

©2013

WENZUNAIRAH WANIBADULLAH

ALL RIGHTS RESERVED

**LIPID PROTEIN INTERACTIONS IN PEANUT BUTTER**

by

**WANZUNAIRAH WANIBADULLAH**

A Dissertation submitted to the Graduate School-New Brunswick,

Rutgers, The State University of New Jersey

In partial of fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Food Science

written under the direction of

Professor Karen M. Schaich

and approved by

---

---

---

---

New Brunswick, New Jersey

(October, 2013)

## **ABSTRACT OF DISSERTATION**

### **Lipid protein interactions in peanut butter**

**by WANZUNAIRAH WANIBADULLAH**

Dissertation Director:

Professor Karen M. Schaich

Lipid co-oxidation of proteins was studied in peanut butter packaged in laminated MRE pouches; incubated at 25, 40, and 60 °C for twelve weeks; and analyzed weekly for physical properties (moisture content, texture, color), lipid degradation (conjugated dienes, hydroperoxides, aldehydes, free fatty acids), and protein characteristics (solubility, intrinsic fluorescence, Schiff base fluorescence, SDS and native polyacrylamide gel electrophoresis, and protein carbonyl products).

Changes in nearly all properties increased with incubation time and were more marked at 60 °C than at the two lower temperatures. Among physical properties, peanut butter darkened and became redder with incubation and texture hardened and became less cohesive and more sticky and gummy. Less than 1% moisture was lost so texture changes were due to chemical modifications rather than dehydration.

Values of conjugated dienes and hydroperoxides cycled (+/–) with a three week periodicity; levels remained low and showed a net decrease during incubation. Low levels of aldehydes formed during the first few days of incubation at 60 °C, then decreased thereafter; aldehydes did not accumulate at lower temperatures. In contrast, fatty acids increased to 3% at 60 °C and 1% at lower temperatures, due at least in part to oxidation of aldehydes.

Peanut proteins showed unique modification of surface residues that resulted in differential loss of Coomassie blue and silver dye binding, structural reorganization of arachins, and shifting of albumins and globulins to SDS-soluble fractions. Surface modifications resulted primarily from extensive protein oxidation revealed in antibody reactions, especially in the SDS-soluble fraction, although low levels of carbonyl-amine reactions (Schiff base or Michael addition) and some decarboxylation of acidic residues were also detected. Only limited disulfide and non-disulfide crosslinking was observed, but there was notable fragmentation or disassociation of arachins. Protein modifications followed the same cycling pattern as lipid oxidation, suggesting that lipid oxidation products did not accumulate because lipid radicals, hydroperoxides, and to a lesser extent aldehydes reacted preferentially with proteins, broadcasting oxidation to these non-lipid molecules. Hardening of the peanut butter appears to be directly related to surface modifications and structural reorganization of the proteins mediated by oxidized lipids.

## **ACKNOWLEDGEMENTS**

I would like to express my deepest gratitude to my advisor, Dr. Karen M. Schaich, for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. Her kindness, knowledge, generosity and patience gave me great help along the journey of my pursuit of Doctoral Degree. I would never be able to achieve what I have done today without her. I am grateful to the privilege of having known and worked with her.

I thank my dissertation committee members, Dr. Chaim Frenkel, Dr. Chi-Tang Ho, Dr. Kit Yam, Dr Michael Tunick for their comments and suggestions about my dissertation and defense.

I acknowledge the financial support from the Scholarship Division, Higher Ministry of Education, Malaysia and Universiti Putra Malaysia.

I would like to thank my labmates Yuan Dong, Xin Tian, Karen Chang, Jia Xie, Tony, Teng Peng, Linhong Yao, Brandon Bogusz, Anna Puganen and Elah Steltzer for their friendship and their help in my research.

Finally, I deeply thank my husband, Mohd Ikhfan, my children, and my parents for their unconditional support, always cheering me up through good times and bad.

## Table of Contents

ABSTRACT OF DISSERTATION .....	ii
ACKNOWLEDGEMENTS .....	iv
LIST OF TABLES .....	ix
LIST OF FIGURES .....	x
1. INTRODUCTION .....	1
2. BACKGROUND .....	4
2.1 Composition of peanuts .....	4
2.1.1 Proximate composition. ....	4
2.1.2 Peanut proteins .....	8
2.1.3 Oil in peanuts .....	11
2.1.4 Carbohydrates in peanuts.....	12
2.2 Peanut butter.....	12
2.2.1 Processing of peanut butter.....	12
2.2.2 Roasting effects on peanuts .....	13
2.3 Degradation of peanut butter by lipid oxidation .....	15
2.3.1 General reactions .....	15
2.3.2 Specific observations in peanut butter.....	19
2.4 Lipid co-oxidation of proteins.....	19
2.4.1 General background.....	19
2.4.2 Mechanisms of lipid co-oxidation of proteins.....	22
2.4.3 Types of protein damage caused by lipid co-oxidation.....	26

3. INFORMATION GAPS, RATIONALE, AND SIGNIFICANCE OF RESEARCH	41
4. HYPOTHESES AND OBJECTIVES	43
4.1 Hypothesis	43
4.2 Specific objectives	43
5. MATERIALS AND METHODS	45
5.1 Overall experimental design	45
5.2 Peanut butter source and incubation	46
5.3 Moisture content of peanut butter	46
5.3 Color	46
5.4 Texture profile analysis (TPA)	47
5.5 Extraction of lipids	49
5.6 Analysis of lipid oxidation	49
5.6.1 Conjugated dienes	49
5.6.2 Lipid hydroperoxides	49
5.6.3 Aldehydes	50
5.6.4 Free fatty acids	50
5.7 Extraction of peanut proteins: procedure optimization	50
5.8 Sequential fractionation of defatted peanut butter proteins	52
5.9 Protein solubility	53
5.10 SDS-PAGE of peanut proteins	54
5.11 Native PAGE	55

5.12	Intrinsic and Schiff Base Fluorescence .....	56
5.13	Protein carbonyl formation.....	57
6.	RESULTS AND DISCUSSION .....	59
6.1	Physical changes .....	59
6.1.1	Moisture content .....	59
6.1.2	Color changes in peanut butter .....	60
6.1.3	Texture characteristics of peanut butter.....	64
6.2.	Chemical changes in peanut butter during storage.....	70
6.2.1	Lipid changes during storage.....	70
6.2.1.1	Lipid extractability .....	70
6.2.1.2	Oxidative stability.....	71
6.2.1.2.1.	Conjugated dienes.....	72
6.2.1.2.2.	Peroxide values.....	73
6.2.1.2.3	Aldehydes .....	75
6.2.1.2.4.	Free Fatty Acids.....	77
6.2.2	Protein modifications in peanut butter.....	79
6.2.2.1	Protein solubility.....	79
6.2.2.2	Crosslinking and fragmentation by SDS-2ME electrophoresis of protein fractions extracted from incubated peanut butter .....	82
6.2.2.3	Native PAGE to detect changes in protein surface charge .....	103
6.2.2.4	Protein carbonyls .....	114
6.2.2.5	Fluorescence – intrinsic emissions from aromatic amino acids .....	121



7. SUMMARY AND CONCLUSIONS .....	129
8. FUTURE WORK.....	134
REFERENCES .....	136

## LIST OF TABLES

Table 1. Composition of peanut kernels (Freeman et al., 1954).....	5
Table 2. Nutrient composition of 100 g edible peanut product (Watt and Merrill, 1963). .....	6
Table 3. Amino acid content of arachin and conarachin fractions of peanut proteins...	9
Table 4. Variations in excitation and emission maxima for Schiff base fluorescence from different amino acid and protein substrates. ....	39

## LIST OF FIGURES

Figure 1. Anatomy of peanut ( <a href="http://google.com/anatomyofpeanut">http://google.com/anatomyofpeanut</a> ). .....	5
Figure 2. Integrated scheme for lipid oxidation showing alternate reactions that compete with classical hydrogen abstraction (red vertical reactions in center)... 18	
Figure 3. Types of damage that occur when oxidizing lipids co-oxidize cellular molecules in foods and living tissue (plant and animal). .....	21
Figure 4. Epoxide reactions with amino acids. ....	25
Figure 5. Diagrammatic representation of amino acids most susceptible to attack by oxidizing lipids. ....	27
Figure 6. Reactions involved in free radical crosslinking induced by oxidizing lipids. .....	31
Figure 7. Experimental flow diagram for investigation of lipid and protein oxidation in peanut butter. ....	45
Figure 8. Nutrition facts and ingredients of peanut butter. ....	46
Figure 9. Texture profile analysis curve showing compression cycles, force peaks, and equations for calculating primary texture parameters.....	48
Figure 10. BSA standard curve for measurement of protein solubility. A separate curve was generated for each batch of Bradford reagent.....	54
Figure 11. Visible color and texture changes in peanut butter incubated in MRE packages for 12 weeks. ....	59
Figure 12. Effect of incubation time and temperature on moisture content of peanut butter stored in MRE packages. ....	60
Figure 13. Effect of time and temperature on L* values of peanut butter incubated in MRE packages for 12 weeks.....	62

Figure 14. Effect of time and temperature on a* values of peanut butter incubated in MRE packages for 12 weeks.....	62
Figure 15. Effect of time and temperature on b* values of peanut butter incubated in MRE packages for 12 weeks.....	63
Figure 16. Effects of incubation time and temperature of hardness of peanut butter over the course of 12 weeks.....	64
Figure 17. Effects of incubation temperature of hardness of peanut butter incubated for 12 weeks.....	66
Figure 18. Effects of incubation temperature of adhesiveness of peanut butter incubated for 12 weeks. ....	68
Figure 19. Effects of incubation temperature on gumminess of peanut butter incubated for 12 weeks.....	69
Figure 20. Effect of temperature on lipid extractability from peanut butter incubated for 12 weeks in MRE packages. ....	71
Figure 21. Effect of temperature on conjugated diene concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages. ....	72
Figure 22. Effect of temperature on hydroperoxide concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages. ....	74
Figure 23. Effect of temperature on aldehyde concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages. ....	76
Figure 24. Effect of incubation temperature on release of free fatty acids in peanut butter incubated for 12 weeks in MRE packages.....	78
Figure 25. Efficiency of protein extraction by different methods. ....	79
Figure 26. Solubility of the total soluble protein fraction extracted from peanut butter incubated at three different temperatures.....	80

Figure 27. Solubility of the albumin fraction extracted from peanut butter incubated at three different temperatures. ....	81
Figure 28. Solubility of the globulin fraction extracted from peanut butter incubated at three different temperatures. ....	81
Figure 29. Solubility of SDS-ME soluble proteins extracted from peanut butter incubated at three different temperatures.....	82
Figure 30. SDS-2ME PAGE of proteins extracted from peanut butter incubated at different temperatures for two weeks. ....	83
Figure 31. SDS-2ME PAGE of peanut butter proteins soluble in SDS showing differential binding of silver (left) and Coomassie blue (right) dyes.....	84
Figure 32. SDS-PAGE patterns of total soluble protein fraction extracted from peanut butter incubated in MRE package at 25°C.....	87
Figure 33. SDS-PAGE of total soluble proteins from peanut butter incubated at 40 °C for 12 weeks.....	88
Figure 34. SDS-PAGE of total soluble proteins from peanut butter incubated at 60 °C for 12 weeks.....	89
Figure 35. SDS-PAGE of albumins from peanut butter incubated at 25 °C for 12 weeks.....	91
Figure 36. SDS-PAGE of albumins from peanut butter incubated at 40 °C for 12 weeks.....	92
Figure 37. SDS-PAGE of albumins from peanut butter incubated at 60 °C for 12 weeks.....	93
Figure 38. SDS-PAGE of globulins from peanut butter incubated at 25 °C for 12 weeks.....	95

Figure 39. SDS-PAGE of globulins from peanut butter incubated at 40 °C for 12 weeks.....	96
Figure 40. SDS-PAGE of globulins from peanut butter incubated at 60 °C for 12 weeks.....	97
Figure 41. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 25 °C for 12 weeks.....	99
Figure 42. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 40 °C for 12 weeks.....	100
Figure 43. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 60 °C for 12 weeks.....	101
Figure 44. Native PAGE patterns of albumins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain. ....	106
Figure 45. Native PAGE patterns of albumins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain.....	107
Figure 46. Native PAGE patterns of globulins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain.. ....	108
Figure 47. Native PAGE patterns of globulins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain.....	109
Figure 48. Native PAGE patterns of SDS-soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain.....	110

Figure 49. Native PAGE patterns of SDS-soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain.....	111
Figure 50. Native PAGE patterns of total soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain.....	112
Figure 51. Native PAGE patterns of total soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain.....	113
Figure 52. Distribution of protein carbonyl oxidation products in the total soluble protein fraction extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks.....	117
Figure 53. Distribution of protein carbonyl oxidation products in albumins extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks.....	118
Figure 54. Distribution of protein carbonyl oxidation products in globulins extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks.....	119
Figure 55. Distribution of protein carbonyl oxidation products in the SDS-soluble fraction extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks.....	120
Figure 57. Distribution of intrinsic fluorescence among the various protein fractions in peanut butter.....	123
Figure 58. Changes in intrinsic fluorescence in different peanut protein fractions during incubation for 12 weeks at 25, 40, and 60 °C.....	124
Figure 59. Typical presumptive Schiff base emission spectra from different fractions of peanut butter proteins. ....	126

Figure 60. Changes in presumptive Schiff base fluorescence (linear adducts) in different peanut butter protein fractions during incubation for 12 weeks at 25, 40, and 60 °C. ....	127
--	-----

Figure 61. Changes in presumptive Schiff base fluorescence (ring adducts) in different peanut butter protein fractions during incubation for 12 weeks at 25, 40, and 60 °C. ....	128
--	-----



## 1. INTRODUCTION

The United States has a long history of sharing its food with the world for emergency and humanitarian assistance. Food aid products provide nutritional support to regions around the globe that are stressed by famine, disaster, and political upheaval (Marchione 2002). According to the VAM (Vulnerability Analysis and Mapping), food aid is viewed as a particularly useful ‘pre-investment’ to development activities in specific sectors, a resource that is most useful to enable young children and expectant and nursing mothers to meet their special nutritional value and nutrition-related health needs, enable poor households to invest in human capital, particularly that of women and girls, through education and training, mitigate the effects of natural disasters in areas vulnerable to recurring crises of this kind and make it possible for poor families to gain and preserve assets (World Food Programme, June 2002).

Food aid uses products that have been designed to deliver total nutrition (such as corn-soy-milk extrudates designed at the USDA-ERRC (Ho and Hanharan, 2010) as well as natural, nutritionally-dense and reasonably stable foods such as peanut butter and processed cheese. A general goal for emergency and supplementary food aid is three year shelf life of products because of unpredictable use patterns, requirement for immediate mobilization upon demand, are transit, storage, and distribution under conditions where extreme high temperatures are the norm (Ho and Hanharan 2010). This puts great stress on stability of the foods, leading to degradation of lipids, proteins, sensory qualities, and particularly nutritional value (Marchione 2002).

Many food aid products have achieved this required stability by limiting fat and water contents combined with vacuum packaging in laminate barrier packaging

(Marchione, 2002). However, fluid and semi-fluid foods have more problems with stability. In addition, there is now increasing recognition that unsaturated fatty acids levels must be increased for nutritional support in food aid products, especially those targeted to support growth and development in children (Mosha and Vincent, 2005). Such formulations present significant challenges in preventing lipid rancidity and also destruction of nutrients and loss of functionality and sensory quality by co-oxidation of non-lipid molecules (Bookwalter et al. 1971).

Peanut butter was selected for this investigation because it is a high protein product rich in oxidizable polyunsaturated fatty acids, and it is a product that currently presents stability problems that decrease palatability and impair nutritional value in military field rations and international emergency food aid. As a nutritionally-dense familiar food, peanut butter has been a very important yet problematic product in U.S. military rations due to active lipid oxidation and co-oxidations that degrade food quality. Some stabilization has been attained using MRE laminate packaging combined with nitrogen flush or vacuum. However, problems with texture and color during long term storage, especially at elevated temperature, remain. Now that peanut butter has been demonstrated to be a critical component in international food aid for children, these limitations need to be addressed, and in addition, maintenance of nutritional quality during shipping and storage needs to be assured. Both of these issues require elucidation of the causes of this product degradation, including lipid oxidation and associated protein co-oxidations. Elucidating reactions underlying quality degradation, with emphasis on protein degradation, is the focus of this dissertation.

Peanut butter presents a complex matrix because it is a semisolid emulsion with relatively high lipid content, low moisture, and high levels of interspersed

proteins. The close proximity of proteins to unsaturated lipids in peanut butter facilitates interactions with lipid radicals as well as hydroperoxide, epoxide, and aldehyde products. The presence of adsorbed moisture in peanut butter complicates the mechanisms of co-oxidation, adding potential for lipid hydrolyses and other reactions in addition to co-oxidation. There is also the additional challenge that very little information is available about any peanut protein degradation processes. Thus, for both practical and scientific reasons, peanut butter offers an important and intriguing material in which to investigate and elucidate protein degradation by lipid co-oxidation, and to connect these reactions to physical changes in peanut butter.

What is learned here should be broadly applicable to co-oxidation mechanisms in other high protein foods. It is hoped that comparisons of other proteins such as wheat and zein with peanut protein behaviour may shed light on factors controlling protein co-oxidation chemistry.

## 2. BACKGROUND

### 2.1 Composition of peanuts

#### 2.1.1 Proximate composition.

Peanut are an annual herbaceous plant belonging to Papilionaceae, a suborder of the larger order Leguminosae. After fertilization of the flowers of the plant, a peduncle develops and grows to reach the soil and push a “peg” 3 to 4 in (Woodroof 1973). below the surface where the fruit or pods are formed. The shell of the pod comprises from 20-30% of the whole nut and may easily separate from the kernels. Peanut kernels are composed of approximately equal weights of fatty and non fatty constituents (Tables 1 and 2), the relative amounts of each depending on the quality of the peanuts (Freeman et al. 1954). A desirable peanut is one that is well matured, uniform in size, with a minimum of shrivelled kernels. It has a full, pleasant, natural flavour with tender texture (Watt and Merrill, 1963). The moisture content of stored raw peanuts varies from 5-7%. The first effect of dry roasting or oil roasting is to reduce moisture content to below 2% (Woodroof 1973).

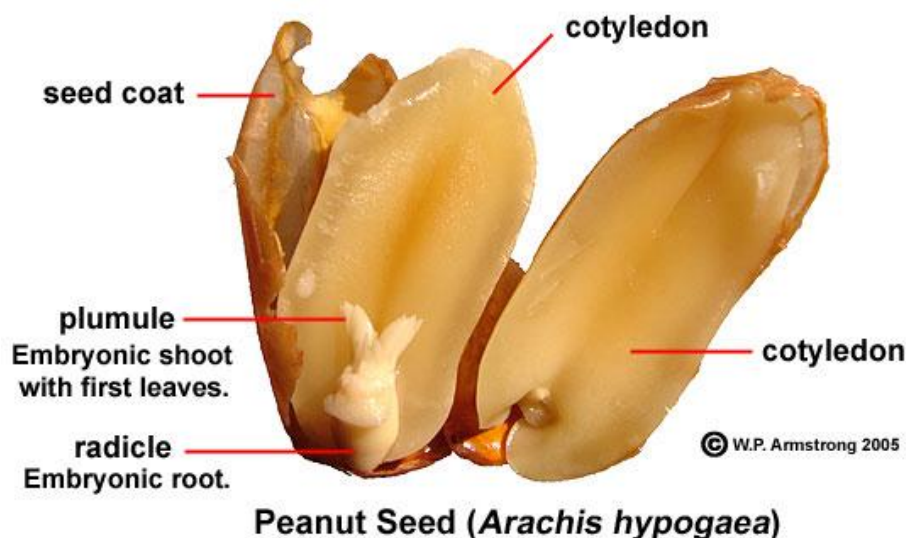


Figure 1. Anatomy of peanut (<http://google.com/anatomyofpeanut>).

Table 1. Composition of peanut kernels (Freeman et al., 1954).

Constituents	Range, %	Average %
Moisture	3.9-13.2	5.0
protein	21.0-36.4	28.5
Lipids	35.8-54.2	47.5
Crude fiber	1.2-4.3	2.8
Nitrogen-free extract	6.0-24.9	13.3
Ash	1.8-3.1	2.9
Reducing sugar	0.1-0.3	0.2
Disaccharide sugar	1.9-5.2	4.5
Starch	1.0-5.3	4.0
Pentosans	2.2-2.7	2.5

Table 2. Nutrient composition of 100 g edible peanut product (Watt and Merrill, 1963).

Item	Raw		Boiled	Roasted		Peanut butter		Spread	
	With skins	Without skins		With skin	Without skin	Added fat, salt	Added fat, salt sugar		
Flour									
Water %	5.6	5.4	36.4	1.8	1.6	1.8	1.7	2.2	7.3
Calories	564	564	376	582	585	581	589	601	371
Protein	26.0	26.3	15.5	26.2	26.0	27.8	25.2	20.3	47.9
Fat, g	47.5	48.4	31.5	48.7	49.8	49.4	50.6	52.1	9.2
Carbohydrate, g	18.6	17.6	14.5	20.6	18.8	17.2	18.8	22.0	31.5
Fiber, g	2.4	1.9	1.8	2.7	2.4	1.9	1.8	1.5	2.7
Ash, g	2.3	2.3	2.1	2.7	3.8	3.8	3.7	3.4	4.1
Calcium, g	69	59	43	72	74	63	59	50	104
Phosphorus, g	401	409	181	407	401	407	380	322	720
Iron, g	2.1	2.0	1.3	2.2	2.1	2.0	1.9	1.5	3.5
Sodium, mg	5	5.0	4.0	5	418	607	605	597	9
Potassium, mg	674	674	462	701	674	670	627	530	1186

Table 3 continued. Nutrient composition of 100 g edible peanut product (Watt and Merrill, 1963).

Item	Raw		Boiled	Roasted		Peanut butter		Spread	
	With skins	Without skins		With skin	Without skin	Added fat, salt	Added fat, salt sugar		
Flour									
Vitamin A, IU	-	-	-	-	-	300	300	-	-
Thiamin, mg	1.14	0.99	0.48	0.32	0.32	0.12	0.12	0.10	0.75
Riboflavin, mg	0.13	0.13	0.08	0.13	0.12	0.12	0.12	0.10	0.22
Niacin, mg	0.13	15.8	10.0	17.1	17.2	14.7	14.7	12.4	27.8
Ascorbic acid, mg	-	-	-	-	-	13	13	-	-

### 2.1.2 Peanut proteins

Peanuts contain about 26% protein, and peanut meal has twice that amount. Hoffpauir and Guthrie (1945) reviewed 94 literature references on the chemical composition of peanuts, mainly proteins. Globulin was isolated from peanuts in 1880, with a yield of 25% in the meal. Of 9.1% nitrogen in peanut material, 8.74% occurred as albuminous substances, including albumins, gluten, and globulins. Arachins precipitated as a sticky, heavy material, and was recovered as a fine white powder.

The amino acid composition of arachins and conarachins in peanuts is given in Table 3 (Guthrie et al., 1949; Murphy and Duan, 1950). Peanut meal protein contains a large amount of nutritionally essential amino acids, particularly the basic amino acid, arginine (Picket, 1941). The basic nitrogen content (6.55%) is among the highest found in seed globulins (Woodroof, 1973), although the most important basic amino acid, lysine, is deficient. Peanut proteins are unusually low in sulphur amino acids. Brown (1941) found that peanut arachin protein was 1.51% cysteine and 0.67% methionine; conarachin was 2.92% cysteine and 2.12% methionine. Conarachins contain about three times as much sulphur as arachins. The greatest nutritional weakness in peanut protein is a low content of two amino acids essential to both human and animal nutrition, lysine and methionine (Woodroof, 1973).



Table 4. Amino acid content of arachin and conarachin fractions of peanut proteins  
(Guthrie et al., 1949; Murphy and Duan, 1950).

Amino acid	Protein Fraction			
	Protein <sup>a</sup>	Total protein <sup>b</sup>	Arachin <sup>b</sup>	Conarachin <sup>b</sup>
Glycine	4.1	5.6	1.8	-
Alanine	-	4.2	4.1	-
Valine	4.7	8.0	1.1	-
Leucine	6.7	7.0	3.9	-
Cystine	-	1.9	1.1	3.0
Aspartic acid	12.0	5.8	5.3	-
Glutamic acid	20.0	19.2	16.7	-
Tyrosine	-	4.4	5.5	2.9
Phenylalanine	5.4	5.4	2.6	-
Proline	-	-	1.4	-
Tryptophan	-	2.0	0.9	2.1
Arginine	14.0	10.6	13.5	14.6
Lysine	3.1	3.4	5.0	6.0
Histidine	2.0	2.1	1.9	1.8
Methionine	1.1	1.2	0.5	2.1
Threonine	3.4	2.9	2.6	2.0
Serine	-	-	5.2	5.0
Hydroxylysine	-	-	0.01	-
isoleucine	4.0	4.3	-	-

From <sup>a</sup> Guthrie et al., 1949, <sup>b</sup> Murphy and Duan, 1950

The coefficient of digestibility for peanut protein is 89% (Pickett, 1941). Since 87% of the nitrogen of peanuts is present as arachins and conarachins, both of which contain 18.3% nitrogen, the factor for calculating protein from nitrogen content is 5.46 (Pickett, 1941).

Peanut seed proteins have been broadly classified into arachins, conarachins and albumins (Johnson et al., 1950; Dawson, 1968; Basha and Cherry, 1976). Arachins and conarachins, which correspond to legumin and vicilin, respectively, of other legume seeds (Johns and Jones, 1916), together compromise approximately about 87% of the seed proteins (Basha and Pancholy, 1981; Mosse and Pernollet, 1983). Arachins account for up to 73% of extractable proteins (Yamada et al., 1979). Purified arachins extracted from peanut seeds by phosphate buffer and characterized on SDS-PAGE showed five major subunits with molecular weights (MW) of 42, 37, 35, 22, and 20 KDa and four minor subunits of MW 66, 31, 29 and 25 KDa (Chiou, 1990). Conarachins contained one major subunit with MW of 64 KDa and three subunits with a MW of 60, 32 and 21 KDa.

The structure, composition, sub-cellular location, association-dissociation and quaternary structure of arachin have been investigated over the last three decades (Basha and Pancholy, 1981; St Angelo and Mann, 1973; Tombs, 1965; Yotsuhashi and Shibasaki, 1973). Two forms of arachins having sedimentation coefficients of 9S and 14S and  $M_r$ 's of 180 and 350 k, respectively, have been demonstrated (Yamada et al., 1973). The native monomer/dimer ratio is cultivar specific (Yamada et al., 1973). The monomeric form (9S) undergoes reversible interconversion to the native dimeric form (14S) by changes in the ionic strength or pH, while (14S) is not affected by pH (Krishna and Mitra, 1987).

The monomeric and the dimeric forms of arachins have similar amino acid

composition and identical subunit pattern consisting of six major subunits (Yamada et al., 1976). The six subunits ( $S_1$ - $S_6$ ) are classified into two groups of hydrophilic ( $S_1$ ,  $S_2$  and  $S_3$ ) and hydrophobic ( $S_4$ ,  $S_5$  and  $S_6$ ) subunits. Apparently there are no disulfide bonds between the members of the two groups (Yamada et al., 1973; Yamada et al., 1976).

Three polymorphic forms A, B, and  $A_1$  were identified in electrophoretic patterns of single seeds (Tombs, 1965). The  $M_r$ 's were 35 kD for the  $\alpha$  and  $\beta$  subunits and 10 kD each for  $\gamma$  and  $\delta$  subunits. All three native forms of arachin had a  $M_r$  of 170 kD and similar amino acid composition (Krishna and Mitra, 1987). The  $A_1$  form was isolated from an African variety and had faster mobility than those of A and B forms (Krishna and Mitra, 1987). However, the inheritance of the polymorphic forms of arachin could not be investigated.

### **2.1.3 Oil in peanuts**

Peanuts contain from 45-49% oil, and peanut butter and oil-roasted nuts contain up to 3% additional oil (Woodroof, 1973). Peanut oil contains 76-82% unsaturated fatty acids, of which to 45% are unsaturated oleic acid, and 30 to 35% are polyunsaturated linoleic acid (Woodroof, 1973). Analyses of 16 varieties and treatments of peanuts using gas chromatography has shown that at least eight nutritionally essential fatty acids are present (Fore et al. 1953).

Peanut oil is fairly stable in that the iodine number, saponification number, acetyl number, and free fatty acids do not change during heat treatments involved in the manufacture of peanut butter or salted peanuts (Woodroof, 1973). Oil of peanut cotyledons is more stable with better flavor than that in the hearts (Dieckert and Morris 1958; Pickett 1946).

#### **2.1.4 Carbohydrates in peanuts**

Peanut cotyledons naturally contain about 18% carbohydrates while the skin contains only 1% (Watt and Merrill, 1963). Peanut meal has about 32% carbohydrates. The starch content of peanuts varies from 0.5 to 5% depending upon the type, growing conditions, and maturity; the starch content of peanut meal is 6.7%. The pectic substance of peanuts is a galactoaraban-pectic acid complex (Freeman et al. 1954). Sucrose is reported to constitute 4-7% of peanuts; phenylosazones of galactose were also found (Woodroof, 1973). The browning reaction accounted for the principal changes occurring in color and flavour during roasting, with hydrolyzed sucrose as the major carbohydrate involved (Woodroof 1973). Peanuts have some crude fiber. Cellulose substances react with amino acids slowly at lower temperature and very rapidly at high temperature (Woodroof, 1973).

### **2.2 Peanut butter**

Peanut butter is an excellent protein supplement and a versatile food with unique and desirable flavor (Young et al., 1974). The caloric value and protein, thiamine, riboflavin, and niacin contents of peanut butter compare favorably with other high-protein food products such as beef and dry beans (Clay, 1941). There are three textures of peanut butter: smooth (very even texture with no perceptible grainy particles), regular (definitely grainy texture with perceptible peanut particles not more than 1/16 in. in any diameter), and chunky where there are partially fine and partially grainy particles with substantial amounts larger than 1/16 in. in diameter (Woodroof, 1973).

#### **2.2.1 Processing of peanut butter**

The processing of peanut butter is relatively simple, consisting of successive steps of shelling, dry roasting, cooling, blanching, picking and inspecting, fine-

grinding, and packaging. Salt is added to improve the flavor, small quantities of other material such as hydrogenated fat and dextrose are usually added, as well as corn syrup solids, glycerine, or lecithin to prevent oil separation, and antioxidants to control rancidity (Woodroof, 1973).

Peanut butters may be made from any variety of peanuts. However, a blend of two parts Spanish and Runner peanuts with one part Virginia peanuts is considered best for the required consistency (Woodroof, 1973). Some peanut butters have a heavy-roast color and flavour, others have light-roast characteristics, and there are many gradations in between (Freeman et al., 1954).

### **2.2.2 Roasting effects on peanuts**

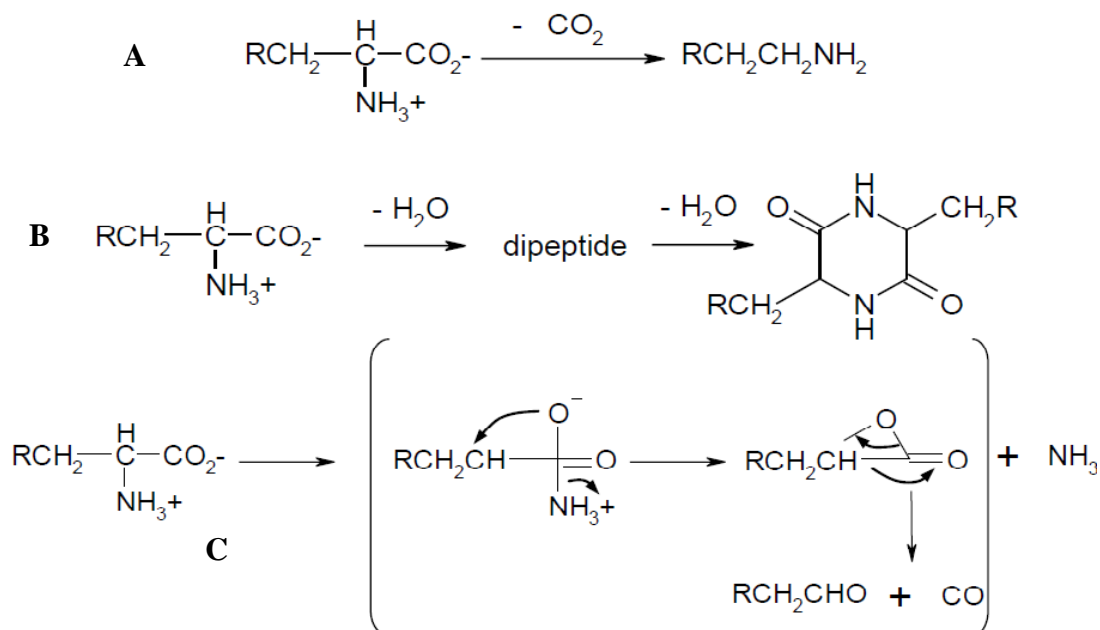
Roasting is the first step in processing of peanut butter. Peanuts used in the manufacture of peanut butter are dry roasted by batch or continuously. The normal moisture level of peanuts is about 5%, but this is reduced rapidly to about 0.5% during roasting (Woodroof et al., 1949b). Drying is followed by the development of oily translucent spots on the surface of cotyledons, called 'steam blisters', caused by oozing of oil from the cytoplasm as free oil. Change in color early in roasting is due to the cell walls becoming wet with oil. This stage is referred to as 'white roast'. The skins, too become wet with oil, and darker in color. The final stage of roasting is the development of a brown color, referred to as brown-roasting (Woodroof and Leahy, 1940). Color and flavor of peanut butter are determined by the extent of brown roasting. Willich et al. (1952) found that the time of roasting peanuts is the most important variable affecting the color of roasted product. The medium-roasted peanuts exhibit the most desirable flavour and flavour retention as compared with butter from light-roasted or dark-roasted peanuts (Morris and Freeman, 1954). Also, the moisture content becomes proportionately lower as the darkness increases.

In addition to its critical role in developing color and flavor in peanuts, heat plays a counter role in reducing nutritional value of peanuts. Table 3 showed the amino acid composition of peanut protein (Woodroof, 1973). At least 16 of these amino acids are found in free form in peanuts, and all appear to be involved in peanut roasting reactions. Proximity of the amino group to the carboxyl group in amino acids influences carbon dioxide evolution in the browning reaction. Moderate heat denatures the proteins, but apparently does not alter their nutritive value. However, high heat markedly degrades many nutrients. The greatest decrease in lysine content occurs when peanuts are subjected to high temperatures for the longest time. Higgin et al. (1941) found that roasting and blanching peanuts for peanut butter reduced the thiamine chloride content from 9.6 mcg per gram to 0.7 while niacin remained unchanged (Woodroof, 1973). For this reason and to avoid development of burnt flavors, high roasting temperatures are undesirable.

High fat and protein contents make peanuts particularly susceptible to degradation by heat and oxidation during roasting and storage, respectively. The effect of roasting heat on peanut protein stability has been extensively studied (Neucere et al., 1969; Ory et al., 1970; Labib et al., 1977). As with lipids, oxygen adds to protein radicals to form protein hydroperoxides that subsequently decompose to protein carbonyls (Kowalik-Jankowska et al., 2004). These can be detected by reaction with dinitrophenyllhydrazine in solution or in antibody reactions on Western blots (Kuzmenko et al., 2001). Both radicals and carbonyls provide hot sites for subsequent reactions.

Pyrolysis of certain amino acids (e.g. Gly, Thr, Ala, and Lys.) to form imidazo quinolines (B, below) is theoretical feasible at roasting temperatures above 430°C. However, under the conditions of normal food processing, imidazo quinolines

compounds are generally present only at very low concentrations (Moldoveanu, 2009). The primary reactions of amino acid pyrolysis are decarboxylation followed by condensation of the resulting carbonyls (A, below) (Ratcliff, 1973). Amino acids containing  $\alpha$ -alkyl substituents also lose ammonia (C) and form intermediate  $\alpha$ -lactones that subsequently yield ketones upon decarbonylation (Ratcliff, 1973).



Racemisation of amino acids from L to D conformation can also occur under extensive heating or treatment with alkali, which may be partly responsible for nutritional loss (Liardon and Hurrell, 1983).

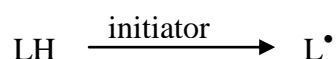
## 2.3 Degradation of peanut butter by lipid oxidation

### 2.3.1 General reactions

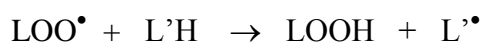
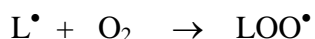
Lipid oxidation is well recognized as the major contributor to oxidative degradation in foods (Schaich, 2005). Polyunsaturated fatty acids are particularly prone to oxidation because the methylene groups between two double bonds have weakly bound hydrogens that can be easily abstracted to form free radicals (Kamal-Eldin and Pokorny, 2005). The most important indicators of lipid oxidation or

'rancidity' are off-odors and off-flavors from lipid oxidation products, particularly aldehydes (Schaich, 2005).

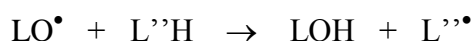
Lipid oxidation has been traditionally understood as a free radical chain reaction that proceeds in three stages -- initiation, propagation and termination. Lipid oxidation, although a facile reaction that appears to occur very rapidly, is not thermodynamically spontaneous and requires an initiator. Initiators of lipid oxidation are ubiquitous in foods and food processing, the most important of which are metals, other free radicals, heat, light (uv and visible with photosensitizers), and lipoxygenase enzyme (Schaich, 2012). These initiators provide the energy required to form the first lipid radicals.



These then add oxygen to form peroxy radicals, which in turn abstract hydrogens from a neighboring lipid molecule to propagate the chain reaction. The products are a lipid hydroperoxide and new lipid radical.



In branching reactions, the hydroperoxides are decomposed by light, heat, or metals to generate alkoxy radicals that accelerate the chain reaction by abstracting more hydrogens at a faster rate than the peroxy radicals (Kamal-Eldin and Pokorny, 2005).



The radical chain is finally terminated by recombination of radicals or other transformation of radicals into non-radical products, e.g.





Products include hydroperoxides, peroxides, epoxides, aldehydes, ketones, and alkanes. These are responsible for “rancid” off-odors and flavours but are also reactive and degrade further (Kamal-Eldin and Pokomy, 2005).

Questions about the validity of this view have been raised based on observed kinetics of reaction and generation of products at all stages of oxidation, not just at termination after the radical reactions have occurred (Schaich, 2005). Extensive free radical research, summarized by Kochi (1973) in what has become a classical text, shows that both peroxy and alkoxy radicals have alternate reactions that compete with hydrogen abstraction and indeed may replace hydrogen abstraction under some conditions. The main alternate reactions are internal rearrangement to epoxides and epoxides, addition to double bonds,  $\beta$ -scission of peroxy oxygen, dismutation of peroxy radicals, and scission of alkoxy radicals. Schaich (2005) presented arguments for existence of these alternate reactions and proposed a scheme that integrates alternate competing reactions into a concerted mechanism for lipid oxidation (Figure 2).

The concept of alternate pathways competing throughout the entire process of lipid oxidation is important for this study in that it brings forward a wide range of intermediates and products from the beginning of lipid oxidation rather than waiting for hydroperoxides to form and decompose. Multiple pathways are more consistent with patterns of damage observed in complex food systems and in model systems. As will be discussed in the next section and will be shown in results, availability of many reactive intermediates and products greatly increases the potential for side reactions



### **2.3.2 Specific observations in peanut butter**

Much research has been conducted on the physical properties of peanut butter because consumer acceptability and judgement of quality is based on peanut butter texture and color (Watters and Young, 1978). Peanut butter must maintain an optimum level of firmness, cohesiveness and spreadability, as well as sufficient adhesiveness to give a characteristic sticky texture. Sensory smoothness, hardness, spreadability, adhesiveness, cohesion, ease of swallowing and preference rating of peanut butter are affected by different levels of grind, size, sucrose and salt concentrations (Crippen et al., 1989).

Lipid oxidation in peanut butter also has been studied previously (St Angelo et al., 1972; St Angelo and Ory et al., 1972, 1973; Gills and Resurreccion, 2000; Felland and Koehler, 1997). Both enzymatic and non-enzymic catalysts are active (St Angelo and Ory, 1972). In particular, water content strongly influenced the stability of peanut butter, and salt and metalloprotein containing iron or copper were major catalysts of peroxide formation in peanut butter (St. Angelo and Ory, 1973). In another study of the physicochemical and sensory changes caused by oxidation of peanut paste, salts and heat-denatured proteins containing iron and copper were observed to catalyze oxidation of peanut paste (Muego-Ghanasekharan and Resurreccion, 1992).

## **2.4 Lipid co-oxidation of proteins**

### **2.4.1 General background**

The sensory effects of lipid oxidation are obvious and definitely lead to rejection of peanut butter and other foods by consumers. However, a less obvious and largely unacknowledged consequence of lipid oxidation is co-oxidation of other molecules present in food, particularly proteins (Schaich, 1980; Schaich, 2008). This

action is potentially more important than oxidation of lipids in the long run because it broadcasts oxidation beyond lipids and is responsible for extensive degradation in food quality and nutrition (Figure 3). Co-oxidations are likely to account for much of the damage that is normally attributed to lipid oxidation, and they provide footprints of lipid oxidation that are not determined in general assays of lipid oxidation (Schaich, 2008).

Although damage to proteins from oxidizing lipids has been recognized for decades, the field has progressed very little. Most early studies used a global approach, associating lipid oxidation with changes in general protein behaviours such as texture deterioration, crosslinking, loss of nutritive value, molecular functionality including enzyme activity, browning and color changes, as well as alteration in molecular function such as cell signalling and gene response and apoptosis in vivo (Figure 2) (Obanu et al., 1980; Dvorak et al., 1968; Horigome and Miura, 1974; Horigome et al., 1974; Kanazawa et al., 1974; Nielsen et al., 1985a, 1985b; Lqari et al., 2003; Schaich, 2008).

Model system studies with the isolated amino acids (Gardner and Weisleder, 1976; Petersen and Doorn, 2002; Wasinwright et al., 1972; Yong and Karel, 1978, 1979; Zamora et al., 1999) and purified proteins (Desai and Tapple, 1963; Zirlin and Karel, 1966; Schaich and Karel, 1975, 1976; Nielsen, 1978; Funes et al., 1982; Hildago and Kinsella, 1989; Hildago et al., 1999; Leaver et al., 1999; Hildago and Zamora, 2000) provided more information about the potential damage processes and reaction mechanisms, but these are not perfect models for reactions in proteins in foods (Schaich, 2008). Mechanisms of physiological protein oxidation have been studied extensively in model systems and cells and tissues (Stadtman, 2004). However, few comparable studies of protein oxidation mechanisms in foods have

been conducted.

