

Value-added food applications utilizing emerging commodities (sorghum, sorghum processing byproducts, *Camelina sativa*) that support sustainable agricultural practices

by

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Abstract

Emerging commodities such as sorghum (*Sorghum bicolor* L. Moench), Camelina sativa (*Camelina sativa* L.), and renewable resources of distillers' dried grains with solubles (DDGS) from ethanol fermentation possess high potential to be utilized in new and value-added food applications. Sorghum (*Sorghum bicolor* L. Moench) is the third commonly grown cereal crop in the United States (USA) and the fifth in the world. It is recognized as a sustainable crop due to its high drought tolerance and quicker adaptability to various environments. There is, therefore, a growing interest in expanding the utilization of sorghum and its byproducts in support of sustainable agricultural practices. In addition to this, DDGS offers great potential as a renewable source for manufacturing biodegradable packaging film materials due to its high protein and fiber content. Sorghum and sorghum DDGS can be utilized to manufacture resin-like viscous materials and biodegradable packaging films. In addition to this, the emerging oil seed crop Camelina sativa has great potential to be used as novel additives for various food applications. It has been transgenically engineered to produce high levels of acetyl-TAG by introducing acetyl-transferase enzymes from different *Euonymus* species. The unusual acetyl-TAG molecules obtained from transgenic Camelina sativa seeds can be used to improve the functional properties of biodegradable packaging films as a plasticizer and to increase the stability and capacity of protein foams and emulsions as an emulsifier or a stabilizer.

The first objective is to develop a biodegradable packaging film using sorghum DDGS and to characterize the effects of milling pretreatment and suitable solvents on physical, mechanical, and thermal properties. In this objective, the films were prepared from sorghum DDGS after Udy and Colloid milling and solubilized using water, chloroform (1:0, 1:1, 1:2, 0:1 (v/v) water: chloroform

ratio) and ethanol. The lowest ($P < 0.05$) moisture solubility ($21.19 \pm 0.03\%$) and the highest tensile strength (19.19 ± 0.03 MPa) were obtained when Udy milling was applied to DDGS using a 1:2 ratio. The melting temperature of the films significantly ($P < 0.05$) increased (134.01 ± 1.1 °C) when DDGS was pretreated using dry milling with the combined use of water, chloroform, and ethanol as compared to only water for solubilization. Overall, dry, and wet milling with a suitable solvent ratio improved film properties.

The second objective is to improve the functional properties of DDGS films by the presence of the *sn*-3 acetyl group of acetyl TAG, obtained from transgenic *Camelina sativa*, and preconditioning via wet milling and suitable solubilization. The 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are unusually structured triacylglycerol (TAG) molecules that possess an *sn*-3 acetate group instead of a long chain fatty acid. This reorganized structure enables Acetyl-TAG to possess different physical properties from regular TAG, including reduced viscosity and improved cold temperature properties. Therefore, high oleic acetyl-TAG from engineered form *Camelina* seeds can be used as a novel and high-yield plasticizer. The moisture solubility of the films pretreated via a ball mill and solubilized with a 1:1 (v/v) solvent ratio decreased by 9% when acetyl-TAG concentration increased from 0.1% to 0.2% (w/v). The elongation at the break increased by 24% when 0.2% (w/v) acetyl-TAG was incorporated into the film prepared with the same conditions as compared to the film without acetyl-TAG. The addition of acetyl-TAG also reduced the wettability and improved the hydrophobic character of the films. Overall, acetyl-TAG with wet milling improved the film properties.

The third objective of this dissertation is to use acetyl-triacylglycerols (acetyl-TAG) that possess *sn*-3 acetate group with high oleic content as an effective stabilizer to the foam at reduced sugar concentrations by increasing the surface viscosity and minimizing foam serum drainage. A

fractional factorial Box-Wilson design was used to investigate the effects of whey protein concentration (WPI) (2-10 wt%), sucrose concentration (SC) (10-30 wt%), and acetyl-TAG concentration (ATC) (0-1 wt%) on overrun (FO), stability against serum drainage (FD) and surface dilatational rheology. Each response was analyzed by linear regression model fitting and a backward elimination algorithm for significance ($\alpha = 0.01$). FO was significantly affected ($P < 0.01$) by WPI and ATC, but not sucrose concentration. The optimum overrun (nearly 700%) was obtained at 9 wt% WPI and 0.6 wt% ATC. Drainage and viscoelastic properties were significantly ($P < 0.01$) affected by all process variables. Dynamic complex modulus ($|E|$) as measured using an optical tensiometer was around 30-40 mN/m when acetyl-TAG was higher than 0.5 wt%. Our findings indicated that the acetyl-TAG can be used to enhance the stability of protein foams in reduced sugar food products, but not at high sugar concentrations (30 wt%).

The fourth objective of this study is to increase emulsion and foam stability at low saturated oil in water emulsions before and after whipping via wild-type acetyl-TAG. In this study, protein-stabilized emulsions using whey protein hydrolysate (WPH) (2.5 % (w/v)), coconut oil (15% (w/v) and 30% (w/v)) and wild-type acetyl-TAG (0, 0.5, 1 % (w/v)) were prepared by a high-pressure homogenizer. Particle size, creaming index, and overrun were used to evaluate the stability and foaming ability of whipped emulsion during aging (21 days at 25°C). Furthermore, the percentage of adsorbed protein, zeta potential, and interfacial rheology were analyzed to investigate the effect of acetyl-TAG on the stability of WPH at the oil-water interfaces and the interaction between acetyl-TAG and coconut oil phase and protein at the interfacial level. The results showed that acetyl-TAG significantly ($P < 0.05$) reduced the particle size and the particle size decreased ($P < 0.05$) further with an increase in the concentration of acetyl-TAG from 0.5% to 1% (w/w) whereas the creaming index decreased ($P < 0.05$) using acetyl-TAG in emulsion preparation.

Furthermore, the capacity (overrun%) of whipped emulsions significantly ($P < 0.05$) increased and drainage of whipped emulsions significantly ($P < 0.05$) reduced with the addition of acetyl-TAG. In conclusion, wild-type acetyl-TAG may be used as an effective surfactant in emulsions stabilized by WPH and improve the whipping characteristics of aerated emulsion systems.

The fifth objective of this dissertation is to obtain oleoresins from waxy burgundy sorghum grain and to characterize the effects of the extraction process and solvents on their composition and functional properties. Oleoresins were obtained by using the following solvents with and without ball milling: two types of novel ionic liquids (IL₁: 1-n-Hexyl-3-methylimidazoliumchloride, IL₂: 1-Ethyl-3-methylimidazoliumchloride), ethanol and dichloromethane. The effects of processing were evaluated for the extraction yield, protein, fat, and total phenolic content, fatty acid composition, particle size and zeta potential, and FTIR spectra. The use of ILs and the ball mill process significantly ($P < 0.05$) affected the extraction yield and physicochemical properties. The highest extraction yields increased ($31.35\% \pm 0.58$) when ball milling was used with IL₂ in comparison to the lowest ($18.37\% \pm 0.77$) obtained by traditional ethanol extraction. Similarly, protein concentration and phenolic content were the highest ($1.37\% \pm 0.05$ and $0.57\% \pm 0.01$, respectively) with ball milling extraction and IL₁. The FTIR spectra indicated higher phospholipids (at 1200 cm^{-1}) and protein-phospholipid bonding (at 1700 cm^{-1}) by ILs, and ball milling as compared to traditional extraction. Overall, wet milling-assisted extraction by using a ball mill and ILs provided control over the composition of the oleoresins important for value-added food applications and higher extraction efficiencies as compared to traditional techniques.

This study showed the oleoresins offering great potential for value added food applications can be successfully extracted with high extraction efficiency from waxy burgundy whole grain sorghum and their composition can be controlled via wet milling-assisted extraction using a ball mill and

novel ionic liquids (ILs). Additionally, renewable biodegradable packaging film alternatives can be manufactured from sorghum DDGS by dry and wet milling preconditioning with a suitable solubilization technique. Furthermore, high oleic acetyl-TAG from engineered form *Camelina* seeds can be used as a novel and high-yield plasticizer to improve the physical, mechanical, and surface hydrophobicity of DDGS films. Moreover, acetyl-TAG isolated from transgenic *Camelina sativa* seeds with high oleic content can serve as an effective protein foam stabilizer at reduced sugar concentrations. Similar to this, wild-type acetyl-TAG can effectively assist whey protein hydrolysate to stabilize coconut oil-based emulsions and promote interfacial adsorption.

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Dedication

I dedicate this dissertation to my family.

Chapter 1 - Plant-based packaging films, emerging crops of sorghum and *Camelina sativa*, and their food applications

Abstract

Renewable biodegradable packaging films have become increasingly popular worldwide in the last decade due to the growing awareness of environmental and sustainability issues. Plant-based sources such as cereals, grains, pseudocereals, ancient grains, crops, and oilseeds are favored for their affordability, sustainability, and variety. In addition to this, emerging crops such as sorghum and *Camelina sativa* can be sustainable plant-based approaches to novel food applications. They can be utilized in biodegradable packaging films and their various fractions (e.g., oleochemicals and by-products) can be great raw material alternatives for packaging materials, commercial plasticizers, emulsifiers, or stabilizers.

In this review, I provide information about plant-based packaging films and an overview of promising food application areas of sorghum, *Camelina sativa*, and some of their components. Furthermore, I discussed the challenges and modifications of plant-based packaging films, extraction of oleochemicals from sorghum, and transgenic triacylglycerol from *Camelina sativa* using some recent studies from the literature.

1. Plant-based packaging applications from cereal and grains

Packaging is essential to preserve the nutritional, organoleptic, and microbiological properties of food during handling, storage, and transportation (Priyadarshi et al., 2021). It is designed to prevent any environmental and health risks causing degradation to food products. The packaging film materials are significant contributors to the safety, quality, and shelf-life of food materials. They

can be classified as primary, secondary, and tertiary depending on the level of contact with the product. Primary packaging films that are the focus of this chapter directly contact with products. Distinctly, secondary, and tertiary packaging films are used as distribution carriers. Packaging films are made of a matrix including macromolecules to create a cohesive structure providing strength, and a plasticizer reducing rigidity and brittleness. In addition to this, they protect against physical and biological damage to food. They also contribute to shelf-life by providing control for the exchange of moisture, gases, and volatile compounds between the material and the surrounding environment.

Traditional packaging films are manufactured from petroleum-based polymers with tunable properties, but well-known environmental drawbacks. For example, the concentration of microplastics fragmented from petroleum-based materials has been increasing to critical levels and is expected to continue to increase over the coming decades. Microplastic contamination in aquatic environments and soils is considered a dominant environmental plastic pollutant to results in serious health concerns for humans and animals (Jang et al., 2020). Nearly 50% of the packaging materials are made up of non-renewable sources such as polyesters or polyolefins (Nguyen et al., 2018). The continuous increase in the use of synthetic plastic may become a threat to the ecological and environmental cycles due to their non-biodegradable nature (Gadhav et al., 2018). For instance, in the United States, more than 55% of polyester, polypropylene, or polystyrene are landfilled and 30% of them are undeclared for the final destination and may become plastic pollution (Nguyen et al., 2018). These persistent plastic pollutants migrate into the environment as chemicals and microplastic components and create serious concerns for aquatic and continental life (Sani et al., 2021).

Alternatively, the adoption of biodegradable films, which are known as materials that can naturally decompose into water, carbon dioxide, methane, or biomass in three to six months, can serve as a viable solution to this problem (Reid et al., 2022; Saravanan et al., 2023). These materials can be obtained from biomass, synthesized from bio-derived monomers, or produced from microorganisms (Abdullah et al., 2022; Bayer et al., 2014). Moreover, these materials can be produced from renewable resources (i.e., cellulosic materials and fibers recovered from process waste-streams) supporting sustainable agriculture practices (Shrestha et al., 2018). There is, therefore, a large need and interest for designing biodegradable, safe, and high-performance packaging materials using biopolymer sources, such as cereals and grains.

Renewable biodegradable packaging films gained popularity over the last decade around the world due to the increase in environmental concerns and sustainability problems (Shrestha et al., 2018). Substituting petroleum-based materials with sustainable ones may reduce the risk resulting from plastic packaging films thanks to their greenie sight (Ciannamea et al., 2018). Environmental concerns encouraged scientists to produce edible, biodegradable, and renewable packaging materials (Giosafatto et al., 2018). Nevertheless, biodegradable films might have limited applicability as well as inadequate physico-mechanical and thermal properties (Shahbazi et al., 2018). The primary disadvantage of these films is their poor moisture resistance, thus, they might require chemical modifications, crosslinking, or physical incorporations to improve their functionality (Briassoulis & Giannoulis, 2018; Popović et al., 2021). Still, natural biopolymers such as proteins, polysaccharides, and lipids are the main biopolymers used to produce biodegradable packaging films and coatings due to their environmentally friendly origin, oxygen barrier ability, and thermo-mechanical characteristics (De Leo et al., 2018). Biopolymers used as packaging materials can be extracted from both animal and plant-based sources (Thankachen et

al., 2021). Yet, plant-based sources such as oilseeds, pulses, cereals, pseudocereals, grains, and ancient grains are preferred due to affordability, sustainability, and diversity. Pulses are edible seeds of plants in the legume (*Leguminosae*) family. They are among the most economically important crops occupying nearly 15% of the world's cultivated land (Bessada et al., 2019). Peas (*Pisum sativum*), lentils (*Lens culinaris ssp. culinaris*), chickpeas (*Cicer arietinum*), kidney beans (*Phaseolus vulgaris*), soya beans (*Glycine max (L.) Merr*) and black beans (*Phaseolus vulgaris*) are known as the most common pulses (Ren et al., 2021). Cereals are plants belonging to the grass family. They are known as plants yielding starchy grains suitable for foods and they refer to the edible grains of certain grasses that are cultivated for food. In addition to this, grains are defined as the edible seeds of cereals and legumes. Maize (*Zea mays L.*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), oat (*Avena sativa*), and rye (*Secale cereale*) are the most-well known cereals and mostly classified as grains (Adebo et al., 2022). Similar to this, pseudo cereals are defined as plants producing fruits and starchy seeds. They are often gluten-free and protein-rich crops (Alvarez-Jubete et al., 2009). Chia (*Salvia hispanica*), quinoa (*Chenopodium quinoa sub sp.quinoa; Chenopodiaceae*), buckwheat (*Fagopyrum esculentum Monch*) and amaranth (*Amaranth caudatus, Amaranth cruentus, Amaranth hypochondriacus*) are the most common pseudocereals (Caselato-Sousa & Amaya-Farfán, 2012; Gorinstein et al., 2007; Kojić et al., 2017). Ancient grains are accepted as wild ancestors of grains nonexposed to any modern breeding or processing (Giambanelli et al., 2013). The most commonly known ancient grains are sorghum (*Sorghum bicolor L. Moench*), mung bean (*Vigna radiata L.*) and teff (*Eragrostis tef*) (Boukid et al., 2018). Cereals, grains, ancient grains and pseudocereals (Figure 1-1). are important categories plant-based sources that present advantages as source of natural bio-based polymers for food packaging applications. They present high adaptability to extreme environmental conditions and

high harvest yield. Therefore they are suitable in various industrial applications (Padulosi et al., 2011; Traugber et al., 2021).

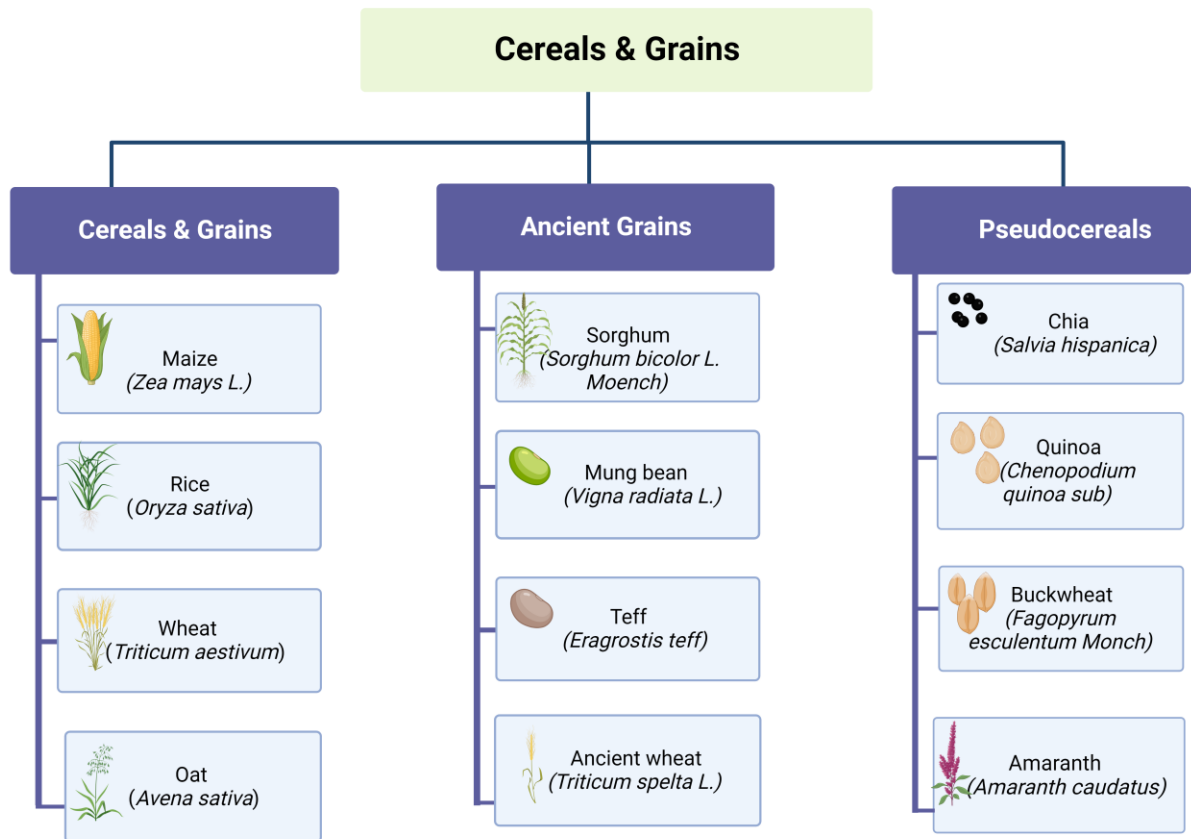


Figure 1-1. Schematic classification of cereals and grains

Packaging materials, especially, biopolymers directly extracted from renewable sources like cereal and grains can be classified based on their source and composition, and have gained attention in recent years due to their flexibility, gas, and moisture barrier abilities (Razzaq et al., 2016). Polysaccharide and protein fractions of cereals and grains are isolated and used for various food packaging applications (Bergo et al., 2006).

Packaging films from cereal and grain sources are manufactured using different methods. The solvent casting method is the most common one and is known as dispersion or wet process in laboratory conditions (Bull et al., 2011). The solvent casting method is the cheapest manufacturing method since specialized equipment is not necessary (Chen, 2008). In addition to this, it provides faster preparation and diverse applicability to various systems (Khambete et al., 2016). This method includes three main steps: i) dispersion of film ingredients into solvents, ii) casting of the solution, and iii) drying of a solution to allow evaporation of solvents. Sorghum starch films were manufactured with solvent casting method and can be used on bakery and confectionary food products (Kusumaningtyas et al., 2018; Mehboob et al., 2020). In addition to this, the extrusion method that is also known as the dry method can be used to manufacture sorghum starch-based packaging films at an industrial scale (Rodríguez-Castellanos et al., 2013). This process has three main steps: i) introduction of material into the zone; ii) remolding zone; and iii) final heating (Calderón-Castro et al., 2018). Extruder-based packaging films are generated with both thermal and mechanical energy during the extrusion process (Fitch-Vargas et al., 2019). The main advantages of the extrusion process are no use of solvent, lower energy consumption, short process time, and improvement of the physicochemical characteristics of film materials. Furthermore, the extrusion method provides easier handling of high-viscosity polymers, and higher efficiency to monitor mechanical characteristics of film materials and mixing (Liu et al., 2009). Nevertheless, this method is suitable for low moisture and temperature tolerant film materials and requires higher manufacturing cost than the casting method due to the use of specialized instruments (Suhag et al., 2020)

1.1.Polysaccharides from cereal and grains as packaging film materials

Polysaccharides obtained from cereals and grains have widely been used due to their abundance and easy handling, since they show permeability resistance to gases (e.g., carbon dioxide, oxygen), and accepted mechanical properties (Román et al., 2016). Nevertheless, because of their hydrophilic origin, they show poor barrier properties against moisture (Rhim et al., 2013).

Cellulose, hemicellulose, and lignin fibers are polysaccharide fractions that are used to prepare films from grain and cereals. (Pereira et al., 2017). However, recent studies focused on the conversion of starch into films. Starch has the advantages of being affordable, available, environmentally friendly, and convenient (Worzakowska, 2020). Furthermore, nearly 60% of grains and cereal kernels are made of starch, therefore representing a convenient and natural source (Thitisaksakul et al., 2012). Polysaccharide-based packaging films might display lower barrier and mechanical properties due to their hydrophilic features than synthetic films. The addition of lipids, surfactants, or other polysaccharides can significantly enhance these properties (Putri et al., 2018). Starch can be obtained and converted into packaging films as described for different ancient grain and pseudocereal commodities.

Maize starch

Starch which is primarily composed of amylose and amylopectin is an important component of maize (Xie et al., 2020). In general, maize starch includes 25-30% amylose while waxy maize starch changes from 0-10% (Bajaj et al., 2018). Maize starch can be used as a material for food packaging purposes. On the other hand, the mechanical and water vapor barrier properties of maize starch need to be improved using plasticizers or physical and chemical modifications (Xie et al., 2013). For example, Zhou et al. (2020) applied electron beam irradiation to increase the linear

chains of waxy maize starch and prepared films with enhanced mechanical properties and increased solubility. Similarly, maize starch recently modified by borax-cross linking and tensile strength and water resistance of the films were improved (Zhai et al., 2021).

Wheat starch

Wheat is composed primarily of starch, which accounts for 65% to 75% of the total dry weight of mature wheat seeds with both amylose (20%–30%) and amylopectin (70%–80%) fractions (Chen et al., 2016). Wheat starch offers a variety of benefits, including access to a wide range of sources, high renewal potential, biodegradable properties, and cost-effectiveness. Therefore, it is an important source for biodegradable packaging applications (Dai et al., 2019b). However, there are still disadvantages associated with the use of wheat starch-based packaging materials, such as inadequate mechanical properties and inadequate moisture resistance. Still, it can be used to make modified starch since it has active hydrogen groups on its surface. For this purpose, chemical modification methods such as esterification, etherification, cross-linking, and grafting can be applied (Zhu & Wang, 2013). For instance, Yousefi et al. (2019) produced novel modified wheat starch-based films by chemical crosslinking using sodium montmorillonite and titanium dioxide. They observed reduced water solubility and improved tensile strength of the films. Besides, plasticizers such as glycerol, fructose, and sorbitol were incorporated into wheat starch-based film formulation to improve the elongation (%) and water resistance (Mohammed et al., 2022).

Rice starch

Rice grain is made up mostly of starch, which makes up about 72-82% of its dry weight and about 90% of the milled rice grain (Toutounji et al., 2019). Rice starch has several advantages over other cereal starches such as wheat, barley, or rye, such as its consumer acceptance, small granules, white color and freeze-thaw and acid resistance as well as its broad spectrum of amylose and

amylopectin (Alves et al., 2007). Rice starch can be used as a raw material in the manufacture of biodegradable films due to excellent film-forming abilities with high amylose content. Similar to the other starch-based biodegradable films, the addition of plasticizers can make the film properties better. For example, rice starch films were reinforced with magnesium oxide nanoparticles and incorporated with glycerols to improve their mechanical properties (Ferreira et al., 2019). In addition to this, Erna et. al (2022) generated rice starch-based films incorporated with curcumin and they reported that the films desired physical and mechanical properties and could be potentially used as an active packaging material for meat products.

Mung bean starch

Mung bean with its high starch composition (50-60% (w/w)) has been widely used as a starch source in the last decade (Li et al., 2011). Various techniques such as ultrasound (Li et al., 2018), microwave or plasma (Shen et al., 2021) well as precipitation using distilled water with NaOH (Beta & Corke, 2001; Liang et al., 2021) can be used to extract starch from mung bean source. On the other hand, mung bean starch represents low solubility and physical stability for film applications (Zou et al., 2019). Therefore, physical or chemical modifications are required to be able to use mung bean starch as a packaging film material (Shen et al., 2021). Besides, the addition of plasticizers, polysaccharides, or other high molecular weight compounds or chemical substitution such as esterification may strength the functional properties of mung bean starch films (Kim et al., 2017). For instance, konjac glucomannan (KGM), a high molecular weight polysaccharide can be added to increase the thickness and smoothness of the films. Fang et al. (Fang et al., 2020) recently observed that mung bean starch and konjac glucomannan blend films showed improved texture, solubility, moisture barrier, and mechanical properties as compared to conventional mung bean starch films without the addition of KGM. After increasing the

functionality of mung bean starch films, they can be manufactured by solvent casting method (Fang et al., 2020).

Teff starch

Gluten-free teff is one of the recently famous starch packaging film sources (Venkatesa Prabhu et al., 2021). Teff starch can be obtained by centrifugation (Bultosaa et al., 2002) or dispersion in sodium metabisulphite-containing solution (Assefa et.al., 2020) after some physicochemical modifications such as pre-gelatinization and can be used as a packaging film material (Wannaphatchaiyong et al., 2017). To improve the film-forming abilities, plant extracts, organic acids or plasticizers can be added to the teff starch film solution and the films can be manufactured with the solvent casting method. For instance, *camu camu* extract that contains polyphenols and vitamin C provides both antioxidant activity and UV-light resistance to teff starch films (Ju & Song, 2019).

Quinoa starch

Quinoa contains a high amount of starch, antioxidants, and protein-free essential oils and both native and modified quinoa starch can be used in packaging films (Korkmaz et al., 2019). For example, gold nanoparticle-incorporated quinoa starch films were manufactured and the films showed promising tensile strength and moisture resistance (Pagno et al., 2015). Similarly, quinoa starch films incorporated with sage and lemon oil can be used effectively to prevent lipid oxidation of rainbow trout fish fillets and provide antioxidant characteristics (Alak et al., 2019). However, before film manufacturing, it might be required to apply steeping (using sodium hydroxide and sodium dodecyl sulfate (SDS)) and soaking processes to eliminate protein, fiber, or saponins (Araujo-Farro et al., 2010; Wright et al., 2002).

Buckwheat starch

Buckwheat starch possesses biodegradability, nutritional value, and various functional characteristics to be effectively used as a packaging material (Sindhu & Singh Khatkar, 2007). However, using buckwheat starch without modification might result in brittle films with low water vapor barrier properties (Zou et al., 2019). Therefore, plasticizers or pretreatments might be needed during film preparation. For example, high-pressure processing (300-600 MPa) was applied to buckwheat starch solution, and the resulting films displayed improved thermal resistance and reduced moisture solubility (Kim et al., 2018).

Amaranth starch

Amaranth is known as a promising packaging film material due to its lower amylose content and smaller granule size. Similar to other starch sources, chemical modifications such as hydroxypropylation can be applied to improve the textural and functional properties of amaranth starch-based packaging films (Pal et al., 2002). Amaranth starch exhibits better textural and functional properties as compared to other cereal-based starch sources due to its amylose composition and smaller granule size. Therefore, amaranth starch films can show better solubility, color, oil, and water-binding characteristics than other cereal-based packaging films (Chandla et al., 2017).

1.2. Proteins from cereal and grains as packaging film materials

Protein sources are important sources for packaging films. The mechanical and physical properties of protein-based films depend mainly on the amino acid composition, elongation degree, sequence of amino acids, and their interactions with neighboring molecules (Huber & Embuscado, 2009).

Proteins have multifunctional features and intermolecular binding characteristics due to their complex structure and composition; Their molecular structure and interactions with other molecules give them the ability to form viscoelastic film structures with better mechanical and gas-barrier properties than polysaccharide and lipid-based films due to covalent, hydrogen bonding and electrostatic interactions (Kashiri et al., 2016; Mojumdar et al., 2011; Patnode et al., 2022; Vieira et al., 2011). Sorghum, mung bean, teff, chia, quinoa, buckwheat, and amaranth are used as packaging film materials (Gao et al., 2006).

Mung bean protein

Mung bean seeds contain nearly 30% (w/w) of protein which can provide antifungal and antimicrobial characteristics in packaging films (Anjum et al., 2011). Mung bean protein can be extracted using ethanol and acid precipitation by HCl (Emmambux & Taylor, 2003; El-Adawy, 2000) and plasticizers are added before casting the film solution into Teflon, glass, or petri plates. Furthermore, *Echium amoenum* extract which is anthocyanin-rich plant extract was included to increase antioxidant and antimicrobial mung bean protein films (Moghadam et al., 2021).

Quinoa protein

Quinoa seeds constitute a considerable amount of globulins and albumin proteins and can be considered as the source of packaging films (Carrillo et al., 2016). Protein fraction can be separated through acid precipitation or physical treatments such as ultrasound that generates acoustic cavitations at high temperature and pressure (Belwal et al., 2018; Safar Razavi Zade et al., 2021). Similar to the other cereal and grain sources, the addition of plasticizers or other polymers can increase the bioavailability and functionality of quinoa protein. For example, quinoa protein based films incorporated with Hsian-tsao gum exhibited improved oxygen resistance and might be alternative packaging films for nuts or meat products (Zhao et al., 2022).

Buckwheat protein

Buckwheat contains 12-15% (w/w) protein. Differently, buckwheat protein is composed of mainly lysine which other cereal proteins lack. (Wu et al., 2021). Buckwheat protein can be extracted similarly to other cereal proteins, using defatting and alkaline precipitation methods, respectively (Wang et al., 2017; Yang et al., 2016). Then, the addition of plasticizers such as sorbitol is needed to improve the functional properties. Sorbitol decreases the interaction between biopolymer molecules and enhances the solubility of the film solution (Jouki et al., 2013; Zhang et al., 2016). Sorbitol-incorporated buckwheat films show enhanced solubility, tensile strength, and thermal stability and are used as sugar or egg coating (Wang et al., 2017). Similar to other protein-based films, buckwheat protein films are produced using solvent casting method (Wang et al., 2017).

Amaranth protein

Amaranth is an excellent source of protein since it contains all the essential amino acids (Das et al., 2021). Amaranth protein can be extracted using pH shifting that brings protein to an isoelectric point (Ventureira et al., 2012), ultrasound that uses cavitation and local heating, thus changing physicochemical characteristics of amaranth protein for packaging film applications (Martínez-Velasco et al., 2018). Similarly, the addition of plasticizers and surfactants such as Tween 80 could help amaranth protein-based films show better transparency and improved thermal properties (Aceituno-Medina et al., 2013).

1.3.Carbohydrate-protein complexes from cereal and grains as packaging film materials

Mucilages are known as hydrocolloids that contain monosaccharides, proteins, and some organic acids (Pejin et al., 2019). They can create sticky and gel-like structures when they disperse in water (Muñoz-tébar et al., 2022; Oliveira et al., 2022). Extraction of mucilages has some advantages over protein and polysaccharides: they can easily be obtained by simple methods with high extraction yields and they have better moisture and gas barrier properties than other biopolymers used in packaging films (Beikzadeh et al., 2020; Jouki et al., 2013). They can be extracted using different methods. Solvent treatment is the most common method to extract mucilage (Nazir et al., 2017). Since plant seed mucilages are hydrophilic, hot distilled water is used to extract mucilage with continuous stirring (Capitani et al., 2016; Dick et al., 2015). Then, filtration and precipitation are done, respectively (Fernandes & Salas-Mellado, 2017). Time, water-to-plant seed ratio, and temperature are important factors that may affect the extraction yield during solvent treatment (Nazir et al., 2017). Agitation and centrifugation are the simplest methods to extract mucilage from plant sources. Seeds are soaked in water and centrifugation can be applied to remove mucilage parts from the whole structure. Then, filtration using cheesecloth can be used to eliminate the tiny particles (de Campo et al., 2017). Nonthermal treatments such as ultrasonication or microwave approach can also be used to extract mucilage from the plant source. Ultrasound creates pores during low-frequency disruption and this enables the solvent to diffuse the pores effectively (Dehghan Sekachaei et al., 2017). Furthermore, the microwave method uses thermal energy to heat the solvents so that sample analytes can diffuse to the solvent (Tavares et al., 2018). This technique provides reduced extraction time and amount of solvent, and increased in extraction yield (Loretta et al., 2019).

Cereals and pseudocereals mucilages can be used for different industrial applications (Chiang & Lai, 2019). Among them, chia is known as the most common source of mucilage. Similar to other biopolymer-based films, the addition of some plasticizers may help to enhance the functional properties of mucilage films. Plant extracts such as *Rhus microphylla* extract, glycerol, gelatin, pectin, and glutaraldehyde can be used to increase thermal, and mechanical properties as well as to give antioxidant characteristics to mucilage-based (Charles-Rodriguez et al., 2020; Luo et al., 2019). When mucilage is extracted, the solvent-casting method is the only method to manufacture polysaccharide-protein-based packaging films (Hossain et al., 2018). Mucilage is poured into petri dishes for drying at certain times. The solvent-casting method is also applicable to protein-based packaging films (Silva et al., 2018).

Food packaging is essential to protect food during transportation and storage (de Koeijer et al., 2017). The use of biopolymers for packaging has gained popularity around the world due to increased environmental and sustainability concerns (Shrestha et al., 2018). The studies about cereal and grain-based packaging applications increased over the years. Nevertheless, more comprehensive research is necessary to continue exploring the worthy potential of cereals and grains in food packaging applications (Jardim et al., 2020; Rakshit et al., 2014).

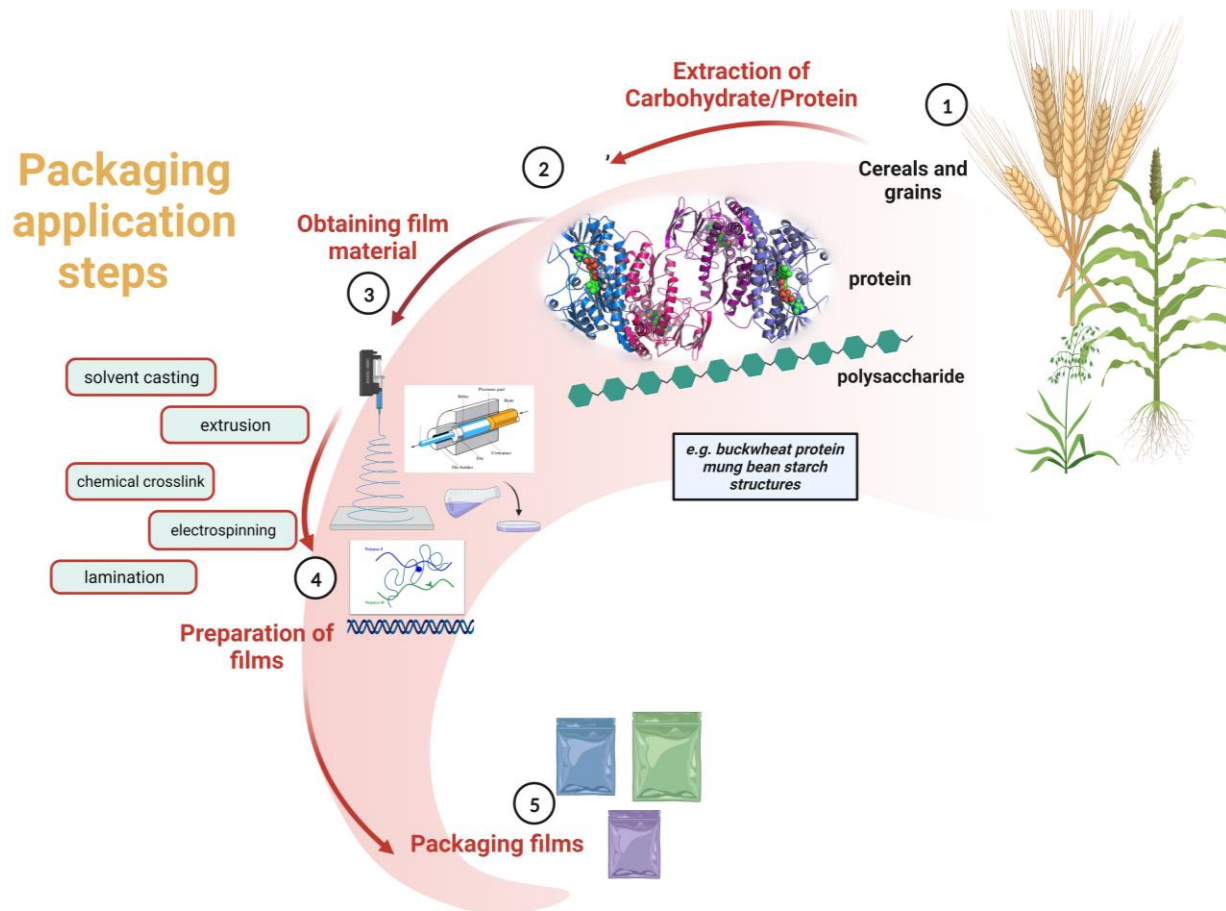


Figure 1-2 General steps and manufacturing methods of cereal and grain-based packaging films

1. Sorghum and Camelina sativa as emerging crops for value-added food applications

1.1. Sorghum

Sorghum, botanically known as *Sorghum bicolor* L., is a carbohydrate-rich crop of the Gramineae family and originated in Africa. It is the third most important cereal crop in the United States after wheat and maize and the fifth in the world, and the United States is the number-one producer and exporter of sorghum (de Moraes Cardoso et al., 2017).

