THE EFFECT OF TEMPERATURE AND EXTRACTION TECHNIQUE ON THE BINDING INTERACTIONS AND HYDROLYSIS OF β-LACTOGLOBULIN WITH MILK FAT GLOBULE MEMBRANE (MFGM)

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COMMITTEE MEMBERSHIP

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ABSTRACT

The Effect of Temperature and Extraction Technique on the Binding Interactions and Hydrolysis of β-Lactoglobulin with Milk Fat Globule Membrane (MFGM)

Corbin R. Kembel

Whey protein, containing predominately β-Lactoglobulin (β-LG), is a popular choice among consumers looking for an excellent protein source. Likewise, fat is a natural component in milk and dairy products. Lipids are packaged within a membrane called the Milk Fat Globule Membrane (MFGM). The MGFM contains a variety of lipids and proteins. Although β-LG has been extensively characterized, the function of the protein is largely unknown. The objectives of this study were to assess the enzymatics of β-LG in an isolated system, evaluate the propensity of β-LG to bind to MFGM, determine the effects of temperature and fat extraction method on the conformation of β-LG, assess the antigenicity of β-LG in an isolated system, and determine the effects of temperature and fat extraction method on the antigenicity of β-LG peptides. Time course hydrolysis of β-LG reveled only slight differences in cleavage rate. A Mass Spectrometry method was developed to detect β-LG peptides. Whey Protein Isolate (WPI) digests in an isolated system yielded on average 8.67 ± 0.33 unique peptides and a protein sequence coverage of 43.67 % ± 1.33. When WPI was a component in a complex system of washed cream, it was found that there was an interaction between temperature and fat extraction method ($P=0.001$) in the individual peptide release. However, it was found that the total number of peptides released was dependent on the extraction method ($P<0.001$). Further, it was found that at control temperatures, MFGM and β-LG form a complex. Due to the natural affinity of β-LG with MFGM, these results suggest that the biological function of β-LG

is to aid in digestion. In addition, it was found that treatment of β-LG with Sc-CO_2 resulted in a decrease in antigenicity. Investigating the binding complex of β-LG and MFGM, utilizing a sensitive analytical instrument and technique, illustrates how β-LG peptides can be accurately detected, quantified, and how conformational changes within the structure protein can be used to infer information regarding the function of the protein.

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CHAPTER 1: INTRODUCTION

The predominant protein in whey, β-Lactoglobulin (β-LG), was first discovered in 1934 (Madureira et al., 2007) and has subsequently been extensively characterized (Farrell et al., 2004). However, the function or purpose of the protein is largely unknown. β-LG is an abundant source of bioactive peptides that elicit a wide range of health benefits throughout the body (Hernandez-Ledesma et al., 2008). However, the hydrolysis of β-LG is critical in releasing the correct peptides, which exhibit these bioactive properties. Therefore, the function of these peptides is completely dependent on the initial conformational state and subsequent hydrolysis of the protein with an appropriate enzyme.

Understanding the changes in conformation within β-LG in the context of various conditions will improve the ability to release desirable, beneficial peptides from β-LG. Furthermore, the hydrophobic core of the β-barrel of β-LG has shown the capacity to bind a wide range of hydrophobic molecules including phospholipids (Puyol et al., 1991; Wu et al., 1999; Wang et al., 1997). Given that lipids are a natural component in milk with whey proteins, it is original to investigate the interaction and binding of Milk Fat Globule Membrane (MFGM) and β-LG. Furthermore, because β-LG has been shown to bind phospholipids, perhaps the function of β-LG involves the binding to MFGM and aiding in digestion.

The work completed attempts to assess the enzymatics of β-LG in an isolated system, the ability of β-LG to form a complex with MFGM, the effects of temperature and fat extraction method on the changes in conformation of β-LG, and the antigenicity of β-LG.

The purpose of this thesis is to contribute to the growing knowledge of β-LG and to the purpose of the protein. Furthermore, to determine how MFGM and various processing conditions influence the release of particular peptides of β-LG.

CHAPTER 2: LITERATURE REVIEW

2.1 Whey Introduction

2.1.1 History

Whey is a co-product of cheese making, where following coagulation of casein by chymosin action, liquid whey is separated (Zadow, 1993). Whey contains a rich source of proteins, peptides, vitamins, minerals, and lactose (Bullerman & Berry, 1966) and subsequently has an extremely high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) that can result in a high polluting factor. Because of the high polluting factor of whey, new legislation and regulations promulgated in the 1970's have encouraged the industry to evaluate production and processing alternatives. Subsequently, a transformation of the cheesemaking industry has occurred. Due to increased environmental regulations, the industry has rapidly transformed with technological advances, scientific support, and nutritional backing (Smithers, 2008).

Technological advances have vastly changed the whey processing environment. These advances include concentration, transformation, fractionation, and dehydration of the whey stream (Smithers, 2008). In addition, more sophisticated technology for whey processing has been developed which includes chromatography, electrodialysis, and membrane processing (Houldsworth, 1980).

2.1.2 Impacts on the Industry

Transformation of the whey steam has taken a dramatic course over the past 50 years. Previously a tremendous nuisance for disposal, whey is now an incredible co-product

from cheesemaking and casein manufacture. Advances in science and technology have transformed whey from a single disposal ingredient into a portfolio of ingredients including Whey Protein Concentrate (WPC), Whey Protein Isolate (WPI), lactose, lactoferrin, individual fractionated Whey Proteins, and hydrosylates for cheesemakers. This expansion in ingredients has opened opportunities for the dairy industry in the baking, confectionary, infant health, adult health, elderly health, and animal health industries. The Whey stream has truly gone from "Gutter to Gold" (Smithers, 2008). In March 2014 alone, the production of whey powder totaled 65.8 million pounds, WPC totaled 46.2 million pounds, WPI totaled 8.4 million pounds, and lactose totaled 100.3 million pounds (NASS, 2014). The utilization of whey has made a significant financial impact on the industry (Smithers, 2008). Processors and scientists alike continue to investigate the beneficial properties of whey by separating out milk and whey components. The first generation of whey ingredients was sweet whey powder. The second generation of whey ingredients was WPC and WPI. The next generation of whey ingredients will undoubtedly be refined whey proteins and whey components such as oligosaccharides, lactoferrin, α-Lactalbumin (α-LA), and β-Lactoglobulin (β-LG) (Barbano, 2014). With the emergence of these new ingredients there has been concurrent research that has highlighted the impacts on health.

2.1.3 Impacts on Health

The nutritional knowledge of whey is constantly being evaluated. It is known that whey protein possesses a higher biological value than egg protein, the previous gold standard. This property is important because the higher the biological value, the higher proportion

of protein becomes incorporated into the body and used in protein synthesis (Smithers, 2008). Whey is also a rich source of amino acids, particularly in branched chain amino acids (BCAAs) that aid in metabolic regulation (Smilowitz et al., 2005). In addition, the biological properties of whey have shown to be antimicrobial, antihypertensive, antioxidant, antithrombotic, opioid agonists, mineral binding, and satiety promoting (Madureira et al. 2007).

2.2 Whey Proteins and β-LG

2.2.1 Introduction

Liquid whey is composed of 65 g of solids per kilogram, which is made up of approximately 50 g lactose, 6 g protein, 6 g ash, 2 g non-protein nitrogen, and 0.5 g fat (Zadow, 1986). The major components of whey include β-LG, α-LA, serum albumin, Ig, and proteose-peptone (Farrell et al., 2004). β-LG is the predominant protein in whey accounting for 50-60% of the protein in whey, while α-LA accounts for 25-30% of the protein in whey (Zadow, 1986).

2.2.2 Whey Processing

The manufacture of WPC generally includes ultrafiltration, diafiltration, and nanofiltration, while the manufacture of WPI includes ultrafiltration, microfiltration, diafiltration, and nanofiltration (Korhonen et al., 1998). Commercial WPC is commonly 34-82% protein while commercial WPI is over 90% protein.

Whey has numerous applications in food and non-food settings and the processing and storage conditions can determine the functionality of the ingredient and which of the

applications whey is best suited. These functionalities include emulsification, foaming, solubility, viscosity, color, texture, taste, and water and fat binding capacity (Jost, 1993). Some of these properties are inherent to the proteins found in whey. For instance, β-LG naturally possesses good foaming, emulsifying, gelation, solubility, and aroma binding properties. On the other hand, α-LA possesses good foaming, emulsifying, and solubility properties with an excellent peptide profile (Hegg, 1982). Due to the high concentration of β-LG in whey protein, the functional properties of β-LG can determine the functionality of the overall whey protein product (Korhonen et al., 1998). Therefore any processing effects on β-LG must be considered.

2.2.3 β-Lactoglobulin

β-LG was first discovered in 1934; however not much about the proteins function has been discovered since then (Madureira et al., 2007). β-LG is synthesized in the mammary gland of ruminant animals (Eigel et al., 1984). To date, 10 genetic variants of β-LG have been characterized (Farrell et al., 2004). The biological function of the protein is unclear. It has been observed that β-LG is very stable at low pH values and has been hypothesized to act as a carrier of nutrition from the mother to the neonate (Cho et al., 1994). Several studies have supported this hypothesis and found that β-LG is capable of transporting retinol (Puyol et al., 1991), palmitate (Wu et al., 1999), Vitamin D and Cholesterol (Wang et al., 1997), and notably fatty acids (Puyol et al., 1991). However, since only ruminant animals produce β-LG, not humans, β-LG has been subject to many allergenic and immunomodulatory studies.

β-LG is a globular protein containing 162 amino acids (Brautnizer et al., 1973) with 8 β-sheet residues arranged in a β-barrel attached to a single α-helix. At milk pH (pH: 6.6-6.8), β-LG exists as a dimer (Papiz et al., 1986). The β-barrel contains a highly hydrophobic core that is capable of binding hydrophobic molecules such as retinol (Wit, 1998). Although β-LG has been subject to allergenic claims, it has also been acknowledged to he of high nutritional and functional value (Chatterton et al., 2006).

2.2.4 Bioactivity

Recent research has focused on the bioactivity of β-LG peptides that provide antihypertensive, antioxidant, antimicrobial, and immunostimulating properties. These β-LG peptides are between two and twenty amino acids in length and the sequence of these peptides greatly influences the peptide function (Hernandez-Ledesma et al., 2008). The release of these peptides is highly depended on the processing conditions and subsequent gastrointestinal digestion within the body. In addition, to the conformation of the protein will often determine what peptides are released and how many peptides are released. Once released, these peptides can exhibit their respective bioactive properties (Hernandez-Ledesma et al., 2008). Therefore, a number of studies have focused on the enzymatic release of bioactive peptides from β-LG. A more complete understanding of the release of these bioactive peptides is needed in the context of whey processing in order to bring additional value to specialty whey products. Further, and understanding of the structure-function relationship of β-LG is needed to understand the purpose of the protein in relation to its structure and how changes in processing conditions influence the conformational changes of the protein.

2.3 Milk Fat Globule Membrane (MFGM) and Whey Lipids

2.3.1 Composition

Milk fat consists mostly of triacylglycols (95%) that are secreted within a tri-layer membrane network called the Milk Fat Globule Membrane (MFGM)(Patton & Keenan, 1975). Milk lipids originate from the rough endoplasmic reticulum of the mammary gland epithelial cells. As the lipids emerge from the apical membrane they are encased in a membrane called the Milk Fat Globule Membrane (MFGM) (Nielsen et al., 1999; Robenek et al., 2006a, Robenek et al., 2006b). The MFGM consists of lipids, triacylglycols, sterols, fatty acids, hydrocarbons, phospholipids, and various proteins (Patton & Keenan, 1975; Keenan et al., 1988). Of these components, proteins only contribute to approximately 1% of the total MFGM mass (Patton & Huston, 1986). However, proteins and phospholipids together make up over 90% of the weight of the membrane (Singh, 2006). The MFGM is arranged with the triacylglycols located within microlipid droplets in the cytoplasm. The triacylglycols are surrounded by a monolayer of phospholipids, proteins, and cholesterol. After secretion, the lipid droplet is encased in a bi-layer of polar lipids. This tri-layer structure contains ordered domains of phospholipids and cholesterol and a heterogeneous distribution of proteins (Singh, 2006). The MFGM can be isolated through a series of centrifugation and washing steps or obtained from buttermilk, a by-product of butter making. The MFGM is incredibly fragile and is can be severely effected by processing treatments such as heating, cooling, homogenization, and spray drying (Walstra, 1995).

Many recent studies have indicated the potential health benefits of the MFGM, most notably due to the relatively high amounts of phospholipids.

2.3.2 Nutritive Value

The MFGM is a very complex system with many proteins, lipids, and sterols. Each of these components varies depending on the individual cow, the season, and stage of lactation (Mondy & Keenan, 1993; Ye et al., 2002). Several studies have highlighted the health promoting effects of the components of the MFGM. Polar lipids and sphingolipids have shown to be anticholesterolemic (Noh & Koo, 2004) and provide maturation to the human neonate's gut (Oshida et al., 2003). Among the phospholipids, phosphotidylserine has been shown to restore normal memory (McDaniel et al., 2003) and phosphotidylcholine has been shown to protect gastrointestinal mucosa from toxins (Anand et al., 1999). Among the MFGM proteins, butyrophilin has been shown to suppress Multiple Sclerosis (Guggenmos et al., 2004), mucin 1 to enlist a protective effect against rotovirus infection (Kvistgaard et al., 2004), and lactadherin to aid in protection from viral gut infection (Kvistgaard et al., 2004).

2.3.3 Presence of MFGM and MFGM Components in Whey

MFGM and its components are in a variety of dairy products, most notably buttermilk. In addition, WPC contains between 3.3-7.38% total lipids and 0.8-1.54% phospholipids (Morr & Foegeding, 1990). In WPC 75, the proportion of phospholipids to triacylglycol is roughly 1:3 (Vaghela & Kilara, 1995). Despite the extensive processing steps involved

in WPC manufacture including Microfiltration and Ultrafiltration, the MFGM components including phospholipids are still present in the Ultrafiltration retentate. This represents an interesting observation that will be considered in this study.

2.4 Emulsions

2.4.1 Introduction

Lipids are rarely isolated within a system and frequently interact with other components such as proteins and carbohydrates. Likewise, proteins are rarely isolated within a system and frequently interact with other proteins, carbohydrates, water, and lipids. While it is important to understand the individual properties of a protein, phospholipid, or MFGM, it is imperative that these individual components be studied in a natural system, not an isolated system.

2.4.2 Protein and Fat Interactions

Protein folding is determined by the lowest free energy and is governed by maximizing polar group interactions with water and minimizing non-polar group interactions with water (Anfinsen, 1973). Protein folding is not static and the protein concentration can greatly influence these transitions to the lowest free energy. These transitions are influenced by pH, temperature, ionic strength, and other components (Mangino, 1984). Thus, many conformations of the same protein can exist given that only slight differences in energy may exist. Incorporation of other components such as lipids and additional activation energy can cause proteins to assume a lower free energy than would be the case in their native state. This is where conformational changes occur (Hettiarachchy &

Ziegler, 1994). Due to the nonpolar and polar regions of proteins, proteins can interact at the interface of fats, exposing hydrophilic and charged regions and burying hydrophobic regions within the bi-layer will decrease the total energy of the protein leading to a favorable interaction (Tanford, 1970). Given sufficient activation energy for unfolding or for a conformational change to occur, the protein will arrive at a lower energy state. Optimal conditions for protein-lipid emulsions to occur have been shown to be around 60°C (Tanford, 1980). MacRitchie (1978) described this process to be irreversible, as the removal of a hydrophobic group from the lipid phase would increase the total energy of the system (MacRitchie, 1978).

The process of protein-lipid interaction begins with the first hydrophobic group inserting into the lipid interface. Depending on the structure of the protein, additional activation energy may need to be applied. This process is called adsorption. In order for adsorption to occur, a minimum of six to eight adjacent hydrophobic amino acid residues on the protein is needed. As this occurs, the misfolding of the protein begins. This process is called spreading. The spreading of the protein is determined by the hydrophobicity and structure of the protein (Graham & Phillips, 1979). This interaction between protein and lipid is expedited by the presence of MFGM due to its excellent emulsification properties (Kanno et al., 1991). Furthermore, when β-LG is present with phosphotidylcholine, β-LG undergoes β-to-α transition in the secondary structure, disrupting the hydrophobic residues allowing for insertion into the lipid layer, stabilizing the α protein component and protecting β-LG from digestion (Zhang and Keiderling, 2006; Zhang et al., 2007).

The emulsification property of MFGM is due to its amphiphilic nature (Kanno et al., 1991). Kanno and others also determined that the foam/emulsion stability, capability, and whippability were dependent on the amount of MFGM material present. However, it must be noted that the stability of these emulsions depends of the heat treatment of the cream. Raw cream without any heat treatment, maintained the best emulsifying properties (Corredig & Dalgleish, 1998).

2.4.3 MFGM and β-LG

Many studies have investigated the association of β-LG with the MFGM. This interaction has been observed to take place between 60°C and 65°C, well before β-LG is denatured by heat (Singh, 2006). There are many proposed methods for this association including sulfhydryl-disulfide interchange (Houllihan et al., 1992) and the displacement of the membrane by which the protein adsorbs to the MFGM surface (Dalgleish et al. 1991). Most notably, it was suggested that the association between β-LG and MFGM was due to the transition of β-LG into monomers prior to the free thiol group exposure. Thus, the free thiol groups initiate the thiol-disulfide interchange allowing the interaction to take place below the denaturation temperature of β-LG (Ye et al., 2004). The amount of β-LG associated with the MFGM increases with heating time above 65°C. However, this association reaches an upper limit of around 1.0 mg of β-LG per gram of fat (Corredig & Dalgleish, 1996). There has not been a scientific explanation why this low limit exists. It has been proposed that β-LG is adsorbed while still maintaining its β-barrel tertiary structure and as more time passes, hydrophobic groups spread throughout the membrane (Hettiarachchy & Ziegler, 1999).

It has been shown that during heat treatments, MFGM proteins Xanthine Oxidase and Butyrophilin remain anchored to the membrane, but PAS 6 and PAS 7 migrate to the serum phase (Houllihan et al., 1992; Kim & Jimenez-Flores, 1995; Ye et al., 2004). Furthermore, in the absence of whey proteins, PAS 7 migration is not observed (Ye et al., 2002). This observation is a potential explanation for MFGM-β-LG complex formation.

2.4.4 Applications

It is clear that β-LG and MFGM form stable interactions with moderate heat treatments through some protein-protein and/or protein-lipid complex. It is unclear what regions of β-LG are responsible for this interaction. Secondly, it is unclear how various processing conditions effect the interaction between MFGM and β-LG and the subsequent protein digestion of β-LG. A great deal of research has separately highlighted the effects of MFGM and β-LG on human health. Given the natural interactions between these two components, it opens up the door for future process innovation to capture the benefits of both components in one product. Given the propensity for these components to interact with one another, perhaps these components should not be used separately in foods. Future applications could include utilization of buttermilk, adaption of WPC production, and further development of the diafiltration permeate stream in the production of WPI.

2.5 Processing Effects

2.5.1 Whey Proteins and β-LG

The processing conditions can greatly influence the properties and structure of β-LG. The

effects of various processing conditions on β-LG have been thoroughly investigated. The denaturation of β-LG occurs between 65-75°C (Bertrand-Harb et al., 2002) and as the temperature increases it leads to an increase in collision frequency. An increase in collision frequency leads to an increase in side chains interactions, leading to extensive cross-linking and a decrease in protein solubility (Walstra et al., 2006). The effect of temperature on β-LG is two fold. The first effect of heat on β-LG is the reversible dissociation of β-LG into monomers (McKenzie, 1971; Mulvihill & Donovan, 1987). The second effect is the partial unfolding of the β-LG monomer, loosing its helical structure (Qi et al., 1997; Prabakaran & Damodaran, 1997). Pressure can also have an effect on β-LG structure. Increased pressure results in the formation of dimers, trimers, octomers, and aggregates (Chicon et al., 2006). While moderate pressure has little effect on the structure of β-LG, extreme pressure can melt the hydrophobic β-barrel (Knudsen et al., 2002).

β-LG structure is also affected by pH, which can lead to one of many transition states. Below pH 3, β-LG dissociates into monomers. Between pH 4 and 5 β-LG undergoes a dimer to octomer transition. Between pH 4.5 and 6 β-LG transitions from a native form to an acidic form that is smaller in radius, less ordered, and with more exposed groups. Between pH 6 and 8, β-LG undergoes a pH transition called the Tanford transition returning to the dimerized form. Above pH 7.5, Glu89 becomes accessible to solvent and is protonated. This results in an increase in protein hydration. Between pH 9 and 13, β-LG begins to transition from a dimer back to a monomer. As the pH increases, β-LG begins to loose its tertiary structure (Taulier & Chalikian, 2001).

2.5.2 MFGM

Much less is known regarding the processing effects on MFGM. MFGM is very fragile and can be broken with very minimal processing (Singh, 2006), reducing the natural emulsion properties (Corredig & Dalgleish, 1998).

2.5.3 MFGM and β-LG

During heating phospholipids along with other MFGM components have been shown to migrate to the serum phase interacting with proteins (Walstra, 1995). Together, β-LG has been shown to exhibit non-native helical structures in the presence of lipids (Lefevre & Subirade, 2000). β-LG forms a α-helical structure upon binding to anionic lipids but not zwitterionic lipids. Furthermore, α-helical residues of β-LG tend to associate parallel to the MFGM, while β-sheet residues of β-LG tend to associate perpendicular to the MFGM (Zhang & Keiderling, 2006). β-LG and MFGM emulsions have yet to be evaluated when subjected to various processing conditions including Supercritical CO_2.

2.5.4 Supercritical CO_2

After its emergence in the 1970's, interest in supercritical fluids soon decreased due to limited application and high cost (Bungert et al., 1998). However, there has been a renewed sense of interest in supercritical fluids with an increasing number of publications in recent years showing promising application. New applications include but not limited to ingredient processing, polymerization, and purification. These applications are possible using supercritical fluids by the adjustment of the solvent power, control of kinetics,

alteration of compound properties, the reduction of viscosities, increase of diffusion coefficients, and an overall increase in mass transfer (Bungert et al., 1998).

The solvent power of supercritical fluids is dependent on density and can be finely adjusted by changing the temperature or pressure. A small increase in pressure or decrease in temperature will increase fluid density (Sihvonen et al., 1999; Palmer & Ting, 1995). This is the attraction of using supercritical fluids as a solvent. Supercritical fluids take advantage of the solvent power near the critical point. At the critical point, gas and liquid phases merge to form a supercritical phase (Palmer and Ting, 1995). No phase boundary is crossed as both the critical temperature and critical pressure is exceeded (Niessen & Woelk, 2007). This phase has both liquid and gas like properties but is neither a gas nor a liquid. In addition supercritical fluids have a higher diffusing coefficient, lower viscosity and surface tension than a liquid solvent which contributes to a more favorable mass transfer (Sihvonen et al., 1999). The density of supercritical fluids is 100-1000 times greater than the density of gases. Therefore, molecular interactions are much stronger (Riekkola & Manninen, 1993). However, an alternative phenomenon has been discovered close to the critical point that does not follow these general observations. At low pressures it has been noted that an increase in temperature actually lowers solubility. This is due to the fact that an increase in solute vapor pressure is not enough to compensate for the reduction in solvent density (Mansoori et al., 1988).

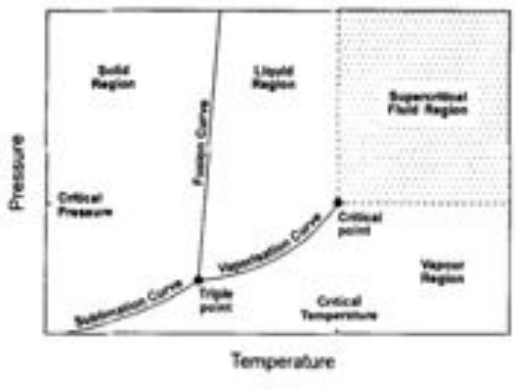

Figure 2.1. Pressure-temperature phase diagram for a pure compound (Palmer & Ting, 1995)

The attractiveness of using carbon dioxide is multifold. The properties of Sc-CO_2 include a supercritical pressure of 72 bar and a supercritical temperature of 31.1°C. Carbon dioxide is nontoxic, nonflammable, inexpensive, environmentally friendly, and can be used in food applications (Sihvonen et al., 1999). The relatively mild conditions and safety of carbon dioxide has increased the appeal in supercritical applications in the past several years. Because of its non-polar nature, it is suited for the extraction of non-polar compounds.

Most supercritical systems utilize carbon dioxide as a solvent because it is inert, inexpensive, nontoxic, nonflammable, recyclable, available in high purity, does not leave residues, and operates at safe temperatures and pressures (Palmer & Ting, 1995). Rizvi and others have shown that carbon dioxide is selective towards low molecular weight lipophilic compounds, less than 500 Daltons, as well as that with increasing molecular weight, the solubility of the compound decreases (Rizvi et al., 1986). There are additional gases that can be used for supercritical extraction, listed in the table below. However,

carbon dioxide offers a practical advantage over other gasses in industrialized applications (Steytler, 1996)

Name	Formula	T_c (°C)	P_c (bar)
Carbon dioxide	CO_2	31.1	73.8
Nitrous oxide	N_2O	36.4	71.5
Ammonia	NH_3	132.4	111.3
Ethane	C_2H_6	32.2	48.2
Propane	C_3H_8	96.6	41.9
Ethylene	C_2H_4	9.2	49.7
Freon 13	$CClF_3$	28.9	38.7

Figure 2.2. Potential gases used for near-critical fluid extraction (Steytler, 1996)

The non-polarity of supercritical carbon dioxide (Sc-CO_2) limits the extraction and solubility of polar compounds. Common methods have used co-solvents (surfactants) that increase the solubility of polar groups/compounds. This allows the extraction of both hydrophobic and hydrophilic materials (Knez, 2009). However, reviews are mixed if in fact co-solvents are effective. Co-solvents complicate the thermodynamics of the system and change the applications that can be used with supercritical fluids (Sihvonen et al., 1999).

The attraction for most industrialized applications is extraction. The main operating variables of a supercritical system are pressure, temperature, flow rate, protein concentration, pH, and holding time (Rozzi & Singh, 2002; Xu et al., 2011). There are two common supercritical systems. One, a supercritical fluid extraction system uses an extraction vessel and separation vessel is used in conjunction to isolate two products. Two, a supercritical fluid fractionation system uses an extraction vessel and multiple separation vessels in conjunction to isolate many products from a raw material (Rozzi &

Singh, 2002). Large-scale supercritical systems have been successfully used for the extraction of hop components and the decaffeination of coffee and tea (Knez et al., 2010).

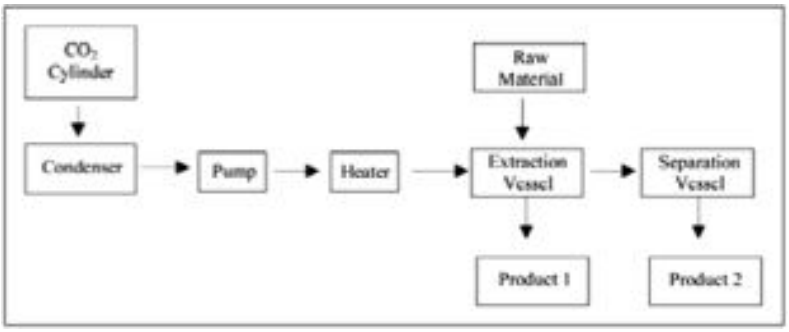

Figure 2.3. Flow diagram of a single-vessel supercritical fluid extraction system (Rozzi & Singh, 2002)

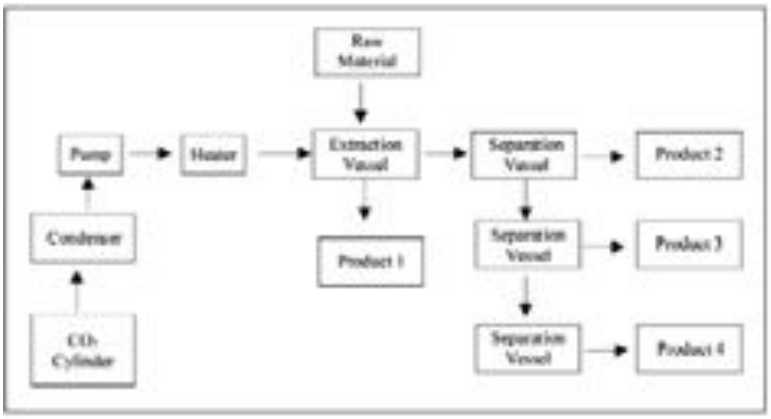

Figure 2.4. Flow diagram of a multiple-vessel supercritical fluid fractionation system (Rozzi & Singh, 2002)

Following extraction, the solute can be removed from the supercritical fluid by two methods. The pressure of the system can be adjusted leading to a non-supercritical solvent or the solute is no longer soluble in the supercritical fluid. This approach can also be accomplished by changing the temperature of the system. On the other hand, the solute can be washed out of the supercritical fluid by using a solvent the can insolubilze the

solute from the solvent. This continuous method is appealing in the industrial setting, where the system is not shut down (Rozzi & Singh, 2002).

Supercritical fluids can also be used for analytical purposes. Supercritical fluid chromatography utilizes supercritical fluid as a mobile phase. The density of the mobile phase can thus be fine tuned, just as in the mobile phase of high performance liquid chromatography (HPLC). However, supercritical fluid chromatography has more variables than can control the analysis as compared to HPLC (Poole, 2000).

Sc-CO_2 is a tool that appears to have many applications. In the context of proteins, Sc-CO_2 has been shown to change the rheological properties of whey proteins. The alteration of the functional properties, including rheological, of a protein is often associated with the modification of the protein structure (Zhong & Jin, 2008). In the context of Sc-CO_2, it has been found that temperature has a significant effect on the functional properties of whey protein. A modification of the conformation of the protein can result in the formation of protein aggregates (Xu et al., 2011).

The principle of denaturation involves the dissociation of intermolecular bonds including hydrogen bonds and disulfide bonds. This can be accomplished by heat, pressure, and/or reducing agents. This is then followed by the synthesis of new bonds between the protein(s) leading to protein aggregation (Fitzsimons et al., 2007).

The treatment of proteins with Sc-CO_2 can result in the oligomerization of proteins even under reducing conditions due to disulfide bridge formation. Treatment of ribonuclease with wet Sc-CO_2 has been reported to form protein aggregates and result in increased trypsin digestibility. The presence of water in the Sc-CO_2 system results in increased digestion of peptides due to the hydrolysis of peptide bonds (Weder, 1980). However, conflicting reports by Stahl et al., 1984 show no evidence of protein aggregation or denaturation from seed meals after treatment with Sc-CO_2 (Stahl et al., 1984). Although this report did not find evidence of aggregation or denaturation from Sc-CO_2 treated proteins, it does little to prove that Sc-CO_2 retains the native conformations of all proteins. Seed meal proteins and ribonuclease protein are completely different and have shown to interact differently under high pressure and temperature conditions. Therefore, protein aggregation and digestibility should be evaluated on a per protein basis as each protein contains different moieties, structures, and sequences.

This phenomenon has also been shown in kappa-casein, where following heat treatment, formations of large k-casein aggregates were visualized. This is caused by the reorganization of disulfide bonding patterns and/or intermolecular sulfhydryl-disulfide interactions. On the other hand, the heat treatment may result in the augmentation of chirality of the k-casein residues (Groves et al., 1992; Cho et al., 2003).

Upon treatment of proteins with Sc-CO_2, the secondary and tertiary structures are disrupted leading to the denaturation of the protein. This is due to the destruction of the hydrogen bonds between polar and non-polar groups (Fitzsimons et al., 2007).

The effect of Sc-CO_2 has also been evaluated on enzyme activity. Enzymatic reactions under Sc-CO_2 conditions offer advantages including increased reaction rates, higher conversion percentages, and subsequent purification of reacted products (Knez & Habulin, 2002). Compared to conventional solvent extraction methods using chloroform or ethyl ether, Sc-CO_2 has less of a negative effect on digestive enzyme activity, lipase activity, and α-amylase activity (Park et al., 2008). The loss of enzyme activity is due to the interactions between carbon dioxide and the enzyme (Knez & Habulin, 2002) forming covalent complexes with free amino groups on the enzyme surface (Park et al., 2008).

The effect of Sc-CO_2 on enzymes and proteins is similar to its effect on whey proteins, which is the topic of study. Sc-CO_2 treated WPI results in a change in secondary structure from a decrease in α-helix content, hydrogen bonds, and an increase in β-sheet content (Xu et al., 2011). Striolo and others also evaluated this, where it was found that an increase of pressure above the threshold pressure increases CO_2 interactions with the protein. This interaction between CO_2 and the carboxyl and amine groups was observed by FT-IR spectroscopy. CO_2 can interact with the amine groups to produce amide bonds as well as interacting with the carboxyl group to a lesser extent. This alteration can change the bioactivity of the protein depending on the pressure. These results show that Sc-CO_2 treated whey proteins can recover the original secondary structure, but also indicate that the secondary structure can widely change, resulting in the change of biological activity (Striolo et al., 2003).

2.5.5 Extraction Methods

Other extraction methods that are commonly used include Hexane and Folch Extractions. These extractions can offer insight into the protein-lipid interactions but are not as favorable to incorporate in industrial applications.

Diethyl Ether extraction is a classic method for lipid extraction. However, hexanes have been commonly substituted. Ether can extract urea and hexoses leading to an inflation of extraction yields (Palmquist & Jenkins, 2003). Hexanes are less polar leading to a reduction in lipid extraction values. Thiex and others compared several methods and concluded that hexanes were a viable substitute (Thiex et al., 2003).

The Folch extraction was originally designed for lipid tissue extractions but has been subsequently used for many other applications including milk lipids (Folch et al., 1957). This method uses a combination of chloroform and methanol, which extracts a wide range of lipid classes. Food samples or tissues are homogenized with chloroform and methanol, a low percent salt solution is added breaking the solution into an aqueous (top) and chloroform (bottom) phase. The top is removed and the chloroform is evaporated from the lower phase (Avalli & Contrarini. 2005).

2.6 Proteolysis

2.6.1 Influences on Hydrolysis

The proteolysis of β-LG is determined by several factors. The overall processing conditions and resulting conformation of β-LG will determine which enzyme-binding

sites are accessible and furthermore, which peptides are released. The release of characterized bioactive peptides is completely dependent on this hydrolysis, as the β-LG peptides are between two and twenty amino acids in length and must be hydrolyzed to exert their respective function (Hernandez-Ledesma et al., 2008). The extent of this hydrolysis can be influenced by temperature, pressure, and enzyme type.

The influence of temperature on β-LG begins with the dissociation of the dimer structure (pH 6-7.5) into monomers with increasing temperature. Temperature increase initially leads to an increase in collision frequency and protein solubility. However, when collision frequency increases, the interactions between side chain groups eventually also increase leading to extensive cross-linking and a decrease in protein solubility (Walstra et al., 2006). Increased temperature decreases solution viscosity and improves interactions between protein and enzyme (Cheison et al., 2010). pH also plays a tandem role with temperature in enzyme hydrolysis. At various pH values, β-LG reassembles itself into one of many quaternary structures as previously described (Taulier & Chalikian, 2001). At pH 3.5-4.5, increasing temperature results in the reduction of monomers and dimers and the formation of large protein aggregates even in the presence of a reducing agent (Bertrand-Harb et al., 2002). These protein aggregates are both insoluble and less susceptible to enzymatic hydrolysis. Pressure also influences the hydrolysis of β-LG. An increase in pressure results in an increased digestibility due to conformational changes within the protein (Hayashi et al., 1987). Conversely, Knudsen and others found that only after extreme pressure (300-400 MPa), did hydrolysis of β-LG increase (Knudsen et al., 2002). Pre-pressurized β-LG (Applied pressure then hydrolysis) resulted in the formation

of dimers, trimers, and octomers. However, pressurized induced hydrolysis did not result in the formation of these protein aggregates (Chicon et al, 2006).

2.6.2 Trypsin

The hydrolysis of β-LG is highly dependent on the type of protease. Whey proteins are relatively resistant to hydrolysis in their native state with the exception to pepsin, trypsin, and chymotrypsin (Jost & Monti, 1977). Trypsin incubation with β-LG for 24 hours resulted in the formation of peptides of less than 2 kDa (Madsen et al., 1997).

Bovine trypsin has a molecular weight of 23.3 kDa and cleaves positively charged side chains of Arginine-X and Lysine-X, except when X is Proline (Olsen et al., 2004). The likelihood of trypsin cleavage of β-LG is dependent on the physical accessibility of the enzyme to the cleavage site and the secondary specificity (5-6 amino acid residues surrounding the site) of the enzyme to the protein (Fernandez & Riera, 2013). Both of these factors contribute to the potential for cleavage. However, the rate-limiting step is the attack of the enzyme on the tertiary structure of the protein (Fernandez & Riera, 2013). Fernandez and Riera observed the rapid release of the external peptides (1-8 & 142-148). They also observed an increase in the degree of hydrolysis after all the intact protein had disappeared (Fernandez & Riera, 2013). This implies that β-LG undergoes an intermediate during hydrolysis and the internal portion of the protein is highly resistant to digestion.

