GAZİANTEP UNIVERSITY GRADUATE
SCHOOL OF NATURAL & APPLIED SCIENCES

EFFECTS OF OZONE ON FUNCTIONAL PROPERTIES OF PROTEINS

M.Sc. THESIS
IN
FOOD ENGINEERING

BY
HİCRAN UZUN
JANUARY 2010

Effects of Ozone on Functional Properties of Proteins

M.Sc. Thesis
In
Food Engineering
University of Gaziantep

Supervisor
Prof. Dr. Esra İBANOĞLU

by
Hicran UZUN
January 2010

T.C.
GAZİANTEP UNIVERSITY
GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES
(Department of Food Engineering)

Name of the thesis: Effects of Ozone on Functional properties of Proteins
Name of the student: Hicran UZUN
Exam date: 18 January 2010

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Ramazan KOÇ
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Sciences

_____

Prof. Dr. Ahmet KAYA
Chairmen of the
Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Sciences

_____

Prof. Dr. Esra İBANOĞLU
Supervisor

Examining Committee Members	**Signature**
Prof. Dr. Osman ERKMEN (Chairman)	_____
Prof. Dr. Esra İBANOĞLU	_____
Prof. Dr. Şenol İBANOĞLU	_____
Assist. Prof. Dr. İlkay PINARLI	_____
Assist. Prof. Dr. Emine ALBEN ERÇELEBİ	_____

TO MY PARENTS

EMİNE & ALİ KEMAL UZUN

ABSTRACT

EFFECTS OF OZONE ON FUNCTIONAL PROPERTIES OF PROTEINS

UZUN, Hicran
M.Sc. in Food Engineering
Supervisor: Prof. Dr. Esra İBANOĞLU
January 2010, 100 pages

Ozone has recently gained the attention of food industries, although it has been used effectively as a primary disinfectant for treatment of water for many years. Egg white, whey proteins have been extensively used in food products to improve textural, functional and sensory attributes. Proteins influence the functional properties in foods such as solubility, emulsion and foam formation and rheological properties. However, there is limited knowledge about the effects of ozone on the food components. In this project, the effects of ozone treatment on the functional properties of whey protein isolate and egg white proteins were investigated. Ozone treatment of proteins was performed either in aqueous solutions or as gas ozonation of pure protein powders. Ozonation influenced foam formation and stability extensively. Ozone treatment affected emulsion activity negatively and reduced the emulsion stability. Thermal denaturation properties of ozonated protein samples were investigated by Differential Scanning Calorimeter (DSC). For whey protein isolate, denaturation temperature increased while denaturation enthalpy decreased. However, denaturation temperature of egg white proteins were influenced less. The effects of gas ozonation on solubility were observed to be more extensive than the effect of ozone treatment in aqueous solutions. The leavorotation of the ozonated protein solutions increased. HPLC chromatograms of ozonated protein samples showed a reduction (42 and 45 %) in the peak areas of whey proteins and egg white proteins, respectively. As a result of ozone treatment, dilute emulsions exhibited more liquid like behavior at low frequencies than concentrated emulsions.

Key Words: Ozone, protein, functionality, emulsion, foam, solubility,optical activity

ÖZET

PROTEİNİN FONKSİYONEL ÖZELLİKLERİ ÜZERİNE OZONUN ETKİSİ

UZUN, Hicran
Yüksek Lisans Tezi, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Prof. Dr. Esra İBANOĞLU
Ocak 2010, 100 sayfa

Ozon, su arıtımında etkili bir dezenfektan olarak uzun yıllardır kullanıldığı halde, gıda endüstrisinde son zamanlarda önem kazanmaya başlamıştır. Yumurta akı proteinleri ve peyniraltı suyu proteinleri gıdalarda yaygın olarak kullanılan proteinlerdir. Proteinler bir çok gıda ürününde hem temel amino asitleri sağladığı hem de çeşitli fonksiyonel özelliklere sahip oldukları için önemlidirler. Proteinlerin gıda sistemlerinde çözünürlük, viskozite, emülsiyon ve köpük oluşturma ile ilgili fonksiyonel özellikleri etkilediği bilinmektedir. Ozon gazı ve/veya ozonlu su ile muamele edilen gıda bileşenlerinin bu muameleden nasıl etkilendiğine dair sınırlı sayıda araştırma mevcuttur. Bu projede peyniraltı suyu protein izolatı ve yumurta akı proteinlerinin ozonla muamele sonucunda fonksiyonel özelliklerinin değişimi incelenmiştir. Projede, toz halindeki protein örnekleri ozonlu su ile ve doğrudan ozon gazı teması ile olacak şekilde iki yolla ozonlanmıştır. Ozon muamelesinin köpük oluşumu ve köpük stabilitesi üzerinde büyük etkisi olduğu görülmektedir. Ozonla muamele emülsiyon aktivitesini olumsuz yönde etkilemiştir ve emülsiyon stabilitesini düşürmüştür.Ozonlanmış örneklerin denatürasyon özellikleri Diferansiyel Sıcaklık Taramalı Kalorimetre (DSTK) ile ölçülmüştür ve peyniraltı suyu protein izolatının denatürasyon sıcaklığının yükseldiği, denatürasyon entalpisinin düştüğü görülmüştür. Aynı etki yumurta akı proteinlerinde daha azdır. Ozonlu solüsyon ile muamele edilen proteinlerin çözünürlüğünde azalma görülürken, bu oran ozon gazı ile muamele edilen proteinlerde daha düşüktür. Proteinlerin optik çevirme gücü sağa doğru artmıştır. Yüksek basınçlı sıvı kromatografisi sonuçlarında proteinlerin denatüre olmaları nedeniyle peyniraltı protein izolatı ve yumurta proteinlerinin piklerinin alanlarında azalma (%42 ve 45) olduğu görülmüştür. Ozon ile muamele sonucunda, seyreltik emülsiyonlar derişik emülsiyonlara göre düşük frekanslarda daha çok sıvıya benzer davranış sergilemiştir.

Anahtar Kelimeler: Ozon, protein, fonksiyonellik, köpük, emülsiyon, optik aktivite.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Prof. Dr. Esra İBANOĞLU for her guidance, interest and patience and also encouragement she provided throughout the course of this study.

I am also very grateful to Prof. Dr. Şenol İBANOĞLU for his guidance and invaluable discussions, but also for his kindness and readiness in assistance throughout this study.

I would like to thank to Prof. Dr. Ahmet KAYA for his help and support.

I would like to thank to chairman of the board of KAHRAMAN TARIM A.Ş. and also head of Southeast Anatolia Exporters Unions, Abdülkadir ÇIKMAZ for his kind help and support.

Special thanks to my family and my coworkers for their kind help and patience at every stage of this study.

CONTENTS

Page

LIST OF FIGURES

Figures **Pages**

LIST OF TABLE

Tables **Pages**

ABREVIATIONS

WPI	Whey protein isolate
ANOVA	Analysis of variance
DSC	Differential Scanning Calorimetry
HPLC	High pressure liquid chromatography
T_d	Denaturation temperature
ΔH	Denaturation enthalpy
EAI	Emulsion activity index
ESI	Emulsion stability index
k	Rate constant
FV	Foam volume
G'	Elastic (storage) modulus
G"	Viscous (loss) modulus
SPSS	Statistical package for social sciences
[α]	Optical Activity

CHAPTER I

INTRODUCTION

Ozone is a powerful antimicrobial substance due to the its potential oxidizing capacity. Ozone use may have many advantages in the food industry. There are suggested applications of ozone in the food industry such as food surface hygiene, sanitation of food plant equipment, reuse of waste water, etc.

In the present work we report our basic research results on the action of ozone on functional properties of proteins. Proteins, as macromolecules, perform important roles in functionality in food systems. Therefore, the growing demand for proteins as important ingredients in formulated food or in pharmaceutical and industrial mixtures has created a necessity for proteins with specific and consistent functional properties. Proteins exhibit many functional properties governed by their physicochemical activities in a bulk liquid phase.

Ozone reacts with proteins and causes the oxidation or the ozonolysis of certain amino acid residues. As a result of this attack the protein molecules undergo changes in their visual folding and binding ability and are denatured.

Although the wide applications of ozone, there is relatively limited information about the basic interaction of ozone with proteins. In literature, studies are also unsatisfied to explain effects of ozone on the functional properties of proteins scientifically.

In this study, whey protein isolate and egg white proteins were chosen since they are widely used as functional ingredients in many food products. Whey proteins are used as food ingredients because of their unique functional characteristics like emulsifying, gelling, thickening, foaming and water binding capacity. Egg white proteins have been extensively used as ingredients in processed foods, besides being desirable ingredients in many foods, such as bakery products, pies, cookies, and meat by-products due to unique functional properties of proteins, such as gel and foam

formation. Thus, whey protein isolate and egg white proteins were used to investigate action of ozone on their functional properties such as solubility, emulsifying, foaming. Furthermore, the rheological behavior of emulsions stabilized whey protein isolate was also investigated to determine the effects of ozone on viscoelastic behavior of emulsions.

The effects of ozone on structures of whey protein isolate and egg proteins were also investigated. Because, action of ozone causes a variation in optical activity of proteins due to an alteration of its secondary and tertiary structure. In literature, structural changes in proteins arising from denaturation or oxidation have been examined by HPLC. Therefore, optical activity measurements and HPLC were used to investigate action of ozone on structure of these proteins. The structural changes introduced by the ozone degradation of proteins could also be examined by thermal analytical techniques.

CHAPTER II

LITERATURE SUMMARY

2.1. OZONE

2.1.1. Chemical and Physical Properties of Ozone

Ozone was first discovered by the European researcher C.F. Schonbein in 1839. It was first used commercially in 1907 in municipal water supply treatment in Nice and in 1910 in St. Petersburg (Kogelschatz, 1988). United States Food and Drug Administration (FDA) accepted generally recognize as safe status for use of ozone in food industry. FDA stated that maximum treatment level was 0.4 mg of ozone per liter of bottled water.

At room temperature, ozone decomposes rapidly and, thus, does not accumulate substantially without continual ozone generation (Miller et al., 1978; Peleg, 1976). At room temperature, ozone is a nearly colorless gas. Ozone has a pungent, characteristic odor described as similar to ‘‘fresh air after a thunderstorm’’ (Coke, 1993). It is readily detectable at 0.01–0.05 ppm level (Mehlman and Borek, 1987; Miller et al., 1978; Mustafa, 1990). It is found in low concentration in nature. Ozone has a longer half-life in the gaseous state than in aqueous solution. Ozone solubility in water is 13 times that of oxygen at 0–30 ^{0}C and it is progressively more soluble in cold water (Rice, 1986). Ozone in pure water rather quickly degrades to oxygen, and even more rapidly in impure solutions (Hill and Rice, 1982). Ozone melts -192.5 ^{0}C and its boiling point is -111.9 ^{0}C. Critical temperature of ozone is -12.1 ^{0}C and critical pressure is 54.6 atm (Manley, 1967; Nebel, 1981).

Fluorine is the most powerful common oxidizing agent and its oxidation potential is 3.06 mV. Ozone is the second most powerful common oxidizing agent. The oxidation potential of ozone is 2.07 mV. The other oxidizing agents are

permanganate, chlorine dioxide, hypochlorus acid and chlorine gas and their oxidation potential is lower than ozone.

Ozone decomposition is faster at higher water temperatures (Rice et al., 1981). Ozone is a blue gas at ordinary temperature. However, the color is not noticeable in concentrations at which it is normally produced. Liquid ozone is easily exploded if greater than 20% ozone to oxygen mixtures occur. Explosions may be detonated by electrical sparks or by sudden changes in temperature or pressure. However, in practical usage explosions of ozone are extremely rare. The three atoms of oxygen in the ozone molecule are arranged at an obtuse angle whereby a central oxygen atom is attached to two equidistant oxygen atoms; the included angle is approximately $116^0$490' and the bond length is 1.278 Å. Four structures of ozone are shown in Fig. 2.1

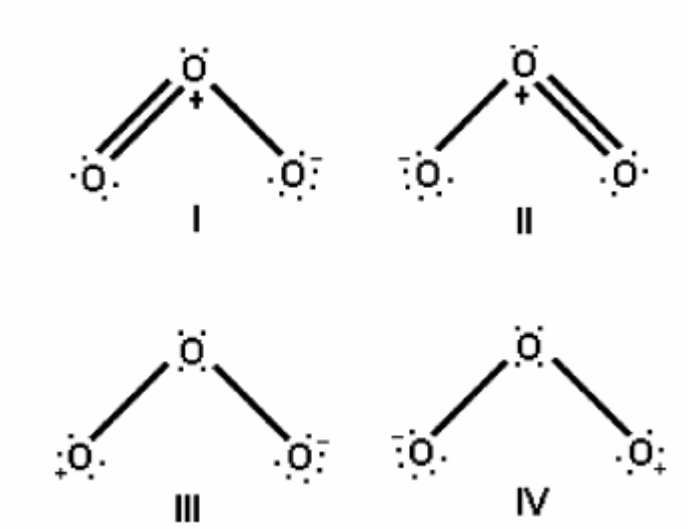

Figure 2.1. Resonance Structures of Ozone Molecule (Oehlschlaeger, 1978).

Although ozone is not an extremely toxic gas in low concentrations, it may be fatal to humans at high concentration. It was found that 0.2 ppm and higher concentrations of ozone can cause varying degrees of damage to the respiratory tract, depending on exposure length (Schwartz et al., 1976). Damage of pulmonary system involves the trachea (Schwartz, et al., 1976), bronchi (Castleman et al., 1973), and alveoli (Schwartz et al., 1976). 50 ppb maximum level recommended by the FDA for protection of human health and reducing the occurrence of potentially adverse health effects (Shaughnessy, 2006).

2.1.2 Ozone Generation

In order to generate ozone, a diatomic oxygen molecule must first be split.

$$e^{-1} + O_2 \rightarrow 2O + e^{-1} \qquad (2.1)$$

The resulting free radical oxygen is thereby free to react with diatomic oxygen to form the triatomic ozone molecule.

$$O + O_2 + M \rightarrow O_3 + M \qquad (2.2)$$

where M is any other molecule in the gas. At the same time, atomic oxygen and electrons also react with ozone to form oxygen.

$$O + O_3 \rightarrow 2\,O_2 \qquad (2.3)$$

$$e^{-1} + O_3 \rightarrow O_2 + O + e^{-1} \qquad (2.4)$$

However, in order to break the O–O bond a great deal of energy is required. Ultraviolet radiation (188 nm wavelength) and corona discharge methods can be used to initiate free radical oxygen formation and, thereby generate ozone. In order to generate commercial levels of ozone, the corona discharge method is usually used (Fig. 2.2).

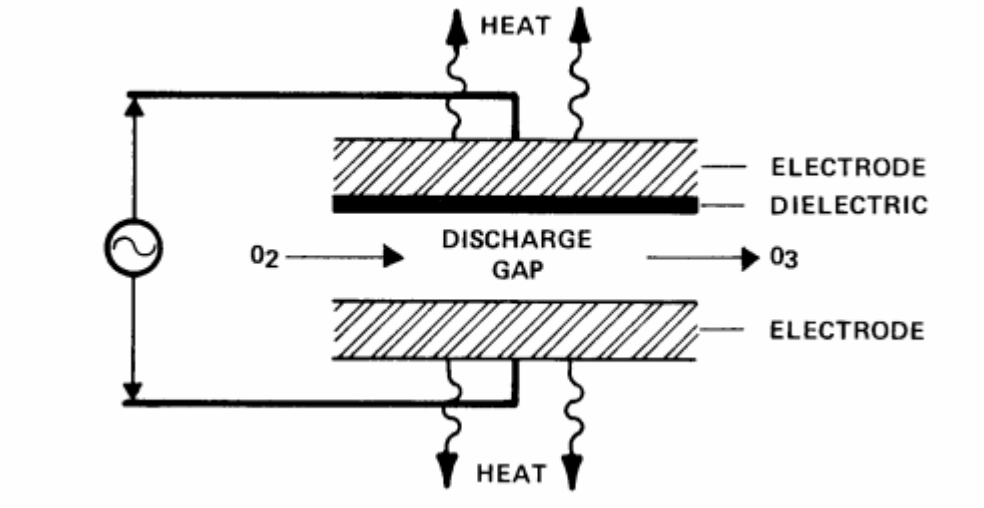

Figure 2.2. Typical Corona cell configuration (Rice et al., 1981).

There are two electrodes in corona discharge, one of which is the high tension electrode and the other is the low tension electrode (ground electrode). Those are

separated by a ceramic dielectric medium and narrow discharge gap is provided (Fig. 2.2). When the electrons have sufficient kinetic energy (around 6–7 ev) to dissociate the oxygen molecule, a certain fraction of these collisions occur and a molecule of ozone can be formed from each oxygen atom. If air is passed through the generator as a feed gas, 1–3% ozone can be produced; however, using pure oxygen allows yields to reach up to 6% ozone (Rice et al., 1981). Consequently, ozone concentration cannot be increased beyond the point that the rates of formation and destruction are equal (Manley and Niegowski, 1967). Ozone gas cannot be stored since ozone spontaneously degrades back to oxygen atoms (Coke, 1993; Kogelschatz, 1988; Wickramanayaka, 1991).

2.1.3. Use of Ozone in Food Industry

Ozone is a powerful antimicrobial substance due to the its potential oxidizing capacity. There are suggested applications of ozone in the food industry such as food surface hygiene, sanitation of food plant equipments, reuse of waste water, lowering biological oxygen demand (BOD) and chemical oxygen demand (COD) of food plant waste (Dosti, 1998; Guzel-Seydim, 1996; Majchrowicz, 1998; Rice et al., 1982). Multifunctionality of ozone application makes ozone a promising agent. Although ozone has not been commonly used in the dairy and food industry, it has found limited applications in a few areas such as conversion of green tea to black tea (Graham et al., 1969), cleaning of shellfish (Anonymous, 1972), and disinfection of poultry carcasses and chill water in the poultry industry (Yang and Chen, 1979; Chang and Sheldon, 1989; Sheldon and Brown, 1986;).

Ozone has been used for disinfecting recycled poultry chill water and disinfection of poultry carcasses (Sheldon and Brown, 1986). According to the Code of Federal Regulations (USDA, 1997), there must be at least 60% reduction in total microorganisms and similar reduction in coliforms, *Escherichia coli*, and *Salmonella spp.* Waldroup et al., (1993) determined that there were no viable *E. coli* or presumptive coliforms after ozonation of recycled chill water and the total aerobic plate count was low. Therefore it has been concluded that the use of ozone for disinfecting poultry chill water met USDA recycling requirements. Application of ozone directly to poultry carcasses destroyed more than 2 log-units of all carcass

microorganisms with no significant lipid oxidation, off-flavor development or loss in carcass skin color (Sheldon and Brown 1986). Ozone treatment has been used to increase the shelf-life of fruits and vegetables (Norton et al., 1968; Rice et al., 1982). Treatment with ozone resulted in the reduction in fungal deterioration of blackberries and grapes (Beuchat, 1992), the decreased mold and bacterial counts in onions without any change in chemical composition and sensory quality (Song et al., 2000) and the decreased bacterial content in shredded lettuce in water (Kim et al., 1999). Ozone has been used experimentally as a substitute for ethylene oxide for the disinfection of whole black peppercorns and ground black pepper (Zhao and Cranston, 1995). Although ozone treatment of ground black pepper resulted in slight oxidation of volatile oil constituents, ozone had no significant effect on the volatile oils of whole peppercorns. No new components were detected as a result of ozonation. However, ozonation successfully reduced microbial loads. In order to control mold on Cheddar cheese surfaces and in cheese rooms (Gibson et al., 1960), ozone appeared to destroy the molds present at high ozone concentrations. However, upon termination of ozonation, mold populations flourished.

Chemical sanitizers are the most commonly utilized for sanitation in the food industry. Chlorinated agents are used worldwide for disinfecting water, waste water and for sanitizing food processing plant equipment. Even though chlorine sanitizers have several disadvantages including being harmful and irritating at high concentrations, being prone to forming carcinogenic compounds and being toxic to the environment, these compounds are economical bactericides that inactivate all types of vegetative cells. Food researchers are searching for alternative cleaning and sanitizing agents, effective against food spoilage and pathogenic bacteria, harmless to humans and the environment. Additionally, these agents must also be non-corrosive to expensive food processing equipment. Ozone is a potential alternative to chlorine for use in the food industry and the use of ozonated water has been proposed as sanitizer for dairy and food plants (Greene et al., 1993). The effectiveness of ozonated water and chlorinated sanitizer for the disinfection of stainless steel surfaces were compared by incubating the equipment with UHT milk inoculated with either *Pseudomonas fluorescens* (ATCC 949) and *Alcaligenes faecalis* (ATCC 337) at 32^0C for 4–24 h. It has been found that ozone was as effective as chlorination against dairy surface attached bacteria, both treatments reduced bacterial populations

by 99%. Ozone, chlorine and heat were compared for effectiveness against food spoilage bacteria in synthetic broth (Dosti, 1998). Ten min ozonation caused the highest bacterial population reduction with a mean reduction over all species of 7.3 log units followed by heat (5.4 log reduction) and chlorine (3.07 log reduction). Guzel-Seydim et al., (2000) studied the use of ozonated water in dairy equipment. Soiled stainless steel coupons were treated with ozonated water as a pre-rinse. Results implied that ozone treatment removed 84% of dairy soil when compared to warm water (40^0C) which only removed 51%. Therefore ozone has been proposed as a promising cleaning and waste treatment agent for the dairy and food industry.

2.1.4. Effects of Ozone on Proteins

Proteins belong to a class of organic compound called polyamides. Polyamides are polymers where the monomer units are held together by amide groups. The monomer units in proteins are called α-amino acids and the amide group –CO-NH- joining two α-amino acids is often called a peptide link. The vast majority of the proteins found in living organisms are composed of only 20 different kinds of amino acids, repeated many times and strung together in a particular order. Each type of protein has its own unique sequence of amino acids; this sequence, known as its primary structure, actually determines the shape and function of the protein.

Protein structure is commonly described as having four levels of organization, primary, secondary, tertiary and quaternary structure. These divisions are somewhat arbitrary because it is the total structure that controls function, but they are a useful way of building the structure.

Protein oxidation is defined as the covalent modification of a protein induced either directly by reactive oxygene species (ROS) or indirectly by reactions with secondary by products of oxidative stress (Tetik et al., 2007). Proteins have many specific functions. Oxidative modification of a protein leads to biochemical consequences. Different forms of oxidative modification have different functional consequences.

Agents responsible for oxidative damage and protein oxidation are chemical reagents (H_2O_2, Fe^{+2}, Cu^{+} etc.), γ-irradiation in the presence of O_2 , UV light, ozone, lipid peroxides (HNE, MDA, acrolein), mithocondrial electron transport chain leakage etc.

In this study, the effects of ozone on functional properties of proteins were examined. With proteins, ozone causes the oxidation or the ozonolysis of certain amino acid residues, for instance, tryptophan, tyrosine and cysteine. As a result of this attack the protein molecules undergo changes in their usual folding and binding ability and are denaturated (Cataldo, 2003).

Oxidation reactions affect the quality of food, but they also have an impact on the charge and conformation of the three-dimensional structure of the protein (exposure of hydrophobic groups, changes in secondary structure and disulfide groups), loss of enzyme activity, and changes in the nutritive value (loss of essential amino acids) (Howell et al., 2001; Karel et al., 1975; Zamora et al., 1999a). In addition, the modified proteins will have different functional properties from those of their unmodified molecules; their emulsifying, foaming, gelling, and water binding properties may be affected as well as the texture of food will be changed (Leaver et al., 1999b).

The most sensitive amino acids toward oxidation are heterocyclic amino acids. In addition, amino and phenolic groups of amino acids are susceptible to oxidation (Desai and Tappel, 1963). Due to their structure tryptophan, histidine, and proline, but also lysine, cysteine, methionine, and tyrosine, are prone to oxidation where the hydrogen atom is abstracted either from OH-, S- or N-containing groups (Baker et al., 1998; Doorn and Petersen, 2002; Gardner, 1979; Karel et al., 1975; Kikugawa et al., 1991; Matoba et al., 1984).

