**Flocculation of a Kaolin Clay Slurry by Utilizing Specified Risk Material**

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Bioresource Technology

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# Abstract

The oil sands are a vital part of the provincial economy in Alberta and oil production in this sector produces large amounts of waste called tailings. The tailings consist of a slurry of clay suspended in process water with residual bitumen present. Reclamation of the land that the tailings ponds occupy is of great concern to the province and the oil producers. One strategy to consolidate this material is to facilitate separation of the solids from the liquid phase using a chemical called a flocculant. Traditional flocculants may not be environmentally sustainable and other alternatives have been sought after. To this end, a waste resource of biological origin called specified risk materials (SRM) has the potential for use in this application.

Specified risk materials are waste proteins from the rendering of cattle that have the potential to contain prion diseases, such as Bovine Spongiform Encephalopathy (mad cow disease). Currently, this material is being land-filled or incinerated and is an economic burden to the industry as well as a liability to the local environment. To first make this material safe to use, it must first be hydrolyzed into its molecular components; peptides. These peptides can then be used in various applications and can also be chemically modified to alter its properties.

In this work, peptides derived from hydrolyzed SRM were tested as a flocculant in a model kaolin clay system. The peptides were then chemically modified using two different approaches. The first modification was an esterification reaction with methanol to modify the functional groups of the peptides. The second modification was a crosslinking reaction with glutaraldehyde to increase the molecular weight of the peptides. The flocculation performance of the modified peptides was tested in flocculation experiments and was compared to the unmodified version. Characterization analyses were also conducted on the various peptide products including Fourier

transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA), carboxylic acid titration, sodium dodecyl sulphate gel electrophoresis (SDS-PAGE), high performance liquid chromatography (HPLC) and elemental analysis.

To summarize, hydrolyzed SRM peptides can act as a flocculant in this model system. Modification of this material by crosslinking with glutaraldehyde improves the flocculation performance of the peptides in this system and is recommended for future use in real world tailing consolidation applications. The characterization tests confirmed that the crosslinking reaction was occurring as theoretically expected. The use of this material could provide a biodegradable alternative to the synthetic polymers used in the industry, while also providing a source of nitrogen for plant growth during reclamation. This has the potential to convert an economic and environmental burden into a material that could benefit the environment by aiding in the reclamation of oil sand tailing ponds.

## Preface

This thesis is an original work by Jesse Yuzik. No part of this thesis has been previously published.

# Acknowledgements

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# List of Abbreviations

ATR - Attenuated Total Reflectance

BSE - Bovine Spongiform Encephalopathy

$2H_2O \cdot CaSO_4$ - Calcium sulphate dihydrate (Gypsum)

COD - Chemical Oxygen Demand

Da - Dalton

DNA - Deoxyribonucleic acid

FFT - Fluid Fine Tailings

FTIR - Fourier Transform Infrared Spectroscopy

GA-Pep - Glutaraldehyde Crosslinked Peptones

HPAM - Hydrolyzed Polyacrylamide

HPLC - High Performance Liquid Chromatography

IR - Infrared

MFT - Mature Fine Tailings

MW- Molecular Weight

NMR - Nuclear Magnetic Resonance

PAM - Polyacrylamide

PEG - Polyethylene Glycol

PVA - Polyvinyl Alcohol

SEC-HPLC - Size Exclusion High Performance Liquid Chromatography

SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SRM - Specified Risk Materials

SPW - Synthetic Process Water

TGA - Thermal Gravimetric Analysis

UV - Ultraviolet

wt - weight

# 1. Introduction

## 1.1. Project Background

Petroleum and petrochemicals accounted for 71.4% of the Alberta provincial exports in 2018 making the viability of the oil sands industry of vital importance (Statistics Canada and Alberta Economic Development and Trade). Production of oil in this sector relies on the use of the Clarke hot water extraction method. In this procedure, oil is separated from the sediments present by injection of hot water into the bitumen-rich layer of the ground. The hydrocarbon-rich bitumen stream is isolated and the remaining byproduct, which consists of water with fine clays and residual bitumen, is called tailings (Allen, 2008). These tailings are stored for long periods of time in tailings ponds, which are large man-made ponds supported by dykes. Consolidation and reclamation of tailings ponds is a considerable challenge that the industry is currently facing and are an environmental and economic burden. With the inventory of these ponds growing by the day, it is essential to find economic and environmentally friendly ways to manage the tailings and reclaim the land that they occupy.

Specified risk materials (SRM) are waste products from the animal meat rendering industry that have the potential to be transformed into high value products. SRM are defined as risk materials because they are the tissues of the livestock that have the potential to contain prion diseases, such as bovine spongiform encephalopathy (BSE), more commonly known as mad cow disease. These tissues include the brain, eyes and spinal column of the animal. In Canada, these materials were once used as animal feed, pet foods and fertilizers, but now must be land-filled or incinerated after the Canadian Food Inspection Agency passed enhanced regulation on this material in 2007. However, this waste material can be made safe to use in other applications after it undergoes a thermal or alkaline hydrolysis. Adding value to SRM would convert a costly, potentially dangerous waste into a product that could ultimately become a revenue source for the rendering industry again.

**1.2. Objectives**

The goal of this research project was to investigate hydrolyzed peptides from specified risk materials (SRM) as flocculating agents for the reclamation of oil sand tailings ponds in Alberta. This would provide an opportunity for utilization of a waste product from one industry to help solve the problem of another industry. The first goal was to test the hydrolyzed peptides as a flocculant in a model kaolin clay system. The next step was to chemically modify the peptides to enhance their flocculation rate through two different methods. Parameter studies were then explored in an attempt to determine the local optimum when varying different reaction and flocculation parameters. Multiple characterization analyses were also performed on the products of these reactions to qualify and quantify any modifications that were occurring. The purpose of this was to attempt to explain the changes in flocculation activity that were observed after a reaction.

# 2. Literature Review

**2.1. Alberta Oil Sand Tailings**

An industry that is vitally important to not only the economy of Alberta, but to Canada as a whole, is the oil sands industry. In the Alberta oil sands, the oil extraction process produces large quantities of waste called tailings, which are kept in ponds. A general schematic of oil sands extraction and the tailings reclamation process is provided below.
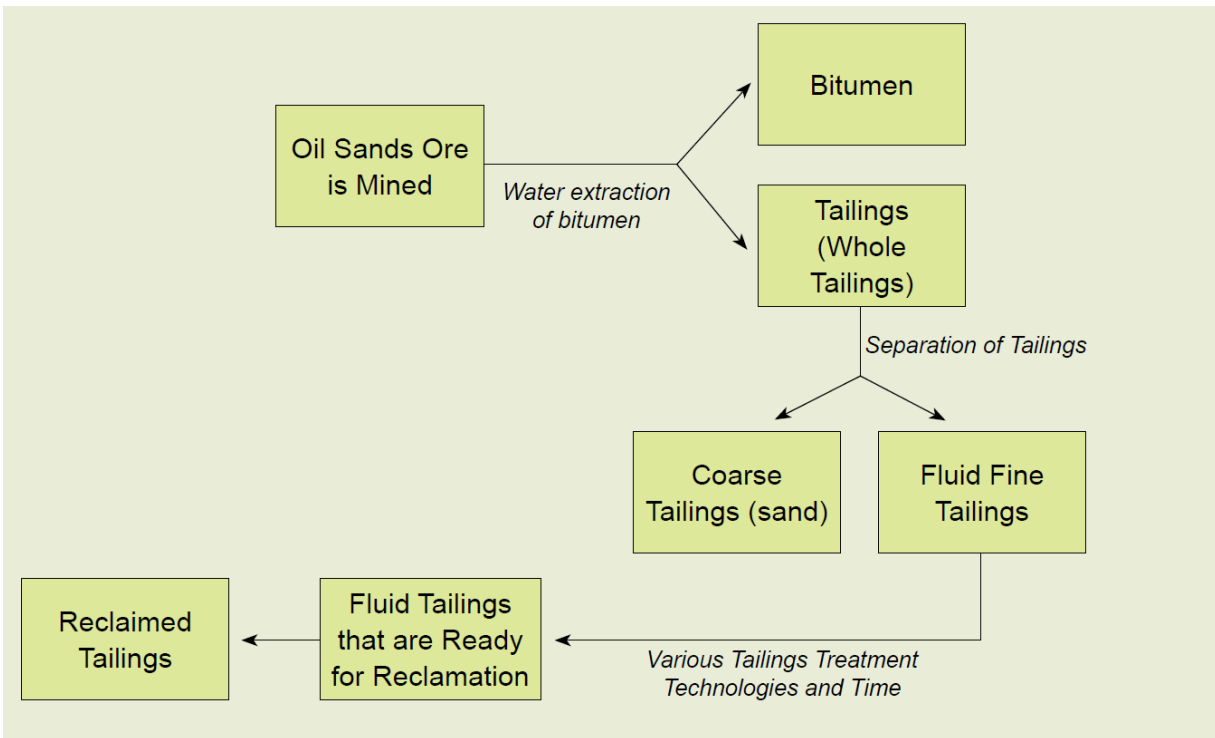
**Figure 1.** Simplified diagram of the oil sands mining and bitumen extraction process. Obtained from the Lower Athabasca Region Tailings Management Framework for the Mineable Athabasca Oil Sands report 2015 (Government of Alberta, 2015). License from: *https://open.alberta.ca/licence*

The tailings consist largely of a slurry of fine clay particles dispersed in process water. After a sufficient settling time of a few years, the larger coarse sand and clay particles settle out of the solution and the remaining material is classified as fluid fine tailings (FFT). FFT are comprised of a colloidal suspension of fine clay particles with the average of 85% being <44 μm (Kaminsky & Omotoso, n.d.). There is not an appreciable amount of settling in FFT due to the small particle size and thus, settling occurs on the order of decades (Allen, 2008). There is a large variability of ion concentrations present in the ponds including $HCO_3^-$, $Na^+$, $SO_4^{2-}$, $Ca^{2+}$, $Mg^{2+}$, $NH_4^+$ and $Cl^-$, which alters the water chemistry of each pond (Allen, 2008). The pH of the ponds is also variable but normally falls within a basic range of 7.7-8.6 (Allen, 2008; Mahaffey & Dubé, 2017). The amount of water present in the tailings is also important and has a large impact on the physical strength that the slurry can withstand, as shown in Figure 2 (Beier, Wilson, Dunmola, & Sego, 2013). From this figure, a higher solids content correlated to a higher strength, which was required to meet reclamation standards. There is also variable levels of residual bitumen,

organics, and other hydrocarbons present in the ponds. The complexity and variability of these ponds makes reclamation a difficult task. Reclamation involves separating the solid clay particles from the aqueous water phase so the water can be recycled and removed, and the land can be returned to its original state.
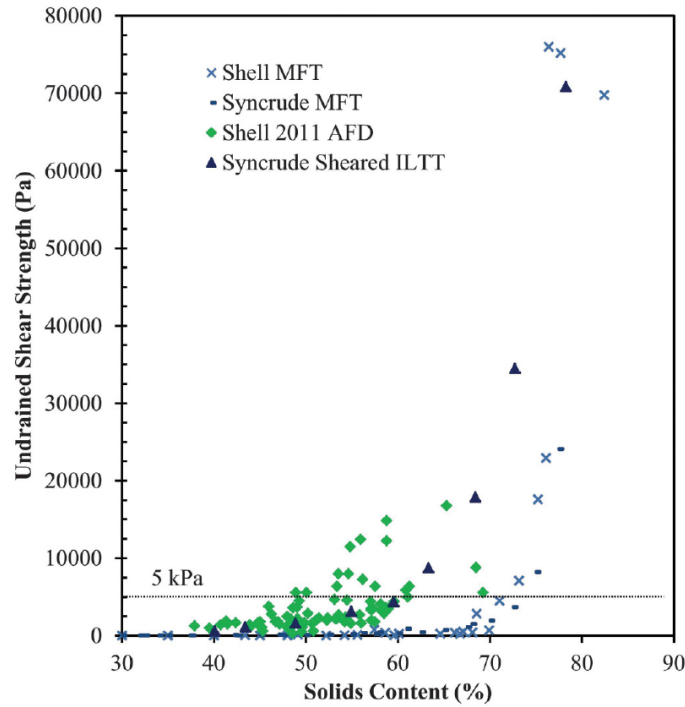


**Figure 2.** The effect of solid content on the shear strength of tailings from different locations (Beier et al., 2013).

In 2015, the Alberta government released a tailings management framework, which states that a tailings pond must be in a ready to reclaim state after 10 years from the end of the project (Government of Alberta, 2015). In 2017, the Alberta Energy Regulator introduced Directive 85, a document that outlined the conditions that oil sand companies must meet for tailings management. One goal of this directive was to outline ways to limit tailings volumes and to provide performance properties that must be met in order to meet ready to reclaim status (AER, 2017). The technologies currently employed for tailings consolidation include centrifugation, composite tailings, in-line thickening, and pond settling (Sobkowicz, 2012). In the cases of centrifugation and in-line thickening, the use of a flocculating agent is often employed to enhance the dewatering of the slurry (Beier et al., 2013). According to the Alberta Government website, at the end of 2013, tailings ponds occupied 88 km² of land with a volume of fine fluid

tailings of 975.6 million m³ (Government of Alberta, 2019). A map to illustrate the area of Alberta that the oil sand tailings ponds occupy is shown in Figure 3.
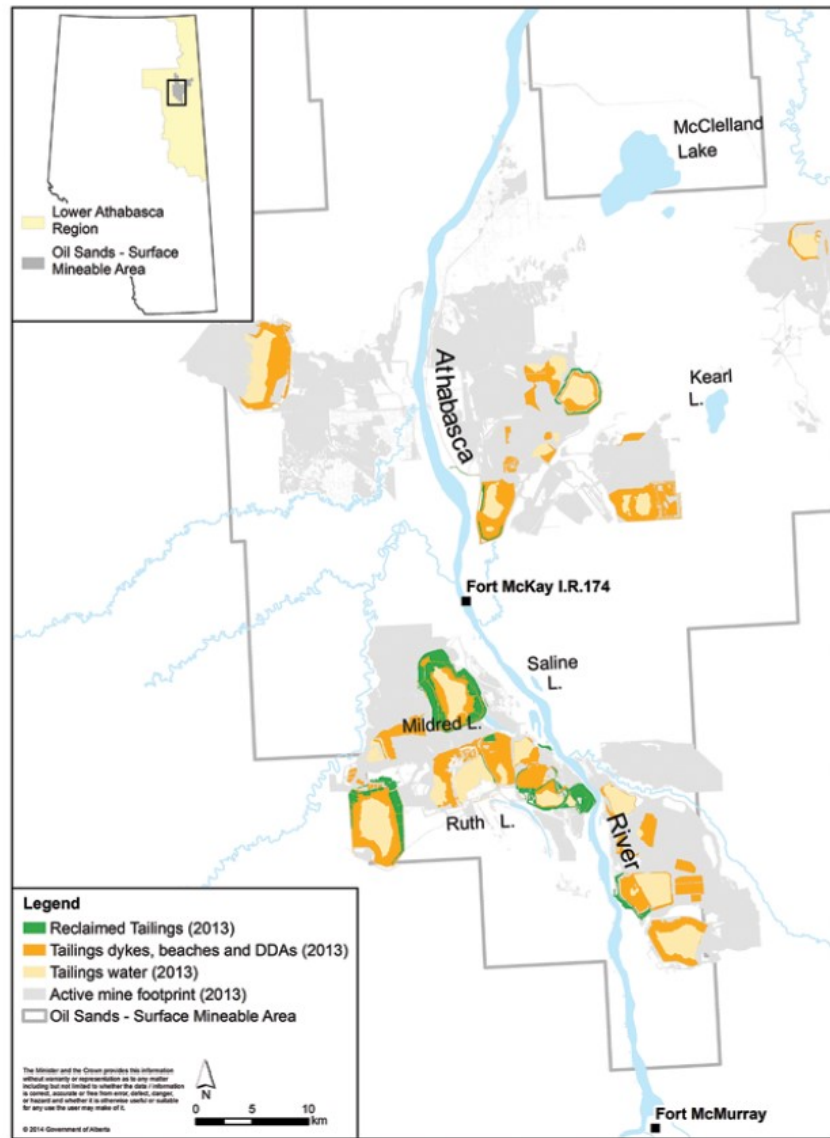


**Figure 3.** Alberta oil sands mineable area with active and reclaimed tailings sites. Obtained from the Lower Athabasca Region Tailings Management Framework for the Mineable Athabasca Oil Sands report 2015 (Government of Alberta, 2015). License from: *https://open.alberta.ca/licence*

Due to this substantial volume, treatment of the tailings ponds through various methods costs the industry a considerable amount of money. The other aspect is that tailings ponds are an environmental concern to northern Alberta due to both the land use and wildlife, particularly

fish, that is negatively impacted by habitat loss and toxic process water exposure (Mahaffey & Dubé, 2017; Matthews, Shaw, Mackinnon, & Cuddy, 2002). Due to the large variability in each tailing pond's composition, finding one chemical that can treat all tailing ponds is unlikely. Instead, a multitude of approaches and chemicals may need to be utilized and tailored to treat ponds on a case by case basis.

## 2.2. Colloidal Suspensions

Colloidal suspensions are classified as two-phase systems where one of the phases is dispersed within another. There can be many types of colloids including liquids dispersed in liquids (i.e. emulsions), gases dispersed in liquids (i.e. foams), liquids dispersed in gases (i.e. aerosols), and solids dispersed in liquids (either sols or colloidal suspensions) (Shaw, 1992). The particles of the dispersed phase have a high surface area to volume ratio, which helps them to remain in suspension (Shaw, 1992). The particle sizes for colloids tend to be from the nano scale ($10^{-9}$ m) to the micro scale ($10^{-6}$ m), but there is not an exact size cut-off, and larger particles can exert colloidal behavior (Kontogeorgis & Kiil, 2016; Partch, Matijevic, Hodgson, & Aiken, 1983). There are two major forces that will determine the stability of a colloidal suspension, the attractive van Der Walls forces and the repulsive electrostatic forces as shown in Figure 4 (Kontogeorgis & Kiil, 2016). The van Der Walls forces are caused by the alignment of the dipoles of the interacting particles, where a random dipole in one particle will induce a dipole in another particle, which are then attracted to each other. The repulsive electrostatic forces are caused by like charges on interacting particles, which repel each other.

## Van Der Walls Attraction

### Atom A          Atom B



Random dipole on Atom A          Induced dipole on Atom B

## Electrostatic Repulsion



Repulsion of like charged particles
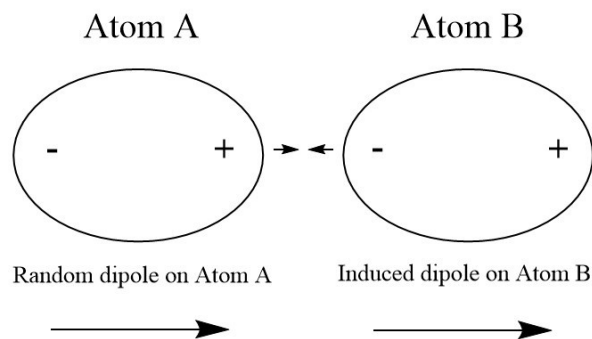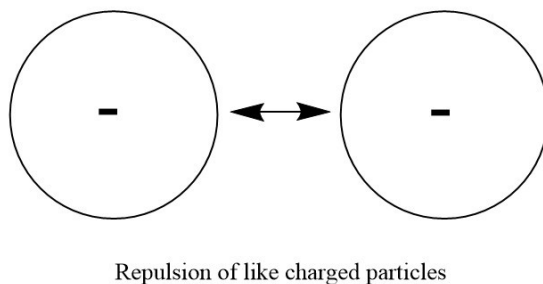
**Figure 4.** Diagram showing van Der Walls forces and electrostatic repulsion amongst particles.

If the attractive forces predominate in the system, then the particles in the system will aggregate and the colloid will lose its stability. On the other hand, if the repulsive forces are dominant in the system, then the colloid will have a greater stability and the particles will remain in suspension. In some cases, there is a third factor that can contribute to an increase in colloidal stability, which is steric repulsion due to large adsorbed molecules that protrude from the particle's surface as shown in Figure 5 (Kontogeorgis & Kiil, 2016). Polymers are commonly used molecules for steric stabilization. Typically, one end of the molecule will favor binding to the surface of the particle and the rest of the molecule will have solvent favorable portions that favor its extension away from the surface. Extension from the surface repels the collision and prevents agglomeration of other particles, thereby increasing the stability of the colloid.

## Steric Stabilization
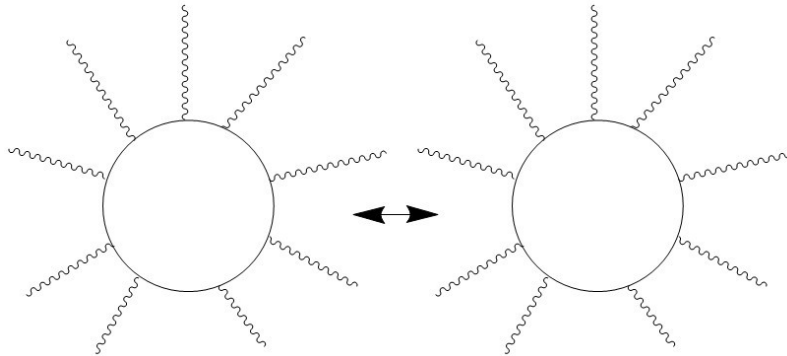


**Figure 5.** Diagram of steric stabilization of two particles coated with large protruding molecules.

### 2.2.1. Application of Colloids

Depending on the field and application there are two main goals when it comes to colloidal suspensions; to increase the stability of the colloid or to decrease the stability of the colloid. In food science, for example, the colloidal stability in various food products, such as milk, can have a large impact on sensory perceptions of those products (Van Vliet, 2010). Maintaining the integrity of a colloidal suspension is essential when it comes to manufacturing of certain substances, paint for example, and the electrostatic repulsion of the particles in the colloid has been shown to be a crucial factor in maintaining stability (Fujitani, 1996). This can be measured by the zeta potential of the colloid, where the larger the value of the zeta potential, the greater the colloidal stability of the colloid. In brief, the zeta potential is defined as the difference in electrical potential between the surface layer of the particle and the medium that it is dispersed in (Kontogeorgis & Kiil, 2016). Another instance where colloidal stability must be regulated and manipulated is in the fermentation process. During fermentation, the cells operate best when they are in suspension in the medium, so that there is better diffusibility of substrates to and from the cell. However, in order to isolate the products of the fermentation from the biomass, a process called flocculation can be used to aggregate the cells for easy separation. An early screening study in this field found that addition of strong polyelectrolytes, specifically the anionic polyelectrolyte polystyrene sulfonate and cationic polyethyleneimine, substantially improved

biomass flocculation in the different broth conditions, achieving 90% cell sedimentation (Gasner & Wang, 1970). While flocculation of yeast cells is an important aspect of the fermentation process, the timing of when the flocculation occurs is crucial for optimal yields of products. One study found a mutant strain of *Saccharomyces cerevisiae* that would suppress or initiate flocculation based on the $Ca^{2+}$ concentration, providing a trigger to initiate flocculation once stationary phase had begun (Kedong Ma, Wakisaka, Sakai, & Shirai, 2009). Although colloidal stability is very important to many industries, in other fields a colloidal suspension can be a huge problem and treatment can be quite costly, including the tailing ponds in the Alberta oil sands.

### 2.2.2. Colloidal Destabilization by Coagulation

Conventional chemical approaches to the reclamation of tailings ponds include destabilizing the suspension of negatively charged clay particles by addition cations in a process called coagulation. In general, clay particles in a solution contain many charges on their surface that are predominantly negative (S. Ali & Bandyopadhyay, 2016; Shainberg & Levy, 2005)·(H. Zhao et al., 2008). This negatively charged surface attracts counter ions, namely cations, which tend to associate with the surface. This causes two opposing forces within the solution: 1) an attraction of counterions to the surface of the particle; and 2) a repulsion of the like charges of the counterions to each other at the surface (Shainberg & Levy, 2005)·(Keren, 1989)·(Chapman, 1913; Gouy, 1910). The net effect of this interaction is the formation of two layers of charge distribution called the double layer as depicted in Figure 6. The first layer is caused by a higher distribution of the total counterions in the solution toward the particles surface, called the surface layer. This effect is enhanced when the concentration of the counterion in the solution is increased as diffusion away from the surface becomes less favorable.
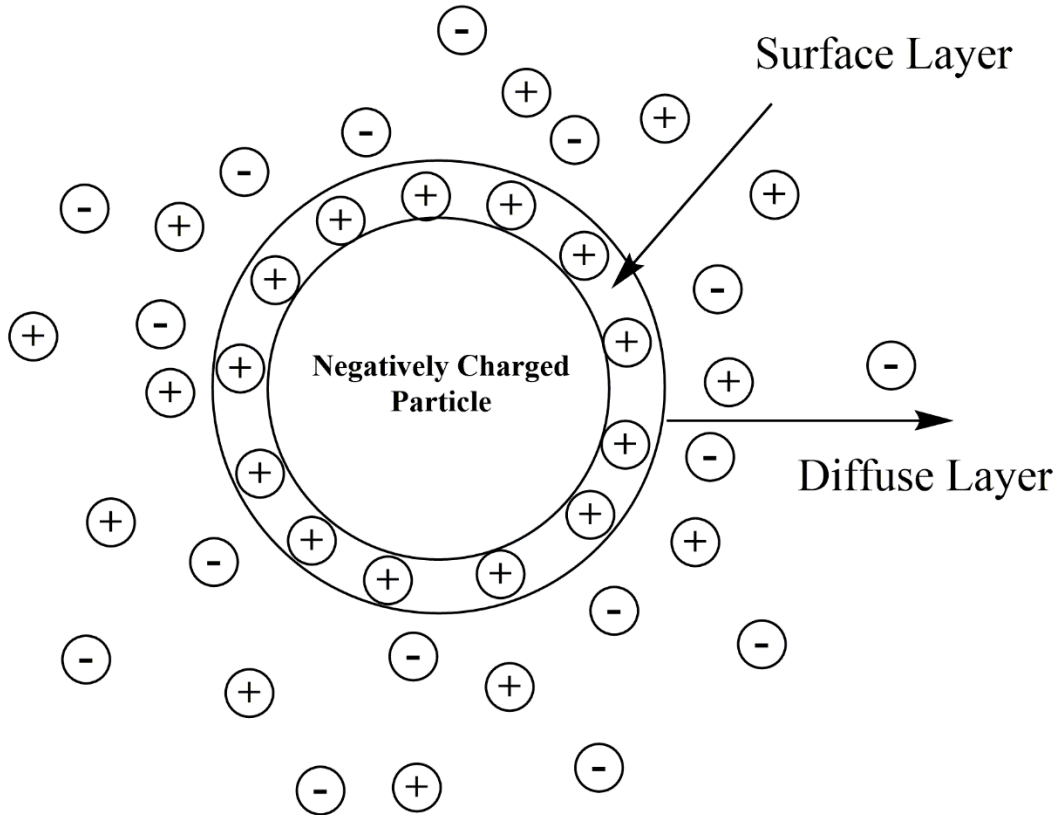
**Figure 6.** Diagram of the double layer of ions that forms on the outside of a negatively charged particle in solution. Idea adapted from (Kontogeorgis & Kiil, 2016), page 399.

To demonstrate the importance of counterions in colloidal stability, one study looked at the influence of ion concentration in a Na-montmorillonite slurry and found that as the NaCl concentration increased to >150 mM the destabilization of the colloidal gel network began and the structure of the slurry collapsed (S. Ali & Bandyopadhyay, 2016). It was shown in another study that $Fe^{3+}$ ions at high concentrations are able to flocculate a kaolin clay slurry by suppression of the double layer and can also act to bond clay particles together by formation of polycations in solution, such as [ $Fe_4O_3(OH_2)_4]_n^{2+n}$, leading to faster settling (Kunsong Ma & Pierre, 1997). The exact concentrations of $Fe^{3+}$ depended on the pH of the solution studied. These studies highlight the importance of ion concentration in the stability of a colloidal suspension. There is a second layer of ions that forms outside of the first surface layer called the diffuse layer. The ions in the second layer are further from the particle surface and are therefore interacting less strongly with the surface, making them more likely to disperse away, as shown in Figure 7. In order for coagulation of particles to occur, the electrostatic repulsion of the clay

particles has to be overcome, and this occurs when the double layer of the two particles can overlap. Divalent counterions are twice as attracted to the particle's surface as a monovalent counterion and lead to a smaller double layer due to their stronger attraction and charge neutralization. The same trend occurs with trivalent ions. This theory is known as Gouy-Chapman's diffuse double layer theory (Chapman, 1913; Gouy, 1910; Shainberg & Levy, 2005).
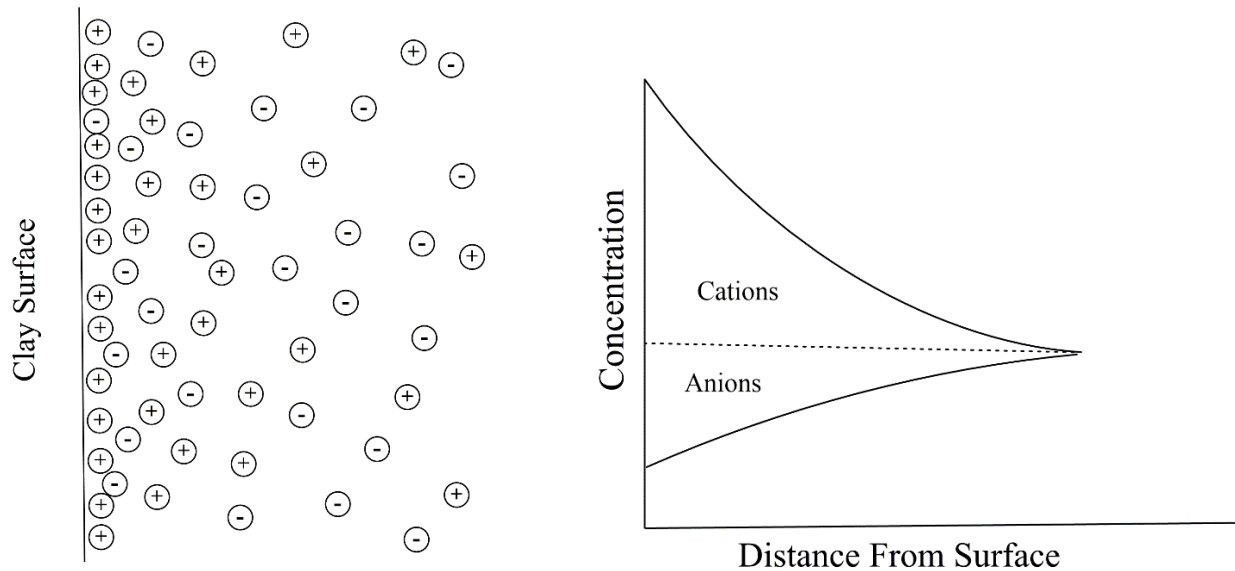


**Figure 7.** Diagram of ion concentration near the clay surface according to the double layer theory. Idea adopted from (Shaw, 1992), page 178.

In a water/clay suspension, destabilization of the colloidal suspension by addition of a coagulant is a key process that can be used to separate the two phases. In the case of oil sand tailings ponds, this would allow for recycling of the pond water and the return of the land back to its natural state. The need for divalent cations in oil sands tailings ponds has been previously demonstrated, and one study revealed that different divalent cations led to different coagulation properties (Sworska, Laskowski, & Cymerman, 2000). In that study, they examined the settling performance of a high molecular weight polyacrylamide (PAM) in various pH ranges and various divalent cation concentrations. They found that $Mg^{2+}$ and $Ca^{2+}$ ions operated optimally at slightly different pH ranges, but the addition of both to the tailing slurry improved the supernatant clarity after settling and improved the ability of PAM to flocculate the slurry at a higher pH. The other important tool that can also be used to expediate the separation process of the solids from the liquid in a colloidal slurry is a flocculating agent.

## 2.3 The Principles of Flocculation Using Polymers

A flocculant is a material that can act as a bridge between particles in a colloid to allow them to interact with each other and form larger particles, called flocs, which can sediment out of solution (O'Gorman & Kitchener, 1974). These materials are traditionally polymers and require a high molecular weight to form long chains that can bridge the gap between the suspended particles (Fleer & Lyklema, 1976). For example, one study looked at the adsorption of the polymer polyvinyl alcohol (PVA) to the suspended particles in a model silver iodide system. It was demonstrated that the adsorption amount increased with an increase in molecular weight of the polymer and this was an irreversible process (Fleer & Lyklema, 1976). The molecular weight (MW) of the polymers in the study were determined by viscosity, and the amount adsorbed changed from 105 mg/m$^2$ with a 3 MW unit polymer to 140 mg/m$^2$ with a 60 MW unit polymer.

Polymers used for flocculation can be cationic, anionic, non-ionic or have multiple charges depending on the molecule and the application (O'Gorman & Kitchener, 1974)'(Feng, Stuart, & Adachi, 2015). There can be large differences in the properties of anionic and cationic polymers when it comes to flocculation of a negatively charged particle slurry. It has been shown that cationic polymers of low molecular weight mainly contribute to charge neutralization on the particles surface, acting as a coagulant, whereas higher molecular weight polymers lead to charge neutralization as well as bridging, acting as a coagulant and a flocculant (Gregory & Street, 1969)'(A. P. Black, F. B. Birlmer, 1966)'(Dixon, fLa Mer, Li, Messinger, & Linford, 1967). It was also shown that cationic polymers may be present in lower quantities than anionic polymers for the same flocculation rates to occur, which is likely due to the reduced electrostatic repulsion by charge neutralization (Gregory & Street, 1969). One recent study found that a cationic polymer bonded irreversibly to the clay surface, whereas the anionic polymer bonded reversibly (Alagha, Wang, Yan, Xu, & Masliyah, 2013). The reversibility of the anionic polymer can be attributed to the strong electrostatic repulsion with the clay surface, which weakens the overall strength of adsorption that occurs by hydrogen bonding with the clay surface. The irreversibility of the cationic polymer is caused by strong electrostatic attraction to the negatively charged clay surface that is improved by hydrogen bonding interactions. Another distinction between the two polymers is that the cationic polymer formed a more compact structure on the

particle surface whereas the anionic polymer stretched out further from the surface. Nevertheless, both were able to provide sufficient bridging for settling to occur.

Regardless of the charge of the polymer, bridging of the space between the particles is the main process that induces rapid flocculation. Bridging is a phenomenon where a polymer extends away from adsorbed particle's surface, far enough for it to interact and/or adsorb onto another particle's surface. This interaction allows for the rapid accumulation of mass of the particles to form flocs, which are able to settle out of the suspended solution due to gravity. Interestingly, there are several tradeoffs when it comes to the chemical functional groups present on a polymer that must be considered when designing a flocculant for a system. For instance, a polymer that has both positive and negative charges associated within its backbone may interact with itself before it can interact with a particle's surface, forming a coiled structure which can limit bridging capability. Figure 8 provides a depiction of possible adsorption mechanisms of polymers to a particle's surface based on the work of (Alagha et al., 2013). The first mechanism (Panel A) is single point attachment, characterized by a single attachment point of the polymer to the surface. This would allow for extension of the polymer into the medium for better bridging capability but would limit adsorption. In Panel B, loop adsorption is occurring, where there are several points of attachment along the backbone of the polymer. More binding sites would allow for a stronger adsorption but may limit the extension into the medium compared to a single point attachment. Panel C depicts a more extreme case of B, called flat extension attachment, where there are multiple regular attachment points to the particle's surface leading to strong adsorption, with extension into the medium being very limited. In Panel D, random coiling is shown, where there are multiple attachment points to the particle's surface as well as multiple random coiled structures forming due to interaction of the polymer with itself. This is the typical mode of interaction with high molecular weight anionic polymers, where extension into the media occurs and polymer adsorption is relatively strong. Depending on the polymers structure, including the number, type, and position of functional groups along the chain, these different modes of adsorption can occur.

A) Single point attachment    B) Loop adsorption

C) Flat extension attachment    D) Random coiling
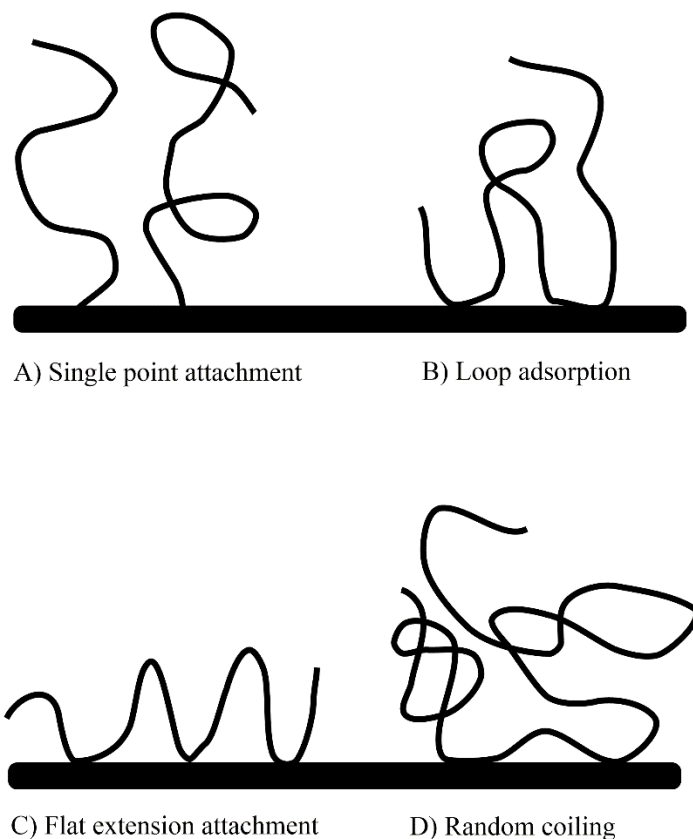
**Figure 8.** Possible configurations of polymers on the clay particle surface. A) single point attachment provides extension into the medium but may be weakly adsorbed to the surface. B) Loop adsorption allows stronger binding with multiple attachments. C) Flat attachment facilitates strong adsorption but with limited extension into the media for bridging. D) Random coiling provides multiple attachments and random coils form in the media where the polymer interacts with itself. Figure adopted from (Alagha et al., 2013).