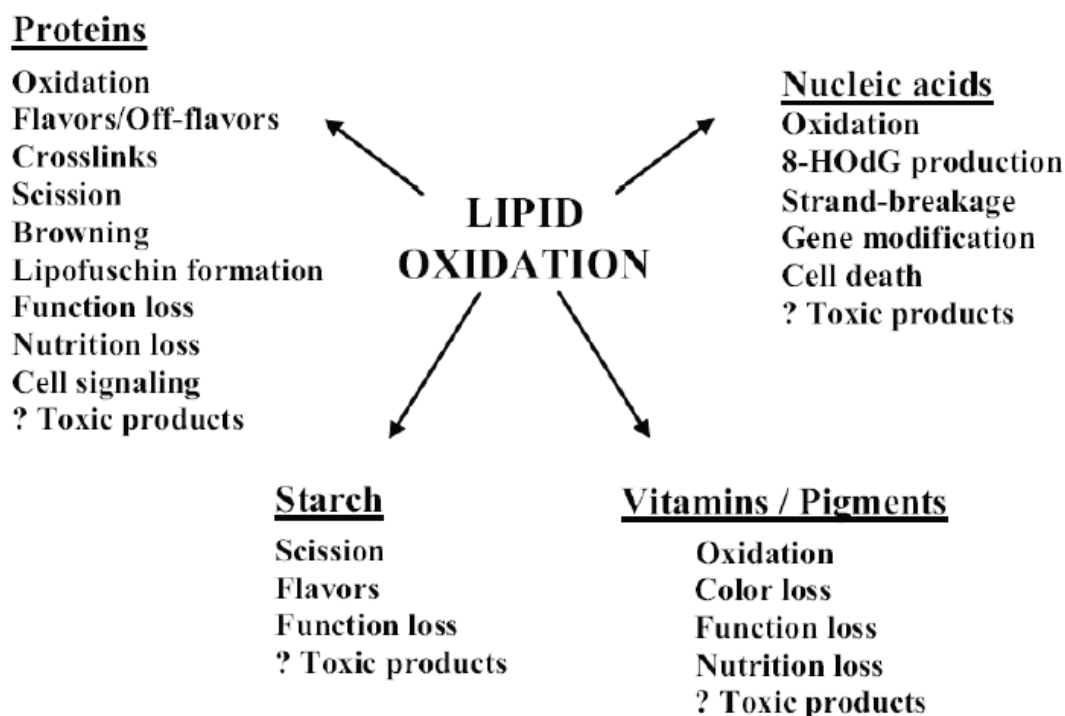


Figure 3. Types of damage that occur when oxidizing lipids co-oxidize cellular molecules in foods and living tissue (plant and animal). From Schaich (2008).

One factor that makes co-oxidation of proteins so difficult to study in foods and tissues is that literally every intermediate and product of lipid oxidation – especially radicals, hydroperoxides, epoxides, aldehydes – is capable of reacting with proteins (Schaich, 2008). Lipid radicals abstract hydrogens from proteins and generate potentially protein radicals that can persist for long periods of time (Schaich, 1976, 1976, 1980, 2008). Hydroperoxides hydrogen bond to proteins leading to induced decomposition in situ and subsequent H abstraction from or lipid radical addition to protein sites (Schaich and Karel, 1976). Epoxides bind to protein and form adducts (Lederer et al., 1996). Carbonyl products, particularly aldehydes, participate in a variety of addition reactions leading to the formation of adducts (Refsgaard, 2000), crosslinking of macromolecules fluorescent products, and browning. Because of the

dynamic nature of lipid oxidation, these four classes of lipid oxidation products react independently and sequentially, leading to crosslinking and polymerization, molecular scissions, surface modifications, changes in solubility, rearrangement of peptide aggregates, and a variety of other effects (Schaich, 2008). That most of the interactions lead to both surface modifications, changes in antibody recognition, and crosslinking, co-oxidation mechanisms cannot be distinguished by measurement of global properties, alone, but will require chemical analyses of the proteins at a level of detail that have not yet been undertaken.

Nevertheless, it is instructive to review what has been about lipid co-oxidation of proteins from model systems to illustrate similarities and differences between product effects and to provide a conceptual framework of expected behaviors that may be useful in interpreting and explaining observations in this and other co-oxidation studies in foods. These model system studies may also provide focal points for extended research in protein co-oxidations in foods.

## **2.4.2 Mechanisms of lipid co-oxidation of proteins**

### **2.4.2.1 Lipid radical reactions with proteins**

Transfer of free radicals from oxidizing lipids to proteins occurs rapidly in dry systems where radicals become stabilized on proteins (Schaich, 1976, 1980), and also in wet systems where they rapidly mediate subsequent reactions (K.M. Schaich, unpublished data). Stable EPR signals of protein radicals induced by lipid oxidation have been observed in a number of dry model systems (Roubal and Tappel, 1966a; Roubal 1970; Schaich and Karel 1975; 1976; Schaich, 1980b) and dry foods (K.M. Schaich, unpublished). The main targets for H abstraction and radical transfer are amino acids with amine or thiol groups, i.e. lysine, histidine, arginine, tryptophan, and cysteine, and EPR signals reflect electron density on the N and S atoms (Schaich

1976, 1980).

EPR signal intensities increase with extent of lipid and protein oxidation and give clear footprints of lipid-mediated damage (Schaich and Karel, 1975; Schaich, 1980a, Saeed et al., 1999, 2006). Consequently, EPR signals may be used in conjunction with lipid oxidation analyses to reveal the extent to which oxidation has been broadcast beyond lipids as well as to improve predictions of quality deterioration during processing and storage (Schaich, 2008).

#### **2.4.2.2 Reactions of lipid hydroperoxides with proteins**

Lipid hydroperoxides are not reactive species per se, unlike lipid radicals, epoxides, and carbonyl oxidation products. However, proteins incubated with lipid hydroperoxides react within minutes (e.g. EPR signals are generated), so possible roles of hydroperoxides beyond decomposition to free, diffusible alkoxyl radicals must be considered (Schaich, 2008). Under certain condition, kinetic analyses argue for the presence of lipid hydroperoxide-protein associations in which there is induced decomposition of LOOH and direct transfer of resulting LO(O)<sup>•</sup> to amino acid targets (Schaich, 1976). Metal contaminants, particularly iron and copper that are always present in tissue and lab reagents, decompose lipid hydroperoxides in solution and release free LO(O)<sup>•</sup> (Schaich, 2008). The unusual sensitivity of metallo-proteins is due in part to binding and reduction of LOOH in reaction cages, leading to oxidation of amino acids, particularly histidine, near the ligand site (Kowalik-Jankowska et al., 2004). Most non-metallo proteins also have metal binding sites (e.g. histidine, glutamic acid, aspartic acid) that can serve as foci for metal-catalyzed reduction of LOOH in cage reactions on protein surfaces (Yuan et al., 2007).

Rapid protein degradation suggested that intensive reactions were involved in substantial losses of tryptophan, methionine, cysteine, proline, valine, and leucine, as

well as fragmentation and crosslinking when lupine conglutins were reacted with MLOOH at pH 9 (Fruebis et al., 1992; Lqari et al., 2003), when butylamine was reacted with LOOH in  $\text{CHCl}_3$  (Zamora and Hildago, 1995), and in the very rapid formation of protein carbonyls and loss of lysine without lipid aldehydes when hydroperoxides of linoleic, linolenic, and arachidonic acids were incubated with bovine serum albumin (Refsgaard et al, 2000).

#### **2.4.2.3 Reactions of lipid epoxides with proteins**

Epoxides, also known as oxiranes, are cyclic products generated by internal rearrangements of lipid hydroperoxides (Hamberg and Gotthammar, 1973), internal additions of peroxy or alkoxy radicals to adjacent double bonds (Gardner, 1989; Schaich, 2005), or reaction between hydroxynonenal and lipid or hydrogen hydroperoxides (Chen and Chung, 1996). Lipid epoxides react extremely rapidly with amine groups on proteins because epoxy functions vicinal to olefin double bonds are particularly susceptible to hydrolysis and nucleophilic attack (Lederer, 1996). When association of a nucleophile (e.g. amine group on proteins) with the epoxide generates a partially charged transition state (Ingold, 1969), the oxirane ring opens easily because the three-member rings is strained and at a higher energy level. The oxygen stays on the less highly substituted carbon and the nucleophile adds to the opposite carbon (the allylic carbon when there is an adjacent double bond) from the backside (Ege, 1999; McMurray, 2000). This basic process is shown in Figure 4 for reaction of lysine, histidine and cysteine (left to right) with a hypothetical isolated epoxide from oxidation of linoleic acid. Lipid epoxide reactions with proteins have been demonstrated in model systems (Lederer, 1996; Lederer et al., 1998; Moll, 1999; Moll et al., 2000) but not clearly in intact foods because of difficulties in tracking products.



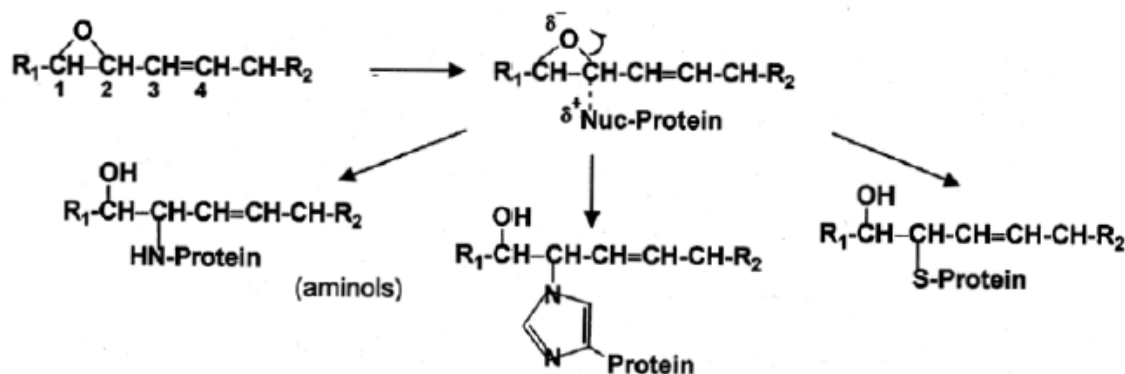


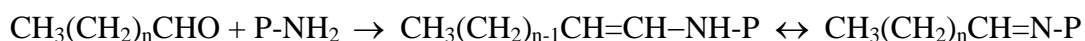
Figure 4. Epoxide reactions with amino acids (from Schaich, 2008).

#### 2.4.2.4 Reactions of lipid aldehydes with proteins

Secondary lipid oxidation products are responsible for the off-odors and flavors associated with rancidity and they also appear to be long lived in biological tissue (Gardner et al., 1977; Gardner and Jursinic, 1981; Yildiz et al., 1998; McMurray, 2000). Most research has focused on aldehyde reactions even though scission reactions of lipid alkoxyl radicals generate a wide variety of other reactive products as well (Suyama and Adachi, 1979; Gardner et al., 1977; Gardner and Jursinic, 1981; Yildiz et al., 1998; Nazir et al., 2000; Schaich, 2008). All aldehydes react with nucleophilic groups on proteins to form adducts, with four possible outcomes of increasing complexity:

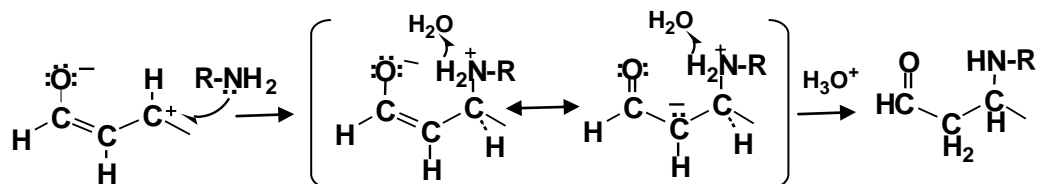
**1) linear adduct formation (1:1 lipid:protein) via Schiff base formation**

(carbonyl-amine condensation),



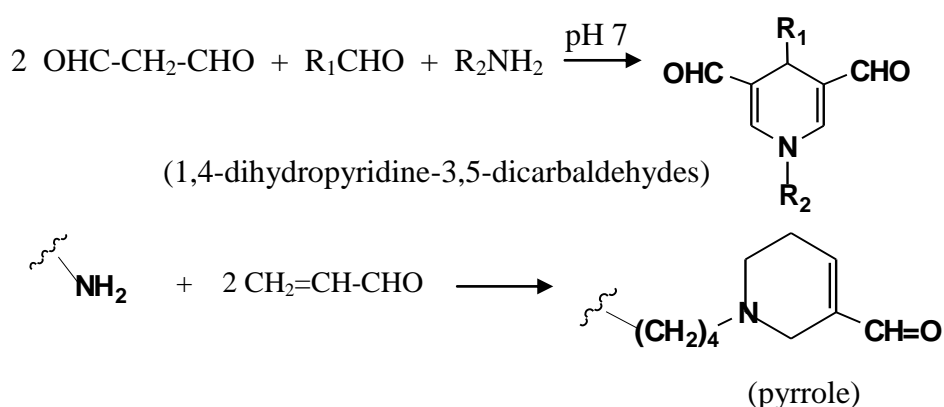
(P-NH<sub>2</sub> is an amine on a side chain of protein P)

**2) Michael addition of amines to unsaturated aldehydes,**



or a combination of both. Such adducts change surface chemistry and protein recognition by antibodies. This is the initial step for all aldehydes.

**3) cyclic adduct formation**, especially dihydropyridines and pyrroles, via multiple aldehydes condensing with one amino acid on a single peptide chain, e.g.



Note that a key result of all Michael additions is addition of a carbonyl to the surface of the reacting protein.

**4) intra- and inter-molecular protein crosslink formation** via Schiff base, Michael addition, ring linkages, or a complex combination of any of these (Schaich, 2008). These reactions will be discussed in detail in Section 2.4.3.4 on crosslinking.

The reaction pathways that occur or dominate in a given system are influenced by the nature of the protein, relative protein-aldehyde concentrations, pH, phase or solvent, oxygen tension, and many other factors (Schaich, 2008).

## 2.4.3 Types of protein damage caused by lipid co-oxidation

### 2.4.3.1 Destruction of amino acids

Numerous studies have documented amino acid losses in protein reacted with oxidizing lipids (Desai and Tapple, 1963; Roubal and Tappel, 1966b; Gamage et al.,

1973; Horigome and Muira, 1974; Kanazawa et al., 1975; Matoba et al., 1984b, Nielsen et al., 1985b). Cysteine, tryptophan, histidine, arginine, tyrosine and methionine which are critical nutritionally always appear with major losses. Interestingly, except for methionine, these amino acids are located primarily on protein surfaces where they are in closest contact with oxidizing lipids (Figure 5). In addition, they have readily abstractable hydrogens so are prime targets for radical transfer from oxidizing lipids. Cysteine, histidine, lysine and arginine all form stable radicals when reacted with oxidizing lipids (Schaich, 1976). These same amino acids are major targets of epoxides and of aldehydes, the latter with formation of Schiff base and Michael adducts as well as cyclic products. Thus, loss of these amino acids can occur at any stage of lipid oxidation, although changing their pathways and final products as oxidation progresses (Schaich, 2008).

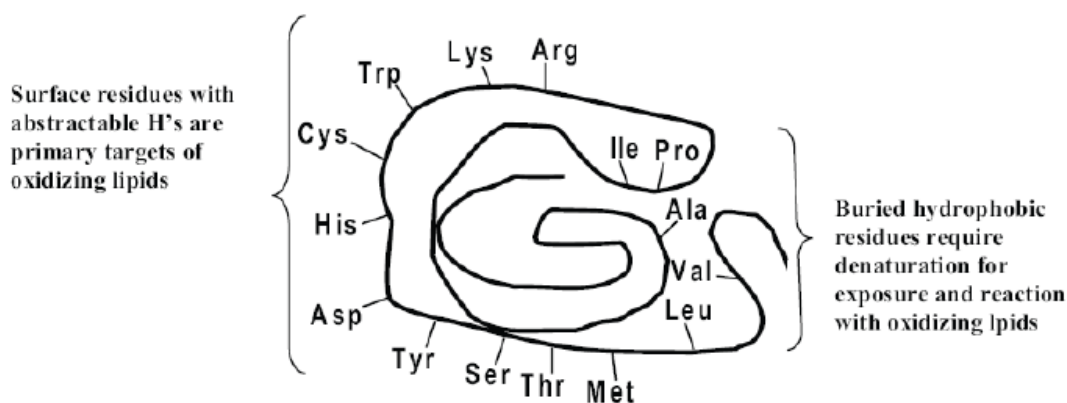


Figure 5. Diagrammatic representation of amino acids most susceptible to attack by oxidizing lipids. From Schaich (2008).

Amino acids such as serine and threonine also show significant losses in proteins reacted with oxidizing lipids (Shi et al., 2007; Halligudi et al., 2000). Although found primarily on protein surfaces, these two amino acids should not be expected *a priori* to be favorable targets for oxidizing lipids because their pKs are greater than 14 and their -OH bond energies are too high for facile H abstraction (Schaich, 2008). However, these amino acids hydrogen bond to lipid hydroperoxides and may thus induce LOOH decomposition by molecular-assisted homolysis. The end result is radical transfer in cage reactions to produce amino acid side chain radicals and subsequent hydroperoxides and breakdown products (Huerta et al., 1997; Shi et al., 2007; Halligudi et al., 2000; Schaich, 2008).

Hydrophobic amino acids such as glycine, alanine, valine, proline, leucine and isoleucine have no readily abstractable hydrogens, do not take part in hydrogen bonding, and are buried in the interior native protein, yet surprisingly show notable losses due to lipid oxidation (Horigome and Miura, 1974; Horigome et al., 1974, Schaich, 1980a; Bobrowski and Schöneich, 1996; Huerta et al., 1997; Guitton et al., 1998). These hydrophobic amino acids do not become exposed without denaturation, so it is not surprising that damage to these amino acids has been reported primarily where denaturation is facilitated (Horigome and Miura, 1974), such as in proteins incubated with oxidizing lipids at higher temperatures (55-60°C). One explanation that has been proposed, based on analogies to radiation chemistry, is that some of the electrons that delocalize along the peptide backbone also migrate to the methylene groups on the side chains of these amino acids (Schaich, 2008). These amino acids also appear to be particularly susceptible to decarboxylation and deamination (Schaich, 1980a, Bobrowski and Schöneich, 1996; Huerta et al., 1997; Guitton et al.,

1998). Thus, another possibility is that they are preferentially damaged when positioned at either end of protein chains.

#### **2.4.3.2 Loss of solubility**

Modifications of amino acids in the course of oxidation, scissions, or adduct formation are reflected in protein properties. One property that is almost universally modified in co-oxidized proteins is decreased solubility. Oxidation can alter protein surface charges which in turn can induce marked changes in conformation or structure of proteins (Haberland, 1982, 1984) that alter intermolecular associations, hydration, and solubility. Increased hydrophobicity caused by denaturation or complexation of lipids to protein (Lea, 1957; Davies and Designore, 1987) decreases water solubility while increasing solubility in SDS and alcohols. Protein extractability is similarly affected by these changes.

Mechanisms contributing to loss of solubility differ with protein, the extent of lipid oxidation or specific lipid oxidation product reacted, and the reaction system (Schaich, 2008). For example, protein solubility was reduced by nearly one-half when soy protein was incubated with soy phospholipids. Changes responsible included protein crosslinking, oxidation of sulfhydryls to products not reducible by mercaptoethanol, and production of protein carbonyls (Boatright and Hettiarachchy, 1995).

#### **2.4.3.3 Surface modification leading to decreased enzyme activity, loss of dye binding, impaired protein functionality**

Destruction of amino acids changes protein conformation and association, hydration, and interactions with surrounding molecules. As a consequence, protein functionalities are also impaired. Protein co-oxidation caused loss in enzyme activity such as sulfhydryl enzymes (Tsen and Tappel, 1958; Little O'Brien, 1967, 1968),


metallo-enzymes (Benderdour et al., 2003; McKnight and Hunter, 1966; Mitchell and Petersen, 1991; Tsuchiya et al., 2005). Other impaired functionalities observed in co-oxidized proteins are impaired baking properties of store flours (Lea, 1957), decreased gel strength and water holding capacity of chicken myofibrils (Smith et al., 1990) and gelatin (Matoba et al., 1984a), and loss of fibril formation in soy proteins (Boatright and Hettiarachchy, 1995). Membrane leakiness and impaired  $\text{Ca}^{2+}$ -accumulation in muscle mitochondria also resulted from protein co-oxidation (Player and Hultin, 1978). Unfortunately, mechanisms responsible for the impairment were not identified in any of these studies.

#### **2.4.3.4 Peptide crosslinking**

Crosslinking is perhaps the most common and dramatic effect of lipid co-oxidation of proteins (Schaich, 2008). Crosslinking can be induced by any of the lipid oxidation intermediates and products and leads to the loss of functionality (Borovyagin et al., 1984; Gebicki and Gebicki, 1999), changes in food texture (El-Gharbawi and Dugan, 1965; Gerrard, 2002), decreased protein solubility (Haberland, 1982, 1984; Huang et al., 2006) and browning (Riley and Harding, 1993; Yuan et al., 2007). The mechanism causing the crosslinking is determined by the nature of protein, the unsaturation of the peroxidizing lipid, the types and levels of lipid oxidation products present, the proportions of lipid and protein, and the reaction system (particularly moisture), and the extent of lipid oxidation. Four fundamental mechanisms are most active and change in balance as oxidation progresses.

*Mechanism 1 – free radical crosslinking.* Crosslinking by free radicals occurs first, even with relatively low levels of total oxidation, fueled by lipid radical transfer to proteins. The general reaction for free radical crosslinking (polymerization) of intact peptides may be described by the following reaction,



where  $\text{P}^\bullet$  are protein radicals (may be  $\text{C}^\bullet$  or  $\text{N}^\bullet$  radicals on a peptide backbone or  $\text{N}^\bullet$ , thiyl- $\text{S}^\bullet$ , or tyrosyl phenoxyl  radicals  $-\text{O}^\bullet$  on amino acid side chains.  $\text{P}^\bullet$  may remain localized on the side chain or migrate along the peptide backbone and become stabilized on glycine or alanine residues (Schaich, 1980b).

Lipid radicals and hydroperoxides are major early sources of protein radicals which recombine to generate dimers, trimers and higher polymers within short incubation reaction times in systems with actively oxidizing polyunsaturated fatty acids and esters. The reactions and some consequences are shown in Figure 6.

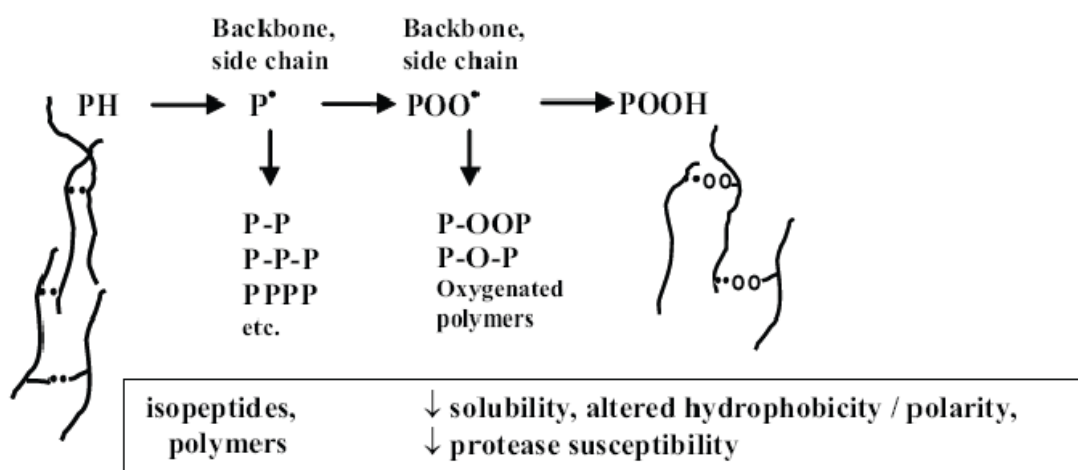
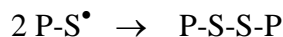
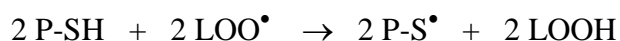


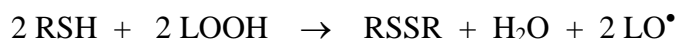
Figure 6. Reactions involved in free radical crosslinking induced by oxidizing lipids. Polymers of intact protein monomers or of protein fragments, with and without oxygen bridges, lead to decreased solubility and changes in physical properties (From Schaich, 2008).

*Disulfide crosslinking* is a very important form of crosslinking, more important than radical peptide crosslinking in high sulphur proteins such as zeins (Dong, 2010). Because thiol groups are strong targets for lipid radical transfer

(Schaich, 1976, 1980), this crosslinking probably proceeds via free radical intermediates.

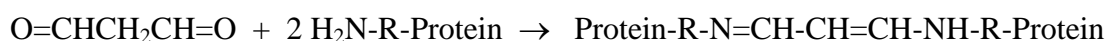


Lipid hydroperoxides also enhance thiol oxidation to disulfides:

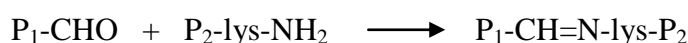


As oxidation progresses, crosslinking by one or more mechanisms involving aldehydes becomes activated. The mechanism that dominates in the system is influenced by pH, relative concentrations of amines and carbonyls, amino acid availability and orientation on the protein surface, and solvent environment (Schaich, 2008).

*Mechanism 2: Schiff base condensation of amines with saturated bifunctional aldehydes or other dicarbonyls to create bridges between protein chains:*



This type of crosslink also forms between amines on one protein chain and carbonyl oxidation sites on a second protein (Schaich, 2008):

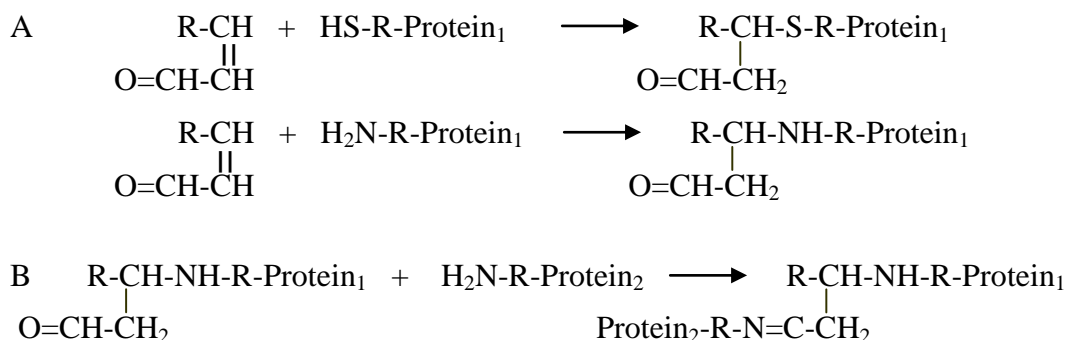


Schiff base crosslinks involve primarily lysine, histidine, glutamine, and cysteine, and are fluorescent with excitation 360 nm and emission 430-480 nm when they have conjugated structure  $\text{-N=CH-CH=CH-}$  (Chio & Tappel, 1969a; Nadkarni and Sayre, 1995).

*Mechanism 3 – crosslinking via Michael addition plus Schiff base.* Direct Michael addition of  $\text{-NH}_2$  or  $\text{-SH}$  groups in proteins generates lipid-protein adducts (A, below) that may or may not be fluorescent. However, the aldehyde carbonyl is left

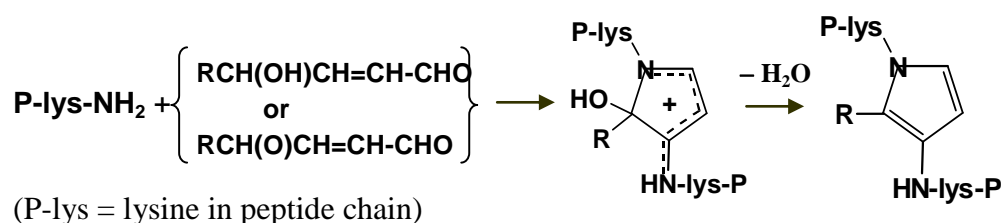


free in the reaction, and when this adds to free amine, imidazole, or thiol on other segment of the same protein or on a neighboring protein via Schiff base formation, a mixed Michael addition-Schiff base crosslink is generated (B, below) (Schaich, 2008).



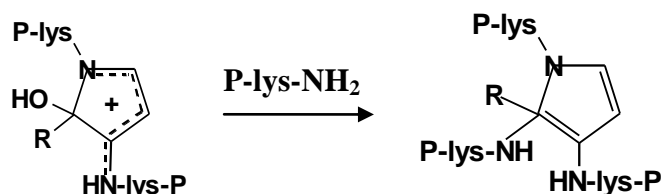
When conjugated structures are present, these linear crosslinks are fluorescent at 430 nm (Chio and Tappel, 1969).

*Mechanism 4 – crosslinking via ring structures such as pyrroles and pyridines formed in Schiff base / Michael addition reactions.* The most complex crosslinking occurs in later stages of oxidation. Both Schiff base and Michael addition products can react further via rearrangement, oxidation, reduction and secondary additions (Schaur, 2003), and variations in their secondary oxidations and rearrangement generate different types of crosslinks in later stages of oxidation. At low levels of secondary products (when peptides are still in excess), pyrrole cross links are formed when hydroxyl or keto derivatives of 2-alkenals (e.g. hydroxyl nonenal and oxononenal) cyclize between two protein nucleophiles such as lysine, histidine and cysteine. One example is shown in following reaction:

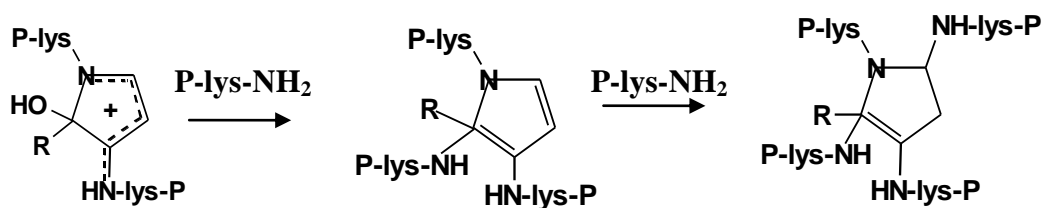


2-pentyl-2-hydroxy- 1,2-dihydropyrrol-3-one iminium link

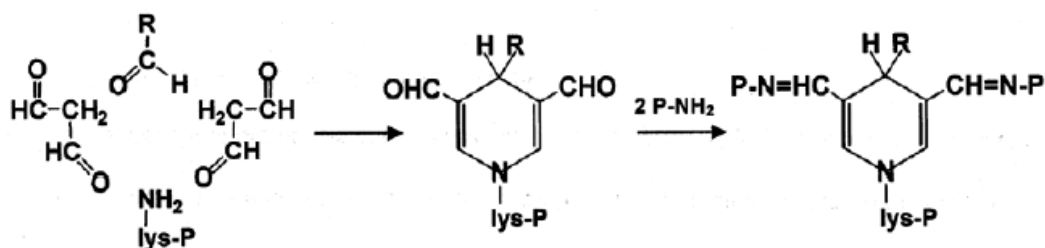
Once this process begins, crosslinking mechanisms can become quite complex as simple Schiff base and Michael addition reactions link additional protein chains to these structures. For example, lysine  $\epsilon$ -NH<sub>2</sub> groups condense with pyrrole -OH to form cyclic or acyclic mixed aminals (Jirousek et al., 1990):



Addition of a third protein nucleophilic link to a double bond in the pyrrole ring creates a protein trimer (Amarnath et al., 1994). Continued reactions of this type may be responsible for some protein aggregation that is observed in extended incubations.



As oxidation progresses further and aldehydes accumulate, pyrrole crosslinks shift to pyridinium crosslinks which are formed by multiple aldehydes reacting with a single amine via sequential Michael addition and Schiff base reactions. Since this ring formation requires malonaldehyde (Esterbauer et al., 1991) which only forms from fatty acids with three or more double bonds (preferably more), it occurs primarily in animal tissues. The exact pyridine structure is determined by the degree of aldehyde excess and nature of aldehyde condensing with malonaldehyde (Beppu, 1996).



Pyrrole (Hidalgo and Zamora, 1993) and pyridinium (Kikugawa and Ido, 1984; Kikugawa and Beppu, 1987) crosslinks are fluorescent with emission at 470 nm.

Considering all these potential pathways, multimodal protein crosslinking should be expected in the most proteins (Schaich, 2008). The dominant mode of crosslinking occurring will vary with the amino acid composition and the configuration of the protein as well as the stage of lipid oxidation and the variety of products present (Schaich, 2008).

#### 2.4.3.5 Peptide fragmentation

The opposite of crosslinking – fragmentation – also occurs in lipid co-oxidation of proteins, although less frequently, and conditions required for fragmentation remain controversial. Extensive scission of apoB-100 into smaller peptides by oxidized LDL has been observed (Fong et al., 1987). Scission occurred rapidly in lyophilized gelatin-methyl linoleate mixtures incubated dry at 50°C, but as moisture content increased, fragmentation changed progressively to crosslinking (Zirlin and Karel, 1969; Matoba et al., 1984a). However, comparable shifts were not observed in other dry lipid-protein systems (Schaich, 1976, 1980; Dong, 2010). Thus, it may be that fragmentation occurs only under extreme conditions and primarily with select proteins that have sensitive amino acid sequence (Schaich, 2008).

By its nature, fragmentation must involve some scission pathway, and scission of alkoxyl radicals provides a good model. Hydroperoxides form in proteins, just as in oxidizing lipids, and decomposition of these hydroperoxides generate protein alkoxyl

radicals. Both  $\alpha$  and  $\beta$  scission of protein alkoxyl radicals occurs, leading to different degradation patterns (Figure 6) (Stadtman and Berlett, 1997; Stadtman and Levine, 2003; Kowalik-Jankowska et al., 2004; Davies, 2005).

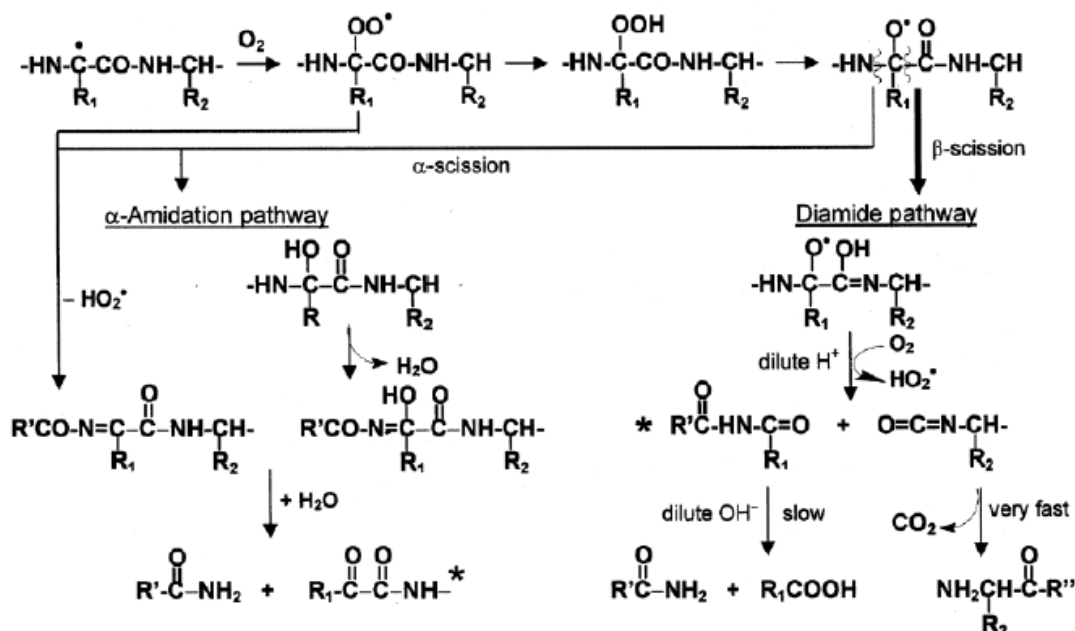


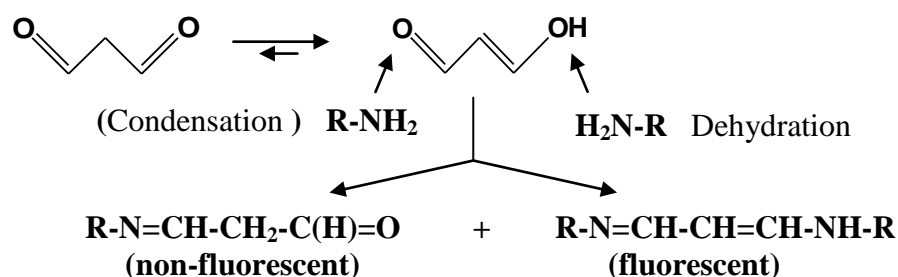
Figure 6. Protein oxidation and fragmentation processes resulting from transfer of free radicals from oxidizing lipids to  $\alpha$ -carbon sites on protein. From Schaich, 2008. Adapted from (Garrison, 1987; Davies et al., 1995; Stadtman and Berlett, 1997; Stadtman and Levine, 2003). R and  $\text{R}_2$  are amino acid side chains;  $\text{R}'$  and  $\text{R}''$  are continuations of the peptide chain.\*denotes carbonyl compounds detected in oxidation assays.

C-C or  $\beta$  scission is an oxidative process that decarboxylates the target amino acid (Garrison, 1987). Although the dominant process in oxygenated systems,  $\beta$ -scission occurs only under conditions of mild hydrolysis. Consequently, there are questions about whether scission occurs in situ or may be induced by sample treatment or preparation for electrophoresis (Hunt et al., 1988; Soyer and Hultin, 2000; Liu and Wan, 2005). In contrast, N-C or  $\alpha$ -scission is a reductive process that

deaminates the target amino acid (Garrison, 1987). Both scission processes generate amides, the carbonyl products determined in standard assays (Schaich, 2008).

#### 2.4.3.6 Formation of fluorescent products.

According to Tappel (1955), fluorescent ceroid age pigments in animal tissues and lipid-protein browning products in foods were co-oxidation products of polyunsaturated lipids and protein. Later research in his group identified N,N'-disubstituted 1-amino-3-iminopropene Schiff base structures generated by reaction of carbonyl lipid oxidation products, particularly MDA, with side chain and terminal amino groups on proteins, as the source of fluorescence (Chio and Tappel, 1969b; Fletcher and Tappel, 1970, Dillard and Tappel, 1971). The reaction mechanism is shown below:



Research in Tappels's group identified lysine as the most reactive side chain, followed by histidine, tryptophan and arginine, but showed that not all Schiff base products are fluorescent. Malshet and Tappel (1973) proposed that adducts are fluorescent only when an electron-donating group is present in conjugation with the imine. Complexes with this structure typically give fluorescence emissions around 430 nm.

However, since that pioneering research, fluorescence has been observed from both adducts and crosslinks formed between proteins and a wide range of lipid oxidation products, including hydroperoxides as well as a range of secondary

products, with many different structures in the fluorescent species (Kikugawa and Beppu, 1987; Hildago et al., 1999; Zamora and Hildago, 2003a). Model systems have identified fluorescence emissions around 470 nm as arising predominantly from cyclic products such as pyrroles and pyridines (Chio and Tappel, 1969a; Fruebis et al., 1992; Yamaki et al., 1992). However, there is considerable variation in exact excitation and emission maxima for different fluorescent structures and with different amino acids involved, as is shown in Table 4. Thus, although this is a common practice, individual emission wavelengths should not be used diagnostically to identify specific structures or even the presence of carbonyl-amine reactions *per se*. The presence of fluorescence with emission maxima about 430 and 470 nm are presumptive evidence for Schiff base and Michael addition reactions, but detailed structure analysis is required to conclusively identify the reactions and lipid/protein reactants involved.

Table 5. Variations in excitation and emission maxima for Schiff base fluorescence from different amino acid and protein substrates.<sup>a</sup>

From Schaich (2008).

Amine	Oxidant	Solvent	Ex (nm)	Em(nm)	Reference
Lysine	MDA	CHCl <sub>3</sub>	395	470	(Chio & Tappel, 1969a)
Lysine	hexanal	10% ethanol	345	415	(Dalsgaard et al., 2006)
Lysine	heptadienal		382	434	(Yamaki et al., 1992)
Lysine	alkanals				
Polylysine	LOOH, AnOOH	pH 7 buffer	330	425	(Fruebis et al., 1992)
Polylysine	MDA	pH 7 buffer	398	470	(Fruebis et al., 1992)
		0.5% SDS,			
Glycine	LOOH	pH 7 buffer	360-370	435-450	(Chio & Tappel, 1969b, Shimasaki et al., 1982)
Glycine	MDA	water	370	450	(Chio & Tappel, 1969b)
Glycine	alkanals+ H <sub>2</sub> O <sub>2</sub>		345-375	415-440	(Chen et al., 1996)
Glycine	heptadienal		397	470	(Yamaki et al., 1992)
Ethylamine	HO-butanal	pH 7 buffer	327	390	(Liu & Sayre, 2003)
Valine	MDA	water	370	450	(Chio & Tappel, 1969b)
Leucine	MDA	water	370	450	(Chio & Tappel, 1969b)
	β-oxyacrolein	neat	390	550	(Buttkus, 1975)
Leucine	β-oxyacrolein	n-butanol	385	460	(Buttkus, 1975)
Glutathione	LOOH	pH 7 buffer	350	440	(Zamora et al., 1989)
Lysozyme	Oxidizing ML	dry emulsion	355	425	(Leake & Karel, 1985)
BSA	LOOH, SP	pH 7 buffer	350	425	(Hidalgo et al., 1999)
BSA	LOOH,	pH 7 buffer	330	425	(Fruebis et al., 1992)
	AnOOH				
BSA	LOOH	pH 7 buffer	360	427	(Shimasaki et al., 1982)
		pH 7 buffer/			
BSA	HNE	MeOH	360	430	(Xu & Sayre, 1998; Xu et al., 1999b)

RNAse	EtAn, MeLn	pH 7 buffer	390,395	470	(Chio & Tappel, 1969b)
RNAse	2-HO-heptanal	pH 7 buffer	325	409	(Liu & Sayre, 2003)
Porcine Myofibrils	oxidizing fibrils	pH 6 buffer	395	485	(Chelh et al., 2007)
Minced meat	5 aldehydes	(solid)	382	450-550	(Veberg et al., 2006)
Frozen sardines	oxidizing lipids	CHCl <sub>3</sub> -MeOH,	327	415	
		MeOH-water	393	463	(Aubourg et al., 1998)
Liver homogenate	oxidizing lipids		397	480	(Li et al., 2006)
LDLox	atherosclerotic plaques	pH 7 buffer	360	430	(Xu et al., 2000)
LDLox	HNE or ONE	pH 7 buffer/ MeOH	366	445	(Xu et al., 2000)
Yellow lipofuscin	in vivo	0.5% SDS,	400	620	(Kikugawa et al., 1994)
		pH 7 buffer			
Blue lipofuscin	in vivo	CHCl <sub>3</sub> -MeOH	350	430	(Kikugawa, 1994)
Amine pyridinium	MDA	pH 7 buffer	403	462	(Kikugawa & Beppu, 1987)
Amine pyridinium	MDA	CHCl <sub>3</sub>	390	446	(Kikugawa & Beppu, 1987)

<sup>a</sup> Abbreviations: MDA, malonaldehyde; LOOH, linoleic acid hydroperoxide; BSA, bovine serum albumin; SP, secondary products of lipid oxidation; AnOOH, arachidonic acid hydroperoxide; HNE, hydroxynonenal; EtAn or MeLn, ethyl arachidonate or methyl linolenate; LDLox, oxidized low density lipoproteins; ONE, oxononenal; SDS, sodium dodecyl sulfate.



### **3. INFORMATION GAPS, RATIONALE, AND SIGNIFICANCE OF RESEARCH**

The food industry is usually more interested in what changes occur in a product to affect consumer acceptability, while academic research has investigated both system effects in intact foods and chemical effects in model systems. A fair amount of information about kinds of changes that occur in parallel with lipid oxidation in high lipid, high protein foods, and some model systems have identified specific damage mediated by individual lipid oxidation products. However, direct causal relationships between lipid oxidation products and protein damage have not been established in foods, and specific lipid oxidation products responsible for different effects on proteins have not been identified. In addition, essentially no attention has been given to influence of food matrix on co-oxidations.

We are aware of no studies other than in our research group that attempt to integrate global food property modifications with specific oxidative chemistry in lipids and proteins to elucidate the chemical reactions underlying oxidative degradation of food qualities. Dong (2010) initiated an extensive program focused on co-oxidation reactions with studies of lipid and protein oxidation in baked vs fried tortilla chips. In this dry, relatively low-lipid food, extensive disulfide crosslinking was the dominant effects, with lesser contributions from free radical crosslinking. Modifications to surface residues were also noted but sources were not identified. In contrast, peanut butter is a high lipid, semisolid matrix in which the proteins come in closer contact with the oxidizing lipids. Detailed co-oxidation has not been previously studied in this type of system or with peanut proteins. Thus, what is learned about co-oxidation processes in peanut butter should provide insights into how food matrices affect lipid oxidation and interactions that lead to protein co-oxidation, how lipid

levels (especially high lipid) alter co-oxidation mechanisms, and how individual protein composition and characteristics determine susceptibility to different lipid oxidation products and direct the dominant effects on the proteins.