In a physiological system, trypsin digestion follows a sequential pattern. The digestion of a protein and amino acids follows these steps after ingestion. After the food particles have entered the stomach and undergone initial chemical and mechanical digestion of large proteins and food particles, they pyloric sphincter releases stomach chyme is released into the duodenum of the small intestine. The low pH of the incoming stomach chyme triggers the secretion of the secretin hormone. This hormone binds to the pancreatic cells stimulating the release of bicarbonate. Another hormone Cholecystokinin (CCK) is also released upon the arrival of amino acids in the duodenum. CCK stimulates the release of many pancreatic enzymes including trypsinogen. Trypsinogen is released through the pancreatic duct upon which is converted to trypsin by enteropeptidase. Trypsin then activates several other pancreatic zymogens. The resulting digest from trypsin is the transport of free amino acids into the epithelial cell lining of the small intestine. These amino acids then travel from the epithelial cell lining entering blood capillaries and then to the liver (Nelson & Cox. 2005).

There are 18 susceptible sites on β-LG for trypsin cleavage (Creamer et al., 2004). Depending on the state of the protein, none, few, or all sites may be cleaved. With 18 possible cleavage sites gives a maximum of 65 possible peptides depending on the degree of hydrolysis. In addition, the formation of peptides is dependent on the type of enzyme selected. As described previously, there is a complex system of hormones, enzymes, sphincters, and organs. In selecting an enzyme in a lab setting, we are attempting to simulate digestion. Due to the complexity of the human digestion system as well as β-LG, a one-enzyme system is commonly used.

2.6.3 Allergenicity

β-LG is a globular protein produced by ruminant animals but absent from human milk. Despite the long list of the benefits in whey including several bioactive properties of β-LG, β-LG has been cited as a major source of milk intolerance and milk allergy in humans (Iametti et al., 2002). In order to address this problem various treatments and enzymes have been used to hydrolyze β-LG into peptides less than 5000 Da leading to a reduction in allergenic potential (Adel-Patient et al., 2012). Previous studies have discovered that β-LG hydrolysis with trypsin resulted in only localized Treg induction, as extensive hydrolysis reduced the initiation of the immune response (Adel-Patient et al., 2012). In order to achieve a more complete digestion, most studies perform irreversible thermal treatment of β-LG. However, upon such treatment β-LG is prone to form aggregates that are resistant to enzyme cleavage and can also elicit an adaptive immune response in certain patients (Iametti et al., 2002). Therefore a sub-denaturing treatment is desired for optimal attack of β-LG. This is supported by the fact that peptides 61-70, 102-124, and 149-162 are susceptible to aggregation (Fernandez & Riera, 2013). These three peptides are one of the several IgE epitopes of β-LG responsible for milk allergy including peptides 41-60, 41-68, 61-70, 92-101, 102-124, 142-148, and 149-162 (Selo et al., 1999).

In an attempt to reduce the allergenicity of β-LG, a sub-denaturing treatment must be used to allow for sequential trypsin digestion but prohibit peptide-protein aggregate formation. Sc-CO_2 at sub-denaturing conditions will be used. Heat and pressure have been used frequently in prior research, but not in tandem with CO_2. As described

previously, this method offers several advantages in changing protein conformation and the ability to expose hydrophobic residues. The effect of Sc-CO_2 on β-LG hydrolysis can be evaluated by various methods including, Enzyme-Linked Immunosorbent Assay (ELISA) and Mass Spectrometry (MS).

2.7 Detection Methods

2.7.1 Traditional Methods

Traditional methods of analysis for peptides and proteins have included Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Kjeldahl, and spectrophotometric kinetic assays to name a few. However, these methods come with many drawbacks. SDS-PAGE was initially used to screen hydrolysis of β-LG, however because of its limited results, little analysis could be performed (Schmidt & Poll, 1991). SDS-PAGE is unable to separate low molecular weight peptides, requires two days of lab work before results are obtained, and quantitation is often inaccurate. Kjeldahl is a very accurate method for protein content determination and proteolysis. However, this method is not able to identify the specific peptides present (Fox et al., 1995). There are also many spectrophotometric kinetic assays available including the OPA/NAC assay. O-phthalaldehyde (OPA) reacts with primary amines to form fluorescent residues that absorb at 335 nm (Hernandez et al., 1990). This kinetic assay monitors the change in absorbance due to the release of amino groups. However, not all peptides released react with OPA, which can underestimate the rate of proteolysis. Due to these combined drawbacks of traditional protein analysis, MS and ELISA have been the preferred tools for protein and peptide analysis.

2.7.2 Mass Spectrometry

MS was first developed in the early 1980's (Alomirah et al., 2000) and not soon after Electrospray Ionization Mass Spectrometry (ESI-MS) was developed by Yamashita and Fenn in 1984 (Yamashita & Fenn, 1984). These instruments quickly became the exclusive tools for the research of proteins and peptides.

MS is an analytical technique that can be used to glean information about a protein or sample mixture including molecular weight and structure. In its simplest form, MS breaks molecules or proteins into smaller pieces, detects these smaller pieces, giving information about the original molecule or protein. The components of a MS include a sample introduction source, an ion source, a mass analyzer, and a detector (Janson, 2011).

Sample introduction can be achieved by HPLC or direct infusion. High Performance Liquid Chromatography (HPLC) is an analytical technique that can be used in many separation analyses. In its simplest form it allows the separation of one molecule from others. The components of an HPLC system include a pump, solvent supply, injection system, column, detector, and recorder (Janson, 2011). These variables allow for the analysis of a wide range of compounds, as any change in one of the variables changes the analysis of the compound. The use of HPLC is a powerful tool used to separate molecules based on their interaction with the stationary phase. However, the detection of the molecules is often limited. Thus, HPLC is frequently coupled with mass spectrometry for further analysis of separated molecules.

Mass spectrometers that are coupled with chromatography instruments commonly use electrospray ionization (ESI) as the ion source. A tight needle is used in conjunction with a high voltage potential. The resulting mobile phase spray contacts the drying gas causing peptides to leave the droplets (Kruve et al., 2010). The ion source is critical in creating ions from the sample introduction that will be detectable. This can be achieved by many different methods including Atmospheric Pressure Photoionization (APPI), Atmospheric Chemical Ionization (APCI), Matrix-assisted Laser Desorption Ionization (MALDI), Electron Ionization (EI), and ESI. ESI operates as a soft ionization technique, limiting pre-MS fragmentation (Mann & Wilm, 1995), and has picomile to femptomole sensitivity (Zaluzec et al., 1994). Ionization occurs within a vacuum through a cycle of solvent evaporation and Coulomb Explosion, whereby a highly charged droplet transitions into a single ion (Bottrill et al., 1999).

After the molecule leaves the ion source as an ion, it is focused and enters the mass analyzer. There are many different mass analyzers including Quadrupole, Time of Flight, and Ion Trap (A. Schilling, University of Illinois at Chicago, San Luis Obispo, CA; Personal Communication). Ion Trap operates under the control of three hyperbolic electrodes that trap ions within three dimensional space using static and RF voltages (Kruve et al., 2010). These voltages are constantly changed to allow ions to enter and exit the trap. Steps for this to occur include accumulation of ions, isolation of ions, excitation, fragmentation, fragment accumulation, and ejection. Fragmentation of the ions provides the basis for detection coupled with helium as the collision gas (A. Schilling, University of Illinois at Chicago, San Luis Obispo, CA; Personal Communication).

After the ion/ion fragments leave the mass analyzer they arrive at the detector. Due to single ions leaving the mass analyzer at a time, amplification is needed. An electron multiplier is used to create an amplification of signal for detection and a charge to mass ratio is recorded (A. Schilling, University of Illinois at Chicago, San Luis Obispo, CA; Personal Communication).

The resulting identification of the molecule is based on the molecular ion from which resulting fragments from the molecular ion result. Peptides are fragmented and the molecular weight of the fragments is recorded. Sequence information can then be determined from this fragmentation pattern. This aids the identification of the peptide/protein usually represented in a vertical bar graph (A. Schilling, University of Illinois at Chicago, San Luis Obispo, CA; Personal Communication).

The initial application of MS to β-LG revealed the three bovine variants A, B, and C (Burr et al., 1996). Furthermore, ESI-MS allows protein interactions to be examined in complex mixtures by extracting the charge state distribution of each protein out of the overall MS profile (Alvarez et al., 2007).

There has been increased interest in understanding the structure-function relationship of β-LG. Given that little is known regarding the function of β-LG, the structure of β-LG needs to be further investigated in isolation and in the context of typical processing conditions. MS is an excellent tool to evaluate the structure and hydrolysis of β-LG.

2.7.3 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has also been used in tandem with MS for screening and quantifying specific β-LG peptides. An Enzyme Linked Immunosorbent Assay (ELISA) is a rapid test for screening and quantifying the presence of an antigen. In its simplest form, proteins in a sample are coated onto a 96 well plate. The nonspecific sites on the plate are blocked with a reagent, commonly non-fat dry milk. A primary antibody is then added to the wells and allowed to interact with the proteins. The primary antibody can be monoclonal, specific for a single epitope of an antigen, or polyclonal, capable of binding at multiple epitopes of an antigen. The wells are washed and a secondary antibody is added and allowed to incubate. A secondary antibody is specific against the primary antibody. The secondary antibody is commonly linked to an enzyme that catalyzes a colorimetric reaction. The wells are washed and a substrate is added. If the secondary antibody is linked to the primary antibody, which is linked to the protein of interest, the enzyme linked to the secondary antibody will convert the substrate leading to the formation of color. The intensity of the color is proportional to the amount of protein present (Nelson & Cox. 2005).

Using an ELISA allows one to quantify the protein of interest in the sample. Using the ELISA, specific allergenic sites on β-LG can be targeted using specific primary antibodies. If hydrolysis occurs within these sites, the signal will be down regulated. If hydrolysis does not occur, the signal will be up regulated. This method is very useful in monitoring the antigenicity of protein samples, simulating the immune response.

2.8 Summary

Whey proteins have been shown to exhibit many biological effects and properties that aid in metabolic regulation (Smilowitz et al., 2005). Furthermore, whey protein possesses the highest biological value, containing a rich source of branched chain amino acids (Smithers, 2008; Smilowitz et al., 2005), persuading consumers to reevaluate their protein source. However, whey proteins do not exist in pure isolation in milk, throughout processing, and in the final WPC or WPI product. Likewise, the MFGM is also responsible for many biological effects. Furthermore, MFGM and its components are present in whey and WPC (Vaghela & Kilara, 1995). Therefore, β-LG should be investigated in tandem with MFGM in the context of natural emulsions. It is unclear how these two components interact under various processing conditions, how structural changes in β-LG can influence this interaction, and if the biological function of β-LG can be explained in the light of the MFGM.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cream Isolation

3.1.1 Milk Collection

Raw, un-homogenized, whole milk was collected from the California Polytechnic State University Dairy immediately following milking. After collection, the milk was brought to room temperature. Three independent replicates were performed in different weeks.

3.1.2 Cream Isolation

The cream isolation was preformed according to the MFGM extraction protocol according to Patton and Huston (1986) and Elías-Argote (2011) with some modifications. 15 mL of phosphate buffer saline (PBS) (1X) (Appendix A) were placed in a 50 mL conical centrifuge tube and then 35 mL of milk was carefully deposited underneath the buffer layer, using a 25 mL disposable pipette (Patton & Huston, 1986). The conical tubes were carefully placed in a centrifuge (Eppendorf, Hauppange, NY) and spun for 20 minutes at 4000 RPM and 21°C (Basch et al., 1985). The cream was removed with a stainless steel spatula and placed in a fresh 50 mL conical tube. The cream was carefully re-suspended with cold diH_2O to a final 40% (w/v) solution. The mixture was placed in the centrifuge and spun for 20 minutes at 4000 RPM and 21°C. The cream was again removed with a spatula, re-suspended with cold diH_2O to a final 40% (w/v) solution, and spun down a total of two times.

3.2 Emulsion Preparation

3.2.1 Whey Protein Isolate (WPI) Emulsion

The washed cream from the cream isolation (3.1.2) was standardized to 40% (w/v) solution with cold diH_2O in a 50 mL conical tube. A 3% (w/v) emulsion was made with WPI (Hilmar Ingredients, Hilmar, CA) by gently stirring. The emulsion was placed on a rotisserie shaker (Barnstead Thermolyne, Boston, MA) for 20 minutes at room temperature. This step was replicated three times as stated in 3.1.1.

3.2.2 β-Lactoglobulin Emulsion

The washed cream from the cream isolation (3.A.II) was standardized to 40% (w/v) solution with cold diH_2O in a 50 mL conical tube. A 3% (w/v) emulsion was made with purified β-Lactoglobulin (Sigma-Aldrich, St. Louis, MO) by gently stirring. The emulsion was placed on a rotisserie shaker for 20 minutes at room temperature. This step was only completed once in the course of the experimentation.

3.3 Heat Treatments

The emulsion was equally divided into 6, 50 mL conical tubes. 2, 50 mL conical tubes were labeled as heat treatment controls and remained at room temperature throughout heat treatment step. 2, 50 mL conical tubes were placed in a water bath (Fisher Scientific, Waltham, MA) at 50°C for 20 minutes. Two 50 mL conical tubes were placed in a water bath at 70°C for 20 minutes. Following the heat treatments, solutions were removed from the water baths and returned to 20°C by placing conical tubes in 4°C diH_2O.

3.4 Fat Extractions

Following each of the heat treatments, each heat-treated emulsion was separated into 4, 50 mL conical tubes, making a total of 12 conical tubes, or 12 individual treatments.

3.4.1 Control Extraction

The emulsion was not extracted following heat treatment. The 2, conical tubes remained at room temperature while the other extractions were performed.

3.4.2 Supercritical CO_2 Extraction

Prior to extraction, the emulsion was placed in a centrifuge and spun for 20 minutes at 4000 RPM and 21°C. The cream was placed in a Whatman #1 filter paper (No. 100150). The filter paper was then placed inside a milk filter bag (Titan) and stapled closed. The water bath (PolyScience, Niles, IL) was turned on 15 minutes prior to extraction and was set at 4°C. The Heat-treated control, 50°C heat treated, and 70°C heat-treated samples were placed inside the Supercritical chamber and packed with sandbags. The automated back pressure regulator (ABPR) (Thar Process, Pittsburgh, PA), temperature controller (Thar Process, Pittsburgh, PA), solvent pump (Thar Process, Pittsburgh, PA), heat exchanger (Thar Process, Pittsburgh, PA), and CO_2 pump (Thar Process, Pittsburgh, PA) was turned on (Figure 3.1). Food grade CO_2 was used (AirGas, Radnor, PA). The CO_2 was turned on and the Superchrom (Thar Process, Pittsburgh, PA) Software was launched. The ABPR was set at 350 bar and the chamber temperature was set at 40°C. The CO_2 pump was set at 50 g CO_2/min until 350 bar was reached. After 350 bar was reached, the flow rate was adjusted to 20 g CO_2/min for the remainder of the run. The

chamber maintained a pressure of 350 bar and a temperature of 40°C ± 2°C for three hours. After the three hours, the method was stopped, the CO_2 was turned off, and the samples were removed. The extracted samples were placed in a fresh 50 mL conical tube and stored at 4°C until enzyme digests were preformed.

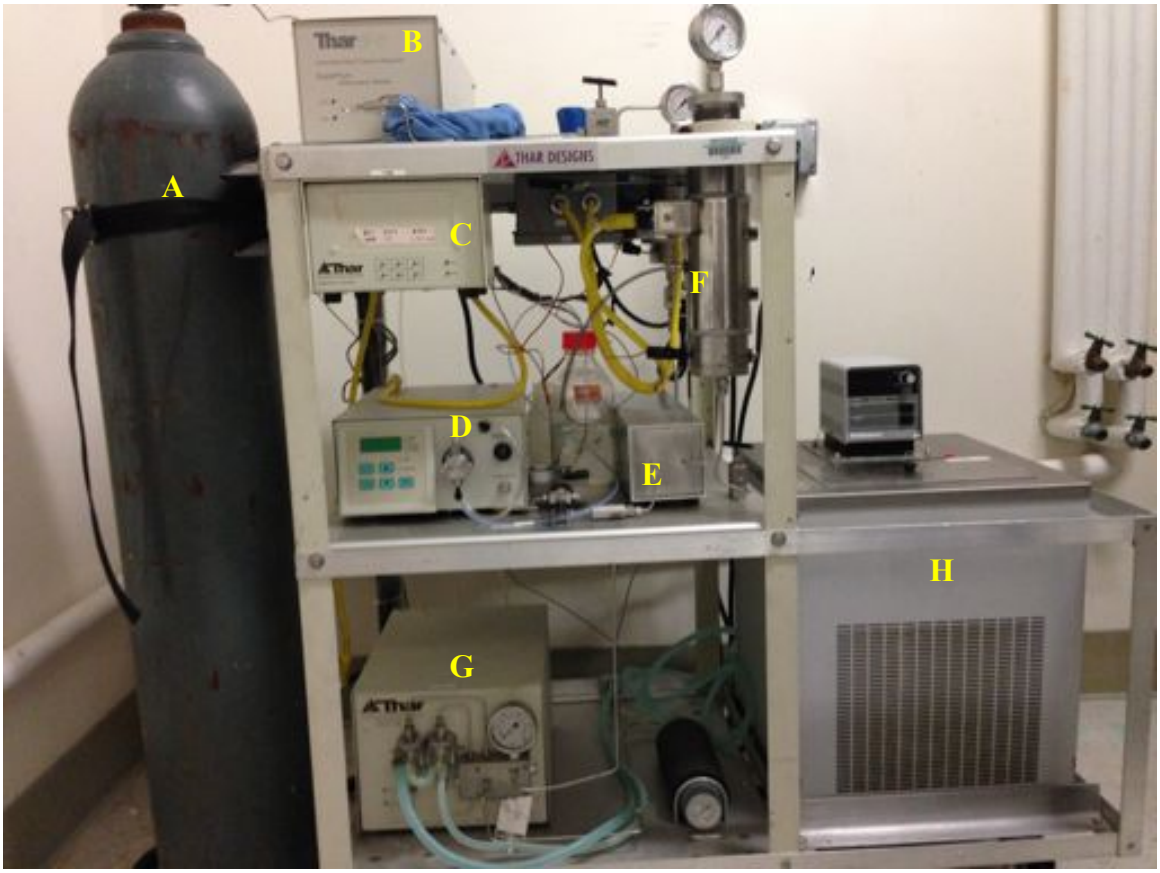

Figure 3.1. Thar SFE unit. A) CO_2; B) Automated Back Pressure Regulator; C) Temperature Controller; D) Solvent Pump; E) Heat Exchanger; F) Chamber; G) High Pressure Pump; H) Water Bath

3.4.3 Hexane Extraction

Hexane extraction was preformed according to Thiex et al., 2003, with some modifications. Prior to extraction, the emulsion was placed in a centrifuge and spun for 20 minutes at 4000 RPM and 21°C. Ten grams of cream was placed in a pre-weighed 125 mL Erlenmeyer flask and 20 mL of HPLC grade Hexane (Fisher Scientific, Waltham,

MA) was added. The hexane-cream solution was poured into a 250 mL separatory funnel. The funnel stopper was inserted, the funnel was inverted, and the stopcock was opened to vent. The stopcock was closed and the funnel was shaken vigorously for 30 seconds before inverting and venting. The funnel was placed on a ring stand and the layers were allowed to separate. The lower layer was drained into the Erlenmeyer flask, not including the boundary layer. The flask and extract was reweighed and the hexane was disposed of in the waste container. The extract was decanted into 3 mL glass amber vials.

3.4.4 Folch Extraction

The Folch extraction was performed according to Folch et al., 1957 with some modifications. Prior to extraction, the emulsion was placed in a centrifuge and spun for 20 minutes at 4000 RPM and 21°C. 1 gram of cream was placed in each of two 50 mL Nalgene chloroform resistant centrifuge tubes (Sigma-Aldrich, St. Louis, MO). 6.6 mL of HPLC grade Methanol (Fisher Scientific, Waltham, MA) was added to each tube and vortexed (Scientific Industries, Bohemia, NY) for 1 minute. 13.3 mL of HPLC grade Chloroform (Fisher Scientific, Waltham, MA) was added to each tube and vortexed for 1 minute. The tubes sat for 15 minutes, were vortexed for 1 minute, sat for 15 minutes, and were vortexed for 1 minute. The tubes were placed in a centrifuge and spun for 5 minutes at 2960 RPM and 20°C to precipitate the solids. 4 mL of NaCl (8.76% NaCl in diH_2O) was added to each tube and vortexed for 1 minute. The tubes sat for 15 minutes, were vortexed for 1 minute, sat for 15 minutes, and were vortexed again for 1 minute. The tubes were placed in a centrifuge and spun for 5 minutes at 2960 RPM and 20°C. The upper phase and interphase was discarded using a glass Pasteur pipette into a waste

container. The lower phase was decanted into a pre-weighed, covered; 250 mL round bottom flask and the chloroform was rotary evaporated with a diagonal condenser (Labconco Corp., Kansas City, MO). The round bottom flask was attached to the condenser with a vacuum of 20 mmHg of pressure. The water bath was kept at 40°C and cold water (4°C) was pumped through the rotary condenser using a peristaltic pump (Cole-Parmer, Vernon Hills, IL). The evaporation of the chloroform was executed to completion before the flask was removed from the vacuum. The flask was then removed, weighed, and the chloroform was discarded into a waste container.

3.5. Enzyme Digests

3.5.1 Standardization and Incubation

Following the fat extractions, each of the 12 treatments was standardized to 5.0 mg/mL of total theoretical protein concentration using diH_2O in a 1.5 mL Eppendorf tube. In addition to the 12 individual treatments, a cream sample and a WPI sample were prepared identically. Trypsin solution (1.0 mg/mL) was prepared using L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) Bovine Trypsin (Thermo Scientific, Waltham, MA) re-suspended in 1% Acetic Acid. Following protein standardization, 25 μL of 1.0 mg/mL of Trypsin solution was added to each digest tube and re-suspended. For each treatment, a control digest tube was also prepared. The same volume of Trypsin added to the digests, a 1% Acetic Acid solution was added to the control tubes. The tubes were re-suspended and incubated at 37°C (Lab-line, Melrose Park, IL).

3.5.2 Filtration

Centrifugal filter units with a 10 kDa molecular cut off (Sigma-Aldrich, St. Louis, MO) were prepared by adding 1 mL diH_2O and placed in a centrifuge and spun for 10 minutes at 4000 RPM and 21°C to remove the glycerol. Digests were added to the filter units placed in a centrifuge and spun for 20 minutes at 4000 RPM and 21°C to collect peptides less than 10 kDa. The filtrate was removed and placed in fresh 1.5 mL Eppendorf tubes and stored at -50°C.

3.5.3 Enzyme Kinetics by SDS-PAGE

SDS-PAGE was used to analyze the kinetics purified β-LG (Sigma-Aldrich, St. Louis, MO) when treated under a variety of conditions. Native β-LG, Sc-CO_2 treated β-LG, heat-treated β-LG, and heat-treated Sc-CO_2 treated β-LG was digested with trypsin. SDS-PAGE reagents were of electrophoresis grade (Bio Rad Laboratories, Hercules, CA). Heat-treated samples were heated at 55°C for 3 hours and then cooled to room temperature. Sc-CO_2 treated samples were run under the same conditions used in the Supercritical CO_2 Extraction protocol. After treatment, protein solutions were standardized to 2.0 mg/mL in diH_2O. A 1:400 enzyme to substrate ratio of TPCK treated trypsin (Fisher Scientific, Waltham, MA) was used. Following digestion, solutions were heated to 95°C for 15 minutes to inactivate trypsin. A 1:2 dilution of each protein sample was prepared with 2X Laemmi (Bio Rad Laboratories, Hercules, CA) in 13% β-mercaptoethanol (Fisher Scientific, Waltham, MA). The samples were resuspended and heated to 95°C for 5 minutes.

15% acrylamide SDS gels were prepared, mounted into an electrophoresis cell, and filled with 1X Tris-Glycine-SDS buffer (Bio Rad Laboratories, Hercules, CA). 15μL of unstained protein standard (Bio Rad Laboratories, Hercules, CA) and 30μL protein sample were loaded into each well. The gels were run at 90V until the protein bands entered the resolving gel and then increased to 110V until the protein bands reached the bottom of the gel.

The gels were destained with destaining solution overnight with shaking. Following destaining, the gels were rinsed once with diH_2O. The gels were oxidized with a 1:10 dilution of oxidizer concentrate (Bio Rad Laboratories, Hercules, CA) for 5 minutes with shaking. The oxidizer was disposed and the gels were rinsed 5 times with diH_2O in a period of 5 minutes. The gels were then washed for 10 minutes in diH_2O changing the water every 5 minutes with shaking. The gels were then stained with a 1:10 dilution of silver reagent (Bio Rad Laboratories, Hercules, CA) for 20 minutes with shaking. The gels were immediately rinsed with diH_2O and a 3.2% (w/v) developing solution (Bio Rad Laboratories, Hercules, CA) was added to the gels, changing every 5 minutes until bands appeared. After bands appeared, a 5% acetic acid solution was added to stop the developer. The gels were imaged on a gel doc (Bio Rad Laboratories, Hercules, CA) in EPI white light.

3.6. Mass Spectrometry

3.6.1 Method Development

Mass Spectrometry was performed on an Agilent 6340 Ion Trap Mass Spectrometer (Agilent Technologies, Santa Clara, CA) connected to a computer equipped with 6300 Series Trap Control Version 6.1 (Bruker, Billerica, MA). A 1 mL glass syringe (Agilent Technologies, Santa Clara, CA) was fixed on an infusion single drive syringe pump (KD Scientific, Holliston, MA). The syringe was coupled to the Electrospray Ionization (ESI) Chamber using a Polyetheretherketone (PEEK) Red Capillary (IDEX, Lake Forest, IL) with an internal diameter of 0.005 inches and an outer diameter of 0.0625 inches (Figure 3.2). LC-MS Grade Helium (AirGas, Radnor, PA) was used as the collision gas. Nitrogen gas was used as the Nebulizer gas and generated by a Nitrogen generator (Dominick Hunter, Lancaster, NY). Prior to data collection, scan calibration was performed using ES Positive Tuning Mix for LC-MSD Ion Trap (Agilent Technologies, Santa Clara, CA)(Mass list: 118.09, 322.05, 622.03, 922.01, 1521.97, 2121.93, 2721.89) with a flow rate of 0.6 mL/hr. The syringe was loaded with 500 μL of a 50/50 mixture of HPLC Grade Methanol (Fisher Scientific, Waltham, MA) and HPLC Grade water (Fisher Scientific, Waltham, MA). The syringe pump was set at 0.6 mL/hr and ran for 10 minutes until source was clean and the baseline peak noise was of similar intensity.

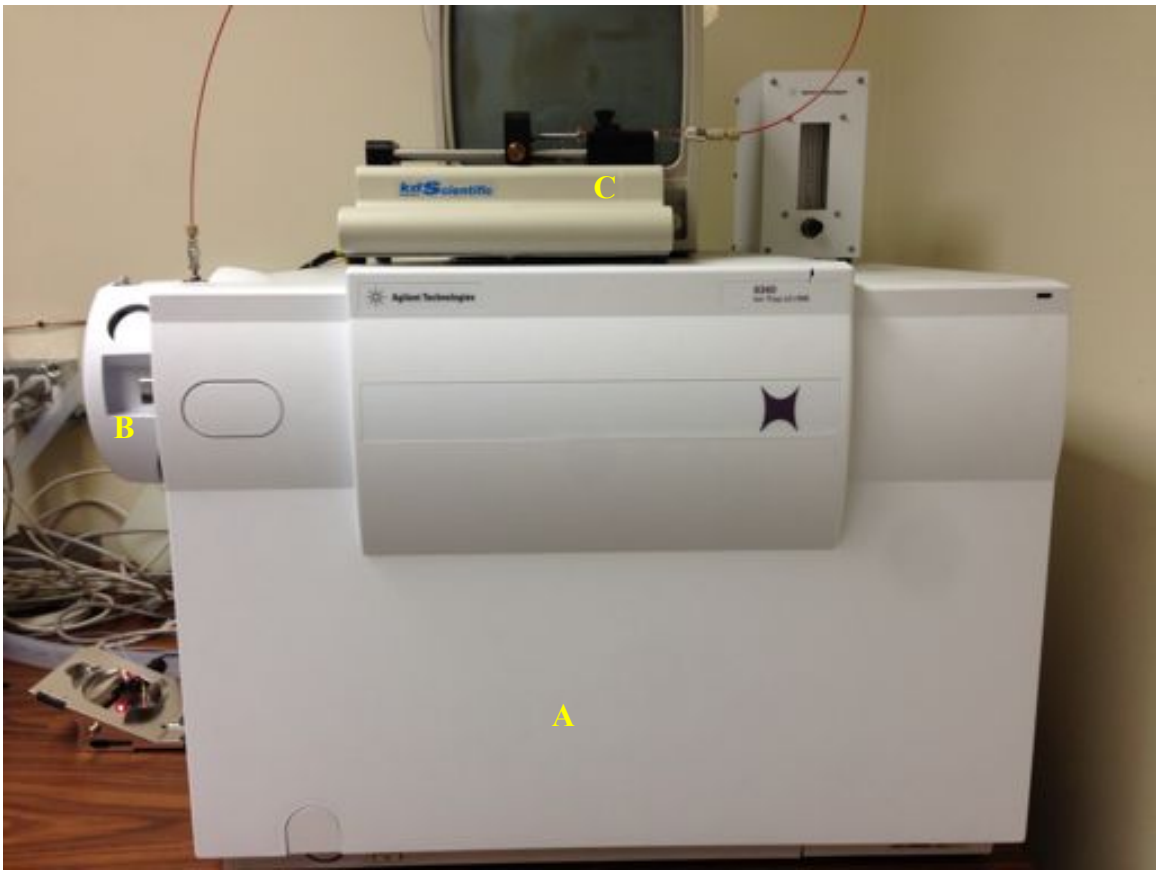

Figure 3.2. Agilent 6340 Ion Trap Mass Spectrometer unit. A). Mass Spectometer; B) Electrospray Ionization Chamber; C) Infusion Single Drive Syringe Pump

Samples were standardized to 1.25 mg/mL of total theoretical protein concentration using a solution of 95% HPLC Grade H_2O (Fisher Scientific, Waltham, MA), 5% HPLC Grade Acetonitrile (Fisher Scientific, Waltham, MA), and 0.1% Fluka Grade Formic Acid (Sigma-Aldrich, St. Louis, MO). 300 µL of the standardized sample was loaded into the syringe and the flow was set at 0.6 mL/hr. The complete run was 10 minutes and the following parameters were selected: Capillary Voltage 3500 V, End Plate Offset -500 V, Nebulizer 15.0 psi, Dry Gas 7 L/min, Dry Temperature 325°C, Skimmer 40 V, Capillary Exit 75.0 V, Oct 1 DC 12 V, Oct 2 DC 2.50 V, Oct RF 200 Vpp, Lens 1 -5.0 V, Lens 2 -60 V, Trap Drive 80 V, Polarity positive, Ion Charge Control (ICC) yes, Smart Target

300,000, Max Accumulation Time 200 ms, Scan 300 to 2200 m/z, Average 2, and Rolling Averaging 2. The following parameters for MS(n) were selected: Auto MS(n) 2, Number of Precursor Ions 2, Threshold Absorbance 5000, Threshold Level 5%, Active Exclusion yes (447.2, 622.1, 922.1, 1522.1), Excluded after 2 spectra, Release after 5 minutes, and MS/MS Fragmentation Amplitude 1.30 V. Following each run, a 50/50 mixture of HPLC Grade Methanol (Fisher Scientific, Waltham, MA) and HPLC Grade water (Fisher Scientific, Waltham, MA) was loaded into the syringe and pumped at 0.6 mL/hr for 10 minutes. For method development, Drying Gas (L/min), Capillary Exit (V), and Trap Drive (V) were optimized.

Table 3.1. Mass Spectrometry Method Development Variables and Optimal Conditions

Variables	**Range**	**Optimal**
Drying Gas	4 L - 8 L	8 L
Capillary Exit	75 V - 100 V	75 V
Trap Drive	60 V - 85 V	80 V

A purified sample of β-LG was used for the method development and the number of unique peptides was counted. The higher the detection rates for a variable, the more optimal the condition. The other parameters were selected through experimentation but not extensive method development (A. Schilling, University of Illinois at Chicago, San Luis Obispo, CA; Personal Communication). The following analyses were preformed using the conditions described previously.

3.6.2 Database Searching and Criteria for Identification

Data analysis was completed using the 6300 Series LC/MS Software 6.1 (Bruker, Billerica, MA). The tandem mass spectra (MS/MS) compound list was extracted and exported. All MS/MS spectra were identified and analyzed using Mascot Server MS/MS Ions Search (Matrix Science Inc., Boston, MA). In a search against the NCBI database the following search parameters were selected: Enzyme Trypsin, Taxonomy Mammalia, Allow up to 3 missed cleavages, Variable Modifications including oxidation of Methionine and methylation of the carboxy-terminus. The peptide tolerance was set at ± 1.2 Da with a MS/MS tolerance of ± 0.6 Da. The peptide charge was set to +1, +2, or +3 and a monoisotopic mass was selected. The instrument type was ESI-TRAP and the Decoy was selected. If protein hits were assigned to the criteria described above, a peptide summary report would identify the peptides and corresponding protein. The Ions Score of -10Log(P), where P is the probability that the observed match is a random event, was used to indicate identity and/or extensive homology. Protein scores are derived from the Ions Score as a non-probabilistic basis for ranking protein hits. Provided the protein hits were peptides of β-LG, the protein view details the protein sequence coverage, the matched peptides, the number of peptides, the false discovery rate (FDR), the number of each peptide, and the peptide sequence. All of which were used in subsequent data analyses.

3.6.3 Peptide Quantitation

The number of each individual β-LG peptide was quantified using the following criteria. If protein hits were assigned to the β-LG protein, a peptide summary report in MASCOT would highlight the number of unique peptides and the number of each peptide detected in the MS/MS spectra. For each treatment, the number of each peptide detected was counted. If protein hits were not assigned to the β-LG protein, even if β-LG peptides were detected but not assigned to protein hits, the peptide was not counted.

Because Mass Spectrometry is limited in linearity, it is necessary to normalize MS parameters to obtain quantitative information about a peptide. Ishihama et al., 2005, determined a way to account for the fact that larger proteins and proteins with many peptides generate more observed peptides (Ishihama et al., 2005). This was named the Protein Abundance Index (PAI). Further, the Exponentially Modified Protein Abundance Index (emPAI) was developed for direct estimation of protein content and was used for data quantitation (Ishihama et al., 2005).

$$PAI = N_{Observed}/N_{Observable} \qquad \text{(Ishihama et al., 2005)}$$

$$emPAI = 10^{PAI} - 1 \qquad \text{(Ishihama et al., 2005)}$$

$N_{Observed}$ is the number of observed peptides per protein and $N_{Observable}$ is the number of peptides that are observable given the conditions, instrument, peptide pI, peptide hyrdophobicity, etc. Further, emPAI is directly proportional to the protein content.

$$\text{Protein content (mol \%)} = emPAI/\Sigma\,(emPAI) \times 100 \qquad \text{(Ishihama et al., 2005)}$$

The $N_{Observable}$ for experimentation was determined to be 12, based on irreversible denaturation of β-LG, followed by overnight Trypsin digest and subsequent MS/MS analysis. For each treatment the $N_{Observed}$ for each peptide was counted using the MASCOT Protein View Protein Hits and the emPAI was calculated, giving a protein quantitation index for data analysis.

3.7 Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

3.7.1 Method Development

A direct ELISA was performed by coating proteins and filtered peptides directly onto the surface of an Immulon 2 HB plate (Thermo Scientific, Waltham, MA). Proteins were diluted to 0 μg/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL in phosphate buffer saline (PBS) (1X) to a total well volume of 100 μL. The plate was covered and incubated overnight at 4°C. Following incubation, the plate was washed three times with phosphate buffer saline with 0.1% Tween-20 (PBST) (1X) (Appendix A). The plate was blocked with 100 μL per well of 1% Fish Gelatin (Sigma-Aldrich, St. Louis, MO) in PBST (1X) (Appendix A). and incubated for 1 hour at room temperature. Following incubation, the plate was washed four times with phosphate buffer saline with PBST (1X). Rabbit anti-β-Lactoglobulin Polyclonal Antibody, Biotin Conjugated (Bioss, Woburn, MA) was diluted to 0 μg/uL, 0.085μg/uL, and 0.2μg/uL with PBS (1X). 100 μL of β-LG antibody was added to each well and incubated for 2 hours at room temperature. Following incubation, the plate was washed four times with PBST (1X). Streptavidin Horseradish Peroxidase (HRP) Conjugate (BD Biosciences, San Jose, CA) was diluted 1:1000 in PBS (1X) and

100 μL was added to each well. The plate was incubated for 1 hour at room temperature in the dark. Following incubation, the plate was washed five times with PBST (1X). A 50/50 mixture of 3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Reagent A (BD Biosciences, San Jose, CA) and TMB Substrate Reagent B (BD Biosciences, San Jose, CA) was prepared and 100 μL was added to each well. The plates were incubated for 15 minutes at room temperature. Following incubation, 100 μL of 2M Sulfuric Acid was added to each well and the absorbance at 450 nm was measured with a spectrophotometer.