### 2.3.1. Synthetic Flocculants

For industries that deal with tailings with finely sized particles, such as negatively charged clays, charged polymers are advantageous because the charges of the polymer can interact with the charges of the particles, forming strong ionic interactions (O'Gorman & Kitchener, 1974)·(Feng et al., 2015). To improve the interaction of negatively charged polymers with clays, the presence of large oppositely charged ions can reduce the electrostatic repulsion between the two and facilitate better polymer adsorption (O'Gorman & Kitchener, 1974). This type of interaction is common with the use of the popular industrial synthetic flocculant, anionic PAM. PAM exhibits

exceptional binding of clay in slurries in the presence of divalent and trivalent cations, such as $Mg^{2+}$, $Ca^{2+}$ and $Al^{3+}$, and leads to rapid settling rates (O'Gorman & Kitchener, 1974). One study investigated PAMs with different charges and molecular weights in a kaolin clay slurry with NaCl as a coagulant, and found that the maximum settling rates achieved by the different variations were between 4.1–7.9 cm/min (Nasser & James, 2006). Another study found that the PAM they synthesized had an initial settling rate of 11.2 cm/min in a kaolin clay slurry and that after alkaline hydrolysis, the hydrolyzed polyacrylamide (HPAM) was able to settle at the same rate but at half the dosage (Strandman, Vachon, Dini, Giasson, & Zhu, 2017). The reduced dosage can be attributed to the improved electrostatic binding to the clay surface compared to the hydrogen bonding interactions that occur with PAM, as well as the intra-electrostatic repulsion of the charged segments of the polymer that facilitate further extension away from the surface. However, there is an optimum hydrolysis percentage, beyond which the electrostatic repulsion becomes too strong and adsorption to the particle surface becomes less favorable. Structures of the various polyacrylamide molecules are shown in Figure 9.



A) Polyacrylamide (PAM)          B) Hydrolyzed Polyacrylamide



C) Partially Hydrolzyed Polyacrylamide (HPAM)
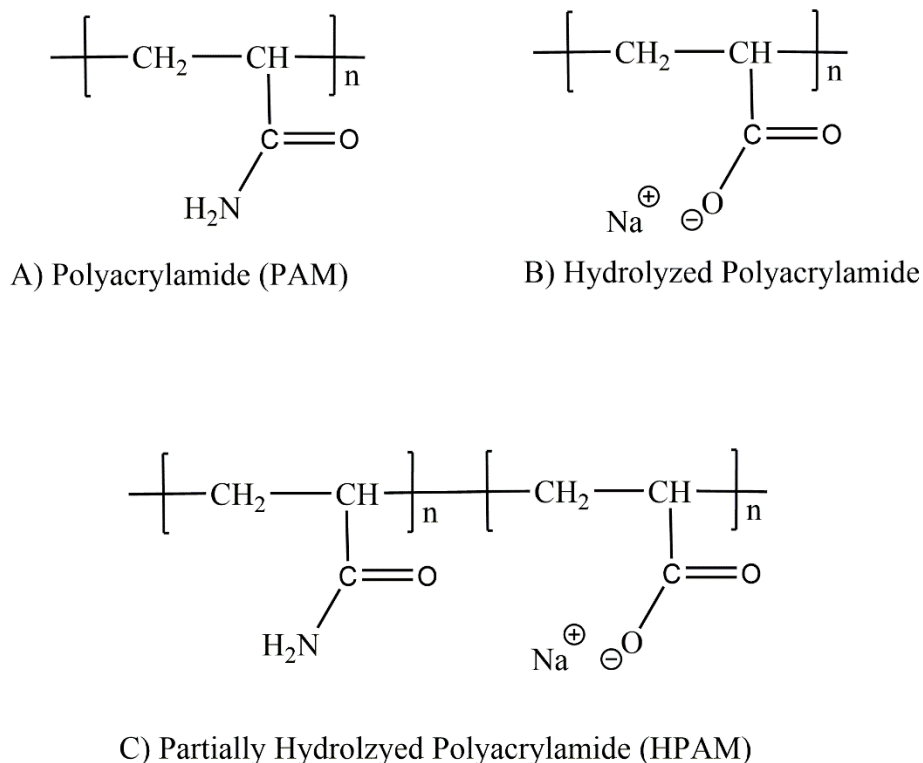
**Figure 9.** Chemical structure of various polyacrylamide molecules. Polyacrylamide (A), hydrolyzed polyacrylamide (B), and partially hydrolyzed polyacrylamide (C).

The rapid settling rates that PAM molecules exhibit is a high benchmark with which other polymers in the flocculation space have to compete. PAM is such a useful and effective flocculant that many other approaches to improving flocculation involve creating flocculants by grafting other polymers and molecules onto PAM or supplementing PAM flocculation with other chemicals (Lu et al., 2016; Tripathy, Bhagat, & Singh, 2001). A downside of using synthetic polymers like PAM is that they are derived from petrochemical sources, which brings into question the long-term sustainability of the use these polymers. Another major issue is the negative environmental impacts associated with them due to their lack of biodegradability and the potential breakdown into its toxic monomer acrylamide via UV irradiation (Caulfield, Hao, Qiao, & Solomon, 2003). Industrial polyacrylamide is also known to contain a small amount of acrylamide monomers in its formulation (0.05%> wt/wt).

Acrylamide is a known carcinogen and neurotoxin. In mice, it has been shown that acrylamide, and four other derivative molecules of acrylamide, are neurotoxic and cause deterioration of neurological tissue (Hashimoto, Sakamoto, & Tanii, 1981). It is also known to form complexes with hemoglobin (with unknown biological consequences) which can be used as a measure of acrylamide exposure (Besaratinia & Pfeifer, 2007). Acrylamide has also been shown to cause DNA damage leading to cancer formation. The route of action for this is largely through the more reactive epoxide form of the molecule called glycidamide. This molecule is produced by the action of cytochrome P450 2E1 and is even more reactive toward DNA and protein than acrylamide, as shown in Figure 10 (Sumner et al., 1999). Once converted to glycidamide, it can form adducts with DNA which cause depurination in the DNA sequence, causing DNA mutagenesis (Gamboa da Costa et al., 2003). The environmental and the health concerns to humans and wildlife of polyacrylamide have sparked the interest of some researchers to investigate potential nontoxic replacements.

**Acrylamide**                                           **Glycidamide**
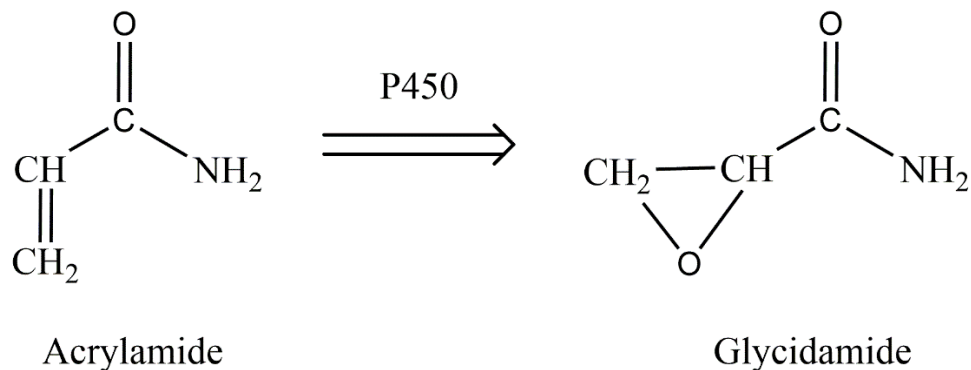
**Figure 10.** General reaction scheme for the conversion of acrylamide to glycidamide.

### 2.3.2. Bio-based Flocculants

Recently, there has been interest in the development of flocculants from a biological origin due to their improved biodegradability and sustainability. In order for a natural polymer to be incorporated as a flocculant industrially, such as in the oil sands, it must be able to do one of the following:

a) compete with the performance of current industrial polymers

b) be more cost effective

c) provide an environmental benefit that is worth the cost

d) supplement current polymers to provide additional benefit (i.e. lower turbidity, more compact sediment etc.)

Popular flocculants in this space are primarily based on polymers of different sugar molecules such as carboxymethylcellulose, guar gum, starch, chitosan and sodium alginate (Renault et al., 2009). Among the biological polymers, chitosan is one of the most popular and highly studied due to its cationic nature. Divakaran and Pillai published a study about the use of cationic chitosan from prawn shells, which showed that the flocculation of a kaolin clay slurry using chitosan, without any coagulant present, was effective in the pH range of 7.0-7.5 (Divakaran & Sivasankara Pillai, 2001). However, the kaolin slurry they used was a very dilute suspension (2 g/L), therefore it is unknown if their chitosan would be as effective at higher slurry concentrations. Another study involving chitosan looked at its use as a flocculant to clean up wastewater from cardboard mills across four seasons, compared to the traditional flocculant

polyaluminium chloride (Renault et al., 2009). They found that the use of chitosan was able to reduce the chemical oxygen demand (COD) by >80% and the turbidity of the wastewater by >85%, which was better than polyaluminium chloride control, at 40-45% for COD and 55-60% for turbidity. This improvement was only seen in fall and winter when lower temperatures were present and there was no difference during summer and spring. A third study looked at using chitosan in the flocculation for beer clarification compared to bentonite and Stabifix, two other coagulants used in the industry (Gassara et al., 2015). They found that at industrial scale, chitosan was able to provide the same flocculation efficiency (~100%) as the other industrial controls at a 160 times lower dosage. Chitosan also had a higher reduction in the total suspended solids (65%) compared to the controls at 32% and 46% respectively.

Other biopolymers may need to be chemically modified to alter the charge of the polymer. In the case of corn starch, one approach to improve its inherent flocculation capacity was to modify the polymer backbone through addition of a cationic group (Pal, Mal, & Singh, 2008). The authors were able to improve the settling rate of the initial starch from 0.46 cm/sec to 0.76 cm/sec after cationic modification, which was in the realm of the industrial flocculants that ranged from 0.51-0.83 cm/sec. In another study, it was found that after grafting sodium alginate onto a polyacrylamide polymer, it was better than industrial polymers at flocculating a high density iron ore slime suspension. It also performed on par with two of the three industrial polymers in a lower density suspension (Tripathy et al., 2001). This showed that the performance of the polymer was dependent on the nature of the suspension it was settling. While a product containing polyacrylamide is inherently unsustainable, the use of this product would help to reduce the amount of synthetic polymer used in the industry. Other researchers showed that modification of cellulose, with a strong base and monochloroacetic acid to form carboxymethylcellulose, created a polymer that was useful in flocculation of industrial waste water streams (Z. M. Ali, Mughal, Laghari, Ansari, & Saleem, 2013). An optimum dosage of 70 mg/L was determined and was shown to effectively reduce between 72-79% of the turbidity in five different waste stream locations. A common theme amongst the research in this field is that modification of the abundant natural feedstock is required to improve its ability to function as a flocculant. A recently published paper using guar gum as a flocculant in a 5% (w/w) kaolin clay slurry without any modification showed that it can act as a flocculant at pH 5 with an 83% transmission rate of infrared light, which indicated lower turbidity. Infrared light is not able to

transmit through a stable suspension and results in backscattering; therefore, transmission indicates lower turbidity. At pH 10, the presence of a high potassium nitrate concentration with the flocculant was able to achieve similar results with an 80% transmission rate (Dwari & Mishra, 2019). Although these natural polymers can be modified to improve their flocculation capabilities, none of them have come close to the flocculation ability of synthetic polyacrylamide.

This may be a challenge for researchers to overcome, but it also provides an opportunity to develop a product that fills a market niche where the use of natural polymers can advantageous. While many potential natural polymers have been discussed, there is another, often overlooked, group of polymers of biological origin that could be used as flocculants, which are peptides, the building blocks of proteins.

### 2.3.3. Peptides as Flocculants

Peptides are a diverse group of biological molecules that are essential to all living organisms. They are polymers of amino acids that are joined together by the bonding of the carboxylic acid end of one amino acid to the amino group of another amino acid, forming a peptide bond. Long chains of peptides are known as polypeptides and proteins consist of one or more of these chains.
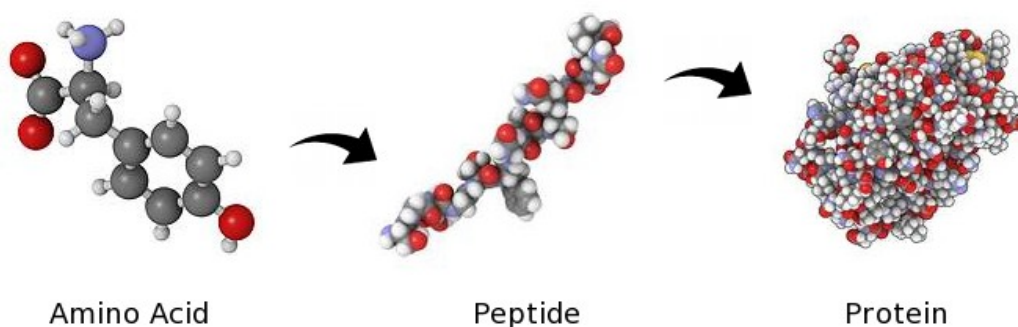


**Figure 11.** Illustration of the difference between an amino acid, a peptide, and a protein. With approval from author PeptideSciences. URL:*https://www.peptidesciences.com/glossary/peptides-vs-proteins/*

Depending on the number and type of amino acids present in the peptide, the chemical features can vary widely, which provides the basis for the vast array and functions of different peptides

and proteins. There are twenty different amino acids in the standard genetic code for eukaryotic organisms, each with different chemical properties. In general, the amino acids are divided into three groups: non-polar, polar, and charged. The different arrangement of the amino acids in a peptide chain will alter the 3D configuration of the polypeptide and therefore the protein.

The charged groups inherent on the peptide backbone provide the opportunity for interaction with a charged clay surface and the basis for their use as flocculants. One of the first studies to look at using peptides and proteins as industrial flocculants was a large screening study done by the United States Department of Agriculture published in 2010 (Piazza & Garcia, 2010). There were many key findings from this study that are of importance when it comes to using proteinaceous material as a feedstock for flocculants. In the study, the feedstocks they were interested in were proteins from bone meal, blood meal, meat meal and feather meal, as they are inexpensive protein sources from the agriculture industry. They also looked at several commercial products known to contain peptides and proteins, such as gelatin. The authors looked at first improving the solubility of the protein feedstocks by hydrolysis through chemical and enzymatic means. The water-soluble peptide and protein fragments that result from hydrolysis are known as peptones. The spray dried samples were then tested as flocculants by addition of the peptides/proteins to a kaolin clay slurry, with and without $CaCl_2$ as a coagulant. The molecular weight of the feedstocks was determined through gel filtration chromatography by HPLC.

It was found that the alkaline hydrolysis treatments performed better as flocculants than the enzymatic treatments, due to larger MW fractions remaining after alkaline hydrolysis. From size determination analyses, it was found that the high molecular weight peptides/proteins had the best flocculation performance but showed decreased activity at higher doses. This was especially true of the commercial gelatin used. The intermediate sized peptides were able to flocculate the slurry but required higher concentrations. Finally, the low molecular weight hydrolyzed materials did not perform as flocculants even at high concentration. These findings help to reinforce the idea that the molecular weight of the polymer is essential when it comes to bridging during flocculation. They also found that at lower pH, the peptides functioned better as flocculants and speculated that this may be due to the net positive charge the peptide/proteins obtain when in an acidic environment due to the protonation of the carboxylic acid group. The

net positive charge of the molecules is likely a factor in this phenomenon, but it is also likely that the additional protons in the solution helped to suppress the double layer of the negatively charged clays, promoting aggregation. The last large takeaway from this study was that the concentration of the dosage required for flocculation in this system was much higher than the dosage for polyacrylamide. The high molecular weight gelatin required twice the dose, whereas the other chemically hydrolyzed materials tested required up to 75 times the concentration. Modification of this material to increase its molecular weight may be necessary to reduce the concentration required to induce flocculation, to an amount closer to that of industrial polymers. This study provides support that hydrolyzed proteins can act as flocculants in a clay slurry, which supports the basis that they have the potential to be used as nontoxic biodegradable replacements for synthetic polymers. A promising source of inexpensive protein that could be used to this end are specified risk materials, a byproduct from the cattle industry.

## 2.4 Specified Risk Materials

Canada's agriculture sector is of great importance to the Canadian economy. In 2016, it employed 2.3 million people and generated $111.9 billion in gross domestic product (Agriculture and Agri-food Canada, n.d.). In the livestock sector, Alberta alone accounted for 40.5% of the Canadian calf and cattle inventory, as of January 2019, at 11.5 million heads (Information, 2019). To make use of all of the material from the livestock sector, a process called rendering is used. Rendering is the process of recycling the parts of livestock that are not used for human consumption, including edible and inedible fats and protein meals. This represents a considerable amount of material and can reach up to 50% for a cow by mass (Ernsthausen, n.d.). Recycling of these products not only provides them with value, by providing materials for other industries, it also helps to reduce the large amount of waste that would otherwise have ended up in landfills. While rendering leads to a reduction of waste from the livestock sector, it has also led to other problems when it comes to disease transfer between animals. The use of rendering material as animal feed for livestock led to the transmission of misfolded protein diseases called prion diseases. This includes the infamous Bovine Spongiform Encephalopathy (BSE), more commonly referred to as "mad cow disease", with the first detection of BSE in Canada occurring in 2003 (Canadian Food Inspection Agency, 2016)·(Report & Island, 2009). Not only can BSE be transferred between ruminants, but it can also be transferred to humans as well. The concerns

of public health and the potential economic loss in international exports caused Canada to review its feed ban policy. The outcome was stricter regulation being passed in 2007 by the Canadian Food Inspection Agency called the enhanced feed ban.

The main effect of the enhanced feed ban was that specified risk materials (SRM) were to be completely removed from the ruminant and human food chain. This includes the skull, brain, trigeminal ganglia, eyes, tonsils, spinal cord and dorsal root ganglia of cattle 30 months or older, and the distal ileum (portion of the small intestine) of cattle of all ages, as these were the organs deemed to have the risk of spreading prion diseases, as shown in Figure 12.
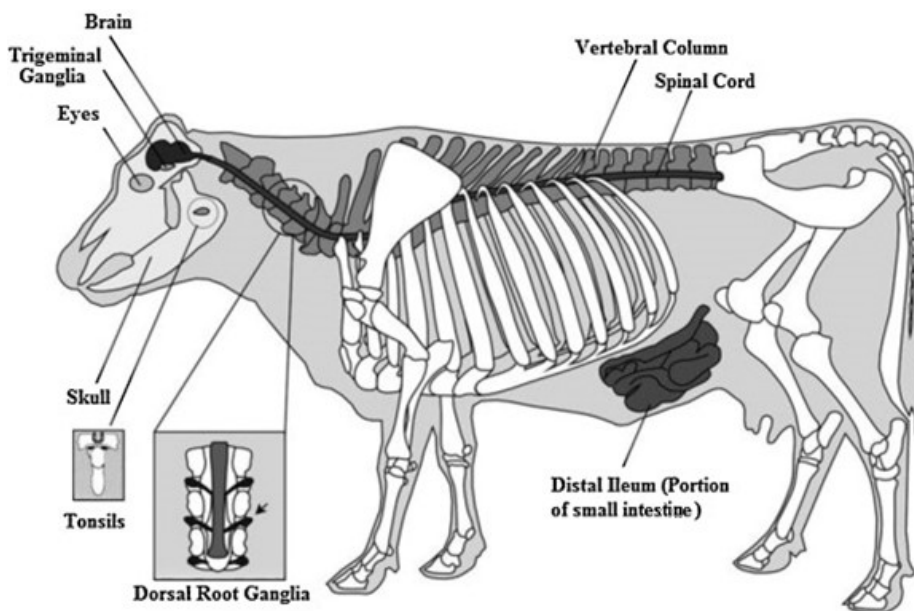


**Figure 12.** Diagram of the areas of livestock that have the potential to contain prion diseases and are deemed as specified risk materials. Republished with permission of Elsevier Science & Technology Journals, from Recovery and characterization of proteinaceous material recovered from thermal and alkaline hydrolyzed specified risk materials, Tizazu H. Mekonnen a, Paolo G. Mussone a, Natisha Stashko a,1, Phillip Y. Choi b, David C. Bressler a∗, Process Biochemistry Volume 48, Issue 5-6, Published in 2013; permission conveyed through Copyright Clearance Center, Inc.

After rendering of the lipid tallow fraction of the waste, the 25% that remains is largely protein and minerals. This accounts for 300,000 tonnes of material annually in Canada (T. Mekonnen,

Mussone, & Bressler, 2016). This material was previously used as animal feed, pet food and fertilizer, but after the enhanced feed ban they now have had to be landfilled or incinerated (Canadian Food Inspection Agency, 2016; Report & Island, 2009). SRM was once a source of revenue for the industry but is now both a financial and environmental burden. Valorization of this material is of great importance to the industry and this involves first making the material safe to use (Report & Island, 2009).

The four approved methods for dealing with SRM in Canada and the United States are landfilling, incineration, thermal hydrolysis, and alkaline hydrolysis (T. Mekonnen et al., 2016). To convert SRM into higher value products, thermal and alkaline hydrolysis were investigated in a 2013 article from Mekonnen *et al*. and the different product streams created were compared (T. H. Mekonnen, Mussone, Stashko, Choi, & Bressler, 2013). The thermal hydrolysis reaction conditions consisted of the addition of water in a 1:1 ratio (wt./wt.) to the dry SRM powder, which was then heated at 180°C at ≥174 psi for 40 minutes in a 5 L Parr reactor. The alkaline hydrolysis reaction conditions were slightly different with heating at 150°C at ≥58 psi for 40 minutes in a 15% NaOH solution. It was found that thermal hydrolysis had the benefit of maintaining higher molecular weight peptides that were more water soluble, at a lower cost than alkaline hydrolysis, without the formation of a salt byproduct during neutralization (T. H. Mekonnen et al., 2013). After thermal hydrolysis, the product consists of a mixture of a broad size range of peptides, mainly in the range of 5-15 kDa, which was analyzed by SDS-PAGE, as shown in Figure 13. As previously discussed, the high molecular weight components that remain after thermal hydrolysis make this process more desirable for use in flocculant applications.
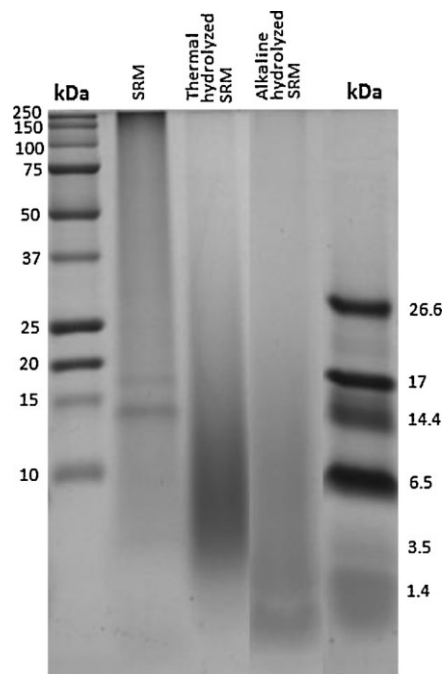
**Figure 13.** SDS PAGE result of analysis of specified risk material (SRM) before hydrolysis, after thermal hydrolysis, and after alkaline hydrolysis (T. H. Mekonnen et al., 2013).

### 2.4.1. Analytical Methods

This brings to light one of the major challenges that one is faced with when working with these peptides; the fact that this is a mixture and is not a pure substance. Many of the analytical techniques used in chemistry and other fields to qualify and quantify novel substances cannot be used with a mixture of substances. For example, nuclear magnetic resonance (NMR) can be a very valuable technique to quantify the changes made to a molecule before and after a reaction. However, the material being analyzed must be very pure with little to no contamination for the measurements to have any interpretation. When working with the peptones derived from SRM, bulk analyses of the mixture as a whole are the only available option. This means that quantification of the properties of the material is quite difficult. Nonetheless, various methods have been employed, which mainly rely on indirect evidence to infer changes in properties, instead of direct evidence. This is the limitation of the feedstock and an issue that can make characterization of this material after reactions difficult.

While there are limitations to what analyses can be done on this material, there are some characterization techniques that can be performed. One such technique is to estimate the

carboxylic acid groups of the molecules by an acid titration method. A pH well above the pKa of the carboxylic acid group is a region where the majority of these groups are charged, i.e. deprotonated. By titrating with a strong acid to a point right before the pKa, the groups will slowly become protonated, providing an estimation of the number of groups. Performing this titration before and after the reaction can provide evidence that a change in the number of carboxylic acid groups has occurred and can provide an estimation of reaction efficiency. Other methods include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) to determine size changes of the molecules, Thermal gravimetric analysis (TGA) to determine changes in thermal breakdown composition, and Fourier transform infrared spectroscopy (FTIR) to determine functional group changes. SDS-PAGE is a commonly used method in biochemistry to determine the molecular weight of peptides and proteins. This involves separation of the molecules by exposing them to SDS to impart a consistent mass-charge ratio. They are then added to a porous gel and an electrical current is applied that pulls the molecules through the gel. Larger molecules take longer to pass through the pores in the gel and migrate slower than smaller molecules, leading to a size separation. The advantage of this technique is that it is fast and does not require expensive equipment to use. A disadvantage is that it is mainly a qualitative technique and an exact quantitative molecular weight determination is not possible. HPLC is another technique that can be used to determine the molecular weight of this material. In this method, a sample is passed through a column that can separate the molecules based on different traits, for example size in a size exclusion column. In this instance, the column is packed with a material, such as porous beads of a determined pore size, that will interact with small molecules and allow larger molecules to pass by. In this way, the larger molecules are eluted from the column first and smaller molecules will be retained longer. An advantage to this method is that it allows a quantitative determination of molecular size range. A disadvantage is that the solvent used as the mobile phase of the column may not fully dissolve all of the product, leaving a portion that cannot be analyzed. TGA is a technique that can be used to examine the thermal stability of products. This is done by heating of a sample at a constant rate while changes in weight are being monitored. Onset of thermal degradation and degradation behavior can be determined. Advantages of this process is that it can operate at a large temperature range and it can be done on pure substances as well as mixtures of compounds. A disadvantage of this technique is that it

may not be able to provide enough sensitivity to determine minor differences among products. FTIR is a very useful technique that can be used to determine functional groups present in a sample. A sample is exposed to infrared radiation and the radiation that is not absorbed by the sample molecules is transmitted through and is detected. This technique has several advantages including a fast analysis time, easily interpretable data, it is inexpensive, and can provide a lot of information. The main disadvantage is that this technique does not provide structural information about the molecules, only the functional groups present. One study looking at the properties of green plastics from soy protein used both TGA and FTIR to characterize their products (Lodha & Netravali, 2005). They found that there was a difference in the onset of thermal degradation measured by TGA after the modification of the soy protein isolate with steric acid. They also found that there was no difference in the FTIR spectra after the reaction, which was expected but did not help them to determine if a reaction did occur.

### 2.4.2. Hydrolysis Process

Before SRM can be characterized or used in value-added applications, the bulk crude hydrolyzed material needs to be processed to isolate the peptides. This includes removing the bone, lipids, and other insoluble material by centrifugation and vacuum filtration. Finally, any residual lipids can be extracted by liquid-liquid extraction with a nonpolar solvent. The remaining material in the aqueous phase can be freeze dried or spray dried to remove water. This process, shown in Figure 14, results in a light brown proteinaceous powder that can then be used for various high value applications.
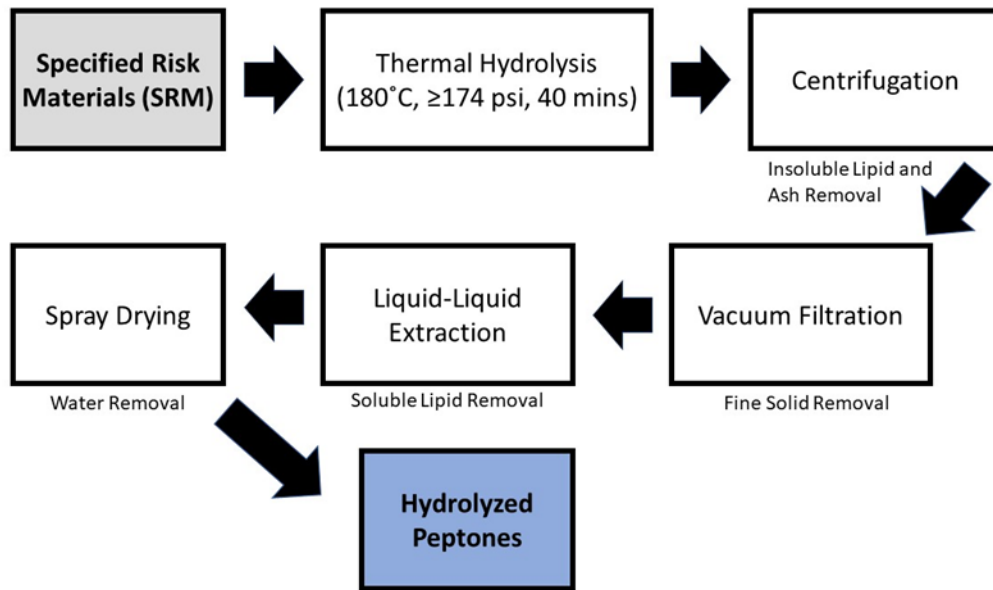
**Figure 14.** Flow diagram of the hydrolysis of specified risk material to peptones.

## 2.4.3. Valorization of SRM

There have been a few studies looking at different approaches to valorizing specified risk materials for various applications. An early study in this field looked at supplementing cow manure with SRM to enhance biogas formation during anaerobic digestion to essentially convert SRM into biofuel (Gilroyed et al., 2010). They found that addition of SRM did improve the production of biogas in the system with a methane yield increasing from $137.5 \pm 13.7$ mL/g with no SRM present, to $359.3 \pm 36.1$ mL/g when a 25% SRM mixture was used. This result was promising; however, the microbial digestion alone was not completely able to degrade the potential prion containing proteins, which would be a problem for commercialization. Approaches by other researchers have been to use hydrolyzed SRM peptides for different applications and to modify the material to enhance its properties. Applications that have been examined include thermosetting bioplastics, adhesives for strand board and plywood, and binders for torrefied wood pellets (Adhikari et al., 2016, 2019; T. H. Mekonnen, Mussone, Choi, & Bressler, 2014; T. Mekonnen, Mussone, El-Thaher, Choi, & Bressler, 2013). One common theme amongst these studies is that they required chemical modification of the hydrolyzed SRM peptides to improve their performance for use in their applications. By chemically modifying the

original peptides, properties such as the water absorbance, solubility, molecular weight, thermal stability, and reactivity can be altered according to the desired traits.

**2.5. Chemical Modifications of Peptides**

One common feature that all amino acids and peptides have is the presence of a carboxylic acid group on one end of the molecule and an amino group on the other end of the molecule. At physiological pH, both of these functional groups are charged, with a positive charge on the amino group and a negative charge on the carboxylic acid group, forming a what is called a zwitterion, as illustrated in Figure 15. The charges of these molecules can be utilized for electrostatic interactions with other charged groups.
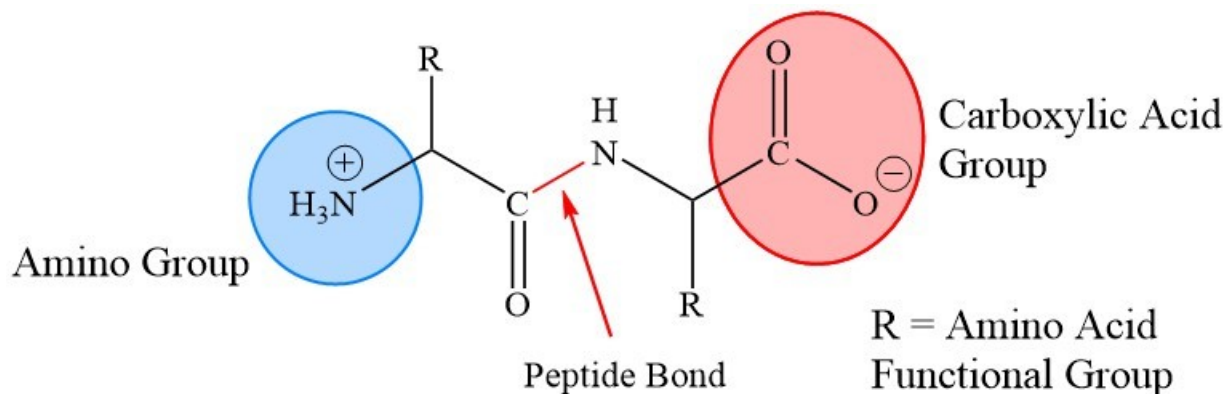


**Figure 15.** Chemical structure of a simple dipeptide consisting of two amino acids bonded together by a peptide bond.

There are many different approaches one can take when it comes to modification of peptides. One approach is to keep the peptide structure intact and focus on modification of the 'R' functional groups that are naturally present on the amino acids of the chain. By altering these functional groups of peptide molecules, their chemical properties can be drastically changed. The two other areas that can be targeted when it comes to functional group modifications are the carboxylic acid and amino groups on the ends of the chain (DeGruyter, Malins, & Baran, 2017).

Modification of proteins can be of great interest to the food industry. For example, one study aimed to increase the solubility and alter other properties of soy bean protein isolate through Milliard reactions, crosslinking, and enzymatic glycosylation modifications of the protein

sequence (Zhang et al., 2018). They found that the crosslinked protein had better water solubility from pH 2-10 and better textural properties, including hardness and adhesiveness, compared to the unmodified protein. These property changes could improve the use of this protein in the food processing industry. Protein modifications are not always desirable and can have negative impacts on food systems as well. A study in the food biochemistry field found that malondialdehyde, an oxidized lipid byproduct, was able to cause significant alterations to proteins from whey protein isolate (Niu et al., 2019). The modifications included crosslinking and aggregation, an increased hydrophobicity, increased carbonyl content, and a loss of thiol and tryptophan content. These changes were speculated to cause a reduced digestibility of the protein.

In terms of large-scale biotechnology processes, another key protein modification involves binding of enzymes to solid supports for easy recycling and removal from the reaction media. Such enzyme immobilization can make the use of expensive enzymes more cost effective and suitable for larger scale operations. Ferrarezi A.L. *et al.* published a study on the immobilization of lipases to various solid supports. They were able to achieve 95% enzyme immobilization, with an increase in enzyme activity by 238%, when it was covalently attached to an octyl-agarose support (Ferrarezi et al., 2013). This article highlights the benefits of modifying enzymes to improve the economic viability of an industrial process. While cost is an important factor in industry, another growing concern is the sustainability and environmental impact of a process. This is where protein modification can be utilized to replace products originally derived from petrochemicals.

The textile industry is one area where this could be applied, as described in one research paper where soy protein was modified with triethanolamine to decrease the intra and inter-molecular forces between the proteins (Y. Zhao, Zhao, Xu, & Yang, 2015). By disrupting these interactions, the authors were able to improve the weaving efficiency by 3% of the protein over polyvinyl alcohol, currently a petrochemical derivative, while maintaining other desirable properties. The protein was also shown to be more biodegradable, as shown by a difference in chemical oxygen demand of $109.5 \pm 6.2$ mg/L compared to $398.3 \pm 8.4$ mg/L of the PVA.

Another industry that is concerned with the modification of protein is the pharmaceutical industry. Initially, using proteins for drug applications was not possible due to the immune

response that their use would cause. However, it was shown that the immunogenicity of the proteins could be reduced or eliminated by chemical modification of the protein by polyethylene glycol (PEG) through reactions with the amine groups of the protein (Abuchowski, Van Es, Palczuk, & Davis, 1977). This opened up the possibility for a new class of drugs and a new field of drug research. Today, protein and peptide therapies are commonplace and crosslinking with PEG is often employed. In general, there are many different types of protein modifications possible and many have been studied and understood for long periods of time.

### 2.5.1 Esterification

A well-known and long understood modification for peptide sequences is an esterification reaction at the carboxylic acid end of the molecule (Fraenkel-Conrat, H and Olcott, 1945; Nudelman et al., 1998; Wilcox, 1972). By reacting a peptide or protein with an alcohol such as methanol or ethanol, in the presence of an acid catalyst, esterification of the carboxylic acid groups can be achieved in a relatively short time (Fraenkel-Conrat, H and Olcott, 1945; Wilcox, 1972). A scheme for this reaction is shown in Figure 16. This reaction results in a reduction in the negative charges on the molecules; increasing their overall positive charge.
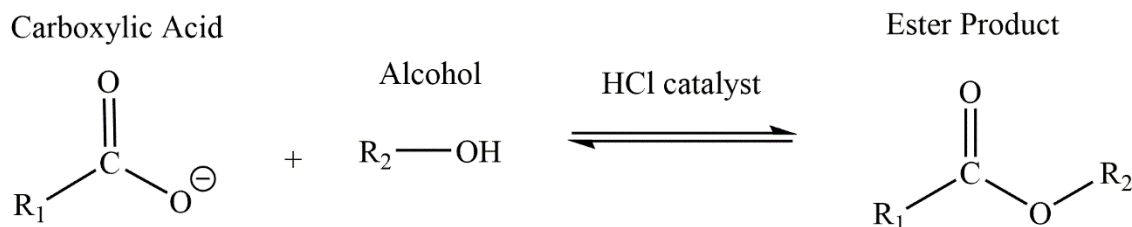


**Figure 16.** Esterification reaction scheme of the reaction of a peptide carboxylic acid group with a primary alcohol to form an ester product.