This study begins with an actual food for three reasons:

- 1) to elucidate reactions responsible for degradation during storage and thus provide information critical to improving stabilization of peanut butter which is very important nutritionally in food aid, military rations, and general diets;
- 2) to demonstrate to the food industry the importance of protein co-oxidations in deterioration of quality in stored foods, and hence the necessity of learning more about co-oxidation reactions and addressing co-oxidations in stabilization strategies;
- 3) to provide a framework for detailed model system studies focused on identifying specific peanut protein modifications mediated by lipid oxidation radicals, hydroperoxides, epoxides, and aldehydes.

What is learned from this study, integrated with Dong's results should be broadly applicable to predicting and stabilizing lipid co-oxidation of proteins, as well as maintaining nutritional value in other foods important for international food aid, military rations, and general consumption.

## 4. HYPOTHESES AND OBJECTIVES

### 4.1 Hypothesis

Roasting establishes some baseline modifications in proteins in peanut butter during processing, but this does not notably affect peanut butter quality. During storage, intermediates and products of lipid oxidation react with proteins in peanut butter, causing changes that go beyond roasting effects and markedly degrade both physical properties and chemical characteristics of peanut butter.

### 4.2 Specific objectives

1. Incubate peanut butter in MRE packaging at 25, 40, and 60 °C for 12 weeks to:
  - a) determine changes in physical properties -- moisture content, browning (color changes), and texture -- occurring in peanut butter during storage,
  - b) measure lipid oxidation as conjugated dienes, hydroperoxides, aldehydes, and free fatty acids to establish extent of oxidation and determine products available for reaction with proteins (or potentially already reacted),
  - c) measure protein modifications that develop during storage,
  - d) correlate lipid oxidation with protein modifications during storage,
  - e) correlate lipid and protein oxidation with physical changes in peanut butter during storage.
  
2. Elucidate the chemical modifications underlying physical changes in the peanut butter by measuring:
  - protein solubility – affected by availability and modification of surface residues, crosslinking vs fragmentation, denaturation
  - surface modification -- revealed in native polyacrylamide gel electrophoresis and in loss of dye binding
  - fragmentation -- presumptive evidence for  $\alpha$  and  $\beta$  peptide scission

crosslinking -- SDS polyacrylamide gel electrophoresis with and without reduction  
with 2-mercaptoethanol to distinguish disulfide crosslinks

differential response of albumin, globulin, and hydrophobic protein fractions –  
identification of protein fractions involved in co-oxidation and types of reactions  
occurring in each

protein oxidation (antibody with protein carbonyls) – verify presence of oxidized  
proteins and peptides involved

tryptophan fluorescence – presumptive evidence for involvement or destruction of  
tryptophan

fluorescence – presumptive evidence for carbonyl-amine reactions by Schiff base  
condensation or Michael addition

3. Correlate protein modifications with lipid oxidation to establish presumptive causal  
relationships.

## 5. MATERIALS AND METHODS

### 5.1 Overall experimental design

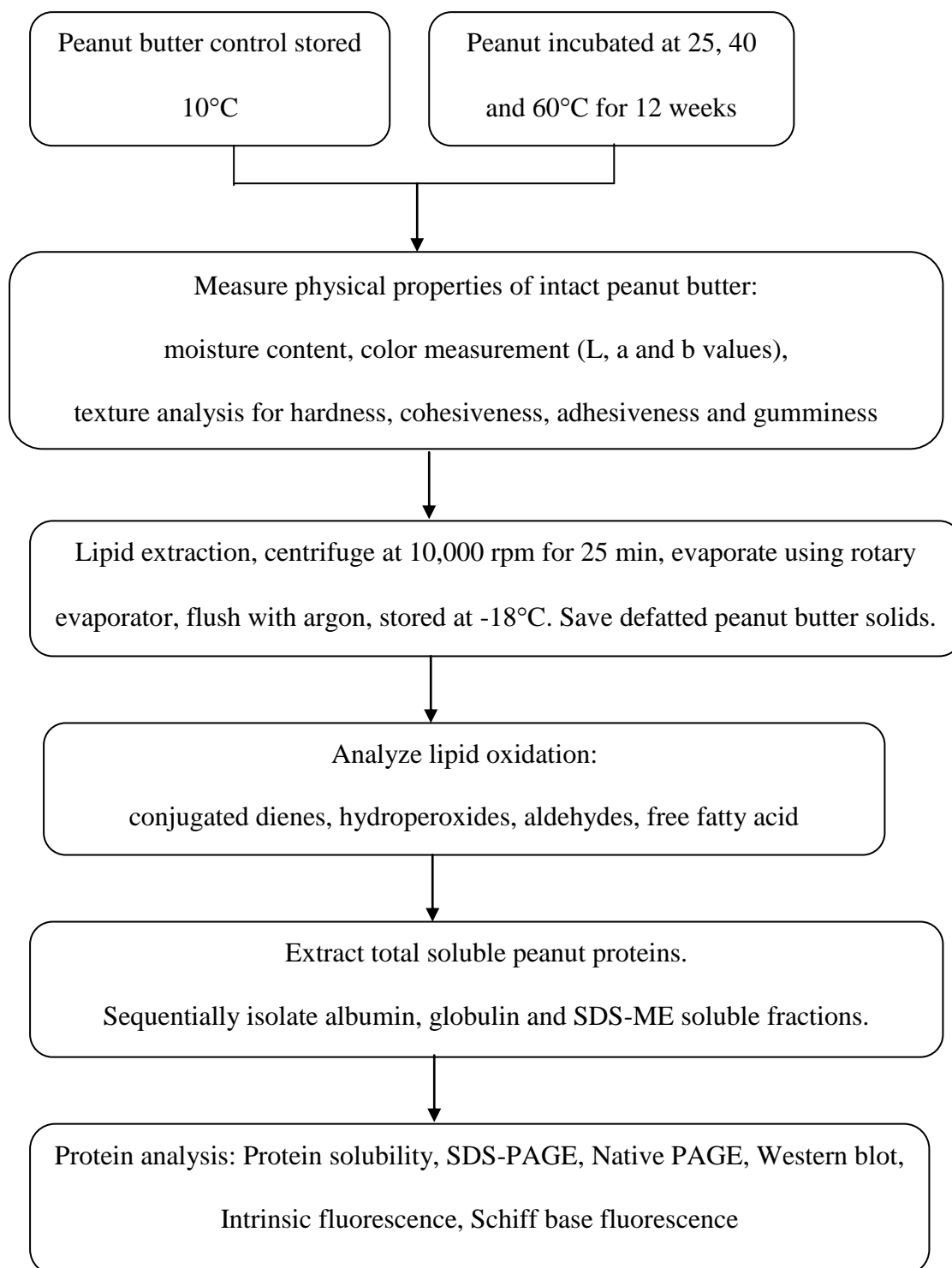


Figure 7. Experimental flow diagram for investigation of lipid and protein oxidation in peanut butter.

## 5.2 Peanut butter source and incubation

Peanut butter in 1.5 ounce laminated MRE packaging was provided by ThermoPac, a commercial supplier. The nutrition label from the product is shown in Figure 8.

All samples were stored at 10 °C until used. For shelf life studies, peanut butter samples were arranged in single layers on trays and incubated at 20, 40 and 60 °C for 12 weeks. Samples were removed weekly for analysis.

<b>Nutrition Facts</b>	
Serv. Size 1 pouch (43g)	
Amount Per Serving	
<b>Calories</b> 250	<b>Fat Cal.</b> 180
%DV*	
<b>Total Fat</b> 21g	<b>32%</b>
Sat. Fat 2.5g	<b>13%</b>
Trans Fat 0g	
<b>Cholest.</b> 0mg	<b>0%</b>
<b>Sodium</b> 200mg	<b>8%</b>
<b>Total Carb.</b> 10g	<b>3%</b>
Fiber 3g	<b>12%</b>
Sugars 4g	
<b>Protein</b> 10g	
Vitamin A 60% • Vitamin C 80%	
Calcium 2% • Iron 4%	
*Percent Daily Values (DV) are based on a 2,000 calorie diet.	

Ingredients: Roasted peanuts, sugar, hydrogenated vegetable oil (rapeseed, cottonseed, and/or soybean oils), salt, vitamin C\*, Vitamin A\*, Vitamin B6\*, thiamine\*.

\* Ingredients not in regular peanut butter. Contains peanut, soy.

Figure 8. Nutrition facts and ingredients of peanut butter.

## 5.3 Moisture content of peanut butter

The moisture content of peanut butter was determined using a HG63 Halogen Moisture Analyzer (Hg63, Mettler-Toledo, Columbus, Ohio). 1 gram samples were loaded onto tared aluminum sample pans and heated at 100°C to constant weight. Three replicates were analyzed for each sample.

## 5.3 Color

Peanut butter color (including visual lightness, redness and yellowness) was measured using a Konica Minolta portable colorimeter (CR-400, Konica Minolta,

Rmasey, USA). 5 g samples were loaded into small glass petri dishes, leveled, and L, a, and b values were recorded in triplicate for each sample.

#### **5.4 Texture profile analysis (TPA)**

Texture profile analysis of peanut butter was performed using a CT3-1500 Texture Analyzer (Brookfield Engineering Labs, Inc, Middleboro, MA, USA) equipped with a 5 kg load cell and TexturePro Ct software. The force and probe were calibrated according to the instrument's operation manuals before all analyses. Peanut butter samples of identical heights about 3 cm were placed in the middle of the texture analyzer platform to ensure precision alignment of the sample and probe. A 50 mm diameter cylindrical plunger with rounded surface was used to compress the sample uniaxially (sample is compressed in one direction and is unrestrained in the other dimensions) to 80% of its original height at a constant compression speed of 1 mm/sec (down-stroke as well as upstroke of the plunger). TPA parameters were obtained from the force-deformation curves. Hardness (N), cohesiveness, adhesiveness and gumminess TPA parameters were measured, as these were considered the most relevant mechanical properties for peanut butter. The hardness value was obtained from the maximum peak force (N) during the first compression cycle (1f), hardness, and gumminess was calculated as  $\text{gumminess} \times (\text{time to peak of compression curve} / \text{time to peak of compression curve 1})$  (Figure 9).

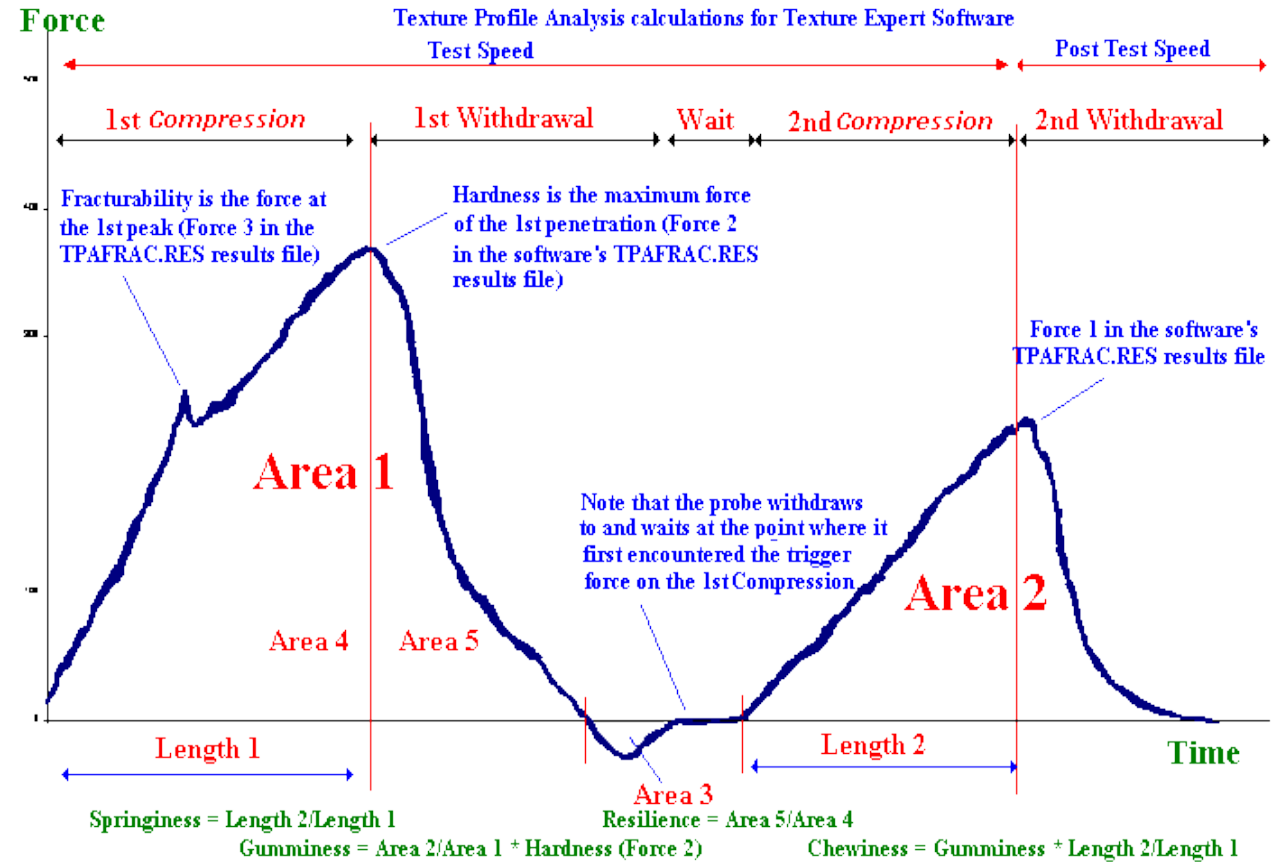


Figure 9. Texture profile analysis curve showing compression cycles, force peaks, and equations for calculating primary texture parameters (Texture Technologies, texture\_profile\_analysis.html).



## 5.5 Extraction of lipids

*Manual:* Peanut butter was mixed with chloroform: methanol (2:1) in 1:5 (w/v) ratio, flushed with argon and allowed to stand for 30 minutes, then centrifuged at 14000 g for 30 min, 4°C. The supernatant was collected, the extraction was repeated, and the two extracts were combined. The meal of peanut butter was dried for 2-3 hrs at room temperature and used for protein analyses.

After extraction, the supernatant was evaporated at 40°C on a Buchi rotary evaporator, and the extracted lipid of peanut butter was weighed to determine % lipid recovery based on initial sample weight. Dried lipid extracts were flushed with argon, sealed, and stored at -18°C until analysis.

## 5.6 Analysis of lipid oxidation

### 5.6.1 Conjugated dienes

Conjugated dienes in the lipid extracts of peanut butter were determined by a modification of AOCS standard method Th 1a-64. Thirty µl oil was diluted to 10 ml in iso-octane (HPLC grade). Absorbance at 234 nm was measured against iso-octane blank using quartz cells. When absorbance exceeded 1, samples were diluted in iso-octane until they read close to 1. Concentrations of conjugated dienes (mM) were calculated from Beer's Law using a molar extinction coefficient of 29500 for iso-octane:

$$\text{CD conc (mM)} = \text{OD} / \epsilon = \text{OD} / 29.5$$

### 5.6.2 Lipid hydroperoxides

SafTests are a set of commercially available kits (MP Biomedicals, Solon, OH) designed to quantitate lipid degradation products (peroxides, aldehydes, and free fatty acids) in samples.

For peroxide analysis, 25  $\mu$ l extracts were mixed with 1000  $\mu$ l PeroxySafe reagent A and 100  $\mu$ l PeroxySafe reagent B and incubated at room temperature (controlled at 25°C in a heating block) for 15 minutes. Absorbance of the reaction solutions was measured using 570/690 nm filter provided with the optical monitoring unit, and concentration was calculated directly by endogenous software, based on a curve prepared from provided standard and saved in the processor.

### **5.6.3 Aldehydes**

For aldehyde analysis, 70  $\mu$ l extracts were mixed with 1000  $\mu$ l AlkalSafe reagent A and 250  $\mu$ l AlkalSafe reagent B and incubated at room temperature (controlled at 25°C in a heating block) for 20 minutes. Absorbance of the reaction mixture was recorded using the 550/660 nm filter provided with the optical monitoring unit; aldehyde concentration was calculated directly by endogenous software.

### **5.6.4 Free fatty acids**

For free fatty acid analysis, 50  $\mu$ l extracts were mixed with 1000  $\mu$ l FA-Safe reagent A and 100  $\mu$ l FA-Safe reagent B and incubated at 37°C-44°C on a heating block for 10 minutes. Absorbance of the reaction, was recorded using the 550/690 nm filter provided with the optical monitoring unit; concentration was calculated directly by endogenous software.

## **5.7 Extraction of peanut proteins: procedure optimization**

Peanut proteins, although hydrophilic, are notoriously difficult to extract fully. Previous methods developed for peanut butter were inefficient with the current peanut butter samples, which it turns out, were roasted longer than previous batches. Hence,

new procedures had to be developed to maximize extraction of proteins. Extraction systems tested include:

*Methods 1a and b (with and without pre-extraction of lipid).* Meals were extracted with 10% sodium chloride (NaCl) in 18 MΩ water at pH 7.4 (adjusted with NaOH) in 1:10 (w/v) ratio for 1 hr with constant shaking at 150 rpm, then the mixture was centrifuged at 14,000 g for 30 min at 4°C. The supernatant was collected, flushed with argon, and stored at -18°C for further analysis.

*Method 2.* No pre-extraction of oil was performed. Peanut butter was mixed with phosphate buffer in 1:5 (w/v) ratio and homogenized using a magnetic stirrer for about 30 min, then the mixture was shaken overnight at 40°C. The mixture was centrifuged at 14 000 g for 30 min at 4°C; the supernatant was then pipetted off and filtered through a Whatman #1 filter paper, flushed with argon, and stored at -18°C until analysis.

*Method 3.* Peanut oil was removed by mixing samples with chloroform: methanol (2:1) in 1:5 (w/v) peanut butter:solvent ratio. The mixture was centrifuged at 14000g for 30 min at 4°C and the supernatant was collected. The extraction procedure was repeated on the remaining solids and the supernatants containing lipids were combined. The peanut butter meal was dried for 2-3 hrs at room temperature. Dried, de-oiled meals were mixed with 10% NaCl containing 1.5% SDS in 18 MΩ at pH 7.4 in 1:10 (w/v) ratio and extracted overnight at 40°C with constant shaking at 150 rpm. The mixture was centrifuged and supernatant containing protein was collected as described above.

*Method 4.* Method 1a (with lipid extraction) was repeated but with overnight extraction at 40°C with constant shaking at 150 rpm.

*Method 5.* The de-oiled peanut butter solids were dried for 2 hrs at room temperature, and the proteins were extracted by mixing 1 g solids with 20 ml 0.2M sodium phosphate buffer, pH 7.9, 0.02% sodium azide for 48 hrs with constant shaking (150 rpm) at room temperature. The mixture was centrifuged at 14000 g for 30 minutes, and the supernatant was transferred to a sample vial, flushed with argon, and stored at -18°C until analyzed.

*Method 5* provided highest yields and was adopted as the standard operating procedure for this study.

## **5.8 Sequential fractionation of defatted peanut butter proteins**

Individual protein fractions were isolated sequentially to more clearly understand which protein fractions were being modified by lipids or heat.

*Albumin fraction:* Defatted peanut butter solids were extracted with Milli-Q water containing 0.02%  $\text{NaN}_3$  by shaking for 2 hrs at 40°C, then centrifuged at 1500 x g for 20 min at 15°C. The supernatants were collected without any filtration and stored at -18°C for further analysis.

*Globulin fraction:* Solids remaining after water extraction were re-extracted with sodium phosphate buffer (0.2 M, pH 7.9) containing 0.02%  $\text{NaN}_3$  at 40°C for 3 hrs. Extracts were centrifuged at 1500 x g for 20 min at 15°C. The supernatants were collected without any filtration and stored at -18°C for further analysis.

*2% SDS, ME fraction:* Solids remaining after sodium phosphate buffer extraction were re-extracted with sodium phosphate buffer (0.2 M, pH 7.9) containing 2% sodium dodecyl sulfate and 2-mercaptoethanol (no azide), then all samples were centrifuged at 1500 x g for 20 min at 15°C. The supernatants were collected without any filtration and stored at -18°C for further analysis.

## 5.9 Protein solubility

After extraction, protein concentrations of the supernatants were determined using the Bradford assay. Bradford reagent: dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use. Prepare bovine serum albumin standards containing 5 to 100  $\mu$ g protein/100 Milli-Q water (Table 5). For reaction, react 5 ml Bradford reagent with 100  $\mu$ l sample in sodium phosphate buffer (0.2 M, pH 7.9, containing 0.02%  $\text{NaN}_3$ ) for 15 min at room temperature. Read absorbance at 595 nm against Milli-Q water. Determine protein concentration from a plot of microgram protein versus absorbance.

Table 5. Serial dilution of standard BSA solution for protein concentration measurement.

Standard	BSA solution ( $\mu$ l)	Distilled water ( $\mu$ l)	Bradford reagent (ml)
1	10	90	5
2	20	80	5
3	40	60	5
4	70	30	5
5	100	0	5

Standard curves were generated by plotting protein concentrations versus A (Figure 9), and best fit (quadratic) regression equations for the curves were calculated using statistical functions in Excel software. Each standard curve was determined from averages of five sets of standard solutions and new standard curves were

generated each time fresh reagent was prepared. The equation was used to calculate protein concentrations in protein extract samples.

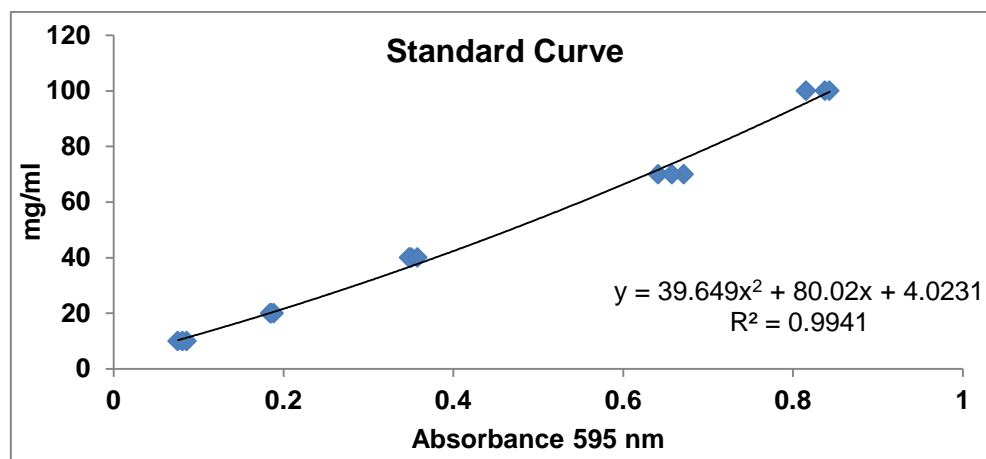


Figure 10. BSA standard curve for measurement of protein solubility. A separate curve was generated for each batch of Bradford reagent.

### 5.10 SDS-PAGE of peanut proteins

Soluble proteins were separated by SDS-PAGE following well-established procedures (Laemmli, 1970). Ten-well mini gels (10 cm H × 10 cm W × 1.0 mm thick) were run at room temperature using a Fisher Scientific Mini Vertical Gel System (Fisher Scientific, Pittsburgh, PA). Protein extracts were diluted to 1 µg protein/ml, then mixed 1:1 (v/v) in a dissociation buffer consisting of a 1 M Tris-HCl pH 6.8, 0.05% bromophenol blue, 35% glycerol, 5% β-mercaptoethanol, 8% (w/v) SDS solution, and heated in boiling water for 5 min; 25 µl aliquots were loaded into each well. Electrophoresis was run on polyacrylamide gels (stacking: 4% and resolving: 12.5%) with a migration buffer consisting of a 0.02 M Tris(hydroxymethyl)-aminomethane, glycine 5 M, SDS (w/v) 0.1% solution, at a constant current of 200 V until the bromophenol blue marker reached the bottom of the gel. Standard high molecular weight protein markers (205, 116, 97, 83, 66, 55, 45,

30, and 21 kDa) (Amershams, Biosciences) were run in one lane of each gel. After elution, gels were stained with silver and Coomassie blue staining.

*Silver staining:* gels were fixed for about 30 min with an alcohol–acetic acid aqueous mixture (Pierce Silver Stain Kit). Color development was halted by adding 5 % acetic acid solution with continuous shaking.

*Coomassie blue staining:* Gels were microwaved for 2 min in the staining buffer, washed several times with Milli-Q water, then microwaved again for 4 min in the destaining buffer. Destaining was completed by continuously shaking the gel on a paper towel inside a container until clear.

Gels were initially recorded by computer scanning or digital photographing then switched to a BioRad gel imager. This eliminates glare and provides a clearer image but records in black and white so color differentiation between dyes was lost.

### **5.11 Native PAGE**

Soluble proteins were separated by Native-PAGE following well-established procedures (Laemmli, 1970). Ten-well mini gels (10 cm H × 10 cm W × 1.0 mm thick) were run at room temperature using a Fisher Scientific Mini Vertical Gel System. Protein extracts were diluted to 1 µg protein/ml, then mixed 1:1 (v/v) in a dissociation buffer consisting of a 1 M Tris–HCl pH 6.8, 0.05% bromophenol blue, 35% glycerol) solution, and heated in boiling water for 5 min; 15 µl aliquots were loaded into each well. Electrophoresis was run on polyacrylamide gels (stacking: 4% and resolving: 12.5%) with a migration buffer consisting of 0.02 M Tris(hydroxymethyl)-aminomethane, glycine 5 M, at a constant current of 200 V until the bromophenol blue marker reached the bottom of the gel. Standard high molecular weight protein markers (205, 116, 97, 83, 66, 55, 45, 30, and 21 kDa) (Amersham

Biosciences, Piscataway, NJ) were run in one lane of each gel. After elution, gels were stained with silver and Coomassie blue staining.

*Silver staining:* gels were fixed for about 30 min with an alcohol–acetic acid aqueous mixture (Pierce Silver Stain Kit, Pierce Biochemicals, Rockford, IL). Color development was halted by adding 5 % acetic acid solution with continuous shaking.

*Coomassie blue staining:* Gels were microwaved for 2 min in the staining buffer, washed several times with Milli-Q water, then microwaved again for 4 min in the destaining buffer. Destaining was completed by continuously shaking the gel on a paper towel inside a container until clear.

## **5.12 Intrinsic and Schiff Base Fluorescence**

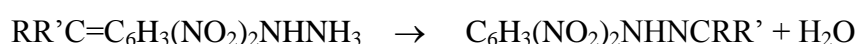
Intrinsic fluorescence from aromatic amino acids (primarily tryptophan) was determined in protein extracts (in extraction buffer) by recording fluorescence emission spectra from 300 to 550 nm, with excitation at 280 nm to detect all aromatics (2) using a SPEX Fluorolog 1 Fluorometer equipped with DM3000F software (HORIBA Jobin Yvon Inc., Edison, NJ). Emission intensity of the peaks at 315 (tyrosine and phenylalanine) and 340 nm (tryptophan) were measured. All spectra were recorded in synchronous mode that presented both excitation and emission curves.

Presumptive formation of Schiff base complexes between lipid carbonyls and protein amino groups was detected by recording fluorescence emission spectra of protein extracts between 400 and 650 nm, with excitation at 350 nm. All spectra were recorded in synchronous mode that presented both excitation and emission curves. Emission intensities were recorded at 430 nm (linear adducts) and 470 nm (ring adducts).



### 5.13 Protein carbonyl formation

An Oxyblot antibody kit (Millipore, Billerica, MA) was used to detect the carbonyl groups in oxidized proteins. 3.5  $\mu$ l protein extracts containing 2.5 to 5  $\mu$ g proteins were mixed with 3.5  $\mu$ l 12% SDS to adjust the SDS content to 6%. Then 7  $\mu$ l 1 $\times$  DNPH (2,4-Dinitrophenylhydrazine) were added to the mixture to convert protein carbonyls to dinitrophenylhydrazone derivatives by the following condensation reaction:



After 15 minutes of incubation at room temperature, 4.5  $\mu$ l neutralization buffer were added to stop the reaction. Peptides in derivatized samples were separated by electrophoresis in a stacking gel of 4% (T), 0.1% (C) and a resolving gel of 12.5% (T), 0.4% (C). Peptide bands separated on the gels were transferred onto PVDF membranes (Millipore, Billerica, MA) in a western blotting unit (Fisher Scientific, Houston, TX) using the following procedure. The PVDF membrane was soaked in methanol, then the polyacrylamide gel plus PVDF and blotting filters were immersed in transfer running buffer (25mM Tris, 192mM glycine, pH 8.3) to ensure electric conductivity when transferring the proteins. The transfer was conducted for 1 hr at a constant voltage of 100 V and the PVDF membrane was blocked with PBS-T% (w/v) BSA on a Model 5900 large reciprocal shaker (Ann Arbor, MI) for 1 hr and 15 minutes at room temperature. The membrane was incubated with 22.5 ml primary antibody (150  $\mu$ l primary antibody mixed with 22.5 ml PBS-T/1 % (w/v) BSA) for 1 hr in a shaker at room temperature. Membranes were washed with PBS-T, incubated with 22.5 ml secondary antibody (75  $\mu$ l secondary antibody mixed with 22.5 ml PBS-T/1% (w/v) BSA) for 1 hr at room temperature, washed again with PBS-T. Chemiluminescent (CL) reagents were prepared according to manufacturer's

instructions (1:1, 3 ml of hydrogen peroxide, 3 ml of luminol reagent) (BioRad, Hercules CA). Membranes were placed in the CL solution for 3 minutes at room temperature, then carbonyl bands were detected by using the Biorad Imager.

## 6. RESULTS AND DISCUSSION

### 6.1 Physical changes

Peanut butter incubated at the three test temperatures exhibited visible darkening in color and stiffening of texture that increased with storage time, particularly at 60 °C (Figure 10). Moisture, color, and texture characteristics were measured to quantitate these changes.

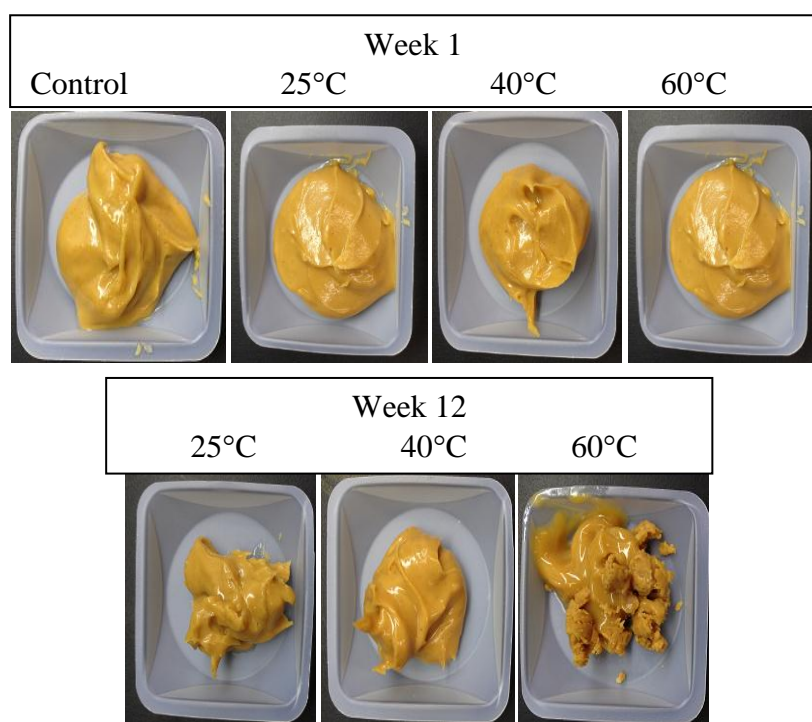


Figure 11. Visible color and texture changes in peanut butter incubated in MRE packages for 12 weeks.

#### 6.1.1 Moisture content

The starting moisture content of peanut butter used in this study was about 1.2%. This low value results from roasting of the peanuts (Pepper, Jr. and Freeman, 1953). Peanut butter stored at 4 °C served as a control. Figure 11 shows how the moisture content of peanut butter samples, determined by drying in a halogen

moisture analyzer, changed during storage under different temperatures. There were no significant differences in moisture contents between controls and samples incubated at 25°C or 40°C. However, at 60°C, the moisture content decreased slightly (~0.2%), starting at week 4 of incubation, then progressively dropped over the remainder of the incubation period. Given such minimal moisture loss, very little change in texture would be predicted.

The reduction of moisture content at 60°C could not have resulted from physical dehydration because the peanut butter is sealed in MRE packaging which consists of four layers of material including a moisture and oxygen barrier. Thus, explanation must be sought in chemical reactions that utilize water.

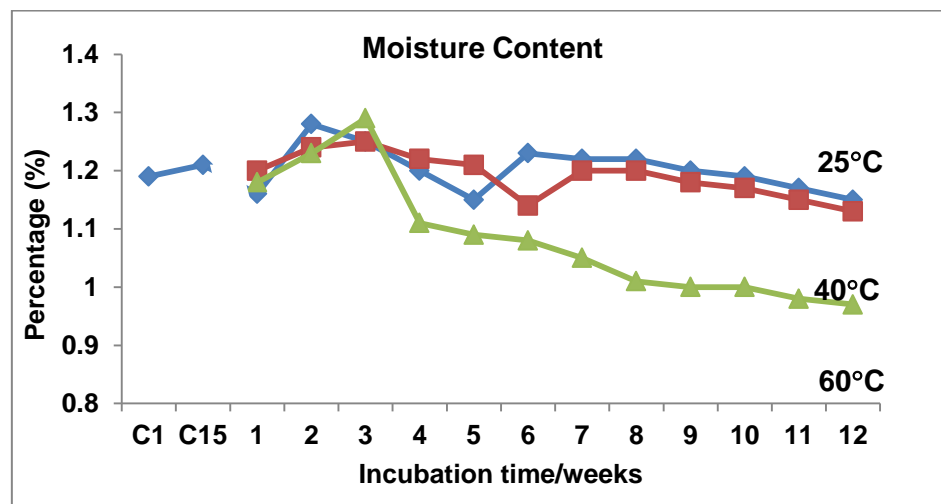


Figure 12. Effect of incubation time and temperature on moisture content of peanut butter stored in MRE packages.

### 6.1.2 Color changes in peanut butter

Raw peanuts are primarily colorless with high L values since the epidermal and parenchymal tissues lack chromoplast or other colored bodies (Abegaz and Kerr, 2006). The characteristic reddish-brown color of peanut butter is generated during

roasting when amino acids react with reducing sugars in a complex series of Maillard reactions, leading to the formation of brown melanoidins (Abegaz and Kerr, 2006). Phenolic browning reactions and oxidation of pigments also alter color in stored foods, as do changes in the physical state and structure of a food product (i.e. opacity), due to reflection, transmission, absorption, and scattering (McClements, 1999).

L\*, a\*, and b\* color values of stored peanut butter samples were measured to assess whether any modifications were occurring, as well as to provide quantitative values for visible changes in color. L values for peanut butter samples incubated in MRE package at three different temperatures for 12 weeks started at about 52 and dropped continuously during incubation to values ranging from 43 to 49 (Figure 12), indicating darkening of the products. Samples were significantly darker at 60 °C ( $p < .05$ ); samples darkened noticeably less at 25 and 40 °C but effects were not significantly different between these two temperatures.

a\* (red-green) and b\* (yellow-blue) color values provide information about color quality and hue. a\* values in peanut butter increased with incubation time and temperature, consistent with progressive browning (Figure 13), although the increase in a\* values of peanut butter stored at 25°C was not significant. In contrast, the yellow b\* values (Figure 14) showed no appreciable changes in peanut butter at any temperature during storage. These results indicate that the browning that developed had redder, rustier tones than produced during roasting and thus probably arose from different reactions.

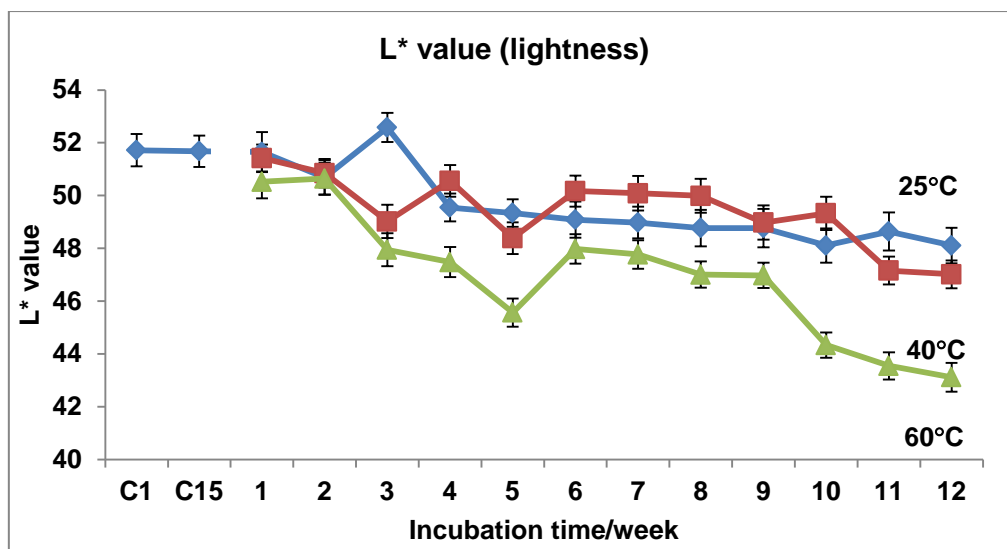


Figure 13. Effect of time and temperature on L\* values of peanut butter incubated in MRE packages for 12 weeks. C1 and C15 are refrigerated controls at 1 and 15 weeks respectively.

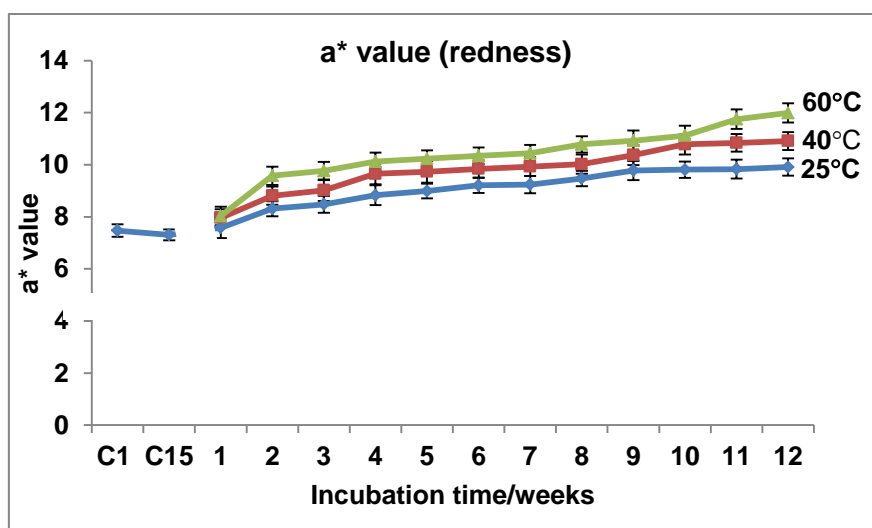


Figure 14. Effect of time and temperature on a\* values of peanut butter incubated in MRE packages for 12 weeks. C1 and C15 are refrigerated controls at 1 and 15 weeks respectively.

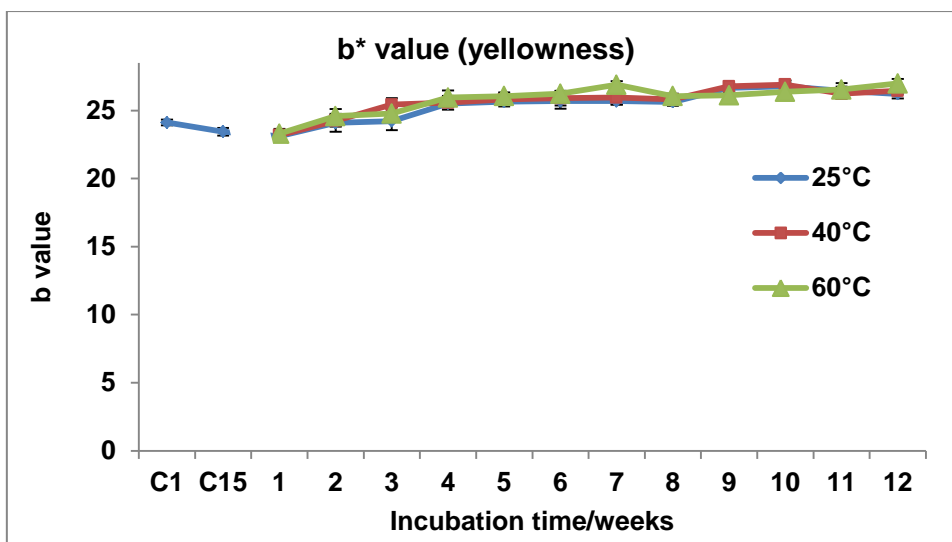


Figure 15. Effect of time and temperature on b\* values of peanut butter incubated in MRE packages for 12 weeks. C1 and C15 are refrigerated controls at 1 and 15 weeks respectively.

Browning during roasting is due largely to Maillard reactions (Mexis et al., 2009), but in incubated peanuts lipid carbonyls can replace sugars as the carbonyl source, and this would shift the color of brown generated Carbonyl- amine condensations in Maillard reactions require a heat source (El-Rawas et al., 2012), which explains partially why browning was greatest at 60 °C and was much lower at room temperature. Maillard reactions also require moisture (Felland and Koehler, 1997). The very low moisture content of the peanut butter thus perhaps favors condensations of carbonyl from lipid oxidation over Maillard reactions for producing new browning during storage. Additional browning may come from aldol condensation of lipid carbonyls catalyzed by the basic groups in proteins (Nawar, 1985).

### 6.1.3 Texture characteristics of peanut butter

Hardness, cohesiveness and adhesiveness are the primary mechanical characteristics in texture evaluation.

**Hardness.** Most notably, the hardness of peanut butter stored at 60°C increased visibly and significantly ( $p < 0.05$ ) during incubation (Figure 15), translating practically into low spreadability. Hardness increased slowly until 8 weeks then rapidly thereafter. In contrast, hardness did not change in peanut butter stored at 25°C, while samples stored at 40°C had only a slight increase in hardness after 8 weeks. A hard outer layer of peanut butter was observed in 40°C samples at week 12 and in 60°C samples at week 9.

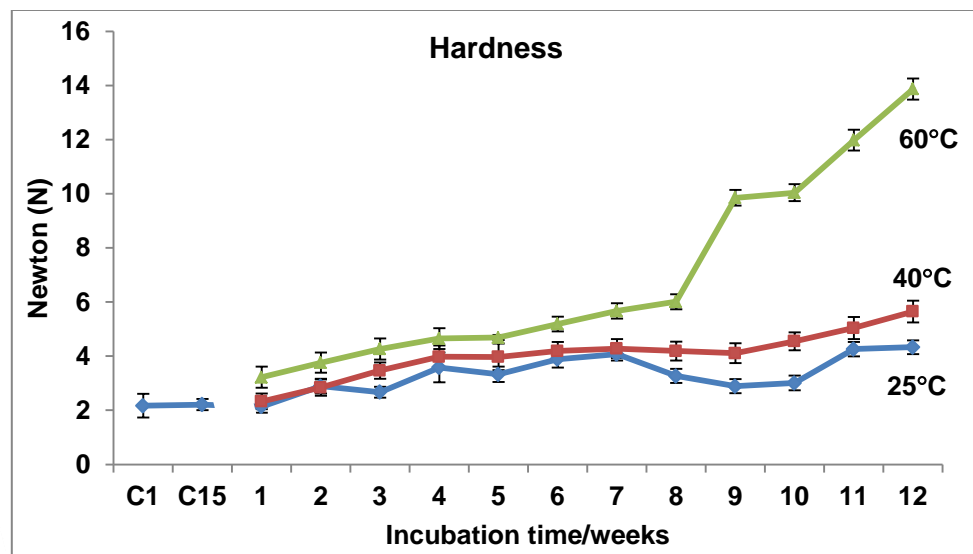


Figure 16. Effects of incubation time and temperature of hardness of peanut butter over the course of 12 weeks.

