ABSTRACT

Funke, Melissa Marie. Characterizing a biomedical hydrogel device. (Under the direction of Dr. Christopher R. Daubert.)

E-Matrix™, a hydrogel principally composed of gelatin and dextran, common food ingredients, is being manufactured with an amino acid formulation and considered as a new medical device. The compound purportedly accelerates the rate of healing once injected beneath a wound. The improved healing is believed to be caused by shifting the healing process from a slower healing adult inflammatory tissue stage to a quicker healing fetal regenerative tissue stage. In addition, gelatin and dextran are anticipated to interact within the medical device to form a stable hydrogel. The objectives of this study were to rheologically characterize E-Matrix™, develop quality control protocols for evaluation of E-Matrix™ and gelatin, investigate the nature of the proposed relationship between gelatin and dextran, and examine rheological properties of E-Matrix™ components.

Rheological techniques using a StressTech Controlled Stress Rheometer (ReoLogica Instruments AB, Lund, Sweden) were used to characterize E-Matrix™, to establish physical properties, and to describe the material flow behavior. Differential scanning calorimetry (PerkinElmer DSC7) was used to determine melt points of E-Matrix™ and a 12% gelatin solution to compare thermal transition temperatures. Rheological protocols were developed for both

E-Matrix™ and the principle ingredient in the material, a 12% gelatin solution. The protocols evaluate specific rheological properties to compare either gelatin lots or manufactured E-Matrix™ batches with established standards. Incorporation of the rheological protocols into a quality control procedure would be a valuable tool for accessing the acceptability of gelatin lots and newly manufactured E-Matrix™ batches. To further understand and characterize E-Matrix™, studies were performed to examine key physical components of the material. Specifically solutions of 12% gelatin, 17% gelatin, 5% dextran, 12% gelatin-5% dextran, and the gelatin-rich domain of E-Matrix™ were rheologically examined and compared to rheological properties of E-Matrix™. In addition the affect of ionic strength and salt valence was also examined through rheological analysis. To determine whether a protein-carbohydrate conjugation resulted from the Maillard reaction, a spectrophotometric technique was performed to determine the degree of covalent conjugation by measuring the change in free amino groups.

E-Matrix™ was rheologically characterized at 37°C and 50°C as having pseudoplastic and Newtonian material flow behaviors, respectively. Differential scanning calorimetry determined the calorimetric melt point of E-Matrix™ (23.9°C) and a 12% gelatin solution (26.0°C) to occur sooner than those determined rheologically (33.7°C) and (32.7°C), respectively. Rheological protocols were developed for quality control evaluation of E-Matrix™ and gelatin. The protocols can be used as a quality control tool by the manufacturer of E-Matrix™, Encelle, Inc. of Greenville, North Carolina. Rheological properties were evaluated for different

components of E-Matrix™; individual components, salt type, and ionic strength concentration. Individual E-Matrix™ components were found to differ significantly in regard to rheological properties. However salt type; monovalent versus divalent, using NaCl and $CaCl_2$ was not found to create significant differences for the properties examined in this study, but ionic strength concentration was found to produce rheological properties of significant difference. In addition, according to spectrophotometry, a hypothesized chemical interaction between gelatin and dextran was not likely occurring.

By understanding the rheological properties of E-Matrix™, the nature of the protein and carbohydrate interaction, and the rheological properties of the E-Matrix™ components, the mechanisms behind the functionality of the wound healing accelerant can be more clearly understood and benefit the product producers through further formulation optimization.

CHARACTERIZING A BIOMEDICAL HYDROGEL DEVICE

By

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DEDICATION

For Mom and Dad

BIOGRAPHY

Melissa Marie Funke, the second daughter of Ron and Judi Funke, was born on September 25, 1981. She grew up in Cincinnati, Ohio with her parents and older sister Betsy. Melissa was an avid swimmer, softball and soccer player. When Melissa was 12 her family moved to Wilmington, North Carolina and she immediately became a beach bunny. Melissa attended John T. Hoggard High School where she continued to swim competitively and play softball. In 1999, she moved to Wolfpack country, Raleigh, North Carolina, to pursue a degree in food science. As an undergraduate at NC State she continued to be very active and was a member of several organizations including an officer on the NCSU Club Swim Team, vice president of Delta Zeta sorority, and participated in a summer internship at TW Garner Foods working with Texas Pete hot sauce for the one summer she was able to pull herself away from the beach and pool, where she was a lifeguard and swim team coach. After graduating in May 2003, with a bachelor's of science degree in food science, Melissa stuck around NC State for a few more years in order to earn a Master's degree in food science and a minor in biotechnology. As a graduate student Melissa continued to be an active student on campus by being a chairman of the food science club, representative for the Institute of Food Technology, and an adviser to Delta Zeta sorority.

Upon completion of her master's degree, Melissa is remaining in the land of sweet tea as she joins Encelle, Inc. in Greenville, North Carolina as a Product Development Scientist.

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CHAPTER 1.

INTRODUCTION

The chapters following the introduction provide a description of the experiments performed by Melissa Funke in the Food Rheology Laboratory at North Carolina State University from 2003 to 2005. Each chapter contains an introductory section with a summary of objectives, scientific approaches, and relevant literature to each particular study. A general overview for each subsequent chapter and the rationale for the project portrayed in the dissertation will be given in this section.

1.1 RHEOLOGICAL CHARACTERIZATION OF E-MATRIX™

Rheological characterization was performed on E-Matrix™ to establish physical properties and to describe material flow behavior. To investigate the properties of the hydrogel, shear rate ramps, mechanical spectra, and temperature ramps were conducted. Shear rate ramps showed E-Matrix™ displayed pseudoplastic flow behavior at 37°C and Newtonian flow behavior at 50°C. As anticipated, shear rate ramps also showed the material was more viscous at lower temperatures. Mechanical spectra were performed to investigate viscoelastic properties: shear storage (G′) and loss moduli (G″) at 37°C and 50°C. The shear loss modulus dominated across a frequency sweep at each temperature, indicating the material possessed more viscous than elastic behavior. Thermal transition points were also identified both rheologically and calorimetrically, using differential scanning calorimetry for the latter. Through rheological and calorimetric techniques, the physical properties of E-Matrix™ were more completely described.

1.2 RHEOLOGICAL PROTOCOL

Rheological protocols were developed for both E-Matrix™ and the principle ingredient in the material, a 12% gelatin solution. The protocols can be used as a quality control tool by the manufacturer of E-Matrix™, Encelle, Inc. of Greenville, North Carolina. The protocols evaluate specific rheological properties to compare either gelatin lots or manufactured E-Matrix™ batches with established standards. These limits would be developed by using the protocol to evaluate numerous E-Matrix™ batches deemed to function successfully. A similar procedure would be used to evaluate gelatin lots used to formulate E-Matrix™. Incorporation of the rheological protocols into a quality control procedure would be a valuable tool for accessing the acceptability of gelatin lots and newly manufactured E-Matrix™ batches.

1.3 EXAMINATION OF E-MATRIX™ COMPONENTS

To further understand and characterize E-Matrix™, studies were performed which examined the key ingredients of the material. Specifically, a 12% gelatin solution and a 5% dextran solution were individually examined since these are the relative amounts of each component in E-Matrix™. To insure the solids content was not the sole source for creating physical property differences, a 17% gelatin solution, the same solids content as E-Matrix™, was also examined. Solutions containing 12% gelatin and 5% dextran were also considered, because these are the relative

concentrations present in E-Matrix™. A monovalent salt, NaCl, and a divalent salt, $CaCl_2$, were added to 12% gelatin solutions to examine ionic strength effect at concentrations below, equivalent, and greater than the ionic strength of E-Matrix™. Thus the effect of a monovalent salt versus a divalent salt on the compound was investigated in addition to the effect of different salt concentrations. Because E-Matrix™ phase separates into a gelatin-rich domain and a dextran-rich domain at 37°C (body temperature), the gelatin-rich domain, hypothesized to be the functioning phase, was also studied. As anticipated, the different components of E-Matrix™ showed different rheological properties, and this knowledge is important to advance the understanding of E-Matrix™ functionality.

As part of the study to examine the components of E-Matrix™, the Maillard reaction was considered because previous studies (Diftis and Kiosseoglou 2004; Ho and others 2000; Kato and others 1993), showed the Maillard reaction was responsible for covalently conjugating proteins to polysaccharides, specifically dextrans to proteins. Therefore, an investigation determined whether gelatin and dextran were covalently conjugated as a result of the Maillard reaction. This investigation was performed using the *O*-Phthaldialdehyde procedure (OPA), and the results indicated the Maillard reaction was not likely occurring.

1.4 SUMMARY

To better understand E-Matrix™ functionality, physical and chemical techniques were used to characterize E-Matrix™. Physical characterization was the focus of this project, specifically with respect to developing rheological techniques to characterize E-Matrix™ and its components. Chemical work was performed to examine possible cross-linking within E-Matrix™ due to the Maillard reaction.

CHAPTER 2.

LITERATURE REVIEW

2.1 INTRODUCTION

Medical devices perform life sustaining functions and biological improvements and consist of an array of products ranging from pacemakers to liquid adhesives. Gelatin and dextran are common components used for such devices. This literature review is relevant to the characterization and application of gelatin and dextran, specifically as ingredients in medical devices. In order to take a closer examination of a specific medical device, E-Matrix™, the subsequent sections describe medical devices, E-Matrix™, the principle components of E-Matrix™, and rheological properties. Methods and principles relevant to physically characterizing biopolymers will also be reviewed. Other analytical techniques were deployed throughout this project, and these methods are reviewed, again with respect to gelatin and dextran.

2.2 MEDICAL DEVICES

Medical devices are defined as products which function through a physical action as opposed to a chemical and metabolic action with the later being a drug (Sall 2004). The Food Drug & Cosmetic Act defines a medical device as "...an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including a component part, or accessory which is: recognized in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them, intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or

other animals, or intended to affect the structure or any function of the body of man or other animals…" Thus medical devices can appear to be both physically and functionally similar to drugs or be physical instruments used in the medical field. Medical devices are used for the purpose of diagnosis, cure, mitigation, treatment, or prevention of disease in humans or other animals or to affect the structure or any function of the body (Sall 2004). Hence there is an array of devices fitting into the medical device category.

To differentiate between various levels of medial devices, the FDA established three classes of medical devices. These device classes are based on the risk posed to the patient and/or the user of the device. Classes are also based on the level of control required to assure the safety and effectiveness of the device. Class I includes devices such as toothbrushes and irrigating syringes which pose the least risks and are subject to general controls (Sall 2004). Moderate risk devices subject to special controls are classified as Class II and include ultrasound imaging systems and pregnancy test kits. The third classification, Class III, are technologically innovative devices posing the greatest risk to the patient or user (Sall 2004). Therefore a medical device may resemble a drug quite closely in appearance or it may be an instrument. Either way, in order for a medical device to be accepted by the Food and Drug Administration (FDA), thorough risk assessment must be performed.

2.3 HYDROGELS

Many medical devices, such as syringes, sample bags, sample tubes, and artificial organs (blood vessels) are hydrogels (Watanabe and others 2004). A hydrogel is commonly defined as a colloidal gel in which the particles are dispersed in a liquid and the dispersion medium is water. Numerous medical devices are composed of hydrogels because these systems swell under conditions of excess water and retain large amounts of water while hydrated, making hydrogels able to function in the human body since they are able to absorb cell culture medium (Watanabe and others 2004). Hydrogels can act as drug delivery carriers, tissue engineering scaffolds, and biomedical devices (Watanabe and others 2004). A foreign body reaction causing an inflammatory or fibrotic reaction is a common problem following injection of materials into the body (Badylak 2004). However the likelihood of a foreign body reaction can be lowered with the use of hydrogels because the body is constructed of network structures (biomacromolecules) and a large percentage of water, similar to the structure of a hydrogel. Further, the surface free energies of the human body and a hydrogel are similar, also lowering the chance of a foreign body reaction (Watanabe and others 2004).

Balakrishnan and Jayakrishnan (2005) state hydrogels derived from natural proteins and polysaccharides mimic the extracellular matrix of the tissue comprised of various amino acids and sugar-based macromolecules, so these natural based hydrogels are ideal for tissue engineering scaffolds. Toxic agents which may be

formed by hydrogels made from or cross-linked with unnatural components can produce detrimental effects if produced in excess amounts or leaked into the body (Balakrishnan and Jayakrishman 2005). Furthermore, hydrogel chemical properties are determined by the polymer backbone, functional side-chain in the monomer unit, and the cross-linking agent (Watanabe and others 2004). Using hydrogels to transport drugs into the body allows stimuli-responsive drug release with stimuli being features such as temperature or pH (Watanabeand others 2004) or the presence of specific enzymes (Kosmala and others 1999). Hence a biomedical device made of a hydrogel can be designed to begin working only once a specific pH, such as that of the human body, is achieved. Because hydrogels are depending on composition can mimic human tissue extracellular matrices, hydrogels make ideal tissue engineering scaffolds (Balakrishnan and Jayakrishnan 2005).

2.4 EXTRACELLULAR MATRIX

Extracellular matrix (ECM) is commonly defined as a scaffold within tissues and organs. This scaffolding is a complex combination of structural and functional proteins and polysaccharides which are aligned in a three-dimensional structure specific to each tissue or organ (Wneck and Bowlin 2004; West and Badylack 2004). Furthermore, the extracellular matrix is vital, dynamic, and a crucial part of all tissues and organs in that it is the body's natural scaffold for tissue and organ repair after injury (Wneck and Bowlin 2004). The extracellular matrix of skin has many functions including a natural method for the body to perform morphogenesis, maintenance,

and reconstruction after tissue or organ injury (Badylak 2004). These properties make the extracellular matrix an excellent scaffold for tissue engineering applications, especially tissue repair and reconstruction (Badylak 2004). While attempting to create a man-made extracellular matrix, it is reasonable to incorporate collagen into man-made extracellular matrix because collagen is the most abundant protein within the natural extracellular matrix (Badylak 2004), so when attempting to reconstruct this matrix it is practical to use a collagen containing material; gelatin.

2.5 E-MATRIX™

Encelle™ Inc. of Greenville, North Carolina developed a medical device, named E-Matrix™, which functions as an extracellular matrix. E-Matrix™ is a hydrogel principally composed of conventional food materials, gelatin (12% w/w) and dextran (5% w/w). Because hydrogels composed of natural polymers mimic the extracellular matrix scaffolding of skin (Balakrishnan and Jayakrishnan 2005) that naturally regenerates and repairs tissue, it makes sense that E-Matrix™ is principally composed of a natural protein found in skin, collagen (gelatin), and a natural polysaccharide (dextran). Hydrogels made of natural proteins and polysaccharides mimic tissue and extracellular matrices.

The gelatin-dextran combination of E-Matrix™, in the presence of an additional amino acid cocktail, purportedly accelerates the rate of healing. The medical device is designed to accelerate the healing rate of wounds by shifting the healing process

from a slower healing adult inflammatory tissue stage to a quicker healing fetal regenerative tissue stage. This switch is crucial as a developing fetus or newborn is able to heal wounds differently and generally better than adults and quicker and more effectively than older adults (Badylak 2004). Potential applications of E-Matrix™ include the following; dermal healing of diabetic foot ulcers and other cutaneous wounds, bone repair through dental surgery, spinal fusion, and non-union fracture repair, connective tissue repair of tendons, ligaments, and cartilage, and soft tissue augmentation in cosmetic surgery, gastroesophageal reflux disease, and urinary incontinence. E-Matrix™ is classified as a Class III medical device and hypothesized to form a matrix under the skin, mimicking an extracellular matrix found in fetal development and supplying a three dimensional scaffolding for tissue reconstruction. Hence, E-Matrix™ has great potential in a variety of areas.

2.6 COMPONENTS OF E-MATRIX™

Since E-Matrix™ is principally composed of gelatin and dextran, importance was placed on examining these key elements as gelatin and dextran properties may directly and indirectly affect E-Matrix™ functionality.

2.6.1 GELATIN OVERVIEW

Gelatin is a derived, soluble protein obtained from partial hydrolysis of collagen, retrieved primarily from connective tissue of pigskins and bovine bones and hides.

Being derived from collagen, gelatin is a protein possessing hydrocolloidal properties permitting functionality in assorted areas. The term hydrocolloid refers to a range of proteins and polysaccharides functioning as thickeners, gelling agents, stabilizing foams, emulsions, and dispersions (Williams and Phillips 2000). A closer examination of collagen will first be discussed, followed by discussion of amino acid composition of gelatin, production, characterization/classification, physical properties, and finally the many uses of gelatin.

2.6.1.1 COLLAGEN

Collagen, the backbone of gelatin, is a crucial component of the structural support system of vertebrates and invertebrates, because it is the major protein constituent of skin, tendon, cartilage, bone, and white fibrous connective tissue in mammals (Balian and Bowes 1977). Collagen is composed of three parallel tropocollagen strands which are chemically crosslinked to form a triple helix, giving collagen the triple helix formation (Figure 2.1). The tropocollagen rod is the fundamental molecular unit of collagen. When heated, the chemical cross-links that bind the tropocollagen monomers together break, and each tropocollagen strand becomes an individual gelatin molecule (Murano 2003). The total molecular weight of tropocollagen is approximately 330,000, it has a 1.5 nm diameter, and a length of approximately 300 nm (Ledward 2000). Mild heating (40°C) causes the triple helix to unfold, producing α-chains, β-chains, and γ-units. The β-chains consist of two covalently bound α-chains, and the γ-units are made of three α-chains (Ledward

2000). Furthermore, collagen contains both intramolecular crosslinks and several intermolecular bonds (Ledward 2000).

The intra and intermolecular links cause animal tissue to have high tensile strength. As animals age, the number of stable linkages increase and these linkages begin to form trivalent (linking three instead of two collagen molecules together) linkages (Ledward 2000). This phenomenon is the cause of tougher collagen in older animals, and these stable linkages result in the need for severe processing of collagen to create a soluble gel. These factors explain why commercial gelatins vary widely in molecular weights from 50,000 to 1,000,000 (Ledward 2000). Hence the initial state of the collagen and conversion from collagen to gelatin strongly dictates the final functional properties of gelatin.

2.6.1.2 GELATIN

Pure collagen cannot be used to produce gelatin, so converting collagen to gelatin and removing the greatest amount of non-collagenous material must occur (Johns 1977), a process crucial for the successful production of gelatin. Note gelatin is the derived protein of collagen, whereas gelatine is the total product produced from collagen- comprised of gelatin as the main component, plus various smaller inorganic and organic impurities (Eastoe and Leach 1977). The structure of gelatin is very similar to that of collagen.

Though structurally the parent collagen is very similar to the resultant gelatin, some differences may exist, such as the amide content, conversion of arginine side chains, and the overall balance of amino acid composition (Eastoe and Leach 1977). If the parent collagen is exposed to an alkaline treatment, the amide content may lower. In addition, prolonged exposure to alkali may increase the conversion of arginine side chains and the gelatins are often richer in the abundant amino acids and poorer in the rarer residues than parent collagens (Eastoe and Leach 1977). Also, when gelatin and collagen are placed in dilute acid or alkali, gelatin will dissolve, but collagen will only swell or hydrate (Ledward 2000). Furthermore, when mildly heating (< 50°C) gelatin and collagen, gelatin again readily dissolves to form a viscous solution at all pHs, but collagen only shrinks and becomes unable to hold water (Ledward 2000). Hence the processing method used to manufacture gelatin plays an important role in the final material properties.

2.6.1.3 GELATIN PRODUCTION

Prior to a mild heat treatment to promote the irreversible break down of the fibrous collagen structure (Eastoe and Leach 1977), gelatin is trapped within the three-dimensional structure of collagen. To obtain gelatin, the secondary and higher structures (Ledward 2000) of collagen must be disrupted, which occurs during gelatin manufacture. Methods of gelatin manufacture include: acid, alkali, due soak, and autoclaving processes (Eastoe and Leach 1977). The most common methods are acid and alkaline processing, followed and/or accompanied by heating in the

presence of water. Acid and base processes produce gelatins commonly referred to as Type A and Type B, respectively. Figure 2.2 is a process flow diagram of these processes, and Figure 2.3 and Table 2.1 identify discrepancies which may result from the different processing method in regard to pH and amino acid composition, respectively. Following these extraction processes, gelatin is dried to form coarse granules, fine powders, or thin sheets, which are odorless, tasteless, and yellowish in color. Granules and powders are commonly produced in America, while sheets are more common in Europe.

Since gelatin is a protein, it is constructed of eighteen amino acids (Kirk-Othmer 1999). Glycine, proline, and hydroxyproline are the most abundant amino acids in gelatin and tend to form the most prevalent amino acid sequence present in collagen and gelatin; Gly-Pro-Hypro-Gly (deMan 1999). Overall, glycine is the most prevalent amino acid in gelatin. Table 2.2 lists the amino acid composition of gelatin, and Figure 2.4 is a common amino acid sequence within gelatin.

All collagens, thus gelatin included, contain a characteristic primary structure with the differences between specific gelatin molecules lying in the small region at the end of the α-chain, called the telopeptide (William and Phillips 2000). The amino acid sequence of gelatin remains the same as that of the parent collagen sequence with the typical sequence being Gly-X-Y as illustrated in Figure 2.4, where X is usually proline and Y is usually hydroxyproline (deMan 1999). Unfortunately, the complete or long sequences of gelatin cannot be determined partly because it is

derived from all three α-chains which each differ (Eastoe and Leach 1977). Although a common structure is present, some unusual structures do exist. Uncommon structures speculated to be present in gelatin include “esterlike” bonds, aldehyde groups, and γ -glutamyl linkages (Eastoe and Leach 1977).

Because gelatin lacks tryptophan and contains only small amounts of some other essential amino acids, it is considered an incomplete protein, but is digestible by humans (Ensminger and others 1994). As a nutritionally incomplete protein, gelatin still has numerous applications. In fact, gelatin possesses several unique physical characteristics which compensate for its nutritional deficiency.

Gelatin applications extend from the food industry as a stabilizer, thickener, and texturizer, to the photographic industry where it is used to make inks, photographic plates, and films (Ensiminger and others 1994). In textiles, gelatin is used as paper sizing, and in the medical industry as adhesives, capsules, and plasma expanders (Ensiminger and others 1994) (Table 2.3). The unique properties characterizing gelatin; melt-in-mouth phenomenon, thermoreversibility, and ability to gel upon cooling, allow the mentioned widespread functionality. Particular to the food industry, the fact gelatin gels melt at relatively low temperatures, such as body temperature 37°C, and are practically odorless and tasteless has expanded gelatin applications as a fat replacer, giving a similar mouthfeel to fat. For example Jell-O® imparts a melt-in-your-mouth sensation, similar to fat. The amino acid building blocks of gelatin permit gelatin to form hydrocolloid gelling agents. Depending on

conditions, gelatin is capable to function as both a protective colloid and as a flocculating agent (Wood 1977). In addition, the clarity of gelatin gels adds to the list of gelatin functionality (de Vries 2004). Furthermore, the unique molecular and physical properties of gelatin are a vital component in making this material functionally versatile.

Just as gelatins span an array of uses, individual gelatin molecules also differ greatly. Commercially desirable physical characteristics of gelatin include gel strength, viscosity, adhesiveness, tack, color, and clarity, while related to the intrinsic chemical composition, molecular weight, and molecular configuration of gelatin is important (Veis 1964). Distinguishable physical characteristics of gelatin as previously noted, determine gelatin classifications such as quality grades. Manufacturing gelatin results in the production of various gelatin grades distinguished by bloom strength, viscosity, and molecular weights which range from ~20,000 to 250,000 (Kirk-Othmer 1999).

Bloom strength is a measure of the rigidity determined by depressing a gelatin gel at standard conditions (Ledward 2000). Bloom strength has been a common quality measurement in the gelatin industry for decades. A bloom gelometer is used to measure the force required to depress a 12.7 mm diameter flat-bottomed cylindrical plunger 4 mm into a gel. This gel must be prepared by standard conditions: placing 7.5 g gelatin into a Bloom jar with 105 g distilled water. This gel must contain 6.67% (w/w) air-dried protein. The gelatin solution next is allowed to swell, and then made

homogenous by heating and stirring at 60°C. The gel is left at ambient conditions for 15 minutes, placed in a 10°C bath for 16-18 hours and finally tested with the Bloom gelometer (Ledward 2000). Unfortunately this test is very sensitive and difficult to repeat. A Texture Analyzer is a more modern instrument used to determine Bloom strength. A bloom value of 100 means a force of 100 g was required to plunge 4 mm into the gel. The higher the bloom value, the higher the grade and price.

The higher grade gelatins have gone through the fewest extractions during processing, and these extractions were performed at the lowest temperatures as higher temperatures cause proteolysis (Ledward 2000). In addition, gelatins of lighter color reflect higher grades (Ledward 2000). As previously mentioned, the strong inter and intra molecular links cause the heterogeneous composition of molecular weights within gelatin. Besides size variation, charge distribution also varies (Williams and Phillips 2000). In addition, moisture content varies and commonly ranges from 9-14% (Eastoe and Leach 1977). More specifically, food grade gelatins range in moisture from 8-12%, have less than 2% ash, and the remaining composition is protein (Ledward 2000). Therefore, there is a wide variability among gelatins.

2.6.1.4 PHYSICAL PROPERTIES OF GELATIN

Physically gelatin can vary from a solid-like gel to a liquid solution, reversing states numerous times without negatively affecting the functional properties of a food

product. Immersion of dry gelatin granules or sheets in hot water can lead to gelatin solubilising and the creation of a random coiled polypeptide solution (Murano 2003). At or above 40°C, gelatin is assumed to exist as a random coil and may still have some helical structure (Williams and Phillips 2000) from the collagen. With time and at the appropriate temperature, a gel will eventually form (Murano 2003). Figure 2.5 illustrates how adding dry gelatin to hot water can lead to the formation of a viscous gelatin solution. Upon cooling the solution, a thermo-reversible gel, can be formed. Transformation between solutions and gels is readily possible because physically, gelatin solutions increase viscosity with a decrease in temperature and can melt with a temperature increase (Murano 2003). This thermo-reversibility is due to gelatin possessing inter and intra-molecular hydrogen bonding (Chatterji 1990). Because junction zones are bound by weak hydrogen bonds, at temperatures of 35-40°C the hydrogen bonds will break causing the gel to melt (de Vries 2004; Abeles and others 1992).

The mechanism of gelation is simple, yet complex (Figure 2.6). At temperatures below 40°C gelatin molecules readily aggregate and gelation occurs to form a gel (Williams and Phillips 2000). As the temperature is lowered, the pyrolidine-rich regions of gelatin chains act as nucleation sites for the formation of potential junction zones (Ledward 2000). Junction zones are ordered structures of two or more molecules (de Vries 2004). The pyrolidine-rich regions, particularly the regions with glycine-proline-proline or hydroxyproline sequences tend to take up the proline-L-proline II helix. A triple helix resembling that of the initial collagen (Figure 2.1) is

then formed (Guo and others 2003; Gekko 1993) when aggregation of three proline-L-proline II helix occurs (Ledward 2000; Gekko and Fukamizu 1991). The triple helix acts as gel junction points or zones, stabilized by inter chain hydrogen bonds. With time and accelerated by a decrease in temperature, additional junction zones form and existing junction zones grow. With junction zone growth and formation, the gel becomes more organized and stronger. In time, gel organization and strength increase. The growth of existing junction zones is believed to be the key contributing factor to increased gel strength.

In addition to time being a factor in increasing gel strength, concentration is also a factor. Increased concentration improves the chances that two or three different chains will form a junction. As the gel becomes more organized with time, the gel also becomes more thermally stable (Ledward 2000). Gekko and Fukamizu (1991) suggest gelatin gels result from large exothermic effects caused by the intermolecular hydrogen bonds overcoming the entropy loss associated with the order of the polypeptide chains. Aggregation and growth of junction zones can continue to form a more mature and stronger gel over time.

Besides concentration and time, chemical factors also influence gelation. Gelatin possesses amphoteric properties, so gelatin solution viscosity is greatly affected by pH. The viscosity of gelatin is smallest at the isoionic point (Hudson 1993). Isoionic point is defined as the pH value at which the net electric charge of an elementary entity in pure water equals zero (IUPAC 1994). However, pH is not a significant

factor within the pH range of 4-10 (Ledward 2000), but outside this range, gelation is greatly inhibited. Gelatin inhibition is most likely due to the high net positive or negative charge carried by the chains, so electrostatic forces prevent the formation of junction zones (Ledward 2000). Therefore at pH 1-3 and 11-14 gelatin will not readily gel.

Gelatin is insoluble in cold water; however, completely soluble in warm water. Swelling of gelatin compares to the swelling of non-ionized crosslinked polymers, but the effects are complicated by charge interactions and the structure of the gelatin network (Wood 1977). Dry gelatin granules are capable of swelling and absorbing 5-10 times their weight in water to form a gel at 35-40°C (Ensminger 1994).

2.6.1.5 GELATIN AS A PHARMACEUTICAL INGREDIENT

Gelatin is not limited to the food, paper, or photographic industries. According to economic reports, gelatin use as a pharmaceutical ingredient is on the rise in the United States (Kirk-Othmer 1999). Gelatin is used most commonly as a drug encapsulating shell (Ledward 2000), permitting smooth oral drug delivery. Gelatin also binds pharmaceutical components in the manufacture of tablets. Furthermore, dentists use gelatin foam cubes to absorb blood during treatment, and in hospitals gelatin is used as blood substitutes to counteract high blood loss (Ledward 2000). In the current study, gelatin was used as a functional component and spacer in a medical device.

2.6.2 DEXTRAN OVERVIEW

Dextran is a polysaccharide composed of D-glucose subunits (Figure 2.7) connected by more than 50% α-1,6 glycosidic linkages with different additional branching through α-1,2, α-1,3, and α-1,4 linkages (Monsan and Auriol 2004; Murano, 2003). Dextran is a non-charged D-glucosyl homopolysaccharide (Monsan and Auriol 2004), lacking strong viscosifying capabilities (Tromp and others 2004). Visually dextran is a white powder. Production of dextran is caused by lactic acid bacteria (Monsan and Auriol 2004) with *Leuconostoc mesenteroides* being used most prevalently for production of clinical grade dextran (de Belder 1996). At the industrial level, production is caused by partial hydrolysis, fractionation, and purification of native dextran produced using the bacterium *Leuconostoc mesenteroides* to make a polymer consisting of approximately 95% α-1,6 linkages (Kennedy and others 1984), making it quite linear and having a small number of side chains. However physical properties of dextran, such as low viscosity, make dextran a beneficial, key ingredient in numerous ways in both the food and pharmaceutical industries. Furthermore, the fact that dextran is biodegradable and a natural polymer prepared from renewable resources, makes incorporating dextran into more products practical for many applications.