The concentrated acid will act to catalyze the reaction by the protonation of the carbonyl oxygen. The carbonyl carbon is susceptible to nucleophilic attack by a lone pair on the oxygen of the alcohol molecule. The newly formed hydroxyl group can then be protonated to form a very good $H_2O^+$ leaving group, which then forms the ester product. By capping the end of the peptide with an ester, the solubility of the peptide will also decrease due its lack of hydrogen bond donating. Of note, this reaction is reversible in the presence of water in acidic conditions, therefore this reaction is solvent dependent. The extent of esterification can be determined by titration of the

peptides with a known concentration of a strong acid from pH 7 to pH 2, before and after the reaction (Wilcox, 1972). The amount of acid added can be correlated to the protonation of the charged carboxylic acid groups to estimate their amount. Esterified peptides are expected to have an increase in binding efficiency to a negatively charged clay surface by reduction of electrostatic repulsion, which could improve their flocculation performance without the need for an inorganic coagulant to be added.

## 2.5.2. Crosslinking Reactions

Crosslinking of two peptide molecules is another approach that can be used in peptide modification. This approach can increase the molecular weight of the peptides by increasing their chain length. As mentioned earlier, crosslinking has been used in many different applications using a variety of chemical crosslinkers, including PEGylation of peptides for use in the medical field (Roberts, Bentley, & Harris, 2012; Veronese & Pasut, 2005). One study used a diglycidyl derivative of PEG, namely poly(ethylene oxide) diglycidyl ether, to crosslink wheat protein and examined how it affected its properties (Kurniawan, Qiao, & Zhang, 2007). They found that after the reaction, the molecular weight of the protein increased from 89,800 g/mol to 153,700 g/mol, indicating that the crosslinking reaction occurred and noted that as the degree of crosslinking increased there was a marked decrease in solubility. They also found that after crosslinking, the protein had a lower glass transition temperature and a higher tan δ, indicating higher viscoelasticity. Other chemical crosslinkers for peptides and proteins are known, but many are expensive or require multi step reactions that may not be desirable for larger scale reactions, such as N-hydroxysuccinimidyl esters (Anderson, Zimmerman, & Callahan, 1964).

Glutaraldehyde is another well-known chemical crosslinker used for proteins and other polymers that can react rapidly at ambient conditions (Korn, Feairheller, & Filachoine, 1972; Okuda, Urabe, Yamada, & Okada, 1991). It has two reactive aldehyde groups on either end of the molecule with a three-carbon chain in between. It can exist in many different forms depending on the solvent it resides in, and the pH of the solution, making it a difficult molecule to characterize (Korn et al., 1972; Okuda et al., 1991)ʼ(Migneault, Dartiguenave, Bertrand, & Waldron, 2004). In fact, depending on the analytical technique used, the structure of the molecule has been shown to change. At a basic pH from 8-11, glutaraldehyde exists mainly as a monomer, whereas in a more acidic pH it has been shown to dimerize and self-polymerize

(Migneault et al., 2004). Furthermore, when considering reactions with peptides and glutaraldehyde, the pH will also impact the charge on the peptide functional groups, which will influence reactivity.

When it comes to reactions with glutaraldehyde the two aldehyde groups present in its structure are known to react readily with amino groups of peptides (Okuda et al., 1991). Glutaraldehyde crosslinking of peptide amino groups and 'R' lysine groups, if present, has been shown to occur at a wide range of temperatures from 4˚C to 150˚C (Okuda et al., 1991)·(Broun, 1976; El-Thaher, Mekonnen, Mussone, Bressler, & Choi, 2013). Originally it was thought that the aldehyde groups will react most rapidly with the less stable amino groups by forming a Schiff base because they are prone to a nucleophilic attack by the amino group, as shown in Figure 17 (Migneault et al., 2004).
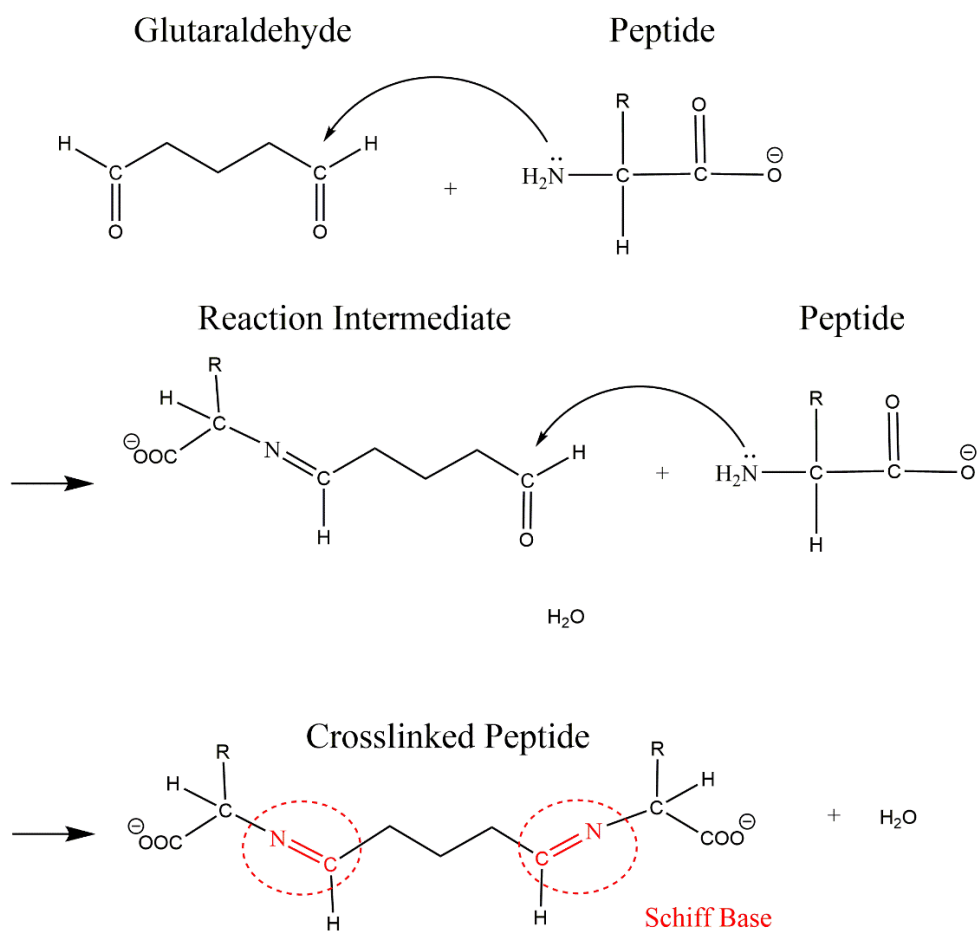


**Figure 17.** Reaction scheme of the glutaraldehyde peptide crosslinking reaction based on Schiff base formation.

This has since been ruled out and the general consensus is that a more complicated set of reaction mechanisms is occurring (Migneault et al., 2004). Other proposed reaction schemes for glutaraldehyde crosslinking, specifically in methanol, are shown in Figures 19-21 below. The general idea behind these reactions is based on review of glutaraldehyde forms and reactions by Isabelle Migneault, Catherine Dartiguenave, Michel J. Bertrand, and Karen C. Waldron, with modifications (Migneault et al., 2004). One important finding that was summarized in this review is that under neutral to slightly acidic conditions, glutaraldehyde exists in a monomeric form, a hemiacetal form and an oligomeric form, as shown in Figure 18.
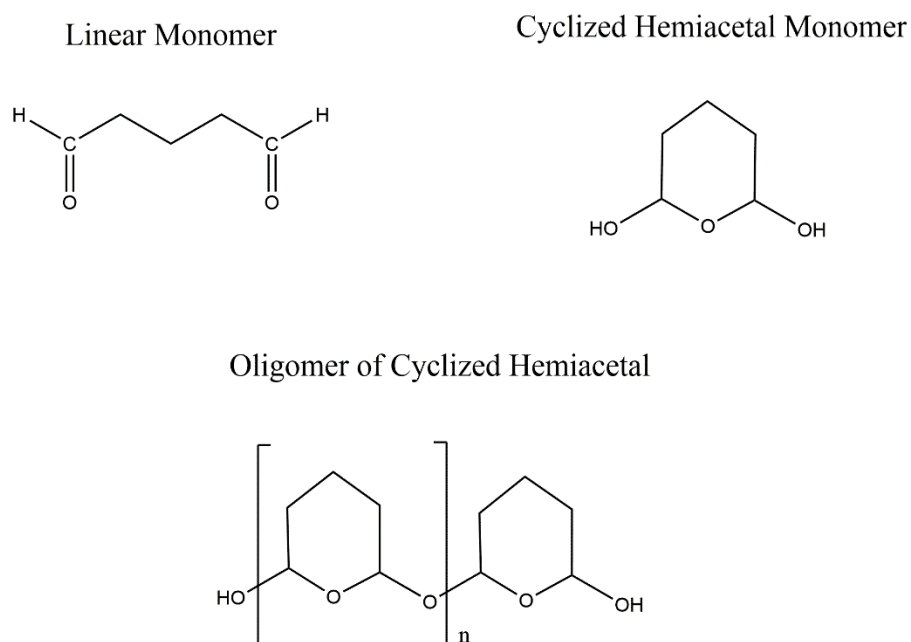


**Figure 18.** Different forms of glutaraldehyde that exist in equilibrium with each other.

These three forms can lead to different reactions and different crosslinked products being formed and exist in an equilibrium. As the pKa of methanol is slightly lower than that of water, 15.5 and 15.7, respectively, methanol is a slightly better acid than water, leading to a solvent environment that could potentially support the hemiacetal and oligomeric hemiacetal forms of glutaraldehyde. A reaction scheme for the glutaraldehyde crosslinking reaction is proposed in Figure 19. The hemiacetal glutaraldehyde can readily react with the amino group of the peptide to form the peptide-glutaraldehyde intermediate molecule. This reaction can occur at the other hydroxyl group on the peptide-glutaraldehyde intermediate resulting in the formation of a crosslinked peptide.
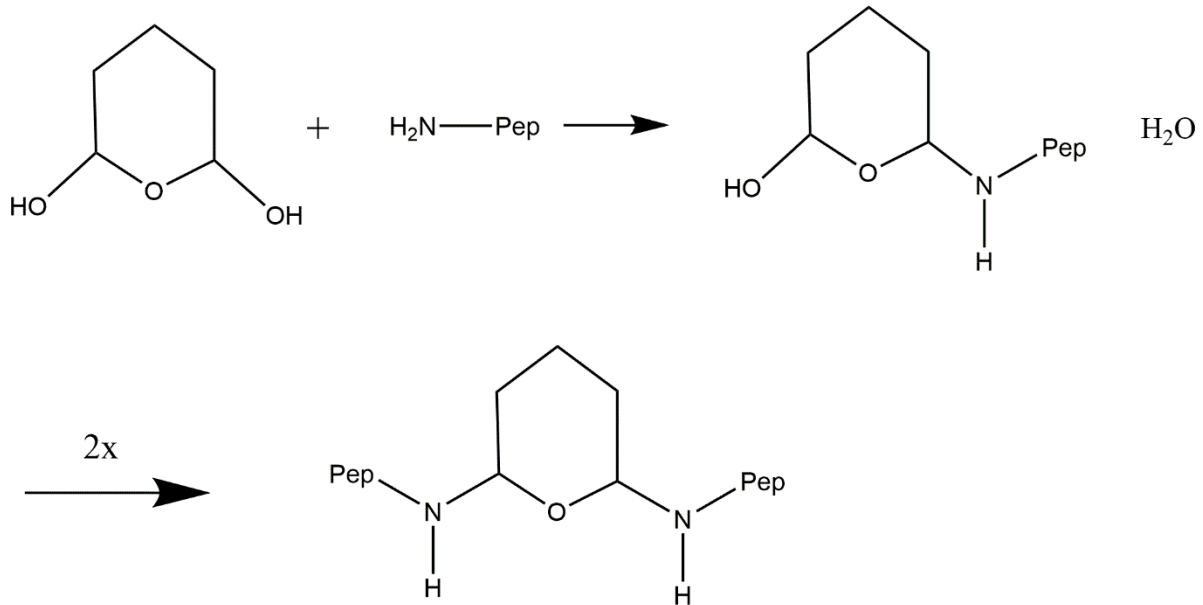
**Figure 19.** Crosslinking reaction scheme of glutaraldehyde. The hemiacetal of glutaraldehyde can react with the amino groups of two peptide molecules to form a larger crosslinked structure. Idea adapted from (Migneault et al., 2004).

Another reaction that could occur with the peptide-glutaraldehyde product is the reaction of another glutaraldehyde molecule with lysine, or potentially another amino acid with nitrogen in its side chain. This can lead to the propagation of a large crosslinked structure involving many glutaraldehyde and peptide molecules as shown in Figure 20.
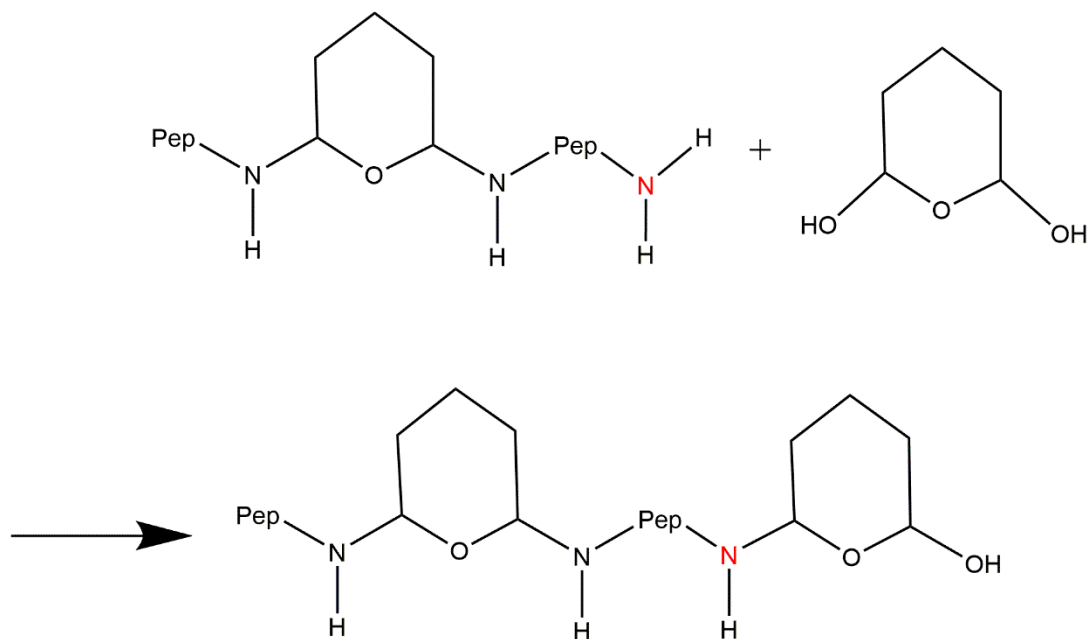
**Figure 20.** Propagation of the crosslinking reaction of glutaraldehyde by the reaction of a second glutaraldehyde molecule with a lysine functional group on the peptide backbone. This can lead to a much larger molecule and can propagate further depending on the nitrogen containing amino acids of the peptides to form a crosslinked structure. Idea adapted from (Migneault et al., 2004).

A third possible reaction, shown in Figure 21, is with an oligomer form of glutaraldehyde and two peptide molecules in a very similar reaction to the one in Figure 19. The main difference is that the final crosslinked product formed will be a longer chain due to the increased amount of glutaraldehyde molecules involved in the product. This would increase the molar ratio of glutaraldehyde used in the reaction compared to the reaction in Figure 19 as well.
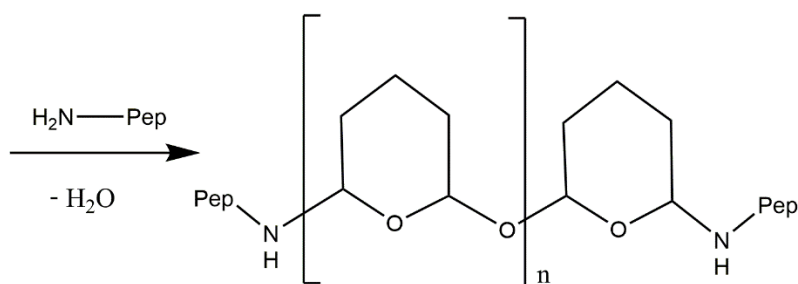
**Figure 21.** The reaction of an oligomer of the hemiacetal of glutaraldehyde to form a linear crosslinked structure with two peptide molecules. Idea adapted from (Migneault et al., 2004).

The result of these reactions is to join two or more peptide molecules together; increasing the chain length and therefore the molecular weight in the product molecule. Increasing the molecular weight of polymers has been shown to improve floc strength and formation rates, as well as flocculation settling rates (Jankovics, 1965). In previous work by Nayef El-Thaher and Tizazu Mekonnen in 2013, crosslinking of SRM-derived peptides with glutaraldehyde required high temperatures for the crosslinking reaction to occur (El-Thaher et al., 2013). In this article the peptides were added directly to a 50% glutaraldehyde solution in an effort to reduce the water present in the reaction and shift the reaction to favor formation of the products. However, the reaction required high temperatures so that water would further be concentrated by evaporation to cause the insoluble crosslinked products to precipitate. Another strategy to achieve a low water concentration in the reaction would be to use a miscible organic solvent, such as methanol, as the reaction medium, as this would allow the water present in the reagent to be diluted without the need for higher temperatures. A less concentrated reaction medium may also allow for a higher peptide to glutaraldehyde ratio to be used due to more solvent being present to dissolve the peptides. Also, a lower temperature would reduce the amount of evaporation of the

glutaraldehyde from solution, allowing more crosslinking to occur and higher molecular weight products to form.

## 3. Materials and Methods

### 3.1. Materials

The specified risk materials were obtained from a large multinational rendering corporation in western Canada. The kaolin clay used was Polygloss 90, which had a median particle size of 0.4 microns, and was obtained from KaMin LLC (Wrens, GA). Calcium sulphate dihydrate (gypsum) (98+%) was obtained from Fisher Scientific (Fair Lawn, NJ). The filter paper used was Whatman no. 4 (pore size 20–25 mm) (GE Healthcare, Chicago, IL) was obtained from Fisher Scientific. The spray drier used was a Buchi B290 Spray Dryer (BUCHI Corporation New Castle, DE). The glutaraldehyde solution (50% Certified), methanol (HPLC grade, 99.9%), and concentrated HCl (Certified ACS Plus, 36.5 to 38.0%) was purchased from Fisher Scientific (Fair Lawn, NJ).

### 3.2. Hydrolysis of SRM

SRM was hydrolyzed as per the protocol described by (T. H. Mekonnen, Mussone, Choi, & Bressler, 2015), and a flow diagram of the process is shown in Figure 22. In brief, hydrolysis took place by addition of distilled water to 1 kg of SRM in a 1:1 ratio by mass, followed by heating at 180°C at ≥174 psi for 40 minutes in a 5.5 L reactor (Parr 4582, Parr Instrument Company, Moline, IL) with constant stirring at 200 rpm, as per the CFIA regulations. After hydrolysis, the material is considered nonhazardous and was further processed to remove lipids and other insoluble material by centrifugation (Avanti J-26 XP high-performance centrifuge, Beckman Coulter Canada LP, Mississauga, ON) at 7000 x g for 30 minutes, followed by a second round of centrifugation at 7000 x g for 10 minutes. After each round of centrifugation, the liquid phase was decanted, and the solids were removed. Residual solids were further removed by vacuum filtration (Whatman 4 filter paper) of the liquid phase. After processing, the peptide mixture was spray dried to remove water, with drying conditions consisting of an inlet

temperature of 170°C and an outlet temperature of 80°C. The final product was a light brown powder.
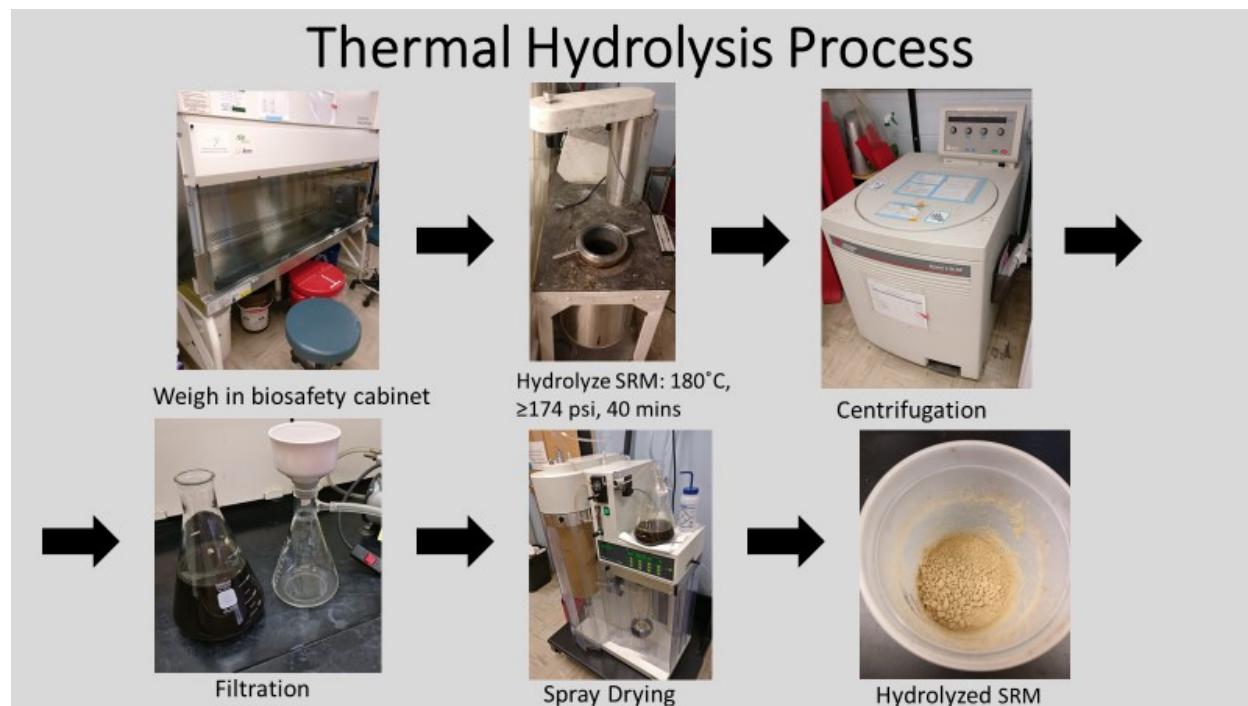


**Figure 22.** Flow diagram of thermal hydrolysis process to convert SRM into peptides.

## 3.3 Preparation of Synthetic Process Water

Synthetic process water (SPW) was used to replicate the ion concentrations of a model oil sands tailing pond. It was prepared by adding the compounds in the proportions found in Table 1 into distilled water. Before the kaolin slurry was prepared, the pH of the SPW was adjusted to 8.00 with concentrated HCl and a pH meter (Fisherbrand™ accumet™ AB15 Basic, Thermo Fisher Scientific, Ottawa, ON).

| Salt | $2H_2O \cdot CaCl_2$ | KCl | NaCl | $Na_2SO_4$ | $6H_2O \cdot MgCl_2$ | $NaHCO_3$ |
|---|---|---|---|---|---|---|
| **Concentration (g/L)** | 0.055 | 0.028 | 0.545 | 0.443 | 0.084 | 0.895 |

**Table 1.** Ion concentrations used for formulation of SPW.

## 3.3 Preparation of Kaolin Slurries

To prepare the 4% (wt/wt) kaolin clay slurries 10 g of kaolin clay was added to 240 g of synthetic process water (SPW). When necessary, the SPW contained 300 ppm of $CaSO_4$ as a coagulant. The slurries were then mixed on a jar tester (PB-900 jar tester from Phipps and Bird, Richmond, VA) at 300 rpm for 30 minutes. The slurry was then left to sit for 90 minutes to allow for full hydration of the clay particles. The mixture was then mixed again for 5 minutes before use.

## 3.4. Flocculation Experiments

Flocculation experiments were carried out in 250 mL graduated cylinders. 250.0 g of slurry, corresponding to 247 mL, was added to the graduated cylinders. Various treatments were added to the cylinders and the contents of the cylinders were then homogenized by plunging 20 times using a custom plunger, made by welding a metal rod to a washer. Flocculants were added on a wt/wt basis. The first number in this ratio represents the weight of the flocculant and the second number is based on the weight of the clay particles present in the column. Settling was recorded by measuring the height of the mudline over time. An example of a completed settling test is shown in Figure 23.
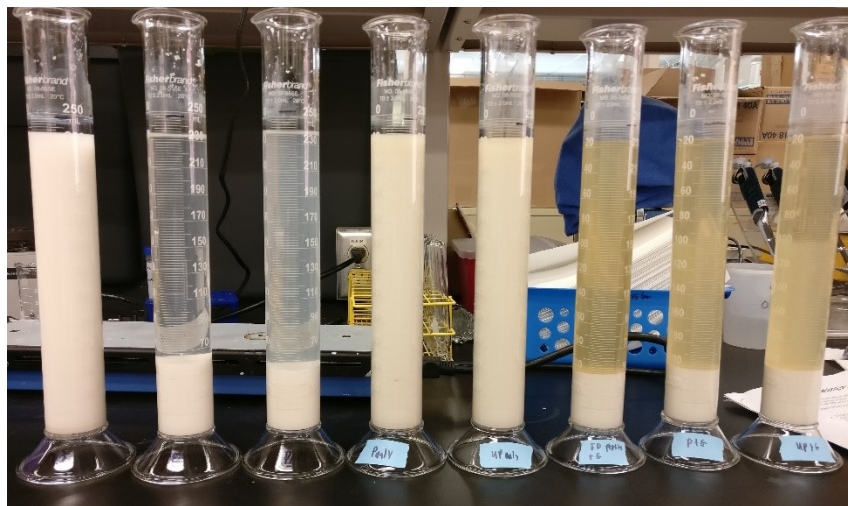


**Figure 23.** Example of a flocculation experiment after completion.

## 3.5. Turbidity Measurements

The turbidity of the supernatant was measured using a turbidimeter (Orion AQ4500 Thermo Fisher Scientific, Grand Island, NY) after 48 hours of settling by withdrawing the supernatant with a pipette approximately halfway between the surface of the solution and the mudline. Samples were measured using the IR ratio mode to eliminate any interference from the color of the sample. The principle behind this measurement is that there is one light source and two detectors to measure the scattering of light. The light source sends a beam of light into the sample and is detected by detector 1, which is situated directly across from the light source, to record a reference measurement. At the same time, a second detector records the light that was scattered at a 90° angle to the light source, which is the active signal. An algorithm then determines the turbidity based on the ratio of these measurements and compensates for the color absorption in the reference beam. The range of the measurement method described by the manufacturer is from 0 – 4000 NTU. The wavelength used for measurement 860 nm.

## 3.6. Esterification of Peptones with Methanol and HCl

Esterification of the peptones was performed by addition of concentrated HCl to the peptones that were dispersed in methanol as a solvent. The HCl was added to make a concentration of 0.2 M and was reacted for 1, 4 or 24 hours. The reaction was halted by neutralization with a 10 M NaOH solution by addition of 20% less than the theoretical amount it should require to fully neutralize the added acid. This was done to not overshoot pH 7, as basic conditions led to a complete reversal of the reaction. It was determined, by titration, that if the majority of the acid had been neutralized and the solution was in the range of pH 1-7 there would be no significant loss of ester groups formed. The strong base NaOH was used instead of a buffer because the effect of the NaCl salt on flocculation of a kaolin slurry is well known and other salts would have added another variable into the experiments. $NaHCO_3$ was also studied to neutralize the reaction, however the resulting pH of the solution was ~8.35, which was high enough to lead to a breakdown of the ester groups formed during the reaction. After neutralization of the acid catalyst in the reaction, the methanol solvent was removed by rotary evaporation. The product was then dissolved in distilled water, the initial pH was taken, and a carboxylic acid titration was

performed to determine the amount of esterification that had occurred. After titration, the water was removed from the products by freeze drying, and the resulting powder was recovered.

### 3.7. Crosslinking Reaction of Peptones with Glutaraldehyde

To increase the molecular weight of the peptones, they were reacted with glutaraldehyde, which is a crosslinking reagent. The peptides were added to methanol and the glutaraldehyde solution was added dropwise. This reaction was carried out at room temperature for 2 hours. The product was recovered by vacuum filtration followed by washing with methanol. Residual solvent was evaporated from the product in a fume hood at room temperature for 48 hours. After drying the resulting solid was ground to a powder with a mortar and pestle. The resulting product was a fine brown powder.

### 3.8. Analytical Methods

### 3.8.1. Carboxylic Acid Titration

A titration method was used based on the pKa of carboxylic acid groups to determine the carboxylic acid content of the peptones before and after a reaction as described by other researchers (Vaz, De Graaf, Reis, & Cunha, 2003). The principle behind this estimation is based on the protonation and deprotonation of a carboxylic acid group depending on the pH of the solution. The pI of a peptide is the point where it is predominantly in its zwitterionic form, which occurs around neutral pH. By titrating from above the pI of the peptide to a value before the pKa of the carboxylic acid group using a strong acid, one can correlate the amount of acid used to the amount of protonated carboxylic acid groups. To do this the peptone sample was solubilized in distilled water and the pH was adjusted to 7 with NaOH. The pH was then brought to 6.00 with HCl and then the volume of a 0.1 M HCl solution that was required to titrate the sample from pH 6.00 to 3.00 was recorded. The volume required per gram of sample was determined and used for comparison.

### 3.8.2. Fourier-Transform Infrared Spectroscopy

To determine the functional groups present in the peptone samples Fourier-Transform infrared spectroscopy (FTIR) was used. Attenuated total reflectance (ATR) was used as it provides a fast

and convenient way to analyze solid samples. The instrument used was an ATR model ALPHA from Bruker Optik GmbH (Ettlingen, Germany).

### 3.8.3. Thermal Gravimetric Analysis

Thermal gravimetric analysis (TGA) was used to determine the thermal stability of the peptone samples. The instrument used was a TGA Q50 from TA instruments (New Castle, DE). The original method used was to ramp the temperature by 10°C/min to 100°C, then remain isothermal for 10 min, and then increase the temperature by 10°C/min to 800°C. The program was altered to end at an earlier endpoint for the reaction parameter experiments in Section 4.5. The method used was to ramp the temperature by 10°C/min to 100°C, then remain isothermal for 10 min, and finally increase the temperature by 5°C/min to 500°C.

### 3.8.4. Ashing Analysis

To measure the mineral content of the peptones and the glutaraldehyde crosslinked peptones an ashing analysis was performed using a muffle furnace. First, the crucibles were dried in a furnace for one hour at 500°C and were left to cool in a desiccator to room temperature. The crucibles were then weighed on an analytical balance and approximately 0.5 g of the samples were added, and the weights were recorded. Samples were then added to the furnace at 500°C overnight. The crucibles were then cooled in a desiccator to room temperature and were weighed again. The ash content was then determined using the weight difference before and after ashing. Samples were analysed in triplicate.

### 3.8.5. Elemental Analysis

Elemental analysis of the unmodified peptones and the crosslinked peptones was performed by CHNS analysis using a Flash 2000 Organic Elemental Analyzer from Thermo Fisher. The reactor was maintained at 1000°C and the detector used was a thermal conductivity detector. Analyses were performed in duplicate.

### 3.8.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The method used for gel electrophoresis was a Tricine-SDS PAGE based on a 2006 protocol published in Nature Protocols and was run on a mini-protean II electrophoresis unit (Bio-Rad

Laboratories, Richmond, CA, USA)(Schägger, 2006). After some trial, error, and optimization, some modifications to the protocol were made to obtain better results with the crosslinked peptones. To improve their solubility and loading into the gel, the samples were incubated in the 1x sample buffer solution and mixed for 2 hours on a nutating mixer prior to gel loading. 20 µL of the samples were loaded into the gel. The concentrations of the peptone sample solutions were also optimized to provide enough sample so that they could be visualized, but not too much sample to avoid overloading causing dark smearing. This required preparing solutions of 5% and 10% for peptones (wt/wt) and 5% for the crosslinked peptones, in the case of the Coomassie staining. For the silver staining lower concentrations were required for proper visualization to occur. 0.5% and 0.1% (wt/wt) solutions were prepared for the peptones and a factor of 10 lower was required for the crosslinked peptones at 0.05% and 0.01% (wt/wt). To obtain the lower concentration samples, dilution with the 1x sample buffer solution was used. Once loaded, the samples were run at 100 V until they left the loading gel and were run at 200 V until the dye band was beginning to leave the bottom of the gel.

For Coomassie staining the protocol used was based on the same 2006 Nature Protocol. The silver staining kit was a Pierce Silver Staining Kit obtained from Thermo Scientific and the method used was defined by the manufacturer (Thermo Fisher Scientific Inc., 2016). After staining, the gels were imaged on an AlphaImager HP gel visualizing system from Alpha Innotech (San Leandro, CA).

### 3.8.7. Size Exclusion High Performance Liquid Chromatography

Size exclusion high performance liquid chromatography (SEC HPLC) was done using a 1200 Series LC System from Agilent Technologies (Mississauga, ON) with a diode array detector to measure in the UV range at 210 nm based on a method from previous research (T. H. Mekonnen et al., 2013). Superdex Peptide 10/300 GL and Superdex 200 Increase 10/300 GL columns (GE Healthcare Biosciences AB, Uppsala, Sweden) were used in series to enhance separation of the molecules. The Superdex Peptide column has a separation range from 100–7000 Da and the Superdex 200 column has a range of 10–600 kDa. The mobile phase used was 0.15 M $Na_2HPO_4$ solution adjusted to pH 9 with NaOH. Sample injection was done at 0.5 mL/min with an injection volume of 20 µL. A series of standards of blue dextran (2000 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4

kDa), aprotinin (6.5 kDa), and Vitamin B-12 (1.36 kDa) were used to standardize the retention times of the products, and were obtained from Sigma Aldrich (St. Louis, MO).

### 3.8.8. Solubility Test

A solubility test was performed to test the relative hydrophobicity of the products. In this test 50 mg of peptones were added to 25 mL of Milli-Q water and stirred for one hour. The solutions were filtered, the filter paper was dried and weighed. The weight of solids remaining on the filter paper were determined.

### 3.8.9. Zeta Potential

The zeta potential of the peptone samples was determined on a Zetasizer Nano ZSP (Malvern Instruments, UK). The samples were in water as a medium with an RI of 1.330, a viscosity of 1.8872 (cP), and a dielectric constant of 78.5 at pH 7. The temperature the measurements were performed at was 25°C. Each sample was analyzed in triplicate.

## 3.9. Reaction and Flocculation Parameter Experiments

### 3.9.1. Varying the Crosslinking Ratio

The ratio of the reactants used during the crosslinking reaction was varied to determine how varying the amount of crosslinker would impact the products. An estimation of the amount of the amino and carboxylic acid groups of the hydrolyzed SRM peptones was determined in a previous study to be 0.6 mmol/g for the amino groups and 1.6 mmol/g for the carboxylic acid groups (Adhikari et al., 2016). The reactant ratio was varied by keeping the peptide amount constant and varying the amount of glutaraldehyde added based on the ratio of the aldehyde groups to the amino groups of the peptides. It has also been shown in the literature that the one amino group containing molecule will react with two glutaraldehyde molecules (Okuda et al., 1991). The reactant ratio was based on the reaction taking place solely with the amino groups, which was shown to occur in the literature (Broun, 1976; Korn et al., 1972; Migneault et al., 2004; Okuda et al., 1991). Therefore, the reactants were varied as shown in Table 2. In theory, the crosslinking reaction should create more hydrophobic molecules by replacing the hydrogen bonding amino groups with carbon-nitrogen bonds.

| Reactant ratio | Peptides (g) | Glutaraldehyde (50% solution) (mL) | Methanol (mL) |
|---|---|---|---|
| 1:2 | 4.00 | 0.426 | 100 |
| 1:4 | 4.00 | 0.852 | 100 |
| 1:8 | 4.00 | 1.704 | 100 |
| 1:16 | 4.00 | 3.408 | 100 |
| 1:32 | 4.00 | 6.816 | 100 |
| 1:64 | 4.00 | 13.632 | 100 |

**Table 2.** Variation of the ratios of the reactants in the glutaraldehyde peptone crosslinking reactions. The ratios were based on the estimated amino groups of the peptones to the aldehyde functional groups of glutaraldehyde. The reactions were performed at room temperature for two hours in methanol with stirring. The resulting material was vacuum filtered to remove the solvent and soluble material from the insoluble product. Reaction were performed in triplicate.

### 3.9.2. Varying Reaction Time of Peptone-Glutaraldehyde Crosslinking

In this experiment the length of the reaction time was varied to determine its effect on the product. As reaction time increases the reactants have more time to react and therefore a higher crosslinking degree should be observed. The reactions in this experiment were conducted with the same conditions as the previous experiment, which was in methanol, at room temperature, and a 1:32 amino to aldehyde reactant ratio was used, as it gave the best results in the previous experiment. The length of the reaction was varied at 30 minutes, 2 hours and 24 hours. In another study, the reaction between protein amino groups and glutaraldehyde has been shown to occur quite rapidly within the first five minutes and had 40-55% of the amino groups consumed by 30 minutes (Okuda et al., 1991). In another study a gel began to form after 10-30 minutes of reaction time when immobilizing a protein with glutaraldehyde, but the reaction progressed for 1-3 hours (Broun, 1976). These studies provide an indication that the crosslinking reaction will occur rapidly at room temperature in water, but this may change with a change of solvent such as the methanol used in these experiments. Therefore, a wide range of times was used to determine when the reaction had gone to completion.

### 3.9.3. Methods for Crosslinked Peptone Addition as a Flocculant

To determine how the method of addition of the peptones to the slurry would affect the flocculation performance different methods were tested in flocculation experiments. One method was to dissolve the peptones in a solution prior to the flocculation experiment. The peptone solution was compared to addition of the peptones as a solid powder in flocculation experiments. Several crosslinked peptone solutions were prepared at different concentrations to determine the optimum dosage for rapid flocculation to occur. This was done by adding different amounts of dry crosslinked peptone powder into 30 mL of SPW and allowing to mix for 1 hour at room temperature.