Sorghum plays an important role in combating hunger and food insecurity and is essential for the food security of many of the world's poor people living in fragile agro-ecological areas. Sorghum is not just one of the most essential carbohydrate-rich crops, but also a staple crop for millions of people living in semi-arid climates. It can adapt to a wide range of conditions, particularly in areas with a lack of water. This versatility makes it a highly useful crop in areas with varying amounts of rainfall and high temperatures (Griebel et al., 2019). It has been observed that sorghum can grow in the tropic and subtropic regions which proved the possibility of cultivating it in a large tropical area. Therefore, it is not surprising to know it as the "King of Millets" in Africa, China, the United States, Mexico, and India (Khaton et al., 2016).

Sorghum is mainly produced as a main crop for animal feeding in countries such as the United States, Brazil, and Australia. There are numerous sorghum strains reported around the world, including grain sorghum, sweet sorghum, and forage sorghum used to make animal feed. It is categorized as brown, black, yellow, white, or red sorghum depending on the color of the pericarp and endosperm (Zhou et al., 2020). Nonetheless, its great nutritional and functional value enables numerous studies on its potential for human consumption (Jardim et al., 2020; Rakshit et al., 2014). It is a nutrient-rich food due to its high content of fiber, protein, fat, carbohydrates, vitamins, phenolic compounds, and minerals (Serna-Saldivar & Espinosa-Ramírez, 2018). In addition to this, sorghum is rich in micronutrients such as phosphorus (P), zinc (Zn), iron (Fe), calcium (Ca), magnesium (Mg), potassium (K), and sodium (Na) (Wu et al., 2018).

1.1.1. Sorghum processing by-product: dried distillers grains with solubles (DDGS)

Besides the use of animal feed and potential human food, sorghum is also used for ethanol production in the United States. According to the most recent statistics, nearly 7 million acres of sorghum are harvested and used mainly as animal feed in United States each year and approximately 25% of the country's sorghum crop is used for ethanol production (U.S Grain Council, 2022). This process produces sorghum-dried distillers grains with solubles (DDGS) (Harron et al., 2017). Sorghum DDGS is the dry fraction obtained after the fermentation of starch to generate ethanol. After this step, ethanol is separated by distillation, and the wet residual is centrifuged to fractionate solids from solubles. Finally, the solubles are concentrated via evaporation and dried with the solid fractions to obtain DDGS (Li et al., 2011). The protein content of sorghum DDGS is approximately 30–40%, making it a cost-effective source of protein (Muhiwa et al., 2017). In addition to its protein content, it contains other complex macromolecules such as carbohydrates, fiber, and oil. Despite its nutrition and affordability, sorghum DDGS is still mostly being turned out in feed, substrates for bio-refineries, or waste. The sorghum processing industry is striving to find alternative ways to minimize the volumes of this by-product (Adriana et al., 2020).

1.1.2. Food applications based on sorghum

The food applications using sorghum have become popular in the United States, Europe, and Australia due to the gluten-free characteristics of sorghum. Sorghum is accepted as a great raw material for wheat-free products due to its neutral flavor and variety of colors depending on the species and low allergenicity. Although sorghum is mainly used for animal feed and ethanol

production in the United States, there is an underrated potential for food applications including as a source of concentrated proteins for gluten-free food formulations.

Among people with celiac disease, sorghum cereal alternatives possess an increasing trend (Visarada & Aruna, 2018). The use of sorghum flour as a substitute for wheat flour in the celiac market is attractive due to its neutral taste and the use of white pericarp hybrids. White-grained lines of sorghum produce a flour resembling wheat flour in texture and do not give off an unusual color. At the beginning of this century, some researchers (Suhendro et al., 2000) produced noodles from decorticated sorghum flour which similar cooking quality such as water absorption and cooking loss as compared to commercial wheat-based pasta. On the other hand, the gluten-free bakery sector presents a particular challenge, and currently, a great deal of research is being conducted on product formulations using composite flours. Despite this challenge, a pregelatinized composite flour with whole sorghum grain which is very similar to wheat flour has been produced by Alves et al. (2021). Stefoska-Needham et al. (2016) produced biscuits from sorghum flour which had an impact on increased satiety resulting in low weight gain. In addition to this, it has been reported that some varieties of sorghum, which have a dense pericarp, can also be cooked and eaten raw, while the taste and smell are comparable to popcorn (Mishra et al., 2015).

Furthermore, sorghum polyphenols are accepted as important ingredients that can be utilized as natural food additives (Girard et al., 2018). It has been studied that pro-anthocyanidins which are condensed tannins of sorghum were more effective in enhancing the dough strength and overmixing as compared to oligomeric tannins from grapes with the help of interactions between gliadin and glutenin fractions (Girard et al., 2016). This showed that sorghum can potentially be used as a food additive for better nutritional and functional characteristics. Moreover, sorghum tannins which act as natural antioxidants successfully reduced lipid oxidation when they were

incorporated into beef burgers (do Prado et al., 2019). However, the acceptance of sorghum and sorghum-derived compounds as food additives depends on a variety of factors, including not only their nutritional properties but also their sensory and technological properties. Sorghum has a lower endosperm function in many food systems, making it less suitable for commercial food processing. It is known that the hydrophobic proteins of sorghum, kafirin, prevent sorghum starch from absorbing water, so longer cooking time is needed which results in a dry and sandy appearance as well as reduced shelf life. This undesired functionality significantly limits the food applications (Wong et al., 2009). The restrictions associated with the endosperm's functionality need to be addressed via genetic or process-driven innovations. Therefore, future research is necessary to develop methods at a commercial level to improve the compatibility of sorghum in food applications (de Mesa-Stonestreet et al., 2010).

1.1.3. Oleochemicals from sorghum

Oleochemicals are known as oils derived from plant or animal sources. Sorghum is also an important source of oleochemicals including fatty acids and triacylglycerols (Yang, 2007). In addition to protein, starch, and polyphenol content, sorghum contains approximately 3.4% lipids which consist primarily of unsaturated fatty acids, with polyunsaturated fatty acids being the most abundant (Xiong et al., 2019). Approximately 90% of total lipids are classified as triacylglycerols with linoleic acid (52%) being the predominant fatty acid (Zhang et al., 2019). Sorghum is also rich in oleic acid (32%) followed by palmitic acid (10%) stearic acid (4%) and linolenic (1%) acid (Baldwin & Sniegowski, 1951).

Sorghum oleochemicals with their high nutritional values can be promising sources for novel value-added applications. Oleosomes and oleoresins are classified as plant-based oleochemicals.

Oil in the form of emulsified droplets in eukaryotes is stabilized by the special protein-phospholipid membrane. These spherosomes can be found in sorghum with a diameter of a few microns and formed during the process of crop development (Tzen, 2012). The most important characteristic of oleosomes is that triacylglycerols as a core material are surrounded by this specialized membrane of the protein-phospholipid mixture. This natural coating protects oleosomes against mechanical and chemical stresses. Oleoresins, on the other hand, are concentrated extracts and do not contain phospholipids. They include fatty acids, terpenoids, phenolic compounds, and essential oils. In contrast to essential oils, they include non-volatile compounds which makes them more complex extracts (Sowbhagya, 2019). Oleosomes and oleoresins are capable of providing texture, aroma, nutritional value, color, and desired emulsifying ability making them interesting to the food industry (Procopio et al., 2022). The various applications may include pre-emulsified products, milk product alternatives, oleosome-based edible films, or natural emulsifiers (Abdullah et al., 2020). To date, plant-based oleochemicals have become popular due to several advantages such as high yield recovery, easy extraction, and safe use in “all natural” products. However, the extraction of complete oleochemicals from sorghum necessitates a comprehensive understanding of their position and interactions with the molecules surrounding them. The extraction of oleochemicals can be achieved through a variety of methods, some of which involve aqueous extraction (Zaaboul et al., 2018), screw pressing (Romero-Guzmán et al., 2020), or enzyme-assisted extraction (Liu et al., 2020).

Ionic liquids (ILs) are liquid molten salts composed of entirely organic cations and inorganic or organic anions, which have a glass transition or a melting point of less than 100°C. ILs can serve as a great alternative solvent with their distinctive properties such as high thermal stability, low

volatility, low vapor pressure, and most importantly favorable solubility in polar and non-polar chemicals (Ventura et al., 2017). ILs are identified as designer solvents that can be tailored to specific applications due to their large number of ionic combinations with cations and anions and the ability to be designed for specific applications. This enables the development of purification platforms that are more selective than the typical volatile organic solvent, thus overcoming the limitation of selectivity. Various bioactive compounds including sorghum oleochemical can be extracted using ILs (Morais et al., 2020; Ventura et al., 2017).

1.1.4. Packaging applications of sorghum

1.1.4.1. Sorghum starch as a packaging film material

Sorghum is primarily composed of starch, which accounts for approximately 68% to 75% of its dry matter content. The amount of starch varies depending on the cultivar, the region, and the climate (Singh et al., 2009). The sustainable starch fraction can be obtained by acid hydrolysis, succinylation (Mehboob et al., 2020), and precipitation method (Kusumaningtyas et al., 2018). After extraction, additional processes are mostly required to functionalize sorghum starch as a packaging material, such as chemical modification (García-Guzmán et al., 2022), extrusion (Hablott et al., 2013; Wu et al., 2021). Among these methods, chemical crosslinking is the most common method applied to disrupt the compositions and structure and enhance the functionality of starch molecules (García-Guzmán et al., 2022). It aims to interconnect the linear or branched starch chains and increase the hydrophobicity of granules and their hydration characteristics. Sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), epichlorohydrin (ECH), phosphorus chloride (POCl_3), and hydrochloric acid are generally used for this purpose (Dai et al., 2019a). Additional modifications such as chemical substitutions can also be performed to enhance

the functionality of starch compounds for packaging films (Kim et al., 2017). Esterification or etherification are the substitutions which include the incorporation of hydroxyl groups (Diop et al., 2011). Substitutions may improve the physicochemical features of starch for packaging film applications (Kim et al., 2017). For instance, in a recent study, acetylation was performed after acid hydrolysis of sorghum starch (Mehboob et al., 2020). These dual modifications on sorghum starch improved the water vapor barrier ability and mechanical properties of films (Mehboob et al., 2020). Similar to chemical substitutions, the addition of plasticizers, sugar alcohols, or organic acids can improve the physicochemical characteristics of sorghum starch films. For instance, the addition of glycerol and citric acid reduces the empty spaces in the film network and helps to reduce the water uptake of films. For example, a lower concentration of sorbitol (20% (w/w)) helped to decrease the moisture solubility and absorption of films. (Kusumaningtyas et al., 2018). In a recent paper, the researchers developed sorghum starch-based packaging films incorporated with sorghum bran extract. In addition to high tensile strength and elongation, the films exhibited antimicrobial activity and lipid oxidation ability which made them suitable for further active packaging applications (Tyagi et al., 2022).

1.1.4.2. Sorghum protein as a packaging film material

Kafirin, the prolamin storage protein of sorghum constituted 50–60% of the total protein (Emmambux et al., 2004). It is structurally similar to zein, but kafirin has a higher degree of hydrophobic activity and a lower degree of digestibility than zein. It is classified as a subunit of the α , β , and γ subunits based on its molecular weight, insolubility, and structural structure (Shull et al., 1991). Kafirin can be extracted using chemical treatments. Chemical treatments include extraction of protein using various solvents such as alkali, acids, or organic solvents (Bose

et al., 2019)., extraction of kafirin can be generally divided into three steps, namely, defatting, extraction, and of protein. N-pentane, n-hexane, and petroleum ether may be used for the first step. Then, non-ionic detergents, salts, and alcohols such as ethanol or denaturing agents can be preferred to extract protein sources. Lastly, ethanol, methanol, hydrochloric acids, or other chemicals are used to precipitate proteins (Lee et al., 2017). Chemical treatments allow the disruption of disulfide crosslinking (Hamada, 1997). Still, kafirin films require plasticizers or cross-linking agents to enhance their functionality since kafirin shows a more hydrophobic and less digestive nature than other plant proteins (Bean et al., 2011). For instance, polyethylene glycol and lactic acid mixture can be added to help the solubilization of prolamin proteins and enhance the tensile strength and oxygen barrier properties of kafirin films (Emmambux et al., 2004). Protein films are produced after isolation and modification processes. The solvent-casting method is also applicable to protein-based packaging films (Hossain et al., 2018). This method presents some limitations such as the amount of film generated, the shape of the film, and the possibility of having the solvent entrap into the protein (Fakhouri et al., 2013). In recent years, kafirin-based films have gained attention due to their functional properties. Dianda et al. (2019) successfully produced kafirin films that demonstrated desired mechanical properties such as high tensile strength and elongation and surface characteristics such as high hydrophobicity. Similar to this, some authors developed kafirin-based films after irradiation which improved thermal and mechanical features as well as water vapor resistance (Huang et al., 2020; Wang et al., 2018). Furthermore, Patil et al. (2020) incorporated plasticizers such as triethyl citrate and polyethylene glycol to improve the thermal, mechanical, and physical properties.

1.2. *Camelina sativa*

Camelina sativa (L.) Crantz also known as Camelina sativa, is an oil seed crop belonging to the family Brassicaceae and has gained so much attention in recent years. It is one of the most promising oil seed crops with many positive characteristics that make it stand out from other oil seed crops. For example, Camelina is versatile and adaptable to a wide range of environments except for organic and heavy clay soils, and this makes it an ideal crop on less productive lands with insufficient rainfall (Gugel & Falk, 2006). Moreover, it has a low water stress tolerance and is therefore less reliant on irrigation. Additionally, some genotypes of Camelina show resistance against *Brassicaceae* diseases such as brown girdling or sclerotinia stem rot (Séguin-Swartz et al., 2009).

Camelina has been used as an oil seed crop since ancient times and the shreds of evidence show that the cultivation of Camelina occurred in the early Iron and Bronze Ages (Bouby, 1998). Nonetheless, the use of Camelina as an oil seed crop decreased during the Middle Ages (Vollmann & Eynck, 2015). The initial field trials conducted in North America occurred in Red River Valley of Manitoba in 1863 and the observations indicated that Camelina was an oilseed plant that should be given serious consideration as a crop option in northern regions due to its earliness, abundant yield, and oil content (Galasso et al., 2015). The agronomic and ecological characteristics of Camelina and the improvements in genetic manipulation tools have increased the interest of scientists and farmers in this underdeveloped crop.

Camelina oil contains α -linolenic acid (18:3n-3) and linoleic acid (18:2n-6) fatty acids. Camelina oil differs from many other oils used for human and animal consumption in that it provides a dietary source of both essential fatty acids due to higher linolenic and lower linoleic acid ratio as

compared to canola, soybean, safflower, and corn oil. Furthermore, Camelina oil is also a rich source of monounsaturated fatty acids of eicosenoic acid (20:1n-9) and erucic acid (22:1n-9). In addition to fatty acid composition, Camelina oil contains tocopherol, flavonoids, and polar phenolic compounds; total tocopherol content reported between 700 and 800 mg/kg of seed, whereas total flavonoid and polar phenolic compounds specified as 439 and 143 mg/kg of seed, respectively (Abramovič et al., 2007) (Matthäus, 2002). Despite the high unsaturated fatty acid content, Camelina oil exhibits oxidative stability which is like other rich unsaturated fatty acid-containing oil seeds due to antioxidant compounds found in the oil. For example, a study showed that Camelina oil is more stable than fish and flax oil but less stable than sesame and corn oil (Eidhin et al., 2003). Camelina sativa oil has been recently used as a binder and may increase the aroma of essential oils and other botanical extracts. In addition to this, it can be used in cosmetics to revive the dullness in hair and promote healthy brightness (Ergönül & Özbek, 2020).

1.2.1. The alteration of lipid metabolism and triacylglycerol (TAG) biosynthesis

Apart from the agronomic benefits of Camelina, the recent advances in tools and resources have made it an excellent platform for the metabolic engineering of oil composition and subsequent development in the field. It is essential to note that Camelina can be transformed quickly and easily through the application of floral-dipping techniques, which are mediated by *Agrobacteria* (Liu et al., 2012). Transgenic seeds can be identified by a selection of markers, such as fluorescence and resistance to herbicides or antibiotics via these simple and quick techniques. Furthermore, the improvements in seed transcriptome and recent genome sequencing make it easy to translate existing and future work in *Arabidopsis* to Camelina (Nguyen et al., 2013).

Triacylglycerols (TAGs) are known as the primary seed storage lipid in the majority of plants including *Camelina* and are utilized to facilitate the provision of nutrients and carbon during seed germination. However, the synthesis of the metabolite TAGs in the seed of the higher plants is complicated and includes multiple metabolite pools within the endoplasmic reticulum (Bates et al., 2013). In short, fatty acids are formed in plastids by the type II fatty acid synthase complex, which is analogous to the type II complex found in prokaryotes. Then, a series of subsequent condensation, reduction, and dehydration reactions adds 2 carbon units to the fatty acid elongating chain, which is attached to an acid carrier protein (ACP). Fatty acids are synthesized to a length of 16 to 18 carbons, although shorter molecules are possible in some species. The formation of a double bond is also possible in the plastid, which is mediated by a Δ^9 -desaturase. The occurred fatty acid is removed from acyl-ACP by acyl-acyl-acyl-acetylthioesterases. Fatty acids are incorporated into the TAG through the Kennedy Pathway, which begins with the sequential catabolism of glycerol-3-phosphate by glycerol-3-phosphate acyltransferases and lysophosphatidic acid acyltransferases using acyl-CoAs, to form phosphatidic acid. After this step, phosphatases further dephosphorylate phosphatidic acid (PA), resulting in the formation of *de novo*-diacylglycerol (DAG). Then, TAG can be produced by diacylglycerol acyltransferases (DGAT) transferring the acyl group from acyl-CoAs to the sn-3 position of DAG (Liu et al., 2012).

1.2.2. Unusual triacylglycerols from *Camelina sativa*: 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs)

Engineering oil seed metabolism can successfully be applied to *Camelina* to produce acetyl-TAGs. The studies showed that the seeds of the burning bush plant (*Euonymus alatus*) are capable of synthesizing 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl TAGs) which are accepted as unusual TAGs

with an sn-3 acetate group (Bagby & Smith, 1967; Kleiman et al., 1967). In addition to this, alteration of DAG composition can generate different fatty acids at the sn-1/2 positions of acetyl-TAGs.

Acetyl-TAGs can incorporate unusual fatty acids, thus enabling the combinatorial approach to be employed to generate a larger number of molecules with desired characteristics and increased value. For example, high oleic acetyl-TAGs are produced using RNAi genes against the genes of CsFAD2 and CsFAE1 and it was transformed with EaDAcT which is known as diacylglycerol acetyltransferase enzyme (Liu et al., 2015).

Acetyl-triacylglycerols (Acetyl TAGs) have several physical characteristics that distinguish them from conventional seed oils, making them suitable for a wide range of uses. For instance, their kinematic viscosity is nearly 40% lower as compared to typical TAGs. This low viscosity enables them to be potentially used as straight vegetable oil (SVO) biofuel (Ryan et al., 1984). Acetyl-TAGs possess also improved cold temperature properties as compared to regular TAGs. This property also provides useful applications of acetyl-TAGs as transformer oils or biodegradable lubricants. In addition to this, these improved properties may facilitate novel food applications including emulsifiers, food coatings, and plasticizers (Liu et al., 2015).

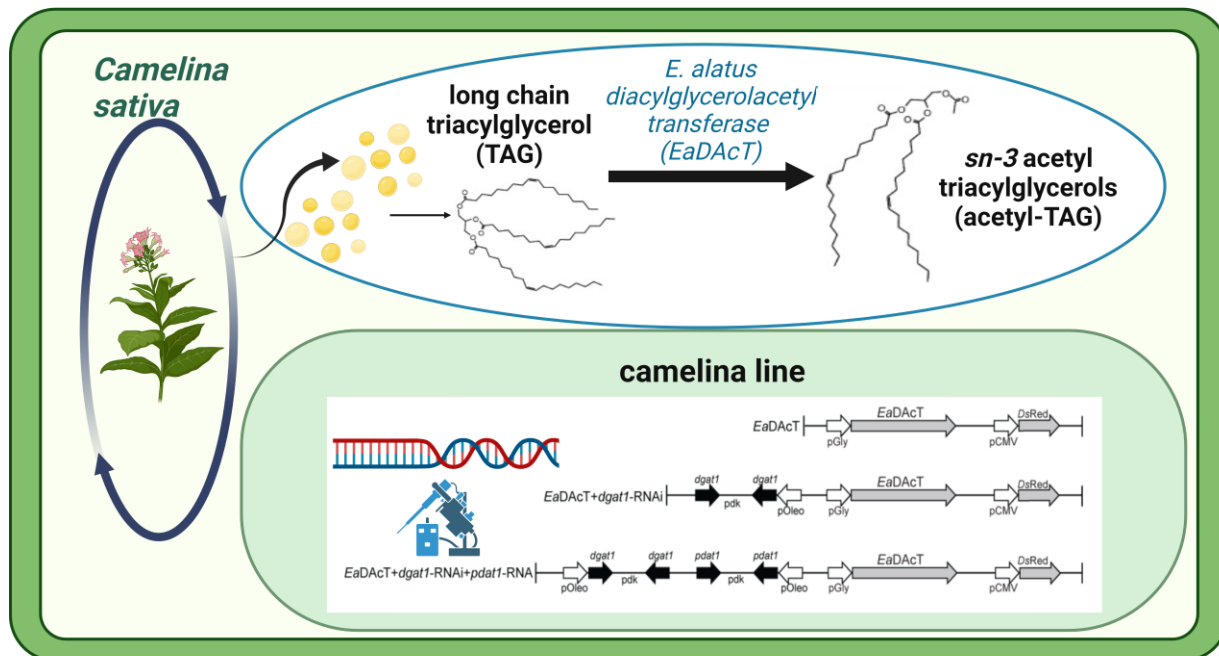


Figure 1-3 Schematic summary of engineered transformation of triacylglycerol (TAG) into acetyl- triacylglycerol (acetyl-TAG)

Conclusion

Food packaging is essential to protect food during transportation, handling, and storage. However, environmental considerations drove researchers to develop packaging materials that are sustainable, biocompatible, and renewable. Plant-based sources meet the renewable packaging film needs with their affordability and diversity. Cereal and grain-based biopolymers can be great alternatives as film raw materials due to their flexibility, gas, and moisture barrier abilities.

Nevertheless, more research is necessary to continue exploring the worthy potential of ancient grains and pseudo-cereal applications in food packaging. Sorghum as an emerging crop possesses high nutritional and functional value making numerous studies possible for potential food applications. For example, sorghum flour was used as a substitute for wheat flour in the celiac market, and protein or starch fractions were successfully used as a raw material for packaging film applications. In addition, sorghum DDGS, produced during ethanol processing has a huge potential as an alternative recycling material with its high protein content. Similar to sorghum, *Camelina sativa* as an emerging oilseed crop can be utilized in various applications. For example, 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl TAGs) which is the engineered oil from *Camelina sativa* is suitable for a wide range of uses due to its improved thermo-physical properties. Besides, it can be facilitated in novel food applications including emulsifiers, food coatings, and plasticizers. The studies highlighted in this review demonstrate the potential of these emerging crops to be applied in different food applications. Still, more research about extraction, application techniques, or technologies is required for further applications.

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Chapter 2 - Knowledge and objectives

Emerging commodities such as sorghum (*Sorghum bicolor* L. Moench) and Camelina sativa (*Camelina sativa* (L.)) and by processing products such as distillers' dried grains with solubles (DDGS) from ethanol fermentation have the potential to be used in packaging films and various food applications. The goal of this dissertation is to show the value-added food applications from sorghum, sorghum processing by-products (distiller's dried grains with solubles), and acetyltriacylglycerols (TAGs) derived from Camelina sativa.

Biodegradable packaging films have gained popularity in recent years due to environmental and sustainability concerns. Distiller's dried grains with solubles (DDGS) are byproducts of grain fermentation in ethanol plants and distilleries. Due to its high protein and fiber content, sorghum DDGS could serve as an alternative renewable source for biodegradable packaging film materials instead of petroleum-based synthetic materials. I hypothesize that renewable sorghum DDGS films would successfully be manufactured as an alternative packaging film using preconditioning with dry and wet milling with suitable solubilization techniques. The objectives of this study were to develop biodegradable packaging materials from sorghum DDGS pretreated with dry and wet milling following a solvent casting approach and evaluate their physical, mechanical, and thermal properties. The film properties would be supported by measuring color, thickness, moisture solubility, oxygen permeability, tensile strength and % elongation, melting temperature, and enthalpy of melting.

Distiller's dried grains with solubles (DDGS), a byproduct of sorghum fermentation can be used as a renewable packaging film source. On the other hand, biopolymers present a particular challenge in terms of moisture resistance and mechanical strength. I hypothesize that high oleic

acetyl-TAG from engineered form emerging Camelina seeds can be used as a novel and high-yield plasticizer. The objective of this study was to improve the functional properties of DDGS films by the presence of the *sn*-3 acetyl group of acetyl TAG, obtained from transgenic Camelina sativa, and preconditioning via wet milling and suitable solubilization. The improved film properties would be supported by moisture solubility, oxygen permeability, tensile strength, % elongation, and surface hydrophobicity.

Protein foam is a two-phase system in which air cells are distributed as elastic pockets in the continuous water phase. Protein foams are the most common type of material used to provide the desired structure to a wide variety of products, including meringues, mousse, whipped creams, ice creams, and marshmallows. However, the physicochemical properties of the material and the large surface area play a role in the problems of film drainage and film coalescence, which may ultimately result in rupture. Therefore, novel surface-active ingredients are required to sustain the stability of the foams. Emerging oil seed crop Camelina sativa has recently been engineered and acetyl-TAG with a high oleic acid was synthesized. The high-oleic fatty acid chain can provide a strong attachment to the foam surface to increase viscosity. Therefore, I hypothesize that the unusual acetyl-TAG molecules obtained from transgenic Camelina sativa seeds with high oleic content can be used to increase the stability and capacity of protein foams. The objective of this study was to understand the effect of whey protein isolate, sucrose, and acetyl-TAG concentrations on foam overrun, stability, and surface rheology (e.g., complex dilatational and elastic modulus) using a Box-Wilson experimental design and response surface methodology (RSM).

Whippable emulsions are an oil-in-water emulsion that can be transformed into a triple-stage process with aeration, where the air bubbles incorporated into the emulsion are encapsulated in a network of lipid granules. They are important structures for whipping cream, toppings, or other

products made from milk fat to give them the texture they need. Whippable creams and emulsions, on the other hand, need to be stabilized with small molecules called surfactants. I hypothesize that wild-type acetyl-TAG can effectively assist as an effective surfactant in oil-water emulsions stabilized by whey protein hydrolysate and improve the whipping characteristics of aerated emulsion systems. The objective of this study was to increase oil in water emulsion stability (particle size, zeta potential, creaming index, absorbed protein concentration) and improve the whipped emulsion characteristics (overrun and drainage) after whipping via wild-type acetyl-TAG obtained from emerging *Camelina* seeds.

Oleoresins are resin-like viscous materials obtained from plants, oilseeds, or spices with functional properties. They constitute terpenoids, fatty acids, phospholipids, essential oils, proteins, and phenolic compounds offering great potential for value-added food applications. However, extraction methods play an important role in the stability, composition, yield, and structural characteristics of oleoresins. I hypothesize that oleoresins from emerging sorghum crops which are rich in polyphenols with strong antioxidant potential can efficiently be extracted and used for value-added food applications. I also hypothesize that the composition and structural properties of the oleoresins can be modulated by using novel solvents of ionic liquids with composite anions and cations.

Chapter 3 - Development and characterization of biodegradable sorghum DDGS films (*submitted to LWT*)

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Abstract

Biodegradable packaging films have gained popularity in recent years due to environmental and sustainability concerns. Distiller's dried grains with solubles (DDGS) are byproducts of grain fermentation in ethanol plants and distilleries. Regarding its high protein and fiber content, sorghum DDGS could serve as an alternative renewable source for biodegradable packaging film materials instead of petroleum-based synthetic materials. The objectives of this chapter were to develop biodegradable packaging film materials using sorghum DDGS and characterize their physicochemical properties (color, thickness, moisture solubility, oxygen permeability, mechanical, thermal, structural properties, and biodegradability). The films were prepared following a solvent casting approach by using water and chloroform (1:0, 1:1, 1:2, 0:1 (v/v) water: chloroform ratio) and ethanol. A wet milling pretreatment using a colloid mill was proposed to modulate film properties by facilitating the dispersibility of the fibers forming the film backbone. Chloroform significantly ($P < 0.05$) reduced moisture solubility. Thermal properties and oxygen barrier ability of the films significantly ($P < 0.05$) improved by the addition of ethanol, whereas ethanol caused a significant reduction ($P < 0.05$) in tensile strength. Moisture resistance and

thermal properties improved ($P < 0.05$) with dry milling, whereas elongation at the break increased ($P < 0.05$) using wet milling.

Introduction

Biodegradable packaging films derived from biological and renewable resources have gained popularity worldwide due to growing environmental and sustainability issues due to the use of petroleum-based synthetic materials. Petroleum-based packaging films have tunable properties, but significant environmental drawbacks. One of the well-known problems is related to the increasing concentrations of microplastics generated from petroleum-based materials and is expected to continue increasing over the coming decades. Microplastic defilement in various ecosystems such as marine and freshwater environments as well as soils is accepted as the major environmental plastic pollutant to causes serious health problems for humans and animals (Arp et al., 2021; Barboza et al., 2018). Thus, biodegradable packaging materials, which are known to naturally decompose into water, carbon dioxide, methane, or biomass within three to six months, are becoming increasingly popular as a solution to these issues.

The natural biopolymers obtained from living plants, animals, and microorganisms, such as proteins, starch, cellulose, and their combinations, often in their purified form, were commonly used to develop biodegradable packaging materials. Protein-based films are generally used as coatings and edible films. Cellulose- and starch-based films were commonly used to develop biodegradable primary packaging materials. There are significant barriers, however, preventing the widespread adoption of biodiverse and biodegradable film technologies in the food industry related to their poor mechanical properties, and low resistance to water vapor and gas permeability (Hosseini et al., 2021).

Among others, biopolymers produced from grains offer great potential as a packaging basis due to their abundance and cheap cost, their versatility, and their potential for acceptable barrier capacity against oxygen, moisture, or light (Perera et al., 2023). Furthermore, renewable sources can further support sustainable agricultural practices. Renewable resources are materials obtained from processing waste streams and replenished, such as the production of packaging film materials. Distillers' dried grains with solubles (DDGS) are by-products of grain fermentation during ethanol production. During the fermentation process sugars and starch are converted to ethanol increasing the protein and fiber content. For example, the protein content of maize DDGS is between 29.5 to 36.08 % and significantly higher than raw maize grains (9.07 to 13.25%) (Semenčenko et al., 2014). Similarly, the protein content of sorghum DDGS is approximately 30–40%, making it a cost-effective source of protein (Muhiwa et al., 2017). DDGS is mainly used as animal feed due to its high protein content and nutritional value. The high protein and fiber content (e.g., cellulose, hemicellulose, lignin, etc.) of DDGS can also offer great potential as a renewable source for manufacturing biodegradable packaging film materials. Sorghum (*Sorghum bicolor* L. Moench) is the third commonly grown cereal crop in the United States and the fifth in the world with a production volume of 4.77 million metric tons in the United States (USDA Foreign Agriculture Service, 2023). It is recognized as a sustainable crop related to its high drought tolerance and quicker adaptability to the various environment, therefore there is a growing interest in expanding the utilization of sorghum and its byproducts in support of sustainable agricultural practices. Therefore, the DDGS obtained from sorghum processing ethanol plants can be used to manufacture biodiverse packaging materials.

The DDGS comes from the ethanol plant as a coarse mass in a heavily aggregated form. Thus, before solubilizing and dispersing its components a particle size reduction is needed. Dry milling

is a conventional approach to reduce and deagglomerate DDGS. In addition, wet milling pretreatment can be used to effectively break down insoluble components as a homogenous mass and linearize the fibrils to increase their dispersibility and solubility. This approach was used as a pretreatment to modify fiber structure and dispersibility, composite fiber assemblies (Feng et al., 2022). Furthermore, wet milling was applied to improve the solubility and extractability of proteins as well (Alpiger & Corredig, 2023).

The identification of film technologies that integrate the potential of protein and polysaccharide sources can potentially lead to improved biodiverse films as alternatives to synthetic materials. The wet milling pretreatment is expected to modulate the physicochemical properties of film materials obtained from sorghum DDGS. The objectives of this chapter were to develop biodegradable packaging materials from sorghum DDGS pretreated with dry and wet milling following a solvent casting approach and evaluate their physical (color, thickness, moisture solubility, oxygen permeability), mechanical (tensile strength and % elongation) and thermal (melting temperature and enthalpy of melting) properties. The solvents used for film preparation were selected based on their miscibility with water.

Materials & Methods

Materials

The DDGS was kindly donated by PureField Ingredients at Russell, KS. Chloroform ($\geq 99.8\%$) and glycerol (ACS reagent, $\geq 99.5\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (99.5%, ACS reagent) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Xanthan gum and locust bean gum were supplied by TCI America (Portland, OR, USA) and CP Kelco (Skensved, Denmark), respectively.

DDGS pretreatment

Before film preparation, the sorghum DDGS was ground with a dry milling approach or pretreated with a wet milling approach. Dry milling was done using a cyclone sample mill (Udy Corporation, Model 3010-018, Fort Collins, CO, USA), where the DDGS sample (50 g) was placed into the grinding chamber and rotated at a speed of 10000 rpm with the belt drive version for 15 minutes. Finely ground DDGS was moved out of the grinding chamber through a screen mesh (100 μ m) into a sample collection bottle. The grinding continued until the desired amount of DDGS (250 g) sample was obtained.

The wet milling was done using a colloid mill (MKO Cone Mill, Model 2000/4, IKA Process Technology, Wilmington, NC, USA). The sample was mixed with water at a ratio of (1:3 w/v) selected based on our preliminary experiments to obtain a viscous mass that can pass through the colloid mill at a rate of 150 mL/min. The rotor was operated at 10000 rpm with 4 cycles of 5 minutes with a gradually decreased rotor gap to yield particle size (between 68.28 to 71.68 nm) (Trappey et al., 2015). The suspension was collected in a glass beaker (500 mL) and kept at 4°C until further use.