Table 3.2. ELISA Method Development Variables and Optimal Conditions

Variables	**Range**	**Optimal**
Protein Concentration	0 μg/mL - 5 μg/mL	5 μg/mL
Blocking Reagent	BSA, Fish Gelatin	Fish Gelatin
Washing Steps	3X - 6X, Step Specific	3X - 5X, Step Specific
Antibody Concentration	0 μg/μL - 0.2 μg/μL	0.2 μg/μL

A purified sample of β-LG and Supercritical CO_2 treated β-LG was used for the method development. For method development the protein concentration, blocking reagent, washing steps, and antibody concentration were optimized. The following analyses were preformed using the conditions described previously.

CHAPTER 4: RESULTS

4.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4.1.1 Enzyme Kinetics of β-LG

SDS-PAGE was used to analyze the enzyme kinetics of β-LG hydrolysis under four different conditions: native β-LG, Sc-CO_2 treated β-LG, heat-treated β-LG, and heat-treated Sc-CO_2 treated β-LG. These four protein samples were then subjected to trypsin hydrolysis of varying times: 0 minutes (undigested control), 5 minutes, 30 minutes, 1 hour, 2 hours, 15 hours, and 19 hours. Following the time-course hydrolysis, trypsin was inactivated by heat. The gels were then run accordingly, insuring that the small peptides (<10 kDa) did not travel beyond the glass. Silver staining was used due to its sensitivity advantage over Coomassie Blue. However, silver staining is not a quantitative stain and therefore the protein and peptide amounts cannot be accurately quantified.

β-LG can be visualized in the SDS polyacrylamide gels shown in Figures 4.1 through 4.4. Figure 4.1 illustrates the time-course hydrolysis of native β-LG. β-LG is shown at approximately 18 kDa. Lanes 3 through 9 represents the enzyme hydrolysis of β-LG with increasing hydrolysis time. Lane 2 represents native β-LG. After the enzyme was added, the amount of hydrolysis time did not substantially affect the hydrolysis of β-LG. Figure 4.2 illustrates the time-course hydrolysis of Sc-CO_2 treated β-LG. β-LG is shown at approximately 18 kDa. Lanes 3 through 9 represents the enzyme hydrolysis of β-LG with increasing hydrolysis time. Lane 2 represents native β-LG. Compared to Figure 4.1, the number of digest products in the Sc-CO_2 treated β-LG was remarkably increased over native β-LG. In addition, it appears that the amount of hydrolysis did increase over time.

Figure 4.3 illustrates the time-course hydrolysis of heat-treated β-LG. β-LG is shown at approximately 18 kDa. Lanes 3 through 9 represents the enzyme hydrolysis of β-LG with increasing hydrolysis time. Lane 2 represents native β-LG. The number of digest products and the amount of hydrolysis appear to be very similar to the Sc-CO_2 treated β-LG (Figure 4.2).

Figure 4.4 illustrates the time-course hydrolysis of heat-treated Sc-CO_2 treated β-LG. β-LG is shown at approximately 18 kDa. Lanes 3 through 9 represents the enzyme hydrolysis of β-LG with increasing hydrolysis time. Lane 2 represents native β-LG. The numbers of digest products and the amount of hydrolysis appears to be greater than the native β-LG, heat-treated β-LG, and Sc-CO_2 treated β-LG. In addition, the rate of hydrolysis appears to be increased compared to the other three treatments.
At 5 minutes there is extensive hydrolysis in heat-treated β-LG, Sc-CO_2 treated β-LG, and heat-treated Sc-CO_2 treated β-LG, which is not seen in the native β-LG. Heat-treated β-LG is comparable to Sc-CO_2 treated β-LG in hydrolysis rate. However, heat-treated β-LG and Sc-CO_2 treated β-LG are not comparable to heat-treated Sc-CO_2-treated in terms of hydrolysis rate.

As hydrolysis rate is increased, the presence of larger protein bands at approximately 25 kDa also increases. These are likely aggregate products of β-LG following digestion.

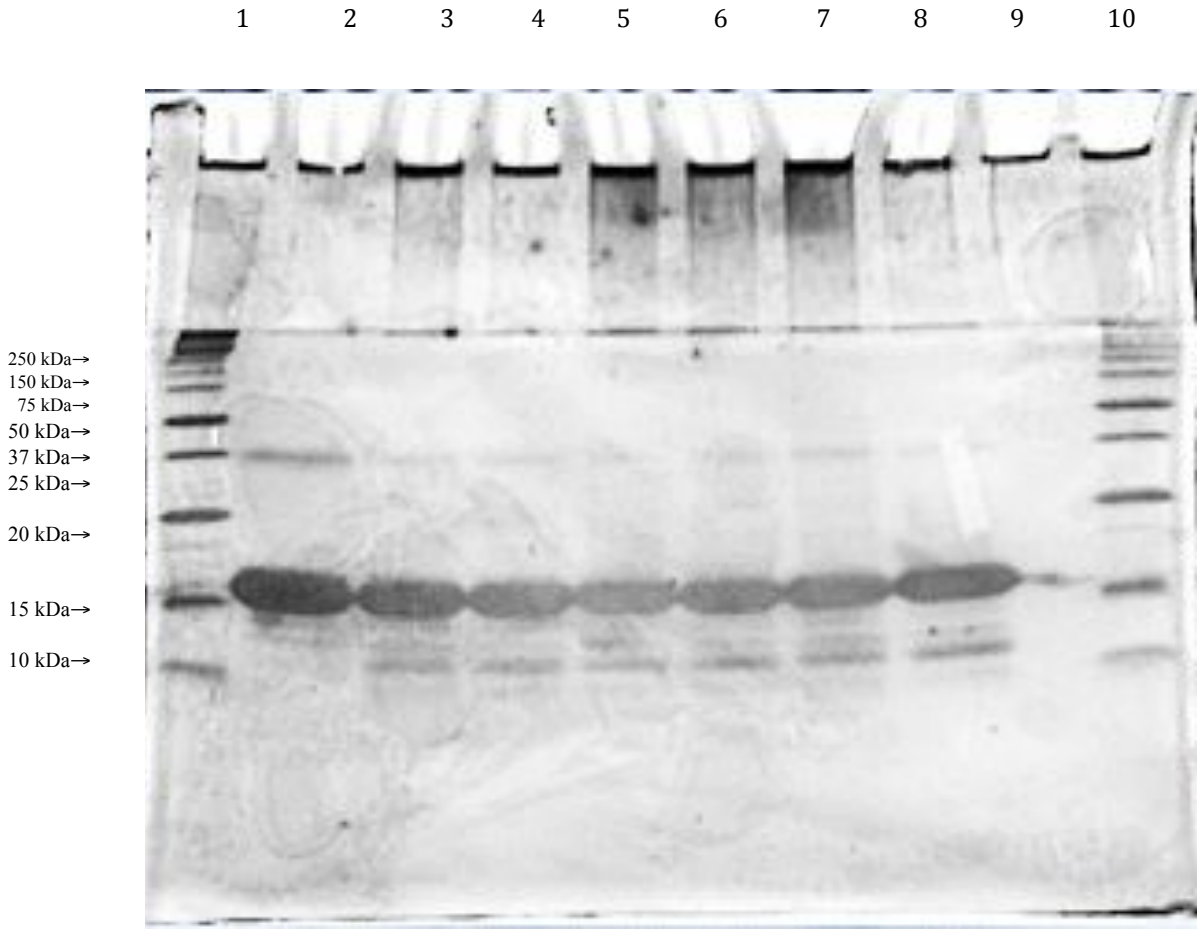

Figure 4.1. SDS-PAGE (15% acrylamide gel) of β-Lactoglobulin time course trypsin hydrolysis. Lane 1. Protein standards; Lane 2. β-LG undigested; Lane 3. β-LG digested for 5 minutes at 37°C; Lane 4. β-LG digested for 30 minutes at 37°C; Lane 5. β-LG digested for 1 hour at 37°C; Lane 6. β-LG digested for 2 hours at 37°C; Lane 7. β-LG digested for 15 hours at 37°C; Lane 8. β-LG digested for 19 hours at 37°C; Lane 10. Protein standards

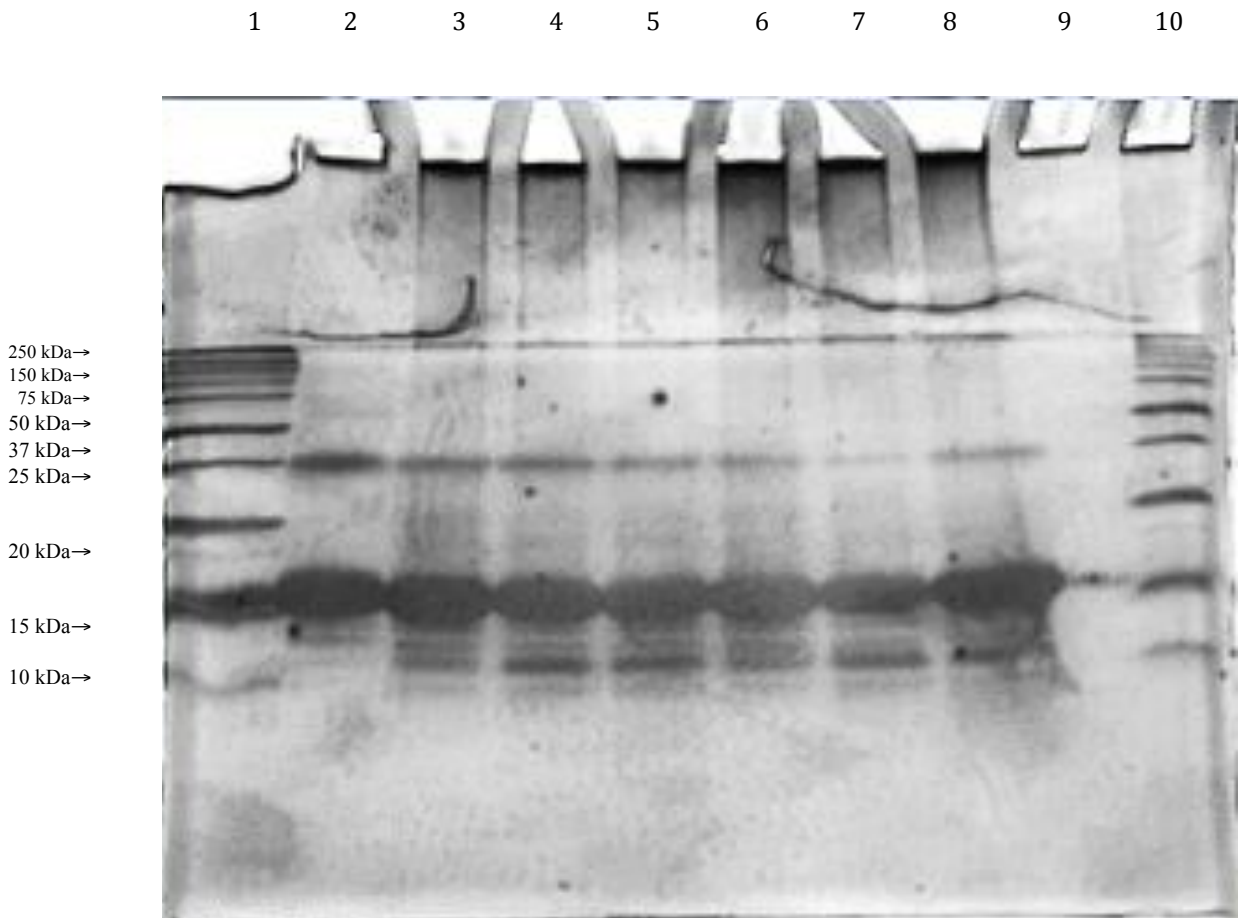

Figure 4.2. SDS-PAGE (15% acrylamide gel) of Supercritical CO_2 (SFE) treated β-Lactoglobulin time course trypsin hydrolysis. Lane 1. Protein standards; Lane 2. Sc-CO_2 β-LG undigested; Lane 3. Sc-CO_2 β-LG digested for 5 minutes at 37°C; Lane 4. Sc-CO_2 β-LG digested for 30 minutes at 37°C; Lane 5. Sc-CO_2 β-LG digested for 1 hour at 37°C; Lane 6. Sc-CO_2 β-LG digested for 2 hours at 37°C; Lane 7. Sc-CO_2 β-LG digested for 15 hours at 37°C; Lane 8. Sc-CO_2 β-LG digested for 19 hours at 37°C; Lane 10. Protein standards

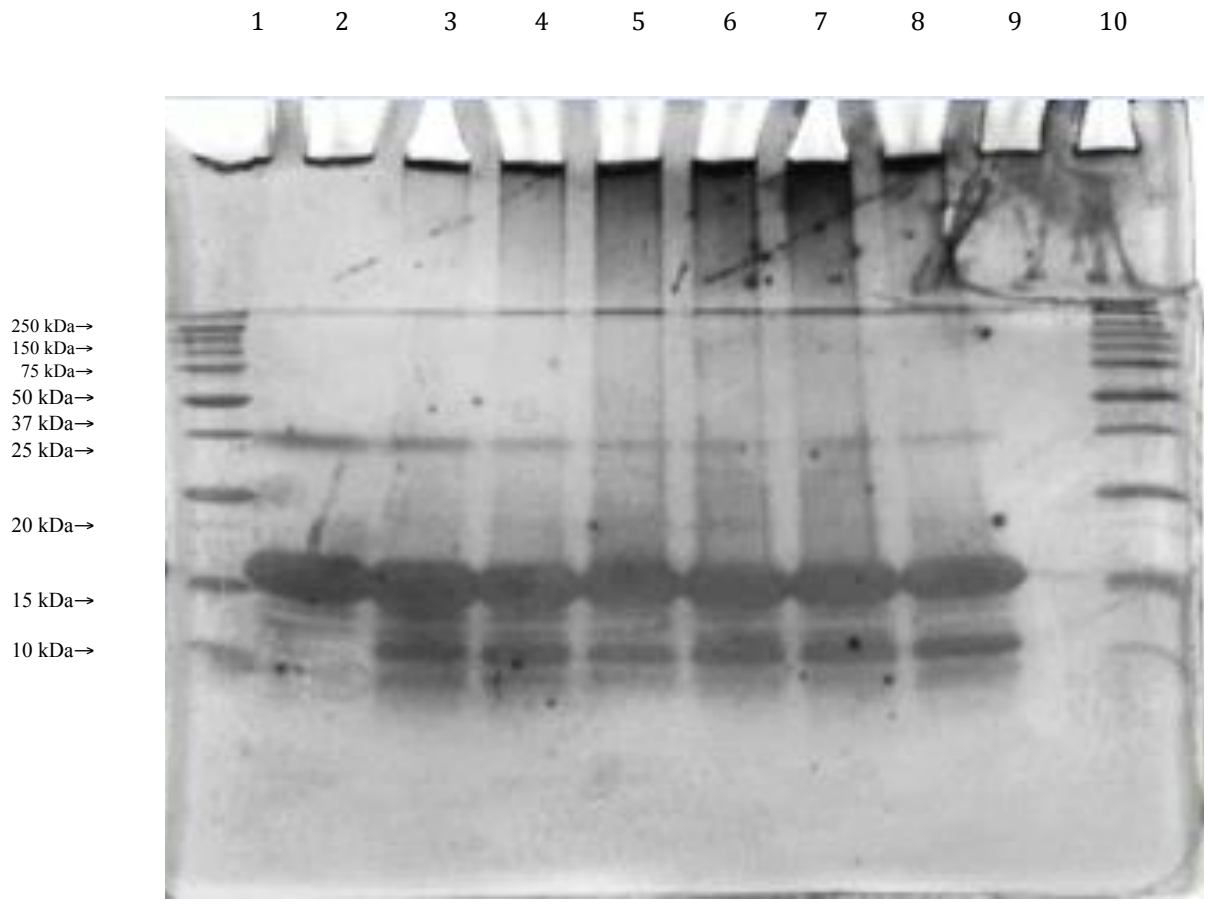

Figure 4.3. SDS-PAGE (15% acrylamide gel) of heat-treated β-Lactoglobulin (55°C for 3 hours) time course trypsin hydrolysis. Lane 1. Protein standards; Lane 2. Heat-treated β-LG undigested; Lane 3. Heat-treated β-LG digested for 5 minutes at 37°C; Lane 4. Heat-treated β-LG digested for 30 minutes at 37°C; Lane 5. Heat-treated β-LG digested for 1 hour at 37°C; Lane 6. Heat-treated β-LG digested for 2 hours at 37°C; Lane 7. Heat-treated β-LG digested for 15 hours at 37°C; Lane 8. Heat-treated β-LG digested for 19 hours at 37°C; Lane 10. Protein standards

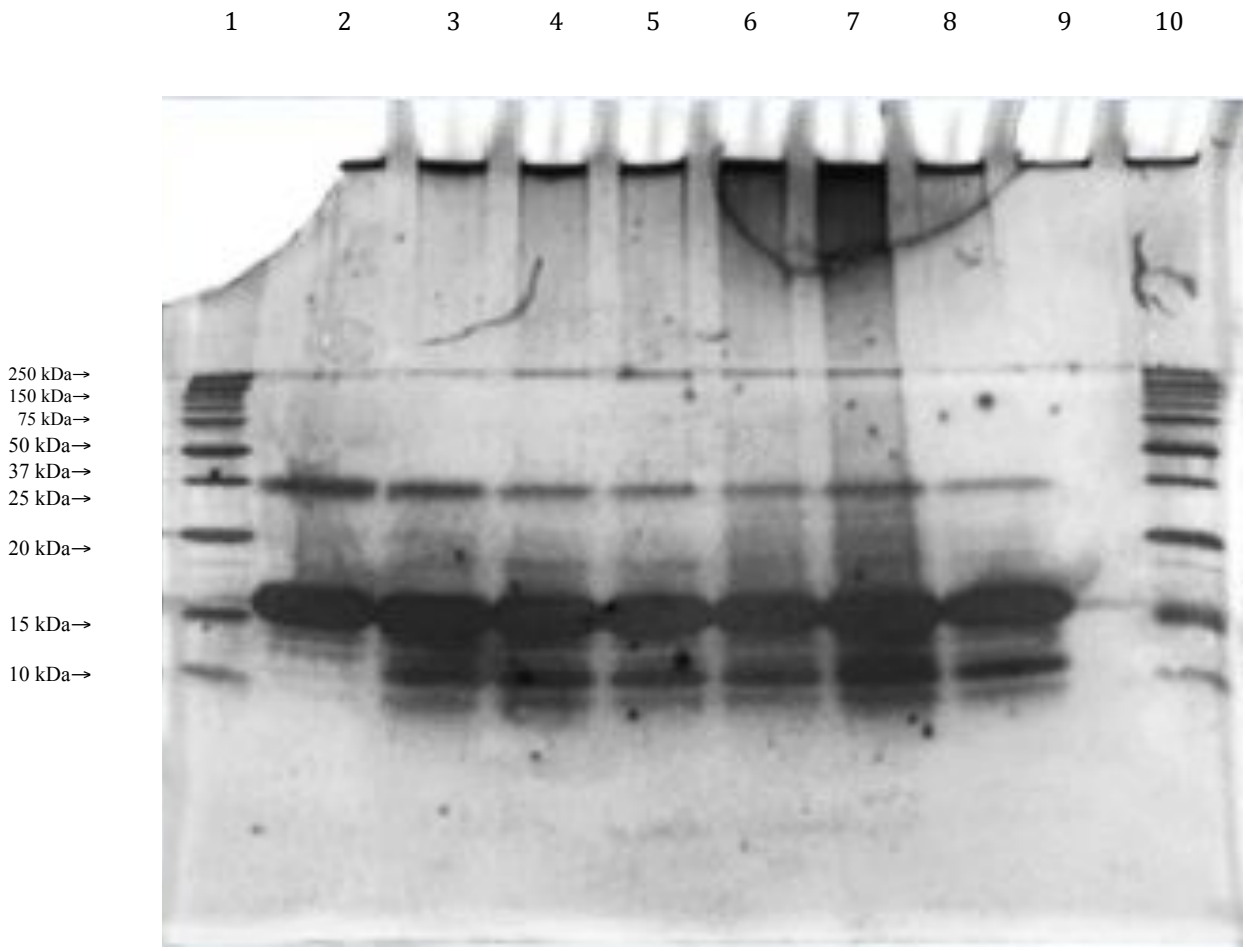

Figure 4.4. SDS-PAGE (15% acrylamide gel) of heat-treated (55°C for 3 hours) and Supercritical CO_2 (SFE) treated β-Lactoglobulin time course trypsin hydrolysis. Lane 1. Protein standards; Lane 2. Heat-treated, Sc-CO_2 β-LG undigested; Lane 3. Heat-treated, Sc-CO_2 β-LG digested for 5 minutes at 37°C; Lane 4. Heat-treated, Sc-CO_2 β-LG digested for 30 minutes at 37°C; Lane 5. Heat-treated, Sc-CO_2 β-LG digested for 1 hour at 37°C; Lane 6. Heat-treated, Sc-CO_2 β-LG digested for 2 hours at 37°C; Lane 7. Heat-treated, Sc-CO_2 β-LG digested for 15 hours at 37°C; Lane 8. Heat-treated, Sc-CO_2 β-LG digested for 19 hours at 37°C; Lane 10. Protein standards

4.2 Mass Spectrometry

4.2.1 Method Development

Mass Spectrometry (MS) was used to analyze β-LG peptides of less than 10 kDa following trypsin digestion of heat-treated and extracted samples. In order to properly and effectively detect the β-LG peptides with reasonable certainty, a MS method was developed using a fully digested β-LG sample. Ultimately, this method was used to detect the presence of certain β-LG peptides from each of the 12 treatments. Furthermore, this method was used to quantify each peptide from each of the 12 treatments. The three main parameters that were evaluated for method optimization was drying gas rate, capillary exit voltage, and trap drive voltage. Variables that were used for assessing method effectiveness were amount of unique β-LG peptides, meaning the number of distinctly different peptides, and β-LG protein sequence coverage (Figure 4.5).

1	LIVTQTMKGL	DIQKVAGTWY	SLAMAASDIS	LLDAQSAPLR	VYVEELKPTP
51	EGDLEILLQK	WENDECAQKK	IIAEKTKIPA	VRKLDAINEN	KVLVLDTDYK
101	KYLLFCMENS	AEPEQSLVCQ	CLVRTPEVDD	EALEKFDKAL	KALPMHIRLS
151	FNPTLQEEQC	HI			

Figure 4.5. β-Lactoglobulin Protein Sequence

Four variations of drying gas rate were evaluated. The drying gas used was nitrogen. During Electrospray Ionization (ESI), drying gas passes across the ionization source causing the charged droplets to undergo solvent evaporation, as shown in Figure 4.6. These charged droplets eventually form sample ions and enter the MS (Bottrill et al., 1999). An increase in drying gas resulted in an increase in both unique peptide number and protein sequence coverage. While drying gas was not the only variable adjusted, an

increase of just 1 l/min (from 6 l/min to 7 l/min) resulted in an increase in protein sequence coverage by 9% (Table 4.1).

Two variations of capillary exit voltage were evaluated. The capillary exit voltage creates a potential for ions to escape the capillary and undergo Coulomb Explosion, as shown in Figure 4.5 (Bottrill et al., 1999). If this potential is negative, positive ions will exit the capillary. Positive ion mode was used for this experimentation. A decrease in capillary exit voltage resulted in an increase in both unique peptide number and protein sequence coverage (Table 4.1). Therefore, a more negative potential was desired to detect more positive ions.

Five variations of trap drive voltage were evaluated. Specifically, the trap drive is the radio frequency voltage applied to the ring electrode shown in Figure 4.6. The ring electrode is responsible for trapping ions within the ion trap (Kruve et al., 2010). Peptides with a higher mass to charge (m/z) ratio require a higher trap drive voltage to be trapped. An increase in trap drive resulted in an increase in protein sequence coverage, but did not have an effect on the number of unique β-LG peptides. Increasing the trap drive from 60 V to 75 V resulted in an increase in protein sequence coverage by 10% (Table 4.1). Further, an increase in trap drive from 75 V to 80 V resulted in an increase in protein sequence coverage by an additional 4% (Table 4.1).

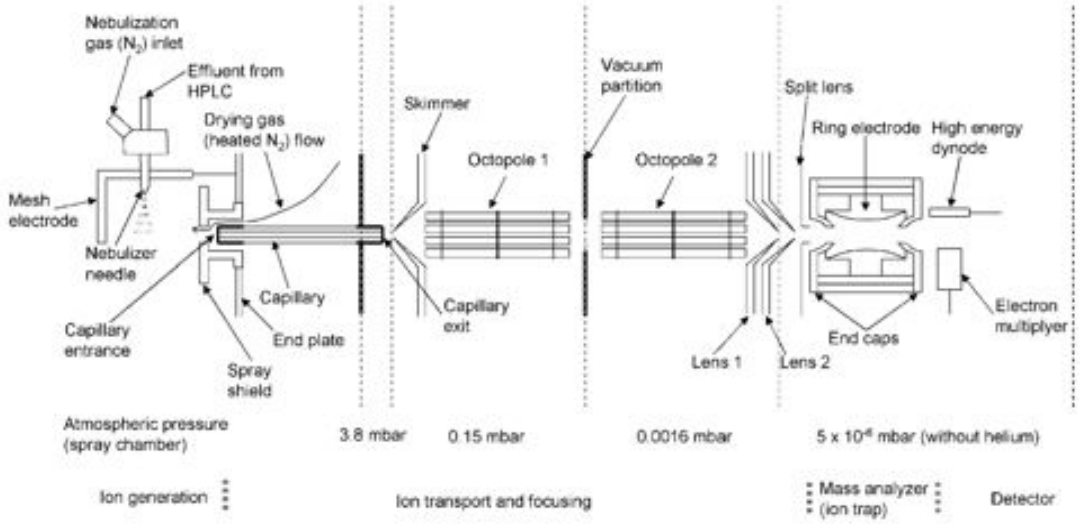

Figure 4.6. Design of the ion mass spectrometer with ESI source, adopted from Kruve et al., 2010

These three parameters must be optimized in order to achieve high sensitivity and reliability. However, there must be a proper balance between each of these parameters, as each parameter has an indirect effect on the other, and also a direct effect on the detection limits. Optimization of each of these three variables resulted in the final MS method as shown in Table 4.2.

Table 4.1. Parameters for Mass Spectrometry method development

Drying Gas (l/min)	Capillary Exit (V)	Trap Drive (V)	Unique β-LG Peptides	Protein Sequence Coverage (%)
4	100	85	4	39
6	75	70	9	33
7	75	70	9	42
8	75	60	10	48
8	75	75	10	58
8	75	80	10	62

Table 4.2. Final Mass Spectrometry parameters

Drying Gas (l/min)	Capillary Exit (V)	Trap Drive (V)	Unique β-LG Peptides	Protein Sequence Coverage (%)
8	75	80	10	62

4.2.2 Whey Protein Isolate (WPI) Controls

Following MS method development, WPI control digests were performed. The digests were then filtered (<10 kDa) and analyzed by MS. The amount of unique β-LG peptides is shown below in Figure 4.7 and the protein sequence coverage is shown in Figure 4.8. On average, 8.66 unique peptides were detected with an average protein sequence coverage of 43.6%.

Table 4.3. Number of unique peptides present and the protein sequence coverage in WPI control Digests

	Average	Standard Error
Unique Peptides Detected	8.67	0.33
Protein Sequence Coverage	43.67	1.33

Furthermore, the exponentially modified protein abundance index (emPAI) was calculated for each of the WPI digests. The emPAI for each peptide is shown below in Figure 4.7. Proportionally, the abundance of β-LG peptides 92-101 and 125-138 was significantly greater than all other peptides. Cleavage at sites 92 or 101 would result in peptides of 10.4 kDa and 7.9 kDa or 11.4 kDa and 6.9 kDa. Cleavage at sites 125 or 138 would result in peptides of 14.1 kDa and 4.2 kDa or 15.6 kDa and 2.7 kDa. In addition, the abundance of peptides 1-8 and 84-91 were significantly greater than peptides 92-100, 41-60, 125-135, 142-148, and 78-83.

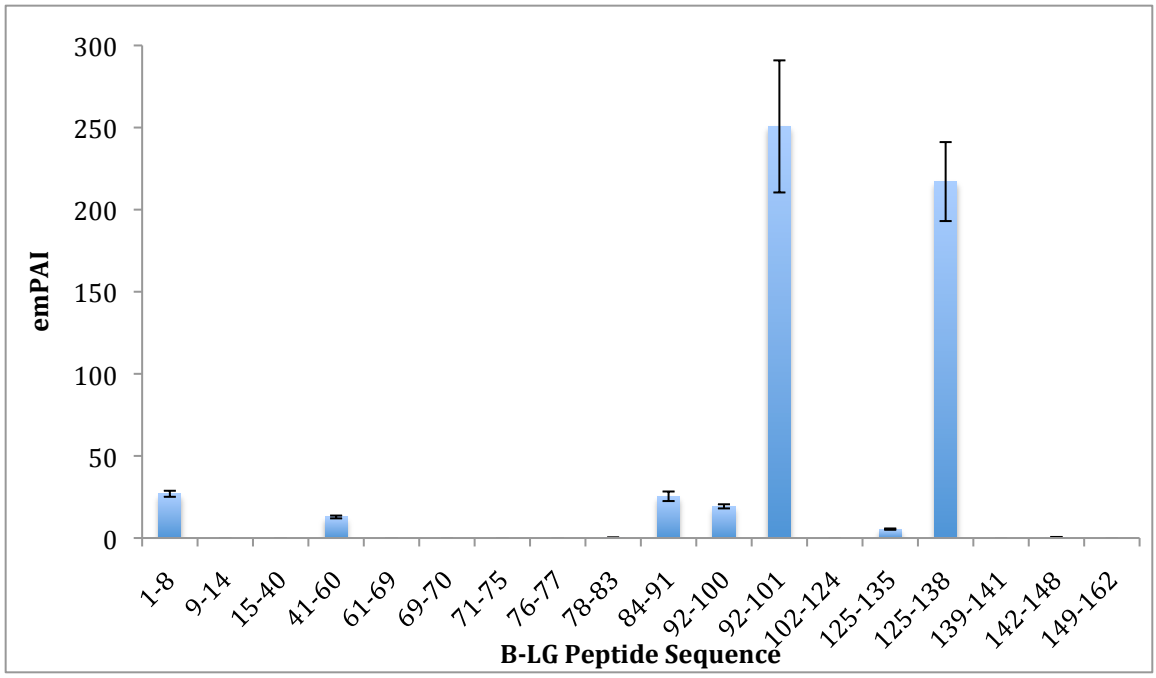

Figure 4.7. Exponentially modified protein abundance index (emPAI) of WPI control digests

4.2.3 Experimental Design

A General Linear Model (GLM) was used for the design of these experiments using heat treatment as a factor with 3 levels and extraction method as a factor with 4 levels.

Table 4.4. 2-Factor experimental design with a total of 12 treatments

		Extraction Method			
		Control	Supercritical CO_2	Hexane	Folch
Heat Treatment	Control	1	4	7	10
	50C	2	5	8	11
	70C	3	6	9	12

Equation 4.1
The model equation for this experimental design:

$$Y_{ijh} = \mu + \alpha_I + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijh}$$

Where:

Y_{ijh} = Quantitative response at the i^{th} and j^{th} treatment levels and the k^{th} replicate
μ = Mean quantitative response
α_I = Main effect of the i^{th} heat treatment level
β_j = Main effect of the j^{th} extraction method level
$(\alpha\beta)_{ij}$ = Interaction effect of the i^{th} heat treatment level and the j^{th} extraction method level
ε_{ijh} = Random error

This GLM was used to determine level of significance in unique peptide number, total emPAI, and domain dependent emPAI across all treatments. The GLM was used in domain independent analysis to investigate the effect of the treatment on specific β-LG protein domains.

4.2.4 β-Lactoglobulin and Cream Treatments

Following the preliminary experiments on WPI and washed cream, purified β-LG was used in place of WPI in an effort to investigate purely β-LG. It was observed that upon adding purified β-LG to the washed cream, the emulsion was unstable. The emulsion required extensive stirring in order for β-LG to go into solution. Following heat treatments, extractions, and digests, the peptide fraction (<10 kDa) was analyzed by MS. The number of unique peptides present was substantially reduced as shown in Figure 4.8. Furthermore, the peptides that were detected were isolated into a few regions of the β-LG protein. These regions included peptides 1-8, 84-81, 92-100, and 125-135 (Figure 4.9).

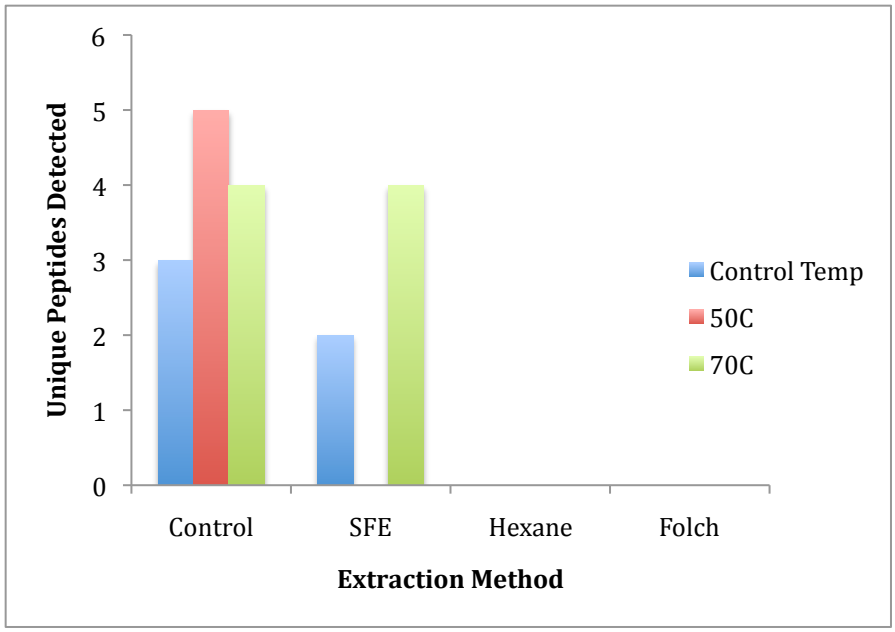

Figure 4.8. Number of unique peptides present in each of the 12 treatment groups of β-LG and cream

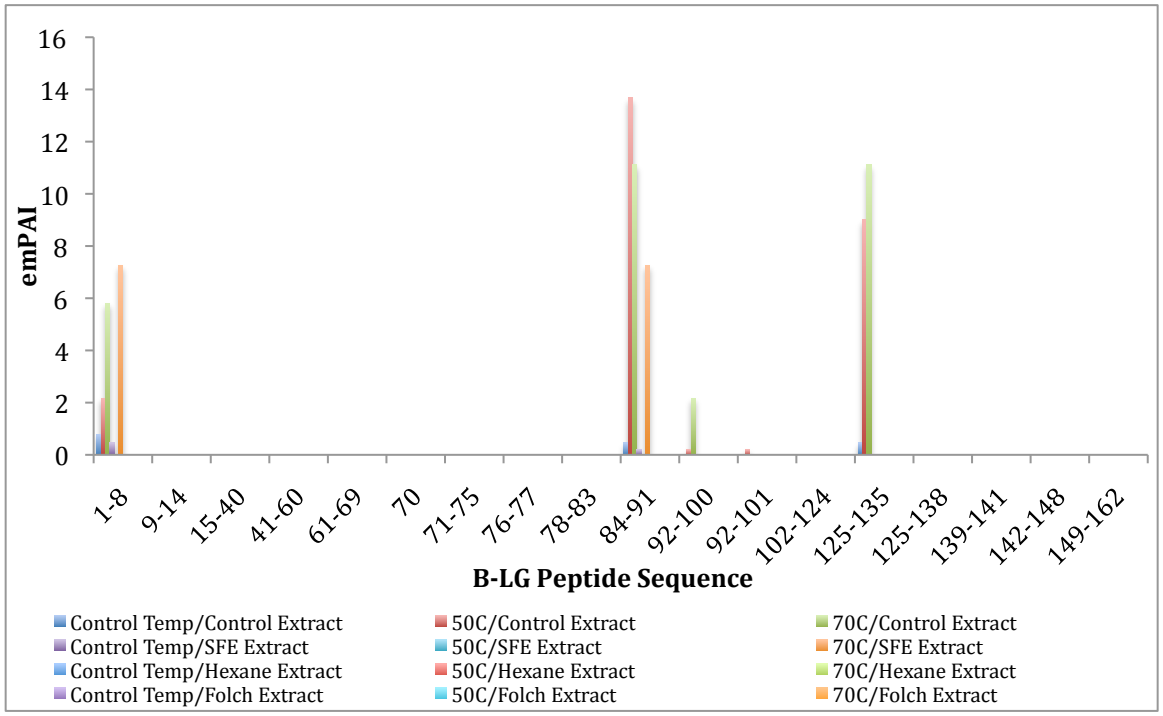

Figure 4.9. Exponentially modified protein abundance index (emPAI) of each peptide in each of the 12 treatment groups of β-LG and cream

The effect of temperature or extraction did not follow a traceable trend, as unique peptides were not detected in 5 out of the 12 treatments (Figure 4.10). Because of the substantial difference between the preliminary experiments, WPI controls, and the purified β-LG and cream treatments, replications were not preformed. As a consequence of the lack of replications, no further conclusions can be drawn.

