Optimizing the Extraction of Phenolic Antioxidant Compounds from Peanut Skins

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Key Words:  polyphenols, antioxidants, resveratrol, peanut skins, solid-liquid extraction, microwave-assisted extraction, response surface methodology, peanut butter, human brain microvascular endothelial cells

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ABSTRACT

Peanut skins are a low-value byproduct of peanut blanching and roasting operations. They have been shown to contain significant levels of phenolic compounds with demonstrated antioxidant properties. In this study, the effects of two types of extraction methods: solid-liquid extraction (SLE) and microwave-assisted extraction (MAE) on the recovery of phenolic compounds from peanut skins were investigated. Response surface methodology was used to optimize the extraction conditions based on total phenolic content (TPC), ORAC (oxygen radical absorbance capacity) activity and \textit{trans}-resveratrol concentration. The protective effect of peanut skin extracts (PSE) against hydrogen peroxide (H$_2$O$_2$)-induced oxidative stress in human brain microvascular endothelial cells (HBMEC) and the effect of PSE on lipid oxidation in commercial peanut butter were evaluated.

In the SLE method, the extraction parameters solvent type, solvent concentration, temperature and time were investigated. EtOH was found to be the most efficient solvent for the extraction of phenolics followed by MeOH, water and EA. Despite EtOH extracts having a higher TPC, samples extracted with MeOH demonstrated slightly higher ORAC activity. Additionally, resveratrol was positively identified in MeOH extracts but was not identified in EtOH, water or EA extracts. The data suggest that MeOH may be the best solvent for the extraction of resveratrol from peanut skins.
The extraction parameters evaluated in the MAE method were microwave power, irradiation time and mass of peanut skins. 30% EtOH was used for all extractions. The maximum predicted TPC under the optimized conditions was 144 mg phenols/g skins compared to 118 mg/g with SLE. The maximum predicted ORAC activity was 2789 μmol TE/g as opposed to 2149 μmol TE/g with the SLE method. MAE was able to extract more phenolic compounds (with higher antioxidant activity) in a faster time while consuming less solvent than the SLE procedure. In addition, the presence of resveratrol was found in PSE derived from MAE although at relatively low levels.

PSE were found to have some protective effects against H₂O₂-induced oxidative stress in HBMEC. Higher doses of PSE appeared to have a slightly cytotoxic effect. However, the data were highly variable which made it difficult to arrive at any definitive conclusions regarding the potential benefits of PSE in preventing oxidative damage to cells. In the PB experiment, although there were significant increases in hexanal concentration over the storage period, hexanal levels were not high enough for the samples to be considered oxidized. The low hexanal concentrations may have been a result of additional procedures performed to stabilize the commercial PB. Despite this, hexanal values of PB samples treated with PSE were lower than the control throughout storage, which suggests that PSE may provide some protection against oxidation of PB. The overall results of this research demonstrated the potential of peanut skins to be an abundant source of natural antioxidants suitable for further development into dietary supplements and various food additives.
ATTRIBUTION

Author, Tameshia S. Ballard, is the major contributor and writer of the manuscripts in chapters 3-6 of this dissertation. Co-authors, Dr. Parameswarakumar Mallikarjunan from the Department of Biological Systems Engineering (Virginia Tech) and Dr. Sean O’Keefe from the Department of Food Science & Technology (Virginia Tech), served as my co-advisors and were involved in the development, implementation and data analysis for each of the manuscripts.

Advisory committee members, Dr. Craig Thatcher from the School of Applied Arts and Sciences (Arizona State University) and formerly of the Virginia-Maryland Regional College of Veterinary Medicine (Virginia Tech) and Dr. Mike Zhang from the Department of Biological Systems Engineering (Virginia Tech) provided additional guidance and support in the development of the manuscripts. Dr. Thatcher, in particular, assisted with methodology development and data analysis for the cell culture portion of the research (Chapter 5).
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**DEDICATION**

This dissertation is dedicated to the loving memory of my aunt, Retha Mae Ballard, whom we recently lost to a hard fought battle with lupus. Although I didn’t want to see you go, I am at peace knowing that you are in a far better place. You will be forever missed!
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Attribution</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td><strong>CHAPTER 1</strong></td>
<td></td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>LITERATURE REVIEW</strong></td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Lipid Oxidation in Foods</td>
<td>7</td>
</tr>
<tr>
<td>2.1.1 Mechanisms of Lipid Oxidation</td>
<td>9</td>
</tr>
<tr>
<td>2.2 Antioxidants</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1 Synthetic Antioxidants</td>
<td>14</td>
</tr>
<tr>
<td>2.2.2 Natural Antioxidants</td>
<td>15</td>
</tr>
<tr>
<td>2.2.2.1 Polyphenolic Compounds</td>
<td>17</td>
</tr>
<tr>
<td>2.2.2.2 Alternative Sources of Natural Antioxidants</td>
<td>20</td>
</tr>
<tr>
<td>2.3 Extraction of Polyphenols from Plant Materials</td>
<td>25</td>
</tr>
<tr>
<td>2.3.1 Solid-liquid Extraction</td>
<td>27</td>
</tr>
<tr>
<td>2.3.2 Supercritical Fluid Extraction</td>
<td>28</td>
</tr>
<tr>
<td>2.3.3 Accelerated Solvent Extraction</td>
<td>31</td>
</tr>
<tr>
<td>2.3.4 Ultrasound-Assisted Extraction</td>
<td>32</td>
</tr>
<tr>
<td>2.3.5 Microwave-Assisted Extraction</td>
<td>33</td>
</tr>
<tr>
<td>2.3.6 Extraction of Antioxidants from Peanut Skins</td>
<td>38</td>
</tr>
<tr>
<td>2.4 Measuring Antioxidant Activity in Food</td>
<td>40</td>
</tr>
<tr>
<td>2.4.1 Thiobarbituric Acid Reactive Substances Assay</td>
<td>41</td>
</tr>
<tr>
<td>2.4.2 Peroxide Value</td>
<td>42</td>
</tr>
</tbody>
</table>
2.4.3 p-Anisidine Value ............................................................................................43
2.4.4 Oxygen Radical Absorbance Capacity Assay ..................................................43

2.5 Polyphenolic Compounds and Human Health .......................................................44
2.5.1 Protective Effect of Polyphenols in Cell Model Systems .................................48

Conclusions .....................................................................................................................49

REFERENCES ...............................................................................................................51

CHAPTER 3
Optimizing the Extraction of Phenolic Antioxidants from Peanut Skins using Response Surface Methodology .................................................................55

Abstract ....................................................................................................................56
Introduction ..............................................................................................................57
Materials and Methods ..........................................................................................59
Results and Discussion ............................................................................................62
Conclusions ..............................................................................................................71
References ................................................................................................................91

CHAPTER 4
Optimizing the Extraction of Phenolic Antioxidants from Peanut Skins using Microwave-Assisted Extraction .................................................................92

Abstract ....................................................................................................................93
Introduction ..............................................................................................................94
Materials and Methods ..........................................................................................96
Results and Discussion ..........................................................................................100
Conclusions ............................................................................................................108
References ..............................................................................................................120

CHAPTER 5
Protective Effect of Peanut Skin Extracts against H₂O₂-Induced Cytotoxicity in Human Brain Microvascular Endothelial Cells ..................................................122

Abstract ..................................................................................................................123
Introduction .............................................................................................................124
Materials and Methods ..........................................................................................126
Results and Discussion ..........................................................................................129
Conclusions .............................................................................................................133
References ..............................................................................................................140
LIST OF TABLES

Table 2.1  Dissipation factor and dielectric constants for solvents commonly used with MAE .................................................................38

Table 3.1  The coded values and corresponding actual values of the optimization parameters used in the response surface analysis ..........................72

Table 3.2  The experimental design for response surface analysis in terms of coded values – MeOH and EtOH ..........................................................73

Table 3.3  The experimental design for response surface analysis in terms of coded values – EA and Water .................................................................74

Table 3.4  Optimum conditions for the extraction of phenolic compounds from peanut skins based on total phenolic content (TPC) and ORAC activity ........75

Table 3.5  Comparison of optimized conditions for the extraction of phenolic compounds from peanut skins reported in the literature ..........................76

Table 4.1  The coded values and corresponding actual values of the optimization parameters used in the response surface analysis. ..............................109

Table 4.2  The experimental design for response surface analysis in terms of coded values .........................................................................................110

Table 4.3  Optimum conditions for the extraction of phenolic compounds from peanut skins using MAE based on total phenolic content (TPC) and ORAC activity .................................................................111

Table 4.4  Comparison of the predicted maximum values for TPC and ORAC activity of PSE using solid liquid extraction (SLE) and microwave-assisted extraction (MAE) under optimized extraction conditions .....................112

Table 5.1  Total phenolic content of PSE (peanut skin extracts) used to treat human brain microvascular endothelial cells (HBMEC). .................................134
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure 2.1</th>
<th>Mechanism of autoxidation</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.2</td>
<td>Mechanism of photo-oxidation</td>
<td>11</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Antioxidant (AH) reactions of free radicals generated during lipid oxidation</td>
<td>12</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Chemical structures of some common synthetic antioxidants</td>
<td>15</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Chemical structures of common flavonoids found in plants</td>
<td>19</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Phase diagram of supercritical CO₂</td>
<td>29</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Antioxidant activity of a tested sample expressed as net area under the curve</td>
<td>44</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Effect of EtOH concentration on TPC of peanut skin extracts</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Response surface of the effect of EtOH concentration and temperature on TPC of peanut skin extracts</td>
<td>78</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Effect of EtOH concentration and temperature on TPC of peanut skin extracts</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Response surface of the effect of MeOH concentration and temperature on TPC of peanut skin extracts</td>
<td>80</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Effect of the treatment interaction of temperature and time on TPC of peanut skin extracts using water as the extraction solvent</td>
<td>81</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Response surface of the effect of temperature and time on TPC of peanut skin extracts using water as the extraction solvent</td>
<td>82</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Effect of extraction temperature on TPC of peanut skin extracts using EA as the extraction solvent</td>
<td>83</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Effect of extraction time on TPC of peanut skin extracts using EA as the extraction solvent</td>
<td>84</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Effect of EtOH concentration on the ORAC activity of peanut skin extracts</td>
<td>85</td>
</tr>
</tbody>
</table>
Figure 3.10  Response surface of the effect of concentration and temperature on the ORAC activity of peanut skin extracts .......................................................... 86

Figure 3.11  HPLC chromatogram of PSE extracted using 30% MeOH at 60 °C and 30 min.......................................................... 87

Figure 3.12  UV-VIS spectra of (a) resveratrol standard and (b) peak of matching retention time in PSE sample extracted with 30% MeOH at 60 °C and 30 min............................... 88

Figure 3.13  HPLC chromatograms of (a) PSE sample extracted with 30% EtOH at 30 °C and 10 min and (b) PSE sample extracted with 90% EtOH at 30 °C and 10 min......................................................... 89

Figure 3.14  Response surface of the effect of temperature and MeOH concentration on resveratrol content................................................................................. 90

Figure 4.1  Response surface of the effect of microwave power and sample mass on TPC of PSE ........................................................................................................ 113

Figure 4.2  The effect of sample mass on TPC of PSE.................................................... 114

Figure 4.3  The effect of microwave power and sample mass on TPC of PSE .......... 115

Figure 4.4  The effect of sample mass on ORAC activity of PSE ................................. 116

Figure 4.5  UV-VIS (a) and mass spectra (b) of resveratrol standard.......................... 117

Figure 4.6  UV-VIS (a) and mass spectra (b) of peanut skin extract sample. Extraction conditions: 90% microwave power, 30 s irradiation time and peanut skin mass of 1.5 g ............................................................ 118

Figure 4.5  Response surface of the effect of microwave power and irradiation time on resveratrol content................................................................................. 119

Figure 5.1  Mean luminescence values of human brain microvascular endothelial cells (HBMEC) insulted with 700 μM H2O2......................................................... 135

Figure 5.2  Mixed model showing the effects of peanut skin extract (PSE) concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10%) on luminescence of HBMEC treated with 700 μM H2O2 .................................................................................................................. 136

Figure 5.3  Mean luminescence values of HBMEC supplemented with PSEW prior to treatment with 700 μM H2O2 .................................................................................. 137
Figure 5.4  Mean luminescence values of HBMEC supplemented with PSEE prior to treatment with 700 μM H₂O₂ .................................................................138

Figure 5.5  Mixed model showing the effects of peanut skin extract (PSE) concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10%) on viability of HBMEC treated with 700 μM H₂O₂ .................................................................139

Figure 6.1  Effect of peanut skin extracts on hexanal concentration in commercial peanut butter stored for 0, 7, 14 and 28 days at 45 °C .................................................................150

Figure 6.2  Comparison of hexanal concentration in control samples and PB samples treated with PSE and stored for 0, 7, 14 and 28 days at 45 °C .................151
CHAPTER 1

INTRODUCTION

Lipid oxidation is a major concern to food processors because it leads to the development of undesirable flavors and odors in food materials. Oxidative deterioration of lipids reduces product shelf-life and renders many foods unacceptable to consumers. The food industry currently uses a variety of synthetic antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butyl hydroquinone (TBHQ) to protect against lipid oxidation. Research [1-3] has suggested that these synthetic antioxidants may be linked to the development of liver disease and cancer. Consumer demand for healthier products containing less synthetic additives is driving research efforts to seek out alternative sources of natural antioxidants.

Polyphenols are natural antioxidant compounds found in a variety of fruits, vegetables and beverages such as tea and wine. These compounds are attracting a great deal of attention due to increasing evidence suggesting that they may prevent chronic conditions such as cancer, atherosclerosis and neurological diseases. One of the most commonly known polyphenols is resveratrol. Resveratrol is reported to have a wide range of beneficial effects including blocking platelet aggregation [4], vasodilatation [5,6] and prevention of cancer [7]. Agricultural waste materials such as peanut skins, hulls and roots and grape seeds and skins are also abundant sources of resveratrol and other polyphenolic compounds. However, peanut skins, unlike grape seeds, have yet to be exploited commercially due to a lack of knowledge of efficient procedures for the extraction of these natural antioxidant compounds.
Peanut skins are low value ($12-$20/ton) byproducts of peanut blanching operations [8]. Several authors [9-12] have reported that peanut skins contain phenolic compounds with demonstrated antioxidant properties. Yu et al. [11] observed three classes of compounds in peanut skin extracts including phenolic acids, flavonoids and stilbene (resveratrol). Some authors [11-14] have employed traditional solid-liquid extraction techniques using different organic solvents to extract antioxidants from peanut skins. Nepote et al. [14] investigated the effects of several parameters on the extraction of phenolic compounds from peanut skins using solid-liquid extraction. In that study, optimum extraction conditions were solely based on the quantity of total phenolic compounds extracted as determined by Folin-Ciocalteu reagent, and no identification of phenolics were reported. Wang et al. [10] extracted phenolics from peanut skins by maceration of the skins with 50% (v/v) aqueous EtOH at room temperature and reported a total phenolics content of 90 mg/g of extract. More research is needed to develop alternative extraction procedures and to obtain a more detailed profile of the phenolic composition of peanut skin extracts.

Interest in microwave-assisted extraction (MAE) has increased significantly over the past few years as a result of its inherent advantages (reduction in extraction time and solvent volume) over more traditional extraction techniques. In MAE, the solvent and sample are contained in sealed extraction vessels under controlled temperature and pressure conditions. The closed vessels allow the temperature of the solvent to rise well above its boiling point which enhances extraction efficiency and shortens extraction time. In addition, the direct heating that occurs with MAE typically allows for a reduction in the volume of solvent needed for extraction. Although the majority of the work on MAE has focused on the extraction of organic compounds from soils, its efficacy in the extraction of phenolic compounds from plant material is now being
investigated [15-17]. To our knowledge, there have been no previous reports on the extraction of polyphenols from peanut skins using MAE.

**Hypothesis**

The hypothesis of this research is that microwave-assisted extraction can be used to increase the extraction efficiency of phenolic antioxidant compounds from peanut skins, and the extracts from peanut skins will reduce oxidation in model food and biological systems.

**Objectives**

The overall goal of this research was to optimize the extraction of phenolic antioxidants from peanut skins. The specific objectives are to:

1. Determine optimal conditions for the extraction of phenolic antioxidant compounds from peanut skins using solid-liquid extraction (SLE) and microwave-assisted extraction (MAE) methods.
2. Identify resveratrol in peanut skin extracts using HPLC and LC-MS procedures.
3. Characterize the antioxidant activity of peanut skin extracts in a model food system.
4. Investigate the protective effects of peanut skin extracts against hydrogen peroxide-induced oxidative stress in human brain microvascular endothelial cells.

**Rationale and Significance**

Lipid oxidation is a huge challenge for the food industry as it reduces product shelf-life and threatens consumer acceptability. Industry’s reliance on synthetic antioxidants to control oxidative reactions must be minimized as consumer demands for healthier products containing fewer synthetic additives are increasing. Polyphenols derived from agricultural byproducts such as peanut skins may be a viable alternative to synthetic antioxidants. However, efficient procedures for the extraction of these natural antioxidant compounds from peanut skins need to
be developed in order to increase commercial appeal. In this way, tremendous value can be added to what would otherwise be considered as waste material. More research is needed in the development of alternative extraction procedures that serve to reduce extraction time and solvent consumption while maximizing the recovery of phenolic compounds. This dissertation focuses on the optimization of the extraction process as well as identification of phenolic compounds derived from peanut skins. This work also investigates potential applications of peanut skin extracts in model food and biological systems.

**Dissertation Layout**

This dissertation consists of seven chapters. Following the introduction in Chapter 1, Chapter 2 consists of a review of the literature pertinent to the research presented. Chapters 3-5 were written as three separate manuscripts to be submitted to peer-reviewed journals. As such, there will be some overlap in topics discussed. Chapters 3 and 4 are focused on the optimization of the two extraction methods, SLE and MAE. Chapters 5 and 6 deal with practical applications of the peanut skin extracts in model food and biological systems. A summary of the results and recommendations for future research are presented in Chapter 7.
REFERENCES

CHAPTER 2

LITERATURE REVIEW

ABSTRACT

Lipid oxidation is a major economic concern for food processors. Oxidation of food lipids severely reduces product shelf-life and renders many foods unacceptable to consumers. The food industry currently uses a variety of synthetic (e.g., BHT and BHA) antioxidants to help protect foods against lipid oxidation. However, consumer demand for natural, healthy products has been increasing in recent years. Researchers are now exploring the potential of naturally derived antioxidants, mainly from agricultural waste materials (e.g., grape pomace and peanut skins and hulls) as an alternative to synthetic compounds. Polyphenols are the major class of natural antioxidants found in these plant byproducts. This review highlights the current state of research as it relates to alternative sources (peanut byproducts, grape byproducts, etc.) of natural antioxidants, conventional (solid-liquid extraction, soxhlet extraction) and novel (supercritical fluid extraction and microwave-assisted extraction) procedures for the extraction of phenolic antioxidant compounds and methods available for analyzing antioxidant activity in food systems. This review also briefly discusses the possible impact of polyphenols on human health.

Key words: lipid oxidation, synthetic antioxidants, natural antioxidants, polyphenols, antioxidant activity, peanut skins, microwave-assisted extraction, solid-liquid extraction
2.1 Lipid Oxidation in Foods

Lipids are the primary components of many foods, and their presence is often needed for the development of flavor, texture and color attributes. However, lipids are highly unstable and are readily attacked by oxygen, leading to a chain of chemical reactions that generate undesirable flavor and odor compounds. These oxidative reactions can be accelerated by metals (e.g., iron, copper), light, temperature, enzymes and bacteria. Lipids can be characterized as saturated or unsaturated fatty acids with the term ‘saturated’ referring to the fact that all carbon atoms are bound to as many hydrogens as possible. Unsaturated fatty acids have one (mono-unsaturated) or more (poly-unsaturated) double bonds between carbon atoms. Foods containing high levels of unsaturated fats such as meat and meat products, dairy, fish and oils are especially susceptible to oxidative reactions as oxygen is able to attack those double bonds, leading to the formation of free-radicals and other oxidation products. Off-flavor resulting from the oxidation of lipids is the most common factor limiting the shelf-life of lipids and lipid containing products [1]. Lipid oxidation, therefore, is a major economic concern as it renders products unacceptable to consumers. The food industry suffers significant losses as a result of decreased product shelf-life caused by “warmed-over flavor” (WOF) development, rancidity and diminished nutritional quality, all of which stem from lipid oxidation.

Tims & Watts [2] first introduced the term warmed-over flavor to describe the rapid development of oxidized/rancid flavor (usually apparent within 48 hours) in refrigerated, cooked meats. Rancidity refers to an unpleasant change in flavor and odor of foodstuffs. It is believed that WOF occurs as a result of the denaturation of iron-containing proteins (hemoglobin and myoglobin) upon heating that causes the subsequent release of ferrous iron (Fe$^{2+}$), which acts as a catalyst for lipid oxidation. Phospholipids appear to be the primary substrate for the formation
of lipid oxidation reaction products that contribute to WOF [3]. WOF development is in marked contrast to the slow onset of rancidity encountered in raw meats, fatty tissue and rendered fat, which is not normally apparent until after several weeks or months of storage [4]. Oxidative rancidity involves lipid oxidation in which various kinds of fats develop oxidized flavors in the presence of oxygen over time and can occur in a wide range of lipid-containing products during storage. Oxidative rancidity is the most important factor that limits the shelf-life of edible oils [5]. In addition, oxidative and hydrolytic rancidity are the major causes of milk quality deterioration. Hydrolytic rancidity refers to the hydrolysis of triglycerides in the presence of water and usually a catalyst such as lipoprotein lipase in milk. The lipase causes the release of free fatty acids which contribute to the rancid, bitter and unpleasant taste in milk [6].

Oxidation of lipids not only affects flavor and odor development, but also impacts food texture, color and nutritive value. Secondary products of lipid oxidation including malondialdehyde (MDA) and 4-hydroxynonenal (4-HN) have been known to interact with proteins and amino acids [1]. These types of interactions can lead to undesirable color and textural changes. For example, foods can experience a darkening in color as a result of a condensation reaction between oxidation products and proteins [7]. Furthermore, textural changes that occur in oxidized products may be attributed to the oxidative induction of protein crosslinks [7]. Lipid oxidation products are also capable of destroying essential fatty acids and lipid-soluble vitamins [1]. In milk, several key nutrients including riboflavin (Vitamin B₂) and ascorbic acid (Vitamin C) are destroyed due to light-induced lipid oxidation (photo-oxidation).
2.1.1 Mechanisms of Lipid Oxidation

**Autoxidation**

Lipid oxidation can occur via three primary mechanisms: autoxidation, photosensitized oxidation and enzyme catalyzed oxidation. The process of autoxidation is of utmost importance when it comes to food products. Autoxidation is a free-radical mediated chain reaction whereby unsaturated fatty acids are attacked by molecular oxygen to form free radicals and a host of other oxidation products that adversely affect taste, texture, safety and nutritional quality of foods. Autoxidation (Fig. 2.1) occurs in three stages: initiation (formation of free radicals), propagation (free-radical chain reaction) and termination (formation of nonradical species).