In the flocculation experiment, the usual 3% (wt/wt) dosage of the solid powder of crosslinked peptones was compared to 0.1 mg/mL, 1 mg/mL, and 10 mg/mL solutions, where 7 mL of each solution was used, leading to the total amount of crosslinked peptides dosed being 0.007% (wt/wt), 0.07% (wt/wt) and 0.7%(wt/wt), respectively. To keep the volumes the same 7 mL of SPW was added to the method blank, gypsum only, and dry powder treatments.

### 3.9.4. Varying of the Reaction Temperature of Peptone Crosslinking

In this experiment, the temperature of the crosslinking reaction was varied from the usual room temperature (22˚C), to a higher temperature of 39˚C and the point at which methanol boiled, which occurred at 56˚C. Another experiment was done to test a lower temperature of 0˚C. This was done to cover a range of temperatures that would be reasonable for industries to scale up in the future. For the higher temperature reactions, the reaction vessels were submerged in an oil bath that was placed on a hot plate. The reaction temperatures were controlled by thermocouples that were submerged in the solution and fed back into the hotplate to regulate temperature, as shown in Figure 24.
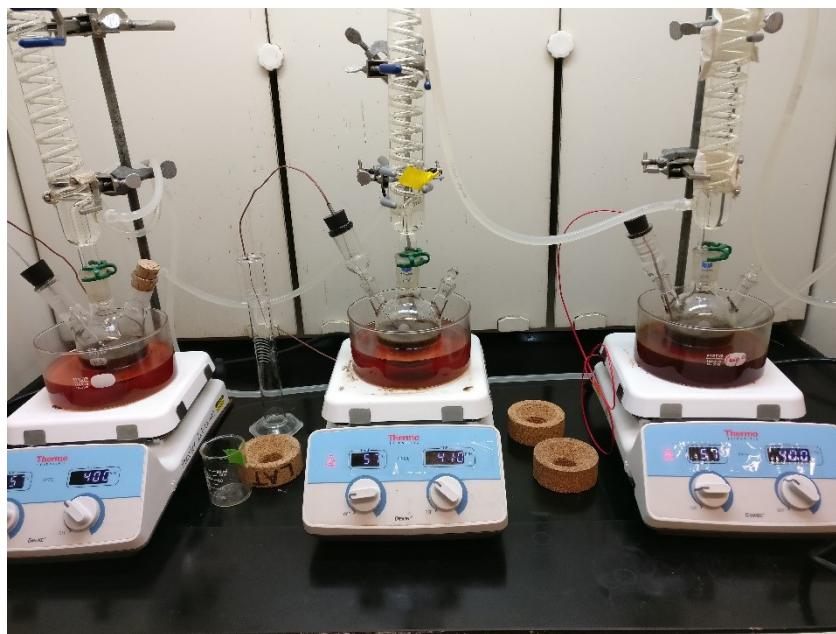
**Figure 24.** Higher temperature reaction apparatus setup.

Evaporated methanol was condensed back into the reaction vessel via a water-cooled condenser. Reactions were conducted for two hours at a 1:32 reactant ratio, as described previously, and products were recovered by vacuum filtration after returning to room temperature. The same procedure was used for the reaction at 0˚C except an ice bath was used instead of an oil bath and the temperature was monitored by a temperature logger. Condensation of methanol was not required.

### 3.10. Statistical Analysis

Statistical Analyses were performed using the R statistical software. Tukey's honest significant difference (HSD) test was used for multiple comparisons of treatments with an $\alpha = 0.5$. The Kruskal-Wallis test was used when the normality assumption was violated. Different letters represent statistically significant differences among groups.

# 4. Results and Discussion

## 4.1. Hydrolyzed Specified Risk Materials as a Flocculant in a Kaolin Clay Slurry

The SRM-derived peptides will from now on be referred to as peptones. The first objective of this research was to determine the effectiveness of the peptones as a flocculant in a model kaolin clay slurry. Based on the work previously addressed in the literature review of G.J. Piazza and R.A. Garcia, peptides from hydrolyzed proteins are able to flocculate a 0.473 % (wt/wt) kaolin clay slurry after 24 hours of settling time (Piazza & Garcia, 2010). Therefore, the peptones of hydrolyzed SRM should be able to perform as flocculants as well, provided they contain high-molecular weight components. As mentioned earlier, it was shown by previous researchers that the peptone mixture does have some high molecular weight molecules within it (T. H. Mekonnen et al., 2013). The other aspect to consider, is whether the flocculant will require the use of a coagulant to first destabilize the colloidal suspension before settling will occur. In the first experiment, peptones were added to a model kaolin clay slurry with gypsum ($2H_2O \cdot CaSO_4$) as a coagulant.

Figure 25 shows the settling over time of the mudline of the clay after addition of different treatments. The first goal of this experiment was to determine if the peptones were able to settle a kaolin clay slurry without the presence of an additional coagulant. The peptone treatment did not have a significant difference in settling rate compared to the method blank at all timepoints throughout the 48-hour experiment, indicating that it was not able to act as a flocculant by itself. The next question that was addressed was whether the peptones could act as a flocculant when a coagulant was added. A gypsum only treatment was used as a control, which showed considerable settling after 120 minutes and was able to settle the slurry after 24 hours. However, addition of the peptones with gypsum together resulted in a synergistic effect where settling occurred faster than either component alone. This provides evidence that the peptones are acting as a flocculant and require a coagulant, such as gypsum, for settling to occur.
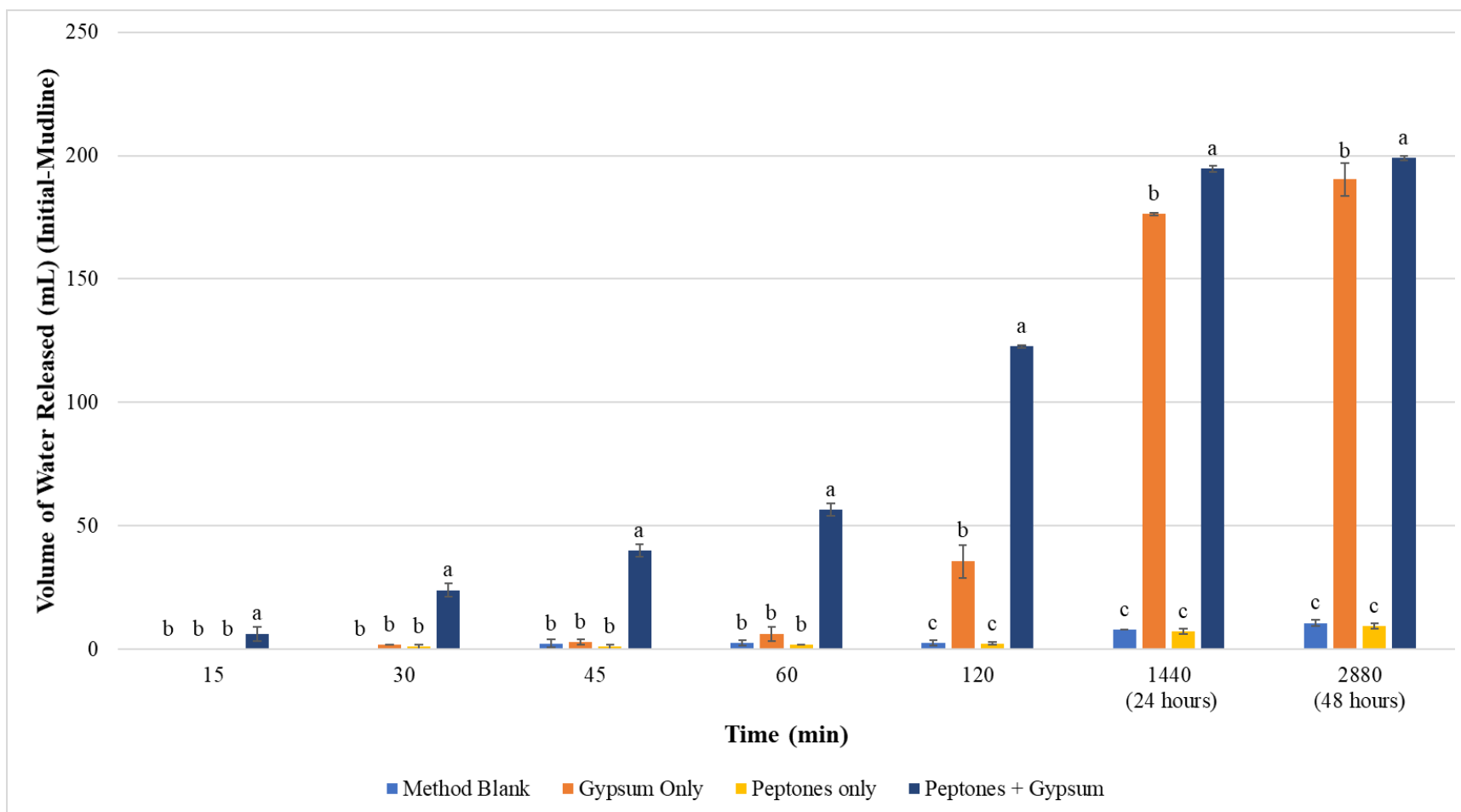
**Figure 25.** Flocculation using peptones derived from the hydrolysis of specified risk materials. Flocculation of a 4% (wt/wt) kaolin clay solution over time. Peptones treatments were dosed at 3% (wt/wt) and gypsum was used at a concentration of 300 ppm for treatments with gypsum. Readings were taken by recording the difference in height of the mudline from the initial height of the slurry. Different letters indicate statistically significant differences in means within time points. Experiments were performed in triplicate.

While the peptones with gypsum can settle a kaolin clay system, the rate is relatively slow compared to industrial flocculants. In one study an industrial HPAM was able to fully settle 100 g of 4.2% (wt/wt) tailings slurry in 30 seconds (Wang, Feng, Xu, & Masliyah, 2010). In order to improve the flocculation rate of the peptones, different chemical modifications were investigated.

## 4.2. Esterification of Peptones

### 4.2.1. Esterification Reaction with Peptones and Methanol

Esterification of the peptones with methanol was the first modification explored to try to increase the interaction rate with the clay surface. The rationale for this was by reducing the amount of negative charges on the peptones, the amount of electrostatic repulsion between the peptone and the clay surface would also be reduced. This would have the potential to increase the amount of binding the peptones would have with the surface of the clay particles in the slurry and improve flocculation. While the esterification reaction is a relatively simple reaction on paper, in practice, there are several challenges that arise. The main issue that was faced when designing this reaction was the removal or neutralization of the strong acid that is required as a catalyst. Different approaches to solving this problem include centrifugation followed by decanting, filtration, or neutralization with a base. After centrifugation and decanting the reaction solution was attempted, a few problems came to light. Firstly, some of the soluble peptone products were lost after decanting. Second, it required many washing steps, which meant product loss was inevitable, and residual acid could persist on the remaining solid. The issue with having residual acid is that once the product is dissolved in water, the remaining acid would drive the reverse of the esterification reaction to occur, and lead to a reduced reaction efficiency. A similar problem was faced with filtration, in that, much of the soluble portion was washed through the filter paper, which led to low product yields. Also, the product would stick to the filter paper, acting like an adhesive, and during separation, flakes of filter paper would end up in the product. The best approach seemed to be the neutralization of the acid with a base, followed by rotary evaporation to remove the methanol.

### 4.2.2. Carboxylic Acid Titration Characterization of Esterified Peptones

In the neutralization method with NaOH, the whole product could be characterized after the reaction and the acid would be converted into salt and water. Initially, the use of a pH meter to determine when the solution was neutralized seemed like an easy way to determine how much base to add to neutralize the acid. However, since the reaction solution was in methanol, and the pH of a solution is determined in water, the reading of pH would be meaningless. Therefore, an experiment was designed where various volumes of 10 M NaOH were added to the solution to neutralize the majority of the acid. After the neutralization, the methanol was first evaporated by rotary evaporation, and the product was dissolved in water. The initial pH of the solution was then determined with a pH meter. A carboxylic acid titration was performed to determine the amount of ester groups formed. In this estimation, a decrease in volume of the 0.1 M HCl, used to titrate from pH 6.00 to pH 3.00, indicates a decreased amount of carboxylic acid groups in the product.

The results of this experiment are shown in Figure 26. The "calculated volume of NaOH" is the volume that it should take to theoretically neutralize all of the concentrated acid with 10 M NaOH. The ±% treatments are a % reduction or increase of the NaOH volume added to neutralize the acid, based on the calculated volume. From the results, it can be seen that adding 10 M NaOH in the range of -35% to -20% of the calculated volume would preserve the formed ester groups. If more NaOH was used, above this volume range, the initial pH of the solution would go above 7.5, which would lead to a loss in the formed ester groups. From these data, as long as the initial pH of the solution, after evaporation of methanol and dissolving in water, was anywhere from a very acidic value of pH ~1, to a neutral value of pH ~7.5, the ester groups formed during the reaction would be stable, and the reverse reaction would not occur to a significant degree. Of note, once the initial pH was measured the solution was brought to pH 7 quickly, to reduce the likelihood of the reverse reaction occurring.
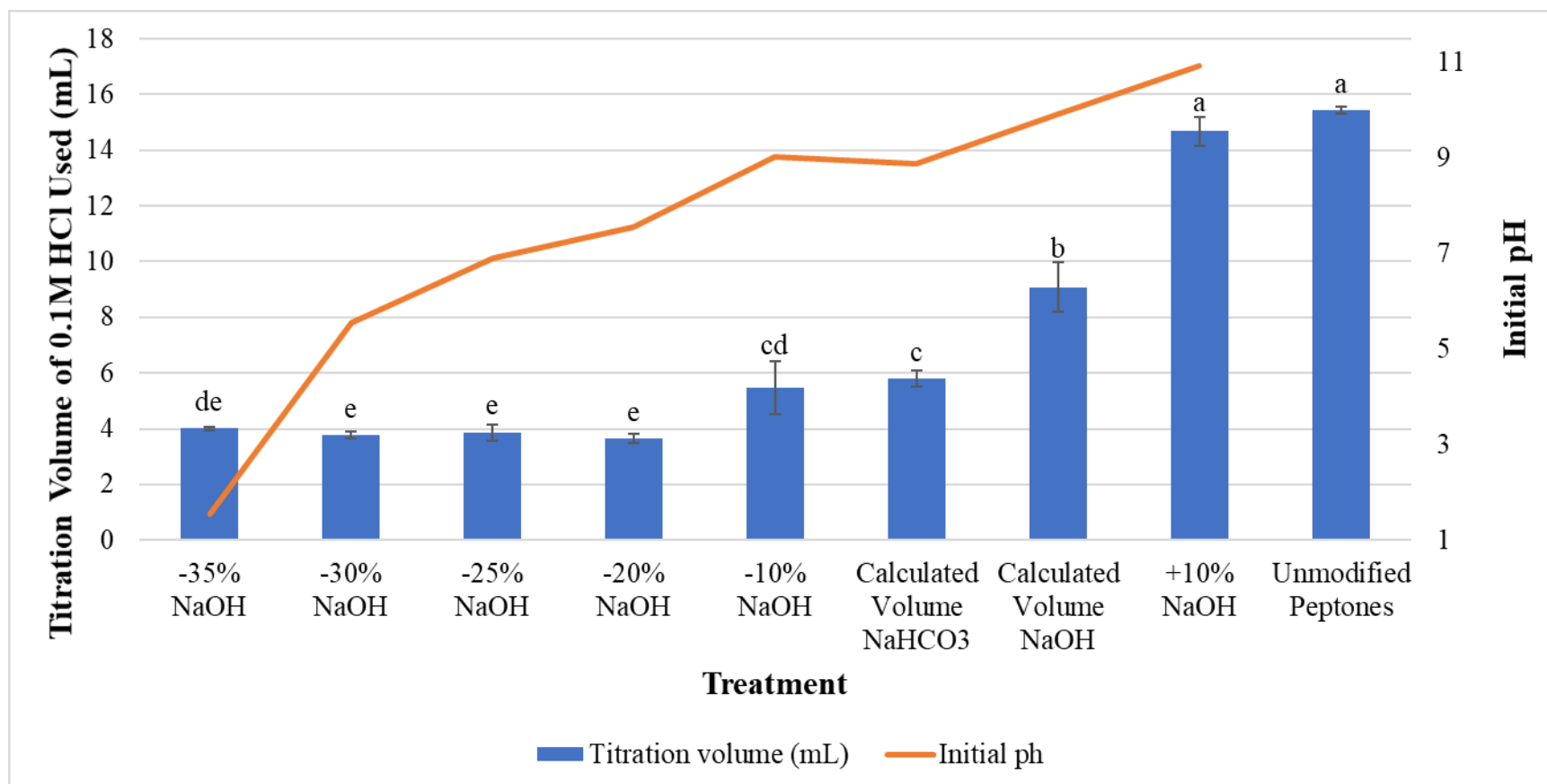
**Figure 26.** Carboxylic acid group titration after esterification reaction of peptones with methanol. The reaction with methanol occurred for 24 hours, at room temperature, with HCl used as a catalyst at a concentration of 0.2 M, followed by neutralization with 10 M NaOH at varying volumes. Methanol was removed by rotary evaporation and the samples were dissolved in water. The initial pH of the solution was taken, followed by a carboxylic acid group titration to determine reaction efficiency. Reactions were performed in triplicate, except -30% NaOH was in duplicate. Different letters represent statistically significant differences in the treatment means.

### 4.2.3. FTIR Analysis of Esterified Peptones

FTIR data from the esterified peptones and the unmodified peptones are shown in Figure 27. Without a proper calibration with many samples, FTIR by ATR should not be used quantitatively. However, it can be used qualitatively to determine the presence and absence of functional groups on different molecules. An ester peak occurs in the range of 1735-1750 $cm^{-1}$. As expected, there is no ester peak on the unmodified peptone sample at this range. However, after the esterification reaction a clear peak is observed in this range, which provides direct evidence that the reaction occurred as theoretically expected.
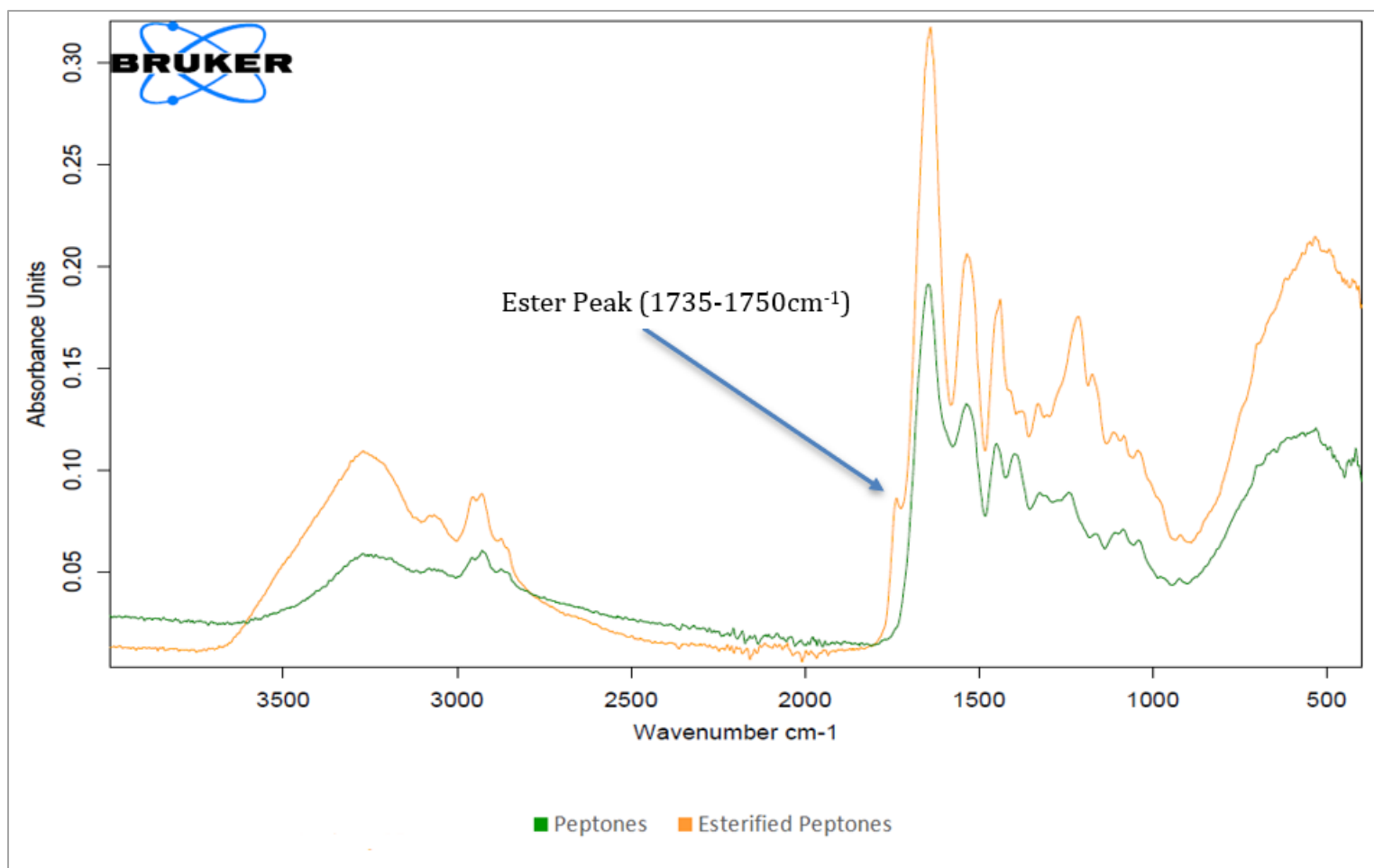
**Figure 27.** FTIR absorbance graph comparing peptones and esterified peptones by ATR. Of note, a peak at 1735-1750 cm$^{-1}$ representing an ester bond was present on the esterified peptone sample and was absent on the peptone sample.

### 4.2.4. Flocculation of a Kaolin Clay Slurry with Esterified Peptones

The esterified peptones were then tested in a flocculation experiment to determine if the modification improved the settling performance compared to the unmodified peptones. The esterified peptone treatment used was from the neutralization conditions with -20% NaOH, as described above. The results from the experiment are shown in Figure 28. The first hypothesis tested in this experiment was that the esterified peptones would improve the settling rate in this system compared to the unmodified peptones. To reiterate, reducing the amount of negative charges on the peptones should allow them to better interact with the clay particles in the slurry, due to a decrease in electrostatic repulsion with the negatively charged clay surfaces. From the graph, the esterified peptones had significantly less settling than the unmodified peptones up to 60 minutes of settling. There was no significant difference between the two treatments after 120 and 180 minutes, but after 24 and 48 hours it again had a significantly lower settling volume. It was also no different than the gypsum control at any time point, meaning it was likely the gypsum that caused the settling, given that the esterified peptones alone led to very minimal flocculation. The evidence from this experiment did not support the first hypothesis. This may be because the electrostatic repulsion was not greatly hindering the interaction of the peptones with the clay surface, as was previously thought. In fact, their may have been a decrease in the amount of interaction occurring by removal of the negative charges. This would make sense, as HPAM has negative charges in it's molecular structure, and it is able to flocculate very rapidly in the presence of a coagulant (Alagha et al., 2013; Nasser & James, 2006; Wang et al., 2010).

The second hypothesis that was tested, was that the esterified peptones would be able to settle the kaolin clay slurry without the presence of a coagulant such as gypsum. However, there was no difference between the method blank and the esterified peptones without gypsum across all time points, therefore this hypothesis could also be refuted. This would also make sense, as the amino groups of the peptones that remained after the esterification reaction would only have a valency one +1, whereas the calcium ions from gypsum would have a valency of +2. The valency of an ion strongly impacts its adsorption ability to a clay. For example, it has been shown that it requires 12.5 times less $CaCl_2$ to induce coagulation compared to NaCl in a kaolin clay slurry (Shainberg & Levy, 2005).
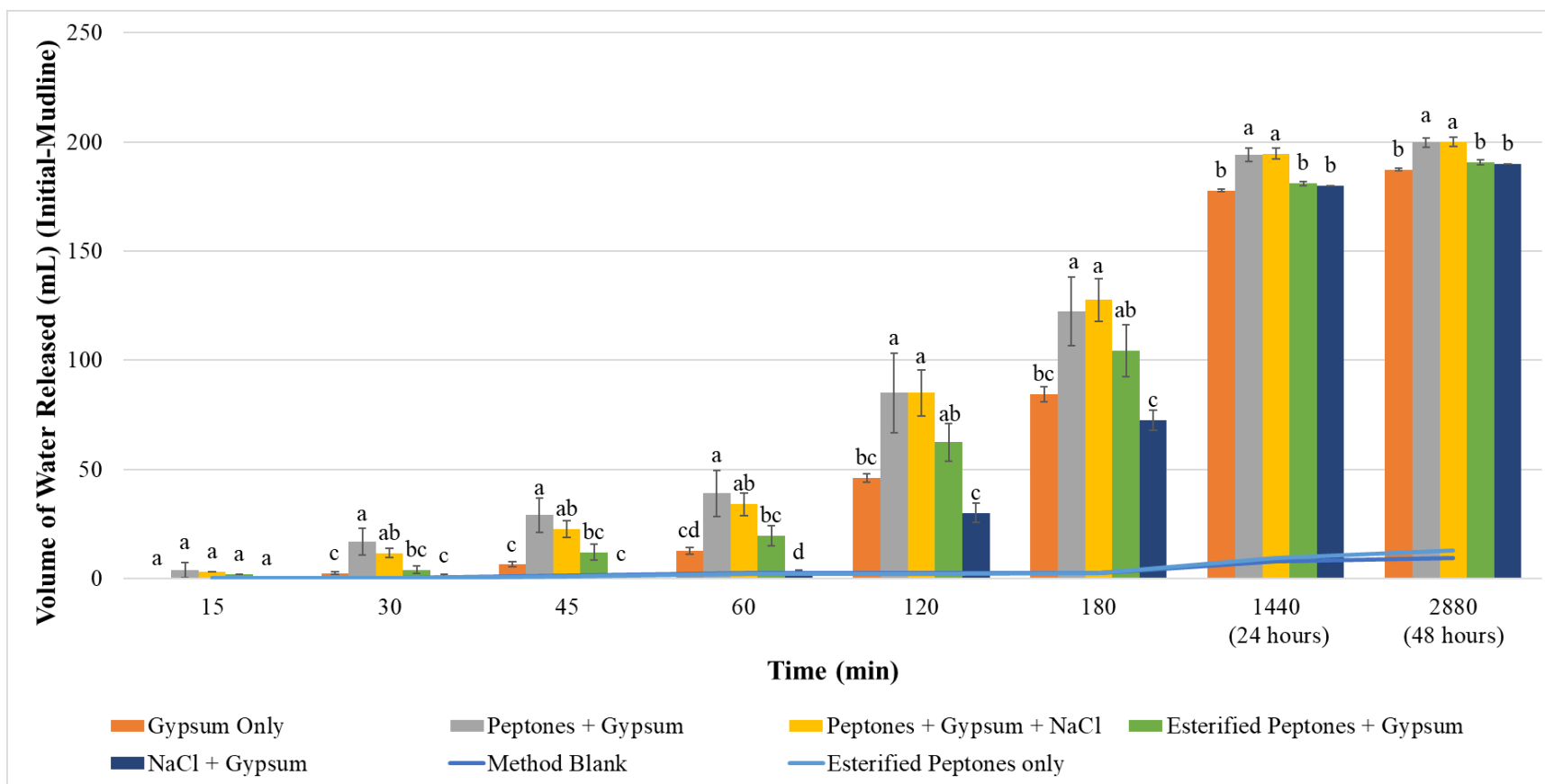
**Figure 28.** Flocculation of a kaolin clay slurry using esterified peptones. A 4% (wt/wt) kaolin clay solution was used. Peptones were dosed at 3% (wt/wt) as a flocculant, and gypsum was used at a concentration of 300 ppm. NaCl controls were used to compensate for the NaCl added to the peptones during esterification, which was 1.82% (wt/wt). Readings were taken by recording the difference in height between the mudline and the initial height of the slurry. Lines represent treatments that were not used for statistical analysis, as minimal flocculation activity was seen. Different letters indicate statistically significant differences in means within time points. Three replicates were performed.

The turbidity measurements after 48 hours are shown in Figure 29. The turbidity was lower in the esterified peptones than the unmodified peptones, however it was no different than gypsum by itself. Without an improvement in settling rate compared to gypsum there would be no advantage to using the esterified peptones.
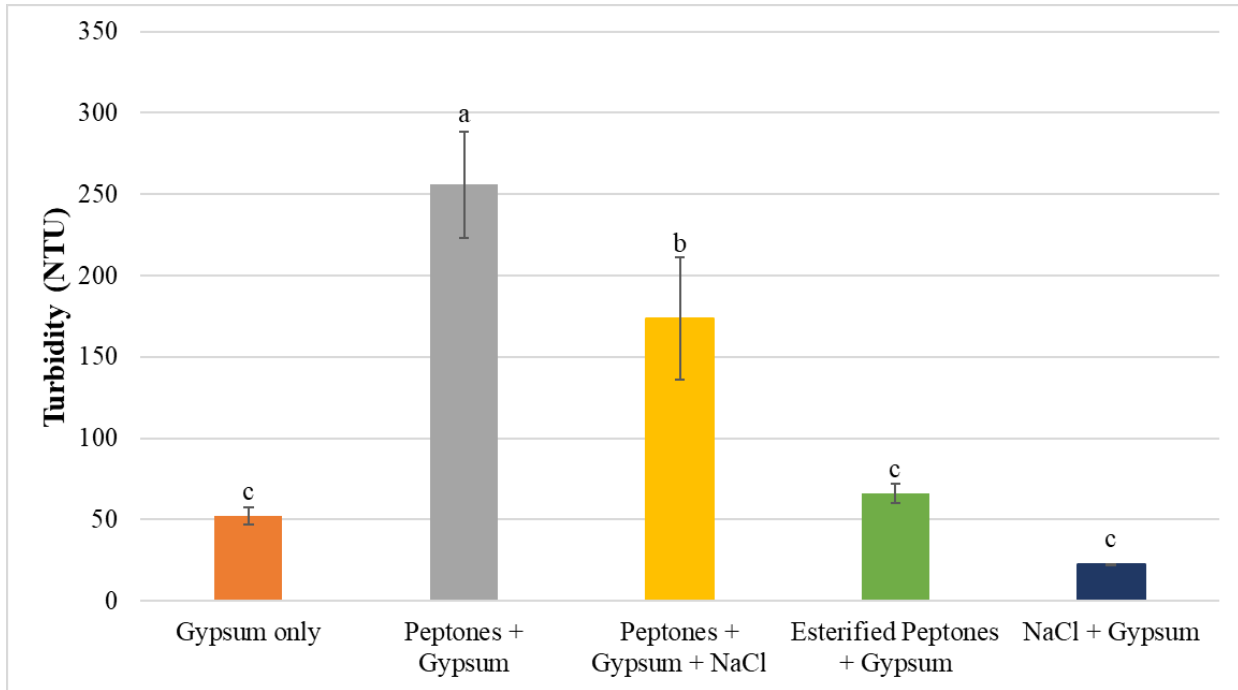


**Figure 29.** Turbidity of the supernatant after flocculation using esterified peptones. The readings were taken after 48 hours of settling by treatment using a turbidimeter. Experiments were performed with three replicates. Different letters indicate statistically significant differences in turbidity.

The results from this experiment indicate that the esterification reaction did not improve the flocculation ability of the peptones and a new type of modification should be considered.

### 4.3. Glutaraldehyde Crosslinking of Peptones

### 4.3.1. Glutaraldehyde Crosslinking Reaction with Peptones

A second reaction that was investigated was a glutaraldehyde crosslinking reaction, with the goal to increase the molecular weight of the peptones, as larger molecules have been shown to have better flocculation characteristics (Fleer & Lyklema, 1976; Jankovics, 1965; Nasser & James, 2006). This was also shown to be the case when it comes to peptide-based flocculants, as was

discussed in the literature review. It was shown that after hydrolysis, lower molecular weight peptides were not able to flocculate a kaolin clay slurry, intermediate molecular weight peptides were able to cause flocculation at high concentrations, and high molecular weight peptides were able to induce flocculation at low concentrations (Piazza & Garcia, 2010). The crosslinking chemical glutaraldehyde was investigated, due to its relatively low cost and high reactivity. When attempting this reaction in water, a similar filter paper issue was encountered when vacuum filtering, as was described in section 4.2.1. when performing the esterification reaction. The recovered products were tightly bound to the filter paper and much of the product was washed through into the filtrate. As a result, a low recovery with contamination from the filter paper occurred in this reaction. Different solvents were then investigated to try to avoid these problems. Methanol was chosen due to its high miscibility in water and its polar protic nature. It has a pKa of 15.5, which is similar to the pKa of water at 15.7. In addition, the peptones were less soluble in methanol compared to water, due to its lower polarity, which allowed for better product recovery.

### 4.3.2. Flocculation of a Kaolin Clay Slurry with Glutaraldehyde Crosslinked Peptones

The crosslinked products were then tested in a flocculation experiment, with the data shown in Figure 30 and the turbidity data in Figure 31. The first objective of this experiment was to determine if the glutaraldehyde crosslinked peptones would have a faster settling rate than the unmodified peptones with or without gypsum as a coagulant. From the results, after five minutes the glutaraldehyde crosslinked peptones had a significantly higher amount of settling, in both the treatment with and without gypsum, than the unmodified peptones and the other treatments. In fact, it took until 120 minutes of settling before the unmodified peptones reached around the same settling volume as the glutaraldehyde crosslinked treatments in methanol after 5 minutes of settling.

Another objective of this experiment was to determine if the products from the crosslinking reaction would perform better in methanol as the solvent compared to water. This experiment confirmed that the peptones crosslinked with glutaraldehyde in water did not exhibit the same rate of settling, across all time points, as when methanol was used as a solvent for glutaraldehyde crosslinking. Also, the peptones crosslinked with glutaraldehyde in water with gypsum was not significantly different than the unmodified peptones with gypsum. This provides evidence that

using methanol as a solvent was a better choice over water. This could be explained by findings of one study, which looked at glutaraldehyde crosslinking of bovine pericardial tissue in solvent environments of decreasing dielectric constant (Gratzer, Pereira, & Lee, 1996). They found that the lysine residues, which were the targets for crosslinking, were still able to react with glutaraldehyde as the dielectric constant of the solvent decreased. This was unexpected, as decreasing the dielectric constant should have promoted the turn of the polar lysine residues inward, decreasing reactivity. However, they speculated that a potential shift of the pKa of the lysine residues may have been occurring in the more nonpolar solvent environments, to increase reactivity. The shift of the pKa of peptide functional groups in solvent environments of lower dielectric constant has been demonstrated by other researchers (Kalman Burger and Bela Noszal, 1981). Specifically, they found that the terminal amino groups of peptides shifted to a lower pKa of 6.77, in a trifluoroethanol-water solution, compared to a water solution where the pKa was 7.27. This shift indicates that a less polar solution favors the electrically neutral amino group, which is more nucleophilic, and therefore would be more reactive towards glutaraldehyde. Other researchers found that changing the solvent environment of glutaraldehyde from water to an anhydrous solvent, chloroform and benzene, caused it to undergo rapid polymerization (Hardy, Nicholls, & Rydon, 1969). If the polymerized glutaraldehyde then underwent a crosslinking reaction with two peptide molecules, it would create a high molecular weight molecule.