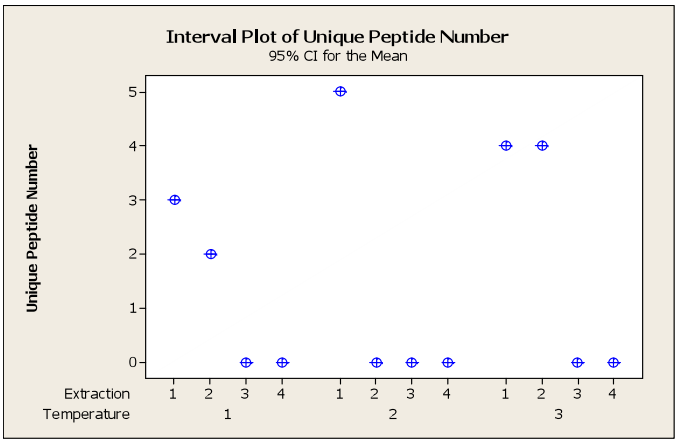

Figure 4.10. Interval plot of unique peptide number in each of the 12 treatment groups of β-LG and cream

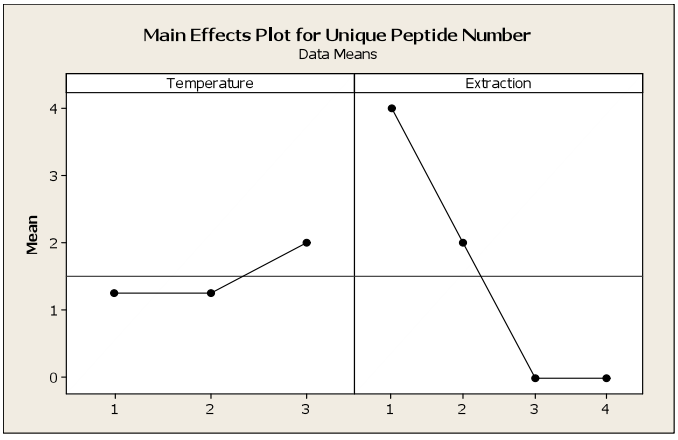

Figure 4.11. Main effects plot of unique peptide number in each of the 12 treatment groups of β-LG and cream

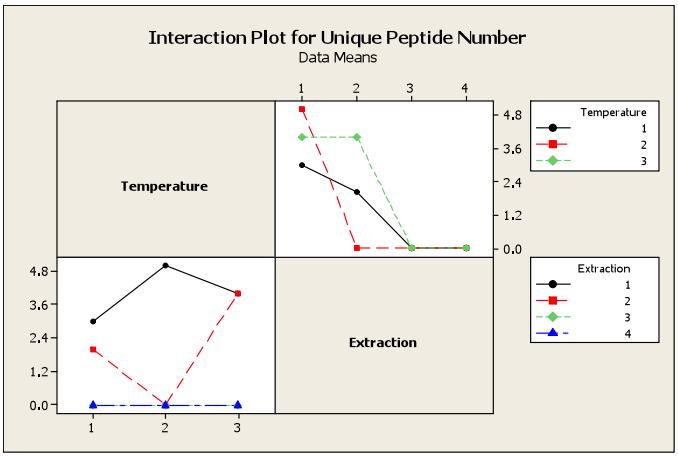

Figure 4.12. Interaction plot of unique peptide number in each of the 12 treatment groups of β-LG and cream

4.2.5 WPI and Cream Treatments

Due to the puzzling and inconsistent emulsions and MS data from the purified β-LG, the experiment was repeated using WPI and replicated three times. The results were remarkably different.

A MS spectra of treatment 4 is shown in Figure 4.13. The top chromatogram represents the Total Ion Chromatogram (TIC) of the entire 10-minute sample run. The middle MS spectra is the MS of compound 125 with a m/z of 467.3. The next spectra is the MS(2) of compound 125 with the same m/z of 467.3, illustrating the fragmentation compounds. The last spectra is the MS of compound 126.

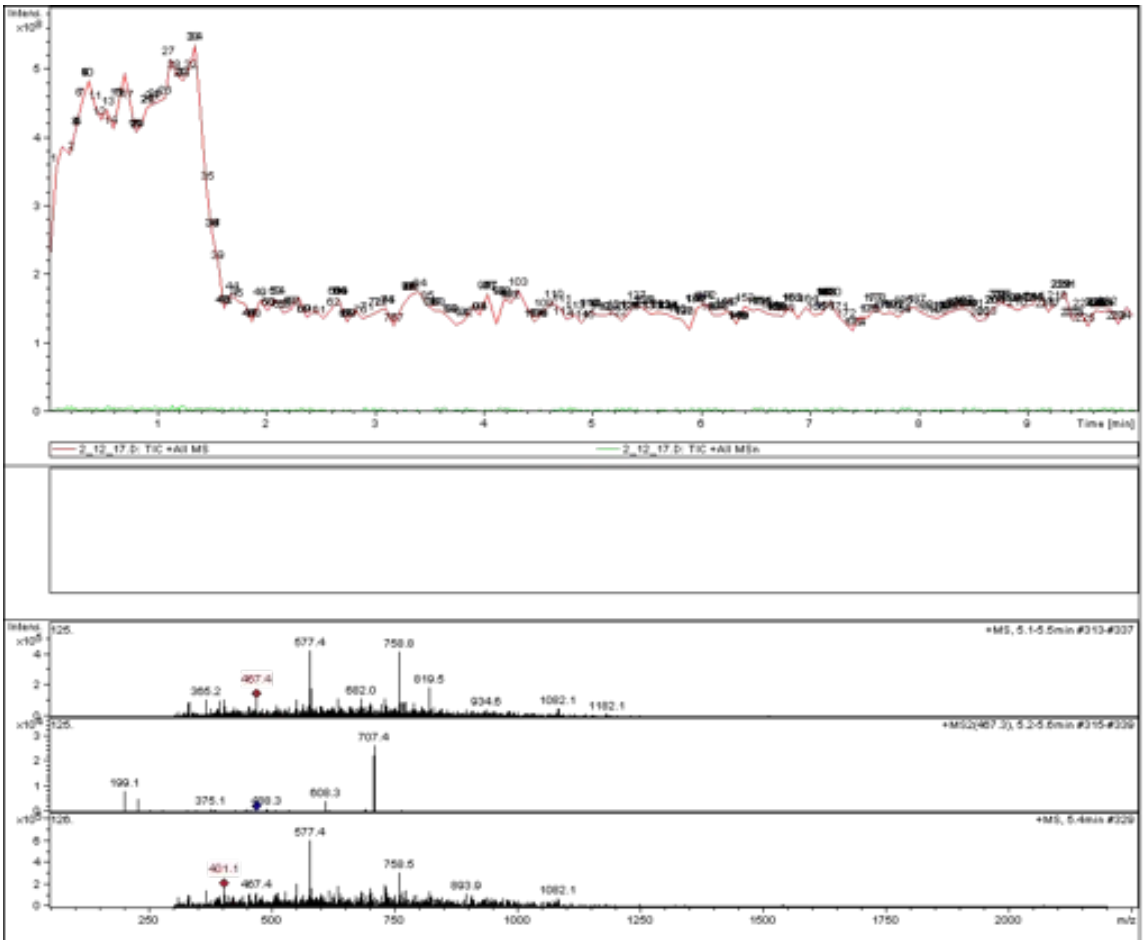

Figure 4.13. Total Ion Chromatogram of Treatment 4 (Replicate 2) with the accompanying MS of compound 125 (m/z = 467.3), MS(2) of compound 125 (m/z = 467.3), and the MS of compound 126

For each compound detected, the accompanying MS spectra for each treatment was uploaded to the Matrix Science Mascot database for MS/MS ions search and identification. The protein view Mascot search results for treatment 4 are shown in Figure 4.14. The identified peptides of β-LG are listed with the observed m/z values and the peptide sequences. Compound 125 previously shown in Figure 4.13, can be identified by the m/z value of 467.3 to be peptide 1-8 with a sequence of IIVTQTMK. Each of the identified peptides is highlighted on the protein map of β-LG.

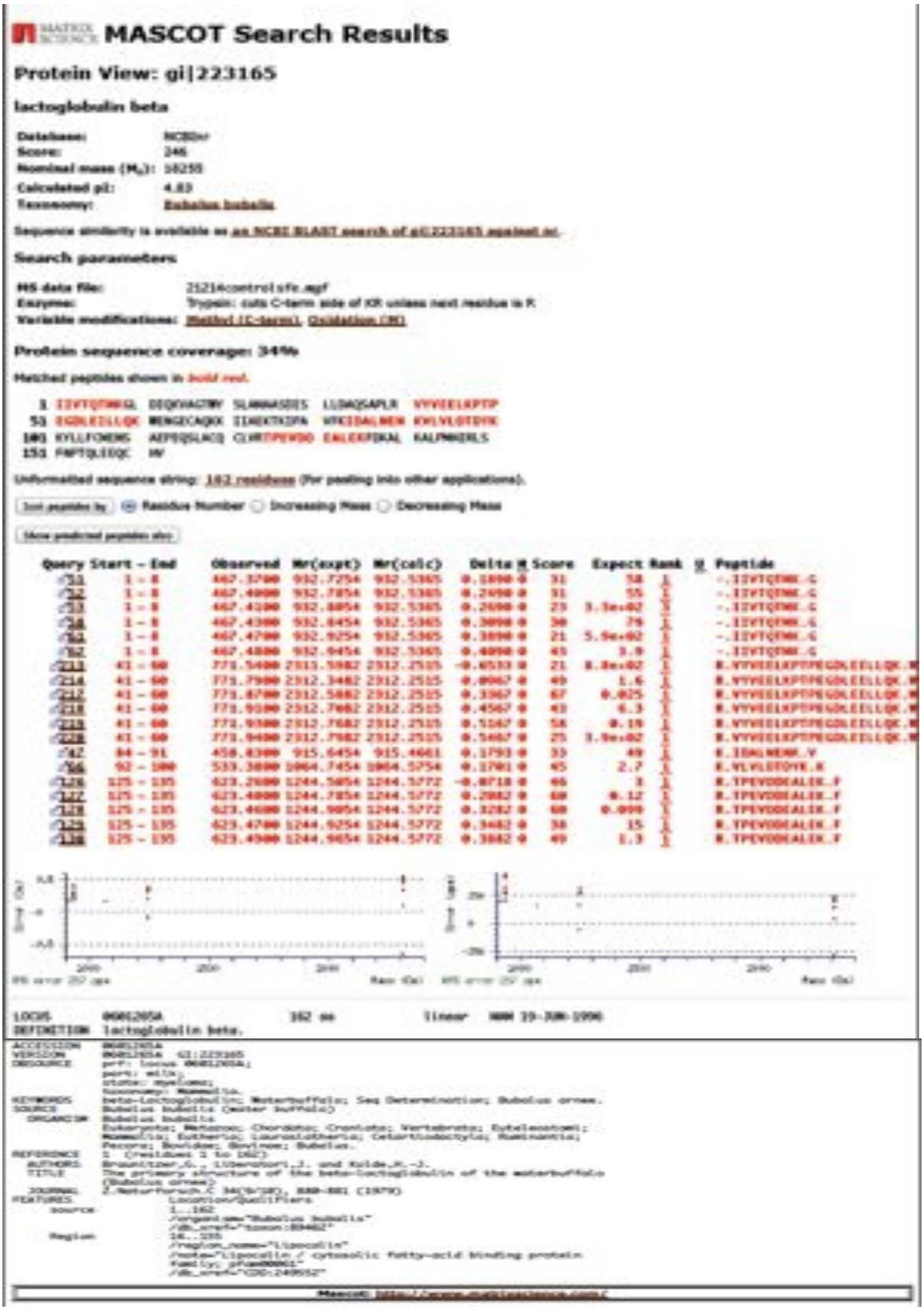

Figure 4.14. Matrix Science Mascot MS/MS Ions Search results from Treatment 4 (Replicate 2) of the detected MS(n) compounds

Following the analysis of all treatments, there was a significant effect of temperature ($P = 0.003$) and extraction ($P < 0.001$) on the unique peptide number (Figure 4.15)(Appendix B). In addition, there was a significant interaction effect of temperature and extraction on the unique peptide number ($P = 0.001$). Therefore, the main effects were not be interpreted due to a significant interaction. Thus, the remainder of analysis will focus only on the interaction effect of temperature and extraction.

Tukey's Honestly Significant Difference (HSD) pairwise comparison was used following GLM analysis to determine which combinations of temperature and extraction resulted in significantly different unique peptide number (Appendix B). Treatments 2 and 3 were significantly greater in unique peptide number as compared to treatments 5, 6, 11, and 12. Treatments 1, 4, 6, 8, 9, and 10 were significantly greater in unique peptide number as compared to treatments 5, 11, and 12. Treatments 4, 5, 6, 10, and 11 were significantly greater in unique peptide number as compared to treatment 12. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data violated the normality assumption (Appendix B).

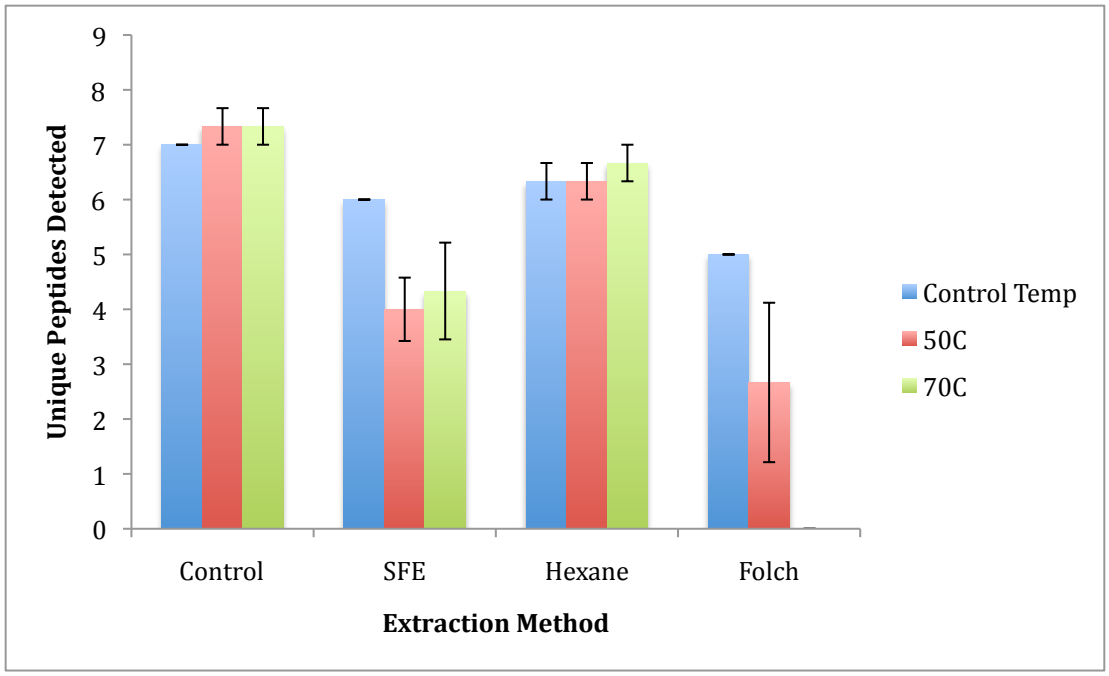

Figure 4.15. Number of unique peptides present in each of the 12 treatment groups of WPI and cream

The average number of unique peptides for each treatment was translated into which trypsin cleavage site was cut. This is illustrated in the β-LG "heat map" in Figure 4.16. Internal peptides required two sites to be cleaved while external peptides require only one site to be cleaved. As the color of the trypsin cleavage site goes from white to red, the cleavage percentage increases from 0% to 100%.

Cleavage Figure Legend

Cleavage Percentage	Site Color
0%	White
33%	Pink
66%	Orange
100%	Red

Figure 4.16. β-LG protein "heat map" illustrating the trypsin cleavage sites and the average cleavage frequency for each of the 12 treatments

The total number of peptides was then investigated using the total emPAI for each treatment. There was a significant effect of extraction ($P < 0.001$) on the total emPAI (Appendix C). Temperature ($P = 0.623$) and the interaction between extraction and temperature ($P = 0.633$) were not significant. Tukey's HSD pairwise comparisons were again used to determine which extraction methods resulted in significantly different total emPAI values (Appendix C). Control extractions and hexane extractions resulted in a significantly higher total emPAI as compared to Supercritical CO_2 (SFE) and Folch extractions. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). No violations of the assumptions were detected (Appendix C).

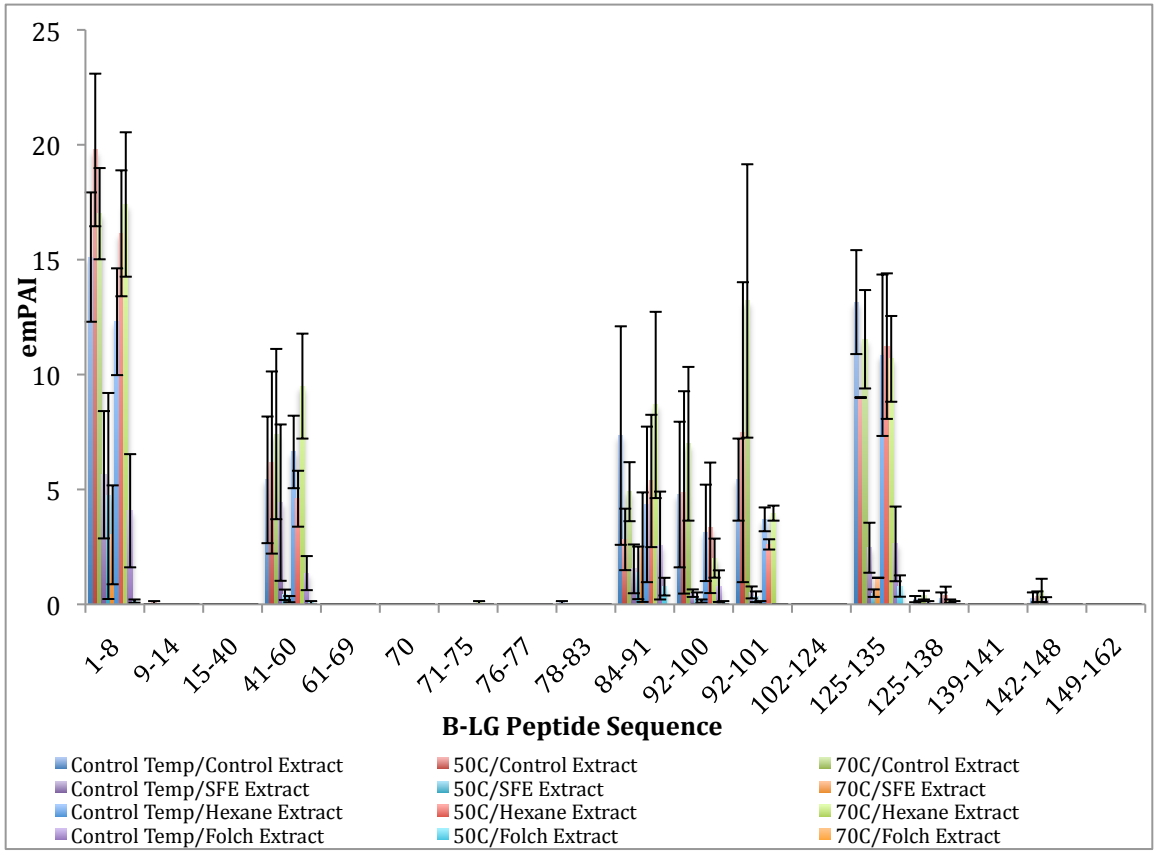

Figure 4.17. Exponentially modified protein abundance index (emPAI) of each β-LG peptide in each of the 12 treatment groups of WPI and cream

4.2.6 Domain Independent Analysis

Following total emPAI analysis (Figure 4.17), β-LG was divided into three distinct sections. Section 1 highlights amino acids 1 through 77, Section 2 highlights amino acids 78-138, and Section 3 highlights amino acids 139-162. The individual emPAI values for each treatment and for each section are shown in Figures 4.18, 4.19, and 4.20.

In β-LG Section 1 (Figure 4.18), there are two notable peptides of interest: peptides 1-8 and 41-60. Within peptide 1-8, control extraction and hexane extraction appear to have similar effects on emPAI, while SFE and Folch appear to have similar effects on emPAI. Within peptide 41-60, a similar trend of extraction method on emPAI is shown.

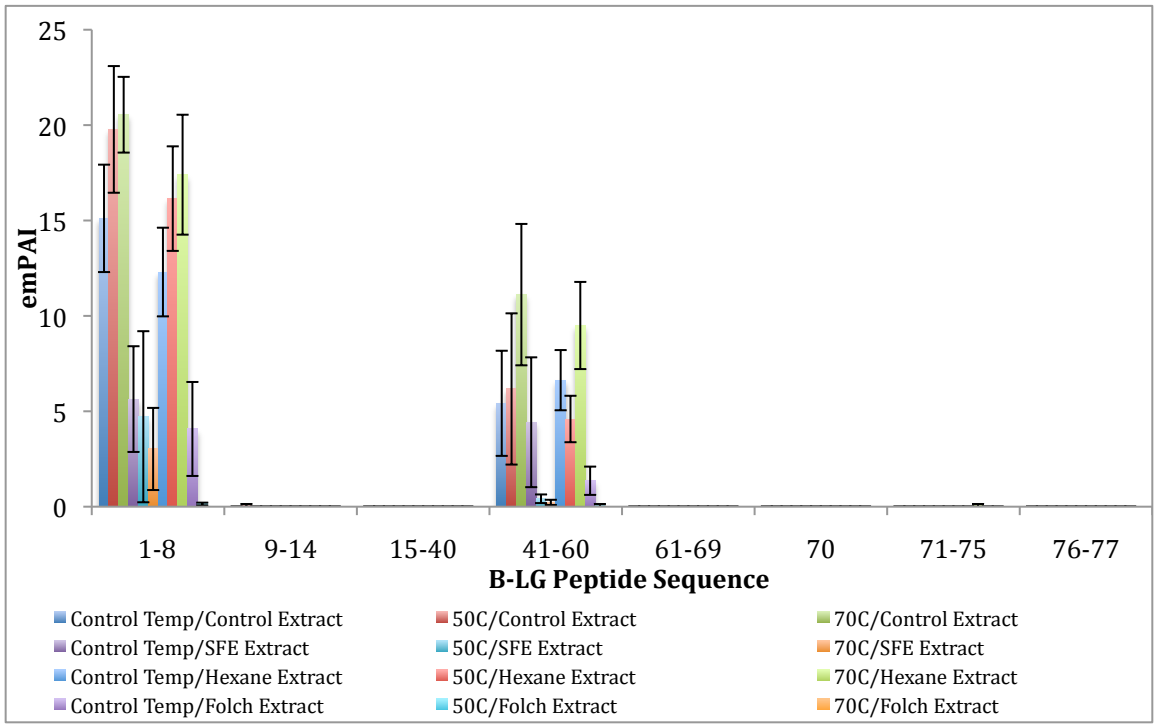

Figure 4.18. Exponentially modified protein abundance index (emPAI) of each β-LG peptide in Section 1 (Amino Acid 1-77) in each of the 12 treatment groups of WPI and cream

In β-LG Section 2 (Figure 4.19), there are five notable peptides of interest, peptides 84-91, 92-100, 92-101, 125-135, and 125-138. In Section 2, the effect of extraction method does not appear to have as significant effect on emPAI, with several treatment groups displaying overlapping standard error bars.

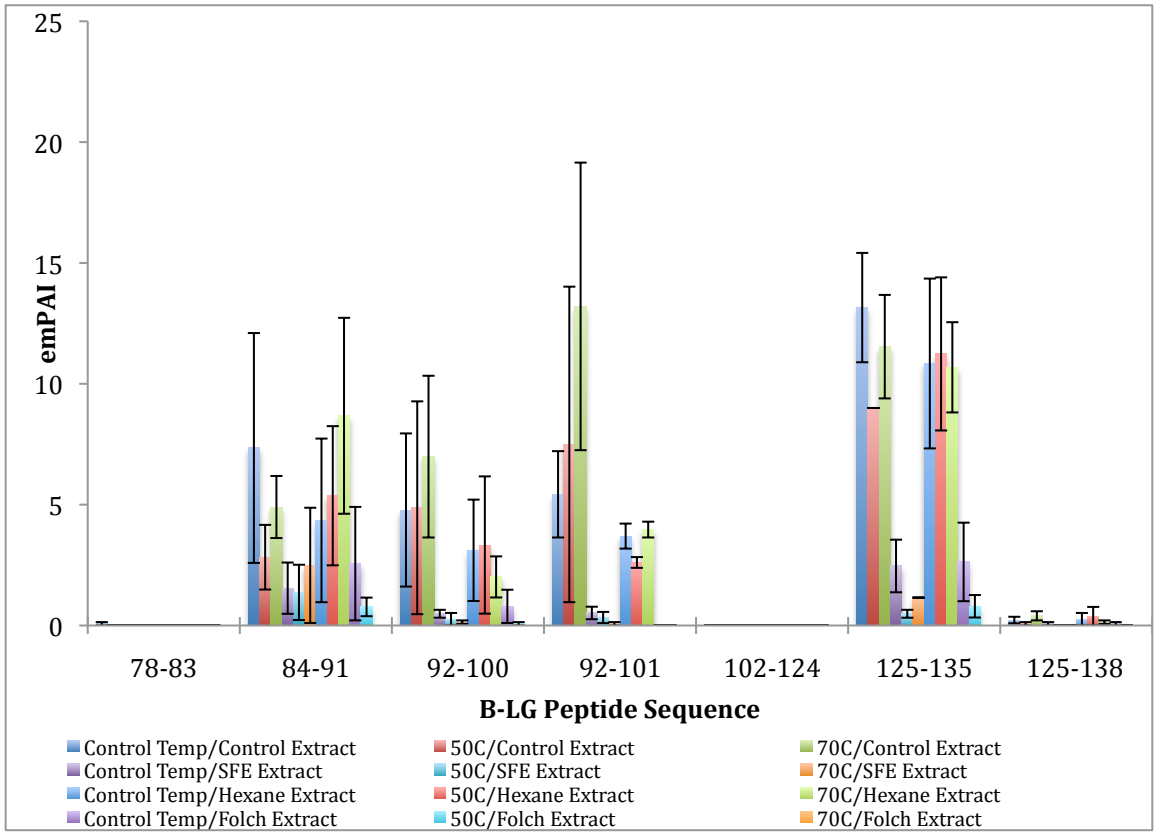

Figure 4.19. Exponentially modified protein abundance index (emPAI) of each β-LG peptide in Section 2 (Amino Acid 78-138) in each of the 12 treatment groups of WPI and cream

In β-LG Section 3 (Figure 4.20), there is only one notable peptide of interest, peptide 142-148. There does not appear to be an effect of extraction method or temperature on emPAI. However, it is important to note the graph scale and the emPAI values are dramatically reduced in Section 3 compared to Section 1 and Section 2.

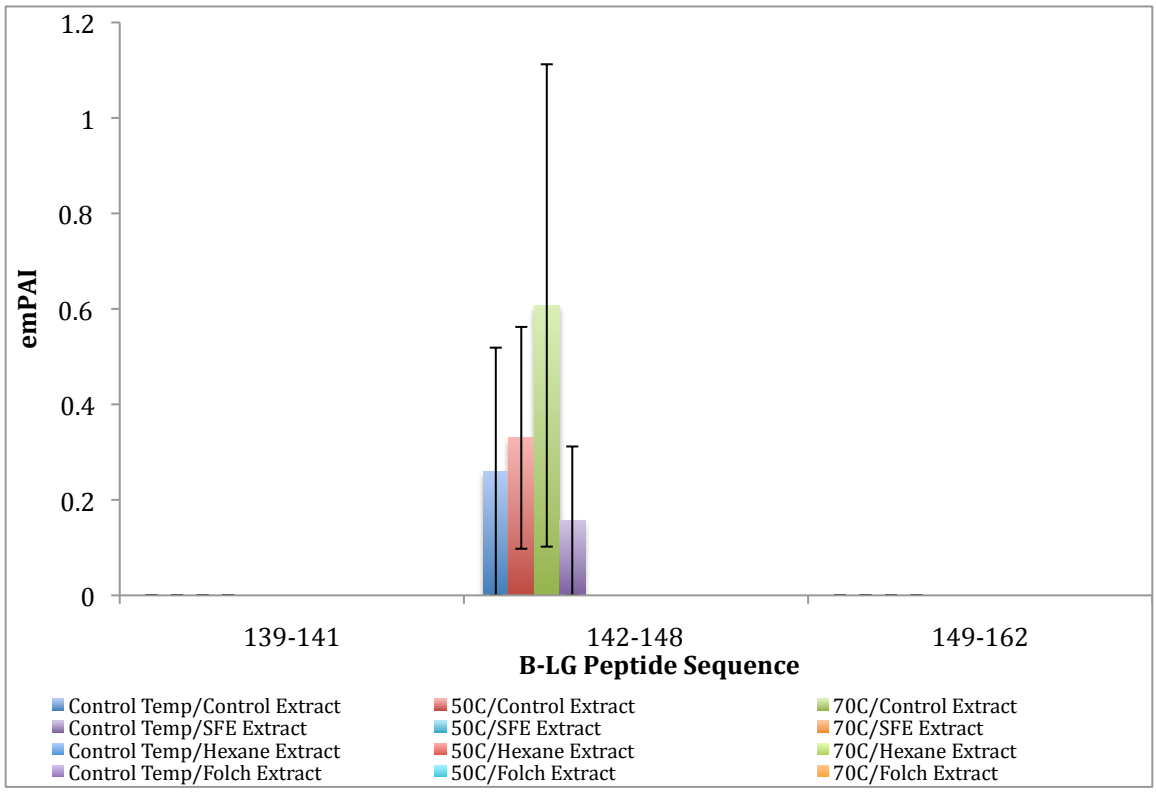

Figure 4.20. Exponentially modified protein abundance index (emPAI) of each β-LG peptide in Section 3 (Amino Acid 139-162) in each of the 12 treatment groups of WPI and cream

Following sectional β-LG emPAI analysis, eight domains of β-LG were selected for domain independent analysis. The domains selected are highlighted in Table 4.5. These domains were selected based on the relative emPAI values from Figures 4.18, 4.19, and 4.20. For each domain, a GLM was performed to identify if extraction, temperature, or an interaction between temperature and extraction had an effect on the individual domain emPAI. Tukey's HSD pairwise comparison was used for each domain following the GLM analysis to determine which factor levels resulted in significantly different emPAI values for each peptide.

In domain 1, there was a significant effect of extraction ($P < 0.001$) on emPAI (Appendix D). Control extraction and hexane extraction were significantly greater in emPAI than SFE extraction and Folch extraction. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data did not violate any assumptions (Appendix D).

In domain 2, there was a significant effect of extraction ($P = 0.002$) on emPAI (Appendix E). Hexane extraction was significantly greater in emPAI than SFE extraction. Control extraction and hexane extraction were significantly greater in emPAI than Folch extraction. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data appeared to violate the Normality assumption (Appendix E).

In domain 3, there was a not a significant effect of extraction ($P = 0.061$) or temperature ($P = 0.666$) on emPAI (Appendix F).

In domain 4, there was a significant effect of extraction ($P = 0.017$) on emPAI (Appendix G). Control extraction was significantly greater in emPAI than SFE extraction and Folch extraction. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data appeared to violate the Normality assumption (Appendix G).

In domain 5, there was a significant effect of extraction ($P = 0.001$) on emPAI (Appendix H). Control extraction was significantly greater in emPAI than SFE extraction and Folch extraction. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data appeared to violate the Normality assumption (Appendix H).

In domain 6, there was a significant effect of extraction ($P < 0.001$) on emPAI (Appendix I). Control extraction and hexane extraction were significantly greater in emPAI than SFE extraction and Folch extraction. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data appeared to violate the Normality assumption (Appendix I).

In domain 7, there was a not a significant effect of extraction ($P = 0.128$) or temperature ($P = 0.928$) on emPAI (Appendix J).

In domain 8, there was a significant effect of extraction ($P = 0.037$) on emPAI (Appendix K). There were no significant pairwise comparisons. This data was analyzed for any ANOVA assumption violations (Normality, Equal Variance, and Independence). This data appeared to violate the Normality assumption (Appendix K).

Table 4.5. Selected domains and sequences of β-LG used for domain independent analysis

Domain	Peptide Sequence	Sequence Number
1	IIVTQTMK	1-8
2	VYVEELKPTPEGDLEILLQK	41-60
3	IDALNENK	84-91
4	VLVLDTDYK	92-100
5	VLVLDTDYKK	92-101
6	TPEVDDEALEK	125-135
7	TPEVDDEALEKFDK	125-138
8	ALPMHIR	142-148

4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

4.3.1 Method Development

An Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze the same β-LG peptides that were previously analyzed by MS. Similarly to MS, an ELISA method was developed using intact, purified β-LG and intact, purified, Sc-CO_2 treated β-LG to reasonably insure effective antigen detection and antigen quantitation. Ultimately, this method was used to detect the presence and amount of antigens from each of the 12 treatments (Table 4.4). The four main parameters that were evaluated for method optimization were protein concentration, blocking reagent, washing protocol, and antibody concentration.

Three variations of protein concentration were evaluated. In a direct ELISA, the protein is bound directly to the ELISA plate. Therefore, the protein concentration can directly affect the signal strength. As the protein concentration was increased the signal strength was increased only slightly with digested samples and Sc-CO_2 treated β-LG. However,

with intact β-LG, as the concentration as increased, the signal strength also increased (Figure 4.21).

Two variations in blocking reagent were evaluated. In a direct ELISA, the blocking reagent is applied following the protein incubation. This step allows the blocking reagent to bind all the unbound sites on the ELISA plate. This ensures that when the antibody is added, the antibody does not bind to the plate. In addition it is important that the antibody does not bind to the blocking reagent. This will result in increased background signal. First, a 1% Bovine Serum Albumin (BSA) in TBS blocking solution was used. This resulted in high background absorbance for all controls and all protein samples (Appendix M). Next, a 1% fish gelatin in TBS blocking solution was used. This significantly improved the background absorbance (Appendix N).

The washing steps were slightly adjusted following secondary antibody incubation. Initial washing steps included wash three times with 1X PBST. This step was increased to five times with 1X PBST, each at one minute. This also improved non-specific signals (Appendix M and N).

Three variations in antibody concentration were evaluated. The primary antibody is added following the blocking step and binds to the protein attached on the ELISA plate. An increase in antibody concentration will increase signal. However, an increase in antibody concentration can also increase background. Antibody concentrations of 0

µg/µL, 0.085 µg/µL, and 0.2 µg/µL were used. An antibody concentration of 0.2 µg/µL provided the highest signal strength, while still limiting background noise.