**Initiation**

Initiation is marked by the formation of free radicals via a hydrogen atom abstraction by an oxidizing agent. Potential oxidizing agents include transition metals, singlet oxygen and free radicals. The abstraction of a hydrogen atom from an unsaturated fatty acid by an initiator leads to the generation of a lipid free radical (L•). L• rapidly reacts with molecular oxygen to form the lipid peroxyl radical (LOO•).

**Propagation**

The propagation stage involves the rapid acceleration of the chain reaction begun in initiation. During propagation, the peroxyl radical abstracts a hydrogen atom from another unsaturated fatty acid, generating a lipid hydroperoxide (LOOH) and another L•. Hydroperoxides are highly unstable primary products of oxidation, but do not contribute to the undesirable flavors and odors commonly associated with rancid foods. However, because of their instability, peroxides continue in the chain reaction and are further degraded into secondary
reaction products such as aldehydes, ketones, acids and alcohols. It is these secondary products of oxidation that are responsible for off-odor and off-flavor development in oxidized foods.

**Termination**

Termination is the stage in which free radicals begin to bind to one another to form more stable, nonradical species, thus completing one cycle of lipid oxidation. However, there can be reinitiation causing the cycle to repeat [1].

![Mechanism of autoxidation](image)

**Photo-oxidation**

Photo-oxidation is a process whereby oxidation occurs due to the reaction of a photosensitizing agent with molecular oxygen in the presence of light (Fig. 2.2). Some common photosensitizers in food products include food dyes (e.g., FD&C Red No. 3), chlorophyll and riboflavin. During photo-oxidation, a photosensitizer (\(^{1}\text{S}\)) absorbs ultraviolet light (hv) and reaches an excited state (\(^{3}\text{S}^*\)). The excited sensitizer is then able to transfer that energy to triplet oxygen, i.e., ground state oxygen (\(^{3}\text{O}_2\)), thereby forming the more highly reactive singlet oxygen species (\(^{1}\text{O}_2\)). The extremely electrophilic nature of singlet oxygen allows it to directly attack unsaturated fatty acids. Photo-oxidation, therefore, happens at a much faster rate than
autoxidation. Consumer preference for transparent packaging and colorful foods creates ideal conditions for exposure of food to light, thus increasing the likelihood of oxidative damage [7]. Photo-oxidation in food primarily occurs through the following mechanism [8]:

\[ ^1S + h\nu \rightarrow ^1S^* \rightarrow ^3S^* \]

\[ ^3S + ^3O_2 \rightarrow ^1O_2 + ^1S \quad \text{(energy transfer)} \]

\[ ^1O_2 + LH \rightarrow LOOH \]

**Fig. 2.2 Mechanism of photo-oxidation**

**Enzyme-mediated Oxidation**

Oxidation of lipids can also be an enzyme-mediated process whereby endogenous enzymes catalyze reactions that lead to the generation of free radical species. The superoxide radical anion \( (O_2^\bullet-) \) along with hydrogen peroxide \( (H_2O_2) \) are often involved in these enzymatic reactions. The enzyme superoxide dismutase, for example, catalyzes a reaction that converts \( O_2^\bullet- \) to \( H_2O_2 \) and \( O_2 \). In the Fenton reaction (shown below), metal ions such as iron react with \( H_2O_2 \) to form the highly reactive hydroxyl radical \( (OH^\bullet) \). The hydroxyl radical can directly attack the double bond in lipids to initiate the process of lipid oxidation.

**Fenton Reaction:**

\[ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^- \]

**2.2 Antioxidants**

It has been known for years that antioxidants help prevent lipid oxidation in foods and play a key role in protecting against the development of cardiovascular disease (CVD) and cancer. The food industry currently uses a variety of synthetic antioxidants including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butyl hydroquinone (TBHQ) and
propyl gallate (PG) to prevent oxidative deterioration of lipids. Natural antioxidants, α-tocopherol, vitamin C and rosemary extracts have also been employed by industry. An antioxidant can be defined as any substance that when present at low concentrations compared with that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate [1]. According to Boskou and Elmadfa [9], an effective antioxidant should:

1. Compete effectively with the substrate for the reactive intermediate.
2. Be able to be readily repaired by the biological system. If the antioxidant is destroyed irreparably at every encounter with the oxidizing species then the system will rapidly reach a state where it is no longer protected.
3. Be accessible to the reactive intermediate in the microenvironment, i.e., the antioxidant has to be located in the same microenvironment as the oxidizing radical species.
4. Be relatively unreactive to the substrate. The products should not be toxic to the system and must not take part in the reaction.
5. Be catalytic in its quenching mechanism. Ideally, the antioxidant should be generated once it has reacted with the radical species.

An antioxidant, AH, reacts with free radicals in the following mechanism:

\[
\begin{align*}
L\cdot + AH & \rightarrow LH + A\cdot & (1) \\
LO\cdot + AH & \rightarrow LOH + A\cdot & (2) \\
LOO\cdot + AH & \rightarrow LOOH + A\cdot & (3) \\
LR\cdot + A\cdot & \rightarrow LA & (4) \\
LO\cdot + A\cdot & \rightarrow LOA & (5)
\end{align*}
\]

**Fig. 2.3** Antioxidant (AH) reactions with free radicals generated during lipid oxidation
The reactions shown in Fig. 2.3 may suggest that adequate levels of an appropriate antioxidant can totally prevent lipid oxidation. However, this is not the case. The reaction of antioxidants with free radicals generated during lipid oxidation is a complex process that depends on a number of factors including the type of lipid under investigation and the corresponding oxidation products that are produced. There is a diverse array of free radical configurations that can be generated during lipid oxidation. Therefore, to learn about the real effects of antioxidants, it is important to obtain specific chemical information about what products of lipid oxidation are inhibited [10]. Currently, there is no evidence that suggests that any one antioxidant, albeit synthetic or natural, outperforms all others in all lipid substrates [11]. The variety of free radicals formed in oxidation illustrates the magnitude of the problem of intercepting all free radicals with one single antioxidant [11].

It has been suggested that perhaps the most effective means of retarding lipid oxidation in foods is to use a combination of antioxidants. Due to the vast number of free radical configurations that can be generated during lipid oxidation, it would stand to reason that some antioxidants are better suited to intercept certain free radicals more than others depending on factors such as steric hindrance and solubility [11]. Therefore, the addition of one or more antioxidants will improve the odds of intercepting more free radical species. It has been reported that two or more antioxidants present in a food can act synergistically to improve the overall oxidative stability of the product [11]. Food systems, however, are inherently complex in nature and therefore, it is difficult to determine the ideal mix of antioxidants and at what levels they should be added for maximum benefit.

The role of antioxidants in food systems is not fully understood, especially in systems that exist as emulsions [12]. A large majority of foods do exist as emulsion. According to
Frankel [12], the interfacial phenomena are the keys to better understanding of antioxidant action in heterogeneous foods and biological systems. Natural antioxidants exhibit complex interfacial affinities between air-oil and oil-water interfaces that significantly affect their relative activities in different lipid systems [12]. Hydrophilic antioxidants have been found to be more effective in bulk oils while lipophilic antioxidants are more effective in oil-in-water emulsions. It is believed that polar antioxidants show greater antioxidant activity (AOA) in bulk oils because they tend to accumulate at the air-oil interface, the location where oxygen concentration is high and subsequent oxidation reactions are greatest. Conversely, the AOA of nonpolar antioxidants is greatest in emulsions as they are retained in the oil droplets and may accumulate at the oil-water interface, the location where interaction between hydroperoxides and pro-oxidants in the aqueous phase occur [13].

2.2.1 Synthetic Antioxidants

Synthetic antioxidants have been widely used to extend the shelf-life of various food materials. The current preference for synthetic antioxidants can be attributed to their proven effectiveness in a variety of food systems and their relative low cost when compared to natural antioxidants. The most commonly used synthetic antioxidants in the U.S. food industry include BHT, BHA and TBHQ (Fig. 2.4). The use of PG has been limited by its tendency to cause undesirable color changes. BHT and BHA are hydrophobic phenolic antioxidants that inhibit free-radical initiated chain reactions. Protection against lipid oxidation may occur as a result of the formation of a BHT radical, which is thought to have a lower reduction potential than that of lipid peroxyl radicals [9]. BHA is commonly used in combination with BHT or PG which creates a synergistic effect [14]. TBHQ is less volatile than BHA and BHT but is stable at higher
temperatures. Due to the stability of TBHQ at elevated temperatures, it has proven to be more effective in polyunsaturated vegetable oils [14].

Although synthetic antioxidants are extremely effective at slowing oxidation, there have been recent consumer concerns over potential adverse health effects associated with these compounds. Studies have reported that BHT and BHA cause a wide range of health problems including, enlarged liver, increased liver microsomal enzyme activity and conversion of some ingested materials into toxic and carcinogenic substances, especially if they are present in excessive amounts [15]. Many processors wish to avoid adding synthetic antioxidants to foods to eliminate these health concerns and to be able to state on the label that the product is “all natural.”

![Chemical structures of BHA, BHT, and TBHQ](image)

**Fig. 2.4 Chemical structures of some common synthetic antioxidants**

### 2.2.2 Natural Antioxidants

In recent years, much research has been focused on identifying sources of natural antioxidants that can be used to replace their synthetic counterparts. Natural antioxidants are presumed safe by consumers because they are naturally found in plant materials and have been used for centuries [10]. Although synthetic compounds remain the antioxidant of choice in the food industry, there are some noteworthy natural antioxidants including tocopherols (vitamin E
and its derivatives) and extracts from herbs and spices such as rosemary, sage, thyme and oregano that have been proven effective in many food systems and are currently being employed by industry. Natural antioxidants including ascorbic acid, β-carotene and other carotenoids have also been used. Natural antioxidants not only reduce lipid oxidation in food systems, but have also been shown to play a significant role in preventing a number of chronic diseases such as heart disease, cancer and Alzheimer’s and Parkinson’s diseases [16-19].

There are conflicting reports in the literature regarding the antioxidant capacity of naturally derived antioxidants (from plant sources) versus that of synthetic antioxidants in preventing lipid oxidation in food matrices. Some studies have shown that natural antioxidants do not inhibit lipid oxidation reactions as well as their synthetic counterparts, while others claim that natural antioxidants can be just as effective as synthetic antioxidants although usually at higher concentrations [15,20-22]. Formanek [20] examined the effects of the addition of commercial rosemary extracts (Duralox NMC and Herbalox type 25) and BHA/BHT on the oxidative stability of beef patties. It was reported that rosemary extracts comprising 2% Duralox and 0.25% Herbalox exhibited similar AOA as 0.01% BHA/0.01% BHT. Despite the conflicting reports, consumer demands are continuing to drive research efforts to seek out alternatives to synthetic antioxidants.

Current research [10,23-28] is investigating the efficacy of using plant materials as sources of natural antioxidants to be incorporated into food products to control lipid oxidation. The majority of these studies have looked into the effects of antioxidants on various meat systems. Britt et al. [24] studied the effects of antioxidant compounds from tart cherries on lipid oxidation in raw and cooked ground beef patties. It was found that cherry extracts significantly reduced thiobarbituric acid reactive substances (TBARS) when compared to the control and the
addition of cherry tissue to ground beef before cooking delayed oxidation in cooked meat.

Mansour and Khalil [29] also investigated the effects of different plant extracts on lipid oxidation in ground beef. In their study, it was also shown that the addition of plant extracts was effective in retarding rancid odor, thiobarbaturic acid reactive species and color change. Furthermore, a correlation was found between sensory perception of rancidity and the addition of extracts. Products containing plant extracts received significantly lower rancid odor scores than the products in which no antioxidant was added.

2.2.2.1 Polyphenolic Compounds

The antioxidant activity of plant extracts can be in large part attributed to the presence of polyphenolic compounds located within the plant tissues. Polyphenols are attracting a great deal of attention due to evidence suggesting that an increase in their consumption in the diet may prevent cancer, strokes and neurological diseases. They are the most abundant antioxidants in our diets and it is estimated that we consume about 1 g of polyphenols per day [30]. Several thousands of natural polyphenols have been identified in plants and plant foods. Polyphenolic compounds are present in high concentrations in a variety of fruits, vegetables and beverages such as tea and wine. They are also abundant in agricultural byproducts such as peanut skins, hulls and roots, grape seeds and skins and in a number of herbs and spices (rosemary, sage, thyme and oregano). Polyphenols are important to plant growth and development and provide a defense mechanism against infection and injury [31]. Many polyphenolic compounds have been found to have a much stronger antioxidant activity than vitamins C and E and \(\beta\)-carotene within the same food [16].

Flavonoids make up the largest class of polyphenolic compounds and can be divided into several subclasses including flavanols (catechin and catechin gallate esters), flavanones, flavones
(luteolin), anthocyanidins and flavonols (quercetin, myricetin, kaempferol) (Fig. 2.5). All flavonoids consist of a 15-carbon \((C_6C_3C_6)\) diphenylpropane skeleton. The 15-carbon backbone takes the form of two benzene rings (A and B) connected to a third heterocyclic ring known as the C ring. Differences in substitution on ring C help distinguish the different classes of flavonoids. Flavonols, for example, lack a carbonyl at the carbon-4 (C-4) position on the C ring \[32\]. The C-4 position in flavonols is occupied instead, by a keto group. The most familiar of the flavanols are the flavan-3-ols, (+)-catechin and (-)-epicatechin that are known to give green tea some of its antioxidant properties.

The existence, number, placement and degree of substitution of hydroxyl groups on the benzene ring provides much of the structural variation found in flavonoids \[32\]. Flavonoids can act as free radical scavengers, singlet oxygen quenchers or metal chelators, depending on their chemical structure (Fig. 2.5). There is much debate in the literature in regards to which structural configuration confers the highest degree of antioxidant activity. It has been postulated that the antioxidant activity of flavonoids can be attributed to the hydroxyl groups positioned at the 3’,4’-OH of ring B and the 2,3-double bond of ring C, and the ability to stop free radical chain reactions increases with the number of OH groups on rings A and B \[12\]. Flavonoids can act as metal chelators by binding metals at two points: the orthodiphenol grouping in ring B and the ketol structure in the C ring of flavonols \[12\]. However, it is likely that different metals exhibit different properties with regard to chelation by flavonoids \[33\].
Fig. 2.5 Chemical structures of common flavonoids found in plants.
2.2.2.2 Alternative Sources of Natural Antioxidants

Peanut Byproducts

Peanuts (*Arachis hypogaea*) are an important agricultural crop in many parts of the world. Peanuts consist of the kernel, skin (seed coat) and hulls. The kernels are used to make a variety of products such as peanut butter, peanut oil and confectionary products while the hulls and skins are treated as waste material. However, several authors [33-39] have reported the presence of polyphenolic compounds in peanut skins, hulls and roots that may have significant antioxidative properties. Chen et al. [34] found that peanut roots contain as high as 1.3 mg/g of resveratrol, which has received widespread attention within the past five years as a potent antioxidant. In a study conducted by Yen *et al.* [39], it was reported that peanut hulls contain high levels of luteolin, a flavonoid commonly found in numerous plants. Furthermore, it was shown that methanolic extracts from peanut hulls (MEPH) of varied maturity exhibited 92.9-94.8% inhibition of peroxidation of linoleic acid.

Rehman [15] compared the effects of methanol extracts from peanut hulls to BHA and BHT on oxidation of sunflower oil used to fry potato chips. No significant differences were found between the synthetic antioxidants (200 ppm) and MEPH (1200 and 1600 ppm) in the inhibition of fried potato chip peroxidation. The author concluded that although the levels of MEPH used in the study were six times that of the synthetic antioxidants, MEPH would be preferred due to minimized health effects. Although peanut hulls contain polyphenolic compounds, they have a low bulk density which makes them poorly suited as a raw material for antioxidant extraction when compared to peanut skins. Additionally, peanut skins have been shown to contain higher levels of phenolic compounds than the hulls [37] which make them a more attractive source for extracting antioxidant compounds.
Peanut skins are a low-value byproduct of the roasting and blanching processes. The world production of peanut skins can be estimated at over 750,000 tons annually [40]. Currently, this agricultural waste material is either incinerated or sold as animal feed for less than one cent per pound (Lee, 1996). However, peanut skins contain significant levels of natural antioxidants, namely polyphenolic compounds that can be extracted and potentially utilized in a variety of food and pharmaceutical applications. Peanut skins have been used for centuries in traditional Chinese medicine to treat chronic haemorrhage and bronchitis [41]. The early work of Karchesy and Hemingway [42] employed several techniques (NMR, TLC and HPLC) to separate and identify procyanidin compounds in peanut skin extracts. Among the compounds identified were the flavan-3-ols catechin, epicatechin and epicatechin-(4β→8;2β→O→7)-catechin.

Lou et al. [35] reported the presence of six A-type proanthocyanidins from the water soluble fraction of peanut skin extracts. Of those six compounds, three were deemed new compounds and were identified as epicatechin-(2β→O→7,4β→6)-catechin, epicatechin-(2β→O→7, 4β→6)-ent-catechin and epicatechin-(2β→O→7, 4β→6)-ent-epicatechin. Lou et al. [43] also isolated eight new flavonoids and two novel indole alkaloids from the water soluble fraction of peanut skins. Yu et al. [44] observed three classes of compounds in peanut skin extracts: (1) phenolic acids including chlorogenic and caffeic acids; (2) flavonoids including epigallocatechin (EGC) and epicatechin and (3) stilbene (resveratrol). Other important flavonoids such as quercetin and luteolin have also been found in peanuts and peanut byproducts [39,45,46]. Studies [47,48] have shown that both quercetin and luteolin have anti-cancer activities.

Phenolic antioxidants have also been isolated from the peanut seed itself, peanut butter and other peanut products. Sobolev and Cole [38] reported that commercial peanuts and peanut products contained resveratrol at levels ranging from 0.06 ppm in roasted peanuts to 5.1 ppm in
boiled peanuts. Similarly, Ibern-Gomez et al. [49] found that natural and blended peanut butters contained significant levels of resveratrol (0.27-0.75 μg/g) and piceid (0.07-0.23 μg/g), a glucoside of trans-resveratrol. Huang et al. [50] extracted peanut seed testa with various solvents and fractionated the extracts using HPLC. It was found that fraction 17 showed the highest antioxidant activity, and the predominate compound isolated from this fraction was identified as ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester). Additionally, Huang et al. [50] observed that roasted peanut kernels exhibited excellent AOA and radical scavenging ability.

The seed testa from other nuts such as cashews [51] and almonds [52] have been reported to contain phenolic compounds that exhibit antioxidative behavior in vitro. Kamath and Rajini [51] were able to extract phenolic compounds from the skins of cashew nuts (Anacardium occidentale L.) using ethanol as an extraction solvent. The total phenolic content of the cashew skin extracts (CSE) was 243 mg gallic acid equivalents/g skin powder. They found that CSE were able to effectively scavenge the ABTS and superoxide radicals, with CSE proving to be equally as potent as BHA in the scavenging of ABTS. Additionally, it was found that epicatechin was the compound largely responsible for the AOA of CSE. Sang et al. [52] isolated and identified nine phenolic compounds from almond skins (Prunus amygdalus Batsch) and they include quercetin, kaempferol, catechin, protocatechuic acid and vanillic acid.

**Grape byproducts**

It is estimated that the wine industry worldwide uses 43 million tons of grapes (Vitis vinifera) for wine production annually, thereby generating approximately 5-7 million tons of grape pomace per year [53]. The pomace consists of grape seeds, skins and some stems that are left over after pressing and fermentation. The presence of polyphenolic compounds in grape
pomace has been documented by a number of researchers [53-58]. The abundance of these potentially health-promoting compounds in grape byproducts has lead to an increased number of products on the market made from grape skins and seeds (e.g., grape seed extracts). Phenols in grape pomace can be classified into three main groups: (1) phenolic acids (mainly benzoic and hydroxycinnamic acids), (2) simple flavonoids (catechins, flavonols and anthocyanins) and (3) tannins and proanthocyanidins [54]. Both grape skins and seeds contain monomeric, oligomeric and polymeric proanthocyanidins with the mean degree of polyphenol polymerization being higher in the skins [55]. The phenolic content of grape skins ranges from 285-550 mg phenols/kg skin depending on grape variety and type of pretreatment used [54].

Yildirim et al. [53] compared the AOA and total phenols content of red and white grapes, pomace, juice, must and wine. The authors found that grape pomace exhibited the highest level of AOA (82.3%) compared to just 9.81% for red grape juice. Furthermore, it was shown that the juice had higher total phenolic content than the pomace despite having lower AOA. This was attributed to the fact that compounds in grape pomace are of much greater potency than those found in the juice. Da Silva et al. [56] investigated the radical scavenging capacity of procyanidins from grape seeds. It was reported that when compared to Trolox (a water-soluble form of vitamin E), procyanidin compounds from grape seeds exhibited potent radical scavenging properties, with procyanidin B₂ 3'-O-gallate being the most effective compound in trapping free radicals.

Herbs and Spices

Spices and herbs have been used for centuries as flavor enhancers for many different types of foods. More recently, spices have been regarded as more than just ingredients to impart flavor, but have garnered considerable attention for their ability to retard lipid oxidation and
subsequently increase the shelf-life of a variety of products. Some of the major antioxidant compounds identified in spices include rosmarnol, epirosmanol, carnosol and carnosic acid. Two herbs from the *Labiatae* family, rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*), have found the most widespread commercial use although spices such as oregano have also been studied. Carnosol and carnosic acid have been found in both rosemary and sage, indicating a close botanical relationship between the two spices [57].