2.6.2.1 MOLECULAR AND PHYSICAL PROPERTIES OF DEXTRAN

This large and neutral polysaccharide comes in a range of molecular weights ranging from 500 to 6000 kDa (Monsan and Auriol 2004; Ebert and Schenk 1968; Groenwall and Ingelman 1948). Large physical size allows dextran to be used widely in the medical field since it is too large to pass through uninjured vessel walls. The majority of dextran physical properties are dependent on the molecular weight distribution (de Belder 1996). For example, the viscosity of dextran is directly proportional to the molecular weight as Equation 1 illustrates (de Belder 1996) with $[\eta]$ representing the intrinsic viscosity.

$$[\eta] = 2.43 \times 10^{-3} M_w^{0.42} \quad \textbf{(1)}$$

Molecularly, dextrans are nearly identical to amylose, as amylose is a linear sequence of glucose molecules linked together (Figure 2.8), but the slight branching of polymeric chains in dextran prevents stacking and causes most dextrans to be soluble in water (Walstra 2003) and electrolytes (de Belder 1996). Biologically, dextran can be degraded once in vivo by degrading enzymes present in animal and human tissues. However most hydrolysis products produced by dextran can be absorbed to rapidly raise the blood sugar and liver glycogen levels. If dextran is orally introduced into the body, it passes through the small intestine and metabolism or degradation occurs by colonic bacterial flora and fermented short-chain fatty acids in the small intestine (Monsan and Auriol 2004; de Belder 1996). However, a disadvantage of dextran is the potential for antigenicity causing trigger of an immune response, among high molecular weight fractions (Dellacherie 1996). Dextran

solutions are thermally-stable, and consequently can be sterilized and used as injectable pharmaceutical components.

2.6.2.2 DEXTRAN AS A PHARMACEUTICAL INGREDIENT

Dextran plays an active role in the pharmaceutical industry. Specifically, unmodified dextran, with a molecular weight ranging from 75,000 ± 25,000, is used as a blood plasma substitute, as 6% solutions have a viscosity value similar to that of human blood plasma (Kennedy and others 1984). This polyglucose has been incorporated as a plasma expander and a drug deliverer. Additionally, iron dextran is injected into muscles for iron deficient patients. Infusing dextran into the patient intravenously is quite beneficial because its size is too large to pass through uninjured vessel walls and it is osmotically active. Furthermore, the emulsion polymerization of dextran with epichlorihydrin leads to insoluble cross-linked dextran beads which swell in water. This property has led to the ability of dextran to separate molecules by size and thus to be used in biological research. Dextran beads are used to treat skin wounds and lesions as they absorb wound exudates in secreting infected wounds and therefore accelerate the wound healing time (de Belder 1996). In the current project, dextran is used as a key component of E-Matrix^{TM} directing cells toward a non-inflammatory wound healing response and leading to tissue regeneration by inhibiting renaturation of gelatin and helping maintain the open structure of the gelatin chain.

2.6.3 GELATIN-DEXTRAN COMPOUND

Gelatin and dextran are natural, biodegradable polymers (Bigi and others 2004; Zhang and Chu 2000; Tabta and Ikada 1998). This feature is important because the gelatin-dextran compound is highly responsive to stimulus and has a good biocompatibility with the body (Kurisawat and Yui 1998). Furthermore, Kosmala and others (2000) state combining gelatin and dextran can produce a selectively enzymatically, but not hydrolytically, degradable hydrogel. Therefore incorporation of gelatin-dextran into drug delivery systems permits selective delivery of the drug only to sites containing the appropriate enzymes. Also hydrogels can aid in three dimensional tissue reconstruction scaffold (Watanabe and others 2004).

The addition of dextran to gelatin, even in small magnitudes, can powerfully increase the gelation rate of the solution and increase the elastic modulus of gelatin-dextran gels (Tolstoguzov 1993). It is thought this gelation rate increase occurs because junction zones leading to gelation form between polymeric chains and with the addition of dextran, the number of available chains forming junction zones increases (Tolstoguzov 1993). Kurisawa and Yui (1997) suggested that physical chain entanglements are present between gelatin and dextran networks in hydrogels prepared below the gelation temperature. Thus the addition of dextran to gelatin can effect gelation and physical entanglements can form between gelatin and dextran.

2.6.3.1 PHASE SEPARATION

Like many polysaccharide and protein mixtures, gelatin and dextran are incompatible in aqueous solutions (Edelman and others 2001). Due to this instability, phase separation, the phenomenon in which the formation of regions of different compositions form, (Walstra 2003) may take place with the introduction of time and temperature (Figure 2.9).

Two types of phase separation may occur within a polymer mixture such as the mixture of gelatin (2), and dextran (3), with water (1) as the solvent. One type is segregative phase separation and the second is associative phase separation (Walstra 2003). The two kinds of phase separation can be distinguished by a value called the second virial coefficient (*B)*. Virial coefficient is a function of physicochemical parameters and denotes the interaction between two polymers (gelatin and dextran) (Vivares and Bonnete 2004). Segregative separation occurs when the second virial coefficient $B_{23}>0$ and the two components separate into a phase rich in one polymer (2) and poor in the other (3) and a second phase rich in 3 and poor in 2 is formed (Walstra 2003). At w/w concentrations above 3% of both components, segregative phase separation occurs (Edelman and van der Linden 2001). Also, in this type of separation, the two polymers avoid each other and the polymers are named incompatible (Walstra 2003). If $B_{23}<0$, associative phase separation occurs as the two polymers attract each other forming a complex coacervate which is a phase rich in both solutes (Walstra 2003). Complex

coacervate also occurs if the polymer concentration is large and B_{23}~0 (Walstra 2003).

For a gelatin-dextran-water solution, at sufficient concentrations, usually above 3% (w/w) for each component, phase separation occurs forming a gelatin-rich region and a dextran-rich region. It is suggested the phase separation of gelatin and dextran is an entropy-driven process and the total polymer concentration at which phase separation occurs is nearly independent of temperature. Although heat is required for the biopolymer to separate into two domains, limited temperature dependence occurs between 40 and 80°C. As would be expected, above gelation temperature (~30°C), gelatin-dextran compounds exhibit normal liquid-liquid phase separation, but once the temperature is lowered below ~30°C and gelation occurs, separation is reduced (Edelman and others 2001). At the warmer temperatures (40-80°C), which are above the gelation temperature, gelation does not occur, and composition and hydrodynamic conditions determine the structure of the resulting two phase system (Packe and others 2003).

In regard to component positioning of a phase separated system, Tromp and others (2004) believe salt concentration determines which phase will be on top, with larger salt concentrations causing the gelatin-rich phase to rise to the top. Additionally, Norton and Frith (2003) argue if the gelling phase; gelatin, is the major component, the non-gelling phase; is the dispersed phase at rest. Therefore prior to phase separation, the gelatin is the continuous phase with dispersions of dextran

molecules throughout. Examination of E-Matrix™ under a microscope revealed gelatin was the continuous phase of the multi phase system. Post phase separation, the dextran molecules may be trapped among the gelatin, causing dextran rich and gelatin rich domains with an amount of trapped dextran molecules remaining in the gelatin rich portion. Additionally, a viscosity difference exists between the gelatin-rich domain and the dextran-rich domain (Edelman and others 2001).

2.6.3.2 EXPLANATION OF MAILLARD REACTION

The Maillard reaction, also referred to as non-enzymatic browning, is a very important chemical phenomena to the food industry. The reaction (Figures 2.10-11) affects food quality in processing and storage, as it may promote color change, aroma, and nutritional decline in foods. The reaction is a sequence of events beginning with the reaction of the amino group of amino acids, peptides, or proteins with a glycosidic hydroxyl group of sugars (Ellis 1959). This sequence terminates with the formation of brown nitrogenous polymers or melanoidins (Ellis 1959). Lysine is usually the most reactive amino acid due to its free ε-amino group (deMan 1999). The Maillard reaction is described by Hurst (1972) as a series of five chemical steps. The first step involves an *N*-substituted glycosylamine being produced from an aldose or ketose, reacting with a primary amino group of an amino acid, peptide, or protein (deMan 1999; Hurst 1972). This step is followed by the glycosylamine being rearranged by a reaction yielding an aldoseamine or ketoseamine, known as an Amadori rearrangement. Next, the ketoseamine is

manipulated again with a second aldose to form a diketoseamine; or aldosamine reacts with a second amino acid to yield a diamion sugar. Amino or nonamino compounds are next produced by the degradation of amino sugars, losing one or more water molecules. Finally condensation of the amino or nonamino compounds, formed in the previous step, with each other or amino compounds, form brown pigments and polymers (deMan 1999). The brown pigments are a characteristic mark of the Maillard reaction having occurred and can be seen in browning of baked cookies.

A number of variables affect the Maillard reaction, including time, temperature, water content, water activity, pH, oxygen, metals, phosphates, sulfur dioxide, concentration of reactants, and the type of reactants and solvents (Miao and Roos 2004; deMan 1999). In addition, the viscosity of the matrix material affects the Maillard reaction rate (Miao and Roos 2004). Glass transition, water content, and water activity also affect the reaction rate (Craig and others 2001; Le Meste 1995; Karmas and others 1992).

Studies have deduced the Maillard reaction was responsible for covalently conjugating proteins to polysaccharides and specifically dextrans to proteins (Diftis and Kiosseoglou 2004; Ho and others 2000; Kato and others 1993). The protein lysozyme, found in egg white, and dextran form a conjugate due to the Maillard reaction (Nakamura and others 1991). Furthermore, Ho and others (2000) used SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Pephadex G-150 filtration

chromatography to confirm the covalent attachment of dextran to ε-polylysine through a Maillard reaction with the ε-polyllysine amino groups and the reducing-end carbonyl group of dextran. Another study also claims the Maillard reaction is responsible for the covalent bond of a protein-carbohydrate substance with dried egg white being the protein and the polysaccharide being galactomannan with the bond occurring between the ε-amino groups in the protein and the reducing-end carbonyl residue in the polysaccharide (Kato and others 1993). Even though the Maillard reaction occurs more readily and rapidly at higher temperatures, it is noted that even at room temperature the reaction between polylysine and dextran takes place (Ho and others 1999). Thus lower temperatures, such as body temperature (37˚C), may be adequate for the Maillard reaction to occur.

2.7 RHEOLOGICAL METHODS

Rheology is the scientific study of the flow and deformation of matter. Principles of rheology are commonly applied to understand and improve the flow behavior and textural attributes of materials and to reveal relationships between the physical properties and the functionality of a material (Steffe 1996). Rheology attempts to build relationships between forces and corresponding deformations, or expressed more fundamentally as stress and strain.

2.7.1 SHEAR STRESS

Stress (σ) is a measurement of force and is defined as a force per unit area (Equation 2). Stress is commonly expressed in Pascals (N/m^2), and Figure 2.12 demonstrates the application of a shear stress onto an object.

$$\sigma = \frac{F}{A} \quad \textbf{(2)}$$

2.7.2 SHEAR STRAIN

Strain is a dimensionless quantity representing the deformation of a physical body under the action of applied forces. The direction of the applied stress to the material surface determines the type of strain; normal strain or shear strain. Normal strain occurs when stress is applied to the surface of the material in a perpendicular (normal) direction which occurs during compression (Daubert and Foegeding 1998). The second type of strain, shear strain, occurs when a stress is applied parallel to the surface of the material (Equation 3). In Equation 3 the shear strain (γ) is the inverse tangent of the change in distance divided by the initial height of the material.

$$\gamma = \tan^{-1}\left(\frac{\delta d}{h}\right) \quad \textbf{(3)}$$

2.7.3 SHEAR RATE

For fluids, a shear stress can induce a unique type of flow called shear flow (Figure 2.13). The differential change of strain with respect to time is known as shear (strain) rate ($\dot{\gamma}$), calculated as Equation 4, with units of sec^{-1}.

$$\dot{\gamma} = \frac{d\gamma}{dt} \quad \textbf{(4)}$$

2.7.4 VISCOSITY

Apparent viscosity (η) is the measurement of the resistance to flow or the fluidity of a material and is mathematically calculated as the ratio of shear stress to shear rate.

$$\eta = \frac{\sigma}{\dot{\gamma}} \quad \textbf{(5)}$$

2.7.5 SHEAR MODULUS

Shear modulus (G) is the constant of proportionality used to relate shear stress with shear strain (Daubert and Foegeding 1998).

$$G = \frac{\sigma}{\gamma} \quad \textbf{(6)}$$

2.7.6 VISCOELASTICITY

Viscoelastic materials exhibit both viscous and elastic properties. Viscous properties possess fluid-like behavior, while elastic properties represent the solid properties of a material. Measurement of viscoelastic properties can be done using dynamic oscillation of shear stress or strain. Oscillation studies are the most common dynamic method for examining viscoelastic behavior (Steffe 1996). Harmonic oscillation of shear stress involves a material being oscillated sinusoidally with varying stress while the resulting strain is measured (Zhong 2003). When performing dynamic tests, four assumptions are assumed: 1.) a constant stress or strain throughout the sample; 2.) no slip of the sample; 3.) sample homogeneity; and 4.) that measurements are performed within the linear viscoelastic region. Furthermore, key functions describe viscoelastic behavior of a material, such as complex moduli, phase angle, storage modulus, and loss modulus.

2.7.6.1 COMPLEX MODULUS

The complex shear modulus (G*) is the ratio of stress and strain amplitudes during the oscillation and gives an indication of the strength of a gel as defined in Equation 7.

$$G^* = \frac{\sigma_o}{\gamma_o} = \sqrt{(G')^2 + (G'')^2} \quad \textbf{(7)}$$

γ_0 = shear strain amplitude

σ_0 = stress amplitude

G′ = storage modulus

G″ = loss modulus

2.7.6.2 PHASE ANGLE

Phase angle (δ) is also related to the viscoelasticity of a material and is directly related to the energy lost per cycle divided by the energy stored per cycle (Steffe1996). Phase angles can vary from 0 to 90°, with 0° indicating an ideal solid material (Hookean solid) and 90° indicating an ideal viscous material (Newtonian fluid). Equation 8 describes the phase angle calculation, and in the current research, phase angle was used to designate gel and melt points.

$$\tan(\delta) = \frac{G''}{G'} \quad \textbf{(8)}$$

2.7.6.3 SHEAR STORAGE AND LOSS MODULI

The shear storage modulus (G′) indicates the degree of elastic behavior in a material. Shear storage modulus is the component (in phase) with the stress, or the elastic behavior defined by Equation 9.

$$G' = G * \cos\delta \quad \textbf{(9)}$$

Shear loss modulus (G") is the component (out of phase) with the strain or viscous behavior. Thus shear loss modulus is an indication of the viscous properties of the material and is defined in Equation 10.

$$G'' = G * \sin\delta \quad \textbf{(10)}$$

2.7.7 SHEAR VISCOMETRY

Rotational viscometry is a key approach for obtaining rheological measurements of viscoelastic fluid materials. Rotational viscometry involves an attachment with known geometry placed in contact with a sample, followed by mechanical rotation to shear the sample (Daubert and Foegeding 1998). Rheological attachments include concentric cylinders, cone and plate, and parallel plate geometries and mixers. The concentric cylinder is commonly referred to as the cup and bob because this apparatus consists of a cup with radius R_c, and a bob with a slightly smaller radius R_b that is placed inside the cup. Figure 2.14 displays the concentric cylinder. Concentric cylinders are constructed in two different configurations; couette in which the cup rotates and the bob is stationary, and searle in which the bob rotates and the cup is stationary. A serrated searle set up was used in the current project. Steffe (1996) states a serrated or roughened surface may effectively prevent slip between the tested material and the cup and bob walls. Furthermore, advantages of the concentric cylinder include: examination of weak viscous materials, the ability of a large surface area to increase the sensitivity at low shear rates, and improved response for suspensions (Daubert and Foegeding 1998). Disadvantages of the concentric cylinder are that the shear rate and stress are a function of location with respect to the bob, potential end effects may occur, and a large sample volume is required.

2.8 CALORIMETRY

Differential scanning calorimetry (DSC) is a thermal analysis technique in which the difference in energy inputs into a substance and a reference material is measured as a function of temperature while the substance and reference are subjected to a controlled temperature program (Hohne and others 2003; Schenz and Davis 1999). Usually the sample is sealed inside a metal or ceramic pan and the reference is an empty pan of the same composition. The DSC technique can be used to detect phase transitions and other thermodynamic reactions within a sample (Resch 2004). The output from a DSC is the combination of all endothermic reactions (occurring when a material absorbs heat) and exothermic reactions (when heat is given off) that occur in the substance as it is heated (Schenz and Davis 1999). Common examples of endothermic reactions are melting, protein denaturation, and starch gelatinization, while common examples of exothermic reactions are crystallization (sugar, ice) and curing (crosslinking due to heat, chemical additives, or UV light). In this project DSC was used to determine a thermal transition point indicative of melting E-Matrix^{TM} and a 12% gelatin solution.

2.9 SUMMARY

The demand for effective and efficient medical devices has caused scientists to continually seek innovative materials to construct such devices. The versatility of gelatin and dextran has led to their growing popularity and application in medical

devices. Furthermore, gelatin and dextran can be utilized independently, or combined as a compound. During this investigation E-Matrix™ was rheologically characterized, quality control protocols were developed for acceptance or rejection of E-Matrix™ and gelatin, and specific physical and chemical effects were studied. These analyzes were conducted to better understand the functionality of E-Matrix™ and its primary components.

2.10 REFERENCES

Abeles RH, Frey PA, Jencks WP. 1992. Biochemistry. Boston: Jones and Bartlett Publishers. 212-217.

Badylak SF. 2004. Extracellular Matrix Scaffolds. In: Wnek GE and Bowlin GL. Encyclopedia of Biomaterials and Biomedical Engineering. NY: Marcel Dekker Inc. 561-567.

Balian G, Bowes JH. 1977. The Structure and Properties of Collagen. In: Ward, AG and Courts, A. The Science and Technology of Gelatin. New York: Academic Press. 1-30.

Balakrishnan B, Jayakrishnan A. 2005. Self-cross-linking biopolymers as injectable in situ forming biodegradable scaffolds. Biomaterials 26(18):3941-3951.

Bigi A, Panzavolta S, and Rubini K. 2004. Relationship between triple-helix content and mechanical properties of gelatin films. Biomaterials 25(25):5675-5680.

Chatterji PR. 1990. Interpenetrating Hydrogel Networks. I. The Gelatin-Polyacrylamide System. J Appl Polym Sci 40(3-4):401-410.

Craig ID, Parker R, Rigby NM, Crairns P, Ring SG. 2001. Maillard reaction kinetics in model preservation system in the vicinity of glass transition: experiment and theory. J Agric Food Chem 49(10):4706-12.

Daubert CR, Foegeding EA. 1998. Rheological Principles for Food Analysis. In: Neilsen, SS editor. Food Analysis 2nd edition. Gaithersburg, MD: Aspen Publishers. 551-569.

de Belder AN. 1996. Medical Application of Dextran and Its Derivatives. In: Dumitriu,S. Polysaccharides in Medicinal Applications. New York: Mercel Dekker, Inc. 505-523.

Dellacherie E. 1996. Polysaccharides in Oxygen-Carrier Blood Substitutes. In: Dumitriu S ed. Polysaccharides in Medical Applications. NY: Marcel Dekker, Inc. 525-544.

deMan JM. 1999. Principles of Food Chemistry. Aspen Publishers, Inc.: Gaithersburg, MD. 120-172.

de Vries J. 2004. Hydrocolloid gelling agents and their applications. In: Williams, PA and Phillips, GO. Gums and Stablilisers for the Food Industry 12.

Cambridge, UK: The Royal Society of Chemistry. 23-31.

Diftis, N, Kiosseoglou V. 2004. Competitive adsorption between a dry-heated soy protein-dextran mixture and surface-active materials in oil-in-water emulsions. Food Hydrocolloids 18:639-646.

Eastoe JE, Leach AA. 1977. Chemical and Constitution of Gelatin. In: Ward AG and Courts A. The Science and Technology of Gelatin. New York: Academic Press. 73-107.

Ebert KH, Schenk G. 1968. Mechanism of biopolymer growth: The formation of dextran and levan. Adv Enzymol 30:179-210.

Edelman MW, van der Linden E, de Hoog E, Hans Tromp R. 2001. Compatibility of Gelatin and Dextran in Aqueous Solution. Biomacromolecules 2:1148-1154.

Ellis GP. 1959. The Maillard reaction. In: Advances in carbohydrate chemistry. Vol. 14, ed. ML Wolfrom and RS Tipson. New York: Academic Press.

Ensminger AH, Ensminger ME, Konlande JE, Robson JRK. 1994. Foods and Nutrition Encyclopedia 2nd ed. Ann Arbor, MI: Pegus Press. 554 and 1057.

Finot PA, Bujard E, Mottu F, Mauron J. 1977. Availability of the true Schiff's base of lysine and lactose in milk. In: Friedman, M. Ed; Protein Crosslinking: Nutritional and Medical Consequences. Plenum: NY. 343-364.

Friedman M. 1982. Chemically reactive and unreactive lysine as an index of browning. Diabetes. 31(Suppl. 3). 5-14.

Gekko K, Fukamizu M. 1991. Effect of pressure on the sol-gel transition of gelatin. Int J Biol Macromol 13:295.

Gekko K. 1993. The sol-gel transition of food macromolecules under high pressure. In: Nishinari, K and Doi, E. Editors. Food Hydrocolloids Structures, Properties, and Functions. NY: Plenum Press. 259-264.

Gelatin Manufacturers Institute of America, Inc. Gelatin. New York, NY, 1993:5-6.

Groenwall AJ, Ingelman BGA. 1948. Manufacture of infusion and injection fluids. US Patent 2,437,518.

Guo L, Colby RH, Lusignan CP, Howe AM. 2003. Physical Gelation of Gelatin Studied with Rheo-Optics. Marcromolecules 36:10009-10020.

Ho YT, Ishizaki S, and Munehiko T. 2000. Improving emulsifing activity of

ε-polylysine by conjugation with dextran through the Maillard reaction. Food Chem 68(4):449-455.

Hodge JE. 1953. Chemistry of Browning Reactions in Model Systems. J of Agric Food Chem 1(15):928-943.

Hohne GWH, Hemminger WF, Flammersheim HJ. 2003. Differential Scanning Calorimetry. 2nd ed. Germany: Springer. 1.

Hudson CB. 1993. Gelatine-Relating Structure and Chemistry to Functionality. In: Nishinari, K. and Doi, E., editors. Food Hydrocolloids Structures, Properties, and Functions. New York: Plenum Press. 353-354.

Hurst DT. 1972. Recent developments in the study of nonenzymatic browning and its inhibition by sulphur dioxide. BFMIRA Scientific and Technical Surveys No. 75. Leatherhaed, England.

IUPAC Compendium of Chemical Terminology. 1997. 2nd edition.

Johns P. 1977. The Structure and Composition of Collagen Containing Tissues. In: Ward, AG and Courts, A. The Science and Technology of Gelatin. New York: Academic Press. 31-72.

Karmas R, Buera MP, Karel M. 1992. Effect of glass transition on rates of non-enzymatic browning in food systems. J Agric Food Chem 40(5):873-9.

Kato A, Minaki K, Kobayahi K. 1993. Improvement of Emulsifying Properties of Egg White Proteins by the Attachment of Polysaccharide through Maillard Reaction in a Dry State. J Agric Food Chem 41(4):540-543.

Kennedy, JF, AJ Griffiths, DP Atkins. 1984. The Application of hydrocolloids: Recent developments, future trends. In: Gums and Stablilsers for the Food Industry 2: Application of Hydrocolloids. New York: Pergamon Press. 417-455.

Kirk-Othmer. Concise Encyclopedia of Chemical Technology 4th ed. A Wiley-Interscience Publication John Wiley and Sons Inc. New York. 1999.

Kosmala JD, Henthorn DB, Brannon-Peppas L. 2000. Preparation of interpenetrating networks of gelatin and dextran as degradable biomaterials. Biomaterials 21(20):2019-2023.

Kurisawa M, Yui N. 1998. Gelatin/dextran intelligent hydrogels for drug delivery: dual-stimuli-responsive degradation in relation to miscibility in interpenetrating polymer networks. Macromolecular Chemistry and Physics 199(8):1547-1554.

Ledward DA. 2000. Gelatin. In: Phillips, G.O. Williams, P.A. editors. Handbook of hydrocolloids. Boston: Woodhead Publishing Limited. 67-86.

Le Meste M. 1995. Mobility of small molecules in low and intermediate moisture foods. In: Barbosa-CanovasG, editor. Food preservation by moisture control: Fundamental and applications. Lancaster, Pa.: Technomic. 209-23.

Lodish H, Arnold B, Zipursky L, Matsudaira P, Baltimore D, Darnell J. 2000. Molecular Cell Biology. NY: W. H. Freeman Publishing.

Martins SIFS, Jongen WMF, van Boekel MAJS. 2001. A review of Maillard reaction in food and implications to kinetic modeling. Trends in Food Sci and Technology 11(3-4):364-373.

Miao S, Roos, YH. 2004. Comparison of Nonenzymatic Browning Kinetics in Spray-dried and Freeze-dried Carbohydrate-based Food Model Systems. JFS. 69(7):E322-331.

Monsan PF, Auriol D. 2004. Dextran and Glucooligosaccharides. In: JR Neeser and JB German. Bioprocesses and Biotechnology for Functional Foods and Nutraceuticals. NY: Marcel Dekker, Inc. 135-149.

Murano PS. 2003. Understanding Food Science and Technology. Belmont, CA: Thomson Wadsworth.

Nakamura S, Kato A, Kobayashi K. 1991. New Antimicrobial Characteristics of Lysozyme-Dextran Conjugate. J Agric Food Chem 39(4):647-650.

Norton IT, Frith WJ. 2003. Phase separation in mixed biopolymer systems. In: Dickinson, E. and Van Vliet, T. Food Colloids Biopolymers and Materials. 292.

Pacek AW, Ding P, Norton T. 2003. Effect of Temperature and Hydrodynamic Conditions on Structure and Drop Size in a Phase-Separated Gelatin+Dextran System. In: Dickinson E and Van Vliet, T eds. Food Colloids Biopolymers and Materials. UK: The Royal Society of Chemistry. 309-318.

Ross-Murphey SB. 1992. Structure and rheology of gelatin gels: recent progress. Polymer 33(12):2622-2627.

Ross-Murphey SB. 1991. Incipient behavior of gelatin gels. Rheologica Acta 30:401-411.

Sall B. 2004. FDA Medical Device Regulation. In: Pisano DJ and Mantus D,

editors. FDA Regulatory Affairs A Guide for Prescription Drugs, Medical Devices, and Biologics. New York: CRC Press. 153-193.

Schenz TW and Davis EA. 1999. Thermal Analysis. In: Nielsen SS, editor. Food Analysis. 2nd ed. Gaithersburg, MD: Aspen Publishers, Inc. 587-598.

Steffe JF. 1996. Rheological Methods in Food Process Engineering. 2nd ed. MI: Freeman Press.

Tabata Y, Ikada Y. 1998. Protein release from gelatin matrices. Advanced Drug Delivery Reviews. 31:287-301.

Tolstoguzov VB. Thermodynamic Aspects of Food Protein Functionality. In: Nishinari, K. and Doi, E. editors. Food Hydrocolloids Structures, Properties, and Functions. New York: Plenum Press. 336-337.

Tromp RH, Edelman, MW, van der Linden E. 2004. Gelatine/dextran solutions- a model system for food polymer mixtures. In: Williams, P.A. and Phillips, G.O.Gums and Stabilisers for the Food Industry. 12. Cambridge, UK: The Royal Society of Chemistry. 201-210.

Veis A. 1964. The Macromolecular Chemistry of Gelatin. New York: Academic Press, Inc.

Vivares D, Bonnet F. 2004. Liquid-Liquid Phase Separations in Urate Oxidase/PEG Mixtures: Characterization and Implications for Protein Crystallization. J Phs Chem B 108(20):6498-6507.

Walstra P. 2003. Physical Chemistry of Foods. NY: Marcel Dekker, Inc. 179-186.

Watanabe J, Kiritoshi Y, Nam KW, Ishihara K. 2004. Hydrogels. In: Wnek GE and Bowlin GL editors. Encyclopedia of Biomaterials and Biomedical Engineering. NY: Marcel Dekker, Inc. 790-801.

West JL. 2004. Biofunctional Polymers. In: Encyclopedia of Biomaterials and Biomedical Engineering. Wnek GE and Bowlin GL. NY: Marcel Dekker Inc. 89-95.

Williams PA, Phillips GO. 2000. Introduction to food hydrocolloids. In: Phillips GO, and Williams PA editiors. Handbook of hydrocolloids. Boston: Woodhead Publishing Limited. 1-20.

Wood PD. 1977. Technical and Pharmaceutical uses of Gelatin. In: Ed. A.G. Ward, and A. Courts. The science and technology of gelatin. New York: Academic Press. 414-437.

Zhang Y and Chu CC. 2000. Biodegradable dextran-polyactide hydrogel network and its controlled release of albumin. J of Biomedical Materials Research 54(1):1-11.

Zhong Q, Daubert CR. 2003. Cooling effects on the functionality and microstructure of processed cheese. Ph.D. Dissertation, NC State University.

