-point, however visualization at low PV was still difficult. The greater differences at these low PV was likely a result of human error in judgement of the end-point. Figure 1 shows a plot of the relationship between the PV obtained by the Official Method and those obtained using the small-scale method. The correlation coefficient (R^2) between the two methods was 0.998.

Oil Set II.

Similarly, a comparison of the methods using samples prepared from sparged soybean oil with various levels of added TBHP to produce a PV range of 0 to 100, resulted in an R^2-value of 0.994 (Fig. 2). The PV obtained using both the AOCS Official and small-scale methods were linear within the 0 to 100 range. This range is expansive enough to encompass PV of most fats and oils during normal and accelerated storage conditions.

Oil Set III.

Because the iodometric titration method is highly empirical, the results and accuracy of the test are strongly dependent on experimental conditions, including sample type. The results from PV determinations of various oil samples using both the AOCS Official Method and the small-scale method are shown in Table 2. Both methods resulted in similar PV for each of the different oils, with relatively small standard deviations.

The two principal sources of error in the iodometric methods for the determination of peroxides are 1) the absorption of iodine at unsaturated bonds of the fatty material, and

2) the liberation of iodine from potassium iodide by oxygen present in the solution being titrated (9). Oxygen in the solution of a sample causes the liberation of iodine from potassium iodide by the following reaction:

$$4I + O_2 (air) + 4H \text{ ------> } 2I_2 + 2H_2O$$

This reaction, which is accelerated in the presence of light and peroxides, is sometimes referred to as the oxygen error, and leads to high results in peroxide determination. The assumption that subtracting a blank determination negates this error may be incorrect because the effect of oxygen is more pronounced in the presence of peroxides.

Because of the large surface area to volume ratio of the samples used for the small-scale method, there was concern that oxygen would be rapidly absorbed into the sample, resulting in elevated PV measurements. To attenuate the risk of oxygen error, each sample was weighed and immediately analyzed before weighing the next sample. Preliminary determinations of PV in RBD soybean oil samples indicated that this precaution reduced coefficients of variation, particularly in samples with high levels of peroxides. For the small-scale method, standardizing the 0.001 N sodium thiosulfate, which was prepared by diluting the 0.1 N solution, also reduced error, particularly at very low (0 to 2 PV) peroxide levels.

In addition, variation in weight of sample, variation in reaction conditions, such as time and temperature, type and grade of solvent used, and the types and reactivity of the peroxides being titrated can significantly influence measured PV. In an effort to test this

method under the most lax conditions, thus maximizing the ease and convenience of the small-scale method, no efforts to control temperature, such as utilization of ice-baths, were made. All samples were analyzed under ambient conditions with both Official Method and small-scale method determinations for individual samples performed on the same day.

The type of peroxide present in a sample also can influence the liberation of iodine. For example, dialkyl peroxides, which may be formed during the termination reaction of fat oxidation, are much less easily reduced than are hydroperoxides (8). For this reason, it is important that validation of tests designed to measure PV include side-by-side methodological comparisons of samples with known storage histories. Simply adding known quantities of peroxides may not be indicative of how a test will perform under real conditions, because as oxidation progresses, several stages of oxidation occur simultaneously. Thus, several different species of peroxides, with varying degrees of reducibility, may exist concurrently in a given sample.

Results from the small-scale method agree closely with those obtained by the AOCS Official Method over a wide PV range with soybean oil as well as plant oils from other sources. The small-scale method is advantageous because its use greatly reduces the use and disposal of organic solvents and is effective for evaluation of small (0.5 g) oil samples.

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Table 1. Peroxide Values of Oxidized Soybean Oils (Oil Set I) Determined by AOCS and Small-Scale Methods

Method	PV (meq/kg) at specified days								Overall mean (%)	CV[a] (%)
	0	2	4	6	8	10	12	14		
AOCS Official	0.4	15.7	41.8	49.9	66.3	78.7	91.6	100.7	55.6	1.3
Small-scale	0.5	15.7	43.8	49.3	64.9	76.2	91.8	100.8	55.4	2.7

[a]CV, coefficient of variation.

Table 2. Peroxide Values of Autoxidized Oils (Oil Set III) Determined by the AOCS and the Small-Scale Methods

Oil	PV[a], meq/kg	
	AOCS Official	Small-Scale
Soybean	5.3 ± 0.15	5.1 ± 0.15
Corn	12.3 ± 0.22	12.6 ± 0.26
Olive	8.9 ± 0.28	9.3 ± 0.21
Sunflower	26.7 ± 0.75	28.0 ± 0.92
Walnut #1	1.2 ± 0.15	1.4 ± 0.20
Walnut #2	9.8 ± 0.25	9.5 ± 0.30

[a]Mean of three replications ± standard deviation.

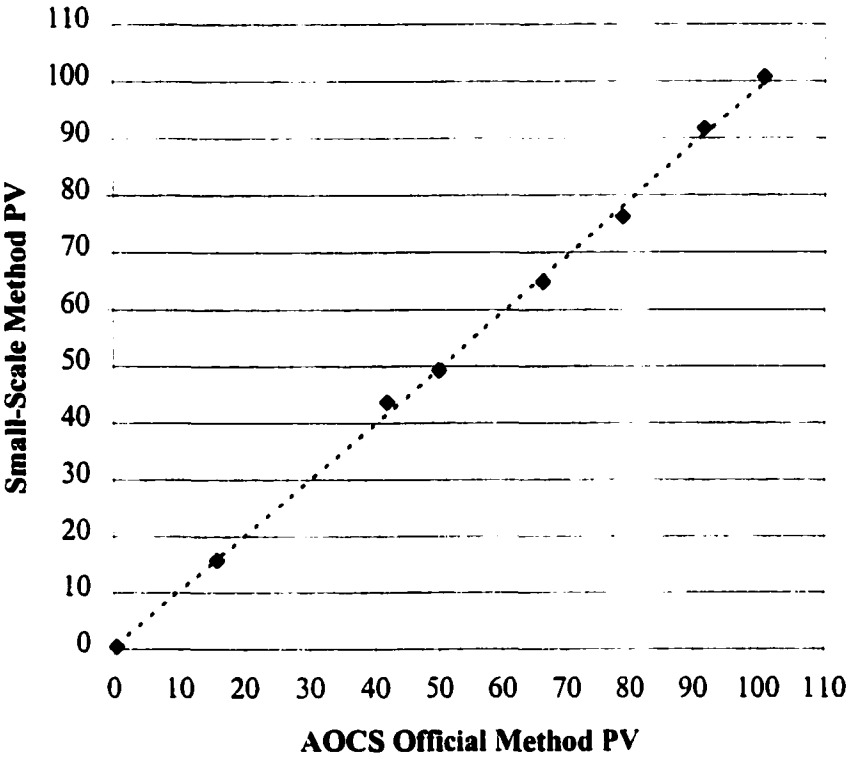

Fig. 1. Relationship Between the AOCS Official Method and the Small-Scale Method for Determination of PV (meq/kg) in Autoxidized Soybean Oil (Oil Set I). The correlation coefficient (R^2 value) between the two methods was 0.998.

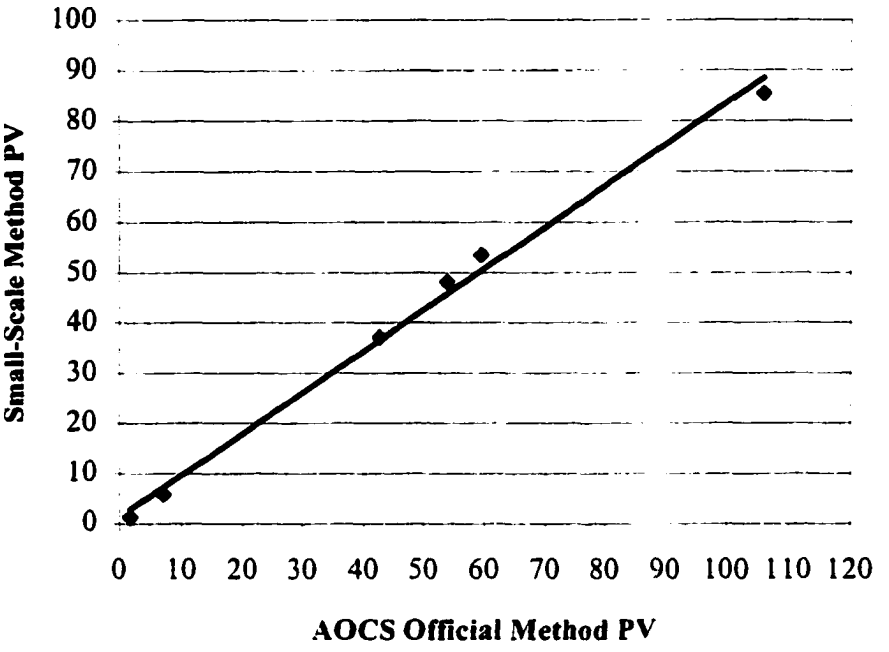

Fig. 2. Relationship Between the AOCS Official Method and the Small-Scale Method for Determination of PV (meq/kg) in Soybean Oil Spiked with *tert*-butyl hydroperoxide (Oil Set II). The correlation coefficient (R^2 value) between the two methods was 0.994.

CHAPTER 4. SOLID-PHASE MICROEXTRACTION AND DYNAMIC HEADSPACE METHODS FOR DETERMINING LIPID OXIDATION

A paper to be submitted to the Journal of the American Oil Chemists' Society

Tammy D. Crowe and Pamela J. White

ABSTRACT:

Measurement of volatile oxidation products is important in the assessment of oil flavor and stability. Solid-phase microextraction (SPME) is a solvent-free, inexpensive, rapid and easy-to-use technique successfully used to analyze volatile compounds in various food and beverage systems. Dynamic headspace (DHS) and SPME were compared to determine the effectiveness of SPME for extracting the headspace volatiles of soybean oil and soybean flakes. Soybean oils and full-fat soybean flakes were stored under accelerated conditions and analyzed by DHS trapping onto Tenax-packed capillary tubes and SPME extraction using a 100 μm poly(dimethylsiloxane) fiber. Volatile compounds were measured by gas chromatography (GC) with a flame ionization detector at zero time and after 8 and 15 days of oil storage. There were both qualitative and quantitative differences between DHS and SPME. Increased peak intensities, total peak area, and a greater number of peaks were measured using DHS; however, SPME had increased reproducibility with a decreased coefficient of variation for the major soybean oil oxidation products. Similar changes with time in most volatile compounds during storage at both 60 °C (dark) and 35 °C (light) were measured by both methods. These

results suggest that SPME may be a viable method for assessing changes in the major oxidation products of soybean oil, soybean flakes and other lipid-containing materials.

INTRODUCTION:

Numerous analytical methods have been reported for measuring volatile components in lipid systems, important contributors to both desirable and undesirable flavors and odors (1-5). Most of these methods are time-consuming, expensive, require special equipment or glassware, and can lead to thermal degradation of volatile compounds in samples because of the high temperatures required for analysis. Of these methods, solid-phase microextraction (SPME) recently has gained considerable attention as a rapid, highly reproducible, quantitative method for measuring volatile lipid components (5-6). The SPME apparatus consists of a fused silica fiber coated with a polymeric film, and a stainless steel plunger mounted to a holder. Several fiber coatings are commercially available and can be used to enhance fiber selectivity for specific compounds. Because SPME extracts volatile compounds at relatively low temperatures, further degradation of hydroperoxides and flavor compounds is avoided. The 100 μm PDMS coated fiber has been used previously for the detection of volatile oxidation compounds in oils (5, 6, 8). The fiber has a high sample capacity, and is not prone to breakage.

The objective of this study was to compare dynamic headspace (DHS) trapping onto Tenax, a widely used technique for the analysis of volatile compounds, with

SPME,for the volatile analyses of soybean oil and soybean flakes oxidized to different levels.

EXPERIMENTAL PROCEDURES:

Refined, bleached and deodorized (RBD) soybean oil was obtained from the Archer Daniels Midland Company (Decatur, IL). To simulate conditions used for oxidative stability testing, the oil was stored at 60 °C in the dark and 35 °C in the light (540 lx). For each storage condition, twelve 100-mL beakers containing 50 mL of oil (surface area to volume ratio 0.03 mm^2/mL) were loosely covered with plastic wrap and stored in a constant temperature oven for 14 days. Stored oil samples were removed at days 7 and 14 for volatile analysis. Full-fat soyflakes (~ 20% fat (w/w)) were obtained from the Center for Crops Utilization Research (Iowa State University, Ames IA). The soy flakes were stored on aluminum trays loosely covered with plastic wrap, at 60 °C in the dark for 14 days. Each storage study was carried out in duplicate with volatile analysis by either SPME or DHS performed in triplicate, and peroxide value (PV) measured in duplicate according to AOCS Official Method Cd 8-53 (7) at each timepoint (0, 7 and 14 days) for each experimental replication.

Solid-phase microextraction (SPME) of soybean oil.

Two milliliters of oil was placed in a 20-mL glass vial sealed with a teflon/silicone septum. After equilibration of the oil sample for 5 min at 50 °C, the 100 μm poly(dimethylsiloxane) (PDMS) SPME fiber was exposed to the vial headspace for

30 min at 50 °C. The PDMS fiber was thermally desorbed in the gas chromatograph (GC) inlet for 1 min.

Solid-phase microextraction (SPME) of soy flakes.

Soyflakes (5 g) were placed in a two-neck round-bottomed flask. One neck of the flask contained a small external fan designed to circulate the headspace above the sample. The SPME device was inserted through the other neck. The flask was placed in a 50 °C water bath, where the sample was equilibrated for 5 min prior to exposure of the SPME fiber (100 μm PDMS) for 30 min.

Dynamic headspace (DHS) onto Tenax.

The DHS method described by Lee *et al.* (4) was used. A 5-mL oil sample was placed in a specially designed volatiles-stripping apparatus, immersed in a 50 °C water bath, and sparged with helium (74 mL/min). The oil sample was equilibrated for 5 min prior to collection of volatile compounds onto a 3-mm o.d. x 72-mm glass tube filled with Tenax for 30 min. The Tenax-filled adsorbent tube was thermally desorbed in the GC inlet. For evaluation of soy flakes, a 5-g sample was placed on a mesh screen inserted into the volatiles-stripping apparatus.

Gas Chromatography/mass spectrometry.