Rosemary possesses the most potent antioxidant activity (AOA) among the common spices [21], and is therefore, the most effective at preventing lipid oxidation. There are two primary compounds that are responsible for an estimated 90% of the AOA of rosemary extracts: carnosic acid and carnosol [58]. Other compounds that have exhibited AOA in rosemary extracts are rosmarnol, epirosmanol and isorosmanol. Mielnik *et al.* [21] evaluated the stabilizing effect of commercial rosemary antioxidants on mechanically deboned turkey meat, and compared the antioxidant capacity of rosemary extracts to several other natural and synthetic antioxidants. It was found that an increase in concentration of rosemary extract (200-800 mg/kg) produced a nearly 8-fold decrease in TBARS values when compared to the control. Additionally, no significant differences in TBARS values were found between rosemary extract, Trolox C and ascorbic acid.

Formanek *et al.* [20] reported that an addition of rosemary extracts improved the oxidative stability of dietary α-tocopherol acetate supplemented beef, and rosemary extracts were just as effective in reducing TBARS as the combination of BHA and BHT. Rosemaridiphenol, a diphenolic terpene isolated from rosemary, is reported to be superior to BHA when tested in lard [14]. Although rosemary extracts are extremely effective antioxidants,
it is a costly ingredient to add to foods [59]. Furthermore, the characteristic aroma of extracts derived from spices limits its widespread commercial appeal.

Oregano (*Origanum vulgare*) is a characteristic spice in Mediterranean cuisine. The dry leaves and flowers of oregano, its extracts and essential oil have all been reported to inhibit lipid oxidation when added to various food systems [23]. In the study conducted by Botsoglou *et al.* [23], chickens were supplemented with dietary oregano and later euthanized and muscle tissue was evaluated for the extent of lipid oxidation. It was observed that dietary oregano essential oil supplementation at a concentration of 100 mg oregano essential oil/ kg feed retarded the effects of lipid oxidation in stored raw and cooked chicken meat.

### 2.3 Extraction of Polyphenols from Plant Materials

Although the concept of using naturally derived antioxidants to prevent lipid oxidation in food products is promising, standard procedures for the extraction of these compounds from plant materials must be developed in order to increase commercial appeal. Researchers have employed a variety of extraction procedures usually based on methods that work best for their particular study. Waterman and Mole [60] list 33 different extraction procedures that have been reported in recent plant biochemical literature. The variation in these procedures involved extraction times ranging from 30 seconds to 96 hours and ratios of solvent volume to sample weight from 2 to 200 [32].

The fact that one single plant may contain up to several thousand secondary metabolites makes it necessary to develop high performance and rapid extraction methods [61]. Currently, extraction is being carried out using traditional methods including Soxhlet extraction, solid-liquid and liquid-liquid extraction. These methods have been associated with high solvent consumption, longer extraction times and an increased risk of thermal degradation of labile
components. Soxhlet extraction has been the most regarded amongst all the conventional extraction methods even though it requires lengthy extraction times (up to 24 hours or more) and high energy consumption. Additionally, the extended extraction times severely decreases sample throughput, which is a tremendous liability in terms of commercial applicability.

Alternative novel extraction procedures are now being sought after that will reduce extraction time and solvent consumption, increase sample throughput and improve analyte recovery. Some of these novel methods include supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), solid-phase microextraction (SPME), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). One of the most promising of these methods is MAE. Gao and Liu [62] compared MAE and UAE to more traditional methods for the extraction of flavonoids from plant tissue. They showed that MAE had the highest extraction efficiency of flavonoids among all tested procedures.

Plant byproducts from peanuts (skins, hulls and roots) and grapes (seeds and skins) represent viable natural sources of antioxidants. However, the use of these agricultural waste materials has been limited by a lack of documented standard procedures for the extraction of valuable phytoconstituents. Optimization and standardization of the extraction process is desperately needed as huge quantities of these waste materials are being generated. In Europe alone, 112 million tons of grapes are processed by the wine industry and an estimated 13% (14.5 million tons) correspond to the byproduct (consisting mainly of seeds and skins) after pressing [55].
2.3.1 Solid-Liquid Extraction

*SLE Theory*

Solid-liquid extraction (SLE), also commonly referred to as leaching, involves the removal of a desired component (solute) from a food using a liquid (usually an organic solvent) capable of dissolving the solute [63]. The food material and the solvent are mixed together and the mass transfer of solutes from the solid material to the solvent occurs in four stages: (1) the solvent penetrates the solid matrix, (2) the solute dissolves in the solvent, (2) the solution moves through the solid matrix to its surface and (3) the solution becomes dispersed in the bulk of the solvent [63]. The time required for the solvent to interact with the solid material is critical to solute recovery and depends not only on the solubility of the solute, but also the temperature of extraction, surface area of the material being extracted, solvent viscosity and flow rate [63].

*Solid-liquid Extraction*

Leaching is one of the oldest extraction techniques as it has been used for many years in the extraction of sugar from sugar beets and the separation of minerals from their ores. Over the years, SLE procedures have been adopted by a number of researchers for the extraction of a wide variety of compounds (including phenolic antioxidants) from numerous sources. Research has shown that flavonoids can be extracted with polar solvents such as ethanol, methanol, water and combinations of these. There are conflicting reports in the literature, however, as to which solvent is the most efficient to use with this procedure. The choice of solvent will depend on a number of factors previously mentioned including the solvent’s ability to solubilize the solute, extraction temperature and particle size. Although SLE is still used in many industrial applications, more viable alternatives to SLE are being sought after due to many of the problems
that plague older extraction methods such as increased extraction time, high solvent consumption and sometimes poor solute recovery.

2.3.2 Supercritical Fluid Extraction

**SFE Theory**

Supercritical fluid extraction (SFE) is an extraction technique that separates desired components from a solid or liquid using a supercritical fluid as the extracting solvent. The term supercritical is used to describe any fluid that exists at a temperature and pressure above its critical point. At the critical point, the liquid and gas phases of a substance are indistinguishable and the fluid exhibits properties of both a liquid and a gas. A phase diagram (the simplest being a pressure-temperature diagram), is often used to represent the phases of a substance and the conditions under which each phase exists. Carbon dioxide is the most commonly used solvent with SFE and its phase diagram is shown in Fig. 2.6. Its low critical temperature (31.06 °C) and pressure (73 atm) make it an extremely attractive solvent for use with SFE.

In the supercritical region, CO₂ diffuses like a gas, allowing it to easily penetrate the solid matrix and is able to dissolve solutes like a liquid. When CO₂ or any other substance is near its critical point, small variations in temperature and pressure have a huge impact on the density of the fluid. The solubility of a compound in a supercritical fluid tends to increase with increasing fluid density. As pressure is increased above the critical pressure, the density of the fluid increases; hence, solubility increases with pressure. In this way, the selectivity of a solvent toward a particular solute can be adjusted simply by making small variations in pressure. The ability to control solubility through pressure is one of the main features that distinguishes supercritical fluids from traditional liquid solvents [64].
The effect of temperature on solubility is not so straightforward. It is generally observed that as temperature increases, the solubility of liquid solvents increases. The situation with supercritical fluids is more complex as both temperature and pressure must be taken into account when determining solubility [64]. Near the critical point, even slight increases in temperature can cause a marked decrease in density and hence dissolving power. However, at or above the critical point, it can be generally stated that solubility of supercritical fluids increases with increasing temperature at constant density [64].

![Phase diagram of supercritical CO2](image)

**Fig. 2.6 Phase diagram of supercritical CO2**

Often supercritical fluids by themselves are not suitable for the extraction of more polar constituents such as polyphenols. This problem can be overcome by the use of an entrainer (also called a modifier or co-solvent) such as ethanol or methanol which will serve to increase solubility of the compounds in the supercritical fluid [65]. Entrainers enhance the solubility of polar solutes in supercritical fluids by introducing stronger molecular interaction in the solvation shell of the solute [64]. However, using an organic modifier could mean that an additional
purification step may be required to remove any residual solvent (e.g., methanol) if the end product is intended for consumption.

*Supercritical Fluid Extraction*

SFE has been used in a wide variety of food and pharmaceutical applications. The high degree of selectivity and control over solubility afforded by pressure (and temperature) variation has led to the introduction of many novel SCF extraction and fractionation processes [64]. Carbon dioxide and water are the two fluids most often used with SFE. The use of supercritical carbon dioxide has many advantages including the fact that it is non-toxic, non-flammable, inert to most materials and readily available with a high degree of purity at minimal cost [66]. Furthermore, it can then be easily removed from the solute after extraction is complete, leaving behind no chemical residue, which can be a problem in traditional extraction methods. Moreover, SFE offers an additional advantage over conventional methods in that extraction occurs in the absence of oxygen and light which reduces the chances of oxidative degradation of phytoconstituents. However, one major hurdle in its wide-scale commercial use is the high financial investment required for SFE equipment.

*Ge et al.* [66] successfully used SFE with CO$_2$ to extract vitamin E from wheat germ. They found that the yield of vitamin E from SFE was significantly higher than that obtained from Soxhlet extraction, with extraction temperature and time being much lower for SFE. Other authors have used SFE to extract phenolics from a number of products including grape seeds [65,67,68] and rosemary [72,73]. Palma and Taylor [65] used SFE to fractionally extract compounds from grape seeds. The authors utilized pure CO$_2$ to extract mainly fatty acids, aldehydes and sterols from grape seeds in the first fraction while using a mixture of CO$_2$ and methanol to extract the phenolic compounds catechin, epicatechin and gallic acid in the second
fraction. Similarly, Murga et al. [68] demonstrated that supercritical carbon dioxide with ethanol as a co-solvent can be used to extract phenols and tannins from grape seeds.

2.3.3 Accelerated Solvent Extraction

**ASE Theory**

Accelerated Solvent Extraction (ASE) utilizes high temperatures (50-200°C) and pressures (5-200 atm) to extract analytes from the sample matrix. It is performed at temperatures well above the boiling point of the solvent. Increasing temperature causes an increase in solubility of solutes, an increase in diffusion rates, a weakening of molecular interactions (van der Waals forces) between the solute and the matrix, and a decrease in the viscosity and surface tension of the solvent, which improves solvent penetration into the matrix leading to improved mass transfer [69]. As a result of the high operating temperatures used in ASE, high pressures (5-200 atm) must be maintained within the extraction cell to ensure that the solvent remains in the liquid state. Utilizing high pressure also serves to increase extraction efficiency by ‘pushing’ solvent into sample pores, thereby increasing the accessibility of the analytes to the solvent [69].

**Accelerated Solvent Extraction**

ASE (also known as pressurized fluid extraction or pressurized liquid extraction) was first introduced into analytical practice in 1995 [69], and is therefore, a relatively new extraction technique. It has mainly been used to remove organic compounds from environmental samples [70]. Some advantages of ASE over conventional extraction methods are that extraction times are significantly reduced (5 to 15 min), and it allows for the use of a wide range of solvents, even those that may not be effective in traditional methods. Furthermore, the high operating pressures permits the extraction of thermo-labile analytes even at high temperatures of the process [69].
Despite the advantages of ASE, it has not been widely used as an extraction technique due to several key limitations including low analyte selectivity, large solvent volume requirements (during the rinsing step) and high equipment costs. At elevated temperature and pressure, extraction occurs much faster but selectivity is significantly reduced as other undesirable components become more soluble as well. As a consequence, an additional clean-up step is often required after extraction.

### 2.3.4 Ultrasound-Assisted Extraction

**UAE Theory**

Ultrasound is high frequency sound (>20 kHz) that is greater than the upper limit of human detection. There are two levels of ultrasound used in the food processing industry: low-intensity (< 1 W/cm²) and high-intensity ultrasound (10-1000 W/cm²). Ultrasound-assisted extraction (UAE) involves passing ultrasonic energy in the form of waves through a liquid solvent containing the solid particles. As the waves hit the surface of the material, a force that is either perpendicular or parallel to the surface is generated. If the force is perpendicular to the surface, a compressive wave is formed whereas if the force is parallel to the surface, a shear wave is produced. This constant production of compressive and shearing forces gives rise to a phenomenon known as cavitation. Júnior et al [71] explains that bubbles grow during the rarefying phase of the sound wave and collapse during the compression phase. On collapse, sonic energy is converted into mechanical energy in the form of shock waves equivalent to several thousand atmospheres of pressure. Cavitation is defined as the whole process of bubble nucleation, growth and collapse [71]. It is believed that these rapid localized increases in pressure and temperature are responsible for the disruption of cellular membranes, thereby
facilitating the leaching of desired components out of the cell and the migration of solvent into the cell.

**Ultrasound-Assisted Extraction**

High-intensity ultrasound has been used for preservation, cleaning of equipment, emulsification, accelerated drying and freezing, and most recently, for extraction of various compounds from food materials. UAE is an attractive means of extraction as it is relatively cheap when compared with other methods (e.g., SFE) and has low instrumental requirements. Extraction is carried out using an ultrasonic probe system or an ultrasonic bath. Rostagno *et al.* [72] used UAE to extract isoflavones from soybeans. They found that maximum recoveries occurred when using 50% EtOH as the extraction solvent at 60°C for 20 min. Additionally, Wang *et al.* [73] was able to utilize UAE to extract phenolic compounds from wheat bran. They used a response surface design to determine the optimum extraction conditions based on total phenolic content. The optimized conditions were 64% EtOH, 60°C and 25 min. for extraction solvent, temperature and time, respectively. Similarly, Rodrigues and Pinto [74] employed response surface methodology to determine the optimum extraction parameters for the removal of phenolics from coconut shell powder using UAE. It was reported that extraction time was the most significant factor influencing extraction efficiency. Maximum recoveries of total phenolics (406 mg/L) occurred at an ultrasound extraction time of 60 min.

### 2.3.5 Microwave-Assisted Extraction

**Microwave Theory**

Microwaves are a form of non-ionizing electromagnetic energy. This electromagnetic energy can be absorbed, reflected or transmitted to a material in the form of waves at a frequency ranging from approximately 300 MHz to 300 GHz. Domestic microwave units typically operate
at a frequency of 2450 MHz while the Federal Communications Commission (FCC) has approved a frequency of 915 MHz for industrial applications. Microwave power that is transmitted through the bulk of a material can be absorbed and converted to heat. There are two proposed mechanisms of microwave heating: dipole rotation and ionic polarization.

The mechanism of dipole rotation is based on the fact that many molecules exist as electric dipoles, meaning that the molecule has a negatively charged end and a positively charged end. Water, for example, consists of two hydrogen atoms carrying a slightly positive charge and one oxygen atom having a slightly negative charge. When placed in an electromagnetic field, dipoles attempt to orient themselves according to the polarity of the field, which changes about $4.9 \times 10^9$ times per second [75]. The constant rotation of the molecules causes frictional heat. Ionic polarization occurs when ionic compounds such as salt move at an accelerated pace in response to an alternating electric field. The kinetic energy generated by the movement of the ions is converted to thermal energy. The mechanisms mentioned above clearly indicate that dielectric materials and solvents having a permanent dipole moment heat most efficiently in microwaves.

The extent to which a given material interacts with an applied electric field is largely dependent on that material’s dielectric properties. The dielectric properties of interest are the dielectric constant and the dielectric loss factor. The dielectric constant ($\varepsilon'$) describes the ability of a material to store electrical energy while the dielectric loss factor ($\varepsilon''$) determines the ability of the material to dissipate electrical energy. The relationship between $\varepsilon'$ and $\varepsilon''$ is given by:

$$\varepsilon' = \varepsilon'' \tan \delta$$
where $\varepsilon'$ = dielectric constant
$\varepsilon''$ = dielectric loss factor
$\tan \delta$ = loss tangent or dissipation factor

The dissipation factor ($\tan \delta$) is an indicator of how well a material can be penetrated by an electromagnetic field and how it dissipates that electrical energy in the form of heat. The heating power per unit volume of material is a function of the dielectric loss factor, microwave frequency and intensity of the electric field and can be calculated using the following equation:

$$ P = 5.56 \times 10^{-4} \times f \times \varepsilon'' \times E $$

where $P$ = absorbed microwave power (W/cm³)
$f$ = microwave frequency (GHz)
$\varepsilon''$ = dielectric loss factor of material
$E$ = electric field intensity for a given volume (volts/cm)

**Microwave-Assisted Extraction**

Interest in MAE has increased in recent years due to its inherent advantages (reduction in extraction time and solvent volume) over more traditional extraction techniques. There are two types of commercially available microwave extraction systems: closed- and open-vessel systems. Closed systems are usually referred to as microwave-assisted extraction and is the most commonly used of the two [76]. In MAE, the solvent and sample are contained in sealed extraction vessels under controlled temperature and pressure conditions. The closed vessels allow the temperature of the solvent to rise well above its boiling point which enhances extraction efficiency and shortens the extraction time. In open systems (also called focused microwave-assisted solvent extraction), the sample and solvent are placed in an open vessel and
only the part of the extraction vessel containing the sample is exposed to irradiation by the microwave [61].

Conventional solvent extraction methods rely on conductive and convective processes to heat the product, and therefore, require longer extraction times. Longer extraction times increases the risk of degradation of thermolabile constituents. In contrast, MAE uses microwave energy to directly heat the molecules within the material (as previously discussed) often in a matter of seconds. This direct heating allows for a significant reduction in extraction time when compared to traditional extraction techniques. Zohar et al. [77] compared the efficiency of Soxhlet extraction to MAE in the removal of saponins from chickpea, and they found that profiles from the extract obtained at 20 min with the MAE process was similar to those observed after three hours of Soxhlet extraction. Similarly, Martino et al. [78] extracted coumarin from Melilotus officinalis using MAE, Soxhlet extraction and UAE. It was found that UAE gave satisfactory results with an optimum extraction time of 60 min, but MAE proved to be the most efficient method as it produced the highest yields in just 10 min. Soxhlet, on the other hand, was found to be inadequate as extraction with this method lead to lower recoveries and poor reproducibility.

Solvent selection and solvent volume are two key factors that must be given great consideration when performing MAE. Solvents commonly used with MAE include methanol (MeOH), ethanol (EtOH), acetone, hexane, water and composites of these. The high temperatures that can be achieved with microwave heating increases the solvating power of most solvents by decreasing surface tension and solvent viscosity, which improves sample wetting and matrix penetration [79]. The efficiency with which a solvent is heated by means of microwaves depends a great deal on its dissipation factor. EtOH and MeOH, for example, will undergo less
microwave absorption than water due to their lower ε’ values, but the overall heating efficiency for both solvents will remain higher than water due to an increased tan δ value [61]. It is common practice to use a mixture of organic solvent and water at varying ratios to improve recovery of phytoconstituents with MAE. Predicting the correct solvent:water ratio can be difficult as some research has shown that increasing the concentration of organic solvent can actually decrease extraction yield. In a study conducted by Li et al. [80], it was found that the recovery of phenolic acids from *E. ulmodies* increased up to 20% MeOH in water but decreased when higher concentrations of MeOH were used. Similar results were reported by Lee et al. [81] in which it was shown that extraction yield of decursin decreased as the ethanol concentration increased above the maximum value.

The unique method of microwave heating usually allows for a reduction in the volume of solvent needed for extraction. In general, recovery increases with increasing solvent volume in conventional extraction methods. However, this isn’t necessarily the case with MAE in which higher solvent volumes may give rise to lower recoveries possibly due to inadequate stirring of the solvent by microwaves [61]. Li et al. [76] employed a focused microwave-assisted extraction technique to extract geniposidic and chlorogenic acids from *E. ulmodies*. It was shown that as solvent volume increased from 5 to 20 ml g⁻¹ sample, the extraction efficiency of chlorogenic acid decreased.


<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric Constant(\varepsilon'(\mathrm{e''}))</th>
<th>Dielectric loss ((\tan\delta) × (10^{-4}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>20.7</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.3</td>
<td>2500</td>
</tr>
<tr>
<td>Hexane</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>32.6</td>
<td>6400</td>
</tr>
<tr>
<td>2-propanol</td>
<td>19.9</td>
<td>6700</td>
</tr>
<tr>
<td>Water</td>
<td>78.3</td>
<td>1570</td>
</tr>
</tbody>
</table>

*From Mandal et al. [61]

*aDetermined at 20°C

The majority of the work [75,82-84] on MAE has focused on the extraction of organic compounds from soil and essential oils from plants. However, the potential of MAE for the extraction of phytochemicals from plant materials [61,76,77,80,81,85] has sparked greater interest in this area in recent years. The pharmaceutical industry, for example, is a very attractive area to apply MAE for sample preparation [76]. Eskilsson et al. [86] successfully utilized MAE to determine the presence of felodipine and its degradation products in drug tablets. Li et al. [80] studied the effects of focused MAE on polyphenolic acids (gallic acid, protocatechuic acid, chlorogenic acid and caffeic acid) from *E. ulmodies*, a plant widely used in Chinese medicine as a result of its antibacterial, antimutagenic and antioxidant properties. The authors investigated several parameters including microwave power, irradiation time, solvent volume and sample particle size. The best extraction conditions were found to be 50% microwave power, 30 s irradiation time, and a solvent:sample ratio of 10 ml g\(^{-1}\). It was also observed that MeOH produced higher recoveries of phenolic acids than pure water for all measured analytes, which was attributed to the higher value of \(\tan\delta\) for methanol than for water.

### 2.3.6 Extraction of Antioxidants from Peanut Skins

Despite the proven antioxidant properties of peanut skins, they remain an underutilized natural resource mainly due to limited knowledge of efficient extraction procedures for the
removal of these health-promoting compounds. Some authors [37,44,87,88] have used traditional solid-liquid extraction with different organic solvents to extract antioxidant compounds from peanut skins. Nepote et al. [87] investigated the effects of several parameters including ethanol concentration in water, particle size and number of extraction stages on the extraction of phenolics from peanut skins. In that study, optimum extraction conditions (70% v/v ethanol in water, non-crushed peanut skins, shaking for 10 min and three extraction stages) were based on the quantity of total phenolic compounds extracted and no identification procedures were performed to determine exactly what compounds were being extracted. The maximum yield of total phenols observed under the optimum conditions was 0.118 g g⁻¹.

Yu et al. [44] studied the effect of three skin removal methods (direct peeling, blanching and roasting) and extraction solvent on total phenolics and antioxidant activity of peanut skin extracts. The authors reported a total phenolics content of 90-125 mg/g of dry skin, with roasted peanut skins having the highest levels of total phenolics. Additionally, Wang et al. [41] extracted defatted peanut skins with 50% (v/v) aqueous EtOH at room temperature. The total phenolics content of the skins was 90 mg g⁻¹ of extract, and the extracts exhibited strong radical scavenging (DPPH, hydroxyl radical and superoxide anion) and metal chelating abilities compared to BHA and EDTA, respectively. The estimated EC₅₀ (antioxidant concentration required to scavenge 50% of the radicals in the reaction mixture) value of peanut skin extracts in the DPPH assay was 30.8 μg/ml compared to 40 μg/ml for BHA.