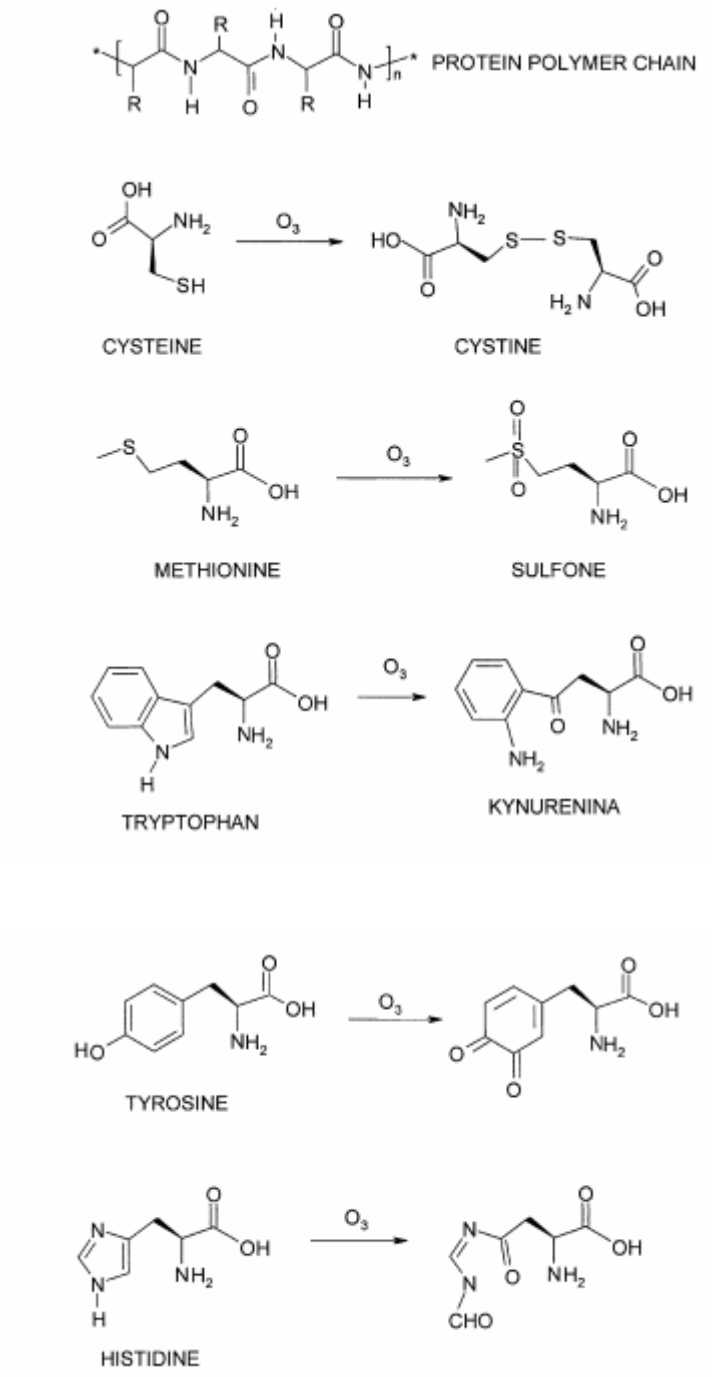

Figure 2.3 Ozone Reactions with Proteins (Cataldo, 2003).

The oxidation and the cleavage of amino acid residues causes significant changes in the supramolecular structure or which is the same, the tertiary and quaternary structures of the proteins changing or hindering their folding ability and therefore destroying their biological activity. The precipitation of a protein from the solution is dependent from the amount if cysteine residues present in the virgin molecule. The action of ozone converts the thiol group of cysteine into disulphides.

The ozone attack is directed mainly toward the aromatic amino acid causing the degradation of the relative moiety. Figure 2.3 shows the oxidation products of ozone with amino acids. Tryptophan has been known as the most sensitive amino acid to ozone and is degraded to formylkynurenine and, by hydrolysis, to kynurenine. Tyrosin is oxidized to an ortho-quinone (Figure 2.3) and by further oxidation the ring is cleaved into aspartic acid. Reaction of phenylalanine with O_3 is much slower in comparison to tyrosine which, in turn, is much less reactive with ozone in comparison to tryptophan. The general amino acid reactivity towards ozone has been reported as follows (Bailey, 1982);

Trytophan > Methionine >> Cystine >> Tyrosine >>> Phenylalanine

2.2. Whey Proteins

Whey proteins are used as food ingredients because of their unique functional characteristics like emulsifying, gelling, thickening, foaming and water binding capacity (Kinsella and Whitehead, 1989). As food ingredient they are used not only because of their functional properties, but also because of their high nutritive value and GRAS status (Bryant and McClements, 1998; Harper, 2000; Hudson et al., 2000).

The most abundant protein in whey is β–lactoglobulin (β–Lg). It comprises 10% of the total milk protein or 58% of the whey protein. The functionality of whey protein isolate (WPI) mainly reflects the functionality of this globular and amphiphilic protein that has the ability to adsorb at the water–oil and air–water interface. It contributes in this way to sauce formation by lowering the interfacial tension, and also by stabilizing the film formation at the interface (Damodaran, 1996). This protein unfolds partially at the interface and forms intermolecular associations, either by hydrophobic interactions or S–S bridges (Funtenberger et al., 1995). β–lactoglobulin contains 162 amino acids with a molecular weight of about 18,300. The molecule contains two disulfide and 1 free sulfhydryl groups and no phosphorus (Swaisgood, 1982). It is a globular protein, and a radius of about 2 nm. The isoelectric point (pI) of the protein is pH 5.1 (Whitney, 1997).

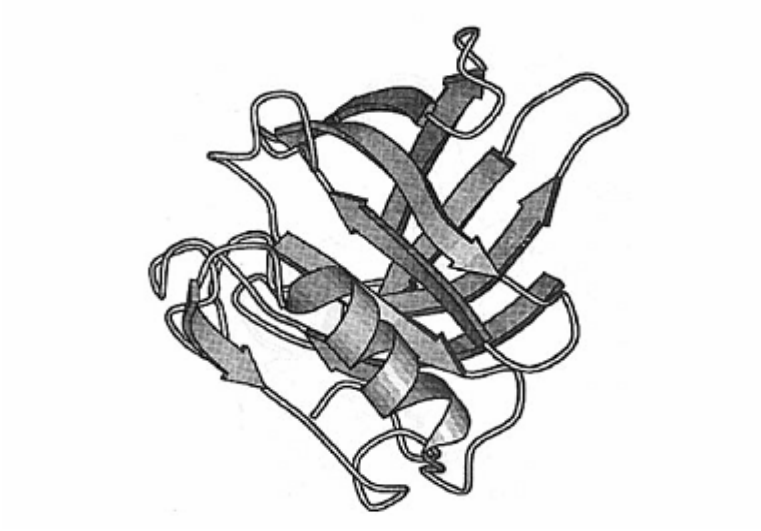

Figure 2.4. Structure of Bovine β–Lactoglobulin (Papiz et al., 1986).

The second most abundant whey protein, α–lactalbumin (α–La), is also reported to have emulsifying and stabilizing properties and it will participate together with β–Lg in the S–S bridging and film formation at the interface (Schokker et al., 2000). It comprises about 2% of the total milk protein that is about 13% of the total whey protein. The molecule consists of 123 amino acids and has a molecular weight of 14,146. The molecule contains four disulfide linkages and no phosphate groups.

α–Lactalbumin unusually is stable to heat in the presence rather than the absence of calcium, although most proteins show increased heat sensitivity in the presence of calcium. This is probably due to the ability of calcium to promote the formation of ionic intermolecular cross-links with most proteins. These crosslinks hold the molecules in proximity and increase the likelihood of aggregation upon heating. α–lactalbumin, on the other hand, uses calcium to form intramolecular ionic bonds that tend to make the molecule resistant to thermal unfolding. Under favorable conditions of calcium and pH, α–lactalbumin can remain soluble after exposure to 100^0C (Swaisgood, 1996).

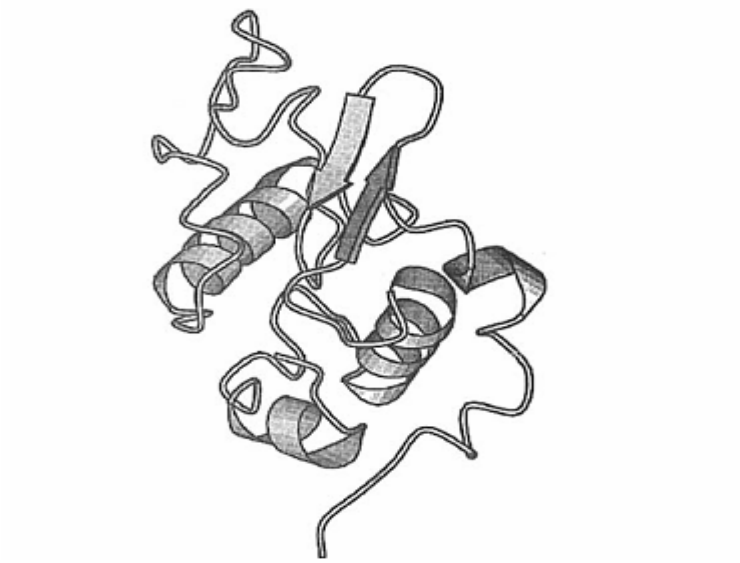

Figure 2.5 Three Dimensional Structure of α-Lactalbumin (Swaisgood, 1996).

The Bovine Serum Albumin (BSA), isolated from milk, is identical to the blood serum molecule. BSA is not synthesized in the mammary gland, it passes into the milk through passive leakage from the bloodstreams. The protein has a molecular weight of 69,000. It contains no phosphorus, 17 disulfides and one free sulfhydryl group. In blood, plasma albumin is a carrier of free fatty acids. The molecule has specific binding sites for hydrophobic molecules and may bind them in milk as well (Brown, 1977).

Whey proteins are now used widely in the food industry, with applications in, for example, processed meats, bakery products, pasta, ice cream, confectionary, infant foods, spreads, dips and beverages (Evans and Gordon, 1979; Mulvihil and Ennis, 2003). Their functions include enhancement of nutritional value, emulsification and foaming. Another important property of whey proteins, however, is their ability to form gels on heating (Singh and Hevea, 2003).

2.3 Egg White Proteins

Egg white is a natural source of proteins of recognized nutritional, biological, and technological potential interest (Awade et al., 1994; Croguennec et al., 2000). Three particularly important proteins present in egg white are lysozyme, ovotransferrin and ovalbumin. Lysozyme (3.5 g/100 g of the total egg white protein) has antibactericidal

properties and is widely used in food preservation and pharmaceutical industry. Ovotransferrin (13 g/100 g of the total egg white protein) is a glycoprotein with an important role in the iron transport and with wide antimicrobial activity. Ovalbumin, the major egg white component, corresponding to 54 g/100 g of the total egg white protein, is a glycoprotein with coagulation and gelation properties (Vachier et al., 1995).

Ovalbumin contains 385 amino acids, and its relative molecular mass is 45 kDa. The molecule contains four free sulphydryl groups and two disulphide bridges. It has four thiol groups which are buried within the core of the protein that become exposed upon heating, leading to intermolecular reactions that stabilize the gel structure (Cheftel et al.,1989).

Conalbumin (or ovotransferrin) is a glycoprotein consisting of two subunits. It has the capacity to bind bi- and trivalent metal cations into a complex. At its isoelectric point, one molecule can bind two cations and take on a red (Fe^{3+}) or yellow (Cu^{2+}) colors. These metal complexes are more thermostable than protein in the native state.

Lysozyme is a single polypeptide chain of 129 amino acids cross-linked by four disulphide bridges, with a molecular weight of ~14.4 kDa and isoelectric point of ~10.5–11.0 (Johnson and Larson, 2004). The excellent foaming properties of egg white and heat stability are caused by electrostatic interactions. Lysozyme, e.g., at the natural pH of fresh egg white, is positively charged and interacts with negatively charged proteins at the interface.

Due to unique functional properties of proteins, such as gel and foam formation, hen egg white proteins have been extensively used as ingredients in processed foods, besides being desirable ingredients in many foods, such as bakery products, pies, cookies, and meat by-products (Mine and Bergougnoux, 1998; Wong et al., 1996). Additionally, the commercialization of egg-derived products has gained great importance in the international market (Stadelman and Cotterill, 1995).

2.4. Functional Properties of Proteins

Proteins are being used as ingredients in man-made food products because they contribute to one or more of the desired characteristics of that food product. These characteristics might be consumer related (e.g. texture, mouthfeel, appearance, taste) or technology related. The latter includes both storage (shelf life, palatability) and processing (e.g. mixing behaviour, foam, and emulsion or gel formation). Proteins contribute to one or more of these characteristics because of their functional properties. This term is mostly used to indicate physicochemical properties that govern the performance and behaviour of a protein in food systems during preparation, processing, storage and consumption (Kinsella and Whitehead, 1989). Also, proteins often are added to foods to improve the nutritional quality.

Generally, the functional properties of food proteins may be classified into three main groups: (a) hydration properties, dependent upon protein–water interactions that have an important bearing on wettability, swelling, adhesion, dispersibility, solubility, viscosity, water absorption and water holding; (b) interfacial properties including emulsification and foaming characteristics; and (c) aggregation and gelation properties, which are related to protein–protein interactions.

2.4.1 Solubility

Among the functional properties of proteins, solubility is of primary importance due to its significant influence on the other functional properties of proteins. In general, proteins used for functionality are required to have high solubility, in order to provide good emulsion, foam, gelation and whipping properties (Nakai and Chan, 1985; Wit, 1989). In other words, a decrease in protein solubility affects in unfavorable manner its functionality (Vojdani, 1996). Solubility of proteins relates to surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interaction; in food case, such solvent is the water, and therefore the protein solubility is classified as a hydrophilic property.

The protein solubility has several definitions, since the proteins, in an aqueous spiritual medium, can form true or colloidal solution or insoluble particles

suspension (Borderias and Monteiro, 1988). Thermodynamically, the protein solubility is the protein concentration in the solvent in a simple or two-phase system (protein solution in liquid–liquid or in liquid–solid phases) in balance state (Vojdani, 1996). Mathematically, the protein solubility degree of a protein is the amount of protein present in liquid phase in relation to the total amount of protein in liquid and solid phases in balance. The protein solubility also can be defined as a certain operational parameter for the protein retention in the supernatant after the solution centrifugation for certain time period and under certain force centrifuge (Morrisey et al., 1982).

The protein solubility is a function of many factors, such as the native or denatured state and environmental factors (i.e. pH, temperature). The pH of the solution affects the nature and the distribution of the protein's net charge. Generally, the proteins are more soluble in low (acids) or high (alkaline) pH values because of the excess of charges of the same sign, producing repulsion among the molecules and, consequently, contributing to its largest solubility.

According with several authors (Kakalis and Regenstein, 1986; Wit, 1989; Mann and Malik, 1996; Vojdani, 1996; Wong et al., 1996), a protein usually has the least solubility at the isoelectric point (pI), i.e. protein–protein interaction increases because the electrostatic forces of the molecules are at a minimum and less water interacts with the protein molecules. This is a favorable condition for protein molecules to approach each other and aggregate, and possibly precipitate. At pH values above and below the pI, where a protein has a net negative or positive charge, more water interacts with the protein charges. Net charges and charge repulsion contribute to greater protein solubility and the protein may stay in the solution. For a great number of proteins, their pI values are in the range of 3.5 and 6.5. At extremely acidic or basic pH values, the protein may unfold, exposing more hydrophobic groups.

Oxidation of certain amino acid residue results from reaction of ozone with proteins. At the end of this reaction, several changes can occur in protein molecules such as in their visual folding and binding ability and they are denatured. (Cataldo, 2006) The

action of ozone converts the thiol groups into disulfides. The S-S crosslinks formed denature the protein and change its solubility (Cataldo, 2003).

2.4.2 Foaming Properties

Food foams are dispersions of gas bubbles in a continuous liquid or semisolid phase composed of water, proteins, saccharides, lipids, and all other food components. These gas bubbles are responsible for the desirable texture of many food products, including milk shakes, whipped cream, ice cream, merinques, beer froth, cakes, snacks, and bread. Thus, size and distribution of bubbles and the stability of the foam may be important criteria of food quality. In different products, the bubbles are generated due to fermentation, e.g. beer, wine and bread dough, or air is incorporated into the food matrix by aeration like in modern bread making during the mixing of the dough.

The gas bubbles in food foams are separated by membranes of the continuous phase, composed of two films of proteins adsorbed on the interface between a pair of gas bubbles, with a thin layer of liquid in between. Stabilization of the system is due to lowering of the gas-liquid interfacial tension and formation of rupture-resistant, viscoelastic protein films surrounding the bubbles, as well as by the viscosity of the liquid phase. The foams, if not fixed by the heat-setting of the protein network, may be destabilized by drainage of the liquid from the inter sheet space due to gravity, pressure or evaporation by coalescence of the bubbles resulting from the rupture of the protein films, and by diffusion of the gas from the smaller to the larger bubbles. There is a pressure gradient (ΔP) over the gas liquid interface:

$$\Delta P = 2\gamma \cdot r^{-1} \qquad (2.5.)$$

where:

γ = interfacial tension

r = radius of the bubble

that is higher in smaller gas cells compared to that in larger ones. This leads to diffusion of the gas from the smaller to the larger bubbles and finally to disappearance of the smaller ones.

The efficiency of proteins as foaming agents depends on the factors affecting the rate of migration of the molecules to the interface and on the ability of the protein to form strong viscoelastic films around the gas bubbles. The molecules in the adsorbed layer are held together by ionic and hydrogen bonds as well as by hydrophobic interactions. The foaming capacity or foaming power, i.e., the ability to promote foaming in a system, measured by the increase in volume, is affected mainly by the surface hydrophobicitiy of the protein. The stability and strength of the foam, measured by the rate of drainage and the resistance to compression, respectively, depend on the flexibility and the mechanical strength of the protein film. Other components of the system, predominantly salts, sugars and lipids affect the foam formation and stability by either changing the physicochemical state of the proteins or the viscosity of the continuous phase (Campbell et al., 1999).

2.4.3. Emulsifying Properties

Proteins are often used in food systems to stabilize the coexistence of two or more phases. In most food emulsions small oil/fat droplets are distributed throughout the water phase. When no stabilizer is added, the oil droplets will flow together and form an oil layer (Walstra 1987). The role of the proteins that act as surfactants is to prevent the separation process and to retain fine oil droplets or air bubbles as dispersed (Dickinson, 1992; Friberg, 1997). Different food proteins are used in food systems for this functionality.

Proteins such as milk, egg and soya proteins, triglycerides, and polar lipids such as lecithin are commonly used stabilizers in food emulsions. Repulsive forces between the droplets with adsorbed proteins may be electrostatic, steric, and hydration forces. These interaction forces are influenced by the extent of adsorption of proteins and emulsifier, pH, ionic strength, etc.(Halling, 1981).

The most important aspect for the stability of emulsions is the prevention of coalescence (Dickinson, 1992).Whey protein; egg protein and soy protein are some of the commonly employed proteins in food emulsions. Proteins are invariably involved in stabilizing food emulsion. Because they contain both hydrophobic and

hydrophilic functional groups, proteins are inherently surface active, i.e., they tend to adsorb at air/water and oil/water interfaces. An interfacial adsorbed protein layer stabilizes the emulsion via two mechanisms. First, the adsorbed protein layer provides steric repulsion between droplets, resulting in an energy barrier to flocculation and subsequent coalescence. Protein molecules can lower their free energy by adsorbing at the O/W interface and exposing their hydrophobic functional groups to the oil phase and their hydrophilic groups to the aqueous phase. This decrease in the free energy is the driving force for their adsorption. Protein molecules diffuse from bulk solution to the interface. Consequently, their rate of adsorption is equal to their rate of diffusion. Second, adsorption of proteins provides interfacial viscosity and interfacial elasticity high enough to retard film drainage and rupture. Experimental observation of interfacial viscosity of adsorbed proteins (Graham and Philips, 1980) showed that globular proteins such as bovine serum albumin and lysozyme provided much better mechanical properties than flexible random coil proteins, such as β-casein. Therefore, flexible random coil proteins are more surface active, but globular proteins tend to provide better stability to emulsion. The surface activity and stabilizing ability of different proteins depend on their tertiary structure. Proteins, being macromolecules, tend to adsorb much more slowly than small molecule surfactant. Consequently, the kinetics of adsorption of proteins are important. In addition, competitive adsorption of mixture of proteins and surfactants is extremely important in complex interaction in such systems (Narsimhan, 1991).

The viscosity of the bulk phase also plays a role in stabilizing emulsions. The rate of creaming or sedimentation is directly related to this viscosity. As a result of creaming or sedimentation, the part of the product containing droplets becomes smaller, thereby decreasing the mean distance between the droplets, and enhancing coalescence and disproportionation. Coalescence and disproportionation phenomena depend on surface rheological properties as well as bulk rheological behaviour. In the presence of proteins the bulk rheological properties are relatively less important than in case of low molecular weight surfactants (Wilde, 2000).

Whey protein isolate (WPI) is widely used as a pertinacious emulsifier in food emulsions. WPI contains both hydrophobic and hydrophilic regions and can be rapidly adsorbed on the oil–water interface in the form of a protective film,

consequently providing structural support for oil droplets through a combination of electrostatic and steric interactions (Gwartney et al., 2004).

In this study, we investigated that effect of ozone on emulsifying properties of whey protein isolate stabilized emulsions. Whey proteins are used as food ingredients because of their unique functional characteristics like emulsifying, gelling, thickening, foaming and water binding capacity (Kinsella & Whitehead, 1989). Ozonation changes some specific monomeric unit and causes protein denaturation (Cataldo, 2003). Denaturation is an important factor affecting the emulsifying properties of proteins due to conformational changes of proteins (Kato et. al, 1982).

2.4.4. Rheological Properties

2.4.4.1 Definition of rheology

Rheology is defined as the science of deformation and flow of matter. Rheology is applicable to all types of materials, from gases to solids. Rheology is used in food science to define the consistency of different products. Rheologically the consistency is described by two components, the viscosity (“ thickness”, lack of slipperiness) and the elasticity (“stickiness”, structure). In practice, therefore, rheology stands for viscosity measurements, characterization of flow behaviour and determination of material structure. Basic knowledge of these subjects is essential in process design and product quality evaluation (Fox, 1982).

Food emulsions are particularly interesting because their physical and chemical properties affect quality aspects like ‘‘texture’’ and shelf life in a complex way. When designing a new product, a relationship between macroscopic parameters (related to perceived properties) and material microstructure is necessary to understand the effects of relevant ingredients (such as fats and emulsifiers) aiming to obtain a formulation with controlled characteristics avoiding a long ‘‘trial and error’’ approach.

Emulsion stability and ‘‘texture’’ (i.e. rheological properties) are probably the most important characteristics to be considered when a new product is studied. Stability affects product processing (e.g. shear induced separation during pumping), unit

operation design (stirring systems, pumps, etc.) and shelf life (potential phase separation before commercial limits). Rheological properties are necessary to design unit operations properly (e.g. pumping systems) and determine the organoleptic characteristics perceived by the consumers (Gabriele et al., 2009).

2.4.4.2 Rheological instruments

Instruments which measure rheological properties are called rheometers. Common instruments capable of measuring fundamental rheological properties of fluids and semi-solid foods may be placed into two general categories: rotational type and tube type. Viscometer is more limiting term referring the devices that only measure viscosity. Rotational instruments may be operated in the steady shear or oscillatory mode. Rotational systems are generally used to investigate time-dependent behavior because tube systems only allow one pass of the material through the apparatus. Generally, dynamic (oscillatory) measurements have been studied for the characterization of emulsions (Steffe, 1996).

In oscillatory instruments, samples are subjected to harmonically varies stress and strain. This testing procedure is the most common dynamic method for studying the viscoelastic behavior of food. Results are very sensitive to chemical composition and physical structure so they are useful in a variety of applications including gel strength evaluation, monitoring starch gelatinization, studying the glass transition phenomenon, observing protein coagulation or denaturation, evaluating curd formation in dairy products, cheese melting, texture development in bakery and meat products, shelf life testing, and correlation of rheological properties to human sensory perception. Food scientists have found oscillatory testing instruments to be particularly valuable tools for product development work (Whorlow, 1992).

Oscillatory testing may be conducted in tension, bulk compression, or shear. Typical commercial instruments operate in the shear deformation mode and this is the predominant testing method used for food. Shear strain may be generated using parallel plate, cone and plate or concentric cylinder fixtures. Dynamic testing instruments may be divided into two general categories: controlled rate instruments where the deformation (strain) is fixed, stress measured, and controlled stress

instruments where the stress amplitude is fixed and deformation is measured. Both produce similar results (Rao and Skinner, 1986).