The third objective of this experiment was to determine if the crosslinked peptones required a coagulant for flocculation to occur, as is the case with HPAM. The settling data showed that the peptones crosslinked with glutaraldehyde in methanol did not require gypsum as a coagulant to settle the slurry. Interestingly, it also showed that the peptones crosslinked with glutaraldehyde in methanol without gypsum had a faster settling rate than the treatment with gypsum. However, when comparing the turbidity data after 48 hours of settling, the treatment without gypsum had a much higher turbidity than the treatment with gypsum. This indicates that there is a trade-off between a slightly faster settling rate or a lower final turbidity, when gypsum is absent or present. This trade-off has also been shown in other flocculation studies. For example, one study that looked at two versions of PAM, one cationic and one anionic for tailings settling. They found that the PAM with the higher initial settling rate, 43 m/hr, had a higher turbidity, 470 NTU, compared to the other PAM, which had slower initial settling rate, 20 m/hr, but a lower turbidity, 68 NTU (Wang et al., 2010). When the turbidity of the supernatant is higher it

indicates that there are some particles remaining in solution, therefore, when gypsum is present fewer particles are remaining in the solution afterward. Ultimately, the use of gypsum will depend on the application, and whether the settling rate or turbidity is the more important factor.
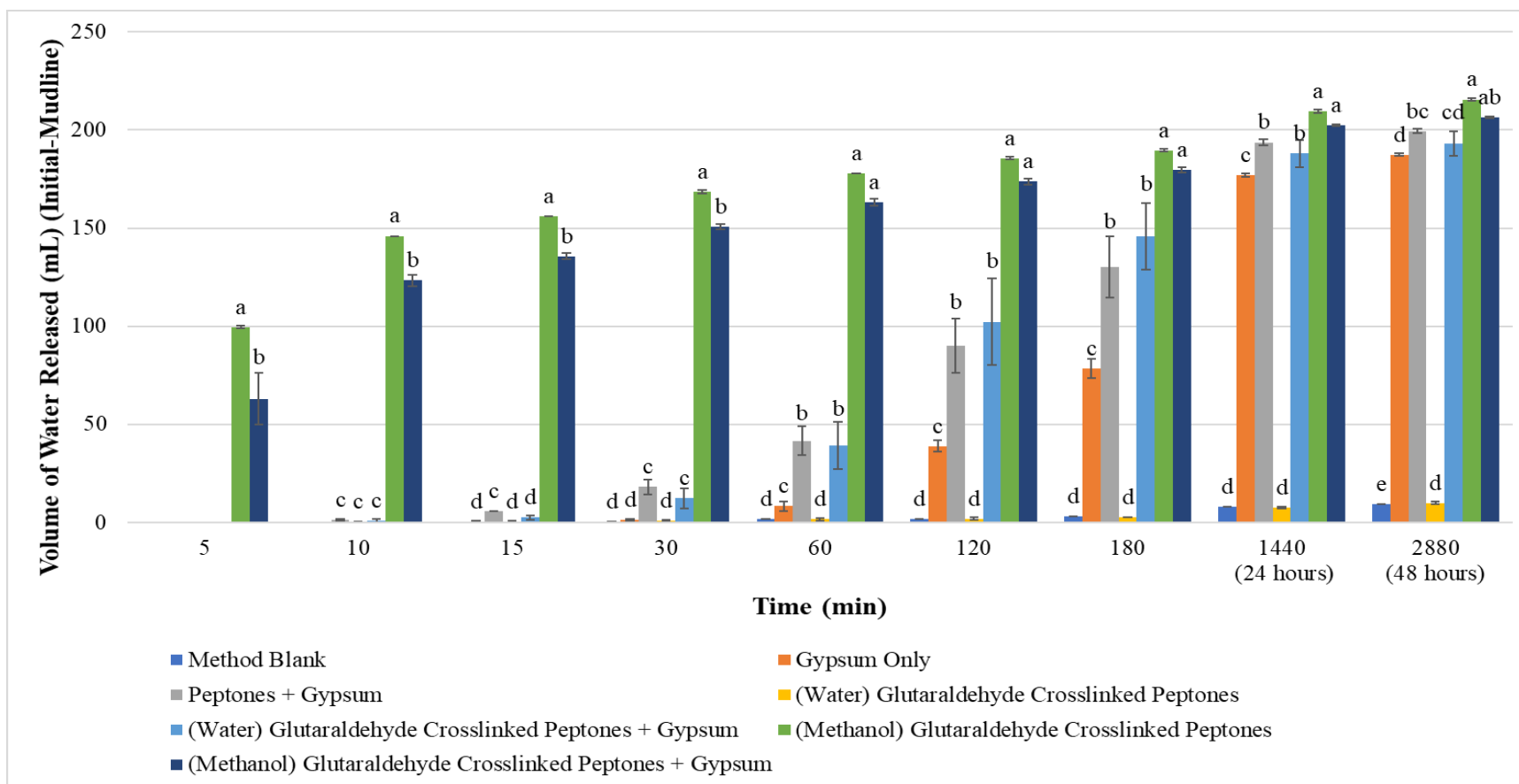
**Figure 30.** Flocculation using glutaraldehyde crosslinked peptones. A 4% (wt/wt) kaolin clay slurry was used. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at a 3% dosage (wt./wt.), and the difference of the mudline and the initial height of the slurry was recorded as the water released. The solvent used for the crosslinking reaction is indicated in brackets. Experiments were performed in triplicate, except for (Methanol) Glutaraldehyde Crosslinked Peptones, which was performed in duplicate due to an experimental error. Different letters represent statistically significant differences of groups within timepoints.
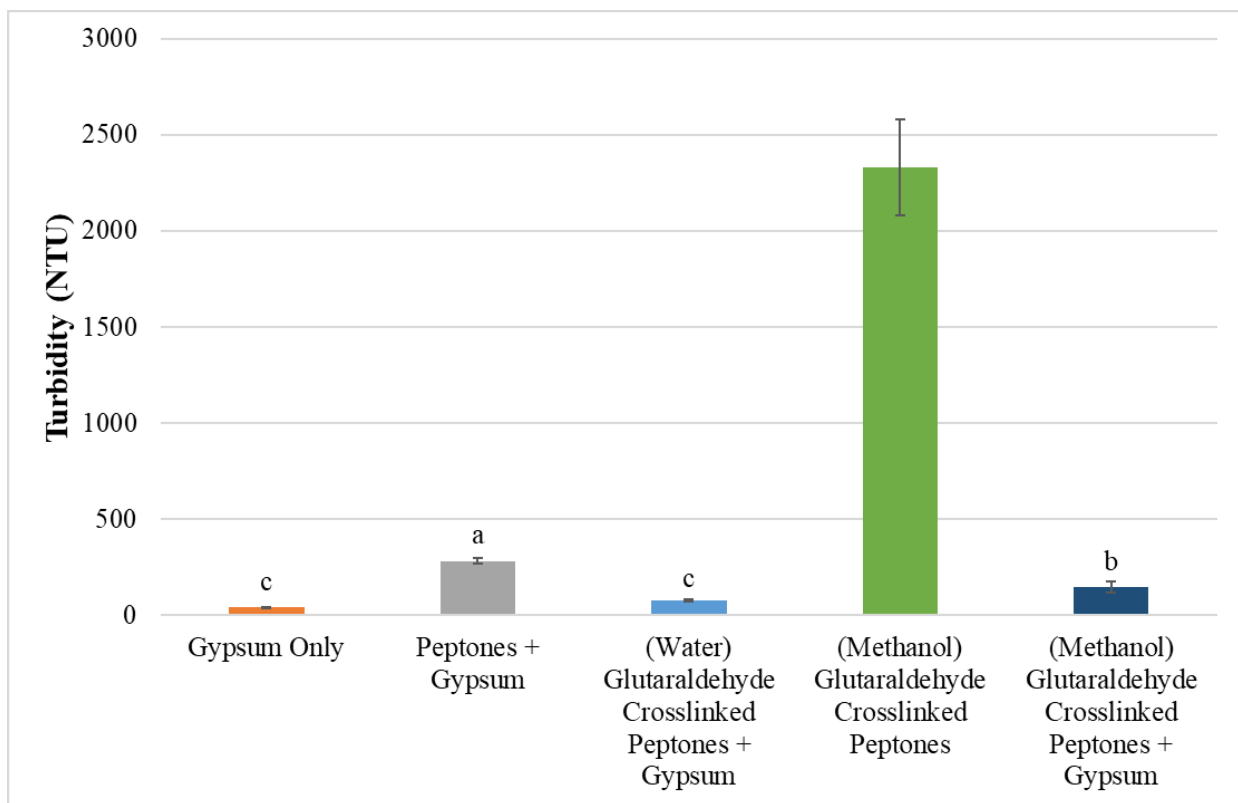
**Figure 31.** Turbidity after flocculation with glutaraldehyde crosslinked peptones. The turbidity averages of the different treatments, after 48 hours of settling, in a 4% (wt/wt) kaolin clay system. Gypsum was added as a coagulant at a concentration of 300 ppm. The solvent used for the crosslinking reaction is indicated in brackets. Experiments were performed in triplicate, except for (Methanol) Glutaraldehyde Crosslinked Peptones was in duplicate due to an experimental error and was left out of the statistics. Different letters represent statistically significant differences in turbidity.

## 4.4. Characterization of the Glutaraldehyde Crosslinked Peptones

After the results from the flocculation experiments, characterization analyses were conducted to attempt to identify any changes to the structure of the peptones that occurred after the glutaraldehyde crosslinking reaction. Theoretically, the crosslinking reaction should have occurred at the amino groups of the peptones to create more thermally stable, higher molecular weight products. By determining these changes, other strategies for improving the flocculation performance of the peptones could be investigated in the future. Several analytical techniques were used to attempt to characterize the peptone-glutaraldehyde crosslinked products. This

includes FTIR, TGA, carboxylic acid titration, SDS-PAGE, HPLC, ashing analysis, and CNHS analysis. For all the characterization studies, the methanol solvent crosslinking system was used, as this system was shown above to have superior flocculation performance.

### 4.4.1. FTIR Analysis of Glutaraldehyde Crosslinked Peptones

In Figure 32 the results from the FTIR are shown. Although no new peaks were expected to form in this reaction, FTIR is a fast and cheap method to analyze the products and can confirm that no unexpected functional groups formed during the reaction. While there are some small peak shift differences between the two samples, no clear discernable new peaks were formed or were absent after the crosslinking reaction. However, when considering the proposed reaction schemes from Figures 19-21, in section 2.4, no new functional groups should have formed. This also reinforces the idea that Schiff base formation (Figure 17 in section 2.4) was not the mechanism of glutaraldehyde crosslinking, as described in the literature (Migneault et al., 2004). There was no C=N peak formed in the crosslinked product to indicate a Schiff base, which is normally represented with a medium intensity peak from 1690-1640 $cm^{-1}$. In both cases there was a peak in the range of 1650-1580 $cm^{-1}$, which is indicative of N-H bending from amine groups. The peak in the unmodified peptones at 1644 $cm^{-1}$ overlaps these two ranges, but there isn't expected to be C=N in natural peptides, meaning it is likely N-H stretching. The results from the FTIR don't necessarily show that the reaction occurred, but they are consistent with what was expected from the reaction.
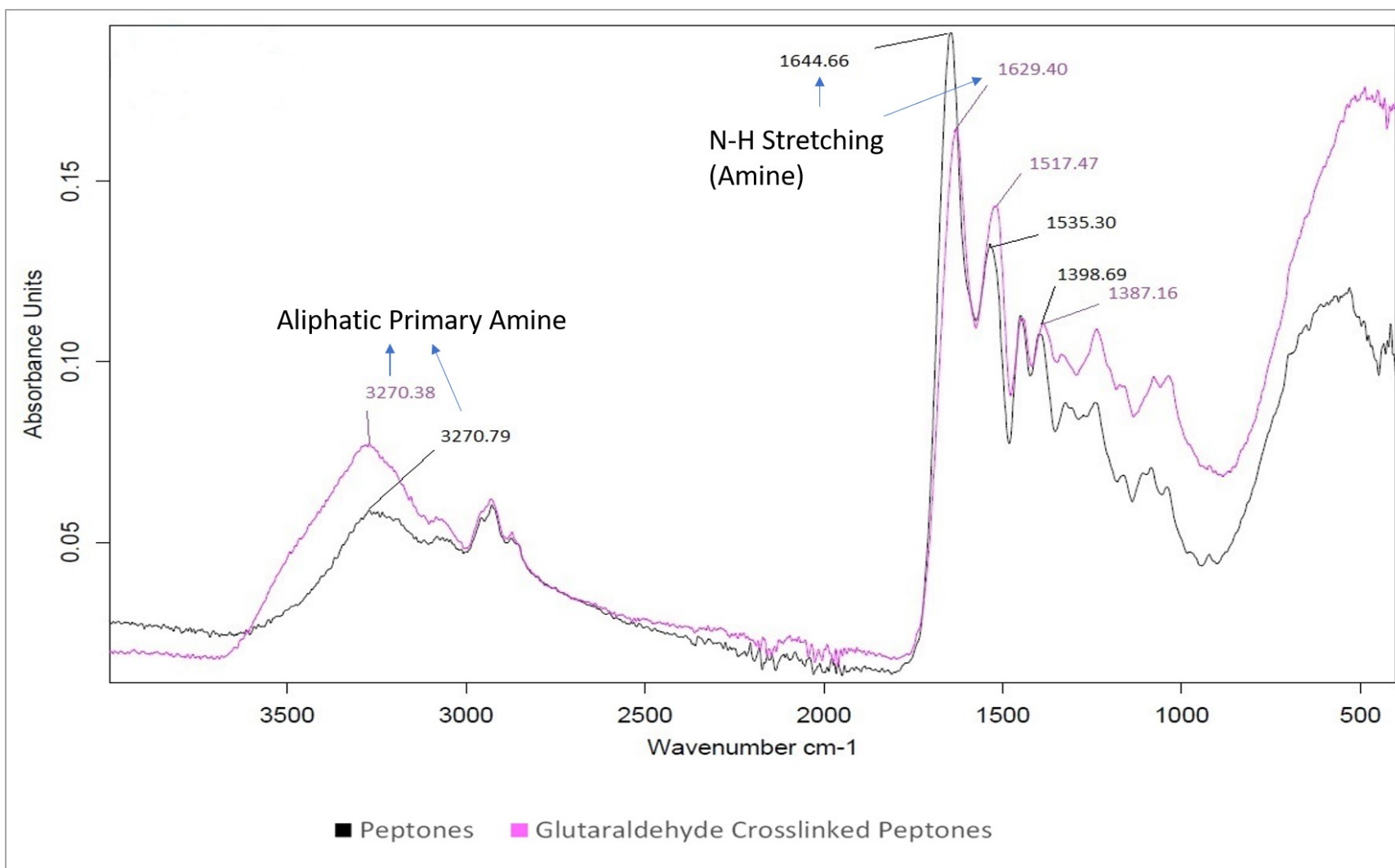
**Figure 32.** FTIR absorbance graph comparing peptones and glutaraldehyde crosslinked peptones by ATR.

## 4.4.2. TGA of Glutaraldehyde Crosslinked Peptones

Another analysis that was used to characterize the glutaraldehyde crosslinked peptones was thermal gravimetric analysis (TGA) as shown in Figures 33. The onset of degradation can be determined by finding the intercept of two extrapolated lines, one from the flat base before decomposition, and the other from the sharpest slope, as described by ASTM standard method E2550–17. For the unmodified peptones the onset temperature was 249˚C and for the glutaraldehyde crosslinked peptones it was 263˚C. This shift signifies that the glutaraldehyde crosslinked peptones were more thermally stable and required a higher temperature to break the increase in bonding that occurred during the reaction with glutaraldehyde. Another interesting note is that at 500˚C the unmodified peptones had a lower weight % remaining than the crosslinked peptones, indicating that the material had higher thermal stability at high temperature due to the increase in bonding after the reaction. The results from the TGA indicate that there was an increase in thermal stability of the glutaraldehyde crosslinked peptones compared to the unmodified peptones, which indicates that additional bonds were formed after the reaction. The addition of more bonds indicates that a reaction did occur between the peptones and the glutaraldehyde. This would help to explain the improvement in flocculation performance, as higher molecular weight peptides have been shown to act as better flocculants, as discussed previously (Piazza & Garcia, 2010).
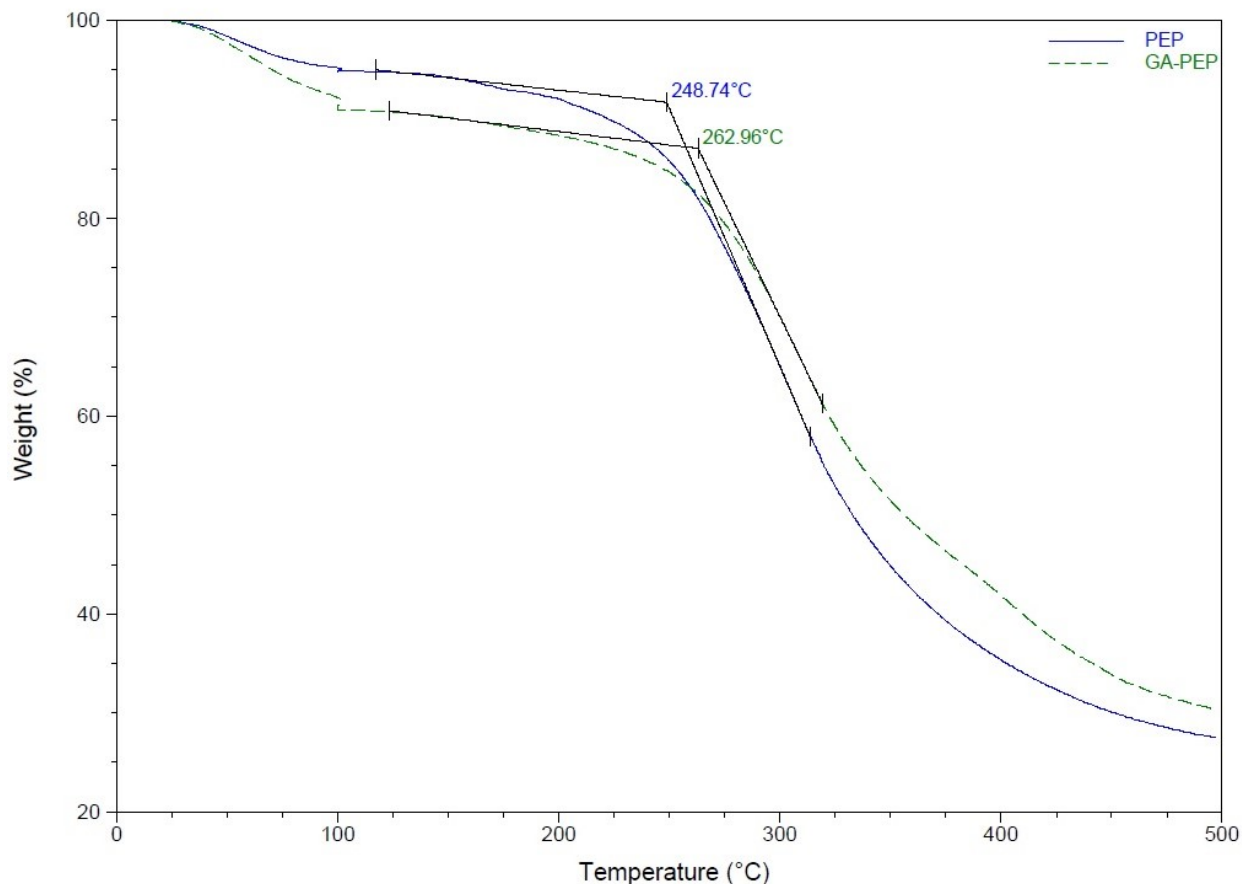
**Figure 33.** TGA data from peptones (PEP) and glutaraldehyde crosslinked peptones (GA-PEP). The crosslinked peptones were reacted at room temperature for two hours in methanol. The onset of the thermal degradation was determined by the ASTM standard method E2550-17.

### 4.4.3. Carboxylic Acid Titration of Glutaraldehyde Crosslinked Peptones

Carboxylic acid titration was another approach used to confirm that the reaction was not occurring at the carboxylic acid groups of the peptones, as it was expected to occur at the amino groups of the peptones based on the literature (Migneault et al., 2004; Okuda et al., 1991). The results from the analysis are shown in Figure 34. In this test, a decrease in the titration volume, to take the pH from 6.00 to 3.00, would indicate that there are less carboxylic acid groups present to buffer the acid. There was no decrease in the titration volume, and therefore in the carboxylic acid groups, which confirms that the crosslinking reaction with glutaraldehyde did not occur with these functional groups. However, there was a small, but significant increase in the volume of acid required to titrate the crosslinked peptones after the crosslinking reaction. To speculate, this

may be due to some unknown side reaction products formed during the reaction. The main takeaway from this test is that the crosslinking reaction did not reduce the carboxylic acid groups present on the molecules, which are likely playing a large role in their functionality as a flocculant.
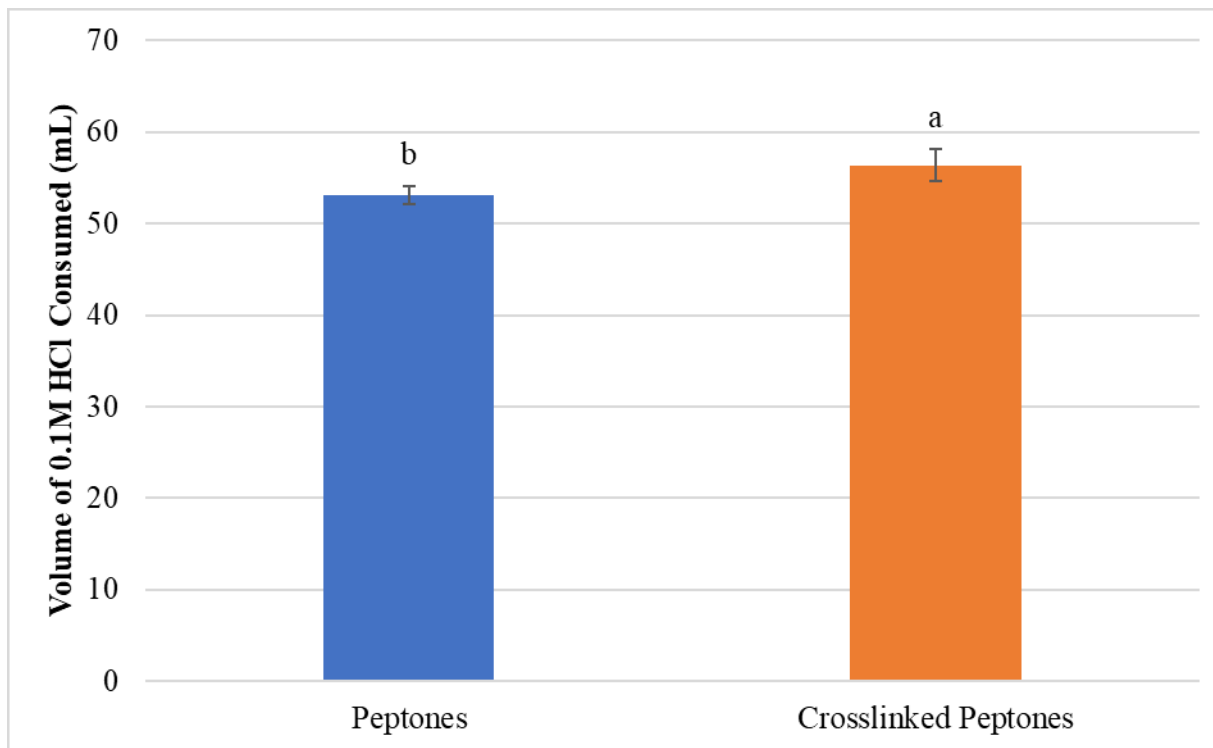


**Figure 34.** Carboxylic acid titration data on hydrolyzed peptones and glutaraldehyde crosslinked peptones. The pH of the solution was brought to ~7 with NaOH, then to 6.00 using HCl. The volume of 0.1 M HCl required to adjust the pH from 6.00 to 3.00 was recorded. Experiments were performed in triplicate. Letters represent different statistically significant groups.

### 4.4.4. Elemental Analysis of Glutaraldehyde Crosslinked Peptones

One of the potential reasons for the flocculation ability of the peptones, and the improvement in flocculation performance with the glutaraldehyde crosslinked peptones, is that they are providing a source of ions from the salt present in the starting material. To rule this out, CHNS elemental analysis and ashing analysis were performed to determine the elemental composition and mineral content of the materials, as shown in Table 3 and Table 4, respectively. From the elemental analysis, a substantial amount of the material in both the peptones and the crosslinked peptones are made up of carbon, nitrogen and hydrogen, which is expected in a proteinaceous material.

There is a marked difference in the nitrogen and carbon content of the two samples, with a reduction in the nitrogen content and an increase in carbon content after crosslinking. This was expected, as the crosslinking reaction with glutaraldehyde should incorporate more carbon from the glutaraldehyde molecules into the molecular structure of the peptones. By adding more carbon, the % of nitrogen would inherently decrease. Based on this logic the sulphur amount should have shown a significant decrease as well, but it did not. This may be due to the sulphur content being near the limit of quantitation for the instrument, which was 0.2 wt.%.

From the ashing analysis, there is a significant decrease in the mineral content of the crosslinking peptones compared to the unmodified peptones. This is likely due to the soluble salts that are present in the starting peptone material that are removed during filtration after the crosslinking reaction. Although the salts are not as soluble in methanol as they are in water, they are still able to dissolve to a certain degree. The ashing analysis provides evidence that a large portion of the peptone samples are not just minerals, which means that coagulation from the addition of salt ions is likely not the cause of improved flocculation performance.

| Sample | %N | %C | %H | %S |
|---|---|---|---|---|
| Peptones | $14.40 \pm 0.06$ | $48.37 \pm 0.04$ | $6.52 \pm 0.00$ | $0.58 \pm 0.03$ |
| Crosslinked Peptones | $13.02 \pm 0.06$ | $50.23 \pm 0.03$ | $6.66 \pm 0.01$ | $0.60 \pm 0.01$ |

**Table 3.** Elemental analysis results from CHNS analysis. % composition based on weight % of sample. Analyses were performed in duplicate.

| Sample | Percent Ash |
|---|---|
| Peptones | $3.89 \pm 0.24$[a] |
| Crosslinked Peptones | $1.49 \pm 0.01$[b] |

**Table 4.** Ashing analysis results of peptone samples with and without glutaraldehyde crosslinking. Samples were heated at 500°C for 24 hours in a muffle furnace and ash % was determined by weight before and after heating. Experiments were performed in triplicate.

### 4.4.5. SEC-HPLC Analysis of Glutaraldehyde Crosslinked Peptones

The goal behind using a crosslinker, like glutaraldehyde, is to increase the molecular weight of the products. An increase in molecular weight has been shown to improve a flocculants performance in settling rate, making the characterization of this property a key analysis

(Jankovics, 1965; Piazza & Garcia, 2010). To determine the changes in the molecular weight of the peptones after the glutaraldehyde crosslinking reaction, SEC-HPLC was attempted. When attempting to analyze the glutaraldehyde crosslinked peptones by this method, some notable problems were encountered. The main issue was that during glutaraldehyde crosslinking reaction, the peptones are becoming less and less solvent soluble as the reaction occurs. This is beneficial, as it indicates that the reaction is occurring and provides a method for isolating the product. However, it is also an issue because SEC-HPLC requires that the product being analyzed is completely soluble in the mobile phase, which the glutaraldehyde crosslinked peptones were not. Only a portion of the products were able to be dissolved and the insoluble, likely highly crosslinked, material had to be removed by filtration before the sample could be analyzed to avoid destruction of the column. The material that was soluble was analyzed and the results of the analysis are shown in Figure 35. From these data, the soluble portion of the glutaraldehyde crosslinked peptones are distributed in a higher molecular weight region than the unmodified peptones. However, this approach likely did not provide an accurate range of the molecular weight of the peptones after the reaction with glutaraldehyde, and therefore, another technique was attempted.
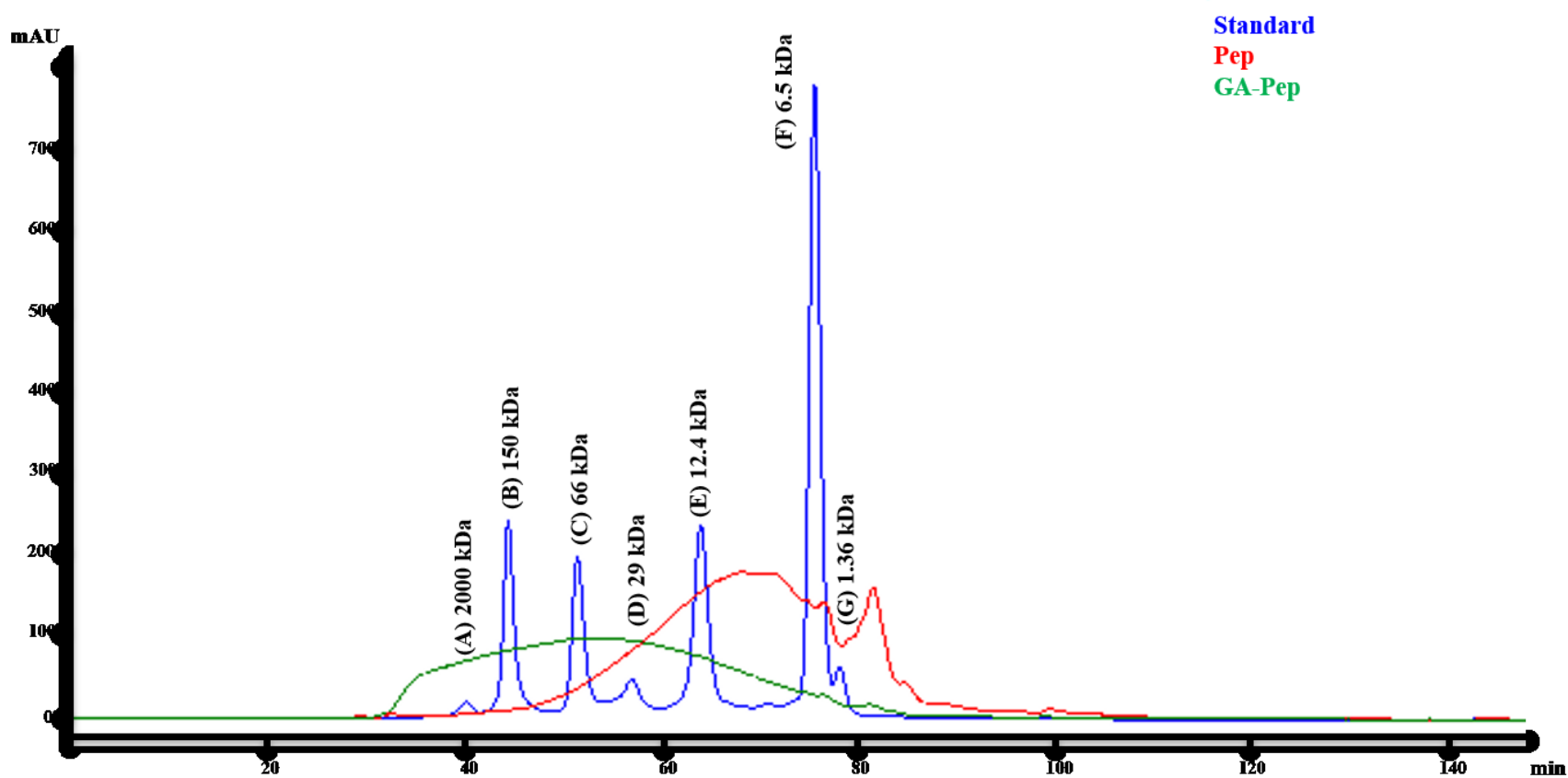
**Figure 35.** Results from the SEC-HPLC analysis of the peptones (Pep) and the soluble portion of the glutaraldehyde crosslinked peptones (GA-Pep). A 0.15 M $Na_2HPO_4$ solution was used as the mobile phase using HPLC grade reagents. Standards used to standardize the retention times of the products were (A) blue dextran (2000 kDa), (B) alcohol dehydrogenase (150 kDa), (C) albumin (66 kDa), (D) carbonic anhydrase (29 kDa), (E) cytochrome C (12.4kDa), (F) aprotinin (6.5 kDa), and (G) Vitamin B-12 (1.36 kDa)

## 4.4.6. SDS-PAGE Analysis of Glutaraldehyde Crosslinked Peptones

To overcome the problems of characterizing the molecular weight of the products, a commonly used molecular biology technique was tried, namely sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). One of the advantages of SDS-PAGE over SEC-HPLC, is that it uses a surfactant, sodium dodecyl sulphate (SDS), which is normally added to help denature the protein structure. After much trial and error, it was found that the SDS was also able to improve the solubility of the crosslinked peptones in solution. The protocol for this analysis was then modified to improve the solubility of the glutaraldehyde crosslinked peptones. The modification involved incubating the samples in the sample buffer, containing SDS, for 2 hours prior to loading onto the gel. In the case of the crosslinked peptones, this helped them to fully dissolve, so that a more accurate analysis could be performed.

Two types of staining procedures were used to stain the gel after SDS-PAGE was performed, which were Coomassie staining and silver staining. Silver staining is a more intense staining method, which can better stain lower concentration bands and make visualization easier. The bands seen using these two staining methods are not in the typical discrete bands commonly found in SDS PAGE gels, instead they are more smeared or streaked across a region of their lane. This is a result of the hydrolysis reaction when creating the peptones, as it produces a mixture of high to low molecular weight products, which have a broad molecular weight distribution, as shown in previous studies (T. H. Mekonnen et al., 2013). Based on the gels one can get an idea of the range of molecular weights of the mixture. The results from the Coomassie staining are shown in Figures 36. From this gel, the crosslinked peptones have a streak that is in a higher molecular weight range, compared to the streak of the unmodified peptones, which is in the ≤25 kDa range. In the gel, the glutaraldehyde crosslinked peptones loaded at 10% (10% GA-Pep) were overloaded, leading to a darker streak over much of the lane, compared to the unmodified peptones loaded at the same amount (10% Pep). This result was surprising, however, it has been found that the Coomassie dye binds to the basic and hydrophobic amino acid residues on peptide and proteins (Tal, Silberstein, & Nusser, 1985). It has also been noted in the literature that Coomassie dye does not produce color with amino acids, and low molecular weight peptides and proteins (Wrolstad, 2004). Therefore, to speculate, the reason the dye binds better to the crosslinked peptones than the unmodified peptones, is either due to the presence of more

hydrophobic groups in the crosslinked peptones, or, more likely, is due to the low binding efficiency of the dye to the lower molecular weight peptones.

To try to get a better visualization of the gels, a silver staining method was used. The advantage of this method is that the intensity of the staining is controllable based on how long the gel is left in the solution. By staining for longer, the less intense peptone bands were able to be visualized. In the silver stained gel in Figure 37, two loading amounts of both the peptone and glutaraldehyde crosslinked peptone treatments were analyzed to try to obtain a better visualization. The lower concentration in both cases was the best for visualization as they were not overloaded. From the gel, the 0.01% glutaraldehyde crosslinked peptone treatment (0.01% GA-Pep) was in the ≥75 kDa range, which was much higher than the range of the 0.1% peptone treatment (0.1% Pep) at ≤25 kDa. This indicates that the glutaraldehyde crosslinked peptones were of a much higher molecular weight than the unmodified peptones. This provides evidence that the crosslinking reaction with glutaraldehyde was occurring as theoretically predicted.

Also of note, in both gels there is a visible amount of material in the crosslinked peptone samples found in the upper loading gel, which likely has a molecular weight range that is ≥250 kDa. This material may be part of the insoluble material that was not able to dissolve in the HPLC mobile phase and was therefore not analyzed. This would explain why there was a difference in the range of the molecular weights from the SDS-PAGE and the SEC-HPLC. This demonstrates the advantage to using this technique when analyzing the glutaraldehyde crosslinked peptones.
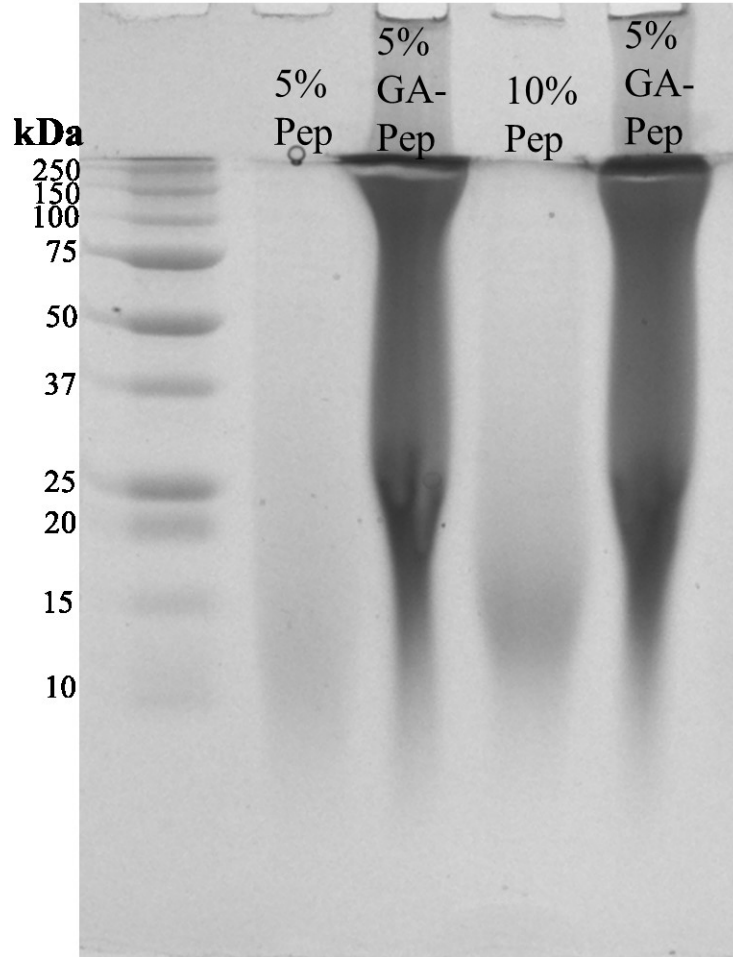
**Figure 36.** SDS-PAGE results of unmodified peptones (Pep) vs. glutaraldehyde crosslinked peptones (GA-Pep) using Coomassie staining. 5% and 10% solutions by weight were prepared by adding 50 mg and 100 mg of sample, respectively, to 1 mL of 1x sample buffer. Samples were dissolved in sample buffer for 2 hours prior to loading. 20 μL was loaded on the gel corresponding to 1 mg and 2 mg respectively.
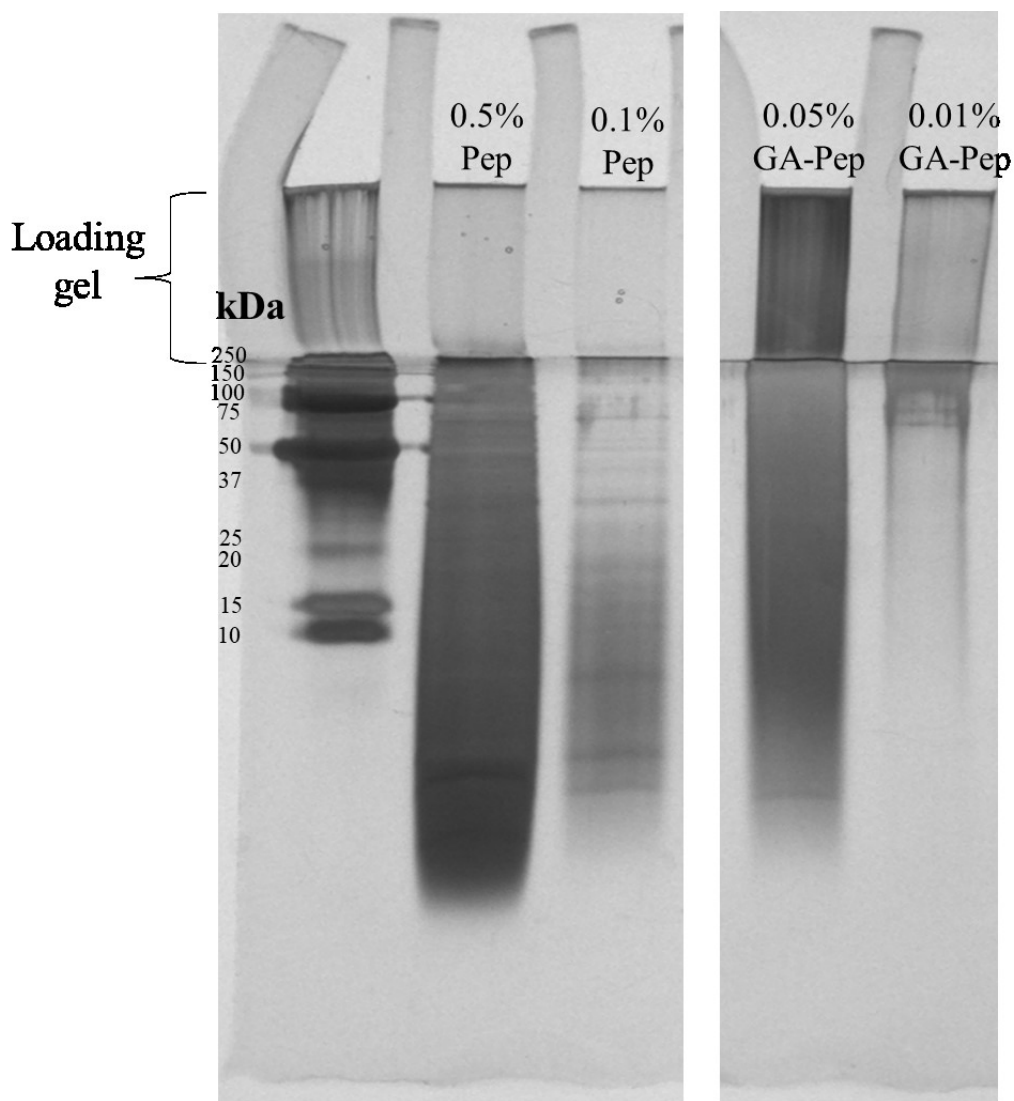
**Figure 37.** SDS-PAGE results of unmodified peptones vs. glutaraldehyde crosslinked peptones after silver staining. Solutions were prepared on a weight basis. The samples include unmodified peptones (Pep), and the glutaraldehyde crosslinked peptones (GA-Pep). 0.5% and 0.05% solutions by weight were prepared by adding 5 mg and 0.5 mg, respectively, of sample to 1 mL of 1x sample buffer. The 0.1% and 0.01% solutions were prepared by dilution of the 0.5% and 0.05% solutions, respectively. Samples were dissolved in sample buffer for 2 hours prior to loading. 20 µL was loaded on the gel corresponding to 0.1 mg and 0.02 mg for the 0.5% and 0.1% solutions, respectively, and to 0.01 mg and 0.002 mg for the 0.05% and 0.01%, respectively.  Two pictures of the same gel are shown where the middles lanes are removed, as they contained samples that were not relevant to the discussion.