Volatile compounds were desorbed onto a Hewlett-Packard 5890 Series II GC (Palo Alto, CA) with an inlet temperature of 230 °C, and transferred in helium at 1.7

mL/min onto a Supelco SPB-1 fused-silica capillary column (30 m, 0.25-mm i.d., 0.25 μm film thickness; Bellefonte PA). The GC was held for 3 min at an initial temperature of 30 °C, increased to a final temperature of 210 °C at 6 °C/min, with a final hold time of 5 min. Peaks were detected by a flame ionization detector (FID) held at 220 °C. Standard response curves were prepared for the major volatile compounds in oxidized oils using authentic standards added to stripped soybean oil (sparged at 50 °C with helium for 16 h) in concentrations from 10^{-3} to 10^{-7}. For mass spectrometry, a Hewlett-Packard 5970 mass-selective detector was used in place of the FID. Peaks were identified by comparing their retention times and mass spectra with those of known compounds.

Statistical analysis.

Data were analyzed by analysis of variance (ANOVA) using the General Linear Model procedure of SAS. Significance was established at $P < 0.05$.

RESULTS AND DISCUSSION:

In the soybean oils, both the DHS and SPME methods were carried out at identical temperatures and sampling times, 50 °C for 30 min. At this temperature decomposition of peroxides occurs slowly (9), and therefore the volatile compounds measured by GC should be representative of the compounds actually present in the oil sample. Total integrated peak areas (Table 1) and number of peaks were significantly greater for DHS than for SPME. This difference was expected because the SPME fiber

offers greater selectively for specific types of compounds than does DHS Tenax-trapping. Dynamic sampling also results in more complete extraction of volatile and semivolatile compounds present in the sample.

Preliminary analysis of soy flakes using various sample sizes in a 20-mL vial did not result in good extraction or adsorption of volatile compounds. Extraction of volatile compounds improved when a 5-g sample was added to a two-neck, round-bottomed flask; however, the GC response was extremely low. The addition of a fan to one neck of the flask greatly increased the GC response, even with "intact" soy flakes. Experiments using this system with walnuts revealed excellent detection of oxidation products without grinding or crushing influence. The ability of this system to extract volatile compounds from food products with minimal disruption, coupled with the low temperatures used, may be important for flavor analysis using SPME.

Quantitative comparisons between DHS and SPME yielded similar results for selected compounds (Fig. 1-7). Higher analyte concentrations, as measured by GC peak area, were generally observed using DHS. Changes in concentrations of selected volatile compounds over time, however, were similar (Table 2). For the purpose of this study volatile compounds to be compared were selected based on the following criteria: representative of a range of oxidation products (e.g. various chain lengths); compounds for which authentic standards were available; previous literature indicating changes with time when subjected to autoxidative or photooxidative conditions. Pentane, although an important indicator of oil oxidation, was not included because of its high flavor threshold (340 ppm; (10)). Hexanal was the major compound in both autoxidized and photo-

oxidized soybean oil, and autoxidized soy flakes. Incomplete recovery of compounds with five or fewer carbons also was reported for the DHS method used in this study (4). Snyder *et al.* (1) reported relatively lower concentrations of pentane using DHS compared with static headspace. Snyder *et al.* (1) also reported higher quantities of heptadienal and decadienal using dynamic versus static headspace.

The relatively small number of steps and decreased sample manipulation required for SPME use resulted in increased precision, with fewer background peaks than DHS. The relative formation of artifact or interference peaks from the food matrix was low with both the DHS method used here, and SPME, because volatile compounds were able to reach equilibrium without being heating to elevated temperatures. Proper conditioning of the SPME fibers before initial use, and routine desorption of fibers during use, greatly reduced background peaks. The relatively large SPME needle also tended to cause small pieces of the GC injector septa to be deposited in the GC injection liner. Frequent cleaning of the liner was necessary to achieve consistent results. Silicate compounds resulting from SPME fiber bleed were noted as described previously (11-12). These compounds generated reproducible and distinctive chromatograms, and were easily distinguished from other analytes of interest.

Coefficients of variation were 20.3 and 10.2% for DHS and SPME, respectively. Steenson *et al.* (13) reported a coefficient of variation of 3.2% for SPME analysis of oxidized soybean oil following optimization and practice using the technique, although initial CV's were as high as 20%. Similarly, we found that increased familiarization with, and use of, the SPME apparatus and sampling techniques significantly increased

analytical precision. In particular, strict adherence to procedures that ensured a "clean" fiber prior to sampling, such as using blank runs and thermal desorption of the fiber, greatly reduced variability. Marsili (14) compared DHS with SPME for the analysis of photo-oxidation products in milk and reported greater precision for SPME than for DHS, with coefficients of variation for individual compounds extracted using DHS two to four times greater than SPME. The small number of steps and decreased sample manipulation used with SPME significantly reduce experimental and sample to sample variation. Much of the increased error observed with DHS can likely be attributed to sampling issues associated with the purging, trapping and desorption steps.

Static headspace sampling is routinely used because it is rapid, and requires minimal sample preparation and clean-up (1). One problem with static headspace sampling is dilution of compounds by headspace gases. Because SPME is based on the adsorption of analytes onto a solid phase (i.e. fiber), the influence of volatile compound dilution is minimized. In general, however, only compounds of relatively high vapor pressure (i.e. low boiling point) are suitable for analysis by static headspace. Snyder *et al.* (1) compared direct injection, static headspace, and dynamic headspace GC for the volatile analysis of soybean oil oxidized to different peroxide levels. Higher molecular weight compounds, primarily isomers of 2,4-decadienal, were the major components found by direct injection and dynamic headspace analyses. Static headspace favored lower molecular weight compounds (e.g. acrolein, propanal and pentane). SPME analysis greatly improved recovery of oxidized flavor compounds in various juices

compared with traditional static headspace sampling, with SPME peaks ~ 1800 times larger than gas-sampled peaks (15).

Detection limits are comparable with current analytical methods (16), however, fiber selectivity limits the number of compounds adsorbed. Therefore, the PDMS fiber may not be well suited for quantitative analysis because these fibers strongly discriminated against more polar and very volatile compounds. The PDMS coating favored adsorption of longer, more lipophilic compounds in apple fruit than did GC/time-of-flight/MS (17). A sampling time of 30 min. was previously found to be sufficient for most soybean oil oxidation compounds when analyzed by SPME using a 100 μm PDMS fiber (5). The PDMS fiber had the lowest coefficient of variation for analysis of soybean oil oxidation compounds among the fibers tested (13).

Sensitivity also is limited by the nature of the sample matrix. Yang and Peppard (11) reported that sensitivity is generally lower in oil samples than with similar compounds in aqueous conditions because compound solubility is generally much higher in oil. Elmore et al. (12) identified 55 and 48 compounds in regular and diet colas, respectively, using DHS, and 25 and 15 compounds using SPME. DHS favored more volatile compounds and there was an increase in medium polarity compounds such as alcohols. The wide variation in SPME performance reported in the literature indicates that successful SPME analysis is strongly dependent on the sample composition and the experimental conditions.

Traditionally, DHS analysis has been considered optimal for quantitative studies of volatile oxidation compounds, because of its high sensitivity compared with that of

static headspace methods. However, DHS is also labor-intensive with respect to sample preparation, analysis and clean-up, and requires expensive specialized equipment or glassware. In addition, complete desorption of adsorbent polymers is often difficult, resulting in sample carryover. Lower-boiling point compounds may be lost during purging, while less volatile compounds such as hexanal, heptadienal and decadienal are concentrated on the trap, thus altering the relative ratios of these compounds (1). The primary advantages of SPME over DHS are ease of use, rapid sampling times and low cost. The selectivity offered by the SPME fibers is particularly useful when the compounds of interest are known, e.g. when screening samples for oxidation markers.

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Table 1. Total peak area (integrator counts) of volatile compounds from soybean oils analyzed by DHS and SPME

Time	DHS	SPME
Day 0	1,295,107	632,661
Day 7 (light)	2,684,206	964,233
Day 14 (light)	6,955,885	1,275,136
Day 7 (dark)	4,564,905	984,657
Day 14 (dark)	25,215,734	5,542,383

Table 2. Correlation between DHS and SPME measurements of selected volatile compounds in soy flakes

Compound	Correlation Coefficient (R^2)	p-value
t-2 pentenal	0.829	0.071
hexanal	0.811	0.082
t-2 heptenal	0.791	0.096
nonanal	0.882	0.041
2,4-decadienal	0.808	0.084

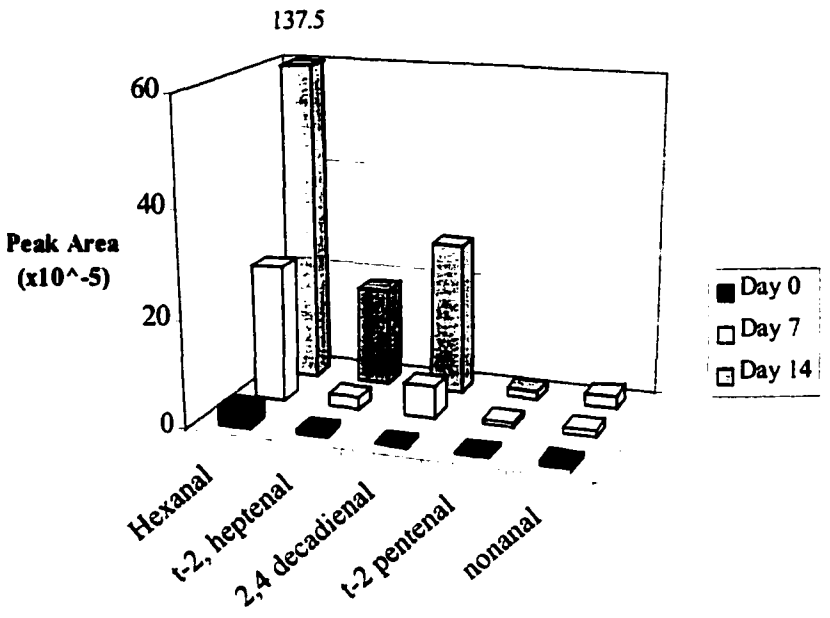

Fig. 1. Peak Areas of Selected Volatile Compounds in Soy Flakes Measured Using DHS

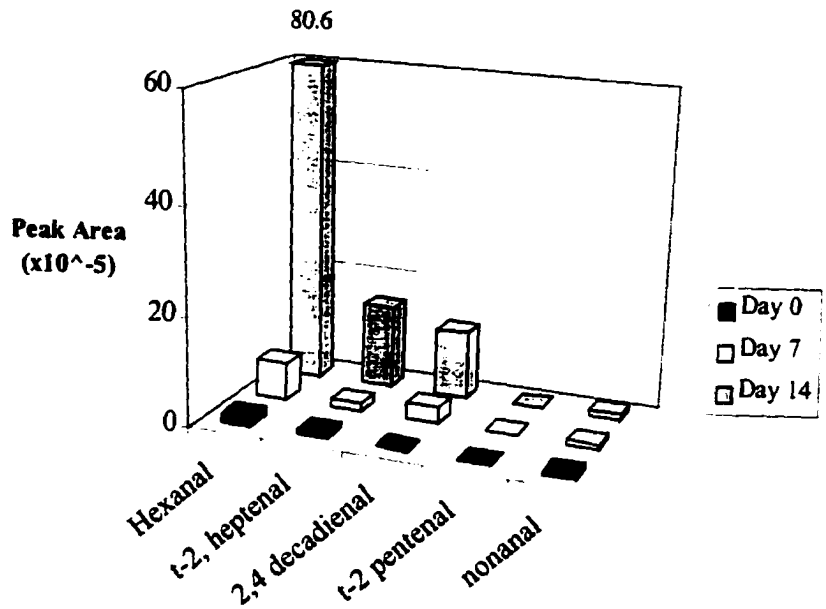

Fig. 2. Peak Areas of Selected Compounds in Soy Flakes Measured by SPME.

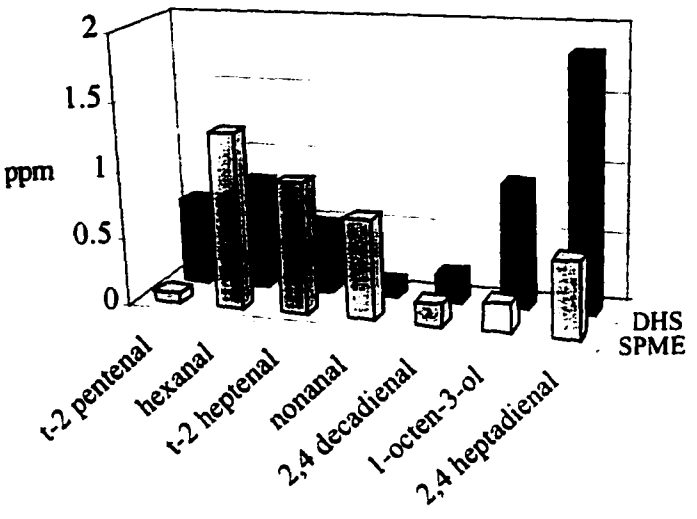

Fig. 3. Concentration of Selected Volatiles in Soybean Oil Measured by DHS and SPME at Day 0.

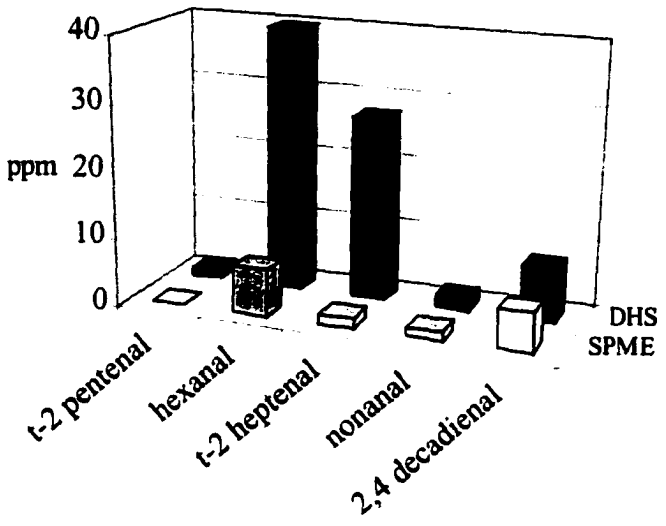

Fig. 4. Concentration of Selected Volatiles in Soybean Oil Measured by DHS and SPME at Day 7 (60 °C, Dark Storage).