Film preparation

The films were prepared following the solvent-casting technique described in (Tonyali et al., 2020) with modifications. The pretreated sorghum DDGS (~10 g) was solubilized in solvents (20 mL) made of water and chloroform (1:0, 1:1, 1:2, 0:1 water: chloroform) with and without ethanol (50%). The mixture was stirred on a magnetic stirrer for 30 minutes at 25 °C until a homogeneous mixture was observed. Glycerol (0.1 g) was added to the solution as a plasticizer, and xanthan (0.01 g) and locust bean gum (0.01 g) were added as stabilizers. The final solution was further stirred for 20 minutes at 25 °C, then poured onto high borosilicate glass Petri dishes (diameter of

90 mm) and dried under a fume hood for 48 hours at 25 °C. The films were stored in Zipper-top poly bags (101 mm x 152 mm) at 25 °C until further use. Each film was characterized for physicochemical properties within two weeks of preparation.

Physical Characterization of the Films

Color measurement

Color measurements were performed by using a Mini Scan Hunter LAB colorimeter (MiniScan, Model EZ 4500 L, Reston, VA, USA). The instrument was calibrated with the white standard calibration plate, then a film sample (70 mm in diameter) was placed on it for the color reading over the LAB color scale: L = 0 (black) to L = 100 (white); -a (greenness) to +a (redness); and -b (blueness) to +b (yellowness) with 0 as a neutral. Four measurements were taken from each film sample (Hunter, 1978). The color (ΔE) was calculated by using the following equation. ΔE was calculated based on the Equation 1:

$$\Delta E = \sqrt{(\Delta a)^2 + (\Delta b)^2 + (\Delta L)^2} \quad (1)$$

Thickness of the films

The thickness of DDGS films was measured using Vernier digital micro caliper (Gyros Digi-Science Accumatic Digital Caliper, Melnick Drive Monsey, NY, USA). An average of five measurements at different locations were used in data analysis.

Moisture solubility

The moisture solubility of the films was analyzed according to the method of Jangchud & Chinnan (1999) with minor modifications. A piece of dry film (20 mm x 20 mm) with a known initial mass (100 mg) was immersed into a beaker filled with distilled water (20 mL), and sealed. After 24 hours at 25°C, the solution was poured over a Whatman® cellulose-based filter paper (Grade 40,

Sigma Aldrich, Inc., Model Z240079, St. Louis, MO, USA). The remaining film parts on the filter paper were recovered by rinsing with distilled water (10 mL) and dried in disposable aluminum plates with the inner base and inner top diameters of 46 mm and 62 mm, respectively (Fisher Scientific, Waltham, MA, USA) for 24 h at room temperature. Finally, a dry mass of films was recorded. Measurements were performed quadruplicate. The percent solubility (S%) of films was calculated using Equation 2, where m_0 is the initial dry mass after first drying and m_1 is the final dry mass after second drying.

$$\%S = \frac{m_0 - m_1}{m_0} \times 100\% \quad (2)$$

Oxygen permeability

Oxygen permeability was measured using a custom-designed system. A small and a big chamber (depth: 8 cm, diameter: 30 mm and depth: 10 cm, diameter: 50 mm, respectively) were placed at the bottom and top respectively (Fig. 3-1). The film samples (diameter: 70 mm) were placed between two chambers sealed with an O-ring closure at the connection. An oxygen meter (CY-12C Pro O₂ Oxygen Concentration Content Tester Oxygen Detector Monitor Meter, China) was installed in the upper chamber, which was hermetically sealed. Pure oxygen gas was supplied from the inlet valve of the bottom chamber at point A (Fig 1) at a flow rate of 10 mL/min and passed through the chamber to exit at point B to keep a constant oxygen concentration of 100% in the bottom chamber. The change in the oxygen concentration was recorded at 15-minute intervals. Measurement was continued until a constant oxygen concentration of 70%. Assuming Fickian diffusion, the diffusion coefficient was calculated using the Equation 3 where J is the diffusion flux, D is the diffusion coefficient and l is the dimension which is length. Then the permeance of the films (P) was calculated using Equation 4 where D is the diffusion coefficient and p is the

partial pressure of oxygen. Finally, the oxygen permeability coefficient (PO_2) is calculated using Equation 5.

$$J = D \times \frac{\partial C}{\partial l} \quad (3)$$

$$P = \frac{D}{p} \quad (4)$$

$$PO_2 = P \times l \quad (5)$$

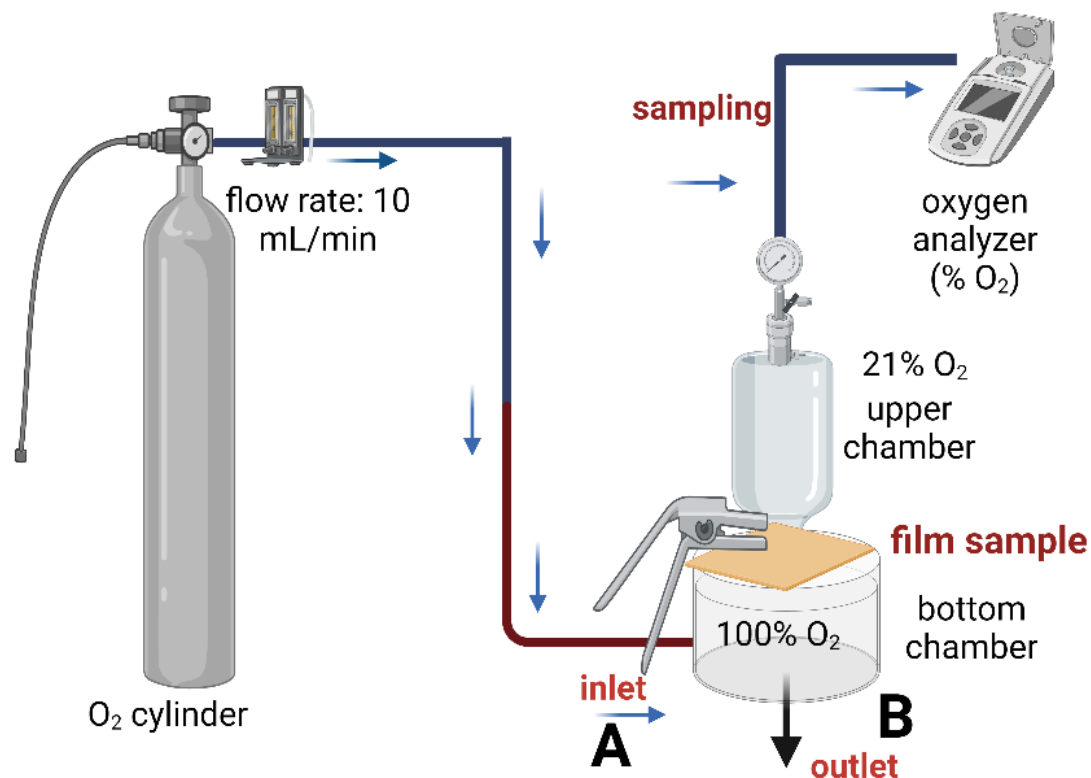


Figure 3-1 Schematic representation of the setup used for oxygen permeability analysis

Biodegradability of films

The biodegradability of films was analyzed following the method described by (Shaikh et al., 2021). The film piece (40 x 40 mm) was weighed and placed in a glass beaker (150 mL) containing

soil at a depth of 10 cm from the top at 25°C. The film degradation was tracked at regular time intervals (2 weeks) for a total of 8 weeks by removing the film samples from the soil without any soil adhering to the surface. The percentage weight loss of the samples for each week was recorded as a quantitative measure of degradation. The weight of the sample was estimated using Equation 6 where W_o and W_1 are the initial weight and residual weight after degradation at each time.

$$\text{Weight loss (\%)} = \frac{W_o - W_1}{W_o} \times 100 \quad (6)$$

Mechanical characterization of films

The mechanical properties of the films were determined using a texture analyzer (TA-XT2 Texture Analyzer, Stable Micro Systems, Haslemere, England) following the standard test method of D882-9 suggested by the manufacturer (ASTM, 2018). Force calibration was done by using a standard weight (500 g). The initial distance of the gauge was set to 15 mm. The speed of the crosshead was adjusted to 5 mm/min. The film samples were cut into rectangular pieces (10 x 50 mm). The samples were clamped between two tensile grips. The films were pulled up and stretching was produced at a given crosshead speed. Deformation (mm) and the maximum force (N) were recorded. Tensile strength was calculated by dividing the maximum force (F) by the initial cross-sectional area (A) of the films using Equation 7. The percentage elongation at the break (E%) was also measured using Equation 8 where d_{before} and d_{after} were the distances between grips holding the film sample before and after the break, respectively.

$$\text{Tensile strength (MPa)} = \frac{F \text{ (N)}}{A \text{ (mm}^2\text{)}} \quad (7)$$

$$E \text{ (\%)} = \frac{d_{\text{after}} - d_{\text{before}}}{d_{\text{before}}} \times 100 \quad (8)$$

Thermal characterization of films

Thermal stabilities of the films were characterized by using a differential scanning calorimeter (Shimadzu DSC-60, Lenexa, KS, USA). A small piece of film (ca. 10 mg) weighed into a hermitically sealed aluminum pan with a diameter of 6 mm and a height of 1.5 mm (Shimadzu Aluminum Crimp Pan, Model 201-52943-00, Shimadzu, Columbia, MD, USA). An empty aluminum pan was used as the reference. The sample and reference pans were heated from 25°C to 180°C at a heating rate of 5°C/min controlled by nitrogen gas (0.4 L/min). Thermal denaturation temperatures (T_m) and enthalpies (ΔH) were calculated from DSC curves using the instrument's integrated software.

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR analysis was performed at the Center for Grain and Animal Health Research (USDA-ARS, Manhattan, KS, USA). Attenuated total reflectance (ATR)-FTIR spectra collected at 400–4000 cm^{-1} range with a resolution of 4 cm^{-1} , data spacing of 0.482 cm^{-1} , and 32 scans using a Thermo Nicolet iS50 FTIR (Thermo Fisher Scientific Co., Waltham, MA) spectrometer equipped with a single-bounce diamond crystal and a deuterated triglycine sulfate detector. Experiments were repeated triplicate for each film sample at ambient temperature. The quantitative analysis was done by “peak fitting” procedure and individual component bands according to a Gaussian curve fit (GRAMS/AI™ Spectroscopy Software Suite, Thermo Scientific™, Waltham, MA, USA).

Statistical analysis

Experiments were performed in quadruplicate, and the values were expressed as mean $\pm$ standard deviation. The statistical analysis was done by using Minitab (version 16.2.4.4, LLC, State

College, PA, USA). The one-way ANOVA and post-ANOVA Tukey's multiple comparison tests were used to analyze differences among samples at significance level $\alpha = 0.05$.

Results and Discussion

Physical characterization of films

The color and appearance of the films play an important role since they directly affect consumer satisfaction and acceptance of the end use of packaging films. The appearances of the films are shown in Fig 3-2. They were translucent with a color resembling that of sorghum DDGS due to the presence of natural pigments and polyphenols. As expected, the films in this study were darker in color and showed opacity as compared to colorless and transparent isolated protein or starch films such as soy protein or corn starch-based films (Liu et al., 2021). The color measurements of the films over the LAB scale are shown in Table 1. The films prepared without any milling pretreatment were not successful with brittle and non-uniform structures, therefore they were not considered further in our study.

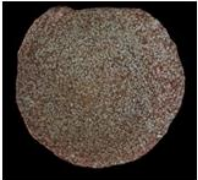
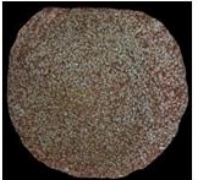
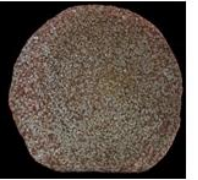
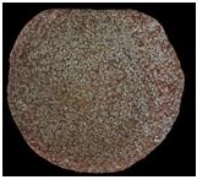
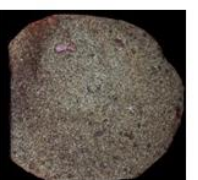
Water:chloroform ratio (v/v)		1:0	1:1	1:2	1:2 + etOH
Pretreatment	Udy mill DDGS				
	Colloid mill DDGS				

Figure 3-2 Images of DDGS films

The solvent ratio had a significant ($P < 0.05$) effect on the overall color (ΔE) of the films. The films produced using only water were coarse, with a non-homogeneous particle distribution and a dark appearance (max $\Delta E = 29.65 \pm 0.06$). The physical properties of these films were inferior as compared to the films prepared with organic solvent mixtures due to the limited solubility of the insoluble polymers and fibers preventing the formation of a successful polymer network. The addition of chloroform matrix helped with solubilization to obtain films with improved characteristics. The color of the films became significantly ($P < 0.05$) lighter with the addition of chloroform. The lowest ΔE was observed when the films were solubilized using a 1:1 (v/v) water: chloroform ratio after Udy mill pretreatment. On the other hand, the addition of ethanol significantly ($P < 0.05$) increased ΔE for both dry and wet mill pretreated films.

For the films solubilized using water and chloroform, the color of the films pretreated with dry and wet milling were significantly ($P < 0.05$) different, where ΔE was higher ($\sim 10\%$) with wet milling

as compared to dry milling. This was observed since the colloid mill provided ultrafine crushing of DDGS via shear and friction force as well as high-frequency vibration that may release the natural pigments solubilized in solvent and resulted in higher ΔE similar to the study by Mihaela Jipa et. al (2012).

The solvent ratio and pretreatment type significantly ($P < 0.05$) affected the thickness of the films (Table 1). Overall, the combined use of water and chloroform decreased ($P < 0.05$) the thickness of the films (min: $325.01 \pm 5.03 \mu\text{m}$) as compared to ($490.02 \pm 10.07 \mu\text{m}$) the ones solubilized only with water.

In parallel, the films prepared with the addition of ethanol had a thickness lower ($P < 0.05$) than water and higher ($P < 0.05$) than water-chloroform mixture. Similarly, Vostrejs et al. (2020) showed that chloroform helped to solubilize the film ingredients such as polyhydroxyalkanoates (PHAs) and lignin from grape seeds and reduced the overall film thickness. Similarly, Wen et al. (2021) studied that polyvinyl alcohol effectively solubilized carboxymethyl chitosan by a cross-linked network, and the thickness of chitosan films was significantly reduced. The combined effects of these two phenomena were further discussed for gas permeability, tensile strength, and elasticity of the films. The results were probably related to the formation of a denser film matrix with a higher extent of crosslinking and limited availability of water. The type of milling pretreatment also affected ($P < 0.05$) the film thickness (Table 3-1). At the highest chloroform content (1:2 water/chloroform) the dry milling pretreatment resulted in a thinner film ($325.01 \pm 5.03 \mu\text{m}$) as compared to colloid milling ($335.04 \pm 10.01 \mu\text{m}$). The thinner film structure indicates a denser polymer network due to the evaporation of solvent.

Table 3-1 Thickness and color properties of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between the types of processing conditions (nontreated, Udy and Colloid milling). The roman letter shows the differences between the solvent ratio for each processing condition

Processing Type	Thickness (μm)				Color difference (ΔE)			
	Water-chloroform ratio (v/v)				Water-chloroform ratio (v/v)			
	1:0	1:1	1:2	1:2 + etOH	1:0	1:1	1:2	1:2 + etOH
Udy mill DDGS	490.02 $\pm$ 10.07 ^{a,i}	340.04 $\pm$ 10.02 ^{b,III}	325.01 $\pm$ 5.03 ^{b,IV}	370.02 $\pm$ 10.07 ^{b,II}	26.92 $\pm$ 0.07 ^{b,i}	19.63 $\pm$ 1.05 ^{b,III}	20.14 $\pm$ 1.08 ^{b,III}	24.95 $\pm$ 2.07 ^{a,II}
Colloid mill DDGS	385.01 $\pm$ 15.02 ^{b,i}	348.02 $\pm$ 5.09 ^{b,II}	335.04 $\pm$ 10.01 ^{a,III}	378.12 $\pm$ 10.02 ^{a,i}	29.65 $\pm$ 0.06 ^{a,i}	21.59 $\pm$ 1.07 ^{a,III}	20.82 $\pm$ 1.09 ^{a,III}	22.98 $\pm$ 2.08 ^{b,II}

Finally, similar to other physical properties, the moisture solubility and oxygen permeability of the films were affected ($P < 0.05$) by the types of solvent and pretreatment (Table 3-2). Moisture and oxygen barrier capability are important for packaging films to preserve short shelf life goods and eliminate food spoilage (Samantaray et al., 2022). The water solubility of the films indicates the water resistance of the films. Moisture solubility was the lowest with 1:2 (v/v) water: chloroform ratio, surpassing the highest values obtained with only water as the solvent by ca. 31% and 33% for dry and wet milling, respectively. This was not surprising, since the addition of chloroform and ethanol resulted in a more effective dissolution of film ingredients and a more homogenous film matrix. The denser film structure was also observed as thinner films, which increased the barrier properties of the films. Zhong et al. (2019) used chloroform and dichloromethane for improved gas permeability of polylactic acid-based films. Moreover, the water solubility of the films produced from sorghum DDGS in our study was lower than the bio-composite films based on buckwheat DDGS (i.e., ranging from 53.91% to 60.08%) developed by Wang et. al (2017). The higher moisture resistance of our sorghum DDGS films can be attributed to the relatively higher hydrophobic nature of kafirin, the dominant protein fraction in DDGS, as compared to proteins found in soybean, corn, or buckwheat DDGS.

A parafilm and a plastic stretch film were used as commercial reference materials and their oxygen diffusion coefficient values were calculated as $0.05 \pm 0.01 \times 10^{-11}$ and $0.08 \pm 0.01 \times 10^{-12}$ mol /m² s Pa, respectively. The oxygen permeability values of DDGS films were significantly ($P < 0.05$) higher (between $0.35- 0.43 \times 10^{-12}$ mol /m² s Pa) than parafilm and plastic stretch films. This is as expected due to the nature of biopolymers confirming with the existing literature (Yang et al., 2016); however, DDGS films in this study showed improved oxygen barrier properties as compared to other studies where corn fiber residual, arabinoxylan-based composite film showed

higher oxygen permeability (Salvada et al., 2022). Surprisingly, the increasing chloroform ratio and addition of ethanol slightly but significantly increased ($P < 0.05$) the oxygen permeability. The addition of organic solvents might enhance free volume and mobility in the film network, which is directly proportional to the oxygen permeation. A similar pattern was also reported by Byun et al. (2011) for the effect of solvent mixture (chloroform, methylene chloride, and acetonitrile) on the oxygen permeability of polylactic acid films.

Table 3-2 Moisture solubility and oxygen diffusivity of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between the types of processing conditions (nontreated, Udy and Colloid milling). The roman letter shows the differences between the solvent ratio for each processing condition

Processing Type	Moisture Solubility (%S)				O ₂ Permeability Coefficient (PO ₂) ($\times 10^{-12}$ mol /m ² s Pa)			
	Water-chloroform ratio (v/v)				Water-chloroform ratio (v/v)			
	1:0	1:1	1:2	1:2 + etOH	1:0	1:1	1:2	1:2 + etOH
Udy mill DDGS	30.88 $\pm$ 0.03 ^{a,I}	23.74 $\pm$ 0.01 ^{a,III}	21.19 $\pm$ 0.03 ^{a,IV}	24.12 $\pm$ 0.02 ^{a,II}	0.35 $\pm$ 0.07 ^{a,III}	0.40 $\pm$ 0.05 ^{a,II}	0.42 $\pm$ 0.08 ^{a,I}	0.39 $\pm$ 0.07 ^{a,II}
Colloid mill DDGS	32.79 $\pm$ 0.02 ^{a,I}	26.92 $\pm$ 0.02 ^{a,IV}	22.34 $\pm$ 0.03 ^{a,III}	27.49 $\pm$ 0.04 ^{a,II}	0.39 $\pm$ 0.6 ^{a,II}	0.43 $\pm$ 0.07 ^{a,I}	0.42 $\pm$ 0.09 ^{a,I}	0.38 $\pm$ 0.08 ^{a,III}

Mechanical characterization of films

The mechanical properties of the films were characterized by their tensile strength at the break (σ) and elongation (%) based on their tensile stress-strain curves. The solvent ratio and pretreatment type had a significant ($P < 0.05$) effect on the mechanical properties of the DDGS films (Table 3-3). Overall, the addition of chloroform doubled ($P < 0.05$) the tensile strength of the films as compared to the use of water alone. The higher tensile strength was expected as chloroform helped to solubilize the film ingredients and made a homogeneous and strong film network that showed more resistance to deformation. Thus, the 1:2 (v/v) water-chloroform solvent combination resulted in the strongest films. On the other hand, the addition of ethanol reduced ($P < 0.05$) the tensile strength by 69% and 48% for dry and wet milling, respectively. The addition of ethanol might increase mobility and decrease intermolecular forces, thus decreasing the toughness or tensile strength (Turhan & Şahbaz, 2004).

The elongation at the break (%) was also significantly ($P < 0.05$) affected by solvent type (Table 3). Contrary to the effect observed for tensile strength, the addition of chloroform significantly ($P < 0.05$) decreased the elongation, and the films prepared with 1:2 water: chloroform (v/v) ratio had the lowest elongation at break. Following the same trend, the addition of ethanol increased ($P < 0.05$) the elongation to its highest value. This could be explained by the ability of ethanol to conformational effect on the film-forming polymers to result in a plasticizing effect. The observation was consistent with a recent study where the authors highlighted a similar mechanical effect of ethanol addition during the preparation of porcine gelatin-based films (Peng et al., 2022). The tensile strength was also significantly ($P < 0.05$) affected by the processing type and there was a significant difference ($P < 0.05$) between wet and dry milling: as in the study where the authors observed similar results of dry Wiley mill on keratin films (Poole & Church, 2015). In

contrast, there was only a significant ($P < 0.05$) difference between wet and dry milling on elongation at break (%) for the films solubilized using a 1:2 (v/v) water: chloroform ratio and the elongation at break (%) was higher ($P < 0.05$) for the films pretreated via wet milling as compared to dry milling using the same water: chloroform ratio. This might be expected since wet milling pretreatment improved the dissolution of DDGS in the film matrix more effectively so that continuous and extensible films can be achieved.

Table 3-3 Mechanical properties (tensile strength and % elongation) of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between the types of processing conditions (nontreated, Udy and Colloid milling). The roman letter shows the differences between solvent ratio for each processing condition

DDGS Processing	Tensile Strength (σ) (MPa)				% Elongation			
	Water-chloroform ratio (v/v)				Water-chloroform ratio (v/v)			
	1:0	1:1	1:2	1:2 + etOH	1:0	1:1	1:2	1:2 + etOH
Udy mill DDGS	8.21 $\pm$ 0.37 ^{a,IV}	16.74 $\pm$ 0.01 ^{a,II}	19.19 $\pm$ 0.03 ^{a,I}	6.03 $\pm$ 0.02 ^{b,III}	27.88 $\pm$ 0.03 ^{a,II}	20.74 $\pm$ 0.01 ^{a,III}	17.16 $\pm$ 0.03 ^{b,IV}	29.28 $\pm$ 0.02 ^{a,I}
Colloid mill DDGS	7.91 $\pm$ 0.38 ^{b,IV}	13.92 $\pm$ 0.02 ^{b,II}	16.40 $\pm$ 0.03 ^{b,I}	8.49 $\pm$ 0.04 ^{a,III}	26.89 $\pm$ 0.02 ^{a,I}	21.92 $\pm$ 0.02 ^{a,II}	19.74 $\pm$ 0.03 ^{a,III}	26.49 $\pm$ 0.04 ^{b,I}

Thermal characterization of films

The DSC thermograms represented a common endothermic peak for all the films (Figure 3-3). This peak indicated the thermal decomposition of homogeneous films, thus the resistance of the films up to 180°C. The solvent ratio and pretreatment type had a significant ($P < 0.05$) effect on the thermal stability (Table 3-4). The addition of chloroform increased ($P < 0.05$) the melting temperature and energy for both milling treatments. This increase was related to the higher extent of cross-linking among polymers such as protein, fat, and fiber and increased film density. In specific, the interactions between kafirin chains which represent the most abundant protein in sorghum DDGS, and solvents (water, chloroform, ethanol) via Van der Waals, polar, ionic, charged, and hydrogen bonds play a role in increasing thermal resistance of the films (Ma et al., 2008). In addition to this, the non-polar interactions between fat, chloroform, and ethanol improved the thermal resistance of the films. In support of this hypothesis, the addition of ethanol increased ($P < 0.05$) the melting temperature and enthalpy of the films.

Table 3-4 Thermal properties (melting temperature & peak enthalpy) of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between the types of processing conditions (nontreated, Udy and Colloid milling). The roman letter shows the differences between the solvent ratio for each processing condition

DDGS Processing	Melting T (°C)				Peak Enthalpy (ΔH) (J $\times$ g ⁻¹)			
	Water-chloroform ratio (v/v)				Water-chloroform ratio (v/v)			
	1:0	1:1	1:2	1:2 + etOH	1:0	1:1	1:2	1:2 + etOH
Udy mill DDGS	129.41 $\pm$ 0.07 ^{a,III}	132.01 $\pm$ 1.03 ^{a,II}	131.84 $\pm$ 0.08 ^{a,II}	138.17 $\pm$ 1.04 ^{a,I}	90.32 $\pm$ 0.03 ^{a,IV}	96.48 $\pm$ 0.09 ^{a,III}	97.31 $\pm$ 0.05 ^{a,II}	109.31 $\pm$ 1.06 ^{a,I}
Colloid mill DDGS	126.58 $\pm$ 0.09 ^{b,III}	130.01 $\pm$ 1.03 ^{b,II}	130.66 $\pm$ 0.08 ^{a,II}	134.01 $\pm$ 1.01 ^{b,I}	86.51 $\pm$ 0.03 ^{b,III}	95.44 $\pm$ 0.05 ^{b,II}	95.77 $\pm$ 0.06 ^{b,II}	102.21 $\pm$ 1.05 ^{b,I}

There was a significant difference between wet and dry milling on melting temperature and enthalpy of melting (Table 3-4). Both thermal properties were higher ($P < 0.05$) for dry milling as compared to wet milling. Dry milling led to a stronger film network which was also consistent with tensile strength results. Therefore, higher energy was required to disrupt the films which was supported by Briones et al. (2020) in their recent study where they found that liquefied corn husk film pretreated using a commercial dry cutting mill showed increased ($P < 0.05$) melting temperature.

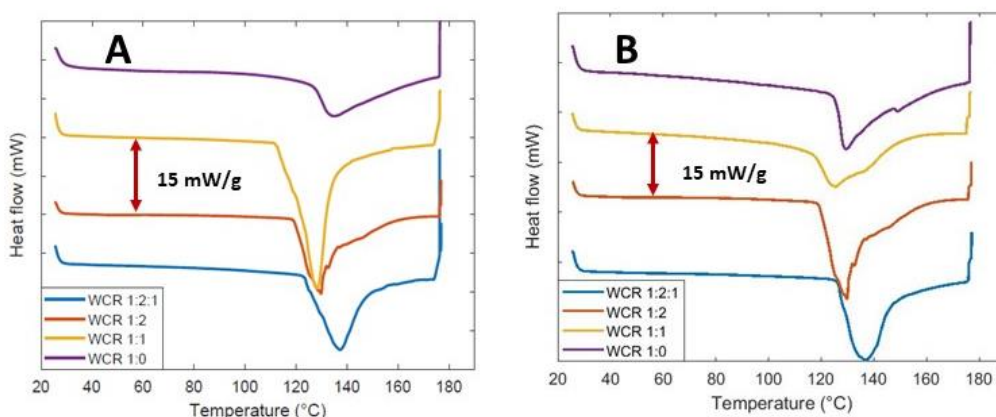


Figure 3-3 DSC curves (melting temperature & peak enthalpy) of sorghum DDGS films. The plots are based on processing types of A) Udy and B) Colloid milling

FT-IR analysis of films

The film architecture was characterized by using FT-IR spectroscopy. (Figure 3-4). The stretching band of C-O around $1050\text{--}1150\text{ cm}^{-1}$ was assigned to solvents and plasticizer interactions (Dianda et al., 2019). The weak amide I peak due to C=O and C=C stretching around 1500 and 1700 cm^{-1} was assigned to protein moieties. The typical absorption band in the range of $2800\text{--}2900\text{ cm}^{-1}$ was assigned to --CH_3 and --CH_2 alkyl aliphatic side chains of the amino acid R groups of the DDGS

proteins and the N-H stretching between 3050-3400 cm^{-1} in the DDGS protein (Byaruhanga et al., 2006). The films solubilized with only water showed lower intensity peaks of C-O and $-\text{CH}_3$ and $-\text{CH}_2$ stretching around 1050-1150 cm^{-1} and 2800-2900 cm^{-1} , respectively as compared to films prepared with the addition of chloroform and ethanol as solvents. This observation supported our previous hypothesis that the evaporation of organic solvents limited the availability of water and resulted in a denser film structure. Similarly, Dianda et al. (2019) observed similar effects on the FT-IR spectra for the addition of ethanol and t-butanol to the solvent mixture used for the preparation of the biodegradable films from kafirin, the most abundant protein in sorghum.

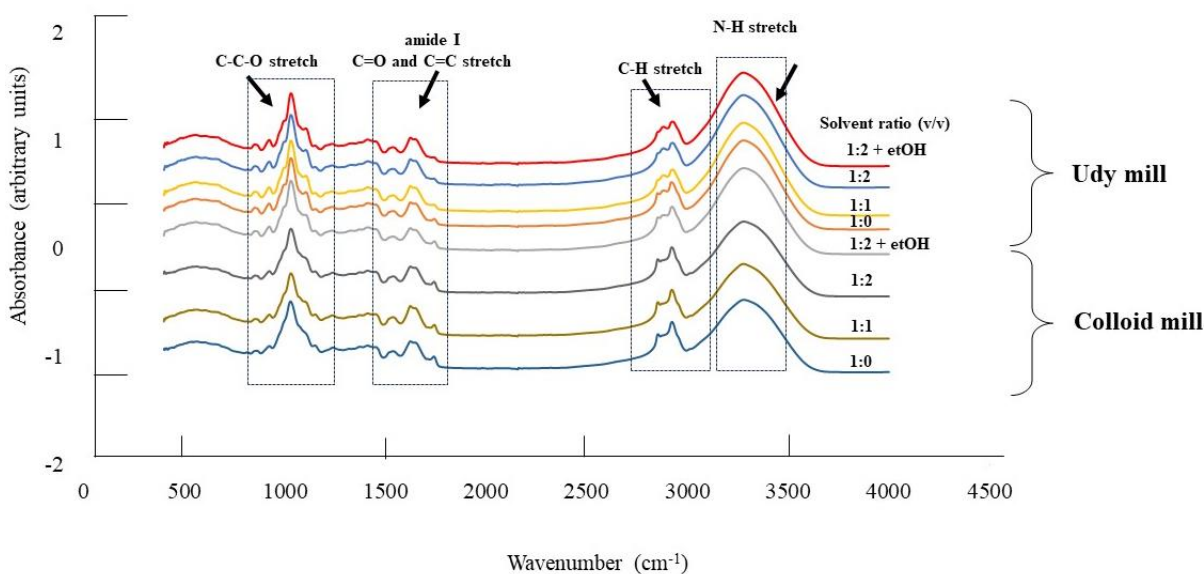


Figure 3-4 FT-IR spectra of DDGS films

In addition to this, there was a slight difference between wet and dry milling on the shape and intensity of some of the peaks. For example, the intensity of the peak due to $-\text{CH}_3$ and $-\text{CH}_2$ stretching around 2800-2900 cm^{-1} was relatively higher in wet milling as compared to dry milling

showing improved dissolution of film ingredients, especially DDGS, thus increased sharpness of the peak. This confirmed that wet milling pretreatment helped to dissolve the film ingredients and a similar effect of wet milling on amaranth-based films was reported by Elizondo et. al (2009). The FT-IR results were also consistent with physical and mechanical analysis.

Biodegradability

The biodegradability of the films was verified by using a soil degradation test, where the films were buried in potting soil. The DDGS film pretreated via Udy mill and solubilized using 1:2 (v/v) water: chloroform ratio was selected for the soil burial test based on the physical analysis results. The films started to degrade by day 15, and almost completely disappeared within 60 days. The supplementary figure representing the soil test is given in Figure S1. The percentage weight loss of the film was recorded as compared to the original dry weight (11.78 ± 0.03 g) related to the number of days of soil burial. An approximate weight loss of 39.5% (w/w), weight loss of 78.1% (w/w), and weight loss of 98.8% (w/w) were observed after day 15, day 30 and day 60. In addition to this, the surface of the film became slightly rough with tiny cavities at the end of day 15. The roughness of the surface increased with the burial period.

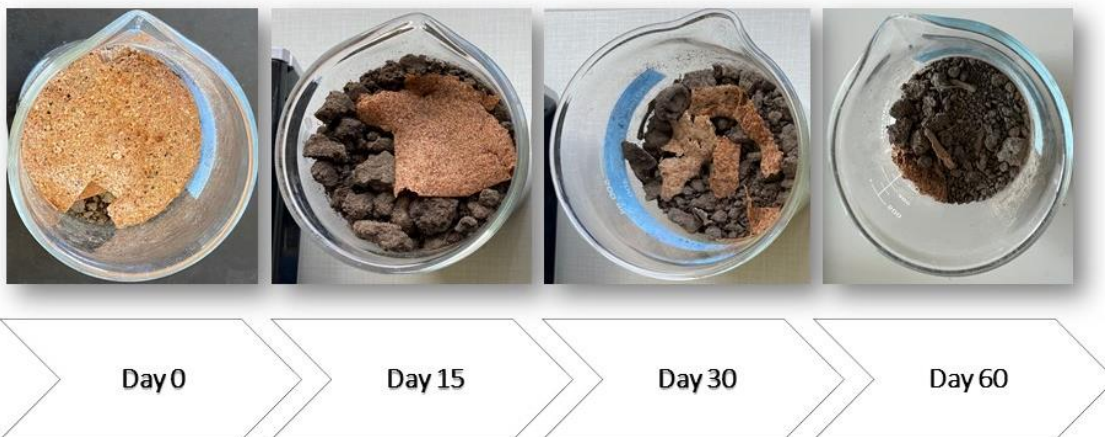


Figure 3-5 Soil degradation for the DDGS film pretreated with Udy mill using a 1:2 (v/v) water-chloroform ratio

Conclusion

The results in this chapter are original to show manufacturing renewable biodegradable film alternatives from sorghum DDGS preconditioned with dry and wet milling with suitable solubilization techniques. The physical, mechanical, and thermal characteristics of the films can be improved by adding chloroform to water as a co-solvent. For example, the addition of chloroform dissolved the film ingredients effectively and increased ($P < 0.05$) the barrier properties of the films: the moisture solubility was the lowest with 1:2 (v/v) water: chloroform ratio and Udy preconditioning, exceeding the highest values obtained with only water as the solvent by *ca.* 31%. Preconditioning via both dry and wet milling significantly improved the structural integrity to form a suitable film material. This was reflected in a significant increase in mechanical and thermal properties such as tensile strength and melting temperature.

Acknowledgements

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Chapter 4 - Effect of high oleic acetyl triacylglycerol (acetyl-TAG) on functional properties of biodegradable sorghum DDGS packaging film

Abstract

In recent years, there has been a surge in demand for packaging films that are biodegradable and are derived from a variety of biological sources, due to the growing awareness of environmental and sustainability issues. In our earlier chapter, we showed that a distiller's dried grains with solubles (DDGS), a byproduct of sorghum fermentation can be used as a renewable packaging film source. mechanical strength. The objective of this chapter was to improve the functional properties of DDGS films by the presence of the *sn*-3 acetyl group of acetyl TAG, obtained from transgenic *Camelina sativa*, and preconditioning via wet milling and suitable solubilization. The moisture solubility of the films pretreated via a ball mill and solubilized with a 1:1 (v/v) solvent ratio decreased by 9% when acetyl-TAG concentration increased from 0.1% to 0.2% (w/v). The elongation at the break increased by 24% when 0.2% (w/v) acetyl-TAG was added into the film prepared with the same conditions as compared to the film without acetyl-TAG. The addition of acetyl-TAG also reduced the wettability and improved the hydrophobic character of the films. Overall, acetyl-TAG with wet milling improved the film properties.