Commercially available oscillatory instruments will operate in numerous modes. A strain or stress sweep, conducted by varying the amplitude of the input signal at a constant frequency, is used to determine the limits of linear viscoelastic behavior by identifying a critical value of the sweep parameter.

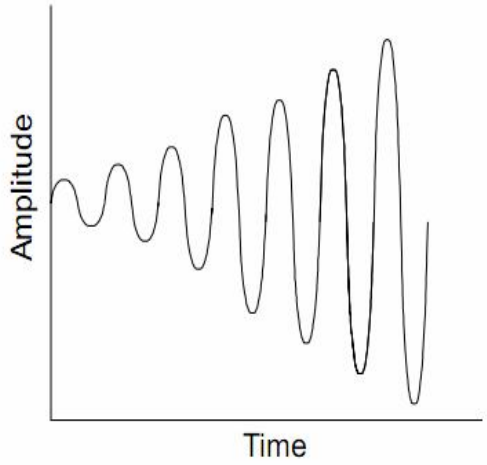

Figure 2.6. Strain or stress sweep mode in oscillatory testing (Steffe, 1996).

The frequency sweep is probably the most common mode of oscillatory testing because it shows how the viscous and elastic behavior of the material changes with the rate of application of strain or stress. In this test the frequency is increased while the amplitude of the input signal (stress or strain) is held constant. Frequency sweeps are very useful in comparing, sometimes called “finger printing”, different food products or in comparing the effects of various ingredients and processing treatments on viscoelasticity. Materials usually exhibit more solid like characteristics at higher frequencies. In this study, frequency sweep mode is used to understand viscous and elastic behavior of our samples (Steffe, 1996).

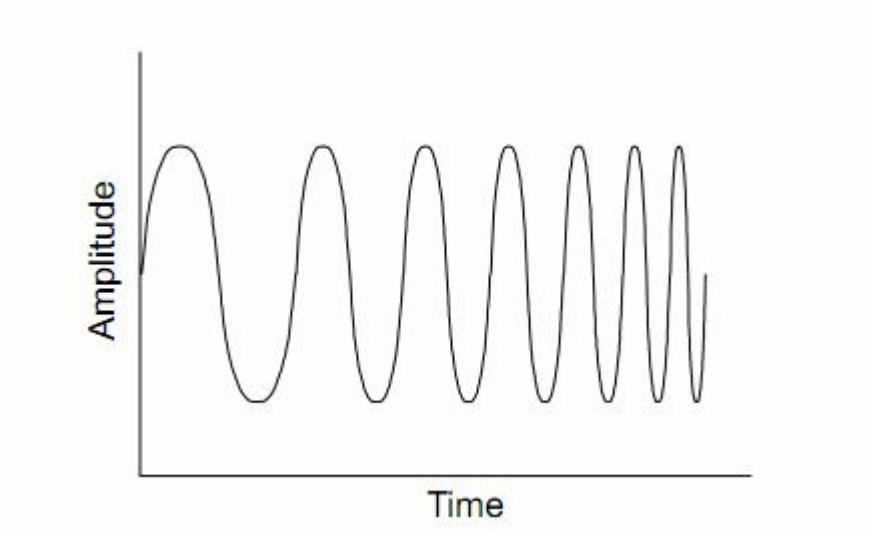

Figure 2.7. Frequency sweep mode in oscillatory testing (Steffe,1996).

2.5. Optical Activity

The optical rotation of a substance may be regarded as the sum of contributions arising from the mutual interactions of all the groups present in the molecule (Kauzmann, 1957a, b). For the folded form of polypeptide consisting of amino-acid residues, the optical rotation will depend upon the composition and configuration (i.e. whether L- or D-) of the individual residues, the disposition of the residues relative to one another within the same molecule, i.e. the chain configuration and environmental factors, e.g. solvation and ionization effects and intermolecular interactions. Native proteins are made up of L- amino acids and are always laevorotatory. The specific rotation $[\alpha]_D$ of globular proteins generally lies between -30^0 and -60^0 (Doty and Geiduscheck, 1953), while the values for fibrous proteins are usually less negative (Cohen and SzentGyorgyi, 1957). Upon 'denaturation', which is believed to involve a change in the spatial configuration of the molecule without an accompanying fall in molecular weight (Putnam 1953), the laevorotation of proteins invariably increases (Doty and Geiduscheck, 1953; Jirgensons 1952a, b) to a value for $[\alpha]_D$ usually in the region of -80 to -120 in aqueous media, which is believed to be characteristic of the random configuration in proteins (Cohen 1955b; Cohen and Szent-Gyorgyi, 1957; Yang and Doty, 1957).

Proteins are characterized by their optical activity. The optical activity arises for two different reasons: the fact that the polyamide chain is composed of chiral amino acids and for the helicity of the chains and other superstructural features of the proteins

(secondary and tertiary structure). All amino acids, except glycine, have chiral carbon atoms and their optical activity is influenced by protein's structural changes (Kongraksawech et al., 2007). Specific optical activity of native proteins that are composed of L-amino acids depends on specifically active amino acids in their structure, secondary (helical structure) and tertiary structure (White et al., 1978). Some of aromatic amino acids such as trytophan, tyrosine, phenylalanine and also histidine, methionine are very reactive with ozone. Ozonation causes changes in some monomeric units, the first contribution to the variation of specific optical rotation of proteins could be derived from these structural changes (Cataldo,2006). Additionally, a further contribution to the variation of specific optical rotation of proteins can be derived from the protein denaturation i.e. the variation in the secondary and tertiary structure induced by the chemical changes introduced in certain monomeric units (Cataldo 2007).

CHAPTER 3

3. MATERIAL AND METHODS

3.1. Materials

Powdered whey protein isolates (98 %) (WPI, BIOPRO, Lot No. JE 030-3-420) was obtained from Davisco Foods International (Le Sueur, MN, USA). Hen egg albumen powder (sample no.1392) (egg white) were kindly supplied from NIVE (Nederlandse Industrie van Eiproducten, Holland). Buffer salts, potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate and sodium dodecyl sulfate (SDS) were purchased from Analar analytical reagent; BDH Chemical Ltd. Sodium chloride (JT Baker), sodium hydroxide (Riedel-de-Haen), were used. Copper sulfate, sodium potassium tartrate, potassium iodide, hydrochloric acid, trifloroacetic acid, acetonitrile were obtained from Merck Company. All chemicals used were analytical grade.

3.2. Ozone Generator and Equipments

Ozone gas was generated by coronal-discharge method using OMS Model Ozone Generator. The generator consists of diffuser mixing part, degasser (removing of undissolved ozone in water), redox control (ORP) system, and integrated oxygen unit. Oxygen required for ozone generation was provided from the air.

Ozone generator was designed to mix different samples with different concentrations of gas or dissolved ozone. For the ozone generator which has a maximum ozone production capacity of 60 g/h, oxygen was provided from air. Oxygen in the air was decomposed by a concentrator with a flow rate of 350L/h and converted to the ozone gas by the aid of three generators. Samples were treated with ozone gas in a 1 L glass gas washing bottle.

Gas is directed from generator to the bottle by a connection and the sample is treated with ozone at a rate of 60 g/h consistently.

Since the solubility of ozone gas changes with temperature, the temperature of glass equipment was controlled by a thermostatic water bath while dissolving ozone gas in the solvent used. The solvent (buffer/double distilled water) was poured into the bottle and the bottle was placed into the thermostatic bath which was set to a desired temperature. The solvent was left in the thermostatic bath until the equilibrium was established. Then, the ozone gas was directed into the bottle through the glass tube whose end was equipped with a gas disperser which creates bubbles to increase the solubility. Dissolved ozone concentration was determined by oxidation/ reduction potential (ORP). PTFE or silicon tube was used for ozone generator and equipments. Undissolved ozone gas was driven to the atmosphere through a discharge tube, after being heated to reduce the concentration. Ozone concentration measured as ORP was converted to ppm by using a calibration table.

Figure 3.1. Photograph of Ozone Generator

3.3. Ozonation of Protein Samples

Protein samples were treated with ozone gas by two different procedures. The ozone gas was either dissolved in double distilled water/buffer (pH 7.0) which was used to prepare the aqueous protein solutions which, in turn, was used to analyze functional properties of proteins or dry protein powder was exposed to ozone gas in a glass gas

washing bottle at a rate of 60g/h and then the protein solutions to be used in the experiments were prepared by using ozonated proteins samples. In the former, double distilled water was used as solvent for some protein samples to observe the effect of maximum ozone concentration that can be obtained under laboratory conditions. Because it was seen in preliminary experiments that the amount of dissolved ozone reduced when buffer salts were present in the aqueous medium. Ozonation was carried out for different period of time (1, 5, 10, 15 and 30 min for solvent and gas ozonation). ORP value of the solution was measured to determine dissolved ozone concentration in the distilled water/ buffer (pH 7.0) by ORP measurement device. Protein samples were dissolved in the solution in different concentrations. The samples which were ozonated either in an aqueous medium or in the atmosphere were analyzed about 1-2 hours after ozonation, a time judged sufficient for a complete reaction of ozone gas with protein samples.

Figure 3.2. Photograph of glass gas washing bottle

3.4. Preparation of Stock Protein Solutions

Phosphate buffer was prepared by dissolving potassium dihydrogen orthophosphate (3.76 g) and disodium hydrogen orthophosphate (3.44 g) in 2 liters distilled water and pH was adjusted to 7.0 by using 0.1M NaOH and 0.1M HCI. 0.01 % (w/w)

sodium azide was added to the buffer as an anti-microbial agent for emulsion experiments. Whey protein isolate (WPI) was dissolved in phosphate buffer (ionic strength 0.05M and pH 7.0) or double distilled water at room temperature while stirring with magnetic stirrer for 1 hour.

Egg white powder was prepared by dissolving powder in phosphate buffer containing 0.17M NaCI solution.

3.5. Determination of Protein Content

Protein content of samples to be used in solubility and optical activity measurements were determined by Biuret method. For the preparation of Biuret reagent, 1.5 g copper sulfate ($CuSO_4.5H_2O$) and 6.0 g sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) were dissolved in sufficient distilled water separately and then they mixed and 300 ml 10% sodium hydroxide (NaOH) was added to this mixture with constant swirling. A 1.0 g potassium iodide (KI) was added into the mixture and the solution was diluted to 1.0 liter with distilled water.

Standard protein solution (0.0-2 mg/ml) were prepared by using bovine serum albumin as a standard protein with a final volume of 1.0 ml. Each solution was added by 4.0 ml of Biuret reagent and mixed thoroughly. They were kept at room temperature for 30 min and the absorbance was read in spectrophotometer (Pharmacia Biotech, Novaspec II, UK) at 540 nm against the blank (water). Standard curve was prepared from the data by plotting absorbance versus concentration (Figure A1).

For solubility and optical activity measurements, 1.0 ml of sample was taken and protein content was determined by following the above procedure.

3.6. Differential Scanning Calorimetry

Ozonated or untreated whey protein isolate (10.0% w/v) and egg white (10% w/v) solutions were prepared. A 50 μL sample was placed into aluminum DSC pan and the pan was sealed. The samples were placed in the DSC (Perkin-Elmer DSC 6

equipped with a Pyris software, Perkin-Elmer Inc., Wellesley USA) and scanned from 15 to 110°C at a heating rate of 5°C/min using an empty pan as a reference. The enthalpy of denaturation (ΔH) and the denaturation temperature (Td) were determined from each curve (Relkin et al., 1999; Relkin and Sourdet, 2005).

3.7 Emulsifying Properties

3.7.1. Preparation of Emulsions

The emulsifying properties of whey protein isolate were determined by the modified Pearce and Kinsella (1978) method. To prepare emulsions, 25.0% (v/v) soybean oil and 75.0% (v/v) of protein solution were mixed roughly for 15 s and then homogenized using Soniprep 150 Ultrasonic Disintegrator at 23 kHz the amplitude of 15 (microns) for 15 s or 1 min to give final protein concentration of 0.1, 0.05% (w/w) in WPI stabilized emulsions. Emulsions were also prepared from ozonated solutions to observe the effect of ozone gas on emulsifying properties of proteins. Emulsions were prepared in duplicate.

3.7.2. Emulsifying Activity and Emulsion Stability

A 0.1 ml aliquot of the emulsion was taken from the bottom of the container at different time intervals and diluted with 5ml of 0.1% (w/v) SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm by spectrophotometer (Pharmacia Biotech, Novaspec II, UK). The emulsifying activity (EAI) was determined from the absorbance measured immediately after emulsion formation and the emulsion stability index (ESI), was estimated by following the time-dependent change in the absorbance readings. The result was expressed as surface area per unit weight of protein (m^2/mg) used in the emulsions. Emulsion experiments were replicated two times.

$$EAI(m2/mg) = 2T\left[\frac{A0xdilutionfactor}{CxΦx10000}\right] \qquad (3.1.)$$

where T=2.303, c=weight of protein per unit volume (mg/ml) of protein aqueous phase before emulsion formation, Φ = oil volume fraction of the emulsion (0.25 in this case) and the dilution factor was 4000 (Bernard et al., 2007)

3.8. Foam Formation and Stability

Foaming properties of whey protein isolate, egg white were investigated. Stock protein solutions were diluted to 0.01% (w/w). The samples were foamed in a glass sintered column of 250mm in length and 35mm in internal diameter. Air was fed into the column by an air pump (EYELA, Tokyo, Japan) at a constant flow rate of 0.5 nl/min and 5ml of sample was sparged for 15 sec. Immediately after turning off the gas, the foam volume (FV) was calculated. The time for the collapse of the foam to half of its initial value was measured and expressed as foam stability. The foaming experiments were also done for the ozonated solutions to observe the effects of ozone gas on foaming properties of the proteins. Foaming experiments were replicated three times.

3.9. Solubility

The solubility of whey protein isolate and egg white were determined by the modified Kakalis and Regenstein (1986) method. Protein samples were dissolved in phosphate buffer (pH 7.0). Phosphate buffer containing 0.17M NaCI (pH 7.0) was used for egg white proteins. Ozone gas was dissolved in the buffer by means of a disperser in a gas washing bottle.

The protein samples were also treated directly with ozone gas. Dry whey protein isolate or egg white powder is exposed to ozone gas for 1, 5, 10, 15, 30 min in a closed container, and then the protein solutions (0.01, 0.05, 0.1, 0.2, 0.5, 0.1% w/w) to be used in the experiments were prepared by using ozonated proteins samples. After samples were kept at the room temperature for about 1 hour, they were filtered with the aid of a filter paper (Whatman No:2). A 1.0 ml of sample was taken and the protein content was determined by Biuret method. The experiments were replicated twice. The soluble protein percentage was calculated through the following equation:

$$P.S. = \left[\frac{A(g/L)50}{W(g)S/100}\right]100 \qquad (3.2)$$

where P.S. is the soluble protein content in the sample (g/100 g), A is the supernatant protein concentration (g/L), W is the sample mass (g), S is the sample protein concentration (g/100 g).

3.10. Optical Activity

Optical activity of the whey protein isolate determined by using polarimeter (PolAAr 3000, Optical Activity Ltd., England). The samples were prepared by dissolving protein (0.1, 0.2 (%w/w)) in double distilled water (pH:7.0) through which ozone gas is passed at 8^{o}C for 15 min. The solutions were left under magnetic stirring for about 1 hour. Then, they were filtered by using filter paper (Whatman No:2). Protein concentration of the filtered solution was determined by Biuret method. Optical activity of the sample was measured by polarimeter. Optical activity was calculated as specific optical activity from the following equation;

$$[\alpha\ ]_D = \alpha V L^{-1} m^{-1} \qquad (3.3.)$$

where [α]D is , the specific optical activity (°), α is optical rotation (°), measured by polarimeter , L is a cell length (in dm) and V is a volume of solution (in ml) and m is a dissolved solute amount (in g).

Alternatively, protein samples were treated directly with ozone gas using a different approach by admitting ozone in an evacuated separatory funnel for 15 min. The separatory funnel was closed by a stopper. The protein solution was poured into the funnel slowly and stopper was closed immediately. Then, the mixture of protein solution and ozone was mixed at constant rate for about 1 hour to provide complete reaction between ozone and protein solution. After mixing process, the solution was filtered by using filter paper (Whatman No:2). Protein concentration of the solution was determined by Biuret method. Optical activity of the sample was measured.

3.11. HPLC Analysis

Ozonated whey protein isolate and egg white samples were analyzed by reversed phase HPLC. HPLC system consisted of quadratic pump (model LC-10ADVP; Shimadzu, Japan) equipped with a column (Supelcosil LC-318)**;** with an accompanying guard column (2cm length and 4-mm id) of the same phase and an ultraviolet (UV) detector (Hewlett Packard Series 1100). Elution was monitored by UV absorbance at 220 nm. The mobile phase consisted of solvent A (0.1%(v/v) trifloroacetic acid) and solvent B (0.1% (v/v) trifloroacetic acid and 99.9% (v/v) acetonitrile) with a flow rate of 0.8 mL/min. The column temperature was set at 25^{o}C with a column heater (Eppendorf CH-30 column heater). 0.1% (w/w) samples were prepared by procedure mentioned in part 3.3. and filtered with 0.45µm prior to injection. Injection volume was 25µL. A software integration system (Chem Station Rev. A.09.03 [1417], Agilent Technologies) was used for data collection and integration. All results are given in percentage area.

3.12 Rheological Measurement

Dynamic rheological measurements were performed at 25±0.01 ^{0}C, with a RheoStress RS-1 controlled stress rheometer (HAAKE, Karlsruhe, Germany), using a parallel plate geometry (35 mm diameter , 1mm gap). For each measurement, 2.0 ml of emulsion were carefully deposited over the pleateau of the rheometer. After the plateau has been contact with the plate, the exposed surface of sample was covered with a thin layer of silicone oil to prevent evaporation during the measurement. Frequency sweep tests(1 Pa stress value at 25^{0}C) were done inside linear viscoelastic region in a frequency range of 0,1 to 100 rad/ sec and the elastic (G') and viscous (G'') moduli were recorded against frequency. Data analysis software (Reowin Pro Data Manager Version 2.64) was used to obtain the experimental data (elastic, viscous modulus, phase angle etc.).

3.13. Statistical Analysis

The SPSS (Statistical package for social sciences) Statics 16.0, version 2.0 (2006), (SPSS Inc., Chicago) was used for all statistical analysis. Experimental results were

subjected to one-way analysis of variance (ANOVA). In order to determine the data that are significantly different from each other, Duncan multiple range test method was applied. Trends were considered significant when means of compared parameters differed at $P < 0.05$ significance level.

These parameters are;

- Effects of ozone on foam stability and foam capacity of whey protein isolate.

- Effects of ozone treatment on solubility of whey protein isolate and egg white proteins.
- Effects of ozone treatment on emulsifying properties of whey protein isolate.
- Effects of ozone treatment on denaturation temperature and transition enthalpy of whey protein isolate and egg white proteins.
- Effects of ozone treatment on transition enthalpy of whey protein isolate.
- Effects of ozone treatment on denaturation temperature of egg white proteins.
- Effects of ozone treatment on transition enthalpy of egg white proteins.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Foaming Properties

The ability of a protein to form a foam and emulsion is related to its ability to adsorb at the oil-water interface and to stabilize the film (Pearce and Kinsella, 1978). In a part of this study, the effect of ozone treatment on the foaming properties of whey protein isolate (WPI) and egg white proteins, which are known as good foaming agents, were investigated. The foaming properties of these protein mixtures were expressed in terms of foam capacity (foam volume) and foam stability.

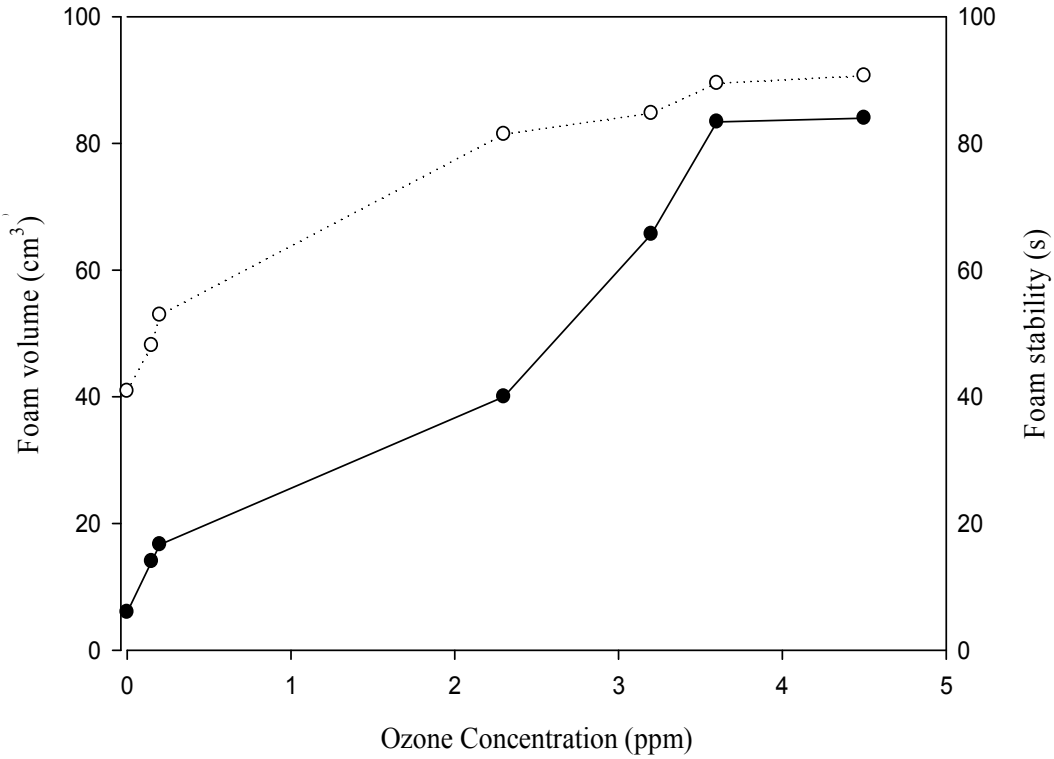

Figure 4.1.Effects of ozone gas dissolved in distilled water (pH 7.0) on foaming properties of whey protein isolate. Protein solutions (0,01%w/w) were prepared in the ozonated distilled water. (●; foam stability, ○; foam volume)

Figure 4.1. shows the effects of ozone gas on foaming properties of WPI. The results indicated the change in the foam volume and foam stability of WPI after protein was treated with various concentrations of ozone gas in an aqueous solution. Different ozone concentrations in double distilled water (pH 7.0) were obtained by dissolving ozone gas by means of a disperser in a gas washing bottle. Thus, ozonated water was used to prepare the aqueous protein solutions which were used to perform foaming experiments. The reason why double distilled water was used as solvent was to observe the effect of maximum ozone concentration that can be obtained under laboratory conditions. Because it was seen in preliminary experiments that the amount of dissolved ozone reduced when buffer salts were present in the aqueous medium. The figure shows a dramatic change in the foam volume and foam stability when the WPI was treated with ozone. Also, the effect of different ozone concentrations on foam volume and foam stability of protein were significantly different ($p<0.05$). Both, foaming ability and foam stability increased when ozone concentration of the aqueous solution was increased. However, foam stability of WPI seemed to be affected more by ozone gas than foaming ability of protein. It increased gradually with ozone concentration. Although the foam volume of WPI increased 2.25 times as compared with that of untreated protein, foam stability appeared to increase 15 times when protein was treated with 4.5 ppm ozone in an aqueous medium. Cataldo (2003) stated that ozone causes denaturation of the proteins and introduces changes in their secondary and tertiary structure. It has been noticed that the foaming power of proteins increased due to slight increase in surface hydrophobicity (Kato et al., 1985). Therefore, the emulsifying and foaming properties may be improved as the surface hydrophobicity of proteins increases with denaturation, due to a reduction in the surface tension. However foam stability has been stated to be related to the extend of denaturation rather than surface hydrophobicity (Kato et al., 1982). In general, foam stability of proteins increases as the denaturation temperature increases. The ability to dissociate and form a film due to denaturation may be essential for foam stability of proteins.