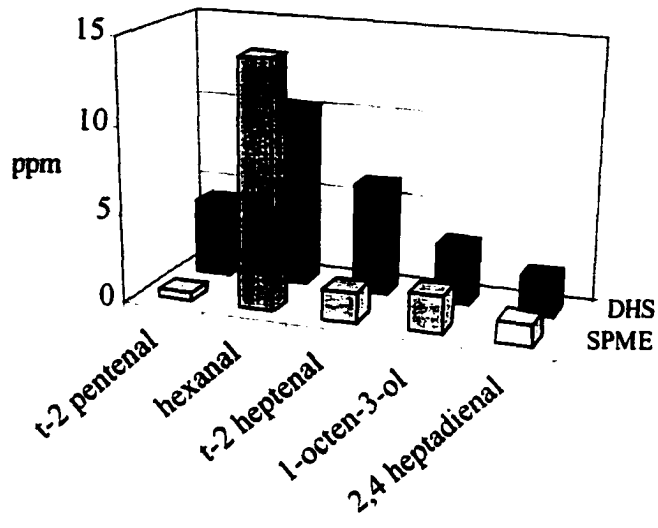

Fig. 5. Concentration of Selected Volatiles in Soybean Oil Measured by DHS and SPME at Day 7 (35 °C, Light Storage).

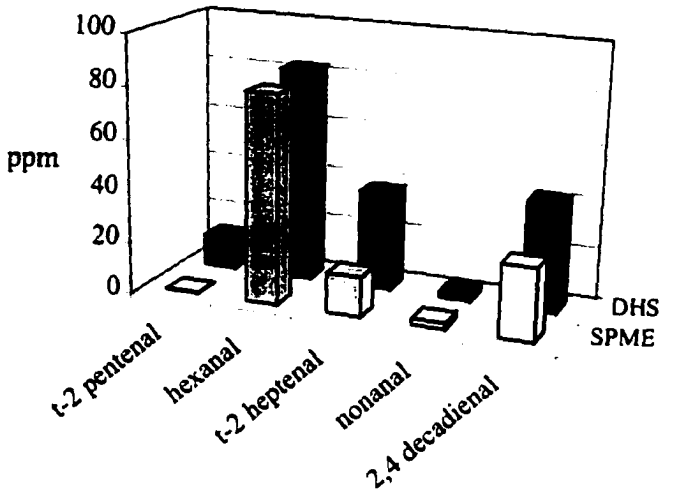

Fig. 6. Concentration of Selected Volatiles in Soybean Oil Measured by DHS and SPME at Day 14 (60 °C, Dark Storage).

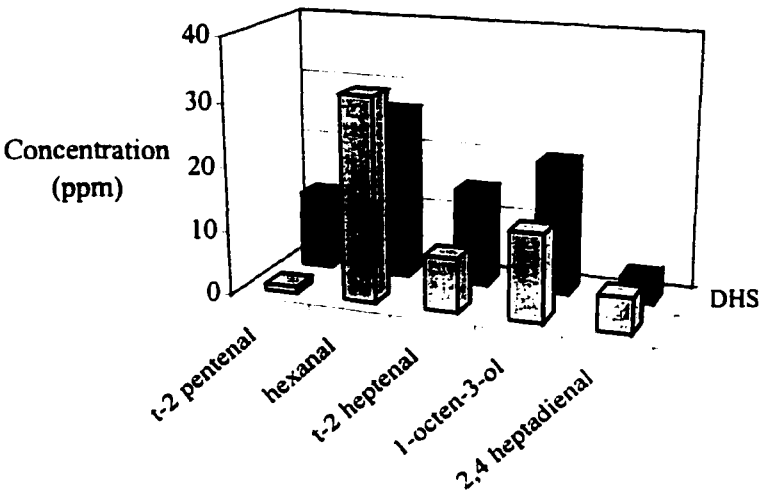

Fig. 7. Concentration of Selected Volatiles in Soybean Oil Measured by DHS and SPME at Day 14 (35 °C, Light Storage).

CHAPTER 5. IMPACT OF EXTRACTION METHOD ON LIPID OXIDATION PRODUCTS FROM OXIDIZED AND UNOXIDIZED WALNUTS

A paper submitted to the Journal of the American Oil Chemists' Society

Tammy D. Crowe, Troy W. Crowe, Lawrence A. Johnson, and Pamela J. White

ABSTRACT:

Oxidation is a great concern in fat-containing foods because of its impact on consumer acceptability and product shelf-life. The quality of fat in these foods is generally assessed by measuring indices of lipid oxidation and deterioration. Accurate measurement of these oxidation products may be problematic because of the influence of extraction solvents or conditions that potentially promote lipid oxidation or degradation, particularly in products where oxidation has already occurred. The objective of this study was to measure and compare differences in oxidized products of oil extracted from unoxidized and oxidized walnuts using five different extraction methods: mechanical pressing, or solvent extraction with hexane, methylene chloride, chloroform-methanol, or supercritical carbon dioxide (SC-CO_2). Of the extraction methods evaluated, only chloroform-methanol and methylene chloride provided reasonable results for all parameters measured (total lipid yield, fatty acid profile, peroxide value, conjugated dienes, free fatty acids content, and volatile content); however, chloroform-methanol extracted significantly greater levels of volatile compounds. The SC-CO_2 extraction with

compounds, as these materials are lost during the lipid extraction. Pressing was neither quantitative nor qualitative, and hexane extraction retrieved significantly lower levels of volatiles than the other methods, except for SC-CO_2.

INTRODUCTION:

The qualitative and quantitative extraction of lipids is imperative to the success of analytical procedure in examining the shelf-life of high-fat foods, such as walnuts. The quality of fat-containing foods may be assessed by measuring indices of lipid oxidation, such as peroxide value, conjugated dienes, free fatty acids, and volatile compounds. The methods employed to extract lipids from oxidized foods may influence the values obtained for these oxidative products. Extraction conditions, e.g. temperature, time, and type of solvent, have been shown to affect sensory quality (1), volatile content (2) and oxidative stability of the extracted oil (3,4).

For laboratory-scale extraction, using chloroform-methanol as the extraction solvent (5-6) is generally considered optimal for removal of all lipid classes (7). But, direct comparisons of this and other methods, including solvent-free extraction techniques such as pressing and supercritical CO_2, with regard to complete removal of oxidation products and potential artefact production during extraction, have not been published. The purpose of this study was to compare the oxidation products present in the oil extracted by several different methods from both unoxidized and oxidized walnuts.

EXPERIMENTAL PROCEDURES:

Food-grade premium walnut pieces were obtained from Diamond Walnut Growers, Inc. (Stockton, CA) and stored at –20 °C prior to use. For oxidized samples, walnuts were stored on loosely covered aluminum trays at 60 °C in the dark for 8 days. Following extractions, oil samples were stored at –20 °C under nitrogen. All extractions were performed in triplicate.

Methylene chloride extraction.

Lipids were extracted according to the method described by O'Keefe *et al.* (8) with modifications. For each extraction, 100 g of walnuts and 300 mL of methylene chloride were blended for 1 min at high speed in a Waring blender. The slurry was vacuum filtered through Whatman No. 1 filter paper (Maidstone, England), and reextracted with 200 mL methylene chloride. Solvent was removed by rotary evaporation under vacuum at 40 °C.

Chloroform methanol extraction.

The Bligh and Dyer method (6) with modifications was employed. Walnuts (50 g) were homogenized using a Polytron homogenizer (Kinematica, GmbH, Switzerland, distributed by Brinkman Instruments, Westbury, NY) with 180 mL of water, 200 mL of methanol, and 100 mL of chloroform for 2 min. An additional 100 mL of chloroform

was added to the mixture and blended for two more min. The homogenate was centrifuged at 500 x *g* for 10 min, the upper layer was removed by aspiration, and the bottom layer was vacuum filtered through Whatman No. 1 filter paper, reextracted with 200 mL of chloroform and refiltered. The chloroform-lipid extract was passed through anhydrous sodium sulfate (Na_2SO_4), and the Na_2SO_4 was rinsed with 100 mL of chloroform. Solvent was removed by rotary evaporation under vacuum at 30 °C.

Hexane extraction.

A pilot-scale hexane extraction method described by Shen *et al.* (9) was used. For each extraction, 700 g of walnuts previously ground for 1 min at high speed in a Waring blender were placed in a glass vessel and extracted at 60 °C at a ratio of 2:1 (hexane:walnuts). Walnuts from each replication were extracted six consecutive times with fresh hexane for 6 min of recirculation, plus 3 min of drainage, and the collected solvent pooled. The total extraction time was approximately 45 min. Walnut oil was desolventized in a rotary evaporator under vacuum at 60 °C.

Supercritical CO_2 (SC-CO_2) extraction.

Walnut pieces were ground to pass through a 10-mesh screen, and a weighed amount (2.5 g) was placed inside an extraction vessel. Diatamaceous earth (0.5 g) was placed above and below the sample. The extraction vessels were placed in a FastFat9(HT) (ISCO Inc., Lincoln, NE) supercritical fluid extractor, where the contents

were extracted with either welding grade (Praxair Inc., Danbury, CT) or Coleman grade CO_2 (Air Products Inc., Allentown, PA) at 10,000 psi for 35 min, a CO_2 flow rate of 5.0 mL/min, and chamber and restrictor temperatures of 85 °C and 80 °C, respectively. The extracted oil was collected into an evaporator flask fitted with a glass wool plug and cooled in an ice bath. A total of twenty runs were performed, with the oil pooled for each of the triplicate extractions.

Pressing.

Walnuts (20 g) were placed between two 25 cm x 25 cm aluminum plates and mechanically pressed (Black Brothers Laminating and Gluing Equipment, Mendota, IL) at 1,000 psi for 2 to 3 min. Extracted oil was collected using a rubber spatula and filtered through cheesecloth prior to storage. A total of ten presses were performed, with the oil pooled for each of the triplicate extractions.

Analytical methods.

Peroxide values (PV) and conjugated dienes were determined by the American Oil Chemists' Society (AOCS) official methods Cd 8-53 and Ti 1a-64, respectively (10). Free fatty acids (FFA) were measured according to a modification of method Ca 5a-40 (AOCS 1989) proposed by Rukunudin *et al.* (11) using a 0.5 mL oil sample. Total oil content was determined gravimetrically after the extract was dried to a constant weight as outlined in method Ca 2c-25 (10). Fatty acids were prepared for analysis as fatty acid

methyl esters (FAMES) as previously described (12). The FAMES were injected onto a Hewlett-Packard 5890 Series II gas chromatograph (Kennett Square, PA) equipped with a flame-ionization detector. A J&W Scientific DB-23-fused-silica capillary column (15m, 0.25 mm i.d., 0.25 μm film thickness; Rancho Cordiva, CA) was used for isothermal analysis at 200 °C, with a helium carrier gas flow rate of 100 mL/min. All analyses were performed in duplicate and the results averaged.

Volatile analysis was performed using solid phase microextraction (SPME) as described by Snyder *et al.* (13). Briefly, a 2-mL sample of oil with 1 ppm dodecane added as an internal standard was placed in a 20-mL clear glass vial from Supelco with a teflon-lined silicone septa. The vial was placed in a 50 °C water bath and allowed to equilibrate for 5 min. The SPME fiber (polydimethylsiloxane, Supelco Inc., Bellefonte, PA) was exposed to the headspace for 30 min, thermally desorbed in the inlet of a Hewlett-Packard 5890 Series II GC (Palo Alto, CA) at 230 °C, and transferred in helium at 1.7 mL/min onto a Supelco SPB-1 fused-silica capillary column (30 m, 0.25-mm i.d., 0.25 μm film thickness). The GC was held for 3 min at an initial temperature of 30 °C, increased to a final temperature of 210 °C at 6 °C/min with a final hold time of 5 min. Peaks were detected by a flame ionization detector (FID) held at 220 °C. For mass spectrometry, a Hewlett-Packard 5970 mass-selective detector was used in place of the FID. Peaks were identified by comparing their retention times and mass spectra with those of known compounds. Standard response curves were prepared for the major

volatile compounds in oxidized oils using authentic standards added to stripped soybean oil (sparged at 50 °C with helium for 16 h) in concentrations ranging from 10^{-3} to 10^{-7}.

Statistical analysis.

Statistical analyses were performed using the General Linear Model Procedures of SAS 6.06 (SAS, 1991). Tukey's (HSD) Studentized t-test was used to test for significant differences between mean values of analytical procedures among treatments, with significance established at a level of $P < 0.05$.

RESULTS AND DISCUSSION:

The oil yields by each of the extraction methods are presented in Table 1. As expected, oil extraction by pressing did not result in a quantitative yield. Oil recoveries from walnuts using organic solvents and SC-CO_2 were not significantly different from each other. Previously, similar results were reported for oil yields from SC-CO_2 extraction when compared with ether/hexane extraction (14), and acid hydrolysis/solvent extraction (15).

Fatty acid composition was not influenced by extraction method, with similar relative percentages of the predominant fatty acids in walnut oil achieved by all methods (palmitic, stearic, oleic, linoleic and linolenic acids) (Table 2). Also, there were no significant differences in fatty acid composition of the oxidized versus unoxidized walnuts among extraction methods.

Values for primary oxidation products (PV and conjugated dienes) were not significantly different among the solvent extraction methods used in this study, except for welding grade SC-CO_2. The PV obtained using Coleman-grade CO_2, however, were similar to those from the solvent extraction methods. Calvo *et al.* (4) reported that oxidative stability of supercritically extracted sunflower oil could be improved by using ultra-pure CO_2. The increase in primary oxidation products would be expected because of oxidation caused by CO_2 impurities. The PV from oxidized walnut oil extracted with chloroform-methanol tended to be greater than the PV obtained using the other solvent extraction methods and SC-CO_2 (Coleman), possibly because it is more efficient than the other solvents in extracting polar oxidation products. Peroxide values obtained from pressed walnuts for both unoxidized and oxidized walnuts were significantly lower than PV obtained for the other extraction methods (Table 3). Oil extraction by pressing was not quantitative and, therefore, may not have resulted in a representative sample, particularly in the presence of polar compounds such as hydroperoxides.

Among the extraction methods evaluated, conjugated diene values were significantly greater in both oxidized and unoxidized walnuts oils extracted with welding grade CO_2 (Table 3). No significant differences in conjugated diene values of oil from the unoxidized or oxidized walnuts were measured among the other five extraction methods (Table 3).