### 4.4.7. Zeta Potential Determination with Glutaraldehyde Crosslinked Peptones

The zeta potential of the kaolin clay slurry was determined before and after addition of the peptone treatments to identify any differences in surface charge of the kaolin clay particles that may occur. The results are shown in Table 5. There was a significant difference in zeta potential after the two treatments were added to the slurry compared to the slurry alone. However, there was not a significant difference in the strength of the zeta potential between the two samples. Therefore, no differences in surface charge of the kaolin particles can be inferred after the crosslinking reaction with glutaraldehyde.

| Treatment | Zeta Potential (mV) |
|---|---|
| Kaolin Slurry | $-46.4 \pm 3.4^b$ |
| Peptones | $-35.5 \pm 3.0^a$ |
| Crosslinked Peptones | $-28.9 \pm 3.6^a$ |

**Table 5.** Results from the zeta potential analysis of kaolin clay slurry before and after treatment with peptone samples.

### 4.5. Glutaraldehyde Crosslinking Reaction Parameter Studies

After the results from the flocculation experiment with the glutaraldehyde crosslinked peptones, it was clear that increasing the molecular weight of the peptones was a more important modification than modifying the functional groups on the peptones, as was the case of the esterification reaction. The next step was to attempt to further improve the flocculation performance of the glutaraldehyde crosslinked peptones in methanol by studying the parameters of the reaction and flocculation conditions.

When optimizing a reaction there are many different strategies that a researcher can utilize to get the optimal product. A full factorial design of the parameters would be a way to determine the optimal conditions of this reaction and obtain the optimal flocculation results. However, this type of design requires $n^k$ experiments, where, n = number of levels for each factor and k = number of parameters. With an initial estimation of 6 factors with 3 levels each, this would require 729 experiments, and if each was performed in triplicate then 2187 experiments. Due to time, material, and financial limitations this approach is not always feasible. Another approach for

optimization is to use surface response methodology. In this approach, one builds a model based on factors deemed to be important by determination in a screening experiment. One then begins testing variations amongst those parameters and recording the responding variable. A model of the surface can be built from this data and a way toward the peak or valley of the response variable can be determined. In one study in the flocculation field, researchers looked into modeling the effect that altering the $Na^+$, $Ca^{2+}$, polyacrylamide dosage and clay particle size would have on flocculation in MFT (Motta, Gaikwad, Botha, & Soares, 2018). Using surface response methodology, they found optimum conditions based on three output criteria, CST (a filterability parameter), normalized mudline height, and solids content of the sediment. Using surface response methodology requires a great understanding of your system and what factors are important for optimization. In the case of the glutaraldehyde crosslinked peptones, the parameters that were essential for flocculation performance were not yet known. Therefore, a series of parameter screening experiments was performed to get an initial understand of how the parameters of the reaction and the flocculation experiments would influence the flocculation performance in this system. In this approach, often called the one factor at a time method, all factors are held at a constant value except for one which is varied. The conditions of the varied variable that produce the best results is the optimal condition for that parameter. Normally this can take either a peak or valley shape when graphing the resultant data, or a plateau, where altering the condition further does not result in further improvements to the desired result. This type of approach would determine which factors were of the greatest importance and could provide future researchers with the basis for performing a full factorial or other larger optimization experimental designs. The factors investigated in this research for the peptone glutaraldehyde crosslinking reaction were the ratio of the reactants, the length of reaction and the temperature of reaction. The method of the flocculant addition to the slurry and the dosage of the material to the slurry were also investigated.

### 4.5.1. Varying Reactant Ratio During Peptone Crosslinking

The ratio of the two reactants, glutaraldehyde and peptones, was differed and their effect on settling performance was studied. In these experiments, the amount of the peptone amino groups was kept consistent and the amount of glutaraldehyde was varied. The first number in the ratio describes the molar amount of amino groups in the peptones and the second number describes

the molar amount of the aldehyde groups of glutaraldehyde. The results are shown in Figures 38-43. Figures 38 and 39 show the results of the lower glutaraldehyde ratios of 1:2 and 1:4. Figures 40 and 41 show the results for the 1:8 and 1:16 ratios. Finally, Figures 41 and 42 show the results of the experiment with higher glutaraldehyde ratios of 1:16, 1:32 and 1:64.

The results from Figure 38 and 39 show that as the glutaraldehyde in the reactant ratio increased, so did the settling rate. The 1:4 ratio treatment had the fastest settling rate at every time increment. There was not a significant difference in turbidity among the two treatments after 48 hours. Since a plateau or peak in settling had not yet been reached, this indicated that a local optimum for the reactant ratio may not yet have been reached, and higher glutaraldehyde amounts in the reactant ratio were then investigated.

The results from Figure 40 and 41 showed a similar trend as the previous reactant ratios, where the settling rate increased with an increase in the amount of glutaraldehyde in the reactant ratio. The 1:16 ratio had a higher amount of settling compared to the 1:8 ratio for the first 10 minutes of settling. After this point there was no longer a statistically significant difference between the groups until 48 hours of settling, where the 1:8 ratio had settled more than the 1:16 ratio. For the turbidity after 48 hours of settling, the supernatant clarity improved with an increase in glutaraldehyde in the reactant ratio. A plateau or peak in settling had still not yet been reached, and another set of treatments with higher amounts of glutaraldehyde in the reactant ratio were then investigated.

The results of the next experiment are shown in Figure 42, where the glutaraldehyde in the reactant ratio was further increased. An increase in settling performance was observed when increasing the ratio from 1:16 to 1:32, up to the 60-minute timepoint. After this point, there was no longer a significant difference between these two treatments in settling performance. This was accompanied by a significant decrease in performance with an increase to the 1:64 ratio treatment across all time points, which indicated that a local optimum in settling performance was reached with the 1:32 treatment.

However, as seen in Figure 42, the 1:16 ratio had a lower turbidity after 48 hours than the other two treatments. This indicated that a local optimum for supernatant clarity was at a different ratio than the settling performance. Depending on the end application, the trade-off between settling rate and final turbidity would have to be examined and the appropriate ratio could be chosen.

**Figure 38.** Flocculation performance of glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:2 and 1:4 reactant ratios). Molar ratios of 1:2 and 1:4 peptone amino functional groups to glutaraldehyde functional groups were compared. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at 3% (wt/wt). The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences of groups within timepoints.

**Figure 39.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:2 and 1:4 reactant ratios). Averages in turbidity of the different treatments after 48 hours of settling in a 4% (wt./wt.) kaolin clay system. Comparison of varying the ratio of the reactant functional groups during a glutaraldehyde crosslinking reaction. Molar ratios of 1:2 and 1:4 peptone amino functional groups to glutaraldehyde functional groups were compared. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.
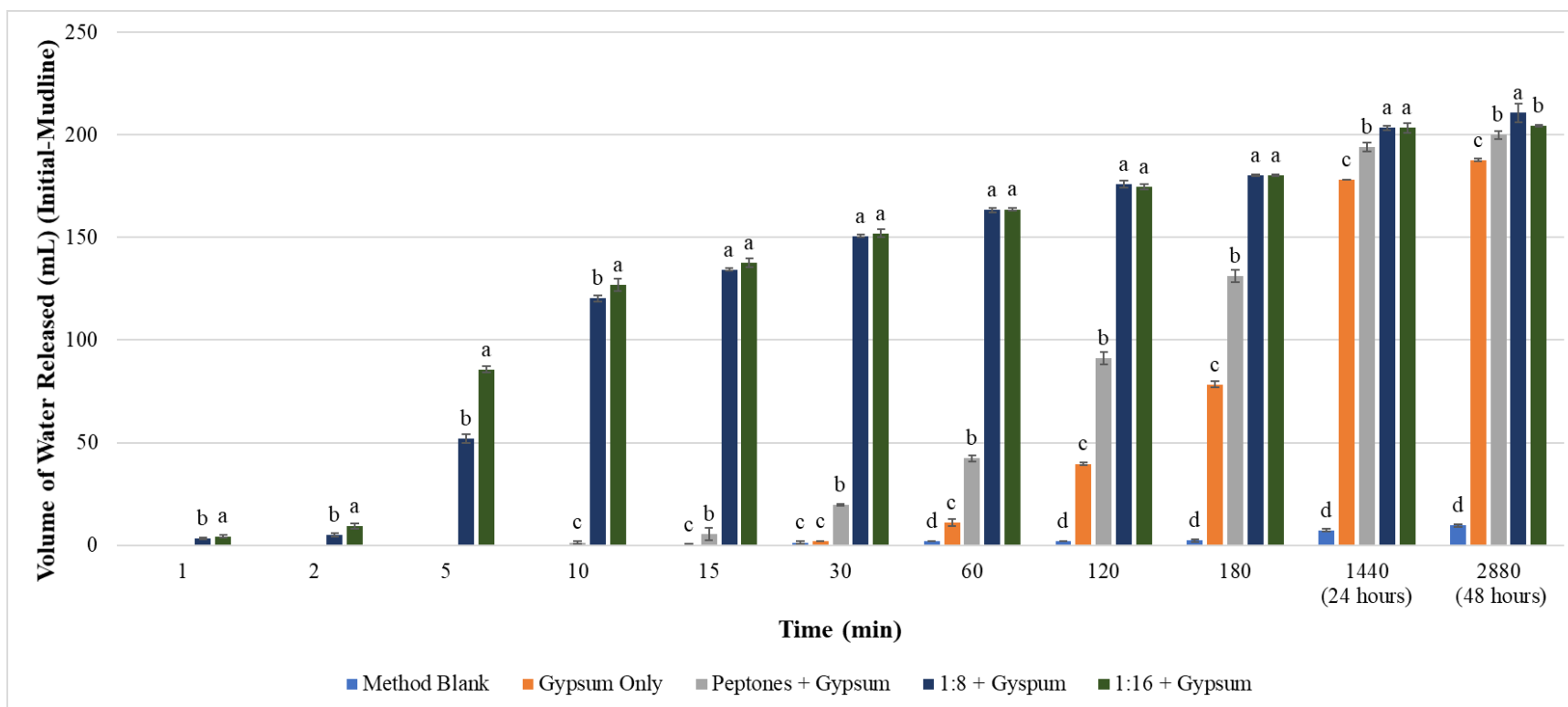
**Figure 40.** Flocculation performance of glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:8 and 1:16 reactant ratios). Molar ratios of 1:8 and 1:16 peptone amino functional groups to glutaraldehyde functional groups were compared. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at 3% (wt/wt). The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences of groups within timepoints.
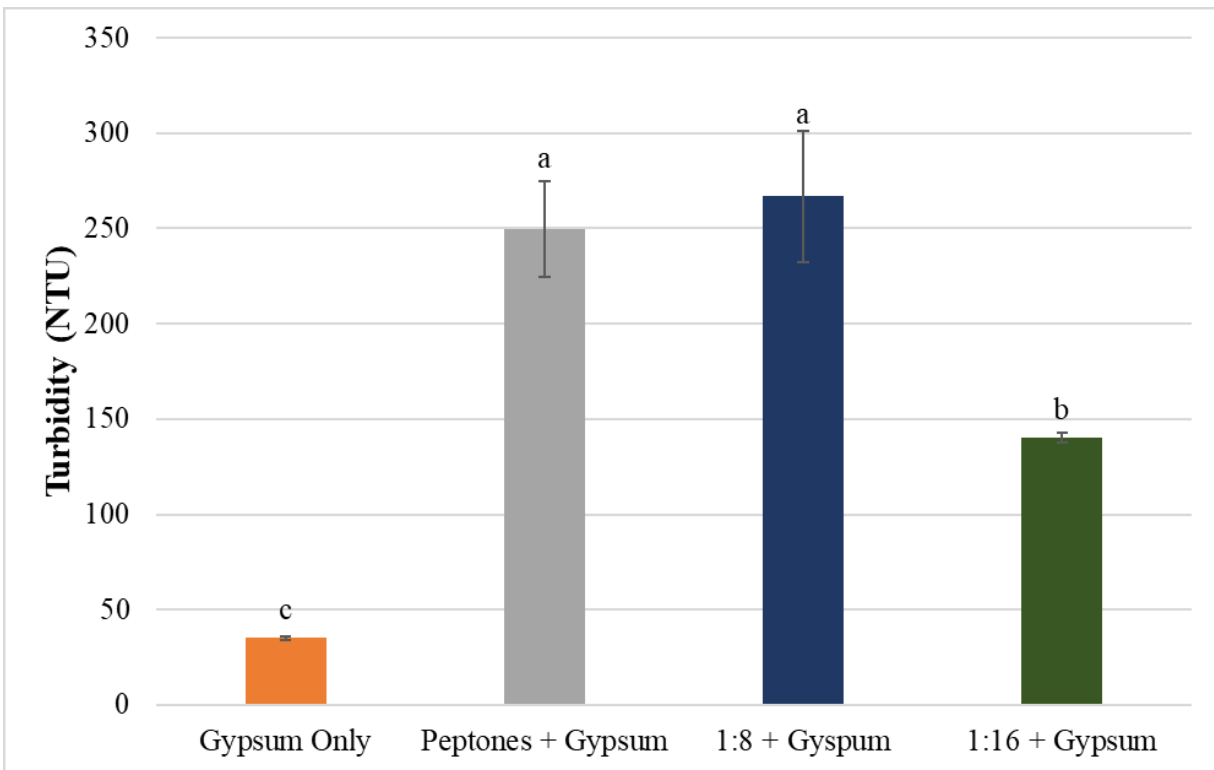
**Figure 41.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:8 and 1:16 reactant ratios). Averages of turbidity in the different treatments after 48 hours of settling in a 4% (wt./wt.) kaolin clay system. Comparison of varying the ratio of the reactant functional groups during a glutaraldehyde crosslinking reaction. Molar ratios of 1:8 and 1:16 peptone amino functional groups to glutaraldehyde functional groups were compared. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.
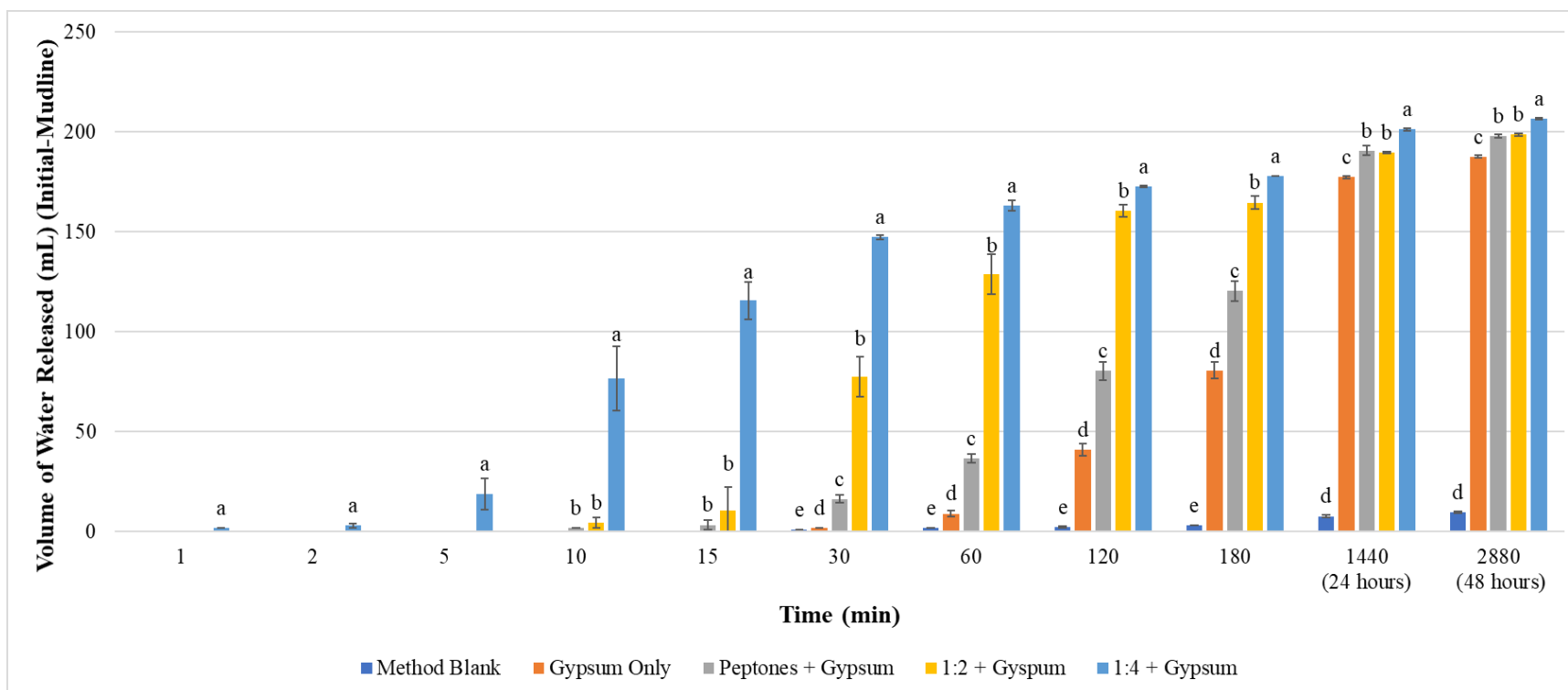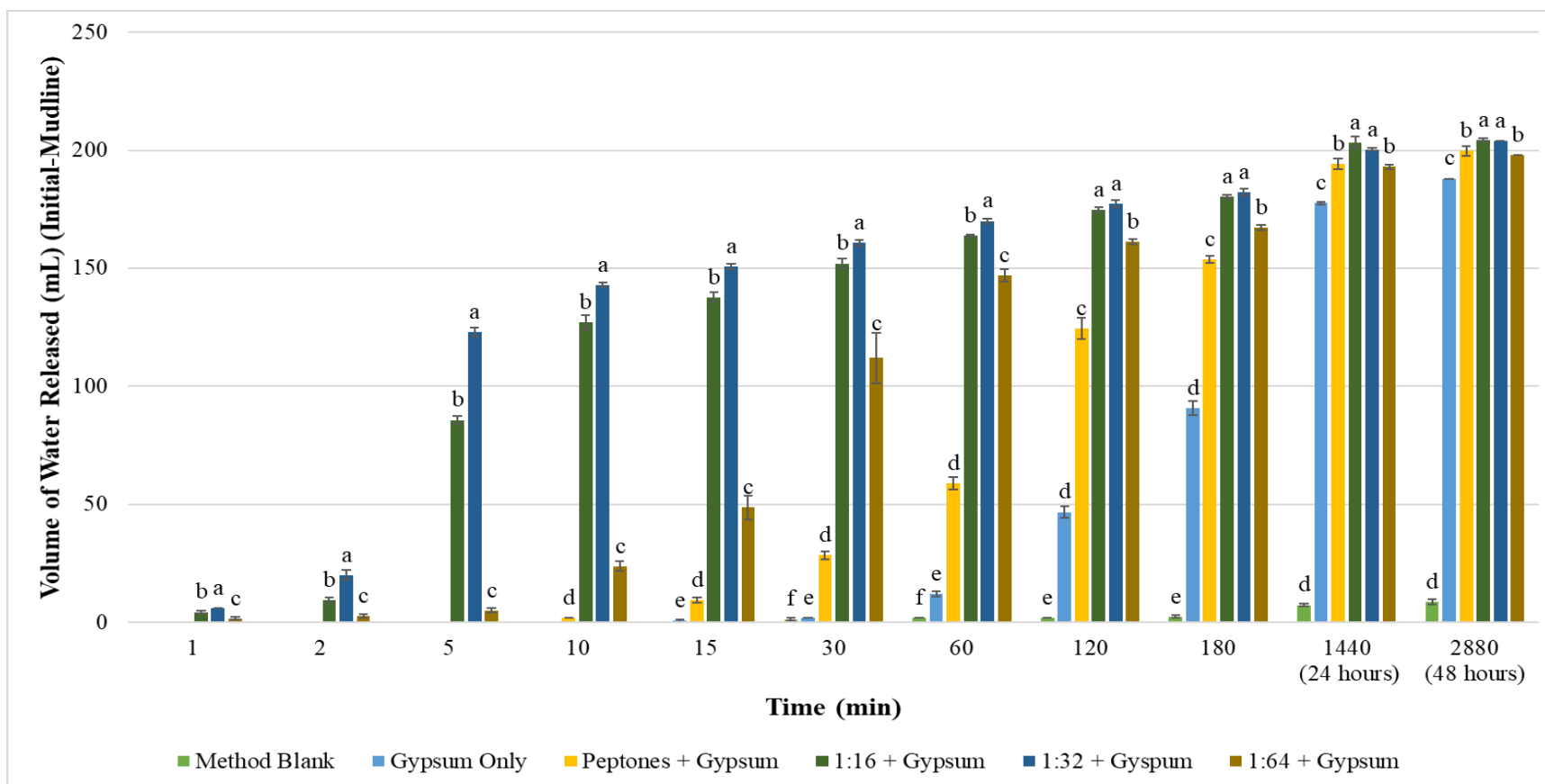
**Figure 42.** Flocculation performance of glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:16, 1:32 and 1:64 reactant ratios). A molar ratio of 1:16, 1:32 and 1:64 peptone functional groups to glutaraldehyde functional groups were compared. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at 3% (wt/wt). The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences of groups within timepoints.
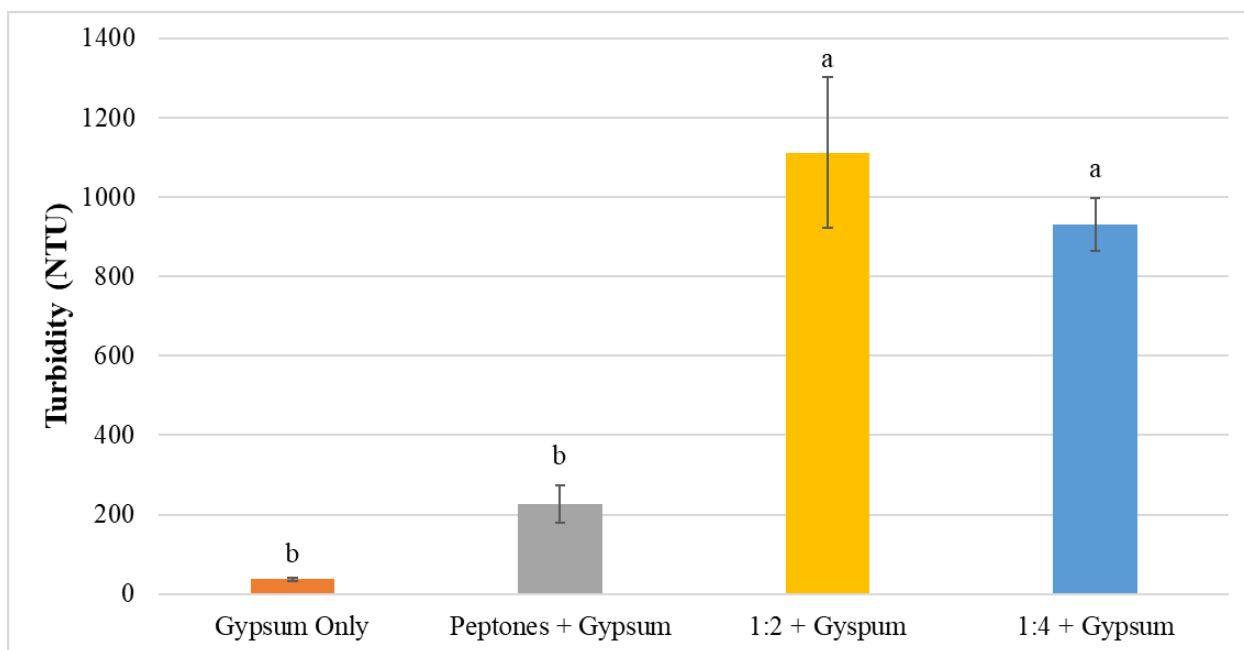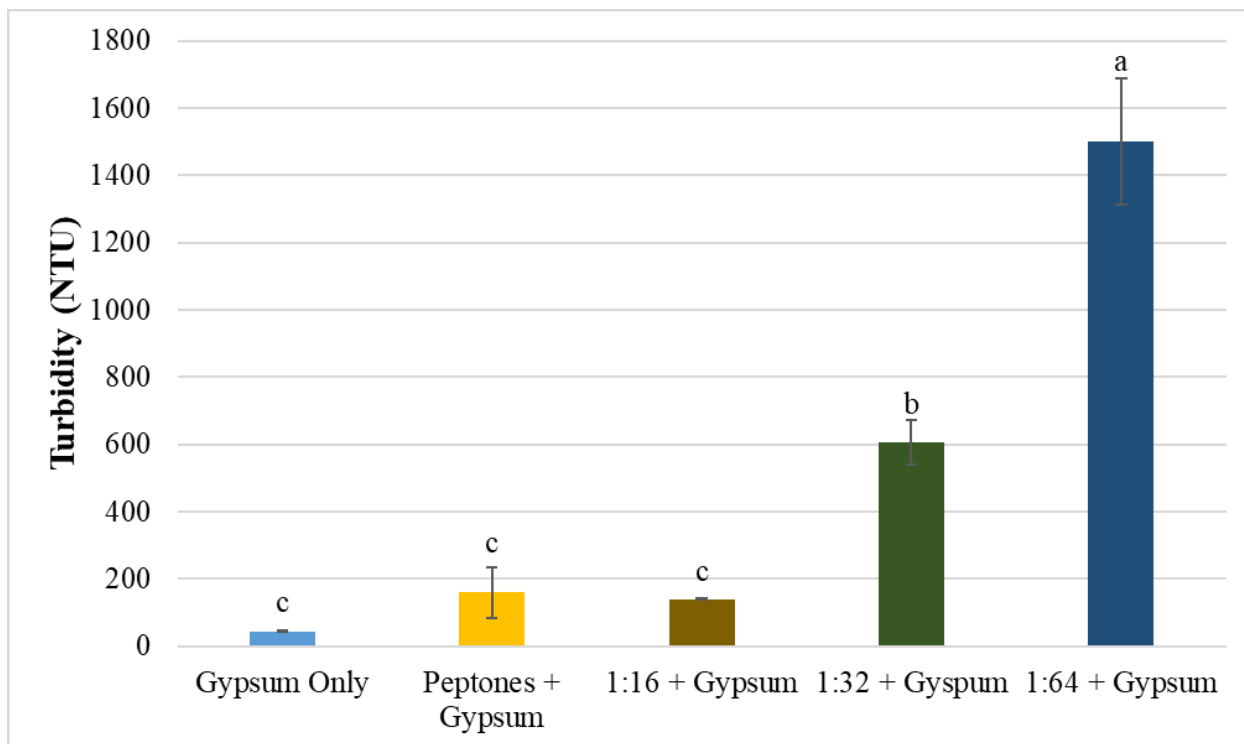
**Figure 43.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different reactant functional groups ratios (1:16, 1:32 and 1:64 reactant ratios). Averages in turbidity of the different treatments after 48 hours of settling in a 4% (wt./wt.) kaolin clay system. Comparison of varying the ratio of the reactant functional groups during a glutaraldehyde crosslinking reaction. Molar ratios of 1:16, 1:32 and 1:64 peptone amino functional groups to glutaraldehyde functional groups were compared. Experiments were performed in triplicate. Different letters represent statistically significant differences amongst groups in turbidity.

To attempt to determine any differences in the amount of crosslinking among the different reactant ratio products they were analyzed using TGA and FTIR. Unfortunately, there were no discernable differences were detected between the products by FTIR, so TGA was investigated. The results of the TGA analyses are shown in Table 6. It was expected that there would be a large difference in the onset of degradation temperature amongst the products when analyzed by TGA, as a higher degree of crosslinking was hypothesized to have occurred. There was a general trend of increasing onset of degradation temperature, except for the 1:16 ratio, which had a decrease in this value. While there were differences, they were not as drastic as was expected. Perhaps TGA may not be sensitive enough to determine the differences among the samples.

After some research in the literature, one study showed that polymers of similar composition have similar onset of thermal degradation temperatures, which would explain these results (Hongbo, Yanping, Wen, & Siqing, 2016). The differences in flocculation performance were apparent, however characterization data to identify these differences were elusive.

| Treatment | Thermal Onset of Degradation Temperature (˚C) |
|-----------|----------------------------------------------|
| 1:4 | 274 |
| 1:8 | 274 |
| 1:16 | 271 |
| 1:32 | 275 |
| 1:64 | 276 |

**Table 6.** Summary table of the thermal onset of degradation temperature after TGA of the glutaraldehyde crosslinked peptones with different reactant functional groups ratios. The onset of the thermal degradation was determined by the ASTM standard method E2550-17.

**4.4.2. Varying Reaction Time of Peptone-Glutaraldehyde Crosslinking**

The length of time of the glutaraldehyde crosslinking reaction was varied from 30 minutes to 24 hours, the data are shown in Figure 44. The hypothesis for this experiment was that as the reaction time increases, the settling rate will also increase. This would theoretically be due to an increased amount of the crosslinking reaction with glutaraldehyde occurring. After the first 5 minutes of settling, there is an increase in the settling rate with the 30-minute reaction time treatment compared to the 2-hour and 24-hour reaction time treatments. This increase is still apparent after 15 minutes of settling but becomes not statistically significant after 30 minutes of settling between the 30 minute and 2-hour reaction time treatments. After 2 hours there is no longer a statistically significant difference between the three treatments. For the turbidity of these treatments after 48 hours of settling, in Figure 45, there was no significant difference between the 30-minute and 24-hour treatments. There was a significantly lower turbidity in the 30-minute treatment compared to the 2-hour treatment, although the difference was relatively small, compared to the other control treatments.

The 30-minute reaction appears to have a better initial settling rate than the treatments with a longer reaction time, which refutes with the original hypothesis. To speculate, this may be due to the reduction of water solubility of the crosslinked peptone products as the reaction with glutaraldehyde progresses. Therefore, the increased hydrophobicity could be causing the product to interact less effectively with the slurry. If this is the case, the method that the crosslinked peptone flocculants are added to the system will be important.
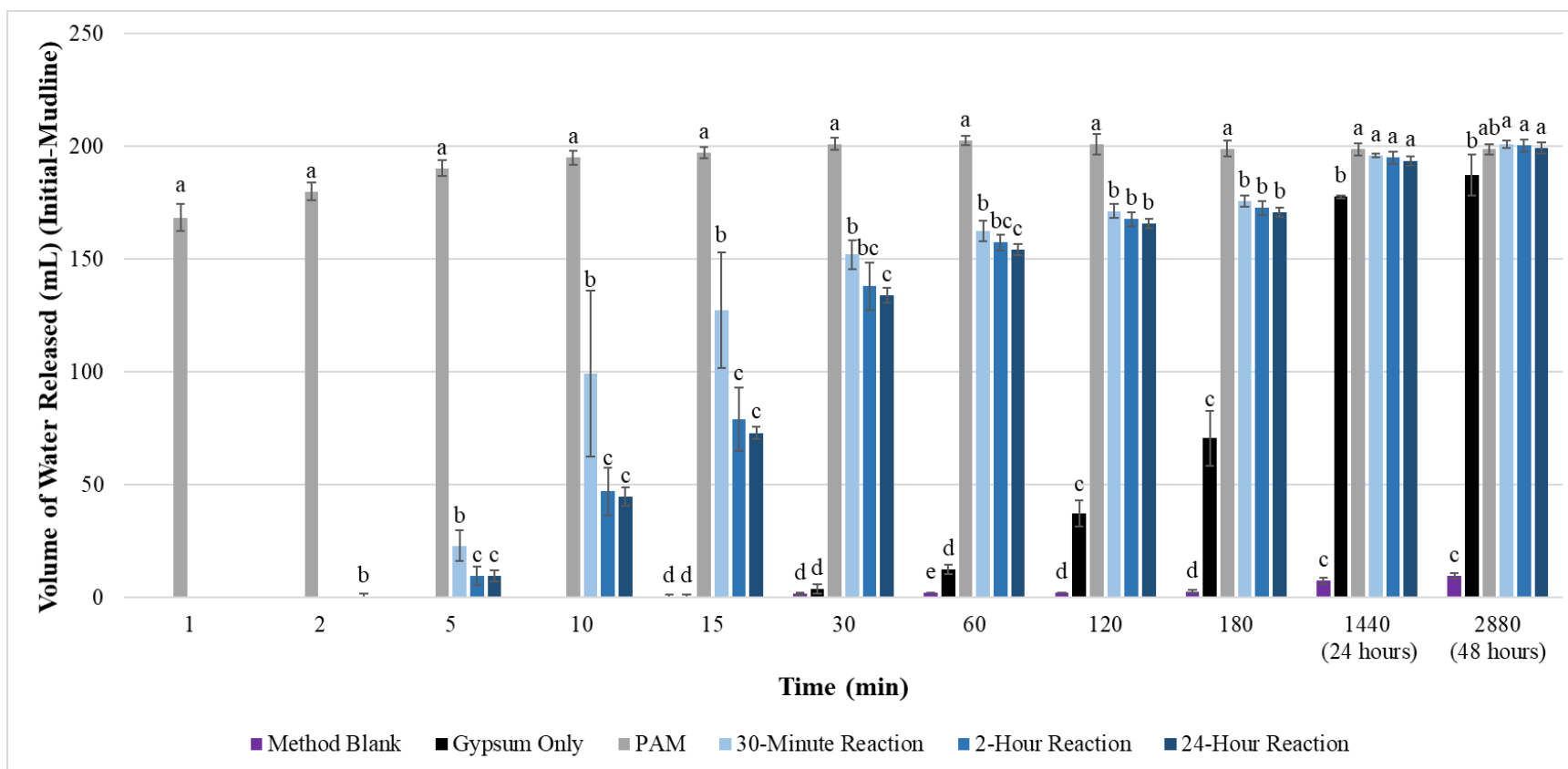
**Figure 44.** Flocculation performance of glutaraldehyde crosslinked peptones with different reaction times. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Flocculants were added at 3% (wt/wt). Gypsum was added as a coagulant at a concentration of 300 ppm. The time of the glutaraldehyde crosslinking reaction was varied. The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences within timepoints.
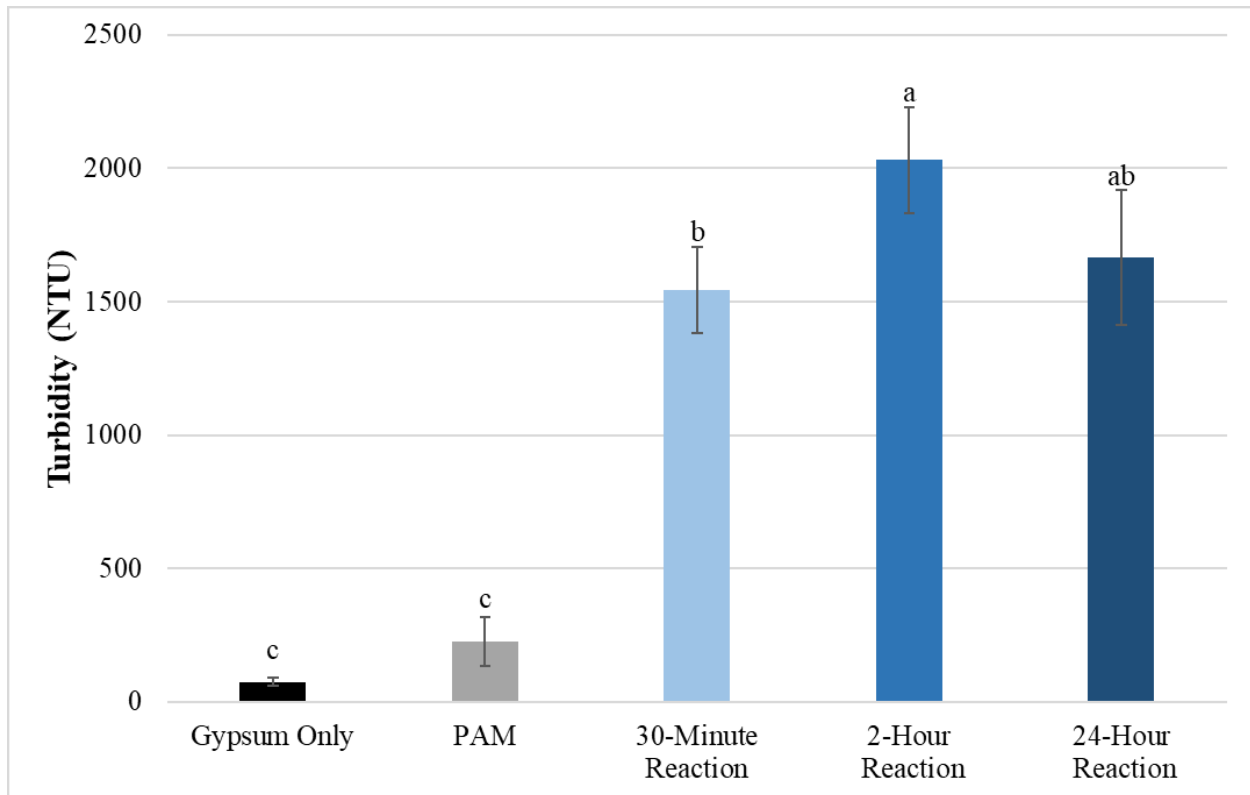
**Figure 45.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different reaction times. Turbidity averages of the different treatments after 48 hours of settling time in a 4% kaolin clay system. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.