Introduction

Biopolymers in the development of packaging materials are considered promising due to the low risk of toxicity or the generation of harmful chemicals, which readily decompose into harmless end products such as biomass, water, carbon dioxide, and methane and integrate into the soil. Plant-based biopolymers such as polysaccharides, lipids, and proteins from grains and peels of

fruits and vegetables were generally used to develop renewable food packaging materials. Despite their benefits and advantages, natural biopolymer-based films show poor mechanical and moisture barrier properties as well as high moisture sensitivity (Liu et al., 2022).

The 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are unusually structured triacylglycerol (TAG) molecules that possess an *sn*-3 acetate group instead of a long chain fatty acid. This reorganized structure enables acetyl-TAG to possess different physical properties from regular TAG, including reduced viscosity and improved cold temperature properties (Bansal et al., 2018). Various plant species, including different members of the *Euonymus* genus, naturally accumulate high levels of acetyl-TAG in their seeds, but these plants are not good sources for the large-scale production of these unusual TAG molecules (Tran et al., 2017). Recently, the emerging oil seed crop *Camelina sativa* has been transgenically engineered to produce high levels of acetyl-TAG by introducing acetyl-transferase enzymes from different *Euonymus* species (Alkotami et al., 2021). Furthermore, camelina seeds that synthesize acetyl-TAG with a high oleic acid content have been developed by suppressing endogenous desaturase and elongase activities (Liu et al., 2015). Acetyl-TAG with improved functional properties facilitates the use of this modified oil in food applications as an emulsifier (Gaupp & Adams, 2015), foam stabilizer (Lundsgaard et al., 2009), or plasticizer in food packaging products (Coltro et al., 2014).

Bioresources obtained from waste biomass or by-products can be great sustainable materials for utilizing packaging films (Viel et al., 2019). Distillers' dried grains with solubles (DDGS) are a by-product of grain fermentation during ethanol production. DDGS is mainly used as animal feed with high protein and fiber content. However, it can also be utilized as a starting material for the development of value-added products such as packaging films. This intense agglomerated structure can be used after a linearized fibril network and increased solubility are achieved (Gao

et al., 2023). This can be possible with suitable pretreatment methods. Wet milling via a cone mill, colloid mill, or ball mill is one of the effective preconditioning methods that helps in the proper diffusion of the solvent into the target components to reduce the particle size and improve the physical properties of the final product (Long et al., 2023). For example, ball mill pretreatment was applied to corn DDGS fillers to reduce the particle size and improve the rheological and textural properties of biocomposites made of corn DDGS and polypropylene (Paleri et al., 2021). Earlier, we demonstrated that distiller's dried grains with solubles (DDGS), a byproduct of sorghum fermentation in distilleries and breweries, can be pretreated via wet or dry milling and used as a renewable source for packaging films. On the other hand, a general challenge with sorghum DDGS packaging film was resistance against moisture and mechanical strength. In this study, we hypothesize that these functional properties can be improved by the presence of the *sn*-3 acetyl group of TAG, obtained from transgenic *Camelina sativa* seeds as a novel and high-yield plasticizer.

Materials & Methods

Materials

The sorghum DDGS was kindly donated by PureField Ingredients, an ethanol at Russell, KS. Chloroform ($\geq 99.8\%$) and glycerol (ACS reagent, $\geq 99.5\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (99.5%, ACS reagent) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Xanthan gum and locust bean gum were supplied by TCI America (Portland, OR, USA) and CP Kelco (Skensved, Denmark), respectively. High oleic acetyl-TAG was kindly donated by the Department of Biochemistry, Kansas State University.

DDGS pretreatment

Before film preparation, the DDGS was ground with two different wet milling methods. The first wet milling was done using a colloid mill (MKO Cone Mill, Model 2000/4, IKA Process Technology, Wilmington, NC, USA): the DDGS was mixed with water at a ratio of (1:3 w/v) selected based on our preliminary experiments to obtain a viscous mass that can pass through the colloids mill at a rate of 150 mL/min. The rotor was operated at 10000 rpm with 4 cycles of 5 minutes. The second wet milling was performed using the DDGS and the same amount of water by using a ball mill (Retsch, PM 100 type, Retsch GmbH, Germany) equipped with a milling bowl (250 mL) and ceramic balls (diameter:10 mm; quantity: 100 pieces) and operated at 500 rpm with 2 cycles of 10 min.

Film preparation

The films were prepared following the solvent-casting technique described in (Tonyali et al., 2020) with minor modifications. The pretreated sorghum DDGS (10 g) was solubilized in solvents (20 mL) made of water and chloroform (1:1, 1:2 (v/v) water: chloroform ratio). The mixture was stirred on a magnetic stirrer for 30 minutes at 25 °C until a homogeneous mixture was observed. Glycerol (0.1 g) was added to the solution as a plasticizer, and xanthan (0.01 g) and locust bean gum (0.01 g) were added as stabilizers. Then, the high oleic acetyl-TAG was added to the film mixture as a plasticizer (0, 0.1, 0.2% w/v). The final solution was further stirred for 30 minutes at 25 °C, then poured onto high borosilicate glass Petri dishes (diameter of 90 mm) and dried under a fume hood for 48 hours at 25 °C. The films were stored in Zipper-top poly bags (101 mm x 152 mm) at 25 °C until further use.

Physical analysis of the film

Color measurement

Color measurements were performed by using a Mini Scan Hunter LAB colorimeter (MiniScan, Model EZ 4500 L, Reston, VA, USA). The instrument was calibrated with the white standard calibration plate, then a film sample (70 mm in diameter) was placed on it for the color reading over the LAB color scale: L = 0 (black) to L = 100 (white); -a (greenness) to +a (redness); and -b (blueness) to +b (yellowness) with 0 as a neutral. Four measurements were taken from each film sample (Hunter, 1978). The color (ΔE) was calculated by using the following equation. ΔE was calculated based on the Equation 1:

$$\Delta E = \sqrt{(\Delta a)^2 + (\Delta b)^2 + (\Delta L)^2} \quad (1)$$

Thickness of the films

The thickness of DDGS films was measured using Vernier digital micro caliper (Gyros Digi-Science Accumatic Digital Caliper, Melnick Drive Monsey, NY, USA). An average of five measurements at different locations were used in data analysis.

Moisture solubility

The moisture solubility of the films was analyzed according to the method of Jangchud & Chinnan (1999) with minor modifications. A piece of dry film (20 mm x 20 mm) with a known initial mass (100 mg) was immersed into a beaker filled with distilled water (20 mL), and sealed. After 24 hours at 25°C, the solution was poured over a Whatman® cellulose based filter paper (Grade 40, Sigma Aldrich, Inc., Model Z240079, St. Louis, MO, USA). The remaining film parts on the filter paper were recovered by rinsing with distilled water (10 mL) and dried in disposable aluminum

plates with the inner base and inner top diameters of 46 mm and 62 mm, respectively (Fisher Scientific, Waltham, MA, USA) for 24 h at room temperature. Finally, a dry mass of films was recorded. Measurements were performed quadruplicate. The percent solubility (S%) of films was calculated using Equation 2 where m_0 is the initial dry mass after first drying and m_1 is the final dry mass after second drying.

$$\%S = \frac{m_0 - m_1}{m_0} \times 100\% \quad (2)$$

Oxygen permeability

Oxygen permeability was measured using a custom-designed system. A small and a big chamber (depth: 8 cm, diameter: 30 mm and depth: 10 cm, diameter: 50 mm, respectively) were placed at the bottom and top respectively (Fig. 3-1). The film samples (diameter: 70 mm) were placed between two chambers sealed with an O-ring closure at the connection. An oxygen meter (CY-12C Pro O₂ Oxygen Concentration Content Tester Oxygen Detector Monitor Meter, China) was installed in the upper chamber, which was hermetically sealed. Pure oxygen gas was supplied from the inlet valve of the bottom chamber at point A (Fig 4.1) at a flow rate of 10 mL/min and passed through the chamber to exit at point B to keep a constant oxygen concentration of 100% in the bottom chamber. The change in the oxygen concentration was recorded at 15-minute intervals. Measurement was continued until a constant oxygen concentration of 70%. Assuming Fickian diffusion, the diffusion coefficient was calculated using Equation 3 where J is the diffusion flux, D is the diffusion coefficient and l is the dimension which is length. Then the permeance of the films (P) was calculated using Equation 4 where D is the diffusion coefficient and p is the partial pressure of oxygen. Finally, the oxygen permeability coefficient (PO_2) is calculated using Equation 5.

$$J = D \times \frac{\partial C}{\partial l} \quad (3)$$

$$P = \frac{D}{p} \quad (4)$$

$$PO_2 = P \times l \quad (5)$$

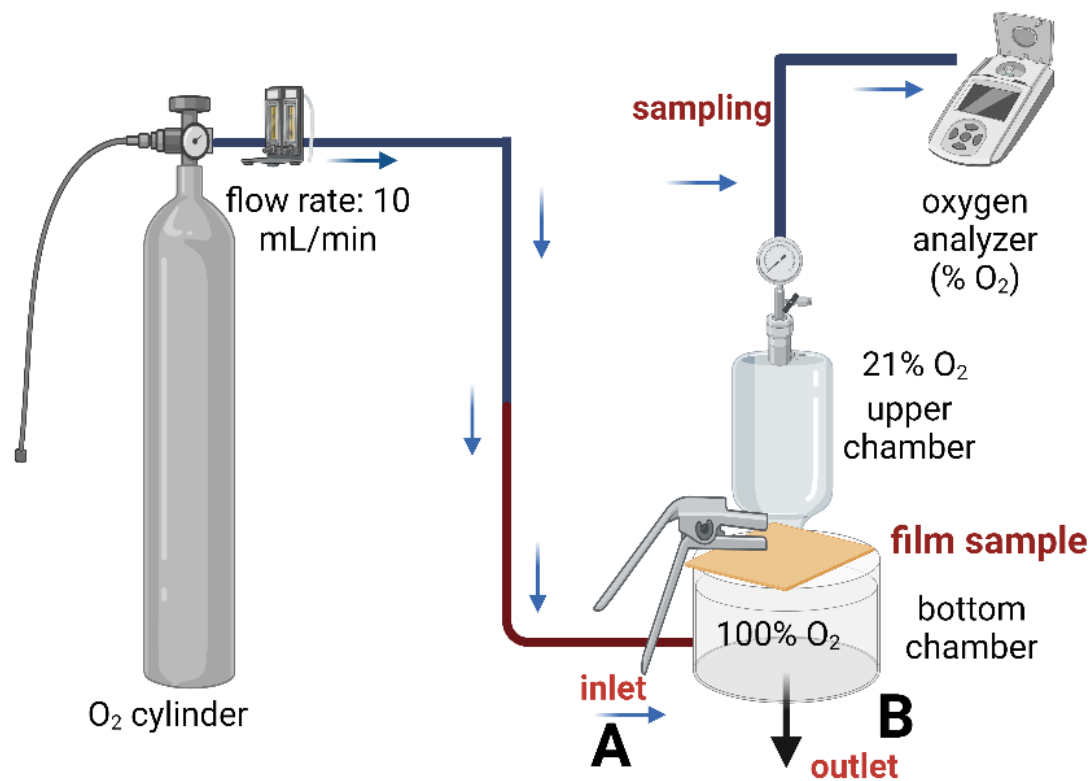


Figure 4-1 Schematic representation of the set up used for oxygen permeability analysis

Mechanical characterization of films

The mechanical properties of the films were determined using a texture analyzer (TA-XT2 Texture Analyzer, Stable Micro Systems, Haslemere, England) following the standard test method of D882-9 suggested by the manufacturer (ASTM, 2018). Force calibration was done by using a standard weight (500 g). The initial distance of the gauge was set to 15 mm. The speed of the crosshead was adjusted to 5 mm/min. The film samples were cut into rectangular pieces (10 x 50 mm). The samples were clamped between two tensile grips. The films were pulled up and stretching was produced at a given crosshead speed. Deformation (mm) and the maximum force (N) were recorded. Tensile strength was calculated by dividing the maximum force (F) by the initial cross-sectional area (A) of the films using Equation 7. The percentage elongation at the break (E%) was also measured using Equation 8 where d_{after} and d_{before} were the distances between grips holding the film sample after and before the break, respectively.

$$\text{Tensile strength (MPa)} = \frac{F \text{ (N)}}{A \text{ (mm}^2\text{)}} \quad (7)$$

$$E \text{ (\%)} = \frac{d_{after} - d_{before}}{d_{before}} \times 100 \quad (8)$$

Surface hydrophobicity

The hydrophobicity of DDGS films was evaluated using contact angle and interfacial tension measurements by using an optical drop tensiometer (Attension Theta, Biolin Scientific, Phoenix, Arizona, USA). The protocols were established using the instrument guidelines. Prior to analysis, the instrument is calibrated by camera adjustments to focus on a metal ball standard (4mm in diameter) at a zoom angle of *ca.* 143°. All sessile drop measurements were performed at room temperature. The contact angle and interfacial tension measurements were conducted with a sessile

droplet of (12 μ L) of deionized water using a needle (1 mL Luer tip, gastight Hamilton syringe) on DDGS films. Contact angle values were calculated using the Young-Laplace equation by OneAttension software (NanoScience Instruments, Phoenix, AZ, USA).

Statistical analysis

Experiments were performed in triplicate, and the values were expressed as mean $\pm$ standard deviation. The statistical analysis was done by using Minitab (version 16.2.4.4, LLC, State College, PA, USA). One-way ANOVA and Tukey's multiple comparison tests were used to analyze differences among samples at significance level $\alpha = 0.05$.

Results and Discussion

Physical characterization of films

In the previous chapter related to sorghum DDGS films, it has been observed that films solubilized with 1:1 and 1:2 (v/v) water: chloroform ratios have shown better physical and mechanical properties as compared to different solvent ratios. Therefore, they were considered to prepare the films and further analysis in this study.

Color is accepted as an important physical parameter since it directly influences appearance and has a strong impact on consumer satisfaction and acceptance of the end use of packaging film. The captured images of the DDGS films are presented in Fig 4-2.

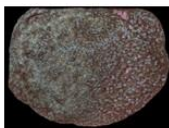
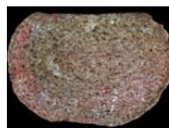
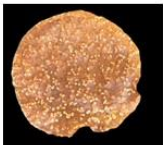
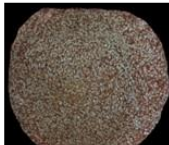
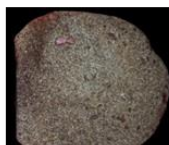
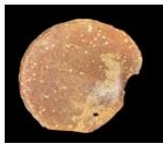
Water:chloroform ratio (v/v) Acetyl-TAG concentration (%w/v)	Ball mill		Colloid mill	
	1:1	1:2	1:1	1:2
No acetyl-TAG				
0.1% acetyl-TAG				
0.2% acetyl-TAG				

Figure 4-2 Images of DDGS films

The films were opaque with a color of yellow to brown which is due to the characteristics of natural pigments and polyphenols and DDGS or acetyl-TAG. The color values of the films as a function of the addition of acetyl-TAG, processing type, and solvent ratio are shown in Table 4-1. The films prepared without pretreatment were not successful with brittle and non-uniform structures, therefore were not further considered in our analysis.

Table 4-1 Thickness and color properties of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower and upper superscripts indicate significant difference ($P < 0.05$) for each column and row, respectively

Thickness						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	292.1 $\pm$ 0.8 ^{b,C}	289.1 $\pm$ 1.0 ^{b,C}	297.4 $\pm$ 0.9 ^{b,B}	295.7 $\pm$ 0.7 ^{b,B}	299.4 $\pm$ 1.0 ^{b,A}	298.2 $\pm$ 0.8 ^{b,A}
Colloid mill	348.1 $\pm$ 1.2 ^{a,C}	335.1 $\pm$ 1.2 ^{a,D}	359.3 $\pm$ 1.2 ^{a,B}	354.1 $\pm$ 1.1 ^{a,B}	365.3 $\pm$ 0.5 ^{a,A}	361.1 $\pm$ 0.6 ^{a,A}

Color difference (ΔE)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	17.26 $\pm$ 1.2 ^{b,A}	16.49 $\pm$ 1.3 ^{b,B}	16.39 $\pm$ 1.3 ^{b,B}	15.98 $\pm$ 1.4 ^{b,C}	15.92 $\pm$ 1.6 ^{b,C}	15.39 $\pm$ 1.1 ^{b,D}
Colloid mill	21.59 $\pm$ 1.4 ^{a,E}	20.82 $\pm$ 1.5 ^{a,E}	20.48 $\pm$ 1.5 ^{a,C}	20.22 $\pm$ 1.1 ^{a,D}	19.64 $\pm$ 1.3 ^{a,A}	19.37 $\pm$ 1.7 ^{a,B}

The incorporation of acetyl-TAG had a significant ($P<0.05$) effect on the overall color (ΔE) of the films. The color change of the films prepared with the addition of acetyl-TAG reduced ($P<0.05$) which might be related to the natural yellow color of acetyl-TAG extracted from Camelina seed. Furthermore, the films prepared with the addition of acetyl-TAG had smoother and more uniform surfaces as compared to the films prepared without acetyl-TAG. This showed homogeneous acetyl-TAG droplet distribution throughout the film matrix without aggregation during the drying step.

The processing type also significantly ($P<0.05$) affected the color of the films. The color difference with colloid milling was significantly ($P<0.05$) higher as compared to ball milling. The highest color was observed when DDGS was pretreated via a Colloid mill and solubilized in 1:1 (v/v) water: chloroform mixture without the addition of acetyl-TAG.

In addition to this, the color of the films was affected ($P<0.05$) by the solvent ratio: the values were significantly ($P<0.05$) lower when a 1:2 (v/v) solvent ratio was used for the solubilization. The effectiveness of the combined use of solvents to dissolve insoluble polymers and fibers was the reason for this significant change is similar to a recent study (Cardoso et al., 2022) where the authors developed oregano oil incorporated butylene adipate-co-terephthalate based biodegradable films using water and chloroform as solvents.

The addition of acetyl-TAG and processing type showed effects ($P<0.05$) on film thickness (Table 1). Overall, the incorporation of acetyl-TAG increased ($P<0.05$) the thickness of the films. Thicker films were expected due to the amount of additives incorporated. In addition to this, an increase in acetyl-TAG concentration was followed by an increase in the thickness of the films. Salvada et al. (2022) showed similar results with the addition of oleic acid into arabinoxylan-beeswax multilayer film (Salvada et al., 2022).

In parallel, the thickness was also significantly ($P < 0.05$) affected by the processing type as discussed in the following section (Table 4-1). There was a significant ($P < 0.05$) difference between the two different types of wet milling. The wet milling process with a ball mill resulted in a thinner film (min: $289.1 \pm 1.0 \mu\text{m}$) as compared to wet milling with a colloid mill (min: $335.1 \pm 1.2 \mu\text{m}$). Ball milling could be more effective due to multidirectional beatings with shear force, collision, and friction which resulted in the generation of high dynamic energies to reduce the particle size as compared to colloid mill (Kumar et al., 2020). This was supported by a recent study (Arun et al., 2022) where the authors showed that the thickness of the cellulose nanofiber films from coconut waste significantly ($P < 0.05$) reduced via ball mill pretreatment so that there was no significant difference between nanocomposite films pretreated with ball mill and polyvinyl alcohol polymer matrix as a control.

It was anticipated to find out that the addition of acetyl-TAG had a considerable ($P < 0.05$) impact on moisture solubility in tandem with the results for color and thickness. Moisture solubility results are given in Table 4-2. Moisture resistance quantified with solubility is one of the important features of biodegradable packaging films since it represents their efficiency in preserving foods with high water activity. The addition of acetyl-TAG significantly ($P < 0.05$) increased the moisture resistance of the films. This was foreseen since the addition of acetyl-TAG embedded within the film matrix and the functional acetate group interacted with DDGS polymers and other film ingredients, thus, reducing the voids for capturing water molecules. Similar to our study, Sani et al. (2018) reported that the incorporation of rosemary essential oil into cellulose nanofiber and whey protein isolate (WPI) based composite film caused a significant ($P < 0.05$) decrease ($38.33 \pm 1.65\%$) as compared to WPI film ($46.66 \pm 2.85\%$) (Alizadeh-Sani et al., 2018). Moreover, the increase in composition of acetyl-TAG resulted in a significant ($P < 0.05$) decrease in moisture

solubility: the solubility of the films decreased by increasing acetyl-TAG concentration from 0.1% to 0.2% (w/v) to about 9% for the films solubilized using 1:1 (v/v) water: chloroform ratio and pretreated via ball mill. The increase in hydrophobicity due to an increase in acetyl-TAG concentration was concurrent with decreased wettability which will be discussed in the following section. A recent study (Vishnu Priya et al., 2021) found similar results where the incorporation of 0.6% (v/v) *Plectrathus amboinicus* oil significantly increased the moisture resistance of the chitosan-based films as compared to 0.2% (v/v). This significant change was interpreted as reduced interaction between hydroxyl and free amine groups of chitosan and water molecules with the help of embedded oil droplets.

In addition to this, there was a significant ($P < 0.05$) difference between processing type on moisture solubility of the films. Colloid mill pretreatment with a combined water-chloroform mixture helped to soluble film ingredients through a narrow gap of the mill using intense shearing and friction forces. Nevertheless, the solubility of the films was lower (min: $17.4 \pm 1.6\%$) ($P < 0.05$) with the ball mill as compared to the colloid mill (min: $21.6 \pm 1.5\%$). This was observed since the ball mill reduced the particle size and broke down agglomerations of DDGS to obtain dispersion in the film matrix in a more effective way as compared to the colloid mill. This provided a homogeneous and compact film network with a less porous structure which improved the moisture resistance (Jantasrirad et al., 2021).

It is also crucial to consider the gas permeability properties of the films for food packaging applications to ensure that packaged food degrades less. In parallel to the moisture solubility results, acetyl-TAG had a significant ($P < 0.05$) effect on the oxygen permeability of the films (Table 2). On the other hand, an increase in acetyl-TAG concentration from 0.1% to 0.2% (w/v) did not ($P > 0.05$) reduce the oxygen permeability of the films as predicted. This might be related

to the solubility of oxygen in the non-polar acetyl triacylglycerol phase which may contribute to the transfer of nonpolar oxygen molecules in the plasticized polymer matrix. In addition to this, the plasticization effects of acetyl-TAG might create free volume which enables molecular diffusion (Arfat et al., 2018).

Similar to the moisture solubility results, oxygen permeability values were significantly ($P<0.05$) different between milling types. The permeability values were lower ($P<0.05$) in the ball mill because of its efficiency in reducing the particle size and creating a stronger barrier to reduce the gas permeation as compared to the colloid mill for all film samples which was by other physical film properties.

Table 4-2 Moisture solubility and oxygen diffusivity of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower and upper superscripts indicate significant difference ($P < 0.05$) for each column and row, respectively

Moisture solubility (%)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	22.2 $\pm$ 1.5 ^{b,A}	22.5 $\pm$ 1.6 ^{a,A}	19.3 $\pm$ 1.3 ^{b,B}	19.1 $\pm$ 1.6 ^{b,B}	17.7 $\pm$ 1.2 ^{b,C}	17.4 $\pm$ 1.6 ^{b,C}
Colloid mill	26.9 $\pm$ 1.7 ^{a,A}	22.3 $\pm$ 0.9 ^{a,C}	26.2 $\pm$ 1.5 ^{a,B}	21.9 $\pm$ 1.5 ^{a,C}	25.5 $\pm$ 1.7 ^{a,B}	21.6 $\pm$ 1.5 ^{a,C}

O ₂ Permeability Coefficient (PO ₂) (x10 ⁻¹² mol /m ² s Pa)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	0.38 $\pm$ 1.1 ^{b,A}	0.42 $\pm$ 0.8 ^{b,A}	0.31 $\pm$ 1.8 ^{b,B}	0.39 $\pm$ 1.3 ^{b,A}	0.31 $\pm$ 1.9 ^{b,B}	0.37 $\pm$ 1.1 ^{b,AB}
Colloid mill	0.43 $\pm$ 1.3 ^{a,B}	0.47 $\pm$ 1.1 ^{a,A}	0.42 $\pm$ 1.1 ^{a,B}	0.44 $\pm$ 1.4 ^{a,B}	0.40 $\pm$ 0.8 ^{a,B}	0.42 $\pm$ 0.7 ^{a,B}

Mechanical properties of the films

The mechanical properties of the films, namely tensile strength at the break (σ) and elongation (%) are shown in Table 4-3. Tensile strength is a quantitative measure of film strength, whereas elongation at break is a measure of film stretchability prior to breakage. The incorporation of acetyl-TAG markedly ($P < 0.05$) affected both tensile strength and elongation: tensile strength significantly ($P < 0.05$) decreased while elongation increased ($P < 0.05$) when the acetyl-TAG concentration increased. Acetyl-TAG incorporated into the film matrix could reduce the interactions between DDGS biopolymers, glycerol, and stabilizers mixed in the film formulation and probably hinder the chain-chain interaction of polymers, which eventually leads to a decrease in tensile strength (Shen & Kamdem, 2015). Jamroz et al. (2018) supported this finding in their study where they observed that the incorporation of lavender oil reduced the tensile strength of potato starch-furcellaran-gelatin films due to interrupting interactions between polymer chains.

The films without acetyl-TAG were more firm and less extensible than the films incorporated with acetyl-TAG. The addition of acetyl TAG and increase its concentration significantly ($P < 0.05$) improved the flexibility with increased elongation at the break (%) due to improved compatibility and extensibility of the film network. For example, elongation at the break increased by 24% when 0.2% (w/v) acetyl-TAG was added into the film pretreated with ball mill and solubilized in 1:1 (v/v) water: chloroform ratio as compared to the film prepared with the same conditions but without acetyl-TAG. The results of elongation at the break (%) were supported by a recent study conducted by Venkatachalam et al. (2023) where they demonstrated that addition of garlic, kaffir lime or galangal oils significantly ($P < 0.05$) resulted in more elongation of rice-starch based films as compared to the control ones without oil incorporation. They explained that addition of oil might

induce intense chemical reactions between the functional groups and the film polymer and lead to efficient transfer of stress throughout the polymer layers and creating stronger film elasticity (Venkatachalam et al., 2023).

Following the effect of acetyl-TAG, the processing type had a significant ($P < 0.05$) effect on elongation at the break values. Overall, elongation at the break was higher ($P < 0.05$) for the films pretreated via ball mill in comparison to the colloid mill. This was not surprising since ball milling pretreatment improved the dissolution of DDGS in the film matrix more effectively so that continuous and extensible films could be achieved. In a recent study, Jing et al. (2022) observed that elongation at break showed an upward trend ($P < 0.05$) for the microfibrillated cellulose films which became more extensible after ball mill pretreatment as compared to control film without pretreatment.

Table 4-3 Mechanical properties (tensile strength and %elongation) of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower and upper superscripts indicate significant difference ($P < 0.05$) for each column and row, respectively

Tensile Strength (σ) (MPa)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	12.9 $\pm$ 0.4 ^{b,B}	15.9 $\pm$ 0.2 ^{b,A}	10.9 $\pm$ 0.9 ^{b,CD}	11.5 $\pm$ 0.6 ^{b,C}	8.5 $\pm$ 0.2 ^{b,E}	10.4 $\pm$ 0.7 ^{b,D}
Colloid mill	14.9 $\pm$ 0.4 ^{a,C}	17.1 $\pm$ 0.5 ^{a,A}	13.8 $\pm$ 0.3 ^{a,D}	16.4 $\pm$ 0.9 ^{a,B}	11.2 $\pm$ 0.5 ^{a,E}	14.9 $\pm$ 1.1 ^{a,C}

% Elongation						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	22.1 $\pm$ 1.5 ^{a,BC}	20.1 $\pm$ 1.8 ^{a,C}	27.1 $\pm$ 1.5 ^{a,AB}	26.6 $\pm$ 1.8 ^{a,AB}	29.1 $\pm$ 1.5 ^{a,AB}	28.8 $\pm$ 1.8 ^{a,A}
Colloid mill	21.9 $\pm$ 0.2 ^{b,AB}	19.7 $\pm$ 0.3 ^{b,B}	22.1 $\pm$ 1.7 ^{b,AB}	20.3 $\pm$ 1.9 ^{b,AB}	23.8 $\pm$ 1.7 ^{b,A}	23.3 $\pm$ 1.4 ^{b,AB}

Surface hydrophobicity of the films

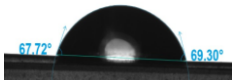
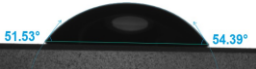
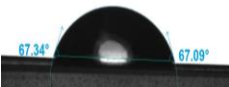
One of the essential characteristics of packaging materials is their attributes related to hydrophobicity, hydrophilicity, and wettability of films for further application in industry. Water contact angle ($^{\circ}$) which is an indicator of the penetration and evaporation of water in film matrices was measured to interpret the surface behavior of the films. In general, a contact angle which is less than 90° is an attribute of hydrophilic materials (Pirnia et al., 2022).

All film samples had contact angle values less than 90° which reflected hydrophilic characteristics (Table 4-2). Acetyl-TAG had a significant ($P < 0.05$) effect on contact angles. Regarding the data presented in Table 4-4, there were nearly $9\text{--}10^{\circ}$ and $23\text{--}24^{\circ}$ ($P < 0.05$) increases in contact angle for the films upon the addition of acetyl-TAG at concentrations of 0.1% and 0.2% (w/v), respectively. It is known that contact angle increases via increasing surface hydrophobicity; thereby, contact angle increment as a function of acetyl-TAG addition is attributed to its hydrophobic nature. It is known that the wettability of the films can be interpreted by the contact angle; the higher contact angle indicates that the interaction between the film surface and water is unfavorable (Li et al., 2022). This clearly showed that the addition of acetyl-TAG reduced the wettability and improved the hydrophobic character of the films. When the physical characterization of the films was considered, the observed effect of acetyl-TAG on hydrophobicity and wettability was entirely in line with reduced moisture solubility shown in Table 4-2. Gao et al. (2020) reported similar results via observing the effect of soybean oil on the contact angle of corn and octenylsuccinated starch based biodegradable films.

Furthermore, processing type had a significant ($P < 0.05$) effect on the contact angle ($^{\circ}$) of the films prepared using 0.1% and 0.2% (w/v) acetyl-TAG. Contact angle ($^{\circ}$) was higher ($P < 0.05$) for the films pretreated using a ball mill as compared to a colloid mill which showed improved hydrophobicity of the films prepared via the ball mill. The effect of the ball mill was coherent with the other physical and mechanical results throughout the study. Deng et al (2016) also supported this by showing the similar effects of ball milling to improve the hydrophobicity of cellulose films.

Table 4-4 Water contact angles (°) with images of sorghum DDGS films. The results are expressed as mean value $\pm$ SD (n = 3). The lower and upper superscripts indicate significant difference ($P < 0.05$) for each column and row, respectively.

Contact angle (°)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill	47.2 $\pm$ 1.1 ^{a,F}	48.1 $\pm$ 1.3 ^{a,E}	55.6 $\pm$ 1.2 ^{a,D}	58.2 $\pm$ 0.5 ^{a,C}	68.7 $\pm$ 0.1 ^{a,B}	70.1 $\pm$ 0.8 ^{a,A}
Colloid mill	45.2 $\pm$ 1.5 ^{a,F}	47.6 $\pm$ 1.1 ^{a,E}	52.3 $\pm$ 0.5 ^{b,D}	55.1 $\pm$ 1.7 ^{b,C}	64.6 $\pm$ 0.5 ^{b,B}	66.3 $\pm$ 0.7 ^{b,A}

Contact angle (°)						
Composition						
Processing Type	No acetyl-TAG		0.1% acetyl-TAG		0.2% acetyl-TAG	
	Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)		Water-chloroform ratio (v/v)	
	1:1	1:2	1:1	1:2	1:1	1:2
Ball mill						
Colloid mill						

Conclusion

The present study showed that sorghum DDGS films incorporated with high oleic acetyl-TAG from engineered form Camelina seeds and preconditioning via wet milling with suitable solubilization technique can be successfully produced with improved physical, mechanical, and surface hydrophobicity. The films represented low moisture solubility, oxygen permeability, high elongation at the break (%), and low wettability. For example, the addition of acetyl-TAG and ball mill pretreatment significantly ($P < 0.05$) increased the moisture resistance of the films reducing the volume for water molecule intake; the solubility of the films decreased by increasing acetyl-TAG concentration from 0.1% to 0.2% (w/v) to about 9% for the films solubilized using 1:1 (v/v) water: chloroform ratio and pretreated via ball mill. The increase in hydrophobicity with the addition of acetyl-TAG was by decreased adsorption (interfacial tension) and wettability. Mechanical properties were affected similarly. The elongation at the break (%) increased for the films prepared via 0.2% (w/v) acetyl-TAG addition and ball mill pretreatment. In conclusion, high oleic acetyl-TAG from engineered form Camelina seeds can be used as a novel and high-yield plasticizer.

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Chapter 5 - Improving the whey protein foam structures by using novel acetylated triglycerides (acetyl-TAG): A response surface methodology (RSM) approach (*published in JAOCS*)

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Abstract

Protein foams are common in foods, such as meringue, whipped cream, and mousses. Stability is a challenging issue determined by fat and sugar content. We hypothesize that acetyltriacylglycerols (acetyl-TAG) that possess *sn*-3 acetate group with high oleic content can provide stability to the foam at reduced sugar concentrations by increasing the surface viscosity and minimizing serum drainage. A fractional factorial Box-Wilson design was used to investigate the effects of whey protein concentration (WPI) (2-10 wt%), sucrose concentration (SC) (10-30 wt%), and acetyl-TAG concentration (ATC) (0-1 wt%) on overrun (FO), stability against serum drainage (FD) and surface dilatational rheology. Each response was analyzed by linear regression model fitting and a backward elimination algorithm for significance ($\alpha = 0.01$). FO was significantly

affected ($P<0.01$) by WPI and ATC, but not sucrose concentration. The optimum overrun (nearly 700%) was obtained at 9 wt% WPI and 0.6 wt% ATC. Drainage and viscoelastic properties were significantly ($P<0.01$) affected by all process variables. Dynamic complex modulus ($|E|$) as measured using an optical tensiometer was around 30-40 mN/m when acetyl-TAG was higher than 0.5 wt%. Our findings indicated that the acetyl-TAG can be used to enhance the stability of protein foams in reduced sugar food products, however not at high sugar concentrations (30 wt%).

Introduction

Protein foams are two-phase systems, where air cells are dispersed as elastic pockets in a continuous aqueous phase. They are the commonplace class of materials that give the desired structure to a broad range of products, such as meringue, mousses, whipped cream, ice cream, and marshmallows. The physicochemical characteristics and large surface area contribute to the instability issues through film drainage and coalescence, eventually leading to rupture (Wierenga et al., 2023). There are several factors affecting foam stability. The type of polymer and surface-active ingredients added to the foam formulation might influence the foam's quality and stability. The movement of the free fluid due to gravity is known as drainage and causes the main instability problem in foam systems. Furthermore, the rate of drainage depends on several factors such as temperature, viscosity of the liquid phase, whipping time, and whipping conditions (Fu et al., 2020).

Proteins are the most common group of ingredients used for foam preparation. They increase the surface viscosity to slow down the gravity-driven film drainage, which leads to thinning of the gap between air bubbles. They also provide a steric hindrance to prevent the coalescence of adjacent bubbles. Whey protein isolates (WPI) are widely used in various foam-based food products (Kristensen et al., 2021; Nooshkam et al., 2023).

When adsorbed at the air-water interface, they unfold by the orientation of hydrophilic and hydrophobic groups toward water and air, respectively. A recent study showed that WPI reduces the drainage rate and stabilizes the air bubbles via Pickering stabilization which provides a mechanical barrier against coalescence (Bansal et al., 2018). Similar results were observed by Yang et al. (2021) who highlighted the strong viscoelastic behavior of WPI at the air-water interface.