Unlike the results from the other analytical tests, the level of FFA in the lipids from unoxidized walnuts extracted with SC-CO_2 (Welding) was not different from the

so the FFA level would not be expected to change very much with walnut oxidation. Some enzymes present in the walnuts, however, might catalyze the fatty acid cleavage (16), as would water. FFA values were not different among extraction methods for the unoxidized walnuts, however, for oxidized walnuts, oil extracted with SC-CO_2 (Welding) had greater FFA than did hexane-extracted oils, which had significantly greater FFA than did oils from all other extraction methods (Table 3). The relatively high extraction temperature and length of solvent exposure to the walnuts during the hexane extraction method may have either solubilized more of the FFA that were present, or actually accelerated hydrolytic rancidity. The latter seems likely given that levels of FFA in oil from the oxidized walnuts were increased only after extraction with either hexane or welding-grade CO_2, and because impurities in the welding-grade CO_2, particularly water, might increase hydrolytic rancidity. As expected, FFA were greatest in the lipids from oxidized walnuts extracted using the lower quality, welding-grade CO_2.

Chloroform-methanol was the most effective among all methods for extraction of total volatile compounds, particularly from the oxidized walnuts (Table 3). Similar increases in hexanal, an important marker of oxidative flavor deterioration, with walnut oxidation was observed for both methylene chloride and pressing (Table 3). The total volatile content of the hexane-extracted walnut oil was lower by approximately an order of magnitude in both the unoxidized and oxidized samples, most likely indicating volatile loss during the extraction or desolventizing step. No volatiles were detected in oils extracted using SC-CO_2 from either the unoxidized or oxidized walnuts. King *et al.* (2) also reported relatively few volatile compounds in the lipid fraction from SC-CO_2 -

oxidation was observed for both methylene chloride and pressing (Table 3). The total volatile content of the hexane-extracted walnut oil was lower by approximately an order of magnitude in both the unoxidized and oxidized samples, most likely indicating volatile loss during the extraction or desolventizing step. No volatiles were detected in oils extracted using SC-CO_2 from either the unoxidized or oxidized walnuts. King *et al.* (2) also reported relatively few volatile compounds in the lipid fraction from SC-CO_2-extracted raw beef samples using milder SC-CO_2 extraction conditions than those presented here. These extraction conditions strip the sample of volatiles as a result of the procedure. If desired, the volatile compounds could be captured for further analysis. Several investigators have reported various methods for the collection of volatile compounds from lipids extracted using SC-CO_2, including Tenax traps (2), direct extraction to a gas chromatograph (17), and extraction into ethanol (18).

Of the extraction methods evaluated, only chloroform-methanol and methylene chloride provided reasonable results for all parameters (total lipid yield, fatty acid profile, PV, conjugated dienes, FFA and volatile content). The Bligh and Dyer chloroform-methanol extraction and the pilot-scale hexane-extraction methods were the most time consuming, in terms of both time (~ 2 h) performing the extractions and clean-up of equipment and glassware. SC-CO_2 extraction using high purity (Coleman grade) CO_2 was the most rapid and efficient method of oil extraction, requiring only grinding of the sample and relatively little clean up. SC-CO_2 extraction offers the additional advantage of being a solvent-free method. But, special procedures would be required for the

collection of volatile compounds when using this method, and quantitative extraction of lipids may require sample-specific extraction conditions for optimization, such as changes in extraction temperatures, pressures, and time (14). Several researchers have found SC-CO_2 to be a suitable replacement for traditional organic solvent extractions for determination of total lipids, and analysis of specific lipid components (14, 19-21), particularly in comparison with results obtained with less environmentally harmful nonpolar solvents.

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Table 1. Quantitative Yields (w/w) of Oil Extraction of Unoxidized Walnuts by Various Methods.

Extraction Method	Total Lipids[a], %
Hexane	67.2[a]
Methylene Chloride	69.8[a]
Pressing	32.2[b]
Chloroform Methanol	69.9[a]
SC-CO_2 (Coleman[b])	68.2[a]
SC-CO_2 (Welding[b])	68.2[a]

[a] Values within a row with the same letter are not significantly different, $P<0.05$.
[b]Coleman (high purity) and Welding grade CO_2

Table 2. Fatty Acid Composition of Oil Extracted from Unoxidized and Oxidized Walnuts.

	Fatty Acid Compostion[a], Relative Area %				
	16:0	18:0	18:1	18:2	18:3
Unoxidized Walnuts					
Hexane	6.5	2.5	15.2	60.2	14.9
Methylene-chloride	6.8	2.5	15.1	60.2	14.8
Pressing	6.8	2.6	15.5	60.3	14.5
Chloroform-methanol	6.7	2.6	15.5	60.4	14.9
SC-CO_2 (Coleman)	6.5	2.6	15.4	60.6	14.4
SC-CO_2 (Welding)	6.4	2.6	15.7	60.5	14.4
Oxidized Walnuts					
Hexane	6.6	2.9	15.5	60.2	14.3
Methylene-chloride	6.2	2.7	16.0	60.0	14.1
Pressing	6.4	2.9	14.8	60.8	14.8
Chloroform-methanol	6.5	2.7	15.7	60.4	14.4
SC-CO_2 (Coleman)	6.5	2.6	15.2	60.0	14.3
SC-CO_2 (Welding)	6.6	2.6	15.0	59.1	14.4

[a] No significant differences ($P<0.05$) in fatty acid composition among extraction methods.

Table 3. Peroxide Values (meq/kg), Conjugated Dienes (%), Free Fatty Acids (%), Hexanal Content (ppm) and Total Volatiles (Integrator Counts) of Oil Extracted by Various Methods from Unoxidized and Oxidized Walnuts.

	Peroxide Values (meq/Kg)	Conjugated Dienes (%)	Free Fatty Acids (%)	Hexanal (ppm)	Total Volatiles ($x10^{-5}$)
Unoxidized Walnuts					
Hexane	0.91[b]	0.13[a]	0.20[a]	0	2.8[a]
Methylene Chloride	1.01[b]	0.12[a]	0.26[a]	0	7.1[b]
Pressing	0.06[a]	0.11[a]	0.24[a]	0	10.3[b]
Chloroform Methanol	1.02[b]	0.12[a]	0.27[a]	0	14.5[c]
SC-CO_2 (Coleman)	0.99[b]	0.13[a]	0.23[a]	ND[b]	ND
SC-CO_2 (Welding)	1.58[c]	0.19[b]	0.22[a]	ND	ND
Oxidized Walnuts					
Hexane	9.41[b]	0.50[a]	0.40[b]	90.9 (19.1)[a]	5.3[a]
Methylene Chloride	8.61[b]	0.53[a]	0.23[a]	252.7 (5.8)[b]	25.4[b]
Pressing	5.02[a]	0.46[a]	0.27[a]	554.0 (10.3)[c]	31.5[b]
Chloroform Methanol	10.43[b]	0.50[a]	0.29[a]	627.2 (2.5)[d]	160.0[c]
SC-CO_2 (Coleman)	8.73[b]	0.51[a]	0.28[a]	ND	ND
SC-CO_2 (Welding)	12.37[c]	0.82[b]	0.59[c]	ND	ND

[a] Values within a column for each category (unoxidized or oxidized) with the same letter are not significantly different, $P<0.05$.
[b]ND, not detectable. Values in parentheses are percentages relative to total peak area.

CHAPTER 6. OXIDATIVE STABILITY, FLAVOR AND TEXTURE OF WALNUTS REDUCED IN FAT CONTENT BY SUPERCRITICAL CARBON DIOXIDE

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ABSTRACT:

During the past several years, walnuts have gained popularity because of their good taste, high n-3 fatty acid content, and reported hypocholesterolemic effects. Two negative components of walnut consumption are the relatively high fat content of this commodity (~70%), and the lack of oxidative stability caused by the high level of fat and its polyunsaturated nature. The objectives of this study were to 1) use supercritical carbon dioxide (SC-CO_2) extraction to decrease the total lipid content of walnuts, thus improving dietary fat content, and 2) determine the effects of SC-CO_2 lipid extraction on the oxidative stability, flavor and textural characteristics of the reduced-fat walnuts immediately after extraction, and after storage. The relative fat content of English walnut pieces was reduced by 25 and 40% using a pilot-scale SC-CO_2 extraction system. Full-fat, 25- and 40%-reduced-fat walnuts were stored at 25 °C and 40 °C for 8 weeks. Fatty acid profiles were not different for reduced-fat and full-fat walnuts. Peroxide values (PV) and volatile compounds were significantly greater in full-fat walnuts than in reduced-fat walnuts at both storage temperatures. Reduced-fat walnuts were less astringent, and had

less walnut and rancid flavors, as judged by a trained sensory panel. Full-fat walnuts were determined, by both sensory and instrumental texture profile analysis, to have greater hardness than reduced-fat walnuts. In general, reducing the relative fat contents of walnuts by 25 and 40% improved oxidative stability, while maintaining a high level of consumer acceptance.

INTRODUCTION:

During the past several years, walnuts have gained popularity because of their good taste, high n-3 fatty acid content (linolenic acid) and reported hypocholesterolemic (1-2) and antihypertensive effects (1). One negative component of walnut consumption is the relatively high total fat content of this commodity (~70%). In addition, the highly polyunsaturated nature of walnut lipids makes them prone to oxidative instability. Although supercritical carbon dioxide (SC-CO_2) extraction has been used as a tool to reduce fat in other foods (3-4), little attention has been paid to the fatty acid profile, oxidative stability and flavor attributes of the reduced-fat products. The objectives of this study were to use SC-CO2 to decrease the total lipid content of walnuts and to determine the effects of SC-CO_2 lipid extraction on the oxidative stability, flavor and textural characteristics of the reduced-fat walnuts.

EXPERIMENTAL PROCEDURES:

Premium walnut pieces were provided by Diamond Walnut Growers Inc. (Stockton, CA). The walnuts were randomly divided into three treatment groups, 25 %

reduced-fat (fat extracted by SC-CO_2), 40% reduced-fat (fat extracted by SC-CO_2), and full-fat (no fat extracted). The 25% and 40% reduced-fat walnuts represent relative reductions of the total fat content (i.e. 25% less total fat than the full-fat walnut pieces). Preliminary data from our laboratory suggested that oil reductions greater than 40% resulted in high levels of walnut fracture and product loss.

Supercritical CO_2 extraction.

The pilot-scale semicontinuous SC-CO_2 extraction system located at the ARS Northern Regional Research Center (Peoria, IL) and described by Friedrich *et al.* (5) was used. Extractions for each reduction level (25% and 40%) were performed in duplicate at 10,000 psi and 80 °C with a CO_2 flow rate of .33 lb/min. The walnuts were flushed with nitrogen and stored at –20 °C prior to analysis. Preliminary experiments were performed using a custom-assembled supercritical fluid extraction system (6) at various operating pressures and temperatures.

Total fat content.

The Bligh and Dyer method (7) with modifications was employed for determination of total fat content. Walnuts (25 g) were homogenized using a Polytron homogenizer (Kinematica, GmbH, Switzerland, distributed by Brinkman Instruments, Westbury, NY) with 90 mL of water, 10 mL of methanol and 5 mL of chloroform for 2 min. An additional 5 mL of chloroform was added to the mixture and blended for two more min. The homogenate was centrifuged at 500 x *g* for 10 min, the upper layer was

removed by aspiration and the bottom layer was vacuum filtered through Whatman No. 1 filter paper (Maidstone, England), reextracted with 10 mL of chloroform and refiltered. The chloroform-lipid extract was passed through anhydrous sodium sulfate (Na_2SO_4) and the Na_2SO_4 was rinsed with 50mL of chloroform. Solvent was removed by rotary evaporation under vacuum at 30 °C.

Analytical procedures.

Tocopherol content was determined by HPLC according to AOCS Official Method Ce 8-89 (8). Fatty acid methyl esters were prepared and analyzed as described by Hammond (9). Moisture contents were determined according to the 2-hr oven drying method described in AOCS Official Method Ba-38 (8). Crude protein was measured using a Perkin Elmer Series II Nitrogen Analyzer 2410 (Perkin Elmer Corp., Norwalk, CT). Nitrogen was multiplied by a factor of 6.25 for estimating crude protein content.

Color measurements.

Color was measured using a Color Difference Meter (Model D25A-2, Hunter Laboratories, Inc., Reston, VA). Walnuts with the pellicles removed were placed in a petri dish and the readings were recorded by rotating the sample vessel in 90° increments. A total of 6 samples were evaluated in triplicate for each experimental replication.

Texture profile analysis.

The hardness of fresh walnut meats was evaluated using a TA-XT2 texture analyzer (Texture Technologies Corp., Scarsdale, NY). A 1.2 cm square was cut from walnut pieces, and the thickness was measured using calipers. Cut pieces were grouped according to thickness with 8 and 9 mm used for analysis. A 1.5" anvil was used to determine 50% compression at a compression rate of 0.5 mm/sec. A total of 15 samples were evaluated and the results averaged for each extraction replicate to account for surface differences and to reduce standard error among experimental replications.

Scanning electron microscopy.

Internal walnut structures were prepared for scanning electron microscopy (SEM) by physically snapping the edges of freshly broken walnut pieces. Samples were mounted with aluminum stubs using carbon adhesive tape. Silver paint was applied around sample edges to promote conductivity and aid in attachment. The samples were sputter-coated using a Denton Desk II Cold Sputter/etch coating unit (Denton Vacuum, Inc., Morestown, NJ). Walnuts were viewed using a gold/palladium (60/40) alloy target and images were collected with a JEOL 5800LV scanning electron microscope.

Storage studies.

SC-CO_2 extracted (25% reduced-fat and 40% reduced-fat) and full-fat walnuts were randomly divided for storage under two different conditions, 25 °C and 40 °C, both

in the dark for 8 weeks. Walnuts were placed in aluminum pans and loosely covered with plastic wrap during storage.

Oxidation measurements.

Peroxide values (PV) were measured weekly using the modification of AOCS method Cd 8-53 described by Crowe *et al.* (10). For PV analysis, oil was extracted by small-scale SC-CO_2 extraction, in which ground walnut (2.5 g) was placed in the extraction vessel and extracted at 10,000 psi for 35 min at a flow rate of 5.0 mL/min with chamber and restrictor temperatures of 85 °C and 80 °C, respectively. All PV analyses were performed in duplicate and the results averaged.