### 4.4.3. Methods for Addition of Crosslinked Peptones as a Flocculant

To test the hydrophobicity hypothesis, an experiment was conducted where the crosslinked peptones were first dissolved in solution prior to a flocculation experiment and were compared to the original method of adding them as a dry powder. Dissolving a flocculant prior to its addition to a slurry is not a new concept. For instance, when PAM is used as a flocculant, a solution of the polymer is first required to ensure a homogenous distribution throughout the slurry during flocculation. The concentration of the PAM solution has been shown to affect the viscosity of the solution, which would impact its mixing and performance during flocculation (Amir, Mohd Saaid, & Mohamed Jan, 2018). Therefore, using glutaraldehyde crosslinked peptones in solution may be able to improve its distribution throughout the slurry, and improve its performance as a flocculant.

Initially, a screening experiment was performed to identify the solution conditions that would best dissolve/disperse the crosslinked peptones. Solutions tested included Milli-Q water, SPW at pH 6, SPW at pH 8.35, methanol, 1:1 methanol:water and a 0.1 M surfactant solution of didodecyldimethylammonium bromide. The water and process water solutions were chosen because they are inexpensive and could be easily used in industry. Methanol solutions were tested as they would be easy to implement after the crosslinking reaction occurred, as methanol was the solvent used. A surfactant was chosen because in the SDS PAGE characterization, it was found that the addition of the surfactant, SDS, increased the solubility of the crosslinked peptones. By using a surfactant with a positive charge, it was thought that it may be able to act as a coagulant in the system, while also aiding in the product dissolution. The crosslinked peptones used in this test were the products of the crosslinking reaction in which the peptone amino group to glutaraldehyde aldehyde groups was 1:32, as mentioned previously. This ratio was chosen due to its superior settling rate, which was shown in section 4.4.1. It was found that the SPW at pH 8.35 and the 0.1 M surfactant solution were equally able to almost entirely dissolve the crosslinked peptones, as determined by visual inspection. The small amount that was not dissolved consisted of fine fluffy particles that remained well dispersed for several hours. Due to cost and ease of use, the SPW at pH 8.35 solution was explored further.

The hypothesis for this experiment was that having the peptones in solution would facilitate improved settling rates compared to adding the peptones as a powder. The rationale behind this hypothesis came from observations made in previous experiments, where after mixing, a portion of the glutaraldehyde crosslinked peptone treatments would remain undissolved and float to the top of the cylinder. The undissolved portion slowly dissolved over the course of the 48 hours during the settling test. This observation prompted investigation into dissolving the peptone treatments in a solvent before addition to the slurry.

Different concentrations of crosslinked peptone solutions were prepared, to vary the dosage while keeping the volume the same, and the results of this experiment are shown in Figure 46. There was no difference in settling rate across all timepoints between the 0.7% (wt/wt) solution dosage and the 3% (wt/wt) solid powder dosage, despite the lower amount of product used. However, there was a large, significant difference in the final turbidity of the supernatant of the columns after 48 hours of settling time as shown in Figure 47. The 0.7% (wt/wt) solution dosage

had a much lower turbidity compared to the 3% (wt/wt) solid powder dosage. The 0.7% (wt/wt) solution dosage had no significant difference in turbidity compared to the gypsum control as well. When comparing these dosages, adding the peptones as a solution did not significantly impact settling rate compared to adding it as a solid powder and enhanced the final clarity of the supernatant of the water released, with a lower amount of product required. After this experiment, it was evident that a local optimum for the dosage was not reached, as there was not yet a peak or plateau in the settling rate when increasing concentration. Therefore, higher concentration solutions were also tested.

The results of the higher concentration experiments are shown in Figure 48. A 5x and 10x increase to the dosage was originally chosen to be tested as that would be a logical step increase. However, the 10x solution was too concentrated to dissolve all of the solids, but the 5x solution was able to dissolve the products. Therefore, an intermediate 3x solution was chosen to test as well. This corresponded to a 2.1% (wt/wt) solution and a 3.5% (wt/wt) solution for the 3x and 5x increases, respectively. From the data, after 5 minutes of settling there was a significant difference between the three dosages, with the lower 0.7% (wt/wt) dosage having a faster settling rate. After 10 minutes, there was no longer a significant difference between the three treatments. However, after 15 minutes the trend switched, and the 2.1% (wt/wt) and 3.5% (wt/wt) dosages settled significantly more than the 0.7% (wt/wt) treatment. This difference was no longer significantly different after 60 minutes of settling but was again significant after 180 minutes of settling. After 24 hours of settling there was no longer a significant difference between the treatments. It appears that a plateau was reached at the 2.1% (wt/wt) solution dosage, where further improvements in settling were not seen at higher concentration. Based on the findings from this study, it was decided that using the crosslinked peptones in a solution was the better method and the 2.1% (wt/wt) solution dosage would be incorporated into future experiments.
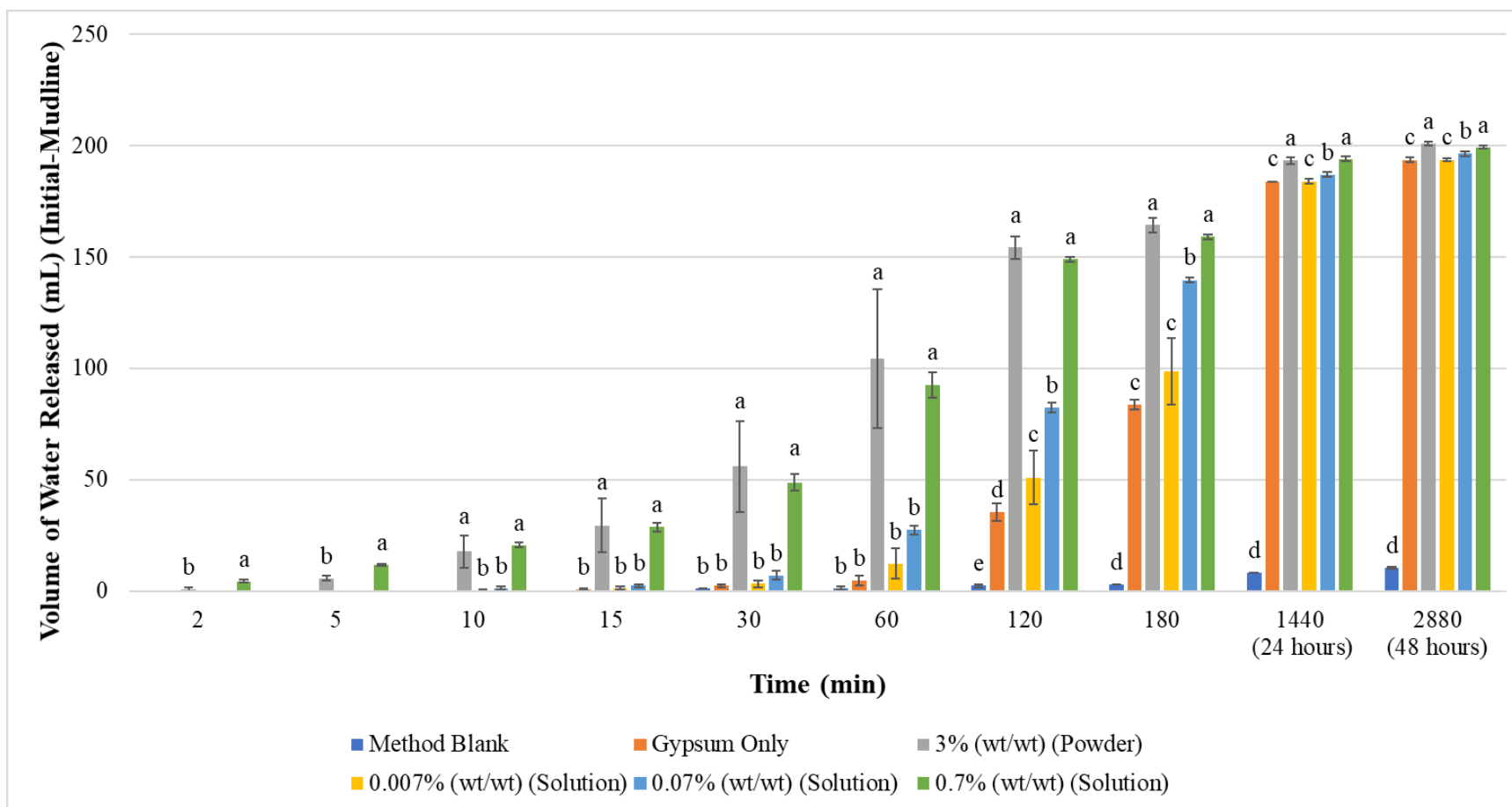
**Figure 46.** Flocculation performance of glutaraldehyde crosslinked peptones with different of methods for addition to the slurry. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at varying concentrations and as a powder or a solution. The method of addition is indicated in brackets. The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences within timepoints.
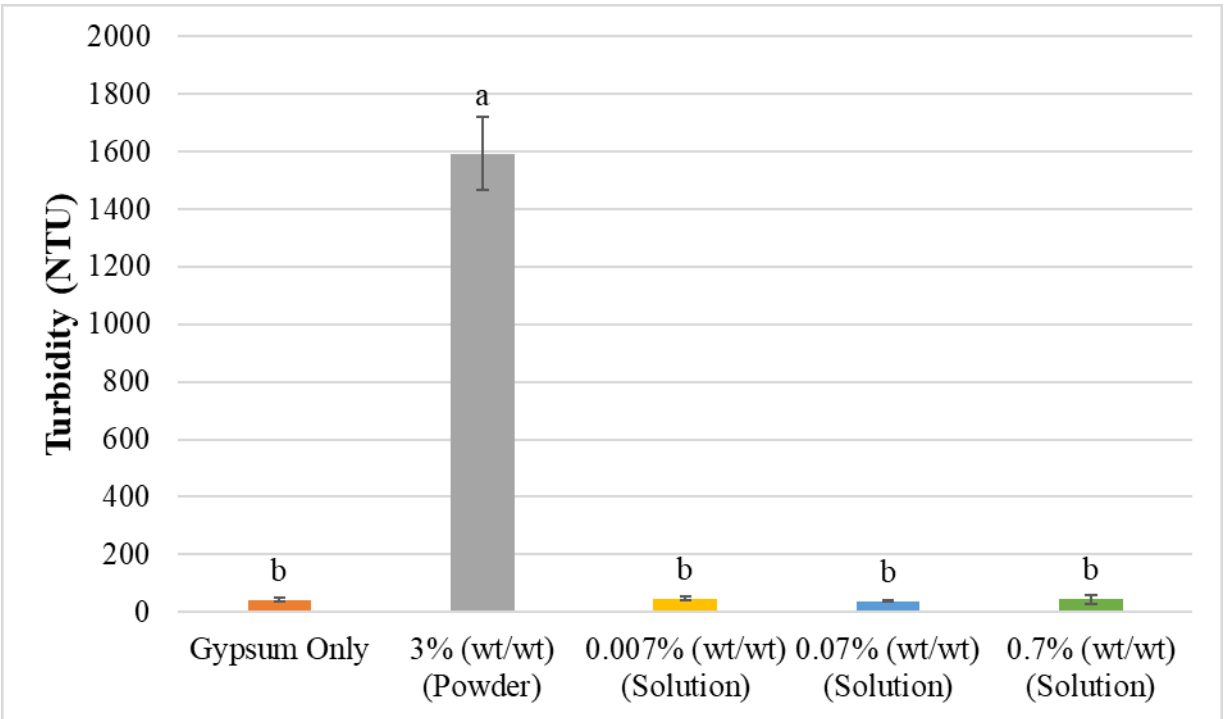
**Figure 47.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different of methods for addition to the slurry. Flocculants were added at varying concentrations and as a powder or a solution. The method of addition is indicated in brackets. Turbidity averages of the different treatments after 48 hours of settling time in a 4% kaolin clay system. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.
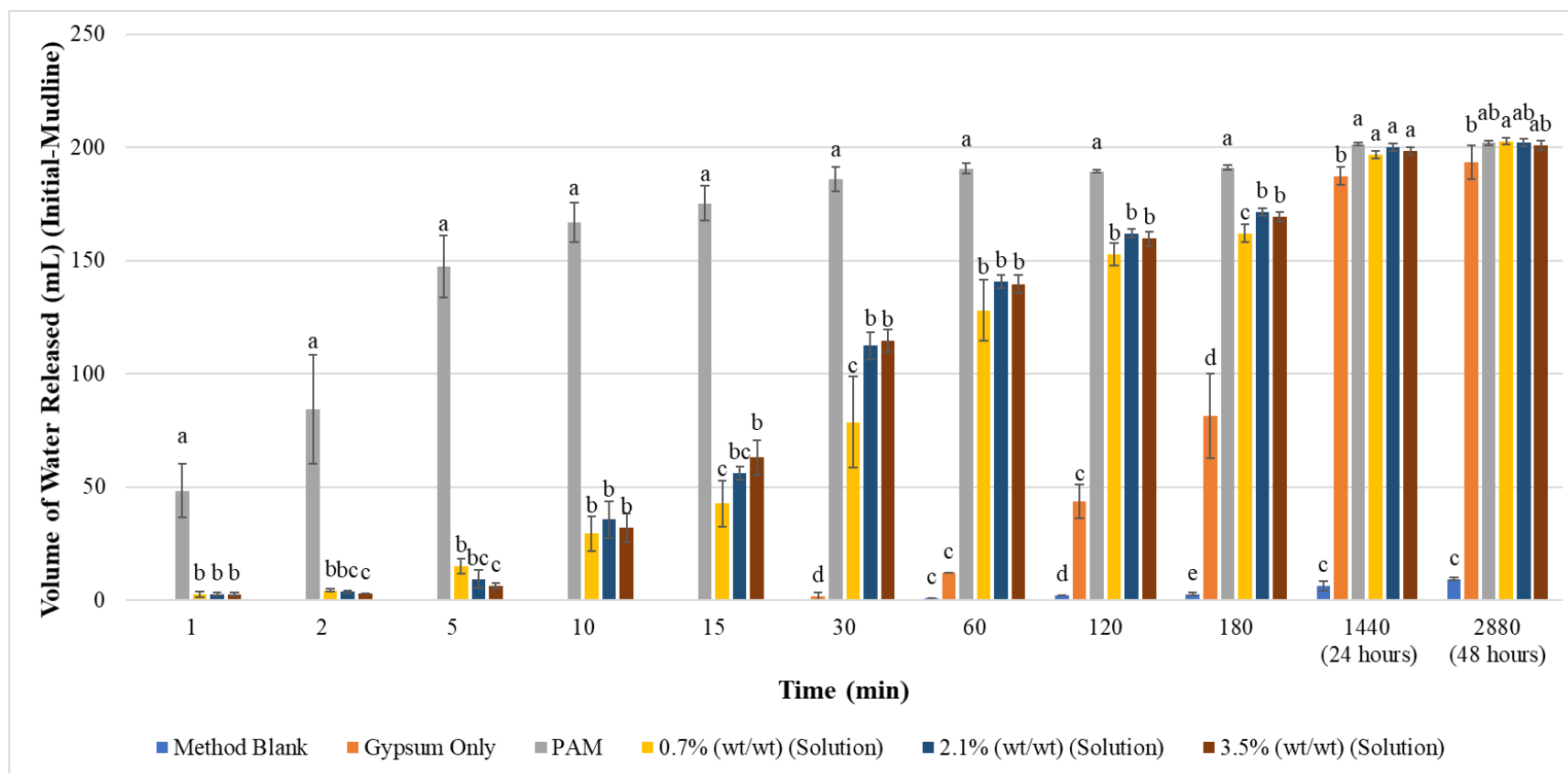
**Figure 48.** Flocculation performance of glutaraldehyde crosslinked peptones with different solution concentrations over time. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. Flocculants were added at varying concentrations in solution. The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences within timepoints.

### 4.4.4. Varying of the Reaction Temperature of Peptone Crosslinking

The last variable examined during the parameter experiments was the temperature of the crosslinking reaction. The temperature of the reaction can have a large impact on the reaction products depending on the type of reaction. In general, increasing the temperature of a reaction will increase the reaction rate, as it imparts free energy into the system, which allows the energy barrier of the reaction to be overcome. Increasing the temperature of a reaction can also promote side reactions to occur that would not be thermodynamically favored at lower temperatures. This would lead to a decrease in the formation of the desired reaction products, and an increase in contamination by the side products, resulting in a lower purity. Lowering the reaction temperature will cause the kinetically favored products to form in a reaction, which are not necessarily the most stable products, but will have a lower energy barrier to their reaction. At lower temperature there is less energy available for the reverse reaction to occur as well. At higher temperatures the thermodynamically favored products are formed, which are typically the most stable products that can form during a reaction but have a higher energy barrier. At high temperature, the kinetically favored products would still form, but there is enough energy available for the reverse reaction to occur, leading to the product with the lowest energy to form. The temperature of the reaction can be altered depending on which product is more desirable.

To test the influence of the temperature of the crosslinking reaction the reaction was performed at three different temperatures. Room temperature was the original temperature used for this reaction and was used as a control. The highest temperature possible would be at the boiling point of the solvent, which was the next temperature chosen. Finally, a third temperature that was in between these two boundaries was used, which was 39°C.  In theory, increasing the temperature of the reaction there would either: lead to the crosslinking reaction occurring quicker, due to an increase in reaction rate, with the same amount of crosslinking after the reaction, or it would lead to a new reaction occurring where a more thermodynamically favored product was formed. If a new thermodynamically favored product was formed, the products of the reaction could potentially have different flocculation behavior.

The results of the settling experiment with higher temperature reaction products is shown in Figure 49. There were no significant differences in settling between the three temperatures of reaction until 15 minutes of settling. At that point there was an increased amount of settling in

the room temperature reaction compared to the 39°C reaction, but there was not a significant difference with the boiling point reaction. This trend was also seen after 30 minutes of settling. However, after 60 minutes there was no longer any significant differences between the groups. There was no difference between the room temperature reaction and the boiling point reaction treatments at any time points. The results of the turbidity measurement after 48 hours are shown in Figure 50. There were no significant differences between the turbidites of the supernatants of the three treatments.

Therefore, there was no improvement in flocculation when increasing the temperature of the crosslinking reaction. The only benefit may be to increase the rate of the reaction. There was a small, but significant decrease in settling performance of the 39°C reaction temperature compared to the room temperature reaction at 15 and 30 minutes. The results from this experiment were surprising because in previous research, increasing the temperature of the crosslinking reaction between SRM derived peptones and glutaraldehyde in water, exhibited an improvement in the thermal stability of the products, as shown by TGA, which was correlated to an improved crosslinking degree (El-Thaher et al., 2013). To speculate, this may be because the reaction had gone to completion at all three temperatures and no new thermodynamically favored reaction occurred at higher temperature. After this result, the next step was to determine if there would be an effect when the temperature was lowered, as the lowest temperature (room temperature) reaction had a slight improvement in the settling rate. Potentially, lowering the reaction temperature could lead to the formation of new kinetically controlled reaction products.

The results of the flocculation experiment with the lower reaction temperature crosslinked peptones are shown in Figure 51. In this experiment, it was expected that as the temperature of the crosslinking reaction was lowered, the rate of reaction would be lowered. This would either lead to the reaction occurring slower, perhaps to a lesser degree, or to the formation of kinetically favored reaction products. A product that was less crosslinked would theoretically have a decreased hydrophobicity, which could more easily be dispersed in the clay slurry and could improve its flocculation performance. From the data, there was no significant difference in the settling of the room temperature and the 0°C treatments in the first 5 minutes. However, from 10 minutes to 120 minutes of settling, the 0°C treatment had a significantly higher amount of settling than the room temperature treatment. After 180 minutes of settling, there was no longer a

significant difference between the two groups. This increase in settling rate was interesting and several characterization studies were performed to try to identify the cause of the increase. From the turbidity data in Figure 52, after 48 hours of settling, there was a significantly higher turbidity in the 0˚C treatment than in the room temperature treatment. This again shows the tradeoff that occurs between clarity of the release water and the settling rate when changing the reaction conditions.
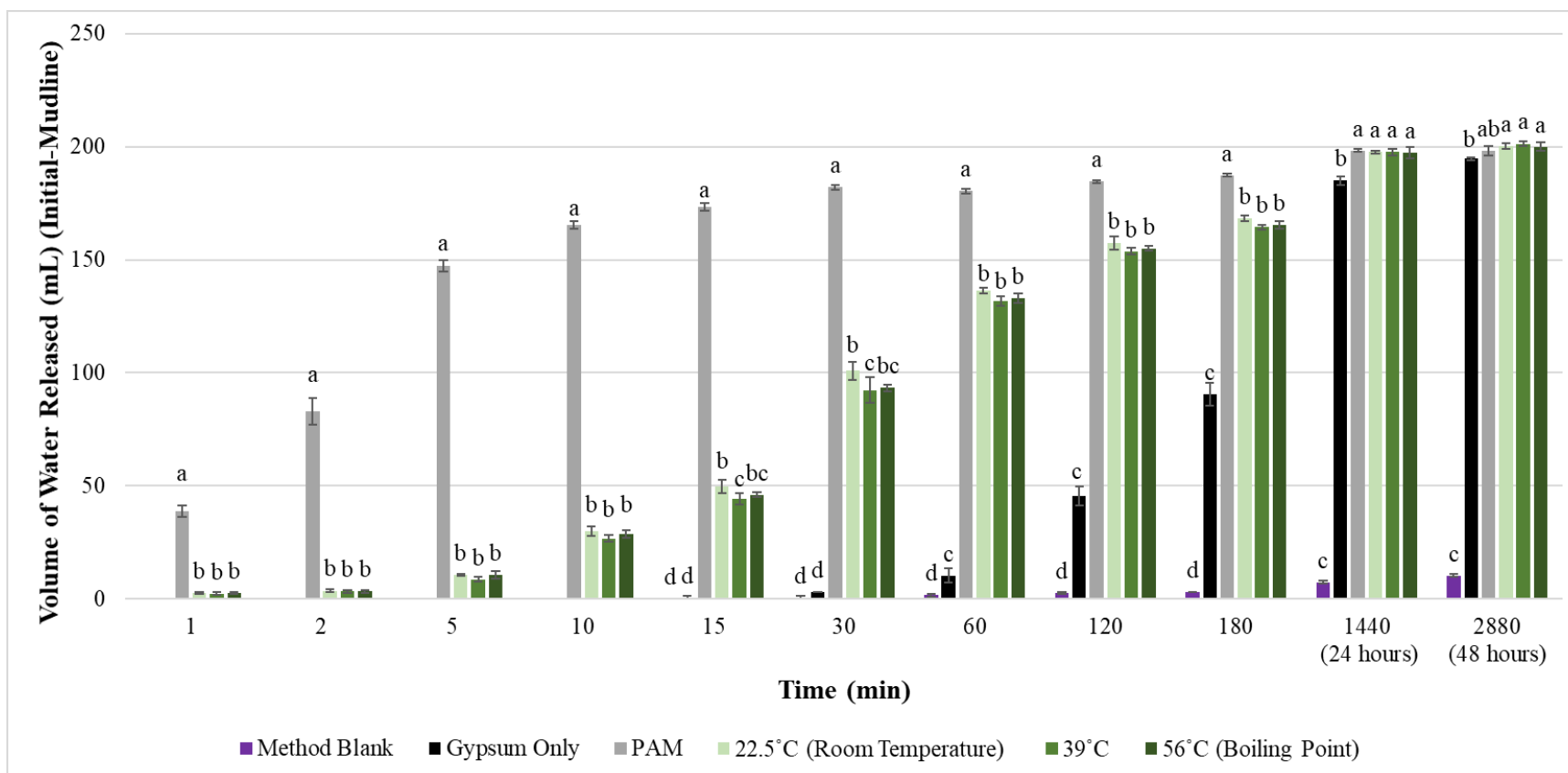
**Figure 49.** Flocculation performance of glutaraldehyde crosslinked peptones with different reaction temperatures. Flocculation of a 4% (wt/wt) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. The temperature of the crosslinking reaction was varied in the three treatments. Flocculant treatments were added at a 2.1% (wt/wt) dosage in 7 mL solutions. The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences within timepoints.

**Figure 50.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones with different reaction temperatures. Turbidity averages of the different treatments after 48 hours of settling time in a 4% kaolin clay system. The temperature of the crosslinking reaction was varied. Flocculant treatments were added at a 2.1% (wt/wt) dosage in 7 mL solutions. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.
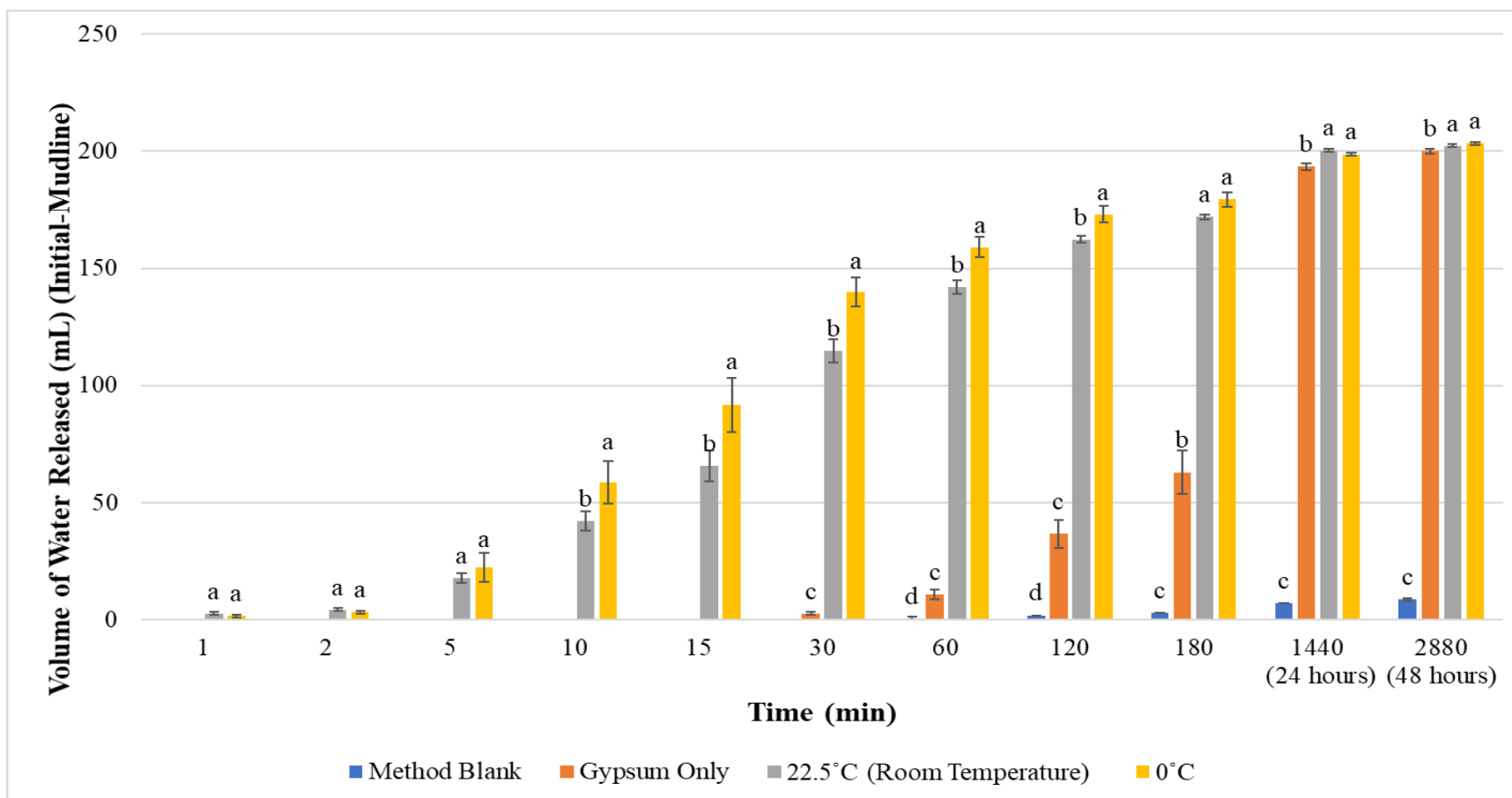
**Figure 51.** Flocculation performance of glutaraldehyde crosslinked peptones after lowering the reaction temperature. Flocculation of a 4% (wt./wt.) kaolin clay slurry over time. Gypsum was added as a coagulant at a concentration of 300 ppm. The temperature of the crosslinking reaction was lowered. Flocculant treatments were added at a 2.1% (wt/wt) dosage in 7 mL solutions. The difference of the mudline and the initial height of the slurry was recorded as the water released. Experiments were performed in triplicate. Different letters represent statistically significant differences within timepoints.
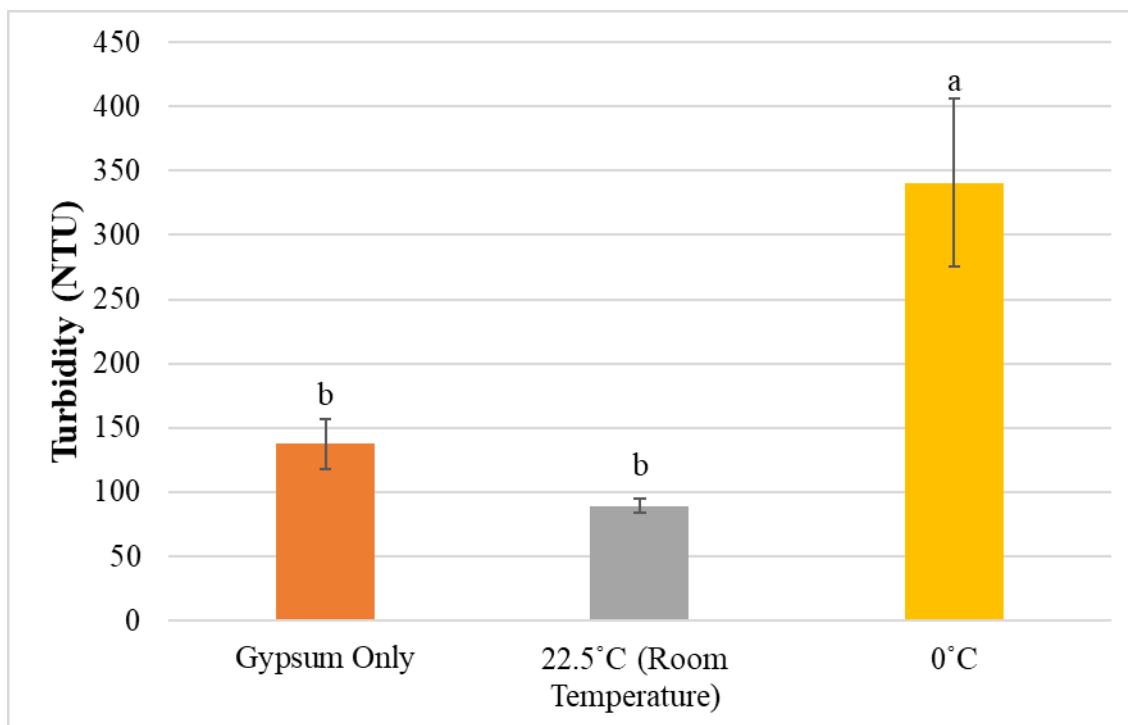
**Figure 52.** Turbidity of supernatant after flocculation using glutaraldehyde crosslinked peptones after lowering the reaction temperature. Turbidity a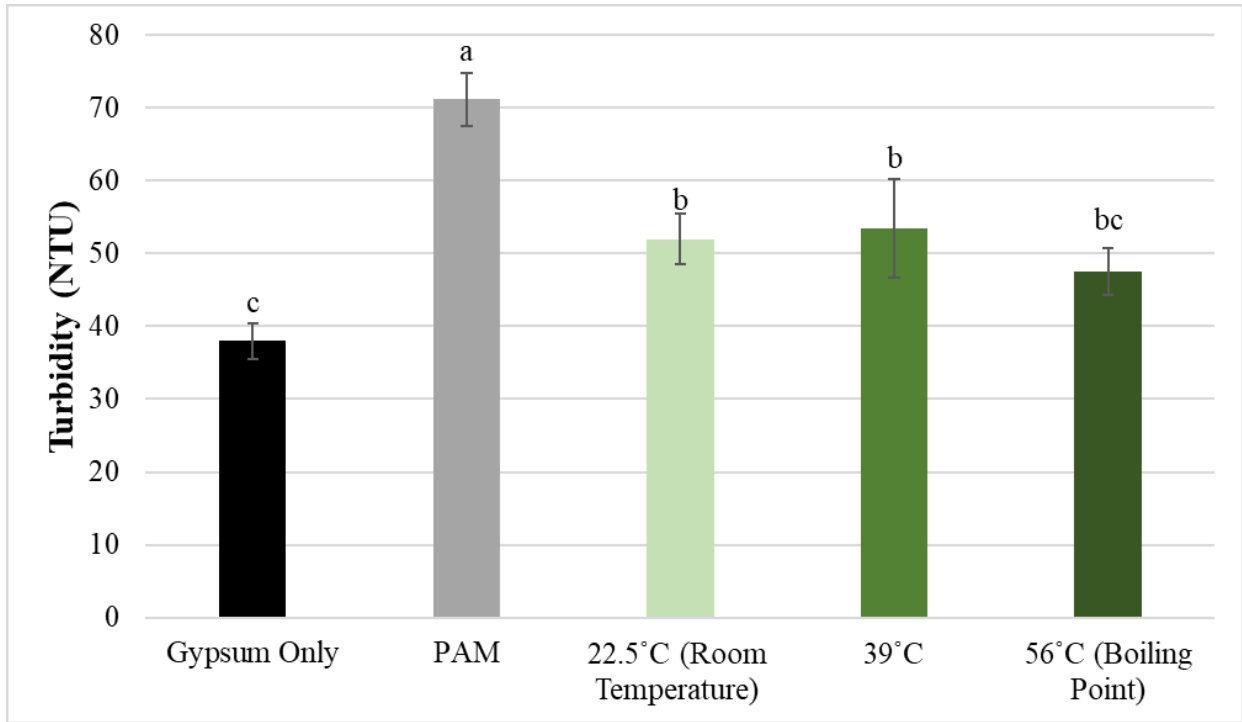verages of the treatments after 48 hours of settling time in a 4% kaolin clay system. The temperature of the crosslinking reaction was lowered. Flocculant treatments were added at a 2.1% (wt/wt) dosage in 7 mL solutions. Experiments were performed in triplicate. Different letters represent statistically significant differences in turbidity.