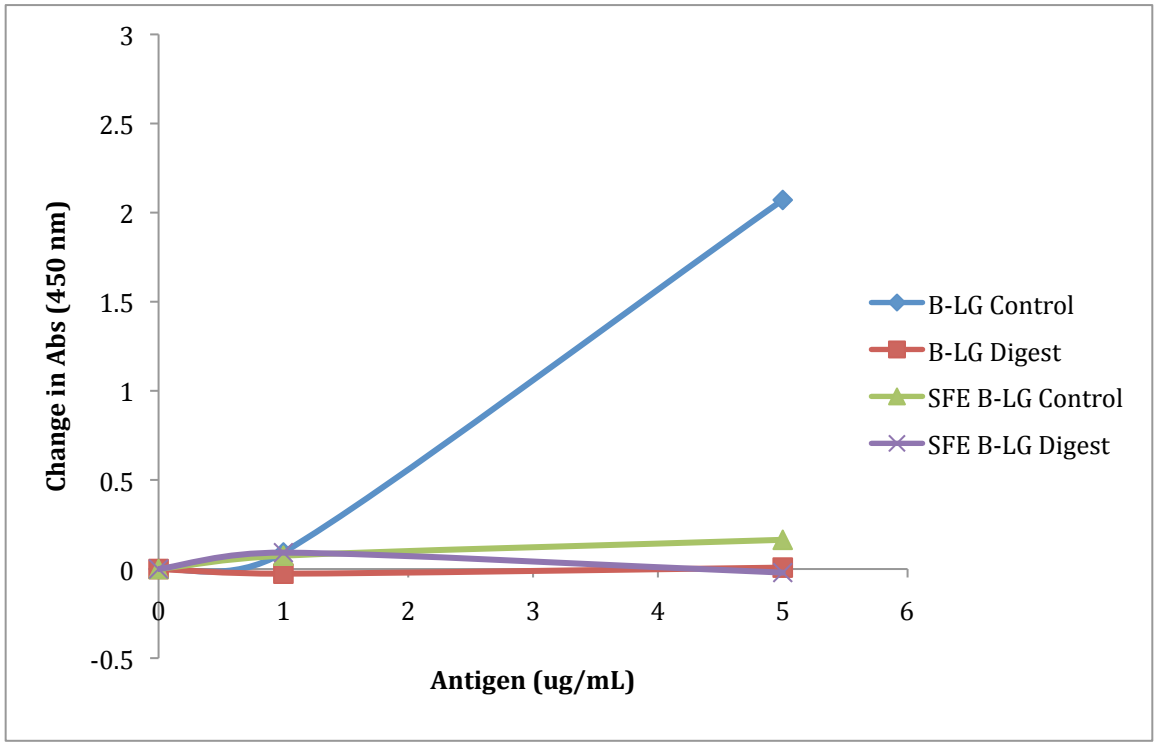

Figure 4.21. The effect of antigen concentration on the change in absorbance of each of the four protein samples with an antibody concentration of 0.085 µg/µL

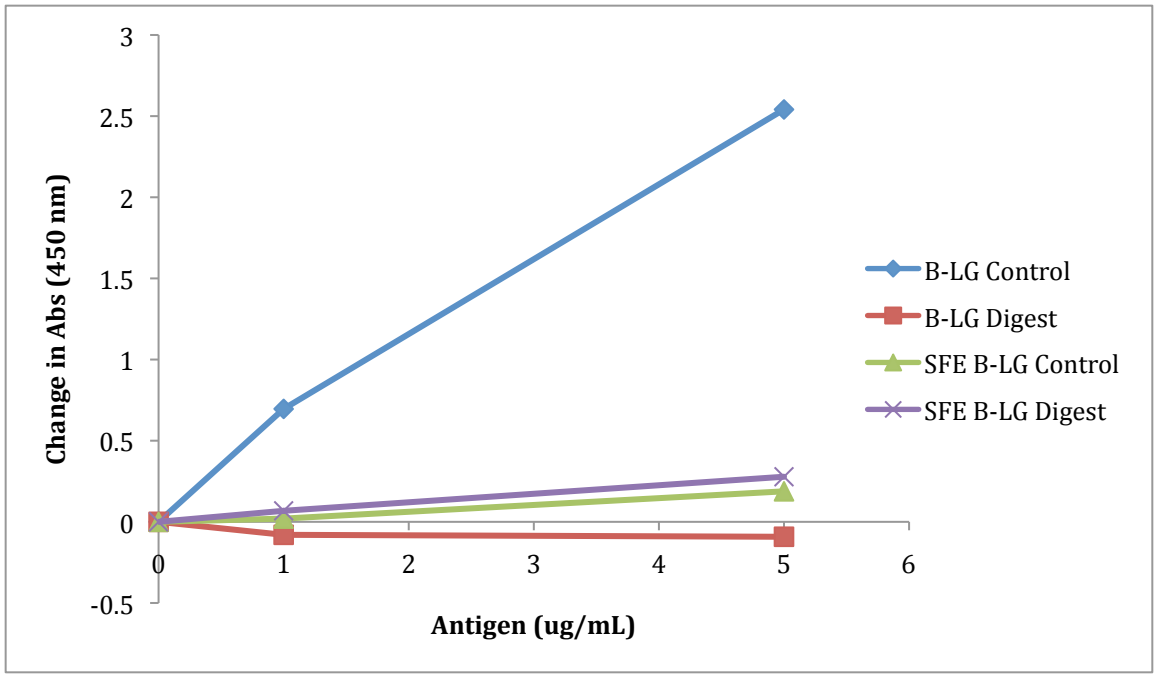

Figure 4.22. The effect of antigen concentration on the change in absorbance of each of the four protein samples with an antibody concentration of 0.2 μg/μL

4.3.2 WPI and Cream Treatments

Following ELISA method development, treatments 1 through 12 were diluted in 1X PBS to a concentration of 5 μg/mL. The peptide solutions were plated and an ELISA was performed. The digest controls were plated alongside the peptide digests and the raw absorbance values were subtracted to obtain a change in absorbance value at 450 nm for each treatment. In light of the MS results, all treatments should reveal a positive net absorbance value.

Analysis of the peptide fraction revealed dramatically different results, as compared to intact native β-LG protein. As shown in Figure 4.23, 5 out of the 12 treatments revealed a negative change in absorbance (Appendix O). Within each temperature and extraction factor, there do not appear to be any data trends that can explain this negative change in

absorbance. A possible reason for these results could be due to the fact that certain eptitopes of β-LG are undergoing conformational changes. Due to these changes, certain treatments will experience a loss of epitopes following hydrolysis, while other treatments, epitopes will still be accessible for antibody binding.

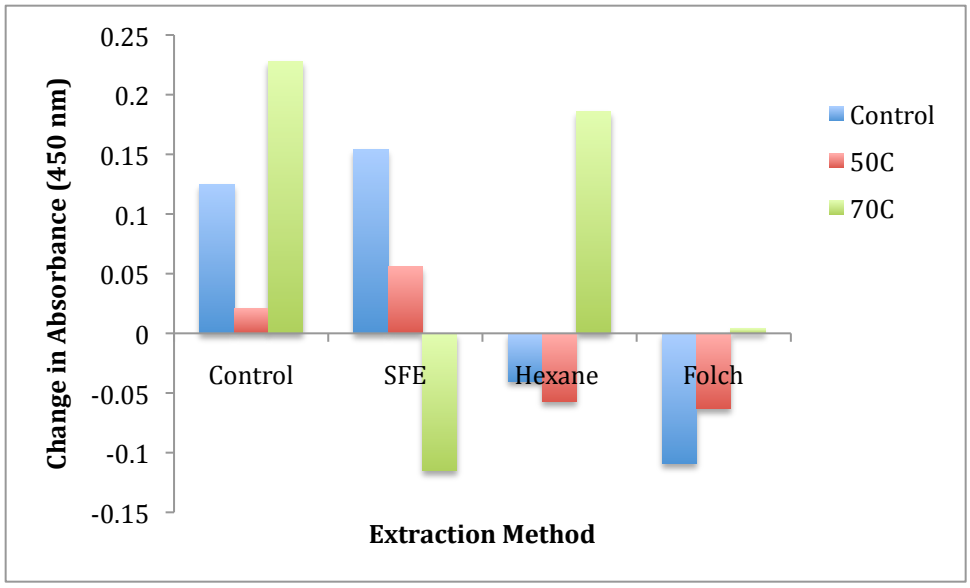

Figure 4.23. ELISA results on the effect of extraction method and temperature on the Change in absorbance between digest treatments and digest controls with a protein concentration of 5 ug/uL and an antibody concentration of 0.2 ug/uL. Replicate 1

In the second replicate shown in Figure 4.24, 6 out of the 12 treatments revealed a negative change in absorbance (Appendix P). When compared to replicate 1, there appears to be limited similarities and limited data trends between the factors.

Due to the ambiguity in the ELISA results with the polyclonal antibody, no further analysis was completed.

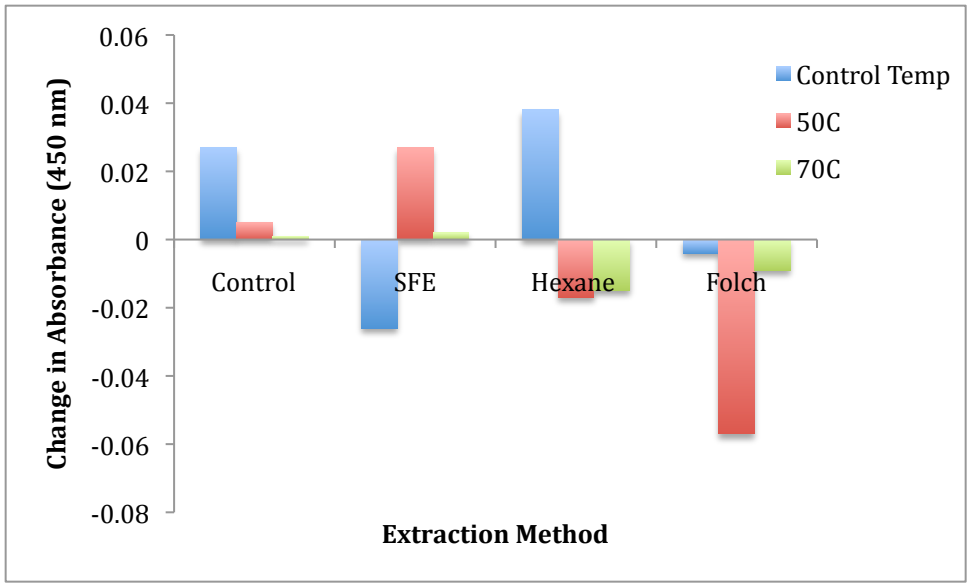

Figure 4.24. ELISA results on the effect of extraction method and temperature on the Change in absorbance between digest treatments and digest controls with a protein concentration of 5 ug/uL and an antibody concentration of 0.2 ug/uL. Replicate 2

CHAPTER 5: DISCUSSION

5.1 Enzyme Kinetics of β-LG

5.1.1 Explanation of Kinetic Differences

β-LG time-course hydrolysis exemplifies some moderate differences between untreated β-LG and treated Sc-CO_2 β-LG, as well as heat-treated β-LG and heat-treated Sc-CO_2 β-LG (Figures 4.1-4.4). β-LG is well characterized both physically and chemically. However, the function of the protein is largely unknown (Olsen et al., 2000). Therefore, the structure of β-LG was investigated during the enzymatic kinetics, in hopes to glean something regarding the function of the protein. In each of the digests, there was a slight difference in the rate of peptide production between untreated β-LG and treated Sc-CO_2 β-LG, as well as heat-treated β-LG and heat-treated Sc-CO_2 β-LG. However, at 5 minutes of hydrolysis, there were visible peptides in all treatment groups. In addition, the majority of the intact protein did not change over 19 hours of hydrolysis, showing no decrease in protein band intensity. This is in accordance to the literature, as the C and N terminal areas are easily digested, while the internal portion of the protein is highly resistant to digestion (Fernandez & Riera, 2012).

Initial protein enzymatics using traditional Coomassie Blue staining revealed few if any peptides of β-LG. Final enzymatics using silver staining revealed slightly more peptides of β-LG. However, peptides below 10 kDa could not be elucidated and the molecular weight and sequence of each peptide could not be identified using SDS-PAGE. Traditional tools for protein visualization have included SDS-PAGE. Never the less, limitations in analysis have forced scientists to supplement SDS-PAGE results with other

techniques. Therefore, the extent of analysis was limited and further protein analysis was completed using Mass Spectrometry.

However, these results were confirmed by the WPI method validation results. The abundance of β-LG peptides 92-101 and 125-138 was significantly greater than all other peptides. Cleavage at sites 92 or 101 would result in peptides of 10.4 kDa and 7.9 kDa or 11.4 kDa and 6.9 kDa. Cleavage at sites 125 or 138 would result in peptides of 14.1 kDa and 4.2 kDa or 15.6 kDa and 2.7 kDa. This was shown on the SDS polyacrylamide gels.

5.2. Emulsion of WPI and Cream

5.2.1 Temperature

Following SDS-PAGE, a method was optimized for the detection of B-LG peptides using MS to determine the effects of temperature and extraction method on the interaction of β-LG with MFGM and on the conformational changes of β-LG.

When analyzed for unique peptide number, there was a significant interaction between temperature and extraction method. When analyzed for emPAI, temperature was also not a significant factor in peptide release. These results bring to light that the conformation of β-LG does not significantly change under various temperatures when in complex with MFGM.

5.1.2 Extraction Method

On the other hand, extraction method was a significant factor in emPAI and in Domain Independent Analysis. This analysis highlights the fact that β-LG behaves differently under SFE and solvent extractions when in complex with MFGM.

Violations of the ANOVA normality assumption were observed. This is not surprising that a very small sample size was taken. A randomization test would be completed in future research to determine if the departures from normality had a significant effect on the p-values. The equal variance and independence assumptions were satisfied.

5.2.3 Reasons for Cleavage and non-Cleavage – 2° Protein Structure

Cleavage of a particular peptide is dependent on several factors including secondary structure, hydrophobicity, and location. Figure 5.1 shows the relative structure, hydrophobicity, and location of peptides along the length of the protein. According to previous research, a β-to-α transition in β-LG secondary structure can occur, allowing for insertion into the lipid bilayer protecting the protein from digestion (Zhang et al., 2007; Zhang & Keiderling, 2006). This is a possible explanation for peptide 142-148 (Region 8), where little of the peptide is detected by MS upon interaction with MFGM due to its slight hydrophobic character. This is also a possible explanation for peptides 92-101 and 125-138 where a reduction in protein abundance is observed when cream is introduced into the system as compared to the WPI control sample.

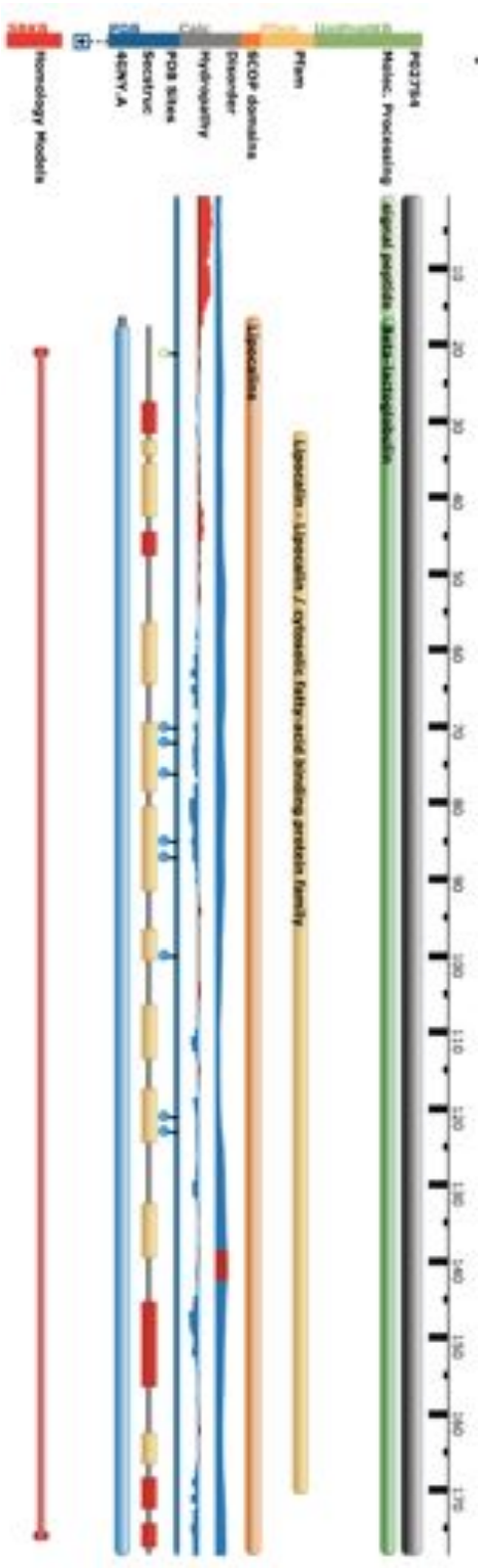

Figure 5.1. β-LG protein view site characterization. Adopted from Gutierrez-Magdaleno et al., 2013

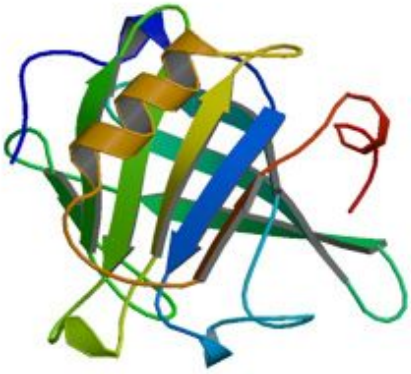

Figure 5.2. Bovine β-LG protein view. Adopted from Gutierrez-Magdaleno et al., 2013

5.2.4 Domain Independent Analysis

Following the unique peptide number analysis and the total emPAI analysis, eight out of 17 possible domains were selected for further analysis. Out of the eight domains, temperature was not a significant factor in a single domain. Extraction method was a significant factor in 6 out of the 8 domains. On the other hand, extraction method had a significant effect on the behavior of different β-LG domains. Most notably, in SFE the total number of peptides was reduced. In addition, SFE extraction showed a reduction in peptide cleavage in each of the eight domains. These results imply that SFE encourages MFGM and β-LG complex formation and furthermore that SFE encourages β-LG spreading of hydrophobic regions within the MFGM lipid bilayer. This processing aid changes the domain interactions of β-LG with MFGM and represents a future processing tool for value added ingredients and supplements.

These results are similar to the unique peptide number and total emPAI results in that temperature does not have a significant effect on β-LG hydrolysis when in complex with

MFGM. However, the control temperature treatment illustrates that β-LG forms a complex with MFGM without any activation energy. This was not shown in previous literature as sub-denaturing temperatures of 60°C and 65°C were used to form complexes (Singh. 2006). This is important to highlight that β-LG is shown naturally associating with MFGM without any additional processing techniques. These results beg the question regarding the biological function of β-LG and its role in co-digestion with the MFGM. Furthermore, the increased resistance to digestion of β-LG when complexed with MFGM supports the role of β-LG as a transport protein for digestion. In addition, MFGM and β-LG association has been characterized with an upper limit of 1.0 mg of β-LG per 1 gram of fat (1:1000 w/w)(Corredig & Dalgleish, 1996). This would equate to around 0.156 grams of β-LG associated with MFGM per gallon of whole milk.

5.3 Influence on Immune Activity

5.3.1 Native Proteins and Peptides

Contradictory to the biological benefits of β-LG, β-LG has elicited negative attention regarding its allergenic properties (Iametti et al., 2002; Adel-Patient et al., 2012). Therefore, it was desired to investigate the antigenicity of β-LG with and without Sc-CO_2 treatment and to investigate the peptides following digestion of the MFGM and β-LG complex. Thus, an ELISA method was created and optimized to investigate this property of β-LG.

The change in absorbance was the highest with the intact β-LG protein. The Sc-CO_2 treated β-LG showed a large reduction in absorbance as compared to the native β-LG

protein. It was also interesting to note that the change in absorbance between the Sc-CO_2 treated β-LG and Sc-CO_2 treated β-LG peptides was very similar. These results illustrate that in native β-LG protein, the epitopes are available for antibody binding and thus produce a large absorbance value. As digestion occurs, these epitopes of the intact protein are destroyed and the absorbance is reduced. In Sc-CO_2 treated β-LG, the change in absorbance is similar to the digested samples. This signifies that the protein confirmation has changed and the epitope accessibility has been reduced, therefore decreasing the absorbance value.

The application of this ELISA method to the β-LG and MFGM system did not perform as expected. It was shown that several of the treatments had a negative change in absorbance values. This is not in accordance with the MS results due to the fact that no peptides were observed in the digest control samples. There are two reasons for this discrepancy. First, the amount of peptides in each of the 12 treatment samples was remarkably lower than the samples used during the method development. Second, a polyclonal antibody was used. Therefore, the antibody has the capacity to bind to several sites on β-LG, thereby producing inconsistent absorbencies depending on the presence or absence of certain peptides or due to the retention or loss of certain epitopes. The retention or loss of particular epitopes is dependent on the change in conformation of β-LG. Consequently; upon digestion the absorbance value is not entirely indicative of the total amount of peptides. In order to properly investigate the antigenicity of the 12 treatments, a targeted monoclonal antibody to a specific β-LG peptide or several monoclonal antibodies targeting several β-LG peptides would need to be developed.

5.4. Industrial Relevance

5.4.1 Enzyme or Substrate Modification

Scientific advancement can occur in one of two realms. In an isolated system, a single variable and a single response can be studied. This approach is simplistic and conclusions can easily be drawn regarding the subject and variable. However, an isolated system is commonly very different than the native environment of study. Therefore, translating findings from an isolated system is very challenging and can result in scientific impediment. Changing the native conditions of a protein or peptide can change interactions, hydrolysis, secondary, tertiary, or quaternary structure. The second way in which scientific advancement can occur is in a complex system examining one response with many variables. This approach is complex and conclusions are much more difficult to draw due to the interaction between variables. On the other hand, breakthroughs in research in complex systems can easily be translated and have a great impact on the industry. Consequently, β-LG was not only investigated in a pure, isolated state but also investigated in a complex system.

Likewise, the hydrolysis of β-LG can be studied by one of two methods either by modifying the enzyme or by modifying β-LG. Due to the extensive proteomic research and database support done with trypsin, it was decided to modify the substrate, β-LG. In addition, given that the human digestive system is comprised of a multitude of enzymes, β-LG was modified using a set of different conditions. Therefore, the overarching

question was, are there some conditions that would change the structure of β-LG and if so, how do the conditions affect the number and type of peptides produced?

5.4.2 Structure and Function of β-LG

Utilizing a complex system of MFGM and WPI allowed for the investigation of both the structure and possible function of β-LG. An isolated system with pure β-LG was used in preliminary research and limited information was obtained regarding the structure of β-LG and even less information was obtained regarding the function of β-LG. Therefore, by investigating the interaction between MFGM and β-LG, both of which are components in milk, structure and function was evaluated.

5.4.3 Tryptic Mapping

It was found that the structure of β-LG is divided into three distinct sections. These sections vary in trypsin cleavage percentage, secondary structure, and hydrophobity. This was illustrated in the tryptic mapping where by the percentage of cleavage along each site of the β-LG protein was determined. In addition, it was found under native conditions that MFGM and β-LG formed a complex. This was not shown in previous research, as it was thought that activation energy was needed to encourage MFGM and β-LG interaction. Subsequently, it was found that β-LG domains behave differently under SFE and solvent extraction methods when in complex with MFGM. Therefore, by investigating β-LG in a complex system, important information regarding the structure of the protein was gained and groundbreaking information regarding the protein function was established.

5.4.4 Production of Novel Bioactive Peptides

β-LG has been extensively characterized both functionally and structurally. Within the protein, β-LG contains a multitude of bioactive peptides (Hernandez-Ledesma et al., 2008). The release of these peptides is dependent on the processing conditions. The challenge with bioactive peptide production from β-LG is that the internal core of β-LG is fairly resistant to digestion, as shown in Figure 4.1-4.4 (Fernandez & Riera, 2012). Moreover, when β-LG is complexed with MFGM, β-LG is even more resistant to digestion than in a native state. These results support the proposed role of β-LG as a transport protein to aid in digestion. Further, these results suggest that β-LG, when in complex with MFGM, is digested much further into the gastrointestinal tract.

Lastly, SFE was shown to increase the rate of hydrolysis of β-LG (Figure 4.1-4.4). However, in complex with MFGM, SFE was shown to decrease the number of β-LG peptides. In SDS-PAGE, SFE is exposing more trypsin sites for cleavage. In the context of MFGM, SFE is causing β-LG to unfold, interacting with the MFGM lipid bilayer, concealing trypsin cut sites. Therefore, β-LG is changing confirmation in both experiments. However, in one experiment β-LG is made more available to enzyme degradation and in the other experiment β-LG is made more available to MFGM.

It is apparent that digestion needs to be further investigated with the incorporation both lipases and trypsin to determine if digestion is facilitated by the presence of MFGM.

CHAPTER 6: CONCLUSIONS & LIMITATIONS

In milk products and milk processing, components are not present in isolation and frequently interact with one another. Therefore, it makes logical sense to consider this natural system and incorporate as many of the components as possible into dairy research.

In this research, the question that was to be answered was how does the effect of temperature and fat extraction method affect the hydrolysis of β-LG?

In the preliminary experiments, it was found that Sc-CO_2 treatment of β-LG had a slight effect on the rate of protein hydrolysis. After incorporating washed cream, it was found that β-LG is divided into three distinct sections. These three sections vary in cleavage selectivity or frequency. Cleavage frequency is dependent on the secondary structure, the relative hydrophobicity, and its propensity to interact with MFGM.

The fat extraction method was a significant factor in the amount of each peptide released. It was found that conformationally, β-LG behaves differently under SFE than under other solvent extraction methods when in complex with MFGM. Under SFE, the number of total peptides and the number of peptides in each domain were reduced. It was shown that SFE promotes complex formation and the spreading of the hydrophobic regions of β-LG within the lipid layer of MFGM. SFE represents an interesting processing aid that changes the domain interactions of β-LG and may perhaps be a processing aid for value added ingredients and supplements.

It is possible that the interaction between MFGM and β-LG was initiated by the transition of peptide 142-148 (Region 8) from β-to-α allowing for insertion into the MFGM bilayer. This is supported by the limited detection of the peptide in the emPAI analysis, the hydrophobic character of the region, in agreement with previous research (Zhang et al., 2007; Zhang & Keiderling, 2006). Further, this interaction was seen in the control heat treatment groups. These results suggest that this interaction is biologically innate and that the biological function of β-LG is a transport protein to aid in digestion, whereby the complex of β-LG-MFGM is co-digested. This would suggest that digestion takes place further down the gastrointestinal tract, supporting the previously characterized satiety factors of β-LG. Lastly; the allergen potential of β-LG was assessed. It was found that Sc-CO_2 treatment of β-LG reduced the allergenic response as compared to native β-LG.

In summary, MFGM and β-LG appear to have a natural affinity under many different conditions. This interaction could certainly help explain the function of β-LG. SFE represents a very promising technique that can be used in process for many applications. Further research will be needed to confirm the role of β-LG as well as the industrial impacts of SFE. However, these findings indicate that the procedure established from this work can be used for further exploration of bioactive peptide production. We also demonstrated that β-LG is not easily digested and digestion is further hindered within the presence of MFGM. Furthermore, the conformation of β-LG is significantly changed following treatment with SFE in the presence or absence of MFGM.

The limitations of this research were multifold. The isolation and preparation of washed cream was done in several batches due to the size of the centrifuge. This could be improved and accelerated by using a larger centrifuge and would have limited the amount of down time. Secondly, the temperature controls of the Supercritical unit were collaged and needed constant monitoring by hand. This resulted in a wide range of temperatures during the extraction step. This variation could have impacted the SFE extraction results. Next, the liquid chromatography (LC) source to the MS was plagued with problems including reliability, poor separation, and costly repairs. Due to these problems, the LC source was replaced with a direct infusion pump. This solved the problem but did not replace the capabilities of the LC unit. A lack of separation prior to fractionation could have impacted the quantitation power of the results. Lastly, the polyclonal antibody used was not specific to a single epitope of β-LG isolated by one of the 12 treatments. Due to the conformational changes occurring as a result of these treatments, the presence of epitopes varied among the treatments. Therefore, the peptide ELISA results was not reliable. The incorporation of a monoclonal antibody to this method would have greatly improved these results.

CHAPTER 7: DIRECTIONS FOR FUTURE RESEARCH

- Peptide fractionation and microbial plating to determine antimicrobial effects of hydrolyzed peptides under Sc-CO_2 treatment.
- Atomic Force Microscopy – Infrared Microscopy (AFM-IR) to visualize undigested β-LG interactions with MFGM over a wide range of temperatures.
- Targeted ELISA using specific primary epitope monoclonal antibodies to determine reduction in antigenicity of hydrolyzed peptides under Sc-CO_2 treatment.
- Revise Sc-CO_2 instrumentation to perform in-process digestion.
- Quantum dots and Circular Dichroism Spectroscopy of β-LG to track changes in confirmation during the interaction with MFGM.
- Determine the aggregation properties of native β-LG and β-LG peptides.
- Include Diafiltration permeate (Pro-Cream) into the experimental design.
- Determine innovative way for detecting Section 1 peptides.
- Continue Invitro and explore Invivo digestion studies of undigested MFGM and β-LG complex.
- Determine proteins and phospholipids of MFGM responsible for β-LG interaction.
- Determine why α-LA does not share the same affinity as β-LG for MFGM.

CHAPTER 8: REFERENCES

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CHAPTER 9: APPENDIX

APPENDIX A: Buffer Protocols

a. Phosphate Buffer Saline (PBS) (1X)
    i. A 1X solution was made by adding 8 g of NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 was added to a one-liter bottle and filled with deionized water up to 800 mL. The solutes were dissolved and the pH was adjusted with 1N NaOH to 7.40. The final volume was adjusted to one liter.
b. Phosphate Buffer Saline with 0.1% Tween-20 (PBST)(1X)
    i. A 1X solution was made by adding 8 g of NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 1 mL Tween-20 was added to a one-liter bottle and filled with deionized water up to 800 mL. The solutes were dissolved and the pH was adjusted with 1N NaOH to 7.40. The final volume was adjusted to one liter.
c. Phosphate Buffer Saline (PBS) (1X) with 1% Fish Gelatin
    i. A 1X solution was made by adding 8 g of NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 10 mL Fish Gelatin was added to a one-liter bottle and filled with deionized water up to 800 mL. The solutes were dissolved and the pH was adjusted with 1N NaOH to 7.40. The final volume was adjusted to one liter.
d. Phosphate Buffer Saline (PBS) (1X) with 1% Bovine Serum Albumin (BSA)
    i. A 1X solution was made by adding 8 g of NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 10 g BSA was added to a one-liter bottle and filled with deionized water up to 800 mL. The solutes were dissolved and the pH was adjusted with 1N NaOH to 7.40. The final volume was adjusted to one liter.
e. 2M Sulfuric Acid
    i. 21.72 mL of HPLC grade Sulfuric Acid was added to 178.28 mL of deionized water to a final volume of 200 mL.

APPENDIX B: Unique Peptide Number Data

a. Raw Data

Treatment	Temperature	Extraction	Unique Peptide Number	RESI1	Observation order
1	1	1	7	0	1
2	2	1	7	-0.33333	2
3	3	1	7	-0.33333	3
4	1	2	6	0	4
5	2	2	5	1	5
6	3	2	6	1.666667	6
7	1	3	7	0.666667	7
8	2	3	7	0.666667	8
9	3	3	7	0.333333	9
10	1	4	5	0	10
11	2	4	5	2.333333	11
12	3	4	0	0	12
1	1	1	7	0	13
2	2	1	8	0.666667	14
3	3	1	8	0.666667	15
4	1	2	6	0	16
5	2	2	3	-1	17
6	3	2	4	-0.33333	18
7	1	3	6	-0.33333	19
8	2	3	6	-0.33333	20
9	3	3	7	0.333333	21
10	1	4	5	0	22
11	2	4	3	0.333333	23
12	3	4	0	0	24
1	1	1	7	0	25
2	2	1	7	-0.33333	26
3	3	1	7	-0.33333	27
4	1	2	6	0	28
5	2	2	4	0	29
6	3	2	3	-1.33333	30
7	1	3	6	-0.33333	31
8	2	3	6	-0.33333	32
9	3	3	6	-0.66667	33
10	1	4	5	0	34
11	2	4	0	-2.66667	35
12	3	4	0	0	36

b. Unique Peptide Number Data Summary

	Control	SFE	Hexane	Folch	
Control	7	6	6.33	5	6.0825
50ºC	7.33	4	6.33	2.66	5.08
70ºC	7.33	4.33	6.66	0	4.58
Average	7.22	4.776667	6.44	2.553333	5.2475

c. Unique Peptide Number General Linear Model Statistics

General Linear Model: Unique Peptide N versus Temperature, Extraction

```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Unique Peptide Number, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS  Adj MS      F      P
Temperature              2   14.000   14.000   7.000   7.41  0.003
Extraction               3  115.194  115.194  38.398  40.66  0.000
Temperature*Extraction   6   30.889   30.889   5.148   5.45  0.001
Error                   24   22.667   22.667   0.944
Total                   35  182.750


S = 0.971825   R-Sq = 87.60%   R-Sq(adj) = 81.91%


Unusual Observations for Unique Peptide Number

      Unique
     Peptide
Obs   Number      Fit   SE Fit  Residual  St Resid
  6  6.00000  4.33333  0.56108   1.66667      2.10 R
 11  5.00000  2.66667  0.56108   2.33333      2.94 R
 35  0.00000  2.66667  0.56108  -2.66667     -3.36 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
1            12   6.1  A
2            12   5.1    B
3            12   4.6    B

Means that do not share a letter are significantly different.
```

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Unique Peptide Number
All Pairwise Comparisons among Levels of Temperature
Temperature = 1 subtracted from:

Temperature	Lower	Center	Upper	-----+---------+---------+---------+-
2	-1.990	-1.000	-0.0097	(---------*---------)
3	-2.490	-1.500	-0.5097	(---------*---------)
				-----+---------+---------+---------+-
				-2.0 -1.0 0.0 1.0

Temperature = 2 subtracted from:

Temperature	Lower	Center	Upper	-----+---------+---------+---------+-
3	-1.490	-0.5000	0.4903	(---------*---------)
				-----+---------+---------+---------+-
				-2.0 -1.0 0.0 1.0

Grouping Information Using Tukey Method and 95.0% Confidence

Extraction	N	Mean	Grouping
1	9	7.2	A
3	9	6.4	A
2	9	4.8	B
4	9	2.6	C

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Unique Peptide Number
All Pairwise Comparisons among Levels of Extraction
Extraction = 1 subtracted from:

Extraction	Lower	Center	Upper	----+---------+---------+---------+--
2	-3.708	-2.444	-1.181	(----*----)
3	-2.041	-0.778	0.486	(----*----)
4	-5.930	-4.667	-3.403	(----*----)
				----+---------+---------+---------+--
				-5.0 -2.5 0.0 2.5

Extraction = 2 subtracted from:

Extraction	Lower	Center	Upper	----+---------+---------+---------+--
3	0.403	1.667	2.9300	(----*----)
4	-3.486	-2.222	-0.9588	(----*----)
				----+---------+---------+---------+--
				-5.0 -2.5 0.0 2.5

Extraction = 3 subtracted from:

Extraction	Lower	Center	Upper	----+---------+---------+---------+--
4	-5.152	-3.889	-2.626	(----*----)
				----+---------+---------+---------+--
				-5.0 -2.5 0.0 2.5

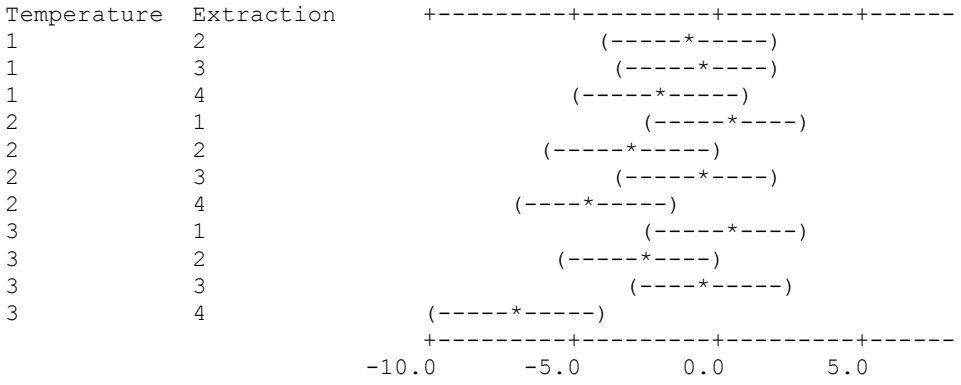
Grouping Information Using Tukey Method and 95.0% Confidence

Temperature	Extraction	N	Mean	Grouping
3	1	3	7.3	A
2	1	3	7.3	A
1	1	3	7.0	A B
3	3	3	6.7	A B C
2	3	3	6.3	A B C
1	3	3	6.3	A B C
1	2	3	6.0	A B C
1	4	3	5.0	A B C D
3	2	3	4.3	B C D
2	2	3	4.0	C D
2	4	3	2.7	D E
3	4	3	0.0	E

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Unique Peptide Number
All Pairwise Comparisons among Levels of Temperature*Extraction
Temperature = 1
Extraction = 1 subtracted from:

Temperature	Extraction	Lower	Center	Upper
1	2	-3.862	-1.000	1.862
1	3	-3.528	-0.667	2.195
1	4	-4.862	-2.000	0.862
2	1	-2.528	0.333	3.195
2	2	-5.862	-3.000	-0.138
2	3	-3.528	-0.667	2.195
2	4	-7.195	-4.333	-1.472
3	1	-2.528	0.333	3.195
3	2	-5.528	-2.667	0.195
3	3	-3.195	-0.333	2.528
3	4	-9.862	-7.000	-4.138



```
Temperature = 1
Extraction = 2  subtracted from:

Temperature  Extraction   Lower  Center   Upper
1            3           -2.528   0.333   3.195
1            4           -3.862  -1.000   1.862
2            1           -1.528   1.333   4.195
2            2           -4.862  -2.000   0.862
2            3           -2.528   0.333   3.195
2            4           -6.195  -3.333  -0.472
3            1           -1.528   1.333   4.195
3            2           -4.528  -1.667   1.195
3            3           -2.195   0.667   3.528
3            4           -8.862  -6.000  -3.138

Temperature  Extraction      +---------+---------+---------+------
1            3                          (-----*----)
1            4                       (-----*-----)
2            1                            (-----*----)
2            2                     (-----*-----)
2            3                          (-----*----)
2            4                  (----*-----)
3            1                           (-----*----)
3            2                     (-----*----)
3            3                          (----*-----)
3            4            (-----*-----)
                          +---------+---------+---------+------
                        -10.0      -5.0       0.0       5.0


Temperature = 1
Extraction = 3  subtracted from:

Temperature  Extraction   Lower  Center   Upper
1            4           -4.195  -1.333   1.528
2            1           -1.862   1.000   3.862
2            2           -5.195  -2.333   0.528
2            3           -2.862   0.000   2.862
2            4           -6.528  -3.667  -0.805
3            1           -1.862   1.000   3.862
3            2           -4.862  -2.000   0.862
3            3           -2.528   0.333   3.195
3            4           -9.195  -6.333  -3.472

Temperature  Extraction      +---------+---------+---------+------
1            4                       (----*-----)
2            1                           (-----*-----)
2            2                     (----*-----)
2            3                        (-----*-----)
2            4                  (-----*----)
3            1                           (-----*-----)
3            2                     (-----*-----)
3            3                          (-----*----)
3            4            (----*-----)
                          +---------+---------+---------+------
                        -10.0      -5.0       0.0       5.0
```

```
Temperature = 1
Extraction = 4  subtracted from:

Temperature  Extraction   Lower  Center   Upper
2            1          -0.528   2.333   5.195
2            2          -3.862  -1.000   1.862
2            3          -1.528   1.333   4.195
2            4          -5.195  -2.333   0.528
3            1          -0.528   2.333   5.195
3            2          -3.528  -0.667   2.195
3            3          -1.195   1.667   4.528
3            4          -7.862  -5.000  -2.138

Temperature  Extraction      +---------+---------+---------+------
2            1                                (-----*----)
2            2                           (-----*-----)
2            3                                (-----*----)
2            4                      (----*-----)
3            1                                (-----*----)
3            2                           (-----*----)
3            3                                (----*-----)
3            4                  (-----*-----)
                                +---------+---------+---------+------
                             -10.0      -5.0       0.0       5.0


Temperature = 2
Extraction = 1  subtracted from:

Temperature  Extraction   Lower  Center   Upper
2            2           -6.19  -3.333  -0.472
2            3           -3.86  -1.000   1.862
2            4           -7.53  -4.667  -1.805
3            1           -2.86   0.000   2.862
3            2           -5.86  -3.000  -0.138
3            3           -3.53  -0.667   2.195
3            4          -10.19  -7.333  -4.472

Temperature  Extraction      +---------+---------+---------+------
2            2                       (----*-----)
2            3                           (-----*-----)
2            4                  (-----*----)
3            1                            (-----*-----)
3            2                       (-----*-----)
3            3                            (-----*----)
3            4               (----*-----)
                             +---------+---------+---------+------
                          -10.0      -5.0       0.0       5.0


Temperature = 2
Extraction = 2  subtracted from:

Temperature  Extraction   Lower  Center   Upper
2            3          -0.528   2.333   5.195
2            4          -4.195  -1.333   1.528
3            1           0.472   3.333   6.195
3            2          -2.528   0.333   3.195
3            3          -0.195   2.667   5.528
3            4          -6.862  -4.000  -1.138
```

```
Temperature  Extraction      +---------+---------+---------+------
2            3                              (-----*----)
2            4                        (----*-----)
3            1                                  (-----*----)
3            2                           (-----*----)
3            3                                (----*-----)
3            4                     (-----*-----)
                                   +---------+---------+---------+------
                                -10.0       -5.0        0.0        5.0
```

Temperature = 2
Extraction = 3 subtracted from:

Temperature	Extraction	Lower	Center	Upper
2	4	-6.528	-3.667	-0.805
3	1	-1.862	1.000	3.862
3	2	-4.862	-2.000	0.862
3	3	-2.528	0.333	3.195
3	4	-9.195	-6.333	-3.472

```
Temperature  Extraction      +---------+---------+---------+------
2            4                     (-----*----)
3            1                               (-----*-----)
3            2                        (-----*-----)
3            3                             (-----*----)
3            4                  (----*-----)
                                   +---------+---------+---------+------
                                -10.0       -5.0        0.0        5.0
```

Temperature = 2
Extraction = 4 subtracted from:

Temperature	Extraction	Lower	Center	Upper
3	1	1.805	4.667	7.5282
3	2	-1.195	1.667	4.5282
3	3	1.138	4.000	6.8615
3	4	-5.528	-2.667	0.1949

```
Temperature  Extraction      +---------+---------+---------+------
3            1                                       (----*-----)
3            2                                 (----*-----)
3            3                                    (-----*-----)
3            4                           (-----*----)
                                   +---------+---------+---------+------
                                -10.0       -5.0        0.0        5.0
```

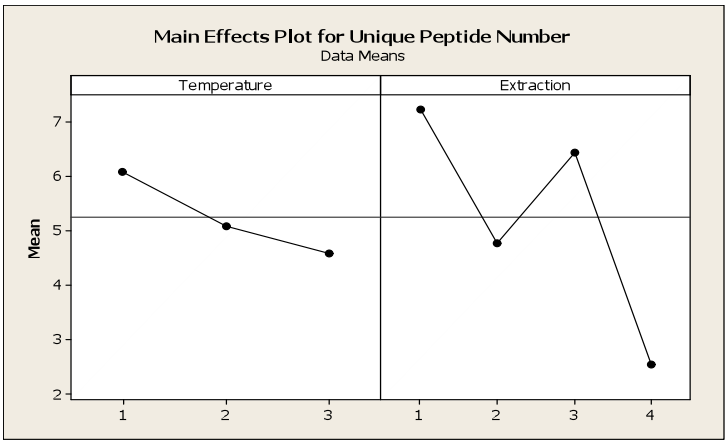
Temperature = 3
Extraction = 1 subtracted from:

Temperature	Extraction	Lower	Center	Upper
3	2	-5.86	-3.000	-0.138
3	3	-3.53	-0.667	2.195
3	4	-10.19	-7.333	-4.472

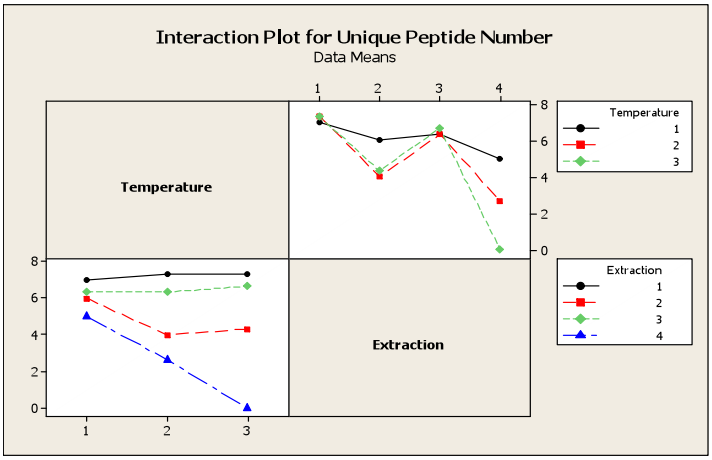
```
Temperature  Extraction      +---------+---------+---------+------
3            2                            (-----*-----)
3            3                                 (-----*----)
3            4                    (----*-----)
```

```
                          +---------+---------+---------+------
                        -10.0      -5.0       0.0       5.0


Temperature = 3
Extraction = 2  subtracted from:

Temperature  Extraction   Lower  Center   Upper
3            3           -0.528   2.333   5.195
3            4           -7.195  -4.333  -1.472

Temperature  Extraction      +---------+---------+---------+------
3            3                                (-----*----)
3            4                      (----*-----)
                          +---------+---------+---------+------
                        -10.0      -5.0       0.0       5.0


Temperature = 3
Extraction = 3  subtracted from:

Temperature  Extraction   Lower  Center   Upper
3            4           -9.528  -6.667  -3.805

Temperature  Extraction      +---------+---------+---------+------
3            4                (-----*----)
                          +---------+---------+---------+------
                        -10.0      -5.0       0.0       5.0
```

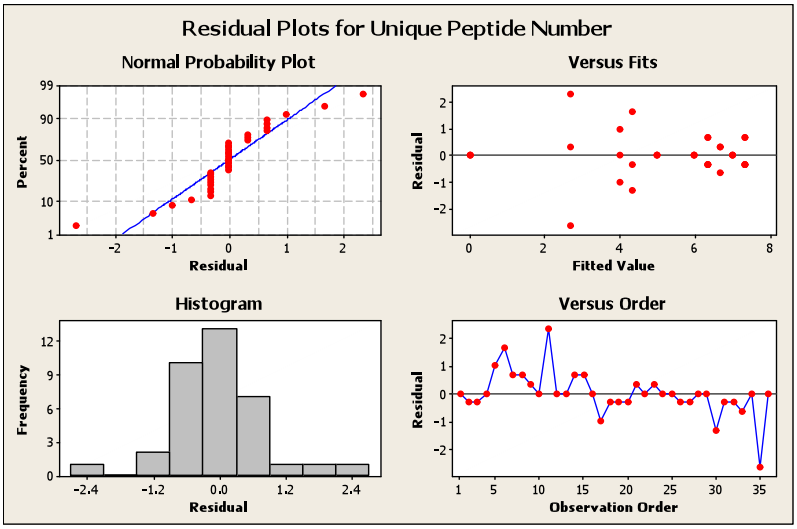
d. Unique Peptide Number Main Effects Plot



e. Unique Peptide Number Interaction Plot



f. Unique Peptide Number Residual Plots



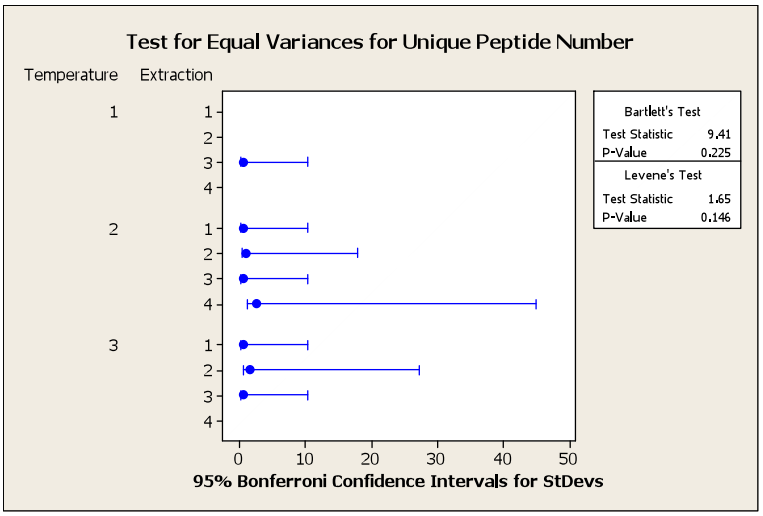
g. Unique Peptide Number ANOVA Assumptions

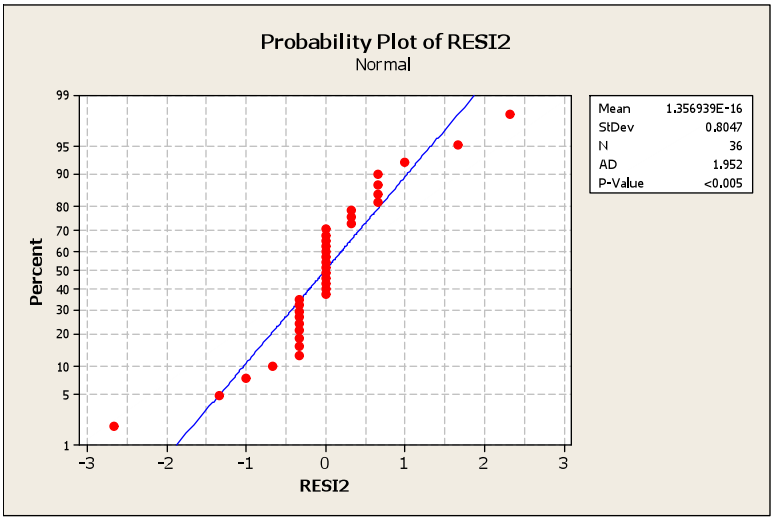
Test for Equal Variances: Unique Peptide Number versus Temperature, Extraction

```
95% Bonferroni confidence intervals for standard deviations

Temperature  Extraction  N    Lower    StDev    Upper
          1           1  3        *  0.00000        *
          1           2  3        *  0.00000        *
          1           3  3  0.24039  0.57735  10.3199
          1           4  3        *  0.00000        *
          2           1  3  0.24039  0.57735  10.3199
          2           2  3  0.41637  1.00000  17.8746
          2           3  3  0.24039  0.57735  10.3199
          2           4  3  1.04783  2.51661  44.9833
          3           1  3  0.24039  0.57735  10.3199
          3           2  3  0.63601  1.52753  27.3038
          3           3  3  0.24039  0.57735  10.3199
          3           4  3        *  0.00000        *


Bartlett's Test (Normal Distribution)
Test statistic = 9.41, p-value = 0.225


Levene's Test (Any Continuous Distribution)
Test statistic = 1.65, p-value = 0.146
```

Regression Analysis: RESI1 versus observation order

```
The regression equation is
RESI1 = 0.686 - 0.0371 observation order

Predictor              Coef  SE Coef      T      P
Constant             0.6857   0.2430   2.82  0.008
observation order  -0.03707  0.01145  -3.24  0.003


S = 0.713921   R-Sq = 23.5%   R-Sq(adj) = 21.3%


Analysis of Variance

Source          DF       SS      MS      F      P
Regression       1   5.3375  5.3375  10.47  0.003
Residual Error  34  17.3292  0.5097
Total           35  22.6667


Unusual Observations

     observation
Obs        order   RESI1     Fit  SE Fit  Residual  St Resid
 11         11.0   2.333   0.278   0.147     2.055      2.94R
 35         35.0  -2.667  -0.612   0.223    -2.055     -3.03R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 2.00325
```


Test for Equal Variances for Unique Peptide Number
Temperature
Extraction
1
2
3
4
0
10
20
30
40
50
95% Bonferroni Confidence Intervals for StDevs
Bartlett's Test
Test Statistic 9.41
P-Value 0.225
Levene's Test
Test Statistic 1.65
P-Value 0.146


Probability Plot of RESI2
Normal
Percent
99
95
90
80
70
60
50
40
30
20
10
5
1
-3
-2
-1
0
1
2
3
RESI2
Mean 1.356939E-16
StDev 0.8047
N 36
AD 1.952
P-Value <0.005

APPENDIX C: Total emPAI

a. Total emPAI raw data

Treatment	Temperature	Extraction	emPAI	RESI1	Observation Order
1	1	1	72.11	20.32	1
2	2	1	73.93	23.33	2
3	3	1	68.78	6.736667	3
4	1	2	17.93	2.633333	4
5	2	2	19.65	12.07667	5
6	3	2	16.29	9.19	6
7	1	3	55.31	14.12333	7
8	2	3	59.82	16.15333	8
9	3	3	62.73	10.28667	9
10	1	4	27.06	15.58	10
11	2	4	3.39	1.546667	11
12	3	4	0	0	12
1	1	1	42.48	-9.31	13
2	2	1	48.9	-1.7	14
3	3	1	57.51	-4.53333	15
4	1	2	6.56	-8.73667	16
5	2	2	1.2	-6.37333	17
6	3	2	3.44	-3.66	18
7	1	3	38.3	-2.88667	19
8	2	3	43.24	-0.42667	20
9	3	3	53.3	0.856667	21
10	1	4	2.96	-8.52	22
11	2	4	2.14	0.296667	23
12	3	4	0	0	24
1	1	1	40.78	-11.01	25
2	2	1	28.97	-21.63	26
3	3	1	59.84	-2.20333	27
4	1	2	21.4	6.103333	28
5	2	2	1.87	-5.70333	29
6	3	2	1.57	-5.53	30
7	1	3	29.95	-11.2367	31
8	2	3	27.94	-15.7267	32
9	3	3	41.3	-11.1433	33
10	1	4	4.42	-7.06	34
11	2	4	0	-1.84333	35
12	3	4	0	0	36

b. Total emPAI Data Summary

	Control	SFE	Hexane	Folch	
Control	51.79	15.29	41.18	11.48	29.935
50ºC	50.6	7.57	43.66	1.84	25.9175
70ºC	62.04	7.1	52.44	0	30.395
	54.81	9.986667	45.76	4.44	28.74917

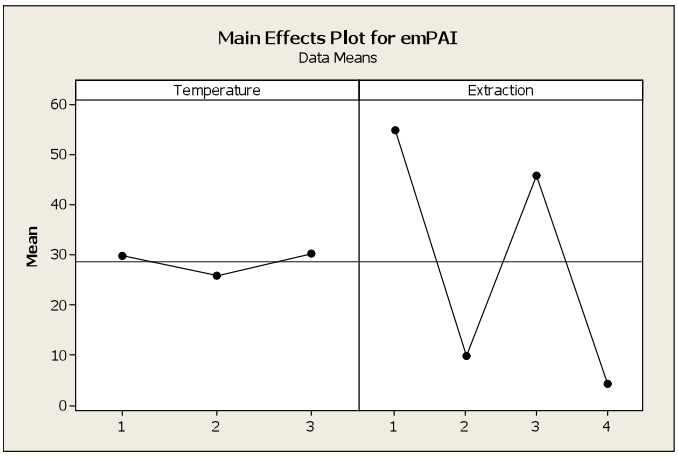
c. Total emPAI General Linear Model Statistics

General Linear Model: emPAI versus Temperature, Extraction

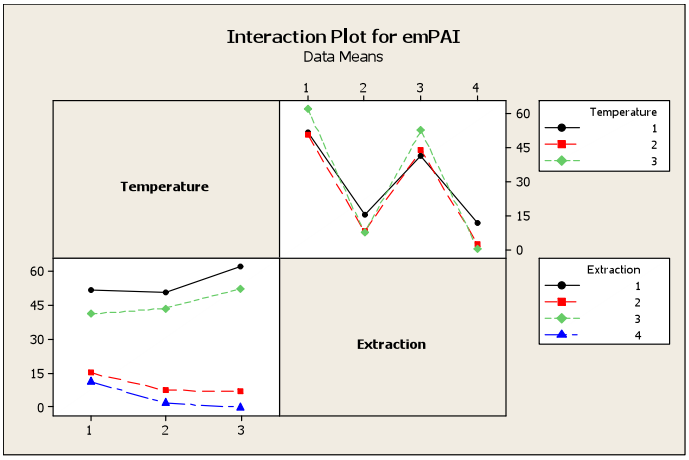
```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for emPAI, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS  Adj MS      F      P
Temperature              2    145.5    145.5    72.8   0.48  0.623
Extraction               3  17204.1  17204.1  5734.7  38.03  0.000
Temperature*Extraction   6    657.0    657.0   109.5   0.73  0.633
Error                   24   3618.8   3618.8   150.8
Total                   35  21625.5


S = 12.2795   R-Sq = 83.27%   R-Sq(adj) = 75.60%


Unusual Observations for emPAI

Obs    emPAI      Fit  SE Fit  Residual  St Resid
  1  72.1100  51.7900  7.0895   20.3200      2.03 R
  2  73.9300  50.6000  7.0895   23.3300      2.33 R
 26  28.9700  50.6000  7.0895  -21.6300     -2.16 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
3            12  30.4  A
1            12  29.9  A
2            12  25.9  A

Means that do not share a letter are significantly different.
```

```
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable emPAI
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower  Center   Upper  -------+---------+---------+---------
2           -16.53  -4.018   8.496  (------------*-----------)
3           -12.05   0.458  12.971       (-----------*------------)
                                     -------+---------+---------+---------
                                          -10         0        10


Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper  -------+---------+---------+---------
3           -8.037   4.476  16.99           (-----------*------------)
                                    -------+---------+---------+---------
                                         -10         0        10


Grouping Information Using Tukey Method and 95.0% Confidence

Extraction  N  Mean  Grouping
1           9  54.8  A
3           9  45.8  A
2           9  10.0    B
4           9   4.4    B

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable emPAI
All Pairwise Comparisons among Levels of Extraction
Extraction = 1  subtracted from:

Extraction   Lower  Center   Upper  ---------+---------+---------+-------
2          -60.78  -44.82  -28.86    (---*----)
3          -25.01   -9.05    6.92              (---*----)
4          -66.33  -50.37  -34.41  (----*---)
                                   ---------+---------+---------+-------
                                          -35         0        35


Extraction = 2  subtracted from:

Extraction   Lower  Center  Upper  ---------+---------+---------+-------
3            19.81  35.776  51.74                           (---*----)
4          -21.51  -5.549  10.41              (---*----)
                                  ---------+---------+---------+-------
                                         -35         0        35


Extraction = 3  subtracted from:

Extraction   Lower  Center   Upper  ---------+---------+---------+-------
4          -57.29  -41.32  -25.36     (---*----)
                                   ---------+---------+---------+-------
                                          -35         0        35
```

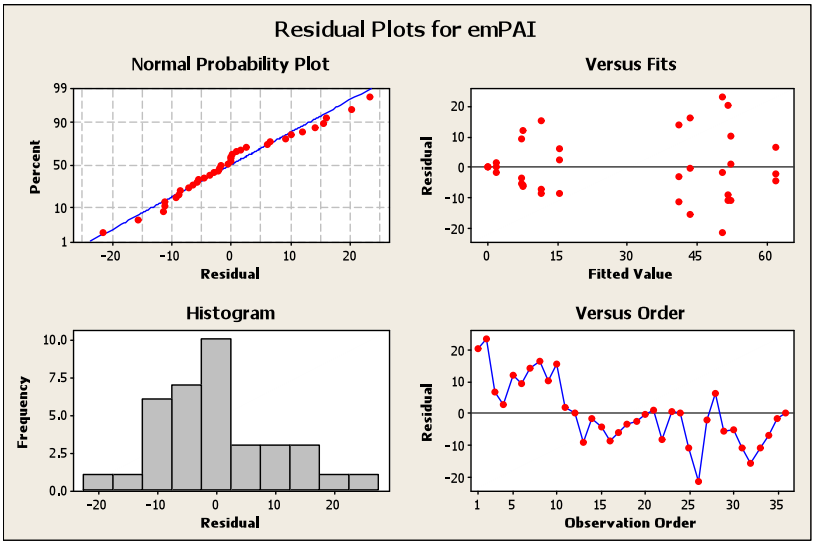
d. Total emPAI Main Effects Plot



e. Total emPAI Interaction Plot



f. Total emPAI Residual Plots



g. Total emPAI ANOVA Assumptions

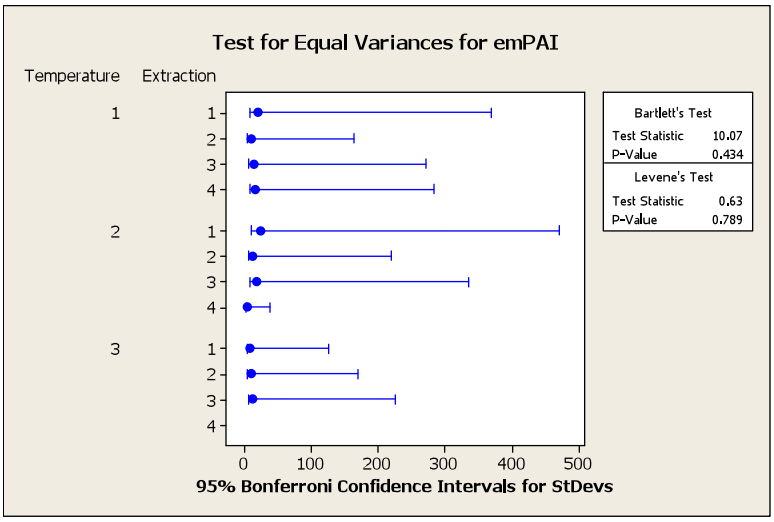
Test for Equal Variances: emPAI versus Temperature, Extraction

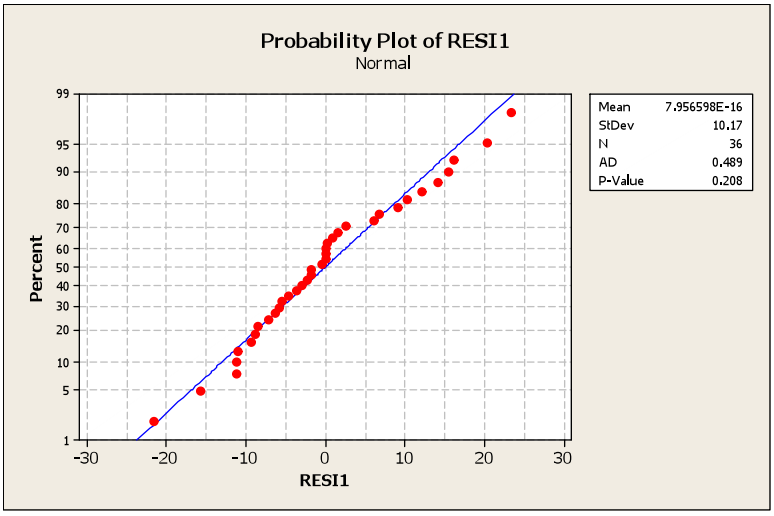
```
95% Bonferroni confidence intervals for standard deviations

Temperature  Extraction  N    Lower    StDev    Upper
          1           1  3  7.14113  17.6182  369.351
          1           2  3  3.14638   7.7626  162.736
          1           3  3  5.23849  12.9241  270.944
          1           4  3  5.47695  13.5124  283.278
          2           1  3  9.13129  22.5282  472.286
          2           2  3  4.24138  10.4641  219.371
          2           3  3  6.46266  15.9443  334.260
          2           4  3  0.69488   1.7144   35.940
          3           1  3  2.41142   5.9493  124.723
          3           2  3  3.24810   8.0135  167.997
          3           3  3  4.35349  10.7407  225.170
          3           4  3        *   0.0000        *


Bartlett's Test (Normal Distribution)
Test statistic = 10.07, p-value = 0.434


Levene's Test (Any Continuous Distribution)
Test statistic = 0.63, p-value = 0.789
```

Regression Analysis: RESI1 versus Observation Order

```
The regression equation is
RESI1 = 12.5 - 0.676 Observation Order


Predictor             Coef  SE Coef      T      P
Constant            12.512    2.505   4.99  0.000
Observation Order  -0.6763   0.1181  -5.73  0.000


S = 7.36003   R-Sq = 49.1%   R-Sq(adj) = 47.6%


Analysis of Variance

Source          DF      SS      MS      F      P
Regression       1  1777.1  1777.1  32.81  0.000
Residual Error  34  1841.8    54.2
Total           35  3618.8


Unusual Observations

     Observation
Obs        Order   RESI1    Fit  SE Fit  Residual  St Resid
 26         26.0  -21.63  -5.07    1.51    -16.56     -2.30R

R denotes an observation with a large standardized residual.
```

Durbin-Watson statistic = 1.07266





APPENDIX D: Domain Independent Analysis – Domain 1

a. Domain Independent Analysis Statistics

General Linear Model: Domain 1 versus Temperature, Extraction

```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 1, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS  Adj MS      F      P
Temperature              2     6.16     6.16    3.08   0.15  0.864
Extraction               3  1665.71  1665.71  555.24  26.41  0.000
Temperature*Extraction   6   111.81   111.81   18.64   0.89  0.520
Error                   24   504.62   504.62   21.03
Total                   35  2288.30


S = 4.58539   R-Sq = 77.95%   R-Sq(adj) = 67.84%


Unusual Observations for Domain 1

Obs  Domain 1     Fit  SE Fit  Residual  St Resid
  5   13.6800  4.7160  2.6474    8.9640      2.39 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
2            12  10.2  A
3            12   9.4  A
1            12   9.3  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 1
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower   Center  Upper  --------+---------+---------+--------
2            -3.760  0.91233  5.585        (---------------*---------------)
3            -4.598  0.07433  4.747     (--------------*---------------)
                                    --------+---------+---------+--------
                                         -3.0       0.0       3.0


Temperature = 2  subtracted from:

Temperature   Lower   Center  Upper  --------+---------+---------+--------
3            -5.511  -0.8380  3.835  (--------------*---------------)
                                    --------+---------+---------+--------
                                         -3.0       0.0       3.0
```

Grouping Information Using Tukey Method and 95.0% Confidence

Extraction	N	Mean	Grouping
1	9	17.3	A
3	9	15.3	A
2	9	4.5	B
4	9	1.4	B

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 1
All Pairwise Comparisons among Levels of Extraction
Extraction = 1 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
2	-18.80	-12.83	-6.874	(----*----)
3	-7.97	-2.01	3.948	(----*----)
4	-21.85	-15.89	-9.930	(----*----)
				--------+---------+---------+--------
				-12 0 12

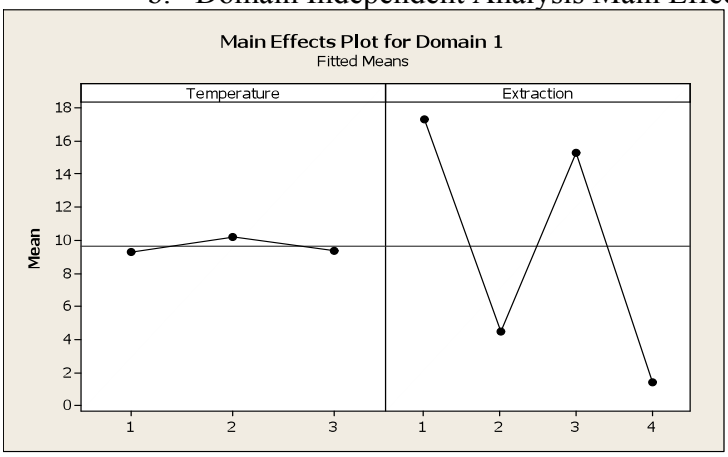
Extraction = 2 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
3	4.860	10.821	16.782	(----*----)
4	-9.017	-3.056	2.905	(----*----)
				--------+---------+---------+--------
				-12 0 12

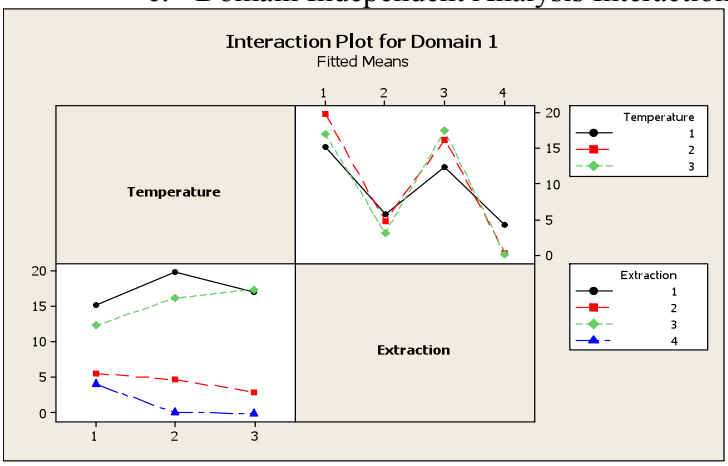
Extraction = 3 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
4	-19.84	-13.88	-7.916	(----*----)
				--------+---------+---------+--------
				-12 0 12

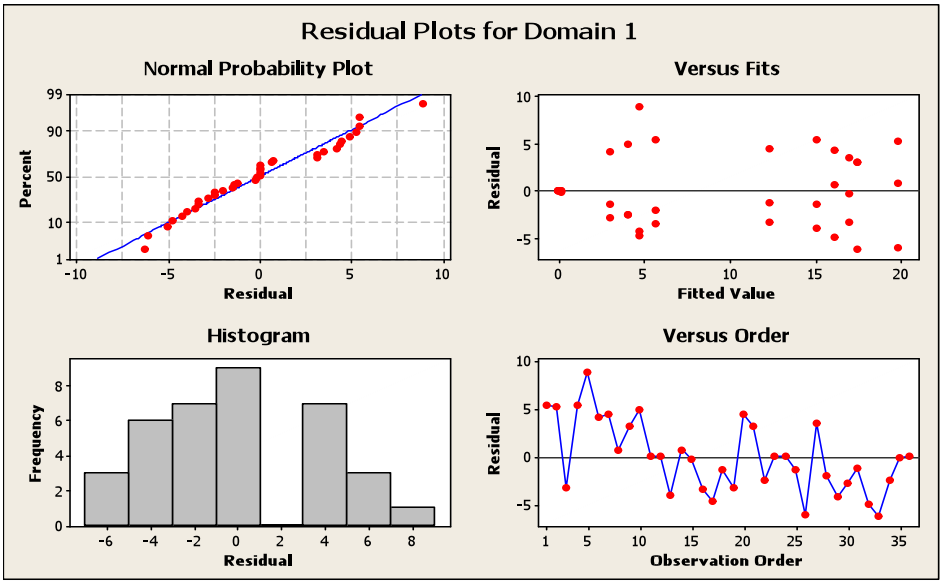
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

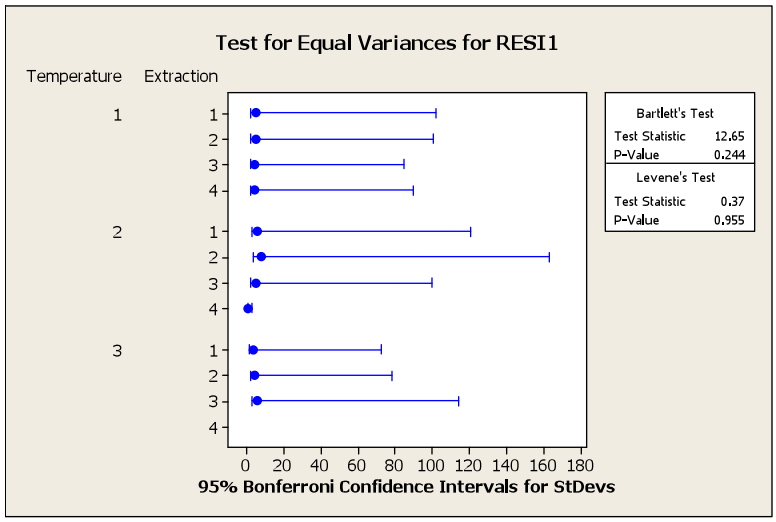


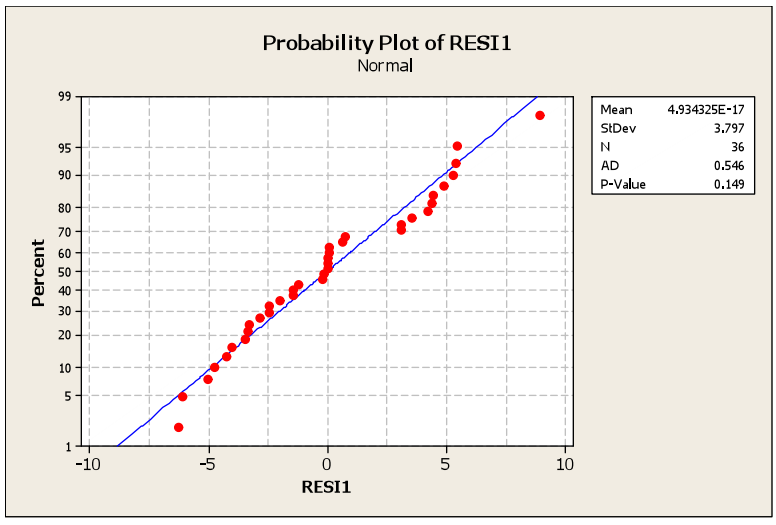
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

```
Unusual Observations

     Observation
Obs         order    RESI1     Fit   SE Fit   Residual   St Resid
  3           3.0   -3.320   3.231    0.941     -6.551     -2.18R
  5           5.0    8.964   2.814    0.859      6.150      2.03R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 1.76337
```