A number of factors may contribute to the observed hardening. Textural properties of peanut butter are significantly influenced by the peanut selection, processing conditions, addition of ingredients and type of peanut butter. Peanut butter



is a colloidal suspension of lipids and water in a protein meal phase. According to Butnett et al. (2000), peanut butter contains 55% fat, 25-30% protein, and 0.5 to 2% moisture. The high protein content in peanut butter maintains the consistency of the continuous peanut butter oil phase. In this peanut butter, hydrogenated vegetable oil (rapeseed, cottonseed or soybean) was also added to prevent oil separation. Thus, one potential contribution to hardening may be disruption and reorganization of the continuous fat phase and other components (Abegaz and Kerr, 2006). Separation of phases often results at the higher temperatures, leading to insufficient liquid oils in the peanut solids matrix (Aryana et al., 2000). In addition, when fat is heated above its melting point, existing crystal nuclei are destroyed and the fat may crystallize into any one or a mixture of possible forms (Freeman et al., 1954). Recrystallization of glycerides in original or modified form, e.g.  $\alpha$  or  $\beta'$  converting to  $\beta$  crystals, may be induced as well by changes in matrix proteins (Moran, 1994) or by lipid-protein interactions. Lipid phase properties need to be measured in future work, but the instrumentation required for these analyses was not available for this study.

A second obvious potential source of hardening is loss of moisture during storage. Physical loss of moisture can be eliminated as a cause since the decrease in moisture is too small (0.2% max) to account for the notable hardening observed (Section 6.1.1). However, chemical loss of moisture by consumption in reactions remains a possibility. Interactions between sugar and moisture the peanut butter has been proposed (Abegaz and Kerr, 2006), but more likely is the dehydration known to occur in Maillard reactions (Muego-Ghansekharan and Resurreccion 1992). Indeed, increased browning and slightly decreased moisture were observed in the 60 °C samples where hardening occurred, but not at room temperature where samples did not harden.

Finally, increased protein crosslinking or changes in protein organization could enhance hardening. This possibility will be addressed in Section 6.2.2.2 (SDS electrophoresis of proteins).

**Cohesiveness.** Cohesiveness values in peanut butter samples decreased continuously during incubation, and the effects increased with temperature (Figure 16). Peanut butter stored at 25 and 40 °C showed slow, low decreases in cohesiveness, dropping from 1.18 to 0.90 and 0.84 after 12 weeks at 25 and 40 °C, respectively; difference between controls and incubated samples at these temperatures were not significantly different ( $p < 0.05$ ) until Week 7. In contrast, cohesiveness in peanut butter incubated at 60°C decreased significantly ( $p < 0.05$ ) and continually throughout the incubation period, dropping to a value of 0.38 after 12 weeks.

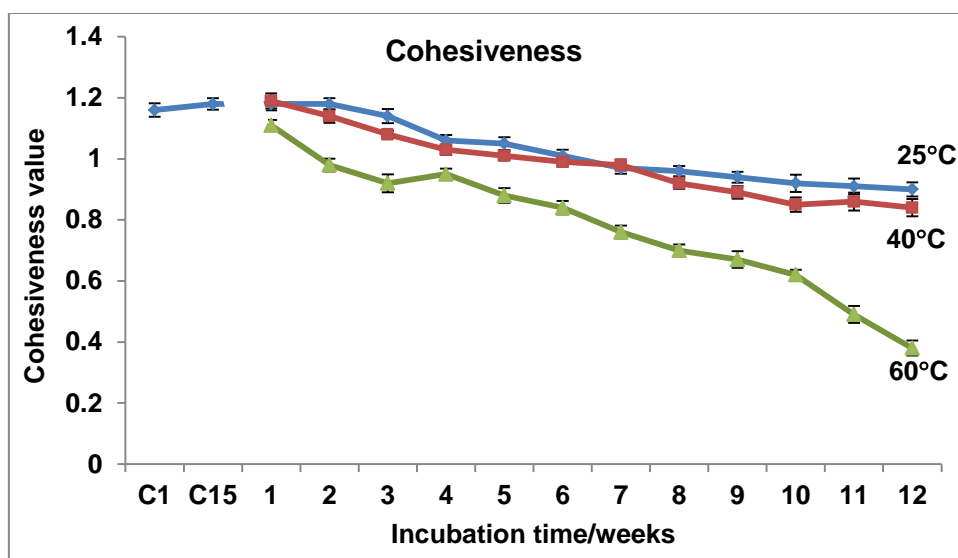


Figure 17. Effects of incubation temperature of hardness of peanut butter incubated for 12 weeks.

Cohesiveness reflects the extent to which a material can be deformed before it ruptured (Cevill and Szczesniak, 1973) which in turn is controlled by the strength of

intermolecular interactions within a material (Abegaz and Kerr, 2006). The decrease in cohesiveness as storage time and temperature increased indicates that the peanut butter samples had decreasing connectedness and possessed some hardening so deformed more and more rapidly. This behaviour has been attributed to changes in intermolecular associations between sugar and water as well as to disruption of the lipid-protein matrix and separation of the oil phase in peanut butter (Abegaz and Kerr, 2006). Such changes also affect the elastic modulus and viscoelastic behaviour of peanut butter, which in turn modify deformability (Abegaz and Kerr, 2006).

***Adhesiveness.*** Adhesiveness is defined as the work necessary to overcome attractive forces between the surface of the food sample and an external surface in contact with the food (such as tongue or palate) (Szczesnick, 1963a). Adhesiveness values for peanut butter in MRE packages stored at 25° and 40 °C showed minimal changes for the first few weeks but began to increase consistently after about Week 5 (Figure 17). In contrast, peanut butter stored at 60 °C started with much higher adhesiveness values, which then decreased for the first three weeks before dramatically increasing thereafter. Thus, the amount of work need to overcome the attractive forces between peanut butter surface and the plastic or metal probe was substantially increased in peanut butter samples incubated at all “elevated” temperatures relative to the controls stored at refrigerated temperatures. Translating into practical terms, this means the peanut butter should become increasingly difficult to clear the mouth and swallow (Syarief et al., 1985; Crippen et al., 1989). Similarly, increased adhesiveness impairs the spreadability of peanut butter and thus reduces its quality. According to How and Young (1985), many consumers dislike sticky and overly adhesive peanut butter. In this study, adhesiveness scores are consistent with

visual evaluations of stiffening to the point where acceptability to consumers would be questionable.

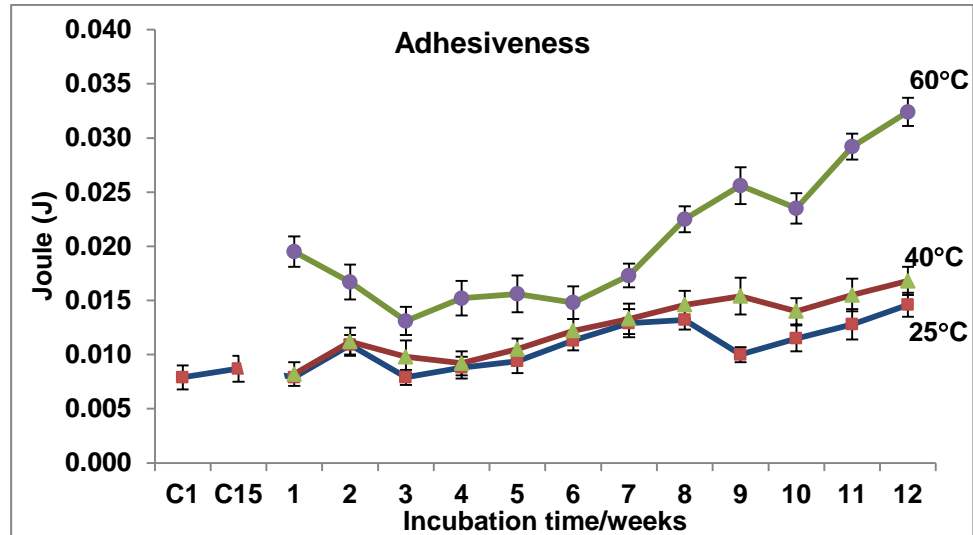


Figure 18. Effects of incubation temperature of adhesiveness of peanut butter incubated for 12 weeks.

**Gumminess.** Gumminess is a springy and rubbery secondary or derived characteristic of food samples. Temperature effects on gumminess were relatively minor until 8 weeks, after which gumminess markedly increased (Figure 18). Gumminess scores for peanut butter stored at 25°C and 40°C (~3 to 4 Newtons) are generally associated with moderate gumminess (Muego-Gnanasekharan and Resurricon, 1992). In contrast, peanut butter stored at 60°C showed consistently higher gumminess than other samples over the entire period, and at the end of 12 weeks incubation, values were significantly more gummy ( $p < 0.05$ ) than samples stored at all lower temperatures. Gumminess values of 6-7 Newtons would be rated as quite gummy, and correspondingly lower quality and consumer acceptability would be expected. Low moisture contents can contribute to rubbery character due to

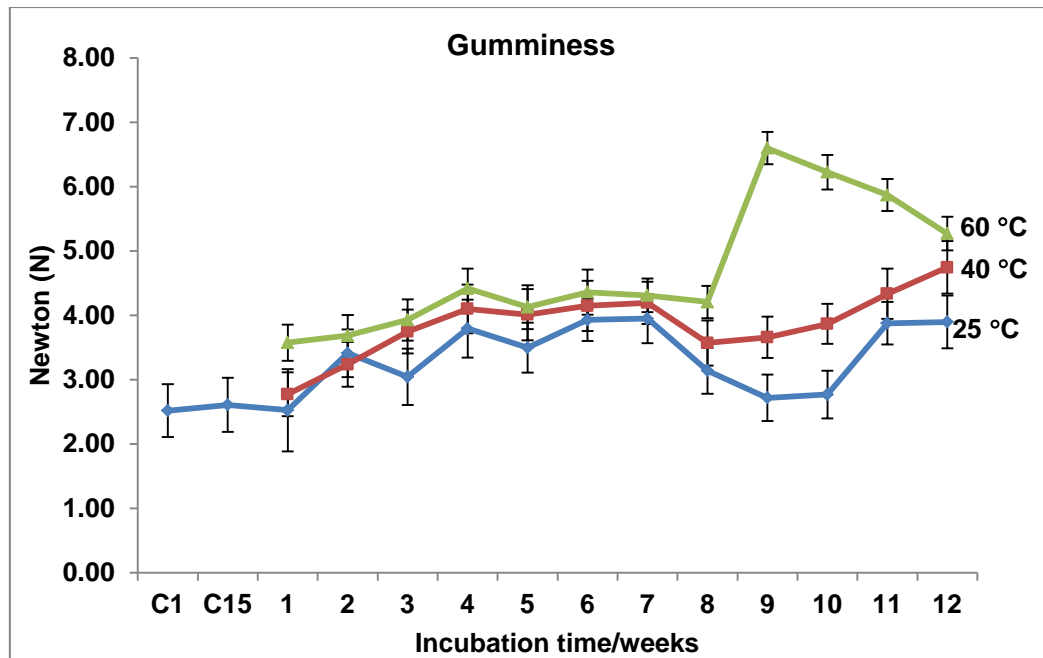


Figure 19. Effects of incubation temperature on gumminess of peanut butter incubated for 12 weeks.

immobilization of amorphous matrices (Slade and Levine, 1995). However, since moisture changes in these samples were minimal, moisture and glass transitions are not likely explanations for the observed texture.

Since gumminess and adhesiveness both followed the same trend as hardness in their relationship to temperature, and protein conformations and interactions are strongly affected by temperatures, it seems productive to examine possible roles of protein modifications in these texture changes. Peanut butter has high contents of proteins and lipids, which are both susceptible to increased oxidation and other modifications at higher temperatures. Lipid oxidation and protein modifications determined in peanut butter samples incubated at the different temperatures and described in the following sections should shed some light on possible roles of lipid co-oxidation processes in this distinctive degradation of texture in peanut butter.

## **6.2. Chemical changes in peanut butter during storage**

### **6.2.1 Lipid changes during storage**

#### **6.2.1.1 Lipid extractability**

Lipids were extracted from peanut butter samples manually to determine losses by potential lipid binding or entrapment versus lipid release via phase separations, as well as to determine extent of oxidation manual extractions of peanut butter. At all incubation temperatures, there was a gradual increase in lipid extractability over the incubation period, with a trend towards increased lipid yields with increased temperature (Figure 19). Lipid yields at 60°C were significantly higher than at 25°C and 40°C at the end of the incubation time. There also appeared to be a moderate cyclic pattern in lipid extractability over the time, similar to the cycling we have observed in chocolate and nuts (Rossi-Olsen, 2009), tortilla chips (Dong, 2010), pet foods (unpublished data), and methyl linoleate (Denis and Brandon AOCS presentations, 2012).

The increased lipid extractability may be due to partial disruption of the peanut butter emulsion, which increases oil separation from peanut butter and accessibility to solvent. Peanut butter now has hydrogenated vegetable fat routinely incorporated to prevent the separation of natural peanut oil. Hard fat is added only at levels sufficient to increase viscosity of the oil phase and to ensure the presence of sufficient crystals at room temperature to entrap the natural oil; both effects slow movement of oil droplets and prevent oil separation. Oil separation was observed at 60°C, during the incubation time. In peanut butter, oil separation may be an indication that is not fresh since the free oil is more susceptible to oxidation than in granular form during milling to stabilize the peanut butter. Oil separation was visibly detectable in samples stored at 60°C probably because the melted fat crystals and

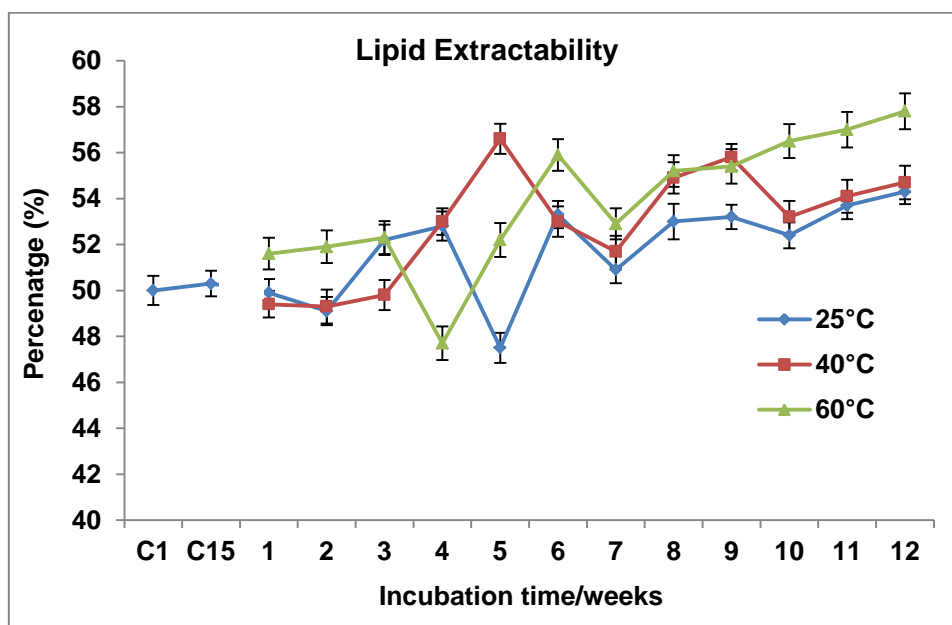


Figure 20. Effect of temperature on lipid extractability from peanut butter incubated for 12 weeks in MRE packages.

peanut oil rose to the surface. There is a natural tendency for free oil to rise to the surface of peanut butter without stabilizers after standing for a few weeks even at room temperature (Woodroof and Thompson, 1994).

Another possible contribution to increased oil extractability is modification of the protein matrix in peanut butter at higher temperatures, e.g. by crosslinking or aggregation, such that entrapped oil can no longer be held and is thus released without completely breaking the peanut butter emulsion. This process can be visualized as a version of syneresis, only with oil instead of water. Protein modifications identified in incubated peanut butters will help distinguish these two mechanisms.

#### 6.2.1.2 Oxidative stability

Conjugated dienes, peroxide values, aldehydes, and free fatty acids were analyzed in lipid extracts from peanut butter to determine extent of lipid oxidation,

recognizing at the same time that reaction of these lipid oxidation products with proteins removes them from the analytical stream. Thus, in interpreting causality or relationship, we may be looking for obvious holes in lipid oxidation products rather than increased production.

#### 6.2.1.2.1. Conjugated dienes

Overall, conjugated dienes decreased during incubation for all temperatures (Figure 20). There was also an indication of cyclic increase and decrease in CD levels at all temperatures, with cycles lasting approximately three to four weeks. CDs were rising during the last four weeks of the incubation period.

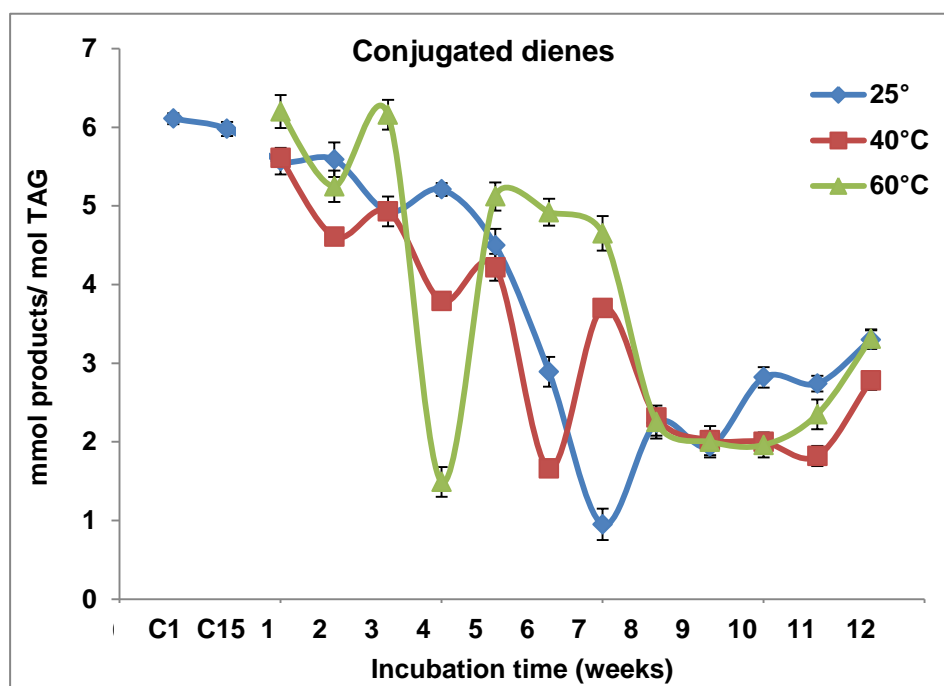


Figure 21. Effect of temperature on conjugated diene concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages.

This was not the pattern expected for active lipid oxidation. One way that the results may be interpreted is that instead of accumulating, the oxidation products were rapidly decomposing or transforming to other products. Since hydroperoxides retain



the conjugated diene structure, if CDs were decreasing, hydroperoxides should be decreasing in parallel and aldehydes or other secondary products from hydroperoxide decomposition should be increasing at the same time. This pattern was observed, in general, as will be shown in the following sections. However, the relationship is not perfect because aldehydes increased initially at 60 °C but barely increased at the other two temperatures. Thus, other reaction pathways must also be active.

Taking another view, peanut proteins or antioxidants (e.g. tocopherols) in peanuts may be quenching lipid radical reactions and inhibiting oxidation (Burton and Ingold, 1981; Min and Boff, 2002). In this case, all downstream products should also have been inhibited, which did not happen, while active oxidation should ensue when the antioxidant was consumed (Fourie and Bason, 1989, Senesi et al., 1991; Yao et al., 1996; Lima et al., 1998), for which we have no data. Nevertheless, if proteins quench lipid free radicals, i.e. if lipids transfer their free electrons to proteins rather than to other lipids molecules, this is active radical transfer and parallel reactions or “footprints” must be looked for in proteins rather than in lipids.

#### **6.2.1.2.2. Peroxide values**

Peroxide values showed the same decrease-increase-decrease cycling that was observed for conjugated dienes (Figure 21). However, the overall trend for samples at 25°C was to increase over the incubation period, while at 40°C and even more so at 60°C, peroxide values decreased significantly ( $p < 0.05$ ) with storage time. Highest peroxide levels were found in samples incubated at 25°C.

Once again, these results may seem to be counter what should be expected. However, it must be remembered that levels of any oxidation products are instantaneous balances between production and decomposition or transformation. At lower temperatures, peroxides do not form as fast but they are more stable, so they

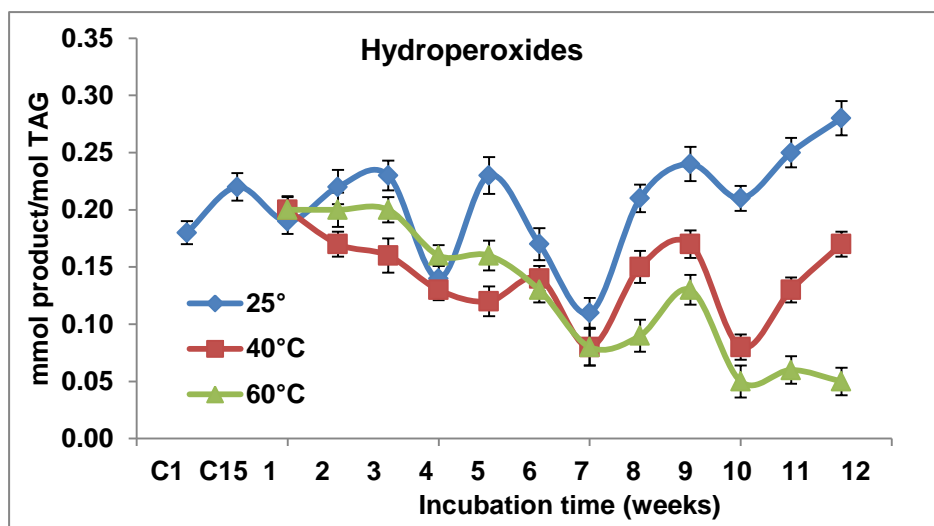


Figure 22. Effect of temperature on hydroperoxide concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages.

can accumulate to higher levels. Above 40 °C, however, lipid hydroperoxide decomposition rates begin to increase dramatically while at the same time hydroperoxides are forming more rapidly. By 60 °C, hydroperoxide decomposition rates generally exceed formation rates, so overall peroxide values decrease paradoxically (K.M. Schaich, unpublished data). This is the most probable reason why peroxide values decreased with incubation temperature.

A new cycle of active oxidation began after seven weeks of incubation, with hydroperoxides at 25 °C increasing dramatically, at 40 °C decreasing less, and at 60 °C increasing temporarily (for two weeks) then decreasing. This marked differences in patterns is consistent with different reactions or different reaction pathways being active at each temperature. Examples of other reactions include formation of volatile compounds that are released to the headspace and not detected (Schieberle and Gorsch, 1981), non-volatile oxidized dimers, trimers, or polymers (Choe and Min, 2005), or epoxides (Gardner and Kleiman, 1981; Gardner et al., 1985). If alternate

reactions are competing, analyzing the same simple hydrogen abstraction products for all conditions may give erroneous and misleading results. Thus, additional product analyses are needed to supplement the ones reported here in order to elucidate reaction processes.

#### **6.2.1.2.3 Aldehydes**

Aldehydes are critical products in that they are usually viewed as evidence for hydroperoxide decomposition, and the odors generated are sensed at low levels and recognized by consumers. The concentrations of aldehydes detected in lipid extracts of peanut butter incubated at the three different temperatures are presented in Figure 22. Aldehydes were not detectable in peanut butter controls, and at Week 1 for 25°C and 40°C samples. At Week 2, aldehydes increased to 1 mmol/mol triacylglycerols (TAG) in peanut butter samples incubated at 25°C and 40°C but did not change significantly after that. In contrast, at 60°C, aldehydes began to increase rapidly as soon as incubation commenced, and peaked at week 4 with aldehyde levels of ~5 mmol/mol TAG; there was a statistically significant ( $p < 0.05$ ) accumulation of aldehydes. However, after five weeks of incubation, aldehyde concentrations decreased dramatically during incubation and ended the experiment with levels lower than the other two temperatures.

The rapid increase in aldehydes early in oxidation does not parallel either conjugated dienes or hydroperoxides. This suggests that there may be different mechanisms for forming aldehydes at 60 °C, those not involving conjugated dienes and hydroperoxides as precursors. At lower temperatures, the unusually low steady-state production of aldehydes may result because decomposition occurs, yielding products not measured in this study, e.g. ketones or epoxides, or because all the aldehydes formed rapidly react with the peanut proteins present, via Schiff base or

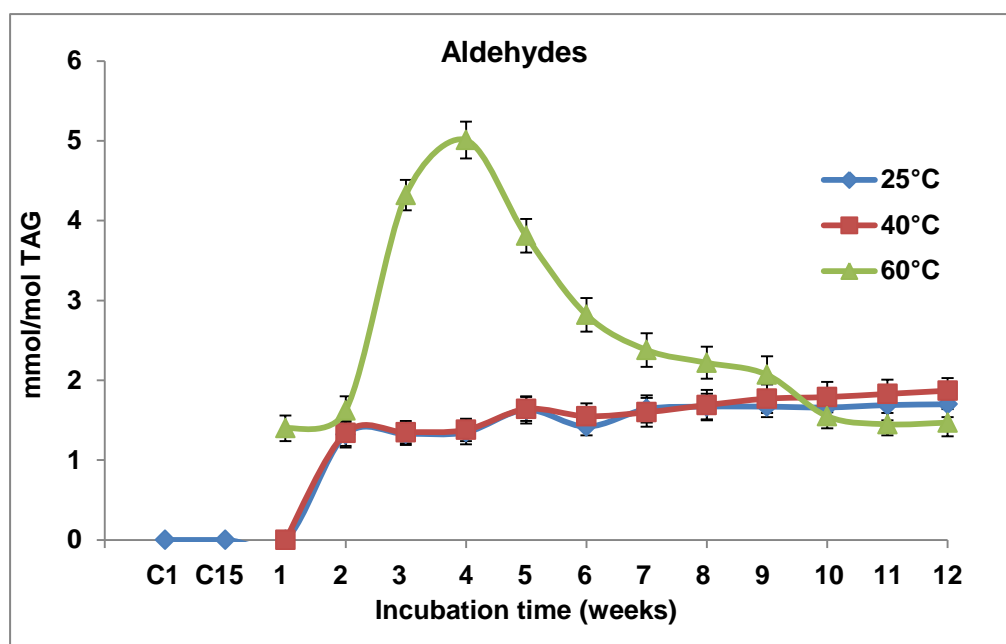


Figure 23. Effect of temperature on aldehyde concentrations in lipid extracts from peanut butter incubated for 12 weeks in MRE packages.

Michael addition. These two possibilities (or others) cannot be determined without additional detailed product analyses. However, some clues may be available from fluorescence analyses reported in Section 6.2.2.5

Additional alternate pathways may be mediated by variations in oxidation catalysts. Peanuts are a complex molecular matrix with potential for a number of catalytic mechanisms. In high oil/protein product like peanut butter in which the peanut had been heated, lipid degradation is more likely catalyzed by nonenzymic agents such as hemoproteins and metals than by lipoxygenase (Ory and St Angelo, 1982). When peanuts are roasted, these hemoproteins lose their enzyme activity, yet being more dissociated have increased ability to catalyze linoleic acid oxidation to greater degree than when unheated (Eriksson, et al., 1970). Salts and metalloprotein containing iron or copper are primary catalysts of fatty acid peroxidation in peanut

butter storage, and depending which were present in the peanut butter studied, can greatly modify decomposition pathways at different temperatures.

Aldehyde lipid oxidation products result in off-flavors, rancid odors and discoloration in meat (Fogerty and others 1989); they may also affect protein stability. Protein modification by aldehydes is believed to play a central role in many pathophysiological conditions traditionally associated with free radical damage (Esterbauer and others 1991a; Szweda and others 1993) as  $\alpha,\beta$ -unsaturated aldehydes modify sulfhydryl groups, primary amino groups, histidine, cysteine and lysine residues (Uchida and Stadtman 1992). These adducts can be formed through the Michael addition (Uchida and Stadtman 1992) or Schiff base reactions (Szweda and others 1993). Lipid oxidation of foods can be delayed by packaging systems which limit oxygen and moisture permeation. This can be achieved by the use of high oxygen barrier packaging film or edible coating. Peanut butter in MRE package showed that the lipid oxidation can be reduced during storage at room temperature but not suitable for high temperature.

#### **6.2.1.2.4. Free Fatty Acids**

Fatty acids (carboxylic acids) are formed by hydrolysis of phospholipids and triacylglycerols which is promoted by food moisture (Al-Harbi and Al-Kabtani, 1993) and, less well-known, also by oxidation of aldehydes (McMurray, 2000; Tian, 2013). Thus, increases in fatty acids, in combination with other products, can provide some interesting snapshots into degradation processes in peanut butter.

Free fatty acid levels detected in the lipid extracts, expressed as % total lipid, increased significantly ( $p < 0.05$ ) at all temperatures, but dramatically at 60°C (Figure 23). Unlike other products, fatty acids increased linearly with incubation time, with the rate increasing with temperature, while no fatty acids could be detected in controls

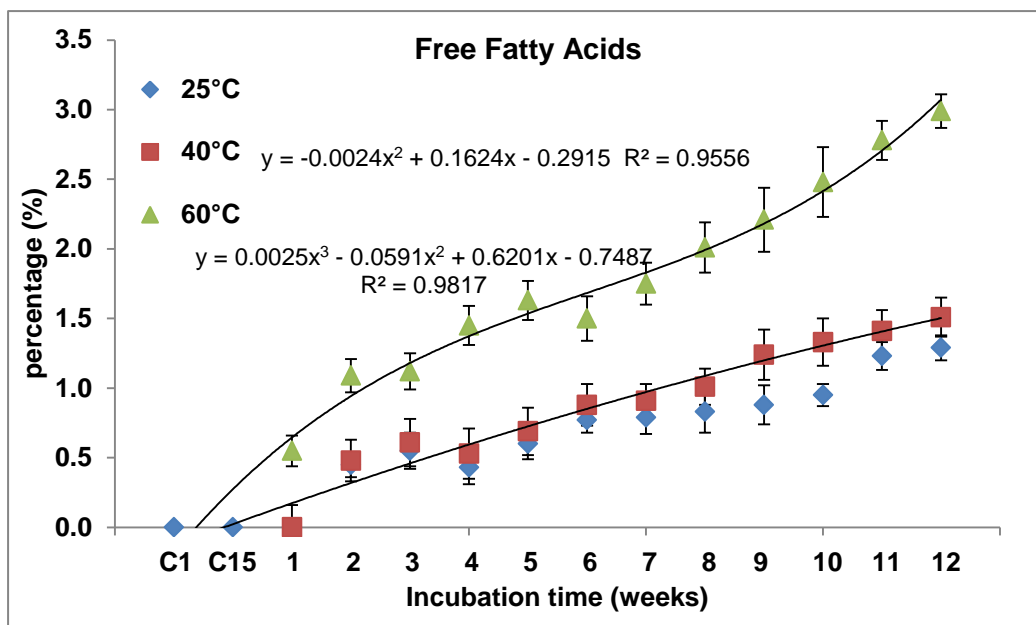


Figure 24. Effect of incubation temperature on release of free fatty acids in peanut butter incubated for 12 weeks in MRE packages.

before incubation and after 15 weeks of incubation. If these fatty acids arise from oxidation of aldehydes, the reaction mechanism is independent of hydroperoxides and other oxidation pathways since patterns are so different. It does not seem reasonable to expect that the 1% moisture present should be sufficient to catalyze such marked hydrolysis, especially since fatty acid levels at room temperature reach >1% over the incubation period – the level that is legally mandated for dilution or disposable of frying oils. Water alone does not usually cause hydrolysis, but requires acids or heat as catalysts. Thus, in this system, there must be an alternate mechanism or a combination hydrolysis-oxidation mechanism that generates free fatty acids. Detailed LC-MS analysis of fatty acids produced in peanut butter is needed to identify specific products and distinguish active mechanisms for their formation.

## 6.2.2 Protein modifications in peanut butter

### 6.2.2.1 Protein solubility

Protein solubility was measured following extraction with the five procedures detailed in method section. As shown in Figure 24, extraction with phosphate buffer at pH 7.9 (no NaCl) extraction provided significantly increased protein yields. Both the buffer/pH and the contact time appear to be critical for the increased yields. While not 100%, these yields are the best we have observed in our laboratory and found in literature reports. Thus, this extraction procedure was used as the base for all subsequent studies.

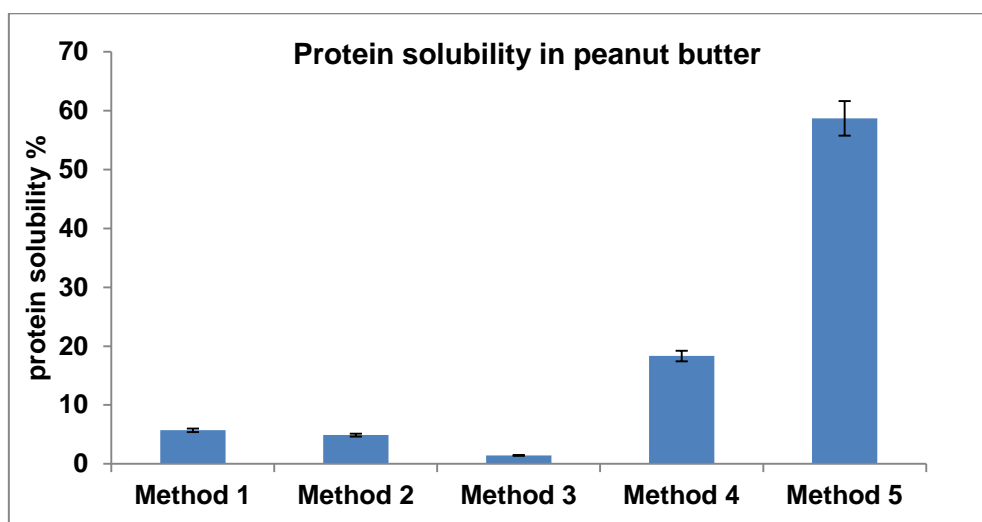


Figure 25. Efficiency of protein extraction by different methods.

Solubility of the incubated total soluble protein fractions significantly decreased relative to controls during incubation, although the differences between temperatures were not great (Figure 25). A cyclic pattern with the same periodicity observed in lipid oxidation products was present for protein solubility at all temperatures. Intensity of excursions appeared to be greater at 60°C than at the lower temperatures.

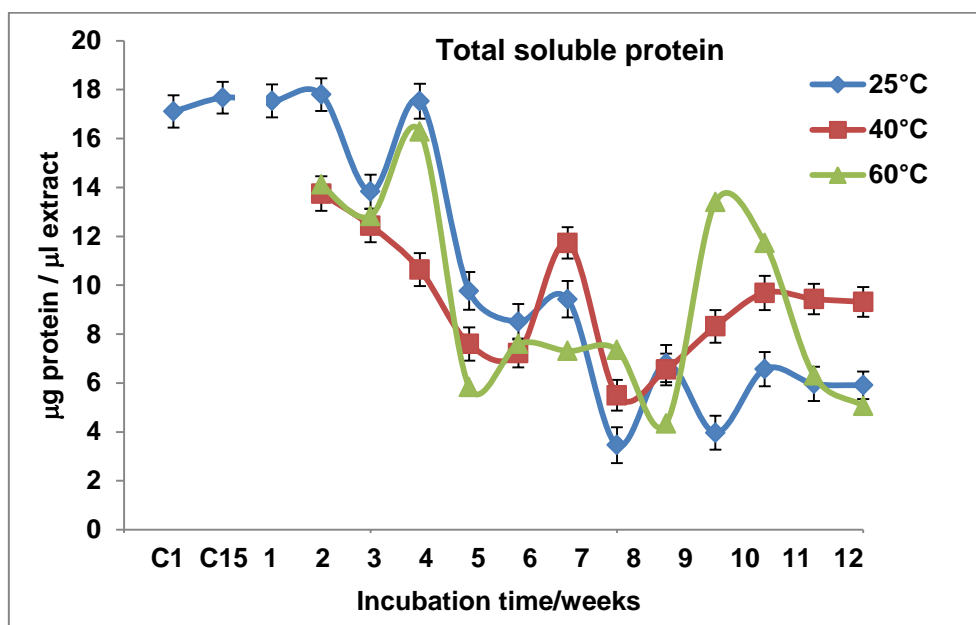


Figure 26. Solubility of the total soluble protein fraction extracted from peanut butter incubated at three different temperatures.

Albumins (the water soluble protein fraction) had the highest solubility in raw peanuts but the lowest at 60°C. For all temperatures, protein solubility decreased over the time of incubation (Figure 26). Moderate cycling was present.

Globulins showed a more pronounced cyclic pattern in protein solubility with some shifts in periodicity (Figure 27). The tendency was for greater insolubilization at 60 °C than at the lower temperatures.

Interestingly, solubility of proteins in the SDS-ME barely changed at any temperatures over the incubation period (Figure 28).

When peanut is roasted, peanut proteins undergo denaturation (Chiou et al., 1991; Luis et al., 2005) and other structural conformation changes that can ultimately result in decreased solubility (Kopper et al, 2005). Generally, higher temperatures and longer heating times reduce protein solubility more extensively, possibly due to cross-



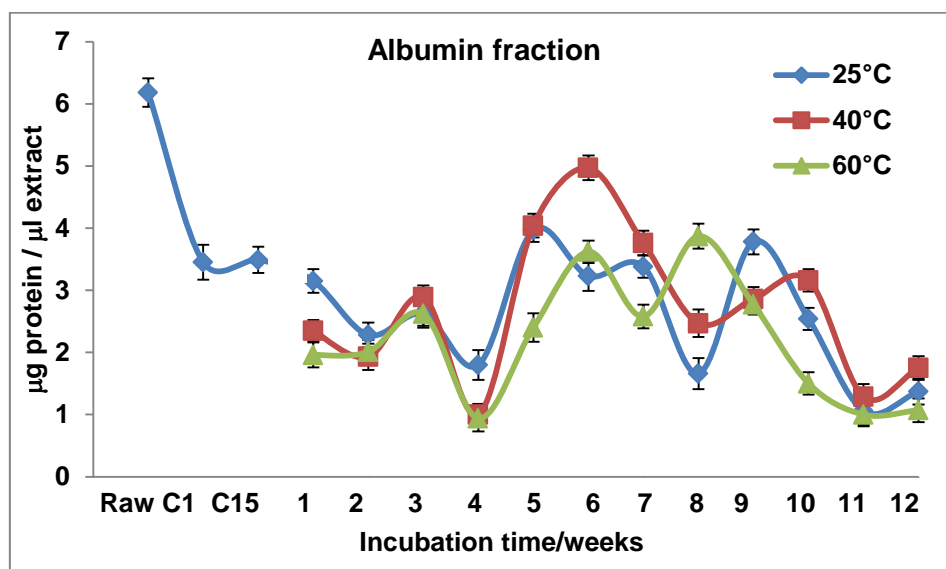


Figure 27. Solubility of the albumin fraction extracted from peanut butter incubated at three different temperatures.

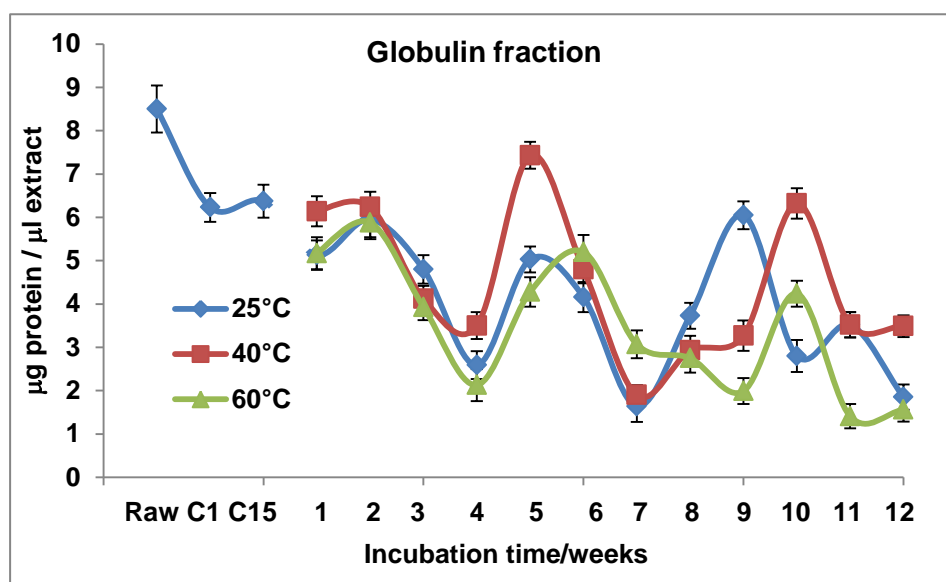


Figure 28. Solubility of the globulin fraction extracted from peanut butter incubated at three different temperatures.

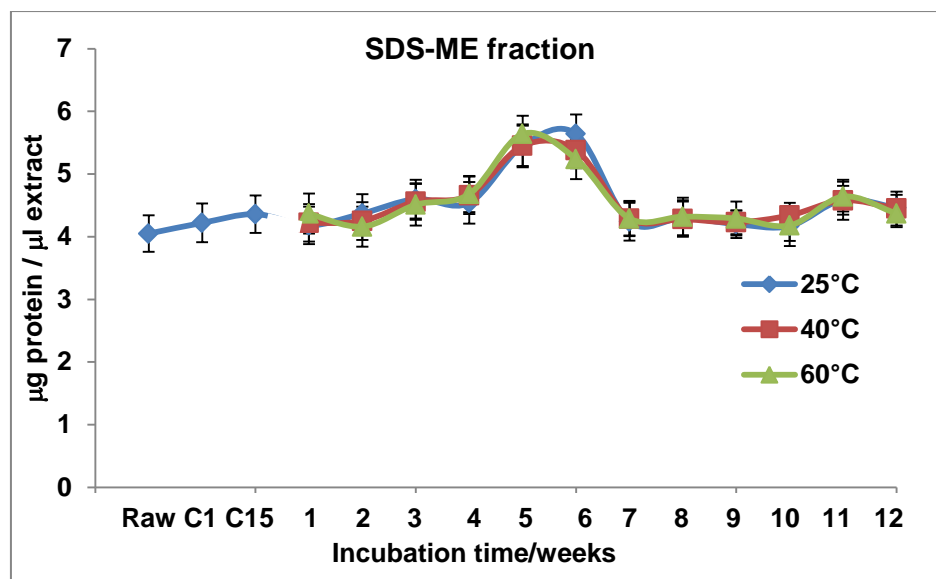


Figure 29. Solubility of SDS-ME soluble proteins extracted from peanut butter incubated at three different temperatures.

linking between carbohydrates and glycoproteins in Maillard reactions. However, the reduction in solubility during incubation go well beyond changes induced by heat alone. The periodicity in protein solubility that parallels lipid oxidation strongly supports a direct causal relationship, i.e. that lipid oxidation products react with proteins and cause changes that impair solubility.

#### 6.2.2.2 Crosslinking and fragmentation by SDS-2ME electrophoresis of protein fractions extracted from incubated peanut butter

Electrophoresis of peanut butter proteins documented changes in proteins unlike any patterns observed previously in this laboratory. Most commonly, the dominant changes are various forms of crosslinking (mixtures of free radical and S-S, with some Schiff base or Michael addition) accompanied by loss of solubility. Peanut proteins showed a number of anomalies compared to this. First, peanut peptides were not “lost” by running through the gel too fast or not entering gel due to large size.