In addition to protein, sugar is another common ingredient that contributes to foam stability by increasing the bulk phase viscosity, which slows down the film drainage rate. Sugar can interact with proteins to further influence foam stability. This interaction may increase or decrease foam stability depending on the sugar concentration (Sun et al., 2022). For example, some of the earlier research showed that the presence of sucrose higher than a critical limit can deplete surface proteins from the surface leading to instability of protein foams (Goralchuk et al., 2020; Nastaj et al., 2021). In contrast, this behavior was not observed in foams prepared with small molecule surfactants, such as polysorbate 80, where foam stability increased with increasing sucrose concentration (Kubbutat & Kulozik, 2021). Therefore, it is critical to control and elucidate the effect of sugar concentration on the stability of protein foams. This also relates to the current public health strategies that promote sugar reduction. Overall, protein and sugar are important for foam stability, which can further be enhanced by using novel surface-active ingredients through modulation of the interfacial rheology at altered protein and sugar concentrations.

The 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are unusually structured triacylglycerol (TAG) molecules that possess an *sn*-3 acetate group instead of a long chain fatty acid. Acetyl-TAG possesses different physical properties from regular TAG, including reduced viscosity and improved cold temperature properties (Bansal et al., 2018). Several plant species, including different members of the *Euonymus* genus, naturally accumulate high levels of acetyl-TAG in their seeds, but these plants are not good sources for the large-scale production of these unusual TAG molecules (Tran et al., 2017). Recently, the emerging oil seed crop *Camelina sativa* has been transgenically engineered to produce high levels of acetyl-TAG by introducing acetyl-transferase enzymes from different *Euonymus* species (Alkotami et al., 2021). In addition, camelina seeds that synthesize acetyl-TAG with a high oleic acid content have been developed by suppressing

endogenous desaturase and elongase activities (Liu et al., 2015). The high-oleic fatty acid chain can provide a strong attachment on the foam surface to increase viscosity. In this chapter, we hypothesize that the unusual acetyl-TAG molecules obtained from transgenic *Camelina sativa* seeds with high oleic content can be used to increase the stability and capacity of WPI foams. The objective of this study was to understand the effect of whey protein isolate, sucrose, and acetyl-TAG concentrations on foam overrun, stability, and surface rheology using a Box-Wilson experimental design and response surface methodology (RSM).

Materials and Methods

Materials

The WPI was obtained from Milk Specialties Global (Milk Specialties Global, NE, USA). Granulated pure cane sugar was procured from a local market. Deionized water used to prepare foams was obtained using Barnstead Nanopure II purification system (Barnstead™ PCS, Dubuque, Iowa, USA). High oleic acetyl-TAG oil derived from transgenic camelina plants was a kind gift from John Ohlrogge (Michigan State University). Acetyl-TAG was extracted from this oil using silica gel chromatography with a step-gradient of hexane and diethyl ether (Bansal et al., 2018). The purity and the fatty acid composition of the acetyl-TAG were confirmed by thin-layer chromatography (TLC) and gas chromatography (GC).

Foam Preparation

The WPI (2, 4, 6, 8, 10 wt%) was initially hydrated in deionized water (220 mL) for 1 h at ambient temperature under constant stirring and kept at refrigerated conditions for 1 hour until used. Then, sucrose (10, 15, 20, 25, 30 wt%) and acetyl- TAG (0, 0.25, 0.5, 0.75, 1 wt%) were added into the protein solution. The foams were prepared by whipping the solution (200 mL) using a 6-Speed

Hand Mixer (Hamilton Beach Brand, Glen Allen, Virginia, USA) at the highest speed at 5 minutes intervals four times. The whipping duration was determined based on preliminary experiments to obtain uniform bubbles and adequate volume without overbeating.

Physical characterization of foams

Foam images and bubble size determination

Foam images were taken using a Canon PowerShot digital camera (CanonSD1100 IS, Zoom Lens 3x IS) immediately after preparation. The pictures of the foam surfaces were taken directly from the surface of the glass bowl (diameter: 152.4 mm and height: 73 mm) that they were prepared. The images were taken from a constant distance (7 cm) stabilized by a tripod. The camera zooms focused on the center of the bowl and covers all the foam surfaces to take representative pictures. Before the imaging, the Canon camera lens was calibrated using an autofocus micro adjustment and color correction features. The image stabilization function was also activated. The pictures were taken in a photo section with a black background to eliminate any reflection. Bubble size distribution was analyzed using ImageJ (NIH, Bethesda, MD, USA). The measurements were taken using a scale bar by drawing a straight line with a known distance (18.9 pixels) on the image. The unit of the length was converted to mm and the scale calibration was applied to all images. The images were adjusted by subtracting the background and splitting channels around bubbles to obtain clear and separate images. Dark lines on the surface plateau borders were reduced by identifying pixels below a threshold value. The total number of bubbles was calculated per area and the average bubble size was measured in millimeters.

Foaming capacity

The foaming capacity was quantified as foam overrun (FO) using the protocol described by Campbell and Mougeot with some modifications (Campbell & Mougeot, 1999). The freshly prepared foam and remaining serum phase were separated and transferred into beakers. The weights of each phase and unwhipped solution were measured to calculate FO (%) using Equation 1:

$$FO \% = \frac{(weight\ of\ unwhipped\ solution)-(weight\ of\ whipped\ sample)}{(weight\ of\ whipped\ product)} \times 100 \quad (1)$$

Foam stability

The stability of foam was determined by measuring foam drainage (FD) under gravity using the protocol described by Fredrick et al. with minor modifications (Fredrick et al., 2013). The freshly prepared foam (15 mL) was placed in a glass funnel with top and stem diameters of 75 and 8 mm, respectively. The funnel was positioned over a graduated cylinder (250 mL) at room temperature for 1 h. The weight of the serum collected in the graduated cylinder was used to calculate FD:

$$foam\ drainage\ (\%) = \frac{weight\ of\ serum\ collected}{weight\ of\ whipped\ cream} \times 100 \quad (2)$$

Surface characterization of foams

The properties of the air/liquid interface of the foams were evaluated using the interfacial tension and rheology measurement by using an optical drop tensiometer (Attension Theta, Biolin Scientific, Phoenix, Arizona, USA). The protocols were established using the instrument guidelines and help of the technical help from Biolin Scientific as follows. Prior to analysis, the instrument is calibrated by camera adjustments to focus on a metal ball standard (4mm in diameter)

at a zoom angle of *ca.* 143°. All surface characterization measurements were performed at room temperature.

The equilibrium surface tension measurements were conducted with a pendant drop (15 µL) formed at the tip of a needle (1 mL Luer tip, gastight Hamilton syringe). The equilibrium surface tension was calculated using the equation below.

$$\gamma = \frac{\Delta\rho * g * R_o}{\beta} \quad (3)$$

where γ is the surface tension, $\Delta\rho$ is the density difference between air and liquid phases, g is the gravitational acceleration constant, R_o is the curvature drop radius at the apex, and β is the droplet shape factor.

The surface rheology of the air/liquid interface was analyzed using a similar pendant drop (15 mL) under dynamic conditions. The piezoelectric pump of the tensiometer automatically fluctuated the drop volume as sinusoidal oscillations with the following parameters: frequency (0.5 Hz), waveform, amplitude (0.5), and time (the 60s). Preliminary experiments were done with the help of Biolin Scientific scientists to determine the ideal oscillatory-drop parameters considering the significant interfacial area changes.

Changes in the droplet shape (μL), surface area (ΔA), and phase angle difference (γ) were automatically recorded and correlated to complex surface dilatational modulus ($|E|$, [mN/m) by the equation below:

$$|E| = E' + E'' = \frac{d\gamma}{d\ln A} = E_0 + i * \omega * \eta \quad (4)$$

where $|E|$ is the complex dilatational modulus, E' is the elastic modulus, E'' is the viscous modulus, γ is the surface tension, A is the droplet area, ω is the angular velocity and η is the viscosity.

Statistical analysis and design of the experiment

Box-Wilson experimental design and response surface methodology

The statistical analysis was conducted at the Department of Statistics, Kansas State University. A Box-Wilson central composite design was used to determine the experimental conditions. The experiment was carried out with a total of 20 runs: out of which 6 sets of experiments were at the center points for every variable. All experimental conditions are shown in Table 5-1.

Table 5-1 The Box-Wilson central composite design with the run order.

Test Run	Whey Protein	Sucrose	
Order	Isolate%	Concentration%	Acetyl-TAG %
	(w/v)	(w/w)	(w/v)
Control*	10	30	0
1	6	20	0.5
2	6	10	0.5
3	6	30	0.5
4	6	20	0.5
5	4	15	0.75
6	6	20	0.5
7	4	25	0.75
8	8	25	0.25
9	2	20	0.5
10	6	20	0.5
11	8	25	0.75
12	8	15	0.75
13	8	15	0.25
14	6	20	1
15	6	20	0.5
16	6	20	0
17	10	20	0.5
18	4	15	0.25
19	6	20	0.5
20	4	25	0.25

*Control run is in addition to the Box-Wilson design to verify the model efficacy.

The independent variables with multiple levels of WPI concentration (2, 4, 6, 8, 10 wt%, sucrose concentration (SC) 10, 20, 30 wt%, and acetyl-TAG concentration (ATC): 0, 0.25, 0.5, 0.75, 1 wt%) were investigated for their effect on the response parameters: overrun (FO), stability against serum drainage (FD) and surface rheology. After defining independent and dependent parameters, the order of runs was designed by the Box-Wilson method responsive to the analysis of experimental data using the response surface methodology. A control sample without acetyl-TAG was prepared to verify the response parameters.

Then, the RSREG procedure using the SAS software (Version 9.4 TS Level 1M5, Statease, Minneapolis, MN, USA) was used to assist in the design of the experiment and compute the statistical analysis. A multiple linear regression model was used to evaluate the effect of independent variables on each response. Backward elimination was performed to simplify the models by removing the variables with the highest p-value. This process was repeated until the significant variables with a p-value below 0.01 were left. ANOVA was also conducted to validate the quality fit of the final model which was expressed below as a second-degree polynomial equation (Yadav et al., 2018).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} (X_i)^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j \quad (4)$$

where Y is the dependent variable, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the two factors interaction coefficient.

Finally, response surfaces were created to understand the behavior of each response with the changes in significant variables by 3D response surface counter plots. It was conducted to make model interpretations and see the interactional impact of variables.

Results and Discussion

The linear regression models are shown in Table 5-2. Each response has a characteristic equation generated by second-order polynomial regressions. These equations were composed of linear, quadratic, and two-factor interaction effects determined by backward elimination. The predicted values for each response were similar to the values obtained from the model with high correlation coefficients (R^2) to prove the reliability of the model. The individual data points generated using the experimental design are shown in Table 5-3.

Table 5-2 The ANOVA models derived by linear regression for each response.

Response	Polynomial Equation	R ²
Foam Overrun	$Y_1 = -298.81 + 193.39*WPI + 185.17*ATC - 11.62*WPI^2 - 409.91*ATC^2 + 37.38*(WPI)(ATC)$	0.9354
Foam Drainage	$Y_3 = 116.75 - 21.03*WPI - 1.06*SC + 7.87*ATC + 0.95*WPI^2 + 0.24*(WPI)(SC)$	0.8025
Dilatational Modulus	$Y_5 = -55.66 + 16.44*WPI + 43.08*ATC + 1.76*SC - 0.94*WPI^2 - 36.78*ATC^2 - 0.09*(WPI)(SC) - 0.05*SC^2$	0.9239

Table 5-3 Actual measurement values for foam capacity (FO), foam drainage (FD) and complex dilatational modulus ($|E|$) based on Box-Behnken experimental order

Test Run Order	Whey Protein Isolate (WPI) % (w/v)	Sucrose Concentration (SC)% (w/w)	(ATC) % (w/w)	Foam Overrun (%)	Foam Drainage (%)	$ E $ [mN/m]
control 1	10	30	0	498.4	41.9	22.2
control 2	2	10	0	108.5	65.2	5.2
1	6	20	0.5	548.1	35.7	33.1
2	6	10	0.5	500.8	31.2	34.6
3	6	30	0.5	503.4	42.5	27.8
4	6	20	0.5	545.5	35.1	33.9
5	4	15	0.75	307.4	48.1	15.9
6	6	20	0.5	544.6	35.6	33.9
7	4	25	0.75	281.8	53.5	14.4
8	8	25	0.25	586.0	30.5	35.9
9	2	20	0.5	110.5	66.5	4.5
10	6	20	0.5	549.7	34.5	34.1
11	8	25	0.75	625.7	39.5	38.1
12	8	15	0.75	644.4	31.4	41.8
13	8	15	0.25	599.4	24.9	37.5
14	6	20	1	530.6	36.1	35.9
15	6	20	0.5	549.8	34.8	32.8
16	6	20	0	449.5	32.6	23.5
17	10	20	0.5	660.8	31.7	44.1
18	4	15	0.25	327.4	49.3	13.9
19	6	20	0.5	546.2	34.5	34.1
20	4	25	0.25	330.5	47.5	14.2

Foam formation and morphology

The foam morphology was visualized with imaging (Fig. 5-1), and foam formation capacity was evaluated as FO (Fig. 5-2). The FO analysis showed that SC was not a significant ($P > 0.01$) factor in foam formation (Fig. 5-2). The foam size and distribution were affected by both WPI and ATC (Fig. 5-1). Foams prepared using 2 wt% WPI did not form stable bubbles due to insufficient amount of protein. Increasing protein concentration resulted in smaller and uniform bubbles (Fig. 5-1). The maximum FO was ca. 700% found at the WPI above 8 wt% and ATC above 0.75 wt%. The control measurements (10 wt% WPI, 30 wt% SC, and 0 wt% ATC) were ca. 500% confirming the surface graph value in Fig 2. WPI, ATC, and interaction of WPI and TC had a significant ($P < 0.01$) effect on FO (Table 5-2).

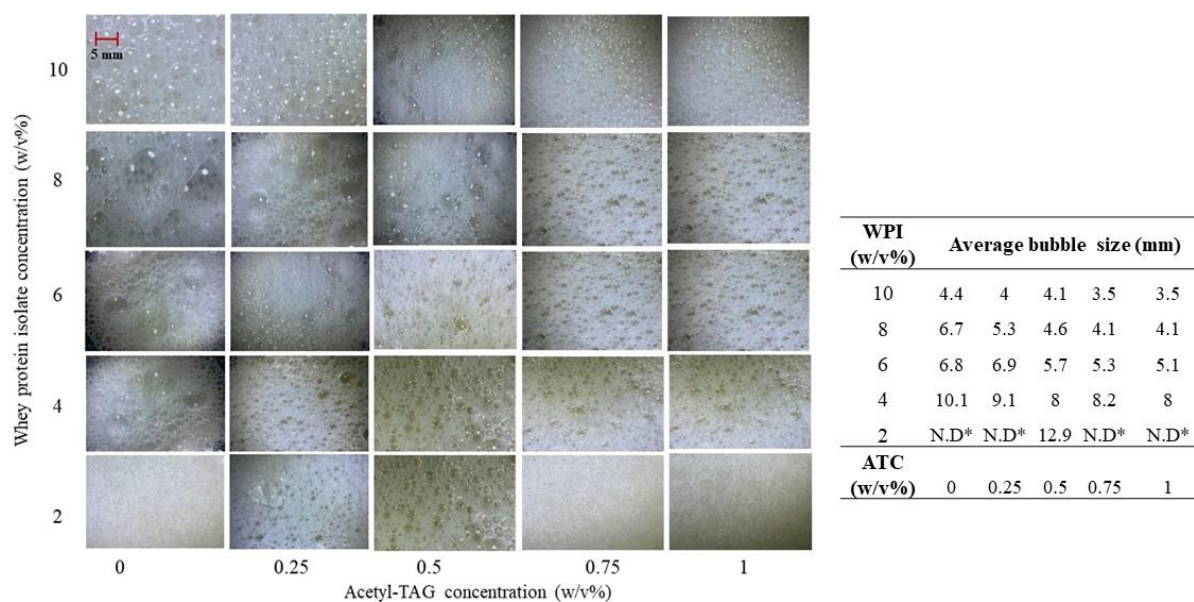


Figure 5-1 Images and average particle size of the non-fat whey protein foams with a pooled standard deviation of 0.8 with respect to whey protein isolate concentration (WPI) (2-10%) and acetyl-TAG (ATC) (0-1%) with constant sucrose concentration of 30% (w/v). N.D* denotes non-determined particle size due to insufficient bubble formation.

Increasing the WPI, increased FO to the maximum obtained at 10 wt% WPI. In parallel, the total number of bubbles per area (mm^2) increased by 60% when WPI increased from 4 wt% (0.37# of bubbles/ mm^2) to 10 wt% (0.62# of bubbles/ mm^2) along with a decrease in average bubble size (56%) as determined by ImageJ analysis (Fig. 5-1). The result was expected since the increase in protein concentration from 4% to 10% resulted in sufficient coverage of the droplet interface. Similar observations were found in others (Delahaije & Wierenga, 2022; Tan et al., 2016; Xiong et al., 2020). Increasing WPI up to 8 wt%, increased FO; however, a further increase to 10 wt% did not show a significant ($P < 0.01$) difference in the absence of ATC. This is expected since whey protein molecules unfold and adsorb to the air-aqueous interface and surround air bubbles to form foam measured as overrun. At the threshold concentration of 8 wt% WPI, surface saturation is reached, and further increase did not show a difference. Similarly, Grossmann et.al (2020) observed an increase in foaming capacity with increasing the concentration of selected proteins (i.e., egg albumen, gelatin protein, Na-caseinate); however, up to a threshold level determined by the protein type (i.e., at 2 wt% for egg albumen and gelatin, and 5 wt% for sodium caseinate).

The addition of acetyl-TAG significantly ($P < 0.01$) increased FO (Fig. 5-2). This is in parallel with the morphology observations (Fig. 5-1). The addition of acetyl-TAG decreased bubble size and increased the total number of bubbles per area at a given protein concentration responsible for the formation of a larger surface area. For example, an increase in ATC from 0.25 wt% to 0.75 wt% significantly ($P < 0.05$) increased the total number of bubbles per area (mm^2) by 20% (0.8 bubbles/ mm^2 at 0.75 wt%) while reducing the average bubble size by 13% (3.5 mm at 0.75 wt%). The effect of ATC on FO was not significant ($P > 0.05$) above 0.75 wt%. These observations can be explained by the ability of small molecule surfactants, such as acetyl-TAG, to cover the newly forming surfaces at a faster rate as compared to large protein polymers during foam preparation to

result in more volume and smaller droplets (Yang et al., 2022). Furthermore, Yu et al., (2019) claimed that an increase in surfactant concentrations after a threshold level may even reduce the foaming capacity. For example, an earlier study showed that an increase in Persian gum (GM) and xanthan gum (XG) concentrations from 0.05 to 0.15 wt% increased foam density by trapping less air leading to a decrease in the foaming capacity of the egg white protein foams (Dabestani & Yeganehzad, 2019). Our observations were also supported by the changes in the equilibrium surface tension at the air-liquid interface.

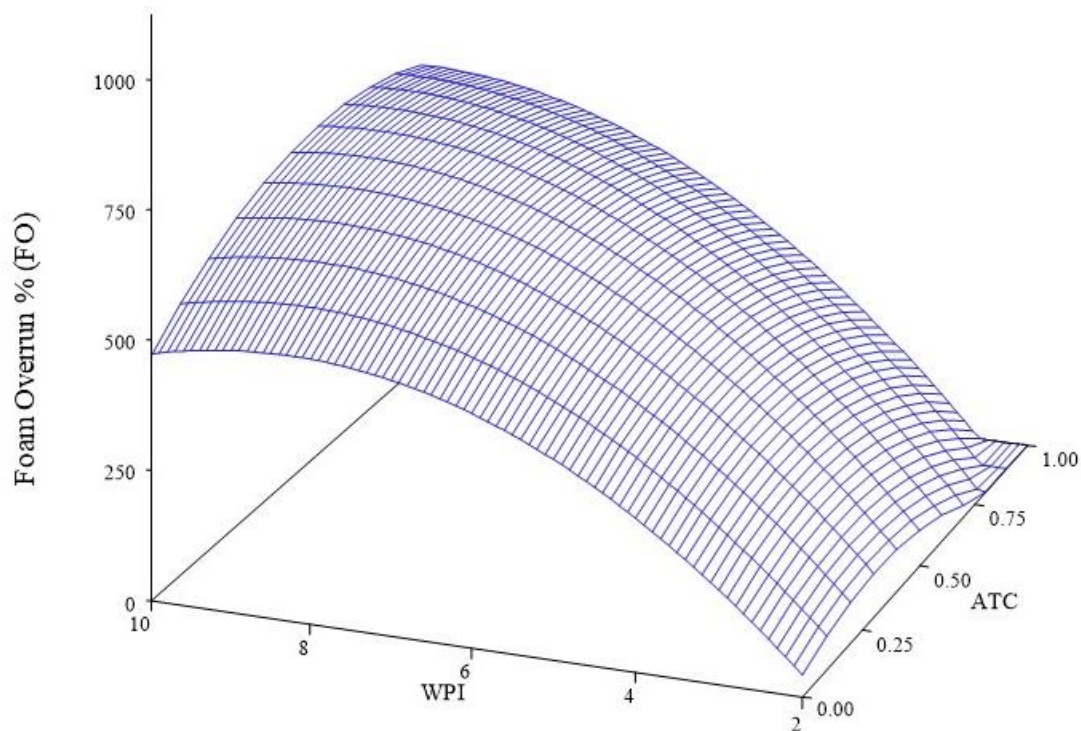


Figure 5-2 The 3-dimensional response surface graph for foam overrun (FO) as a function of whey protein isolate (WPI) concentrations (2-10 wt%) and acetyl-TAG (ATC) (0-1 wt%)

The control whey protein solution prepared with 10 wt% WPI concentration (no acetyl TAG) had a surface tension of 65.3 ± 0.57 mN/m. The surface tension decreased from 43.85 ± 0.07 to 38.9 ± 0.28 mN/m by increasing acetyl-TAG concentration from 0.25 to 0.75 wt%. A recent study conducted by Nooshkam et al. (2022) showed that increasing the surfactant concentration of licorice extract from 0.1 wt% to 0.5 wt% gradually increased the adsorption at the interface and decreased the surface tension of WPI foams.

Foam stability

The stability of the foams was assessed via FD measurements (Fig. 5-3). Foam drainage is the flow of the aqueous serum separating adjacent air bubbles through the thin interstitial space, which results in the thinning of the gap between bubbles and leads to coalescence and foam breakdown. This phenomenon is driven by gravity and slowed by bulk viscosity and surface viscosity, and the lower drainage rate indicates higher stability against gravity. The experimental FD value for the control condition (10 wt% WPI, 30 wt% SC, 0 wt% ATC) was *ca.* 42%, which correlated with the reading from the three-dimensional surface graph (Fig. 3). All the variables WPI, ATC, SC, and the interaction of WPI and SC had significant ($P < 0.01$) effect on FD (Table 5-2). The lowest FD was less than 20% at the highest WPI (10 wt%) and the lowest SC (10 wt%). The interaction effect between protein and sugar (WPI*SC) is given in Table 5-2.

Intermolecular attraction or repulsion at a molecular level can cause both negative and positive influences on foam stability depending on the type of sugar and protein (Davis & Foegeding, 2004). The sugar might allow the increased extent of protein aggregation which is involved in film stabilization and reduces the serum drainage (Rodríguez Niño et al., 1997). For example, Alavi et al (2020) observed that in the presence of sucrose, due to the strengthening of the protein-protein net attractive interactions, significant aggregation of egg white protein occurs leading to a decrease

of the surface activity and increased film stabilization and reduced the drainage (Alavi et al., 2020). On the other hand, the addition of sugar can limit protein unfolding and the development of protein–protein attractive interactions since sucrose is known to create a less favorable thermodynamic environment for protein unfolding. This results in a decrease in foam stability and increases drainage (FD) (Campbell & Mougeot, 1999). For instance, Yang et. al (2010) used whey protein isolate and they highlighted that the addition of sugar reduced the attractive forces between protein–protein molecules, and this was observed as an increase in drainage which was also observed in our study.

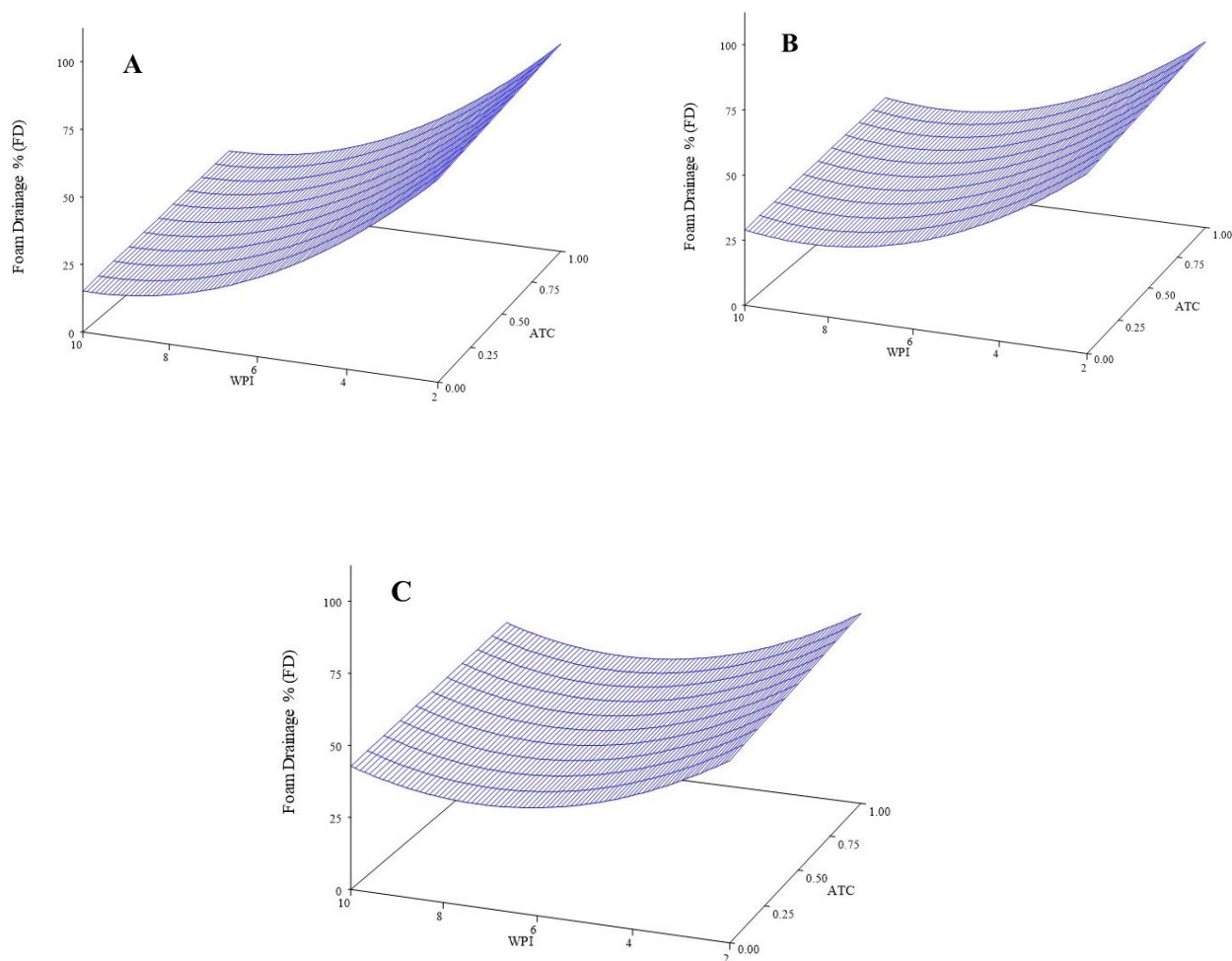


Figure 5-3 The 3-dimensional response surface graph for foam drainage rate (FD) as a function of whey protein isolate (WPI) concentrations (2-10 wt%) and acetyl-TAG (ATC) (0-1 wt%) at SC of A) 10 wt%, B) 20 wt%, and C) 30 wt%.

As expected, increasing WPI helped the formation of thicker films slowed the liquid drainage, and increased the stability of the foams. A recent study showed similar effects of protein concentration significantly ($P < 0.05$) increasing the foam stability due to the adsorption and coverage of the micellar proteins at the air-liquid interface (Saragih et al., 2022). In parallel to, our observations for FO, WPI did not significantly ($P > 0.05$) affect FD at concentrations above 8 wt% based on the surface plot generated using the regression equation (Fig. 5-3). The confidence intervals were

calculated at $\alpha = 0.05$ at a given point using the variability for that point and compared to the confidence intervals at the subsequent points. At the concentration above 8 wt% WPI (e.g., 10wt%), the confidence intervals were overlapping to indicate no significant difference. This observation, therefore, is following our earlier explanation where after the threshold concentration of 8 wt% WPI surface coverage was reached.

Our hypothesis for the effect of sugar on the foam stability was based on the increase in bulk viscosity with increasing SC to slow down FD. Earlier research showed that the sugar-WPI interactions can lead to a multilayer cohesive film at the interface with enhanced thickness of the surface film, which slows down the serum drainage and prevent foam collapse (Davis & Foegeding, 2004). Our results indicated that FD was significantly ($P < 0.05$) increased with the addition of sucrose from 10 wt% (22%) to 30 wt% (43%) in the absence of acetyl-TAG. The FD at 30 wt% SC and 8wt% WPI concentration was quite similar to the control (10 wt% WPI, 30 wt% SC, 0 wt% ATC) at which FD was obtained ca. 42%. This also demonstrated the threshold concentration of 8 wt% for WPI where FD remained almost constant. In a recent study, Goralchuk et al. (2020) observed a similar situation when they used higher than 10 wt% crystal sugar in a whipped topping formulation (Goralchuk et al., 2020). The results can be explained through the attractive interactions of sugar molecules with proteins adsorbed at the interface to form carbohydrate-protein complexes and alter the surface activity of proteins. For example, increasing sugar may negatively affect the diffusion of protein molecules toward the interface by reducing attractive protein-protein interactions and limit protein unfolding at the interface through complexation. This could lead to disruption of liquid lamella phase surrounding air bubbles and cause weaker foam microstructure (Lau & Dickinson, 2005).

Increasing the ATC significantly ($P < 0.01$) decreased FD which can be seen from the surface plot (Fig. 3). Indeed, acetyl-TAGs are small molecules that co-adsorb at the increasing surface film density and eventually surface rheology as investigated in the next section. The interfacial area is expected to be enhanced during the foam formation via the faster diffusion of acetyl-TAG than proteins, which are eventually responsible for the foam formation and stability. The rapid adsorption of acetyl-TAG reduced the surface tension at the air-water interface and improved the stability (Goralchuk et al., 2020). As discussed in foam morphology, increasing ATC resulted in smaller bubbles due to faster adsorption kinetics of acetyl-TAG. The smaller bubble size was also responsible for the increased foam stability related to increased curvature, thus the higher energy required for surface film rupture.

Interfacial rheology

The dilatational rheology measurements were used to investigate the changes in surface elasticity and mobility as a function of ingredient concentrations. The complex dilatational modulus ($|E|$, mN/m) was significantly ($P < 0.01$) affected by all ingredients (protein, sugar, and acetyl-TAG) and the interaction of WPI and SC. The $|E|$ value reading for the control condition from the surface graph was 21.3 mN/m and similar to the experimentally measured value (Fig. 5-4). The dilatational rheology can be strongly correlated with the stability of foam (Arabadzhieva et al., 2011; Braunschweig et al., 2016). As in the case of foam stability, interactions between sugar and whey protein may disrupt the presence of protein molecules at the air-liquid interface by affecting structural rearrangement of them. Increasing the concentrations of WPI increased $|E|$ (Fig. 5-4). This observation conforms with the foam stability. Other scientists observed similar effects as well (Rullier et al., 2008). In comparison, the highest viscoelasticity (45 mN/m) was observed at the

highest WPI, ATC, and the lowest SC. This is related to the interaction between ATC and SC as further discussed below.

Similar to the discussion for foam stability, protein-sugar interactions are complex and do not necessarily follow a linear pathway. There was no considerable difference in $|E|$ between 10% and 20% SC in the absence of ATC, but $|E|$ decreased significantly (*ca.* 30%) when the SC was further increased to 30 wt% (Fig. 5-4). This is attributed to the ability of sugar molecules to weaken the adsorption of proteins and therefore the overall protein layer above a threshold concentration. Davis and Foegeding (2007) and Rodriguez Nino et al. (1997) observed similar behavior. Davis and Foegeding (2007) showed that the addition of 25 wt% sucrose causes a decrease in the interfacial elasticity of WPI. In addition to this, Rodriguez Nino et.al (1997) demonstrated that interfacial viscoelasticity of bovine serum albumin (BSA) decreased with increasing sucrose concentration: suggesting possible reduced protein–protein interactions in the adsorbed layer, so fewer protein molecules involved in film formation at interfaces, resulting in lower interfacial viscoelasticity.

The ATC showed two distinct effects on surface rheology as a function of SC. In the first case with the low SC (10 wt%) increasing ATC increased the dilatational viscoelasticity (Fig. 5-4A). This observation is expected since increasing the number of acetyl-TAG molecules in the solution increases the surface excess pressure with the adsorption of more acetyl-TAG molecules at the surface. Therefore, a higher density of molecules at the surface increases the film's dilatational viscoelasticity. The surface elasticity can contribute to the foam stability via two mechanisms: 1) increasing surface elasticity hinders the gravity-driven liquid flow by providing a non-slip boundary; 2) restoring the thickness of thinning films by slowing Gibbs-Marangoni mechanism (Zhang et al., 2023). For example, ordinary small molecule surfactants, such as sodium dodecyl

sulphate (SDS), Tween 20, and Tween 40, were shown to prevent bubble coalescence and stabilize foams through similar mechanisms (Petkova et al., 2020). A recent research used cetyltrimethylammonium bromide to retard the Gibbs-Marangoni effect and improve the stability of skim milk protein foams (Ho et al., 2023).

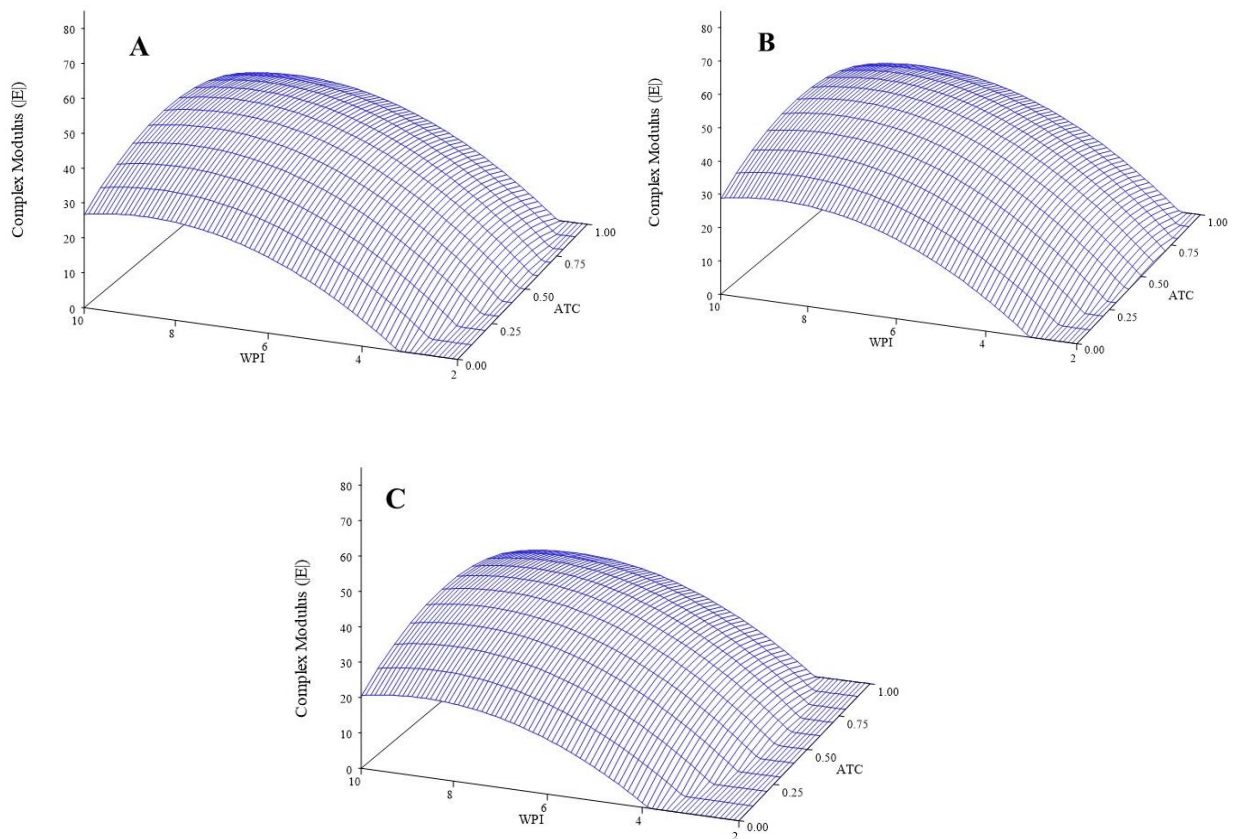


Figure 5-4 The 3-dimensional response surface graph for complex dilatational modulus ($|E|$) as a function of whey protein isolate (WPI) concentrations (2-10 wt%) and acetyl-TAG (ATC) (0-1 wt%) at SC of A) 10 wt%, B) 20 wt%, and C) 30 wt%.