Table 2.1. Amino acid composition of gelatin and collagen-residues per 1000 residues

Amino acid	Type I collagen (bovine)	Type A gelatin (acid processed pigskin)	Type B gelatin (alkali processed bone)
Alanine	114	112	117
Arginine	51	49	48
Aspargine	16	16	---
Aspartic acid	29	29	46
Glutamine	48	48	---
Glutamic acid	25	25	72
Glycine	332	330	335
Histidien	4	4	4
4-Hydroxyproline	104	91	93
ε-Hydroxylysine	5	6	4
Isoleucine	11	10	11
Leucine	24	24	24
Lysine	28	27	28
Methionine	6	4	4
Phenylalanine	13	14	14
Proline	115	132	124
Serine	35	35	33
Threonine	17	18	18
Tyrosine	4	3	1
Valine	22	26	22

(Redrawn from Ledward 2000)

Table 2.2. Amino acid composition of gelatin

Amino acid	g amino acid/100g protein
Glycine	27.5
Alanine	11.0
Valine	2.6
Leucine	3.3
Isoleucine	1.7
Serine	4.2
Threonine	2.2
Methionine	0.9
Phenylalanine	2.2
Tryosine	0.3
Proline	16.4
Hydroxyproline	14.1
Aspartic acid	6.7
Glutamic acid	11.4
Lysine	4.5
Arginine	8.8
Histidine	0.8

(Redrawn from deMan 1999)

Table 2.3. Uses of gelatin and dextran

Gelatin	Dextran
Ice and sugar crystallization inhibition in frozen cream products	Plasma volume expander
High quality gels with clean melt in mouth texture	Blood flow improvement
Golf ball core material	Thrombosis prophylaxis
Emulsifying agent in cosmetics, pharmaceuticals, water paints, and disinfectants	Organ perfusion and preservation
Sedimentation agent for defibrinated blood	Distending media for hysteroscopy
Cork binding agent	Decrease blood viscosity
Viscosity increaser	Increase blood flow
Water holding capacity utilized in low calorie sweets and spreads	Disappearance of platelet aggregation
Retain juiciness of meat products	Increase in capillary pressure
Beer clarification	Decrease in tissue pressure
Controls crystal growth in electroplating and electrolyte metal refining	Decrease in friction between red cells and capillaries
Bonding agent for photosensitive silver bromide in photography	Anti-sludge effect on red cells
Floculating agent in extraction of uranium ore	Protection against the apparition of intravascular coagulation
Stabilizing agent and emulsifier for emulsions and foams	
Hard and soft capsule shells, tablets, pastilles, protective dressings, gelatin sponge, surgical powder, suppositories, plasma expender	
Sizing agent for paper, paper coating for carbon printing, gelatin composition rollers for printing, gummed paper tape, glues in the coated abrasive industry and carton/box manufacture, book binding	

(Ledward 2000; de Belder 1996; Dellacherie 1996; Ensiminger and others 1994; Wood 1977)

Figure 2.1. Triple helix structure of collagen (Lodish and others 2000)

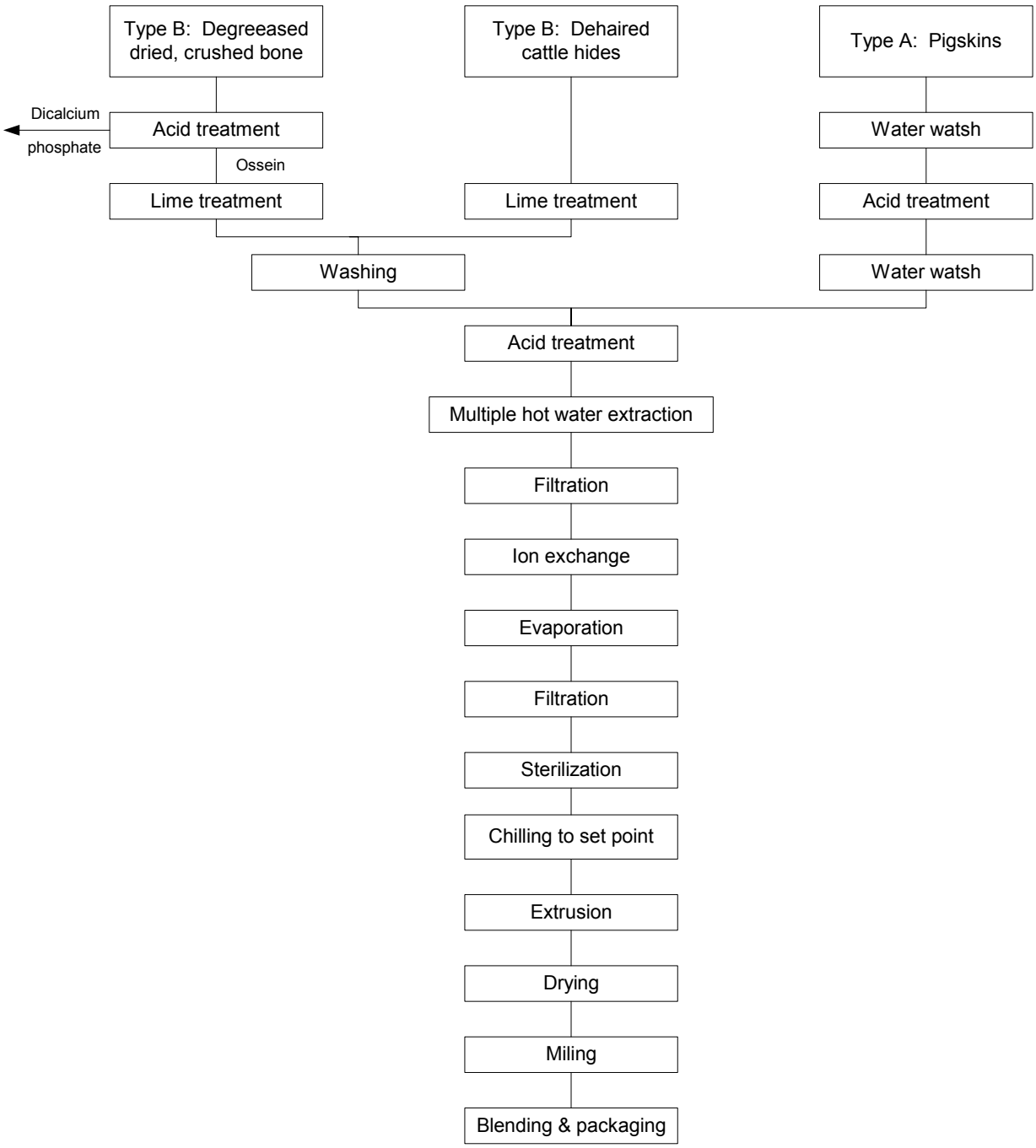


Figure 2.2. Process flow diagram of Type A and B manufacturing (Gelatin Manufacturers Institute of America 1993)

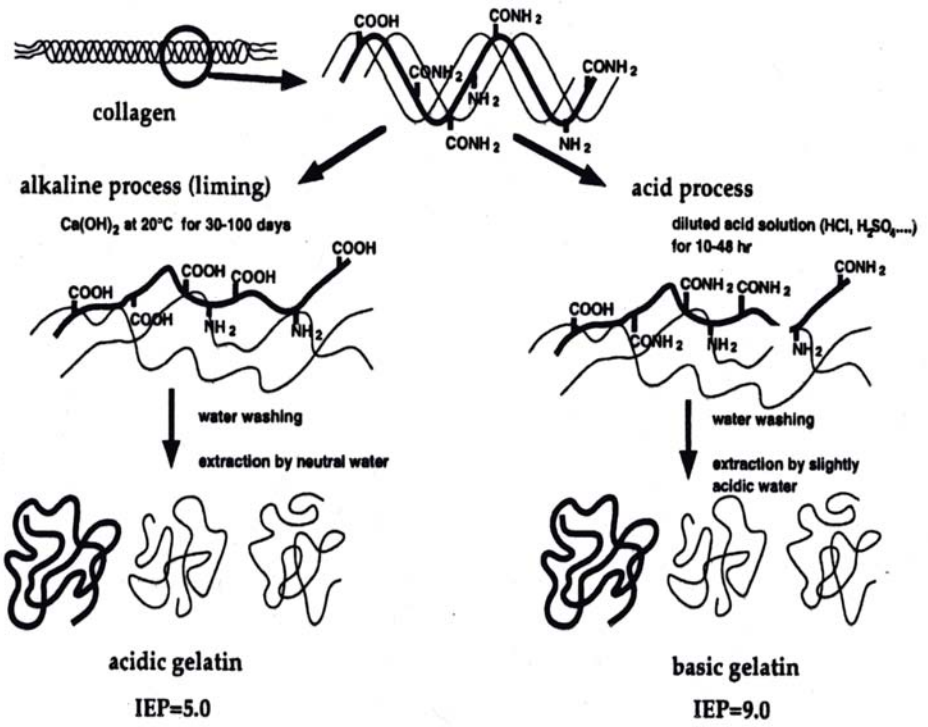


Figure 2.3. pH discrepancies between gelatin produced by the acidic or basic method (Tabata and Ikada 1998)

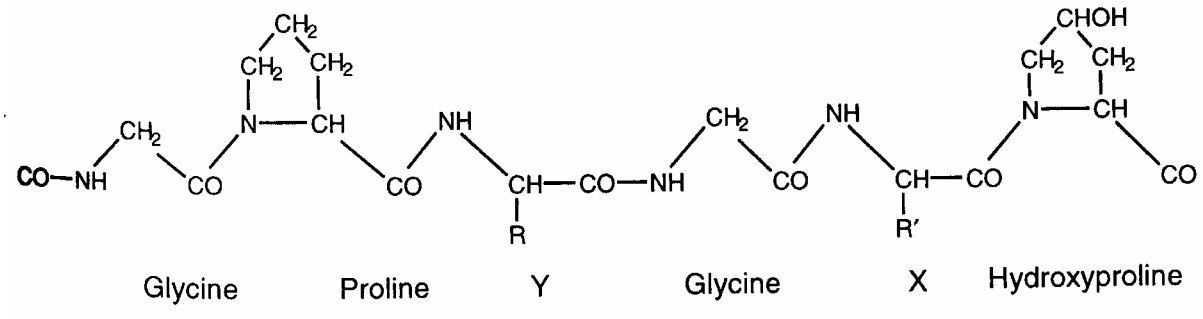


Figure 2.4. Amino acid sequence in gelatin (deMann 1999 and Gross 1961)

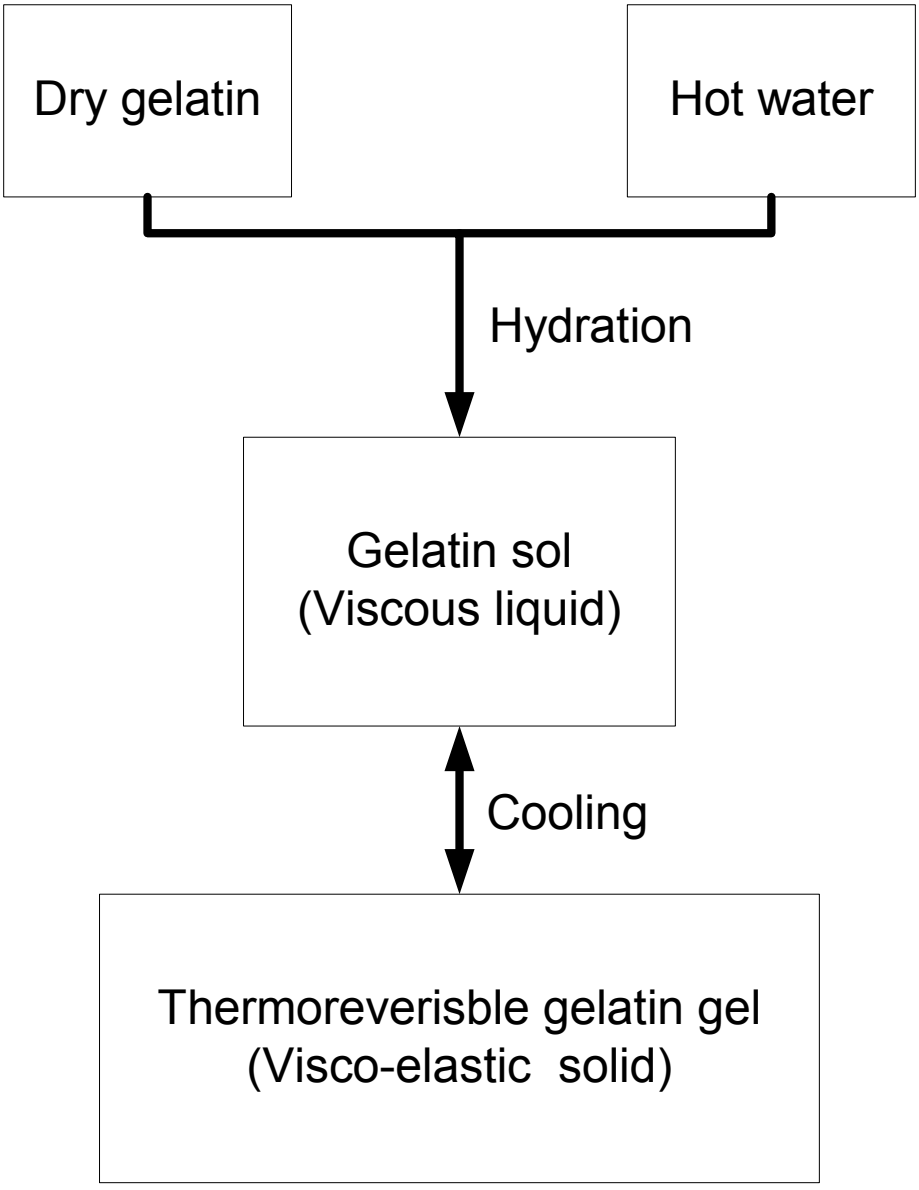


Figure 2.5. Gelation of gelatin in hot water (redrawn from Murano 2003)

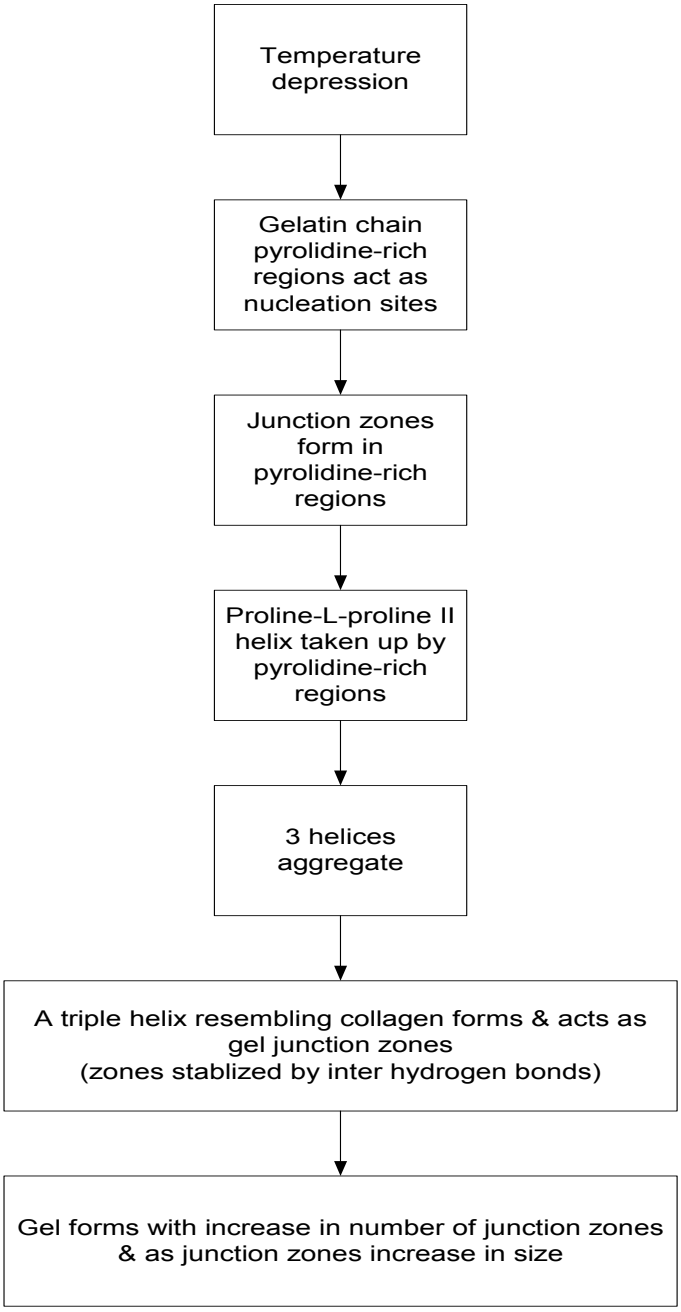


Figure 2.6. Mechanism of gelatin gelling (Ledward 2000)

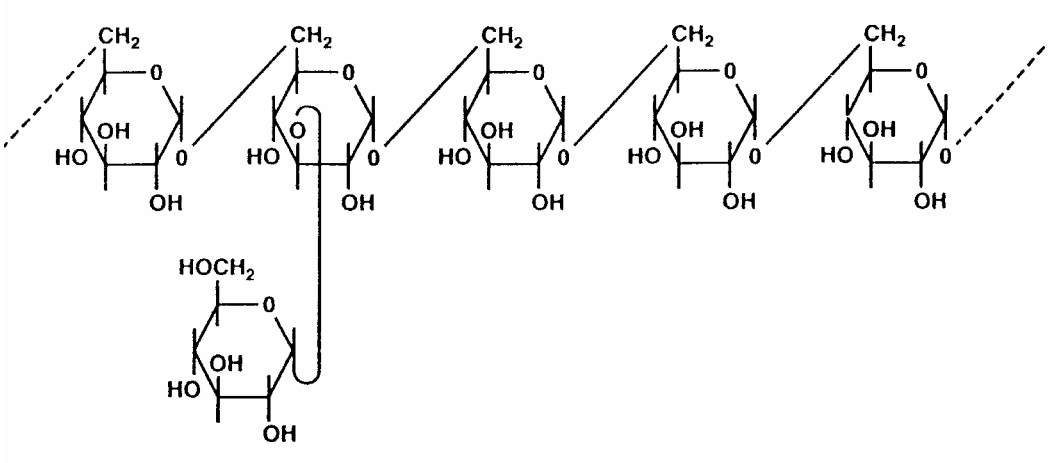
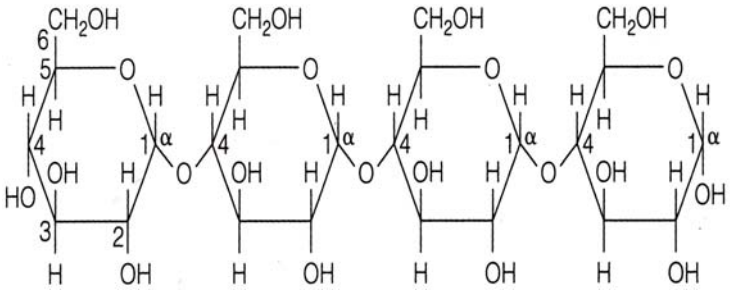


Figure 2.7. Molecular diagram of dextran B512F (de Belder 1996)

Nonreducing end



Reducing end

Figure 2.8. Molecular diagram of amylose (Murano 2003)

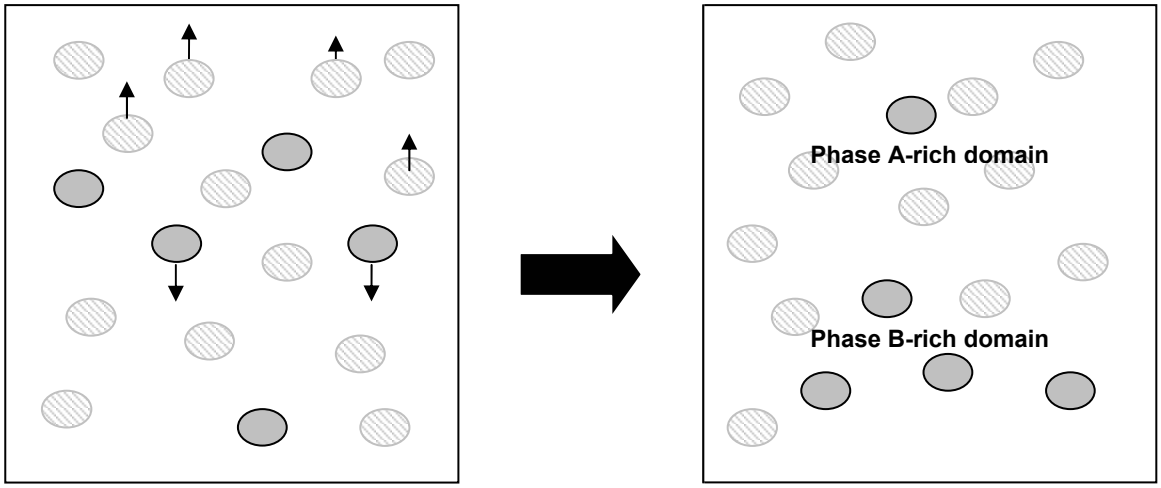
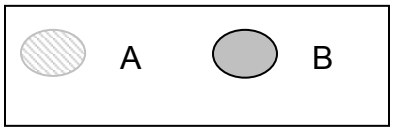


Figure 2.9. Phenomenon of phase separation occurring with the introduction of time and temperature into the system. The A molecules rise to the top of the container and the B molecules fall to the bottom of the container creating phase A-rich and phase B-rich domains



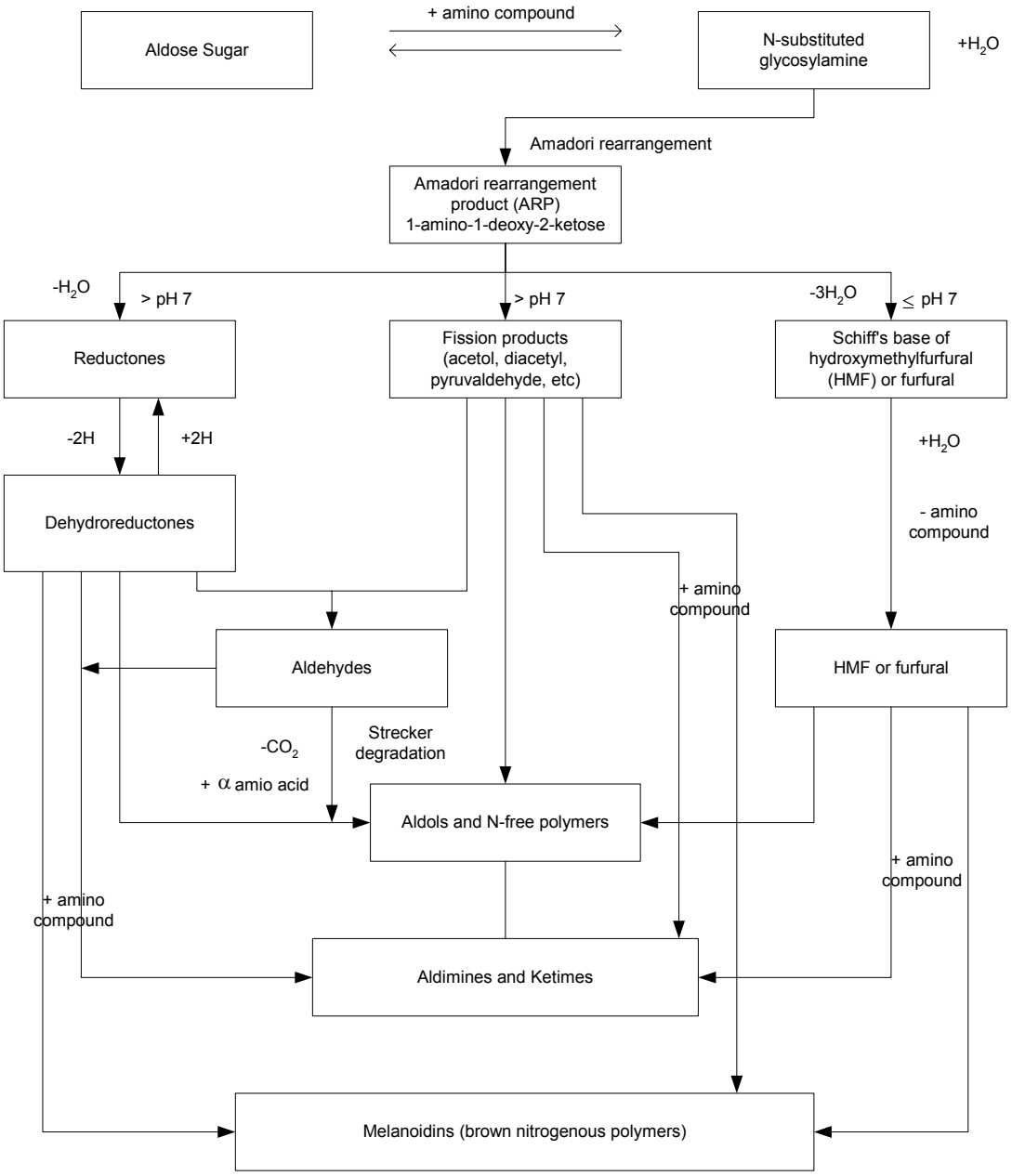


Figure 2.10. Maillard reaction scheme (redrawn from Hodge 1953; Martins and others 2001)

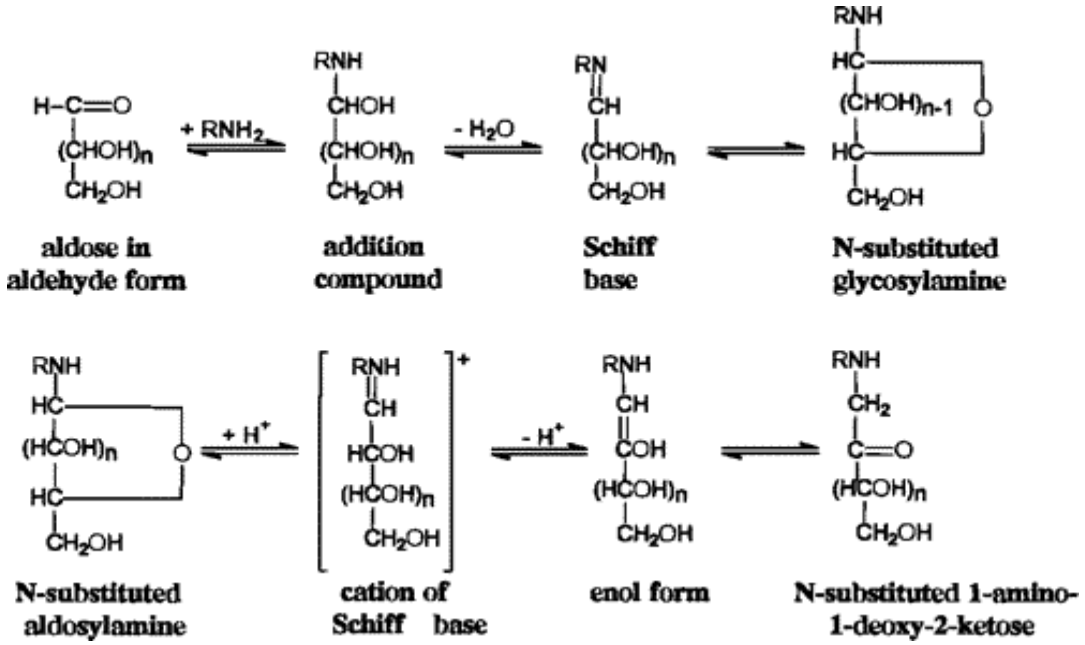


Figure 2.11. Molecular events in the initial stages of the Maillard reaction (Finot 1977; Friedman 1982)

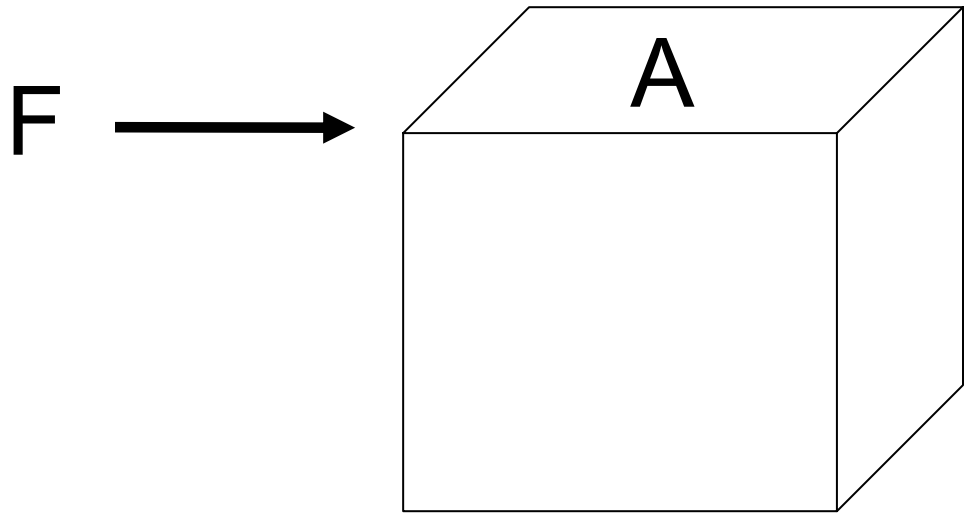


Figure 2.12. Diagram of shear stress on a cube

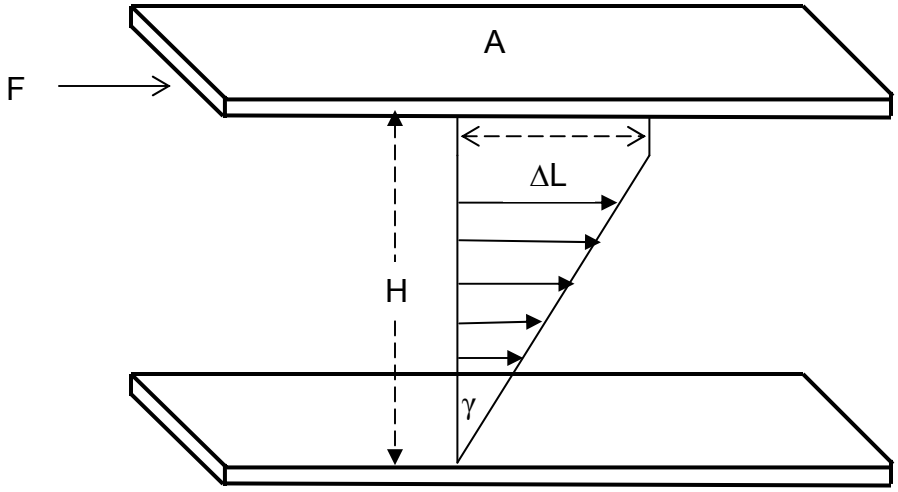


Figure 2.13. Shear flow between parallel plates

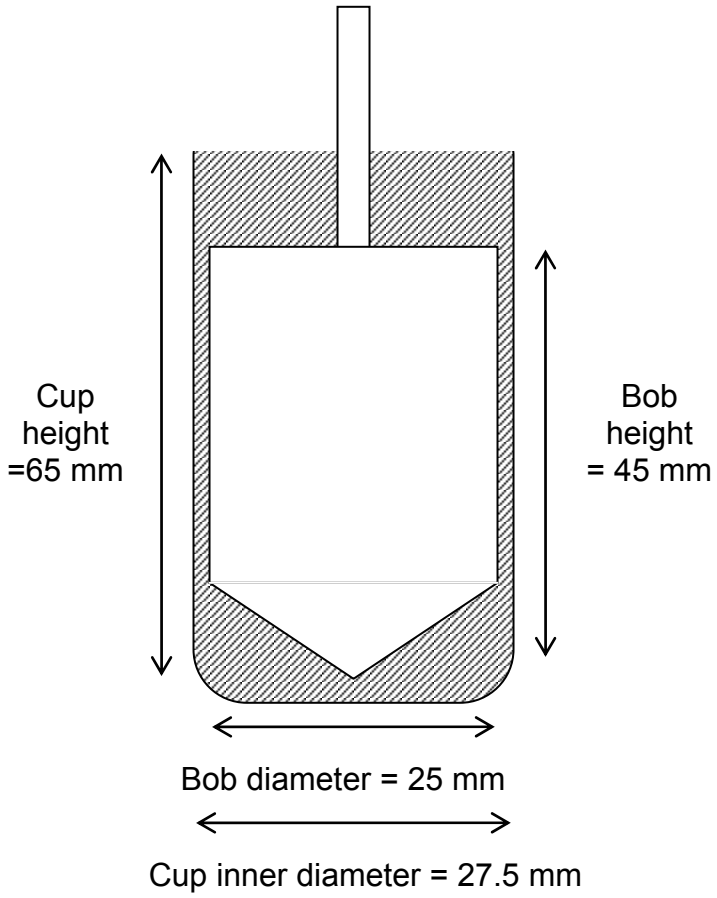


Figure 2.14. Schematic of concentric cylinder

CHAPTER 3.