Rather, they appeared to be reorganized, both by intermixing of arachin sub-units and by random modifications of peptides, e.g. by free radical transfer from lipids. This became quite evident when proteins were extracted sequentially as different classes.

Before incubation, patterns of total soluble protein, albumins, globulins, and SDS-soluble were substantially different from each other (Figure 29). As will be shown below, protein modifications led to intermixing of these fractions during incubation, and at time albumins looked very much like globulins, and even the SDS-soluble fraction began to show similarities. Transitions between fractions have been noted previously and are related to modifications of surface residues, particularly acid, thiol, and amine groups (Cherry and Ory, 1973; Basha and Cherry, 1976; Basha and Pancholy, 1982).

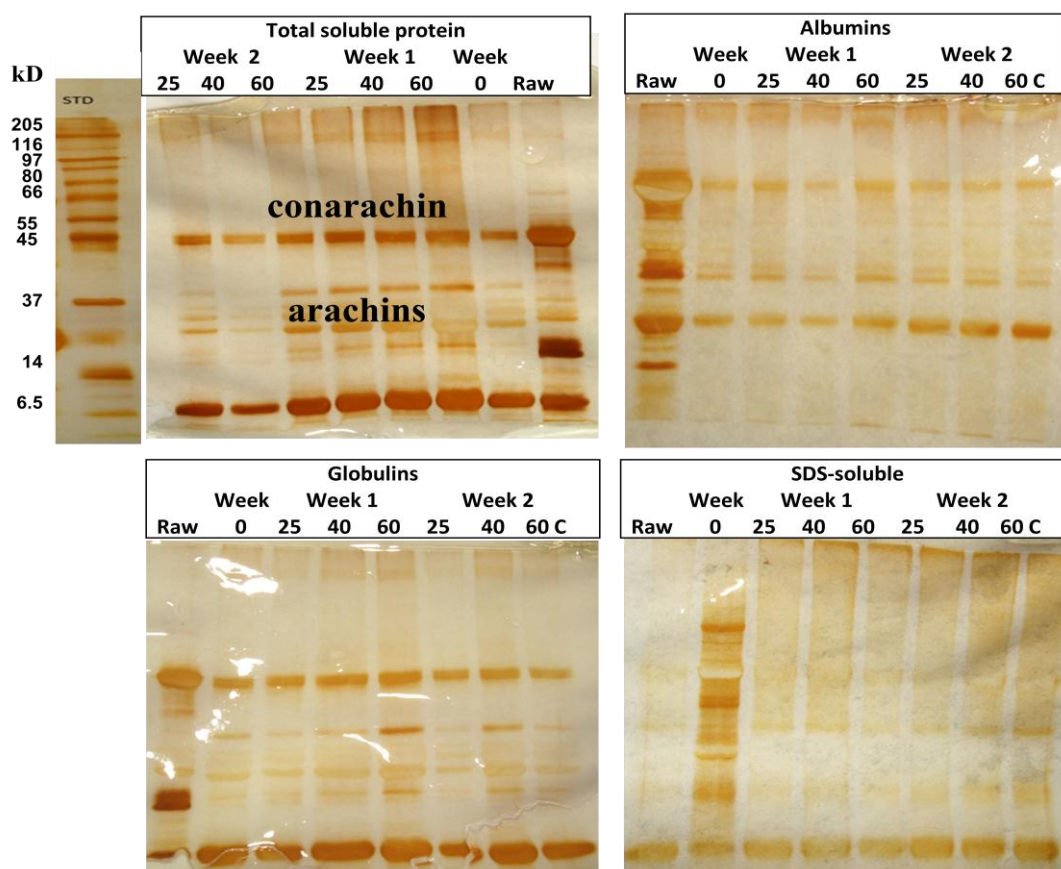


Figure 30. SDS-2ME PAGE of proteins extracted from peanut butter incubated at different temperatures for two weeks. 10% gels.

In addition, silver and Coomassie Blue (CBB) stains revealed very different protein patterns when used to stain duplicate gels (Figure 30). Normally, silver staining is more sensitive and just accentuates the peptide pattern already shown by CBB. With peanut proteins, however, even with pre-extraction of lipids, CBB and Ag gave peptide patterns with little similarity. This may be partially explained by differences in binding sites for the two dyes. CBB binds preferentially to hydrophobic and basic residues of proteins (his, arg, lys, trp). In contrast, silver binds most strongly to the carboxylic acid groups in acidic residues (Asp and Glu), plus imidazole (His), sulfhydryls (Cys), and amines (Lys). Usually, enough binding residues are present in each protein that both dyes detect the same peptides. However, peanut proteins have fractions that are strongly acidic or basic, and their amino acid compositions are correspondingly skewed, altering dye binding. Interestingly, major modifications occurred in the acidic proteins of all fractions, i.e. they are clearly evident in Ag-stained gels while CBB-stained gels showed much less change.

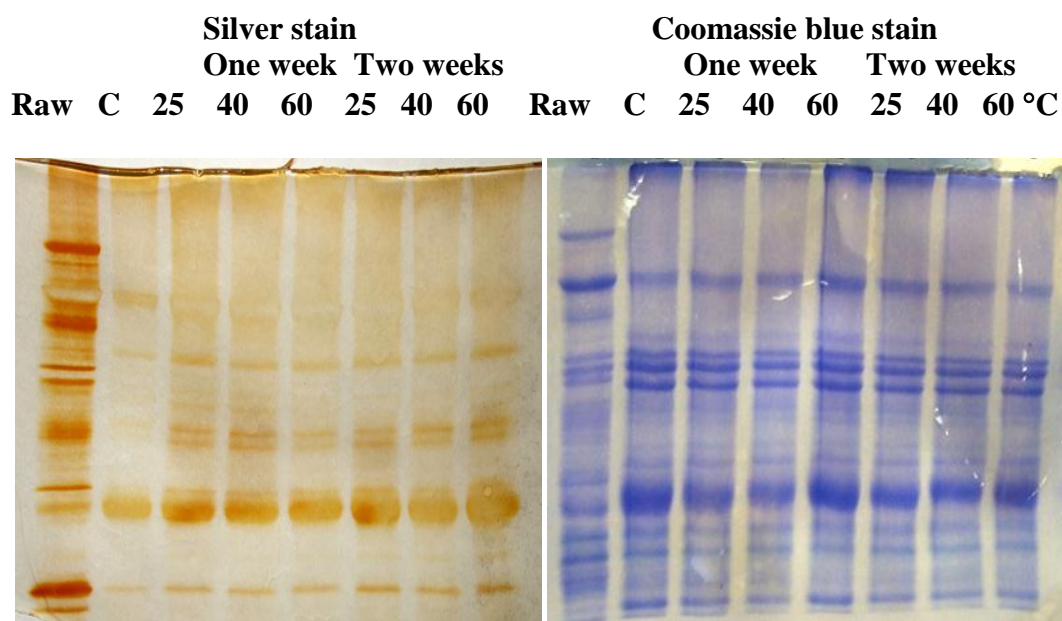


Figure 31. SDS-2ME PAGE of peanut butter proteins soluble in SDS showing differential binding of silver (left) and Coomassie blue (right) dyes. Other fractions showed the same behaviour.

The functional groups responsible for the acidic and basic properties that alter dye binding are also the ones most susceptible to modification by oxidation and heat, hence creating surface effects that contribute to the strong propensity for internal restructuring rather than crosslinking. In addition, many of the arachins and conarachins are glycoproteins containing substantial neutral and amino sugar contents. The sugar components may also modify co-oxidation patterns relative to other proteins such as zeins, gliadins, and glutenins. We will return to this point in later discussions.

Shifting from general effects to specific modifications in each fraction provides some insights into the peptide fractions and chemical mechanisms involved in lipid co-oxidation of peanut proteins.

**Total soluble proteins.** In gels stained by Coomassie blue, PAGE patterns of total soluble proteins retained approximately the same fractions throughout incubation (Figures 32-34), i.e. no fractions were obviously lost as was observed with zeins in tortilla chips (Dong, 2011). However, there was a fair amount of shifting among dominant bands (amount of material in bands changed), with increased intensity of a triplet of conarachin bands (40-44 kDa, A in Figure 3) and one major arachin (20 kDa, B in Figure 3), as well as appearance of two high mol wt bands near the wells (>200 kDa, C in Figure 3). There was also increased “fuzziness” of bands as low levels of peptides with non-specific molecular weights accumulated in the background. Most notably, the mol weights of all fractions decreased over time. This effect was reproducible through many repeat extractions and gel preparations. Two possible explanations for this behavior may be offered: 1) Arachins and conarachins are complexes of proteins built from small molecular monomer units of ~10,000 mol wt. Dissociation of the complexes monomer by monomer would yield a continual mol

wt decline. 2) Release of sugar components from the proteins by hydrolysis or oxidative scission would result in lower molecular weights. The latter is more consistent with the mol wt shifts observed. However, detailed analysis will be required for definitive identification of the changes responsible for mol weight loss.

In contrast, proteins in silver stained gels showed substantial changes over time. Initially, only two bands bound silver stain. However, the numbers of bands and complexity of band patterns increased with incubation time, and there was continual shifting among molecular weights, particularly among fractions less than 70 kDa. The bands remained discrete rather than losing distinction and becoming diffuse and fuzzy, suggesting that the changes involved reorganization of arachin subunits rather than random degradation.

There were no clear differences of temperature on protein changes.

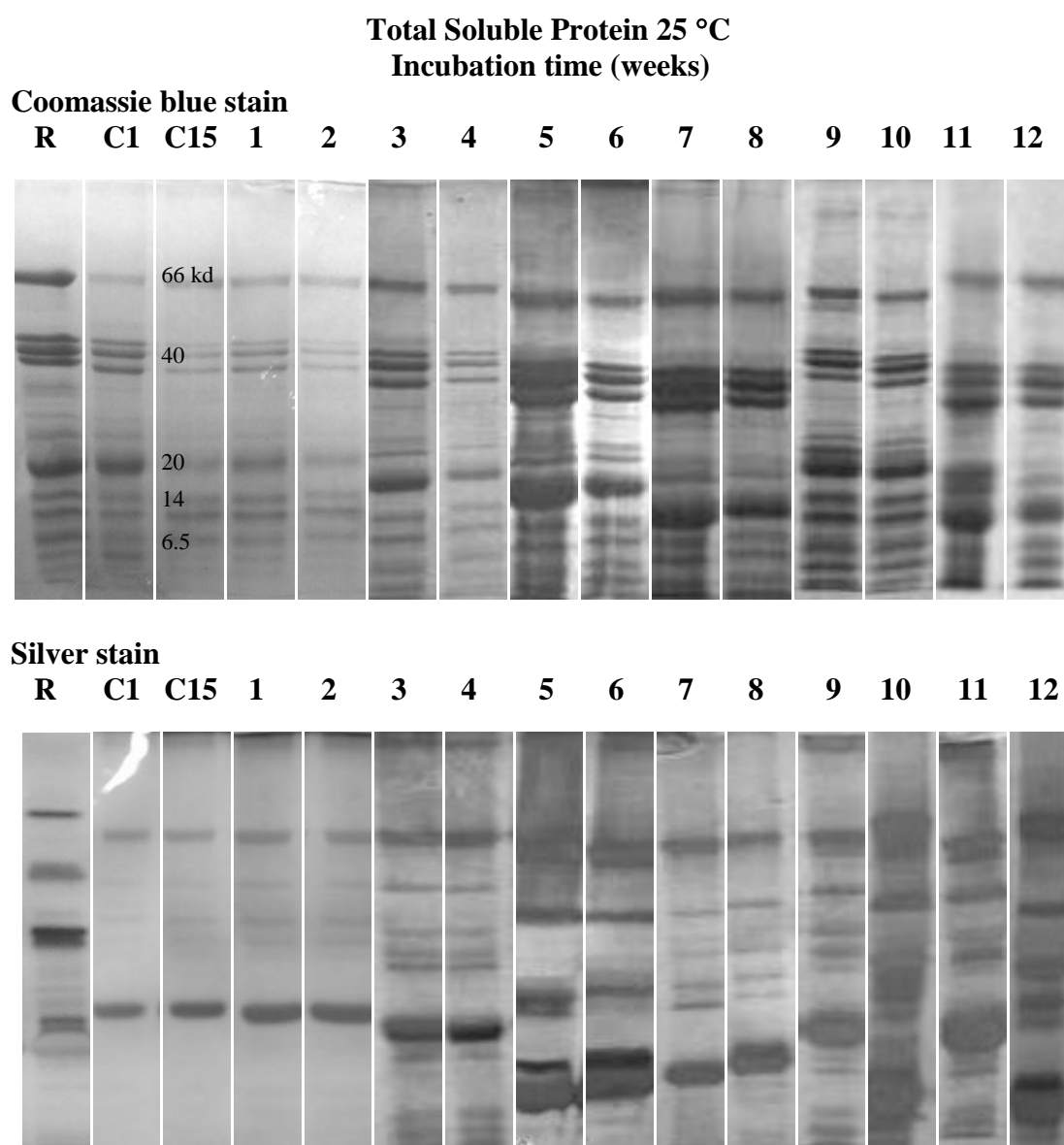


Figure 32. SDS-PAGE patterns of total soluble protein fraction extracted from peanut butter incubated in MRE package at 25°C. C1 and C15 are controls at 1 and 15 weeks of incubation, respectively.

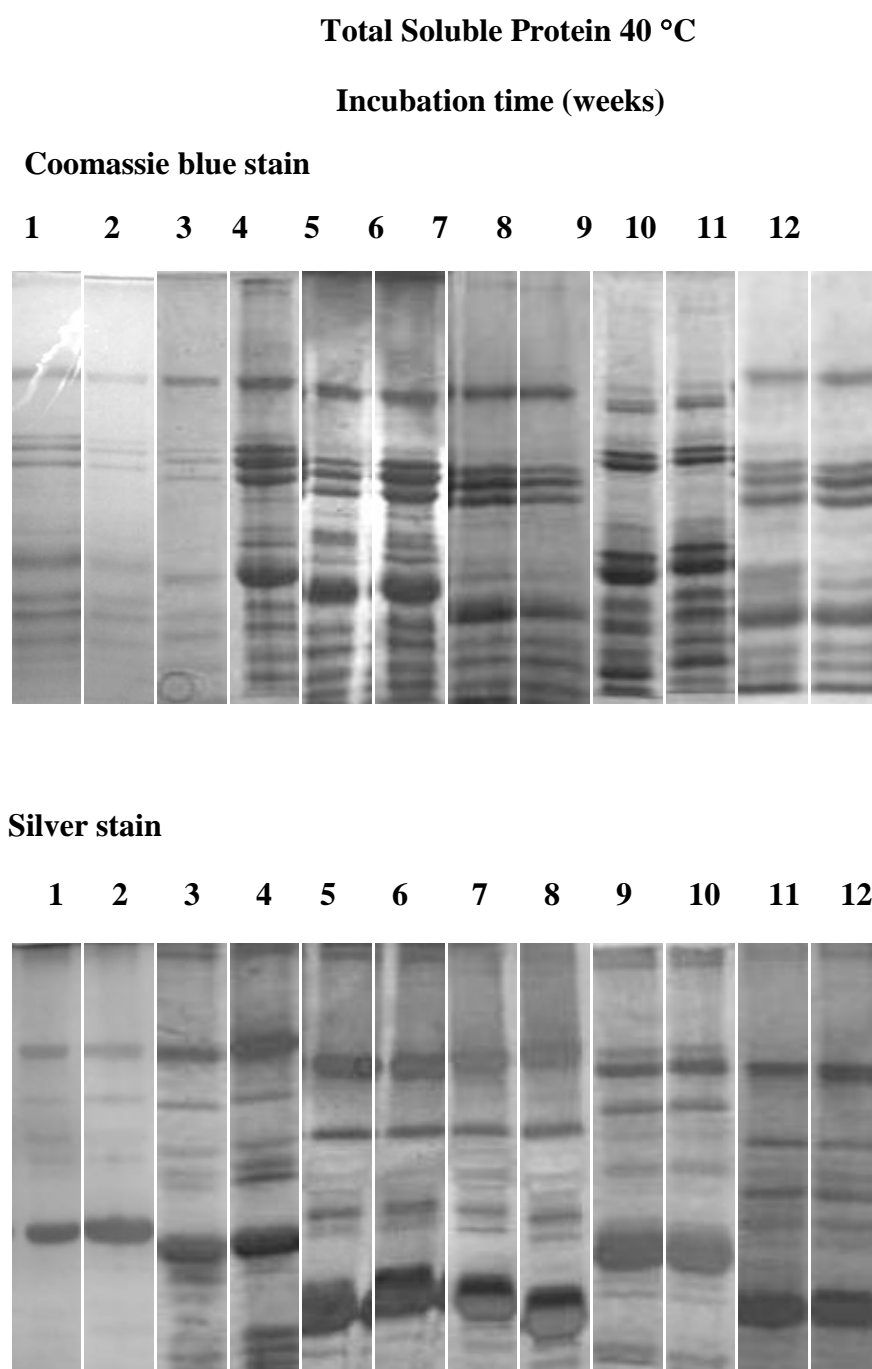


Figure 33. SDS-PAGE of total soluble proteins from peanut butter incubated at 40 °C for 12 weeks.



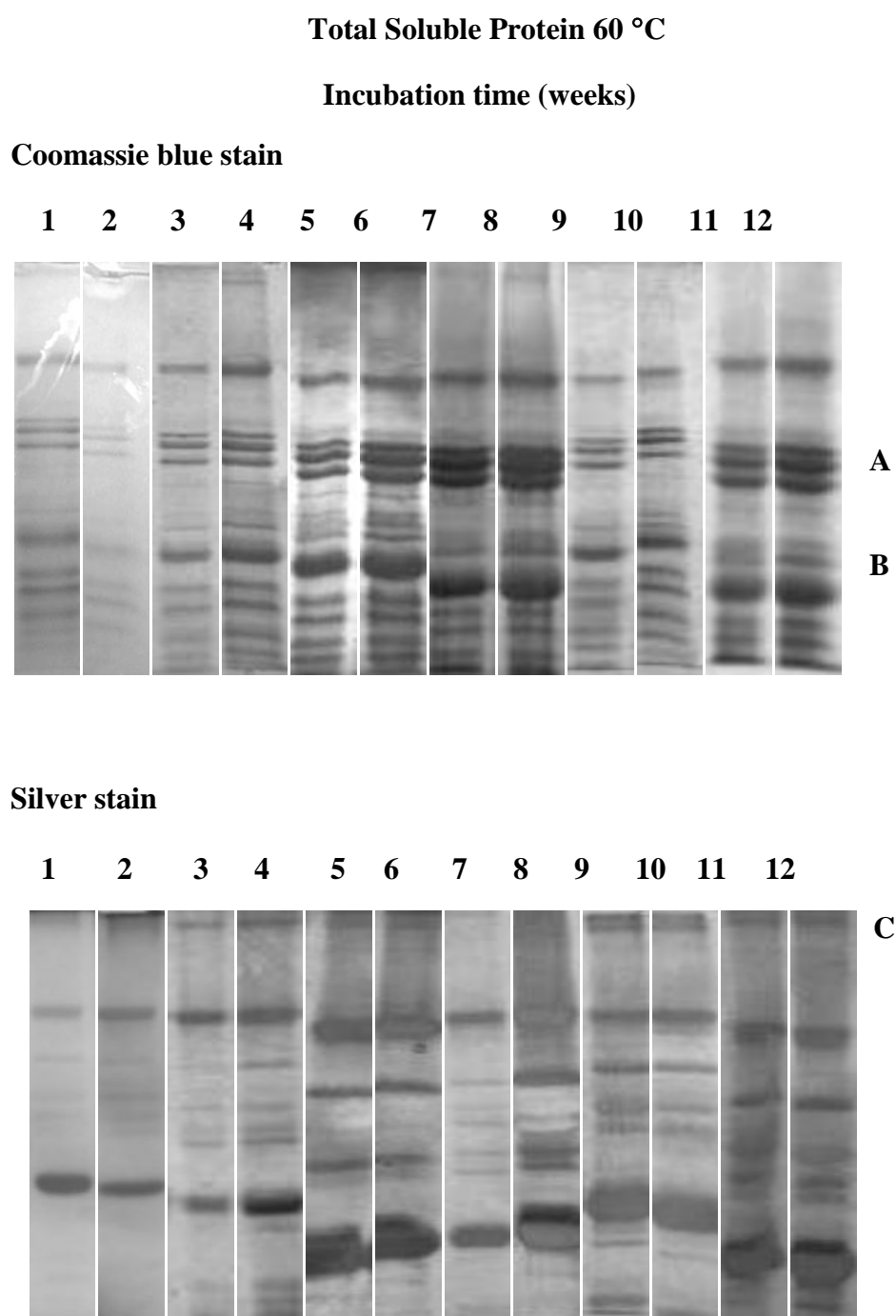


Figure 34. SDS-PAGE of total soluble proteins from peanut butter incubated at 60 °C for 12 weeks.

**Albumins.** Albumins, which include the most soluble metabolic proteins and enzymes, presented the largest number of discrete bands of the three subfractions analyzed. All of the basic peptides stained by Coomassie blue showed gradual decreases in molecular weights (Figures 35-3 top; 25, 40 and 60 °C, respectively), with some loss of the lowest mol wt bands. Acidic proteins that bound silver stain showed more difference (Figures 35-37, bottom). During the first two weeks, only three bands bound the silver stain: ~64, 37, and 20 kDa. With longer incubation, many new bands that bound Ag appeared in both arachin and conarachin regions, and these bands were different than those binding Coomassie blue. There are perhaps two ways this could happen—1) acidic side chains developed or became available on the proteins, perhaps by deglycosylation, and 2) acidic peptides not originally soluble in water only were modified and shifted solubility. Changes were not progressive, but rather showed different patterns every two weeks. The conarachin band at 64 kDa was relatively constant, but the arachin bands showed continual reorganization. Most of the rearrangements of peptides in the gels occurred in the lower mol wt arachins.

Changes occurred earlier at higher incubation temperatures, but otherwise were quite similar at all incubation temperatures.

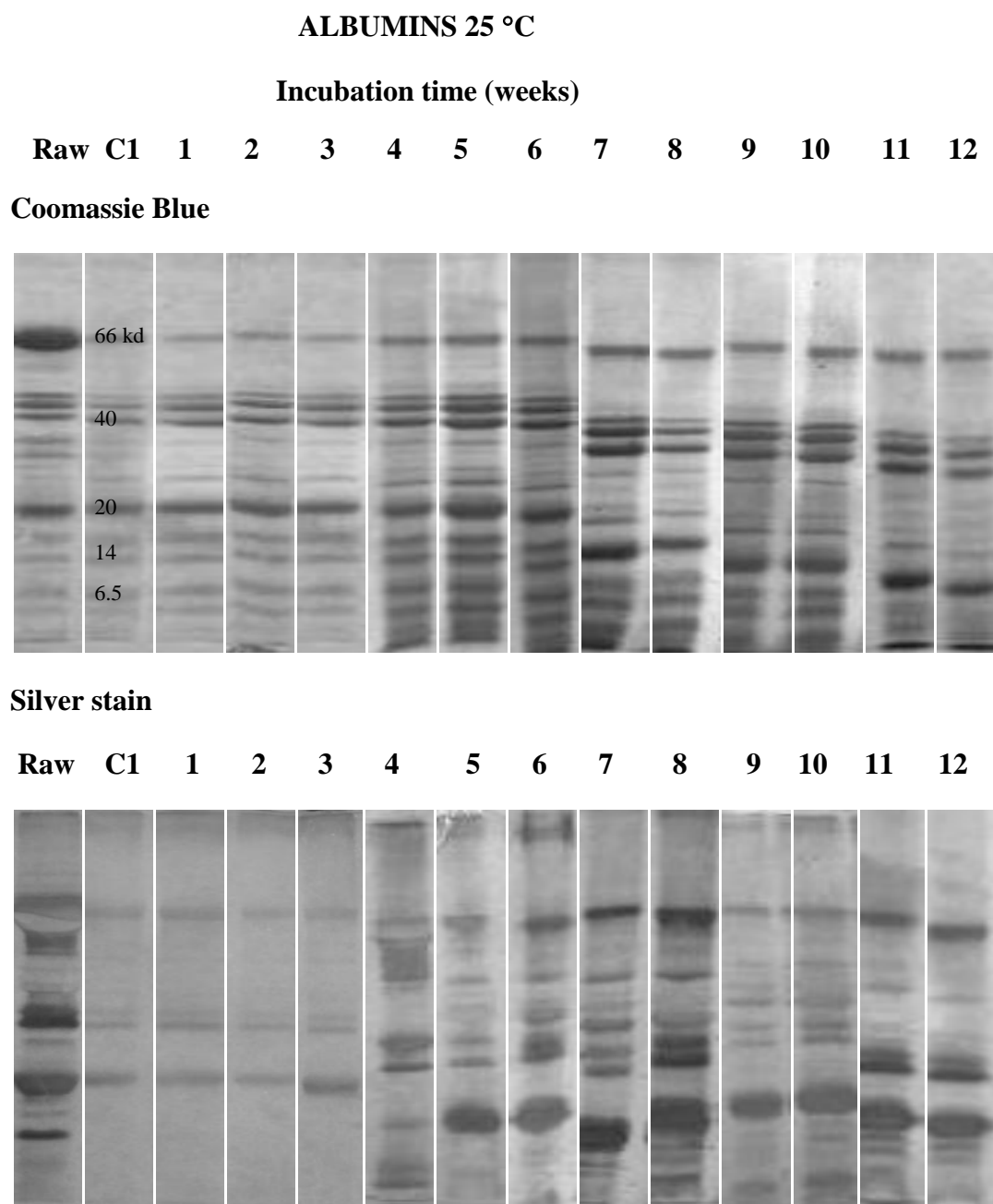


Figure 35. SDS-PAGE of albumins from peanut butter incubated at 25 °C for 12 weeks.

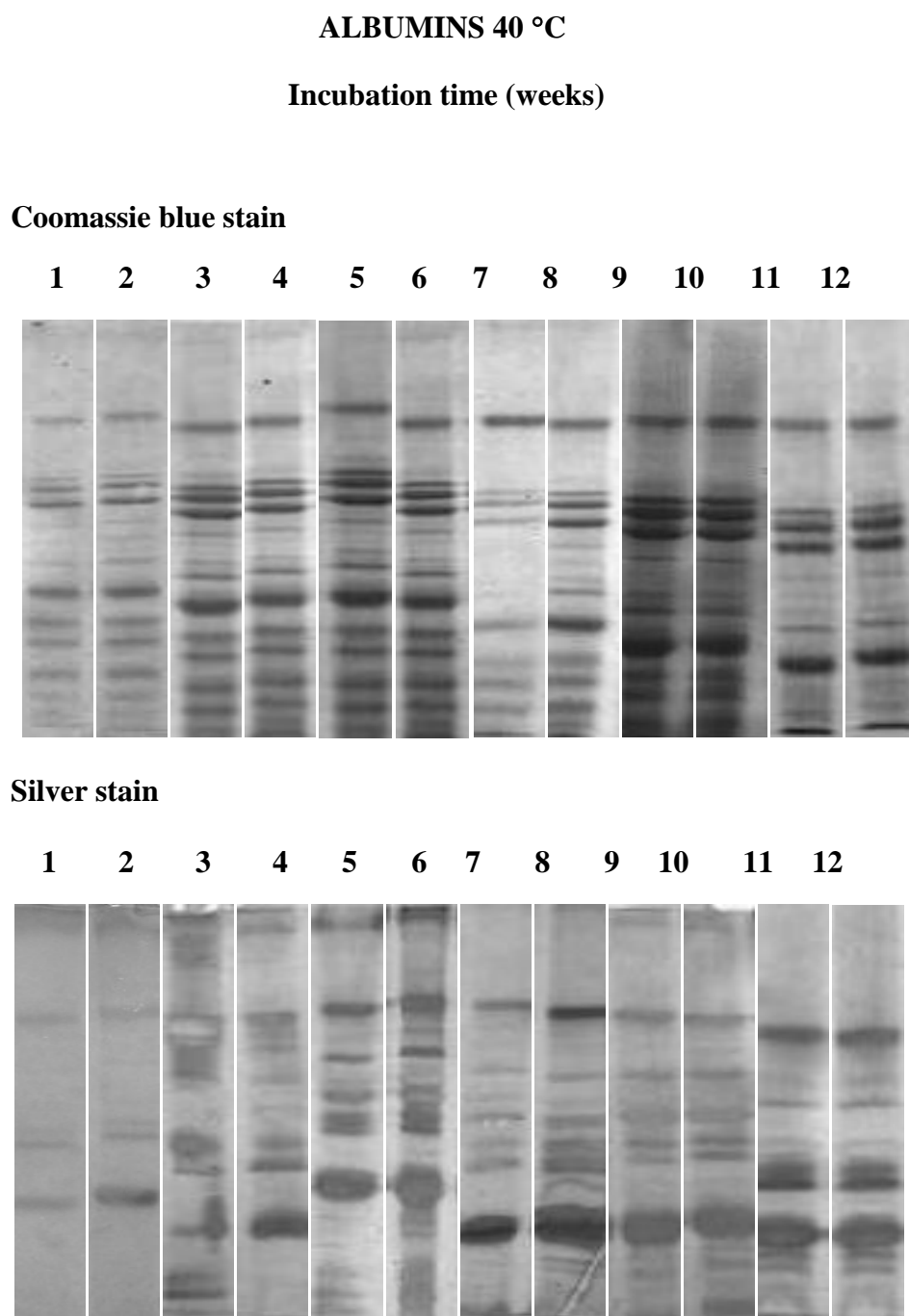


Figure 36. SDS-PAGE of albumins from peanut butter incubated at 40 °C for 12 weeks.

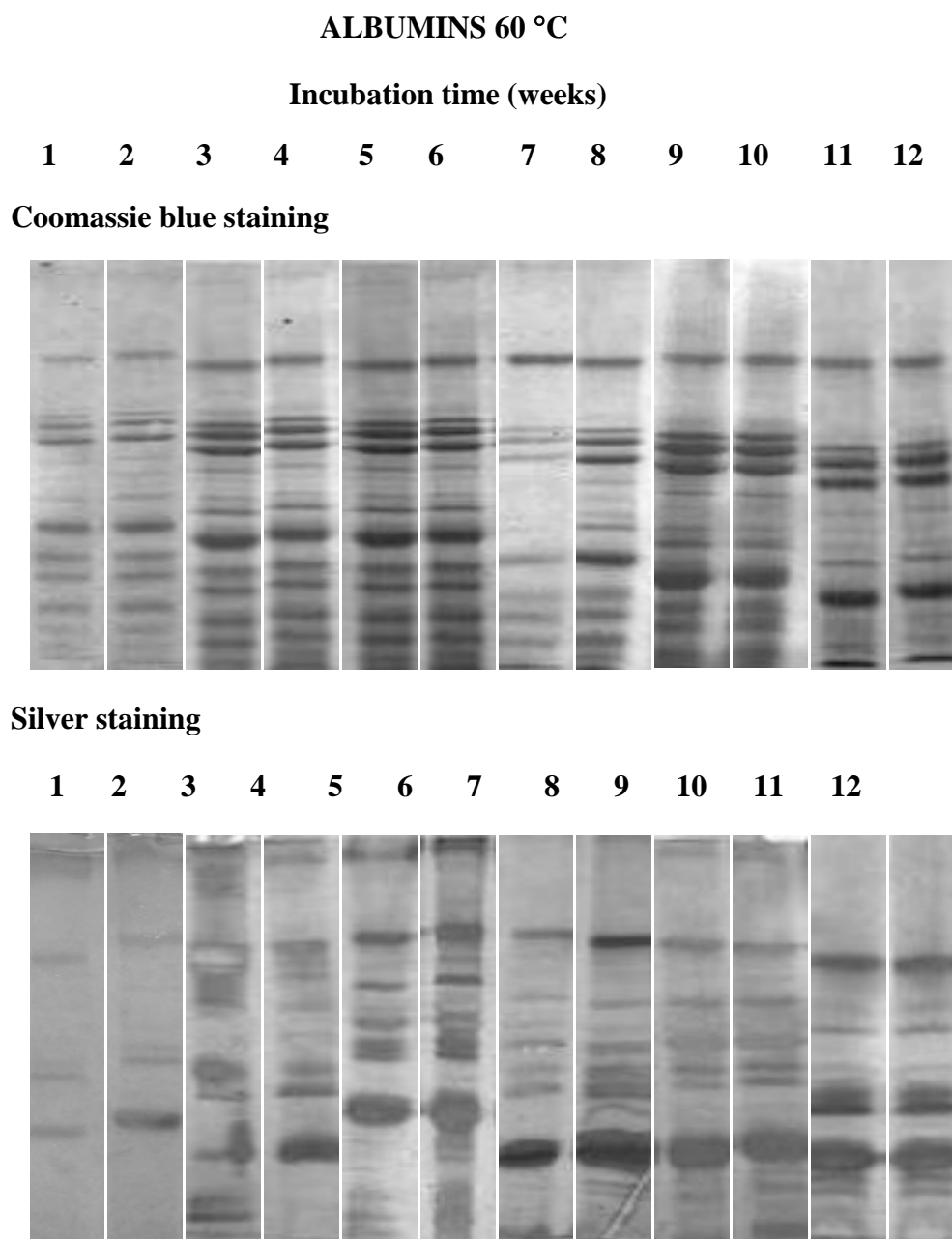


Figure 37. SDS-PAGE of albumins from peanut butter incubated at 60 °C for 12 weeks.

**Globulins.** At first glance, the proteins stained with Coomassie blue appear to have the same pattern as in globulins, but the molecular weights and dominant bands are not the same (Figures 38-40). The major effect was continual decrease in molecular weight among the arachins; the conarachin band did not change. Unlike the albumins, with higher accumulation of proteins in four specific bands – the triplet ~40-45 kDa, and the single band that started at about 27 kDa and dropped to about 15 kDa.

Silver-stained gels showed one main bands, ~22 kDa, that was present in fresh peanut butter, and a second main band at ~66 kDa that grew in with incubation. There were also a number of minor bands in mid-range and low molecular weights, plus two polymeric bands near 225 kDa. The mid-range peptides were relatively constant during incubation, but the low mol wt bands (<22 kDa) showed continual shifting and reorganization during incubation. The most noticeable effect was an increase in the intensity of the two main bands up to 8 weeks, followed by decreasing intensity of the bands. There were no remarkable differences between temperatures.

Overall, it appears that some arachin subunits may be disassociating, followed by partial reorganization with preferential formation of the two major bands.

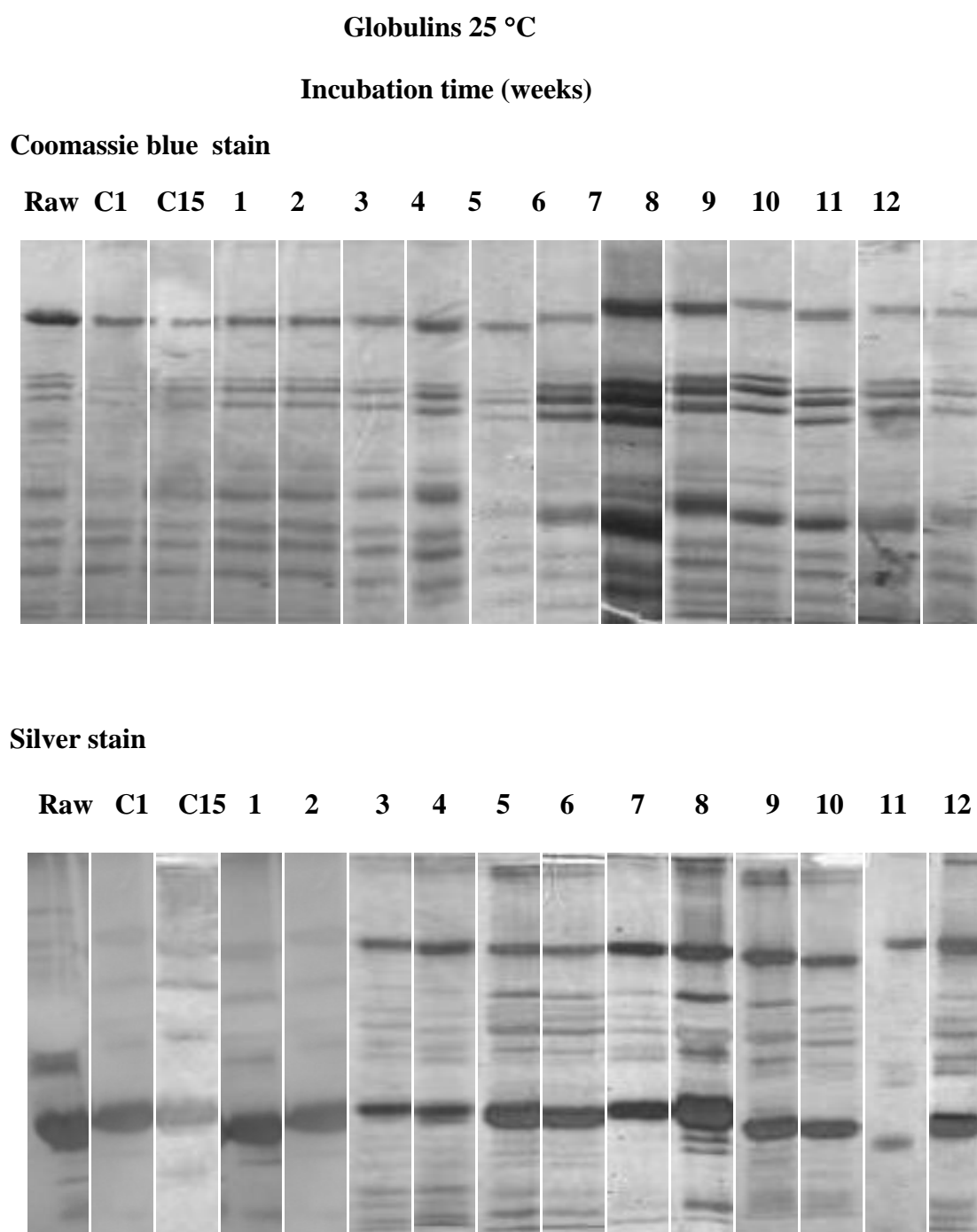


Figure 38. SDS-PAGE of globulins from peanut butter incubated at 25 °C for 12 weeks.

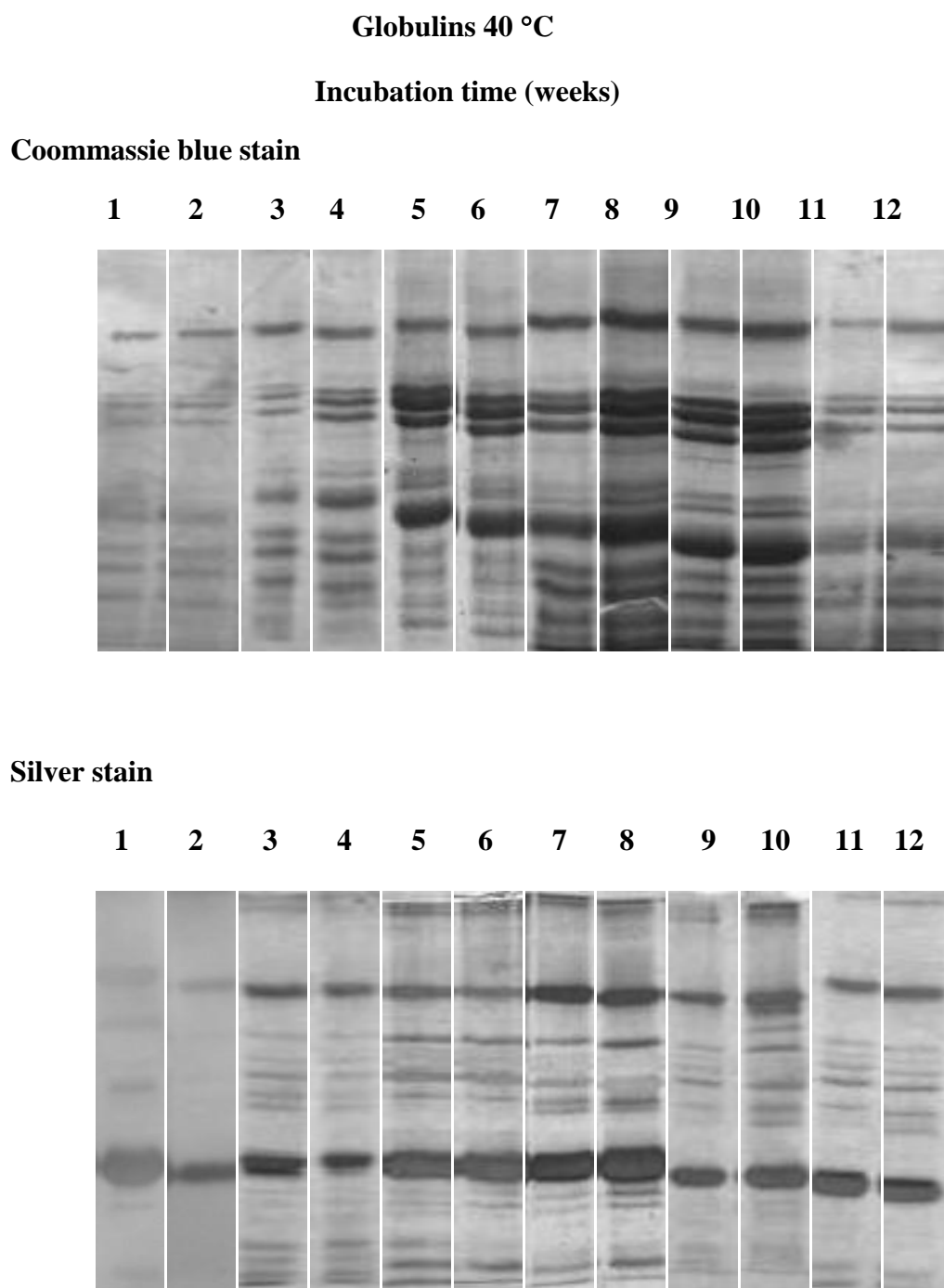


Figure 39. SDS-PAGE of globulins from peanut butter incubated at 40 °C for 12 weeks.



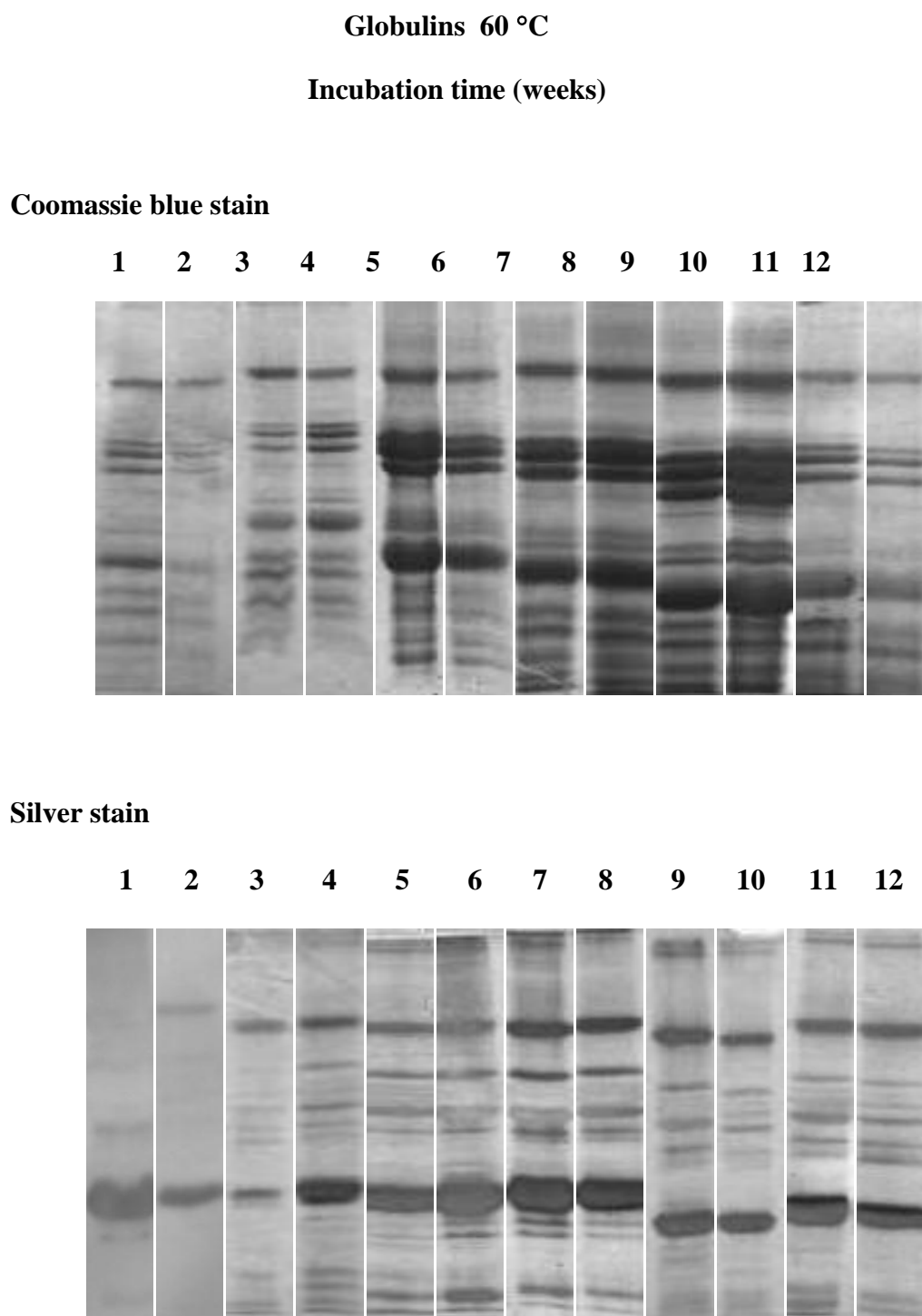


Figure 40. SDS-PAGE of globulins from peanut butter incubated at 60 °C for 12 weeks.