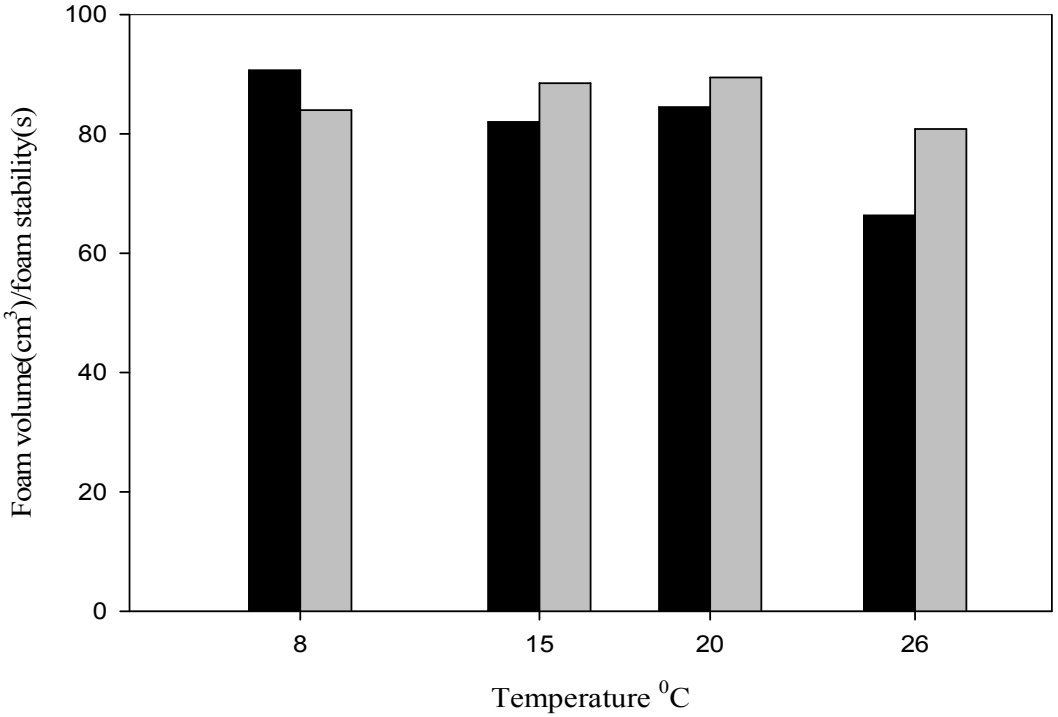

Figure 4.2. The effects of ozone treatment at different temperatures on foaming properties of whey protein isolate. The concentration of ozone and protein solution were 4.5 ppm and 0,01% w/w respectively.(dark bars; foam stability(s) ; light bars; foam volume(cm^3))

Ozone solubility in water is 13 times that of oxygen and it is progressively more soluble in cold water (Rice, 1986). As the temperature increases, the solubility of ozone and the half-life for ozone degradation in water decrease (Rice et al., 1981). Figure 4.2. shows the effect of ozone treatment at different temperatures on the foam volume and foam stability of WPI solutions. The foam volume and foam stability of were measured by foaming of the protein solutions which were prepared at different temperatures (8°C, 15°C, 20°C, 26°C) using double distilled water (pH 7.0) keeping the initial ozone concentration (4.5 ppm) as constant. It was determined that the effect of different initial temperatures of solution on foam volume was not significantly different ($p<0.05$). However, the effect of temperature on foam stability was found to be significantly different ($p<0.05$) in the way that a high initial solution temperature exhibited a low foam stability. This may show a low probability of protein molecules to get in contact with ozone in solution at high initial solution temperatures due to a short half-life and a fast degradation rate of ozone. This may

also explain that the ozone influences the foaming ability of protein in a short treatment time compared with foam stability.

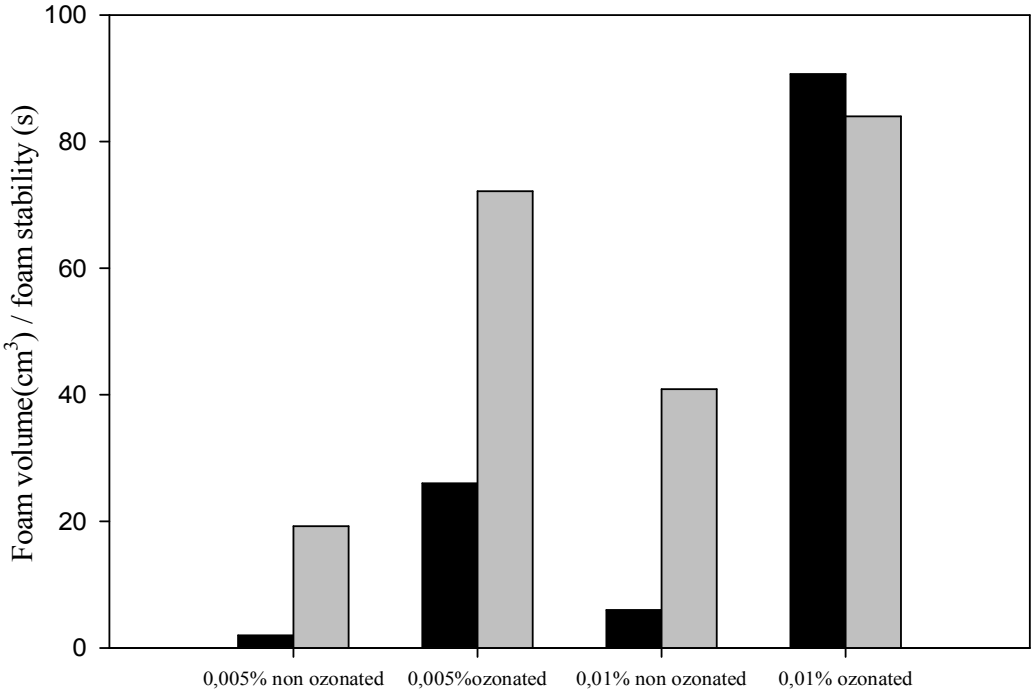

Figure 4.3. The effects of concentration of ozonated protein on foaming properties of whey protein isolate. The concentration of protein solutions were 0,005, 0,01% w/w.(dark bars; foam stability(s) ; light bars; foam volume(cm^3))

Figure 4.3. shows the change in the foam volume and foam stability of WPI with the concentration of ozonated protein. By keeping the initial ozone concentration constant (4.5 pm), two WPI solutions having different protein concentrations (0.005 and 0.01 % w/w) were prepared and were foamed. The results arising from the differences in protein concentration were significantly ($p<0.05$) different. At the low protein concentration, the foam volume obtained with ozonated sample was 3.7 times that of untreated sample, while the foam stability increased 13.0 times. However, when the protein concentration was doubled, foam volume and foam stability increased 2.1 times and 15.1 times, respectively. In the latter, more protein molecule were influenced from ozone and the foam stability shifted. However, the ratio of increase in the foam volume was less than the ratio of increase in foam stability. This can be explained by the compensation during foam formation by increased number of protein molecules due to concentration in solution. The comparison of the foamability and foam stability of ozonated samples shows that foaming capacity

increases by 1.2, while the foam stability increases by 3.48. This result correlates well with the previously observed one that ozonation influences the foam stability of protein solution more than the foamability of protein solutions.

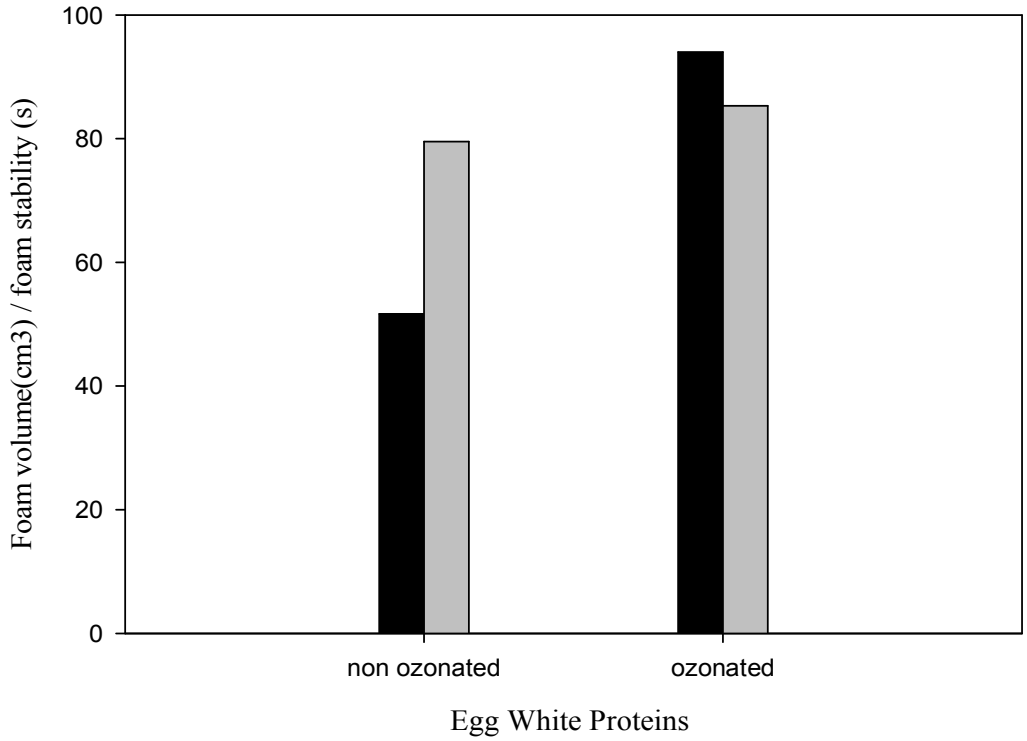

Figure 4.4. The effects of ozone on foam volume and foam stability of egg white proteins (0,01% w/w).The concentration of ozone in the protein solution was 4,5 ppm.(dark bars; foam stability(s) ; light bars; foam volume(cm^3))

Egg white proteins have been extensively used as ingredients in processed foods due to their unique functional properties such as gel and foam formation (Mine and Bergougnoux, 1998; Wong et al., 1996). In this study, effects of ozone on foaming properties of egg white proteins were also studied. Effects of ozone gas on foaming properties of egg white protein solutions were shown in Figure 4.4. An improvement in the foaming properties of egg white proteins was observed. However, it does not seem to be as much as the improvement in foaming properties of whey protein isolate by ozone treatment. While foam volume and foam stability of WPI solutions increased by 2.21 and 15, respectively (Figure 4.4.), the ratios of increase were 1.1 and 1.8 for foam volume and foam stability of egg white proteins, respectively, under the same conditions. Similar to the result observed with WPI, ozone treatment influenced the foam stability of egg white proteins more than foaming ability.

However, the extent of change was different for both, WPI and egg white proteins. This difference may arise from the structure and amino acid composition of component proteins in protein mixtures. Because functional properties of proteins are influenced by factors such as amino acid composition, conformation, sequence of amino acids, hydrophobic groups on surface (Damodaran, 1994).

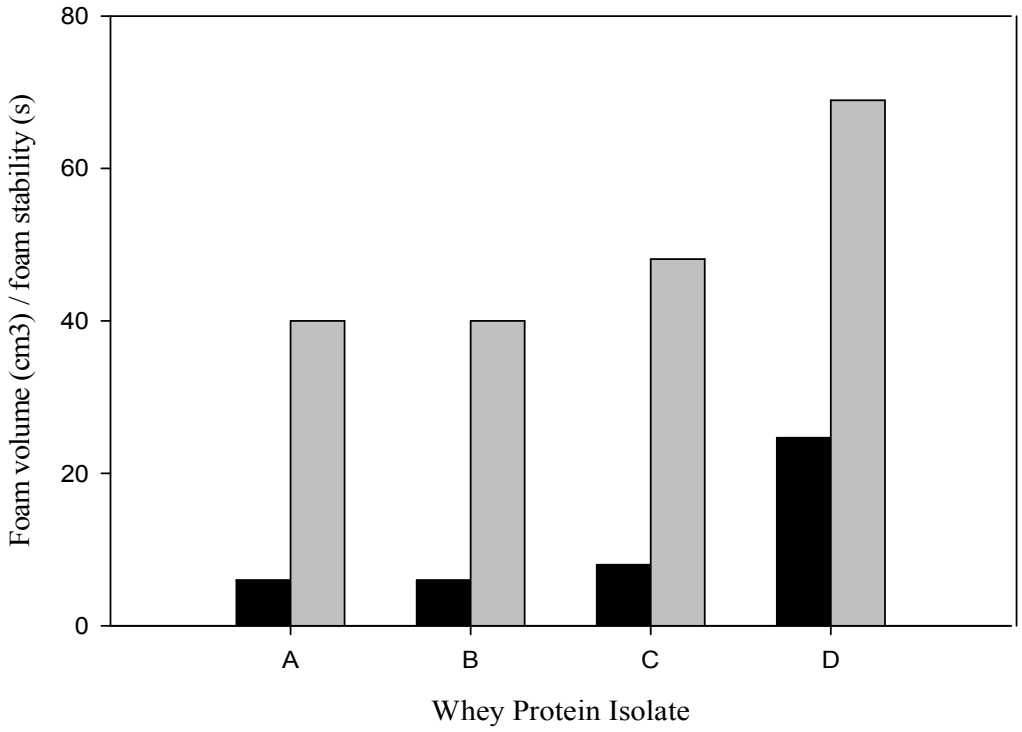

Figure 4.5.Effects of ozone on foam volume and foam stability of whey protein isolate. A: control sample (non ozonated, B:0,01 g of protein treated with ozone (4,5ppm capacity) and then 0,01g of protein was used for preparation of a solution (0,01% w/w), C: 0,2 g of protein treated with ozone and then a solution (0,01%w/w) were prepared, D:0,01 g of protein treated with ozone and then 0,01 g of protein was used for preparation of a solution (0,01 w/w). Samples of C and D were treated with ozone (60 kg/capacity ozone) for 15 minutes. .(dark bars; foam stability(s) ; light bars; foam volume(cm^3))

The foaming experiments were repeated after WPI was treated directly with ozone gas. Dry WPI powder is exposed to ozone gas in a closed container, then the protein solutions to be foamed were prepared by using ozonated proteins samples. The experiments were carried out to determine the difference between the efficiency of ozonation in an aqueous medium and in the atmosphere. Figure 4.5. represents the foam volume and foam stability of WPI solutions (0.01 %w/w) prepared after protein powder was kept in the ozone atmosphere. The effect of different ozonation methods

on foaming properties of WPI were significantly different ($p<0,05$). This figure shows the foaming properties of four solutions (0.01%w/w, pH 7.0). A was a control sample (not treated with ozone). B (0,01 g protein) was treated with ozone gas in the atmosphere (4.5 ppm) for 2 hours and then protein solution was prepared. A 4.5 ppm is the maximum ozone concentration that are provided under our lab conditions in the aqueous medium and this sample was prepared to observe the difference between ozonation in an aqueous medium (Figure 4.1.) and gas medium. Sample C (0.2 g protein) and D (0.01 g protein) were ozonated for 15 min at 60 g/sa ozone capacity in a closed container. These samples were prepared to see the influence of the protein quantity on the efficiency of ozonation process. As seen in the graph, foaming properties of sample B is resembling those of sample A. The comparison of two figures (Figure 4.1. and 4.5.) reveals that the same amount of ozone, used in the aqueous medium and gas medium does not exhibits the same improvement in the foaming properties, the former influences the protein's behaviors toward air/water interface more that the latter. Therefore, it can be concluded that ozone treatment in the aqueous environment is more effective than ozone atmosphere. Furthermore, the quantity of protein treated with ozone was important in terms of ozone effectiveness. As shown in Figure 4.5., effects of ozone on the samples of C and D samples were different ($p<0.05$) from each other. The ozone gas affected the foam volume and foam capacity of sample of D (0.01 g protein) more than those of sample C (0.2 g protein). On a molecular basis, the functional properties of protein seemed to be affected more as the number of ozone molecules increases. Although an increase was observed on foam volume and stability of both samples (C,D), the improvement was more pronounced with sample D.

The foaming experiments were performed with egg white protein, which is also a protein mixture similar to WPI. Although no visible change in the solubility of WPI has been noticed, the solubility of egg white was reduced considerably when the protein was dissolved in ozonated solvent or the protein was treated with ozone atmosphere. Since the protein solubility is the primary factor that influences functional properties such as emulsification and foam formation, gelation (Nakai and Chan, 1985; Wit, 1989), foaming experiments have not been considered to be done with egg white proteins.

4.2 Solubility of Proteins

Among the functional properties of proteins, solubility is vital in choosing the protein ingredient to be used in drinks, fluid food, foams and emulsions, under different processing conditions (Kinsella 1982; Fennema 1993; Pelegrine and Gasparetto 2005; Sousa et al. 2007). In this study, the effects of ozone gas on solubility properties of WPI and egg white proteins were examined. Proteins used for functionality are required to have high solubility, in order to provide good emulsion, gelation and whipping properties (Nakai and Chan, 1985; de Wit, 1989). In other words, a decrease in protein solubility affects protein's functionality in unfavorable manner (Vojdani, 1996). Solubility of proteins is related to surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interactions, and in foods, such solvent is the water. Therefore the protein solubility is classified as a hydrophilic property (Borderias and Monteiro, 1988).

Effects of ozone on solubility of the whey protein isolate and egg white proteins were investigated after the protein samples were treated with ozone in the solution or directly in the atmosphere as described in the previous chapter (Chapter 3). Figure 4.6. illustrates the change in the solubility of egg white proteins and whey protein isolate as a function of ozonation period for aqueous and gas ozonation methods. In the measurements, either dry powder protein was treated with gas ozone or the solvent (water for WPI, buffer containing 0.17 M NaCI for egg white proteins) was ozonated at different periods of time and then the protein solutions were prepared at a constant concentration (0.1% w/w) either with ozonated dry protein or by dissolving native protein in ozonated solvent. As seen in the graph, the solubility of proteins changed as the method of ozonation was changed. Statistical analysis also showed that there was a significant difference ($p<0.05$) between the solubilities of samples exposed to different ozonation medium. It was observed that solubility of the protein samples were reduced more by gas ozonation method than by ozonation of solvent. Also, the loss of solubility was more pronounced in egg white proteins. While the solubility of egg white proteins decreased by 25,3 % by gas ozonation that of WPI decreased as %18,85. In aqueous ozonation, the loss in solubility were 16,5 and 19,48 % for WPI and egg white proteins, respectively.

The amount of dissolved ozone in the solution is a function of temperature of solution (Rice, 1986). It has been detected in the preliminary experiments that the concentration of soluble ozone that could be maintained in the solvent under our laboratory conditions was 4,5 ppm at 8^0C. Therefore, the gas ozonation provides a much higher concentration (60 g/sa) of ozone to the medium depending on the duration of ozonation. Effectiveness of gas ozonation process could be explained by the number of ozone molecules to which protein was exposed. Consequently, ozone influenced more protein molecules with time during gas ozonation causing an extensive change in the protein structure. The substantial loss in the solubility of egg white may arise from the structural differences of two protein samples.

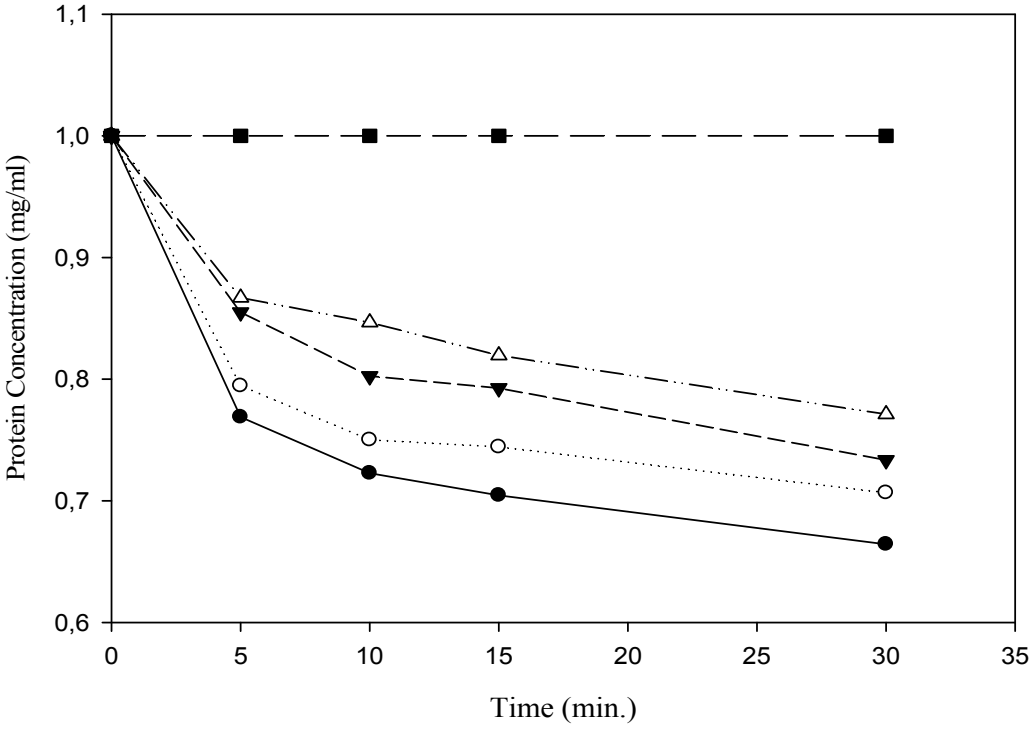

Figure 4.6.Effects of ozone on solubility of whey protein isolate and egg white protein at constant protein concentration (0,1% w/w).○ egg white ozonated with aqueous ozonation method (4,5 ppm); ● egg white ozonated with gas ozonation method (60 g/sa); ▼ WPI ozonated with gas ozonation method (60 g/sa); Δ whey protein isolates ozonated with aqueous ozonation method (4,5 ppm); ■ pure(non treated with ozone) protein solution.

Figure 4.7. illustrates the change in solubility of protein solutions involving different protein concentrations when ozonation was carried out at a fixed period. Dry powder protein or solvent was ozonated with ozone gas for 15 min and then solution having

various protein concentrations were examined for the loss in the solubility. The x-axis of the graph shows the concentration of actual solution while y-axis shows the detected concentration of solution after loss in solubility. ANOVA results showed that results of both ozonation methods were significantly ($p<0.05$) different. As seen in the figure, the gas ozonation method was more effective on solubility than aqueous ozonation method. This may arise from the substantial effect of ozone in gaseous state on structure of proteins since ozone has a longer half-life in the gaseous state than in aqueous solution (Rice, 1986). Consequently, the amount of ozone in the gaseous state may be higher than that in the aqueous state and solubility of protein molecules in the solution might be influenced more during gas ozonation for a fixed period of time.

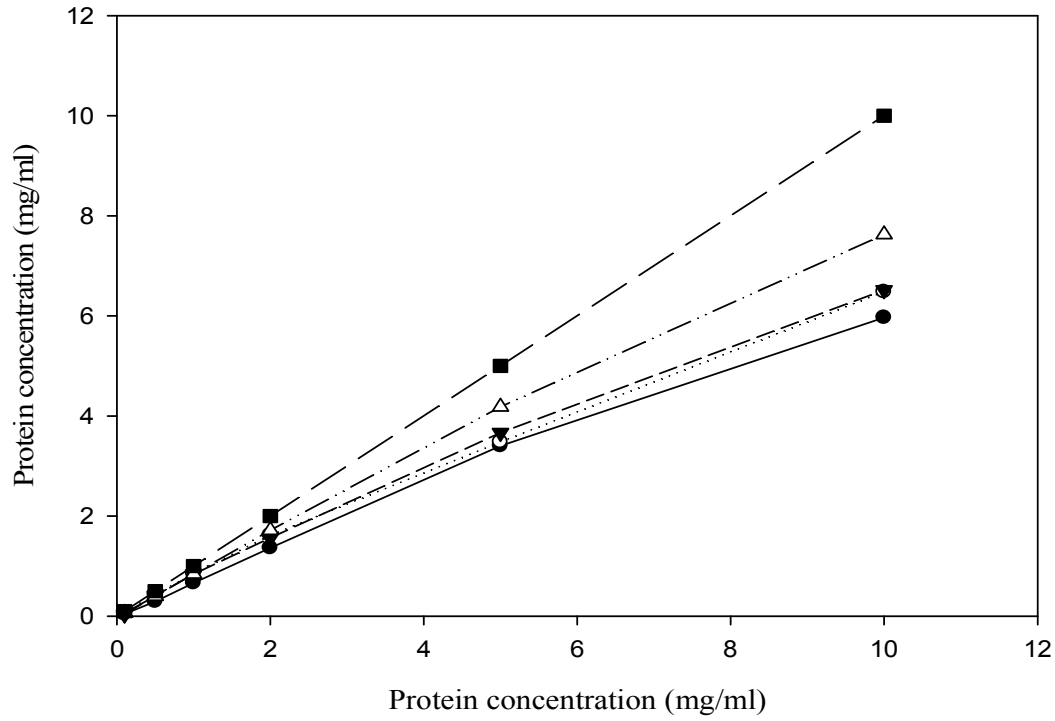

Figure 4.7. Effects of ozone on solubility of whey protein isolate and egg white protein at fixed time of ozone treatment (15 min.).○ egg white ozonated with aqueous ozonation method (4,5 ppm); ● egg white ozonated with gas ozonation method (60 g/sa); ▼ WPI ozonated with gas ozonation method (60 g/sa); Δ whey protein isolates ozonated with aqueous ozonation method(4,5 ppm); ■ pure(non treated with ozone) protein solution.