Volatile lipid components were measured at 0, 4 and 8 weeks of storage by solid phase microextraction (SPME), a technique previously applied to measuring lipid degradation volatiles in vegetable oils (11). Walnuts (5 g) were ground in a Waring blender at high speed for 10 sec and placed in a two-neck round-bottomed flask. One neck of the flask contained a small external fan designed to circulate the headspace above the sample. The SPME device was inserted through the other neck. The flask was placed in a 50 °C-water bath, where the sample was equilibrated for 5 min prior to exposure of the SPME fiber (100 μm polydimethyl siloxane (PDMS)) for 30 min. The SPME fibers were thermally desorbed for 1 min in the inlet of a Hewlett-Packard 5890 Series II GC (Palo Alto, CA) at 230 °C and transferred in helium at 1.7 mL/min onto a Supelco SPB-1 fused-silica capillary column (30 m, 0.25-mm i.d., 0.25 μm film thickness; Bellefonte, PA). The GC was held for 3 min at an initial temperature of 30 °C, increased to a final

temperature of 210 °C at 6 °C/min with a final hold time of 5 min. Peaks were detected by an FID held at 220 °C. For mass spectrometry, a Hewlett-Packard 5970 mass-selective detector was used in place of the FID. Peaks were identified by comparing their retention times and mass spectra with those of known compounds.

Sensory analysis.

Walnuts from each storage condition were evaluated by a 10-person trained panel. Sensory panelists were familiarized with evaluation methods during three one-hour training sessions using walnut samples oxidized at different levels, AOCS Recommended Practice Cg 2-83 reference samples for rancid and painty flavor (8), and texture references for hardness. At weeks 0, 4 and 8, samples were presented in duplicate at two different sittings and evaluated using a 15-cm line scale, with the low end of the scale indicating none and the high end indicating extreme for each attribute (rancidity, paintiness, astringency, and hardness). Sample evaluation was performed by panelists in individual sensory booths under red light. Additionally, consumer acceptance testing was conducted for the 25% reduced-fat and full-fat walnuts by 33 untrained panelists using a 9-point balanced hedonic scale.

Statistical analysis.

Statistical analysis were performed by ANOVA with repeated measures and Pearson's correlation using the General Linear Model procedures of SAS 6.06 (Cary, In) to determine significant differences among treatments at $P < 0.05$.

RESULTS AND DISCUSSION:

Preliminary experiments using a custom assembled supercritical fluid extraction system (6) demonstrated the feasibility of SC-CO_2 lipid extraction from walnut pieces, with lipid reduction levels between 25 and 40% (w/w). Lipid extraction beyond 40% of total fat resulted in product fracture and powdering of the walnut pieces. Initial studies also demonstrated that slow pressurization and depressurization were necessary to minimize damage to the walnut pieces.

Relative fat and protein percentages of full-fat, and 25- and 40%-reduced fat walnuts were significantly ($P < 0.05$) different among treatments (Table 1). Total fat contents decreased from 69% for the full-fat walnuts to 54% and 42%, respectively, for the 25- and 40% reduced-fat walnuts, with expected commensurate increases in the relative protein percentages. Moisture contents were similar among all three walnut fat levels (Table 1). Fatty acid compositions were not significantly different (Table 2), with linoleic acid being the predominant fatty acid of all three walnut treatments. Similar relative percentages of the predominant fatty acids in walnut oil (palmitic, stearic, oleic, linoleic and linolenic acids) were measured for each of the three extraction levels. Alexander *et al.* (3) reported significant effects of SC-CO_2 extraction conditions on the fatty acid composition, particularly the oleic/linoleic acid ratio, of pecans. The solubilities of individual fatty acids differ in CO_2 (6), however, the relatively low ratio of saturated:unsaturated fatty acids in walnuts likely made fractionation difficult, particularly under the extraction conditions used in this study, which were designed to optimize extraction time.

Both total tocopherol and individual tocopherol isomer contents were significantly lower in both the reduced-fat walnut treatments than in the full-fat walnuts (Table 3). Previous studies using SC-CO_2 extraction in soybeans, reported only limited extraction of tocopherols and suggested that lower tocopherol levels may be partly responsible for the decreased oxidative stability of SC-CO_2 extracted oils (12). The predominant tocopherol isomer, gamma, was significantly lower in the 25% reduced-fat walnuts than in the 40% reduced-fat walnuts. The SC-CO_2 procedure extracted lipids from the outer portion of the walnut first. The outer area of the walnut may have a relatively greater concentration of tocopherols that naturally attenuates surface oxidation, thus explaining why greater relative reductions in tocopherol occurred in the 25% reduced-fat product. To our knowledge, however, distributions of tocopherols within walnuts have not been measured.

Peroxide values for the full-fat walnuts increased faster than did the PV for both the reduced-fat walnut treatments when stored at both 25 and 40 °C (Figs. 1a and 1b). The 25% reduced-fat walnuts had a significantly greater PV after 5 weeks at 40 °C, than did the 40% reduced-fat walnuts. The differences between the two reduced-fat treatments were not significant at 25 °C-storage. Despite the highly unsaturated nature of the walnut oil, the PV's were relatively low at both storage temperatures. After reaching a PV of ~ 15 meq/Kg, a decline in PV was noted in both the full-fat and 25% reduced-fat walnuts. Similar trends in oxidizing walnuts have been reported by other investigators (13-14). At this point during oxidation the rate of peroxide degradation to secondary oxidation compounds evidently was greater than the rate of peroxide

formation, a fact supported by the sharp increase in hexanal contents between 4 and 8 weeks for both the full-fat and 25% reduced-fat treatments (Figs. 2a and 2b).

Volatile oxidation compounds were present initially at similar levels among all walnut treatment groups (data not shown). Most compounds increased significantly with time at both storage temperatures for each of the three walnut fat levels, and were generally significantly greater in the full-fat walnuts. Hexanal was the primary volatile oxidation product measured in all three walnut treatments. As shown in Table 2, linoleic acid is the predominant fatty acid in walnuts, therefore the relatively high levels of hexanal are reasonable since this compound is formed primarily from the breakdown of linoleic acid hydroperoxides. The amount of hexanal formed by 8 weeks at 25°C and generally after 2 weeks at 40°C was significantly greater in the full-fat walnuts than in both reduced-fat treatments (Fig. 2a and 2b). After 4 weeks at both temperatures, the 40% reduced-fat walnuts were significantly lower in hexanal contents than the other two treatments.

Human sensory scores conducted on walnuts stored at 25 and 40 °C for both rancidity ($r = 0.85$ (25 °C); $r = 0.87$ (40 °C) and paintiness ($r = 0.95$ (25 °C); $r = 0.89$ (40 °C) correlated well with hexanal formation at both 25 and 40 °C, with both oxidized flavor and hexanal content increasing with increased walnut fat level (Table 4). No significant differences in rancidity or paintiness were measured between the 25 and 40% reduced-fat walnuts stored at 40 °C. Astringency scores were significantly less in both the 25 and 40% reduced-fat walnuts than in the full-fat walnuts at all sampling times. The lower astringency scores were likely the result of SC-CO_2 extraction of ellagic acid

from the walnut pellicle (15). Consumer acceptance hedonic scores of 25% reduced fat walnuts were not significantly different ($P < 0.05$) than full-fat walnut scores (6.5 and 7.0, respectively).

Partial removal of fat from the walnuts by using SC-CO_2 extended walnut shelf-life based on PV, hexanal and human sensory data. Partial fat removal also has been reported to increase the shelf-life of pecans (16) and peanuts (17). The authors suggested that the decrease in available lipid substrate was responsible for the increased stability of these commodities. Because exposure of lipid to oxygen is an important oxidation mechanism, it is also feasible that the removal of lipid from the external surface of the walnut pieces decreased the accessibility of oxygen to the remaining lipid, hence decreasing the overall rate of oxidation.

Texture of the fresh walnut meats, as determined by both human sensory and instrumental analyses, significantly decreased in firmness with decreasing walnut fat level (Figs. 3a and 3b). Instrumental and sensory evaluations of hardness were strongly correlated among all three walnut treatment groups ($r = 0.95$). Significant decreases in breaking intensity and hardness measured by rheometry for partially defatted peanuts (4), and decreased hardness and increased fracturability of supercritically extracted pecans (18) previously were reported. These differences in hardness may be understood by viewing scanning electron micrographs, which showed clear differences in cell wall structure among each of the walnut fat levels (Figs. 5a-5f). Cell walls of the 25% reduced-fat walnuts were distorted, but remained intact, whereas the 40% reduced-fat walnuts displayed evidence of cell wall collapse. Preliminary studies in our laboratory

indicated unacceptably high levels of product loss with greater than 40% fat reduction in walnuts, indicating probable complete cell wall collapse at these fat reduction levels.

Color of walnuts was affected ($P < 0.05$) by SC-CO_2 extraction (Table 5). The L-values, indicating lightness/darkness of a surface, were significantly less in the full-fat walnuts than in the reduced-fat treatments, meaning that the reduced-fat walnuts had a whiter appearance. Hunter a- and b-values were not significantly different among all walnut treatments. Similar color changes with SC-CO_2 extraction of pecans and peanuts have been reported by other investigators (19-20). The extracted walnut oil had an amber color, which may have contributed to the darker color of the walnuts prior to SC-CO_2 extraction of the oil and accompanying lipid-soluble pigments.

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Table 1. Composition of Walnuts.

Walnut Treatment (% Reduced Fat of Walnuts)	% Fat[a]	% Crude Protein	% Moisture
Full Fat	68.8[a]	13.8[a]	3.7[a]
25%	50.4[b]	21.2[b]	2.5[a]
40%	40.5[c]	26.9[c]	2.7[a]

[a]Values within a column with the same letter are not significantly different, $P<0.05$.

Table 2. Fatty Acid Composition of Walnuts.

Walnut Treatment (% Reduced Fat of Walnuts)	Fatty Acid Composition (Relative %)				
	16:0	18:0	18:1	18:2	18:3
Full Fat	7.0	2.0	16.5	61.0	13.6
25%	6.9	2.2	16.4	60.7	13.8
40%	6.7	2.0	16.5	61.1	13.7

Table 3. Total and Individual Tocopherol Contents of Walnuts.

Walnut Treatment (% Reduced Fat of Walnuts)	Tocopherol Content (ppm)[a]			
	Total	Alpha	Gamma	Delta
Full Fat	202[a]	8.4[a]	139[a]	12.8[a]
25%	108[b]	5.6[a]	93[b]	7.5[b]
40%	108[b]	4.4[a]	96[b]	7.9[b]

[a]Values within a column with the same letter are not significantly different, $P < 0.05$.

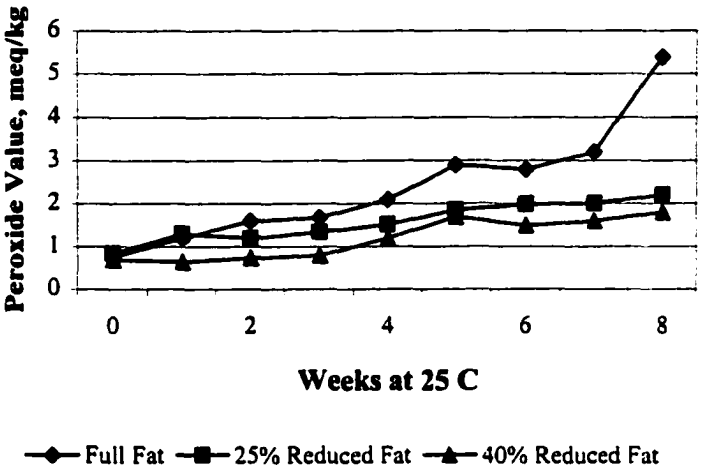

Fig. 1a. Peroxide Values of Walnuts Stored at 25 °C.

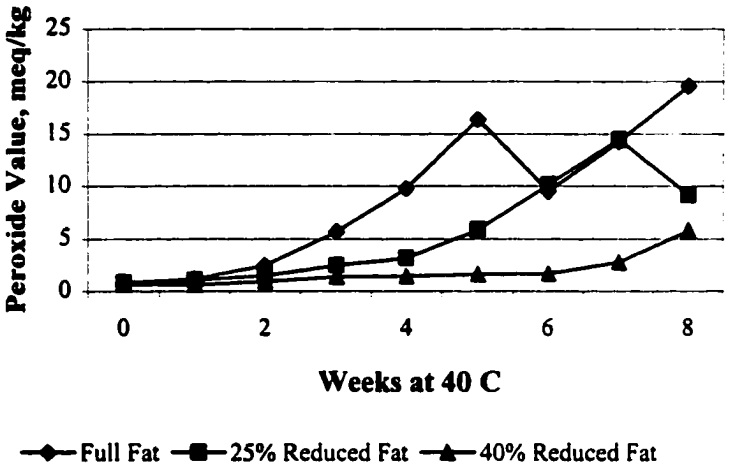

Fig. 1b. Peroxide Values of Walnuts Stored at 40 °C.

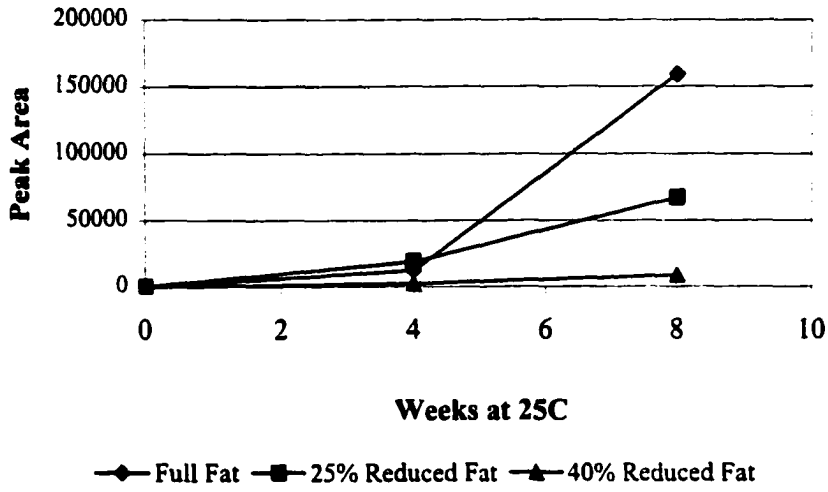

Fig. 2a. Hexanal Contents of Walnuts stored at 25 °C for 8 weeks.