APPENDIX E: Domain Independent Analysis – Domain 2

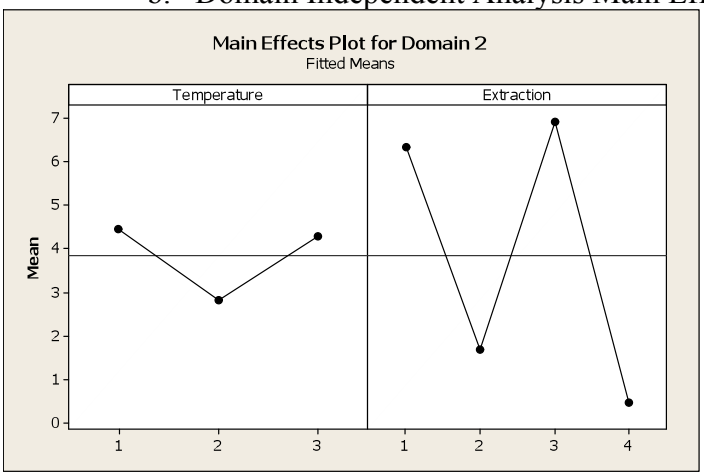
a. Domain Independent Analysis Statistics

General Linear Model: Domain 2 versus Temperature, Extraction

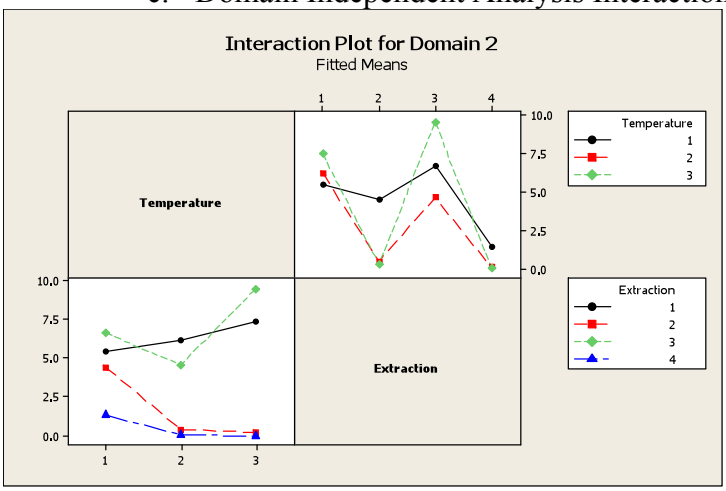
```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 2, using Adjusted SS for Tests

Source                  DF  Seq SS  Adj SS  Adj MS     F      P
Temperature              2   19.61   19.61    9.81  0.67  0.521
Extraction               3  284.16  284.16   94.72  6.48  0.002
Temperature*Extraction   6   60.15   60.15   10.03  0.69  0.663
Error                   24  350.69  350.69   14.61
Total                   35  714.61


S = 3.82258   R-Sq = 50.93%   R-Sq(adj) = 28.43%


Unusual Observations for Domain 2

Obs  Domain 2     Fit  SE Fit  Residual  St Resid
  3    0.0000  7.4133  2.2070   -7.4133     -2.38 R
 14   13.6800  6.1717  2.2070    7.5083      2.41 R
 28   11.1200  4.4273  2.2070    6.6927      2.14 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
1            12   4.5  A
3            12   4.3  A
2            12   2.8  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 2
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower  Center  Upper  --------+---------+---------+--------
2            -5.541  -1.646  2.250  (------------*-----------)
3            -4.070  -0.175  3.721      (------------*------------)
                                    --------+---------+---------+--------
                                         -3.0       0.0       3.0
```

```
Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper  --------+---------+---------+--------
3            -2.424   1.471  5.366           (------------*------------)
                                   --------+---------+---------+--------
                                        -3.0       0.0       3.0


Grouping Information Using Tukey Method and 95.0% Confidence

Extraction  N  Mean  Grouping
3           9   6.9  A
1           9   6.3  A B
2           9   1.7    B C
4           9   0.5      C

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 2
All Pairwise Comparisons among Levels of Extraction
Extraction = 1  subtracted from:

Extraction   Lower  Center    Upper  ---------+---------+---------+-------
2            -9.61  -4.645   0.3248     (-------*--------)
3            -4.40   0.574   5.5436              (-------*-------)
4           -10.83  -5.858  -0.8884   (-------*--------)
                                     ---------+---------+---------+-------
                                           -6.0       0.0       6.0


Extraction = 2  subtracted from:

Extraction   Lower  Center   Upper  ---------+---------+---------+-------
3            0.249   5.219  10.188                     (--------*-------)
4           -6.183  -1.213   3.756           (-------*-------)
                                   ---------+---------+---------+-------
                                         -6.0       0.0       6.0


Extraction = 3  subtracted from:

Extraction   Lower  Center   Upper  ---------+---------+---------+-------
4           -11.40  -6.432  -1.463  (-------*--------)
                                   ---------+---------+---------+-------
                                         -6.0       0.0       6.0
```

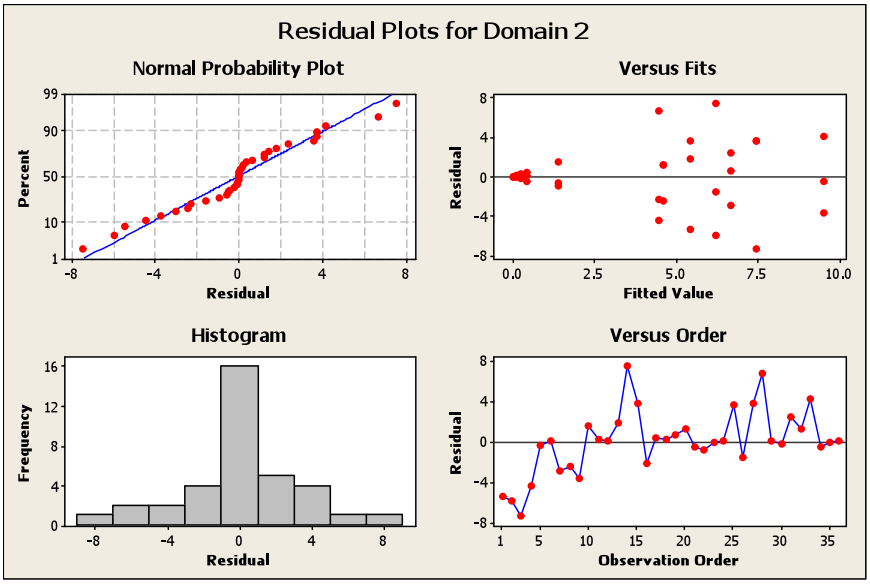
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

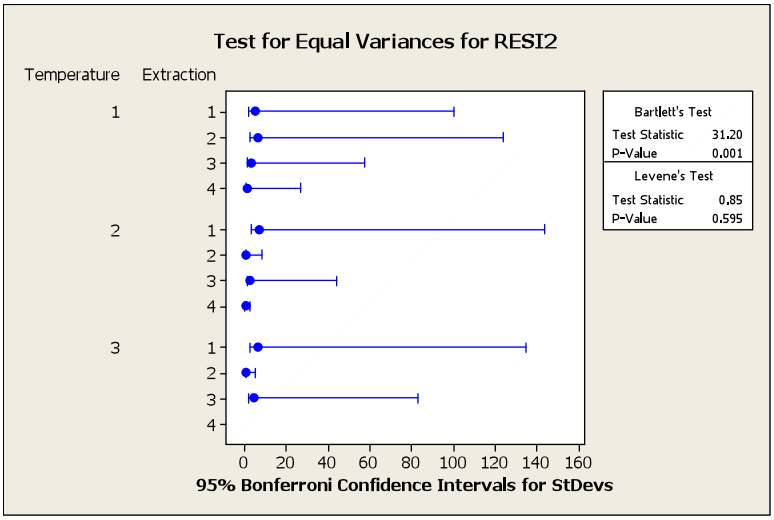


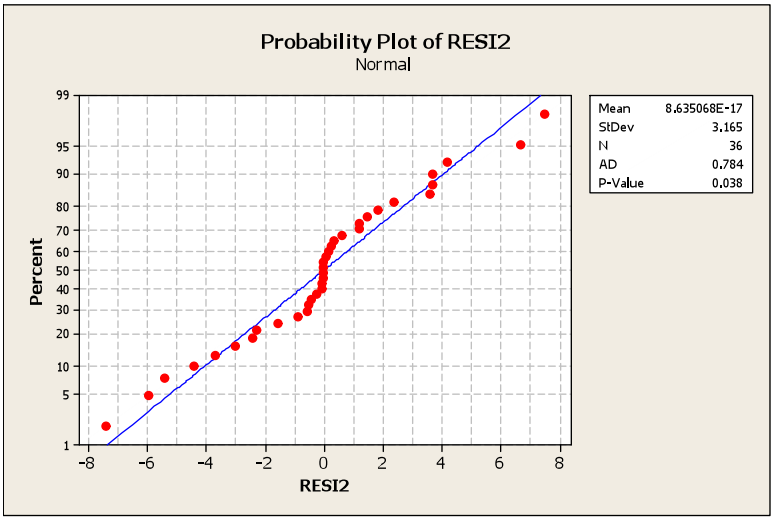
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

```
Unusual Observations

     Observation
Obs        order  RESI2      Fit  SE Fit  Residual  St Resid
 14         14.0  7.508   -0.702   0.498     8.211      3.04R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 1.25877
```





APPENDIX F: Domain Independent Analysis – Domain 3

a. Domain Independent Analysis Statistics

General Linear Model: Domain 3 versus Temperature, Extraction

```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 3, using Adjusted SS for Tests

Source                  DF  Seq SS  Adj SS  Adj MS     F      P
Temperature              2   15.70   15.70    7.85  0.41  0.666
Extraction               3  160.66  160.66   53.55  2.82  0.061
Temperature*Extraction   6   58.23   58.23    9.70  0.51  0.794
Error                   24  456.01  456.01   19.00
Total                   35  690.59


S = 4.35893   R-Sq = 33.97%   R-Sq(adj) = 3.70%


Unusual Observations for Domain 3

Obs  Domain 3     Fit  SE Fit  Residual  St Resid
  1   16.7830  7.3449  2.5166    9.4381      2.65 R
  9   16.7830  8.6766  2.5166    8.1064      2.28 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
3            12   4.0  A
1            12   3.9  A
2            12   2.6  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 3
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower  Center  Upper  -------+---------+---------+---------
2            -5.807  -1.366  3.076  (------------*------------)
3            -4.374   0.068  4.510       (-----------*------------)
                                   -------+---------+---------+---------
                                       -3.5       0.0       3.5


Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper  -------+---------+---------+---------
3            -3.008   1.433  5.875          (------------*------------)
                                   -------+---------+---------+---------
                                       -3.5       0.0       3.5
```

Grouping Information Using Tukey Method and 95.0% Confidence

Extraction	N	Mean	Grouping
3	9	6.1	A
1	9	5.0	A
2	9	1.8	A
4	9	1.1	A

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 3
All Pairwise Comparisons among Levels of Extraction
Extraction = 1 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
2	-8.890	-3.224	2.443	(---------*--------)
3	-4.559	1.107	6.774	(---------*--------)
4	-9.582	-3.915	1.752	(--------*---------)
				--------+---------+---------+--------
				-6.0 0.0 6.0

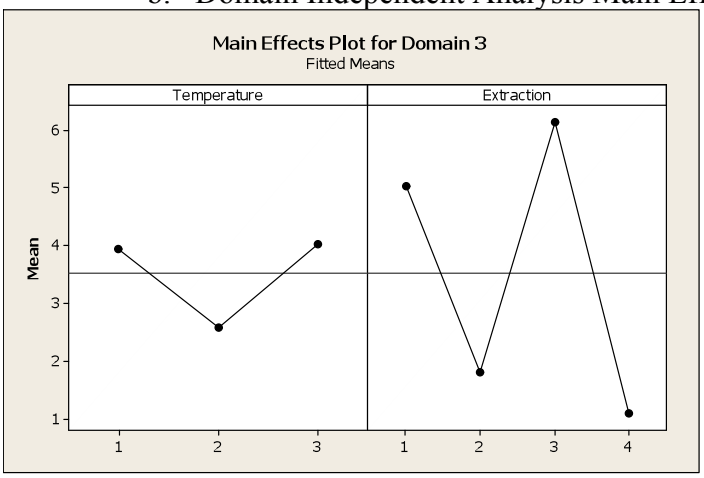
Extraction = 2 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
3	-1.336	4.3311	9.998	(--------*---------)
4	-6.358	-0.6912	4.975	(---------*--------)
				--------+---------+---------+--------
				-6.0 0.0 6.0

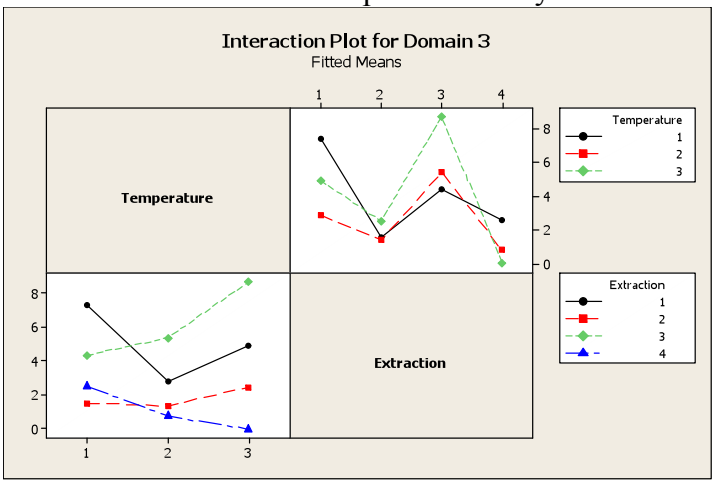
Extraction = 3 subtracted from:

Extraction	Lower	Center	Upper	--------+---------+---------+--------
4	-10.69	-5.022	0.6444	(---------*--------)
				--------+---------+---------+--------
				-6.0 0.0 6.0

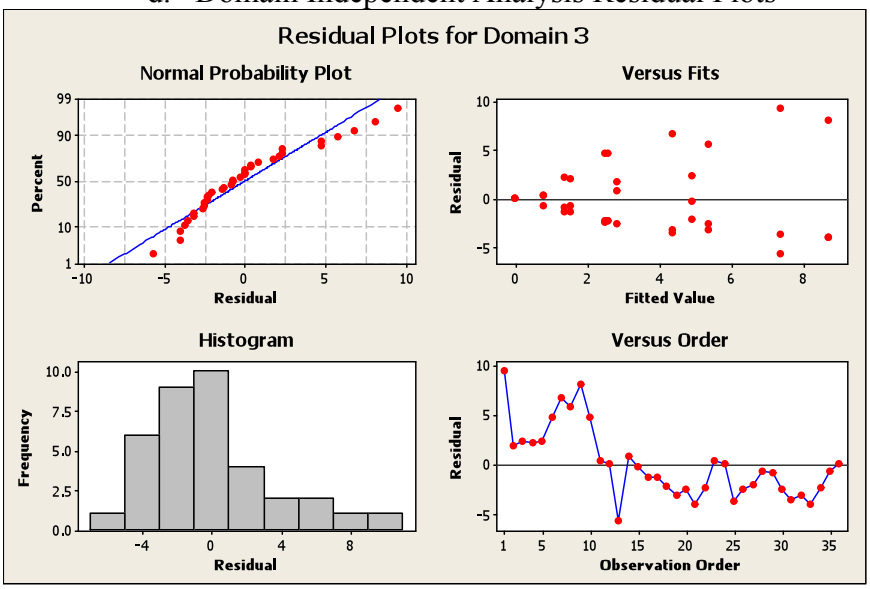
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

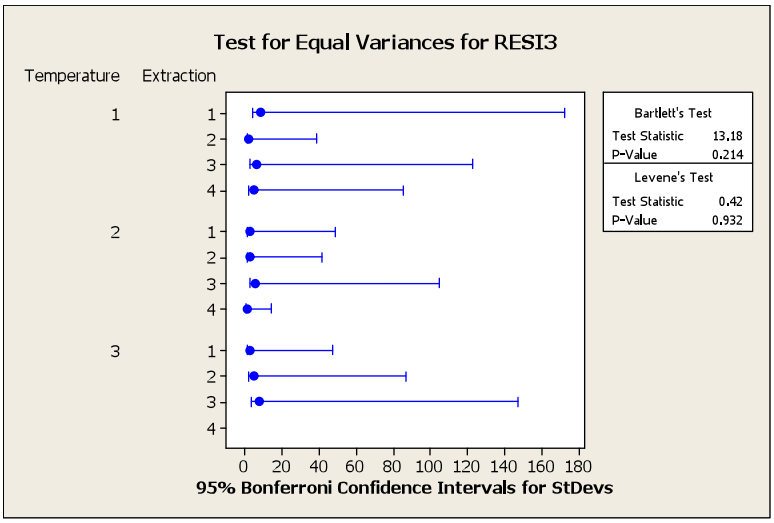


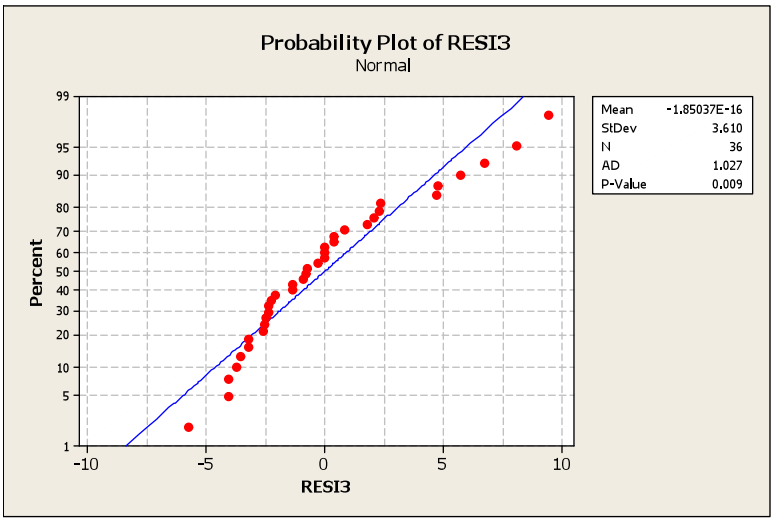
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

```
Unusual Observations

     Observation
Obs         order    RESI3     Fit   SE Fit   Residual   St Resid
  1           1.0    9.438   4.049    0.882      5.389      2.11R
  9           9.0    8.106   2.198    0.610      5.908      2.25R
 13          13.0   -5.735   1.273    0.509     -7.007     -2.64R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 0.903045
```





APPENDIX G: Domain Independent Analysis – Domain 4

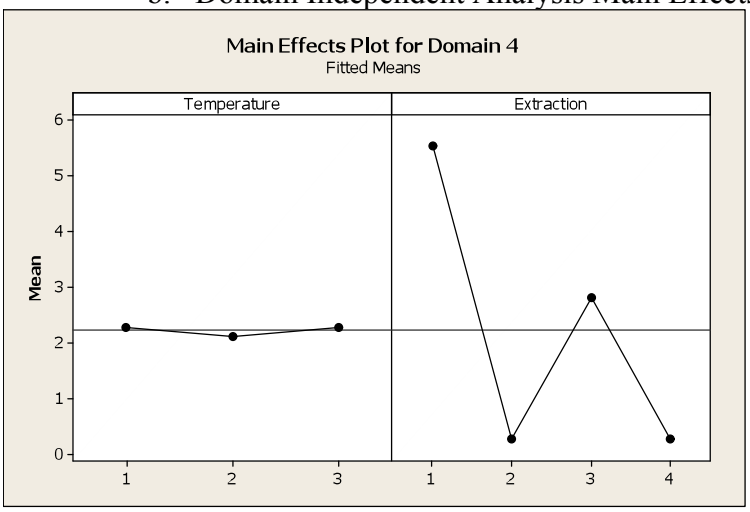
a. Domain Independent Analysis Statistics

General Linear Model: Domain 4 versus Temperature, Extraction

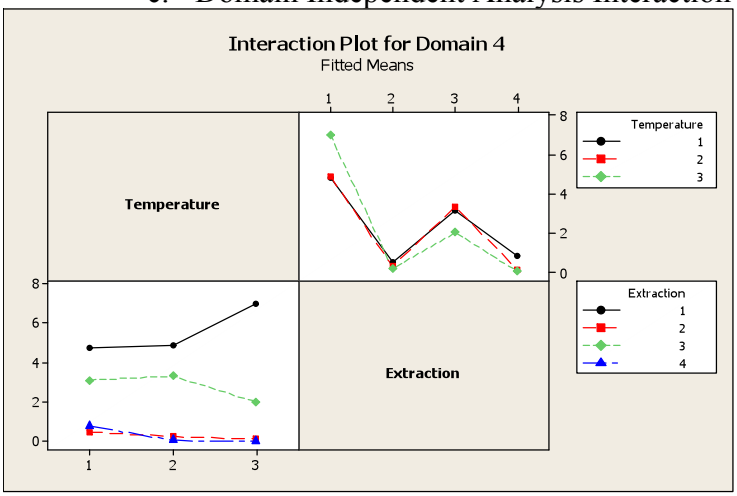
```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 4, using Adjusted SS for Tests

Source                  DF  Seq SS  Adj SS  Adj MS     F      P
Temperature              2    0.19    0.19    0.10  0.01  0.993
Extraction               3  169.69  169.69   56.56  4.16  0.017
Temperature*Extraction   6   13.51   13.51    2.25  0.17  0.984
Error                   24  326.31  326.31   13.60
Total                   35  509.70


S = 3.68733   R-Sq = 35.98%   R-Sq(adj) = 6.64%


Unusual Observations for Domain 4

Obs  Domain 4     Fit  SE Fit  Residual  St Resid
  1   11.1150  4.7785  2.1289    6.3365      2.10 R
  2   13.6780  4.8712  2.1289    8.8068      2.93 R
  3   13.6780  6.9871  2.1289    6.6909      2.22 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
1            12   2.3  A
3            12   2.3  A
2            12   2.1  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 4
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower   Center  Upper  ------+---------+---------+---------+
2            -3.916  -0.1588  3.599  (--------------*--------------)
3            -3.765  -0.0070  3.750   (--------------*--------------)
                                     ------+---------+---------+---------+
                                        -2.5       0.0       2.5       5.0
```

```
Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper  ------+---------+---------+---------+
3            -3.606  0.1518  3.909    (--------------*--------------)
                                    ------+---------+---------+---------+
                                       -2.5       0.0       2.5       5.0


Grouping Information Using Tukey Method and 95.0% Confidence

Extraction  N  Mean  Grouping
1           9   5.5  A
3           9   2.8  A B
2           9   0.3    B
4           9   0.3    B

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 4
All Pairwise Comparisons among Levels of Extraction
Extraction = 1  subtracted from:

Extraction   Lower  Center    Upper      +---------+---------+---------+------
2           -10.04  -5.250  -0.4566      (--------*---------)
3            -7.52  -2.729   2.0648           (---------*--------)
4           -10.05  -5.258  -0.4648      (--------*---------)
                                         +---------+---------+---------+------
                                      -10.0      -5.0       0.0       5.0


Extraction = 2  subtracted from:

Extraction   Lower    Center  Upper      +---------+---------+---------+------
3           -2.272   2.52144  7.315                   (---------*---------)
4           -4.802  -0.00818  4.785              (---------*---------)
                                         +---------+---------+---------+------
                                      -10.0      -5.0       0.0       5.0


Extraction = 3  subtracted from:

Extraction   Lower  Center  Upper      +---------+---------+---------+------
4           -7.323  -2.530  2.264           (---------*---------)
                                         +---------+---------+---------+------
                                    -10.0      -5.0       0.0       5.0
```

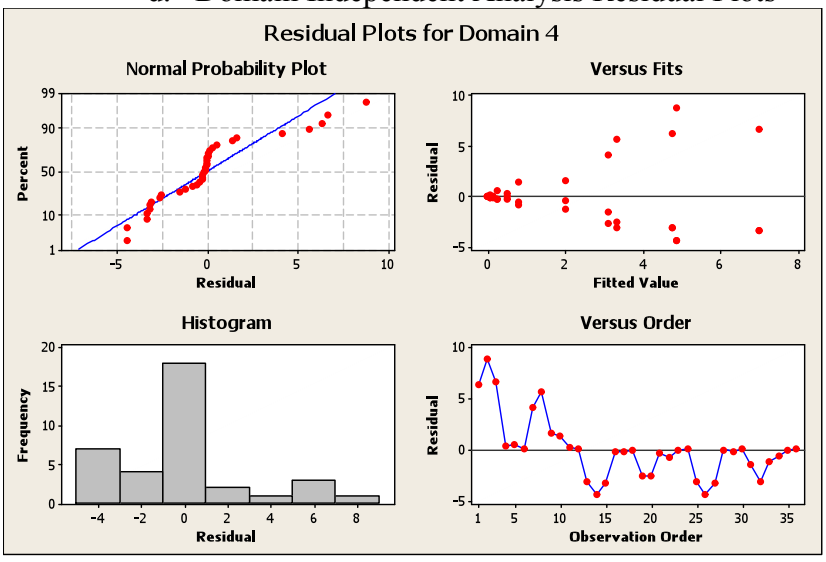
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot



d. Domain Independent Analysis Residual Plots



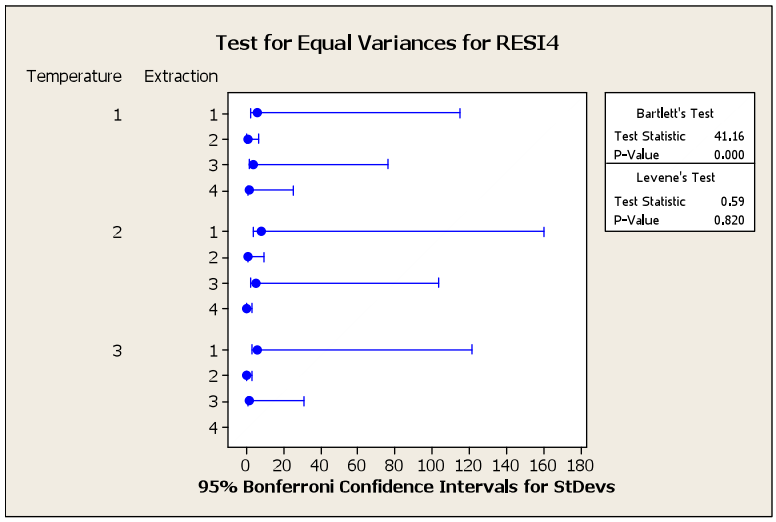
e. Domain Independent Analysis ANOVA Assumptions

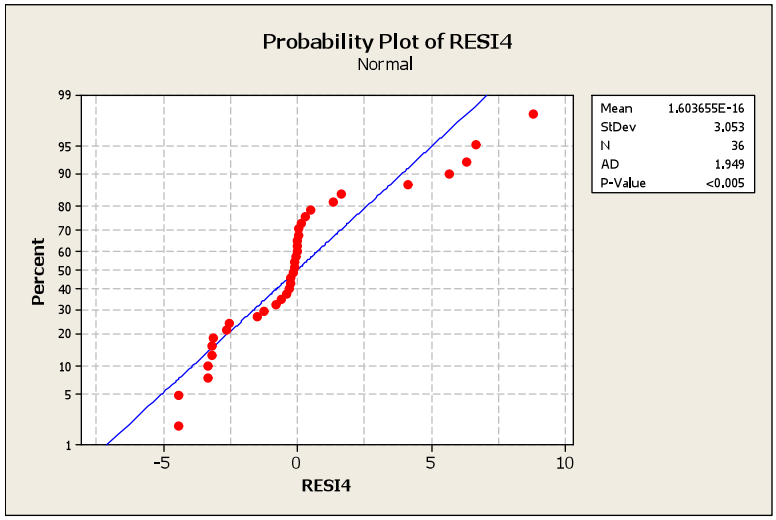
Unusual Observations

Obs	Observation order	RESI4	Fit	SE Fit	Residual	St Resid
2	2.0	8.807	2.716	0.798	6.090	2.51R
14	14.0	-4.403	0.741	0.463	-5.144	-2.05R

R denotes an observation with a large standardized residual.

Durbin-Watson statistic = 0.698681





APPENDIX H: Domain Independent Analysis – Domain 5

a. Domain Independent Analysis Statistics

General Linear Model: Domain 5 versus Temperature, Extraction

```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 5, using Adjusted SS for Tests

Source                  DF   Seq SS  Adj SS  Adj MS     F      P
Temperature              2    26.19   26.19   13.09  0.64  0.536
Extraction               3   440.70  440.70  146.90  7.19  0.001
Temperature*Extraction   6    74.57   74.57   12.43  0.61  0.721
Error                   24   490.41  490.41   20.43
Total                   35  1031.87


S = 4.52038   R-Sq = 52.47%   R-Sq(adj) = 30.69%


Unusual Observations for Domain 5

Obs  Domain 5      Fit  SE Fit  Residual  St Resid
  2   20.5440   7.4922  2.6098   13.0518      3.54 R
  3   25.1020  13.2033  2.6098   11.8987      3.22 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
3            12   4.3  A
2            12   2.6  A
1            12   2.4  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 5
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower  Center  Upper  -----+---------+---------+---------+-
2            -4.410  0.1963  4.803  (---------------*--------------)
3            -2.707  1.8993  6.506        (--------------*---------------)
                                    -----+---------+---------+---------+-
                                      -3.0       0.0       3.0       6.0


Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper  -----+---------+---------+---------+-
3            -2.903   1.703  6.309       (---------------*--------------)
                                    -----+---------+---------+---------+-
                                      -3.0       0.0       3.0       6.0
```

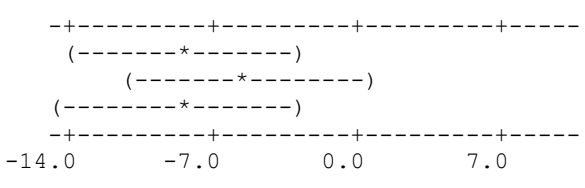
Grouping Information Using Tukey Method and 95.0% Confidence

Extraction	N	Mean	Grouping
1	9	8.7	A
3	9	3.4	A B
2	9	0.3	B
4	9	0.0	B

Means that do not share a letter are significantly different.

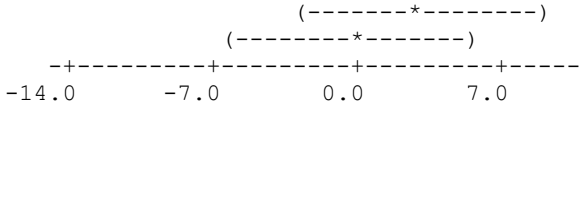
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 5
All Pairwise Comparisons among Levels of Extraction
Extraction = 1 subtracted from:

Extraction	Lower	Center	Upper
2	-14.28	-8.401	-2.525
3	-11.16	-5.282	0.594
4	-14.58	-8.708	-2.831



Extraction = 2 subtracted from:

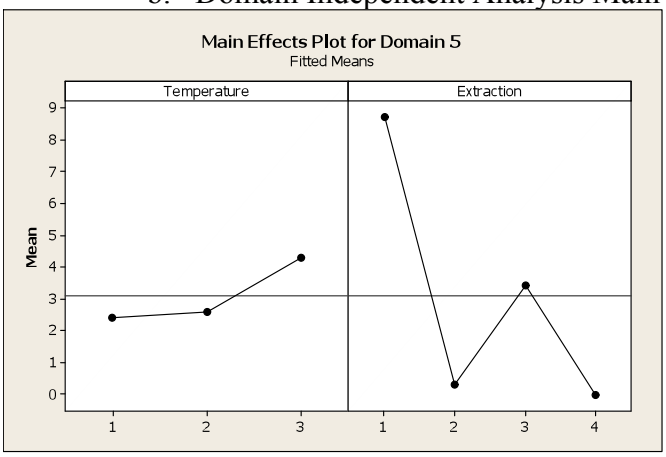
Extraction	Lower	Center	Upper
3	-2.758	3.1188	8.995
4	-6.183	-0.3064	5.570



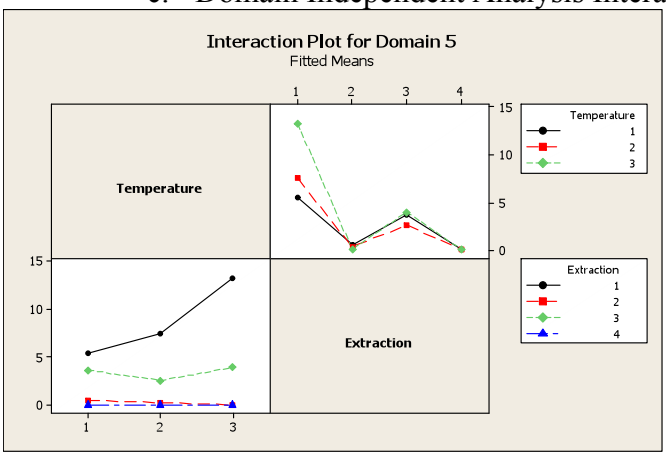
Extraction = 3 subtracted from:

Extraction	Lower	Center	Upper
4	-9.302	-3.425	2.451

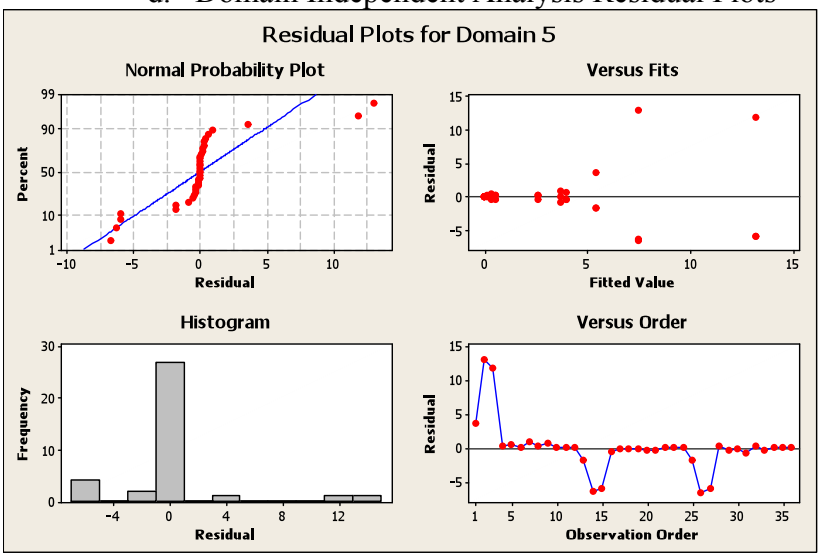
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

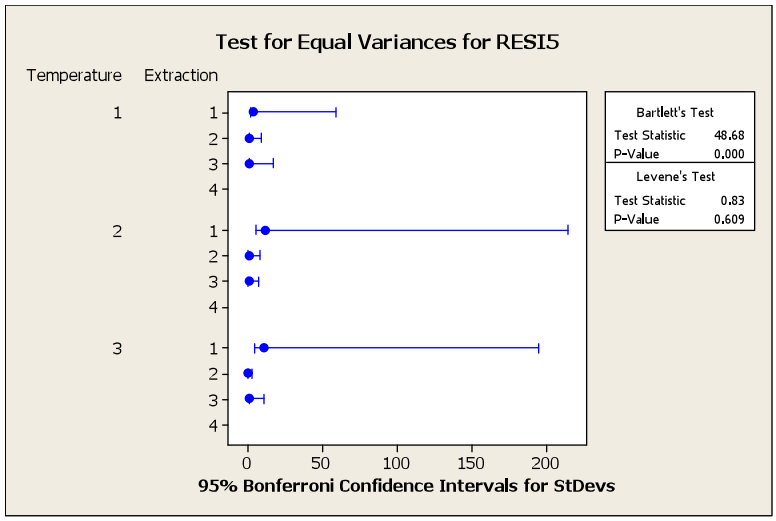


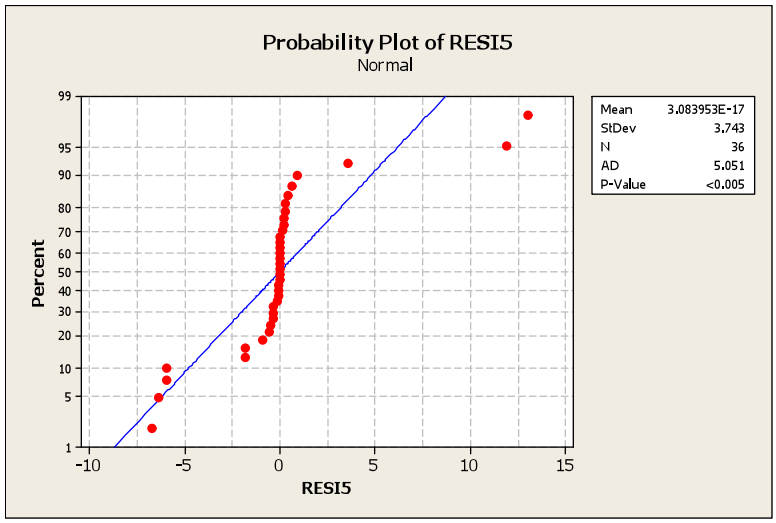
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

```
Unusual Observations

     Observation
Obs         order    RESI5     Fit   SE Fit   Residual   St Resid
  2           2.0   13.052   2.382    1.086     10.670      3.24R
  3           3.0   11.899   2.238    1.039      9.661      2.92R
 14          14.0   -6.338   0.650    0.630     -6.987     -2.05R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 0.858348
```