O’Keefe and Wang [89] extracted raw peanut skins with aqueous MeOH and determined the effects of the peanut skin extracts on oxidation of beef products. It was reported that the extraction rate of phenolic compounds from peanut skins was approximately 3.8% (38 mg of gallic acid equivalents per gram of skins). In addition, it was found that 200-400 ppm extract
appeared to be optimal for the reduction of lipid oxidation in the beef products upon 14 days storage. The addition of peanut skins at 200 ppm had a similar inhibitory effect on lipid oxidation in honey roasted peanuts [90].

2.4 Measuring Antioxidant Activity in Food

There are a number of chemical assays that have been used to measure the antioxidant activity (AOA) of wide variety of compounds. According to Huang et al. [91], these assays can be roughly divided into two main categories depending on the type of reaction that is involved: assays based on hydrogen atom transfer (HAT) and assay based on electron transfer (ET). The majority of HAT-based assays apply a competitive reaction scheme in which the antioxidant and substrate compete for thermally generated peroxyl radicals [91]. HAT-based assays include oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP). ET-based assays measure the capacity of an antioxidant to reduce an oxidant. The oxidant changes color when reduced and the degree of color change is correlated to the antioxidant concentration present in the sample. ET-based assays include the Folin-Ciocalteu total phenols assay, Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and DPPH.

The diversity of the testing systems, methods and conditions used for oxidation is a major factor in the difficulty of interpreting the literature regarding antioxidant capacity of natural antioxidants derived from plant extracts [12]. The complexity of the topic of antioxidants coupled with the improper use of questionable methods has lead to a state of disarray in the antioxidant research community and industry [91]. Although these assays have been used quite extensively by many researchers to evaluate AOA, their usefulness in predicting AOA in food and other biological systems is not clear.
The activity of antioxidants in food systems is not only dependent upon the chemical reactivity (e.g., free radical scavenging and chelation) of the antioxidant but also on factors such as physical location, interaction with other food components and environmental conditions [13]. The results from these chemical assays are valid only for the specified reaction conditions used in the assay, and those conditions are usually not accurate representations of real food systems. For example, the activity of antioxidants in food systems is largely dependent upon their partitioning behavior in lipids and water [13]. Many of the assays are performed in the absence of lipids, which means that the impact of antioxidant partitioning is not evaluated, thereby possibly leading poor correlation between the results of in vitro assays and antioxidant performance in foods [13].

Despite the drawbacks associated with the current methods of measuring AOA, there are several methods that have been viewed as industry standards when it comes to assessing oxidative deterioration in food systems. These methods of assessment include thiobarbituric acid reactive substances (TBARS) assay, peroxide value (PV), p-anisidine value (pAV), active oxygen method, Rancimat tests and sensory analysis. Although TBARS and PV are the most frequently used, there are some limitations to both tests. Most recently, the use of the ORAC assay to assess oxidation in food has increased in popularity. However, sensory analysis remains the most reliable method as the task of assessing the acceptability and preference of products is best carried out by consumers [92].

### 2.4.1 Thiobarbituric acid reactive substances assay

TBARS is the method of choice for the assessment of lipid oxidation in food and biological systems. In this assay, a thiobarbituric acid reagent is mixed with a lipid solution and the reaction of secondary products, mainly malondialdehyde (MDA) with thiobarbituric acid
results in a pinkish-colored chromophore with an absorbance maximum at 530-532 nm. The intensity of the pink color is directly proportional to the concentration of TBA-reactive species present. The TBARS assay has received some criticism because a large body of evidence suggests that food components other than MDA can react with the thiobarbituric acid reagent to generate the same chromophore [7]. Furthermore, the lack of reproducibility of the data has caused some concern over use of this method.

2.4.2 Peroxide value

Peroxides and hydroperoxides are the primary products of lipid oxidation. The PV test is used to measure the concentration of these primary oxidation products in a given sample. PV is based on the ability of peroxides to liberate iodine from potassium iodide in a biphasic system with a thiosulfate solution as shown in the reactions below [93]:

\[
ROOH + 2H^+ + 2I^- \rightarrow I_2 + ROH + H_2O
\]

\[
I_2 + 2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2I^-
\]

The more peroxides present in the lipid sample, the more iodine that will be liberated. The extent of oxidation is determined by the amount of iodine released. This approach, however, is restricted by the chemical instability of peroxide and hydroperoxide compounds. After their concentration reaches a maximum level, it decays as a function of temperature, the presence of other food components, etc. [7]. Another potential drawback of this method are absorption of iodine at unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be titrated [93].
2.4.3 p-Anisidine value

As the free-radical chain reaction in lipid oxidation continues, hydroperoxides are further broken down into secondary oxidation products including aldehydes, ketones and alcohols, which are responsible for the objectionable flavors and odors associated with rancid foods. The p-Anisidine value (p-AV) is a measure of the extent of lipid oxidation based on the presence of aldehydes, mainly 2-alkenals and 2,4-dienals. In this method, lipids are mixed with an acetic acid solution and aldehydes present in the mixture react with a p-anisidine reagent, thereby forming a yellow pigment. The absorbance is measured spectrophotometrically at a wavelength of 350 nm.

2.4.4 Oxygen radical absorbance capacity assay

ORAC has most commonly been used to study antioxidant capacity in human and animal systems, but its use in the food industry has been increasing in recent years. ORAC has been so well received that some nutraceutical manufacturers are beginning to include ORAC values on product labels [94]. The ORAC assay measures the ability of antioxidant compounds to compete with a fluorescent probe (fluorescein) for a thermally generated peroxyl radical. An azo radical initiator, 2,2´-azobis-(2-amidinopropane) hydrochloride (AAPH), is used to initiate the reaction. As the reaction of fluorescein with the peroxyl radical progresses, fluorescein is consumed and subsequently, the fluorescence intensity decreases over time. The presence of antioxidants delays the decay in fluorescence [91]. The antioxidant capacity of the sample is quantitated using the area under the fluorescence decay curve (AUC) (Fig. 2.7). A standard curve of Trolox (water-soluble analogue of vitamin E) concentration versus AUC is generated and used for comparison of antioxidant samples. Hence, ORAC values are typically expressed as micromoles of Trolox equivalents per liter or gram of sample.
An advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phase [91]. This method of analysis employed by ORAC is particularly useful for food samples, which often contain multiple ingredients and have complex reaction kinetics [91]. Another advantage is that the method is readily automated and the extended reaction period ($\geq 30$ min) avoids underestimation of AOA by accounting for the effects of secondary antioxidant products [94]. Although once limited to the measurement of the antioxidant capacity of hydrophilic antioxidants against only peroxyl radicals, it can now be used with lipophilic antioxidants as well.

Fig. 2.7 Antioxidant activity of a tested sample expressed as net area under the curve. From Prior et al. [94]

2.5 Polyphenolic Compounds and Human Health

Polyphenolic compounds are not only capable of preventing lipid oxidation in food matrices but evidence has shown the these compounds also play a key role in reducing oxidative stress in humans. Oxidative stress can be defined as a disturbance in the prooxidant-antioxidant
balance in favor of the former, leading to potential damage [95]. According to Frei [95], there are four endogenous sources that account for most of the reactive oxygen species (ROS) generated by cells:

1. Normal aerobic respiration; mitochondria consumes molecular oxygen; leakage of electrons from the electron transport chain cause a one electron reduction of oxygen, forming the superoxide radical (O$_2$•-).

2. Phagocytic cells destroy bacteria or virus infected cells with an oxidative burst of nitric oxide (NO•), O$_2$•-, H$_2$O$_2$ and OCl$^-$. 

3. Peroxisomes, which are organelles responsible for degrading fatty acids produce H$_2$O$_2$ as a byproduct.

4. Cytochrome P-450 enzymes; the induction of these enzymes prevent acute toxic effects from foreign chemicals, but also result in oxidant byproducts that damage DNA.

Oxidative stress is thought to be a precursor to the development of a number of chronic diseases including cancer, coronary heart disease and Alzheimer’s and Parkinson’s diseases. Research [18,96,97] has suggested that flavonoids may be influential in the prevention of these diseases. It has been found that flavonoids act as scavengers of ROS, thus preventing oxidative damage to macromolecules such as DNA, proteins and lipids. In a review conducted by Butterfield et al. [96], it was suggested that the consumption of polyphenolic compounds may provide an approach to slow the onset and progression of Alzheimer’s disease. There are several flavonoids (e.g., resveratrol, quercetin, anthocyanins and catechins) that have been widely studied for their potential in preventing various diseases.
**Resveratrol**

Resveratrol (3,5, 4' trihydroxystilbene) is a stilbene phytoalexin that is produced by plants in response to a microbial challenge or some other exogenous stimuli [38]. The major dietary sources include grapes, wine soy, peanuts and peanut products [98]. Fresh grape skins contain about 50-100 μg resveratrol/g and the concentration in red wine is in the range of 1.5 to 3 mg/L [99]. Resveratrol can be found in at least 72 plant species [99] and is reported to have a wide range of beneficial health effects including blocking platelet aggregation [100], vasodilatation [101,102] and prevention of cancer [99]. It is believed that resveratrol is the compound primarily responsible for the ‘French Paradox’, which refers to the fact that the French experience lower incidences of cardiovascular disease despite consuming a diet high in saturated fats due to their frequent consumption of red wine.

**Quercetin**

Quercetin is the most commonly consumed flavonol in the human diet. It is a potent antioxidant capable of chelating metal ions to prevent the Fenton reaction and is also effective at scavenging hydroxyl radicals [103]. Significant dietary sources of quercetin include onions, apples, tea and red wine [103]. Numerous in vitro studies have revealed diverse biological effects of quercetin, including apoptosis induction, antimutagenesis, protein kinase C inhibition, superoxide dismutase-like activity, modulation of cell cycle, angiogenesis inhibition and inhibition of angiotensin converting enzyme II [104]. Epidemiological data have demonstrated an association between a diet that is rich in quercetin and a significant reduction in the risk of mortality from coronary heart disease and a reduced risk of stroke.
Anthocyanins

Anthocyanins are a class of flavonoids present in colorful flowers and fruits [18]. Anthocyanins are among the most abundant components of red wine due to their presence in the skin of grapes. In a study conducted by Tedesco et al. [17], it was found that anthocyanins lower ROS and methemogloblin production in human erythrocytes treated with H$_2$O$_2$. Youdim et al. [19] examined both the oxidative and anti-inflammatory properties of anthocyanins and hydroxycinnamic acids (HCA) present in blueberries. It was reported that both anthocyanins and HCA protect vascular endothelial cells against ROS and inflammatory insults, with anthocyanins proving to be more protective than HCA. Furthermore, it was found that anthocyanins may protect against age-related neurological dysfunctions.

Catechin and its derivatives

Catechins are the most abundant polyphenols found in green tea and are believed to be a significant contributor to the many health benefits associated with the consumption of tea. Catechins belong to the sub-class of flavonoids known as flavanols. There are several catechin derivatives including epigallocatechin (EGC), epicatechin (EC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG), with EGCG being the major component of green tea. The catechol structure, which possesses two hydroxyl groups at neighboring positions, is remarkably superior to other dispositions in electron donating ability. Therefore, flavonoids containing catechol structure can exert powerful radical scavenging activities [104]. In fact, catechins have been found to be more efficient radical scavengers than vitamins E and C [18]. Weinreb et al. [18] published a review of the effects of green tea polyphenols on Alzheimer’s and Parkinson’s diseases which reported that recent epidemiological studies have shown reduced risk of Parkinson’s disease (PD) associated with consumption of two or more cups of tea daily. It was
concluded that *in vivo* studies are needed to clarify whether EGCG and its metabolites, at sufficient concentrations, can reach the brain and alter signaling pathways.

### 2.5.1 Protective effect of polyphenols in cell model systems

It is known that flavonoids affect basic cell functions such as growth, differentiation and apoptosis [97] but the levels at which they become beneficial in terms of disease prevention and overall health has not been elucidated. Polyphenolic compounds can act as either antioxidants or pro-oxidants depending on the concentration present in the cell. Research has suggested that when acting as antioxidants, polyphenolic compounds may prevent anti-inflammatory insults, CVD, AD, diabetes and cancer. However, when acting as pro-oxidants, polyphenols can damage macromolecules such as DNA, proteins and lipids. Several authors [19,105,106] have used animal cell model systems to investigate the protective effect of various phenolic antioxidants against oxidative stress in a number of cell lines. One of the most often used cell models for these types of experiments are endothelial cells. Vascular endothelial cells, in particular, tend to be good candidates as they are constantly in contact with ROS generated by neutrophils and monocytes [19]. The physiological response of HMVEC to ROS is critical because an inadequate response can lead to the development of atherosclerosis and possibly neurodegenerative disorders [19].

Youdim *et al.* [19] studied the antioxidant and anti-inflammatory effects of blueberry and cranberry anthocyanins and hydroxycinnamic acids (HCA) against hydrogen peroxide ($H_2O_2$)-induced damage to human microvascular endothelial cells (HMVEC). It was found that following a 24 hr. incubation period with 100 $\mu$M $H_2O_2$, cells not supplemented with polyphenols exhibited a 58% decline in viability and that supplementation with anthocyanins and HCA provided significant protection against $H_2O_2$. In a similar study, Youdim *et al.* [106]
investigated the protective effects of elderberry extract against oxidative insult in bovine aortic endothelial cells. The authors reported that elderberry extract at concentrations of 0.05, 0.1 and 0.5 mg/ml provided significant protection against all H$_2$O$_2$ concentrations (75, 150, 300 μM).

Sasaki et al. [105] investigated the protective effects of nine flavonoids including kaempferol, luteolin, quercetin, rutin and 3-hydroxyflavone against linoleic acid hydroperoxide (LOOH). They pre-incubated (with flavonoid followed by LOOH) or co-incubated (simultaneous incubation of flavonoid and LOOH) rat pheochromocytoma PC12 cells and found that upon pre-incubation, only luteolin demonstrated protective effects similar to α-tocopherol. However, upon co-incubation, it was shown that kaempferol, quercetin, 3-hydroxyflavone and luteolin had much higher protective effects than α-tocopherol. Additionally, luteolin was found to be the most effective regardless of incubation conditions. Watjen et al. [97] used rat H4IIE cells to determine the protective effects of seven flavonoids including quercetin, myricetin, catechin and rutin against H$_2$O$_2$-induced DNA fragmentation. It was reported that pre-incubation with 10 μM quercetin decreased strand breaks by about 50% and that protective effects of quercetin could be detected at levels as low as 10-25 μM. The authors also noted that quercetin became cytotoxic at concentrations ranging from 50 to 250 μM.

**CONCLUSIONS**

Lipid oxidation remains a huge challenge for the food industry. It is imperative that we come to fully understand the mechanisms that give rise to these oxidative reactions and how to best control them. Industry’s reliance on synthetic antioxidants must be minimized as consumer demands for healthier products containing fewer synthetic additives are increasing. Polyphenols derived from agricultural byproducts such as peanut skins may be a viable alternative to synthetic antioxidants. However, efficient, cost-effective procedures for the extraction of these
natural antioxidant compounds need to be developed in order to increase commercial appeal. In this way, tremendous value can be added to what would otherwise be considered as waste material. More research is needed in the development of alternative extraction procedures that serve to reduce extraction time and solvent consumption while maximizing the recovery of phenolic compounds. This dissertation will focus on the optimization of two extraction techniques (solid-liquid extraction and microwave-assisted extraction), identification of phenolic antioxidant compounds, and assessment of antioxidant activity in two model systems (peanut butter and human cell line).
REFERENCES

CHAPTER 3

OPTIMIZING THE EXTRACTION OF PHENOLIC ANTIOXIDANTS FROM PEANUT SKINS USING RESPONSE SURFACE METHODOLOGY†

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ABSTRACT

Peanut skins are a byproduct of peanut blanching and roasting operations and contribute very little to the value of peanuts. They have been shown to contain significant quantities of polyphenols that act as natural antioxidants. The effect of four solvents: methanol (MeOH), ethanol (EtOH), water and ethyl acetate (EA), alcohol concentration (30, 60, 90%), extraction temperature (30, 45, 60 min) and extraction time (10, 20, 30 min) on total phenolic content (TPC), ORAC (oxygen radical absorbance capacity) activity and recovery of trans-resveratrol from peanut skins was investigated. Response surface methodology (RSM) was used to determine the optimum extraction conditions for each of the solvents. EtOH was the most efficient solvent for the extraction of phenolic compounds followed by MeOH, water and EA. The maximum predicted TPC of EtOH extracts under the optimized conditions (30.8%, 30.9°C, 12 min) was 118 mg GAE/g skins. MeOH extracts had the greatest ORAC activity followed by EtOH and water. The maximum predicted ORAC activity of MeOH extracts was 2149 μmol TE/g skins under the optimized conditions of 30% MeOH concentration, 52.9 °C and 30 min. EA was not effective at extracting phenolic compounds. Resveratrol was identified in MeOH extracts but could not be identified in samples extracted with EtOH, water or EA.

Key words: polyphenols, antioxidants, peanut skins, resveratrol, ORAC, extraction, response surface methodology
INTRODUCTION

Peanut skins are a low-value byproduct of the peanut processing industry. The world production of peanut skins can be estimated at over 750,000 tons annually [1]. Currently, this agricultural waste material is either incinerated or sold as animal feed for less than one cent per pound [2]. However, peanut skins contain significant levels of natural antioxidants, namely polyphenolic compounds that can be extracted and potentially utilized in a variety of food and pharmaceutical applications. Peanut skins have been used for centuries in traditional Chinese medicine to treat chronic haemorrhage and bronchitis [3]. Despite these beneficial properties, peanut skins remain an underutilized natural resource mainly due to limited information on efficient extraction procedures for the removal of these health-promoting compounds.

Phenolics derived from other natural sources such as grapes, wine, and tea have been widely studied by a number of researchers. Grape skins, for example, have been shown to contain high concentrations of resveratrol [4,5]. Resveratrol is a stilbene phytoalexin that is produced by plants in response to a microbial challenge or some other exogenous stimuli [6]. It is reported to have a wide range of beneficial effects including blocking platelet aggregation [7], vasodilatation [8,9] and prevention of cancer [10]. It is believed that resveratrol is the compound primarily responsible for the ‘French Paradox’, which refers to the French having lower incidences of cardiovascular disease despite consuming a diet high in saturated fats due to their frequent consumption of red wine. Sobolev and Cole [6] reported that commercial peanuts and peanut products contained resveratrol at levels ranging from 0.06 ppm in roasted peanuts to 5.1 ppm in boiled peanuts.

The early work of Karchesy and Hemingway [11] employed several techniques (NMR, TLC and HPLC) to separate and identify procyanidin compounds in peanut skin extracts.
Among the compounds identified were the flavan-3-ols catechin, epicatechin and epicatechin-
(4β→8;2β→O→7)-catechin. Lou et al. [12] reported the presence of six A-type proanthocyanidins from the water soluble fraction of peanut skin extracts. Of those six compounds, three were deemed new and were identified as epicatechin-(2β → O → 7, 4β → 6)-catechin, epicatechin-(2β → O → 7, 4β → 6)-ent-catechin and epicatechin-(2β → O → 7, 4β → 6)-ent-epicatechin. Yu et al. [13] observed three classes of compounds in peanut skin extracts: (1) phenolic acids including chlorogenic and caffeic acids; (2) flavonoids including epigallocatechin (EGC) and epicatechin and (3) stilbene (resveratrol). The presence of other flavonoids including quercetin and luteolin in peanuts and peanut byproducts has also been reported [14-16].

Although a number of compounds in peanut skins have been identified, many remain unknown. In addition, there have been few studies to determine the best extraction conditions for the removal of these antioxidant compounds from peanut skins. Nepote et al. [17] investigated the effects of several parameters including ethanol concentration in water, particle size and number of extraction stages on the extraction of phenolic compounds from peanut skins. In that study, optimum extraction conditions were solely based on the quantity of total phenolic compounds extracted as determined by Folin-Ciocalteu reagent, and no identification of phenolics were reported. The maximum yield of total phenols observed under the optimum conditions was 118 mg total phenols/g peanut skins. Yu et al. [13] studied the effect of three skin removal methods (direct peeling, blanching and roasting) and extraction solvent (water, ethanol and methanol) on total phenolics and antioxidant activity of peanut skin extracts. The authors reported a total phenolics content of 90-125 mg/g of dry skin, with roasted peanut skins extracted using 80% EtOH having the highest levels of total phenols.
The objectives of this study were to determine optimal conditions for the extraction of phenolic antioxidant compounds from peanut skins, to positively identify resveratrol using high performance liquid chromatography and to measure the antioxidant activity of peanut skin extracts using the Oxygen Radical Absorbance Capacity (ORAC) assay. Optimization parameters tested were solvent type, solvent concentration, temperature and time.

MATERIALS AND METHODS

Materials

Peanut skins obtained from blanching were a gift from Tidewater Blanching Company (Suffolk, VA). The skins were stored in a freezer at -4°C in sealed plastic bags until analysis. Food grade ethanol (95% Everclear, Luxco Distilling Co., St. Louis, MO) was obtained from a local distributor. Methanol and ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ). A trans-resveratrol standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (FL) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 2,2'-azobis(2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals U.S.A. (Richmond, VA).

Extraction Procedure

The phenolic compounds were extracted using 30, 60 and 90% (v/v) methanol (MeOH) and ethanol (EtOH) in water, 100% ethyl acetate (EA) or distilled water. 50 ml of solvent was added to 2.5 g of peanut skins (20 ml solvent/g skins) and placed in a reciprocal shaking water bath (Model 50, Precision Scientific, Chicago, IL) at a speed of 150 rpm. Samples were heated to temperatures of 30, 45 and 60°C for 10, 20 and 30 min. The crude extracts were allowed to cool to room temperature before centrifugation at 5000 rpm for 10-30 min. The supernatant was
collected, placed in 15 ml glass centrifuge tubes and flushed with nitrogen gas. Crude extracts were stored at -20°C until analysis. Each solvent extraction was run in triplicate.