The reason why the solubility of egg white proteins were influenced more by ozone than WPI can be explained by amino acid composition of these proteins which may

show different reactivity for ozone. When ozonation period was kept constant, the loss in solubility of egg white proteins was 33,5 % while when this ratio was 26,7 % for WPI after 30 minute ozone treatment. The solubility of the protein is intensively influenced by the amount cysteine residue since the action of ozone converts the thiol group of cysteine into disulfides (Cataldo, 2003). The formation of S-S crosslinks may substantially reduce the solubility. Amino acids exposed to ozone have shown that the most susceptible amino acids were cysteine, tryptophan, methionine, histidine and tyrosine (Pryor,1984).

The major proteins in WPI are β-lactoglobulin and α-lactalbumin, which constitute, approximately 50% and 12% of the total (Fox and McSweeney, 1998). The remainder is immunoglobulins, bovine serum albumin, proteose peptones and other minor proteins. On the other hand, three particularly important proteins present in egg white are lysozyme (3.5 % of the total egg white protein), ovotransferrin (13 % of the total egg white protein) and ovoalbumin (54 % of the total egg white protein) (Vachier et al., 1995). Table 4.1. reports reactivity of amino acids with ozone (Cataldo, 2003). According to Table 4.1., arginine, cysteine, histidine, methionine, phenylalanine, tyrtophan and tyrosine are very reactive with ozone. Table A.20. and A.21. shows that amino acid compositions of β-lactoglobulin and ovoalbumin (Table A.20 and A.21). From these tables, we calculated the amount of amino acid content which reactive with ozone of these proteins. As a result, we obtained that the amino acid content reactive with ozone of ovalbumin was 24.6% and amino acid content of β-lactoglobulin which reactive with ozone was 18.8%. Consequently, it could be explained that amino acid composition of these proteins may show different reactivity for ozone. And also, we could say that effects of ozone on solubility property of egg white proteins were higher than whey protein isolate.

Table 4.1. Amino Acids reactivity with Ozone (Cataldo, 2003).

Amino Acids	Reactivity with Ozone
Alanine	Not Reactive
Arginine	Reactive
Asparagine	Not Reactive
Aspartic Acid	Not Reactive
Cystine	Very reactive
Glycine	Not reactive
Glutamic Acid	Not Reactive
Glutamine	Not Reactive
Histidine	Reactive
Isoleucine	Not Reactive
Leusine	Not Reactive
Lysine	Not Reactive
Methionine	Very Reactive
Phenylalanine	Very Reactive
Proline	Not Reactive
Serine	Not Reactive
Threonine	Not Reactive
Trytophan	Very Reactive
Tyrosine	Very Reactive
Valine	Not Reactive

4.3. Differential Scanning Calorimetry (DSC) Measurement

Heat treatment is of great importance in production, processing and concentration of proteins. Heat treatment has a pronounced effect on the structures and functional properties of proteins (de Witt, 1981). Heat denaturation of globular proteins is associated with destruction of some of the forces that stabilize native conformations such as hydrogen bonds and hydrophobic interactions. The distruption of electrostatic and van der Walls interactions are observed to occur to a lesser extent (Relkin, 1994).

Thermal denaturation of globular proteins has been extensively studied and thermodynamic parameters have been determined (Baeza and Pilosof, 2002; Li et al., 2004; Sreema et al., 2000). Differential scanning calorimetry (DSC) has been established as a sensitive technique for studying thermal denaturation and conformational transitions of proteins (Arntfield et al., 1990; Boye and Alli, 2000; Hendrix et al., 2000; Paulsson et al.,1985; Relkin 1994) providing qualitative and quantitative information as to the thermodynamic properties of proteins.

In this study, thermal stability of whey protein isolate and egg white proteins was examined after the proteins were treated with ozone. Thermal denaturation of the proteins was determined by differential scanning calorimeter. The enthalpy of thermal transition (ΔH) was calculated from the area under the peak. Denaturation enthalpies correspond to the loss of favorable intramolecular interactions within the protein molecule (Welzel, 2002).

Table 4.2. shows the denaturation temperature (Td) and the enthalpy changes (ΔH) for heat denaturation of native protein (whey protein isolate and egg white proteins) solutions and protein solutions treated with different concentrations of ozone. The ozonation of proteins was carried out by two different methods; ozonation of solvent before protein solution was prepared and direct treatment of protein by ozone gas.

Table 4.2. The denaturation temperature (Td) and enthalpy changes (ΔH) for heat denaturation of whey protein isolate and egg white proteins. Concentration of protein solutions was %10. A; pure protein solution (non-treated with ozone), B; Protein solution treated with 4,5ppm ozone (solution ozonation method), C; Protein treated with ozone gas 5min.(gas ozonation method), D; Protein treated with ozone gas 15min.(gas ozonation method), E; Protein treated with ozone gas 30min.(gas ozonation method).

	Sample	Td_1 (^{0}C)	Td_2 (^{0}C)	ΔH (J/g)
Whey Protein Isolate	A	78.73		15.06
	B	80.65		12.68
	C	81.84		11.64
	D	85.08		4.39
Egg White Proteins	A	64.03	84.74	12.75
	B	65.51	84.51	12.15
	C	64.59	84.42	10.62
	D	65.77	83.61	8.61
	E		83.67	6.46

Native WPI solution had a single thermal transition at 78.73 ^{0}C in the DSC thermograms. A single peak for transition of whey protein concentrate has been reported by Boye and Alli (2000). It was observed that denaturation temperature of WPI solutions increased depending on the increase in the amount of ozone used for protein treatment and this value was 85.05 ^{0}C for sample exposed to ozone gas for 15 min. Thus, thermal stability of the solution may increase with the increase in the amount of the ozone. ANOVA results revealed that ozone had a significant effect ($p<0.05$) on denaturation temperature and enthalpy of whey protein isolate solutions. The comparison of the denaturation enthalpies of ozone-treated and untreated protein solution showed that the denaturation enthalpy decreases as the quantity of ozone increases. Aggregation and disruption of hydrophobic interactions have been reported as exothermic reactions, which lower the observed enthalpy (Boye et al., 1996). The enthalpy values measured by DSC exhibits the sum of the exothermic and endothermic transitions. Thus, the reduction in the enthalpy change could be explained by the disruption of hydrophobic interactions and aggregation of protein associated with the unfolding of protein.

Thermal stability of egg white proteins were also examined by DSC. Egg white proteins exhibited two main thermal transition at temperatures 64.03 and 84.74^{0}C. While the dominant latter is due to ovalbumin, conalbumin and lysozyme appear as a single peak in the former as concealed by each other (Donovan et al., 1975). The thermograms and the denaturation enthalpies of egg white proteins correlated well with the results of Perez and Pilosof (2004). It was observed that thermal stability and denaturation temperature of egg white proteins were affected significantly ($p<0.05$) by ozone treatment. Denaturation thermograms changed depending on the ozone gas used for treatment. The first peak in the thermogram was observed to disappear after treatment for 30 min. However, the effect of gas ozonation on thermal stability of egg white proteins was not pronounced much as on that of whey protein isolate.

The change in denaturation enthalpies of egg white proteins with ozone treatment was also examined. The results showed that ozone had a significant effect ($p<0.05$) on the enthalpy values. A reduction in the enthalpy values of egg white proteins was observed with the increase in the amount of ozone gas as consistent with the results

obtained by WPI. The results of DSC measurements correlate well with the results of foaming experiments in such a way that ozone influences WPI more than egg white proteins. The difference between the behavior of WPI and egg white proteins depends on the structural differences of components proteins in the mixtures.

4.4. Optical Activity

Native proteins are made up of L- amino acids and are always laevorotatory. The specific rotation $[\alpha]_D$ of globular proteins generally lies between -30^0 and -60^0 (Doty and Geiduscheck, 1953), while the values for fibrous proteins are usually less negative (Cohen and SzentGyorgyi, 1957).

In this study, optical activity of ozonated protein was measured to determine effects of treatment using gas and aqueous ozonation methods. These experiments were carried out with WPI solutions since egg white proteins exhibited low solubility and turbidity which deflects the optical activity measurements. In a previous study, specific optical rotation of invertase has not been measured after ozonation because the solution became completely turbid. This result was explained that ozone causes the oxidation of the thiol groups of cysteine residues in the chain with consequent formation of disulfite bonds (crosslinks) and so solution can form a precipitate (Cataldo, 2003). At first, ozonation has been performed by applying previous methods those we have used for foaming measurements.

Figure 4.8. shows that optical activity values of whey protein isolate solutions prepared at the different concentration (0,1 and 0,2% (w/w)) were changed with ozone treatment using gas and aqueous ozonation method. As seen in the figure, gas ozonation had strong effect on optical activity of these protein solutions.

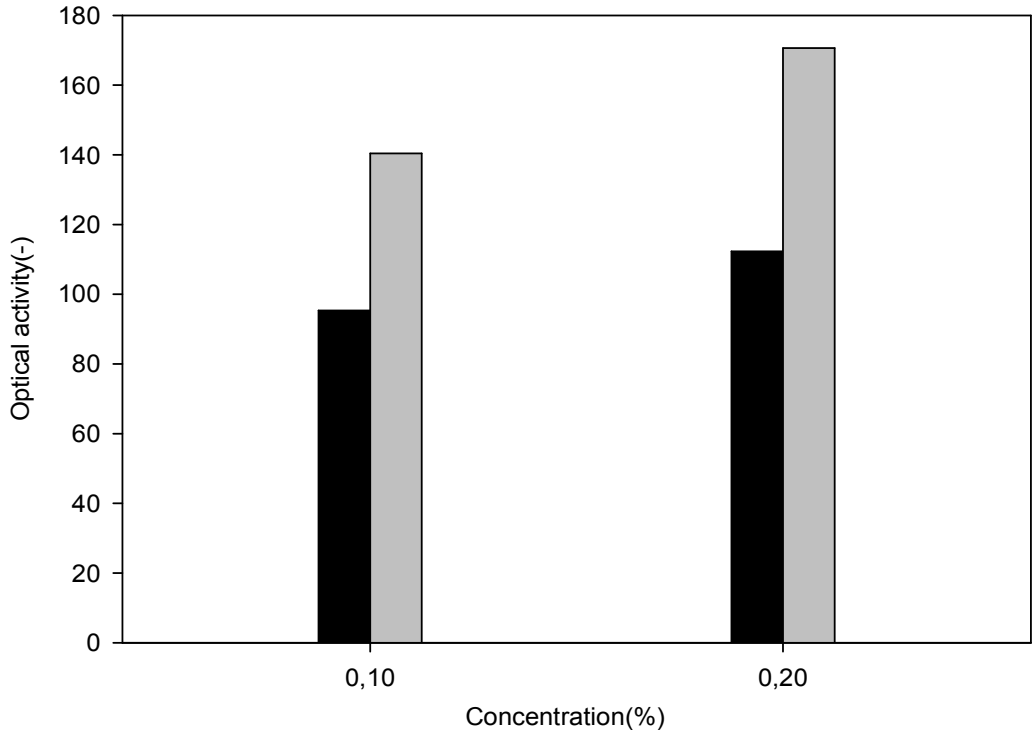

Figure 4.8. Effects of ozone treatment on optical activity of whey protein isolate (0,1 and 0,2% w/w). Dark bar; aqueous ozonation method, light bar; gas ozonation method.

However, changing the procedure to shaking gently (at a fixed amplitude) fresh native protein solution of known concentrations (0.1% and 0.2% w/w) in a separator funnel, evacuated and then filled with ozone gas, to provide mixing of gas ozone with the protein solution resulted in a considerable change in the optical activity between treated and untreated protein solutions as described by some researchers (Cataldo 2007). It has been stated by these researchers that ozone had been more reactive with protein molecules using this method than aqueous ozonation method and the optical activity of gelatin had changed to laevorotatory form. In the separator funnel, we kept the solutions at different periods of time and then the optical activities were measured.

Figure 4.9. shows that optical activities of WPI solutions ozonated for different periods of time, where the dark coloured bar shows solution containing 0.1%w/w WPI, while the light coloured bar shows solution containing 0.2% WPI. As seen in the figure, the laevorotatory forms of the protein solutions were increased with ozonation time. The change in the laevorotatory form was higher in the 0.2%

(w/w) protein solution than 0.1% (w/w) solution. The changes in the optical activities were 17.2 % and 29.0 % for 0.1 and 0.2 % (w/w) solutions, respectively.

As stated in a previous section, ozone has a longer half-life in the gaseous state than in aqueous solution (Rice, 1986).Thus, during mixing period of the protein solution with ozone gas in the funnel; the ozone in gaseous state transferred completely from gas phase to aqueous solution and reacted with more protein molecules in the solution. Consequently, more protein molecules were influenced by ozone and changes in optical activity of the protein molecules were higher due to structural changes in protein molecules caused by ozone in gaseous state.

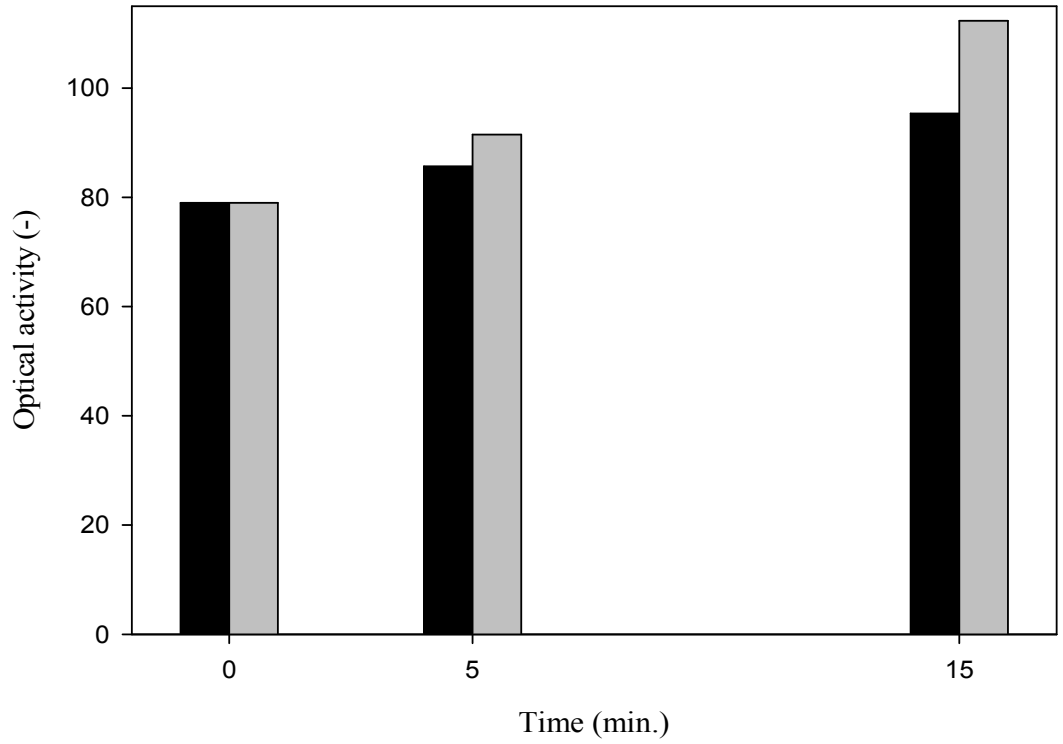

Figure 4.9. Changes in Optical activity values of whey protein isolate with ozonation (gas ozonation) time. dark bar; WPI solution (0,1% w/w) , light bar; WPI solution (0,2% w/w).

Some of aromatic amino acids such as trytophan, tyrosine, phenylalanine and also histidine, methionine are very reactive with ozone. Ozonation causes changes in some monomeric units, the first contribution to the variation of specific optical rotation of proteins could be derived from these structural changes (Cataldo, 2006).

Additionally, a further contribution to the variation of specific optical rotation of proteins can be derived from the protein denaturation i.e. the variation in the secondary and tertiary structure induced by the chemical changes introduced in certain monomeric units (Cataldo 2007).

In some previous studies (Putnam, 1953; Jirgensons, 1952 a, b; Doty and Geiduscheck, 1953) it has been reported that denaturation of proteins caused the changes in the spatial configuration of the protein molecules without accompanying fall in molecular weight and the laevorotation of proteins invariably increased. As seen in figure 4.9., we observed that the specific optical activity of protein solutions increased in laevorotatory form with ozonation treatment which also may indicate the denaturation process.

4.5. High Pressure Liquid Chromatography (HPLC) Analysis

Ozonated and non-ozonated solutions of WPI and egg white proteins were examined by reverse phase HPLC. HPLC chromatograms for ozonated protein solutions were compared with those of native protein solutions considering that structural changes and protein denaturation induced by the oxidation of sensitive amino acid residues may increase local flexibility or rigidity to the protein chain causing an alteration of its secondary and tertiary structure (Cataldo, 2003).

Figure 4.10. shows the reverse phase HPLC chromatograms for WPI solutions which represents the marked peaks of α-lactalbumin, lactoferrin, bovine serum albumin (BSA), β-lactoglobulin B and A and immunoglobulin, respectively. β-lactoglobulin (50%) and α-lactalbumin (20%) are regarded as major proteins with the percentages of 50 and 20 %, respectively (Hambling et al., 1992), as seen in the chromatograms. Similar chromatograms for WPI have been obtained in previous studies (Almecija et al., 2007; Elgar et al., 2000).

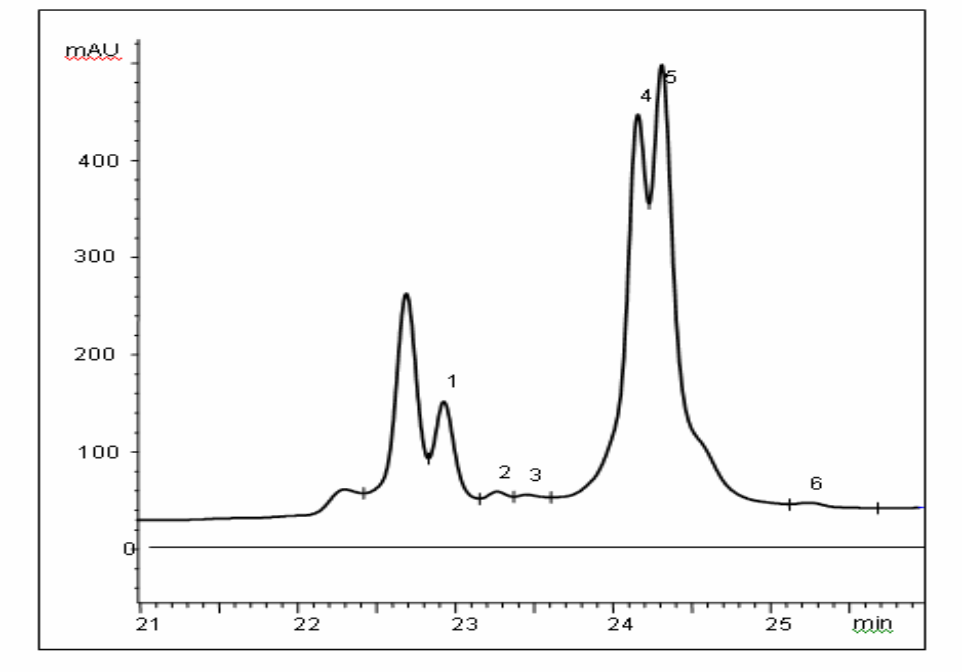

Figure 4.10. Reverse phase HPLC chromatography for native whey protein isolate solution (0.1% w/w) (non treated with ozone).1; α-lactalbumin, 2;lactoferrin, 3; bovine serum albumin (BSA), 4,5; β-lactoglobulin B and A, 6; immunoglobulin.

The chromatogram of whey protein isolate solution treated with ozone is given in Figure 4.11. The figure reflects a similarity with HPLC chromatogram of denaturation of whey proteins with temperature (Kiokias et al., 2007) which exhibited a decrease in peak area for native whey proteins with increasing temperature. A decrease in peak area of native WPI after ozone treatment was observed from Figure 4.11. The area under the peaks of α-lactalbumin, β-lactoglobulin A and B were reduced by 45 % and the peaks of lactoferrin, bovine serum albumin (BSA) were observed to disappear. In addition, a new peak appeared at 23.8 min. This new peak may be explained by the cross binding of some protein molecules. Native β-lactoglobulin has two disulphide bonds and one free thiol group which is buried within the protein structure. α-Lactalbumin is a small compact globular protein stabilized by four disulphide bonds and it does not contain a free thiol group (Brew and Grobler, 1992). In the solubility section we have seen that ozone treatment caused precipitation in protein solutions which may be caused by the formation of crosslinks between adjacent protein chains with a consequent drop in solubility (Cataldo 2003). Thus, ozone treatment may result in the oxidation of thiol groups (-SH) of cysteine with consequent formation of disulfide bonds (crosslinks).

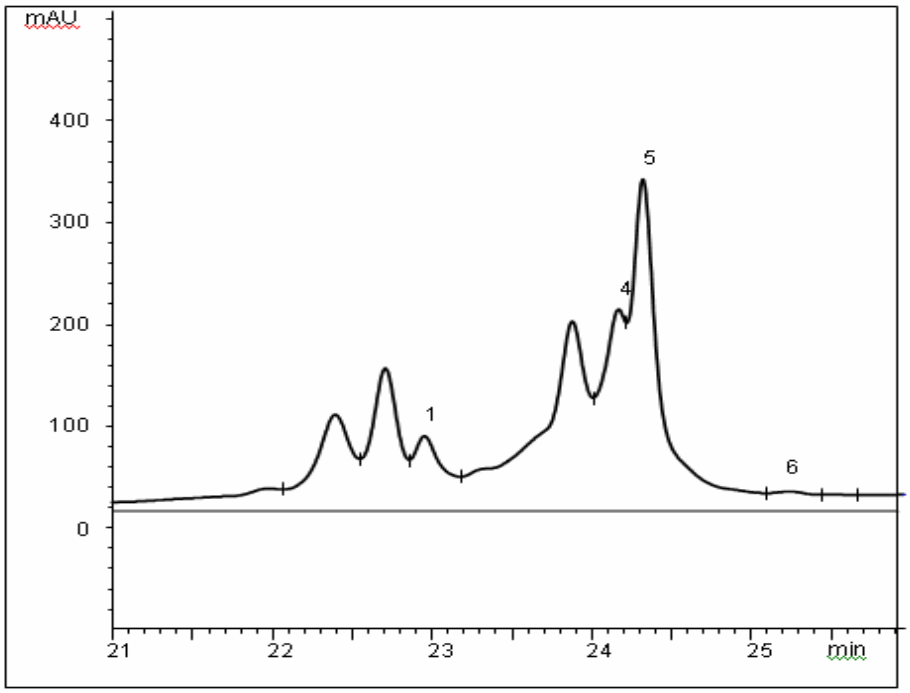

Figure 4.11. Reverse phase HPLC chromatography for whey protein isolate solution (0.1% w/w) treated with ozone.1; α-lactalbumin, 2;lactoferrin, 3; bovine serum albumin (BSA),4,5; β-lactoglobulin B and A, 6; immunoglobulin.

Reverse phase chromatography for native egg white proteins (non-treated with ozone) were shown in Figure 4.12. The marked peaks on the chromatogram belong to lysozyme, ovotrasferrin and ovalbumin, respectively. A similar result of reverse phase HPLC chromatogram for egg white proteins was obtained in a previous study (Guerin-Dubiard, 2005). Among as many as 40 different proteins contained in the egg white, the major proteins involved are ovalbumin (54%), conalbumin (12%), ovomucoide (11%) and lysozyme (3.5%) (Donovan et al., 1975). The higher stability of lysozyme is regarded due to the compactness of this protein, since it has four disulfide cross-linkages and no free thiol group. Ovalbumin has four thiol groups which are buried within the core of the protein that become exposed upon heating, leading to intermolecular reactions (Cheftel et al., 1989).