APPENDIX I: Domain Independent Analysis – Domain 6

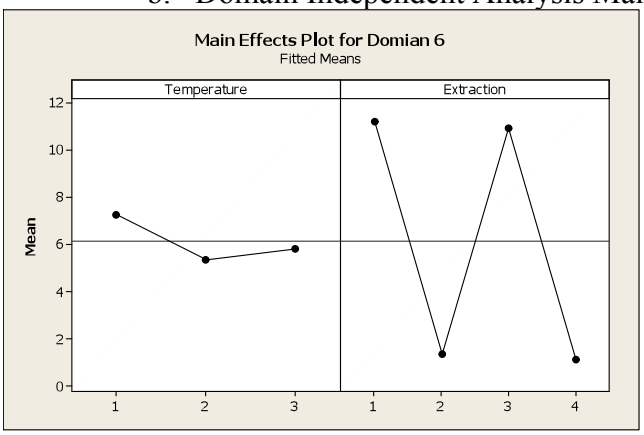
a. Domain Independent Analysis Statistics

General Linear Model: Domian 6 versus Temperature, Extraction

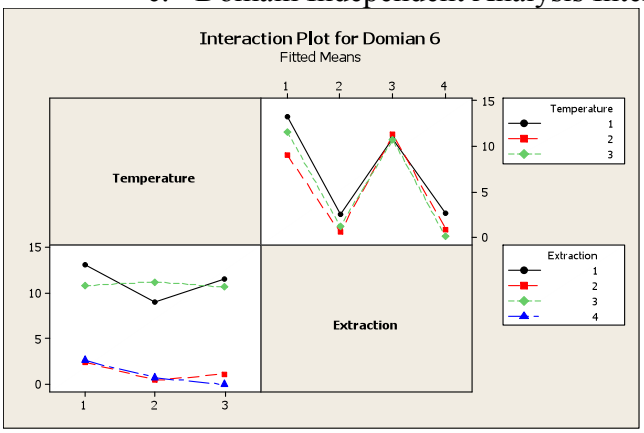
```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domian 6, using Adjusted SS for Tests

Source                  DF    Seq SS   Adj SS   Adj MS      F      P
Temperature              2    23.340   23.340   11.670   1.18  0.325
Extraction               3   868.574  868.574  289.525  29.22  0.000
Temperature*Extraction   6    20.434   20.434    3.406   0.34  0.907
Error                   24   237.772  237.772    9.907
Total                   35  1150.121


S = 3.14757   R-Sq = 79.33%   R-Sq(adj) = 69.85%


Unusual Observations for Domian 6

Obs  Domian 6      Fit  SE Fit  Residual  St Resid
  8   16.7830  11.2370  1.8172    5.5460      2.16 R
 19   16.7830  10.8405  1.8172    5.9425      2.31 R
 31    4.6234  10.8405  1.8172   -6.2171     -2.42 R
 32    5.8129  11.2370  1.8172   -5.4241     -2.11 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
1            12   7.3  A
3            12   5.8  A
2            12   5.4  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domian 6
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature   Lower  Center  Upper     +---------+---------+---------+------
2            -5.099  -1.892  1.315     (-----------*-----------)
3            -4.636  -1.428  1.779      (-----------*-----------)
                                       +---------+---------+---------+------
                                     -5.0      -2.5       0.0       2.5
```

```
Temperature = 2  subtracted from:

Temperature   Lower  Center  Upper     +---------+---------+---------+------
3            -2.744  0.4636  3.671              (------------*------------)
                                      +---------+---------+---------+------
                                    -5.0      -2.5       0.0       2.5


Grouping Information Using Tukey Method and 95.0% Confidence

Extraction  N  Mean  Grouping
1           9  11.2  A
3           9  10.9  A
2           9   1.4    B
4           9   1.1    B

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domian 6
All Pairwise Comparisons among Levels of Extraction
Extraction = 1  subtracted from:

Extraction   Lower  Center   Upper  --------+---------+---------+--------
2           -13.95   -9.86  -5.771   (----*----)
3            -4.40   -0.31   3.782              (-----*----)
4           -14.18  -10.09  -5.996  (----*-----)
                                    --------+---------+---------+--------
                                         -8.0       0.0       8.0


Extraction = 2  subtracted from:

Extraction   Lower   Center   Upper  --------+---------+---------+--------
3            5.460   9.5523  13.644                           (----*----)
4           -4.317  -0.2255   3.866               (----*----)
                                     --------+---------+---------+--------
                                          -8.0       0.0       8.0


Extraction = 3  subtracted from:

Extraction   Lower  Center   Upper  --------+---------+---------+--------
4           -13.87  -9.778  -5.686   (----*----)
                                    --------+---------+---------+--------
                                         -8.0       0.0       8.0
```

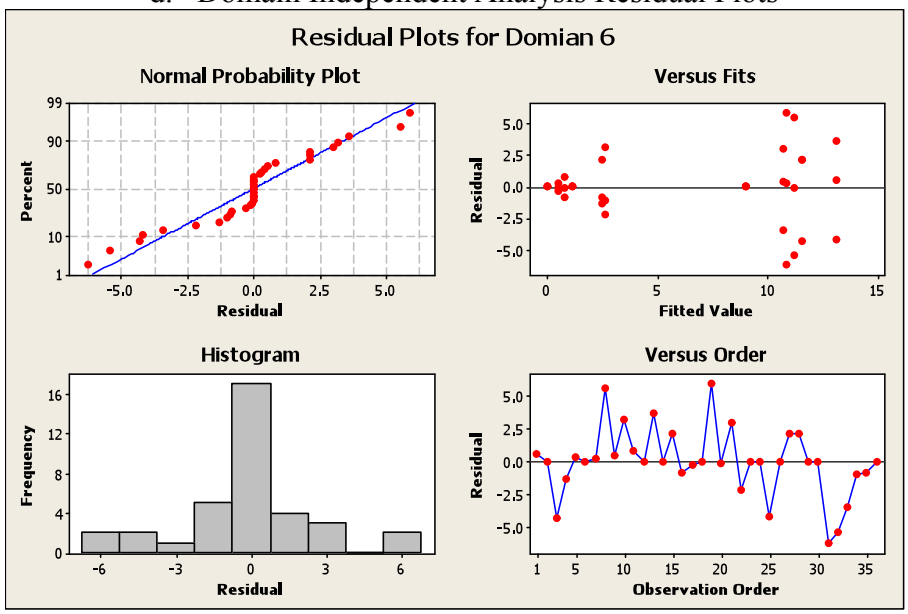
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot



d. Domain Independent Analysis Residual Plots



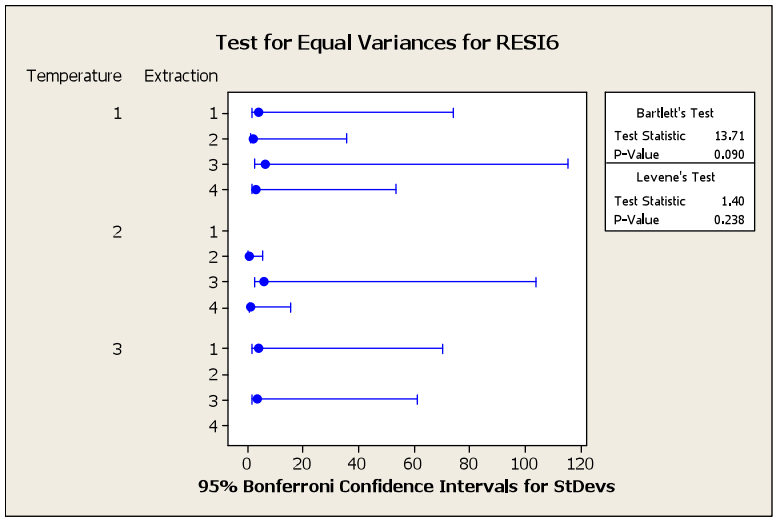
e. Domain Independent Analysis ANOVA Assumptions

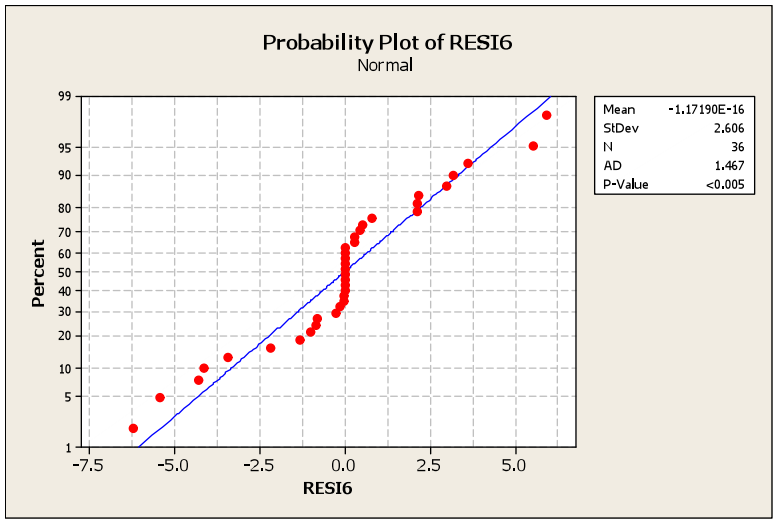
Unusual Observations

Obs	Observation order	RESI6	Fit	SE Fit	Residual	St Resid
3	3.0	-4.283	1.064	0.761	-5.347	-2.21R
19	19.0	5.943	-0.034	0.424	5.977	2.39R
31	31.0	-6.217	-0.858	0.662	-5.359	-2.18R

R denotes an observation with a large standardized residual.

Durbin-Watson statistic = 1.57188





APPENDIX J: Domain Independent Analysis – Domain 7

a. Domain Independent Analysis Statistics

General Linear Model: Domain 7 versus Temperature, Extraction

```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 7, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS   Adj MS     F      P
Temperature              2  0.01087  0.01087  0.00543  0.08  0.928
Extraction               3  0.45461  0.45461  0.15154  2.09  0.128
Temperature*Extraction   6  0.26035  0.26035  0.04339  0.60  0.728
Error                   24  1.73661  1.73661  0.07236
Total                   35  2.46244


S = 0.268996   R-Sq = 29.48%   R-Sq(adj) = 0.00%


Unusual Observations for Domain 7

Obs  Domain 7      Fit   SE Fit  Residual  St Resid
  7   0.77830  0.25943  0.15530   0.51887      2.36 R
  8   1.15440  0.38480  0.15530   0.76960      3.50 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
1            12   0.2  A
3            12   0.1  A
2            12   0.1  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 7
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature    Lower    Center   Upper  ------+---------+---------+---------+
2            -0.3167  -0.04256  0.2316  (-------------*-------------)
3            -0.2955  -0.02136  0.2528   (-------------*-------------)
                                        ------+---------+---------+---------+
                                           -0.20      0.00      0.20      0.40


Temperature = 2  subtracted from:

Temperature    Lower   Center   Upper  ------+---------+---------+---------+
3            -0.2529  0.02120  0.2953     (-------------*-------------)
                                        ------+---------+---------+---------+
                                           -0.20      0.00      0.20      0.40
```

Grouping Information Using Tukey Method and 95.0% Confidence

Extraction	N	Mean	Grouping
3	9	0.3	A
1	9	0.2	A
2	9	0.0	A
4	9	0.0	A

Means that do not share a letter are significantly different.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 7
All Pairwise Comparisons among Levels of Extraction
Extraction = 1 subtracted from:

Extraction	Lower	Center	Upper	-------+---------+---------+---------
2	-0.5591	-0.2094	0.1403	(---------*---------)
3	-0.3209	0.0288	0.3785	(---------*---------)
4	-0.5591	-0.2094	0.1403	(---------*---------)
				-------+---------+---------+---------
				-0.35 0.00 0.35

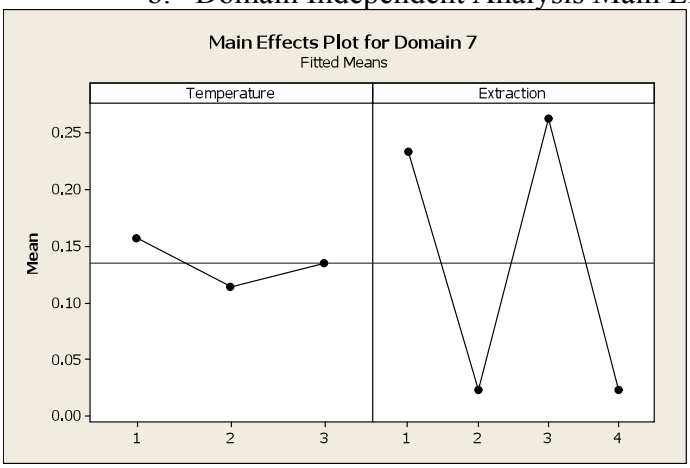
Extraction = 2 subtracted from:

Extraction	Lower	Center	Upper	-------+---------+---------+---------
3	-0.1115	0.238244	0.5879	(---------*---------)
4	-0.3497	-0.000000	0.3497	(---------*---------)
				-------+---------+---------+---------
				-0.35 0.00 0.35

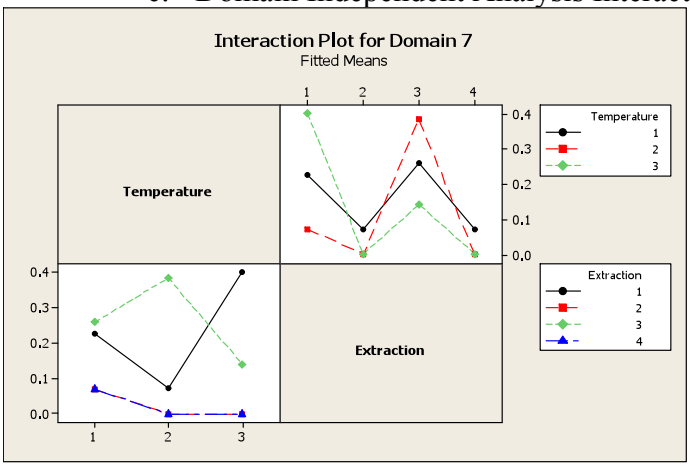
Extraction = 3 subtracted from:

Extraction	Lower	Center	Upper	-------+---------+---------+---------
4	-0.5879	-0.2382	0.1115	(---------*---------)
				-------+---------+---------+---------
				-0.35 0.00 0.35

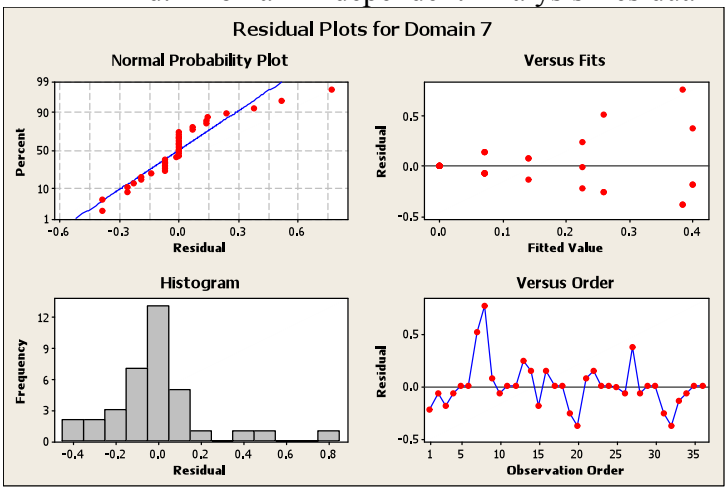
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

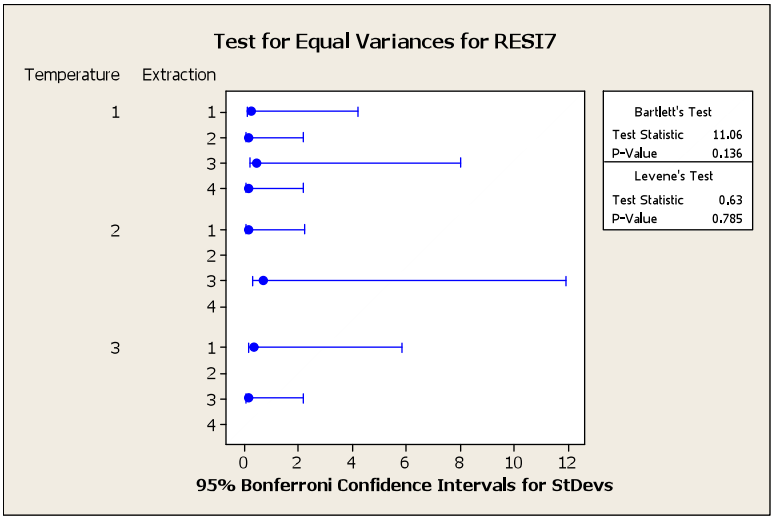


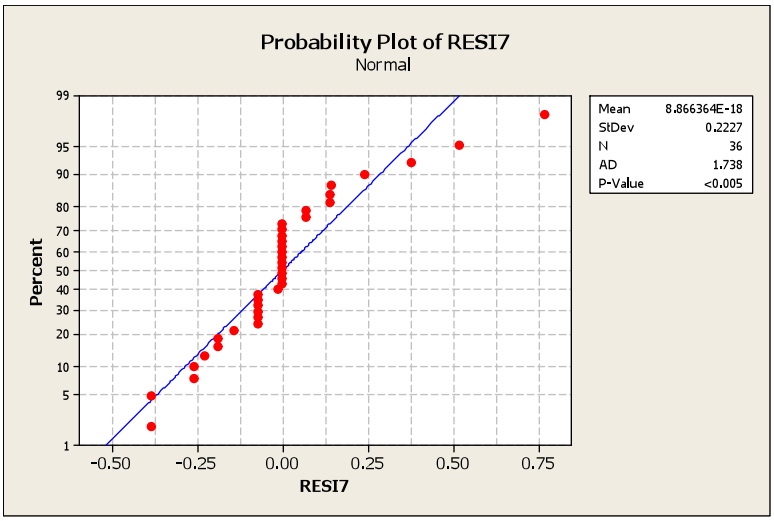
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

```
Unusual Observations

     Observation
Obs        order    RESI7      Fit   SE Fit  Residual  St Resid
  7          7.0   0.5189   0.0485   0.0551    0.4703      2.19R
  8          8.0   0.7696   0.0443   0.0525    0.7253      3.37R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 1.25416
```





APPENDIX K: Domain Independent Analysis – Domain 8

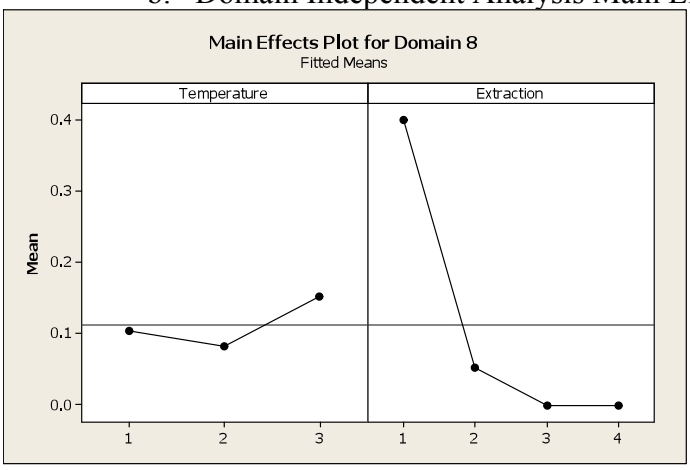
a. Domain Independent Analysis Statistics

General Linear Model: Domain 8 versus Temperature, Extraction

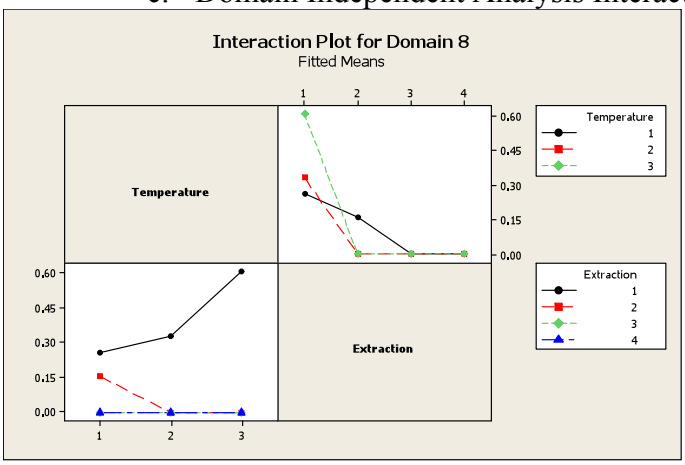
```
Factor       Type   Levels  Values
Temperature  fixed       3  1, 2, 3
Extraction   fixed       4  1, 2, 3, 4


Analysis of Variance for Domain 8, using Adjusted SS for Tests

Source                  DF  Seq SS  Adj SS  Adj MS     F      P
Temperature              2  0.0303  0.0303  0.0151  0.15  0.861
Extraction               3  0.9988  0.9988  0.3329  3.32  0.037
Temperature*Extraction   6  0.2212  0.2212  0.0369  0.37  0.892
Error                   24  2.4049  2.4049  0.1002
Total                   35  3.6552


S = 0.316552   R-Sq = 34.21%   R-Sq(adj) = 4.05%


Unusual Observations for Domain 8

Obs  Domain 8      Fit   SE Fit  Residual  St Resid
  1   0.77830  0.25943  0.18276   0.51887      2.01 R
  3   1.61020  0.60723  0.18276   1.00297      3.88 R
 27   0.00000  0.60723  0.18276  -0.60723     -2.35 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95.0% Confidence

Temperature   N  Mean  Grouping
3            12   0.2  A
1            12   0.1  A
2            12   0.1  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 8
All Pairwise Comparisons among Levels of Temperature
Temperature = 1  subtracted from:

Temperature    Lower    Center   Upper  -------+---------+---------+---------
2            -0.3439  -0.02136  0.3012  (---------------*---------------)
3            -0.2746   0.04797  0.3705     (---------------*----------------)
                                        -------+---------+---------+---------
                                            -0.20      0.00      0.20
```

```
Temperature = 2  subtracted from:

Temperature    Lower   Center   Upper  -------+---------+---------+---------
3            -0.2532  0.06932  0.3919      (---------------*----------------)
                                        -------+---------+---------+---------
                                            -0.20      0.00      0.20


Grouping Information Using Tukey Method and 95.0% Confidence

Extraction  N  Mean  Grouping
1           9   0.4  A
2           9   0.1  A
4           9   0.0  A
3           9   0.0  A

Means that do not share a letter are significantly different.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Domain 8
All Pairwise Comparisons among Levels of Extraction
Extraction = 1  subtracted from:

Extraction    Lower   Center    Upper  ---+---------+---------+---------+---
2           -0.7584  -0.3469  0.06463   (-----------*-----------)
3           -0.8104  -0.3989  0.01265  (-----------*----------)
4           -0.8104  -0.3989  0.01265  (-----------*----------)
                                       ---+---------+---------+---------+---
                                       -0.70     -0.35      0.00      0.35


Extraction = 2  subtracted from:

Extraction    Lower    Center   Upper  ---+---------+---------+---------+---
3           -0.4635  -0.05198  0.3595           (-----------*----------)
4           -0.4635  -0.05198  0.3595           (-----------*----------)
                                       ---+---------+---------+---------+---
                                       -0.70     -0.35      0.00      0.35


Extraction = 3  subtracted from:

Extraction    Lower    Center   Upper  ---+---------+---------+---------+---
4           -0.4115  0.000000  0.4115            (-----------*-----------)
                                       ---+---------+---------+---------+---
                                       -0.70     -0.35      0.00      0.35
```

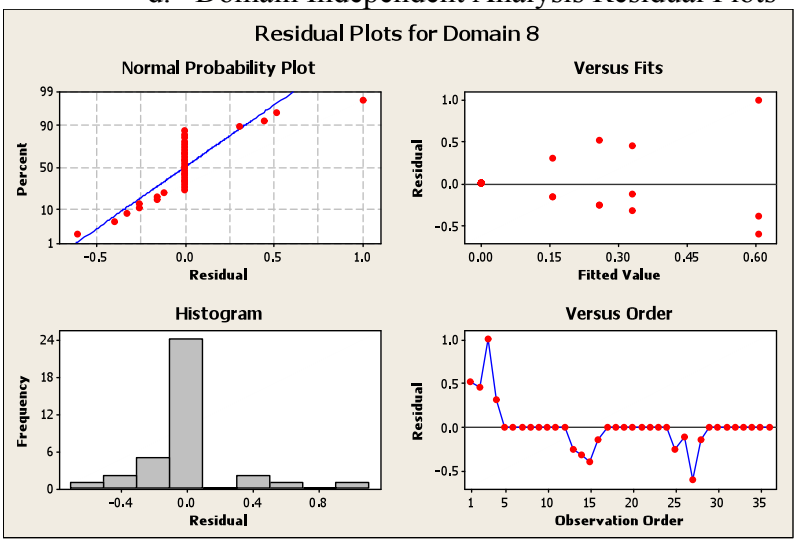
b. Domain Independent Analysis Main Effects Plot



c. Domain Independent Analysis Interaction Plot

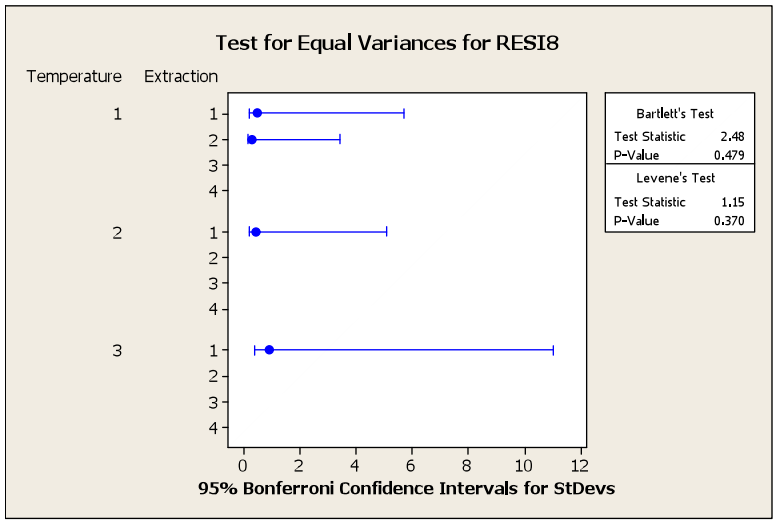


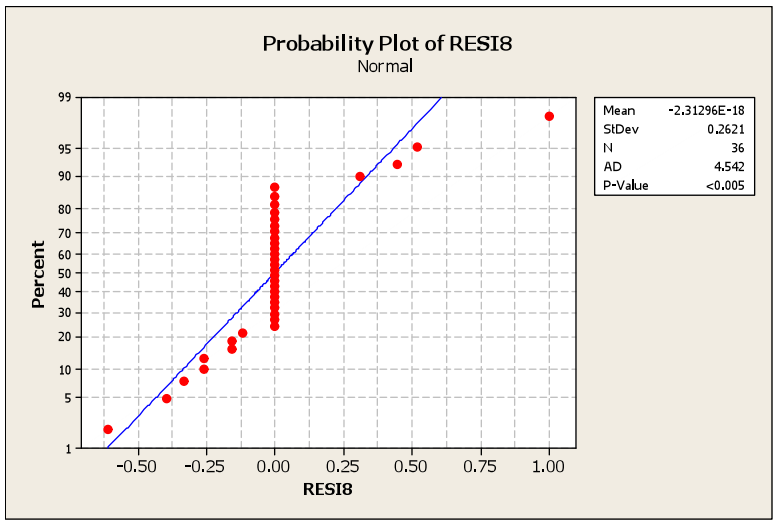
d. Domain Independent Analysis Residual Plots



e. Domain Independent Analysis ANOVA Assumptions

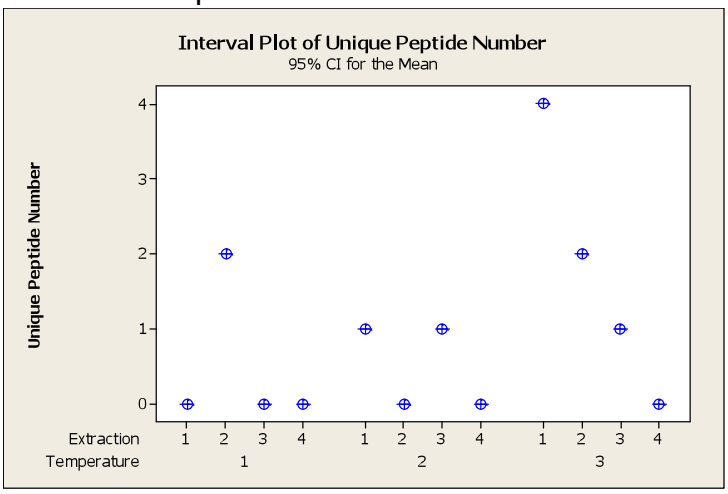
```
Unusual Observations

     Observation
Obs        order     RESI8       Fit   SE Fit  Residual  St Resid
  3          3.0    1.0030    0.1639   0.0721    0.8391      3.65R
 27         27.0   -0.6072   -0.0899   0.0518   -0.5174     -2.20R

R denotes an observation with a large standardized residual.


Durbin-Watson statistic = 0.808331
```



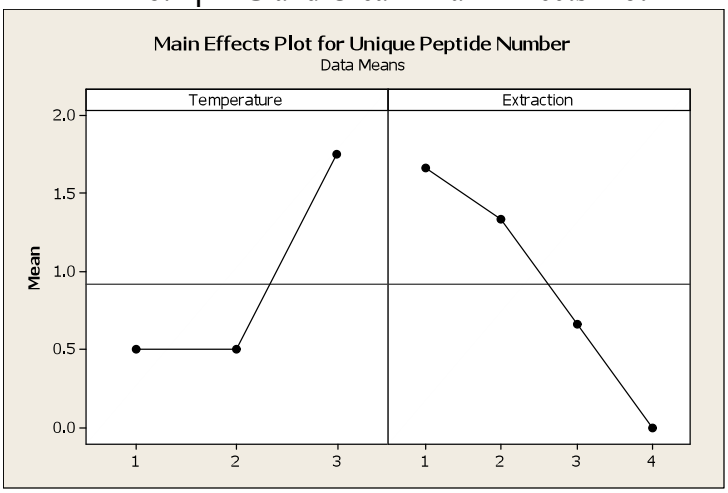


APPENDIX L: β-LG and Cream

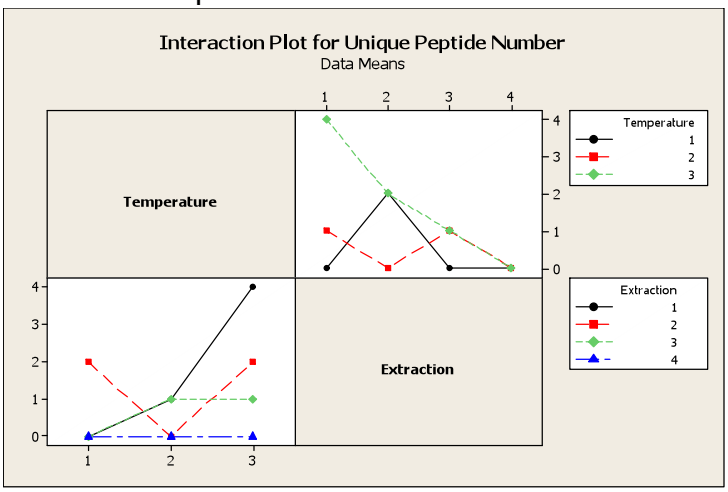
a. β-LG and Cream Interval Plot



b. β-LG and Cream Main Effects Plot



c. β-LG and Cream Interaction Plot



APPENDIX M: ELISA Method Development – 1% BSA as Blocking Reagent

a. ELISA Raw Data and Contents

ELISA Results - 450nm						
	1	2	3	4	5	6
A	1.109	0.92	0.962	0.946	0.968	0.877
B	1.099	0.972	0.946	1.036	0.964	0.868
C	1.126	1.078	1.097	0.974	0.899	1.099
D						
E						
F						
G						
H						
Contents						
	1	2	3	4	5	6
A	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest
B	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest
C	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest
D						
E						
F						
G						
H						
Protein Concentration						
	1	2	3	4	5	6
A	0	1	5	0	1	5
B	0	1	5	0	1	5
C	0	1	5	0	1	5
D						
E						
F						
G						
H						
Antibody Concentration						
	1	2	3	4	5	6
A	0	0	0	0	0	0
B	0.085	0.085	0.085	0.085	0.085	0.085
C	0.2	0.2	0.2	0.2	0.2	0.2
D						
E						
F						
G						
H						

APPENDIX N: ELISA Method Development – 1% Fish Gelatin as Blocking Reagent

a. ELISA Raw Data and Contents

ELISA Results - 450nm

	1	2	3	4	5	6						
A	0.089	0.103	0.07	0.064	0.075	0.072	0.058	0.068	0.068	0.07	0.064	0.076
B	0.383	0.476	2.454	0.391	0.365	0.399	0.385	0.46	0.549	0.391	0.483	0.371
C	0.695	1.391	3.236	0.785	0.705	0.693	0.78	0.8	0.968	0.773	0.841	1.053
D												
E												
F												
G												
H												

Contents

	1	2	3	4	5	6						
A	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest
B	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest
C	β-LG	β-LG	β-LG	β-LG Digest	β-LG Digest	β-LG Digest	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest	Sc-CO_2 β-LG Digest
D												
E												
F												
G												
H												

Protein Concentration

	1	2	3	4	5	6						
A	0	1	5	0	1	5	0	1	5	0	1	5
B	0	1	5	0	1	5	0	1	5	0	1	5
C	0	1	5	0	1	5	0	1	5	0	1	5
D												
E												
F												
G												
H												

Antibody Concentration

	1	2	3	4	5	6						
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085
C	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
D												
E												
F												
G												
H												

APPENDIX O: ELISA – Replicate 1

a. ELISA Raw Data and Contents

ELISA Results - 450nm

	1	2	3	4	5	6						
A	0.871	0.751	1.004	0.936	0.758	0.816	0.732	0.736	1.006	0.724	0.779	0.827
B	0.746	0.73	0.776	0.782	0.702	0.931	0.772	0.793	0.82	0.833	0.842	0.823
C												
D												
E												
F												
G												
H												

Contents - Treatment

	1	2	3	4	5	6						
A	1	2	3	4	5	6	7	8	9	10	11	12
B	1- Control	2- Control	3- Control	4- Control	5- Control	6- Control	7- Control	8- Control	9- Control	10-Control	11- Control	12- Control
C												
D												
E												
F												
G												
H												

Protein Concentration (µg/mL)

	1	2	3	4	5	6						
A	5	5	5	5	5	5	5	5	5	5	5	5
B	5	5	5	5	5	5	5	5	5	5	5	5
C												
D												
E												
F												
G												
H												

Antibody Concentration (µg/µL)

	1	2	3	4	5	6						
A	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
B	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C												
D												
E												
F												
G												
H												

APPENDIX P: ELISA – Replicate 2

a. ELISA Raw Data and Contents

ELISA Results - 450nm

	1	2	3	4	5	6						
A	0.117	0.089	0.086	0.076	0.106	0.083	0.114	0.081	0.086	0.091	0.08	0.082
B	0.09	0.084	0.085	0.102	0.079	0.081	0.076	0.098	0.101	0.095	0.137	0.091
C												
D												
E												
F												
G												
H												

Contents - Treatment

	1	2	3	4	5	6						
A	1	2	3	4	5	6	7	8	9	10	11	12
B	1- Control	2- Control	3- Control	4- Control	5- Control	6- Control	7- Control	8- Control	9- Control	10-Control	11- Control	12- Control
C												
D												
E												
F												
G												
H												

Protein Concentration (µg/mL)

	1	2	3	4	5	6						
A	5	5	5	5	5	5	5	5	5	5	5	5
B	5	5	5	5	5	5	5	5	5	5	5	5
C												
D												
E												
F												
G												
H												

Antibody Concentration (µg/µL)

	1	2	3	4	5	6						
A	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
B	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
C												
D												
E												
F												
G												
H												