RHEOLOGICAL CHARACTERIZATION OF E-MATRIX™

3.1 INTRODUCTION

Rheological characterization applies mathematical models to develop physical properties and to describe the flow behavior of a material. The specific material examined for this project was E-Matrix™, a thermo-reversible medical device. E-Matrix™ is a hydrogel that purportedly functions as a wound healing accelerant when injected under human skin around a target area to repair or regenerate diseased or damaged tissue. The medical device is principally composed of gelatin (12% w/w), dextran (5% w/w), and a formulation of amino acids with an ionic strength of 0.1279 M. Thus its rheological behavior depends on the properties of the stated protein, carbohydrate, and any interaction between the components.

To investigate the properties of the hydrogel, shear rate ramps at body (37°C) and manufacturing (50°C) temperatures were conducted to determine appropriate rheological models for describing the product. In addition, mechanical spectra were performed at 37°C and 50°C to determine the shear storage (G') and loss moduli (G''). To better understand the physical properties of E-Matrix™, thermal transition points were also identified both rheologically and calorimetrically, using differential scanning calorimetry for the latter. The following chapter describes the rheological and thermal characterization of E-Matrix™.

3.2 MATERIALS AND METHODS

The material examined in this project, E-Matrix™, was manufactured and supplied by Encelle, Inc. (Greenville, NC). The proximate composition of E-Matrix™ was determined by Microbac Laboratories, Inc. Southern Testing and Research Division (Wilson, NC), compiled in Table 3.1. The protein content was determined by the Kjeldahl method (Bradstreet 1965) to compute the nitrogen content, then multiplying this value by the nitrogen to protein conversion factor (5.55) for gelatin (Jones 1941). The 5.5 factor was used since E-Matrix™ is principally composed of gelatin. Moisture was determined by using a forced draft oven drying method (Bradley 1998); lipid content was determined by hydrolytic extraction gas chromatography (AOAC 996.06 GC-FID) for total fat; ash was determined by a muffle furnace technique (AOAC 923) (Harbers 1998), and carbohydrate content was calculated as the remaining difference.

E-Matrix™ was delivered in 15 ml sealed glass vials in a cooler, and upon receipt the samples were transferred to a 7°C refrigerator (Holiday NC156P, Norge, Indianapolis, IN). Prior to analysis, the sample vials were removed from refrigeration and placed in a pre-heated (39°C) heating block (Multi-Block® Heater, LAB-LINE) for 30-90 minutes to prepare the material for injection with a syringe or to perform subsequent analysis. A minimum of 30 minutes was required to melt the sample, and beyond 90 minutes, network structure changes occurred. Before injection or analysis, the vial was removed from the block and inverted 180 degrees ten times

for appropriate mixing, as per manufacturing instructions. All sample preparation methods mimicked manufacturer's instructions for clinical use of the product.

3.2.1 SHEAR VISCOMETRY

E-Matrix™ was tested using steady and oscillatory shear rheological tests. Shear rate ramps, temperature ramps, and frequency sweeps were conducted using a StressTech Controlled Stress Rheometer (ReoLogica Instruments AB, Lund, Sweden) with a serrated concentric cylinder attachment (CCE) (Figure 2.14). A serrated or roughened surface may effectively prevent wall slip between the test material and the cup and bob walls (Steffe 1996). The cylindrical bob has an outer diameter of 25 mm, height of 45 mm, and the cup has an inner diameter of 27.5 mm, and a height of 65 mm. All rotational viscometry tests were performed in triplicate.

3.2.1.1 STRESS SWEEPS

Prior to performing all oscillatory, small strain tests, stress sweeps were conducted to determine the linear viscoelastic region (LVR) at the frequencies examined throughout the analysis: 0.01, 0.1, and 10 Hz, while measuring the complex shear modulus (G*) at 8°C, 37°C, and 50°C. The stress was increased from 0.001 Pa to 1000 Pa (Figures 3.1a-c). Performing rheological tests at frequencies below 0.01 Hz is time consuming. The linear viscoelastic region is defined as the range of stresses where the complex shear modulus is independent of stress magnitude.

3.2.1.2 SHEAR RATE RAMPS

Shear rate ramps (5-150 s^{-1}) were conducted to determine the stress and shear viscosity profiles. Appropriate pre-shearing was performed at each temperature prior to initiating shear rate ramp analysis. Fresh samples were tested at 37°C and 50°C in triplicate.

3.2.1.3 FREQUENCY SWEEPS

A small amplitude oscillatory shear (SAOS) technique was used to determine network characteristics of E-Matrix™. This type of testing procedure subjects the material to harmonically varying stresses or strains and is the most common dynamic method for studying viscoelastic behavior (Steffe 1996). In addition, oscillatory testing is very sensitive to the chemical composition and physical structure of the materials being examined (Steffe 1996). For the SAOS tests, the samples were subjected to a frequency ranging from 0.01 to 10 Hz and the storage and loss moduli (G′ and G″) were recorded (Figure 3.3). All samples were evaluated within the LVR at a stress of 0.05 Pa, and analysis was conducted in triplicate.

3.2.1.4 TEMPERATURE RAMPS

Oscillatory methods were also used to rheologically determine two, thermal transition characteristics of the samples: gel point and melt point. The tangent of the

phase angle (δ) is greater than 1 for a viscous fluid and less than 1 for an elastic solid (Tung and Dynes 1982). Furthermore, a material possessing a 90° phase angle is considered to have ideal liquid (Newtonian fluid) behavior and a 0° phase angle is considered to have ideal solid (Hookean solid) behavior (Bryant and McClements 2000; Daubert and Foegeding 1998). This relationship can be expressed using Equation 1.

$$\tan\delta = \frac{G''}{G'} \quad \textbf{(1)}$$

Using the above equation, the point at which tan δ equals 1 occurs when $G' = G''$, and the phase angle is 45°. The point at which $G' = G''$ is commonly called the crossover point for viscoelastic fluids, because at frequencies below the crossover point frequency, the material is viscous-like and above the crossover point frequency, the material is elastic-like (Daubert and Foegeding 1998). Furthermore, Michon and others (1993) state the relationship expressed in Equation1 is a critical point which may be precisely located when performing heating or cooling ramps. Hence, the point where $G' = G''$ and the phase angle is 45° represents a hypothetical transition point between a viscous fluid and an elastic solid. It is the midpoint between an ideal solid (0°) and an ideal liquid (90°) and can be deemed the gel point or melt point when examining data of phase angle versus temperature or time. Therefore for practical reasons of this research, gel point was defined as the temperature at which a 45° phase angle was achieved while cooling at -1°C/min from 50°C to 25°C. Likewise, the melt point was defined as the temperature at which a 45° phase angle was achieved upon heating at 1°C/min from 8°C to 33°C.

Temperature ramps were performed in triplicate by placing samples under a constant stress of 0.05 Pa and a frequency of 0.10 Hz when cooled from 50°C to 10°C and constant stress of 200 Pa and a frequency of 0.10 Hz when heated from 10°C to 39°C. The material displayed varying properties across the temperature range, complicating stress selection for oscillatory analysis. Therefore, the selected frequency-stress combinations were optimal and within the LVR for the initial temperature of the temperature ramp (50°C for cooling and 8°C for heating).

3.2.2 CALORIMETRY

Differential Scanning Calorimetry (DSC) was performed using a PerkinElmer DSC7 with Pyris software equipped with an Intracooler II refrigeration unit and dry box (PerkinElmer Corp, Norwalk, CT) to determine the melt point of E-Matrix™ when heating. The DSC was calibrated using indium (onset = 156.6°C; enthalpy change = 28.45 J/g) and mercury (onset = −38.83°C) as standards. Aluminum DSC pans were filled with an accurately weighed sample of E-Matrix™ or 12% gelatin solution at 39°C. The hermetically sealed material was subsequently placed in the DSC chamber and held at 39°C for a 5 minute equilibrium period. The pans were then cooled in the DSC chamber from 39°C to 4°C at a rate of 5°C/min and subsequently held at 4°C for 2 minutes. This step was performed to create a consistent and known thermal history for each sample because polymer thermal history influences melting characteristics (Hatakeyama and Quinn 1997). Next, each pan was individually placed in an ice filled cooler and transported to a 4°C incubator (Percival

Scientific Model I-36NL Perry, IA) for approximately 48 hours. Pans were next removed from the incubator, transported to the DSC chamber via the ice filled cooler, and heated to 39°C at a rate of 5°C/min. An empty aluminum pan was used as the reference and nitrogen (flow rate = 24 cc/min) was used as the purge gas.

An average value of the triplicate samples was reported for the onset temperature, peak temperature, and enthalpy change. Onset temperature is the temperature at the intersection between the tangent to the maximum rising slope of a DSC peak and the extrapolated sample baseline (Hatakeyama and Quinn 1994). Onset temperature is the first observed deviation from the linearity of the sample baseline (Hatakeyama and Quinn 1994). The midpoint peak temperature is the maximum temperature difference between the measured curve and the baseline (Hohne and others 2003). For the current project, this temperature was considered the melt point and compared to the rheologically determined melt point. Melt point comparison was made using the peak temperature because this corresponds to the temperature at which half the sample has melted. The change in enthalpy was also reported.

A scan rate of 5°C/min was used with the DSC instead of 1°C/min as in the rheometer because using a larger scan rate with the DSC generates a more prominent and detectable peak for analysis (Schenz and Davis 1998). A detectable peak at the slower scan rate (1°C/min) was unable to be produced under the previously described conditions due to lack of DSC sensitivity. Furthermore, a larger

peak was generated at the faster scan rate because the melting peak area is directly proportional to the heating rate (Hatakeyama and Quinn 1997). Therefore at a scan rate of 5°C/min, the peak was five times larger than at 1°C/min.

3.3 RESULTS AND DISCUSSION

Shear rate ramps, mechanical spectra, and thermal transition analyses were performed on E-Matrix™ to rheologically characterize and determine physical properties of the material.

3.3.1 STRESS SWEEPS

Stress sweeps identified the linear viscoelastic region to insure data was appropriately analyzed. Figures 3.1a-c display the complex shear modulus (G*) for E-Matrix™ samples during stress sweeps at 8°C, 37°C, and 50°C. The linear viscoelastic region at 8°C (Figure 3.1a) was between 1-1000 Pa. At 37°C (Figure 3.1b) the linear viscoelastic region was between 0.01-1.0 Pa, with the lower frequencies (0.01 and 0.10 Hz) having an extended LVR between 0.001-1.0 Pa. Finally, at 50°C the LVR is between 0.001-5.0 Pa for all three frequencies, but extending between 0.001-10.0 Hz for the lower frequencies of 0.01 and 0.10 Hz (Figure 3.1c). At the lower temperatures, 8°C and 37°C , a linear viscoelastic region could clearly be established at all three frequencies; 0.01, 0.1, and 10.0 Hz. However, at the higher temperature, 50°C, it was more difficult to establish a clear,

linear viscoelastic region at the higher frequency of 10.0 Hz as illustrated in Figure 3.1c.

3.3.2 SHEAR RATE RAMPS

Shear rate ramps examined the effects of temperature and shear on the stress and viscosity of E-Matrix^{TM}. Figure 3.2a compares the flow behavior of E-Matrix^{TM} at 50°C and 37°C. E-Matrix^{TM} exhibited Newtonian flow behavior at 50°C, but pseudoplastic (shear thinning) flow behavior at 37°C. Hence at the higher temperature, viscosity is independent of shear rate, however at the lower temperature, viscosity is a function of the shear rate.

A general relationship to describe the behavior of a fluid is the power law equation. Logarithmic plots of shear stress versus shear rate and the incorporation of the power law equation (Equation 2) were used to represent a general relationship describing the E-Matrix^{TM} flow behavior, where σ is shear stress or force per area (Pa), K is a material constant called the consistency coefficient (Pa s^n), $\dot{\gamma}$ is shear rate or the change in shear strain with respect to time (s^{-1}), and n is another unitless material constant called the flow behavior index.

$$\sigma = \kappa(\dot{\gamma})^n \qquad \textbf{(2)}$$

The power-law model can be used to fit experimental results of shear stress versus shear rate, when n = 1 the material is deemed Newtonian; when 0 < n < 1 the material is shear-thinning (pseudoplastic); and when 1 < n < ∞ the material is shear-

thickening (dilatent). At 37°C, E-Matrix™ has a flow behavior index (n) less than 1 (n = 0.83). Therefore, E-Matrix™ is a shear thinning material at 37°C, as the viscosity diminishes when an increased shear rate is applied to the material. Furthermore, at 50°C, the flow behavior index is 1.0, so the material is considered Newtonian and, thus the flow behavior is independent of shear rate. This phenomenon may be due to the fact E-Matrix™ contains a protein which gels when cooled. Therefore at a higher temperature (50°C) the material is a liquid with little structure. However, at lower temperatures (37°C) aggregation among gelatin helices may be occurring and a weak gel is likely forming. This weak gel is however broken with an increase in shear rate causing the material to thin at increased rates. Pseudoplasticity is recognized to represent an irreversible structural breakdown, and the decrease in viscosity occurs as a result of molecular alignment that takes place within the material (Glicksman 1969). This behavior is common to food hydrocolloid solutions, for example 1% carrageenan exhibits more Newtonian-like behavior at higher temperatures ($n = 0.78 \pm 0.003$) and non-Newtonian behavior at lower temperatures ($n = 0.54 \pm 0.01$) (Marcotte and others 2001), similar to E-Matrix™.

Comparing viscosities at 37°C and 50°C in Figure 3.2b shows E-Matrix™ is more viscous at low temperatures as was anticipated. A decrease in viscosity with a temperature increase was expected because many materials predominantly composed of physical bonds exhibit this change. Hydrogen bonds are thermo-reversible bonds that are broken when heated (Abeles and others 1992). Additionally, hydrogen bonds are relatively stronger at low temperatures (Jeffrey

1997). E-Matrix™ is principally composed of gelatin, and hydrogen bonds are present within gelatin (Ledward 2000). Consequently hydrogen bonds are likely responsible for the viscosity dependence on temperature since association of helices in the junction zones within gelatin are stabilized by temperature sensitive hydrogen bonds which break at 35-40°C resulting in melting (Bigi and others 2004; Ledward 2000). As temperature is increased, the hydrogen bonds break, causing disruption of junction zones critical for the formation of elastic-like properties in gelatin, leading to a decrease in viscosity at higher temperatures as illustrated in Figure 3.2b. Examining the rheological properties of another material bound with hydrogen bonds, water, the following comparisons were made. Water is a Newtonian fluid and the viscosity of water also decreases with temperature like E-Matrix™. At 10°C water has a viscosity of 1.31×10^{-3} Pa s, at 50°C 5.47×10^{-4} Pa s, and at 90°C 3.15×10^{-4} Pa s (Weast and others 1984). In comparison, the viscosity of E-Matrix™ at 50°C, 62.0 ± 0.7 mPa s, is more than 100 times as viscous as water. Hence water and E-Matrix™ are both Newtonian at 50°C, are both composed of hydrogen bonds, and both decrease in viscosity with a temperature increase.

3.3.3 FREQUENCY SWEEPS

Information about the viscoelastic nature of the material was investigated by evaluating the mechanical spectra at 37°C and 50°C (Figure 3.3). The loss modulus (G'') was higher than the storage modulus (G') throughout the frequency range deployed, confirming the viscous nature of the material. Hence, there is a greater

viscous contribution and a smaller elastic contribution present in the product. However as the frequency increased to approximately 2.0 Hz at 50°C, G′ crossed over G″ and G′ began to dominate the spectra. A similar trend occurred at 37°C, where G″ dominated at lower frequencies and a crossover occurred at approximately 7.0 Hz. The crossover is the point at which G″ = G′ and is the point at which the phase angle (δ) equals 45° (Steffe 1996). Steffe (1996) reports materials usually exhibit stronger elastic characteristics at higher frequencies, so the results shown are common for viscoelastic fluids. Additionally, materials adjust to the deformation and rheological measurements are taken over time. If a material is given a longer period of time to adjust to a deformation such is the case at small frequencies, the material will have more fluid-like behavior versus if the material is only given a short amount of time to adjust to the deformation as is the case at high frequencies where solid-like behavior will occur. This relationship can be compared to moving a hand through water quickly, where the water presents a more forceful resistance- solid-like; as opposed to moving a hand through water slowly, where the water presents little resistance-fluid-like (Daubert and Foegeding 1998). Consequently the pronounced dependence on frequency coupled with the observed crossover is indicative of weak gel structures or networks (Ahmend and others 2005). For strong gels, G′ is consistently larger than G″ over a range of frequencies (Steffe 1996). Since the G′- G″ crossover is indicative of gel formation, a crossover only at a higher frequency indicates a weak gel because if the gel was strong G′ would have been larger than G″ over the entire range of frequencies, not just at the higher frequencies.

A caramel study revealed similar viscoelastic property results to those of E-Matrix™. Ahmed and others (2005) observed viscous behavior at low frequencies in caramel as a result of the molecules having more time to rearrange themselves, thus becoming more viscous. However, at the higher frequencies the molecules have less time to adapt to the imposed field or entangle/rearrange, resulting in a more solid-like property (Ahmed and others 2005). This principle may be applied to the flow behavior of E-Matrix™, as the material follows a similar trend of fluid-like behavior at low frequencies and subsequently more solid-like behavior at higher frequencies. Furthermore, E-Matrix™ is composed of two large molecular weight molecules; gelatin (1100 kDa) and dextran (668 kDa). Depending on molecular orientation and shape, large molecular weight molecules must untangle or rearrange themselves in order to flow and have viscous-like behavior. In conclusion, without rearrangement the molecules will not flow freely and will possess more solid-like behavior.

3.3.4 TEMPERATURE RAMPS

Rheological stability of E-Matrix™ between 50°C and 25°C was examined while cooling at a constant stress of 0.05 Pa and a constant frequency of 0.10 Hz (Figure 3.4). The gel point of the material appeared to be approximately 27.9°C when cooled at a constant rate of -1°C/min. In addition, E-Matrix™ was heated from 8°C to 33°C at a constant stress of 200 Pa and a constant frequency of 0.10 Hz (Figure

3.5). Investigating E-Matrix™ between 8°C and 33°C, while heating at a constant rate of 1°C/min, showed the melt point appeared at approximately 33.7°C. Hence the gel point and the melt point occur at different temperatures, and the thermal history (if the material was a gel or liquid at the initiation of the examination) of the material influences the behavior of the material. For instance, at a given temperature within the window of melt and gel points (27.9°C - 33.7°C) the thermal history determines if the material will act more viscous or elastic-like.

Knowing E-Matrix™ properties at body temperature is of concern, because these are the conditions at which the product must function as a wound healing accelerant. At body temperature (37°C), E-Matrix™ is considered a viscoelastic fluid because 37°C is above the gel and melt points.

Molecular weight, concentration, and thermal history influence the melt and gel points of gelatin. The lower the molecular weight, the greater number of network interactions are required per volume to form a gel, so low molecular weight gels have lower melting points (Gilsenan and Ross-Murphy 2000). Thus the size of the raw ingredients, specifically gelatin and dextran molecules, used to manufacture E-Matrix™ influences the gel and melt points of the end product. Solids concentration of a material also affects the gel and melt points of a material. A higher solids concentration means there are more particles available to form networks or junctions. The more abundant the molecules, the more readily networks form.

Finally, thermal history affects gel and melt points, specifically the organization and structure of junction zones, that form during gel formation. With time, junction zones become larger and stronger (Ledward 2000). Therefore, a gel with a thermal history that either cooled the material at a slower rate or allowed the material time to mature (held at a constant low temperature), will be a stronger gel as maturity is frequently associated with increased gel strength. A stronger gel requires more energy to melt and will display a higher melt point.

3.3.5 CALORIMETRY

A melt point of 23.9°C for E-Matrix™ (Table 3.2) was determined from the midpoint of the endothermic peak on the DSC curve (Figure 3.6). This temperature differed from the rheologically determined thermal transition point of 33.7°C as a result of several factors. First the DSC and rheometer are different instruments which measure different properties. The DSC measures heat flow (Schenz and Davis 1998) to determine thermal transition points, whereas the rheometer measures physical property changes to detect thermal transition points. Therefore the DSC may be considered a more sensitive instrument for detecting thermal transitions. The DSC melt point temperature was lower for E-Matrix™, (23.9°C) than the melt point determined rheologically (33.7°C). Contrarily, rheological melt point detection is delayed when heating rate is increased (Resch 2004). In addition, the test variables; heating rate, stress, and oscillatory frequency, with the latter two

implemented only during the rheological determination, influence the thermal transition points (Hamann and others 1990). Therefore, using a different stress and/or frequency may have produced a rheological melt point value more similar to those produced calorimetrically. Additionally, different scan rates were also used; 5°C/min in the DSC and 1°C/min in the rheometer. The DSC measures the heat flow, so faster heating rates produce larger, more detectable signals (Schenz and Davis 1998). The different rates were selected in an effort to use the best scan rate for the instrument used. Hence, different frequency, stress, and scan rates may have influenced the discrepancies between the rheological and calorimetric melt points along with the discrepancies in instrument and thermal history between the two techniques.

Furthermore, Kasapis and others (2003) claim the glass transition point determined rheologically and calorimetrically cannot easily be compared as a clear reference point for comparison is lacking. Depending on the polymer examined, the rheological and calorimetric glass transition points may differ up to 30° (Deszczynski and others 2002). Thus, it is reasonable that differences may also occur between the thermal transition point of melt point determined rheologically and calorimetrically as found in the current project. Besides it is also unclear as to which calorimetric temperature should be compared to the rheological melt point. In this project the calorimetrically determined peak temperature was chosen to be compared to the rheologically determined melt point. However, the DSC onset temperature is also an inclination of melt point because it is the temperature at which the initial signs of

melting begin to occur. Therefore, it may be argued that either the onset or peak temperature is a more appropriate point to compare to the rheologically determined melt point.

Table 3.2 lists the average enthalpy change, onset, and peak temperatures for triplicate runs of E-Matrix™ and a 12% gelatin solution. Figure 3.6 shows the averages of triplicate heating curves for E-Matrix™ and a 12% gelatin solution. The 12% gelatin solution was presented with the E-Matrix™ results because 12% is the approximate composition of gelatin in E-Matrix™. Thus a comparison between the principle component and E-Matrix™ shows E-Matrix™ required slightly more energy (7.26 J/g) than gelatin (5.27 J/g) to produce a signal and melted at a lower temperature (23.9°C) than gelatin, (25.6°C). Graphically, E-Matrix™ may have had a smaller DSC signal than the gelatin solution, because the additional components of E-Matrix™ may have attributed to additional interactions. These interactions may have involved both endothermic and exothermic interactions. The upward DSC curve presented represents the endothermic interaction. However an exothermic interaction would have caused a downward curve, so the combination of exothermic and endothermic curves would lead to a less defined curve as found with E-Matrix™.

3.4 CONCLUSIONS

The rheological characterization of E-Matrix™ determined the flow behavior of the material as pseudoplastic at 37°C and Newtonian at 50°C. As anticipated the

viscosity of the material was higher at lower temperatures, due to the breaking of hydrogen bonds with an increase in temperature. Frequency sweeps indicated the material was more viscous as opposed to elastic at 37°C and 50°C. Temperature ramps rheologically determined the melt point; 33.7°C. Finally, calorimetry and rheology were used to produce thermal transition points. Calorimetry produced a melt point (23.9°C) differing from the thermal transition point produced rheologically (33.7°C). The rheological characterization of E-Matrix™ allows the producers of this product to more clearly understand the behavior of the product, advantageous to understanding the mechanisms occurring within the materials throughout processing.

3.5 REFERENCES

Abeles RH, Frey PA, and Jencks WP. 1992. Biochemistry. London: Jones and Bartlett Publishers, Inc. 217.

Ahmed J, Ramaswamy HS, Pandey PK. 2005 in press. Dynamic rheological and thermal characteristics of caramels. LWT - Food Science and Technology.

Bigi A, Panzavolta S, Rubini K. 2004. Relationship between triple-helix content and mechanical properties of gelatin films. Biomaterials 25(25): 5675-5680.

Bradley RL. 1998. Moisture and Total Solids Analysis. In: Nielsen SS. Food Analysis 2nd ed. Gaithersburg, MA: Aspen Publishers, Inc.

Bradstreet RB. 1965. The Kjeldahl Method for Organic Nitrogen. NY: Academic Press.

Bryant CM, McClements DJ. 2000. Influence of xanthan gum on physical characteristics of heat-denatured whey protein solutions and gels. Food Hydrocolloids 14(4): 383-390.

Daubert CR, Foegeding EA. 1998. Rheological Principles for Food Analysis. In: Nielsen SS, editor. Food Analysis. 2nd ed. Gaithersburg, MD: Aspen Publishers, Inc. 551-569.

Deszczynski M, Kasapis S, MacNaughton W, Mitchell JR. 2002. High sugar/polysaccharide glasses: resolving the role of water molecules in structure formation. Int J Biol Marcromol 30(1): 279-282.

Gilsenan PM, Ross-Murphey SB. 2000. Viscoelasticity of thermoreversible gelatin gels from mammalian and piscine collagens. J Rheol 44(4): 871-883.

Glicksman M. 1969. Rheology, Texture, and Gums. In: Gum technology in the food industry. Chapter 3. Academic Press; NY. 56-93.

Hamann DD, Purkayastha S, Lanier TC. 1990. Thermal Scanning Rheology of Food Gels. In: Harwalkar VR, MA C-Y, editors. Thermal Analysis of Foods. NY: Elsevier Science Publishers LTD. 312.

Harbers LH. 1998. Ash Analysis. In: Nielsen SS. Food Analysis 2nd ed. Gaithersburg, MA: Aspen Publishers, Inc. 141-165.

Hatakeyama T, Quinn FX. 1994. Thermal Analysis Fundamental and

Applications to Polymer Science. New York: John Wiley and Sons. 34-35; 65-68.

Hohne GWH, Hemminger WF, Flammersheim HJ. 2003. Differential Scanning Calorimetry. 2nd ed. Germany: Springer. 116-117.

Jeffrey GA. 1997. An Introduction to Hydrogen Bonding. NY: Oxford University Press Inc. 213.

Jones, DB. 1941. Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Protein. United States Department of Agriculture, Circular No. 183.

Kasapis S, Al-Marhoobi IM, Mitchell JR. 2003. Testing the validity of comparisons between the rheological and the calorimetric glass transition temperatures. Carbohydrate Research 338(8): 787-794.

Ledward, DA. 2000. Gelatin. In: Phillips, G.O. Williams, P.A. editors. Handbook of hydrocolloids. Boston: Woodhead Publishing Limited. p 67-86.

Marcotte M, Taherian Hoshahili AR, Ramaswamy HS. 2001. Rheological properties of selected hydrocolloids as a function of concentration and temperature. Food Research International 34(8):695-703.

Michon C, Cuvelier G, Launay B. 1993. Concentration dependence of the critical viscoelastic properties of gelatin at the gel point. Rheologica Acta 32:94-103.

Resch JJ. 2004. Processing parameter effects on the functional properties of derivatized whey protein ingredients. Ph.D. Dissertation, NC State University.

Schenz TW, Davis EA. 1998. Thermal Analysis. In: Nielsen SS, editor. Food Analysis. 2nd ed. Gaithersburg, MD: Aspen Publishers, Inc. 587-598.

Steffe, JF. 1996. Rheological Methods in Food Process Engineering. 2nd ed. MI: Freeman Pres.

Tung CYM, Dynes PJ. 1982. Relationship between Viscoelastic Properties and Gelation in Thermosetting Systems. J of Applied Polymer Sci 27(2): 569-574.

Weast RC, Astle MJ, Beyer WH. 1985. CRC Handbook of Chemistry and Physics. CRC Press, Inc. Boca Raton, FL.