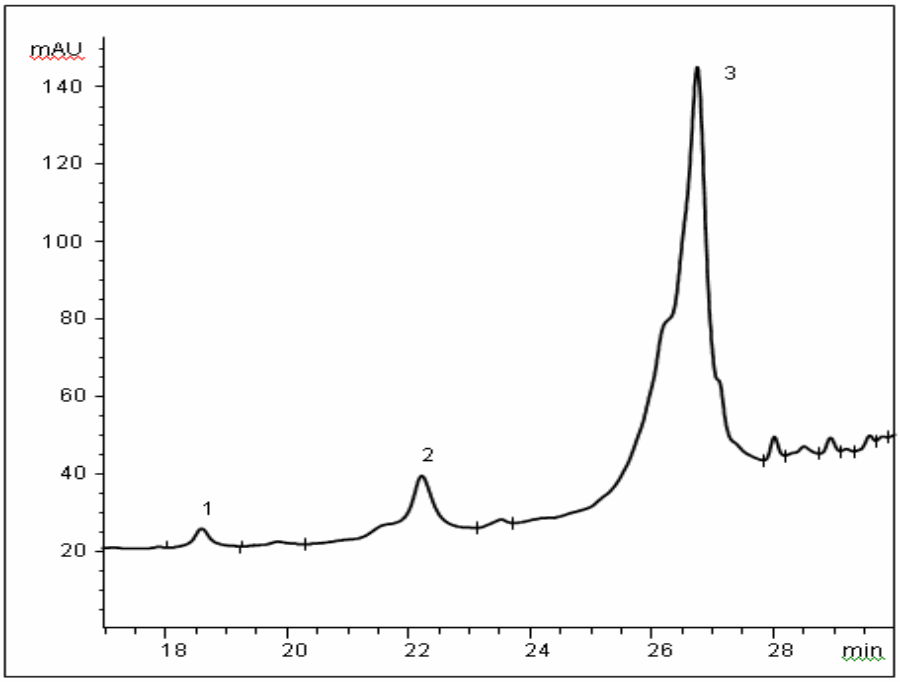

Figure 4.12. Reverse phase HPLC chromatography for native egg white protein (non treated with ozone) (0.1% w/w).1; lysozyme, 2; ovotransferrin, 3; ovalbumin.

Figure 4.13 showed chromatogram of egg white proteins after oxidation by ozone. According to comparison of chromatograms in Figure 4.12 and 4.13, a 42 % decrease in the area under the peaks was determined. In addition, a new peak was observed on the left hand side of ovalbumin peak. The new peak widens the bottom of ovalbumin peak. As a result of ozone oxidation, protein molecule may undergo various changes which is likely to be in aromatic amino acid residues (Cataldo, 2003). In addition, oxidation of thiol groups, products formation from protein oxidation and conversion of some amino acid residues to carbonyl derivatives might be possible changes. Also, cleavage of polypeptide chain and formation of cross-linked protein aggregates may result from oxidation (Kayalı and Çakatay 2004). Some alterations may occur in protein conformation and lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, susceptibility to proteolysis, and diminution of normal function (Cataldo, 2006).

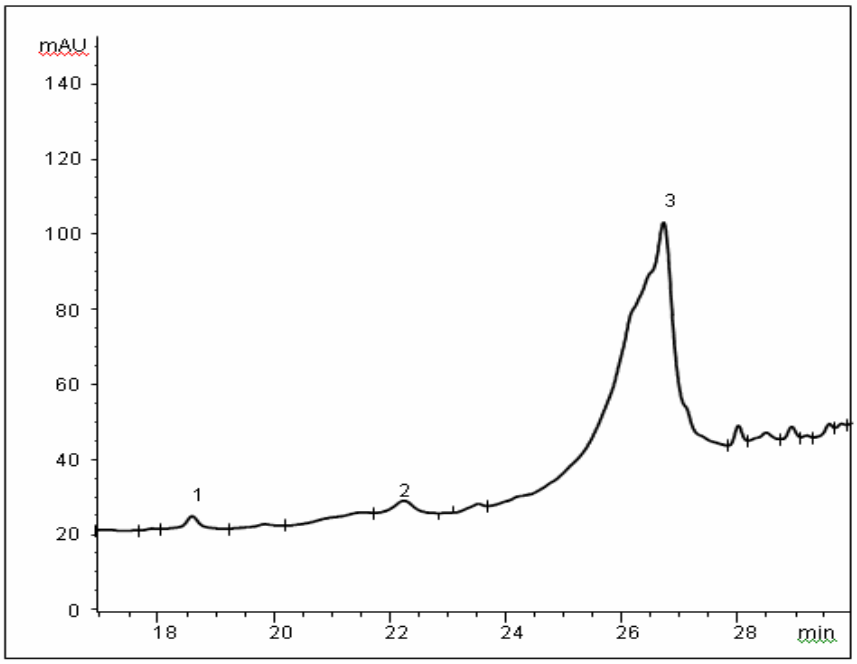

Figure 4.13. Reverse phase HPLC chromatography for egg white protein (0.1% w/w) treated with ozone.1; lysozyme, 2; ovotransferrin, 3; ovalbumin.

These chromatograms may not give idea about the oxidation products of protein. A further research might be needed for analyzing soluble aggregates formed during protein ozonation by LC-MS.

4.6. Emulsifying Properties

In this study, emulsifying properties of whey protein isolate were examined at two different protein concentration (0,1 and 0,05% w/w). The samples were treated with ozone using aqueous ozonation method as described in previous sections. Emulsions were prepared by an ultrasound disintegrator (15 amplitudes for 1 min or 15 s). Guzey (2001) reported that high intensity ultrasonic processing improves emulsifying properties of whey protein isolate. In some studies (Pongsawatmanit et al., 2006; Gulseren et al., 2007) the emulsions have been treated with high intensity ultrasonic waves to disrupt any flocculated droplets.

Emulsifying properties of whey protein isolate were expressed in terms of emulsifying activity index (EAI) and emulsion stability index (ESI) and were determined by the modified Pearce and Kinsella (1976) method. The EAI and ESI of

the emulsions were measured for long period (about 7 days) to observe behavior of droplets in the emulsion.

Figure 4.14 showed that EAI of emulsions prepared using whey protein isolate at different protein concentrations and duration of ultrasound treatment The statistical analysis of data showed that EAI of emulsions were significantly different ($p<0,05$) for three applications (1 min ultrasonic treatment for 0.1% and 0.5 % w/w, 15 s treatment for 0.05 % w/w). EAI was measured as 2.347 m^2/mg for emulsion containing 0.1% (w/w) protein concentration without ozone treatment and 2.312 m^2/mg for emulsion (% 0.1 (w/w) protein concentrations) after ozone treatment. EAI values for emulsion containing % 0.05 protein concentration without ozone treatment and after ozone treatment were 2.097 and 2.029 m^2/mg, respectively. EAI values for emulsion containing %0.05 protein concentrations for 15 s ultrasound treatments with/ without ozone treatment were 1,927 and 2,009 m^2/mg, respectively. It was observed that ozone treatment affected emulsion activity negatively due to oxidation and denaturation of protein molecules. Cataldo (2006) reported that with proteins, ozone causes the oxidation. As a result of this attack the protein molecules undergo changes in their usual folding and binding ability and are denaturated. It was observed that the ozone treatment has a negative effect on emulsion activity which is more pronounced at the concentrated emulsion (involving 0.1 %w/w protein). In a previous study, reduction in the emulsion activity of β-lactoglobulin and bovine serum albumin in proportion to heat denaturation has been correlated with the decrease in the surface hydrophobicity of these proteins and surface hyrophobicity is certainly a main factor governing the emulsifying properties of proteins (Kato, et al., 1982).

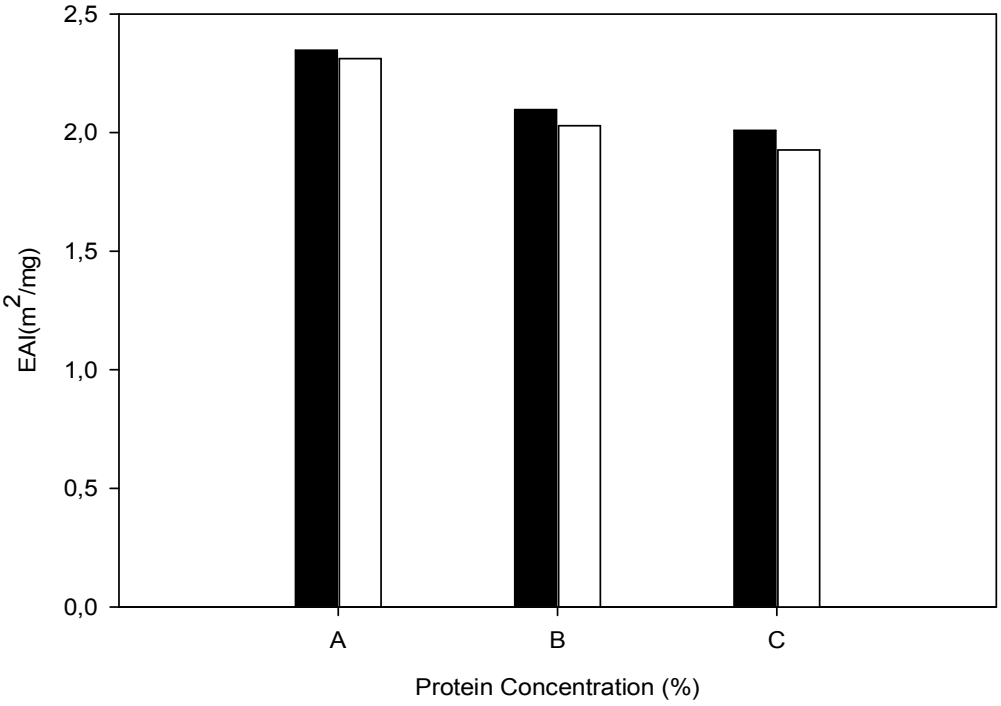

Figure 4.14. Emulsion Activity of whey protein isolate stabilized emulsions. A: 0.1% (w/w) WPI emulsion, ultrasound treatment 1min., B: 0.05% (w/w) WPI emulsion, ultrasound treatment 1min., C: 0.05% (w/w) WPI emulsion, ultrasound treatment 15 s. (dark bars: non ozonated , light bars: ozonated)

Emulsion stability index (ESI) of emulsions were determined by following the time-dependent change in the absorbance of emulsions of WPI with/without ozone treatment and they exhibited a significant difference ($p<0.05$) statistically. Figure 4.15. shows the change in ESI time. ESI initially reduces fast for approximately 60 min, and then gradually slows down and reaches to almost a plateau value. The ESI curve resembles the inverse of an exponential curve. The ozone treatment reduces the emulsion stability. The initial stability decreases sharply as the concentration of protein in the emulsion increases. The lowest value was observed for the emulsion prepared with 0.5 % w/w protein and 15 s of ultrasound treatment. A better stability, when 1 min emulsification was applied, may be related with the interface denaturation of protein together with ultrasound denaturation at a longer treatment time.

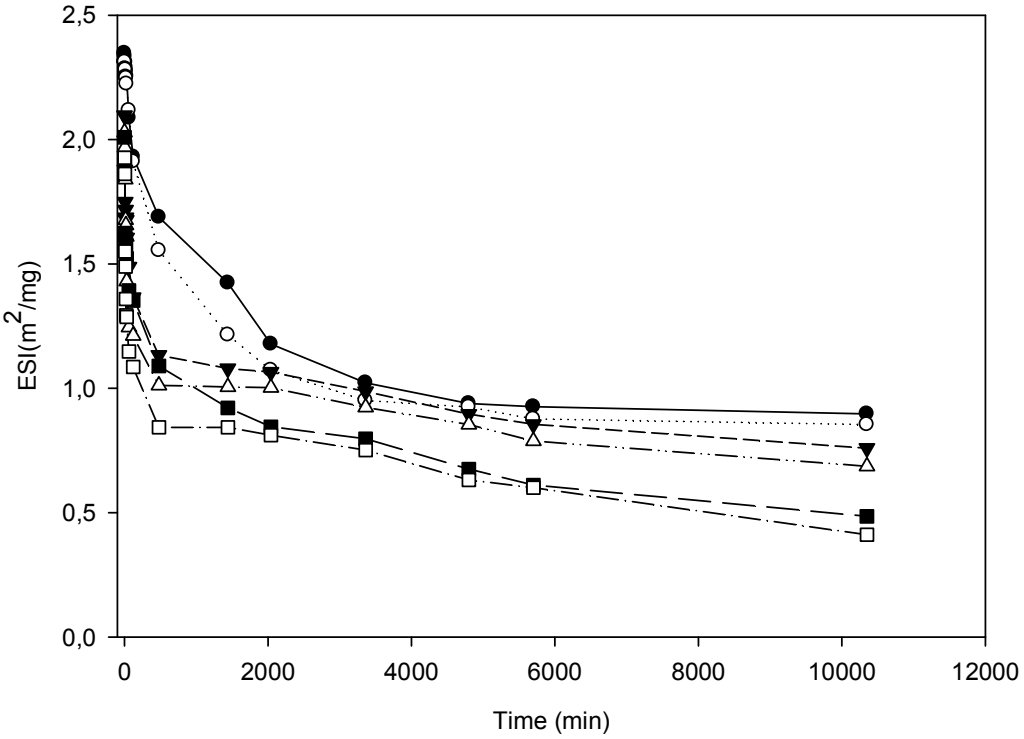

Figure 4.15. Emulsion Stability of whey protein isolate stabilized emulsion. (○ ozonated emulsion containing WPI %0,1 (w/w) for 1min. ultrasound treatment ● emulsion containing %0,1 (w/w) WPI for 1min. ultrasound treatment ▼ emulsion containing %0,05 (w/w) WPI for 1min. ultrasound treatment Δ ozonated emulsion containing %0,05 (w/w) WPI for 1min. ultrasound treatment ■ emulsion containing %0,05 (w/w) WPI for 15sec. ultrasound treatment □ ozonated emulsion containing %0,05 (w/w) WPI for 15sec. ultrasound treatment.

The ESI data was evaluated by applying first order and second order kinetic models. It was observed that the data fitted well to first order kinetic rate (by comparing r^2, error mean square values). A first order kinetic model was used previously to examine thermal denaturation of egg white proteins (Nohara et al., 1998). Also it has been noticed that the stability curve was biphasic. Consequently, the curve was examined in two stage and kinetic rate constants (k values) for each stage were calculated from first order kinetic model (Table A.21.). The first stage was from 0 to 420 min. where a sharp decrease in absorbance values was seen while the second stage was in between 540-13350 min which exhibits a slow decrease in absorbance values.

Table 4.3. Kinetic constant values for each curve and for two stages (t= 0- 420 min. first phase, t= 540- 13350min. second phase), k_1 : k values for first phase, k_2: k values for second phase.

Protein concentration(%)	**k_1**	**k_2**
%0,1 (non treated with ozone, 1min. ultrasound treatment)	$8x10^{-4}$	$7x10^{-5}$
%0,1 (treated with ozone, 1min. ultrasound treatment)	$8x10^{-4}$	$8x10^{-5}$
%0,05 (non treated with ozone, 1min. ultrasound treatment)	$12x10^{-4}$	$9x10^{-5}$
%0,05 (treated with ozone, 1min. ultrasound treatment)	$18x10^{-4}$	$7x10^{-5}$
%0,05 (non treated with ozone, 15 sec. ultrasound treatment)	$10x10^{-4}$	$10x10^{-5}$
%0,05 (treated with ozone, 15 sec. ultrasound treatment)	$16x10^{-4}$	$7x10^{-5}$

Table 4.3. shows the kinetic rate constant values for each curve and for two stages, separately. k values were significantly different ($p<0,05$) for emulsions stabilized by different protein concentrations. Also, there was a significant difference ($p<0,05$) between k values for first stage. Comparison of k values for first stage indicates that, emulsion stability decreases with decrease in protein concentration. However, comparison of ultrasound treatment times (1min. and 15 s) for 0,05% w/w emulsions indicates that emulsion stability decreased with increase in treatment time. For the second stage we observed that the change in emulsion stability was decreasing slowly and k values for second stage were similar to each other virtually. Consequently, statistical analysis showed that there is no significant difference ($p>0,05$) between k values of the second stage. Previously, not only emulsifying activity but also emulsion stability has been correlated with surface hydrophobicity (Kato et al., 1982). The structure and also the surface hydrophobicity of proteins may greatly change during denaturation as observed with β-lactoglobulin. Consequently, emulsion stability decreased with denaturation due to ozonation of protein in this study.

4.7 Rheological Properties

In oscillatory instruments, samples are subjected to harmonically varying stress or strain. This testing procedure is the most common dynamic method for studying the viscoelastic behavior of foods. The method is useful in a variety of applications including gel strength evaluation, monitoring starch gelatinization, studying the glass transition phenomenon, observing protein denaturation and coagulation, evaluating curd formation in dairy products, cheese melting, shelf life testing, etc. (Steffe, 1996). The effects of ozone on rheological behavior of whey protein isolate stabilized emulsions (0.1% w/w and 0.05% w/w) were examined in this study. Dynamic frequency sweep tests were performed in the linear viscoelastic range to determine the frequency dependence of the elastic and viscous moduli.

Figure 4.16, 4.17, 4.18. and 4.19 showed the mechanical spectra describing the viscoelastic behavior of whey protein isolate stabilized emulsions prepared with ozonated and native protein solutions (non treated with ozone). At low frequencies a more fluid-like behaviour was observed, where the loss modulus, G", was higher than the storage modulus, G'. The crossover between G" and G' curves was observed at the frequency range of 0.1–0.2 Hz in Figures 4.16 and 4.18 and 4.19.At frequencies bigger than the crossover frequency, a solid-like behaviour was observed where G' was higher than G". According to Steffe (1992), this description of the mechanical spectra is characteristic of the viscoelastic fluids. Similar results were observed by Ikeda et al. (2001) that analysed the mechanical spectra of WPI suspensions prepared in a concentration range of 0.1%–10% w/w WPI in 0.1 M NaCl, having a crossover between G" and G' at the lower end of the frequency range (<0.3 Hz).

From the figures, it was observed that ozone was more effective on rheological behavior of WPI stabilized emulsions containing a low protein concentration (0,05% w/w) than those having a high protein concentration (0.1 % w/w). That correlates well with the results mentioned in previous sections (4.1, 4.6) as described by the enhanced effect of ozone on a low number of protein molecules. Storage modulus, G', showed little change with ozone treatment and the change was even lower for emulsion containing 0.1% w/w protein than for emulsion containing 0.05% w/w

protein. In contrast, loss modulus, G", increased with ozone treatment and the increase was 78 and 52 % for emulsions containing 0,05 and 0,1% w/w protein, respectively. It was determined that the change in G" was higher than G'. It has been previously observed that G' decreases but G" increases with heat treatment for whey protein concentrate suspensions (Meza et al., 2008). It can be concluded that ozone treatment affects liquid-like behaviour more extensively than solid-like behavior.

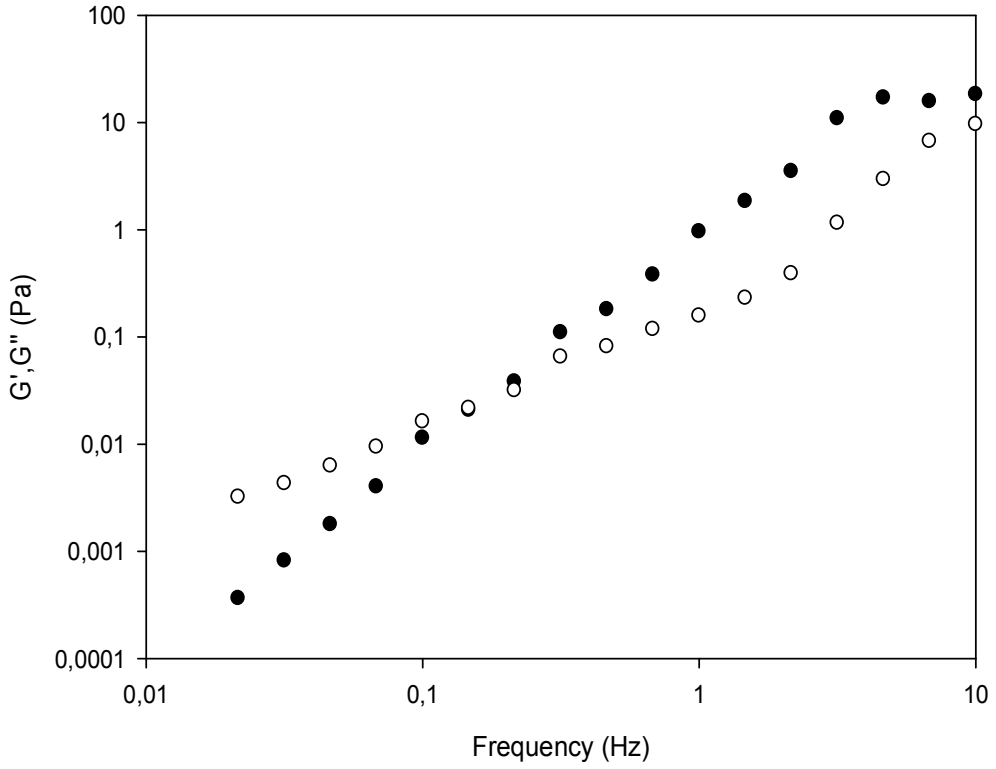

Figure 4.16. Viscoelastic moduli (G ' – filled symbols, G"- open symbols) for WPI (0,05% w/w) stabilized emulsions (non treated with ozone).

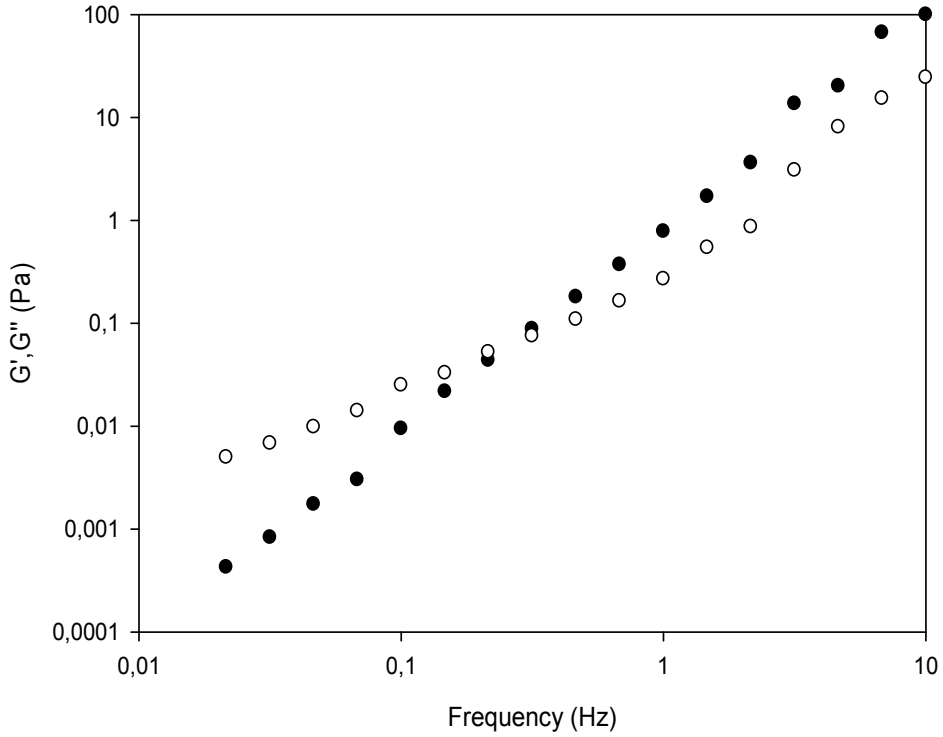

Figure 4.17. Viscoelastic moduli (G ' – filled symbols, G''- open symbols) for WPI (0,05% w/w) stabilized emulsions (treated with ozone).