**SDS-soluble proteins.** SDS solubilizes proteins broadly but especially more hydrophobic proteins. Since peanut proteins are highly polar and the SDS fraction of acidic proteins from raw, control, and short incubation peanuts contains relatively little material, this fraction may be considered a repository of modified proteins. As shown in Figures 41-43, alkaline proteins (CBB stained) consisted of a dominant triplet (38, 40, 42 kDa) plus two monomers at 14 and 66 kDa, polymeric material just under the well (225-250 kDa), and some weak minor bands that grew in during incubation (between 14 and 66 kDa, and < 14 kDa). These showed some changes in mol wts (mostly decreased) but retained original banding patterns throughout. Extraction without 2-mercaptoethanol as a reducing agent revealed that all of these fractions were involved in disulfide crosslinking to form different peptides, even though cysteine/cystine is present at only low concentrations in peanut proteins.

In contrast, the acidic proteins (silver stained) accumulated a number of distinctive new bands and these changed over time, indicating the protein co-oxidation is a serial rather than linear reaction. In other words, the proteins do not work towards a single modification accumulating over time, but undergo a series of transformations that vary with time and perhaps lipid oxidation products present during the analysis period. Once again, PAGE patterns with some common mol wts pointed to a reorganization of existing peptides rather than fragmentation or creation of totally new peptides. However, smears (particularly in the high mol wt region) increased with incubation, indicating that random damage was also occurring. Modified bands appeared earlier at higher incubation temperatures.

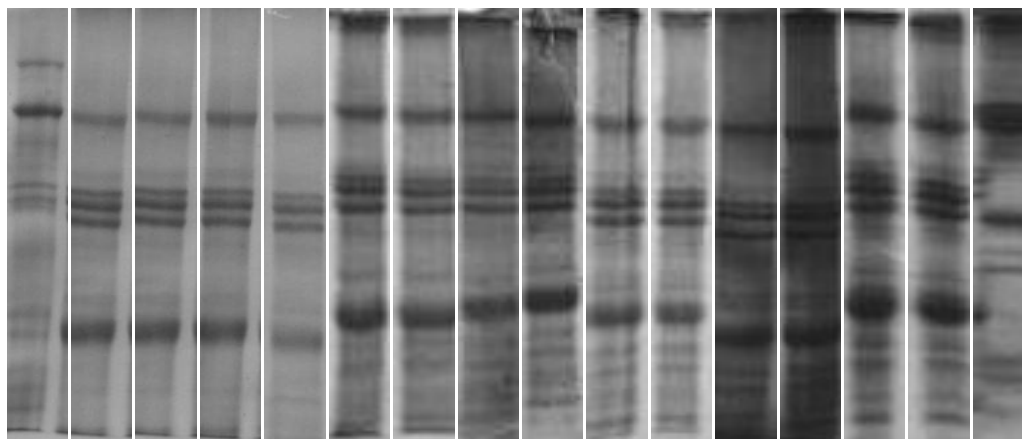
That the peptide mol wts in the SDS-soluble fraction did not exactly match mol wts of albumins or globulins and that additional bands accumulate in the SDS fraction with incubation time supports SDS as a repository of modified proteins.

# SDS-2ME Soluble Proteins 25 °C

Incubation time (weeks)

Coomassie blue stain

Raw C1 C15 1 2 3 4 5 6 7 8 9 10 11 12 NR



Silver stain

Raw C1 C15 1 2 3 4 5 6 7 8 9 10 11 12 NR

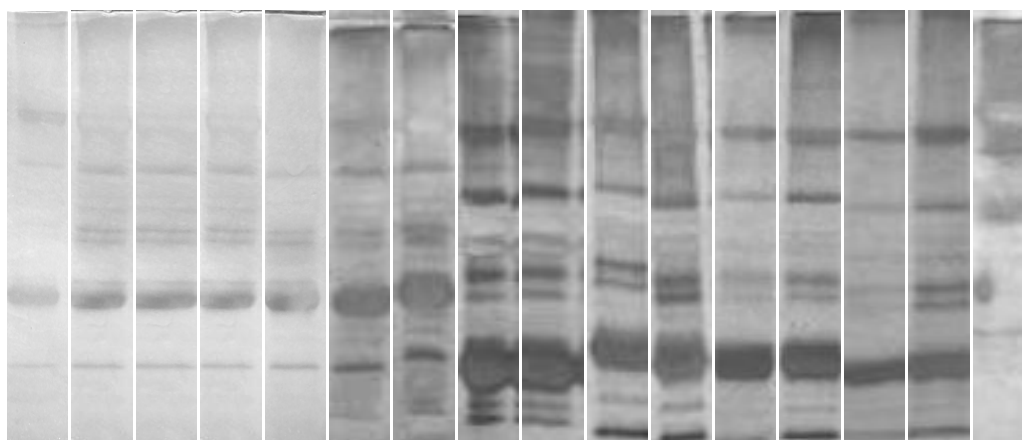


Figure 41. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 25 °C for 12 weeks.

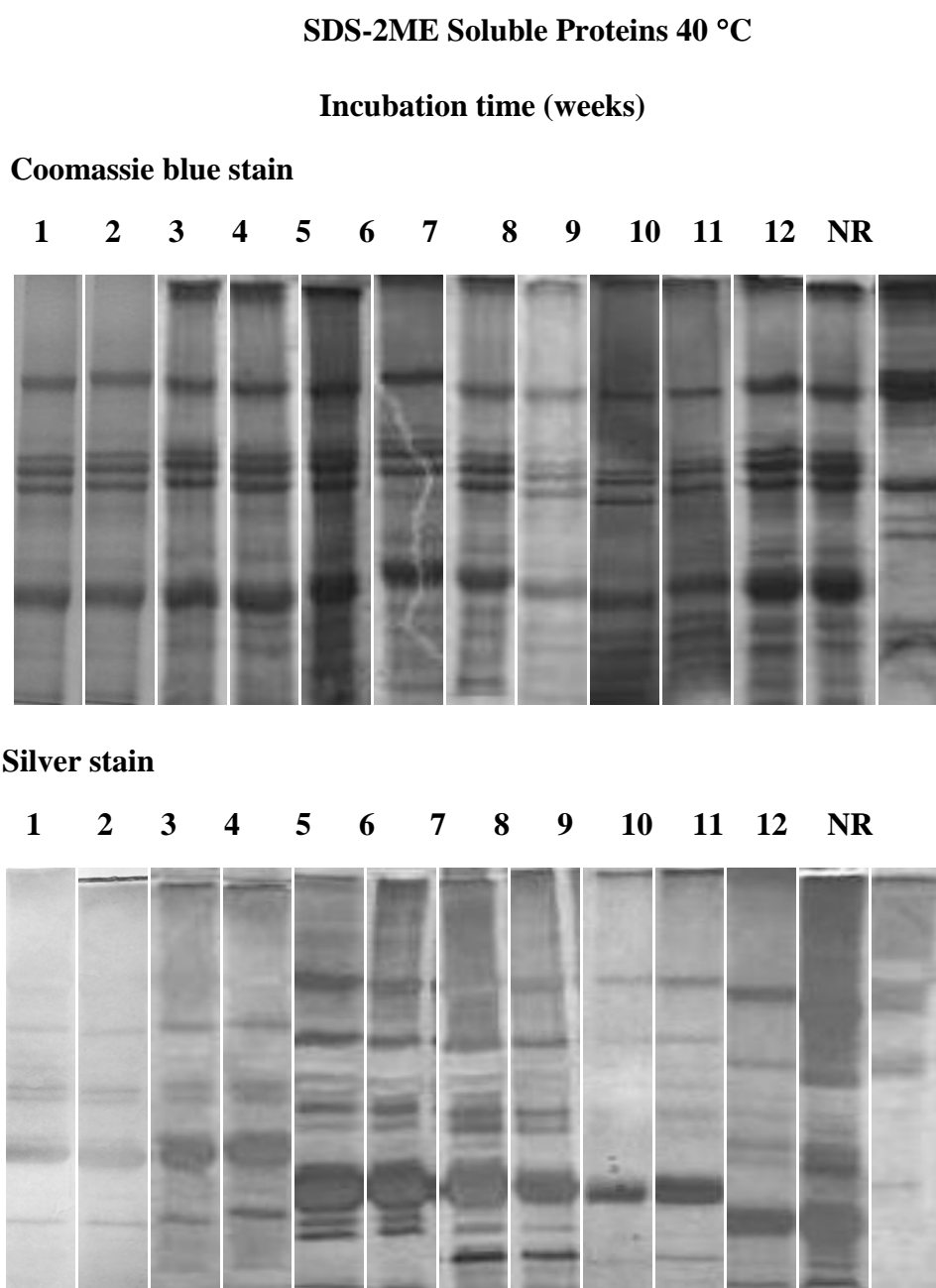


Figure 42. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 40 °C for 12 weeks.

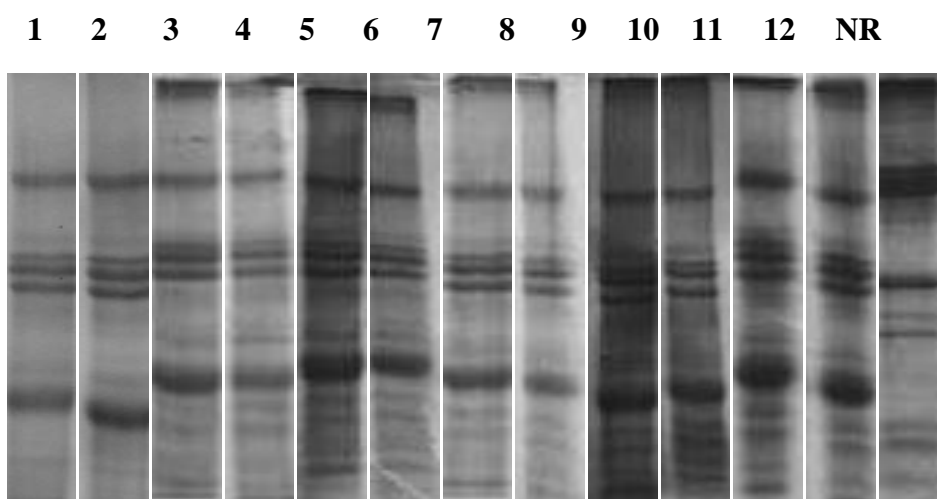
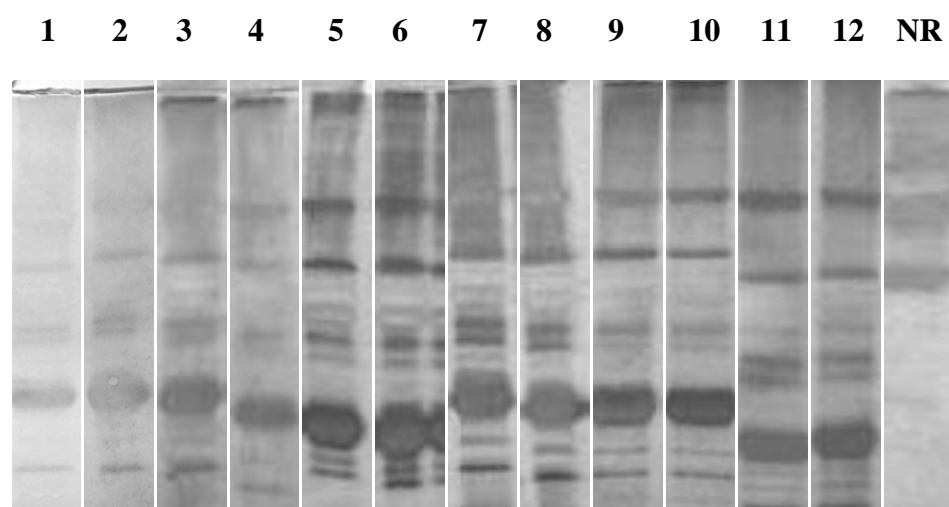
**SDS-2ME Soluble Proteins 60 °C****Incubation time (weeks)****Coomassie blue****Silver stain**

Figure 43. SDS-PAGE of SDS-soluble peptides from peanut butter incubated at 60 °C for 12 weeks. NR indicates protein were extracted without reducing agent.

Summary of PAGE changes:

- ↓ mol wt of basic albumins and globulins, especially in lower mol wt range, indicating peptide fragmentation or reorganization of protein subunits
- Rearrangement among acidic albumins; surface modifications (perhaps deglycosylation) among acidic globulins leading to appearance of new bands binding Ag
- Modified fractions from both alb/glob become hydrophobic (surface or configuration changes ?), move to SDS-soluble fraction
- Both specific (leading to discrete peptides) and non-specific (generating diffuse smears) crosslinking occurred, particularly in the SDS-soluble fraction, but aggregates remain relatively small (<225 kDa); some S-S involved.
- Surface changes decreased CBB dye binding.

Peanut protein co-oxidation patterns differed greatly from those observed previously in corn and wheat where polymerization and formation of high polymers dominated. It is not clear whether these differences arise from variations in protein characteristics (zeins vs gliadin/glutenin vs arachins) or system characteristics (moist, high lipid semisolid vs dry matrices with lower lipid contents).

### 6.2.2.3 Native PAGE to detect changes in protein surface charge

Native PAGE neither denatures nor dissociates proteins, but rather uses a glycine ion buffer to migrate proteins by intrinsic charge to mass ratio rather than by the anionic SDS bound to the proteins. Proteins are prepared in a non-reducing, non-denaturing sample buffer which maintains the protein secondary structure and native charge density. The proteins retain their native conformation, and their mobility is governed by the ratio of electric charge to hydrodynamic friction (Arakawa et al., 2006). Because charge is the driving force for migration, native gels can detect changes in protein charge, e.g. by complexation, deamination or decarboxylation, or aggregation. In the electrophoresis system used, proteins distributed between the anode in the upper solvent reservoir and the cathode in the lower solvent reservoir. Thus, acidic proteins should migrate farther into the gels while basic proteins remain near the top of the gel.

In general, patterns in native gels were consistent with those in SDS gels in suggesting that acidic amino acids were major targets for modification. Coomassie blue and silver stains again gave different peptide patterns, but both showed obvious and marked changes in the most negatively-charged proteins and much greater stability among the positively-charged peptides that barely migrated into the gels.

In the albumin fraction, the most distinct staining was with Coomassie blue. Some strong (+)-charged bands in raw peanuts were lost during roasting, most likely reflecting loss of lysine to Maillard reactions. Several more (–)-charged bands were present, including on very acidic band migrating at the solvent front. These acidic bands were lost during incubation, with losses increasing with temperature. Silver staining revealed on very (+)charged protein band and two strongly acidic bands in raw peanuts; these were lost during roasting. The same diffuse (–)-charged smearing

detected by CBB staining was also detected by silver stain. This was lost during incubation, although at 9 and 10 weeks of incubation new strongly acidic peptides appeared transiently.

In the globulin fraction, Coomassie blue stain showed more discreet and distinct bands among both (+) and (-)-charged proteins. The (-)-charged material tended to decrease with incubation time and increase with temperature. Two strongly (+) bands were relatively constant. Silver stain again detected the same (-)-charged proteins as CBB in raw peanuts and control peanut butter. These bands were lost with higher temperature and oxidation. However, new (-)-charged material appeared at 8 weeks and by the end of the incubation period, Ag bound to peptides that were distributed over the entire length of the gel. This pattern suggests that acidic groups were lost, e.g. by blocking or by decarboxylation, and then were unblocked or reformed at higher concentrations and in different fractions, e.g. by aldehyde oxidation.

The SDS-soluble fraction was paradoxical in its behavior. Coomassie blue staining revealed extensive intense smearing in gel regions that should contain (-)-charged proteins, even though this dye binds to basic amino acids. Banding in this smear became more distinct at higher temperatures. This smeared region decreased with incubation time, progressively losing the most negative peptides, and little of the material remained at the end of the study, showing clearly that major modifications of proteins were occurring. Surprisingly, these peptides that migrated as if they had greater negative charge were not detected by silver stain, which binds preferentially to acidic amino acids. Instead, silver detected only smears of what should be positively-charged proteins. These results are in direct contradiction to observations in SDS gels, where the SDS-soluble fraction showed considerable shifting and reorganization



among peptides stained with silver but mainly molecular weight loss among peptides stained with Coomassie blue. As one possible explanation, peptides unwind and reveal acidic groups in SDS, but in aqueous polar media such as glycine buffer , unglycosylated peanut proteins are prone to aggregation due to their high content of nonpolar amino acids (Narchi et al., 1991; Arakawa et al., 2001). Aggregation may block access to silver binding groups. Clearly, surface modifications are involved in this strange paradox, and the chemistry underlying these must be elucidated to understand overall behavior of peanut proteins.

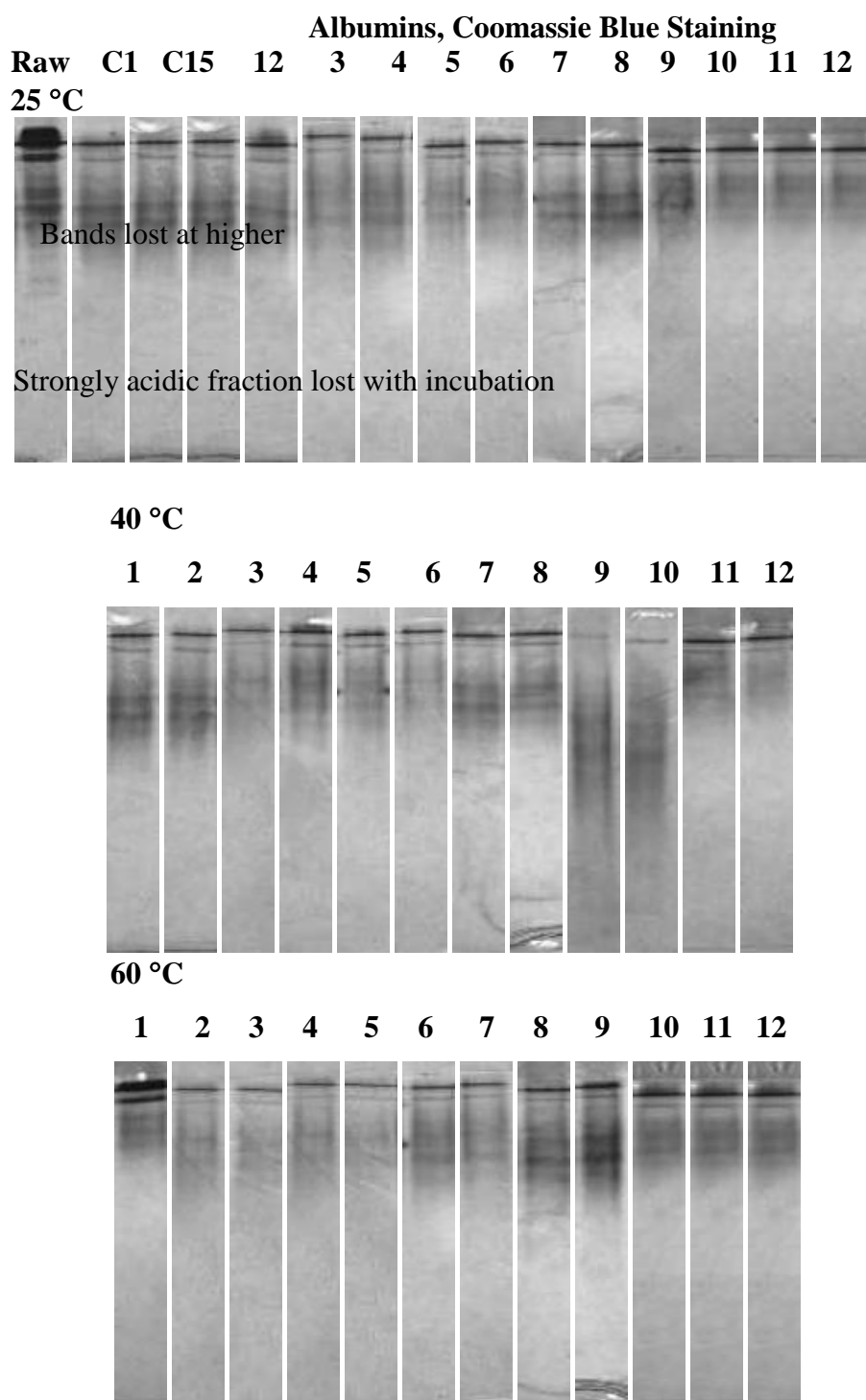


Figure 44. Native PAGE patterns of albumins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain. C1 and C15 are controls at 1 and 15 weeks, respectively.

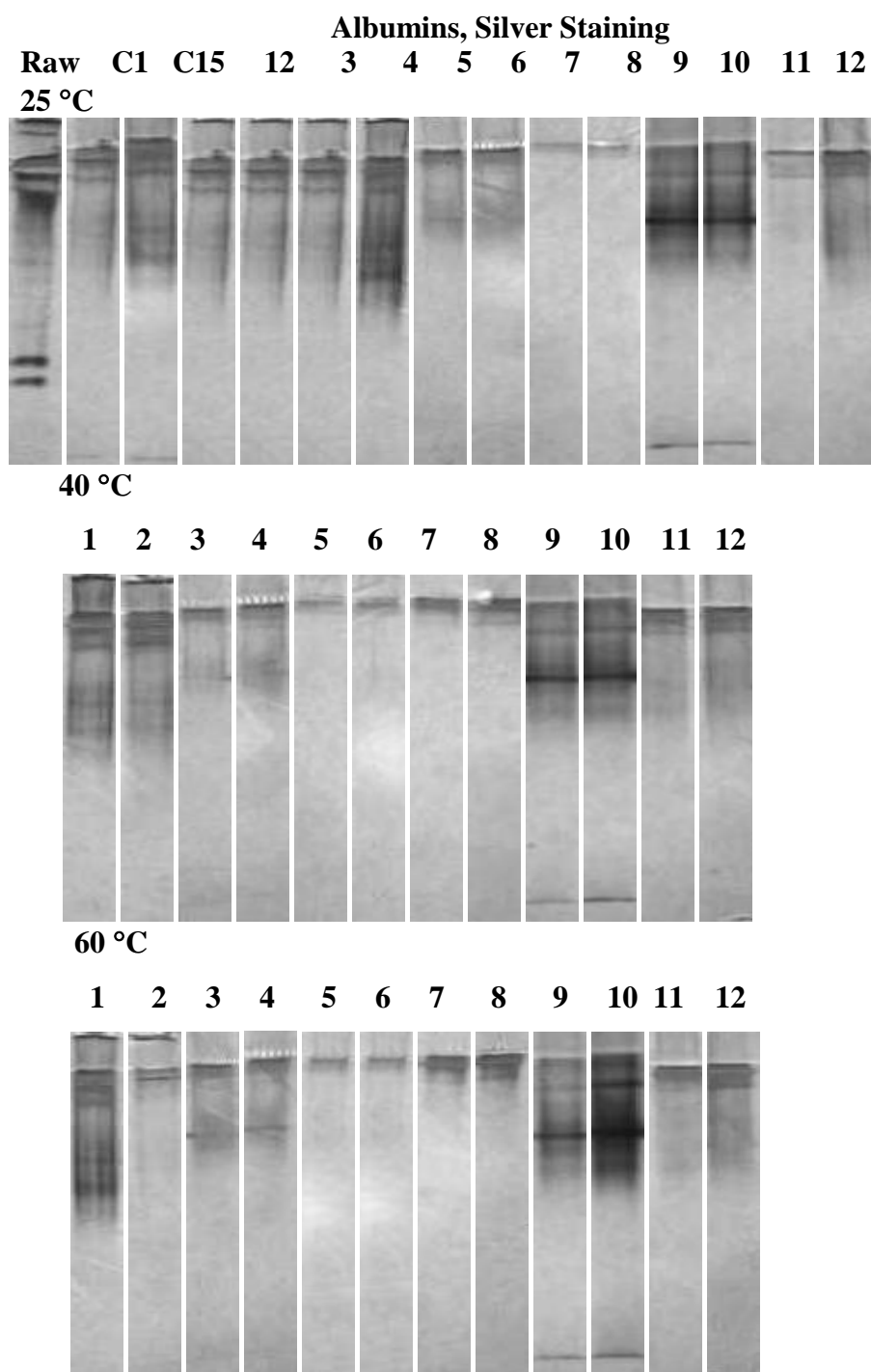


Figure 45. Native PAGE patterns of albumins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain. C1 and C15 are controls at 1 and 15 weeks, respectively.

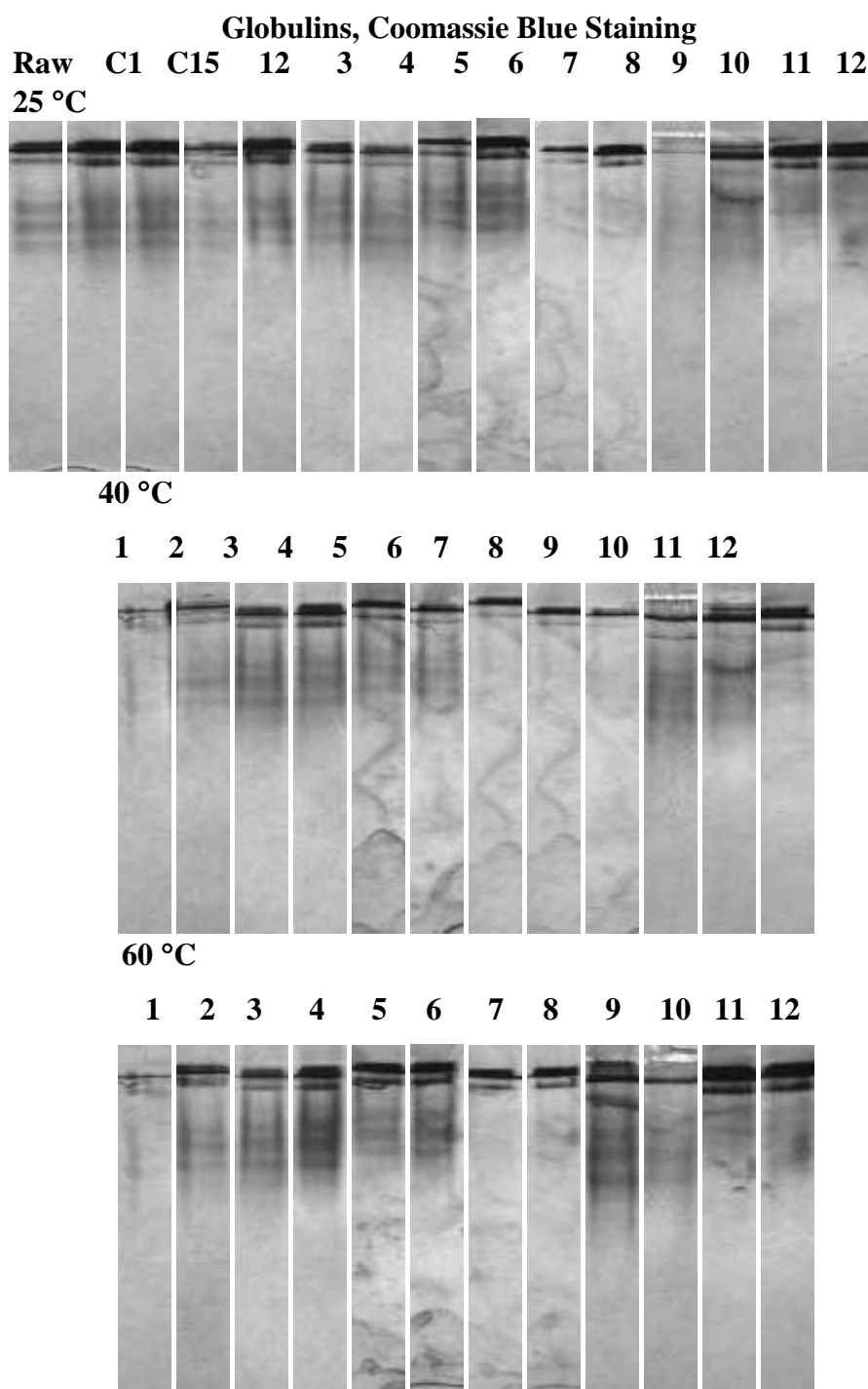


Figure 46. Native PAGE patterns of globulins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain. C1 and C15 are controls at 1 and 15 weeks, respectively.

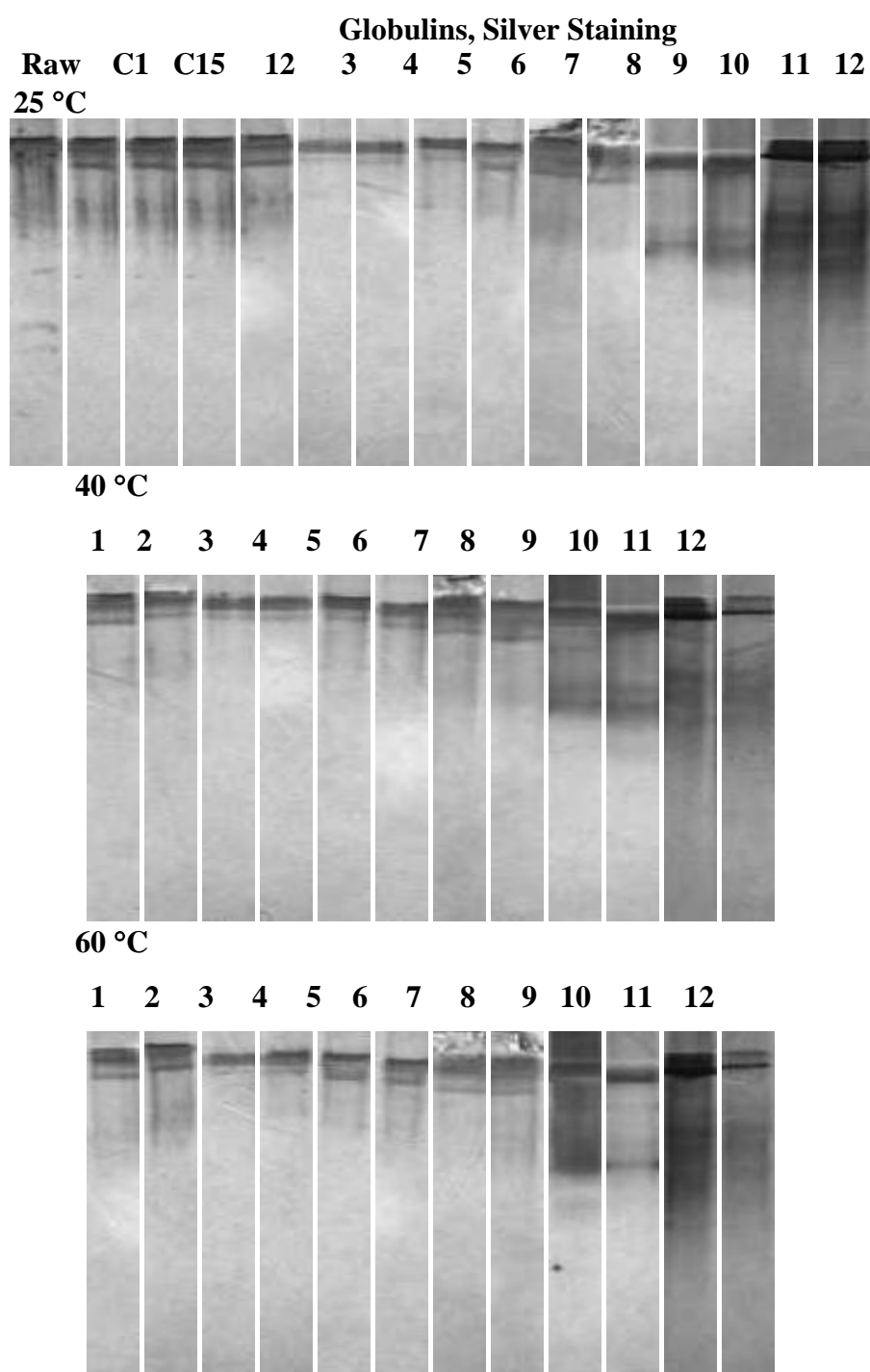


Figure 47. Native PAGE patterns of globulins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain. C1 and C15 are controls at 1 and 15 weeks, respectively.

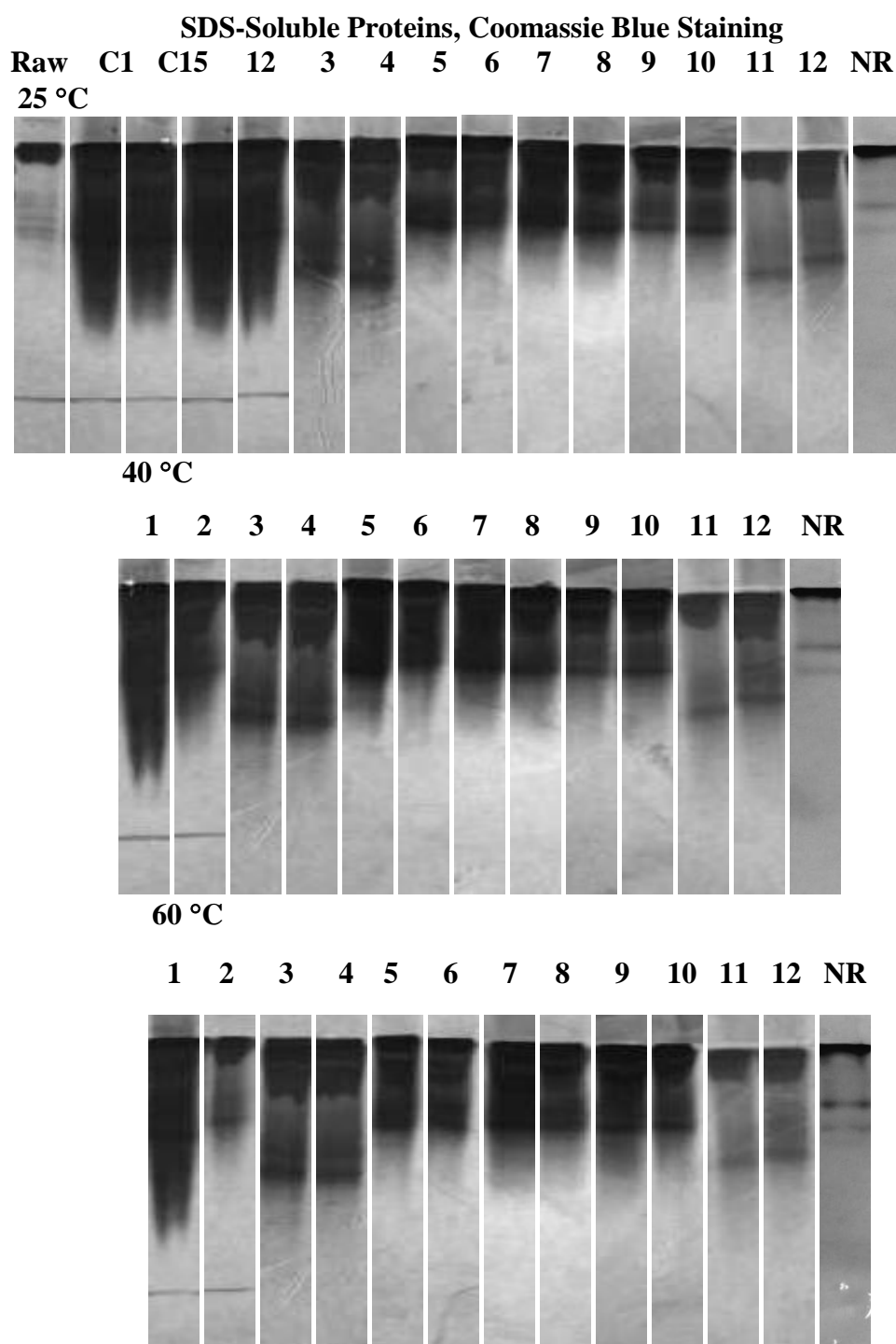


Figure 48. Native PAGE patterns of SDS-soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain. C1 and C15 are controls at 1 and 15 weeks, respectively. NR – extraction without reducing agent.

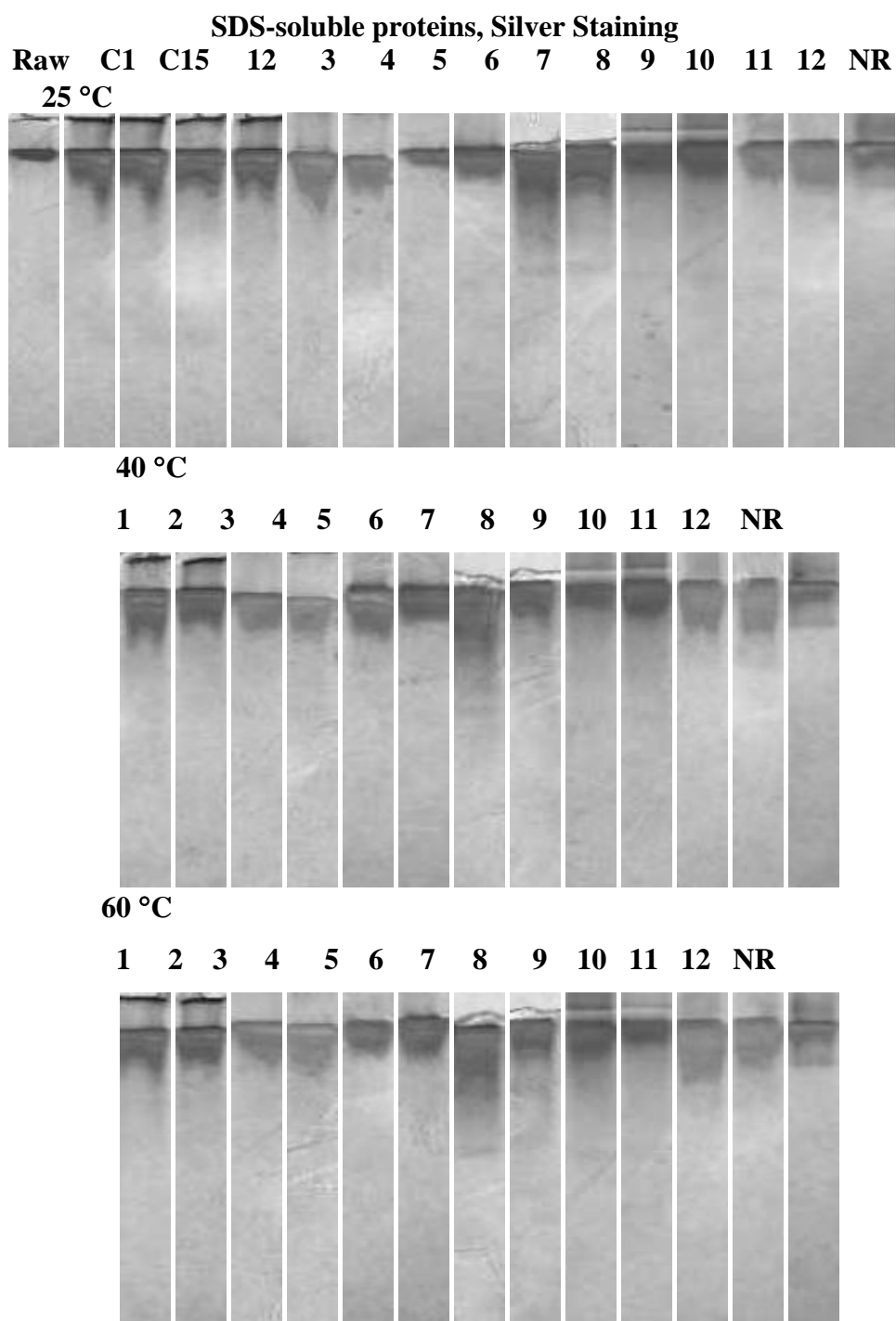


Figure 49. Native PAGE patterns of SDS-soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain. C1 and C15 are controls at 1 and 15 weeks, respectively. NR – extraction without reducing agent.

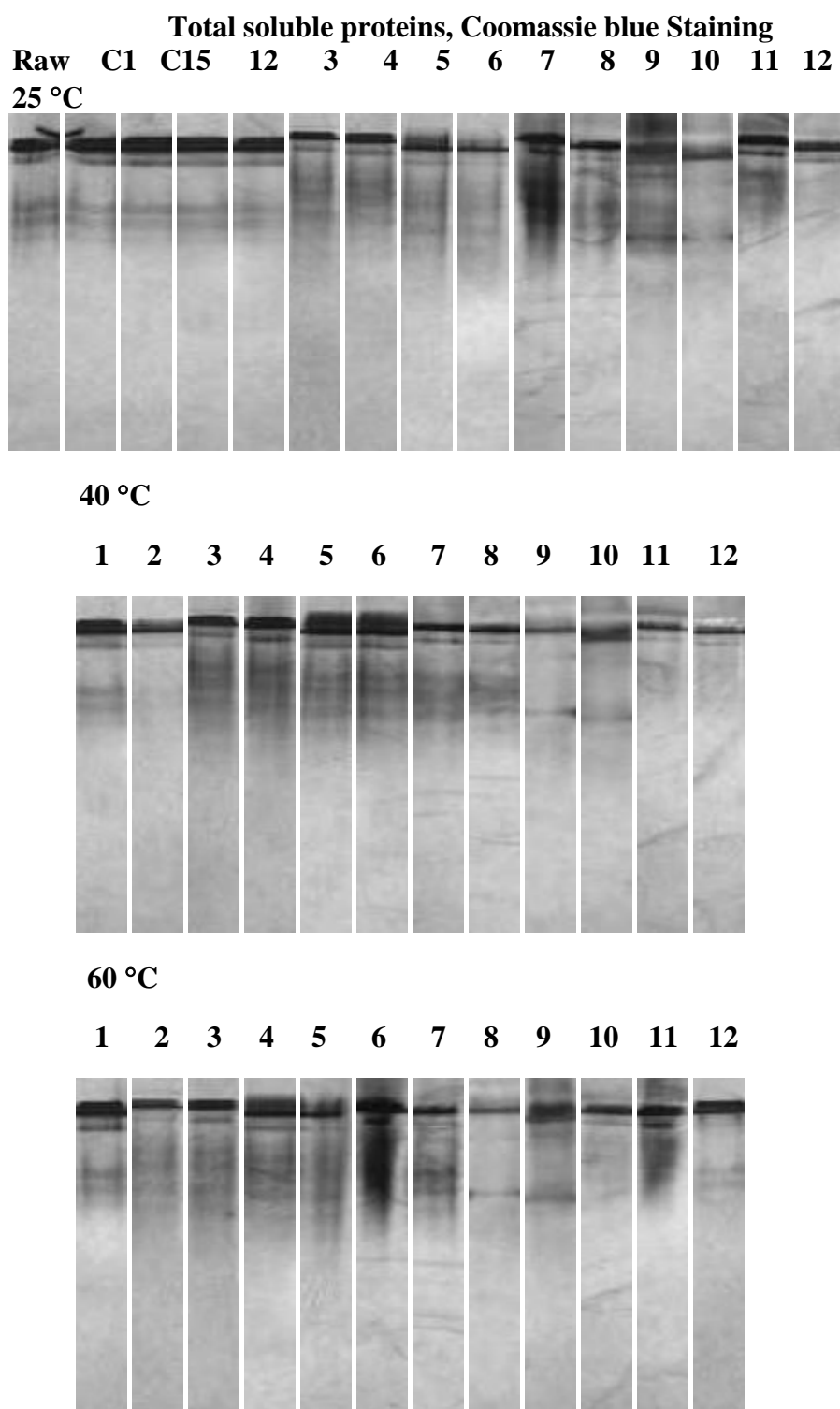


Figure 50. Native PAGE patterns of total soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Coomassie blue stain. C1 and C15 are controls at 1 and 15 weeks, respectively.