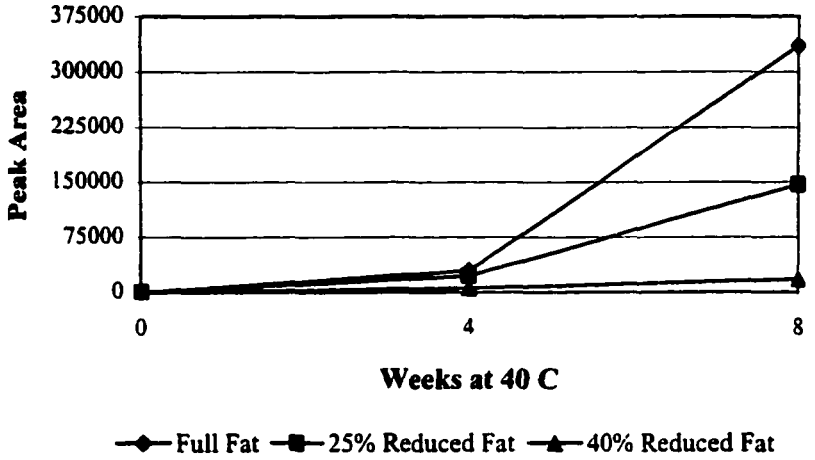

Fig. 2b. Hexanal Contents of Walnuts stored at 40 °C for 8 weeks.

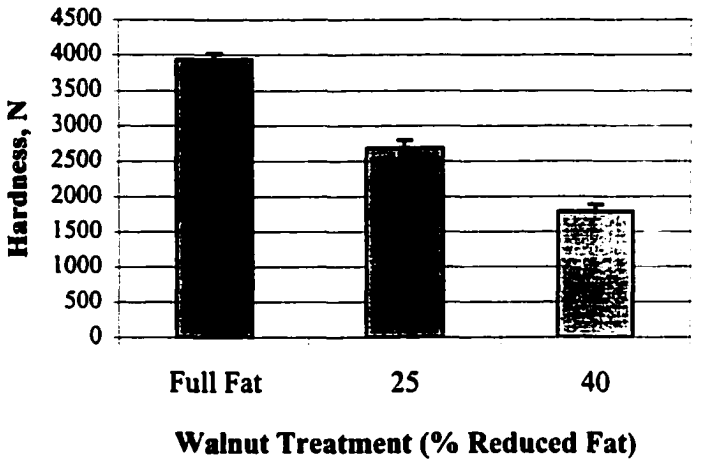

Fig. 3a. Hardness of Walnuts (Instrumental).

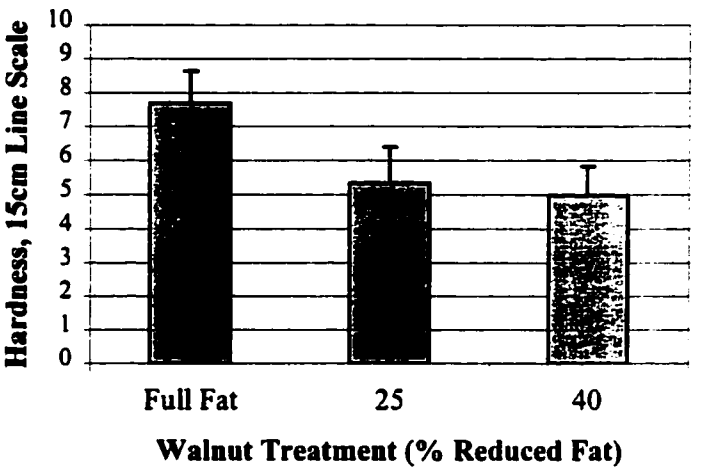

Fig. 3b. Hardness of Walnuts (Sensory; 0 = not hard, 15 = extremely hard).

Table 4. Sensory Attributes of Walnuts Stored at 25 and 40 °C.

Sensory Attribute	Time, weeks at 25 °C	Walnut Treatment (% Reduced Fat of Walnuts) Full Fat	25%	40%
Rancidity[a]				
	0	0[a]	0[a]	0[a]
	4	2.5[a]	0.3[b]	0.4[b]
	8	4.3[a]	1.8[b]	1.1[a]
Paintiness[a]				
	0	0[a]	0[a]	0[a]
	4	2.5[a]	1.1[b]	0.4[b]
	8	4.3[a]	2.3[b]	1.9[b]
Astringency[a]				
	0	8.9[a]	3.9[b]	3.3[b]
	4	8.4[a]	3.5[b]	3.7[b]
	8	8.9[a]	3.8[b]	3.2[a]
	Time, weeks at 40 °C			
Rancidity[a]				
	0	0.0[a]	0.0[a]	0.0[a]
	4	3.7[a]	1.6b	0.9[b]
	8	8.2[a]	2.0[b]	2.7[b]
Paintiness[a]				
	0	0.0[a]	0.0[a]	0.0[a]
	4	3.8[a]	1.8[b]	1.1[b]
	8	7.7[a]	3.5[b]	2.8[b]
Astringency[a]				
	0	8.9[a]	3.9[b]	3.3[b]
	4	8.5[a]	4.1[b]	3.0[b]
	8	8.7[a]	3.4[b]	3.5[b]

[a]Values within a row with the same letter are not significantly different, $P<0.05$.
0 = none, 15 = extreme; n = 10 panelists

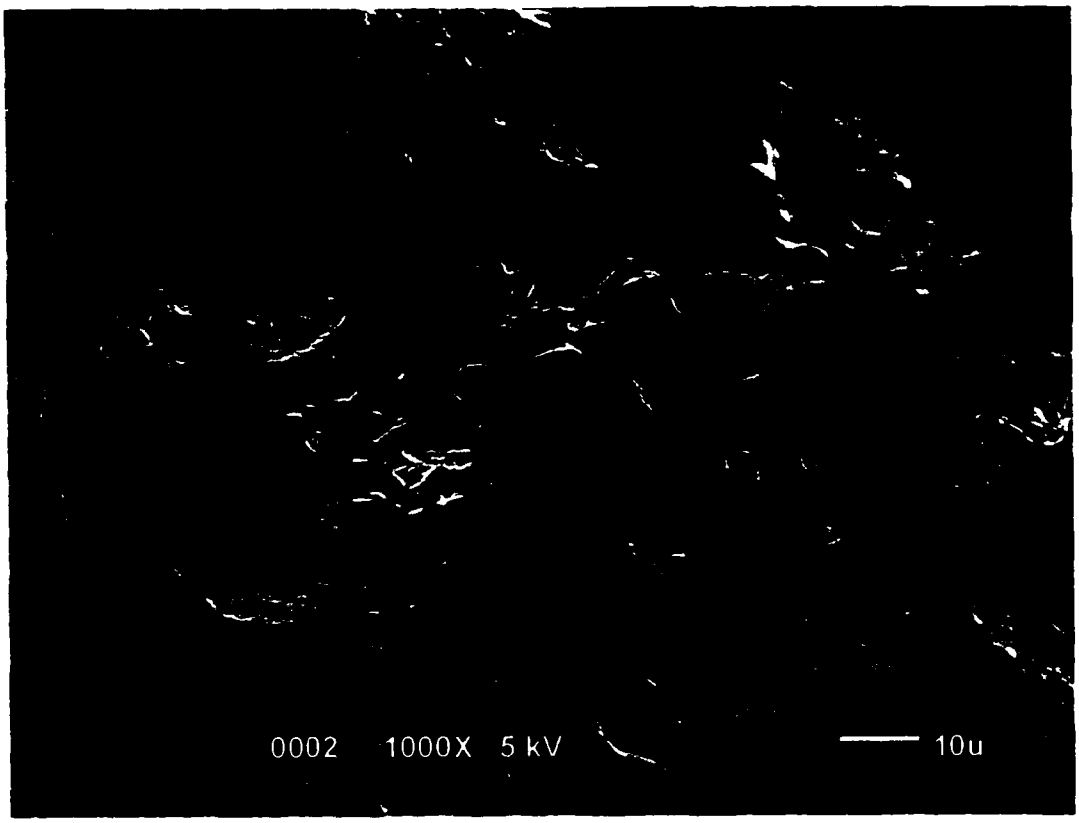

Fig. 4a. SEM of Full Fat Walnuts (External View).

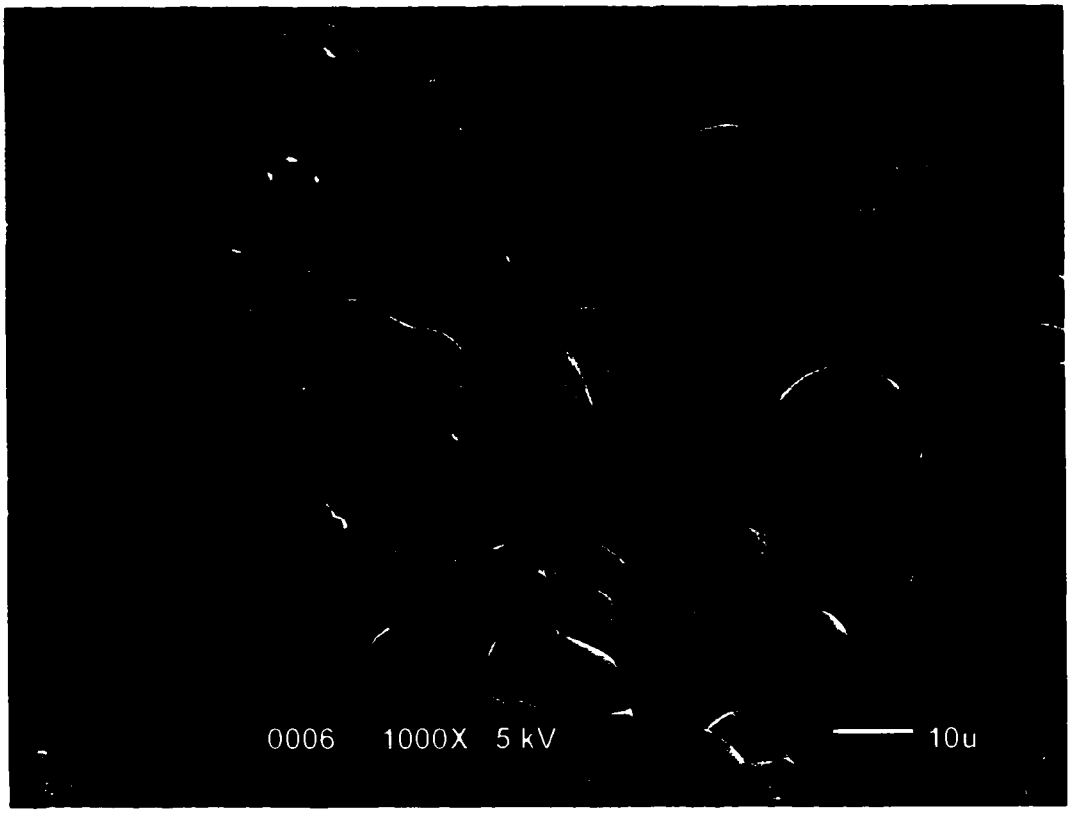

Fig. 4b. SEM of 25% Defatted Walnuts (External View).

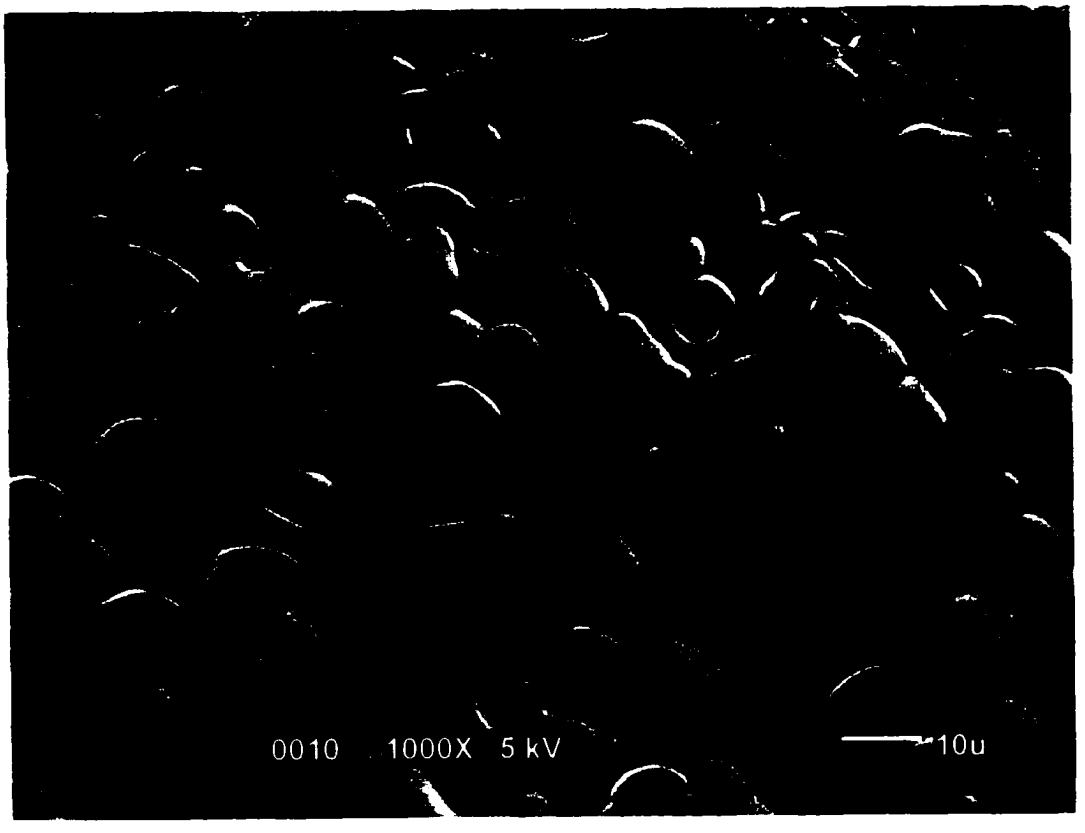

Fig. 4c. SEM of 40% Defatted Walnuts (External View).