In the second case with the high SC (30 wt%), increasing ATC decreased the dilatational viscoelasticity (Fig. 5-4C). The difference between the low SC and high SC can be attributed to the two probable mechanisms: increasing the SC increases the bulk viscosity of the liquid phase, and increasing SC alters the phase equilibria for adsorption of the acetyl-TAG on the surface. The actual mechanism can be explained in comparison with the foam stability. The increasing SC was shown to decrease foam stability (increasing drainage rate) as opposed to what is expected at higher viscosity, which therefore eliminates the former mechanism for SC and acetyl-TAG interaction. Therefore, the second mechanism is the probable explanation for the effect of SC and acetyl-TAG interaction on surface viscosity, where a concentration gradient between the surface and the bulk phase stimulated acetyl-TAG transfer from the surface, reducing the concentration gradient on the surface and weakening film dilatational viscoelasticity. Similarly, Zhang et. al (2015) observed that the addition of sodium dodecyl sulfate (SDS)-polyacrylamide decreased the dilatational viscoelasticity of starch-based surfactant molecules.

Conclusions

Physical and surface characterization of WPI foams showed that the addition of acetyl-TAG isolated from transgenic *Camelina sativa* seeds with high oleic content was an effective stabilizer at reduced sugar concentrations. Foam size and distribution results exhibited that smaller size and uniform bubble distribution were obtained with an increase in WPI and ATC. Physical characterizations indicated that air bubble surfaces were surrounded with acetyl-TAG, leading to a higher overrun and better stability of foams. In addition to this, our surface rheology results showed that acetyl-TAG at reduced sugar concentrations lowered the surface tension and improved the dilatational viscoelasticity of foams, thereby supporting the potential of using acetyl-TAG as a foam stabilizer.

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Chapter 6 - The application of wild type acetyl-TAG in aerated whey protein hydrolysate stabilized emulsion: effect on emulsion properties and whipping characteristics

Abstract

Whippable emulsions are O/W (oil in water) emulsions that can be converted into a three-stage system with aeration where the incorporated air bubbles are trapped within a network of fat granules. They constitute a critical part of whipped creams, toppings, or milk fat-based products to give the desired texture. On the other hand, whippable emulsions require small-molecule surfactants to be stabilized. The objective of this study was to increase oil-in-water emulsion stability (particle size, zeta potential, creaming index, absorbed protein concentration) and improve the whipped emulsion characteristics (overrun and drainage) after whipping via wild-type acetyl-TAG. Protein-stabilized emulsions using whey protein hydrolysate (WPH) (2.5 % (w/v)), coconut oil (15% (w/v) and 30% (w/v)) and wild-type acetyl-TAG (0, 0.5, 1 % (w/v)) were prepared by a high-pressure homogenizer. Acetyl-TAG significantly ($P < 0.05$) reduced the particle size and the particle size decreased ($P < 0.05$) further with an increase in the concentration of acetyl-TAG from 0.5% to 1% (w/w) whereas the creaming index decreased ($P < 0.05$) using acetyl-TAG in emulsion preparation. Furthermore, the capacity (overrun%) of whipped emulsions significantly ($P < 0.05$) increased and drainage of whipped emulsions significantly ($P < 0.05$) reduced with the addition of acetyl-TAG. Overall, the results show that wild-type acetyl-TAG can effectively assist as an effective surfactant in oil-water emulsions stabilized by WPH and improve the whipping characteristics of aerated emulsion systems.

Introduction

The whippable emulsions are oil-in-water (O/W) emulsions that can be transformed into a three-phase system with aeration, where incorporated air bubbles are held in a network of fat globules (Liu et al., 2022). They are the primary components of aerated semi-crystallized units that enable the desired smooth texture and firmness to whipped creams, toppings, and milk fat-based products. The O/W emulsions are generally stabilized by macromolecules such as proteins or low molecular weight emulsifiers. Proteins are the most common group of ingredients used in whippable emulsion preparation. Depending on their amphiphilicity and membrane-forming ability at the oil-water interface, they can reduce the interfacial tension and stabilize the interface. Whey protein hydrolysate (WPH) is commonly used in various aerated emulsion formulations. A recent study showed that whey protein hydrolysate was used to stabilize canola oil in water emulsions and utilized in reduced milk fat-stirred yogurt (Trujillo-Ramirez et al., 2022).

Whipping and aeration can enhance the interactions between protein particles and change the interactions between particles, emulsifiers, and dispersed phases in whippable O/W emulsions. During whipping, large air bubbles are beaten into the cream and protein rapidly adsorbs onto the water interface. Following this, air bubbles are broken up into smaller bubbles and fat globules displace some of the proteins from the bubble interface. This mechanical damage results in a partial coalescence of fat globules and air bubbles are eventually surrounded and stabilized by a network of coalesced fat globules (Huppertz et al., 2008). For example, whippable oil in water emulsions was obtained using sodium caseinate, zein colloidal particles, glycerol monostearate, and anhydrous milk fat with stabilized and reinforced foam structure (Liu et al., 2022b).

On the other hand, the relatively poor stability of aerated emulsions is a major challenge due to their inherent thermodynamic instability as well as their complex structure at multiple length scales

(Liu et al., 2022). Air bubbles within the aerated emulsion would gradually collapse, resulting in serum loss and changes in the internal structure of the foam, which directly deteriorate foam stability (Wang et al., 2023). Therefore, improving both emulsion and foam performances is of great significance.

The 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) are unusually structured triacylglycerol (TAG) molecules that possess an *sn*-3 acetate group instead of a long chain fatty acid. Acetyl-TAG possesses different physical properties from regular TAG, including reduced viscosity and improved cold temperature properties (Bansal et al., 2018). Recently, the emerging oil seed crop *Camelina sativa* has been transgenically engineered to produce high levels of acetyl-TAG by introducing acetyl-transferase enzymes from different *Euonymus* species (Alkotami et al., 2021). The fatty acid chain of acetyl-TAG can act as an emulsion stabilizer by minimizing the interfacial tension at the oil-water interface and providing a strong attachment on the foam surface by increasing viscosity.

The first objective of this study is to further understand the surface rheology of wild-type acetyl-triacylglycerol at the oil/water interface. Then, the chapter aims to investigate the effect of wild-type acetyl-triacylglycerol (acetyl-TAG) concentrations (0, 0.5, 1 % (w/v)) on aerated emulsions at various oil concentrations (15, 30% (w/v)) and evaluate their physical properties (creaming index, particle size, zeta potential, percentage of adsorbed protein, overrun and drainage).

Materials and Methods

Materials

Whey protein hydrolysate (Lactalbumin Hydrolysate) was purchased from Fisher Scientific (Fisher Scientific, Waltham, MA, USA). Pure fractionated liquid coconut oil was obtained from a local market (Now Foods, Bloomingdale, IL, USA). Wild-type acetyl-TAG oil derived from transgenic camelina plants was a kind gift from John Ohlrogge (Michigan State University). Acetyl-TAG was extracted from this oil using silica gel chromatography with a step-gradient of hexane and diethyl ether (Bansal et al., 2018). The purity and the fatty acid composition of the acetyl-TAG were confirmed by thin-layer chromatography (TLC) and gas chromatography (GC). Deionized water used to prepare foams was obtained using Barnstead Nanopure II purification system (Barnstead™ PCS, Dubuque, Iowa, USA).

Interfacial tension and dilatational viscoelasticity measurement

Dynamic interfacial tension (γ) between whey protein hydrolysate solution (heavy) (2.5% (w/v)) and the oil (light) phase composed of coconut oil and different concentrations of acetyl-TAG (0, 0.5, and 1% (w/w)) was measured using an optical drop tensiometer (Attension Theta, Biolin Scientific, Phoenix, Arizona, USA). The protocols were established using the instrument guidelines and preliminary experiments. Before starting the analysis, the instrument is calibrated by camera adjustments to focus on a metal ball standard (4mm in diameter) at a zoom angle of *ca.* 143°. All measurements were performed at room temperature. Protein solution (3 mL) was charged to a Quartz cuvette (model C208A, Biolin Scientific, Phoenix, Arizona, USA). Then, oil droplets (8 μ L) at variable concentrations of acetyl-TAG were generated at the tip of a needle (1 mL Luer tip, gastight Hamilton syringe). The droplets were submerged into the heavy aqueous phase and the images of the droplets were recorded for 1800 s. The interfacial tensions were calculated from

the analysis of the shape of droplets using the Young–Laplace equation by OneAttension software (NanoScience Instruments, Phoenix, AZ, USA). A reference baseline for γ of oil-water was initially obtained.

In addition to the interfacial tension, the surface rheology of the liquid/liquid interface was analyzed using a similar pendant drop (8 μL) immersed in protein solution under dynamic conditions. The piezoelectric pump of the tensiometer automatically fluctuated the drop volume as sinusoidal oscillations with the following parameters: frequency (0.5 Hz), waveform, amplitude (0.5), and time (60s). Preliminary experiments were done to determine the ideal oscillatory-drop parameters considering the significant interfacial area changes.

Changes in the droplet shape (μL), surface area (ΔA), and phase angle difference (γ) were automatically recorded and correlated to complex surface dilatational modulus ($|E|$, [mN/m) by Equation 1 where $|E|$ is the complex dilatational modulus, E' is the elastic modulus, E'' is the viscous modulus, γ is the surface tension, A is the droplet area, ω is the angular velocity and η is the viscosity.

$$|E| = E' + E'' = \frac{d\gamma}{d\ln A} = E_0 + i^* \omega^* \eta \quad (1)$$

Preparation of emulsions

The aqueous phase was prepared by dispersing whey protein hydrolysate (2.5% (w/v)) in distilled water (300 mL) under continuous stirring at ambient temperature for 2 h. Protein dispersions were stored overnight at 4°C to allow protein hydration. After hydration, protein dispersions were equilibrated at ambient temperature before mixing with the lipid phase. Then, the coconut oil was dispersed into the protein solution (15, 30 % (w/v)) and wild-type acetyl-TAG (0, 0.5, 1 % (w/v))

was introduced into this solution with vigorous stirring for 15 min. A high-speed mechanical blender was used to mix the dispersion at a rate of 250 rpm for 1 min at ambient temperature before it was homogenized using a 2-stage valve homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) at 500 bar (where the second stage is set to 50 bar and the total pressure is set to 500 bar with the first stage) for 2 passes.

Emulsion characterization

Determining the creaming index of emulsions

The creaming index of emulsions was tested to analyze stability following the method described in (McClements, 2007) with some slight modifications. The emulsions (15 mL) were weighed and sealed in glass vials with flat bottoms (20 mL) (Sigma Aldrich, Burlington, Massachusetts, USA) and stored for 21 days at ambient temperature. The images of the samples are given in Fig 4. The serum layer thickness (H_s) and the total emulsion height (H_e) were measured using ImageJ software (version 1.38e, Microsoft Java, NIH, Bethesda, MD, USA). The creaming index (CI) was calculated using Equation 2 where H_s and H_e are the serum layer and total emulsion thicknesses, respectively.

$$CI (\%) = \frac{H_s}{H_e} \times 100\% \quad (2)$$

Particle size and zeta potential of emulsions

Particle size and zeta potential of the emulsions were analyzed using a dynamic light scattering (DLS) particle size analyzer (Delsa Max Pro, Beckman Coulter, Kraemer Blvd. Brea, CA, USA) at 25 °C. Prior to analysis, emulsions were diluted in ultra-pure water at the 1:100 (w/v) ratio to avoid multiple scattering effects. The measurements were taken on days 0, 7, 15, and 21 and repeated twice for each replication and their average was used.

Percentage of adsorbed protein

The concentration of adsorbed protein at the oil-water interface was determined according to a modified protocol described by Lowry et. al (1951). Emulsion samples (10 mL) at day 0 and day 21 were centrifuged at 3000 x *g* for 30 min and the bottom (non-adsorbed) phase (3.5 ±0.1 g) was collected carefully using a borosilicate glass Pasteur pipette (Fisher Scientific, Waltham, Massachusetts, USA). The protein content was determined using the LECO TruMac N Nitrogen Analyzer (LECO Corporation, St. Joseph, MI, USA). Total protein concentration was calculated by multiplying the total combustion nitrogen with a factor of 6.38. The instrument was calibrated using a glycine standard. The percentage of adsorbed whey protein was calculated using the following Equation 3 where C_b and C_e are the protein concentrations and V_b and V_e are the volumes of the bottom phase and emulsion, respectively.

$$\text{Adsorbed protein (\%)} = \left(1 - \frac{C_b \times V_b}{C_e \times V_e}\right) \times 100 \quad (3)$$

Preparation of whipped emulsions

To prepare whipped samples, the emulsions were stored in refrigerated conditions overnight after homogenization. Then, the emulsions were aerated to form the foam using a 6-Speed Hand Mixer (Hamilton Beach Brand, Glen Allen, Virginia, USA) at the highest speed at 5-minute intervals three times. The aeration time was determined based on preliminary experiments.

Determination of whipped cream overrun

The overrun of whipped samples was calculated using the protocol described our earlier with some modifications (Campbell & Mougeot, 1999). The aerated creams and remaining serum phase were separated and transferred into beakers. The weights of each phase and unwhipped solution were

measured to calculate overrun (%) using Equation 4 where W_1 and W_2 are the weights (g) of unwhipped and whipped samples, respectively.

$$\text{Overrun (\%)} = \frac{W_1 - W_2}{(W_2)} \times 100 \quad (4)$$

Foam stability of whipped cream

The stability of whipped emulsions was determined by measuring foam drainage (FD) under gravity using the protocol described by Fredrick et al. (2013) with slight modifications. The freshly prepared samples (15 mL) were placed in a glass funnel with top and stem diameters of 75 and 8 mm, respectively. The funnel was positioned over a graduated cylinder (100 mL) at room temperature for 1 h. The drainage was calculated using Equation 5 where W_s and W_t are the weights (g) of the serum collected and whipped cream, respectively.

$$\text{Drainage (\%)} = \frac{W_s}{(W_t)} \times 100 \quad (5)$$

Statistical analysis

All samples were prepared in triplicate. The experimental data were analyzed using Minitab (version 17.1, LLC, State College, PA, USA) and the values were expressed as mean $\pm$ standard deviation. A one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to analyze significant differences among samples at $\alpha = 0.05$.

Results and Discussion

Interfacial rheology at oil-water interface

Interfacial tension is important for the characterization of surface properties, including interactions between surfactants and proteins and it is related to the formation and stabilization of emulsions. The dynamic interfacial tension at the oil-water interface was analyzed in a time-dependent manner

in Fig 6-1. The water-coconut oil interface was measured as a reference point. It was observed that the change of water with whey protein hydrolysate solution reduced the interfacial tension indicating that protein dictates the behavior of the interface. All the samples showed a decreasing trend of interfacial tension with increasing time of adsorption and reached adsorption equilibrium until the experimental period of 500 sec. The samples prepared with the addition of acetyl-TAG showed lower interfacial tension than the ones prepared without acetyl-TAG. In addition to this, increasing acetyl-TAG concentration from 0.5% to 1% (w/w) significantly reduced the interfacial tension. This can be attributed to the adsorption of acetyl-TAG at the interface. Similarly, Zhu et. al (2020) observed that soy glycinin and soyasaponin as a low molecular weight surfactant showed similar interfacial adsorption, and the interfacial tension reduced when soyasaponin concentration increased from 0.25% to 0.5%.

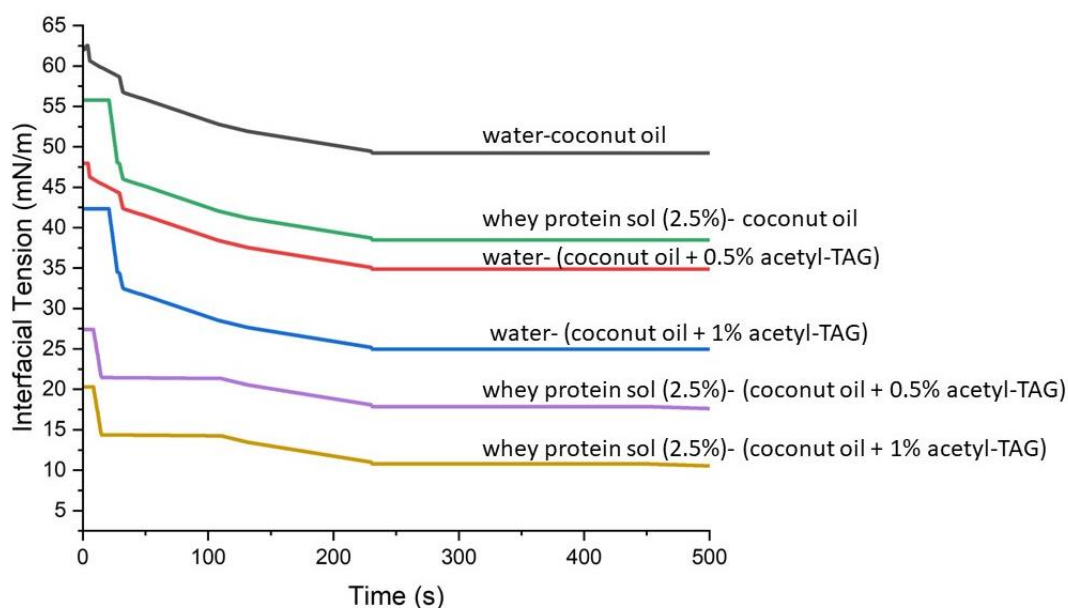


Figure 6-1 Changes in interfacial tension between coconut oil and acetyl-TAG oil phase mixture and aqueous phase (water or whey protein solution) at different acetyl-TAG concentrations (0, 0.5 and 1% (w/w)) as a function of adsorption time.

Interfacial rheology of adsorbed layers demonstrates adsorption of proteins and surfactants at the oil-water interface. Moreover, the viscoelastic properties of protein adsorbed layer at oil-water interface may reflect the emulsion stability (Kalogianni et al., 2017). The dilatational elastic modulus and complex surface dilatational modulus are given in Table 6-1.

The complex surface dilatational modulus ($|E|$) may reflect the mechanical strength of the protein interfacial adsorbed layer. $|E|$ values significantly ($P < 0.05$) higher in the protein-oil interface as compared to the water-oil interface which was also reflected in other stability results mentioned in the following sections. Furthermore, the addition of acetyl-TAG and increase in concentration from 0.5% to 1% (w/w) significantly enhanced ($P < 0.05$) $|E|$ values. These results were as expected due to adsorption and improved intermolecular strength by surface active whey protein and acetyl-TAG. In parallel to $|E|$ values, dilatational elastic modulus (E') values significantly increased with whey protein and the addition of acetyl-TAG which helped to strengthen the interfacial film and increase the viscoelasticity. A recent study also supported that the addition of polymeric lignin surfactant significantly increased the adsorption at the xylene-water interface, thus improving the elasticity (Ghavidel & Fatehi, 2021).

Table 6-1 Elastic and complex dilatational modulus between coconut oil and acetyl-TAG oil phase mixture and aqueous phase (water or whey protein solution) at different acetyl-TAG concentrations (0, 0.5 and 1% (w/w)). The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between samples

Aqueous and Oil Phase	Elastic modulus (E', MPa)	Complex Surface Dilatational Modulus (E , mN/m)
Control 1 (water-coconut oil)	19.81 $\pm$ 1.13 ^f	33.92 $\pm$ 0.17 ^d
Control 2 (whey protein sol-coconut oil)	22.64 $\pm$ 0.53 ^e	39.86 $\pm$ 0.09 ^{cd}
water-coconut oil + 0.5% acetyl-TAG	31.60 $\pm$ 1.02 ^d	40.13 $\pm$ 0.03 ^c
water-coconut oil + 1% acetyl-TAG	32.98 $\pm$ 0.74 ^c	45.21 $\pm$ 0.11 ^b
whey protein sol-coconut oil + 0.5% acetyl-TAG	36.65 $\pm$ 0.44 ^b	45.30 $\pm$ 0.19 ^b
whey protein-coconut oil + 1% acetyl-TAG	40.28 $\pm$ 0.41 ^a	56.78 $\pm$ 0.08 ^a

Emulsion characterization

Particle size and zeta potential are critical factors that relate to their emulsion stability. The addition of acetyl-TAG and storage time had a significant ($P<0.05$) effect on particle size and zeta potential of emulsions. The particle size of emulsions ranged between 179-535 nm (Fig. 6-2A). Overall, the addition of acetyl-TAG significantly ($P<0.05$) reduced the particle size and the particle size decreased ($P<0.05$) further with an increase in the concentration of acetyl-TAG from 0.5% to 1% (w/w). For instance, the average particle size was reduced by nearly 15% when 1% acetyl-TAG was added into the 15% (w/v) coconut oil phase to prepare fresh emulsions. This indicated that acetyl-TAG with its surface activity served as an emulsifier and was tightly adsorbed at the oil-water interface, preventing aggregation of coconut oil globules, and reducing interfacial tension which was also confirmed in the interfacial rheology section. This observation was consistent with a recent study where the authors demonstrated a similar emulsifier effect of glyceryl monooleate at the interface of anhydrous milk fat-coconut oil-based emulsions (Cheng et al., 2020). Furthermore, the particle size of storage emulsions gradually increased ($P<0.05$) as compared to fresh emulsions which was due to the droplet coalescence. On the other hand, among all emulsion samples, emulsion prepared with 15% (w/v) coconut oil and the addition of acetyl-TAG showed the lowest ($P<0.05$) increase in particle size with 1% (w/w) acetyl-TAG having the smallest average particle size. This indicated that acetyl-TAG was able to stabilize the emulsions during storage time.

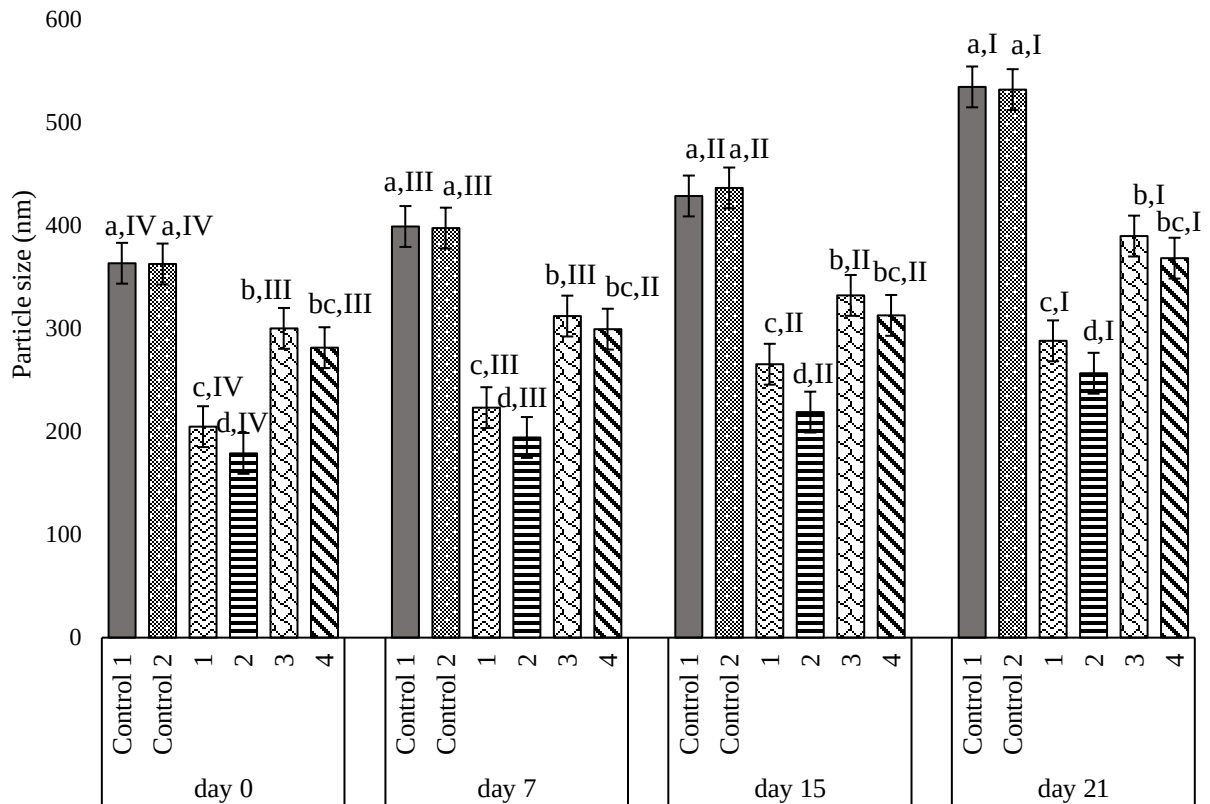


Figure 6-2 Particle size (nm) of emulsions prepared with different concentrations of acetyl-TAG with respect to storage time. Control 1 and control 2 represent emulsions prepared with 15% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v) and 30% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v), respectively 1: 15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 2: 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG, 3: 30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 4: 30% (w/v) coconut oil and 1% (w/w) acetyl-TAG. The results are expressed as mean value $\pm$ SD ($n = 3$). The lower letter superscript indicates the significant differences between emulsion samples in each storage time. The roman letter shows the differences between storage time for each emulsion

In addition, zeta potential indicates the magnitude of particle charge and reflects the strength of the electrostatic interactions between particles; stronger electrostatic repulsion contributes to the stability of particles (Low et al., 2020). The zeta potential values ranged from -19 mV to -51 mV (Fig. 6-2B). In parallel to the particle size data, small particles of emulsions showed higher zeta

potential values, which supported greater stability. Similar to particle size data, the addition of acetyl-TAG and storage time had also a significant ($P<0.05$) effect on the zeta potential of emulsions. The addition of acetyl-TAG and further increase in concentration from 0.5% to 1% (w/w) significantly ($P<0.05$) increased the zeta potential indicating enhanced electrostatic interactions between whey protein hydrolysate molecules, coconut oil, and acetyl-TAG. Acetyl-TAG probably acts as a bridging agent for the whey protein molecules resulting in promoted aggregation of coconut oil droplets via electrostatic attraction with smaller particle size and the higher zeta potential. Similarly, Yan et. al (2022) observed that the addition of polyglycerol esters (0.7%) as emulsifiers significantly ($P<0.05$) reduced the particle size and increased the absolute value of zeta potential of anhydrous milk fat-cellulose microcrystal emulsion. Moreover, it was not surprising to observe that storage time had an adverse effect on the stability of emulsions: zeta potential decreased ($P<0.05$) for all emulsions with the storage time which was parallel with the increase in particle size.

Protein adsorption is another important parameter to understand the protein-small molecule interactions at the oil-water interface and the results are given in Fig 6-4. Overall, the addition of acetyl-TAG at both low and high concentrations (0.5 and 1% (w/w)) significantly ($P<0.05$) reduced the protein adsorption at the oil-water interface. For example, fresh (day 0) whey protein emulsion prepared with the addition of 30% coconut oil and 1% (w/w) acetyl-TAG (sample 4) exhibited nearly 38% lower adsorbed protein concentration as compared to fresh emulsion prepared with the same amount of oil without the addition of acetyl-TAG (control 2).

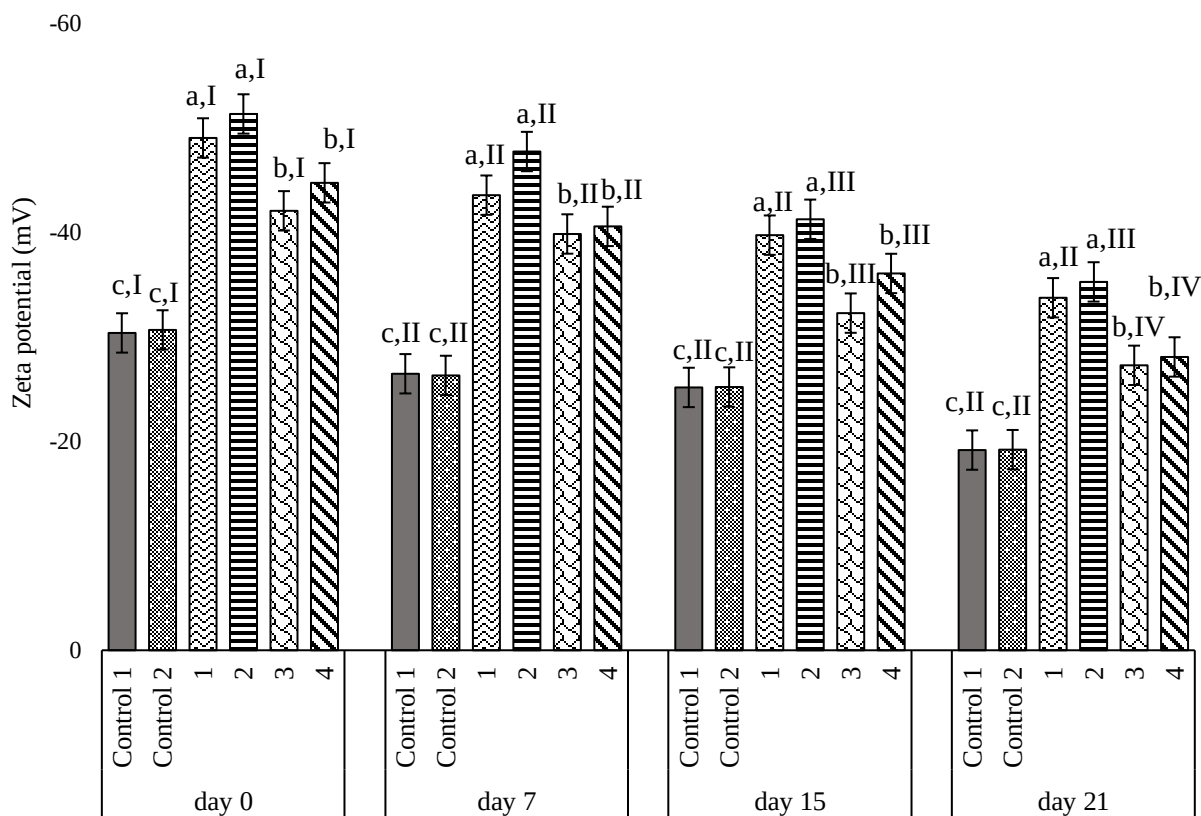


Figure 6-3 Zeta potential (mV) of emulsions prepared with different concentrations of acetyl-TAG with respect to storage time. Control 1 and control 2 represent emulsions prepared with 15% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v) and 30% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v), respectively. 1: 15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 2: 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG, 3: 30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 4: 30% (w/v) coconut oil and 1% (w/w) acetyl-TAG. The results are expressed as mean value $\pm$ SD ($n = 3$). The lower letter superscript indicates the significant differences between emulsion samples in each storage time. The roman letter shows the differences between storage time for each emulsion

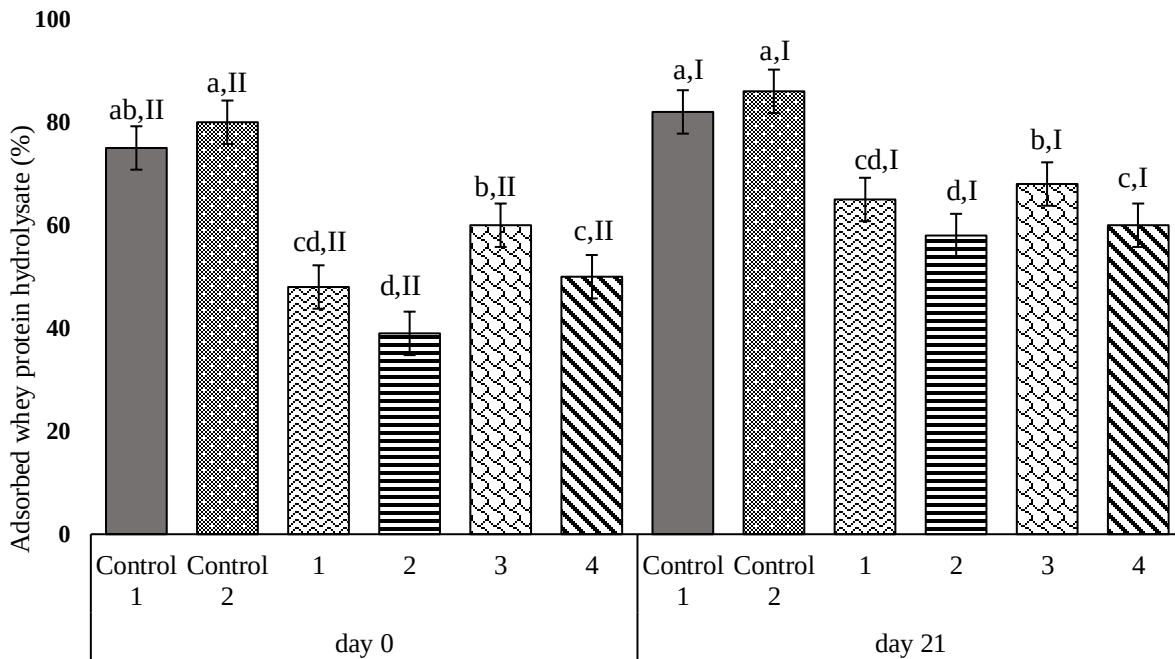


Figure 6-4 Adsorbed protein (%) of emulsions prepared with different concentrations of acetyl-TAG at day 0 and day 21. Control 1 and control 2 represent emulsions prepared with 15% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v) and 30% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v), respectively. 1: 15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 2: 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG, 3: 30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 4: 30% (w/v) coconut oil and 1% (w/w) acetyl-TAG. The results are expressed as mean value $\pm$ SD (n = 3). The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between emulsion samples in each storage time. The Roman letter shows the differences between storage times for each emulsion.

Acetyl-TAG molecules demonstrate a fast adsorption rate and a low surface tension and tend to have high surface activity (Liu et al., 2023). Therefore, they might act in the displacement of protein from the interface (Zhao et al., 2014). Due to increased electrostatic repulsion between protein, oil, and acetyl-TAG particles adsorption of protein might reduce at the oil-water interface which can be also correlated with the creaming index (Figure 6-5). Our results were in accordance with a study where the authors showed a similar effect of sodium stearoyl lactylate on caseinate emulsions (Jiang et al., 2018). In addition to this, storage time ($P < 0.05$) significantly increased the

adsorbed protein (%) and this could be supported by reduced stability and creaming occurring as the top layer. Following the protein adsorption (%) results, the creaming index describes the upward motion of droplets due to their low density as compared to the aqueous phase. Similar to the other physical analysis results, the creaming index of emulsions was significantly affected by acetyl-TAG. The addition of acetyl-TAG significantly reduced ($P<0.05$) reduced the creaming index of emulsions which was the indicator of higher stability against gravity.