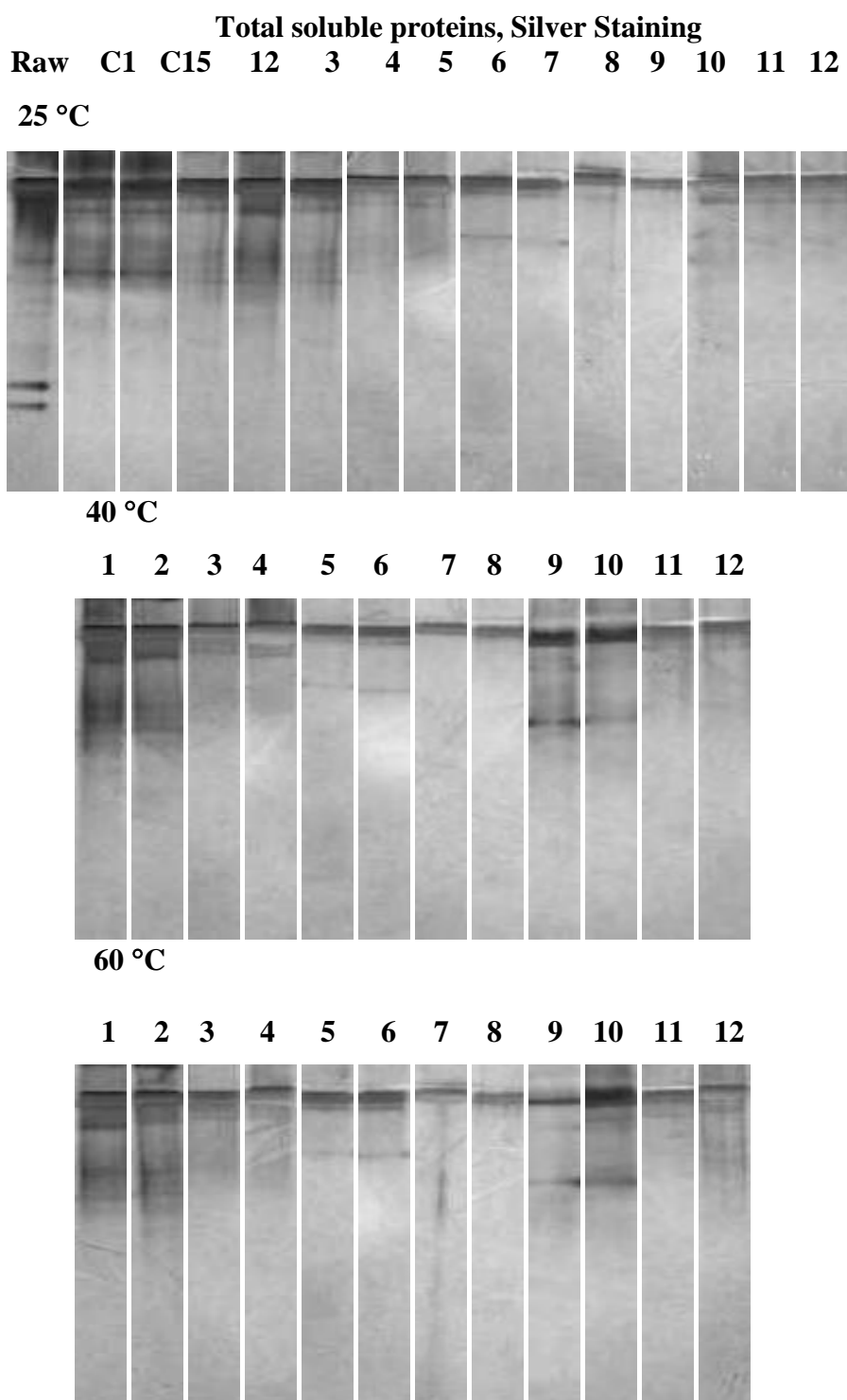


Figure 51. Native PAGE patterns of total soluble proteins extracted from peanut butter incubated in MRE package at 25, 40, and 60 °C for up to 12 weeks. Silver stain. C1 and C15 are controls at 1 and 15 weeks, respectively.

#### 6.2.2.4 Protein carbonyls

Oxidation of proteins modifies the side chains of methionine, histidine, and tyrosine and forms cysteine disulfide bonds (Stadtman, 1993; Uchida and Kawakishi, 1990; Heinecke, 1993). Metal catalyzed oxidation of proteins introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues in a site-specific manner. Carbonyls can also be formed in proteins by peptide scission and deamidation (Stadtman, 2006), by reaction with dialdehydes (Buttkus 1970; Li and King 1999), and by Michael addition of amino acids to unsaturated aldehydes, which forms carbonyl adducts on protein surfaces (Stadtman, 1993; Farber and Levine, 1986; Climent et al., 1989; Levine, 1993). To detect the presence of oxidized proteins, protein carbonyls were analyzed by reaction with dinitrophenyl hydrazine (DNPH) (Levine et al., 1990), and the adducts thus formed were detected in Western blots by specific antibodies raised against DNPH-labeled proteins.

Western blots of control peanut butters (even at 15 weeks) showed only very faint bands of protein carbonyls at about 40 and 80 kDa in globulins and about 14, 20, and 120 kDa in SDS-soluble proteins (Figures 52-55).. Therefore, the appearance of protein carbonyls was specifically associated with oxidative processes during incubation, and did not arise from heat during roasting.

Interestingly, protein oxidation was quite specific in incubated samples. Initially, only narrow bands at about 40, 80, and 120 kDa showed oxidation in total soluble proteins (Figure 52). Least oxidation occurred at 25 °C, where the amount of material in these bands increased some with incubation time, and additional oxidized protein trailing behind each band also appeared. After incubation at 40 or 60 °C, there was much more antibody reaction revealing many more peptides oxidized, and smears of oxidized proteins appeared at mol wts below each of the main oxidized bands. As

might be expected, protein oxidation increased with incubation temperature.

Blots of individual fractions revealed that albumins showed only traces of oxidation until 9 weeks of incubation, and then only peptides at about 37 and 45 kDa were affected (Figure 53). Oxidation increased with temperature, and at 60 °C additional oxidized bands appeared at about 80 and then also 97 kDa.

Globulins were notably more altered (Figure 54). Oxidation still centered on the same peptides noted above, but bands broadened and became more diffuse. Oxidation increased with time, and extensive smearing behind the two higher mol wt bands developed after 9 weeks of incubation. Oxidation also increased with temperature, with smearing appearing earlier (8 days) and more extensively.

In contrast to the specific limited oxidation in albumins and globulins, peptides in the SDS-soluble fraction were dramatically and extensively oxidized (Figure 55). Smears of oxidized proteins covered the entire gel, even in the stacking gel and at lower mol wts where Coomassie blue and silver did not detect peptides. This observation is one more piece of evidence that extensive surface modification occurred in peanut proteins during co-oxidation, and that modified proteins shifted into the SDS-soluble fraction. It also provides new evidence for free radical crosslinking of peanut proteins. The only clear banding occurred at lower molecular weights, mostly less than 20 kDa. Interestingly, a sample that had been extracted without 2-mercaptoethanol to reduce disulfide bonds also showed oxidized bands at lower mol wts not detected by Coomassie blue and silver stains. Oxidation was so extensive that temperature effects could not be determined without densitometer measurements (these were not available).

Overall, these results demonstrate that oxidation of proteins is extensive and a major source of modification and degradation in peanut butter. Protein carbonyls

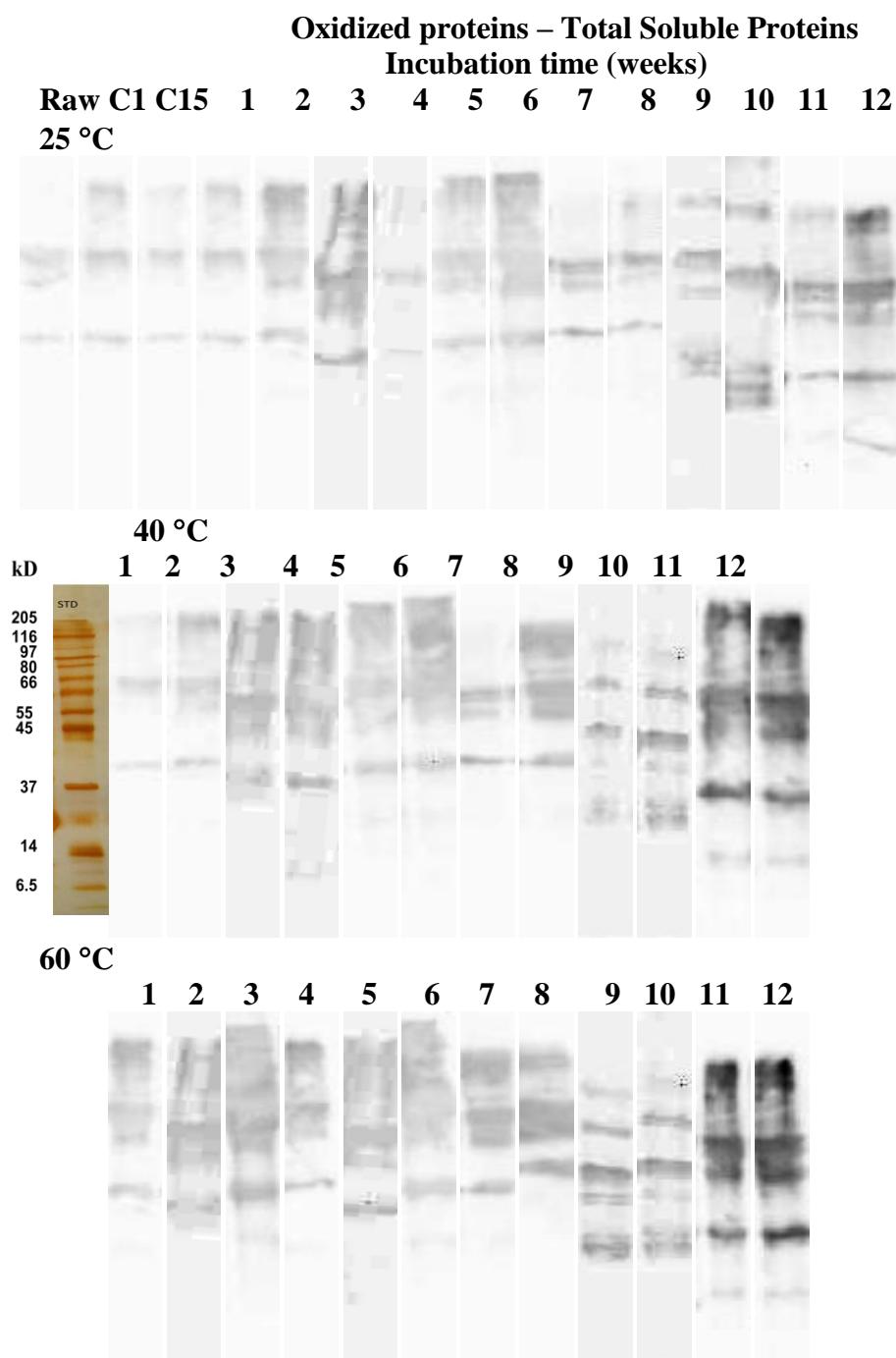


Figure 52. Distribution of protein carbonyl oxidation products in the total soluble protein fraction extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks. C1 and C15: controls stored at 10 °C for 1 and 15 weeks, respectively.

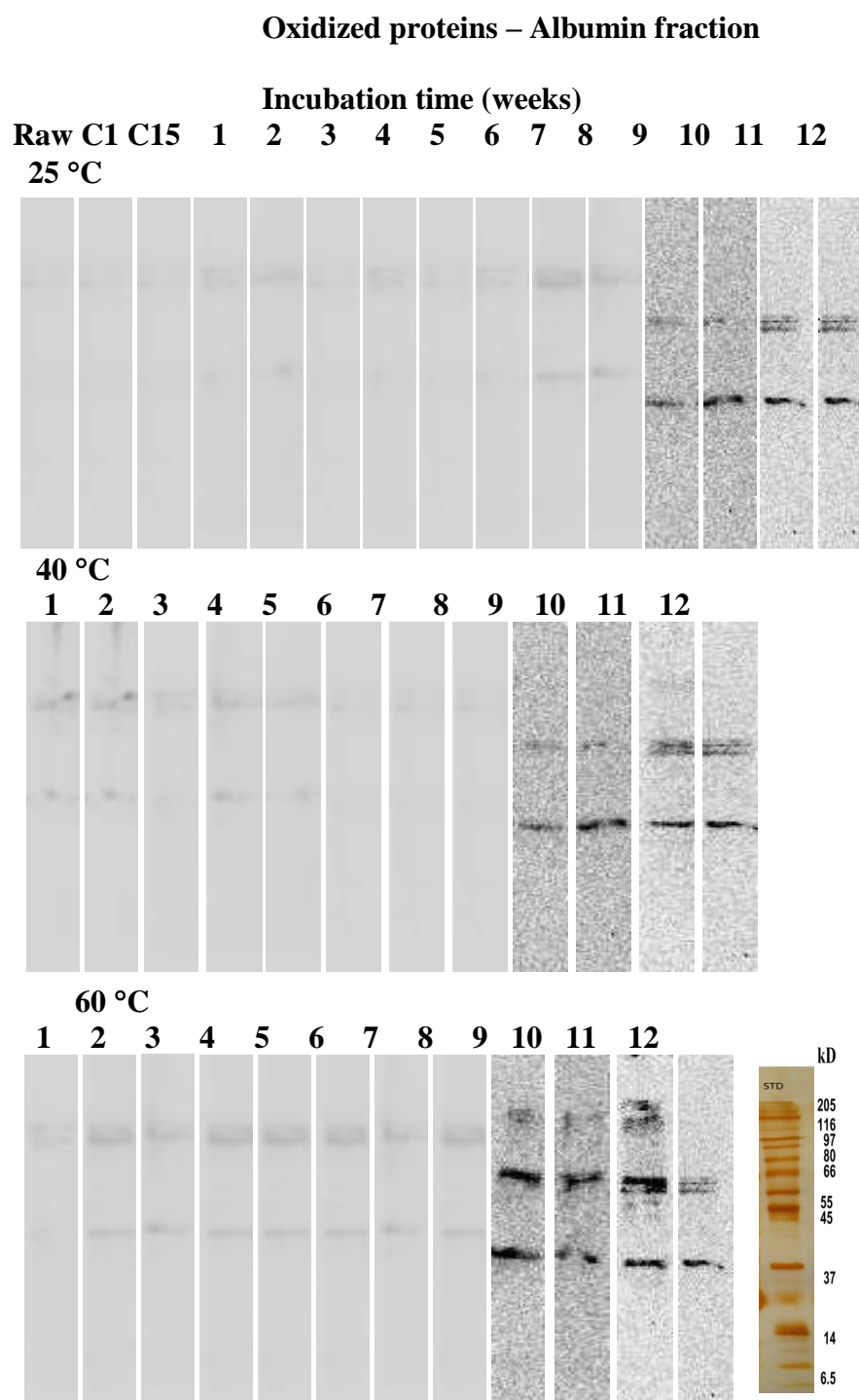


Figure 53. Distribution of protein carbonyl oxidation products in albumins extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks. C1 and C15: controls stored at 10°C for 1 and 15 weeks, respectively.

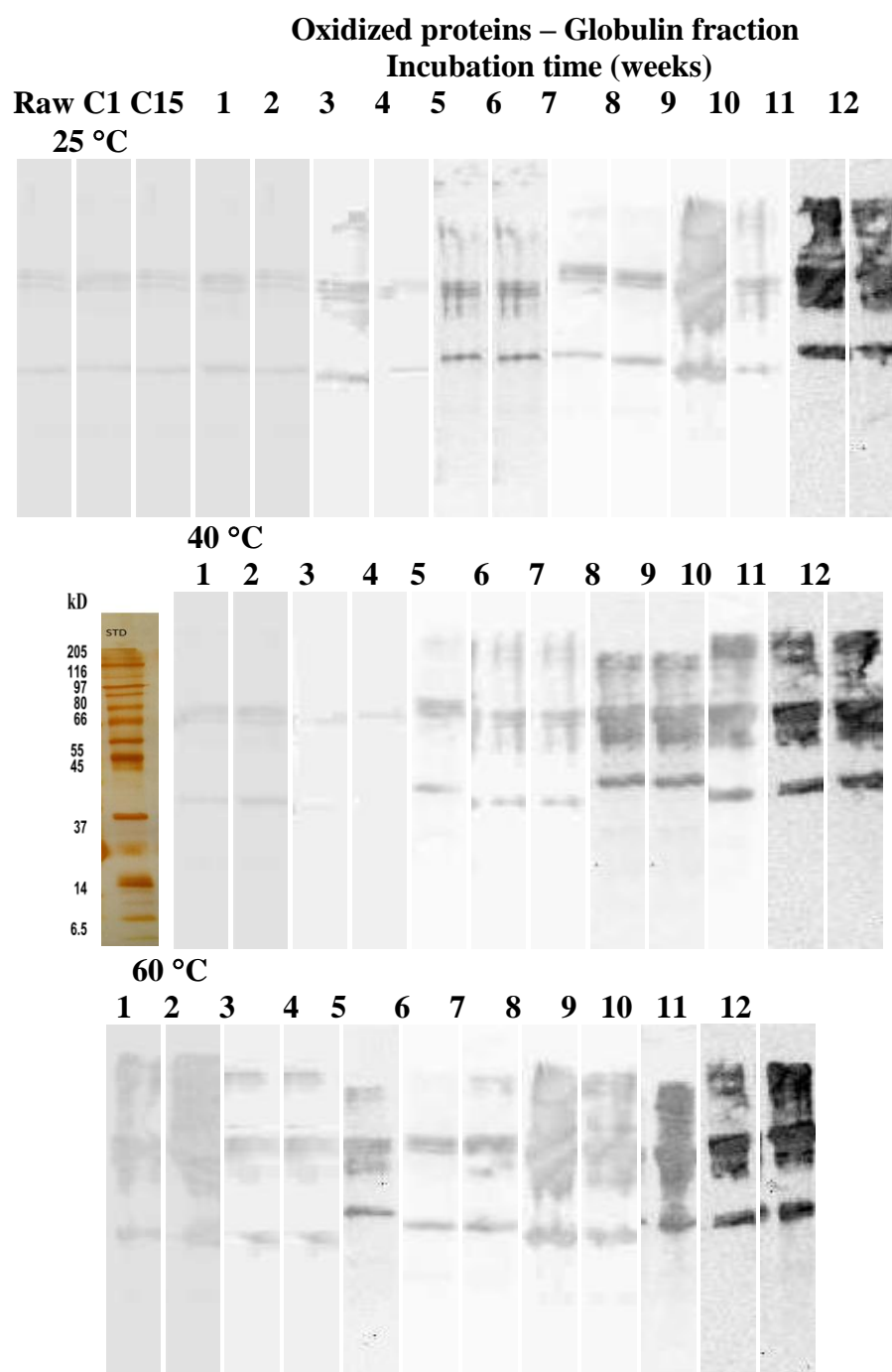


Figure 54. Distribution of protein carbonyl oxidation products in globulins extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks. C1 and C15: controls stored at 10°C for 1 and 15 weeks, respectively.

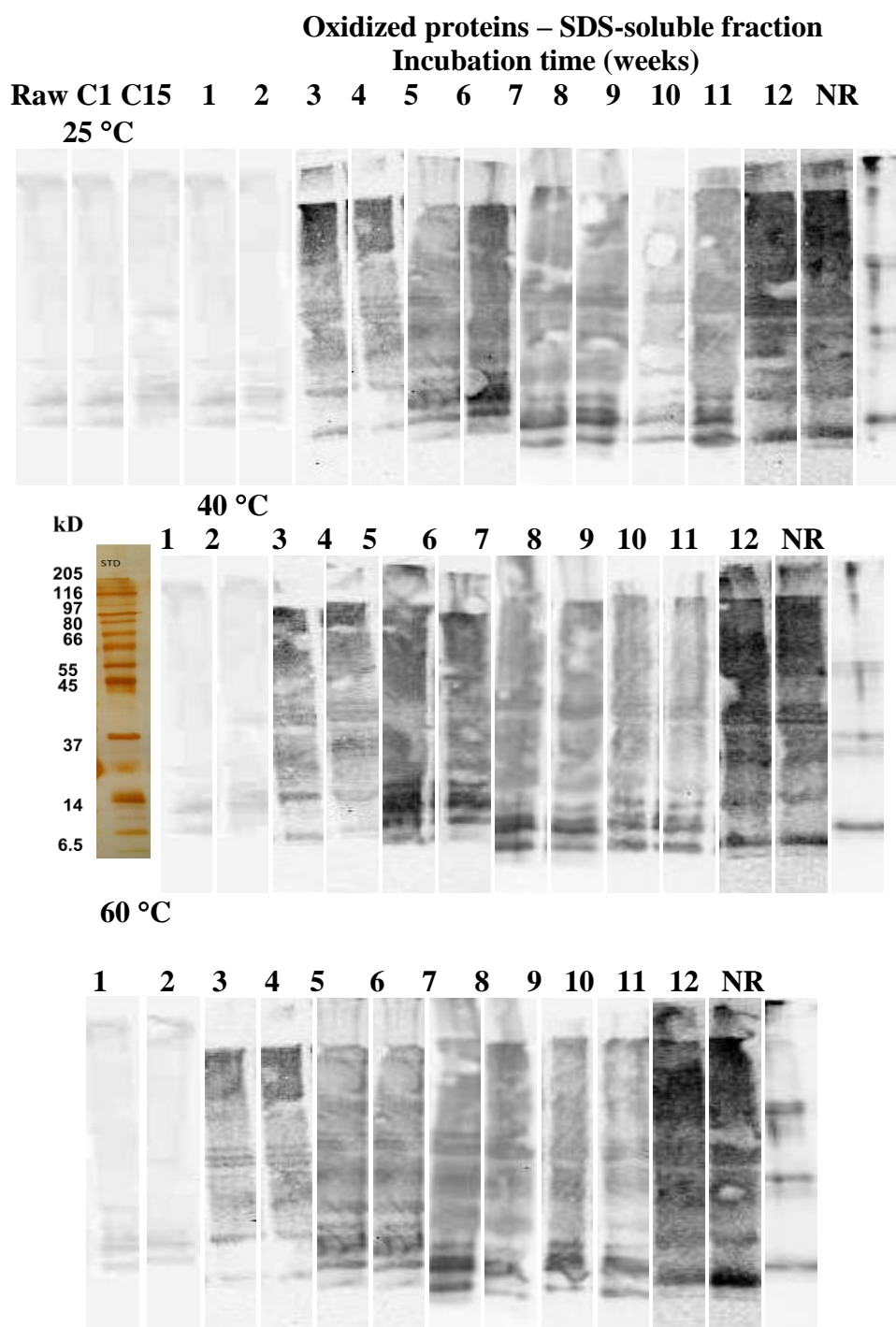


Figure 55. Distribution of protein carbonyl oxidation products in the SDS-soluble fraction extracted from peanut butter incubated at 25, 40, and 60 °C for 12 weeks. C1 and C15: controls stored at 10 °C for 1 and 15 weeks, respectively.



#### **6.2.2.5 Fluorescence – intrinsic emissions from aromatic amino acids**

Proteins contain three aromatic amino acid residues (tryptophan, tyrosine, phenylalanine) which may contribute to their intrinsic fluorescence. Even though the content of all three aromatic amino acids in peanuts is very low, intrinsic fluorescence is present in raw peanuts and it may be used to track protein unfolding and conformational changes (Turoverov and Kuznetsova 2003). In addition, tryptophan is a major binding site for Coomassie blue dye, and it contributes to hydrophobicity of denatured proteins. Thus, intrinsic fluorescence may serve as a marker for changes that contribute to shifting of peptides into the SDS fraction and reduce the intensity of Coomassie blue staining. Generally, 280 and 295 nm excitation are used to monitor total and tryptophan intrinsic fluorescence, respectively. However, we found that with 295 nm excitation, there was greater interference from Schiff base-type fluorescence at higher wavelengths. Thus, 280 nm excitation was used for quantitation analyses, recognizing that the emissions were not tryptophan specific under these conditions.

Intrinsic fluorescence was present at low levels in protein extracts from raw peanuts, as expected from the low concentrations of aromatic amino acids. However, intrinsic fluorescence was selectively present only in the globulin and SDS-soluble fractions and not in the albumins or total soluble proteins (Figure 60). Either aromatic residues were concentrated in these fractions, these fractions retained more of their original configuration (unfolded less), or the solvents used quenched less of the fluorescence. Fluorescence emissions at higher wavelengths in the albumins and total soluble protein fractions, with emission maxima greater than 400 nm, most likely arose from condensation products such as Schiff bases formed in Maillard reactions. These will be discussed further in the next section.

Roasting alone resulted in significant reduction in intrinsic fluorescence (data not shown). However, the aromatic amino acids were not totally destroyed by the heat, because there was an initial increase in intrinsic fluorescence during incubation, followed by a slow decrease to barely detectable levels over time (Figure 61). This suggests that there was an initial disruption of protein configuration followed by oxidation or other permanent modification of the aromatic residues. Such modification could contribute to reduced binding of Coomassie blue dye by modified proteins.

It must be noted that the levels of intrinsic fluorescence for incubated samples were low, close to the limits of detection with the instrumentation used, so useful information is limited to a) aromatic residues appear to be concentrated in globulins, and b) there appear to be multiple sources of fluorescence at higher wavelengths in all fractions.

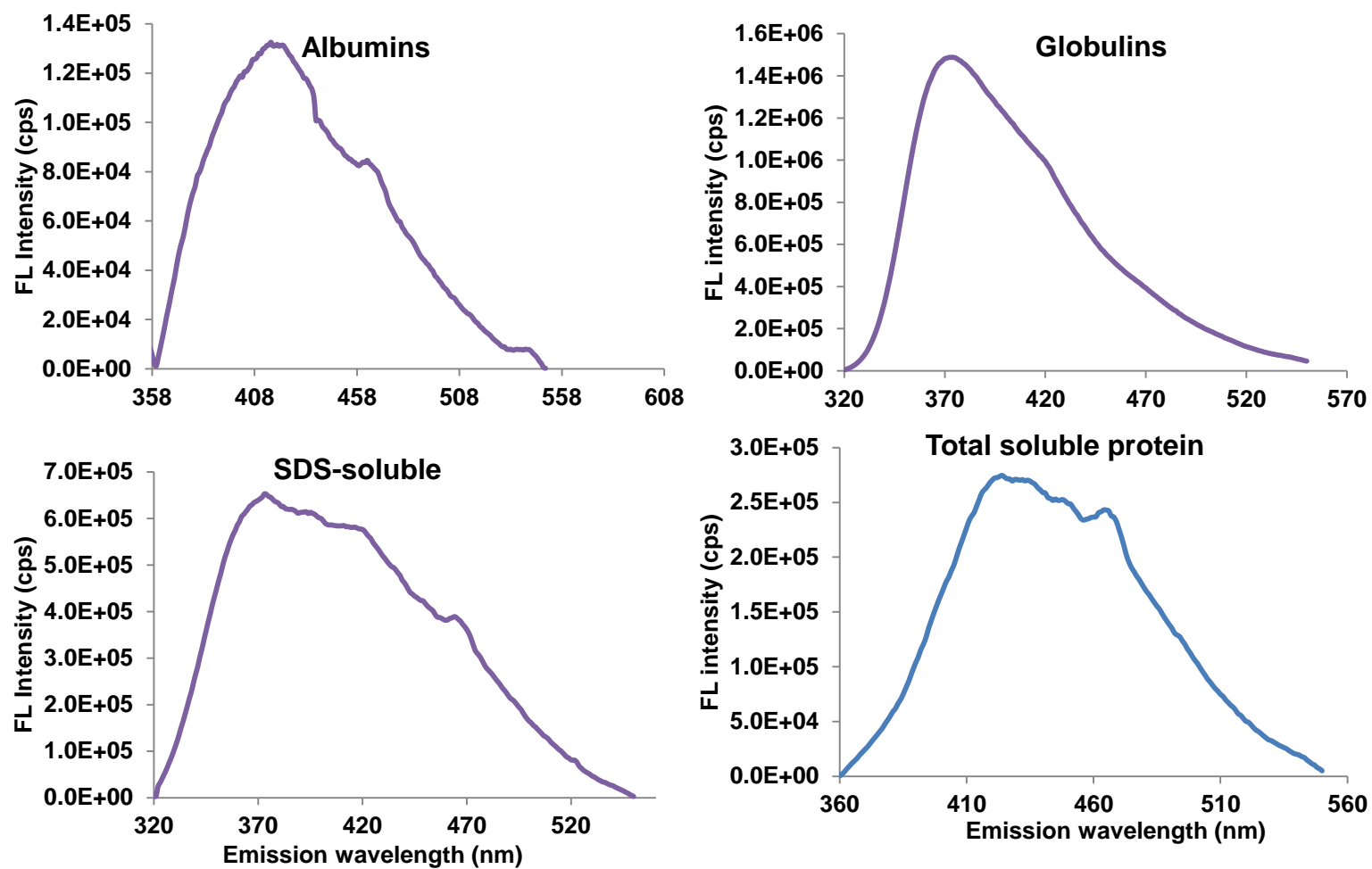


Figure 56. Distribution of intrinsic fluorescence among the various protein fractions in peanut butter.

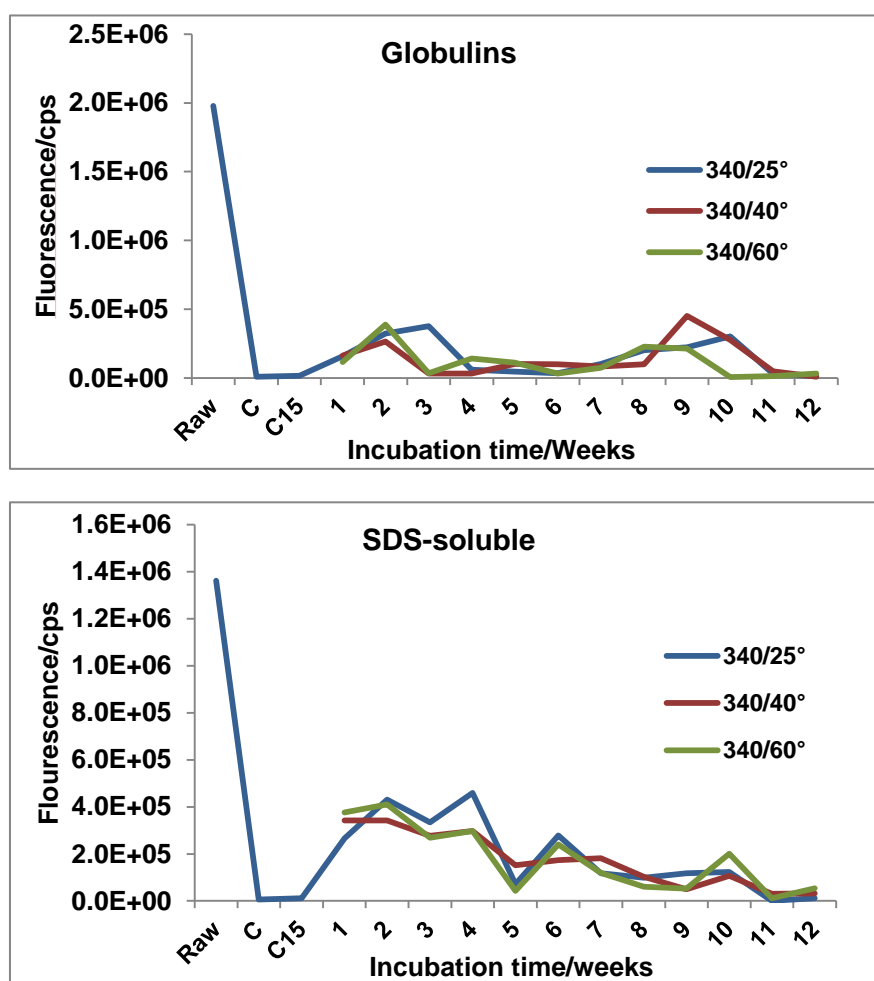


Figure 57. Changes in intrinsic fluorescence in different peanut protein fractions during incubation for 12 weeks at 25, 40, and 60 °C.

#### 6.2.2.6 Fluorescence -- Schiff base formation

Carbonyl-1-amine condensations of Maillard reactions are well known to produce Schiff products that are fluorescent, with linear products emitting near 430 nm and ring products emitting near 470 nm. To detect these products, emission spectra of protein extracts were recorded with excitation at 350 nm.

Extracts from raw peanuts showed relatively narrow emission peaks with emission maxima near 470 nm. With incubation, the emission spectra broadened considerably, and the emission maxima shifted to 430 nm (Figure 62). The asymmetrical shape of the peaks reflects multiple species emitting with different intensities at different wavelengths. Linear adducts were dominant (430 nm emission), with lesser amounts of ring or other species emitting at higher wavelengths.

Fluorescence intensity was present at comparable levels in all protein fractions (Figures 63, 430 nm, and 64, 470 nm), and it varied cyclically almost in parallel with the lipid oxidation cycling noted in Section 6.2.1. However, each protein fraction cycled with a slightly different periodicity. In all samples, fluorescence initially increased with incubation up to about three weeks, then decreased with extended incubation. Presumably, this decrease reflected continued reactions of products responsible for the fluorescence. Surprisingly, fluorescence varied little with temperature, even though heat is known to catalyze carbonyl-amine condensations and also lipid oxidation that produces carbonyl products for the reaction. Either the temperatures used were insufficient to drive the condensations forward, or different reactions were generating the fluorescent species. Overall, Schiff base-type adducts very likely contribute to surface modifications and shifting and reorganization among fractions, but do not appear to be a dominant reaction under conditions of this study since fluorescence levels remained low throughout the incubation period.

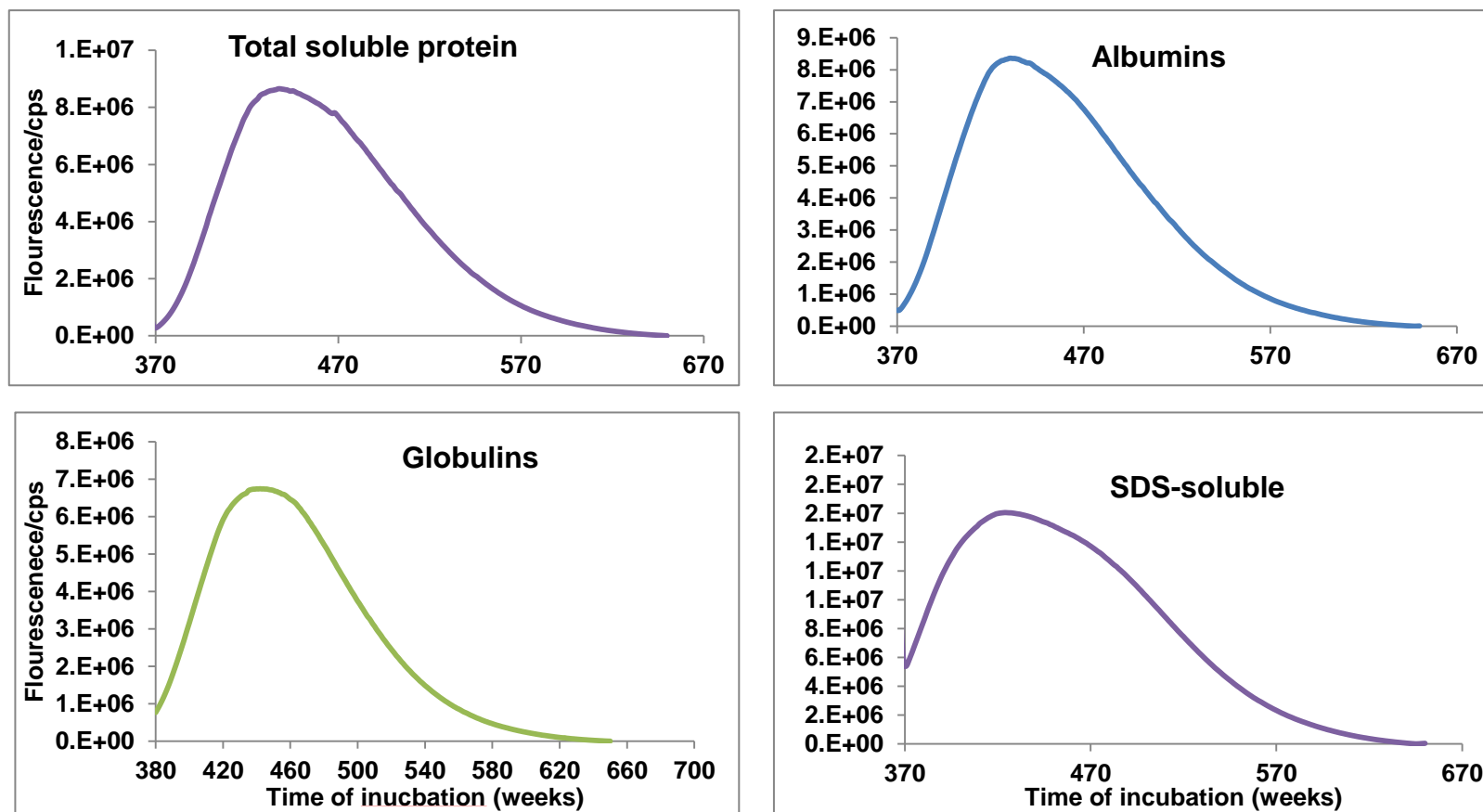


Figure 58. Typical presumptive Schiff base emission spectra from different fractions of peanut butter proteins.

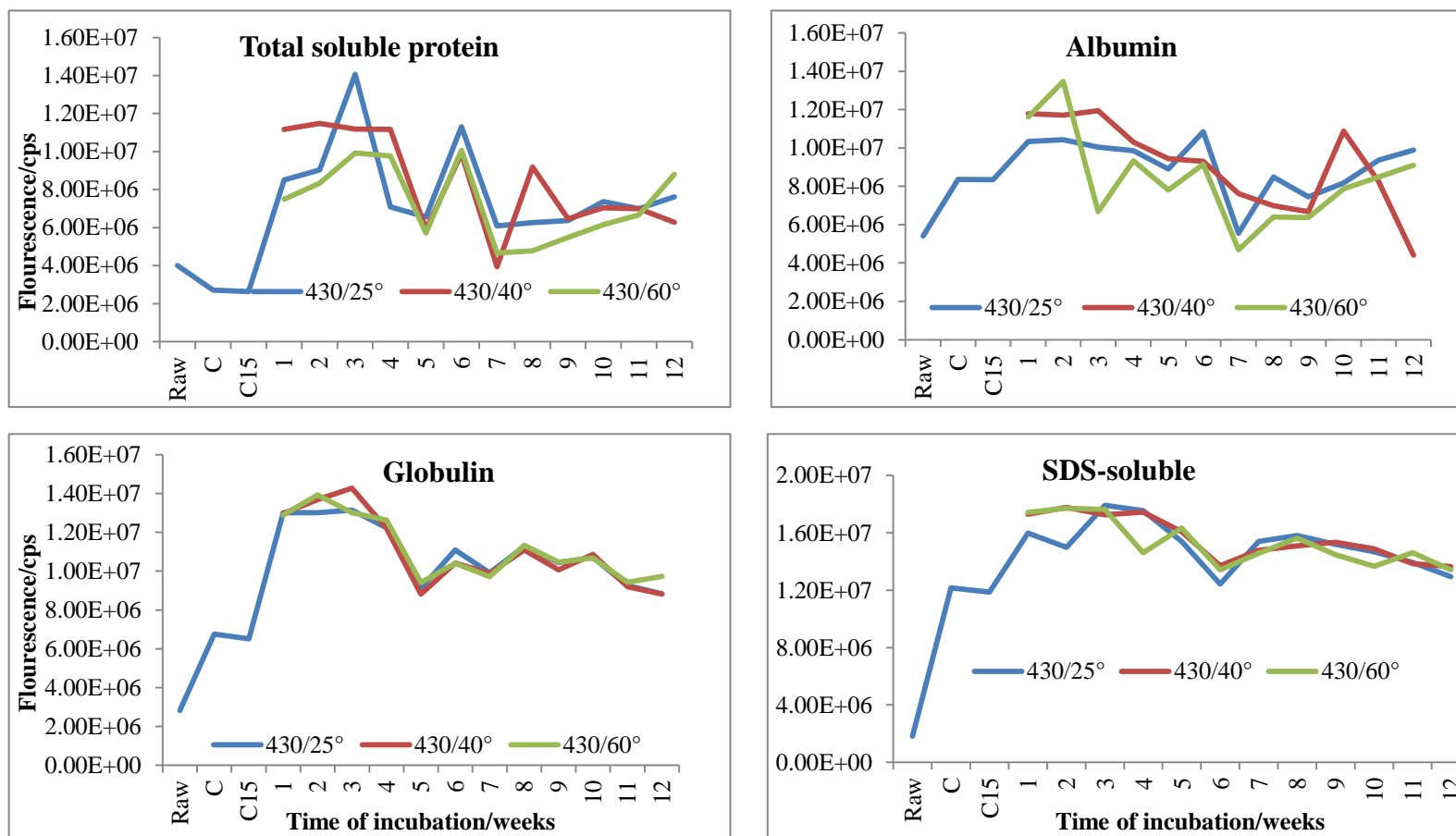


Figure 59. Changes in presumptive Schiff base fluorescence (linear adducts) in different peanut butter protein fractions during incubation for 12 weeks at 25, 40, and 60 °C.