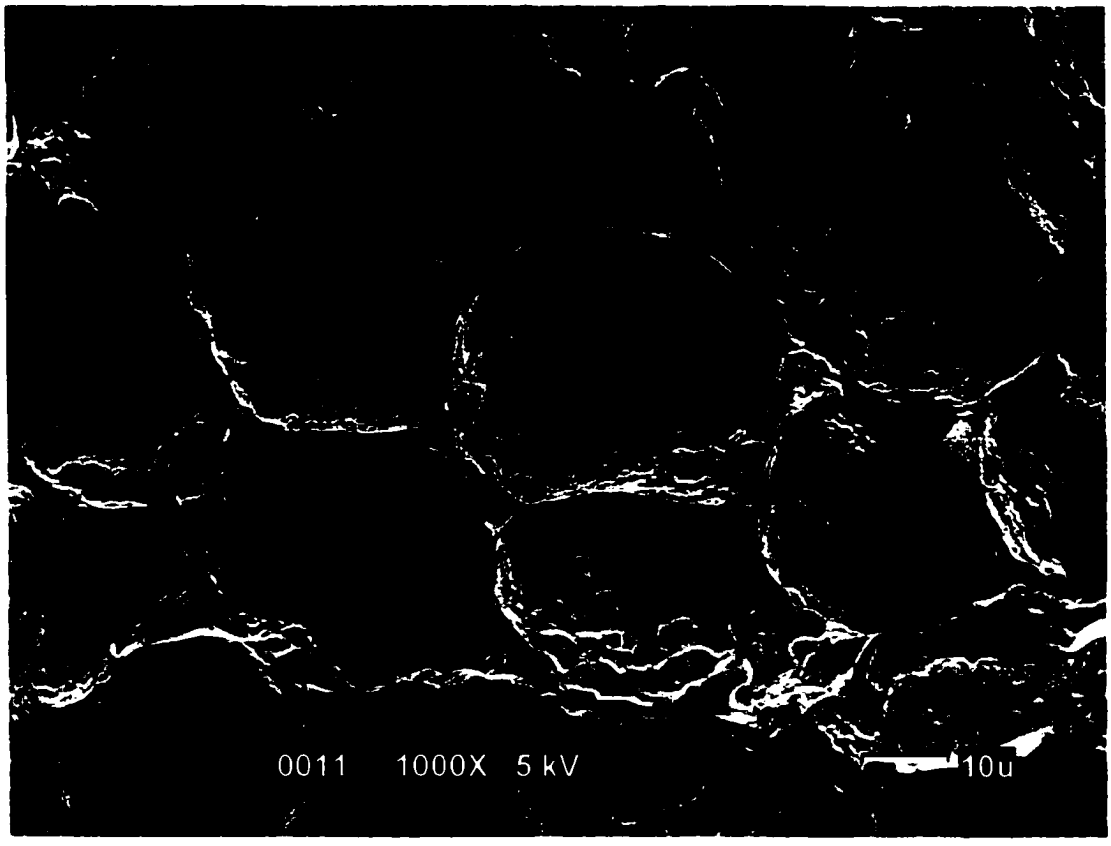

Fig. 4d. SEM of Full Fat Walnuts (Internal View).

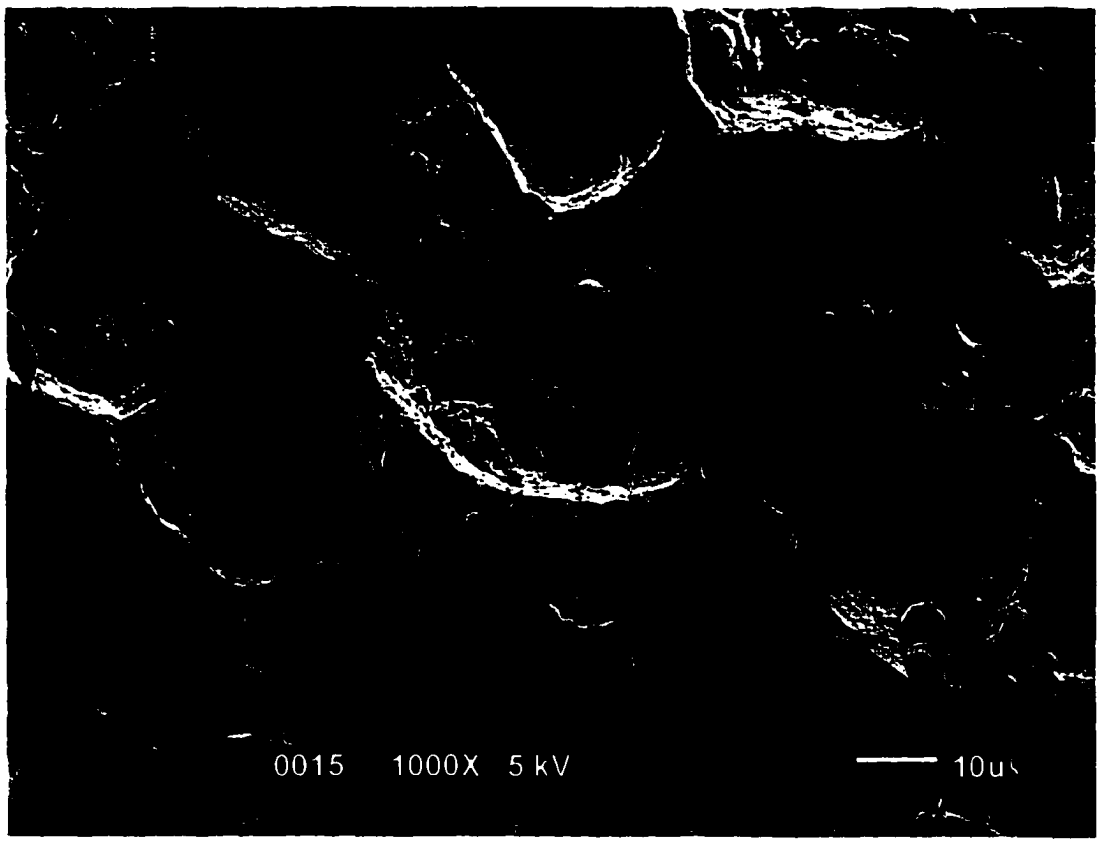

Fig. 4e. SEM of 25% Defatted Walnuts (Internal View).

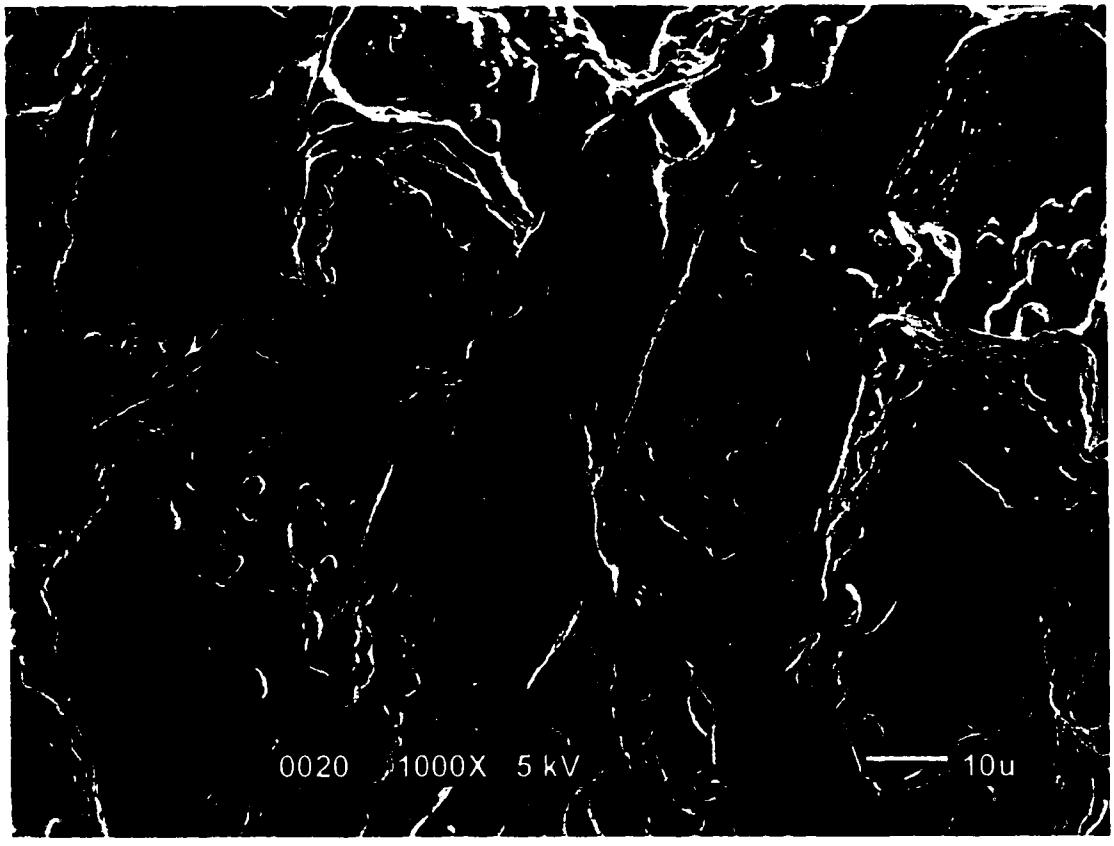

Fig. 4f. SEM of 40% Defatted Walnuts (Internal View).

Table 5. Hunter Color Values of Walnuts.

Walnut Treatment (% Reduced Fat of Walnuts)	Hunter Color Values[a]		
	L	a	b
Full Fat	40.9[b]	1.3[a]	11.7[a]
25%	60.0[a]	1.2[a]	12.3[a]
40%	64.9[a]	1.2[a]	13.6[a]

[a]Values within a column with the same letter are not significantly different, $P < 0.05$.

CHAPTER 7. OXIDATIVE STABILITIES OF WALNUT OILS EXTRACTED WITH SUPERCRITICAL CARBON DIOXIDE

A paper submitted to the Journal of the American Oil Chemists' Society

Tammy D. Crowe and Pamela J. White

ABSTRACT:

Supercritical CO_2 (SC-CO_2) was used to partially defat walnuts, and the oxidative stability of the extracted walnut oils was assessed. The SC-CO_2-extracted oils were less stable during accelerated storage in the dark than was pressed walnut oil, as determined by peroxide value, headspace analysis and sensory methods. The SC-CO_2-extracted oils, however, exhibited greater photo-oxidative stability than pressed walnut oil by all the above methods, probably because of the presence of chlorophyll in the pressed oil. Oxidative stability indices, and tocopherol contents were significantly lower in the SC-CO_2 extracted oils than in pressed oil.

INTRODUCTION:

In other work conducted in our laboratory supercritical carbon dioxide (SC-CO_2) was used to decrease the total fat content of walnuts by 25 and 40% (1). Walnut oil is prized as a specialty oil because of its characteristic flavor and aroma, and potential health benefits (2-3), and is, therefore, a valuable co-product from this process. Several studies have reported decreased thermal oxidative stability of SC-CO_2-extracted oils (4-6); however, SC-CO_2-extracted perilla oil was found to be more stable during storage at 50°C than either hexane-extracted or mechanically pressed oil (7). Savage *et al.* (8) measured the oxidative stability, based on Rancimat data, of various walnut oils, and reported significant differences among oils from different walnut cultivars. To our

knowledge, the photo-oxidative stability of SC-CO_2-extracted oils has not been evaluated. This information may be particularly important in the case of walnut oils, which are often stored in clear glass bottles.

The objective of this study was to compare the stabilities of SC-CO_2-extracted walnut oil with commercially pressed oil under photo-oxidative and accelerated dark conditions.

EXPERIMENTAL PROCEDURES:

Commercially pressed oil from premium walnuts was obtained from Diamond Walnut Growers Inc. (Stockton, CA). The SC-CO_2-extracted oils from 25 and 40% relative fat reduction of premium walnut pieces were processed using the pilot-scale semi-continuous SC-CO_2 extraction system located at the ARS Northern Regional Research Center (Peoria, IL) (9). Oil extractions at each reduction level (25% SC-CO_2 and 40% SC-CO_2) were performed in duplicate at 10,000 psi and 80°C, using analytical grade CO_2 with a CO_2 flow rate of .33 lb/min. The walnut oils were flushed with nitrogen and stored at –20°C prior to analysis.

Storage conditions.

Walnut oils from each extraction were divided to provide duplicate samples, and stored at 60°C in the dark and 35°C in the light (540 lx). For each storage condition, 100-mL beakers containing 50 mL of oil (surface area to volume ratio 0.03 mm^2/mL) were loosely covered with plastic wrap and stored in a constant temperature oven for 15 days.

Analytical procedures.

Peroxide values (PV) were conducted on the oils stored under each condition every three days using the modification of the AOCS method Cd 8-53 described by Crowe *et al.* (10). Fatty acid methyl esters were prepared and analyzed as outlined by Hammond (11). Oxidative stability indices (OSI) were determined by the AOCS standard method Cd 12b-92 (12) with an Oxidative Stability Instrument (Omnion, Rockland, MA) operating at 100°C. Tocopherols were measured by high-pressure liquid-chromatography (HPLC) according to the AOCS method Ce 8-89 (12). Chlorophyll was determined according to the AOCS method Cc 13i-96 (12). All analyses were performed in duplicate at time zero and the data averaged for each experimental replication.

Volatile analysis.

Two milliliters of oil was placed in a 20-mL glass vial sealed with a teflon/silicone septum. After equilibration of the oil sample for 5 min at 50°C, the 100 μm poly(dimethylsiloxane) (PDMS) SPME fiber (Supelco Inc., Bellafonte PA) was exposed to the vial headspace for 30 min at 50°C. The volatile compounds collected on the PDMS fiber were thermally desorbed for 1 min in the inlet of the gas chromatograph (GC) (Hewlett-Packard 5890 Series II GC; Palo Alto, CA) with an inlet temperature of 230°C, and transferred in helium at 1.7 mL/min onto a Supelco SPB-1 fused-silica capillary column (30 m, 0.25-mm i.d., 0.25 μm film thickness). The GC was held for 3 min at an initial temperature of 30°C, increased to a final temperature of 210°C at 6°C/min, with a final hold time of 5 min. Peaks were detected by a flame ionization detector (FID) held at 220°C. Standard response curves were prepared for the major volatile compounds in oxidized oils using authentic standards added to stripped soybean oil (sparged at 50°C with helium for 16 h) in concentrations from 10^{-3} to 10^{-7}. For mass

spectrometry, a Hewlett-Packard 5970 mass-selective detector was used in place of the FID. Peaks were identified by comparing their retention times and mass spectra with those of known compounds. Volatile compounds in each replicate oil treatment were analyzed in duplicate at time zero and 15 days, and the data from each day averaged.

Sensory evaluation.

Five panelists with previous walnut and oil evaluation experience were trained in two sessions for familiarity in identifying off-flavors in oxidized oils. During training panelists were presented with walnut oils autoxidized to various degrees, and standard references for "painty" and "rancid" flavors in oils according to the AOCS method Cg-2-83 (12). The term "off-flavor" was determined to be the most appropriate descriptor for the flavor changes in walnut oil during oxidation. Prior to sensory evaluation, samples were placed in screw-cap glass vials and equilibrated to 25°C in the dark. Panelists were instructed to swirl the oil in the vial before opening the cap, sniff each sample first, and then taste the oils in order of increasing off-odor. Deionized water and unsalted crackers were provided to cleanse the palate between samples. Samples were evaluated using a 15-cm line scale with "no off-flavor" and "extreme off-flavor" as anchors. Samples from each experimental replication were evaluated in duplicate on day zero and day 15 and the data from each day averaged.