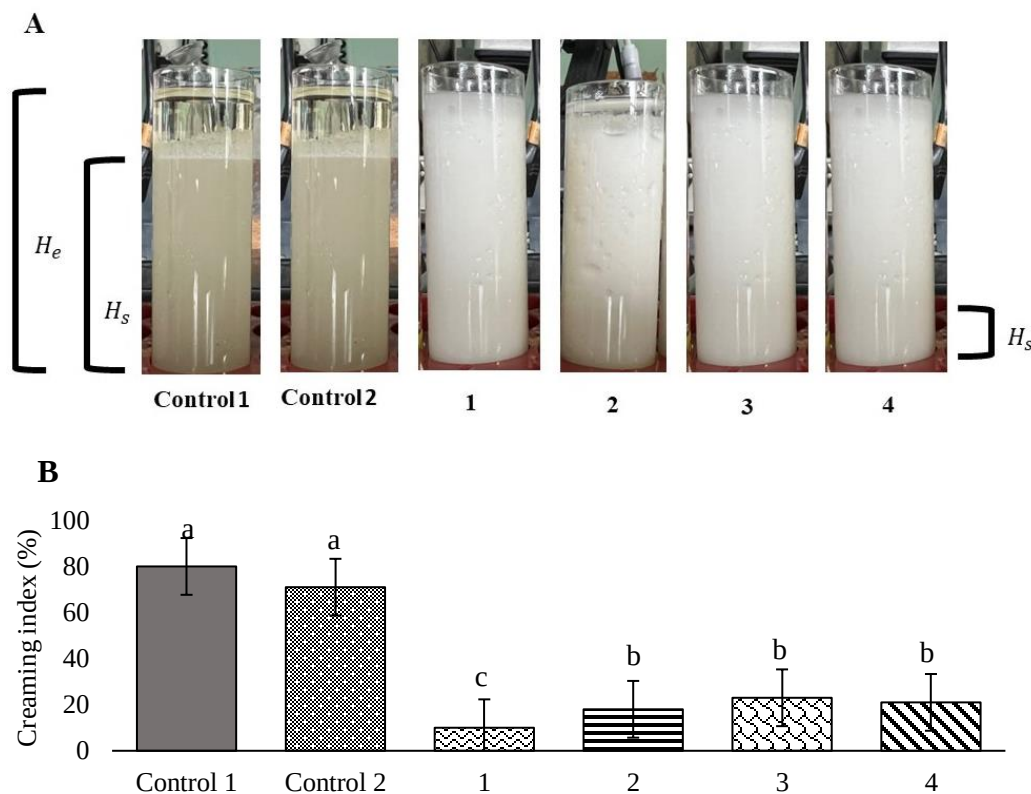


Figure 6-5 Creaming index of emulsions prepared with different concentrations of acetyl-TAG at the end of the storage time (day 21). Control 1 and control 2 represent emulsions prepared with 15% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v) and 30% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v), respectively, respectively. 1: 15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 2: 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG, 3: 30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 4: 30% (w/v) coconut oil and 1% (w/w) acetyl-TAG. The results are expressed as mean value $\pm$ SD ($n = 3$). The lower letter superscript indicates the significant differences between emulsion samples at the end of day 21.

Whipped emulsion characterization

Whipping of oil in water emulsion provided arrangement of particles through the interface layer and produced air bubbles stabilized by the agglomeration of proteins and fat globules (van Lent et al., 2008). The whipping properties such as overrun, and serum drainage are important factors reflecting the stability of aerated emulsions. The overrun and drainage values of whipped emulsions are given in Table 6-2.

Overrun is an essential parameter showing the capacity of air bubbles entrapped during whipping. The overrun (%) was significantly ($P<0.05$) affected by the addition of acetyl-TAG. The addition of acetyl-TAG significantly ($P<0.05$) increased the overrun and reduced ($P<0.05$) the drainage of whipped emulsions indicating that acetyl-TAG helped to stabilize the oil droplets and enable air bubbles to develop quickly. The highest overrun (%) was observed when emulsion was prepared using 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG. This can be explained by the ability of a small molecule surfactant, acetyl-TAG, to cover the newly forming surfaces at a faster rate as compared to large protein polymers during whipping to result in more volume and smaller droplets which was also consistent with our particle size analysis (Yang et al., 2022). A recent study also showed a similar effect of low-molecule emulsifiers such as glycerol monostearate on whipped sodium caseinate emulsion (Liu et al., 2022b). On the other hand, overrun values significantly ($P<0.05$) reduced when the coconut oil concentration increased from 15% to 30% (w/v) with the addition of 1% (w/w) acetyl-TAG. This was probably due to the large aggregation of free oils that caused disruption of bubbles thereby resulting in a decline of overrun (Zhao et al., 2013).

Foam drainage is the flow of the aqueous serum separating adjacent air bubbles through the thin interstitial space, leading to thinning of the gap between bubbles and leading to coalescence and

foam breakdown. This phenomenon is driven by gravity and slowed by bulk viscosity and surface viscosity, and the lower drainage rate indicates higher stability against gravity. In parallel to overrun, the addition of acetyl-TAG ($P < 0.05$) reduced the drainage against gravity. For example, the drainage decreased by 35% when 0.5% (w/w) acetyl-TAG was introduced to the 15% (w/v) coconut oil phase due to reduced rate of film drainage and rupture in foams (Dickinson, 2003). Acetyl-TAGs are small molecules that co-adsorb at the interface and increase surface film density. In addition to this, they enhanced the interfacial area via rapid diffusion during foam formation which increases the curvature and, thus, improves the stability. This was in clearly accordance with the interfacial rheology results. On the other hand, an increase in both coconut oil (30% (w/v)) and acetyl-TAG (1% (w/w)) increased drainage probably due to bubble coalescence and collapse of the foam.

Table 6-2 Capacity (overrun %) and stability (drainage %) of whipped emulsions. Control 1 and control 2 represent emulsions prepared with 15% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v) and 30% (w/v) coconut oil-whey protein hydrolysate (2.5% w/v), respectively. 1: 15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 2: 15% (w/v) coconut oil and 1% (w/w) acetyl-TAG, 3: 30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG, 4: 30% (w/v) coconut oil and 1% (w/w) acetyl-TAG. The results are expressed as mean value $\pm$ SD (n = 3). The lower letter superscript indicates the significant differences between emulsion samples in each storage time. The roman letter shows the differences between storage time for each emulsion.

whipped emulsions	Overrun (%)	Serum drainage (%)
Control 1	233.64 $\pm$ 0.34 ^d	38.34 $\pm$ 0.32 ^{bc}
Control 2	224.41 $\pm$ 0.52 ^e	42.73 $\pm$ 0.61 ^b
15% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG	300.39 $\pm$ 1.14 ^b	24.80 $\pm$ 0.33 ^d
15% (w/v) coconut oil and 1% (w/w) acetyl-TAG	325.33 $\pm$ 0.93 ^a	21.28 $\pm$ 1.28 ^d
30% (w/v) coconut oil and 0.5% (w/w) acetyl-TAG	242.12 $\pm$ 0.82 ^c	31.81 $\pm$ 0.82 ^c
30% (w/v) coconut oil and 1% (w/w) acetyl-TAG	201.75 $\pm$ 0.97 ^f	52.27 $\pm$ 0.51 ^a

Conclusion

The findings in this study are original and demonstrate that wild-type acetyl-TAG can be used as an effective emulsion stabilizer and improve the whipping characteristics of aerated oil in water emulsions. The interfacial rheology at oil water interface and unwhipped and whipped emulsion characteristics can be improved by using acetyl-TAG. For example, acetyl-TAG can significantly reduce the interfacial tension and increase ($P < 0.05$) the mechanical strength of the protein interfacial adsorbed layer. In addition to this, an increase in the concentration of acetyl-TAG from 0.5% to 1% (w/w) significantly ($P < 0.05$) reduced the creaming index and average particle size by *ca.* 15 % as an indicator of effective stabilizer in oil in water emulsion systems. In addition to this, the addition of acetyl-TAG significantly ($P < 0.05$) increased the overrun and reduced the serum drainage of whipped emulsions. This indicated that acetyl-TAG with its surface activity served as an effective emulsifier and stabilizer and tightly adsorbed at the oil-water interface, preventing aggregation of coconut oil globules, and preserving the foam stability in whipped emulsions.

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Chapter 7 - Sorghum Oleoresins: Effect of Extraction on Compositional and Structural Characteristics (*published in JAOCS*)

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Abstract

Oleoresins are resin-like viscous materials obtained from plants, oilseeds, or spices with functional properties. The objectives of this study were to obtain oleoresins from waxy burgundy sorghum grain and to characterize the effects of the extraction process and solvents on their composition and functional properties. Oleoresins were obtained by using the following solvents with and without ball milling: two types of novel ionic liquids (IL₁: 1-n-Hexyl-3-methylimidazoliumchloride, IL₂: 1-Ethyl-3-methylimidazoliumchloride), ethanol and dichloromethane. The effects of processing were evaluated for the extraction yield, protein, fat, and total phenolic content, fatty acid composition, particle size and zeta potential, and FTIR spectra. The use of ILs and the ball mill process significantly ($P < 0.05$) affected the extraction yield and physicochemical properties. The highest extraction yields increased ($31.35\% \pm 0.58$) when ball milling was used with IL₂ in comparison to the lowest ($18.37\% \pm 0.77$) obtained by traditional ethanol extraction. Similarly, protein concentration and phenolic content were the

highest ($1.37\% \pm 0.05$ and $0.57\% \pm 0.01$, respectively) with ball milling extraction and IL₁. The FTIR spectra indicated higher phospholipids (at 1200 cm^{-1}) and protein-phospholipid bonding (at 1700 cm^{-1}) by ILs, and ball milling as compared to traditional extraction. Overall, wet milling-assisted extraction by using a ball mill and ILs provided control over the composition of the oleoresins important for value-added food applications and higher extraction efficiencies as compared to traditional techniques.

Introduction

Oleoresins are phytocomplexes extracted from plant sources, herbs, and oilseeds as oil-rich viscous materials. These resinous extracts include terpenoids, fatty acids, phospholipids, essential oils, proteins, and phenolic compounds offering great potential for value-added food applications (Napoli et al., 2019). Oleoresins carry surface active molecules, such as phospholipids and proteins. The interactions between these proteins, triacylglycerols, and phospholipids are responsible for the integrity and stability of their dispersions and emulsions (Wijesundera & Shen, 2014).

The extraction method plays an important role in the stability, composition, yield, and structural characteristics of oleoresins. Conventional solvent extraction involves the use of organic solvents, such as ethanol, hexane, or ethyl acetate, to simultaneously obtain volatile and non-volatile phytochemicals with functional properties (Khasanah et al., 2017). Wet milling strategies, such as ball milling, can be used to improve extraction efficiency by breaking solid enclosures where the oleoresins are trapped. For example, ball milling was used to extract sesame paste from seeds with improved viscosity and reduced particle size (Jin et al., 2022).

In addition to the extraction method, the type of solvent determines the extraction efficiency and physicochemical properties of the oleoresins. The poly-ionic liquids (ILs) are molten organic salts below 100°C, which combine the characteristics of different ionic chemicals, and received considerable attention as substitutes for volatile organic solvents due to their versatility, less toxicity, remarkable solubilization for organic and inorganic compounds (Tolesa et al., 2017). ILs offer great potential to enhance the extraction efficiencies for complex materials, such as oleoresins containing biopolymers, surface active molecules and small phytochemicals and to provide control over their composition. Among the four general groups (imidazolium, phosphonium, pyridinium, and ammonium), imidazolium based ILs were widely used for their low viscosity and stability in oxidative and reductive conditions. In addition, they can also provide a high affinity for lipids and phenolic molecules as compared to conventional organic solvents, such as ethyl acetate, methanol, and hexane. The objectives of this study are to investigate the combined effects of wet milling (i.e., ball milling) and novel ILs (1-n-Hexyl-3-methylimidazolium chloride, 98%, and 1-Ethyl-3-methylimidazolium chloride, 97%) on the extraction efficiency of oleoresins from waxy sorghum rich in phenolics and evaluate their yield, composition (protein, lipid, fatty acid, phenolic contents) and structural properties of their dispersions (particle size, zeta potential, and FT-IR spectrum) in comparison to conventional solvent extraction (i.e., ethanol and dichloromethane).

Materials and Methods

Materials

The extraction solvents ethanol (99.5%, ACS reagent), dichloromethane (99.5%, ACS reagent), and ionic liquids (ILs) (IL₁: 1-n-Hexyl-3-methylimidazolium chloride, 98% and IL₂: 1-Ethyl-3-methylimidazolium chloride, 97%) were purchased from Fisher Scientific (Fisher Scientific,

Waltham, MA, USA). The selection of ionic liquids was based on previous literature for higher affinity for polar lipids and phenolic molecules. The solutions of ILs (40 mL) were prepared by mixing two ionic liquids with ethanol (99%) as co-solvent at a 1:2 (w/v) ratio. The waxy burgundy whole grain sorghum was kindly donated by Nu Life Market LLC (Nu Life Market LLC, Scott City, KS, USA).

Preparation of oleoresin

Oleoresin was extracted from ground waxy burgundy whole sorghum grain with or without wet milling using traditional (ethanol, dichloromethane) and novel solvents (IL₁ and IL₂). The traditional extraction without ball milling was done following the method of Khasanah et al. (2017) with some modifications. A batch of whole grain sorghum (50 g) was soaked in NaHCO₃ (0.1 M) at a 1:1 (w/v) ratio of 12 h at 21 ± 2 °C. The soaked sorghum grains were ground using a high-shear blender (Waring Commercial, Model 7011BU, McConnellsburg, PA, USA) at the highest speed (22000 rpm) with three-time intervals of 5 min. The ground sorghum (10 g) and the solvent (40 mL) were mixed using a magnetic stirrer for 30 min. The slurry was filtered through a cheesecloth to remove the undissolved solids. The filtrate was centrifuged at 3000 x *g* for 15 min at 20 ± 2 °C to separate the oleoresin-rich organic top layer, which was collected in hermetically sealed glass tubes. The extraction from the filtrate was repeated three times until no sediment was visible. The weight of sediment and the organic phase were recorded in each dilution step for the calculation of the extraction yield. The oleoresins were concentrated by removing the solvent using a refrigerated vapor trap (Savant™ SpeedVac™, RVT5105, Model SPD300DDA, Thermo Fisher Scientific, Marietta, OH, USA). The wet milling extraction was done using the same sorghum and solvent mixture by using a ball mill (Retsch, PM 100 type, Retsch GmbH, Germany) equipped with a milling bowl (250 mL) and ceramic balls (diameter:10 mm; quantity: 100 pieces) and

operated at 500 rpm with 2 cycles of 15 min. The filtrate containing the oleoresin was separated and concentrated similar to traditional extraction.

Oleoresin characterization

Extraction percentage yield

The extraction yield was calculated following the Equation 1:

$$\text{Extraction yield (\%)} = \frac{wt_e}{wt_a} \times 100 \quad (1)$$

where wt_e is the total weight of the extracted oleoresin and wt_a is the weight of the whole grain sorghum.

Total protein content

The total protein content was analyzed with the Dumas technique at the Soil Testing Laboratory, Kansas State University (LECO-Corp, 2005). The oleoresins (0.15 ± 0.01 g) were combusted, and the nitrogen content was measured on a weight percent basis by using a LECO TruSpec CN Combustion Analyzer (TrueSpec Micro, LECO, St. Joseph, MI, USA). EDTA was used as the calibration protein, and the total protein content of samples was calculated by multiplying the total combustion nitrogen with a factor of 6.25.

Total lipid analysis

Total lipid content was determined following a gravimetric method (Yucel et al., 2023). An aliquot of oleoresins (1 g) was added in a dry glass tube (15 mL) with chloroform (2 mL) and methanol (4 mL) and sealed with Teflon-lined screw caps. The tube was shaken well for 10 min at 325 rpm. Then, additional chloroform (2 mL) and water (2 mL) were added, and the tube was shaken for another 10 min. Then, the tube was centrifuged at 2300 rpm for 10 min to separate the organic

phase containing the lipid from the aqueous phase. The organic top layer was collected using a Fisherbrand™ borosilicate glass Pasteur pipet (Fisher Scientific, Waltham, MA, USA). Additional chloroform (4 mL) was added to the tube, centrifuged at 2300 rpm, and the top layer with lipid collected. The solvent was evaporated at 60 °C, and the total lipid in the oleoresin sample was calculated using Equation 2:

$$\text{Total lipid content (wt\%)} = \frac{(W_2 - W_1) \times V_1 \times 100}{(V_a \times S_x)} \quad (2)$$

where W_2 is the total weight of the glass tube and the extract (g), W_1 total weight of the empty dried glass tube (g), V_1 is the total volume of the chloroform layer (mL), V_a is the volume of the extract (mL) and S_x is the weight of the oleoresin (g).

Fatty acid composition

The fatty acid composition was measured using the fatty acid methyl esters (FAME) method described by Yucel et al. (2023). In brief, an aliquot of oleoresin (1 mL) was transferred into a glass test tube (15 mL) with boron trifluoride (14%) in methanol (3 mL) and heated at 100 °C for 40 min. Saturated NaCl solution (15 mL) and hexane (3 mL) were added, and the tube was inverted 3 times. The mixture was allowed to settle for a few minutes for the separation of organic and aqueous phases. The organic top layer was collected using a Pasteur pipette into a clean test tube, where a small amount (1 g) of anhydrous sodium sulfate was added to remove any residual moisture. The methylated organic layer (1 µL) was injected into a GC-MS system (HP 5890–5973 MS, Agilent) equipped with an HP-23 FAME capillary column (30 m length x 0.25 mm i.d. x 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA, USA). Helium (1 mL/min) was used as the carrier gas. The initial oven temperature was 60 °C for 0.5 min, then brought to 150 °C at a rate of 5 °C / min, followed by an increase of 15 °C /min to a final temperature of 230 °C. The

program was finalized with a hold at 230 °C for 4 min. In addition to the National Institute of Standards and Technology (NIST) 2014 database, a FAME standard (> 95 %, FAME Standard Mixture, St, Louis, MO, USA) over a concentration range of 1.25-10 mg/mL was used for identification and quantification. The quantification was performed by using the peak areas.

Phenolic content and composition

The total phenolic content was determined using the Folin Ciocalteu method (Lamuela-Raventós, 2017). The calibration curve was plotted by mixing aliquots (1 mL) of Gallic acid solutions over a range of concentrations (800, 400, 200, 100, 50, 25, 12.5, and 6.25 µg/mL) with Folin Ciocalteu reagent (5 mL) and anhydrous sodium carbonate solution (4 mL). The phenolic content of the oleoresin was measured by mixing the sample (2.5 mL) and Folin Ciocalteu-anhydrous sodium carbonate reagents at the 1:1 (v/v) ratio. The solution was kept for 1 h at 25°C. The absorbance was measured at 725 nm using a spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of oleoresin (mg GAE/g oleoresin) using Equation 3:

$$C = C_1 \times \frac{V}{m} \quad (3)$$

where C is the total phenolic content (mg/g) in GAE, C_1 is the concentration of gallic acid established from the calibration curve in mg/mL, V, and m are the volume and weight of oleoresin, respectively.

The phenolic compounds were determined using the protocol described by (Tohma et al., 2017). The samples were prepared by mixing oleoresin (20 mg) with a mixture (2mL) of methanol: ethanol (90:10 v/v) in a glass tube with a screw top (15 mL) and shaken in a wrist shaker for 1 h. Then, the solution was filtered with a PTFE filter (0.45 µm) and transferred to a 1 mL polypropylene vial (12 mm x 32 mm). The analysis was conducted using an HPLC system (Agilent

Technologies, 1100 series, Santa Clara, CA, USA) equipped with a C18 column (TOSOH Bioscience LC, TSKgel ods-80, 4.6 mm ID×25 cm, 5µm Millipore Sigma, MA, USA) and a diode-array detector (DAD) (Hewlett-Packard, Palo Alto, CA, USA) operated at 260-430 nm. The mobile phase (elution rate of 1.0 ml/min) was composed of acetonitrile (solvent A), methanol (solvent B), and water with acetic acid (solvent C) with a gradient system as follows: 0-5 min 5-8% A, 5-8% B and 90-84% C; 5-15 min 8-10% A, 8-10% B and 84-80% C; 15-25 min 10-25% A, 10-0% B and 80-75% C; 25-35 min 25-30% A, 75-70% C; 35-45 min 30-60% A and 70-40% C. The injection volume was 20 µL and the column temperature was 30°C. Each phenolic compound was identified by its retention time in comparison with standard compounds. The peak areas were quantified using Chem Station software as areas under the curve and calibration curve prepared by their analytical standards. The concentration of each phenolic compound in oleoresin samples was determined using Equation 4:

$$\text{Concentration of compound} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard} \quad (4)$$

Fourier transform infrared (FTIR) analysis

The FTIR analysis was conducted at the Center for Grain and Animal Health Research (USDA-ARS, Manhattan, KS, USA). Attenuated total reflectance (ATR)-FTIR spectra collected at 400–4000 cm⁻¹ range with a resolution of 4 cm⁻¹, data spacing of 0.482 cm⁻¹, and 32 scans using a Thermo Nicolet iS50 FTIR (Thermo Fisher Scientific Co., Waltham, MA) spectrometer equipped with a single-bounce diamond crystal and a deuterated triglycine sulfate detector. Measurements were repeated twice for each sample at ambient temperature. The quantitative analysis was done by “peak fitting” procedure and individual component bands according to a Gaussian curve fit (GRAMS/AI™ Spectroscopy Software Suite, Thermo Scientific™, Waltham, MA, USA).

Particle size and zeta potential of oleoresin

Particle size and zeta potential were analyzed using a dynamic light scattering (DLS) particle size analyzer (Delsa Max Pro, Beckman Coulter, Kraemer Blvd. Brea, CA, USA) at 25 °C. Before analysis, oleoresins were diluted in ultra-pure water at the 1:100 (w/v) ratio and dispersed using an ultrasound bath for 10 min and a high-shear homogenizer (Omni THModel TH-115, Omni International, Kennesaw GA, USA) for 10 min with inner and outer probe diameters of 7 mm x 125 mm (de Aguiar et al., 2021). The measurements were repeated twice for each replication and their average was used.

Statistical analysis

All samples were prepared in triplicate. The experimental data were analyzed using Minitab (version 17.1, LLC, State College, PA, USA) and the values were expressed as mean $\pm$ standard deviation. A one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to analyze significant differences among samples at $\alpha = 0.05$.

Results and Discussion

Effect of extraction conditions on yield

The solvent type had a significant ($P < 0.05$) effect on the extraction yield of oleoresins (Fig. 1). Overall, ILs increased ($P < 0.05$) the extraction yield (25.11 ± 0.84 wt% for IL₁ as compared to 18.37 ± 1.34 wt% for ethanol) via traditional extraction (i.e., without ball milling) (Fig. 7-1). The higher extraction efficiencies with the imidazolium based ILs were as expected due to the combined extractive power of the composite systems. Similarly, other researchers showed that imidazolium based ILs can increase the extraction yield of bioactive phytochemicals, such as flavonoids, alkaloids, caffeine, etc., from plant tissue (Bogdanov & Svinyarov, 2013; Claudio et al., 2013). The increase in the extraction efficiencies can be explained by the combination of dipole, ionic, and hydrophobic interactions of the IL components.

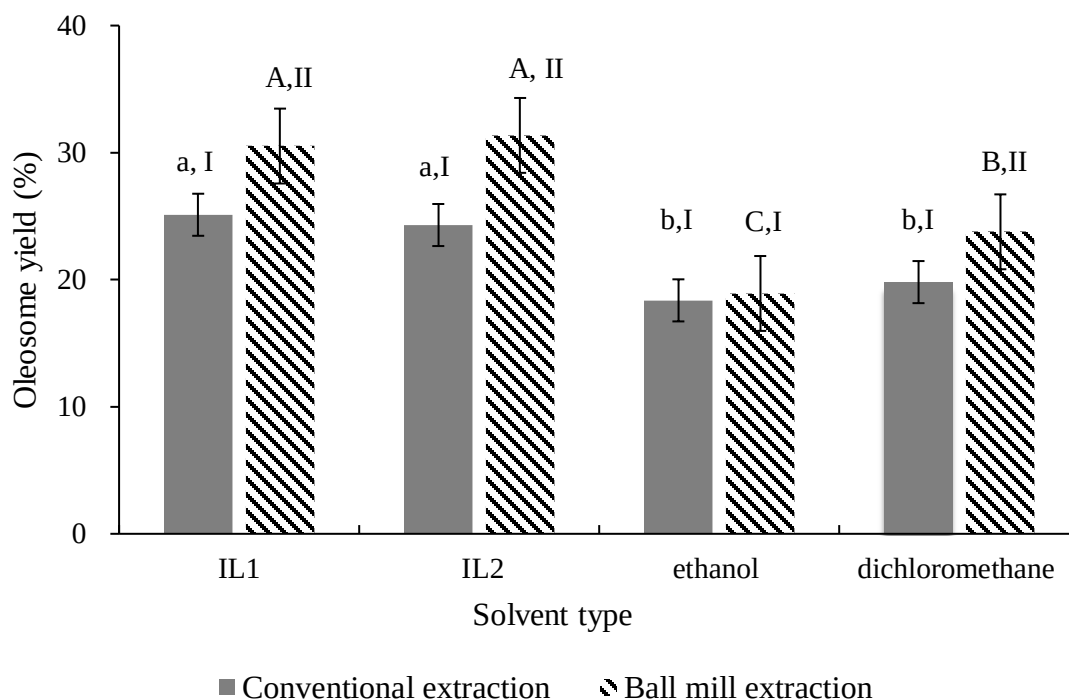


Figure 7-1 Oleoresin extraction yield (%). The results are expressed as mean value $\pm$ SD ($n = 3$). The lower and upper superscripts indicate the significant differences between the types of solvent in each extraction process (conventional or ball mill extraction). The Roman letter shows the differences between the type of extraction for each solvent. The bars show the standard deviation for each extraction condition and solvent type.

In parallel to the extraction efficiency, lipid, protein, and phenolic compositions were also significantly ($P < 0.05$) affected by the extraction conditions as discussed in the following sections (Table 7-1). The efficiency of traditional solvent extraction with ethanol and dichloromethane was the same ($P > 0.05$); however, the compositions of their oleoresin extracts were different. There was no significant difference ($P > 0.05$) in the extraction yield of the two ILs as well (Fig. 7-1). Thus, the difference between the type of charged groups in ILs was not responsible for breaking the solid enclosure required for extraction. In a recent study, Cheng et al. (2020) used different ILs with 11 anions and 8 imidazolium-based cations including the ILs in our study for extraction of isoflavone compounds from *Puerariae lobatae*. They showed that the extraction efficiencies of

the ethyl and hexyl substituted imidazolium ILs were the same yet changed with ion concentration in their solution. In addition, the chemical difference between the two ILs affected the oleoresin composition (Table 1) as discussed later. The wet milling process with a ball mill significantly ($P < 0.05$) increased the extraction yield as compared to traditional solvent extraction. For example, the extraction yield increased by more than 50% when IL₂ was used with a ball mill (31.36 ± 1.01 wt%) as compared to traditional batch extraction (18.92 ± 0.68 wt%) (Fig. 7-1). This can be explained by the ability of the ball mill to break down the integrity of the hard enclosure trapping the oleoresins via the omnidirectional impact of the beads and associated high shear.

Oleoresin Composition

In parallel with the extraction efficiency results, it was not surprising that the solvent type significantly ($P < 0.05$) affected the total lipid, protein, and phenolic content of oleoresins. Following the traditional solvent extraction approach, the protein, lipid, and phenolic contents were the highest with IL₁, surpassing the lowest values obtained with ethanol by ca. 85%, 30%, and over 100%. (Table 1). The higher increase in protein extraction capacity can be explained by the ionic interactions between proteins and anionic and cations of the ILs. Wet milling extraction using a ball mill increased ($P < 0.05$) the total lipid (ca. 20%), protein (ca. 100%), and phenolic content (ca. 20%) of the oleoresins when IL₁ was used as compared to the traditional extraction (Table 7-1). Similarly, Jin et al. (2022) also found that using ball mills increased the overall content of protein, fat, and volatile compounds in the sesame seed extracts as compared to colloid and stone mills. There was no difference ($P > 0.05$) between IL₁ and IL₂ for the extraction of lipid, protein, and phenolic fractions.

Table 7-1 Total protein (wt%), total lipid (wt%) and phenolic (mg GAE/g) contents of oleoresins extracted from sorghum grain using different methods. The results are expressed as the mean $\pm$ standard deviation. The lower and upper superscripts indicate the significant differences between the solvent type for conventional solvent or ball mill-assisted extraction processes, respectively. The Roman numerals show the differences between the type of extraction for each solvent.

Solvent type	Conventional extraction			Ball milling		
	Total protein (wt %)	Total lipid content (wt %)	Total phenolic content (mg GAE/g)	Total protein (wt%)	Total lipid content (wt%)	Total phenolic content (mg GAE/g)
IL ₁	0.67 $\pm$ 0.03 ^{a,I}	69.22 $\pm$ 0.04 ^{a,I}	48 $\pm$ 0.01 ^{a,I}	1.37 $\pm$ 0.05 ^{A,II}	82.19 $\pm$ 0.07 ^{A,II}	57 $\pm$ 0.01 ^{A,II}
IL ₂	0.58 $\pm$ 0.01 ^{a,I}	66.33 $\pm$ 0.06 ^{a,I}	42 $\pm$ 0.01 ^{b,I}	1.32 $\pm$ 0.06 ^{A,II}	80.26 $\pm$ 0.08 ^{A,II}	52 $\pm$ 0.02 ^{A,II}
dichloromethane (GC grade, 99%)	0.36 $\pm$ 0.02 ^{c,I}	57.26 $\pm$ 0.02 ^{b,I}	29 $\pm$ 0.02 ^{c,I}	1.06 $\pm$ 0.01 ^{C,II}	76.32 $\pm$ 0.04 ^{B,II}	32 $\pm$ 0.01 ^{B,II}
ethanol (GC grade, 99%)	0.43 $\pm$ 0.01 ^{b,I}	53.12 $\pm$ 0.08 ^{c,I}	21 $\pm$ 0.02 ^{d,I}	1.15 $\pm$ 0.11 ^{B,II}	70.41 $\pm$ 0.09 ^{C,II}	28 $\pm$ 0.02 ^{C,II}

The fatty acid composition of the oleoresins was analyzed by using gas chromatography and shown in Table 7-2. Oleic acid, palmitic acid, palmitoleic acid, and myristic acid were the most abundant fatty acids in all the oleoresins. Octadecanoic acid was present only in trace amounts in all extracts with a concentration range between 0.292% and 1.792%. This aligned with the fatty acid content of raw sorghum varieties as reported in previous studies (Espitia-Hernández et al., 2022). It is worth mentioning that the fatty acid composition is not discriminative to the free fatty acids and esterified fatty acids; however, the free fatty acid content of sorghum grain was shown to be in negligible amounts, comprising ca. 3% of the total lipids (Moreau et al., 2016).

The solvent type had a significant ($P < 0.05$) effect on the fatty acid composition. The unsaturated fatty acids (oleic and palmitoleic acids) increased more than saturated ones (myristic and palmitic acids) when ILs were used instead of dichloromethane and ethanol. In another study, Motlagh et al. (2019) showed that higher omega-3 polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic acids) extract from microalgae biomass were obtained with ILs in comparison to organic solvents, such as hexane and chloroform. This was probably related to the double bonds of unsaturated fatty acids that provided lower free energy for temporal ionization and interacted with cations and anions of ionic liquids (Motlagh et al., 2019). In addition, the oleic, palmitic, and myristic acid contents were not significantly ($P > 0.05$) different when IL₁ and IL₂ were used in the traditional extraction process, while there was significant ($P < 0.05$) difference in the palmitoleic acid content. In contrast, the percentage of these fatty acids was significantly ($P < 0.05$) different when dichloromethane or ethanol was used. The extraction method also had a significant ($P < 0.05$) effect on the fatty acid composition (Table 7-2). The highest concentration of oleic acid, the most abundant unsaturated fatty acid in oleoresins, was ca. 24% with the combined action of ball milling and IL₁. The oleic and palmitoleic acid contents significantly (P

< 0.05) and 35% and 15% increased with ball milling as compared to traditional extraction. The increase in unsaturated fatty acids reflected a significant decrease ($P < 0.05$) in the saturated myristic and palmitic acid contents. The use of ILs also increased ($P < 0.05$) the total phenolic content of the oleoresins.

Table 7-2 Fatty acid composition (wt%) in oleoresins extracted from sorghum grain using different methods. The results are expressed as the mean $\pm$ standard deviation. The lower and upper superscripts indicate the significant differences between the solvent type for conventional solvent or ball mill-assisted extraction processes, respectively. The Roman numerals show the differences between the type of extraction for each solvent

Solvent type		Myristic acid (C14)	Palmitic acid (C16)	Palmitoleic acid (C16:1 (Δ^9))	Octadecanoic acid (C18:0)	Oleic acid (C18:1 ω 9-cis)	Elaidic acid (C18:1 ω 9-trans)	Linoleic acid (C18:2)
Conventional extraction	IL ₁	32.946 $\pm$ 0.003 ^{a,I}	16.819 $\pm$ 0.675 ^{a,I}	16.188 $\pm$ 0.292 ^{a,I}	1.646 $\pm$ 0.172 ^{c,I}	17.249 $\pm$ 0.329 ^{a,I}	1.553 $\pm$ 0.115 ^{d,I}	13.599 $\pm$ 1.202 ^{a,I}
	IL ₂	32.888 $\pm$ 0.006 ^{a,I}	17.240 $\pm$ 0.723 ^{a,I}	12.855 $\pm$ 0.265 ^{b,I}	1.577 $\pm$ 0.126 ^{c,I}	15.619 $\pm$ 0.215 ^{a,I}	6.465 $\pm$ 0.137 ^{c,I}	13.356 $\pm$ 1.047 ^{a,I}
	dichloromethane (99%)	30.959 $\pm$ 0.018 ^{c,I}	15.758 $\pm$ 0.231 ^{b,I}	10.411 $\pm$ 0.261 ^{ab,I}	7.482 $\pm$ 0.212 ^{b,I}	13.141 $\pm$ 0.243 ^{c,I}	10.439 $\pm$ 0.317 ^{a,I}	11.81 $\pm$ 1.082 ^{c,I}
	ethanol (99%)	31.582 $\pm$ 0.004 ^{b,I}	13.983 $\pm$ 0.277 ^{c,I}	8.130 $\pm$ 0.266 ^{c,I}	9.993 $\pm$ 0.173 ^{a,I}	14.704 $\pm$ 0.211 ^{b,I}	9.586 $\pm$ 0.122 ^{b,I}	12.022 $\pm$ 0.937 ^{b,I}
Ball mill	IL ₁	26.064 $\pm$ 0.006 ^{A,II}	12.058 $\pm$ 0.007 ^{A,II}	19.275 $\pm$ 0.477 ^{A,II}	1.792 $\pm$ 0.002 ^{C,I}	23.663 $\pm$ 0.003 ^{A,II}	3.499 $\pm$ 0.017 ^{D,II}	13.649 $\pm$ 0.025 ^{A,I}
	IL ₂	26.402 $\pm$ 0.041 ^{A,II}	11.478 $\pm$ 0.69 ^{A,II}	19.717 $\pm$ 0.291 ^{A,II}	1.723 $\pm$ 0.233 ^{C,I}	22.787 $\pm$ 0.891 ^{A,II}	4.338 $\pm$ 1.711 ^{C,II}	13.555 $\pm$ 1.7 ^{A,I}
	dichloromethane (99%)	21.868 $\pm$ 0.043 ^{C,II}	9.836 $\pm$ 0.674 ^{B,II}	18.290 $\pm$ 0.281 ^{B,II}	8.637 $\pm$ 0.347 ^{B,II}	20.427 $\pm$ 0.876 ^{B,II}	8.654 $\pm$ 1.887 ^{A,II}	12.288 $\pm$ 1.336 ^{B,I}
	ethanol (99%)	23.491 $\pm$ 0.005 ^{B,II}	8.203 $\pm$ 0.007 ^{C,II}	17.633 $\pm$ 0.456 ^{C,II}	10.638 $\pm$ 0.188 ^{A,I}	18.990 $\pm$ 0.371 ^{C,II}	7.432 $\pm$ 1.112 ^{B,II}	13.613 $\pm$ 1.13 ^{A,I}

Oleoresins contained predominantly eight phenolic compounds: caffeic acid, coumaric acid, ferulic acid, apigenidin, luteolidin, 7-methoxy apigeninidin, eriodictyol, and narencenin (Table 7-3). Caffeic acid, coumaric acid, and luteolidin were the major phenolic compounds present in all oleosome samples. Among these, caffeic acid followed by coumaric acid was the most abundant and they remained so with all types of solvents. The solvent type significantly ($P < 0.05$) affected the total phenolic content and composition (Table 7-3). For example, the caffeic acid content increased by 74.84% when IL₁ was used as compared to dichloromethane with the traditional extraction process. Following the trend observed for protein and lipid contents, the extraction method also had a significant ($P < 0.05$) effect on the phenolic content and composition (Table 7-3). For example, the increase in caffeic acid, the most abundant phenolic molecule, was 28.93% and 50.16% when ball milling was combined with IL₁ and dichloromethane respectively, as compared with the traditional extraction process using the same solvents. However, the extent of the increase in different phenolic molecules was not the same. For example, the increase in luteolinidin was 21.78% and 57.68% when ball milling extraction was performed with IL₂ and ethanol respectively, as compared with traditional extraction. On the other hand, the increase in the same phenolic compound was 3.45% and 15.43% when ball milling extraction was conducted with IL₁ and dichloromethane, respectively as compared with traditional extraction. Similarly, Talekar et al. (2019) showed that total phenolics and antioxidant “punicalagin” increased when ball-mill pre-treatment was used for pomegranate peel waste, and it was more efficient than ultrasound-assisted and Soxhlet extraction.