Table 3.1. Composition of E-Matrix™

Analysis	(%)	Method
Protein	11.40	Kjeldahl
Carbohydrates	5.18	Calculation
Moisture	82.5	Forced Draft Oven
Ash	0.77	AOAC 923
Total Fat	0.15	AOAC 996.06 GC-FID

*Determined by Microbac Laboratories, Inc. Southern Testing and Research Division, (Wilson, NC)

Table 3.2. Differential scanning calorimetry values for E-Matrix™ and a 12% gelatin solution

Sample	Onset Temperature	Peak Temperature	Enthalpy Change
E-Matrix™	12.4°C (0.5)	23.9°C (0.0)	7.26 J/g (1.12)
12% Gelatin solution	13.1°C (1.5)	25.6°C (0.1)	5.27 J/g (0.81)

*Mean ± (standard error)

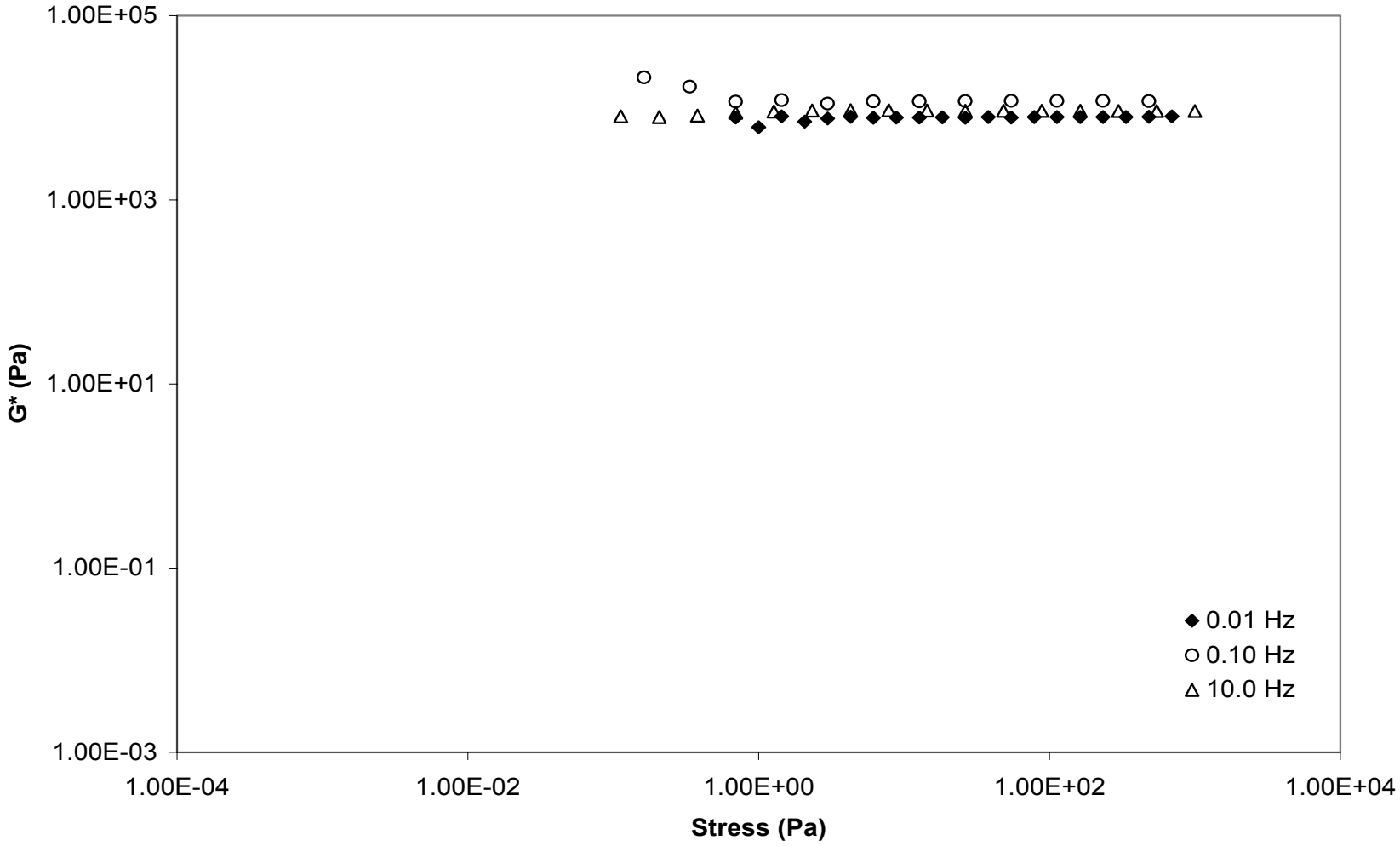


Figure 3.1a. Complex shear modulus (G*) for E-Matrix^{TM} samples during stress sweeps at 8°C

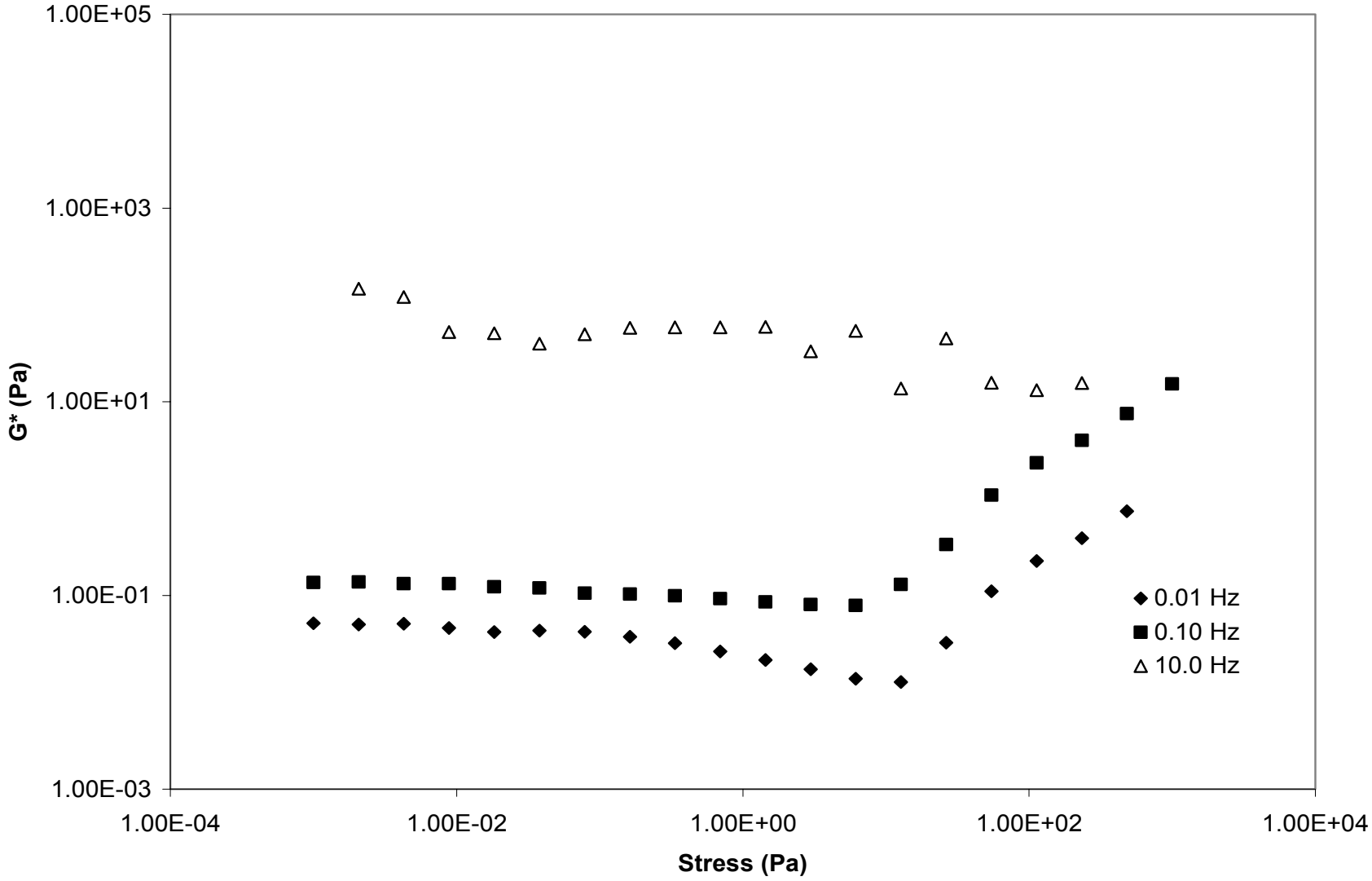


Figure 3.1b. Complex shear modulus (G*) for E-Matrix™ samples during stress sweeps at 37°C

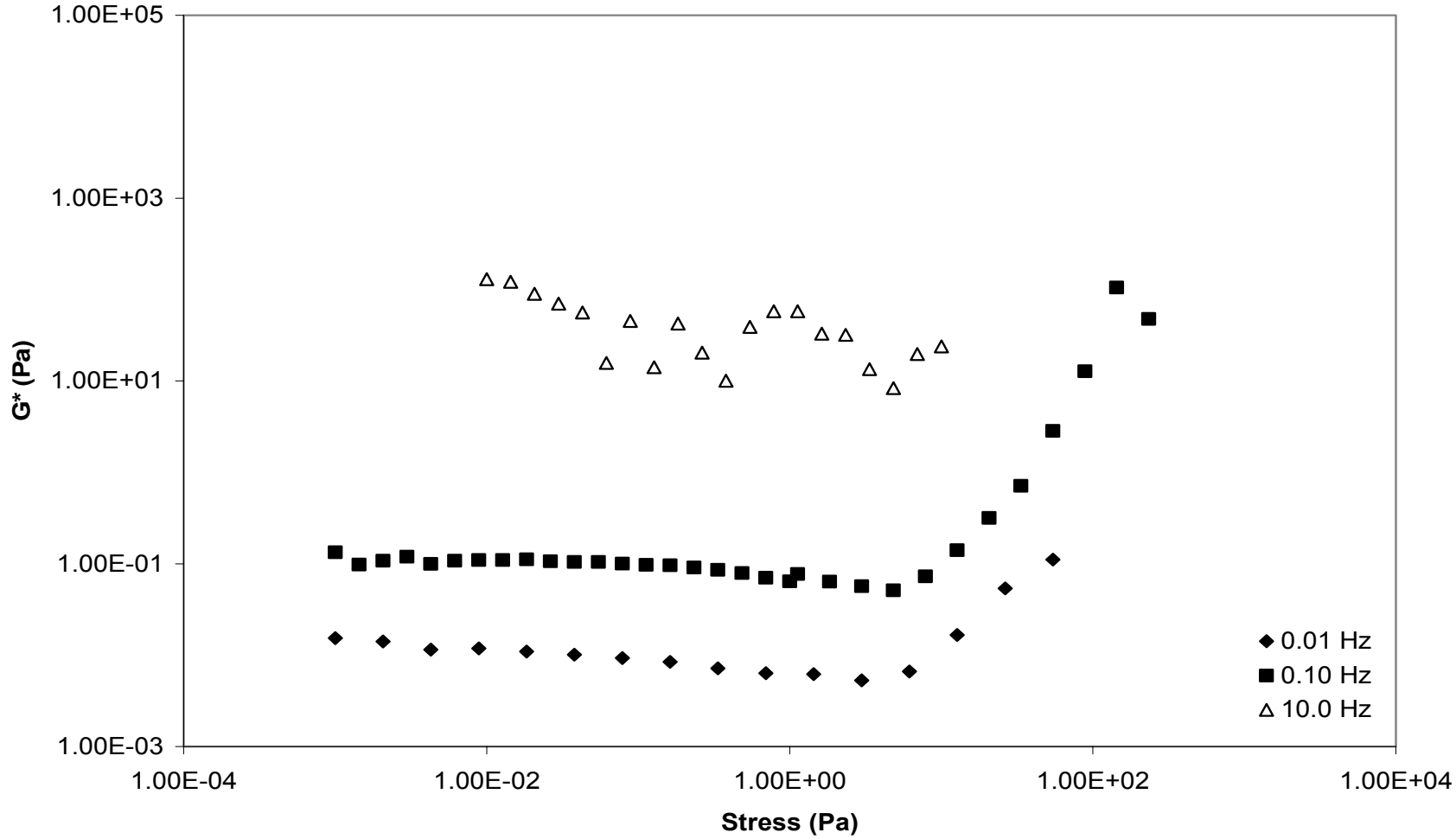


Figure 3.1c. Complex shear modulus (G*) for E-Matrix^{TM} samples during stress sweeps at 50°C

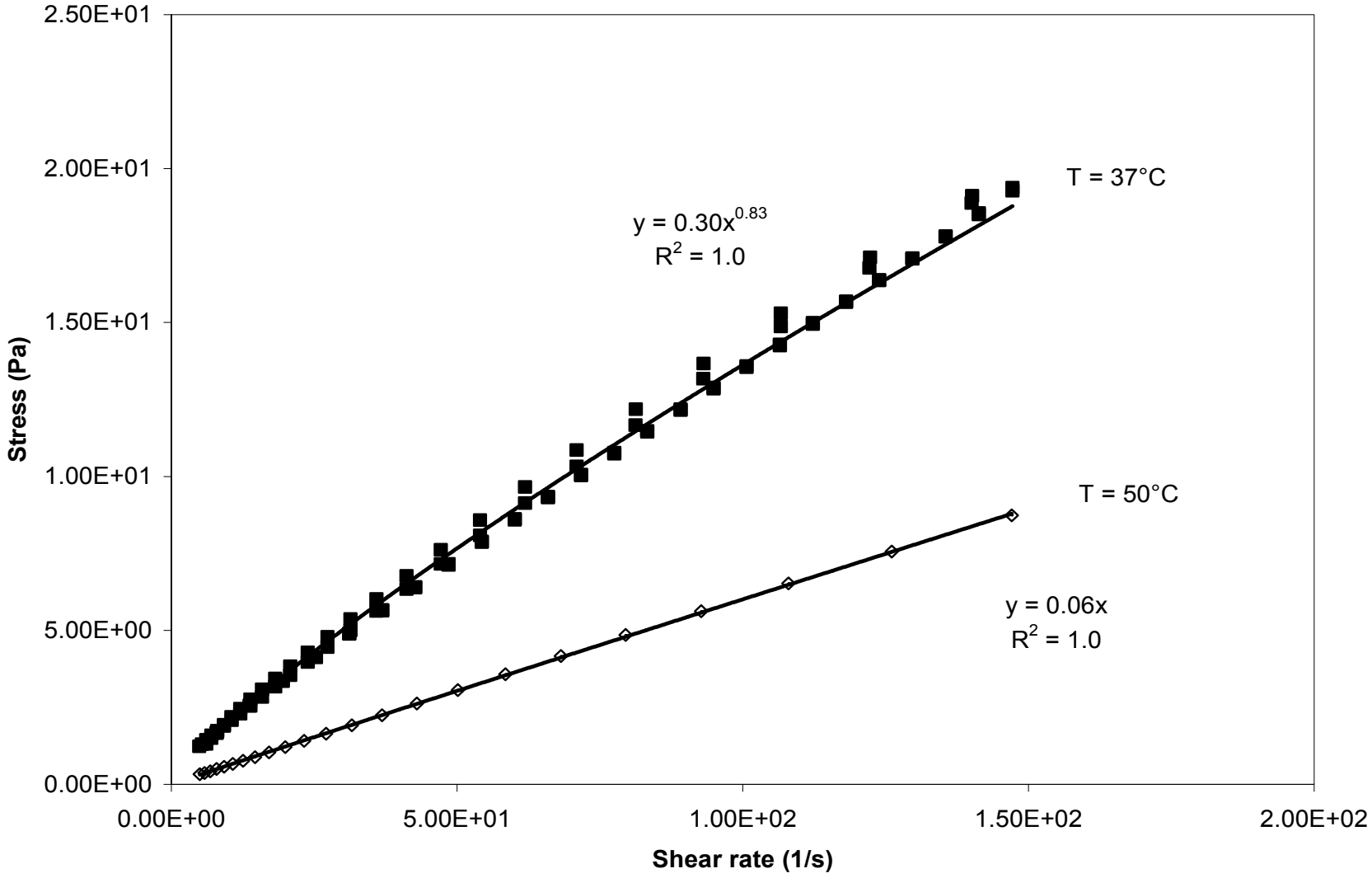


Figure 3.2a. Stress for E-Matrix™ samples during shear rate ramps from 5-150 s^{-1} at 37°C and 50°C

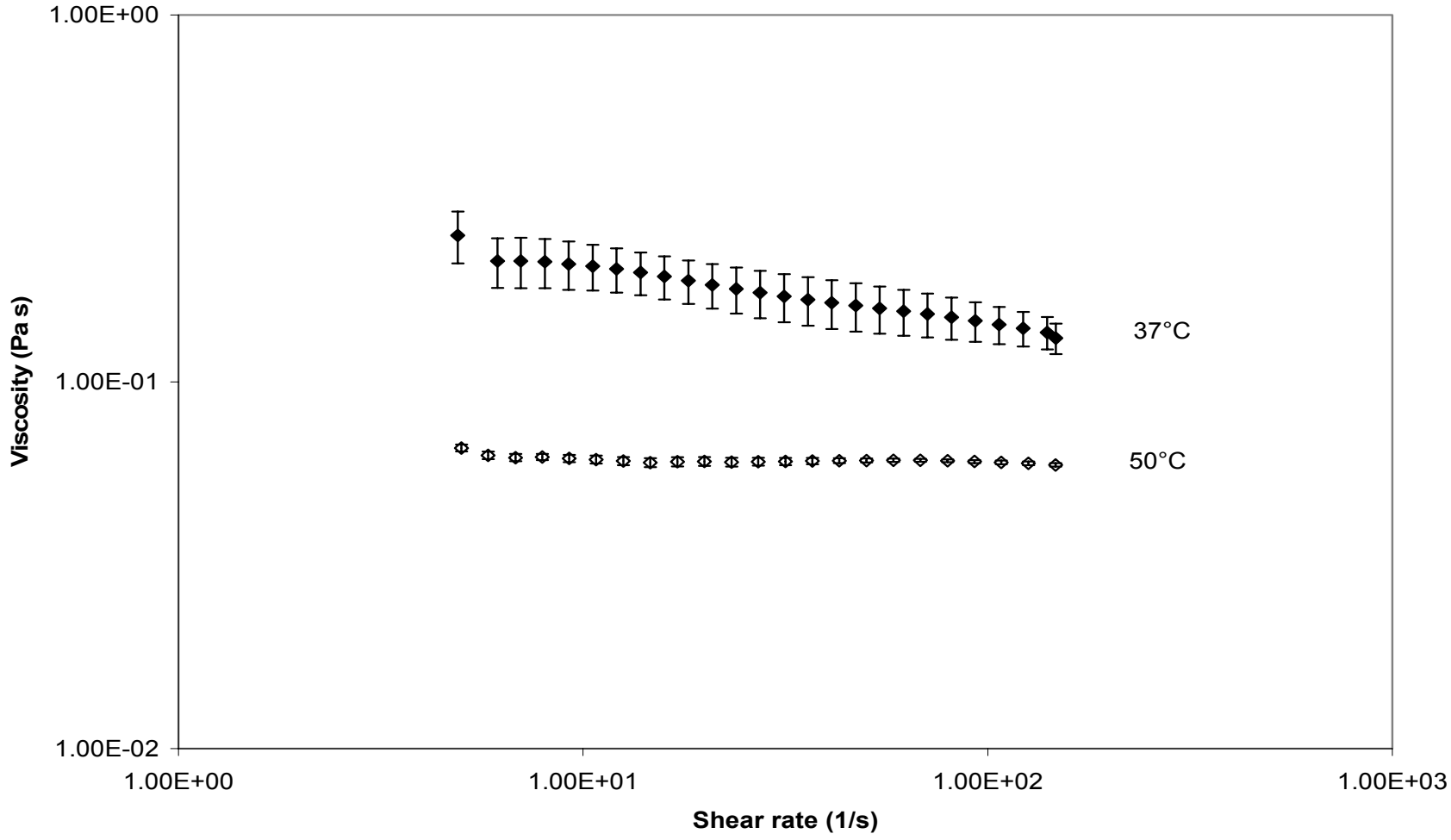


Figure 3.2b. Viscosity for E-Matrix^{TM} samples during shear rate ramps from 5-150 s^{-1} at 37°C and 50°C
The error bars represent 1 standard deviation of samples in triplicate

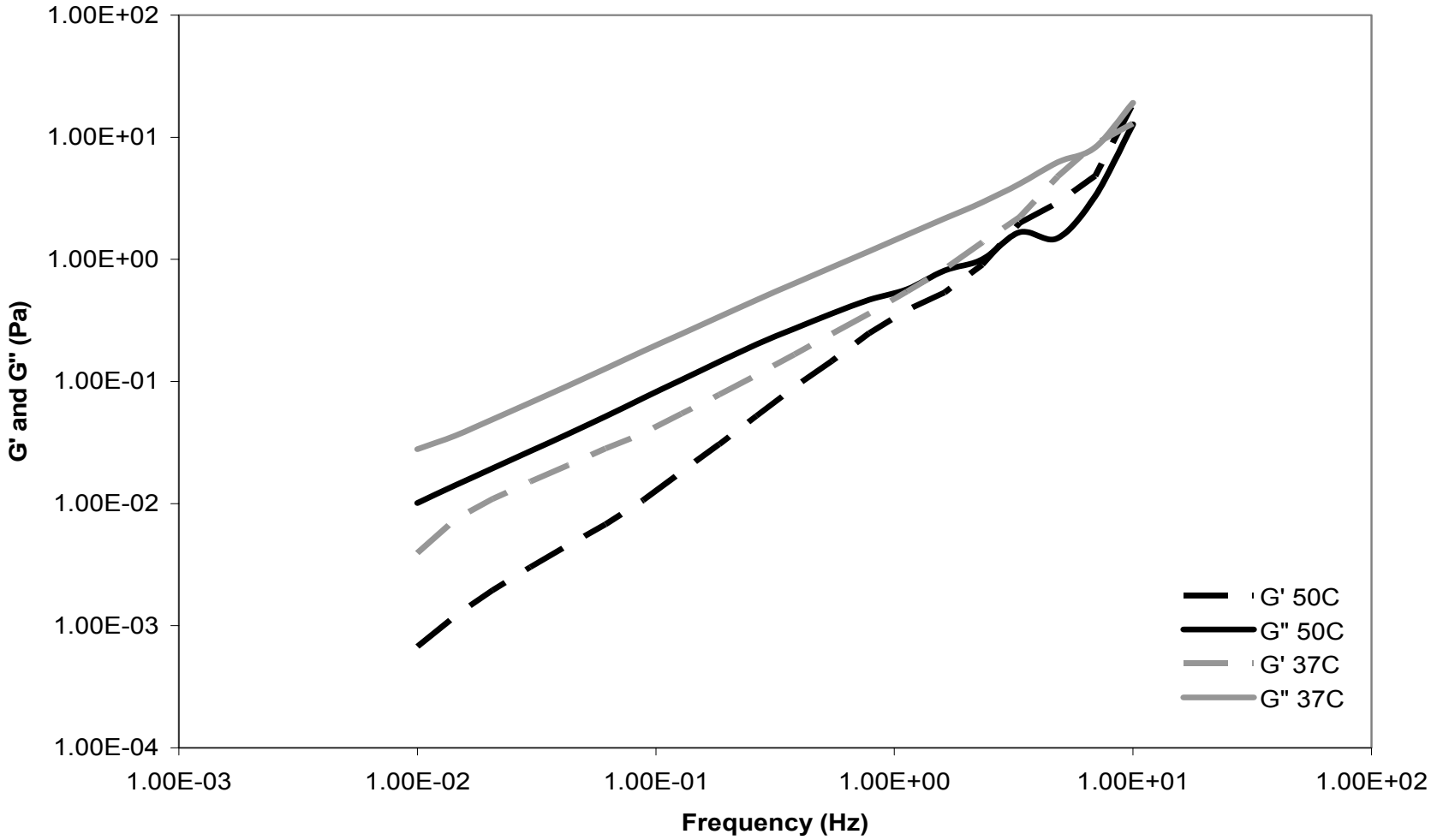


Figure 3.3. Triplicate averages of storage (G′) and loss (G″) moduli for E-Matrix™ samples during a frequency sweep from 0.01 to 10 Hz at 37°C and 50°C

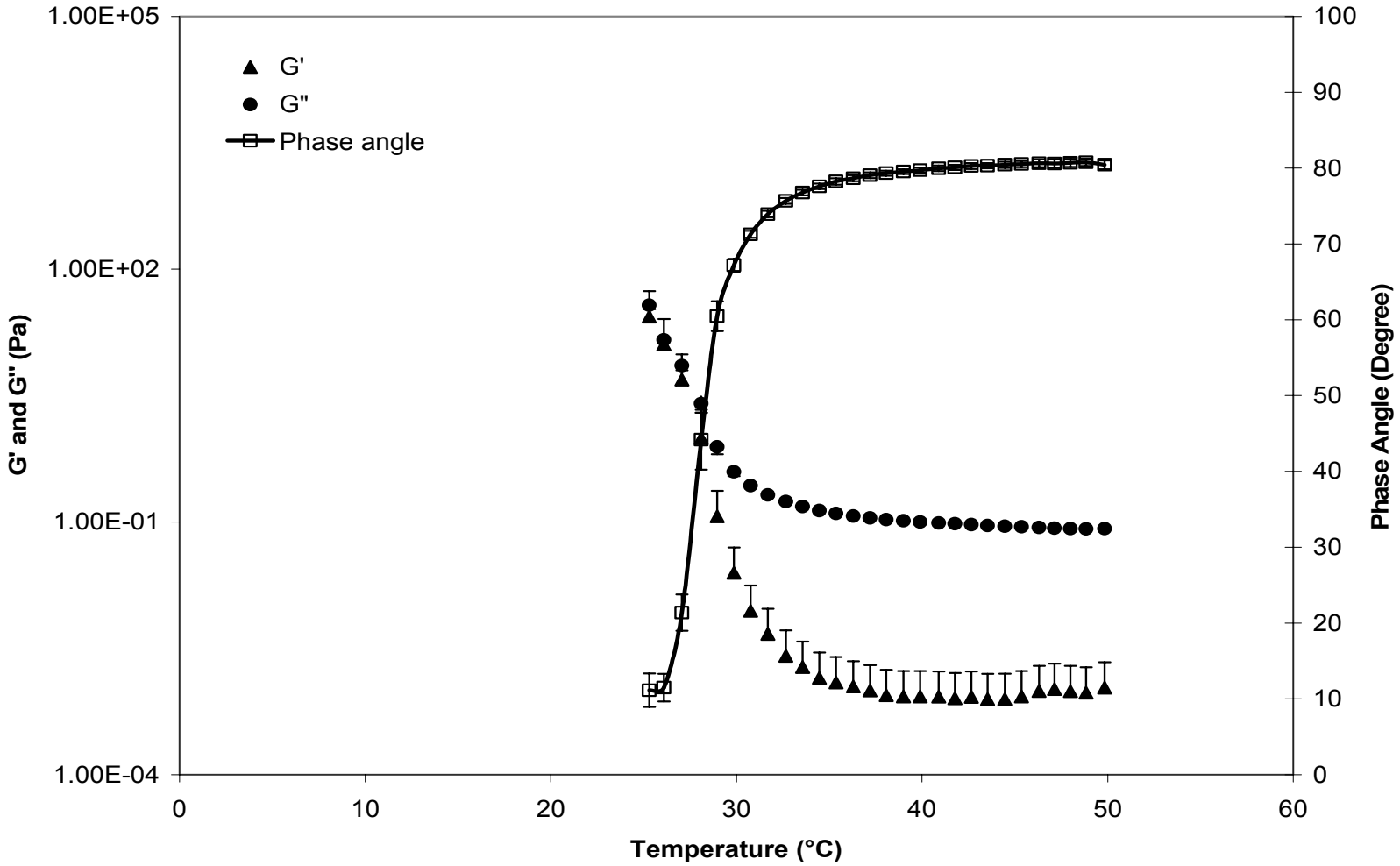


Figure 3.4. Phase angle, storage (G′) and loss (G″) moduli for E-Matrix^{TM} samples during cooling (50°C to 25°C) at -1°C/min
The error bars represent 1 standard deviation of samples in triplicate

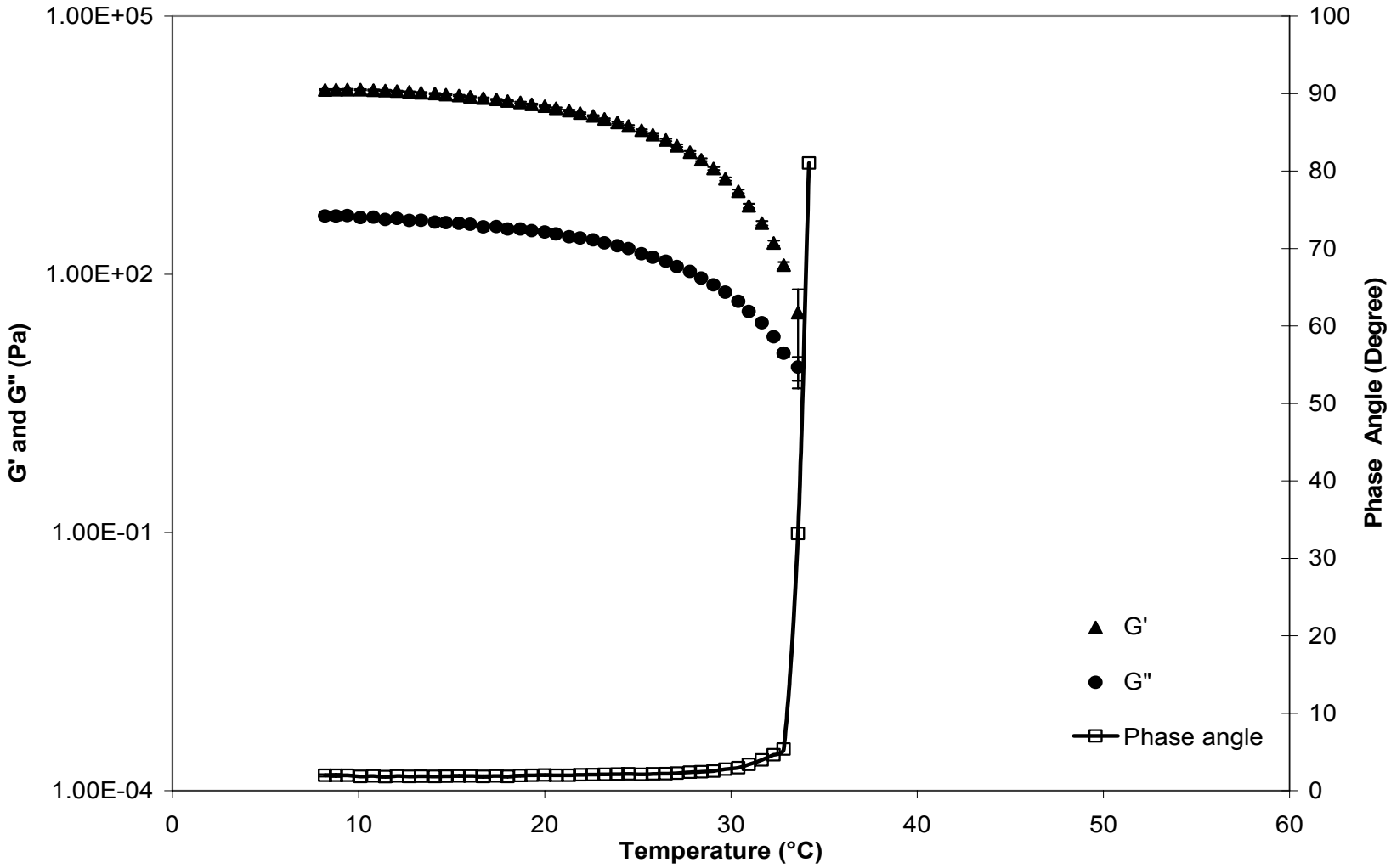


Figure 3.5. Phase angle, storage (G′) and loss (G″) moduli for E-Matrix™ samples during heating (8°C to 35°C) at 1°C/min
The error bars represent 1 standard deviation of samples in triplicate

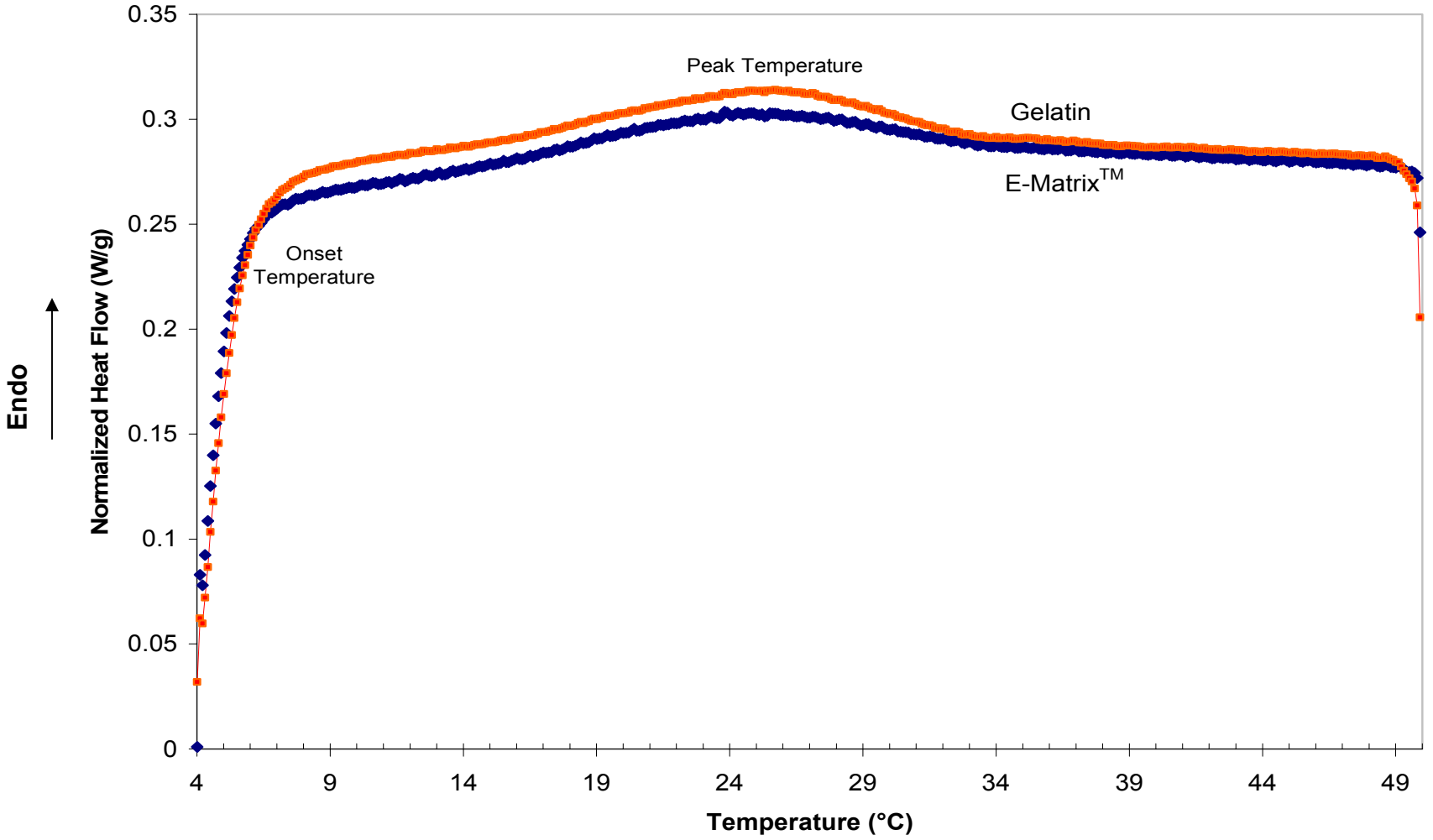


Figure 3.6. Comparison of a 12% gelatin solution and E-Matrix™ heat flow versus temperature using differential scanning calorimetry

CHAPTER 4.