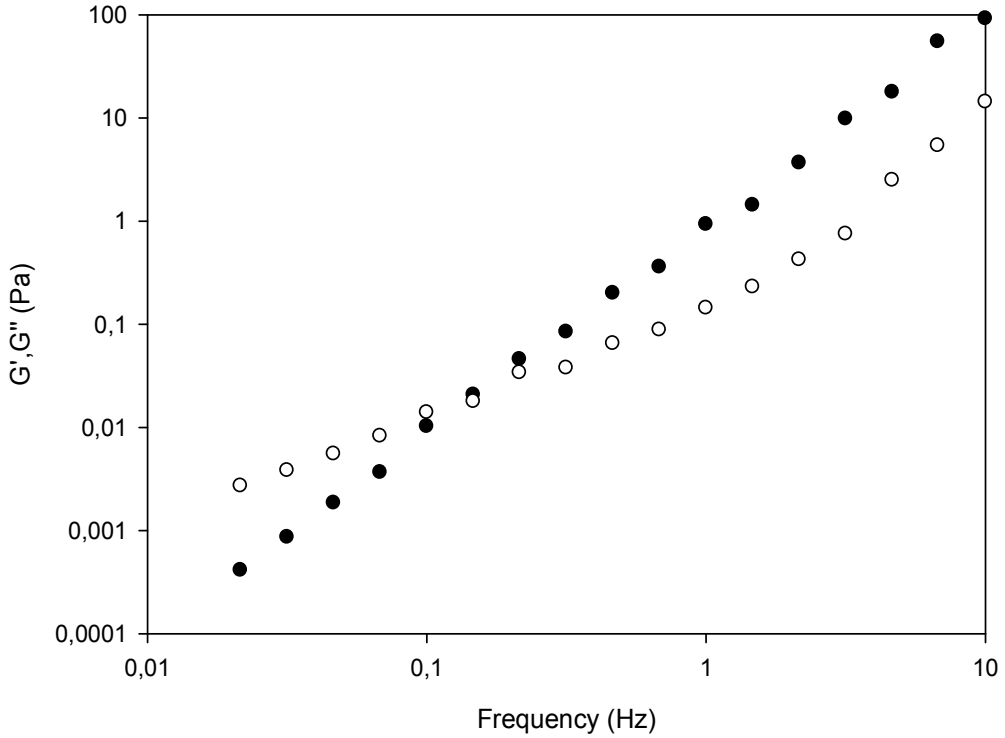

Figure 4.18. Viscoelastic moduli (G ' – filled symbols, G''- open symbols) for WPI (0,1% w/w) stabilized emulsions (non treated with ozone).

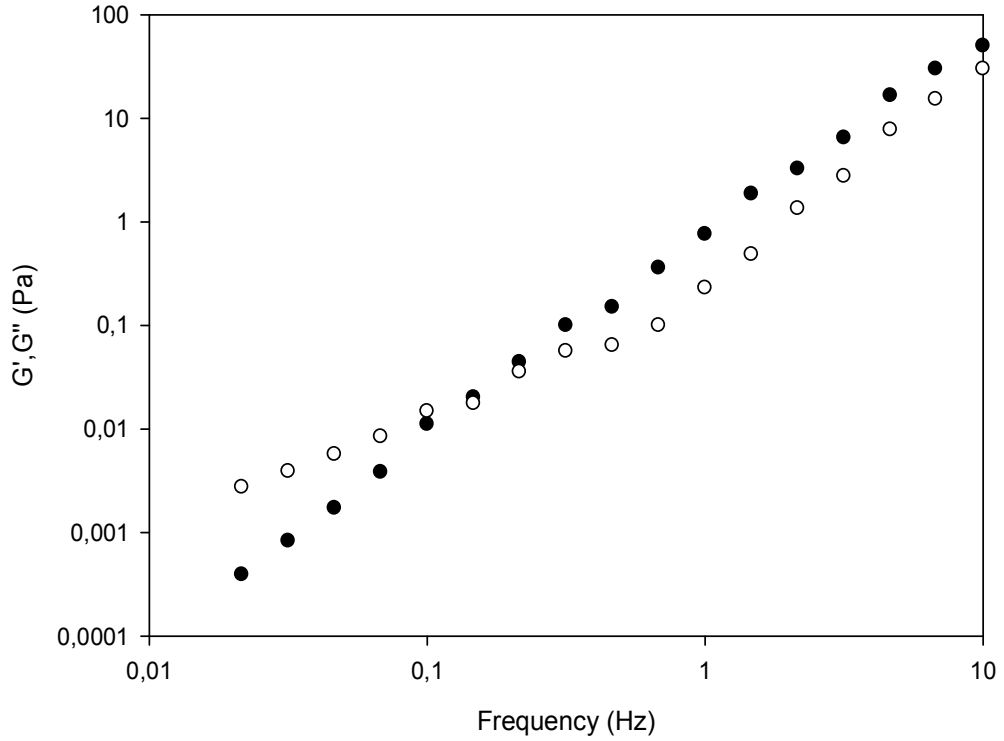

Figure 4.19. Viscoelastic moduli (G ' – filled symbols, G''- open symbols) for WPI (0,1% w/w) stabilized emulsions (treated with ozone).

The crossover between G'' and G' curves was observed to be at the frequency range of 0.1–0.2 Hz for emulsions prepared from non-treated protein (Figure 4.16). The range was the same for emulsions containing 0.1% w/w protein treated with ozone (Figure 4.17). However, the range was observed as 0,2-0,3 Hz for emulsions including 0,05% protein treated with ozone (Figure 4.19). Since the ozone causes denaturation of the proteins, i.e. introduces changes in their secondary and tertiary structure (Cataldo, 2003) the cross over between G'' and G' after ozone treatment might have been observed at a high frequency. Thus, ozone treatment might cause functional and structural changes on protein molecules due to denaturation. As a result of ozone treatment, dilute emulsions (having 0,05%w/w protein) exhibited more liquid like behavior until a high frequency compared with concentrated emulsions (having 0,1 %w/w protein).

CONCLUSION

In this study, the effects of ozone on functional properties of whey protein isolate and egg white proteins were investigated and the following results were obtained.

Foaming Properties;

Foaming ability and foam stability of WPI increased when ozone concentration of the aqueous solution was increased. However, foam stability of WPI seemed to be affected more by ozone gas than foaming ability of protein.

Effect of different initial temperatures of solution on foam volume was not significantly different. However, the effect of temperature on foam stability was found to be significantly different.

Foam volume and foam stability of ozonated samples increased when protein concentration of the solution was increased. However, the ratio of increase in the foam volume was less than the ratio of increase in foam stability.

An improvement in the foaming properties of egg white proteins was observed. However, it does not seem to be as much as the improvement in foaming properties of whey protein isolate by ozone treatment.

The effects of different ozonation methods on foaming properties of WPI were significantly different. The ozone treatment in the aqueous environment is more effective than ozone atmosphere.

Solubility;

At a constant concentration, the solubility of proteins changed as the method of ozonation was changed. Solubility of the protein samples were reduced more by gas ozonation method than by ozonation of solvent.

The gas ozonation method was more effective on solubility than aqueous ozonation method when ozonation was carried out at a fixed period.

Solubility of egg white proteins were influenced more by ozone than WPI because of amino acid composition of these proteins which may show different reactivity for ozone.

DSC Measurements;

Denaturation temperature of WPI solutions increased depending on the increase in the amount of ozone used for protein treatment. Thermal stability of the solution may increase with the increase in the amount of the ozone.

A reduction in the enthalpy values of egg white proteins was observed with the increase in the amount of ozone gas as consistent with the results obtained by WPI. However, the effect of gas ozonation on thermal stability of egg white proteins was not pronounced much as on that of whey protein isolate.

Optical Activity;

The laevorotatory forms of the protein solutions were increased with ozonation time.

Gas ozonation had strong effect on optical activity of WPI. Optical activity measurements were not carried out with egg white proteins since they exhibited low solubility and turbidity which deflects the optical activity measurements.

HPLC Analysis;

Chromatogram of native WPI after ozone treatment showed that a decrease in the peak area. The area under the peaks of α-lactalbumin, β-lactoglobulin A and B were reduced by 45 % and the peaks of lactoferrin, bovine serum albumin (BSA) were observed to disappear. In addition, a new peak appeared at 23.8 min.

Chromatogram of egg white proteins after oxidation by ozone showed that there was 42 % decrease in the area under the peaks. In addition, a new peak was observed on the left hand side of ovalbumin peak.

Emulsifying Properties;

The ozone treatment has a negative effect on emulsion activity which is more pronounced at the concentrated emulsion.

The ozone treatment reduces the emulsion stability. The initial stability decreases sharply as the concentration of protein in the emulsion increases.

The ESI data was also evaluated by applying first order kinetic model.

Rheological Properties;

At low frequencies emulsions behaved like a fluid and at higher frequencies they showed a solid or elastic behaviour.

Ozone was more effective on rheological behavior of WPI stabilized emulsions containing a low protein concentration than those having a high protein concentration.

Storage modulus showed little change with ozone treatment. In contrast, loss modulus, increased with ozone treatment.

REFERENCES

Almecija, M.C., Ibanez, R., Guadix, A., Guadix, E.M. (2007). Effect of pH on the fractionation of whey proteins with a ceramic ultrafiltration membrane, *Journal of Membrane Science*, **288**, 28-35.

Anonymous. (1972). Use of ozone in sea water for cleansing shellfish. *Effluent Water Treatment Journal,* **12**, 260–262.

Arntfield, S.D., Ismond, M.A.H. and Murray E.D.(1990). Thermal Analysis of Food Proteins in Relation to Processing Effects,. Harwalkar, V. R and. Ma C.-Y (Eds.), *Thermal Analysis of Foods*, Elsevier Applied Science, New York, 51-91.

Awade, A. C., Moreau, S., Molle, D., Brule, G., & Maubois, S. J. L (1994). Two-step chromatographic procedure for the purification of hen egg white ovomucin, ovotransferrin and ovalbumin and characterization of purified proteins. *Journal of Chromatography A*, **677**, 279–288.

Baeza, R.I., and Pilosof, A.M.R. (2002). Calorimetric studies of thermal denaturation of β-lactoglobulin in the presence of polysaccharides, *Lebensmittel-Wissenschaft und-Technologie*, **35**, 393-399.

Bailey, P.S. (1982). Ozonation in organic chemistry, Vol. 2: *Nonolefinic compounds*. San Diego, USA: Academic Press.

Baker, A., Äidek, L., Wiesler, D., Chmelik, J., Pagel, M. and Novotny, M.V. (1998). Reaction of N- acetylglycyllysine methyl ester with 2-alkenals: an alternative model for covalent modification of proteins. *Chemistry Res. Toxicology* **11**, 730-740.

Bernard, E.C., Alistairs S.G.(2007). Some functional fractionated soy protein isolates obtained by microfiltration. *Food Hydrocolloids* **21**, 1379-1388.

Beuchat, L.R. (1992). Surface disinfection of raw produce. *Dairy, Food and Environmental Sanitation*, **12**, 6–9.

Borderias, A. J., and Monteiro, P. (1988). Fundamentos de la funcionalidad de las proteinas en alimentos, *Revista Agroquimicay Tecnologia de Alimentos*, **28**, 159-169.

Boye, J.I., Alli, I. (2000).Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study, *Food Research International,* **33**, 673-682.

Brew, K. and Grobler, J.A. (1990).α-Lactalbumin. Fox, P.F. (Ed.)*Advanced Dairy Chemistry 1: Proteins*.(2nd ed.) London, Elsevier Applied Science, 191-229.

Brown, J.R. (1977). Serum albumin. Amino acid sequence. *In Albumin structure 94 Proteins in food processing Function and Uses.* Rosenoer, V.M., Oratz, M. and Rothschild, M.A. (Eds.) Oxford Pergamon Press, 27

Bryant, C. M., and McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science and Technology*, **9**, 143–151.

Campbell, G.M., Webb, C., Pandiella, S.S.,Niranjan, K.(1999). "Preface", in Bubbles in Foods, Campbell, G.M., Webb, C., Pandiella, S.S.,Niranjan, K.(Eds.).Minnesota; Eagan Press.

Castleman, W. L., Dungworth, D. L., and Tyler, W. S. (1973). Cytochemically detected alterations of lung acid phosphatase reactivity following ozone exposure. *Laboratory Investigation*, *9*, 310–319.

Cataldo, F. (2006). DNA degradation with ozone, *International Journal of Biological Macromolecules*, **38**, 248-254.

Cataldo, F. (2007).On the action of ozone on gelatin, *International Journal of Biological Macromolecules*, **41**, 210-216.

Cataldo, F. (2003). On the action of ozone on proteins, *Polymer Degradation and Stability*, **82**, 105-114.

Chang, Y. H., & Sheldon, B. W. (1989). Application of ozone with physical waste water treatments to recondition poultry process waters. *Poultry Science*, **68**, 1078–1087.

Cheftel, J. C., Cuq, J. L., and Lorient, D. (1989). Las proteinas del huevo Cheftel, . J. C., Cuq, J. L and Lorient, D. (Eds.), Proteinas alimentarias Zaragoza, Espana: Editorial Acribia SA. 167–177.

Cohen, C.(1955 b). Optical rotation and polypeptide chain configuration in proteins. *Nature,* Lond.**175**, 129-130.

Cohen C. ,Szent-Gyorgyi A.G. (1957). Optical Rotation and Helical polypeptide chain configuration in α-proteins, *Journal of American Chemical Society*, **248**, 79-85.

Coke, A. L. (1993). Mother nature's best remedy: Ozone. *Water Conditioning and Purification*, (October) 48–51.

Croguennec, T., Francoise, N., Stephane, P., and Gerard, B. (2000). Simple rapid procedure for preparation of large quantities of ovalbumin. *Journal of Agricultural and Food Chemistry*, **46**, 4883–4889.

Damodaran, S. (1994). Structure-function relationship of food proteins, Hettiarachchy, N.S., Ziegler, G.R.(Eds.) *Protein Functionality in Food Systems*, Marcel Dekker Inc., NY.

Damodaran, S. (1996). *Amino acids, peptide and proteins*. New York: Dekker.

Desai, I.D. and Tappel, A.L. (1963). Damage to proteins by peroxidized lipids. *Journal of Lipid Res*. **4**, 204-207.

De Wit, J.N. (1989). Functional properties of whey proteins. *In Developments in Dairy Chemistry-4.*. Fox, P.F. (Ed.) London, Elsevier Applied Science, 285-322.

Dickinson, E. (1992). *An Introduction to Food Colloids*, Oxford University Press, Oxford, UK,

Donovan, J. W., Mapes, C. J., Davis, J. G., and Garibaldi, J. A. (1975). A differential scanning calorimetric study of stability of egg white to heat denaturation. *Journal of Science and Food Agriculture*, **26**, 73–83.

Doorn, J.A. and Petersen, D.R. (2002). Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. *Chemical Resource Toxicology* **15**,1445-1450.

Dosti, B. (1998). Effectiveness of ozone, heat and chlorine for destroying common food spoilage bacteria in synthetic media and biofilms. Thesis. Clemson Universtiy, Clemson, SC, 69.

Doty, P., Geiduschek, E. P. (1953). Optical Properties of Proteins, Part II, Optical Rotation, Neurath, H., Bailey, A. (Eds.). *The Proteins,* Vol:1, Part A, Academic Press, New York , 408-416.

Elgar D.F., Norris, C.S., Ayers, J.S., Pritchard, M., Otter, D.E., Kate, P.P. (2000).Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene, *Journal of Chromatography A,* **878**, 183-196.

Evans, M. T. A., and Gordon, J. F. (1979). Whey proteins. Grant, A. (Ed.), *Applied protein chemistry*, London: Applied Science. 31–67.

Fennema, O.R. (1993). *Food Chemistry*, Marcel Dekker, New York, NY.

Fox P.F. (1982). *Developments in Dairy Chemistry*, Volume 1, Applies Science Publisher, London and New York.

Fox, P. F., and McSweeney, P. L. H. (1998). Milk proteins. Fox, P. F. and McSweeney, P. L. H. (Eds.), *Dairy chemistry and biochemistry*. London: Blackie Academic and Professional. 146–237.

Friberg, S. (1976).*Emulsion Stability In Food Emulsions*, Friberg, S. (Ed.). Marcel Dekker, New York.

Funtenberger, S., Dumay, E., and Cheftel, J. C. (1995). Pressure-induced aggregation of X-lactoglobulin in pH 7 buffers. *Lebensmittel Wissenschaft und Technologie*, **28**, 410–418.

Gabriele, D., Migliori, M., Rossi, C.O.,Ruffolo, S.A. (2009). Characterisation of dairy emulsions by NMR and rheological techniques. *Food Hydrocolloids* **23**, 619–628.

Gardner, H.W.(1979). Lipid hydroperoxide reactivity with proteins and amino acids: A review. *Journal of Agricultural Food Chemistry,* **27**, 220-229.

Gibson, C. A., Elliot, J. A., and Beckett, D. C. (1960). Ozone for controlling mold on Cheddar cheese. *Canadian Dairy and Ice Cream Journal*, **14**, 24–28.

Graham, H. N., Struder, V. V., and Gurkin, M. (1969). Conversion of green tea using ozone. *US patent*, **3**,484,247.

Graham, D.E. and Philips, M.C. (1980). *Journal Colloid Interface Science*. **76**, 240.

Greene, A. K., Few, B. K., and Serafini, J. C. (1993). Ozonated vs chlorinated sanitization of stainless stell surfaces soiled with milk spoilage organisms. *Journal of Dairy Science,* **76**, 3617–3620.

Guerin-Dubiard, C., Pasco, M., Hietanen, A., Quiros del Bosque A., Naua F. Croguennec T.(2005).Hen egg white fractionation by ion-exchange chromatography. *Journal of Chromatography A*, **1090**, 58–67.

Gulseren, I., Guzey, D., Barry, D., Bruce, C. (2007). Structural and functional changes in ultrasonicated bovine serum albumin solutions, *Ultrasonics Sonochemistry* ,**14**,173–183.

Guzel-Seydim, Z. B. (1996). The use of ozonated water as a cleaning agent in dairy processing equipment. Thesis.

Guzel-Seydim, Z. B., Wyffels, J. T., Greene, A. K., and Bodine, A. B. (2000). Removal of dairy soil from heated stainless steel surfaces: Use of ozonated water as a prerinse. *Journal of Dairy Science*, **83**, 1887–1891.

Guzey, D., (2001). High-intensity ultrasonic processing improves emulsifying properties of proteins. *Colloidal and Interfacial Food Science Laboratory*, **18**,967–975.

Gwartney, E.A., Larick, D.K. and Foegeding, E.A. (2004). Sensory texture and mechanical properties of stranded and particulate whey protein emulsion gels. *Journal of Food Science*, **69**, 333-339.

Halling, P.J. (1981).Crit. Rev. Food Science and Nutrition 155.

Hambling, S.C., McAlpine, A.S. and Sawyer, L. (1992). Beta-lactoglobulin In Advanced Dairy Chemistry Vol.1. *Proteins.* Fox, P.F., (Ed.). Elsevier Applied Science. London and New York, 141-190.

Harper, W. J. (2000). Biological properties of whey components: A review. Chicago, IL: American Dairy Products Institute.

Hendrix, T., Griko, Y.V. and Privalov, P.L. (2000).A calorimetric study of the influence of calcium on the stability of bovine α-lactalbumin, *Biophysical Chemistry*, **84**, 27-34.

Hill, A. G., and Rice, R.G. (1982). Historical background, properties and applications. Rice, R. G. (Ed.), Ozone treatment of water for cooling application. Ann Arbor, MI: Ann Arbor Science Publishers, 1–37.

Howell, N.K., Herman,, H. and Li-Chan. E.C.Y. (2001). Elucidation of protein-lipid interactions in lysozyme – Corn oil system by fourier transform raman spectroscopy. *Journal of Agricultural* Food Chemistry, **49**, 1529-1533.

Hudson, H. M., Daubert, C. R., & Foegeding, E. A. (2000). Rheological and physical properties of derivitized whey protein isolate powders. *Journal of Agricultural Food Chemistry,* **48**, 3112–3119.

Ikeda, S., Nishinari, K., and Foegeding, E. A. (2001). Mechanical characterization of network formation during heat-induced gelation of whey protein dispersions. *Biopolymers*, **56**, 109–119.

Johnson, E. A, and Larson, A. E (2004). Lysozyme. *In Antimicrobials in food*. Boca Raton, FL: Taylor and Francis.

Jirgensons, B. (1952). Optical rotation and viscosity of native and denatured proteins. II-Influence of temperature and concentration,. *Arch. Biochem. Biophys*. **41**, 333-344.

Kakalis, L. T., and Regenstein, J. M. (1986). Effect of pH and salts on the solubility of egg white protein, *Journal of Food Science*, **51**, 1445–1455.

Karel, M., Schaich, K. and Roy, R,B. (1975). Interaction of peroxidizing methyl linoleate with some proteins and amino acids. Journal of Agric. Food Chemistry, **23,** 159-163.

Kato A., Takahaski, A., Matsudomi, N., Kobayashi, K. (1982). *Journal of Food Science, 48*, 62-65.

Kato A., Takahaski, A., Matsudomi, N., Kobayashi, K.(1985). Relationship between surface functional properties and flexibility of proteins detected by proteases susceptibility. *Journal of Agricultural Food Chemistry*, **33**, No:5.

Kauzmann W. (1957). The physical chemistry of proteins, *Annual Reviews in Physical Chemistry*, **8**, 413-438.

Kayalı, R. and Çakatay, U. (2004).Protein oksidasyonunun ana mekanizmaları, *Cerrahpaşa Tıp Dergisi*, **35**, 83-89.

Kikugawa, K., Kato, T. and Hayasaka, A. (1991). Formation of dityrosine and other fluorescent amino acids by reaction of amino acids with lipid hydroperoxides. *Lipids* **26**, 922-929.

Kim, J. G., Yousef, A. E., and Dave, S. (1999). Application of ozone for enhancing the microbiological safety and quality of foods: A review. *Journal of Food Protection,* **62(9),** 1071–1087.

Kinsella, J.E. (1982). *Structure and functional properties of food proteins*, Elsevier Applied Science, London, U.K.,72–85.

Kinsella, J. E., and Whitehaead, D. M. (1989). Proteins in whey: Chemical, physical, and functional properties. Kinsella, J. E. (Ed.). Advances in food and nutrition research.Vol. 33, San Diego: Academic Press., 343–348.

Kiokias, S., Dimakou, C., Oreopoulou, K.(2007). Effect of heat treatment and droplet size on the oxidative stability of whey protein emulsions. *Food Chemistry* **105** , 94–100.

Kongraksawech, T., Vazquez, P., Huerta-Ruelas, J. and Torres, J.A.(2008). Optical techniques to determine thermal effects on proteins.*International Journal of Food Science and Technology,* **43**, 1922–1928.

Leave,r J., Law, A.J.R., Brechany, E.Y. and McCrae, C.H. (1999b).Chemical changes in b-lactoglobulin structure during ageing of protein-stabilized emulsions. *Journal of Food Science and Technology*, **34**, 503-508.

Linden, G., Lorient, D.(1999).New Ingredients in Food Processing. Lewis M.D. Ed.,Washington: CRC Press

LI, M.S., Klimov, D.K. and Thirumalai, D. (2004).Thermal denaturation and folding rates of single domain proteins: size matters, *Polymer,* **45**, 573-579.

Kogelschatz, U. (1988). Advanced ozone generation. Stucki, S.(Ed.), *Process technologies for water treatment.* NewYork: Plenum Publishers, 87–120.

Nohara, D., Mizutani, A., Sakai, T. (1999).Kinetic Study on Thermal Denaturation of Hen Egg-White Lysozyme Involving Precipitation. *Journal of Bioscience and Bioengineering* Vol. 87, **2**, 199-205.

Majchrowicz, A. (1998). Food safety technology: a potential role for ozone. *Agricultural Outlook, Economic Research Service/USDA*, **3**,13–15.

Manley, T.C., Niegowski, S.J. (1967). Ozone. *In Encylopedia of chemical technology,* Vol:14 2nd Ed., Wiley, New York, Ny, 410-432.

Mann, B., and Malik, R. C. (1996). Studies on some functional characteristics of whey protein–polysaccharide complex. *Journal of Food Science and Technology*, **33(3),** 202–206.

Matoba, T., Yonezawa, D., Nair, B.M. and Kito, M. (1984). Damage of amino acid residues of protein after reaction with oxidizing lipids: estimation by proteolytic enzymes. *Journal of Food Science*, **49,** 1082-1084.

Mehlman, M. A., and Borek, C. (1987). Toxicity and biochemical mechanisms of ozone. *Environmental Research*, **42**, 36–53.