### 4.4.5. Characterization of Lower Temperature Reaction Glutaraldehyde Crosslinked Peptones

### 4.4.5.1. SDS-PAGE Analysis of Lower Temperature Reaction Glutaraldehyde Crosslinked Peptones

To understand why the low temperature reaction was improving the flocculation performance, an SDS-PAGE analysis was performed to examine any differences in molecular weights of the reaction products. From the results, shown in Figure 53, there are bands present in the lower molecular weight range that are found in both the unmodified peptones and the 0˚C crosslinked peptones, but are not present in the room temperature crosslinked peptones. This indicates that there was some unreacted material in the 0˚C treatment indicating a lower reaction efficiency. A lower reaction efficiency, theoretically, would lead to a product with a lower hydrophobicity. A

lower hydrophobicity could allow the crosslinked peptones to interact more effectively with the clay slurry, which could be the reason behind the faster settling rate observed. This would indicate that there is a trade-off between the hydrophobicity imparted by the glutaraldehyde crosslinking reaction and the increase in molecular weight, when it comes to flocculation performance.
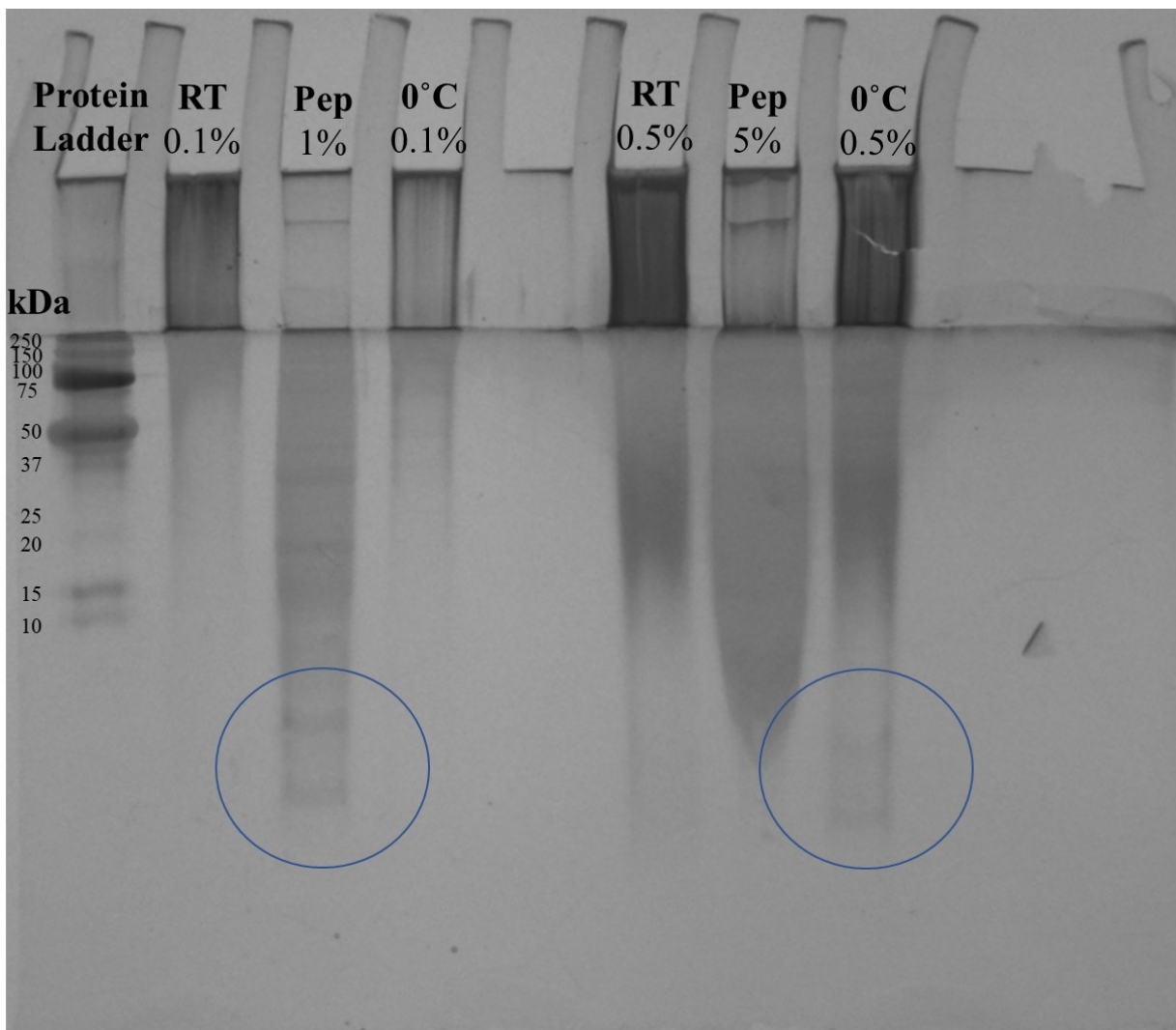


**Figure 53.** SDS PAGE results comparing glutaraldehyde crosslinked peptones reacted at room temperature (RT) and at 0˚C (0˚C) with peptones (Pep). The samples were dissolved in sample buffer containing SDS for 2 hours. Samples were loaded on the gel at 20 µL.

**4.4.5.2. Solubility Test of Lower Temperature Reaction Glutaraldehyde Crosslinked Peptones**

The results from the SDS-PAGE provided some evidence of a difference in molecular weight of the lower temperature glutaraldehyde crosslinking reaction, compared to the room temperature reaction, which could be used to explain the improvement in settling rate. To attempt to identify a difference in the relative hydrophobicity between the products of the two reactions, a simple solubility test was performed, as depicted in Figure 54. In this test, a weighed amount of each reaction product was added to 30 mL of Mill-Q water and was stirred for 1 hour to allow it to dissolve. The beaker was then filtered, and the filter paper was dried in an oven. After drying, the weight of the filter paper and the product remaining was recorded. From this, the amount of product that did not dissolve was determined.

There was not a large difference in the amount of product that remained on the filter paper between the two samples. However, there was a noticeable difference in behaviour when the two samples were added to the water. The room temperature crosslinked peptones formed a hydrophobic film on the top of the solution, whereas the 0˚C crosslinked peptones were well dispersed in the solution. The increased dispersion of the product could potentially allow for better interaction in the kaolin clay slurry and faster settling rates. This would indicate that a lower efficiency of the crosslinking reaction with glutaraldehyde and the peptones was occurring, as a less hydrophobic product was formed. This, coupled with the results of the SDS-PAGE, would indicate that the lowering the temperature of the reaction lowered the reaction rate, and the reaction was not able to go to completion to the same extent as the room temperature reaction after 2 hours.
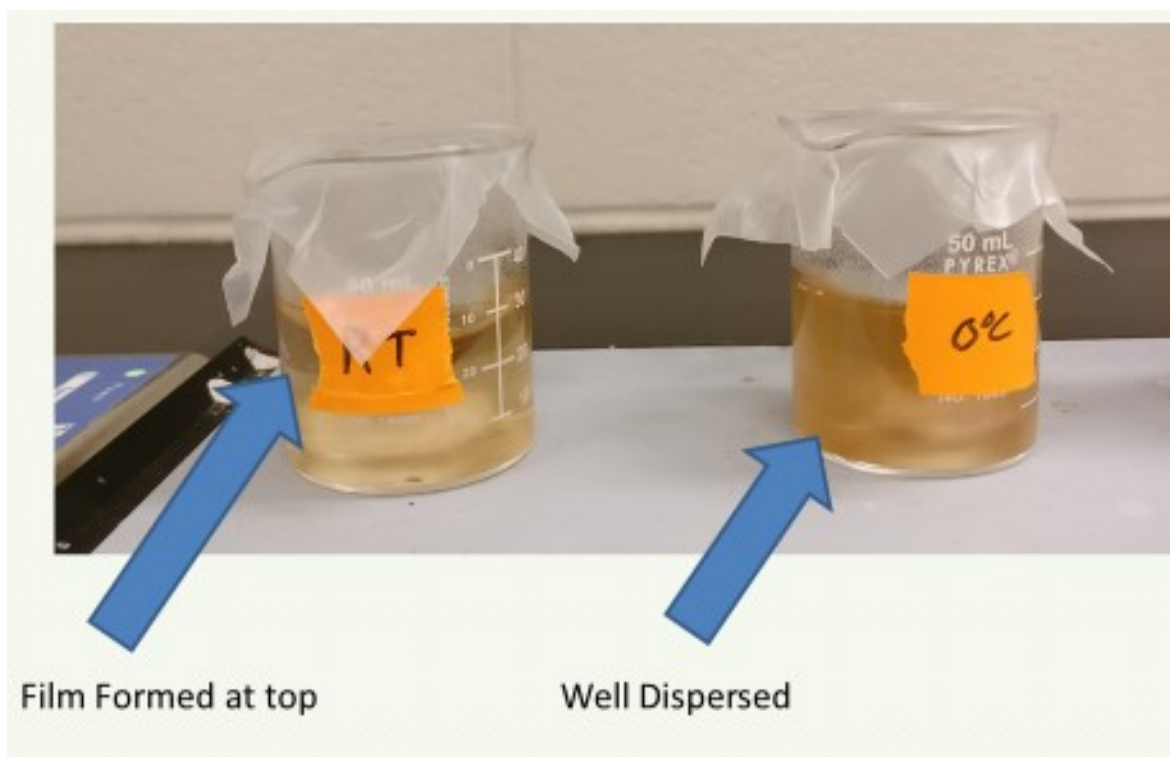
**Figure 54.** Picture showing the difference in dispersion in Milli-Q water of the room temperature crosslinked peptones and the 0˚C crosslinked peptones.

## 5. Conclusions and Future Directions

The Alberta oil sands produce a large amount of tailings waste during oil production in this sector. The treatment of this material and reclamation of the land the waste occupies is a large challenge faced by the industry. Specified risk material (SRM) is a proteinaceous material from the livestock industry, that is currently a waste product but has potential for use in high value applications. In this work, the use of peptones, derived from the hydrolysis of SRM, as flocculating agents have been demonstrated in a model kaolin slurry. In order for flocculation to occur in this system, the presence of a coagulant, such as gypsum, was required. This demonstrated that this material has the potential for use in oil sand tailing pond applications.

To attempt to improve the flocculation performance of these peptones, two types of chemical modifications were investigated. The first modification was an esterification reaction to convert the carboxylic acid groups of the peptones into esters, by reacting them with methanol in acidic conditions. The modification was shown to have occurred at a high efficiency by a carboxylic

acid group titration method, and qualitatively by FTIR. Unfortunately, the esterified peptones did not perform any better than the unmodified peptones in a flocculation experiment. This indicated that this type of modification is not necessary when trying to improve the flocculation performance of this type of material.

The second modification that was studied was with the chemical crosslinking agent glutaraldehyde, to increase the molecular weight of the peptones. The reaction was confirmed to have occurred as intended by several characterization methods, including TGA, elemental analysis, SDS-PAGE and SEC-HPLC. After the reaction, the settling rate of the crosslinked peptones was greatly improved compared to the unmodified peptones and they also had lower supernatant turbidity after 48 hours. This demonstrates that this type of reaction was successful in improving the flocculation ability of the peptones. Increasing the molecular weight with this type of modification is vitally important to improving this materials flocculation performance and should be focused on in future research. The glutaraldehyde crosslinked peptones have a great opportunity for use in future tailings settling applications.

Modification of several parameters of the glutaraldehyde crosslinking reaction were then investigated and local optima in the settling rate and turbidity of the supernatant after settling were found. For the crosslinking ratio, a 1:32 molar ratio of peptone amino groups to glutaraldehyde aldehyde groups was found to provide the best result. For the reaction time, a shorter reaction time of 30 minutes was found to be the local optimum, likely due to its relatively decreased hydrophobicity compared to the products with a longer reaction time. When comparing the method of addition to the slurry of the crosslinked peptone products, adding them as a solution showed no difference in settling than as a solid powder, at the tested dosages. However, adding them as a solution improved the turbidity after 48 hours, while using a lower dosage of flocculant. Finally, increasing the temperature of the reaction did not improve the settling rate or the turbidity in the system. Lowering the temperature of the reaction led to an increase in settling rate, but at a tradeoff of a higher turbidity of the supernatant after 48 hours of settling. Depending on the desired traits, the reaction conditions can be altered to favor either the settling rate or a lower turbidity of the supernatant after settling. These studies demonstrate different conditions for the parameters of the reaction and flocculation tests that could be used to obtain better results in their flocculation performance when used in future systems.

In order for the glutaraldehyde crosslinked peptones to be used in oil sand tailings consolidation applications the cost of the product will have to be competitive with current industrial polymers and will have to be produced in large enough quantities. Assuming a cost of $0 to obtain the SRM, as it is currently a waste stream, the thermal hydrolysis process would be the main cost for the feedstock. The cost for the crosslinking reaction would be up to two factors, the price of the methanol used as a solvent and the price of glutaraldehyde. An online retailer sells bulk glutaraldehyde at $500 for 19 L (https://www.chemworld.com). If using the 1:32 ratio described previously, it would take 6.82 mL/g of peptones. This means that it would take 6.82 L/kg and therefore, $179.47/kg of crosslinked peptones made. For the methanol, online a distributor had a bulk methanol price of 208 L for $613 (https://www.rightpricechemicals.com). If the ratio of the reactants was kept the same upon scale up from the lab scale experiment, it would take 100 L of methanol per kg of crosslinked peptones produced. For the initial purchase it would cost $294.71/kg. The methanol could be recycled during the production process to reduce the cost of methanol drastically. In this scenario when the methanol is recycled, the only ongoing expense for the glutaraldehyde crosslinked peptones would be the glutaraldehyde and the process would cost approximately $179.47/kg. This estimate will change largely depending on the price of the reactants. This estimate is only for the raw materials and does not include other production costs.

For the quantity that could be potentially produced, assuming we could use all of the material, the amount of SRM produced annually in Canada is over 300,000 tonnes (T. H. Mekonnen et al., 2013). After thermal hydrolysis and processing, the recovery rate of the peptones is 34% (Adhikari et al., 2019). In this work with the 1:32 ratio crosslinked peptones, after the glutaraldehyde crosslinking reaction, the recovery rate of the material was 33%. This means that a maximum of 33,660 tonnes or 33.66 million kg of glutaraldehyde crosslinked peptones could be produced annually. According to the Alberta Government website, the tailings volume is increasing by approximately 50 million $m^3$ annually. Researchers have found the density of MFT to be 1310 kg/$m^3$ (Roshani, Fall, & Kennedy, 2017). At 30% solids content, there would be 19,650 million kg of tailings solids to treat annually. In this research, it took a 0.7% (wt/wt) dosage of the crosslinked peptones for settling a 4% solids content slurry. Assuming the dosage scaled 1:1, it would take a 5.25% (wt/wt) dosage to treat the 30% solids content of tailings. This would require 1032 million kg of the crosslinked peptones each year. The maximum amount of the crosslinked peptones that could be produced is only 3.3% of what would be required to treat

all of the tailings produced. This means that the crosslinked peptones would only be able to supplement total required polymers for tailings treatment each year.

Future studies involving flocculation with peptones derived from specified risk materials could explore a few different avenues. One study could involve trying different chemical crosslinkers to increase the molecular weight of the peptones, which could improve the hydrophilicity of the products, settling rate and/or supernatant clarity, compared to the glutaraldehyde crosslinked products. One crosslinker in particular that would be interesting, that was mentioned in the funding proposal, but was not investigated in this work, is poly(ethylene glycol) diglycidyl ether. Its use in crosslinking proteins was described in the literature review, and it should work well for crosslinking of SRM derived peptones. Another future study could be to do a full factorial design optimization experiment with all of the reaction factors to find the true optimum of the system. Another study would be to test and optimize the glutaraldehyde crosslinked peptones that are dissolved in solution on different clay slurries, including oil sands tailings. This would provide an understanding of how they would perform in real world reclamation efforts. The use of the glutaraldehyde crosslinked peptones as a powder on oil sands tailings has already been done by the partners for the project at NAIT, but after the results from the method of flocculant addition experiment in section 4.4.4, it may be worth exploring the solution version as well. Finally, one area that should to be studied is the environmental impact that the peptones will have after their use as a flocculant. It has been assumed that after using the peptones in flocculation applications, it would act as a fertilizer and promote plant growth during reclamation, because of its nitrogen content, but this should be tested and proven. In addition, although the peptones and glutaraldehyde individually are easily broken down in the environment, the breakdown of the glutaraldehyde crosslinked peptones should be studied as well.

This research has shown that peptones derived from specified risk materials can be used as a flocculant in a model system and has the potential for future use in real world applications. The chemical crosslinking of peptones by glutaraldehyde provided a faster settling rate than using the peptones without modification. These crosslinked peptones could provide an alternative to the current polymer flocculants that are used in the industry and may provide benefits to land reclamation and ecosystem recovery due to the nitrogen content of the peptones.

# References

A. P. Black, F. B. Birlmer, and J. J. M. (1966). The Effect of Polymer Adsorption on the Electro-Kinetic Stability of Dilute Clay Suspensions. *Journal of Colloid And Interface Science*, *21*, 626–648.

Abuchowski, A., Van Es, T., Palczuk, N. C., & Davis, F. F. (1977). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *Journal of Biological Chemistry*, *252*(11), 3578–3581.

Adhikari, B. B., Appadu, P., Kislitsin, V., Chae, M., Choi, P., & Bressler, D. C. (2016). Enhancing the adhesive strength of a plywood adhesive developed from hydrolyzed specified risk materials. *Polymers*, *8*(8). https://doi.org/10.3390/polym8080285

Adhikari, B. B., Chae, M., Zhu, C., Khan, A., Harfield, D., Choi, P., & Bressler, D. C. (2019). Pelletization of torrefied wood using a proteinaceous binder developed from hydrolyzed specified risk materials. *Processes*, *7*(4), 1–12. https://doi.org/10.3390/pr7040229

AER. (2017). *Directive 085: Fluid Tailings Management for Oil Sands Mining Projects*. (October), 1–13. Retrieved from https://www.aer.ca/documents/directives/Directive085.pdf

Agriculture and Agri-food Canada. (n.d.). *Overview of the Canadian Agriculture and Agri-Food System 2017*.

Alagha, L., Wang, S., Yan, L., Xu, Z., & Masliyah, J. (2013). Probing adsorption of polyacrylamide-based polymers on anisotropic basal planes of kaolinite using quartz crystal microbalance. *Langmuir*, *29*(12), 3989–3998. https://doi.org/10.1021/la304966v

Ali, S., & Bandyopadhyay, R. (2016). Effect of electrolytes on the microstructure and yielding of aqueous dispersions of colloidal clay. *Soft Matter*, *12*(2), 414–421. https://doi.org/10.1039/c5sm01700a

Ali, Z. M., Mughal, M. A., Laghari, A. J., Ansari, A. K., & Saleem, H. (2013). Polymeric Cellulose Derivative : Carboxymethyl-Cellulose as useful Organic Flocculant against Industrial Waste Waters. *International Journal of Advancements in Research & Technology*, *2*(8), 14–20.

Allen, E. W. (2008). Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. *Journal of Environmental Engineering and Science*, *7*(2), 123–138. https://doi.org/10.1139/S07-038

Amir, Z., Mohd Saaid, I., & Mohamed Jan, B. (2018). An optimization study of polyacrylamide-polyethylenimine-based polymer gel for high temperature reservoir conformance control. *International Journal of Polymer Science*, *2018*. https://doi.org/10.1155/2018/2510132

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964). The Use of Esters of N-Hydroxysuccinimide in Peptide Synthesis. *Journal of the American Chemical Society*, *86*(9), 1839–1842. https://doi.org/10.1021/ja01063a037

Beier, N., Wilson, W., Dunmola, A., & Sego, D. (2013). Impact of flocculation-based dewatering on the shear strength of oil sands fine tailings. *Canadian Geotechnical Journal*, *50*(9), 1001–1007. https://doi.org/10.1139/cgj-2012-0262

Besaratinia, A., & Pfeifer, G. P. (2007). A review of mechanisms of acrylamide carcinogenicity. *Carcinogenesis*, *28*(3), 519–528. https://doi.org/10.1093/carcin/bgm006

Broun, G. B. B. T.-M. in E. (1976). Chemically aggregated enzymes. In *Immobilized Enzymes* (Vol. 44, pp. 263–280). https://doi.org/https://doi.org/10.1016/S0076-6879(76)44022-3

Canadian Food Inspection Agency. (2016). Prions. Retrieved from Inspection.gc.ca website: http://www.inspection.gc.ca/food/archived-food-guidance/meat-and-poultry-products/manual-of-procedures/chapter-5/eng/1395150894222/1395150895519?chap=4#s30c4

Caulfield, M. J., Hao, X., Qiao, G. G., & Solomon, D. H. (2003). Degradation on polyacrylamides. Part I. Linear polyacrylamide. *Polymer*, *44*(5), 1331–1337. https://doi.org/10.1016/S0032-3861(03)00003-X

Chapman, D. L. (1913). LI. A contribution to the theory of electrocapillarity. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, *25*(148), 475–481. https://doi.org/10.1080/14786440408634187

DeGruyter, J. N., Malins, L. R., & Baran, P. S. (2017). Residue-Specific Peptide Modification: A Chemist's Guide. *Biochemistry*, *56*(30), 3863–3873.

https://doi.org/10.1021/acs.biochem.7b00536

Divakaran, R., & Sivasankara Pillai, V. N. (2001). Flocculation of kaolinite suspensions in water by chitosan. *Water Research*, *35*(16), 3904–3908. https://doi.org/10.1016/S0043-1354(01)00131-2

Dixon, J. K., fLa Mer, V. K., Li, C., Messinger, S., & Linford, H. B. (1967). Effect of the structure of cationic polymers on the flocculation and the electrophoretic mobility of crystalline silica. *Journal of Colloid And Interface Science*, *23*(4), 465–473. https://doi.org/10.1016/0021-9797(67)90192-0

Dwari, R. K., & Mishra, B. K. (2019). Evaluation of flocculation characteristics of kaolinite dispersion system using guar gum: A green flocculant. *International Journal of Mining Science and Technology*, *29*(5), 745–755. https://doi.org/10.1016/j.ijmst.2019.06.001

El-Thaher, N., Mekonnen, T., Mussone, P., Bressler, D., & Choi, P. (2013). Effects of electrolytes, water, and temperature on cross-linking of glutaraldehyde and hydrolyzed specified risk material. *Industrial and Engineering Chemistry Research*, *52*(14), 4987–4993. https://doi.org/10.1021/ie303416h

Ernsthausen, W. (n.d.). *The Source Essential, High-Quality Products*. Retrieved from http://hdl.handle.net/2060/19930094591

Feng, L., Stuart, M. C., & Adachi, Y. (2015, December 1). Dynamics of polyelectrolyte adsorption and colloidal flocculation upon mixing studied using mono-dispersed polystyrene latex particles. *Advances in Colloid and Interface Science*, Vol. 226, pp. 101–114. https://doi.org/10.1016/j.cis.2015.09.004

Ferrarezi, A. L., Pivetta, D. H., Bonilla-Rodriguez, G. O., Silva, R. da, Guisan, J. M., Gomes, E., & Pessela, B. C. (2013). Partial purification, immobilization and preliminary biochemical characterization of lipases from Rhizomucor pusillus. *Advances in Enzyme Research*, *01*(04), 79–90. https://doi.org/10.4236/aer.2013.14009

Fleer, G. J., & Lyklema, J. (1976). Polymer adsorption and its effect on the stability of hydrophobic colloids. III. Kinetics of the flocculation of silver iodide sols. *Journal of Colloid And Interface Science*, *55*(1), 228–238. https://doi.org/10.1016/0021-

9797(76)90029-1

Fraenkel-Conrat, H and Olcott, H. (1945). Esterification of Proteins with Alcohols of Low Molecular Weight. *Journal of Biochemistry*, (161), 259–268.

Fujitani, T. (1996). Stability of pigment and resin dispersions in waterborne paint. *Progress in Organic Coatings*, *29*(1–4), 97–105. https://doi.org/10.1016/S0300-9440(96)00661-3

Gamboa da Costa, G., Churchwell, M. I., Hamilton, L. P., Von Tungeln, L. S., Beland, F. A., Marques, M. M., & Doerge, D. R. (2003). DNA Adduct Formation from Acrylamide via Conversion To Glycidamide in Adult and Neonatal Mice. *Chemical Research in Toxicology*, *16*(10), 1328–1337. https://doi.org/10.1021/tx034108e

Gasner, L. L., & Wang, D. I. C. (1970). Microbial cell recovery enhancement through flocculation. *Biotechnology and Bioengineering*, *12*(6), 873–887. https://doi.org/10.1002/bit.260120603

Gassara, F., Antzak, C., Matheyambath, C., Jyoti, S., Kaur, S., & Verma, M. (2015). Chitin and chitosan as natural flocculants for beer clarification. *Journal of Food Engineering*, *166*, 80–85. https://doi.org/10.1016/j.jfoodeng.2015.05.028

Gilroyed, B. H., Reuter, T., Chu, A., Hao, X., Xu, W., & McAllister, T. A. (2010). Anaerobic digestion of specified risk materials with cattle manure for biogas production. *Bioresource Technology*, *101*(15), 5780–5785. https://doi.org/10.1016/j.biortech.2010.02.077

Gouy, M. (1910). Sur la constitution de la charge électrique à la surface d'un électrolyte. *Journal de Physique Théorique et Appliquée*, *9*(1), 457–468. https://doi.org/10.1051/jphystap:019100090045700

Government of Alberta. (2015). *Lower Athabacsa Region: Tailings Management Framework for the Mineable Athabasca Oil Sands*.

Government of Alberta. (2019). Oil sands facts and statistics. Retrieved from https://www.alberta.ca/oil-sands-facts-and-statistics.aspx

Gratzer, P. F., Pereira, C. A., & Lee, J. M. (1996). Solvent environment modulates effects of glutaraldehyde crosslinking on tissue-derived biomaterials. *Journal of Biomedical Materials*

*Research*, *31*(4), 533–543. https://doi.org/10.1002/(SICI)1097-4636(199608)31:4<533::AID-JBM14>3.0.CO;2-H

Gregory, J., & Street, G. (1969). Flocculation of Polystyrene Particles with Cationic Polyelectrolytes. *Transactions of the Faraday Society*, *65*, 2260–2268.

Hardy, P. M., Nicholls, A. C., & Rydon, H. N. (1969). The nature of glutaraldehyde in aqueous solution. *Journal of the Chemical Society D: Chemical Communications*, (10), 565–566. https://doi.org/10.1039/C29690000565

Hashimoto, K., Sakamoto, J., & Tanii, H. (1981). Neurotoxicity of acrylamide and related compounds and their effects on male gonads in mice. *Archives of Toxicology*, *47*(3), 179–189. https://doi.org/10.1007/BF00368678

Hongbo, T., Yanping, L., Wen, Z., & Siqing, D. (2016). Synthesis, Optimization, Property, Characterization, and Application of Dialdehyde Cross-Linking Guar Gum. *International Journal of Polymer Science*, *2016*. https://doi.org/10.1155/2016/6482461

Information, R. (2019). *Alberta / Canada Highlights*. (March), 2018–2020.

Jankovics, L. (1965). Effect of agitation and molecular weight on polymer adsorption and deflocculation. *Journal of Applied Polymer Science*, *9*(2), 545–552. https://doi.org/10.1002/app.1965.070090216

Kalman Burger and Bela Noszal. (1981). Protonation And Complexation Equilibria of Macromolecular Bioligands in Aqueous and Mixed Solvent Solutions. The Solvent Effect. *L. Eotvos University, Department of Inorganic and Analytical Chemistry*, 139–148.

Kaminsky, H., & Omotoso, O. (n.d.). *Variability in Fluid Fine Tailings Introduction-Formation of FFT*.

Keren, R. (1989). Effect of clay charge density and adsorbed ions on the rheology of montmorillonite suspension. *Soil Science Society of America Journal*, *53*(1), 25–29. https://doi.org/10.2136/sssaj1989.03615995005300010005x

Kontogeorgis, G. M., & Kiil, S. (2016). *Introduction to Applied Colloid and Surface Chemistry*. Retrieved from http://ebookcentral.proquest.com/lib/ualberta/detail.action?docID=4462511

Korn, A. H., Feairheller, S. H., & Filachoine, E. M. (1972). Glutaraldehyde: Nature of the reagent. *Journal of Molecular Biology*, *65*(3), 525–529. https://doi.org/10.1016/0022-2836(72)90206-9

Kurniawan, L., Qiao, G. G., & Zhang, X. (2007). Chemical modification of wheat protein-based natural polymers: Grafting and cross-linking reactions with poly(ethylene oxide) diglycidyl ether and ethyl diamine. *Biomacromolecules*, *8*(9), 2909–2915. https://doi.org/10.1021/bm0703719

Lodha, P., & Netravali, A. N. (2005). Thermal and mechanical properties of environment-friendly "green" plastics from stearic acid modified-soy protein isolate. *Industrial Crops and Products*, *21*(1), 49–64. https://doi.org/10.1016/j.indcrop.2003.12.006

Lu, Q., Yan, B., Xie, L., Huang, J., Liu, Y., & Zeng, H. (2016). A two-step flocculation process on oil sands tailings treatment using oppositely charged polymer flocculants. *Science of the Total Environment*, *565*, 369–375. https://doi.org/10.1016/j.scitotenv.2016.04.192

Ma, Kedong, Wakisaka, M., Sakai, K., & Shirai, Y. (2009). Flocculation characteristics of an isolated mutant flocculent Saccharomyces cerevisiae strain and its application for fuel ethanol production from kitchen refuse. *Bioresource Technology*, *100*(7), 2289–2292. https://doi.org/10.1016/j.biortech.2008.11.010

Ma, Kunsong, & Pierre, A. C. (1997). Effect of interaction between clay particles and Fe3+ ions on colloidal properties of kaolinite suspensions. *Clays and Clay Minerals*, *45*(5), 733–744. https://doi.org/10.1346/CCMN.1997.0450512

Mahaffey, A., & Dubé, M. (2017). Review of the composition and toxicity of oil sands process-affected water. *Environmental Reviews*, Vol. 25, pp. 97–114. https://doi.org/10.1139/er-2015-0060

Matthews, J. G., Shaw, W. H., Mackinnon, M. D., & Cuddy, R. G. (2002). Development of composite tailings technology at Syncrude. *International Journal of Surface Mining, Reclamation and Environment*, *16*(1), 24–39. https://doi.org/10.1076/ijsm.16.1.24.3407

Mekonnen, T. H., Mussone, P. G., Choi, P., & Bressler, D. C. (2014). Adhesives from waste protein biomass for oriented strand board composites: Development and performance.

*Macromolecular Materials and Engineering*, *299*(8), 1003–1012.
https://doi.org/10.1002/mame.201300402

Mekonnen, T. H., Mussone, P. G., Choi, P., & Bressler, D. C. (2015). Development of proteinaceous plywood adhesive and optimization of its lap shear strength. *Macromolecular Materials and Engineering*, *300*(2), 198–209. https://doi.org/10.1002/mame.201400199

Mekonnen, T. H., Mussone, P. G., Stashko, N., Choi, P. Y., & Bressler, D. C. (2013). Recovery and characterization of proteinacious material recovered from thermal and alkaline hydrolyzed specified risk materials. *Process Biochemistry*, *48*(5–6), 885–892. https://doi.org/10.1016/j.procbio.2013.04.015

Mekonnen, T., Mussone, P., & Bressler, D. (2016). Valorization of rendering industry wastes and co-products for industrial chemicals, materials and energy: Review. *Critical Reviews in Biotechnology*, *36*(1), 120–131. https://doi.org/10.3109/07388551.2014.928812

Mekonnen, T., Mussone, P., El-Thaher, N., Choi, P. Y. K., & Bressler, D. C. (2013). Thermosetting proteinaceous plastics from hydrolyzed specified risk material. *Macromolecular Materials and Engineering*, *298*(12), 1294–1303. https://doi.org/10.1002/mame.201200429

Migneault, I., Dartiguenave, C., Bertrand, M. J., & Waldron, K. C. (2004). Glutaraldehyde: behavior in aqueous solution, reaction with crosslinking. *BioTechniques*, *37*(5), 790–802. https://doi.org/10.2144/04375RV01

Motta, F. L., Gaikwad, R., Botha, L., & Soares, J. B. P. (2018). Quantifying the effect of polyacrylamide dosage, Na+ and Ca2+ concentrations, and clay particle size on the flocculation of mature fine tailings with robust statistical methods. *Chemosphere*, *208*, 263–272. https://doi.org/10.1016/j.chemosphere.2018.05.171

Nasser, M. S., & James, A. E. (2006). The effect of polyacrylamide charge density and molecular weight on the flocculation and sedimentation behaviour of kaolinite suspensions. *Separation and Purification Technology*, *52*(2), 241–252. https://doi.org/10.1016/j.seppur.2006.04.005

Niu, X., Wang, X., Han, Y., Lu, C., Chen, X., Wang, T., … Zhu, Q. (2019). Influence of

malondialdehyde-induced modifications on physicochemical and digestibility characteristics of whey protein isolate. *Journal of Food Biochemistry*, (August), 1–9. https://doi.org/10.1111/jfbc.13041

Nudelman, A., Bechor, Y., Falb, E., Fischer, B., Wexler, B. A., & Nudelman, A. (1998). Acetyl chloride-methanol as a convenient reagent for: A) quantitative formation of amine hydrochlorides; B) carboxylate ester formation; C) mild removal of N-t-Boc-protective group. *Synthetic Communications*, *28*(3), 471–474. https://doi.org/10.1080/00397919808005101

O'Gorman, J. V., & Kitchener, J. A. (1974). The flocculation and de-watering of kimberlite clay slimes. *International Journal of Mineral Processing*, *1*(1), 33–49. https://doi.org/10.1016/0301-7516(74)90025-8

Okuda, K., Urabe, I., Yamada, Y., & Okada, H. (1991). Reaction of glutaraldehyde with amino and thiol compounds. *Journal of Fermentation and Bioengineering*, *71*(2), 100–105. https://doi.org/10.1016/0922-338X(91)90231-5

Pal, S., Mal, D., & Singh, R. P. (2008). Characterization of Cationic Starch : An Efficient Flocculating Agent. *Wiley InterScience*. https://doi.org/10.1002/app

Partch, R., Matijevic, E., Hodgson, A. W., & Aiken, B. E. (1983). Preparation of Polymer Colloids By Chemical Reactions in Aerosols - 1. Poly(P-Tertiarybutylstyrene). *Journal of Polymer Science. Part A-1, Polymer Chemistry*, *21*(4), 961–967.

Piazza, G. J., & Garcia, R. A. (2010). Proteins and peptides as renewable flocculants. *Bioresource Technology*, *101*(15), 5759–5766. https://doi.org/10.1016/j.biortech.2010.02.073

Renault, F., Sancey, B., Charles, J., Morin-Crini, N., Badot, P. M., Winterton, P., & Crini, G. (2009). Chitosan flocculation of cardboard-mill secondary biological wastewater. *Chemical Engineering Journal*, *155*(3), 775–783. https://doi.org/10.1016/j.cej.2009.09.023

Report, F., & Island, V. (2009). *Slaughterhouse Waste and Specified Risk Material ( SRM ) Management Study*.

Roberts, M. J., Bentley, M. D., & Harris, J. M. (2012). Chemistry for peptide and protein

PEGylation. *Advanced Drug Delivery Reviews*, *64*(SUPPL.), 116–127. https://doi.org/10.1016/j.addr.2012.09.025

Roshani, A., Fall, M., & Kennedy, K. (2017). A column study of the hydro-mechanical behavior of mature fine tailings under atmospheric drying. *International Journal of Mining Science and Technology*, *27*(2), 203–209. https://doi.org/10.1016/j.ijmst.2017.01.023

Schägger, H. (2006). Tricine–SDS-PAGE. *Nature Protocols*, *1*, 16. Retrieved from https://doi.org/10.1038/nprot.2006.4

Shainberg, I., & Levy, G. J. (2005). Flocculation and Dispersion. *Encyclopedia of Soils in the Environment*, (1999), 27–34. https://doi.org/10.1016/b0-12-348530-4/00363-5

Shaw, D. J. (1992). Introduction to Colloid and Surface Chemistry. 4th ed. In *Oxford: Butterworth-Heinemann*.

Sobkowicz, J. (2012). Oil sands tailings technology deployment roadmaps: Project summary. *Alberta Innovates – Energy and Environment Solutions*, *2*, 243. Retrieved from http://www.cosia.ca/uploads/documents/id10/Tailings Roadmap Volume 2 June 2012.pdf

Strandman, S., Vachon, R., Dini, M., Giasson, S., & Zhu, X. X. (2017). Polyacrylamides revisited: Flocculation of kaolin suspensions and mature fine tailings. *Canadian Journal of Chemical Engineering*, *96*(1), 20–26. https://doi.org/10.1002/cjce.22940

Sumner, S. C. J., Fennell, T. R., Moore, T. A., Chanas, B., Gonzalez, F., & Ghanayem, B. I. (1999). Role of Cytochrome P450 2E1 in the Metabolism of Acrylamide and Acrylonitrile in Mice. *Chemical Research in Toxicology*, *12*(11), 1110–1116. https://doi.org/10.1021/tx990040k

Sworska, A., Laskowski, J. S., & Cymerman, G. (2000). Flocculation of the Syncrude fine tailings Part I. Effect of pH, polymer dosage and Mg2+ and Ca2+ cations. *International Journal of Mineral Processing*, *60*(2), 143–152. https://doi.org/10.1016/S0301-7516(00)00012-0

Tal, M., Silberstein, A., & Nusser, E. (1985). Why does Coomassie Brilliant Blue R interact differently with different proteins? A partial answer. *Journal of Biological Chemistry*, *260*(18), 9976–9980.

Thermo Fisher Scientific Inc. (2016). *Pierce<sup>TM</sup> Silver Stain Kit: Instructions*. *0747*(24612). Retrieved from Thermofisher.com

Tripathy, T., Bhagat, R. P., & Singh, R. P. (2001). Flocculation performance of grafted sodium alginate and other polymeric flocculants in relation to iron ore slime suspension. *European Polymer Journal*, *37*(1), 125–130. https://doi.org/10.1016/S0014-3057(00)00089-6

Van Vliet, T. (2010). Colloidal aspects of texture perception. *Food Engineering and Ingredients*, *35*(OCTOBER), 38–42. https://doi.org/10.1016/j.cis.2009.04.002

Vaz, C. M., De Graaf, L. A., Reis, R. L., & Cunha, A. M. (2003). In vitro degradation behaviour of biodegradable soy plastics: Effects of crosslinking with glyoxal and thermal treatment. *Polymer Degradation and Stability*, *81*(1), 65–74. https://doi.org/10.1016/S0141-3910(03)00063-6

Veronese, F. M., & Pasut, G. (2005). PEGylation, successful approach. *Drug Discovery Today*, *10*(21), 1451–1458. https://doi.org/10.1016/S1359-6446(05)03575-0

Wang, X. W., Feng, X., Xu, Z., & Masliyah, J. H. (2010). Polymer aids for settling and filtration of oil sands tailings. *Canadian Journal of Chemical Engineering*, *88*(3), 403–410. https://doi.org/10.1002/cjce.20283

Wilcox, P. E. (1972). Esterification. *Methods in Enzymology*, *25*, 596–615. https://doi.org/10.1016/S0076-6879(72)25059-5

Wrolstad, R. E. (2004). *Handbook of food analytical chemistry : water, proteins, enzymes, lipids, and carbohydrates.* (Vol. 1). Hoboken, N.J.: J. Wiley.

Zhang, Y., Yin, Y., Lu, S., Yao, X., Zheng, X., Zhao, R., … Zhang, S. (2018). Effects of modified processing methods on structural changes of black soybean protein isolate. *Molecules*, *23*(9). https://doi.org/10.3390/molecules23092127

Zhao, H., Bhattacharjee, S., Chow, R., Wallace, D., Masliyah, J. H., & Xu, Z. (2008). Probing surface charge potentials of clay basal planes and edges by direct force measurements. *Langmuir*, *24*(22), 12899–12910. https://doi.org/10.1021/la802112h

Zhao, Y., Zhao, Y., Xu, H., & Yang, Y. (2015). A sustainable slashing industry using

biodegradable sizes from modified soy protein to replace petro-based poly(vinyl alcohol). *Environmental Science and Technology, 49*(4), 2391–2397. https://doi.org/10.1021/es504988w