**Total Phenolics Determination**

The total phenolic content (TPC) of the extracts was determined spectrophotometrically using the Folin-Ciocalteu total phenol procedure outlined by Spanos and Wrolstad [18] with slight modifications. Gallic acid standard solutions were prepared at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The extracts (0.1 ml) and the gallic acid standards (0.1 ml) were transferred to 15 ml test tubes. 3.0 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO) were added to each test tube and mixed using a vortex mixer. After one minute, 2.0 ml of 9.0% (w/v) Na₂CO₃ in water was added and mixed. The absorbance at 765 nm was read on a spectrophotometer (Spectronic 21, Bausch and Lomb, USA) after 2 h at room temperature. The concentration of total phenolic compounds in the extracts was determined by comparing the absorbance of the extract samples to that of the gallic acid standard solutions. All samples were run in duplicate. TPC was expressed as mg gallic acid equivalents (GAE) per g dry peanut skins.

**Oxygen Radical Absorbance Capacity (ORAC) Assay**

Antioxidant activity (AOA) was determined using an ORAC procedure outlined by Zhou et al. [19]. Trolox standard solutions were prepared at 20, 40, 80, 200 and 400 μM. The Trolox standards (40 μl), crude extracts (40μl) and blanks containing only extraction solvent (40 μl) were added to appropriate wells of a 96-well plate. 200 μl of a 100 nM FL solution was added to each well and the plates were covered and incubated for 20 min in a Victor³ multi-label plate reader (Perkin-Elmer, Turku, Finland) preheated to 37°C. A solution of 0.36 M AAPH (35 μl) was added to each of the wells to generate the peroxyl radicals. Fluorescence readings of the plate were taken every minute using an excitation wavelength of 485 nm and an emission
wavelength of 535 nm until all fluorescence readings declined to less than 5% of the initial values. Samples and standards were run in duplicate.

**HPLC Analysis**

Identification of the phenolic compounds was performed using an Agilent 1200 Series HPLC (Santa Clara, CA) system with a diode array detector. The column used was a Phenomenex Luna C\(_{18}\) (250 mm x 4.6 mm, 5\(\mu\)m particle size) with a guard column. The binary mobile phase consisted of solvent (A) 0.5% aqueous acetic acid and solvent (B) 0.5% acetic acid in methanol. The flow rate of the mobile phase was 0.8 ml/min. The elution gradient started with 100% A and 0% B. A decreased to 95.3% and B increased to 4.7% in 4 min. Over the next 38 min, solvent A decreased to 25.3% while solvent B increased to 74.7%. A was decreased to 5% at 54.5 min before increasing to 100% from 55 to 65 min. All ramps were linear.

Absorbance was measured by a UV-VIS diode array detector at a wavelength of 280 nm. Resveratrol in the PSE was identified by comparing the retention time and UV-spectra of the PSE samples to a \(t\)-resveratrol standard analyzed under the same chromatographic conditions.

**Statistical Analysis**

Response surface methodology (RSM) was used to determine the optimal conditions for extraction. RSM was performed using the Design Expert software (Version 7.1.3, Stat-Ease, Inc., Minneapolis, MN) program. A central composite design was used to investigate the effects of three independent variables (solvent concentration, extraction temperature and extraction time) on the dependent variables (TPC, ORAC activity and resveratrol concentration). The codes used in the response surface analysis and the corresponding parameter values are given in Table 3.1. A quadratic model was used to model the treatment effects and treatment interactions. The complete design using aqueous MeOH and EtOH consisted of 20 experimental points.
including six replications of the center point. The complete design using water and EA consisted of 13 experimental points with five replications of the center point. The coded values for the experimental design are given in Tables 3.2 and 3.3.

RESULTS AND DISCUSSION

Optimum extraction conditions based on TPC

*EtOH*

RSM was used to determine the optimum extraction conditions for each solvent. The independent variable, solvent concentration, and the treatment interaction of solvent concentration and temperature had significant (p< 0.05) effects on the TPC of samples extracted using EtOH. TPC of the extracts decreased by 37.3% when going from 30% EtOH concentration (118 mg/g) to 90% EtOH concentration (74 mg/g) (Fig. 3.1). The maximum predicted TPC of 118 mg GAE/g skins was obtained under the optimum extraction conditions of 30.8%, 30.9 °C and 12 min for EtOH concentration, temperature and time, respectively (Table 3.4).

The treatment interaction of solvent concentration and temperature had a significant (p<0.05) impact on TPC of the extracts (Fig. 3.2). At an EtOH concentration of 30%, the TPC at 30°C and 60°C was 119 mg/g and 117 mg/g, respectively (Fig. 3.3). In comparison, the TPC of the extracts at 30 °C and 60 °C using 90% EtOH was approximately 75 and 92 mg/g, respectively. The samples extracted at 30 °C and 60 °C using 90% EtOH were significantly different, but no significance was found among samples extracted using 30% EtOH (Fig. 3.3). An increase in temperature from 30 °C to 60 °C caused a 22.6% increase in TPC when extracting with 90% EtOH (Fig. 3.3). Although extraction time was not shown to be a significant factor in regards to TPC, it was observed that the TPC of the extracts initially increased with increasing
time until a maximum value, after which the TPC decreased with increasing time. Degradation of some of the thermolabile phenolic compounds may have occurred after the optimum extraction time was reached, thereby leading to a lower concentration of phenolic compounds. Romero-Pérez et al. [20] investigated the effects of solvent type, time and temperature on the extraction of resveratrol from grape skins. It was reported that the maximum recovery of resveratrol occurred using 80% EtOH in water, an extraction temperature of 60 °C and an extraction time of 30 min. At temperatures and times higher than the optimum parameters, degradation of resveratrol was observed.

MeOH

Temperature had a significant (p<0.05) effect on TPC of peanut skins extracted with aqueous MeOH. It can be seen from the response surface shown in Fig. 3.4 that TPC of the extracts increased with increasing temperature. This is in contrast to what was observed with EtOH, where there was an initial increase in TPC with temperature followed by a decrease (Fig. 3.3). The squared term of solvent concentration had a significant impact on the model. TPC of extracts using 30% MeOH as the extraction solvent increased to a maximum MeOH concentration of approximately 64% before declining. The optimum extraction conditions were found to be 63.8%, 57.3 °C and 21.6 min for MeOH concentration, temperature and time, respectively (Table 3.4). The maximum predicted TPC obtained under these optimized conditions was 112 mg/g.

Water

Extraction temperature and the treatment interaction of temperature and time had significant effects (p<0.05) on TPC of samples extracted with water. It can be seen from the response surface shown in Fig. 3.6 that TPC increased with increasing temperature. In the case
of the treatment interaction of temperature and time, TPC of the peanut skin extracts increased by 20.2% when going from 30 °C (63.1 mg/g) to 60 °C (79.1 mg/g) at an extraction time of 10 min (Fig. 3.5). A smaller increase of about 3.4% in TPC occurred at 30 min extraction when temperature increased from 30 °C to 60 °C. The optimum extraction temperature (50.4 °C) and time (10.1 min) yielded a predicted maximum TPC of 81 mg/g (Table 3.4).

EA

The independent variables, extraction temperature and time, had significant effects on TPC of samples extracted using 100% EA. The treatment interaction of temperature and time was not significant. TPC increased from 13.9 mg/g at 30 °C to 18.2 mg/g at 60 °C (Fig. 3.7). Extraction time had a similar effect on TPC as there was a 29% increase in TPC when going from 10 to 30 min (Fig. 3.8). A maximum TPC of 18.2 mg/g was determined under the optimum extraction conditions of 60 °C and 30 min for temperature and time, respectively (Table 3.4).

EA did not prove to be a very effective solvent when compared to MeOH, EtOH and water. In fact, the TPC of samples extracted with aqueous EtOH was nearly seven times higher than that of samples extracted using EA. These results are similar to those reported by Huang et al. [21] in which it was found that EtOH extracted the highest amount of phenolic compounds from peanut skins while EA was shown to be ineffective both in the extraction of phenolic compounds and overall antioxidant activity. EA is a moderately polar solvent, which limits its ability to solubilize polar phenolic compounds, thereby leading to reduced extraction yields.

In the current study, EtOH (30% v/v) was found to be the most efficient solvent for the extraction of phenolic compounds followed by MeOH, water and EA. Yu et al. [13] used 80% EtOH, 80% MeOH and water as solvents for the extraction of phenolics from peanut skins. They found that extracting the skins overnight with 80% EtOH at room temperature and a
solvent:mass ratio of 80 ml/g yielded the highest TPC recovery of 90-125 mg GAE/g of peanut skins (Table 3.5). In our work, it was shown that extraction of peanut skins using just 30% EtOH at 31 °C and a solvent:mass ratio of 20 ml/g for 12 min could be used to extract a maximum of 118 mg GAE/g peanut skins. Hence, we were able to extract comparable levels of phenolic compounds using less solvent and in a much shorter time than that reported by Yu et al. [13]. Nepote et al. [17] observed a maximum TPC of 118 mg/g under the optimized extraction conditions of 70% (v/v) EtOH in water, shaking for 10 min at room temperature, a solvent:mass ratio of 20 ml/g and three extraction stages. The optimization procedure employed in this study resulted in a similar recovery of phenolic compounds using just one extraction stage, thereby allowing for a reduction in solvent consumption and time required for extraction. Based on these results, it appears that increasing the extraction temperature just a few degrees can improve extraction efficiency while maintaining the antioxidant properties of the phenolic compounds.

MeOH was also shown to be an effective solvent although higher extraction temperatures were required when compared to EtOH. The optimum temperature for extraction with MeOH was about 57 °C compared to just 30 °C for EtOH, with corresponding maximum TPC recoveries of 118 and 112 mg/g, respectively (Table 3). Additionally, a higher MeOH concentration (approx. 64%) was required to reach TPC levels comparable to that of samples extracted with EtOH. When considering the cost associated with higher extraction temperatures in addition to potential toxicity issues related to the use of MeOH in food applications, EtOH may be preferred over MeOH. Both MeOH and EtOH proved to be more efficient at extracting phenolic compounds than pure water. Nepote et al. [22] also found water to be less effective in extracting phenols from peanut skins, with skins extracted using water having a TPC of 90.7
mg/g defatted skins compared to 165.6 and 150.4 mg/g for defatted skins extracted with MeOH and EtOH, respectively.

**Optimum extraction conditions based on ORAC activity**

ORAC was used to measure the peroxyl radical scavenging ability of peanut skin extract using Trolox (water-soluble vitamin E analogue) as an antioxidant standard. A major advantage of the ORAC assay in measuring AOA is that it provides a controllable source of peroxyl radicals that model the reaction of antioxidants with lipids in both food and physiological systems [23]. The ORAC values were expressed as micromoles of Trolox equivalents (TE) per gram of sample.

*EtOH*

The treatment effect of solvent concentration had a significant effect (p<0.05) on the ORAC activity of peanut skins extracted with aqueous EtOH. As the EtOH concentration increased from 30% (2049.7 μmol TE/g skins) to 90% (1001.5 μmol TE/g skins), ORAC activity decreased by about 51% (Fig. 3.9). According to these results, higher EtOH concentrations significantly reduce the extraction of those phenolic compounds having the ability to scavenge peroxyl radicals. It was previously stated that higher EtOH concentrations also decreased TPC. Therefore, it appears that increasing EtOH concentration has very little benefit in terms of increasing extraction efficiency and overall AOA of peanut skin extracts.

The optimum extraction conditions that provided maximum ORAC activity (2049.7 μmol TE/g) were determined as 30%, 60 °C and 16.4 min for EtOH concentration, extraction temperature and time, respectively (Table 3.4). Upon comparison of the optimum parameters for total phenolic recovery and ORAC activity, it can be seen that maximum TPC and ORAC activity both occurred at an EtOH concentration of about 30% (Table 3.4). The optimum
extraction time was also comparable. The major difference observed between optimum conditions based on TPC and ORAC activity was extraction temperature. The optimum temperatures were 30.9 and 60 °C for TPC and ORAC activity, respectively. It appears that higher temperatures were required for the extraction of those compounds contributing to the majority of the AOA of peanut skin extracts.

MeOH

The independent variables did not have a significant effect on the ORAC activity of peanut skins extracted with MeOH. However, it was seen that under the optimum extraction conditions (30% MeOH concentration, 52.9 °C and 30 min), the maximum value for ORAC activity was 2149 μmol TE/g, which was slightly higher than that seen under the optimum conditions determined for EtOH (2049.7 μmol TE/g) (Table 3.4). Despite EtOH extracts having a higher TPC, they did not exhibit higher ORAC activity than the MeOH extracts. Yu et al. [13] also found that peanut skins extracted with MeOH demonstrated greater total antioxidant activity than samples extracted with EtOH, despite the fact the EtOH extracts had higher yields of total phenols. O’Keefe and Wang [24] used MeOH to extract phenolic compounds from blanched peanut skins, and it was noted that extracts in which MeOH was used as the extraction solvent showed greater AOA in meat systems than those extracted with 95% EtOH. However, the authors did point out that no optimization of extraction conditions were performed in their study.

These results suggest that more of the active compounds responsible for the AOA of peanut skins may be extracted using MeOH as opposed to EtOH. Contradictory results were reported by Huang et al. [21] in which it was found that peanut skins extracted with EtOH exhibited the highest yield of total phenols and the strongest AOA of five tested solvents including MeOH. This discrepancy may be related to the fact that each study assessed the AOA
of the extracts in different systems. For example O’Keefe and Wang [24] used a meat model system while Huang et al. [21] assessed AOA in a linoleic acid system. It is well known that antioxidants behave differently depending on the type of system and the type of lipid substrate involved, thereby making it difficult to compare results.

**Water**

The independent variables did not have a significant ($p<0.05$) on the ORAC activity of peanut skins extracted using water. The optimum extraction conditions were 60 °C and 24.9 min for temperature and time, respectively. The maximum predicted ORAC activity under the given optimum conditions was 612 μmol TE/g, which represents a 69.2% and 71.5% reduction in ORAC activity when compared to the extracts from EtOH and MeOH, respectively. Extraction of phenolic antioxidants from peanut skins using water proved to be less efficient in terms of increasing total phenolic yield and ORAC activity compared to EtOH and MeOH. Other authors [13,25] have also reported water to be a less efficient solvent for the extraction of phenolic compounds from a number of plant materials including peanut skins.

**EA**

ORAC activity could not be determined for samples extracted with EA. The ORAC assay requires an aqueous environment for the reaction to occur. The extracts were not diluted with water as it was expected that even the undiluted extracts (100% EA) would have very little AOA due to the extremely low TPC.

To our knowledge, there have been no previous reports of ORAC activity of peanut skin extracts in the literature. ORAC values have, however, been reported for some common fruits, vegetables, nuts and spices [26,27]. ORAC values of cranberries and blueberries were determined as 93 and 92 μmol TE/g, respectively compared to 42.3 μmol TE/g for Red Delicious
apples [27]. In the study conducted by Wu et al. [27], it was found that spices such as ground cinnamon (2641 μmol TE/g), ground cloves (1533 μmol TE/g) and oregano leaves (1831 μmol TE/g) had relatively high ORAC activity when compared to fresh fruits and vegetables. However, it should be pointed out that these products along with the peanut skins used in this study have much lower water contents than fresh fruits and vegetables. Yilmaz et al. [25] investigated the antioxidant capacities of three different types of grapes seeds based on the ORAC assay. It was found that grapes seeds from Chardonnay exhibited the highest AOA with an ORAC value of 638 μmol TE/g. In our study, the maximum predicted ORAC activity was 2149 μmol TE/g, which is more than three times higher than the reported ORAC value of grape seeds from Chardonnay [25] and nearly 25 times that reported for blueberries [27]. PSE contain antioxidants that exhibit excellent peroxyl radical scavenging properties in vitro, thereby potentially making it a viable source of natural antioxidant compounds for food and pharmaceutical applications.

**Identification of resveratrol**

The HPLC chromatograms of PSE samples extracted with MeOH and EtOH are shown in Figs. 3.11 and 3.13, respectively. We were able to identify resveratrol in the MeOH extracts based on matching retention times and UV spectra of the samples to that of the standard (Fig. 3.12). Other studies [13,28] have also shown that resveratrol is present in peanut skin extracts. The quadratic model was moderately significant (p=0.0523) with regards to resveratrol concentration (as determined by the peak area) of samples extracted using MeOH. The squared terms of concentration, temperature and time had significant effects (p<0.05) on resveratrol concentration. As MeOH concentration increased from 30% (34.8 mA-U·s) to 60% (59.2 mA-U·s), the resveratrol peak area increased until reaching a maximum around 60% before
decreasing (Fig. 3.14). The same trend was seen with temperature, where the resveratrol concentration initially increased with increasing temperature to a maximum value before declining. The optimum extraction conditions were 59.8%, 44.7 °C and 10 min for MeOH concentration, temperature and time, respectively.

Resveratrol was unable to be identified in PSE samples extracted using aqueous EtOH. In some of the EtOH samples, there were peaks occurring at the same retention time as the resveratrol standard, but the UV spectra did not match. In a large number of the EtOH samples, there was no detectable peak at the retention time of the standard. These inconsistencies may be a result of the detection threshold of the HPLC, which was found to be 809 ng/ml. It is also worth noting that the data generated by the software showed that samples extracted with 90% EtOH had fewer detectable peaks than those samples extracted with 30% EtOH although this is not very noticeable when comparing the two chromatograms (Fig. 3.13). The number of peaks detected using 30% EtOH was 61 compared to just 45 peaks using 90% EtOH as the extraction solvent.

The fact that the levels of resveratrol in the EtOH samples may have been so low as to not be detected by the HPLC suggests that perhaps EtOH is not the most suitable solvent for the selective extraction of resveratrol. Given that we were able to identify peaks in MeOH extracts (Fig. 3.12) seems to indicate that MeOH may be the more effective solvent for extracting resveratrol. Furthermore, these findings may explain why it was shown that despite EtOH extracts having a higher overall TPC, MeOH extracts demonstrated slightly higher ORAC activity. It is possible that the higher resveratrol concentrations in the MeOH extracts lead to the increased ORAC activity.
We could not identify resveratrol in samples extracted with water or EA. As with EtOH extracts, samples extracted with water contained peaks at the same retention time as the standard, but the UV spectra were not the same. This suggests that water, too, is not the best solvent for the extraction of resveratrol. EA samples did not have any peaks at the same retention time as the standard and the chromatogram of EA extracts had noticeably fewer peaks than those of all other extracts. This is not surprising, given that EA was found to be ineffective for the extraction of phenolic compounds.

CONCLUSIONS

EtOH proved to be the best solvent for the extraction of phenolic compounds from peanut skins followed by MeOH, water and EA. Although EtOH extracts contained more phenols, samples extracted with MeOH demonstrated slightly higher ORAC activity. In addition, resveratrol was identified in MeOH extracts, but could not be positively identified in EtOH, water or EA extracts. The data suggest that MeOH may be the more suitable solvent for the extraction of phenolic compounds having the greatest antioxidant activity. In our study, we were able to extract similar levels of phenolic compounds from peanut skins as that previously reported in the literature while using less solvent and reducing extraction time. Although more identification work is needed to determine the exact composition of phenols in peanut skins, this work clearly shows that the extraction efficiency can be improved by altering several key extraction parameters. Perhaps this research will increase interest in utilizing peanut skins as an inexpensive source of natural antioxidants with the potential to be developed into various food additives and dietary supplements.
Table 3.1 The coded values and corresponding actual values of the optimization parameters used in the response surface analysis.

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Table 3.2 The experimental design for response surface analysis in terms of coded values.

**MeOH and EtOH**

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Table 3.3 The experimental design for response surface analysis in terms of coded values

*EA and Water*

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<td>0</td>
</tr>
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<td>6</td>
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</tr>
<tr>
<td>13</td>
<td>-1</td>
<td>-1</td>
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</table>
Table 3.4 Optimum conditions for the extraction of phenolic compounds from peanut skins based on total phenolic content (TPC) and ORAC activity

**TPC**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent Concentration (% v/v)</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>TPC (mg GAE/g skins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>30.8</td>
<td>30.9</td>
<td>12.2</td>
<td>118</td>
</tr>
<tr>
<td>MeOH</td>
<td>63.8</td>
<td>57.3</td>
<td>21.6</td>
<td>112</td>
</tr>
<tr>
<td>Water</td>
<td>100</td>
<td>50.4</td>
<td>10.1</td>
<td>81.0</td>
</tr>
<tr>
<td>EA</td>
<td>100</td>
<td>60.0</td>
<td>30.0</td>
<td>18.2</td>
</tr>
</tbody>
</table>

**ORAC Activity**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent Concentration (% v/v)</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>ORAC Activity (μmol TE/g skins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>30.0</td>
<td>60.0</td>
<td>16.4</td>
<td>2049.7</td>
</tr>
<tr>
<td>MeOH</td>
<td>30.0</td>
<td>52.9</td>
<td>30.0</td>
<td>2149.0</td>
</tr>
<tr>
<td>Water</td>
<td>---</td>
<td>60.0</td>
<td>24.9</td>
<td>612.0</td>
</tr>
</tbody>
</table>
Table 3.5 Comparison of optimized conditions for the extraction of phenolic compounds from peanut skins reported in the literature.