A QUALITY CONTROL PROTOCOL TO CHARACTERIZE GELATIN, AN E-MATRIX™ COMPONENT

4.1 INTRODUCTION

Rheological properties are frequently used to identify quality characteristics of materials, and modern rheometer software permits several tests to be programmed for analysis of a single sample. Sample performance during these tests can become a quality control tool if functionality and sample performance can be connected to certain rheological parameters. Rheology has been recognized to play a role in process design, evaluation, and modeling, and these properties can be measured as quality control indicators (Marcotte and others 2001). A programmed series, or protocol, could be beneficial to a company for the evaluation of raw materials or final products.

A rheological protocol was developed for the principle component of E-Matrix™, a 12% (w/w) gelatin solution. The devised protocol is a series of seven rheological tests consisting of shear rate ramps, mechanical spectra, and temperature ramps. From the series of tests, certain data deemed critical can be analyzed against set standards. Should the sample behavior respond within an acceptable range, the sample would be considered acceptable. The rheological data generated with the rheological protocol can be coupled with additional analytical tests such as pH and moisture content to generate an overall product protocol. With respect to gelatin, this overall product protocol can be used to evaluate new lots prior to formulation. Acceptance and rejection standards can be set for the results of the protocol. If a new lot produces results within the standards, the product would be accepted and

consequently rejected if results did not fall within identified limits of acceptability. This chapter presents a detailed explanation of the quality control protocol developed for gelatin.

4.2 MATERIALS AND METHODS

Gelatin was supplied in dry granule form by Encelle, Inc. (Greenville, NC). The granules were stored at room temperature (23°C) in air tight receiver flasks. A 12% gelatin solution was prepared by weighing 12 g gelatin and adding deionized water until the desired mass (100 g) was achieved. The solution was simultaneously stirred and heated on a stir/hot plate (Barnstead/Thermolyne MIRAK^{TM}, Bubuque, IA) at 50°C for 40 minutes. Temperature was maintained and consistently monitored with a thermal probe (Type K).

The composition of a 12% (w/w) gelatin solution was determined by Microbac Laboratories, Inc. Southern Testing and Research Division (Wilson, NC), and is compiled in Table 4.1. The protein content was determined by the Kjeldahl method (Bradstreet 1965) to compute the nitrogen content, then multiplying this value by the nitrogen to protein conversion factor (5.55) for gelatin (Jones 1941). Moisture was determined by using a forced draft oven drying method (Bradley 1998); lipid content was determined by hydrolytic extraction gas chromatography (AOAC 996.06 GC-FID) for total fat; ash was determined by a muffle furnace technique (AOAC 923)

(Harbers 1998), and carbohydrate content was calculated as the remaining difference.

4.2.1 THE PROTOCOL

The protocol developed for gelatin consists of seven sequential rheological tests or steps, and can be accompanied by additional tests such as pH and moisture. Steps in the rheological sequence of the protocol follow (Table 4.2): (1) initial shear rate ramp at 50°C; (2) frequency sweep at 50°C; (3) temperature ramp (50°C - 8°C); (4) frequency sweep at 8°C; (5) temperature ramp (8°C - 39°C); (6) frequency sweep at 39°C; (7) final shear rate ramp at 50°C. Fresh samples of eleven different gelatin lots were evaluated in triplicate with the protocol.

4.2.2 SHEAR VISCOMETRY

The protocol evaluates solutions using steady and oscillatory shear rheological tests. Shear rate ramps, temperature ramps, and frequency sweeps were conducted using a StressTech Controlled Stress Rheometer (ReoLogica Instruments AB, Lund, Sweden) with a serrated concentric cylinder attachment (CCE) (Figure 2.14). A serrated or roughened surface may effectively prevent wall slip between the tested material and the cup and bob walls (Steffe 1996).

4.2.2.1 STRESS SWEEPS

Prior to performing all oscillatory, small strain tests, stress sweeps were conducted to determine the linear viscoelastic region (LVR) at the frequencies examined throughout the protocol: 0.01, 0.1, and 10 Hz, while measuring the complex shear modulus (G*) at 8°C, 37°C, and 50°C. The stress was increased from 0.001 Pa to 1000 Pa (Figures 3.2a-c). Please refer to section 3.2.1.1 of this thesis for more details.

4.2.2.2 SHEAR RATE RAMPS

Shear rate ramps (5-150 s^{-1}) were conducted at 50°C to determine the stress and shear viscosity at the initiation and conclusion of the protocol. To establish a consistent baseline strain history, all samples were pre-sheared at 1 s^{-1} for 30 s, followed by a 15 s equilibrium period prior to analysis.

4.2.2.3 FREQUENCY SWEEPS

A small amplitude oscillatory shear (SAOS) technique was used to determine network characteristics of E-Matrix™ at three temperatures of interest: 8°C (10°C), 39°C, and 50°C. The aforementioned temperatures were selected because 8°C (10°C) is similar to the refrigerated storage temperature, 39°C is the heating

temperature prior to medical application, and 50°C is the manufacturing temperature of E-Matrix™. Note, 10°C was used in the initial tests and was later refined to 8°C.

Mechanical spectra testing subjects the material to harmonically varying stresses or strains and is the most common method for studying viscoelastic behavior (Steffe 1996). In addition, oscillatory testing is very sensitive to the chemical composition and physical structure of the materials being examined (Steffe 1996). For the SAOS tests, the samples were subjected to a frequency ranging from 0.01 to 10 Hz and the storage, loss, and complex moduli (G′, G″, G*) were recorded (Figure 4.3). All samples were evaluated within the LVR at a stress of 0.5 Pa for the 12% gelatin solution.

4.2.2.4 TEMPERATURE RAMPS

Oscillatory methods were also used to rheologically determine thermal transition characteristics of the samples: gel point and melt point. The tangent of the phase angle (δ) is greater than 1 for a viscous fluid and less than 1 for an elastic solid (Tung and Dynes 1982). Furthermore, a material possessing a 90° phase angle is considered to have ideal liquid (Newtonian fluid) behavior and a 0° phase angle is considered to have ideal solid (Hookean solid) behavior (Bryant and McClements 2000; Daubert and Foegeding 1999). The tangent of the phase angle can be expressed as a ratio of the loss to storage shear moduli, Equation 1, where G′ is the shear storage modulus and G″ is the shear loss modulus.

$$\tan \delta = \frac{G''}{G'} \quad \textbf{(1)}$$

Where tan δ equals 1, is where G′ equals G″ and the phase angle is 45°. The point at which G′ = G″ is commonly called the crossover point. Furthermore, Michon and others (1993) state the relationship expressed in Equation 1 is a critical point which may be precisely located when performing heating or cooling ramps. Hence, the point where G′ = G″ (δ = 45°) represents a hypothetical transition point between a viscous fluid and an elastic solid as it is the midpoint between an ideal solid (δ = 0°) and an ideal liquid (δ = 90°) and can be deemed the gel point or melt point when examining data of phase angle versus temperature or time. For practical reasons of this research, gel point was defined as the temperature at which a 45° phase angle was achieved while cooling at -1°C/min from 50°C to 8°C. Likewise, the melt point was defined as the temperature at which a 45° phase angle was achieved upon heating at 1°C/min from 8°C (10°C) to 39°C.

Temperature ramps were performed in triplicate by placing samples under a constant stress of 0.05 Pa and a frequency of 0.10 Hz when cooled from 50°C to 8°C (10°C); and constant stress of 200 Pa and a frequency of 0.10 Hz when heated from 8°C (10°C) to 39°C. The material displayed varying properties across the temperature range, complicating stress selection for oscillatory analysis. Therefore, the selected frequency-stress combinations were optimal and within the LVR for the initial temperature of the temperature ramp (50°C for cooling and 8°C(10°C) for heating).

4.2.3 PROTOCOL ANALYSIS

The statistical hypothesis for this analysis presumed all eleven gelatin lots as identical, possessing equivalent properties. Two statistical methods were used to analyze this hypothesis. The first method was the determination of a 95% confidence interval based on the means of each property examined. Analysis was then performed to determine which lots fit within the confidence interval for each property examined; pH, viscosity at a specified point during the initial and final shear rate ramps, gel point, melt point, and complex shear modulus at a specified point during frequency sweeps at 8°C, 39°C , and 50°C . Additionally, a more sophisticated analysis was performed using the popular statistical analysis program SAS (Cary, NC). Using Tukey's procedure in incorporation with SAS software, the results generated from the protocol; viscosity at a specified point during the initial and final shear rate ramps, gel point, melt point, and complex shear modulus at a specified point during frequency sweeps at 8°C, 39°C, and 50°C, were analyzed and grouped based on significant differences.

Deciding which statistical analysis method is best for the investigator depends on a few factors. The first factor is resources. Using the SAS method requires the user to have a contract with SAS in order to permit usage of this method. Secondly, if the goal of the user is to determine whether all the samples examined are not significantly different, then the SAS method would be most appropriate. However, if the user does not have access to SAS or is more interested in determining whether

results fall within specified limits, then the confidence interval method is more appropriate. Consequently the most appropriate statistical technique is determined by what the user desires to know about the data.

4.3 RESULTS AND DISCUSSION

The eleven different lots of 12% gelatin solutions were each subjected to the rheological protocol in triplicate. Individual properties from each of the seven phases of the protocol were examined, and comparisons were made between properties of the twelve materials.

The development of the protocol was a process. At the onset, data was recorded for tests that were later developed further. For gelatin sample 1, the frequency sweep ranged from 0.01-0.10 Hz. Further work on the protocol expanded this frequency sweep to cover a broader frequency range; 0.01-10.0 Hz in the final protocol.

4.3.1 SHEAR RATE RAMPS

Shear rate ramps performed at the initiation and end of the protocol (50°C) produced results for viscosity and stress. Figures 4.1a-b are shear stress and viscosity versus shear rate for two gelatin lots. These figures are representative of the response from all eleven gelatin lots examined.

A general relationship used to describe the behavior of these fluids is the power law equation. Plots of shear stress versus shear rate and the incorporation of the power law equation (Equation 2) to represent a general relationship describing the gelatin flow behavior, where σ is shear stress, K is a material constant called the consistency coefficient, $\dot{\gamma}$ is shear rate or the change in shear strain with respect to time, and n is another material constant called the flow behavior index were used to characterize the gelatins.

$$\sigma = K(\dot{\gamma})^n \quad \textbf{(2)}$$

When n = 1, the material is considered Newtonian; when 0 < n < 1, the material is shear-thinning (pseudoplastic); and when 1 < n < ∞, the material is shear-thickening (dilatent). During the initial shear rate ramp at 50°C (protocol step 1), gelatin lot samples 10 and 12 displayed similar rheological properties. Based on the power law equation, both gelatin lots have Newtonian flow behavior at 50°C, since both samples have a flow behavior index equal to 1.0.

Because 50°C is above the gelation temperature, the material is viscous and lacks structure. Marcotte and others (2001) found at a cooler temperature of 20°C, 2%, gelatin solutions displayed Newtonian behavior. Furthermore, at most temperatures aqueous gelatin solutions were shown to be Newtonian (Johnston-Banks 1990). Slightly above the setting point, the viscosity becomes time-dependent due protein aggregation which is accelerated at higher concentrations and higher molecular weights (Johnston-Banks 1990).

For efficient use of a protocol, where quick and simple quality control tests are necessary, viscosity can be evaluated at a specified shear rate. For this project, 23 s^{-1}, was the selected shear rate. Table 4.3a contains viscosity measurements at 23 s^{-1} for the eleven lots of gelatin solutions.

The statistical analyses using the two different methods, confidence interval determination and SAS, produced differing results. Selecting gelatin lots by fitting results within the confidence intervals resulted in eight lots (Sample numbers 1, 2, 3, 4, 10, 11, 12, and 13) not fitting within limits for initial viscosity, determined during the initial shear rate ramp. However, SAS determined the lots were not significantly different with regard to viscosity at 23 s^{-1} for the same test. Examination of the specified viscosity during the final shear rate ramp indicated only lot 10 did not fit within the confidence interval. Furthermore, for these same results, using SAS software, the viscosities were deemed significantly different and grouped into three groups (Tables 4.3a-b). It may be noted that gelatin lot 4 was significantly different from the majority of the other lots, according to the statistical analysis for viscosity. Thus it can be labeled as an outlier and potentially rejected.

Because the initial viscosities were not significantly different, but were significantly different during the final shear rate ramp, this may be an indication that the gelatin lots initially have similar viscosities, but with shear and thermal treatments, differences between the samples shown through viscosity measurements are identifiable. In addition, for quality control purposes, since significant differences

were not detected during the initial shear rate ramp, this response may indicate more emphasis should be placed on the final shear rate ramp viscosities for determination of lots differences because more differences were able to be detected at this later point.

4.3.2 FREQUENCY SWEEPS

Frequency sweeps were performed at 8°C (10°C), 39°C, and 50°C on the eleven gelatin lots. Figure 4.2a is a representative frequency sweep of two gelatin lots analyzed from 0.01 – 0.1 Hz at 39°C. In Figure 4.2a, G″ is predominately larger than G′ at 39°C examined in the gelatin graph for the same frequency. Hence the gelatin solution displayed more fluid-like behavior at these frequencies. The complex modulus (G*) is representative of the network strength of a material. Figure 4.2b displays G* data for two representative gelatin solutions across a frequency sweep at 39°C with sample 10 having a slightly larger G* than sample 12. Therefore sample 10 has a slightly stronger network than sample 12.

For interpretation of results from the protocol; the G* value at 0.1 Hz was determined and examined for each sample evaluated with the protocol as listed in Table 4.3b. Using a 95% confidence window to examine the complex shear moduli values at 50°C showed the majority of the gelatin lots fall outside the confidence window. Additionally, a significant difference exists between the samples at 50°C according to the SAS analysis. For the values evaluated at the specified complex shear

modulus during the frequency sweep at 10°C, all eleven gelatin lots fall outside the confidence interval. However, the SAS analysis showed, the lots do not differ significantly. And finally, at 39°C for the specified complex shear modulus, all gelatin lots fall within the confidence interval, and some were deemed statistically similar.

4.3.3 TEMPERATURE RAMPS

Temperature ramps were performed on the eleven gelatin lots. Gel and melt points were identified for each material and are presented in Table 4.3a. However setting a confidence interval for the gelatin lots showed several gelatin lots fall outside this confidence interval. In addition, the lots were deemed significantly different in regard to gel points. The gelatin lots were determined to not be significantly different for melt point. Therefore, as a mode of detecting differences among samples, it may be implied gel point is a better property to analyze than melt point for detection of differences.

4.3.4 PROTOCOL IMPLEMENTATION

Incorporation of the protocol as a quality control tool can be beneficial to Encelle Inc. For the protocol to be used properly, limits must define acceptable values for the properties examined. To identify a range of acceptability, gelatin lots deemed to produce successful and unsuccessful E-Matrix^{TM} batches must be analyzed by the protocol. Subsequently, acceptable parameters can be established. After

evaluating a new gelatin lot with the protocol and determining the rheological points of interest, the lot would be deemed acceptable or rejected depending on whether the values fall inside or outside the standard parameter limits, respectively.

4.4 CONCLUSIONS

A rheological protocol was developed to evaluate the principle ingredient of E-Matrix™, a 12% gelatin solution. Viscosity at 23 s^{-1}, gel and melt points, and complex moduli were determined through analysis of the protocol. In designing the protocol, all gelatin lots were assumed to possess equivalent properties. Implementation of the newly developed rheological protocol can potentially be very beneficial to the manufacture of E-Matrix™, especially through a quality control perspective. A similar protocol, discussed in the following chapter, was designed for the evaluation of E-Matrix™.

4.4 REFERENCES

Bradley RL. 1998. Moisture and Total Solids Analysis. In: Nielsen SS. Food Analysis 2nd ed. Gaithersburg, MA: Aspen Publishers, Inc.

Bradstreet RB. 1965. The Kjeldahl Method for Organic Nitrogen. NY: Academic Press.

Bryant CM and McClements DJ. 2000. Influence of xanthan gum on physical characteristics of heat-denatured whey protein solutions and gels. Food Hydrocolloids 14(4):383-390.

Daubert CR and Foegeding EA. 1999. Rheological Principles for Food Analysis. In: Nielsen SS, editor. Food Analysis. 2nd ed. Gaithersburg, MD: Aspen Publishers, Inc. 551-569.

Gilsenan PM, Ross-Murphey SB. 2000. Viscoelasticity of thermoreversible gelatin gels from mammalian and piscine collagens. J Rheol 44(4): 871-883.

Harbers LH. 1998. Ash Analysis. In: Nielsen SS. Food Analysis 2nd ed. Gaithersburg, MA: Aspen Publishers, Inc.

Johnston-Banks FA. 1990. Gelatin. In: Food gels. Chapter 7. Elsevier Applied Science; NY. 233-289.

Jones, DB. 1941. Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Protein. United States Department of Agriculture, Circular No. 183.

Mackie AR, Gunning AP, Ridout MJ, Morris VJ. 1998. Gelation of Gelatin Observation in the Bulk and at the Air-Water Interface. Biopolymers 46(4):245-252.

Marcotte M, Taherian Hoshahili AR, Ramaswamy HS. 2001. Rheological properties of selected hydrocolloids as a function of concentration and temperature. Food Research International 34(8): 695-703.

Michon C, Cuvelier G, and Launay B. 1993. Concentration dependence of the critical viscoelastic properties of gelatin at the gel point. Rheologica Acta 32:94-103.

Steffe, JF. 1996. Rheological Methods in Food Process Engineering. 2nd ed. MI: Freeman Pres.

Tung CYM, Dynes PJ. 1982. Relationship between Viscoelastic Properties and

Gelation in Thermosetting Systems. J of Applied Polymer Sci 27(2):569-574.

Table 4.1. Composition of 12% gelatin solution

Analysis	(%)	Method
Protein	10.8	Kjeldahl
Carbohydrates	----	Calculation
Moisture	90.2	Forced Draft Oven
Ash	0.150	AOAC 923
Total Fat	0.114	AOAC 996.06 GC-FID

*Determined by Microbac Laboratories, Inc. Southern Testing and Research Division, (Wilson, NC)
**Sum is greater than 100% because each component was measured using a different solution sample

Table 4.2. Rheological protocol for 12% gelatin solution

Step #	Test	Thermal Rate	Stress	Shear Rate	Frequency	Temperature
1	Initial Shear Rate Ramp			5-150 s^{-1}		50°C
2	Mechanical Spectra		0.5 Pa		0.01-10 Hz	50°C
3	Cooling Ramp	-1°C/min	0.5 Pa		0.1 Hz	50-8°C
4	Mechanical Spectra		200 Pa		0.01-10 Hz	8°C
5	Heating Ramp	1°C/min	200 Pa		0.1 Hz	8-39°C
6	Mechanical Spectra		0.5 Pa		0.01-10 Hz	39°C
7	Final Shear Rate Ramp			5-150 s^{-1}		50°C

Table 4.3a. Comparison of gelatin lots

Sample number	Encelle sample name	pH	Initial Viscosity (50°C at 23 s-1)	Final Viscosity (50°C at 23 s-1)	Gel Point (°C)	Melt Point (°C)
1	Encelle lot 010089	5.23	[a]0.0204 Pa s (0.0006)	[a,b]0.0244 Pa s (0.0024)	[a,b]25.7°C (0.1)	[a]33.8°C (0.2)
2	Encelle lot 020008	5.19	[a]0.0200 Pa s (0.0001)	[b]0.0268 Pa s (0.0015)	[b]25.7°C (0.2)	[a]33.8°C (0.1)
3	RM-3794 Lot 010044 Gelatin 13446	5.16	[a]0.0234 Pa s (0.0036)	[b]0.0225 Pa s (0.0010)	[a]26.0°C (0.1)	[a]33.8°C (0.0)
4	Lot 13467-05	3.65	[a]0.0146 Pa s (0.0004)	[c]0.0147 Pa s (0.0004)	[c]23.5°C (0.3)	[a]31.1°C (0.7)
5	Encelle lot 010046	5.14	[a]0.0215 Pa s (0.0020)	[a,b]0.0214 Pa s (0.0025)	[a,b]25.7°C (0.1)	[a]33.9°C (0.1)
6	GE 105 lot PA 011	5.21	[a]0.0215 Pa s (0.1650)	[b]0.0223 Pa s (0.0008)	[a,b]26.0°C (0.0)	[a]34.8°C (0.1)
8	GE 105 Lot 0X0194	5.20	[a]0.0217 Pa s (0.0011)	[b,c]0.0198 Pa s (0.0015)	[a,b]26.3°C (0.1)	[a]33.9°C (0.2)
10	Gelita Boil 3375 1-A	5.48	[a]0.0266 Pa s (0.0016)	[a]0.0314 Pa s (0.0007)	[a,b]26.9°C (0.0)	[a]34.7°C (0.0)
11	Gelita Boil 3375 1-B	5.41	[a]0.0264 Pa s (0.0012)	[a,b]0.0282 Pa s (0.0013)	[a]26.9°C (0.3)	[a]33.8°C (0.0)
12	Gelita Boil 3375 2-A	5.24	[a]0.0188 Pa s (0.0016)	[b,c]0.0206 Pa s (0.0009)	[a,b]25.1°C (0.0)	[a]33.0°C (0.2)
13	Gelita Boil 3375 2-B	5.37	[a]0.0203 Pa s (0.0020)	[b]0.0235 Pa s (0.0018)	[a,b]26.6°C (0.0)	[a]33.9°C (0.0)
Mean ± 95% confidence interval		5.12 ±0.30	0.0214 ±0.0007	0.0214 ±0.0075	25.9 ±0.2	33.7 ±0.2

*(Standard error)

**Means with the same letter are not significantly different

Table 4.3b. Comparison of gelatin lots

Sample number	Encelle sample name	G* at 0.1 Hz 50°C	G* at 0.1 Hz 10°C	G* at 0.1 Hz 39°C
1	Encelle lot 010089	[a,b]0.0143 Pa (0.0005)	[a]16100 Pa (792)	[a,b,c]0.0224 Pa (0.0020)
2	Encelle lot 020008	[a,b]0.0140 Pa (0.0003)	[a]13800 Pa (181)	[a,b,c]0.0219 Pa (0.0013)
3	RM-3794 Lot 010044 Gelatin 13446	[a,b]0.0145 Pa (0.0096)	[a]14900 Pa (757)	[b,c]0.0210 Pa(0.0009)
4	lot 13467-05	[c]0.00992 Pa (0.00002)	[a]13710 Pa (547)	[c]0.0169 Pa (0.0009)
5	Encelle lot 010046	[a,b]0.0156 Pa (0.0013)	[a]15160 Pa (1050)	[a,b]0.0252 Pa (0.0022)
6	GE 105 lot PA 011	[a,b]0.0144 Pa (0.0003)	[a]13300 Pa (815)	[a,b,c]0.0218 (0.0006)
8	GE 105 Lot 0X0194	[a,b,c]0.0138 Pa (0.0007)	[a]12500 Pa (1110)	[b,c]0.0199 Pa (0.0013)
10	Gelita Boil 3375 1-A	[a]0.0176 Pa (0.0010)	[a]19400 Pa (715)	[a]0.0285 Pa (0.0006)
11	Gelita Boil 3375 1-B	[a]0.0173 Pa (0.0009)	[a]282000 Pa (135000)	[a,b]0.0260 Pa (0.0012)
12	Gelita Boil 3375 2-A	[b,c]0.0133 Pa (0.0010)	[a]385000 Pa (271000)	[b,c]0.0197 Pa (0.0008)
13	Gelita Boil 3375 2-B	[b,c]0.0133 Pa (0.0011)	[a]15600 Pa (506)	[a,b,c]0.0218 Pa (0.0020)
Mean ± 95% confidence interval		0.0144 ±0.0004	72900 ±26600	0.0223 ±0.0066

*(Standard error)
**Means with the same letter are not significantly different

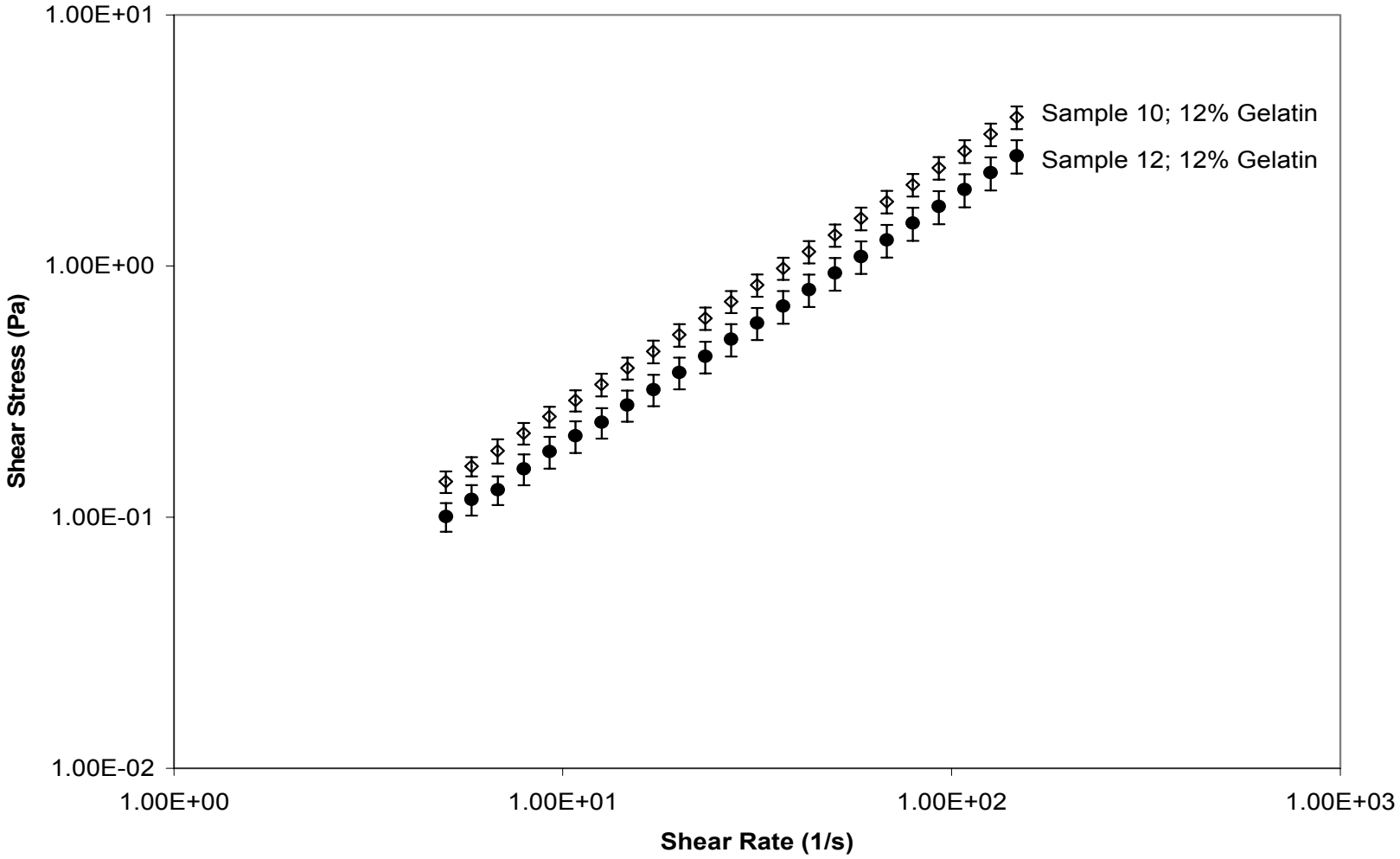


Figure 4.1a. 12% gelatin solution initial shear rate ramps at 50°C
The error bars represent 1 standard deviation of samples in triplicate