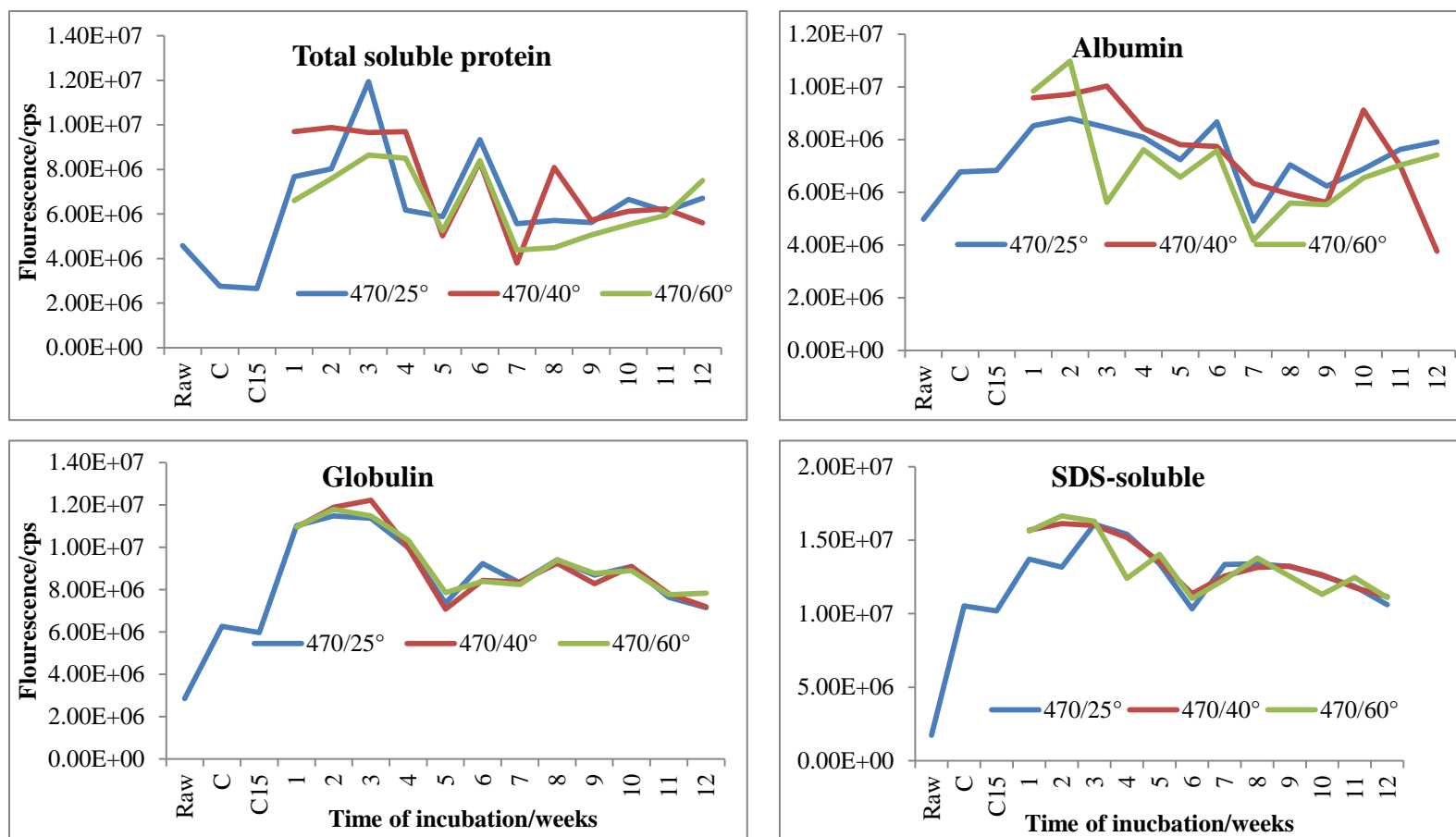


Figure 60. Changes in presumptive Schiff base fluorescence (ring adducts) in different peanut butter protein fractions during incubation for 12 weeks at 25, 40, and 60 °C.



## 7. SUMMARY AND CONCLUSIONS

The main objective of this study was to determine the effects of lipid oxidation and storage temperature on protein oxidation in peanut butter and to begin assessing the potential role of protein co-oxidation in the deterioration of peanut butter quality during storage. This information is critically needed to design more effective formulations, packaging, and storage conditions for stabilizing peanut butter used in military rations and in international food aid.

Peanut butter was incubated in MRE packages at three different temperatures – 25 °C to model normal storage, 40°C to model high temperature environments such as would be encountered in many areas of the world, and 60°C that is commonly used in accelerated shelf life testing. Color, texture, and moisture were measured weekly; lipids and proteins were extracted and analyzed to determine chemical changes underlying physical property changes.

Physically, peanut butter darkened and hardened during storage, particularly at the higher temperatures. Moisture loss was statistically significant, but was too small (less than 1%) to account for texture hardening. Peanut butter also became less cohesive, more adhesive, and more gummy during storage. Some of these texture changes reflected oil separation that occurred during storage of the peanut butter, but for the most part explanations for the texture changes resulted from protein modifications during storage.

Lipid oxidation as measured by conjugated dienes, hydroperoxides, and aldehydes initially increased, then alternately increased and decreased in cycles with a consistent downward trend; product levels remained low throughout incubation. Development of notable off-odors and flavors during incubation demonstrated that low oxidation values occurred not because lipid oxidation did not occur, but because

lipid oxidation products constantly and rapidly transformed to other products or reacted with other molecules in the peanut butter. Oxidation cycles have been observed in a number of other oxidizing systems in this laboratory, particularly when the systems are closed as in the MRE packaging, so they reflect actual phenomena rather than analytical artifacts.

In contrast to primary lipid oxidation products, free fatty acids increased steadily during incubation (no cycles). Since moisture levels were less than 1%, the fatty acids may have been produced by oxidation of lipid aldehydes. This pathway also provides one means of preventing aldehyde accumulation.

Protein modifications including solubility and Schiff base fluorescence appeared to cycle in parallel with lipid oxidation, establishing at least presumptive causality between the two oxidations. In previous studies on foods based on maize and wheat or model systems based on lysozyme, the dominant protein co-oxidation effect was extensive crosslinking and polymerization. In contrast, protein surface modifications appeared to dominate in peanut butter.

The most dramatic and important surface modification was protein oxidation and development of protein carbonyls that became quite extensive over time. Loss of acidic groups on amino acid side chains also occurred, particularly among globulins and SDS-soluble peptides. Some presumptive Schiff base adducts were formed on proteins. Adducts formed during roasting arise from protein- sugar interactions; shifts in peanut butter color and emission maxima during incubation suggest that browning and adducts formed during oxidation involve lipid oxidation products. However, fluorescence levels remained low throughout incubation, so the importance of these adducts in surface modifications cannot be determined definitively. Detection of oxidized peptides by antibodies that had not bound Coomassie or, more often, silver

stain, further demonstrated that unidentified modifications occurred particularly in acidic amino acids.

Consequences of surface modifications included reduction in Coomassie blue dye binding, marked differences in Coomassie blue and silver stain binding to peanut protein fractions, reorganization of peptides within albumin and globulin solubility classes, shifts of albumins and globulins to the SDS-soluble fraction, and marked changes in electrophoretic mobility in native acrylamide gels. That the SDS-soluble fraction is more hydrophobic supports likely formation of lipid adducts with amino acid side chains, or, alternatively, deamination/deamidation, and decarboxylation of side chain functional groups.

Two observations supported the existence of at least some crosslinking and polymerization, even though it was not a dominant effect. Although cysteine/cystine levels in peanut proteins are very low, dramatic loss of peptide bands after extraction without 2-mercaptoethanol as a reducing agent demonstrate involvement of disulfide associations between arachin sub-units and in the observed reorganization of peptides. Also, antibodies detected oxidized peptides in stacking gels of the SDS-soluble fraction. These polymerized proteins were not detected by Coomassie blue or silver dyes, suggesting that the dye-binding residues had been modified, blocked, or destroyed. It remains to be determined whether polymers such as these contribute to hardening of peanut butter during storage.

By all measures analyzed, globulins were much more extensively altered than albumins, and arachins were much more extensively modified than conarachins in all measures.

Overall, the pattern of protein oxidation in high oil, semisolid peanut butter was much different than the crosslinking that dominates in low lipid, dry corn and

wheat products previously studied. It is not known yet whether the shift in damage patterns is driven by the nature of the peanut proteins or by the differences in lipid concentration, physical structure, and molecular organization of peanut butter.

Results of this study, though just a first step, suggest that during relatively short storage periods (a few months), degradation of peanut butter quality is mediated primarily by interactions of lipid radicals and hydroperoxides with proteins. These interactions remove lipid oxidation products from the reaction stream, hence low detection, but still generate off-odors and flavors characteristic of “rancidity”. Radical reactions between oxidizing lipids and proteins or amino acids have been shown in model systems to chemically alter side chain groups, including deamination, deamidation, and decarboxylation, so there is reason to expect comparable reactions in peanut butter. Aldehyde reactions with proteins, although detected, appear to be of minor importance in early oxidations, at least in part because removal of conjugated dienes and hydroperoxides by reaction with proteins severely limits formation of secondary products such as aldehydes. Further studies are needed to determine how this damage pattern changes with long-term storage.

Several important implications arise from this study. First, removal of lipid oxidation products by reaction with proteins or other molecules means that analysis of lipid oxidation alone will not accurately indicate extent of oxidation in peanut butter. Indeed, measuring peroxide values alone or in combination with aldehydes will most likely *UNDER*-estimate oxidation. Analyses of protein oxidation, e.g. in adapted ELISA assays with antibodies, must be added to the analyses for full evaluation of product quality.

Second, if transfer of oxidation from lipids to proteins mediates degradation in peanut butter, antioxidants that interfere with that transfer should protect product

quality. We have preliminary results with controlled release antioxidant packaging that support this approach, i.e. antioxidants delivered from packaging during storage prevented the hardening and browning of peanut butter and also of processed cheese spread. These promising results suggest that modification of antioxidant levels through formulation and/or packaging should effectively maintain product quality and nutrition over extended periods. However, concentrations at which antioxidants convert to pro-oxidants in peanut butter must be determined.

Finally, control peanut butters incubated at 10 °C showed little lipid oxidation or protein modification. This suggests that overall physical degradation can be prevented if lipid oxidation does not occur. Hence, combinations of packaging approaches that limit oxygen dissolved in peanut butter before packaging, as well as in the packaging headspace should effectively prevent lipid oxidation plus associated protein co-oxidation and thus significantly extend shelf life of this food that is so important in nutritional support throughout the world.

## 8. FUTURE WORK

Results of this study have revealed intriguing changes in peanut proteins. Not only have they shown different patterns of protein modification than previously observed, but they have also raised interesting questions about how lipid interactions can induce changes other than crosslinking, and how peanut butter can harden without notable protein crosslinking. Thus, this initial shelf life study offers substantial material for follow-up studies.

To elucidate sources of protein degradation and clearly establish lipid causality, the following chemical questions need to be pursued directly and in detail in peanut butter and in model systems composed of isolated peanut protein fractions and oxidizing methyl linoleate or specific lipid oxidation:

- Which lipid oxidation products react most actively with proteins and what are the consequences?
- What different kinds of surface modifications occur to shift peptide patterns of acidic and basic proteins in PAGE gels?
- How are acidic amino acids modified -- by chemical degradation such as decarboxylation, or rather by complexation with other proteins or with lipids?
- What are the mechanisms of peptide association and dissociation? Are disulfide cross links involved?
- How glycated are peanut proteins and what role do the sugars play in changes such as hardening and browning? Can release of sugar moieties from glycoproteins crystallize and contribute to hardening? What role does glycolylation/deglycosylation play in protein associations and texture properties?

Answering these questions will require application of considerably more sophisticated analytical methods to isolate fractions and track both physical

associations and chemical changes in individual proteins. To provide more detailed information about molecular changes, we intend to apply advanced methodologies of Maldi-TOF LC-MS (in collaboration with the USDA ERRC) to more clearly identify protein crosslinking and fragmentation as well as structures involved, electron paramagnetic resonance to detect lipid radicals and radical transfers to proteins, differential scanning calorimetry to detect changes in lipid and sugar crystal structures, and confocal microscopy to detect spatial distributions of lipid and protein modifications in peanut butter. Determining specific amino acid modifications will require digestion of peanut proteins with immobilized proteases to avoid destroying the amino acids most sensitive to oxidation, followed by LC-MS separation and analysis of modified residues.

## REFERENCES

- Ahmed, E.A., and R.H. Schmidt, Functional properties of and soy proteins as influenced by processing methods, *Peanut Sci.* **6** 1979, 1–6.
- Ahmed, E.M., and Theresa Ali. Textural quality of peanut butter as influenced by peanut seed and oil content. *Peanut Sci.* 1986, 13, 1: 18-20.
- Al-Harbi MM, Al-Kabtani HA. 1993. Chemical and biological evaluation of discarded frying palm oil in commercial restaurants. *Food Chem* **48**:395–401.
- Amarnath, V., Valentine, W.M., Amarnath, K., Eng, M.A., Graham, D.G. The Mechanism, of Nucleophilic Substitution of Alkylpyrroles in the Presence of Oxygen. *Chem Res.Toxicol.* 1994, 7, 56-61.
- Arakawa, T., Philo, J. S., Ejima, D., Tsumoto, K., and Arisaka, F. Aggregation of theurotopic analysis Part1. The general aspects and techniques for assessment. 2006. *Bioprocess International* 4 (10), 42-49.
- Arthur Jr., J.C. Peanut protein isolation, composition and properties, in *Advances in Protein Chemistry* Vol 8, (Eds M.L. Anson, K. Bailey and J.T. Edsall), Academic Press, New York, 1953, 393–414.
- Aryama K.J., Resurreccion, A.V.A, Chinnan, M.S. Beuchat, L.R. Fucntionality of palm oil as a stabilizer in peanut butter. *J. Food Sci.*, 2003 68, 1302-1307.
- Basha, M.M., and Cherry, J.P. Composition, stability and gel lectrophoretic properties of proteins isolated from florunner (*Arachis hypogaea* L.) peanut seed. *J. Agric. Food Chem.* 1976, 24, 359-365.
- Basha, S. M. M. and Pancholy, S. K.. Changes in the polypeptide composition of maturing seeds from four peanut (*Arachis hypogaeas* L.) cultivars. *Peanut Sci* 1981, 8 82: 6-10
- Basha, S.M. and S.K. Pancholy, Composition and characteristics of basic proteins from peanut (*Arachis hypogaea* L.), *Journal of Agricultural and Food Chemistry* **30** (1982), pp. 1176–1179.
- Basha, S.M.M. and Pancholy, S.K. Differences in the seed protein composition of genus *Arachis*. *Canadian Journal of Botany*, 1984, **62**, 105.
- Basha, S.M.M.. Identification of cultivar differences in seed polypeptide composition of peanut (*Arachis hypogaea* L) by two dimensional polyacrylamide gel electrophoresis. *Plant Physio.* 1979, 63, 301–306.
- Beatti, W.R. Making and Using Peanut Butter. USDA Circ. 1936, 384.



- Beppu, B. Role of Heme Compounds in the erythrocyte membrane damage induced by lipid hydroperoxide. *Chem. Pharm. Bull.* 1986, 34, 781-788.
- Berrit C. Stroehla, Lorraine Halinka Malcoe, Ellen M. Velie. Dietary sources of nutrients among rural native American and white children. *J. of the Ame. Dietetic Assoc.*, 2005 105, 12, 1908-1916.
- Boatright W.L. and N.S. Hettiarachchy. Effects of lipids on soy protein isolate solubility. *J. Am. Oil Chem. Soc* 1995 72, 1439-1444.
- Bobrowski K. and C. Schöneich. Decarboxylation mechanism of the terminal glutamyl moiety in  $\gamma$ -glutamic acid and methionine containing peptides. *Radiat. Phys. Chem.*, 1996, 47, 507-510
- Bookwalter G.N; H.A. Moser, W.F. Kwolek, V.F. Prfeifer; E.L. Griffin Jr. Storage stability of CSM:alternate formulations for corn-soy milk. *J. Food Sci.* 1971, 36, 732-736.
- Borovyagin, V.L., A.F. Muronov, V.D. Rumysntseva, Y.S. Tarakhovskii and I.A. Vasilenko. Model membrane morphology and crosslnking of oxidized lipids with proteins. *J. Ultrastruc. Res.* 1984, 89, 261-273.
- Bradford M.M. . A rapid and sensitive method for the quantitaion of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72. 1976 248-254
- Brown, W.L. The Theorinine, serine, cysteine, and methionine content of peanut proteins. *J. Biol. Chem.*, 1941, 142, 299-301.
- Bunnet, S.L. Survival of Salmonella in peanut and peanut butter spread. *J. Appl. Mircobiol*, 2000, 89, 427-477.
- Buttkus H. Accelerated denaturation of myosin in frozen solution. *J. Food Sci.* 1970 35 558-562
- Chen, H.J.C., and Chung F.L. Epoxidation of trans-4hydroxyl-2-nonenal by fatty acid hydroperoxides and hydrogen peroxide. *Chem. Res. Toxicol.* 1996, 9, 306-312.
- Cherry P. and. McWatters, K.H solubility properties of proteins relative to environmental effects and moist heat treatment of full fat peanuts, *J.l of Food Sci.* 40 1975 1257– 1259
- Cherry, J.P.; Dechary, J.M.; Ory, R.L. Gel Electrophoretic analysis of peanut proteins and enzymes.I. Characterization of DEAE-cellulose separated fractions. *J. Agric.FooChem.* 1973, 21, 652-655.
- Chio, K.S., and A.L. Tappel. Inactivation of Ribonuclease and Other enzymes by peroxidizing Lipids and by Malonaldehyde. *Biochemistry*, 1969a, 8, 2827-2832.

- Chiou, R. Y. Y. Effect of heat treatments on peanut arachin and conarachin. *Journal of Food Biochemistry* 1990, 37, 1377-1381
- Civille, G.V. and Szczesniak, A.S. Guidelines to training a texture profile panel. *J. of Text. Studies* 4, 1973, 203-223.
- Clements D.J. *Food Emulsion: Principles, practice and techniques*, 1999, Press, Boca Raton,
- Crippen K.L.; D.D. Hamman C.T. Young. Effects of grind size sucrose concentration and salt concentration on peanut butter texture. *J. Text. Stu.* 1989. 20, 29-41.
- Davies, et al. Protein damage and degradation by oxygen radicals II. Modification of amino acids. *J. Biol. Chem.* 1987 262:9902.
- Davies, K.A.J. and Delsignore, M.E. Protein Damage and Degradation by Oxygen Radicals. III. Modification of Secondary and Tertiary Structure. *J. Biol. Chem.* 1987, 262, 9908-9913.
- Davies, M.J. The Oxidative Environment and Protein Damage. *Biochim. Biophys. Acta* 2005, 1703, 93-109.
- Davies, M.J., Fu, S., Dean, R.T., Protein Hydroperoxides Can Give Rise to Reactive Free Radicals. *Biochem. J.* 1995, 305, 643-649.
- Dawson, R. The nutritive value of groundnut protein. 2. The correlation between electrophoretic pattern and nutritive dechary. *Br. J. Nutr.* 1968, 22, 601-607.
- Desai, I.D. and Tappel, A.L. Damage to proteins by peroxidized lipids. *J. Lipid Res.* 1963, 4, 04-207.
- Dieckert, J.W. and Morris, N.J. Principles of the Peanut. *J. Agric. Food Chem.* 1958, 6, 930- 933.
- Dillard, C.J., and Tappel, A.L. Fluorescent products of lipid peroxidation of mitochondria and microsomes. *Lipids.* 1971, 6, 715-721.
- Dong, Y. 2010. Thermal vs lipid co-oxidation of proteins in baked vs fried tortilla chips. M.S. thesis, Food Science, Rutgers University, New Brunswick, NJ,
- Ege, S.N. *Organic Chemistry: Structure and reactivity*, Houghton: Boston, 517-518, 631-654, 692-693, 725-733, 1999.
- El-Gharbawi, M.I., and L.R. Dugan Jr. Stability of nitrogenous compounds and lipids during storage of freeze-dried raw beef. *J. Food Sci.* 1965, 30 817-822
- El-Rawas, A. Hvizdak, A. Davenport, M. Jaczynski, J. Matak, K. Effect of electron beam on quality indicators of peanut butter over a storage period. *Food Chem.* 2012, 133, 212-219.

- Esterbauer H, Benedetti A, Lang J, Fulceri R, Fauler G, Comporti M. 1986. Studies on the mechanism of formation of 4-hydroxynonenal during microsomal lipid
- Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF. 1982. Separation ADP-Fe<sup>2+</sup> in rat liver microsomes. *Biochem J* 208(1):129-140.
- Esterbauer H, Cheeseman KH. . Determination of aldehydic lipid oxidation lipid products: malonaldehyde and 4-hydroxynonenal. *Meth Enzymol* 1990, 186:407-421.
- Esterbauer H, Dieber-Rotheneder M, Striegl G, Waeg G.. Role of vitamin E in preventing the oxidation of low-density lipoprotein. *Am J Clin Nutr.* 1991b 53(1):314S-321S.
- Esterbauer H, Jurgens G, Quehenberger O, Koller E. 1987. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and
- Esterbauer H, Schaur RJ, Zollner H.. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991a 11(1):81-128.
- Esterbauer H. 1982. Aldehydic products of lipid peroxidation. In: Free radicals, peroxidation and cancer. New York, NY: Academic Press, p 101-122.
- Farber J.M.; R.L. Levine. Sequence of a peptide susceptible to mixed-function oxidation. Probable cation binding site in glutamine synthetase *J. Biol. Chem.* 1986, 261:4574-4578.
- Felland, S.L.; Koehler, P.E. Sensory, chemical and physical changes in increased water activity peanut butter porproducts. *J. Food. Quality.* 1997, 20, 145-156.
- Fletcher, B.L. and A.L. Tappel. Fluorescent modification to serum albumin by lipid peroxidation. *Lipids* 1970, 6, 172-175.
- Fogerty AC, Witfield FB, Svoronos D, Ford GL. 1989. Effect of heat on the fatty acids and aldehydes of veal meat phospholipids. *Int J Food Sci Technol* 24(5):529-534.
- Fong, L.G., S. Parthasarathy, J.L., Witztum, and D. Steinberg. Nonenzymatic oxidative cleavage of peptide bonds in Apoprotein B-100. *J. Lipid. Res.* 1987, 28, 1466-1477.
- Fore, S.P. et al., Factors Affecting the Stability of Crude Oil of 16 Varieties of Peanuts. *J. Am. Oil Chemists Soc.* 1953, 30, 298-301
- Freeman, A.F., Morris, N. J., Willich, R. K. 1954. Peanut butter. U.S. Dept. Agr. AIC-370

- Fruebis, J., Parsasarathy, S., Steinberg, D. Evidence for a concerted reaction between lipid hydroperoxides and polypeptides. *Proc. Nat. Acad. Sci. USA* 1992, 89, 10588-10592.
- Funes, J.A. U. Weiss., and M. karel. Effects of reaction conditions and reactant concentrations on polymerization of lysozyme reacted with peroxidizing lipids. *J. Agric. Food Chem.* 1979, 27, 220-229
- Gamage, P.T., Mori and A. Matsushita. Mechanism of polymerization of proteins by autoxidized products of linoleic acid, *J. Nutr. Sci. Vitaminol.* (Tokyo). 1973, 173-182.
- Gardner, H.W. Oxygen radical chemistry of Polyunsaturated Fatty Acids: A review. *J. Med.* 1989, 7, 65-76
- Garrison, W.M. Reaction Mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem. Rev.* 1987, 87, 391-398.
- Gebicki, S. and J.M. Gebicki. Crosslinking of DNA and proteins induced by protein hydroperoxides. *Biochemical. J.* 1999, 338, 626-636.
- Gerrard, J.A. Protein–protein crosslinking in food: methods, consequences, applications. *Trends in Food Science & Technology*, 2002, 13, 391-399.
- Gill. L.A.; A.V.A. Resurreccion, Sensory and physical properties of peanut butter treated with palm oil and hydrogenated vegetable oil to prevent oil separation. *J. Food. Sci.* 2000, 65, 173-180.
- Guitton, J., F. Tinardon; R. LAmrini; P. Lacan; M. Desage; and A. Francina. Decarboxylation of (1-<sup>13</sup>) leucine by hydroxyl radicals. *Free Radic. Boil. Med.*, 2003, 278, 42027-42036
- Haberland, M.E. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J. Biol. Chem.* 1984, 259, 11305-11311.
- Haberland, M.E. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proc. Nat. Acad. Sci. USA* 1982, 79, 1712-1716.
- Halligudi, N.N.; S.M. Desai; S.K. Mavalangi; and S.T. Nadibewoor. Kitecis of the oxidative degradation of rac-serine bt aqueous alkaline permanganate. *Monatsh. Chem.* 2000, 131 321-332.
- Hamberg M.; B. Gotthammar. A new reaction of unsaturated fatty acid hydroperoxides: Formation of 11hydroperoxy-12, 13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9, 11-octadecdienoic acid. *Lipids*, 1973, 8, 737-744.
- Hamberg, M. and Gotthammar B. A New reaction of unsaturated fatty acid hydroperoxides: formation of 11-Hydroxy-12, 13-Epoxy-9-Octadecenoic Acid from 13-Hydroperoxy-9, 11-octadecadienoic acid. *Lipids* 1973, 8, 737-744.

- Heinecke, J.W., Li W, Daehnke HL 3rd, Goldstein J.A. Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages *J. Biol. Chem.* 1993 1 4069-4077
- Hidalgo, F.J., Alaiz, M., Zamora, R. Effect of pH and temperature on comparative non- enzymatic browning of proteins produced by oxidized lipids and carbohydrates. *J. Agric. Food Chem.* 1999, 47, 742-747.
- Higgins, B.B. et al. Thiamin chloride and nicotinic acid content of peanuts and peanut products. *Georgia Agric. Expt. Sta. Bull.* 1941, 213.
- Ho. M.D.; Hanharan, C.E. International Food Aid programs:Background and issues in Congressional Research Service, 2010, 1-16.
- Hoffpauir, H.C., and Guthrie, J.D. 1945. Chemical composition of peanuts. *Peanut J. Nut World* 24, 6: 26-30
- Horigome, T. and Miura, M. Interaction of protein and oxidized ethyl linolenate in the dry state and nutritive value of reacted protein. *J. Agric. Chem. Soc. Jpn.* 1974, 48, 437-444.
- Horigome, T., Yanagida, T., Miura, M. Nutritive value of proteins prepared by the reaction with oxidized ethyl einoleate in aqueous medium. *J. Agric. Chem. Sco. Jpn.* 1974, 48, 195-199.
- Huerta F.; E. Morallóna, F. Casesb; A.Rodesa; J.L Vazquez and A. Aldaza. Electrochemical behaviour of amino acids on Pt (III). A volumetric and in situ FTIR study. Part II serine and alaninen on Pt(III). *J. Electroanal. Chem.* 1997 431 269-275.
- Hunt, J.V., J.A. Simpson and R.T. dean. Hydroperoxide-mediated fragmentation of proteins. *Biochem. J.* 1988, 6, 581-585.
- Ingold, K.U. *Structure and Mechanism in Organic Chemistry, 2nd edition*, Cornell University Press: Ithaca, NY, 1969, 457-463.
- Jirousek, M.R., Murthi, K.K., Salomon, R.G. Electropilic levuglandin E2-protein adducts bind glycine: A model for proteins cross-linking. *Prostaglandins* 1990, 40, 187-203.
- Johns C. O., Jones B. D. (1916). The proteins of the peanut, *Arachis hypogaea* L. The globulins arachin and conarachin. *Journal of biological Chemistry* 28, 77-78
- Johnson, P.; E.M. Shooter; E.H Rideal. The globulins of the groundnut.II Electrophoretic examnination of arachin system. *Biochem. Biophys. Acta* 1950, 5 376-395.
- Kamal-Eldin, A., Polorny, J. Lipid oxidation products and methods used for their a nalysis. *Analysis of Lipid Oxidation. Chapter 1*, 2005, 1-7.

- Kanazawa, K., G. Danno and M. Natake. Lysozyme damage caused by secondary degradation products during the autoxidation process of linoleic acid. *J. Nutr. Sci. Vitaminol.* (Tokyo) 1975, 21, 373-382.
- Kikugawa, K. and Beppu, M. Involvement of Lipid oxidation products in the formation of fluorescent and cross-linked proteins. *Chem. Phys. Lipids* 1987, 44, 277-296.
- Kikugawa, K. and Ido, Y. Studies on Peroxidized Lipids. V. Formation and characterization of 1,4-dihydrophridine-3,5-dicarbaldehydes as model of fluorescent components in lipofuscin. *Lipids* 1984, 19, 600-608.
- Kikugawa, K., Ido, Y., Mikami, J. Studies on Peroxidized Lipids. VI. Fluorescent products derived from the reaction of primary Amines, malondialdehyde, and monofunctional aldehydes. *J. Am. Oil Chem. Soc.* 1984, 61, 1574-1581.
- Koh, B. K., Karwe, M. V., and Schaich, K. M. 1996. Effects of cysteine on free radical production and protein modification in extruded wheat flour, *Cereal Chem.* 73, 115-122.
- Kowalik-Jankowska, T., Ruta, M., Winiewska, K., Lankiewicz, L., Dyba, M. Products of Cu(II)-catalyzed oxidation in the presence of hydrogen peroxide of the 1-10, 1-16 fragments of human and mouse  $\beta$ -amyloid peptide. *J. Inorg. Biochem.* 2004, 98, 940-950.
- Krishna, T.G. and Mitra R. Arachin polymorphism in groundnut (asrachis hypogaeal). *Phytochemistry*, 1987, 26, 897-902.
- Labib, A.I., Helali, E., Hamza, A.S., and Mady, Y.A. Effect of heat on the nitrogen solubility of defatted groundnut. *Agric. Res. Rev.* 1977, 55, 165-168.
- Lea, C.H. Deteriorative Reactions Involving Phospholipids and Lipoproteins. *J. Sci. Food Agric.* 1957, 8, 1-14.
- Lederer, M.O. Reactivity of Lysine Moieties Toward  $\gamma$ -Hydroxy- $\alpha,\beta$ -Unsaturated Epoxides: A model study on protein-lipid oxidation product interaction. *J. Agric. Food Chem.* 1996, 44, 2531-2537.
- Little C.; and P.J. O'Brien. Products of oxidation of a protein thiol group after reaction with avroius oxidizing agents. *Arch. Biochem. Biophys.* 1976, 122, 406-410.
- Little H.; and P.J. O'Brien. The effectiveness of a lipid peroxide in oxidizing protein and non-protein thiols. *Biochem.* 1968, 106 419-423.
- Liu, W. and J.-Y. Wang. Modifications of protein by polyunsaturated fatty acid ester peroxidation products. *Biochim. Biophys. Acta* 2005, 38, 746-754.

- Liu, Z., Minkler, P.E., Sayre, L.M. Mass Spectroscopic characterization of protein modification by 4-hydroxy-2 (E)-nonenal and 4-oxo-2-(E)-nonenal. *Chem. Res.Toxicol.* 2003, 16, 901-911.
- Lqari, H., J. Pedroche, J. Giron-Calle, J. Vioque and F. Millan. Interaction of *Lupinus angustifolius* L.  $\alpha$  and  $\gamma$  conglutins with 13-hydroperoxide-11, 9-octadienoic acid. *Food Chem.* 2003, 80, 517-523.
- Malshet, M.G. and Tappel, A.L. Fluorescent products of lipid peroxidation. I. structural requirement for fluorescence in conjugated schiff Bases. *Lipids* 1973, 8, 194-198.
- Marchione, T. J., Foods provided through U.S. Government Emergency Food Aid Programs: Policies and customs governing their formulation, selection and distribution, *Journal of Nutrition*, 132, 2104S–2111S, 2002.
- Matoba, T., Kurita, O., Yonezawa, D. Changes in Molecular size and chemical properties of gelatin caused by the reaction with oxidizing methyl linoleate. *Agric. Biol. Chem.* 1984, 48, 2633-2638.
- McMurray, J. Organic Chemistry, Brooks/Cole: Pacific Grove, CA, 2000, pp 718-724, 770-774, 955-960.
- Mexis S.F. Badeka A.V. Riganakos, K.A. Kontominas M.G. Effect of  $\gamma$ -irradiation on the physicochemical and sensory properties of raw unpeeled almonds kernels (*Prunidulcis*). *Innov. Food Sci. and Emer. Tech.* 2009, 10, 87-92.
- Moran D.P.J Fats in spreadable products in Moran D.P.J.and Rajah K.K. Fats in food products , Westport ct avi publishing Co Inc, 1984 55-207.
- Morris, N.J and A.F Freeman. Peanut butter, 4. The effect of roasting on the palatability of peanut butter. *Food Tech.* 1954, 8, 377.
- Mosha, T. C. E., & Vincent, M. M. Nutritional quality, storage stability and acceptability of home-processed ready-to eat composite foods for rehabilitating undernourished preschool children in low income countries. *Journal of Food Processing and Preservation*, 2005 29, 331-356.
- Moss& J. and Pernollet, J. C. in Chemistry and Biochemistry of Legumes (Arora, S. K., cd.) 111. IBH, Oxford 1983.
- Narhi LO, et al. The Effect of Carbohydrate on the Structure and Stability of . *J. Biol. Chem.* 266 1991: 23022–23026.
- Nazir H.; M. Yildiz; H. Yilmaz; M.N. Tahir; and D. Ülkü. Intramolecular hydrogen bonding and tautomerism in Schiff base structure of N-(2-pyridil)-2-oxo-1-naphthylidemethylamine. *J. Mol. Struc.* 2000, 524, 241-250.

- Neucere N.J. Isolation of alpha-arachin, the major peanut globulin. *Anal. Biochem.* 1969, 27, 15-24.
- Nielsen, H.K, J. Löliger, and R.F. Hurrell. Reactions of proteins with oxidizing lipids. 1. analytical measurement of lipid oxidation and of amino acid losses in a whey protein- Methyl linoleate model system *Br J. Nutr.* 1985. 53 61-73
- Nielsen, H.K.; J. Löliger and R.F. Hurrell. Reactions of proteins with oxidizing lipids. 1. Analytical measurements of lipid oxidation and amino acid losses in whey protein –methyl linoleate model system. *Br. J. Nutr.* 1985b, 53, 61-73. of Erythropoietin. *J. Biol. Chem.* 266 1991: 23022–23026.
- Ory, R.L.; Neucere, N.J; Singh, R. and St Angelo, A.J. Stability of peanut proteins to heat and organic solvents. *J. Am. Peanut Res. Educ. Assoc.* 1970, 119-127. oxidation. *Biochim Biophys Acta* 876(1):154-166 oxidation. *Biochim Biophys Acta* 876(1):154-166.
- Partridge, M.A.K., Jiang, Y., Skerritt, J.H., and Schaich, K.M. 2003. Immunochemical and electrophoretic analysis of soluble modified proteins in extruded wheat flour, *Cereal Chemistry* 80(6): 791-798.
- Pepper Jr. M.B. and Freeman A.F. Determination of moisture in peanut butter *J. Americ. Oil Chem. Soc.* 30, 1953, 335-337.
- Pickett, T.A. and Holley, K.T. Peanut roasting studies. *Georgia, Agric. Expt. Tech. Bull.* 1, 1946.
- Pickett, T.A. Chemical Comparison of the Peanut and cotyledon. *Peanut J. Nut. World*, 1946, 25.
- Player, T.J. and Hultin, H.O. The Effect of Lipid Peroxidation on the calcium-accumulating ability of the microsomal fraction isolated from chicken breast muscle. *Biochem. J.* 1978, 174, 17-22.
- Rebello, C.A. and Schaich, K.M. 1999. Extrusion chemistry of wheat flour proteins. II. sulfhydryl/disulfide content and protein structural changes, *Cereal Chem.* 76: 756-763.
- Refsgaard, H.H.R., Tsai, L., Stadtman, E. R. Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc. Nat. Acad. Sci. USA* 2000, 97, 611-616.
- Riley, M., and J.J. Harding. The reaction of malonaldehyde with lens protein and the protective effect of Aspirin. *Biochim. Biophys* 1993, 1158, 107-112.
- Robin, Y.-Y. and Tzong-Tien Tsai. Characterization of peanut proteins during roasting as affected by initial moisture content. *J. Agric. Food. Chem.* 1989, 37, 1377-1381



- Roubal, W.T. and Tappel, A.L., Damage to proteins, enzymes, and amino acids by peroxidizing lipids. *Arch. Biochem. Biophys.* 1966, *113*, 5-8.
- Saeed, S., Fawthrop, S.A., Howell, N. Electron Spin Resonance (ESR) study on free radical transfer in fish lipid-protein interaction. *J. Sci. Food Agric.* 1999, *79*, 1809-1916.
- Saeed, S., Gillies, D., Wagner, G., Howell, N.K. ESR and NMR Spectroscopy studies on protein oxidation and formation of dityrosine in emulsions containing oxidised methyl linoleate. *Food Chem. Toxicol.* 2006, *44*, 1385-1392.
- Schaich, K.M, Shahidi, F., Zhong, Y., and Eskin, N.A.M.. Lipid oxidation. In: *Biochemistry of Foods*, Third Edition, Ed. Eskin, N.A.M., Elsevier, 2012. 419-478.
- Schaich, K.M. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation. Part III: Free radical transfer from oxidizing lipids. *CRC Crit. Rev. Food Sci. Nutr.* 1980 *13*: 189-244.
- Schaich, K.M. and Karel, M. Free radical reactions of peroxidizing lipids with amino acids and proteins: An ESR Study. *Lipids*, 1976, *11*, 392-400.
- Schaich, K.M. and Karel, M. Free radicals in lysozyme reacted with peroxidizing methyl linoleate. *J. Food Sci.* 1975, *40*, 456-459.
- Schaich, K.M. and Rebello, C.A. 1999. Extrusion chemistry of wheat flour proteins. I. Free radical production. *Cereal Chem.* 76: 748-755.
- Schaich, K.M. Co-oxidations of oxidizing lipids: Reactions with proteins, in *Lipid Oxidation Pathways, Chapter 8, Vol 2, ed.*; Kamal-Eldin, A. and Min, D., AOCS Press, 2008, 183-274.
- Schaich, K.M. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation – Part III: Free radical transfer from oxidizing lipids. *CRC Crit. Rev. Food Sci. Nutr.* 1980, *13*, 189-244.
- Schaich, K.M. Lipid oxidation in fats and oils: An Integrated View. In *Bailey's Industrial Fats and Oils, 6th Edn.*; Shahidi, F., Ed.; John Wiley: New York, 2005, p. 2681-2767.
- Schaur, R.J. Basic aspects of the biochemical reactivity of 4-hydroxynonenal. *Mol. Aspects Med.* 2003, *24*, 149-159.
- Shi H.; S. Suna; Z. Liua; and L. Lia. Oxidation of 1-serine and 1-theonine by bis (hydrogen periodato) Argentate (III) complex anion. A mechanistic study. *J. Inorg. Biochem.* 2007, *101*, 1650172.
- Shokraii, E. H, Esen, A. and Mazingo, R. W. 1985. Relation of a 36, 000-dalton arachin subunit to blanchability in peanuts (*Arachis hypogaeal* L.) *J. Agric. Food Chemistry* 33, 1114-1116.

- Smith, D.M., Noomarji, S.H., Price, J.F., Bennink, M.R., Herald, T.J. Effect of lipid oxidation on the functional and nutritional properties of washed chicken myofibrils stored at different water activities. *J. Agric. Food Chem.* 1990, 38, 1307-1312.
- Soyer A., and H.O. Hultin, Kinetics of oxidation of the lipids and proteins of Cod sarcoplasmic Reticulum. *J. Agri. Food Chem.* 2000, 48, 2127-2134.
- St Angelo A.J. R.L. Ory and Brwon, L.E. A comparison of minor constituents in peanut buttres and possible sources of fatty acid peroxidation. *J. Amer. Peanut Res. Edu. Soc.* 1972 4. 186.
- St Angelo, A.J. and R.L. Ory, Investigation of causes and prevention of fatty acid peroxidation in peanut butter. *J. Maric. Peanut Res. Edu. Assoc.* , 1973, 5. 128.
- St. Angelo, A. J. and Mann, G. E. in Peanuts-culture and uses. *Am. Peanut Res. and Educ. Assoc.* 1973
- Stadtman, E. R. Role of oxidation species in aging. *Current Med. Chem.* 2004, 11 1105-1112.
- Stadtman, E.R. and Berlett, B.S. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem. Res. Toxicol.* 1997, 10, 485-494.
- Stadtman, E.R. and Levine, R.L. Free radical –Mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 2003, 25, 207-218.
- Stadtman, E.R. Protein oxidation in aging and age related diseases. *Annals of New York Academy Science.* 2006, 928, 22-28.
- Suyama, K.; and S; Adachi. Reaction of alkanals and amino acids of primary amines. Synthesis of 1,2,3,5- and 1,3,4,5- substituted quaternarypyridinium salts. *J. Agric. Food Chem.* 1979, 44 1417-1420.
- Szczesniak, A.S. Classification of textural properties. *J Food Sci.* 1963 28, 410-421.
- Szweda LI, Uchida K, Tsai L, Stadtman ER. 1993. Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. *J Biol Chem* 268(5): 3342-3347.
- Tappel, A.L. Studies of the mechanism of vitamin E action. III. in vitro copolymerization of oxidized lipids with proteins. *Arch. Biochem. Biophys.* 1955, 54, 266-280.
- Tombs, M. P. and Lowe, M. Amino acid composition of peanut *Biochem. J.* 1967 105, 181.
- Tombs, M.P. 1965. An electrophoretic investigation of groundnut proteins: The structure of arachin A and B. *Biochem. J.* 96: 119-113.

- Totlani, V.M., and Chinnan, M.S., Effect of stabilizer levels and storage conditions on texture and viscosity of peanut butter. *Peanut Sci.*, 34, 1, 1-9.
- Tsen C.C.; and A.A. Tappel. Oxygen lability of cysteine in hemoglobin. *Arch. Biochem. Biophys.* 1958 75 243-250.
- Turverov K.K. and Kuznetsova I.M. Intrinsic fluorescence of actin. *J. of Fluorescence.* 2003, 13, 41-57.
- Uchida K, Stadtman ER. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. USA* 1992, 268 2388-6393.
- Uchida K, Toyokuni S, Nishikawa K, Kawakishi S, Oda H, Hiai H, Stadtman ER. Characterization of epitopes recognized by 4-hydroxy-2-nonenal specific antibodies. *Arch. Biochem. Biophys.* 1995, 324, 241-248.
- Uchida, K. and Kawakishi, S. Site-specific oxidation of angiotensin I by copper(II) and L-ascorbate: conversion of histidine residues to 2-imidazolones. *Arch. Biochem. Biophys.* 1990 283:20.
- VAM Standard Analytical Framework. Role of objectives of VAM activities to support WFP food oriented intervention. World Food Programme, 2002.
- Watt, B. K. and Merrill, A.L. Composition of food. USDA Agricultural Handbook 8.1963.
- Willich, R.K.; A.S. Morris; A.F. Freeman. Peanut butter 1. Roasting, cooling blanching and picking of peanuts. *Food Tech.* 1952, 6, 71.
- Woodroof J.G.; Leahy, J.F Microscopical studies of peanuts with reference to processing. *Georgia Exper. Station. Bull. UGA Agricultural Experiment system* Athens, G.A, 1940 243, 1-39.
- Woodroof, J.G. Peanuts: production, processing and products. 1973 The Avi Publishing Co. Westport. CT.
- Yamada, T., Aibara, S. and Morita, Y. 1979b. Isolation and some properties of arachin subunits. *Agric. Biol. Chem.* 43,2563-2568.
- Yamada, T., Aibara, S., Morita Y. . Dissociation-association behaviour of arachin between dimeric and monomeric. *Agri. Biol. Chem.* 432, 2556.
- Yamaki. S.; T. Kato and K. Kikugawa. Characteristics of fluorescence formed by reaction of proteins with unsaturated aldehydes, possible degradation products of lipid radicals. *Chem. Pharm. Bull.* 1992, 40 2138-2142.
- Yildiz, M.; Z. Kilic; and T. Höelek. Intramolecular hydrogen bonding and tautomerism in Schiff base. Part1. Structure of 18-Di[N-2-oxyphehyl-salicylidene]-3-6-Dioxaoctane. 1998, *J. Mol. Struct.* 441, 1-10.

- Yotsuhashi, K. and Shibasaki K. 1973. Peanut protein I. Dissociation of arachin into subunits. *Japan Soc. Food Sci. Technol.* 20, 321.
- Yuan, Q., Zhu, X., Sayre, L.M. Chemical nature of Stochastic generation of protein based carbonyls: metal-catalyzed oxidation versus modification by products of lipid oxidation. *Chem. Res. Toxicol.* 2007, 20, 129-139.
- Zamora, R. and Hidalgo, F.J. Comparative methyl linoleate and methyl linolenate oxidation In the presence of bovine serum albumin at several lipid/protein ratios. *J. Agric. Food Chem.* 2003, 51, 4661-4667.
- Zirlin, A. and Karel, M. Oxidation effects in a freeze-dried gelatin-methyl linoleate system. *J. Food Sci.* 1969, 34, 160-194.