<table>
<thead>
<tr>
<th>Study</th>
<th>EtOH Concentration (% v/v)</th>
<th>Temperature (°C)</th>
<th>Solvent volume/skins (ml/g)</th>
<th>Time</th>
<th>TPC (mg GAE/g skins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>25</td>
<td>15</td>
<td>overnight</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>25</td>
<td>80</td>
<td>overnight</td>
<td>90-125</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>25</td>
<td>20</td>
<td>10 min</td>
<td>118</td>
</tr>
<tr>
<td>4</td>
<td>30.8</td>
<td>30.9</td>
<td>20</td>
<td>12 min</td>
<td>118</td>
</tr>
</tbody>
</table>

Study (1) Wang et al. [3], (2) Yu et al. [13], (3) Nepote et al. [17], (4) Present work
Fig. 3.1 Effect of EtOH concentration on TPC of peanut skin extracts.
Fig. 3.2 Response surface of the effect of EtOH concentration and temperature on TPC of peanut skin extracts.
Total phenols

- B- 30.000
- B+ 60.000

X1 = A: Concentration
X2 = B: Temperature

Actual Factor
C: Time = 12.23

Fig. 3.3 Effect of EtOH concentration and temperature on TPC of peanut skin extracts.
Fig. 3.4 Response surface of the effect of MeOH concentration and temperature on TPC of peanut skin extracts.
Fig. 3.5 Effect of the treatment interaction of temperature and time on TPC of peanut skin extracts using water as the extraction solvent.
Fig. 3.6 Response surface of the effect of temperature and time on TPC of peanut skin extracts using water as the extraction solvent.
Fig. 3.7 Effect of extraction temperature on TPC of peanut skin extracts using EA as the extraction solvent.
Fig. 3.8 Effect of extraction time on TPC of peanut skin extracts using EA as the extraction solvent.
Fig. 3.9 Effect of EtOH concentration on the ORAC activity of peanut skin extracts.
Fig. 3.10 Response surface of the effect of EtOH concentration and temperature on the ORAC activity of peanut skin extracts.
Fig. 3.11 HPLC chromatograph of PSE extracted using 30% MeOH at 60 °C and 30 min.
Figure 3.12 UV-VIS spectra of (a) resveratrol standard and (b) peak with matching retention time in PSE sample extracted with 30% MeOH at 60 °C and 30 min.
Fig. 3.13 HPLC chromatograms of (a) PSE sample extracted with 30% EtOH at 30 °C and 10 min and (b) PSE sample extracted with 90% EtOH at 30 °C and 10 min.
Fig. 3.14 Response surface of the effect of temperature and MeOH concentration on resveratrol content.
REFERENCES

CHAPTER 4

OPTIMIZING THE EXTRACTION OF PHENOLIC ANTIOXIDANTS FROM PEANUT SKINS USING MICROWAVE-ASSISTED EXTRACTION†

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ABSTRACT

Interest in microwave-assisted extraction (MAE) has increased in recent years due to its advantages (reduction in extraction time and solvent volume) over traditional extraction methods. The current study evaluated the efficacy of MAE for the extraction of polyphenolic antioxidants from peanut skins. A response surface method was used to optimize extraction parameters microwave power (10, 50, 90% nominal), irradiation time (30, 90, 150 s) and mass of peanut skins (1.5, 2.5, 3.5 g). The optimum extraction conditions were based on total phenolic content (TPC), ORAC (oxygen radical absorbance capacity) activity and resveratrol concentration of the peanut skin extracts (PSE). The appropriate mass of peanut skins was extracted with 37.5 ml of 30% aqueous ethanol (EtOH). The maximum predicted TPC under the optimized conditions (90% microwave power, 30 s irradiation time and 1.5 g skins) was 143.6 mg GAE/g skins. The optimum extraction conditions based on ORAC activity were 90% microwave power, 150 s irradiation time and 1.5 g skins, resulting in a predicted ORAC activity of 2789 μmol TE/g skins. LC-MS-MS analysis revealed that PSE contained resveratrol although none of the tested extraction parameters had a significant effect on resveratrol concentration. MAE was found to be a viable alternative to traditional extraction techniques for the extraction of phenolic compounds from peanut skins.

Keywords: polyphenols, antioxidants, microwave-assisted extraction, peanut skins, ORAC, total phenolic content, response surface methodology, resveratrol
INTRODUCTION

Polyphenols have received a great deal of attention in recent years for their ability to act as powerful antioxidants. Polyphenolic compounds are found in plant tissues and are important to plant growth and development as they provide a defense mechanism against infection and injury [1]. Polyphenols are present in high concentrations in a variety of fruits, vegetables and beverages such as tea and wine. Flavonoids make up the largest class of polyphenolic compounds and can be divided into several subclasses including flavanols, flavones, flavanones and flavonols. The existence, number, placement and degree of substitution of hydroxyl groups on the benzene ring confers much of the antioxidant activity of flavonoids [2].

The most popular antioxidants currently being used in the U.S. to prevent oxidative deterioration of many foods are synthetic in nature and include BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tert-butyl hydroquinone) and PG (propyl gallate). There have been increasing concerns over the potential adverse health effects of these synthetic compounds. Naturally derived plant phenols may be an attractive alternative to their synthetic counterparts. Polyphenolic compounds can be extracted from a wide array of plant sources using water and a number of organic solvents including ethanol and methanol. However, standard procedures for the extraction of these compounds from plant materials must be developed in order to increase commercial appeal.

Interest in microwave-assisted extraction (MAE) has increased significantly over the past few years as a result of its inherent advantages (reduction in extraction time and solvent volume) over more traditional extraction techniques (e.g., Soxhlet extraction). Conventional extraction methods have been associated with high solvent consumption, longer extraction times and increased risk of degradation of thermo-labile constituents. In MAE, the solvent and sample are
contained in sealed extraction vessels under controlled temperature and pressure conditions. The closed vessels allow the temperature of the solvent to rise well above its boiling point which enhances extraction efficiency and shortens extraction time. In addition, the direct heating that occurs with MAE typically allows for a reduction in the volume of solvent needed for extraction when compared to traditional extraction methods.

Although the majority of the work [3-6] on MAE has focused on the extraction of organic compounds from soil and essential oils from plants, its efficacy in the extraction of phenolic compounds from plant material is now being investigated [7-9]. Kerem et al. [7] compared the efficiency of soxhlet extraction to MAE in the removal of saponins from chickpea, and it was found that profiles from the extract obtained at 20 min with the MAE process was similar to those observed after three hours of soxhlet extraction. Li et al. [9] studied the effects of focused MAE on polyphenolic acids (gallic, chlorogenic and caffeic acids) from *E. ulmodies*, a plant widely used in Chinese medicine due to its antibacterial, antimutagenic and antioxidant properties. The best extraction conditions were found to be 50% microwave power, 30 s irradiation time, and a solvent volume to skin ratio of 10 ml g⁻¹.

Peanut skins are low value ($12-$20/ton) byproducts of peanut blanching operations [10]. Several authors [11-17] have reported that peanut skins, hulls and roots contain polyphenolic compounds with demonstrated antioxidant properties. Lou et al.[12] identified six A-type proanthocyanidins from the water soluble fraction of peanut skin extracts. Yu et al. [18] observed three classes of compounds in peanut skin extracts: phenolic acids, flavonoids and stilbene (resveratrol). Despite these beneficial properties, peanut skins remain an underutilized natural resource mainly due to limited knowledge of efficient extraction procedures for the removal of these compounds. Some authors [14,18-20] have employed traditional solid-liquid extraction
techniques using different organic solvents to extract antioxidants from peanut skins. Nepote et al. [19] investigated the effects of several parameters on the extraction of phenolic compounds from peanut skins via solid-liquid extraction. The maximum yield of total phenols observed under the optimum extraction conditions was 0.118 g g\(^{-1}\). Wang et al. [21] extracted phenolics from defatted peanut skins by maceration of the skins with 50% (v/v) aqueous EtOH at room temperature and reported a total phenolics content of 90 mg g\(^{-1}\) of extract. To our knowledge, there have been no studies published on the use of MAE to extract phenolic antioxidants from peanut skins.

The objectives of the current study were to investigate the effects of MAE on the extraction efficiency and recovery of polyphenolic antioxidant compounds from peanut skins and to optimize the extraction process. Optimization parameters included microwave power, irradiation time and mass of peanut skins.

MATERIALS AND METHODS

Materials

Peanut skins obtained from blanching operations were a gift from Tidewater Blanching Company (Suffolk, VA). The skins were stored in a freezer at -4°C in sealed plastic bags until analysis. Food grade ethanol (95% Everclear, Luxco Distilling Co., St. Louis, MO) was obtained from a local distributor. trans-Resveratrol, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein (FL) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 2-2′-azobis(2-amino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA).
**Extraction Procedure**

Polyphenolic compounds were extracted from peanut skins using a MES-1000 microwave extraction system (CEM, Matthews, NC). The system supplies 950 W of microwave energy at 100% power. The extraction variables evaluated were microwave power (10, 50, 90% of nominal), irradiation time (30, 90, 150 s) and sample mass (1.5, 2.5, 3.5 g peanut skins). The appropriate mass of peanut skins was placed in a 100 ml Teflon PFA (perfluoroalkoxy) lined extraction vessel. A preliminary study was conducted to determine the effect of solvent volume:skin ratio (5, 10, 15 ml solvent/g skins) on the recovery of phenolics from peanut skins. It was found that 15 ml/g (total volume of 37.5 ml solvent for 2.5 g of peanut skins) provided the maximum recovery of phenolic compounds (data not shown). As pressure differences (caused by differences in solvent volume) may have influenced extraction efficiency in the preliminary study, a constant solvent volume of 37.5 ml was chosen for the current study while varying sample mass. 37.5 ml of 30% aqueous EtOH was added to each extraction vessel. The crude peanut skin extracts (PSE) were allowed to cool to room temperature before centrifugation (Model Avanti J-25I, Beckman Coulter, Inc., Fullerton, CA) at 5000 rpm for 30 min. The supernatant was collected, placed in 15 ml glass centrifuge tubes and flushed with nitrogen gas. PSE were stored at -20°C until analysis. There were three replications of each treatment.

**Total Phenolics Determination**

The concentration of total phenolic compounds was determined spectrophotometrically using the Folin-Ciocalteu total phenol procedure outlined by Spanos and Wrolstad [22] with slight modifications. Gallic acid standard solutions were prepared at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The extracts (0.1 ml) and the gallic acid standards (0.1 ml) were transferred to 15 ml test tubes. 3.0 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, MO) were
added to each test tube and mixed using a vortex mixer. After one minute, 2.0 ml of 9.0% (w/v) Na₂CO₃ in water was added and mixed. The absorbance at 765 nm was read on a spectrophotometer (Spectronic 21, Bausch and Lomb, USA) after 2 h at room temperature. The concentration of total phenolic compounds in the extracts was determined by comparing the absorbance of the extract samples to that of the gallic acid standard solutions. All samples were run in duplicate. Total phenolic content (TPC) was expressed as mg total phenols per g dry peanut skins.

**Oxygen Radical Absorbance Capacity (ORAC) Assay**

Antioxidant activity (AOA) was determined using an ORAC procedure outlined by Zhou et al. [23]. Trolox standard solutions were prepared at 20, 40, 80, 200 and 400 μM. The Trolox standards (40 μl), PSE (40 μl) and blanks containing only extraction solvent (40 μl) were added to appropriate wells of a 96-well plate. 200 μl of a 100 nM FL solution was added to each well and the plates were covered and incubated for 20 min in a Victor³ multilabel plate reader (Perkin-Elmer, Turku, Finland) preheated to 37°C. A solution of 0.36 M AAPH (35 μl) was added to each of the wells to generate the peroxyl radicals. Fluorescence readings of the plate were taken every minute using an excitation wavelength of 485 nm and an emission wavelength of 535 nm until all fluorescence readings declined to less than 5% of the initial values. Samples and standards were run in duplicate.

**HPLC Analysis**

Preliminary identification of resveratrol was carried out using an Agilent 1200 Series HPLC (Santa Clara, CA) system. The column used was a Phenomenex Luna C₁₈ (250 mm x 4.6 mm, 5μm particle size) with a guard column. The binary mobile phase consisted of solvent (A) 0.5% aqueous acetic acid and solvent (B) 0.5% acetic acid in methanol. The flow rate of the
mobile phase was 0.8 ml/min. The elution gradient started with 100% A and 0% B. A decreased to 95.3% and B increased to 4.7% in 4 min. Over the next 38 min, solvent A decreased to 25.3% while solvent B increased to 74.7%. A was decreased to 5% at 54.5 min before increasing to 100% from 55 to 65 min. The absorbance was measured by a UV-VIS diode array detector at a wavelength of 280 nm. Resveratrol in the PSE was identified by comparing the retention time and UV-spectra of the PSE samples to a \( t \)-resveratrol standard analyzed under the same conditions. The resveratrol peak in the sample was collected twelve times to obtain a concentrated sample. After collection, the peak was dried under nitrogen gas and reconstituted in 100 \( \mu \)l of MeOH. LC-MS was used to identify resveratrol in the concentrated PSE sample.

**LC-MS-MS Identification**

Identification of resveratrol was performed using an Agilent 1050 quaternary HPLC pump and multiwavelength UV detector. The HPLC was interfaced with a MicroMass Platform spectrometer (Milford, MA) equipped with an APCI-ES ionization chamber. The HPLC column output was split 1:10 with one part going to the MS and nine parts going to the UV detector. The column used was an Agilent Eclipse C\(_{18}\) column (250 \( \times \) 4.6 mm, 5 \( \mu \)m particle size). The binary mobile phase consisted of solvents (A) 1% aqueous formic acid and (B) CH\(_3\)CN in 1% formic acid. The flow rate of the mobile phase was 0.2 ml/min. The elution gradient started with 100% of A and 0% of B. Mobile phases A and B remained at 0 and 100%, respectively for 25 min. Afterwards, A was reduced to 0% while B was increased to 100% from 25 to 35 min. Mobile phase A was then increased to 100% and B decreased to 0% until the end of the run time which was 36 min. All ramps were linear. Data were collected using both a UV detector at 280 nm and a MS. The MS analysis was conducted in negative ion mode. A scan time of 1.4 s and interscan delay of 0.05 s were used (m/z 200-700 and 500-1200 amu).
**Statistical Analysis**

Response surface methodology (RSM) was used to determine the optimal conditions for extraction. RSM was performed using the Design Expert software (Version 7.1.3, Stat-Ease, Inc., Minneapolis, MN) program. A central composite design was used to investigate the effects of three independent variables (microwave power, irradiation time and sample mass) on the dependent variables (TPC, ORAC activity and resveratrol concentration). The codes used in the response surface analysis and the corresponding parameter values are given in Table 4.1. A quadratic model was used to model the treatment effects and treatment interactions. The complete design consisted of 20 experimental points including six replications of the center point. The coded values for the experimental design are given in Table 4.2.

**RESULTS AND DISCUSSION**

**Optimum extraction conditions based on TPC**

A response surface design was used to optimize the extraction of phenolic compounds from peanut skins based on TPC. The treatment effects of microwave power and sample mass had a significant effect (p<0.05) on the recovery of TPC. As microwave power increased from 10 to 90% at an irradiation time of 30 s, there was a 53.9% increase in TPC (Fig. 4.1). An increase in the mass of the skins from 1.5 to 3.5 g caused a 35.8% reduction in TPC (Fig. 4.2). It was also seen that as irradiation time increased at 90% power, TPC of the PSE decreased independent of the mass of skins used (data not shown) although irradiation time was not a significant factor in terms of TPC. Li et al. [24] investigated the effect of focused MAE on the extraction of geniposidic and chlorogenic acids from *E. ulmodies*. The authors reported that at an irradiation time of 10 s, the extraction efficiency of geniposidic acid was dramatically higher at 90% microwave power versus 50% power. However, when 50% microwave power was used,
extraction efficiency increased with increasing irradiation time [24]. This data suggests that applying a high microwave power for a short time may be the most effective way to extract phenolic compounds from peanut skins and other plant materials using MAE. Extending the irradiation time with higher microwave power may be leading to thermal degradation of the phenols.

The treatment interaction of microwave power and sample mass was found to be significant (p<0.05) with regards to TPC of the extracts (Fig. 4.3). Operating under the extraction conditions of 10% microwave power and sample mass of 1.5 and 3.5 g, TPC of the extracts were 93.3 and 79.1 mg GAE/g skins, respectively. At 90% microwave power and a sample mass of 1.5 and 3.5 g, TPC was determined as 143.6 and 92.5 mg/g. There was a significant difference in sample means for skins extracted at 90% power. A 35.6% reduction in TPC was seen when going from a sample mass of 1.5 g to 3.5 g at 90% microwave power. Additionally, at a sample mass of 1.5 g, there was a 53.9% increase in TPC when increasing the microwave power from 10 to 90%. A similar effect was seen with a sample mass of 3.5 g in which it was found that TPC increased by about 16.9% when the microwave power increased from 10% to 90%. The optimum extraction conditions for the recovery of phenolic compounds from peanut skins using the MAE procedure were 90%, 30 s and 1.5 g for microwave power, irradiation time and mass of skins, respectively. The maximum predicted TPC under the optimized conditions was 143.6 mg/g.

**Comparison of TPC of PSE derived from MAE and SLE**

The maximum recovery of phenolic compounds from peanut skins using solid-liquid extraction (SLE) was 118 mg/g at the optimized conditions of 30.8%, 30.9 °C and 12.2 min for EtOH concentration, temperature and time, respectively [25]. In contrast, the predicted
maximum TPC under the optimized conditions using MAE was approximately 144 mg/g (Table 4.3). The MAE process yielded a 22% increase in TPC when compared to SLE. The higher TPC values found with MAE may be explained by a phenomenon that has been observed in plant cells after exposure to microwave heating. The dried plant material used for extraction contains minute traces of moisture and as microwave energy is absorbed and subsequently converted into heat, the moisture begins to evaporate. The vaporization of water generates pressure within the cell wall that eventually leads to cell rupture, thereby facilitating the leaching out of active constituents into the surrounding solvent and improving extraction yield [26].

Zhou and Liu [27] observed this phenomenon upon investigating the surface of tobacco leaves after MAE treatment using scanning electron microscopy (SEM). The authors found that the surface of the tobacco leaves was severely damaged owing to cellular disruption, which enhanced the extraction of solanesol from the leaves. Similarly, Kratchanova et al. [28] used SEM to observe the effects of microwave heating on fresh orange peels. It was determined that MAE treatment resulted in the destruction of parenchymal cells of fresh orange peels, which increased the extraction yield of pectin. Treatment of the peanut skins with MAE may have initiated cell rupture, which allowed more of the polyphenolic compounds from the skins to be extracted by the solvent. This process is quite different from SLE which relies on the diffusion of the solvent into the solid matrix and extraction of the components by solubilization [3]. Therefore, the yield of total phenolics using SLE was lower than that seen with MAE. It must also be noted that an extraction time of just 2.5 min (150 s) was predicted to give optimal recovery of phenolic compounds from peanut skins compared to 12.2 min for SLE. The MAE process is capable of extracting more phenolic compounds in less time than SLE.
The solvent volume:skin ratio required for optimal extraction also differed when comparing the two techniques. In SLE, the sample mass (2.5 g) and solvent volume to skin ratio (20 ml/g skins) remained constant for all extractions, resulting in a predicted maximum recovery of phenolics of 118 mg/g. The solvent volume to skin ratio used in the SLE procedure was reported to give the highest yield of total phenols in a previous study on the extraction of phenolic compounds from peanut skins by Nepote et al. [19]. In the MAE experiment, 1.5 g of skins in 37.5 ml solvent (25 ml solvent/g skins) was predicted to produce a maximum TPC of 144 mg/g. The MAE process required a higher solvent volume than SLE, which is contradictory to what we would expect given the fact that one of the major advantages of MAE is a reduction in solvent volume. Upon increasing the solvent volume to skin ratio in the SLE procedure from 20 ml/g to 30 and 40 ml/g using 30% EtOH as the extraction solvent, it was determined that the amount of total phenols extracted was 132.7 and 121.7 mg GAE/g skins, respectively. Therefore, a higher solvent volume in the SLE procedure increased the yield of extracted phenolic compounds, although the extraction yield was still not as high as the predicted maximum TPC using MAE.

**Optimum extraction conditions based on ORAC activity**

ORAC was used to measure the peroxyl radical scavenging ability of peanut skin extract using Trolox (water-soluble vitamin E analogue) as an antioxidant standard. The peroxyl radical is the most common free-radical in human biology although the hydroxyl radical, singlet oxygen, superoxide radical and reactive nitrogen species are also all present in biological systems [29]. The ORAC assay provides a controllable source of peroxyl radicals that model the reaction of antioxidants with lipids in both food and physiological systems [30]. The ORAC values were expressed as micromoles of Trolox equivalents (TE) per gram of sample.
The quadratic model was not found to be significant (p<0.05) in terms of ORAC activity of the extracts. The factors, microwave power and irradiation time, did not have a significant effect on ORAC activity at the levels tested in this study. However, the mass of the peanut skins was found to be significant with a predicted optimal value of 1.5 g. As the mass of the skins increased from 1.5 (2789 µmol TE/g) to 3.5 g (1655 µmol TE/g), the ORAC activity of the PSE decreased by about 40.6 % (Fig. 4.4). It would be expected that the ORAC activity of the extracts would increase with increasing amounts of peanut skins simply by virtue of there being more sample from which to extract active compounds. A likely explanation as to why this was not seen in the current study is that increasing the mass of the skins decreased the surface area available for the solvent to penetrate the matrix and solubilize the phenolics, causing the subsequent reduction in extraction yield of these compounds. We suspect that at a mass of 3.5 g, increasing the solvent volume would have yielded an increase in the amount of phenolic compounds extracted. The maximum predicted ORAC activity (2789 µmol TE/g) was found at a microwave power, irradiation time and sample mass of 90%, 150 s and 1.5 g, respectively.

Antioxidant capacities of peanut skin extracts based on the ORAC assay have not been previously reported in the literature. However, several authors have reported ORAC values for a variety of commonly consumed fruits, vegetables, nuts and spices [29,31,32]. In a study by Wu et al. [29], it was found that blueberries and cranberries have ORAC values of 92.1 and 92.6 µmol TE/g, respectively and that ORAC values for fruits were generally higher than those for vegetables (5-20 µmol TE/g). Spices were shown to have relatively high ORAC values with ground cinnamon having the highest value of 2640.8 µmol TE/g [29]. Yilmaz et al. [33] reported ORAC values of 638, 345 and 311 µmol TE/g for Chardonnay, Merlot and Muscadine grape seeds, respectively. In the current study, the maximum predicted ORAC activity of 2789
μmol TE/g peanut skins using MAE is more than four times higher than that reported for grape seeds from Chardonnay [33] and thirty times higher than that of cranberries [29] although cranberries and other fresh fruits have a much higher water content than peanut skins. Extracts from peanut skins appear to contain potent antioxidants that demonstrate excellent activity against the peroxyl radical in vitro. Although in vitro measures of AOA do not always correlate well with AOA in vivo, the high ORAC values for peanut skins suggest that may have some beneficial effects in vivo. In addition, peanut skins may be a suitable raw material for development into dietary supplements to rival grape seed extract which has enjoyed widespread market appeal.