Meza, B.E., Verdini, R.A., and Rubiolo, A.C.(2008). Viscoelastic behaviour of heat-treated whey protein concentrate suspensions. *Food Hydrocolloids* ,**23**, 661–666.

Miller, G. W., Rice, R. G., Robson, C. M., Scullin, R. L, Kuhn, W.,and Wolf, H. (1978). An assessment of ozone and chlorine dioxide technologies for treatment of municipal water supplies. US Environmental Protection Agency Report No. EPA-600/2-78-147. Washington, DC: US Government printing Office.

Mine, Y., and Bergougnoux (1998). European Biochemical Societies, 284(2), 293–294. Adsorption properties of cholesterol reduced egg yolk low-density liprotein at oil-in-water interfaces. *Journal of Agricultural and Food Chemistry,* **46**, 2152–2158.

Morrisey, P. A., Mulvihill, D. M., O'Neill, M. O. (1982). Functional properties of muscle proteins. Hudson, B. J. F. (Ed*.), Developments in food proteins*, Vol. 5, 195–256.

Mulvihill, D. M., and Ennis, M. P. (2003). Functional milk proteins: Production and utilization. Fox, . P. F. and McSweeney, P. L. H. (Eds.), *Advanced dairy chemistry*. Part B, Vol. 1 New York: Kluwer Academic. 1175–1228.

Mustafa, M. G. (1990). Biochemical basis of ozone toxicity. *Free Radical Biology and Medicine,* **9**, 245–265.

Nakai, S., and Chan, L. (1985). Structure modification and functionality of whey proteins: Quantitative structure-activity relationship approach. *Journal of Dairy Science,* **68(10),** 2763–2772.

Narsimhan G. (1991).*Colloids and Surfactant.* (In press).

Nebel, C. (1981).'Ozone' in Encylopedia of Chemical Technology, Vol.14, 3rd ed. New York: John Wiley & Sons. Inc.683-713.

Norton, J. S., Charig, A. J., and Demoranville, I. E. (1968). The effect of ozone on storage of cranberries. *Proceedings of the American Society for Horticultural Science,* **93**, 792–796.

Oehlschlaeger, H. F. (1978). Reactions of ozone with organic compounds. Rice, R. G. and Cotruvo, J. A. (Eds.), *Ozone/chlorine dioxide oxidation products of organic material Cleve-land*, Ozone Press International, 20–37.

Papiz, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C.T., Findlay, J.C.B., Sivaprasadarao, S., Jones, T.A., Newcomer, M.E. and Kraulis, P.J. (1986). The structure of β-lactoglobulin and its similarity to plasma retinol binding protein. *Nature* **324**, 383-386.

Paulsson, M., Hegg, P.O., and Castberg, H.B. (1985). Thermal stability of whey proteins studied by differential scanning calorimetry, *Thermochimica Acta*, **95**, 435-440.

Pearce, K. N., Kinsela, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique, *Journal of Agriculture and Food Chemistry, **26,*** 716-723.

Peleg, M. (1976). Review paper: The chemistry of ozone in the treatment of water. Water Research, 10, 361–365.

Pelegrine, D.H.G., Gasparetto, C.A. (2005). Whey proteins solubility as function of temperature and pH. *LWT-Food Science and Technology*, **38**, 77–80.

Perez, O.E., Pilosof, M.R. (2004).Pulsed electric fields effects on the molecular structure and gelationof b-lactoglobulin concentrate and egg white. *Food Research International* ,**37** ,102–110.

Pongsawatmanit, R., Harnsilawat, T., McClements, D.J.(2006).Influence of alginate, pH and ultrasound treatment on palm oil-in-water emulsions stabilized by β-lactoglobulin *Colloids and Surfaces A: Physicochem. Eng. Aspects,* **287** ,59–67.

Pryor, W.A. (1994). Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radical Biol. Med.* ,**17,** 451–465.

Putnam F.W. (1953). Protein Denaturation, Neurath, H., Bailey, A.(Eds.). *The Proteins,* Vol:I, Part B, Academic Press, New York , 408-416.

Rao V.N.M. , Skinner , G.E., (1986). Rheological properties of solid foods. *Engineering Properties of Foods*, Marcel Dekker, Inc., New York, 215-214.

Relkin, P. (1994). Differential scanning calorimetry: a useful tool for studying protein denaturation, *Thermochimica Acta*, **246**, 371-386 .

Relkin, P., Hagolle, N., Dalgleish, D. G., Launay, B. (1999). Foam formation and stabilization by pre-denatured ovalbumin, *Colloids and Surfaces B: Biointerfaces*, **12**, 409-416.

Relkin, P. (2005). Sourdet, S. Factors affecting fat droplet aggregation in whipped frozen protein-stabilized emulsions, *Food Hydrocolloids,* **19**, 503-511.

Rice, R. G. (1986). Application of ozone in water and waste water treatment. Rice, R. G. and Browning M. J. (Eds.), *Analytical aspects of ozone treatment of water and waste water* Syracuse, NY: The Institute, 7–26.

Rice, R. G., Farguhar, J. W., and Bollyky, L. J. (1982). Review of the applications of ozone for increasing storage times of perishable foods. *Ozone Science and Engineering***, 4**, 147–163.

Rice, R. G., Robson, C. M., Miller, G. W., and Hill, A. G. (1981). Uses of ozone in drinking water treatment. *Journal of the American Water Works Association*, **73(1),** 44–57.

Schokker, E. P., Singh, H., and Creamer, L. K. (2000). Heat-induced aggregation of X-lactoglobulin A and B with -lactalbumin. *International Dairy Journal*, **10**, 843–853.

Schwartz, L. W., Dungworth, D. L., Mustafa M, . G., Tarkington, B.K., and Tyler, W. S. (1976). Pulmonary responses of rats to ambient levels of ozone. *Laboratory Investigation*, **34**, 565–578.

Shaughnessy, R. (2006).Assessing Potential Health Effects and Establishing Ozone Exposure Limits for Ozone-Generating Air Cleaners. *Health Component of Ozone Review,* 3-4.

Sheldon, B. W., and Brown, A. L. (1986). Efficacy of ozone as a disinfectant for poultry carcasses and chill water. *Journal of Food Science*, **51(2),** 305–309.

Singh, H., and Hevea, P. (2003). Thermal denaturation, aggregation and gelation of whey proteins. Fox, P. F. and. McSweeney P. L. H (Eds.), *Advanced dairy chemistry,* Part B, Vol. 1.New York: Kluwer Academic, 1261–1287.

Sousa, R.C.S., Coimpra, J.S.R., Rojas, E.E.G., Minim, L.A.,Oliveira, F.C. and Minim, V.P.R. (2007). Effect of pH and salt concentration on the solubility and density of egg yolk and plasma, *LWT-Food Science and Technolology*, **40**, 1253–1258.

Sreerama, N., Venyaminov, S.Y., Woody, R.W. (2000). Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Inclusion of Denatured Proteins with Native Proteins in the Analysis, *Analytical Biochemistry*, **287**, 243-251.

Stadelman, W. J., and Cotterill, O. J. (1995). *Egg science and technology* (4th ed.). Wesport: AVI Publisher Company.

Steffe J.F. (1996). Rheological Methods in Food Process Engineering (2nd ed.).Freeman Press.

Steffe, J.F. (1992).Rheological Methods in Food Process Engineering, (1sted.), Freeman Press, East Lansing, MI.

Song, J., Fan, L., Hildebrand, P. D., and Forney, C. F. (2000). Biological effects of corona discharge on onions in a commercial storage facility. *Horticulture Technology,* **10(3),** 608–612.

Swaisgood, H.E. (1982). Chemistry of milk proteins. *In Developments in Dairy Chemistry 1. Proteins.* Fox, P.F.(Ed), London, Elsevier Applied Science Publ., 63-110.

Swaisgood, H.E. (1996). Characteristics of milk. In Food Chemistry. Fennema, O. (Ed.).(3rd ed), New York, Marcel Dekker Inc., 842-878.

Tetik S, Yardimci KT.(2007).Oxidized protein and hemostasis. Congress of VIIth Thrombosis, *Hemostasis and Angiology*. Ed. Ulutin ON (ISBN: 978-9944-5184-1-3), 279-291, Çukurova University Medicine Faculty, Adana, 4-6.

USDA. Code of Federal Regulations, Title 9, Part 381.66—poultry products; temperatures and chilling and freezing procedures. (1997). Office of the Federal Register National Archives and Records Administration, Washington, DC.

Vachier, M. C., Piot, M., and Awade, A. C. (1995). Isolation of hen egg white lysozyme, ovotransferrin and ovalbumin, using a quaternary ammonium bound to a highly crosslinked agarose matrix. *Journal of Chromatography* B, **664**, 201–210.

Vojdani, F. (1996). Solubility. Hall, G. M. (Ed.), Methods of testing protein functionality.London: Blackie Academic & Professional, 11–60.

Waldroup, A. L., Hierholzer, R. H., Forsythe, and Miller, M. J. (1993). Recycling of poultry chill water using ozone. *Journal of Applied Poultry Science Research*, **2**, 330–336.

Walstra, P.(1983). Formation of emulsions. *In Encyclopedia of Emulsion Technology,* Vol. 1, Becher, P. (Ed.) Marcel Dekker, New York.

Welzel, P.B. (2002). Investigation of adsorption- induced structural changes of proteins at solid/liquid interfaces by differential scanning calorimetry. *Thermocimica Acta,* **382**, 175-188.

White, A., Handler, P., Smith E. L., Hill, R. L., Lehman I. R. (1978). *Principles of Biochemistry*, Chapter 6, McGraw Hill, New York, USA.

Whitney, R. (1977). Milk Proteins. In Food Colloids. Graham, H.D**. (Ed.),** AVI Publ., Westport, CT., 66-151.

Whorlow R.W. (1992).*Rheological Techniques*.2nd ed. Ellis Horwood, New York.

Wickramanayaka, G. B. (1991). Disinfection and sterilization by ozone. Seymour S. B (Ed.), *Disinfection, sterilization and preservation* (4th ed.) Malvern, PA: Lea and Febiyer,182–190.

Wilde, P J (2000), `Interfaces: their role in foam and emulsion behaviour', *Current opinion in Colloid and Interface Sci*ence, **5**, 176-181.

Wit, J.N. (1989). Functional properties of whey proteins. Fox, P.F.(Ed.), *Developments in dairy chemistry,* Vol. 4. London: Elsevier Applied Science,285–321.

Wong, Y. C., Herald, T. J., and Hachmeister, K. A. (1996). Comparison between irradiated and thermally pasteurised liquid egg white on functional, physical and microbiological properties. *Poultry Science*, **75(6),** 803–808.

Wong, W. S., Camirond, W. M., and Pavlath, A. E. (1996). Structures and functionality of milk proteins. *Critical Reviews in Food Science and Nutrition*, **36(8),** 807–844.

Yang, J.T. and Doty, P. (1957). The optical rotatory dispersion of polypeptides and proteins in relation to configuration. *Journal of American Chemical Society*, **79**, 761-775.

Yang, P. P. W. and Chen, T. C. (1979a). Effects of ozone treatment on microflora of poultry meat., *Journal of Food Processing and Preservation*, **3**, 177–185.

Yang, P. P. W. and Chen, T. C. (1979b). Stability of ozone and its germicidal properties on poultry meat microorganisms in liquid phase, *Journal of Food Science*, **44**, 501–504.

Zamora R, Gallardo E, Navarro JL and Hidalgo FJ. (2005). Strecker-type degradation of phenylalanine by methyl 9,10-epoxy-13-oxo-11-octadecenoate and methyl 12,13-epoxy 9- oxo-11-octadecenoate. *Journal of Agricultural Food Chemistry*, **53,** 4583-4588.

Zhao, J., and Cranston, P. M. (1995). Microbial decontamination of black pepper by ozone and the effect of the treatment on volatile oil constituents of the spice. *Journal of Science Food and Agriculture*, **68**, 11–18.

APPENDICES

Table A.1. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different ozone concentration on foam stability of whey protein isolate.

ANOVA

Foam Stability					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12740,944	5	2548,189	245,280	,000
Within Groups	124,667	12	10,389		
Total	12865,611	17			

Foam Stability

Duncan

Ozone Capacity (ppm)	N	Subset for alpha = 0.05			
		1	2	3	4
0,18	3	1,40000E1			
0,14	3	1,43333E1			
2,5	3		4,00000E1		
3,2	3			6,56667E1	
4,0	3			6,63333E1	
4,5	3				8,33333E1
Sig.		,901	1,000	,804	1,000

Means for groups in homogeneous subsets are displayed.

Table A.2. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different ozone concentration on foam capacity of whey protein isolate.

ANOVA

Foam volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	48,949	5	9,790	74,669	,000
Within Groups	1,573	12	,131		
Total	50,523	17			

Foam volume

Duncan

Ozone Capacity (ppm)	N	Subset for alpha = 0.05		
		1	2	3
0,18	3	5,0000		
0,14	3	5,5000		
4,0	3		8,4000	
2,5	3		8,4667	
3,2	3		8,6000	
4,5	3			9,2667
Sig.		,117	,532	1,000

Means for groups in homogeneous subsets are displayed.

Table A.3. ANOVA and Multiple Comparison Test (Duncan Test) table for the effects of ozone treatment at different temperatures on foaming stability of whey protein isolate.

ANOVA

Foam Stability					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1004,333	3	334,778	9,680	,005
Within Groups	276,667	8	34,583		
Total	1281,000	11			

Foam Stability

Duncan

Temperature (°C)	N	Subset for alpha = 0.05	
		1	2
8	3	66,3333	
15	3		83,3333
20	3		85,6667
26	3		90,6667
Sig.		1,000	,181

Means for groups in homogeneous subsets are displayed.

Table A.4. ANOVA and Multiple Comparison Test (Duncan Test) table for the effects of ozone treatment at different temperatures on foaming capacity of whey protein isolate.

ANOVA

Foam volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,667	3	,556	17,094	,001
Within Groups	,260	8	,033		
Total	1,927	11			

Foam volume

Duncan

Temperature (°C)	N	Subset for alpha = 0.05		
		1	2	3
15	3	8,4000		
8	3		8,8667	
20	3			9,2667
26	3			9,3333
Sig.		1,000	1,000	,663

Means for groups in homogeneous subsets are displayed.

Table A.5. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different protein concentration of solution on foam stability of whey protein isolate.

ANOVA

Foam Stability					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12573,583	3	4191,194	200,376	,000
Within Groups	167,333	8	20,917		
Total	12740,917	11			

Foam Stability

Duncan

Protein Concentration	N	Subset for alpha = 0.05		
		1	2	3
0.01%	3	8,6667		
0.005%	3	15,0000		
0.005% ozonated	3		32,0000	
0.01% ozonated	3			90,6667
Sig.		,128	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Table A.6. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different protein concentration of solution on foam capacity of whey protein isolate.

ANOVA

Foam volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	83,330	3	27,777	651,145	,000
Within Groups	,341	8	,043		
Total	83,671	11			

Foam volume

Duncan

Concentration	N	Subset for alpha = 0.05			
		1	2	3	4
0.005 %	3	2,0600			
0.01%	3		4,2500		
0.005% ozonated	3			7,5000	
0.01% ozonated	3				8,7333
Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Table A.7. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different ozonation methods on foam stability of whey protein isolate.

ANOVA

Foam stability					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11252,250	3	3750,750	106,153	,000
Within Groups	282,667	8	35,333		
Total	11534,917	11			

Foam stability

Duncan

Samples	N	Subset for alpha = 0.05		
		1	2	3
0.01% nonozonated	3	10,3333		
0.2 % gas ozonation	3	10,6667		
0.01% gas ozonation	3		24,6667	
0.01 % aqueous ozonation	3			84,6667
Sig.		,947	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Table A.8. ANOVA and Multiple Comparison Test (Duncan Test) table for the effect of different ozonation methods on foam capacity of whey protein isolate.

ANOVA

Foam volume

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41,876	3	13,959	46,658	,000
Within Groups	2,393	8	,299		
Total	44,269	11			

Foam volume

Duncan

Types	N	Subset for alpha = 0.05		
		1	2	3
0.01% nonozonated	3	4,6000		
0.2 % gas ozonation	3	5,0000		
0.01% gas ozonation	3		7,1667	
0.01 % aqueous ozonation	3			9,2667
Sig.		,397	1,000	1,000

Means for groups in homogeneous subsets are displayed.

Table A.9. ANOVA for the comparison of different ozonation methods on solubility of whey protein isolate at constant concentration (0.1% w/w).

ANOVA

SOLUBILITY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,003	1	,003	12,143	,025
Within Groups	,001	4	,000		
Total	,004	5			

Table A.10. ANOVA for the comparison of different ozonation methods on solubility of whey protein isolate at fixed ozonation time (15 min).

ANOVA

SOLUBILITY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,004	1	,004	66,977	,001
Within Groups	,000	4	,000		
Total	,004	5			

Table A.11. ANOVA for the comparison of different ozonation methods on solubility of egg white proteins at constant concentration (0.1% w/w).

ANOVA

SOLUBILITY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,000	1	,000	30,522	,009
Within Groups	,001	4	,000		
Total	,001	5			

Table A.12. ANOVA for the comparison of two different ozonation methods on solubility of egg white proteins at fixed ozonation time (15 min).

ANOVA

SOLUBILITY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,010	1	,010	28,528	,013
Within Groups	,001	3	,000		
Total	,011	4			

Table A.13. ANOVA and Multiple Comparison Test (Duncan Test) for the effect of different protein concentration and ultrasound treatment on EAI of whey protein isolate.

ANOVA

EAI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,239	2	,119	3,979E4	,000
Within Groups	,000	6	,000		
Total	,239	8			

EAI

	concentration	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	0.05% 15 sec.	3	1,927000E0		
	0.05% 1 min.	3		2,029000E0	
	0.1% 1min.	3			2,312000E0
	Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.14. ANOVA and Multiple Comparison Test (Duncan Test) for the comparison of kinetic constants (k1) in first stage on ESI of whey protein isolate.

ANOVA

ESICONSTANT					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,000	2	,000	84,000	,000
Within Groups	,000	6	,000		
Total	,000	8			

ESICONSTANT

	ESICONCENTRATION	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	0.05% 1min.	3	-1,8000000E-3		
	0.05% 15 sec.	3		-1,6000000E-3	
	0.1% 1min.	3			-8,0000000E-4
	Sig.		1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.15. ANOVA and Multiple Comparison Test (Duncan Test) for the comparison of kinetic constants (k2) in second stage stage on ESI of whey protein isolate.

ANOVA

ESICONSTANT					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,000	2	,000	1,000	,422
Within Groups	,000	6	,000		
Total	,000	8			

ESICONSTANT

			Subset for alpha = 0.05
	ESICONCENTRATION	N	1
Duncan[a]	0.1% 1min.	3	-,0000800
	0.05% 15 sec.	3	-,0000700
	. 0.05% 1min.	3	-,0000700
	Sig.		,281

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.16. ANOVA and Multiple Comparison Test (Duncan Test) for the effect of ozone on denaturation temperature of whey protein isolate.

ANOVA

Thermal Denaturation Temperature (°C)					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	63,915	3	21,305	188,873	,000
Within Groups	,902	8	,113		
Total	64,817	11			

Thermal denaturation temperature (°C)

	Ozonation Time (min)	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	0	3	7,873000E1			
	4.5	3		8,065000E1		
	5	3			8,184000E1	
	15	3				8,508000E1
	Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.17. ANOVA and Multiple Comparison Test (Duncan Test) for the effect of ozone on transition enthalpy of whey protein isolate.

ANOVA

Enthalpy					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	190,183	3	63,394	4,577E3	,000
Within Groups	,111	8	,014		
Total	190,294	11			

Enthalpy

	Ozonation Time (min.)	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	15	3	4,390000E0			
	5	3		1,164000E1		
	4.5	3			1,268000E1	
	0	3				1,506000E1
	Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.18. ANOVA and Multiple Comparison Test (Duncan Test) for the effect of ozone on denaturation temperature of egg white proteins.

ANOVA

Thermal Denaturation Temperature (°C)					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3,194	4	,798	443,583	,000
Within Groups	,018	10	,002		
Total	3,212	14			

Thermal denaturation temperature (°C)

	Ozonation Time (min)	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	15	3	8,361000E1			
	30	3	8,367000E1			
	5	3		8,442000E1		
	4.5	3			8,451000E1	
	0	3				8,474000E1
	Sig.		,114	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.19. ANOVA and Multiple Comparison Test (Duncan Test) for the effect of ozone on transition enthalpy of egg white proteins.

ANOVA

Enthalpy

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80,890	4	20,223	1,605E4	,000
Within Groups	,013	10	,001		
Total	80,903	14			

Enthalpy

	Ozonation Time (min.)	N	Subset for alpha = 0.05				
			1	2	3	4	5
Duncan[a]	30	3	6,460000E0				
	15	3		8,610000E0			
	5	3			1,062000E1		
	4.5	3				1,215000E1	
	0	3					1,275000E1
	Sig.		1,000	1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table A.20 Amino Acid Composition of β-lactalbumin.

Amino acid composition of β-lactoglobulin	
Constituent	%
Phenylalanine	3.78
Leucine	15.50
Isoleucine	5.86
Methionine	3.22
Tyrosine	3.86
Valine	5.62
Proline	5.14
Glutamic acid	19.08
Aspartic acid	11.52
Alanine	7.09
Threonine	4.92
Serine	3.96
Glycine	1.39
Arginine	2.91
Lysine	12.58
Histidine	1.63
Cystine+Cystine	3.40
Trytophan	1.94

Table A.21 Amino Acid Composition of Ovalbumin.

Amino acid composition of Ovalbumin	
Constituent	%
Phenylalanine	8.78
Leucine	6.30
Isoleucine	5.77
Methionine	5.51
Tyrosine	3.71
Valine	5.56
Proline	2.96
Glutamic acid	13.46
Aspartic acid	7.49
Alanine	5.50
Threonine	3.09
Serine	5.91
Glycine	2.48
Arginine	5.13
Lysine	5.30
Histidine	2.08
Cystine+Cystine	3.16
Trytophan	1.10

Correlation Coefficient
0.9987

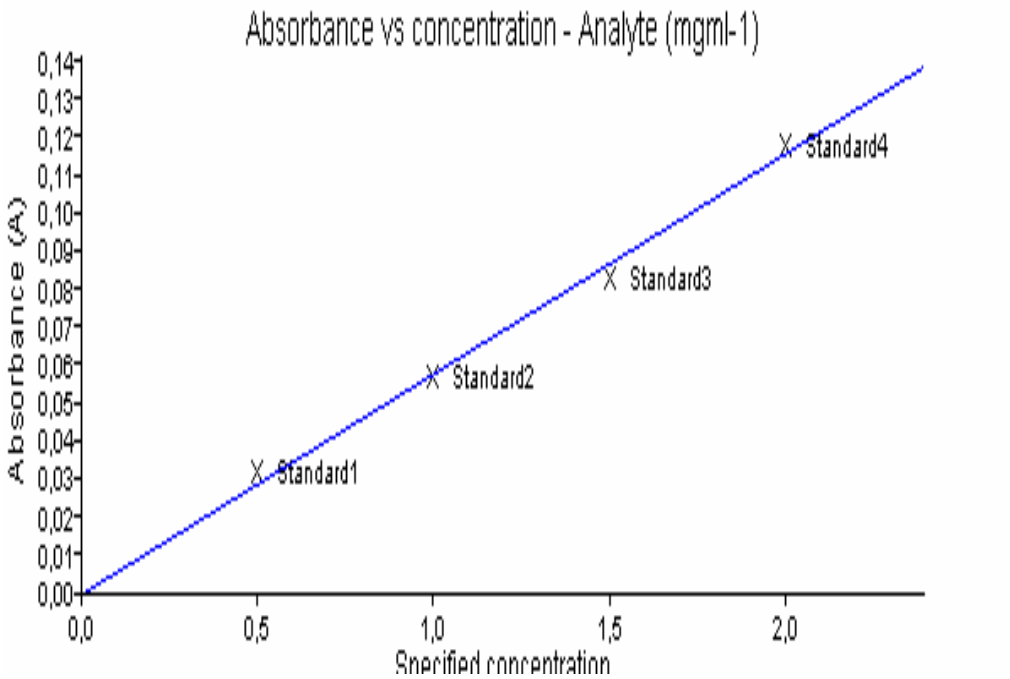

Figure A.1.Standart Curve for protein determination by Biuret method.