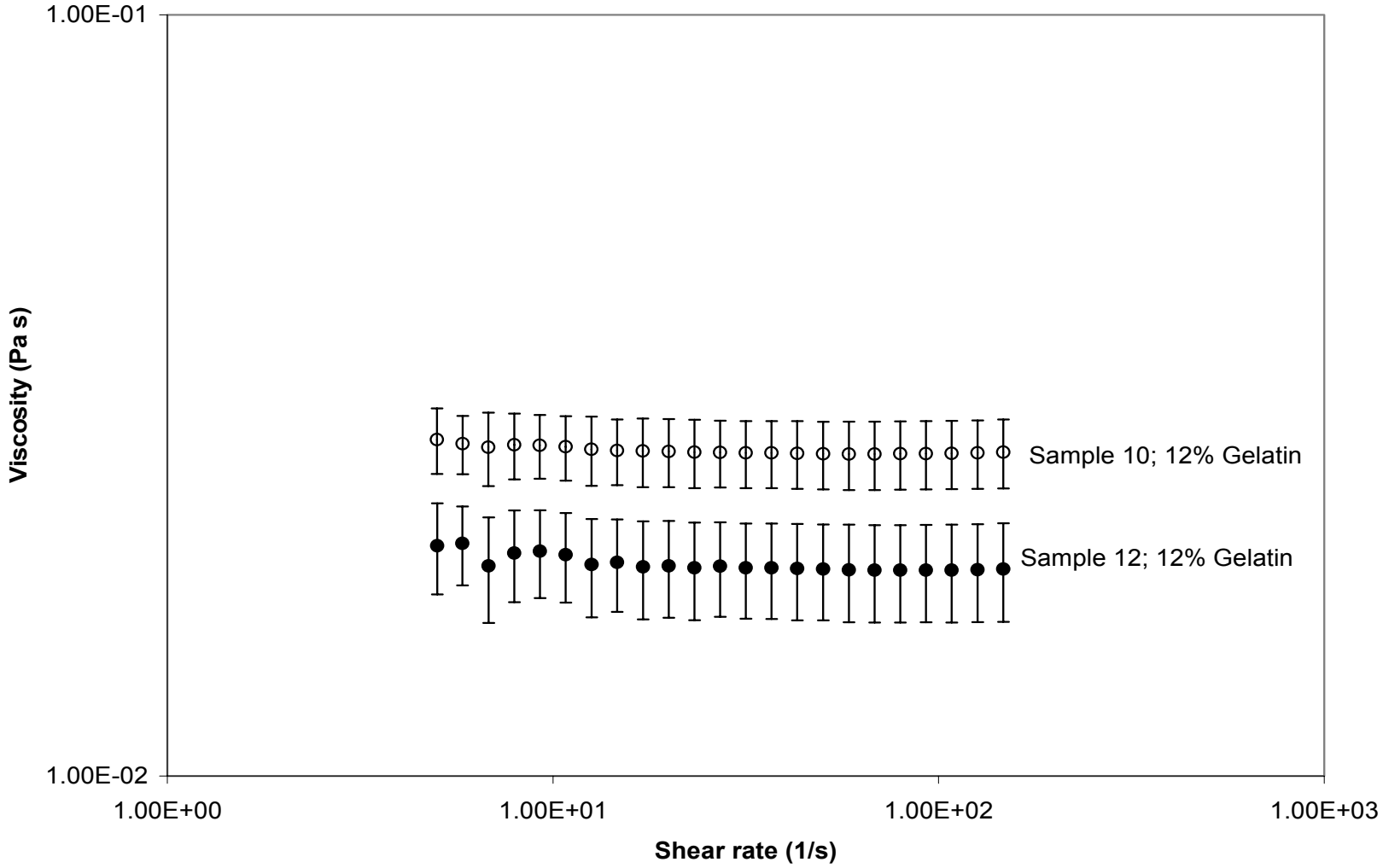


Figure 4.1b. 12% gelatin solution initial shear rate ramps at 50°C
The error bars represent 1 standard deviation of samples in triplicate

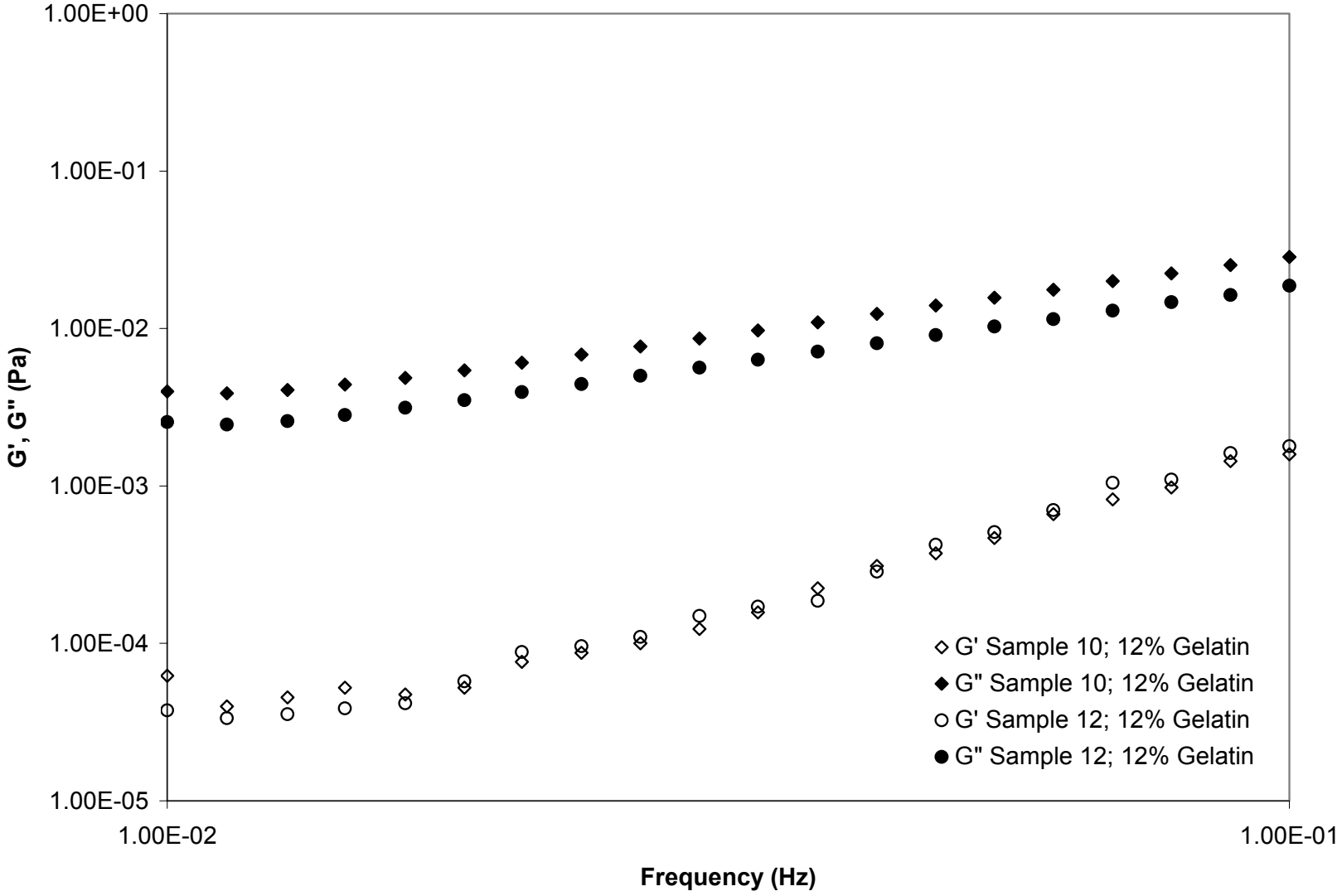


Figure 4.2a. 12% gelatin solution frequency sweeps at 39°C

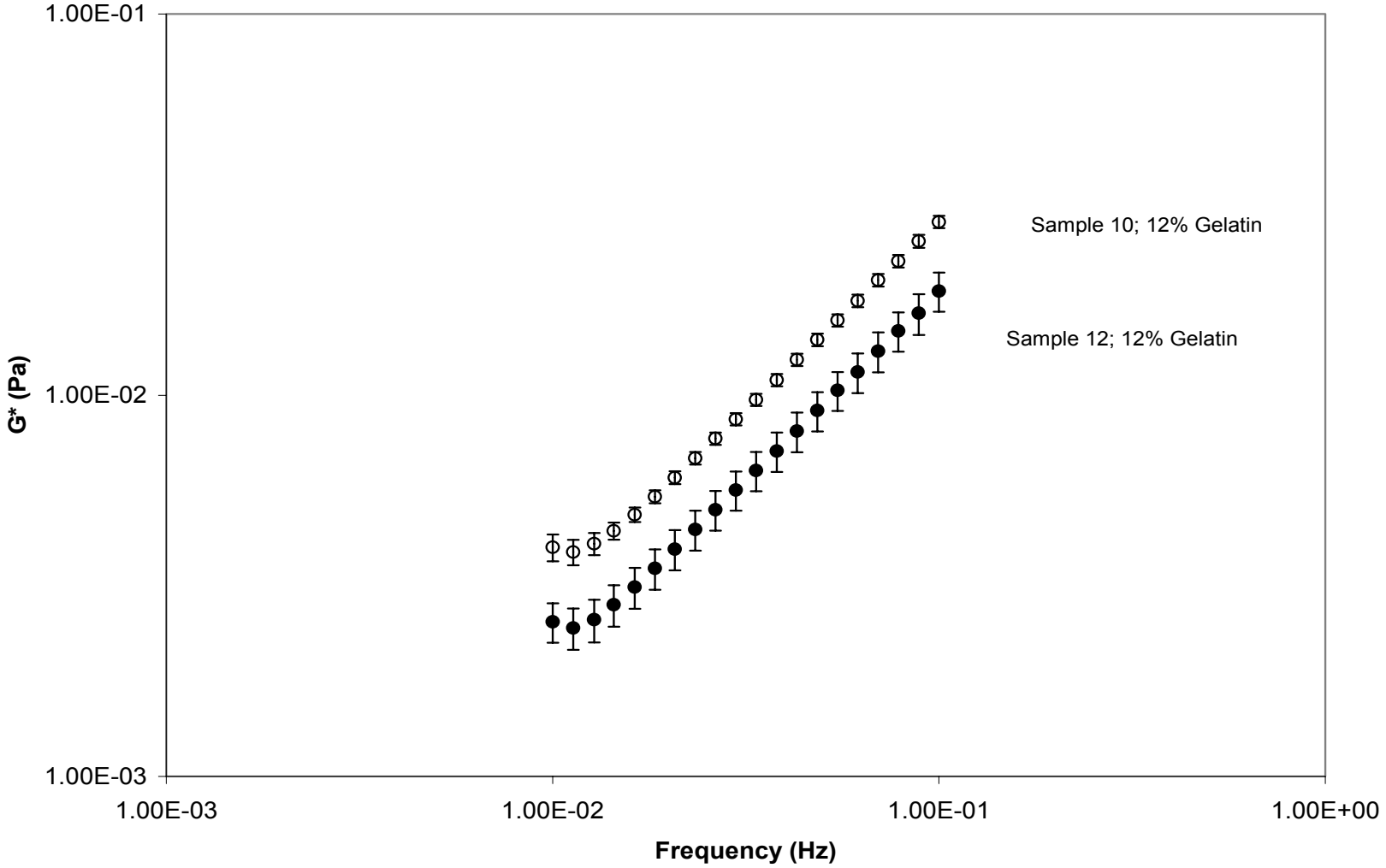


Figure 4.2b. 12% gelatin solution frequency sweeps at 39°C
The error bars represent 1 standard deviation of samples in triplicate

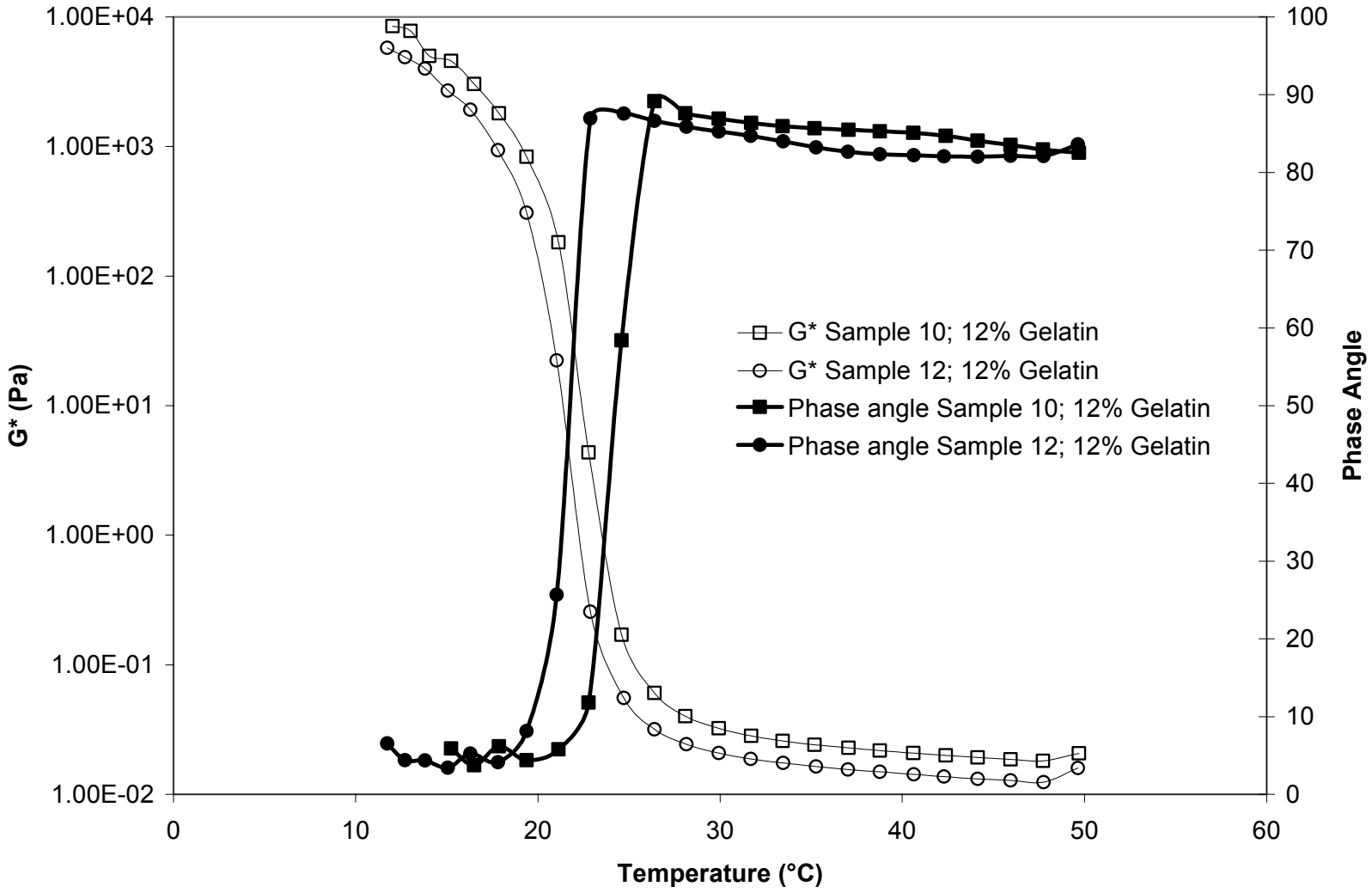


Figure 4.3. 12% gelatin solution cooling ramps from 50°C to 11°C

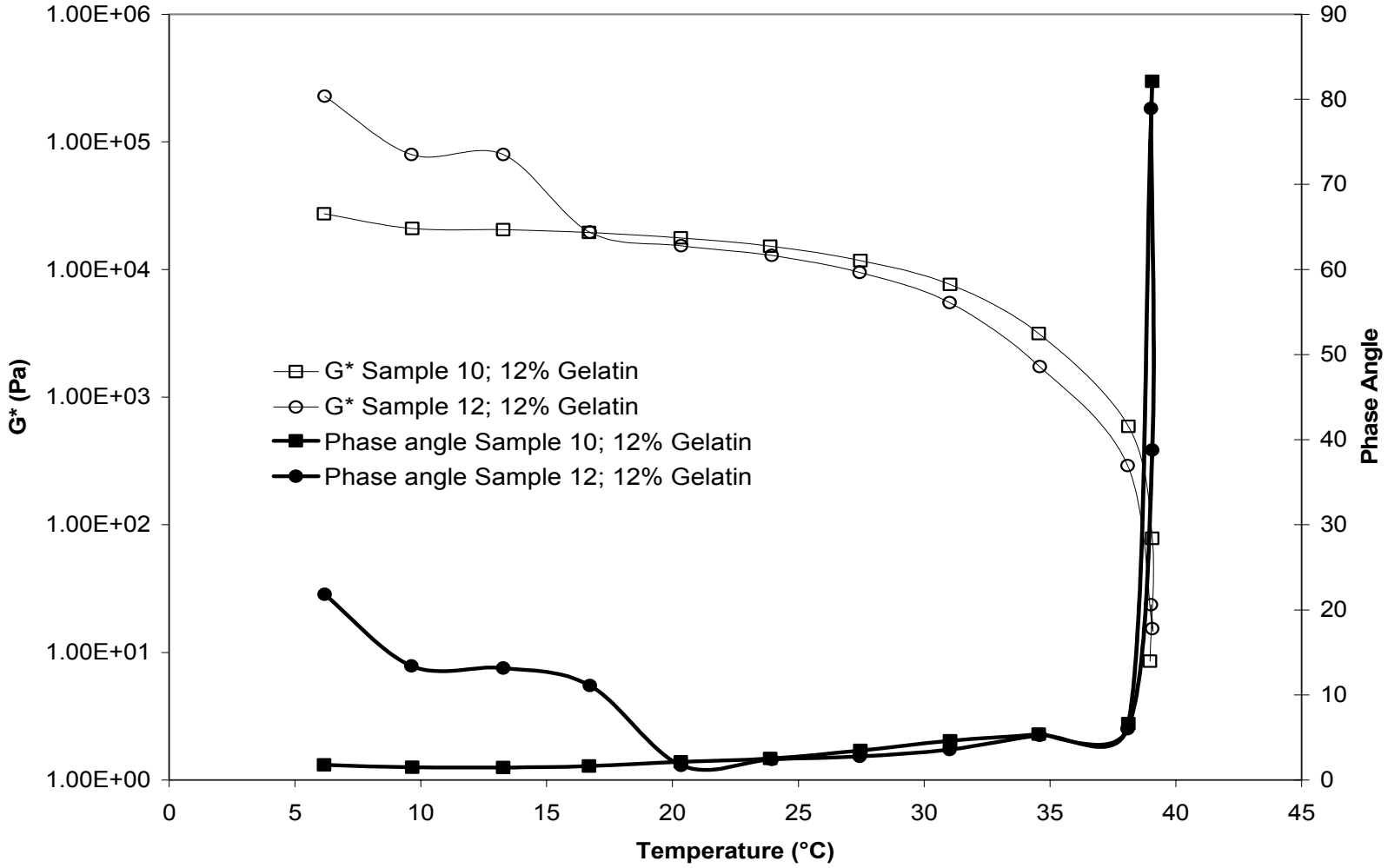


Figure 4.4. 12% gelatin solution heating ramps from 8°C to 39°C

CHAPTER 5.

EXAMINATION OF E-MATRIX™ COMPONENTS

5.1 INTRODUCTION

Studies were performed to understand and characterize E-Matrix™. To do so, the key components constituting E-Matrix™ were examined, specifically, a 12% gelatin solution and a 5% dextran solution. These concentrations were chosen because they represent relative concentrations present in E-Matrix™. E-Matrix™ is approximately 17% solids, and to insure the solids content was not the sole source for differences, a 17% gelatin solution was also considered. Additionally, because E-Matrix™ separates into a gelatin-rich domain and a dextran-rich domain at body temperature (37°C), the domain hypothesized to be the more contributing agent, the gelatin-rich domain, was also evaluated. As anticipated, the different components of E-Matrix™ showed different rheological properties, and this understanding contributes to the knowledgebase for E-Matrix™ function.

To investigate the effect of ionic strength and salt type on E-Matrix™, an ionic strength study was performed. The effect of salt type was considered by examining the effect of a monovalent salt, NaCl, versus a divalent salt, $CaCl_2$, in 12% gelatin solutions at concentrations lower, equivalent, and larger than the ionic strength present in E-Matrix™ through rheological examination.

To investigate interactions between gelatin and dextran, an investigation of the presence of the Maillard reaction was performed. Previous studies (Diftis and Kiosseoglou 2004; Ho and others 2000; Kato and others 1993), showed the Maillard

reaction was responsible for covalently conjugating proteins to polysaccharides and specifically dextrans to proteins. Hence, an investigation was performed in an effort to determine whether the Maillard reaction caused a covalent linkage between gelatin and dextran, using the *O*-Phthaldialdehyde procedure (OPA).

The *O*-Phthaldialdehyde procedure (OPA) technique examines the amount of free amino groups consumed during the Maillard reaction. The Maillard reaction is a non-enzymatic reaction involving the reaction of simple sugars (carbonyl groups) and amino acids (free amino groups). The Maillard reaction was considered because the components necessary for this reaction were present; free amino groups from the protein (gelatin) and a reducing sugar or reducing carbonyl end from the carbohydrate (dextran). Gelatin contains the amino acid lysine (Table 2.2) which is typically the most reactive amino acid in regard to the Maillard reaction because it possesses the ε-amino group (deMan 1999). The Maillard reaction is quite universal in the food industry, and this reaction occurs when most foods are heated and results in reactions that promote browning of cookies, bread, and other baked goods.

If the Maillard reaction was occurring within E-Matrix™, then the absorbance values indicative of the amount of free amino groups present in the compounds having undergone this reaction would decrease. Thus, using the OPA procedure the absorbency of gelatin compared to the absorbency of combinations of gelatin and dextran and E-Matrix™ were evaluated. If the Maillard reaction occurred the

absorbencies of the samples containing gelatin-dextran combinations would be less than from the 12% gelatin solution because the Maillard reaction would have reacted the amino groups, measured with the spectrophotometer.

In addition to the need of the required components for the Maillard reaction to occur, a free amino group and a non-reducing sugar, other factors influence the Maillard reaction such as, time, temperature, water content, water activity, pH, and concentration of the reactants. Although the required molecular components were present within E-Matrix™ the availability of additional environmental, chemical, and physical factors also influences the reaction.

The objective of the research was to examine the components which make up E-Matrix™ in an effort to better understand the properties of the medical device. Through examination of key E-Matrix™ features, such as rheological properties of each key ingredient, examining the effect of ionic strength, salt type, and presence of Maillard reaction, a better understanding of the material can benefit the manufacturer through understanding and potential for formulation changes.

5.2 MATERIALS AND METHODS

5.2.1 *O*-PHTHALDIALDEHYDE

The *O*-Phthaldialdehyde (OPA) procedure, a spectrophotometric procedure, was performed in order to determine the amount of free amino groups used during the Maillard reaction anticipated to occur between gelatin and dextran. The OPA reagent was made by combining 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (w/w) SDS; 40 mg of OPA (dissolved in 1 ml methanol); and 100 µl β-mercaptoethanol and diluted to a final volume of 50 ml with deionized water (Church and others 1983). Twenty micrograms protein was added to 1 ml OPA reagent and absorbance was measured with a spectrophotometer (UV16OU UV-Visible Recording Spectrophotometer; SHIMADZU) at 340 nm after 2 minutes. Solutions were vortexed to ensure proper mixing and homogeneity. A decrease in absorbance values in the complexes compared to the protein sample (12% gelatin) without the carbohydrate would have indicated a decrease in available free amino groups, indicating free amino groups had been used in forming the complex and that a complex had formed.

5.2.2 MONOVALENT AND DIVALENT SALTS

Gelatin (12% w/v) solutions with varying ionic strengths were prepared with the use of the monovalent salt NaCl and the divalent salt $CaCl_2$. Because E-Matrix^{TM} has an

ionic strength of 0.1279M, ionic strengths equivalent, above, and below this value were investigated. Solutions were prepared containing gelatin and either NaCl or $CaCl_2$ at ionic strengths of 0.05, 0.1279, and 0.25M. Deionized water was used as the solvent.

5.2.2.1 THE PROTOCOL

Each sample containing salt was examined using the E-Matrix™ rheological protocol (Table 5.1) similar to the protocol established for gelatin in Chapter 4 of this thesis. Seven specified points (viscosity at 23 s^{-1} during the initial and final shear rate ramps, gel point, melt point, and the complex moduli at 0.1 Hz during frequency sweeps at 50°C, 8°C, and 39°C) were recorded and compared using SAS statistical software (Cary, NC) with Tukey's procedure. Comparisons were made in regard to the affect of a monovalent versus divalent salt, ionic strength levels (0.05M, 0.1279M, and 0.25M), and a general comparison between the six component samples.

5.2.3 E-MATRIX™ COMPONENTS

The following components of E-Matrix™ were prepared and examined: 12% gelatin and 5% dextran, 12% gelatin, 17% gelatin, 5% dextran, and the gelatin-rich domain of E-Matrix™ (Table 5.6). The gelatin-rich domain was prepared by allowing a sealed vial of E-matrix™ to phase separate. Phase separation of E-Matrix™

occurred when the material was heated for an extended period of time. Thus one vial of E-Matrix™ was placed in a heating block (Multi-Block® Heater, LAB-LINE) for approximately 24 hours until a defined phase separation occurred, creating a gelatin-rich domain (Table 5.2) that surfaced to the top of the vial and a dextran-rich domain digressing to the bottom. A pipette was used to extract the gelatin-rich portion from the vial for further examination.

Solutions incorporating dextran were prepared with Dextran 500 Dry Powder (Amersham Biosciences; Uppsala, Sweden) supplied by Encelle, Inc. (Greenville, NC). The powder was stored at room temperature (23°C) in an air tight container. A 5% (w/w) gelatin solution was prepared by weighing 5 g dextran and adding deionized water until the desired weight (100 g) was achieved. The solution was stirred on a stir/hot plate (Barnstead/Thermolyne MIRAK™, Bubuque, IA) for 40 minutes.

The composition of a gelatin-rich domain and 5% (w/w) dextran solutions were determined by Microbac Laboratories, Inc. Southern Testing and Research Division (Wilson, NC), and is compiled in Table 5.2-3, respectively. The protein content was determined by the Kjeldahl method (Bradstreet 1965) to compute the nitrogen content. The nitrogen content was then multiplied by the nitrogen to protein conversion factor for gelatin (5.55) to determine the protein in the gelatin-rich domain and by the general conversion factor (6.25) to determine the protein content in the dextran sample. Moisture was determined by using a forced draft oven drying

method (Bradley 1998); lipid content was determined by hydrolytic extraction gas chromatography (AOAC 996.06 GC-FID) for total fat; ash was determined by a muffle furnace technique (AOAC 923) (Harbers 1998), and carbohydrate content was calculated as the remaining difference.

Gelatin was supplied in dry granule form by Encelle, Inc. (Greenville, NC). The granules were stored at room temperature (23°C) in air tight containers. A 17% (w/w) gelatin solution was prepared by weighing 17 g gelatin and adding deionized water until the desired mass (100 g) was achieved. The solution was simultaneously stirred and heated on a stir/hot plate (Barnstead/Thermolyne MIRAK^{TM}, Bubuque, IA) at 50°C for 40 minutes. Temperature was maintained with a thermal probe. Chapter 4 describes the procedure for preparing a 12% gelatin solution, and Table 4.1 contains the composition of a 12% gelatin solution.

Solutions of gelatin and dextran were prepared with the dextran and gelatin described previously. Dextran was first dissolved into the solvent, followed by heating at 50°C and gradual addition of gelatin. This solution was heated and simultaneously stirred for 40 minutes at 50°C.

5.2.3.1 THE PROTOCOL

Each sample was examined using the E-Matrix^{TM} protocol (Table 5.1). Seven specified points (viscosity at 23 s^{-1} during the initial and final shear rate ramps, gel

point, melt point, and the complex moduli at 0.1 Hz during frequency sweeps at 50°C, 8°C, and 39°C) were recorded and compared using SAS statistical software (Cary, NC) with Tukey's procedure. Comparisons were made between the each of the six samples.

5.3 RESULTS AND DISCUSSION

5.3.1 *O*-PHTHALDIALDEHYDE

Representative results produced from the OPA assay are presented in Table 5.4. Although the average 12% gelatin-5% dextran absorbance value (0.207) was less than that of 12% gelatin (0.231), assumed to not have any amino acids, the 12% gelatin-5% dextran average absorbance value falls within the spread of the two 12% gelatin absorbencies. Therefore, the gelatin-dextran values cannot be considered different than the 12% gelatin values with respect to the amount of free amino groups used during the Maillard reaction. The gelatin-dextran samples prepared with a saline solution to create an equivalent ionic strength to that of E-Matrix™ performed similarly to the gelatin-dextran solution containing no additional salt. The gelatin-dextran sample with salt had an average decreased absorbency compared to the 12% gelatin sample, but once again, looking at the individual absorbency readings, these values fall within the range of the individual 12% gelatin absorbencies.

E-Matrix™ absorbency values were the largest of the samples examined. For this material, the Maillard reaction may in fact still be occurring, using free amino groups and thus reducing the absorbency. However the E-Matrix™ formulation differs from that of 12% gelatin besides the addition of 5% dextran, E-Matrix™ also contains additional amino acids. Thus even though the Maillard reaction may be occurring, causing a reduction in the amount of free amino groups present, the additional amino acids from the formulation also contribute to the spectrophotometric measurements. Consequently, gelatin amino acids are not likely reacting with dextran. Besides, Kosmala and others (2000) argued gelatin and dextran are held together with a physical not chemical crosslink. Therefore, due to the similarity in absorbencies among samples and the additional factor of the amino acid formulation, the OPA procedure results are inconclusive in determining whether the Maillard reaction has occurred within E-Matrix™. Seeing as gelatin and dextran are physically, not chemically, bound, the Maillard reaction is not of significant importance to E-Matrix™.

5.3.2 MONOVALENT AND DIVALENT SALTS

Rheological points of interest throughout the seven step protocol were recorded and examined for each of the six gelatin samples containing salt (Table 5.5). The affects from monovalent and divalent salts on the rheological properties of a 12% gelatin solution was considered. Analysis using the popular SAS software determined the following in regard to the desired comparisons examined. First there was no

significant difference between the rheological properties of the samples containing NaCl versus those containing $CaCl_2$ overall. Hence the use of a monovalent versus divalent salt at the concentrations studied did not make a difference in the rheological properties studied. However, the samples themselves were significantly different from each other, and the ionic strength of the sample produced rheological properties which were significantly different.

Adding salt at the ionic strength concentration equivalent to that in E-Matrix™ (0.1279M), lowered the viscosity of a 12% gelatin solution (Figure 5.1). In addition, an increase in ionic strength concentration caused a decrease in storage moduli (G′) Figures 5.2a-b. Although the trend was not linear with concentration, the lower concentrations (0.05 and 0.1279M) have consistently higher storage moduli than the higher salt concentration (0.25M) at 8°C for both NaCl and $CaCl_2$. This corresponds with work done by Haug and others (2004) using fish gelatin. Various ionic strengths showed increased G′ at low ionic strengths, attributed to electrostatic interactions (Haug and others 2004). The addition of salt caused screening from short range electrostatic interactions which decreased the ability of the α-chains to associate and form electrostatic bridges (Haug and others 2004), hence aggregation was challenged causing a decrease in storage moduli. A decrease in storage moduli, corresponding with ionic strength, was not observed at an elevated temperature (50°C) (Figure 5.2c). Also, Haug and others (2004) found ionic strength affected the gel and melt point. An ionic strength increase, resulted in decreased gel and melt points. This trend was also seen in the present study with regard to gel

point, but not for melt point. If more diverse ionic strengths had been examined, perhaps melt points may have followed a similar trend as Haug and others (2004) found. This decline in gel points is again attributed to a reduced electrostatic interaction preventing attractive inter-chain bridging and gelation (Haug and others 2004). In conclusion these results imply the amount of salt in a 12% gelatin solution affects rheological properties, in general the rheological properties were significantly different from each other, and the addition of salt depresses viscosity, storage modulus, and gel point, suggesting electrostatic interactions are influential in regard to the gelatin gel network formation and stability.

5.3.3 E-MATRIX^{TM} COMPONENTS

Rheological points of interest throughout a seven step protocol were recorded and examined for each of the six samples (Table 5.6). Each of the six samples were statistically compared and found to differ significantly with respect to all rheological properties examined except for the specified point during the frequency sweep at 39°C. The fact that some rheological properties do not show significant differences between samples may imply some properties are more sensitive to the protocol step than others.