Statistical analysis.

Data were analyzed by analysis of variance (ANOVA) using the General Linear Model procedure of SAS (SAS, 1991). Significance was established at $P < 0.05$.

RESULTS AND DISCUSSION:

Analyses of the three walnut oil samples, pressed and SC-CO_2-extracted from 25 and 40% walnut-lipid reductions showed oleic, linoleic, and linolenic acids to be the predominant fatty acids in walnut oil (Table 1). No significant ($P < 0.05$) differences in fatty acids compositions were noted among the oils. Previous studies in our laboratory indicated no differences in the fatty acid composition of pressed- and SC-CO_2-extracted walnut oils (13). The presence of a high level of polyunsaturated fatty acids in walnut oil is a significant contributor to its oxidative instability (14).

Two oil storage conditions were used in this study: 60°C storage in the dark to measure autoxidation, and 35°C storage under fluorescent light to measure photo-oxidation. Oil oxidation was monitored by several methods, each selected to measure different types of products. The PV was used to measure hydroperoxides as initial oxidation products. Volatile analysis was indicative of breakdown products of hydroperoxides (secondary oxidation products), which are important in rancid or off-flavor development. Finally, the OSI was used to provide a rapid, convenient measure to predict overall stability.

The PV of the SC-CO_2-extracted oils were significantly greater than that of the pressed oil at all sampling times except time zero during storage at 60°C for 14 days (Figure 1). As expected, given the relatively high levels of polyunsaturated fatty acids, the PV of all oils increased rapidly under accelerated storage conditions. During storage at 35°C in the light, however, the SC-CO_2 oils exhibited significantly greater stability based on PV than did the pressed oil (Figure 2). No significant differences in PV were measured between the SC-CO_2-extracted oils within each storage condition. The greater rate of oxidation in the SC-CO_2-extracted oils stored at 60°C was probably a result of the greatly reduced levels of tocopherols in these oils (Table 2). The level of chlorophyll

present in the pressed sample, although relatively low (4 ppm; chlorophyll was not detectable in the SC- CO_2 -extracted oils) probably decreased the photo-oxidative stability of that oil. Hall and Cuppett (15) found that low levels of chlorophyll (4.21 ppm) were sufficient to cause a slight increase in the rate of photo-oxidation in soybean oil. The increased stability of SC-CO_2-extracted oils to light-induced oxidation is important because specialty oils, such as walnut, are generally stored in clear glass bottles on store shelves, making them susceptible to photo-oxidation.

The total tocopherol and levels of individual tocopherol isomers were significantly greater in the pressed than in either of the SC-CO_2-extracted oils (Table 2). Total and γ-tocopherol levels of the 40% SC-CO_2 walnut oils were significantly ($P < 0.05$) greater than levels in the 25% SC-CO_2-extracted walnut oil, but the practical differences were probably not important. The longer extraction time for the 40% SC-CO_2-extracted oil than for the 25% SC-CO_2-extracted oil probably facilitated the slightly greater levels of tocopherol recovery. The γ-isomer was the predominant tocopherol in all three oils. The levels of total and individual tocopherol isomers measured in the pressed oil were similar to those reported in the literature (8). But β-tocopherol, which has been reported at low levels (< 10 ppm) in several walnut cultivars (8), was not detected. Tocopherols are important antioxidants, which act by quenching peroxyl radicals in the propagation step leading to the formation of hydroperoxides, and by reacting with alkoxyl radicals, thereby inhibiting further decomposition of hydroperoxides to volatile secondary oxidation compounds (16). Prooxidant effects of high tocopherol levels have been demonstrated in soybean oil, however, this effect has not been examined in walnut oils. Savage *et al.* (8) reported a significant relationship between total tocopherol contents combined with the level of oil unsaturation and

oxidative stability, as determined by a single point peroxide value of walnut oils from various cultivars.

Because of the relatively high levels of polyunsaturated fatty acids present in the oils, an OSI temperature of 100°C rather than 110°C was used to better evaluate differences between the oils. Both of the SC-CO_2-extracted oils had significantly lower OSI (2.5 h and 1.2 h for the 25%and 40% SC-CO_2-extracted oils, respectively) than the pressed oil (12.3 h). Given the ~ 10 times greater level of tocopherols present in the pressed oil, it is not surprising that the OSI was considerably greater in this oil. Also, these findings are consistent with the results of 60°C storage in the dark, in which the pressed oil was more stable than the SC-CO_2-extracted oils. The walnut oil from the 40% SC-CO_2-extraction had a significantly ($P < 0.05$) lower OSI than did the 25% SC-CO_2-extracted oil, despite greater levels of tocopherols in the 40% SC-CO_2 oil. Both SC-CO_2-extracted walnut oils, however, had extremely low OSI values, despite the utilization of lower testing temperatures than typically used for OSI.

The primary volatile compounds identified from SPME oil headspace analysis are shown in Table 4. Hexanal, a product of linoleic acid degradation, was the primary volatile compound in all oils after storage under both conditions. Relatively large amounts of *trans, trans*, and *trans, cis*-2,4-heptadienal were measured in all three oils stored at 60°C in the dark. This volatile compound is an oxidation product of linolenic acid, which makes up about 15% of the fatty acids in walnut oil. Nonanal, which is formed primarily from oleic acid, was greater after light than dark storage. The rate of oleic acid autoxidation is much lower than that of polyunsaturated fatty acids, such as linoleic or linolenic acid, ~ 1:10.3:21.6 for oleic:linoleic:linolenic acids, respectively (17). Photo-oxidation of oleic acid, however, occurs at a much higher rate than does

autoxidation of oleic acid because of singlet oxygen addition directly to the double bond during photo-oxidation (16).

Initial sensory evaluation of the walnut oils at time zero indicated that the SC-CO_2-extracted oils had a significantly lower off-flavor intensity than did the pressed walnut oil (Table 4). After the completion of storage at 60°C in the dark, both SC-CO_2-extracted walnut oils had significantly greater off-flavor intensity scores than did the pressed oil. In contrast, after completion of storage at 35°C in the light, the pressed walnut oil had a significantly greater off-flavor intensity score than did the SC-CO_2-extracted oils. The flavor intensity scores correspond with the levels of hexanal measured in each of the oils following completion of storage under both the light and dark conditions (Tables 3). Total volatile and hexanal contents have been correlated with sensory evaluation of other oils (18). Recently, a significant correlation was found between undesirable sensory scores and the total amount of volatile compounds isolated by SPME (19).

Oil extraction by SC-CO_2 has been associated with greater oxidative instability of the oils than by solvent or expeller extraction methods (4-6). Compared with pressed walnut oil, the oxidative stability of both SC-CO_2-extracted walnut oils examined in this study was less as determined by multiple types of oxidation measurements, including PV, OSI, volatile-compound and sensory analyses. The SC-CO_2-extracted oils showed increased stability to photo-oxidation as determined by PV, volatile and sensory analyses.

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Table 1. Fatty Acid Composition of Walnut Oils.

	Fatty Acid Composition, relative %				
Oil type	16:0	18:0	18:1	18:2	18:3
Pressed[a]	8.3	1.6	16.2	60.9	13.0
25% SC-CO_2[b]	8.0	1.4	16.7	61.6	12.3
40% SC-CO_2[b]	7.9	1.2	17.3	61.0	11.6

[a]Oil obtained from pressed walnut

[b]Oils obtained from walnuts where the oil was reduced by 25% or 40% with supercritical carbon dioxide (SC-CO_2) extraction.

Table 2. Content of Total and Individual Isomers of Tocopherol in Walnut Oils.

	Tocopherols, ppm[a]			
Oil type	Total	Alpha	Gamma	Delta
Pressed[b]	373.0[a]	28.3[a]	306.0[a]	38.4[a]
25% SC-CO_2[b]	32.0[b]	1.7[b]	27.9[b]	2.4[b]
40% SC-CO_2[b]	38.5[c]	2.0[b]	34.1[c]	2.4[b]

[a]Values in the same column with different superscript letters are significantly different ($P < 0.05$).

[b]See Table 1 for description of oil treatments..

Table 3. Volatile Contents (ppm) of Walnut Oils Stored at 60°C in the Dark.

Volatile compound	Pressed[a]		25% SC-CO_2[a]		40% SC-CO_2[a]	
	0d	15d	0d	15d	0d	15d
t-2-pentenal	0.2	2.9	*ND*[b]	4.5	ND	5.1
Hexanal	0.4	19.5	ND	40.2	ND	45.6
t-heptenal	0.2	4.6	ND	14.3	ND	11.2
t,t-2,4 heptadienal	1.4	7.4	ND	17.5	ND	14.2
t,c- 2,4 heptadienal	1.7	11.1	ND	22.4	ND	30.1
1-octen-3-ol	0.3	3.3	ND	5.1	ND	5.6
Nonanal	1.0	1.5	ND	1.8	ND	2.1
t,t, 2,4 decadienal	0.5	9.1	ND	17.6	ND	19.5

[a]See Table 1 for description of oil treatments.
[b]ND, Not Detectable

Table 4. Sensory Evaluation Off-flavor Scores[a,b] of Walnut Oils.

Day	Pressed[c]	25% SC-CO_2[c]	40% SC-CO_2[c]
0	3.0[a]	1.0[b]	1.1[b]
15 (60°C dark storage)	10.1[b]	14.6[a]	13.2[a]
15 (35°C light storage)	14.2[a]	11.6[b]	12.2[b]

[a]A score of 0 = no off-flavor and 15 = extreme off-flavor.
[b]Values in the same row with different superscripts are significantly different ($P < 0.05$).
[c]See Table 1 for description of oil treatments

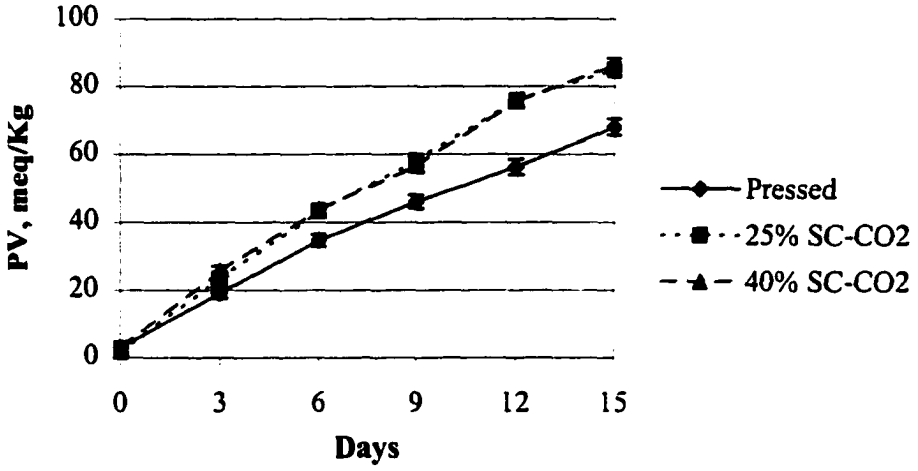

Fig. 1. Peroxide Values of Walnut Oils Stored at 60°C in the Dark

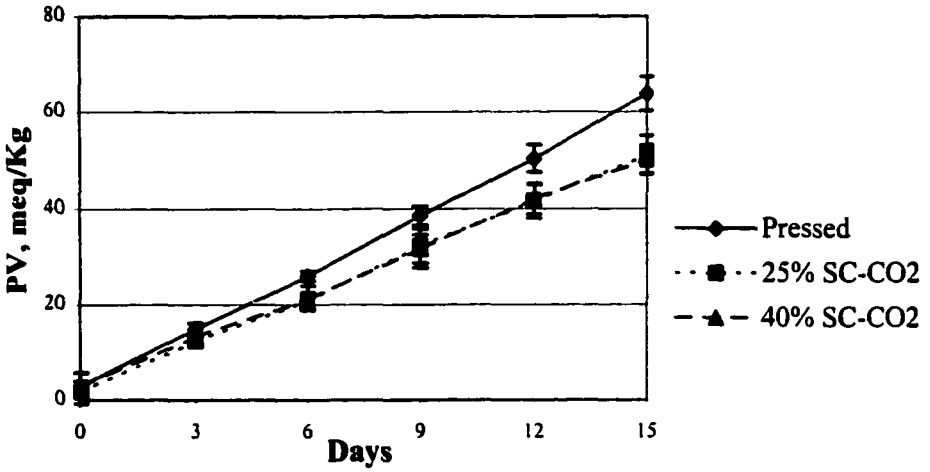

Fig. 2. Peroxide Values of Walnut Oils Stored at 35°C in the Light

CHAPTER 8. GENERAL CONCLUSIONS

The primary objectives of this study were to 1) determine the feasibility, and fat reduction thresholds, of using SC-CO_2 as a chemical-free processing method to decrease the total lipid content of walnuts; 2) determine the SC-CO_2 processing parameters for extraction of 25% and 40% of the lipid components from whole walnut pieces; and 3) to determine the influence of partial lipid extraction by SC-CO_2 on the flavor, triglyceride composition, oxidative stability and textural quality of walnuts.

Each of the primary experimental objectives were met. It was concluded that SC-CO_2 is a viable method for partial defatting of walnuts, and processing parameters for 25% and 40% fat reductions were developed. The reduced-fat walnuts exhibited increased oxidative stability compared with full-fat walnuts, as measured by peroxide value, volatile compound analysis, and human sensory evaluation. The triglyceride composition of the SC-CO_2 extracted walnuts was not different from that of full-fat walnuts, thus maintaining high levels of polyunsaturated fatty acids, including α-linolenic acid which has been linked to potential health benefits (Sabate *et al.* 1993; Abbey *et al.* 1994; Chisholm *et al.* 1998; Lavedrine *et al.* 1999; Iwamoto *et al.* 2000; Zambon *et al.* 2000). Thus, the primary benefit of this study was the production of a relatively low-fat, oxidatively stable source of n-3 fatty acids. Because specialty oils, such as walnut, are valuable commodities in themselves, a secondary objective of this research was to determine the oxidative stability of the SC-CO_2 extracted oils from the partially defatted walnuts. The SC-CO_2 extracted oils were less stable than pressed

walnuts during accelerated storage, as determined by peroxide value, volatile compound analysis, and sensory evaluation. However, SC-CO_2 extracted oils exhibited greater photo-oxidative stability than pressed oil. Because specialty oils are often stored in clear glass bottles for retail sale, these oils may be a potentially valuable co-product from the SC-CO_2 walnut defatting process.

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