When comparing the optimum conditions based on TPC to those obtained from ORAC activity, it was found that 90% microwave power and 1.5 g of skins were optimal for both TPC and ORAC activity (Table 4.3). Therefore, a microwave power of 90% is not only capable of extracting higher quantities of phenols but also extracts larger percentages of those compounds contributing to the AOA of the PSE. There was a difference in the irradiation time required for optimum TPC and ORAC activity. The optimal irradiation times were 30 and 150 s for TPC and ORAC activity, respectively (Table 4.3). Perhaps, the longer extraction times (and subsequent higher temperatures) required for maximum ORAC activity were needed to extract those compounds that are primarily responsible for the overall AOA of the PSE. Similar findings were observed in the SLE experiment in which EtOH concentration and extraction time were essentially the same in regards to providing optimum TPC and ORAC activity, but the extraction temperature for optimum TPC was 30.9 °C compared to 60 °C for ORAC activity [25].
Comparison of ORAC activity of PSE derived from MAE and SLE

The maximum predicted ORAC activity under the optimized conditions using the MAE method was 2789 μmol TE/g skins compared to 2149 μmol TE/g skins using the SLE method. This represents a 29.7% increase in the predicted ORAC activity of PSE from the MAE procedure. It was mentioned earlier that the predicted TPC of extracts obtained using MAE was also higher than that found with the SLE method although there were differences in the ratio of solvent volume to peanut skin mass used in the two procedures. MAE has the potential to extract greater quantities of phenolic antioxidants that exhibit higher AOA than what is seen in traditional extraction methods such as SLE. In addition, the optimal extraction time required for maximum ORAC activity using MAE was just 2.5 min (150 s) versus 30 min for SLE. The MAE method is capable of extracting nearly 30% more phenolic compounds from peanut skins in 1/12 of the time required for optimum ORAC activity using SLE. These findings are consistent with those reported in the literature, which have shown that applying MAE to any number of materials can significantly reduce extraction time compared to conventional extraction methods [7,34]. MAE has even been shown to outperform some of the other more novel extraction techniques such as ultrasound-assisted extraction in terms of decreasing extraction time [34].

Identification of phenolic compounds

The UV-VIS and mass spectra of the resveratrol standard and a PSE sample are shown in Figs. 4.5 and 4.6. Upon comparison of the spectra, it is clearly seen that the PSE sample contained t-resveratrol. As previously stated, the resveratrol peak had to be collected 12 times in order to obtain a concentration high enough for detection by the LC-MS. Given the fact that the levels of resveratrol in the samples extracted with EtOH were apparently very low, it is possible
that EtOH is not the best solvent for the extraction of resveratrol. Ballard et al. [25] speculated that aqueous MeOH may be a more suitable solvent than aqueous EtOH for the extraction of resveratrol from peanut skins. The authors were able to clearly match retention times and UV spectra of samples extracted with MeOH, but were unable to do so for PSE samples extracted using EtOH. Yu et al. [18] reported the presence of resveratrol in peanut skins in addition to flavonoids (epigallocatechin and epicatechin) and phenolic acids (chlorogenic and caffeic acids). In a similar study [20], it was found that peanut skins contain a number of procyanidin compounds including A- and B-type procyanidin dimers and trimers. Limited funding to purchase additional standards and LC-MS services prevented the positive identification of other compounds in this study. Further identification work is needed to obtain a more complete profile of the phenolic compounds present in peanut skins.

The quadratic model was not found to be significant (p<0.05) with regards to the concentration of resveratrol (as determined by the peak area) extracted from the skins. None of the tested factors had a significant effect on resveratrol concentration at the levels investigated in this study. The response surface based on resveratrol peak area is given in Fig. 4.7. The predicted maximum peak area for resveratrol was 107.6 mAU·s under the optimum extraction conditions of 54.6% microwave power, 30 s irradiation time and a sample mass of 3.1 g. Although the factors were not found to be significant, it was observed that at the optimum conditions, as microwave power increased to a maximum of 54.6%, resveratrol concentration increased. After reaching the optimum power, resveratrol concentration decreased with increasing power, suggesting that resveratrol may have been degrading at the higher power levels and subsequent higher temperatures.
CONCLUSIONS

MAE proved to be an attractive alternative to conventional extraction methods such as solid-liquid extraction for the removal of phenolic compounds from peanut skins. The MAE procedure was able to extract more phenolic compounds than the SLE procedure. Furthermore, PSE obtained using MAE exhibited superb ORAC activity (2789 μmol TE/g) especially when compared to the ORAC values reported for some common fruits and spices known to have high antioxidant activity. The extraction time required for optimal phenolic recovery and ORAC activity using MAE was nearly 25 and 12 times less, respectively, than that needed for the SLE procedure. PSE obtained from MAE were found to contain resveratrol although at relatively low levels. More work is needed in this area to obtain a more complete profile of the composition of PSE derived from MAE. In future studies, it is suggested that other solvents be investigated to further improve the extraction efficiency of resveratrol and other phenolic compounds from peanut skins.
Table 4.1 The coded values and corresponding actual values of the optimization parameters used in the response surface analysis.

<table>
<thead>
<tr>
<th>Code</th>
<th>Microwave Power (% nominal)</th>
<th>Irradiation Time (s)</th>
<th>Mass of skins (g)</th>
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Table 4.2 The experimental design for response surface analysis in terms of coded values.

<table>
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<th>Experiment No.</th>
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Table 4.3  Optimum conditions for the extraction\(^1\) of phenolic compounds from peanut skins using MAE based on total phenolic content (TPC) and ORAC activity

<table>
<thead>
<tr>
<th>Microwave Power(^2) (% nominal)</th>
<th>Irradiation Time (s)</th>
<th>Mass of skins (g)</th>
<th>TPC (mg GAE/g)</th>
<th>ORAC Activity (μmol TE/g)</th>
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<tr>
<td>90.0</td>
<td>30.0</td>
<td>1.5</td>
<td>143.6</td>
<td>2543</td>
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<tr>
<td>90.0</td>
<td>150</td>
<td>1.5</td>
<td>135.5</td>
<td>2789</td>
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</tbody>
</table>

\(^1\)Extraction performed using 30% aqueous EtOH as the solvent.
\(^2\)950 W microwave energy generated at 100% power.
Table 4.4 Comparison of the predicted maximum values for TPC and ORAC activity of PSE using solid liquid extraction (SLE) and microwave-assisted extraction (MAE) under optimized extraction conditions

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>TPC (mg GAE/g skins)</th>
<th>ORAC Activity (μmol TE/g skins)</th>
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</thead>
<tbody>
<tr>
<td>SLE(^1)</td>
<td>118</td>
<td>2149</td>
</tr>
<tr>
<td>MAE(^2)</td>
<td>143.6</td>
<td>2789</td>
</tr>
</tbody>
</table>

\(^1\)Optimum extraction conditions based on (TPC): 30.8% EtOH, 30.9 °C extraction temperature, 12.2 min extraction time and (ORAC): 30% MeOH, 52.9 °C extraction temperature, 30 min extraction time.

\(^2\)Optimum extraction conditions based on (TPC): 90% microwave power, 30 s irradiation time, 1.5 g skins and (ORAC): 90% microwave power, 150 s irradiation time, 1.5 g skins
Fig. 4.1 Response surface of the effect of microwave power and sample mass on TPC of PSE.
Fig. 4.2 The effect of sample mass on TPC of PSE.
Fig. 4.3 The effect of microwave power and sample mass on TPC of PSE.
Fig. 4.4 The effect of sample mass on ORAC activity of PSE.
Fig. 4.5 UV-VIS (a) and mass spectra (b) of resveratrol standard
Fig. 4.6 UV-VIS (a) and mass spectra (b) of peanut skin extract sample. Extraction conditions: 90% microwave power, 30 s irradiation time and peanut skin mass of 1.5 g.
Fig. 4.7 Response surface of the effect of microwave power and irradiation time on resveratrol content.
REFERENCES

CHAPTER 5

PROTECTIVE EFFECT OF PEANUT SKIN EXTRACTS AGAINST H$_2$O$_2$-INDUCED CYTOTOXICITY IN HUMAN BRAIN MICROVASCULAR ENDO THELIAL CELLS†

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ABSTRACT

Oxidative damage is thought to play a major role in the etiology of a number of chronic conditions such as cancer and cardiovascular disease. Peanut skins contain natural antioxidant compounds that may help to prevent the development of these diseases. The objective of the present study was to investigate the protective effects of peanut skin extracts (PSE) against hydrogen peroxide (H$_2$O$_2$)-induced cytotoxicity in human brain microvascular endothelial cells (HBMEC). HBMEC were pre-incubated with 0 (control), 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10% (v/v) PSE in experimental media for 2 h followed by treatment with 700 μM H$_2$O$_2$ for 2 h. The CellTiter-Glo assay was used to measure viability of HBMEC before and after H$_2$O$_2$ treatment, which was expressed in terms of luminescence. It was found that cells supplemented with PSE prior to treatment with H$_2$O$_2$ exhibited greater viability than cells not supplemented with PSE. Additionally, higher PSE concentrations (5 and 10%) may have had a small cytotoxic effect on HBMEC. This study showed that PSE may have some protective effects against oxidative damage in HBMEC, but further work is needed in this area.

**Keywords:** oxidative stress, peanut skins, human brain microvascular endothelial cells, hydrogen peroxide, viability, cytotoxicity
INTRODUCTION

Oxidative damage has been implicated in the pathology of a number of chronic conditions including atherosclerosis, cancer and neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases. Oxidative stress by definition is an imbalance between free-radical production and antioxidant defenses [1], in favor of the former, that leads to the damage of vital cellular components such as proteins, lipids and DNA. The human body has a natural antioxidant defense system consisting of endogenous antioxidant enzymes including glutathione peroxidase and superoxide dismutase that help protect against the excessive accumulation of free-radical species. However, the body relies heavily on exogenous antioxidants consumed in the diet to counteract reactive oxygen species (ROS). Vascular endothelial cells are especially susceptible to oxidative attack as they are in constant contact with ROS generated by neutrophils and monocytes [2]. Injury to endothelial cells caused by oxidative damage has been proposed as the initiating event in the etiology of atherosclerosis [3].

Flavonoids are a class of polyphenols that are found in a variety of fruits and vegetables and in beverages such as tea and wine. They are the most abundant polyphenols in the U.S. diet, and it is estimated that we consume about 1 g of these compounds per day [4]. Interest in polyphenols has increased considerably in recent years due to their broad range of antioxidant properties. Some of the more commonly known flavonoids include resveratrol, catechins, quercetin and anthocyanins. Resveratrol is a phytoalexin that has been reported to have a wide range of beneficial health effects including blocking platelet aggregation [5], vasodilatation [6,7] and prevention of cancer [8]. Catechins are thought to be responsible for much of the antioxidant activity of green tea, and recent epidemiological studies have shown that the
consumption of two or more cups of tea daily has been associated with reduced risk of Alzheimer’s and Parkinson’s diseases [9].

Many researchers [1-3,10] have evaluated the effects of flavonoids on oxidative stress in a number of cell lines including vascular endothelial cells. Youdim et al. [2] studied the antioxidant and anti-inflammatory effects of blueberry and cranberry anthocyanins and hydroxycinnamic acids (HCA) against hydrogen peroxide (H₂O₂)-induced damage to human microvascular endothelial cells. It was found that following a 24 hr. incubation period with 100 μM H₂O₂, cells not supplemented with polyphenols exhibited a 58% decline in viability and that supplementation with anthocyanins and HCA provided significant protection against H₂O₂. Watjen et al. [10] reported that pre-incubation of rat H4IIIE cells with 10 μM quercetin decreased H₂O₂-induced DNA strand breaks by about 50%.

Peanut skins are a low-value byproduct of peanut processing operations. Research [11-16] has shown that peanut skins are a rich source of polyphenolic antioxidant compounds including resveratrol, catechins and phenolic acids such as chlorogenic and caffeic acids. Peanut skins have been used for centuries in traditional Chinese medicine to treat chronic haemorrhage and bronchitis [17]. However, the effect of polyphenols in peanut skin extract on oxidative damage in human brain microvascular endothelial cells (HBMEC) has not been previously reported in the literature. The objective of this study was to investigate the possible protective effect of peanut skin extracts against H₂O₂-induced oxidative stress in HBMEC.
MATERIALS AND METHODS

Chemicals

RPMI 1640 with L-glutamine, fetal bovine serum (FBS), Hank’s Balanced Salt Solution (HBSS), trypsin-EDTA, sodium pyruvate, penicillin-streptomycin, nonessential amino acids and vitamins were all obtained from Mediatech, Inc. (Herndon, VA). NuSerum and endothelial cell growth supplement (ECGS) were purchased from BD Biosciences (Bedford, MA) and heparin was obtained from Sigma Aldrich Co. (St. Louis, MO).

Extraction of antioxidants from peanut skins

Peanut skins obtained from blanching operations were a gift from Tidewater Blanching Company (Suffolk, VA). Polyphenolic compounds were extracted from peanut skins using a MES-1000 microwave extraction system (CEM Corp., Matthews, NC) that supplies 950 W of microwave energy at 100% power according to the procedure outlined by Ballard et al. [11]. Peanut skins were extracted with 37.5 ml of 30% aqueous ethanol (95% Everclear, Luxco Distilling Co., St. Louis, MO) under the extraction conditions of 90% (of nominal) microwave power, 150 s irradiation time and a sample mass of 1.5 g. The crude peanut skin extracts (PSE) were allowed to cool to room temperature before centrifugation (Model Avanti J-25I, Beckman Coulter, Inc., Fullerton, CA) at 5000 rpm for 30 min. The supernatant was collected and placed in 10 ml glass centrifuge tubes. To reduce the likelihood of cell death as a result of the high ethanol (EtOH) concentration in the extract, PSE was diluted 1:30 (v/v) PSE to water to obtain a final EtOH concentration of 1% before treating the cells. PSE was flushed with nitrogen gas and stored at -20 °C until needed for the experiments.
Total Phenolics Determination

The concentration of total phenolic compounds in the extract was determined spectrophotometrically using the Folin-Ciocalteu total phenol procedure outlined by Spanos and Wrolstad [18] with slight modifications. Gallic acid standard solutions were prepared at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. PSE (0.1 ml) and the gallic acid standards (0.1 ml) were transferred to 15 ml test tubes. Distilled water (0.9 ml) and 3.0 ml of 0.2 N Folin-Ciocalteu reagent (F-9252, Sigma-Aldrich Co., St. Louis, MO) were added to each test tube and mixed using a vortex mixer. After one minute, 2.0 ml of 9.0% (w/v) Na₂CO₃ in water was added and mixed. The absorbance at 765 nm was read on a spectrophotometer (Spectronic 21, Bausch and Lomb, USA) after 2 h at room temperature. The total phenolic content (TPC) was determined by comparing the absorbance of the extract sample to that of the gallic acid standard solutions. The sample was run in triplicate. TPC was expressed as mg gallic acid equivalents (GAE) per g dry peanut skins.

Cell Culture

Human brain microvascular endothelial cells (HBMEC) were generously provided by the Laboratory of Vascular Biology at Virginia Tech. HBMEC were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The cells were cultured in complete RPMI 1640 media supplemented with 10% FBS, 10% NuSerum, 30 μg/ml ECGS, 15 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM sodium-pyruvate, nonessential amino acid and vitamins. The cells were subcultured every four days after reaching 90% confluence using calcium and magnesium-free HBSS and 0.05% trypsin-EDTA.

Cell Viability

HBMEC were seeded onto 96-well plates at a density of 5×10³ cells/well in complete media for 24 hr to allow cells to become adherent. The media was aspirated and cells were
washed with HBSS. HBMEC were incubated with FBS- and NuSerum-free experimental media containing 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 5.0 and 10% (v/v) PSE in experimental media for 2 h. Following incubation with PSE, experimental media was aspirated and cells were again washed with HBSS. Control cells were treated with experimental media containing 1% EtOH, which was found to have no effect on cell viability. HBMEC were then incubated with 700 μM H₂O₂ for 2 h. Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability assay (Promega Corp., Madison, WI). The CellTiter-Glo assay is based on the luciferase/luciferin reaction that, in the presence of Mg²⁺ and ATP, produces oxyluciferin and releases energy in the form of luminescence. Since the luciferase reaction requires ATP, the luminescence produced is proportional to the amount of ATP present which correlates with the number of viable cells [19]. Briefly, 100 μl of the CellTiter-Glo reagent was added to each well. The plate was placed on an orbital shaker for 2 min and allowed to incubate at room temperature for 10 min to stabilize the luminescence signal. Luminescence was then measured in terms of relative light units (RLU) using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). There were three replications of the experiment.

**Statistical Analysis**

Statistical analyses were performed using the proc mixed function of the SAS software program (SAS Version 9.0, SAS Institute Inc., 2004). A linear mixed model was used to determine the effect of PSE concentration on cell viability, with PSE concentration being treated as a categorical variable. Pairwise differences between PSE levels were examined. Post-hoc multiple comparisons were performed across all PSE levels. The Tukey-Kramer method was used to estimate differences among sample means at the 5% probability level (α=0.05). In
addition, a pre-planned comparison was made to investigate the difference between viability of cells that were not treated with PSE (controls) versus all levels of PSE.

RESULTS AND DISCUSSION

Pairwise comparisons were made in which the difference in luminescence of HBMEC before H$_2$O$_2$ treatment and luminescence after H$_2$O$_2$ treatment were analyzed and compared across all PSE concentrations. Therefore, when the difference in luminescence was large, there was greater cell death and viability was decreased. Conversely, when the difference in luminescence was small, less cell death occurred resulting in greater viability.

It can be seen in Fig. 5.1 that 700 $\mu$M H$_2$O$_2$ proved to be cytotoxic as the viability of the cells not supplemented with PSE prior to H$_2$O$_2$ treatment (C2) was reduced by about 55% when compared to cells not treated with PSE or H$_2$O$_2$ (C1). The results of the pre-planned comparison revealed that there was a significant difference (p $<$ 0.05) between cells not supplemented with PSE and HBMEC treated with all levels of PSE. It was found that cells supplemented with PSE prior to treatment with H$_2$O$_2$ exhibited greater viability than the untreated cells as evidenced by the smaller difference in luminescence (i.e., less cell death) between HBMEC treated with just PSE and those treated with PSE and H$_2$O$_2$ (Fig. 5.2). This data suggests that supplementation with PSE may have had a protective effect against H$_2$O$_2$-induced oxidative stress in HBMEC.

Previous research has also shown that phenolic antioxidants, such as those found in PSE, have the ability to protect cells against oxidative damage. Youdim et al. [20] investigated the protective effects of elderberry extract against oxidative insult in bovine aortic endothelial cells. The authors reported that elderberry extract at concentrations of 0.05, 0.1 and 0.5 mg/ml provided significant protection against all H$_2$O$_2$ concentrations (75, 150, 300 $\mu$M). Sasaki et al. [21] examined the effects of nine flavonoids including kaempferol, luteolin, quercetin and 3-
hydroxyflavone on oxidative stress induced by linoleic acid hydroperoxide (LOOH) in rat pheochromocytoma PC12 cells. It was shown that kaempferol, quercetin, 3-hydroxyflavone and luteolin demonstrated significant protective effects against LOOH.

Upon evaluation of the post-hoc multiple comparisons, it was found that significant differences ($p<0.05$) in luminescence existed among untreated cells and those supplemented with PSE. HBMEC supplemented with 0.1% PSE were significantly different from cells not supplemented with PSE. The difference in luminescence between cells treated with just 0.1% PSE and cells treated with 0.1% PSE and $H_2O_2$ was smaller than that which existed between the control cells, indicating that HBMEC supplemented with 0.1% PSE demonstrated greater viability than the control (Fig. 5.2). In addition, significant differences ($p<0.05$) were found between HBMEC supplemented with 0.1% PSE and those treated with 0.6 and 0.8% PSE. Cells treated with 0.1% PSE showed higher viability than cells treated with either 0.6 or 0.8% PSE. It would appear that supplementation with 0.1% PSE provided the best protection against oxidative damage. However, upon closer examination, it was seen that cells treated with only 0.1% PSE had lower viability when compared to C1. Therefore, supplementation with 0.1% PSE may have in fact, had a slightly cytotoxic effect on the cells. It should also be noted that the data were highly variable, which may have been a result of human error and the inherent variability associated with biological samples. As a result of these factors, it is not completely clear as to whether or not PSE is having a protective effect and if there is an optimal PSE concentration for maximum benefit. More research is needed to optimize treatment and cell culturing conditions to perhaps reduce some of the variability that was seen in the present study.

These inconclusive results were somewhat surprising given that other studies have clearly shown the positive effects of flavonoids against oxidative stress in endothelial cell model
systems. In order to possibly explain the seemingly marginal effect of PSE in the current study, we formulated and tested two hypotheses: (1) the 1% EtOH concentration was interfering with the antioxidant capacity of the phenols present in the PSE and (2) the concentration of phenols was too low to have any significant effect. The total phenolic content (TPC) of the PSE prior to dilution with water (1:30 v/v) was about 6 mg GAE/ml extract (Table 5.1). After the dilution, the total phenolic content was determined as 0.126 mg GAE/ml extract, representing nearly a 98% reduction in total phenols. As a result of the dilution, the cells received a much lower dosage of phenols than that reported in the literature. Kaneko and Baba [3] treated human umbilical vein endothelial cells (HUVEC) with flavonoids at concentrations ranging from 1 to 50 µM and reported that 50 µM luteolin had the greatest protective effect. Similarly, Ou et al. [1] supplemented HUVEC with 0.1, 1 and 5 µM resveratrol and found that 5 µM of resveratrol provided maximum benefit against oxidized LDL-induced cytotoxicity. Moreover, authors [3,21] have reported using EtOH at concentrations less than 0.5% to solubilize the flavonoids prior to treatment of the cells. In both studies, it was found that 0.5% EtOH did not have any beneficial or negative effects on the cells. In the current study, PSE were diluted to 1% EtOH which was not shown to have a cytotoxic effect on HBMEC, but it was unclear as to whether it had an influence on the antioxidant activity of the extracts.

To test the two hypotheses, the PSE sample was freeze-dried to remove all EtOH and was reconstituted in 100% sterile water. A concentration of 1% EtOH was added to a portion of the reconstituted extracts to test the effects of EtOH on the antioxidant capacity of PSE. Therefore, HBMEC were subjected to two PSE treatments: (1) PSE in 100% water (PSEW) and (2) PSE in water and 1% EtOH (PSEE). It was found that the difference between the two PSE treatment types were moderately significant (p=0.0586). HBMEC supplemented with PSEW had slightly
higher differences in luminescence between cells receiving just PSE and cells treated with PSE and H₂O₂ (i.e., greater cell death) than that seen in HBMEC treated with PSEE. Therefore, 1% EtOH did not inhibit the antioxidant activity of PSE but may have in fact, had an enhancing effect.