At the initiation of the protocol, the viscosity determined at a designated shear rate (23 s^{-1}) differed significantly for all the samples except for the E-Matrix^{TM} and 12% gelatin-5% dextran samples. Since the majority of the samples differed significantly,

the viscosity differences were not solely due to the percent solids present in the sample. This observation is likely true because the 17% gelatin and E-Matrix™ sample were significantly different and composed of approximately the same solids content. Making further comparisons, E-Matrix™ and the 12% gelatin-5% dextran sample appeared to not be significantly different in several cases; initial viscosity and complex modulus (G*) during the 50°C and 8°C frequency sweeps. All samples appeared to not have significantly different gel points except for the 12% gelatin solution which differed from all the samples. Therefore, it appears gelatin is responsible for the properties associated with network structure; shear moduli at 8°C, gel point, and melt point.

However dextran and physical interactions between dextran and gelatin contribute the viscous properties, specifically viscosity and those properties when there is little network, shear moduli at 50°C and 39°C. Dextran and the gelatin-dextran interaction appear to be responsible for the viscous properties because the viscosity measurements and shear moduli measurements at 50 and 39°C are not significantly different for E-Matrix™ and 12% gelatin-5% dextran samples.

The six samples were also compared graphically in Figure 5.3. This figure shows the samples have differing viscosities at 50°C during the shear rate ramp run at the end of the protocol. The 5% dextran solution is the least viscous fluid and initially the gelatin-rich domain is the most viscous. However, since the gelatin-rich domain is pseudoplastic, the viscosity decreases with shear rate to a viscosity similar to the

12% gelatin-5% dextran solution. Graphically, the gelatin-rich domain and the 12% gelatin-5% dextran solutions appear similar and the 17% gelatin and E-Matrix™ samples also appear similar in regard to viscosity.

The samples were rheologically characterized (Table 5.7) at 50°C and all six samples exhibit Newtonian flow behavior (n = 1.0) when using the power law equation for comparison (Equation 1) except for the gelatin-rich domain. Plots of shear stress versus shear rate and the incorporation of the power law equation (Equation 1) were used to represent a general relationship describing the flow behavior of the samples, where σ is shear stress or force per area, K is a material constant called the consistency coefficient, $\dot{\gamma}$ is shear rate or the change in shear strain with respect to time, and n is another material constant called the flow behavior index.

$$\sigma = \kappa(\dot{\gamma})^n \quad \textbf{(1)}$$

However the gelatin-rich domain of a gelatin-pullulan mixture showed differing results. Ding and others (2005) found that above the gelling temperature, the gelatin-rich domain of a gelatin/pullulan mixture is approximately Newtonian, but as the temperature is lowered towards the gelation point, the material becomes strongly non-Newtonian and G′ and G″ become time-dependent. Hence the dextran and pullulan components present in the gelatin-rich portions may be influential in the rheological characterization of these samples.

5.4 CONCLUSIONS

It is important to consider the critical components which make up E-Matrix™ to comprehend how the functional and mechanical roles from these components contribute to E-Matrix™ function. Hence it is crucial to examine identifiable components or variables of E-Matrix™. For that reason the rheological properties of these components were determined and compared. The rheological consequence of using a monovalent versus a divalent salt was considered and found to not exhibit a significant difference when implemented into a 12% gelatin solution. However, the amount of salt present in the solution did create a significant difference in the rheological properties by decreasing storage moduli at 8°C and decreasing viscosity and gel point, suggesting ionic strength affects gelatin gel formation and stability. Furthermore rheological properties of similar materials to that of E-Matrix™ were identified and showed significant differences.

5.5 REFERENCES

Church FC, Swaisgood HE, Porter DH, Catignani GL. 1983. Spectrophotometric Assay Using o-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk Proteins. J Dairy Sci 66:1219-1227.

deMan JM. 1999. Principles of Food Chemistry. Aspen Publishers, Inc.: Gaithersburg, MD. 120-121.

Ding P, Packek AW, Frith WJ, Norton IT, Wolf B. 2005. The effect of temperature and composition on the interfacial tension and rheology of separated phases in gelatin/pullulan mixtures. Food Hydrocolloids 19(3):567-574.

Gekko K, Fukamizu M. 1991. Effect of pressure on the sol-gel transition of gelatin. Int J Biol Macromol 13:295.

Haug IJ, Draget KI, Smidsrod O. 2004. Physical and rheological properties of fish gelatin compared to mammalian gelatin. Food Hydrocolloids 18(2):203-213.

Ho YT, Ishizaki S, Munehiko T. 2000. Improving emulsifying activity of ε-polylysine by conjugation with dextran through the Maillard reaction. Food Chem 68(4):449-455.

Kosmala JD, Henthorn DB, Brannon-Peppas L. 2000. Preparation of interpenetrating networks of gelatin and dextran as degradable biomaterials. Biomaterials 21(20):2019-2023.

Table 5.1. Rheological protocol for E-Matrix™

Step #	Test	Thermal Rate	Stress	Shear Rate	Frequency	Temperature
1	Initial Shear Rate Ramp			5-150 s^{-1}		50°C
2	Mechanical Spectra		0.05 Pa		0.01-10 Hz	50°C
3	Cooling Ramp	-1°C/min	0.05 Pa		0.1 Hz	50-8°C
4	Mechanical Spectra		200 Pa		0.01-10 Hz	8°C
5	Heating Ramp	1°C/min	200 Pa		0.1 Hz	8-39°C
6	Mechanical Spectra		0.05 Pa		0.01-10 Hz	39°C
7	Final Shear Rate Ramp			5-150 s^{-1}		50°C

Table 5.2. Composition of gelatin-rich domain

Analysis	(%)	Method
Protein	16.2	Kjeldahl
Carbohydrates	1.23	Calculation
Moisture	81.7	Forced Draft Oven
Ash	0.87	AOAC 923
Total Fat	--	AOAC 996.06 GC-FID

*Determined by Microbac Laboratories, Inc. Southern Testing and Research Division, (Wilson, NC)
**Total fat content was <0.1

Table 5.3. Composition of 5% dextran samples

Analysis	(%)	Method
Protein	0.38	Kjeldahl
Carbohydrates	4.17	Calculation
Moisture	95.2	Forced Draft Oven
Ash	0.10	AOAC 923
Total Fat	0.152	AOAC 996.06 GC-FID

*Determined by Microbac Laboratories, Inc. Southern Testing and Research Division, (Wilson, NC)

Table 5.4. Absorbency of free amino groups present following the OPA procedure

Sample	12% Gelatin	12% Gelatin-5% Dextran	12% Gelatin-5% Dextran 0.1279 Ionic Strength	E-Matrix™ 0.1279 Ionic Strength
Absorbency	0.200	0.205	0.215	0.389
Absorbency	0.261	0.208	0.240	0.389
Average	0.231 (0.031)	0.207 (0.002)	0.228 (0.013)	0.389 (0.000)

*(Standard error)
**340 nm

Table 5.5. Comparison of salts and salt concentrations

Sample	Initial Viscosity (50°C at 23 s^{-1})	Final Viscosity (50°C at 23 s^{-1})	Gel Point	Melt Point	G* at 0.1 Hz 50°C	G* at 0.1 Hz 8°C	G* at 0.1 Hz 39°C
12% Gelatin 0.05M NaCl	[a]0.0178 Pa s (0.0004)	[c]0.0187 Pa s (0.0002)	[a]25.7°C (0.0)	[a]32.6°C (0.4)	[b]0.0143 Pa (0.0003)	[a,b]14300 Pa (31.8)	[a]0.0249 Pa (0.00126)
12% Gelatin 0.1279M NaCl	[a]0.0189 Pa s (0.0003)	[a,b,c]0.0202 Pa s (0.0004)	[a]25.8°C (0.0)	[a]32.0°C (0.1)	[a,b]0.0156 Pa (0.0005)	[a]15000 Pa (142)	[a]0.0304 Pa (0.00213)
12% Gelatin 0.25M NaCl	[a]0.018 Pa s (0.0003)	[a,b]0.0214 Pa s (0.0004)	[b]24.9°C (0.0)	[a]32.0°C (0.2)	[a,b]0.0153 Pa (0.0003)	[b]13400 Pa (164)	[a]0.0291 Pa (0.00119)
12% Gelatin 0.05M $CaCl_2$	[a]0.0185 Pa s (0.0003)	[a,b,c]0.0200 Pa s (0.0003)	[a]25.7°C (0.3)	[a]32.7°C (0.4)	[a,b]0.0151 Pa (0.0006)	[a]14800 Pa (401)	[a]0.0268 Pa (0.00143)
12% Gelatin 0.1279M $CaCl_2$	[a]0.0189 Pa s (0.0004)	[b,c]0.0197 Pa s (0.0002)	[b]24.9°C (0.3)	[a]31.7°C (0.5)	[a,b]0.0150 Pa (0.0001)	[a,b]14100 Pa (117)	[a]0.0265 Pa (0.000460)
12% Gelatin 0.25M $CaCl_2$	[a]0.0191 Pa s (0.0001)	[a]0.0216 Pa s (0.0006)	[b]24.8°C (0.0)	[a]31.6°C (0.1)	[a]0.0162 Pa (0.0003)	[b]13400 Pa (189)	[a]0.0289 Pa (0.000969)

*(Standard error)
**Means with the same letter are not significantly different

Table 5.6. Comparison of materials

Sample	Initial Viscosity (50°C at 23 s^{-1})	Final Viscosity (50°C at 23 s^{-1})	Gel Point	Melt Point	G* at 0.1 Hz at 50°C	G* at 0.1 Hz at 8°C	G* at 0.1 Hz at 39°C
E-Matrix™	[a]0.0938 Pa s (0.0010)	[a,b,c]0.0604 Pa s (0.0010)	[a]27.9°C (0.3)	[a,b]33.7°C (0.2)	[a]0.105 Pa (0.001)	[c]13800 Pa (115.3)	[a]0.0918 Pa (0.00340)
12% Gelatin	[d]0.0210 Pa s (0.00132)	[b,c]0.0214 Pa s (0.0013)	[b]25.7°C (0.0)	[b]32.7°C (0.4)	[d]0.0166 Pa (0.001)	[c]16200 Pa (622)	[a]0.0291 Pa (0.00209)
17% Gelatin	[c]0.0582 Pa s (0.0006)	[a,b,c]0.0643 Pa s (0.0010)	[a]27.8°C (0.2)	[a]35.7°C (1.1)	[c]0.0475 Pa (0.0007)	[a]28700 Pa (133)	[a]0.102 Pa (0.00224)
5% Dextran	[e]0.00404 Pa s (0.00004)	[c]0.00448 Pa s (0.00004)	—	—	[e]0.00688 Pa (0.0001)	[d]4.00 Pa (0.00802)	[a]0.00556 Pa (0.001425)
12% Gelatin-5% Dextran	[a]0.0958 Pa s (0.0016)	[a,b]0.0981 Pa s (0.0170)	[a]28.6°C (0.0)	[a,b]33.7°C (0.2)	[a]0.110 Pa (0.0020)	[c]15943 Pa (82.1)	[a]0.1920 Pa (0.0419)
Gelatin-rich domain	[b]0.0708 Pa s (0.0011)	[a]0.1221 Pa s (0.0409)	[a]28.0°C (0.2)	[a,b]35.2°C (0.5)	[b]0.0635 Pa (0.0024)	[b]22600 Pa (2350)	[a]5.21 Pa (5.08)

*(Standard error)
**Means with the same letter are not significantly different

Table 5.7. Comparison of materials via power law equation

Material	K	n	R^2
12% Gelatin	0.024	0.97	1.0
17% Gelatin	0.068	0.98	1.0
E-Matrix™	0.065	0.98	1.0
5% Dextran-12% Gelatin	0.13	0.91	1.0
Gelatin-rich domain	0.28	0.75	1.0
5% Dextran	0.0046	1.0	1.0

*T = 50°C

** $y = K\dot{\gamma}^n$

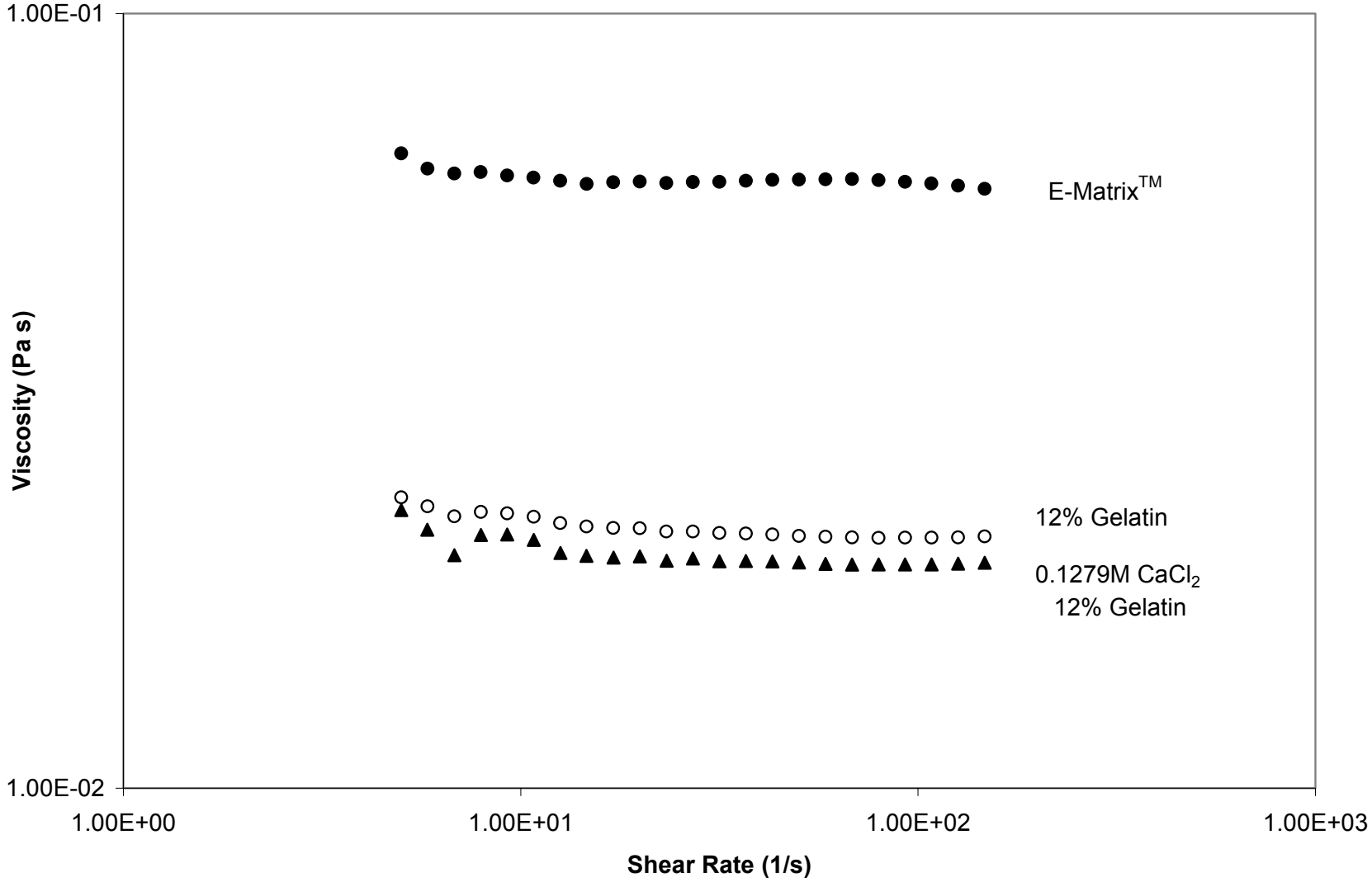


Figure 5.1. Comparison of final shear rate ramps at 50°C

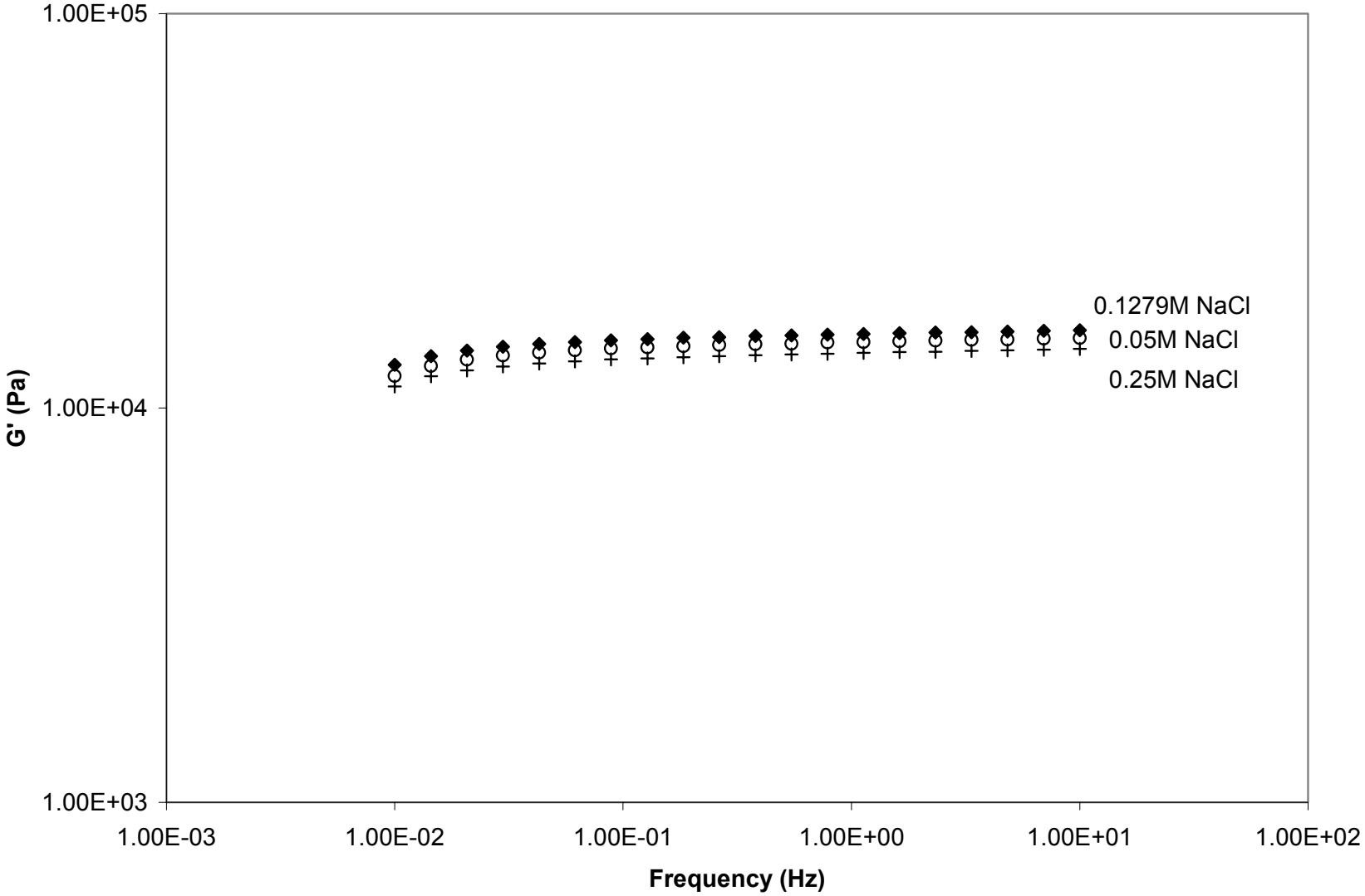


Figure 5.2a. Frequency sweep at 8°C comparing 12% gelatin solutions at differing ionic strength concentrations

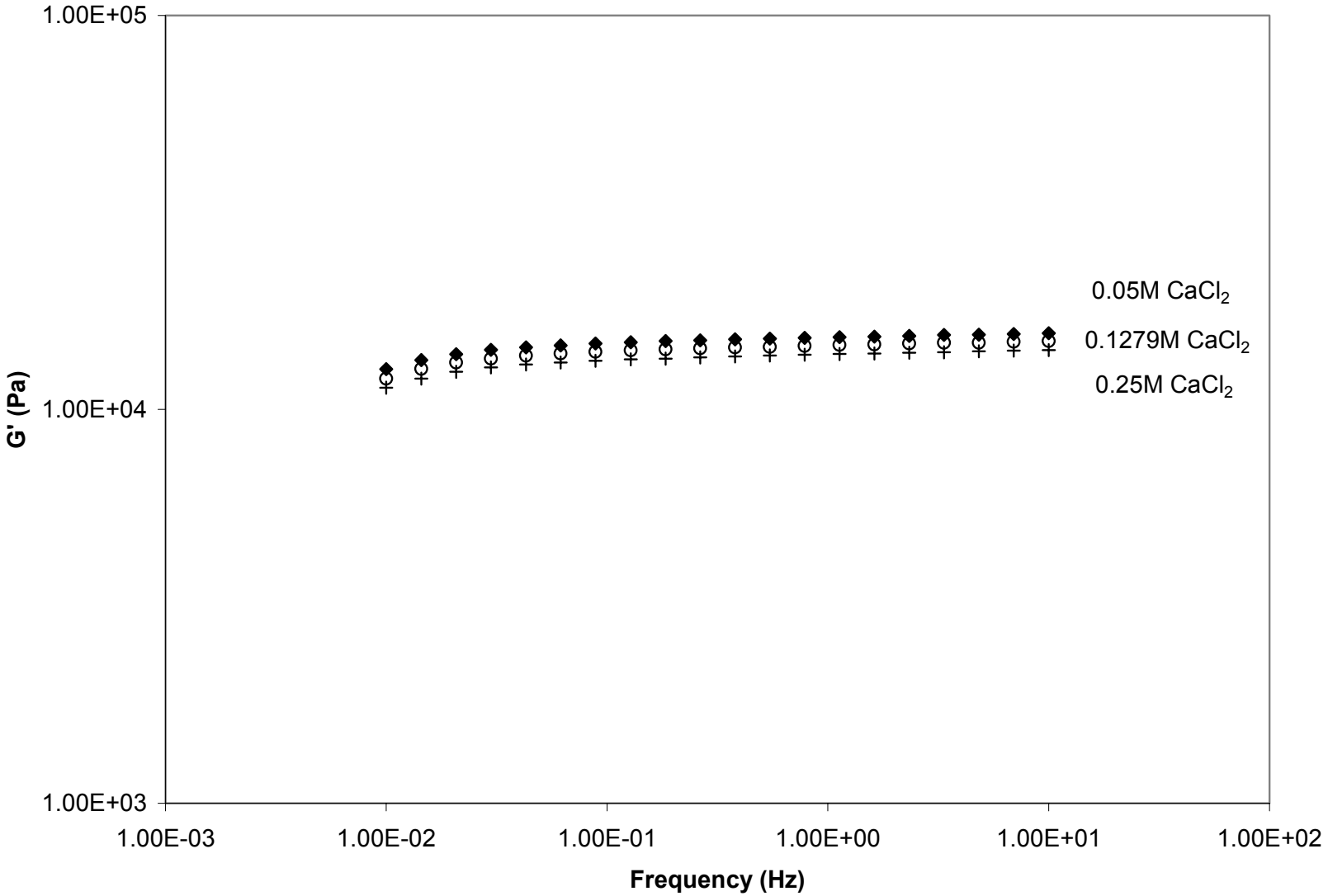


Figure 5.2b. Frequency sweep at 8°C comparing 12% gelatin solutions at differing ionic strength concentrations

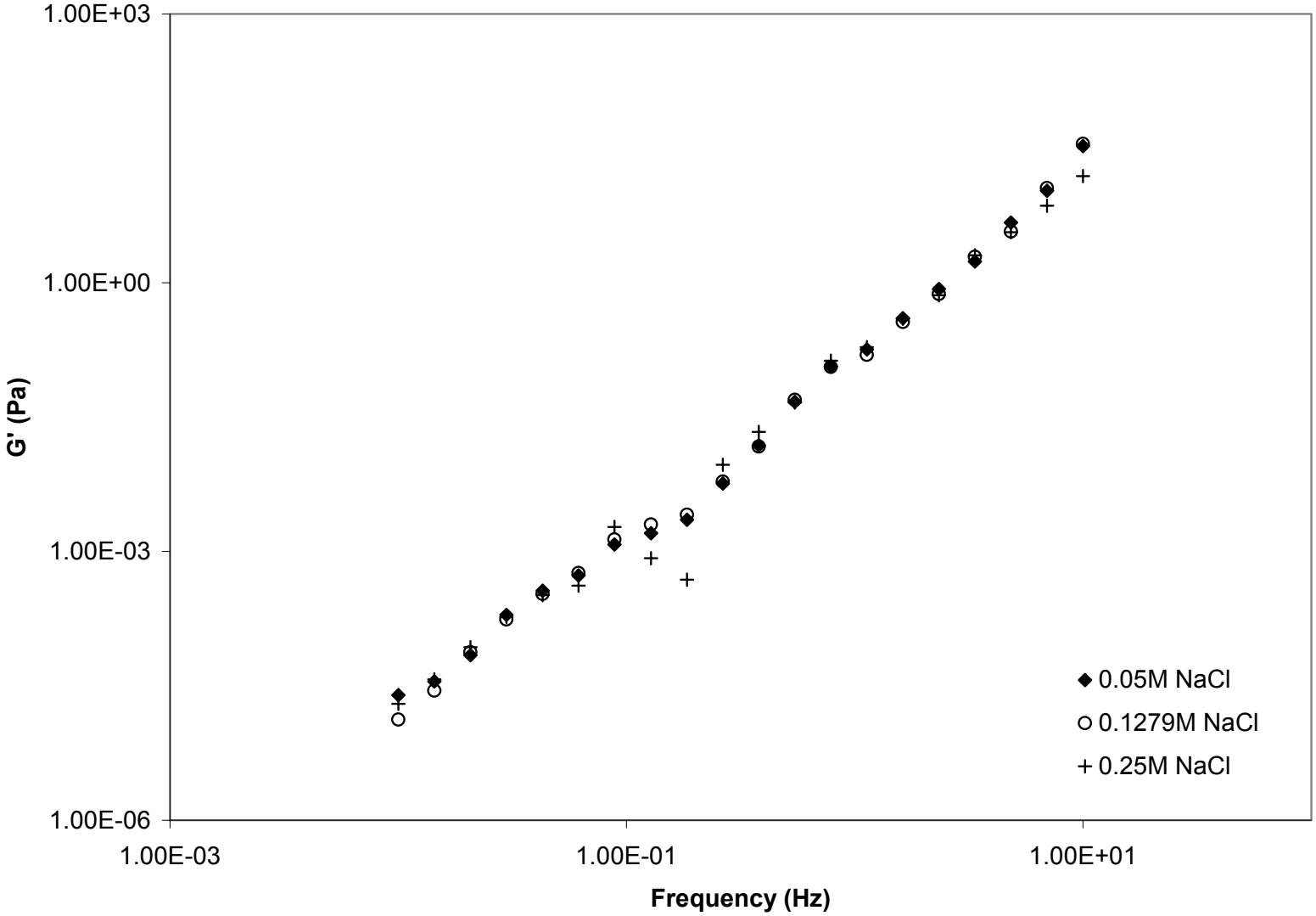


Figure 5.2c. Frequency sweep at 50°C comparing 12% gelatin solutions at differing ionic strength concentrations

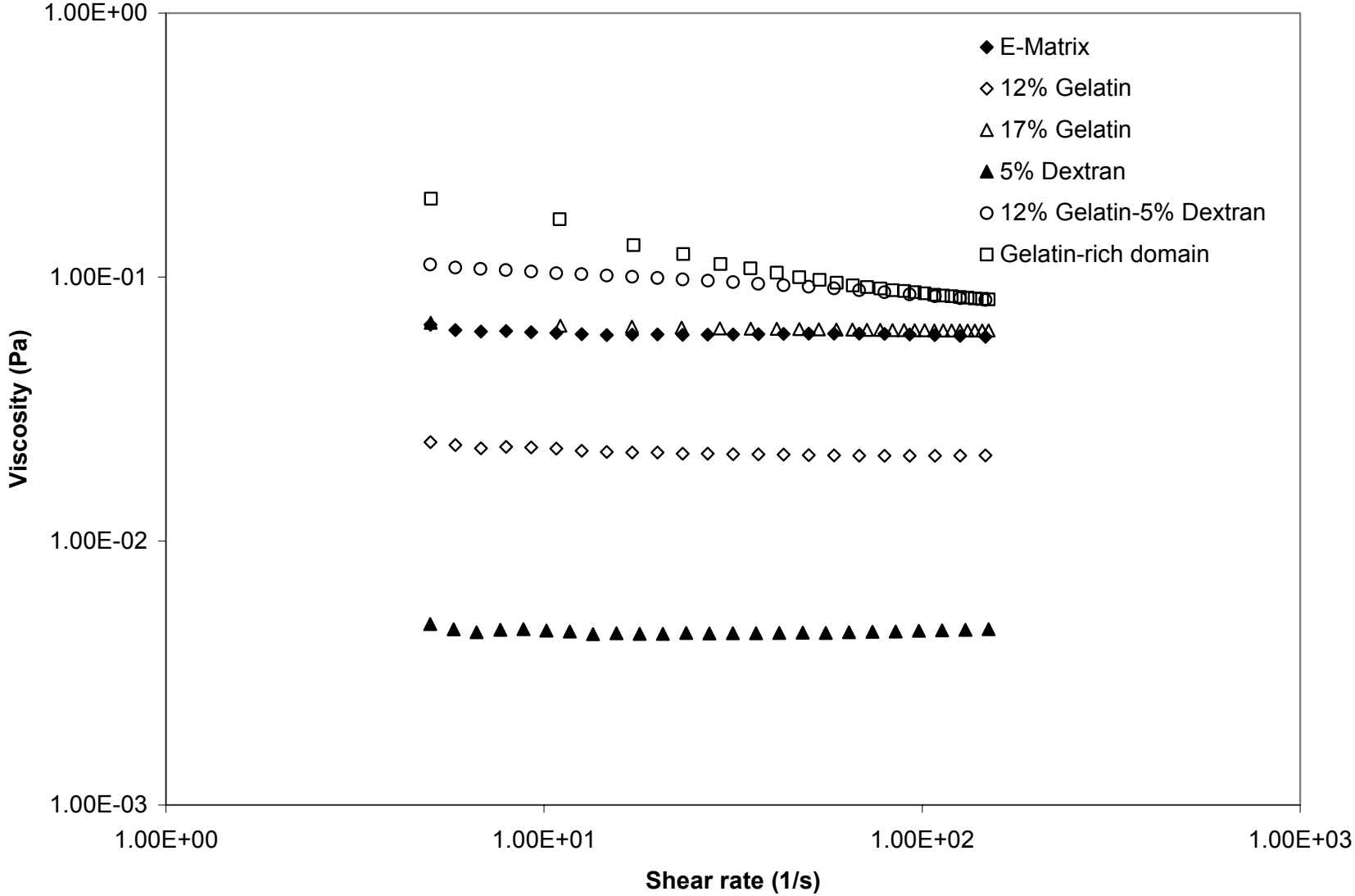


Figure 5.3. Comparison of E-Matrix™ components during final shear rate ramps at 50°C