PSE concentration was also found to have a significant (p<0.05) effect on viability. This result was anticipated given the fact that the cells were receiving a higher dosage of phenolic compounds in the undiluted extracts. HBMEC having no PSE supplementation were significantly different from HBMEC supplemented with 5% PSE. There was greater survival amongst cells treated with 5% PSE (Fig. 5.5). Similarly, it was observed that supplementation with 0.6% PSE provided significantly (p<0.05) less protection against H₂O₂ damage when compared to 5% PSE supplementation (Fig. 5.5). This data suggests that pretreatment of HBMEC with 5% PSE was the most effective at protecting against H₂O₂. However, it was found that PSEW at 5% was somewhat cytotoxic even before treatment with H₂O₂ when compared to control cells (Fig. 5.3). The same trend was seen with PSEE at 5% although to a lesser extent (Fig. 5.4). It can be observed in Figs. 3 and 4 that 10% PSE supplementation also had a slight cytotoxic effect. Higher concentrations of PSE may prove to be cytotoxic to HBMEC. Research has shown that some antioxidant compounds can become pro-oxidants at high concentrations. For example, quercetin and fisetin were found to have protective effects against H₂O₂-induced cytotoxicity, DNA strand breaks and apoptosis at concentrations as low as 10-25 μM in H4IIE rat hepatoma cells [10]. However, these two flavonoids were shown to induce cytotoxicity and DNA fragmentation at concentrations between 50 and 250 μM.
CONCLUSIONS

We found that PSE may have some protective effects against H$_2$O$_2$-induced cytotoxicity in HBMEC. Higher PSE concentrations may have reduced cell viability as the elevated concentrations could have resulted in phenolic compounds acting as pro-oxidants. The high variability in the data inhibits our ability to make any definitive statements regarding the potential benefits of PSE in preventing oxidative damage to cells. In future work, it is suggested that a wider range of PSE concentrations be investigated to determine if there are protective as well as cytotoxic dose ranges of PSE. It is also suggested that other cell lines be explored. A greater protective effect may have been seen in an alternate cell model system. Furthermore, purification of the crude extracts prior to treatment of the cells may improve results. The research findings presented in this study are merely a starting point that we hope leads to further exploration of this topic. This work has shown that peanut skins have the potential to be developed into dietary supplements to rival grape seed extract, which has been widely accepted by consumers for their perceived health benefits.
Table 5.1 Total phenolic content of PSE (peanut skin extracts) used to treat human brain microvascular endothelial cells (HBMEC)

<table>
<thead>
<tr>
<th>PSE Type</th>
<th>Total Phenolic Content&lt;sup&gt;4&lt;/sup&gt; (mg GAE/ml extract)</th>
</tr>
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<tr>
<td>Diluted PSE&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.126 ± 0.004</td>
</tr>
<tr>
<td>PSE in 100% water&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.96 ± 0.005</td>
</tr>
<tr>
<td>PSE in water and 1% EtOH&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.86 ± 0.006</td>
</tr>
</tbody>
</table>

<sup>1</sup>Peanut skins extracted with 30% EtOH and diluted 1:30 (v/v) to obtain a final EtOH concentration of 1%.
<sup>2</sup>PSE were freeze-dried and reconstituted in 100% sterile water.
<sup>3</sup>PSE was freeze-dried, reconstituted in sterile water with 1% EtOH added.
<sup>4</sup>Values represent mean total phenolic content (n=3) ± SD.
Figure 5.1 Mean luminescence values of human brain microvascular endothelial cells (HBMEC) insulted with 700 μM H₂O₂.  
Data shown are the mean luminescence values (n=3) ± SD.  
C1 – control cells containing no PSE or H₂O₂  
C2 – cells treated with only H₂O₂
Figure 5.2 Mixed model showing the effects of peanut skin extract (PSE) concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10%) on luminescence of HBMEC treated with 700 μM H₂O₂. MeanDiff = Difference in mean luminescence between cells supplemented with just PSE and cells treated with PSE and H₂O₂.
Figure 5.3 Mean luminescence values of HBMEC supplemented with PSEW prior to treatment with 700 μM H₂O₂. Data shown are the mean luminescence values ± SD. C1 – control cells containing no PSE or H₂O₂. C2 – cells treated with only H₂O₂.
Fig. 5.4 Mean luminescence values of HBMEC supplemented with PSEE prior to treatment with 700 μM H₂O₂. Data shown are the mean luminescence values ± SD.
C1 – control cells containing no PSE or H₂O₂.
C2 – cells treated with only H₂O₂.
Fig. 5.5 Mixed model showing the effects of peanut skin extract (PSE) concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10%) on viability of HBMEC treated with 700 μM H₂O₂. HBMEC were supplemented with PSEW or PSEE. MeanDiff = Difference in mean luminescence between cells supplemented with just PSE and cells treated with PSE and H₂O₂.
REFERENCES

CHAPTER 6

EFFECT OF PEANUT SKIN EXTRACT ON LIPID OXIDATION IN COMMERCIAL PEANUT BUTTER AS INDICATED BY HEXANAL CONCENTRATION†

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ABSTRACT

Peanut skins are low-value byproducts of peanut processing operations. However, the skins have been shown to be a significant source of natural antioxidants. Peanut-based products such as peanut butter are highly susceptible to lipid oxidation due to high unsaturated fatty acid content. The present study was conducted to determine the effect of peanut skin extracts on lipid oxidation in commercial peanut butter. Hexanal concentration measured by GC-MS was used as an indicator of lipid oxidation. Peanut butter (PB) was treated with 100 or 200 ppm PSE, BHT or PSE/BHT and stored under accelerated conditions of 45 °C for 0, 7, 14 and 28 days. There was not a significant increase in hexanal concentration over the storage period even in samples not treated with PSE or BHT, which may have been a result of additional procedures performed to stabilize the commercial PB. An extension of the storage time to allow sufficient oxidation to occur may reveal greater protective effects of PSE against oxidation in PB.

Keywords: peanut butter, natural antioxidants, lipid oxidation, hexanal, peanut skin extract, BHT
INTRODUCTION

Peanuts (Arachis hypogaea) are an important agricultural crop in many parts of the world. Peanuts consist of the kernel, skin (seed coat) and hulls. The kernels are used to make a variety of products such as peanut butter, peanut oil and confectionary products. Consumers enjoy roasted peanuts and peanut products due to the unique nutty flavors that develop during the roasting process. However, peanuts contain about 50% fat, 80% of which is unsaturated fatty acid, thereby making these products prone to oxidation [1]. Oxidation of unsaturated lipids generates undesirable flavor and odor compounds that reduce product shelf-life and renders foods unacceptable to consumers. Hydroperoxides are highly unstable primary products of lipid oxidation that readily decompose into secondary reaction products including aldehydes such as hexanal, which are responsible for the cardboard and painty flavors associated with oxidized/rancid foods. Hexanal is a major product of linoleic acid oxidation and its presence has been reported in peanuts and peanut products [1,2].

Although peanut kernels are widely used in a number of applications, the skins are largely viewed as waste material with no real value to processors. However, research [3-7] has shown that peanut skins contain significant levels of natural antioxidant compounds such as trans-resveratrol, catechins, and procyanidins. Given that peanuts skins are a good source of natural antioxidants and the fact that the world production of this agricultural material is estimated at over 750,000 tons annually [8], it would behoove researchers to investigate their potential to be added to foods, especially confectionary products that already contain peanuts, to prevent oxidative deterioration and extend product shelf-life.

Industry currently relies on a variety of synthetic antioxidants such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole) and TBHQ (tert-butyl hydroquinone) to
prevent oxidative deterioration of lipids. However, increasing consumer demand for healthier food products is driving research efforts to seek out alternative natural sources of antioxidants. A concern with the use of natural antioxidants is that their effectiveness in food systems compared to synthetic compounds has been questioned. Some research has reported that natural antioxidants are less effective than synthetic compounds, while others claim that natural polyphenols work equally well although higher concentrations of the natural compounds are usually required to obtain similar activity [9-12]. Nepote et al. [13] studied the effects of peanut skin extracts (PSE) on the oxidation of honey roasted peanuts. Although PSE was found to be slightly less efficient than BHT, it did provide significant protection against lipid oxidation as measured by peroxide values, TBARS and sensory panels. To our knowledge, no work has been published on the use of PSE in the prevention of oxidation of peanut butter.

The extent of lipid oxidation in foods is measured using a variety of techniques including the traditional methods of peroxide value and thiobarbituric acid reactive substances assay. However, solid-phase microextraction (SPME) is gaining in popularity as a simple, solvent-free, sensitive and rapid method to assess volatile lipid oxidation products like hexanal [14]. In SPME, an inert fiber is coated with a liquid adsorbent. Upon exposure to the headspace gas, the liquid binds volatile compounds which are then injected into the injection port of a gas chromatograph for analysis.

The objectives of this study were to determine the effect of peanut skin extracts on lipid oxidation of peanut butter stored under accelerated oxidation conditions and to measure hexanal concentration as an indicator of lipid oxidation. The antioxidant activity of PSE was compared to that of a proven synthetic antioxidant, BHT.
MATERIALS AND METHODS

Extraction of Antioxidants from Peanut Skins

Peanut skins obtained from blanching were a gift from Tidewater Blanching Company (Suffolk, VA). Peanut skins were stored in a freezer at -4 °C in sealed plastic bags until analysis. Phenolic antioxidants were extracted from peanut skins using a MES-1000 microwave extraction system (CEM, Matthews, NC) that supplies 950 W of microwave energy at 100% power according to the procedure outlined by Ballard et al. [3]. Peanut skins were extracted with 37.5 ml of 30% aqueous ethanol (95% Everclear, Luxco Distilling Co., St. Louis, MO) under the extraction conditions of 90% (of nominal) microwave power, 150 s irradiation time and a sample mass of 1.5 g. The crude peanut skin extract (PSE) was allowed to reach room temperature before centrifugation (Model Avanti J-25I, Beckman Coulter, Inc., Fullerton, CA) at 5000 rpm for 30 min. The supernatant was collected and placed in 15 ml glass centrifuge tubes. PSE was freeze-dried (Virtis Co., Inc., Gardiner, N.Y.) and reconstituted in 4 ml of 70% ethanol (EtOH) prior to treatment of the PB.

The total phenolic content (TPC) of the extract was determined spectrophotometrically using the Folin-Ciocalteu total phenol procedure outlined by Spanos and Wrolstad [15] with slight modifications. Gallic acid standard solutions were prepared at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. The extract (0.1 ml) and the gallic acid standards (0.1 ml) were transferred to 15 ml test tubes. Distilled water (0.9 ml) and 3.0 ml of 0.2 N Folin-Ciocalteu reagent (F-9252, Sigma-Aldrich Co., St. Louis, MO) were added to each test tube and mixed using a vortex mixer. After one minute, 2.0 ml of 9.0% (w/v) Na₂CO₃ in water was added and mixed. The absorbance at 765 nm was read on a spectrophotometer (Spectronic 21, Bausch and Lomb, USA) after 2 h at room temperature. The concentration of total phenolic compounds in the extracts was determined by
comparing the absorbance of the extract sample to that of the gallic acid standard solutions. All samples were run in duplicate. TPC was expressed as mg gallic acid equivalents (GAE) per g dry peanut skins.

**Peanut Butter Sample Preparation**

A commercial brand of peanut butter (PB) was purchased from a local supermarket. PB was divided into seven 200 g batches. PSE, BHT or a PSE/BHT at concentrations of 100 and 200 ppm were added to each batch and mixed for 1.5 min with a hand mixer. In the combination treatment, PSE and BHT were blended 1:1 (w/w), e.g., the 100 ppm PSE/BHT treatment consisted of 100 ppm PSE + 100 ppm BHT. Four milliliters of 70% EtOH was added to control samples (containing no antioxidants) and samples treated with only BHT. PB batches were further divided into 40 g aliquots, placed in small glass jars and covered with cheesecloth to allow exposure to oxygen. The jars were stored in an incubator at 45 °C for 0, 7, 14 and 28 days to accelerate oxidation. Samples were removed from the incubator at the designated times to perform hexanal analysis. There were three replications of the experiment with three different batches of PB.

**GC-MS Identification/Quantification of Hexanal**

Three samples from each treatment were removed from the incubator at the end of each storage period. Five grams of PB was added to 5 ml glass vials with septum. A Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Valley Forge, PA) with a Combi Pal autosampler (CTC Analytics, Zwingen, Switzerland) was used for the identification and quantification of hexanal. The GC was coupled to a Hewlett Packard 5972 series mass selective detector. Sample compounds were separated using a DB-5 (30 m × 0.25 mm i.d., 0.25 µm film thickness) column. Helium was used as the carrier gas at a flow rate of 1 ml/min. Operating conditions were 35 °C
to 120 °C at a rate of 5 °C/min followed by a heating rate of 20 °C/min to a final temperature of 220 °C.

Solid-phase microextraction (SPME) using a DVB/CAR/PDMS (Divinylbenzene / Carboxen / Polydimethylsiloxane) fiber was used to analyze headspace volatiles. The PB sample was heated to 40°C and held for 30 min before being injected (12 min desorption) into the GC for analysis. The scan mode of data acquisition was used and only compounds in the selected mass range (m/z=35-300 amu) were detected and shown in the resultant total ion chromatogram. A pure standard of hexanal was purchased from Sigma Aldrich Co. (St. Louis, MO). Hexanal standard solutions of varying concentrations were prepared and run in tandem with the PB samples. Hexanal in the sample was identified based on matching retention time to the standard. The concentration of hexanal in the PB samples was determined by the calibration curve method in which the peak area of the samples was measured and compared to that of the hexanal standard of known concentration.

**Statistical Analysis**

Statistical analysis will be performed using the PROC GLM function of the SAS software program (SAS Version 9.0, SAS Institute Inc., 2004). A two-way analysis of variance (ANOVA) will be used to determine the effect of antioxidant concentration and storage time on hexanal concentration. Treatment effects of antioxidant concentration and time will also be evaluated. Fisher’s Least Significant Difference method will be used to estimate differences among sample means at the 5% probability level.

**RESULTS AND DISCUSSION**

According to the results of the Folin-Ciocalteu assay, the PSE used to treat the PB samples contained about 5 mg GAE/ml extract. Hexanal concentrations of PB samples under
accelerated storage conditions are shown in Fig. 6.1. There was a gradual increase in hexanal concentration from day 0 to 14 in all samples regardless of the treatment. There appears to be a significant difference between samples at day 0 and 14. Despite the fact that there were statistical differences between these two groups of samples, the levels of hexanal present in both samples were not high enough for the peanut butter to be considered oxidized. The average consumer would not be able to detect a difference between the PB samples at day 0 and 14. Although hexanal values did not reach levels required for oxidation, it was found that hexanal concentrations in the controls were higher than that seen in PB samples treated with PSE or BHT (Figs. 6.1 and 6.2). Treatment with 200 ppm BHT appeared to provide the best protection against oxidation after 14 days of storage followed by 200 ppm PSE. An unexpected decline in hexanal concentration occurred at 28 days of storage. The hexanal concentration of all PB samples dropped back to the levels seen at day 0. Previous research has reported that there is typically a gradual increase in the levels of oxidation products in peanuts and peanut-containing products over time. Grosso et al. [2] found that hexanal levels in roasted peanuts increased steadily between 0 and 154 days of storage at 23, 30 and 40 °C. However, they also reported inexplicable increases and decreases in hexanal after extended storage.

Although there were slight increases in hexanal concentration over the storage period, we expected to see much greater increases given that the PB was stored at 45 °C with adequate exposure to oxygen. It is not clear as to why even the control samples were not oxidizing at a much faster rate. One explanation may be that the manufacturers of the peanut butter modified the fatty acid composition by including hydrogenated vegetable oils and mono-/diglycerides. In addition, they may have formulated the peanut butter using high oleic peanuts, which would explain the low hexanal content given that hexanal is a product of linoleic acid oxidation. Peanut
oil is composed of about 800 g kg\(^{-1}\) unsaturated fatty acids with 450 g kg\(^{-1}\) being oleic and 350 g kg\(^{-1}\) of linoleic acid [16]. In an early study investigating the stability of high oleic peanuts, O’Keefe et al. [17] were able to show that an increased oleic acid content in peanuts greatly improved oxidative stability. Nepote et al. [16] found that roasted peanuts prepared with high oleic kernels had a 10 times higher shelf-life at 40 °C than normal peanuts.

**CONCLUSIONS**

It was not clear from the present study whether or not peanut skins have a significant effect on oxidation of PB. There was not a significant increase in hexanal concentration over the storage period even in samples not treated with PSE or BHT, which may have been a result of additional procedures performed to stabilize the commercial PB. It was seen that the hexanal content in PB samples treated with PSE were slightly lower than the control throughout the storage period. Therefore, an extension of the storage time to allow sufficient oxidation to occur may reveal some protective effects of PSE against oxidation in PB. A commercial brand of PB should not be used in future studies. This work will be repeated using a peanut paste model system or freshly made peanut butter to ensure that oxidation is not inhibited. It is expected that PSE will have a significant effect on oxidation in the alternate model system. This work warrants further investigation as PSE has the potential to be incorporated in peanut confectionary products as an alternative to synthetic antioxidants.
Fig. 6.1 Effect of PSE and BHT on hexanal concentration in commercial peanut butter stored for 0, 7, 14 and 28 days at 45 °C. Data represent mean (n=3) ± SD.
Fig. 6.2 Comparison of hexanal concentration in control samples and PB samples treated with PSE and stored for 0, 7, 14 and 28 days at 45 °C.
REFERENCES


CHAPTER 7

SUMMARY AND CONCLUSIONS

Oxidation of lipids is a major economic concern for food processors as it negatively impacts sensory and nutritional quality of many food products. Although synthetic antioxidants have been proven effective in a variety of food systems, industry’s reliance on these compounds to prevent oxidative deterioration must be minimized as consumer demand for healthier, all-natural products is steadily on the rise. Research efforts are now focused on identifying sources of natural antioxidant compounds, mainly polyphenols, to replace their synthetic counterparts. Agricultural waste materials such as peanut skins may represent an abundant, inexpensive source of natural antioxidants. It is estimated that over 750,000 tons of peanut skins are generated worldwide each year and a large majority of them are disposed of as waste. The development of more efficient methods for extracting antioxidants from peanut skins is needed in order to increase commercial appeal.

The primary goal of this study was to optimize conditions for the extraction of phenolic antioxidants from peanut skins using one traditional extraction method (SLE) and one novel method (MAE). To achieve this goal, the study was divided into four parts. The objective of the first part of the study was to optimize the extraction conditions for the SLE procedure. The effect of four solvents (EtOH, MeOH, water and EA), alcohol concentration (30, 60, 90%), temperature (30, 45, 60 min) and time (10, 20, 30 min) on total phenolic content (TPC), ORAC activity and recovery of trans-resveratrol from peanut skins was investigated. RSM was used to determine the optimum extraction conditions for each of the solvents. EtOH was found to be the most efficient solvent for the extraction of phenolic compounds followed by MeOH, water and
EA. Despite EtOH extracts having a higher TPC, samples extracted with MeOH demonstrated slightly higher ORAC activity (2149 μmol TE/g). EA was not found to be an effective solvent. Resveratrol was identified in MeOH extracts, but was not identified in samples extracted with EtOH, water or EA. The data suggest that MeOH may be the best solvent for extraction of phenols, especially resveratrol, from peanut skins.

The objective of the second part of the study was to optimize extraction conditions for MAE and to compare the results to those found with SLE. Optimization parameters included microwave power (10, 50, 90% nominal), irradiation time (30, 90, 150 s) and mass of peanut skins (1.5, 2.5, 3.5 g). Optimum extraction conditions were based on TPC, ORAC activity and trans-resveratrol concentration. Peanut skins were extracted with 30% EtOH. The maximum predicted TPC under the optimized conditions was 143.6 mg phenols/g skins compared to 118 mg/g with SLE. The maximum predicted ORAC activity was 2789 μmol TE/g versus 2149 μmol TE/g with the SLE method. MAE was able to extract more phenolic compounds (with higher antioxidant activity) in a faster time while consuming less solvent than the SLE procedure. In addition, the presence of resveratrol was found in PSE derived from MAE although at relatively low levels.

In the first two parts of the study, it was clearly shown that peanut skins contain relatively high levels of phenolic compounds with excellent peroxyl radical scavenging ability (as indicated by ORAC value). As such, peanut skins have the potential to be developed into dietary supplements, similar to grape seed extracts, and into food additives that aid in reducing lipid oxidation. Parts three and four of the study focused on practical applications of PSE. More specifically, we evaluated the antioxidant activity of PSE in two model systems: peanut butter and human brain microvascular endothelial cells (HBMEC).
In part three, the protective effects of PSE on H$_2$O$_2$-induced oxidative stress in HBMEC was investigated. HBMEC were supplemented with PSE at 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5 and 10% v/v PSE in experimental media for 2 h prior to treatment with 700 μM H$_2$O$_2$. It was found that HBMEC not treated with PSE prior to treatment with H$_2$O$_2$ had significantly (p<0.05) higher cell death than cells supplemented with PSE before H$_2$O$_2$ treatment. Higher doses of PSE (5 and 10%) appeared to have somewhat of a cytotoxic effect. However, the data were highly variable which made it difficult to arrive at any definitive conclusions regarding the potential benefits of PSE in preventing oxidative damage to HBMEC.

Part 4 of the study investigated the effects of peanut skin extracts on lipid oxidation in commercial PB. PB was treated with 100 or 200 ppm PSE, BHT or PSE/BHT and stored under accelerated oxidation conditions of 45 °C for 0, 7, 14 and 28 days. It was found that while hexanal concentration did significantly increase over the storage period, the values were not high enough for the PB to be considered oxidized. The lack of oxidation was attributed to additional procedures performed by the manufacturers of the commercial PB to increase product stability. An extension of the storage time to allow sufficient oxidation to occur may reveal some protective effects of PSE against oxidation in PB.

**FUTURE WORK**

It is suggested that in future work the extracts be fractionated to determine which compounds are primarily responsible for the antioxidant activity of PSE. This would be particularly useful with the MeOH extracts as fractionation may reveal if the presence of resveratrol in MeOH extract are responsible for the slightly higher ORAC values. In the MAE procedure, the use of different solvents and combinations of solvents should be explored to further increase extraction efficiency. Solvent composition can also be adjusted to determine if
higher or lower alcohol concentrations prove to be more efficient. For the cell culture work, it is
suggested that treatment and cell culturing conditions are optimized in order to reduce variability
in the luminescence readings. A wider range of PSE concentrations need to be investigated to
determine if there are protective as well as cytotoxic dose ranges of PSE. In addition, other cell
lines should be explored as greater protective effects may have been seen in alternate cell model
systems. A commercial brand of PB should not be used in future studies as product formulation
may have had an influence on oxidation rate, thereby distorting the effect of PSE. It is suggested
that a peanut paste model system or freshly made peanut butter be used instead. Finally, more
identification work is needed to obtain a more complete profile of the exact phenolic
composition of PSE.