Table 7-3 Concentration of phenolic compounds as gallic acid equivalents (ppm) in oleoresins extracted from sorghum grain using different methods. The results are expressed as the mean $\pm$ standard deviation. The lower and upper superscripts indicate the significant differences between the solvent type for conventional solvent or ball mill-assisted extraction processes, respectively. The Roman numerals show the differences between the type of extraction for each solvent

	Solvent type	Caffeic acid	Coumaric acid	Ferulic acid	Apigenidin	Luteolinidin	7-methoxy apicidin	Eriodictyol	Narencenin
Conventional extraction	IL ₁	20.280 $\pm$ 0.007 ^{a,I}	18.409 $\pm$ 0.003 ^{a,I}	10.456 $\pm$ 0.058 ^{a,I}	9.556 $\pm$ 0.003 ^{a,I}	12.673 $\pm$ 0.017 ^{a,I}	8.360 $\pm$ 0.08 ^{a,I}	3.032 $\pm$ 0.005 ^{a,I}	5.061 $\pm$ 0.008 ^{a,I}
	IL ₂	19.208 $\pm$ 0.051 ^{a,I}	13.602 $\pm$ 0.321 ^{b,I}	10.446 $\pm$ 0.291 ^{a,I}	6.319 $\pm$ 1.112 ^{a,I}	10.380 $\pm$ 2.67 ^{a,I}	6.470 $\pm$ 1.342 ^{a,I}	3.158 $\pm$ 0.375 ^{a,I}	4.658 $\pm$ 0.551 ^{a,I}
	dichloromethane	11.599 $\pm$ 0.053 ^{c,I}	10.651 $\pm$ 0.331 ^{d,I}	3.113 $\pm$ 0.281 ^{c,I}	3.096 $\pm$ 1.171 ^{c,I}	4.251 $\pm$ 0.015 ^{c,I}	4.405 $\pm$ 0.371 ^{b,I}	3.085 $\pm$ 0.241 ^{b,I}	4.549 $\pm$ 0.623 ^{c,I}
	ethanol (99%)	14.296 $\pm$ 0.007 ^{b,I}	11.385 $\pm$ 0.038 ^{c,I}	3.315 $\pm$ 0.456 ^{b,I}	4.374 $\pm$ 0.562 ^{b,I}	6.023 $\pm$ 2.131 ^{b,I}	4.486 $\pm$ 0.461 ^{b,I}	3.177 $\pm$ 0.563 ^{ab,I}	3.685 $\pm$ 0.082 ^{b,I}
Ball mill	IL ₁	26.147 $\pm$ 0.005 ^{A,II}	22.965 $\pm$ 0.075 ^{A,II}	6.082 $\pm$ 0.292 ^{A,II}	12.360 $\pm$ 0.081 ^{A,II}	13.111 $\pm$ 1.332 ^{A,II}	4.486 $\pm$ 0.713 ^{A,II}	3.575 $\pm$ 0.211 ^{A,II}	3.272 $\pm$ 0.836 ^{A,II}
	IL ₂	26.075 $\pm$ 0.005 ^{A,II}	20.159 $\pm$ 0.492 ^{A,II}	6.072 $\pm$ 0.265 ^{A,II}	11.927 $\pm$ 0.064 ^{B,II}	13.271 $\pm$ 2.671 ^{A,II}	4.606 $\pm$ 0.136 ^{A,II}	3.312 $\pm$ 0.429 ^{A,II}	2.885 $\pm$ 0.621 ^{A,II}
	dichloromethane	17.417 $\pm$ 0.004 ^{C,II}	13.135 $\pm$ 0.881 ^{C,II}	2.175 $\pm$ 0.261 ^{B,II}	9.278 $\pm$ 1.112 ^{C,II}	4.907 $\pm$ 0.148 ^{C,II}	2.458 $\pm$ 0.722 ^{B,II}	3.145 $\pm$ 0.255 ^{B,II}	1.638 $\pm$ 1.92 ^{B,II}
	ethanol (99%)	19.720 $\pm$ 0.002 ^{B,II}	18.869 $\pm$ 0.320 ^{B,II}	3.376 $\pm$ 0.266 ^{B,II}	11.321 $\pm$ 0.562 ^{B,II}	9.497 $\pm$ 0.156 ^{B,II}	2.540 $\pm$ 0.211 ^{B,II}	3.237 $\pm$ 0.471 ^{AB,II}	1.773 $\pm$ 0.825 ^{AB,II}

Oleoresin structure

The organic moieties containing O-H and bonds, such as phospholipid bilayer, glycosidic bond, and protein-phospholipid complexes, were analyzed by FTIR spectroscopy (Fig. 7-2). The stretching band of C=O at 1100 cm^{-1} and 1600 cm^{-1} was assigned to the polysaccharides and phospholipid bilayers, respectively, the H-OH stretching around $2800\text{-}2900\text{ cm}^{-1}$ to the glycosidic bond between glycoprotein units, and the O-H stretching between $3000\text{-}3500\text{ cm}^{-1}$ to the presence of protein-phospholipid complexes (Li et al., 2022). The FTIR spectra were not affected ($P > 0.05$) by the solvent type but affected ($P < 0.05$) by the extraction process. The intensity of the peak at around 1600 cm^{-1} was lower with ball milling as compared to traditional extraction. This was probably related to the amount of phospholipid compounds extracted and potentially improved the stability of oil bodies. The intensity of the peak around $2800\text{-}2900\text{ cm}^{-1}$ decreased with ball mill treatment. This can be explained by protein-polysaccharide interaction. Ball mill treatment increased the amount of released compounds into the solvent matrix. Among these, soluble polysaccharides became more available for intermolecular interactions with proteins. These interactions were seen as reduced intensity of spectra around $2800\text{-}2900\text{ cm}^{-1}$ representing glycosidic bond between amino group of protein and carboxylic group of polysaccharides. This may prove the formation of protein-polysaccharide complex (Guerrero et al., 2014).

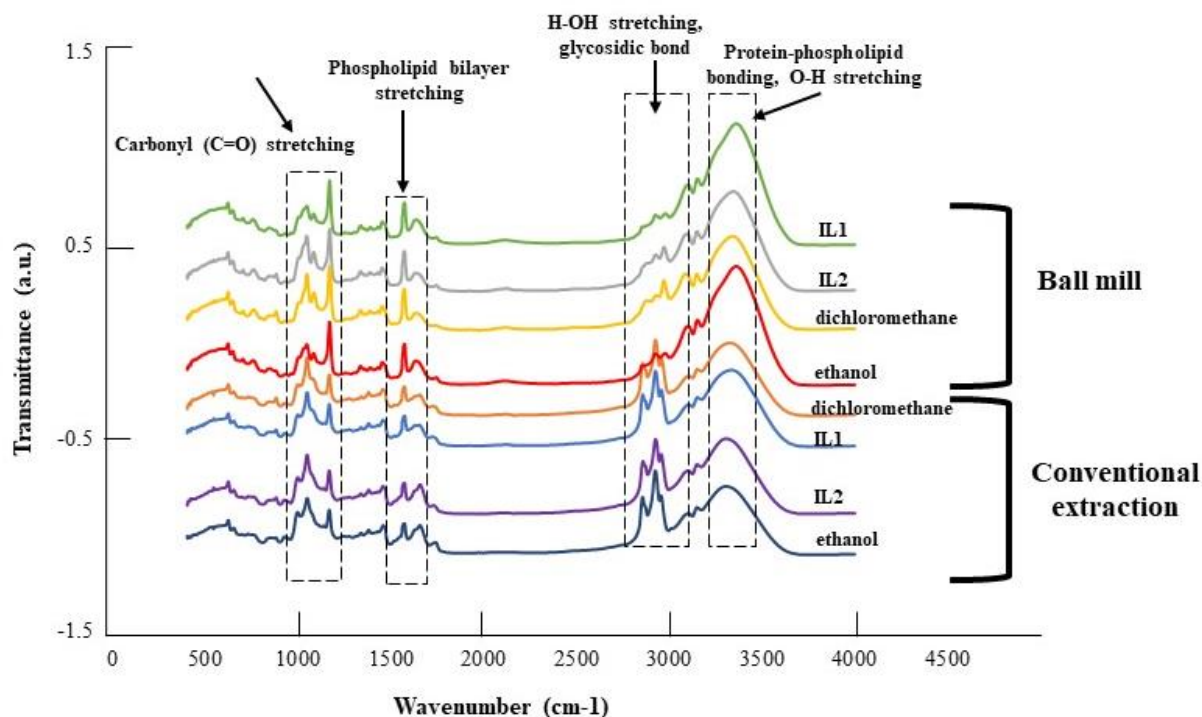


Figure 7-2 FTIR spectra of oleoresins. The results are expressed as mean value $\pm$ SD ($n = 3$). The lower and upper superscripts indicate the significant differences between the types of solvent in each extraction process (conventional or ball mill extraction). The Roman letter shows the differences between the type of extraction for each solvent.

Particle size and zeta potential are important factors that relate to their dispersibility and stability. When oleoresins dispersed in an aqueous environment without additional surfactants, they showed remarkable homogeneity and stability. The particle size of oleoresins ranged between 323-1760 nm (Figure 7-3A). Both solvent type and ball mill treatment significantly ($P < 0.05$) affected the particle size. With traditional extraction, the particle size decreased from a max of 1760 nm to 575 nm when dichloromethane and IL₂ were used, respectively (Fig. 7-3A). This showed that charged groups in ionic liquids were anchored on the interface of oil bodies which resulted in increased electrostatic repulsion and reduced the average particle size. The ball mill process reduced the average particle size of oleoresins. For instance, the average particle size was reduced by nearly 50% when ball mill treatment was applied using ethanol compared to the conventional method. It

was observed that the friction and shear forces during ball milling treatment reduced the particle size. This can be explained in consideration of the FTIR analysis. For oleoresin dispersions of lower particle size, the intensity of the FTIR band at 1100 cm^{-1} , 1600 cm^{-1} , and $2800\text{-}2900\text{ cm}^{-1}$ increased. In addition, the zeta potential was measured as an indicator of the stability and extent of charged polymers, mainly the protein fraction of oleosome structure. The results ranged from -8 mV to -35 mV (Fig 7-3B). In parallel to the particle size data, small particles from ball mill extracts showed higher zeta potential values, which demonstrate greater physical stability. The low molecular weight of oleosins as a major protein fraction plays a critical role in the stability of the oleoresin dispersions. due to their hydrophobic nature, oleosins can penetrate deep into the triacylglycerol core of their emulsions with large detachment energies that reinforce their integrity and stability (Wijesundera & Shen, 2014). Another protein fraction, caleosins have shorter hydrophobic sequences and longer hydrophilic units as compared to oleosin with special N-terminal segments bearing single Ca^{2+} ions responsible for the change in the polarity (Hanano et al., 2023). The difference in the smaller caleosin content of the oleoresins was probably responsible for the formation of smaller particle size as expected with a higher diffusion rate of smaller polymers, and a higher zeta potential observed for them.

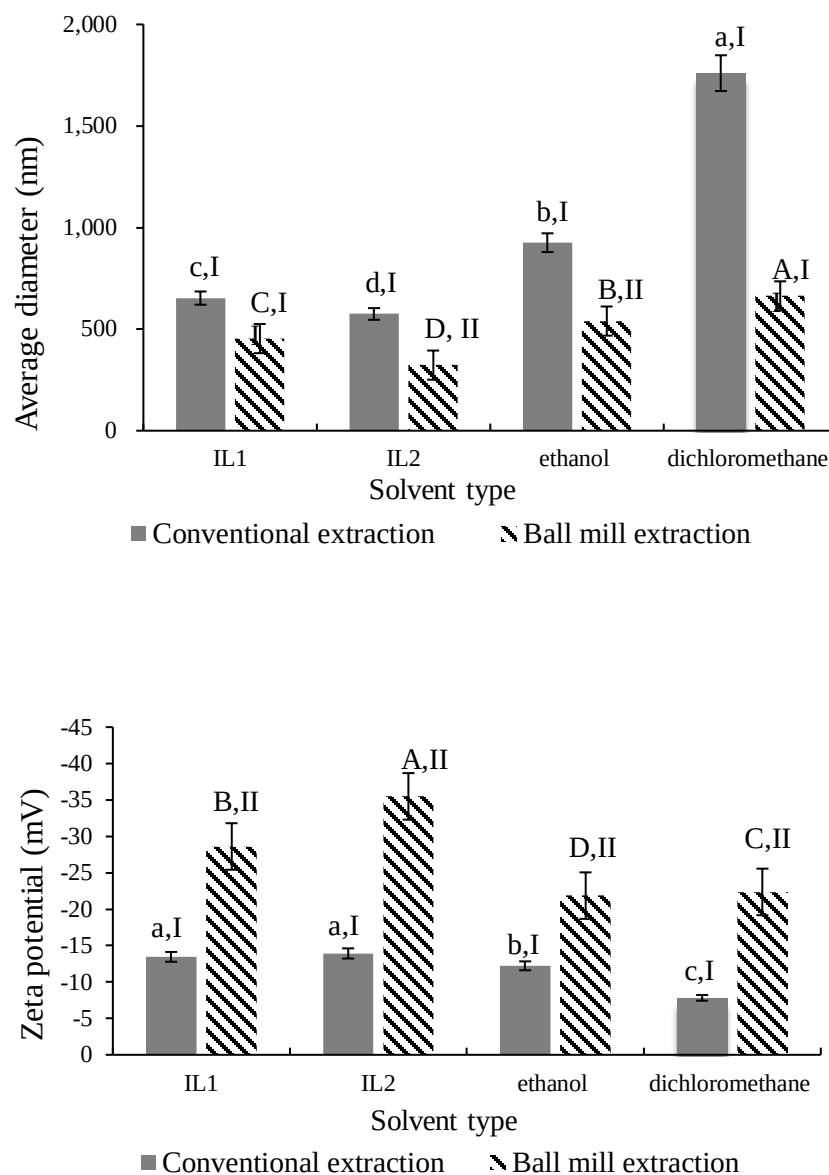


Figure 7-3 Particle size (A) and zeta potential (B) of sorghum oleoresins. The results are expressed as mean value $\pm$ SD ($n = 3$). The lower and upper superscripts indicate the significant differences between the types of solvent in each extraction process (conventional or ball mill extraction). The roman letter shows the differences between the type of extraction for each solvent. The bars show standard deviation for each extraction condition and solvent type.

Conclusions

Our results are original to demonstrate the potential of oleoresins obtained from sorghum grain for value-added food applications. The oleoresins are rich in polyphenols with strong antioxidant potential as expected related to characteristics of the sorghum grain. The composition and structural properties of the oleoresins can be modulated by using novel ILs with composite anions and cations. Extraction yield increased by more than 50% when ball mill is used combined with ILs as compared to traditional batch extraction. This also reflected a significant increase in functional ingredients, such as proteins and phenolic content. Thus, the wet milling process via the use of a ball mill can help to break solid enclosure to improve extraction yield, and at the same time improve protein functionality for higher emulsification power. The use of ILs with composite anions and cations as novel solvents can offer great potential to provide control over the oleoresin composition (e.g., the ratio of saturated and unsaturated fatty acids) as well as phenolic composition as compared to traditional solvent extraction.

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Chapter 8 - Conclusions

Biopolymers made from plant-based sources such as cereals and grains are a great option as film raw materials with their promising gas and moisture properties. However, further investigation is needed to improve the film properties made from grains and cereals in food packaging. Emerging crops such as sorghum, *Camelina sativa* as well as industrial processing DDGS by-products possess considerable potential to be used in food applications.

The recent expansions in ethanol production have increased the availability of sorghum DDGS. Due to its large availability and relatively low cost as compared to biopolymers, it can be a great starting material for packaging film alternatives. Renewable biodegradable packaging films from sorghum DDGS by-products can be manufactured. I showed that suitable solubilization techniques together with wet and dry milling can be effective in successfully processing DDGS. In addition to this, chloroform as a co-solvent can improve the physical, mechanical, and thermal characteristics of the films.

The adaptable nature of *Camelina sativa* to various land conditions as compared to canola or soybean gives a higher production rate for this emerging crop. In addition to this, *Camelina sativa* requires less environmental and agricultural necessities to produce suggesting that the cost of growing *Camelina* oilseed is comparatively lower. This brings about economically feasible acetyl-TAG production as an alternative additive in value-added applications. For example, high oleic acetyl-TAG from *Camelina sativa* as a novel plasticizer can improve the physical, mechanical properties, and surface hydrophobicity of DDGS films. I showed that high oleic acetyl-TAG has great potential as a high-yield plasticizer in biodegradable film production.

Protein foams require a surface-active stabilizer which can improve stability by modulation of the interfacial rheology at altered protein and sugar concentrations. I showed that based on surface

rheology results, high oleic acetyl-TAG at reduced sugar concentrations lowered the foam drainage, surface tension and improved the dilatational viscoelasticity of foams, thereby supporting the potential of using acetyl-TAG as a foam stabilizer. Moreover, whippable emulsion stabilized by whey protein can further be enhanced using the wild type of acetyl-TAG as an effective emulsion stabilizer. I showed that interfacial rheology at oil water interface as well as whipping characteristics of aerated oil in water emulsions can be improved with acetyl-TAG.

Similar to *Camelina sativa*, sorghum as an emerging crop has undeniable potential and its fractions such as oleoresins can be utilized as a novel food additive with their rich phenolic content and antioxidant properties. I showed that sorghum oleoresins can successfully be extracted and both structure and composition can be modulated by using novel ILs with composite anions and cations. Furthermore, wet milling process via the use of ball mill can increase extraction yield by multidirectional beads of ball milling and improve protein functionality for higher emulsification power.

Chapter 9 - Future work

This dissertation includes studies giving insight into sorghum, sorghum by product (DDGS), and acetyl-TAG from *Camelina sativa*. The focus of this dissertation was on new approaches to these emerging crops. The methods, results, and compounds obtained from novel convergences are important contributions to food science and may further promise sustainable solutions for food waste. On the other hand, several different tests, experiments, and further food applications can be suggested as future work. Further studies may include deeper analysis of sorghum DDGS biodegradable packaging films with improved solubilization techniques or pretreatments. It might be helpful to understand the composition of each DDGS fraction such as protein, fiber, and fat. Moreover, new methods to deagglomerate DDGS structure might be another focus point for possible future studies. It could also be interesting to consider each fraction as a starting material for value-added food applications.

In addition to this, it could be interesting to utilize the sorghum oleoresin obtained from different sorghum varieties such as black sumac and white in different real food applications such as chocolate to test its emulsifying ability. This might bring the potential of oleoresin to be used as an alternative instead of commercial and less affordable emulsifiers such as lecithin.

Furthermore, the chapters related to acetyl-TAG applications could also be improved. This dissertation showed that acetyl-TAG can be used as a potential stabilizer, emulsifier, and plasticizer based on given experimental results and literature studies. However, the time was limited to apply acetyl-TAG in real food applications. It might be useful to use the outcomes of this study to further apply it in real food applications.

Regarding the possible application of different fractions from emerging crops sorghum and *Camelina sativa*, it would be useful to extend the research in both extraction and utilization in value-added food applications.

Appendix A - Standard Curves

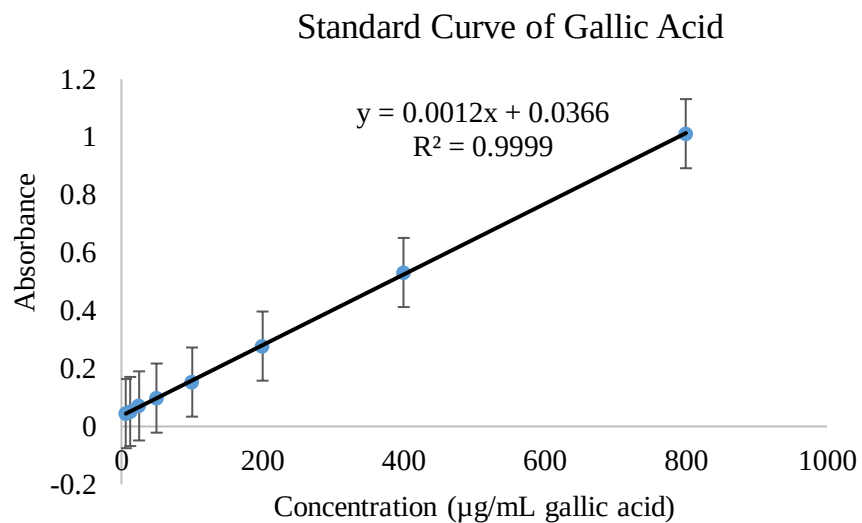


Figure A-1 Standard calibration curve for gallic acid used for total phenolic calculations

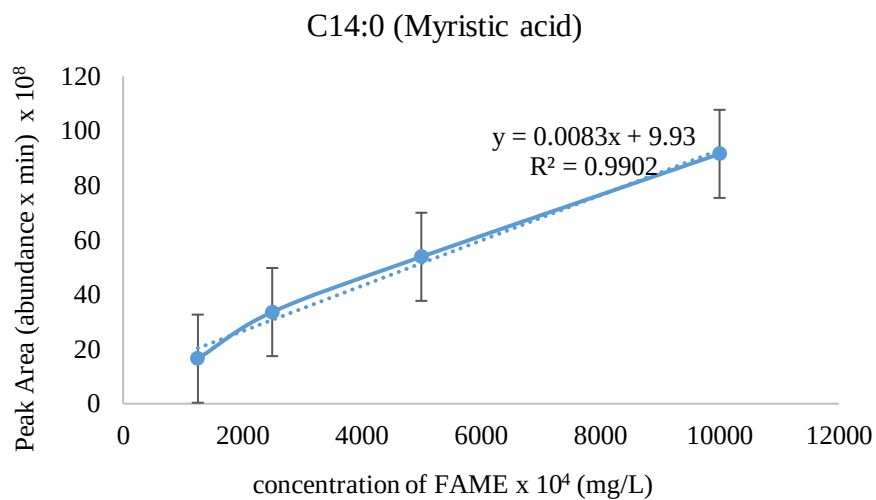


Figure A-2 Standard calibration curve for myristic acid used for FAME calculations

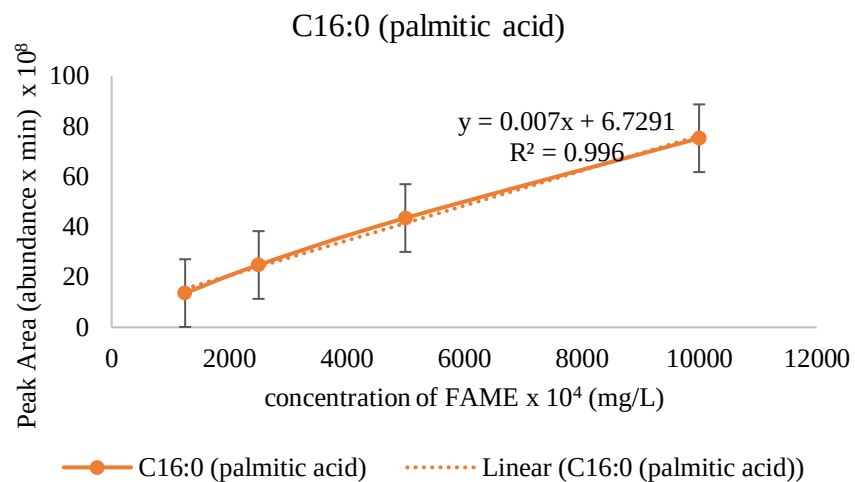


Figure A-3 Standard calibration curve for palmitic acid used for FAME calculations

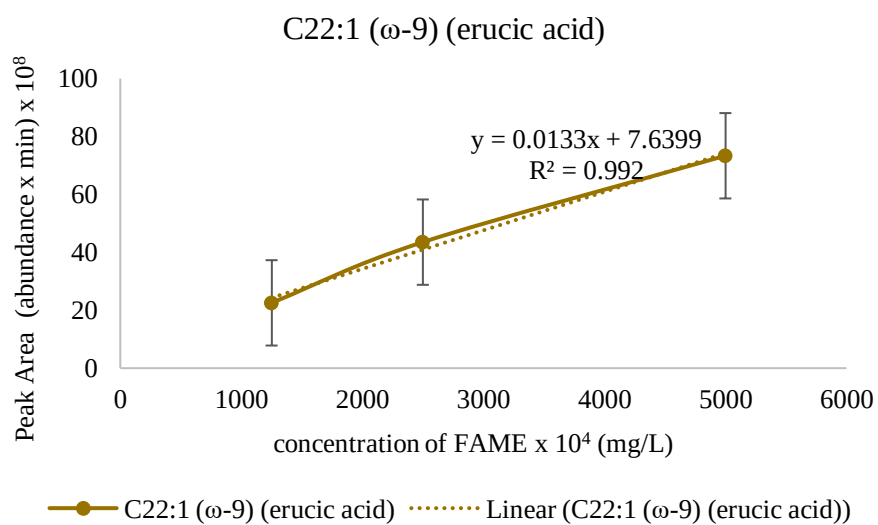


Figure A-4 Standard calibration curve for erucic acid used for FAME calculations

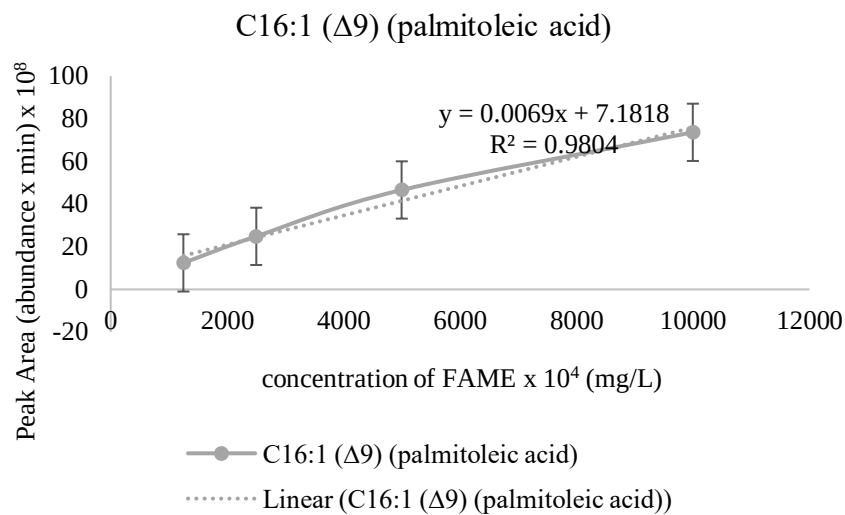


Figure A-5 Standard calibration curve for palmitoleic acid used for FAME calculations

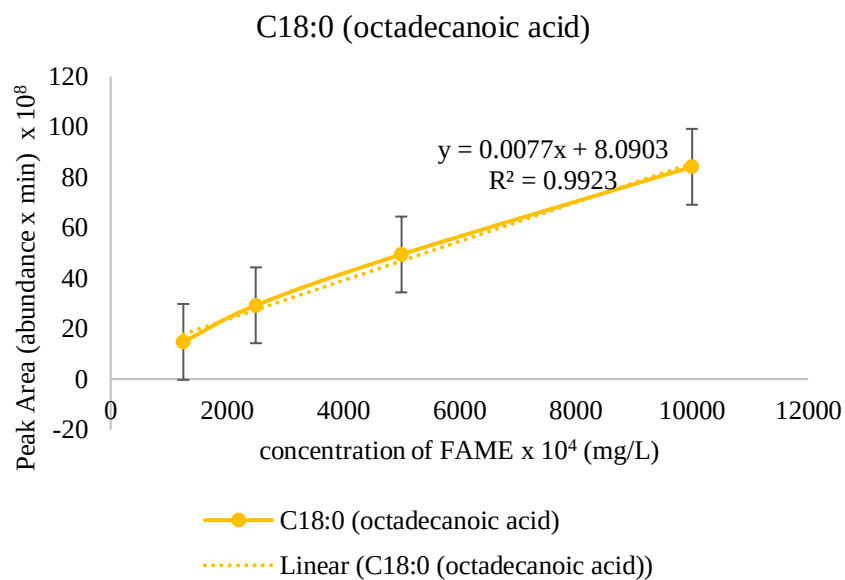


Figure A-6 Standard calibration curve for octadecanoic acid used for FAME calculations

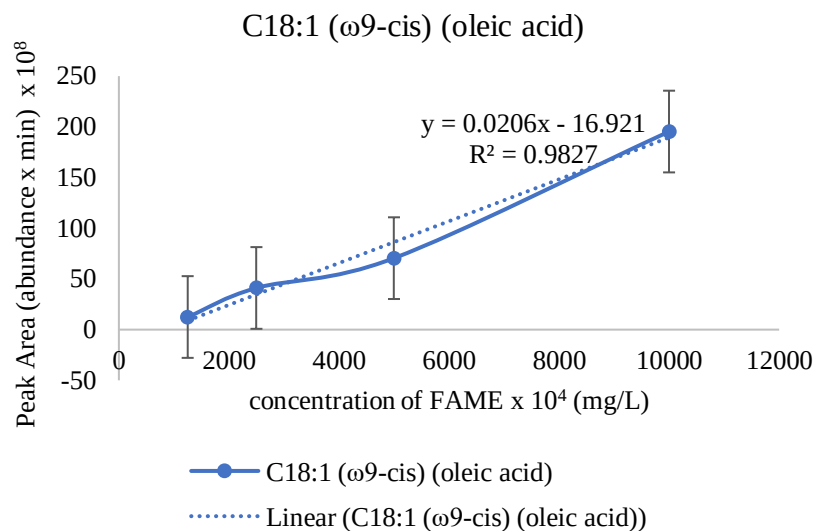


Figure A-7 Standard calibration curve for oleic acid used for FAME calculations

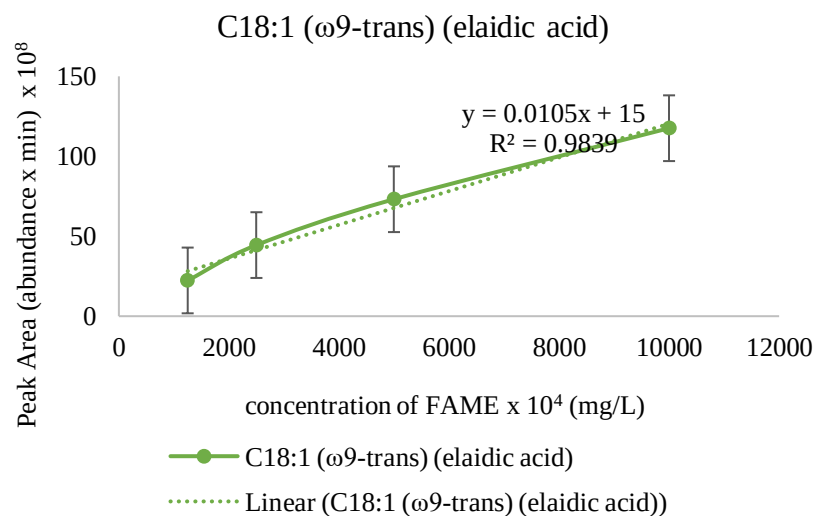


Figure A-8 Standard calibration curve for elaidic acid used for FAME calculations

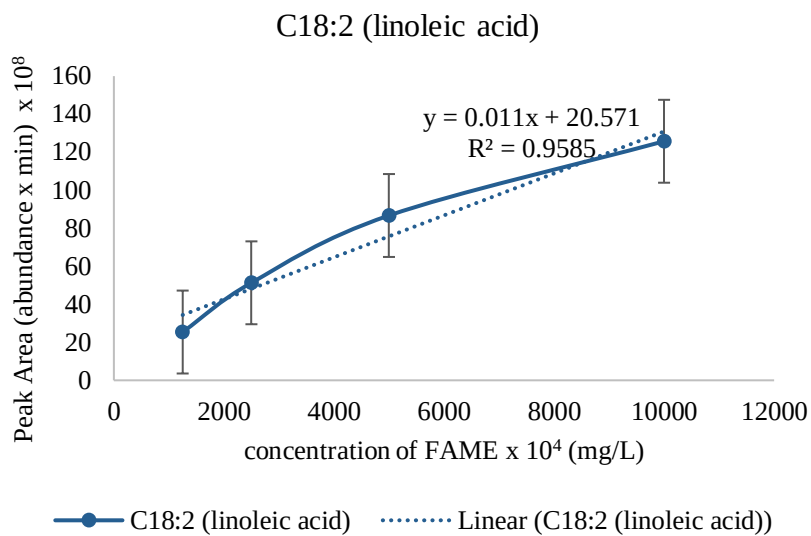


Figure A-9 Standard calibration curve for linoleic acid used for FAME calculations

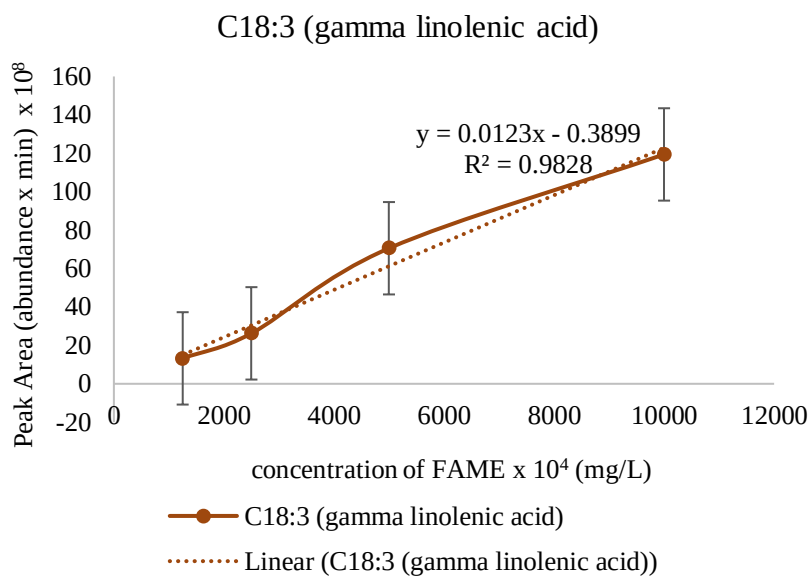


Figure A-10 Standard calibration curve for gamma linolenic acid used for FAME calculations

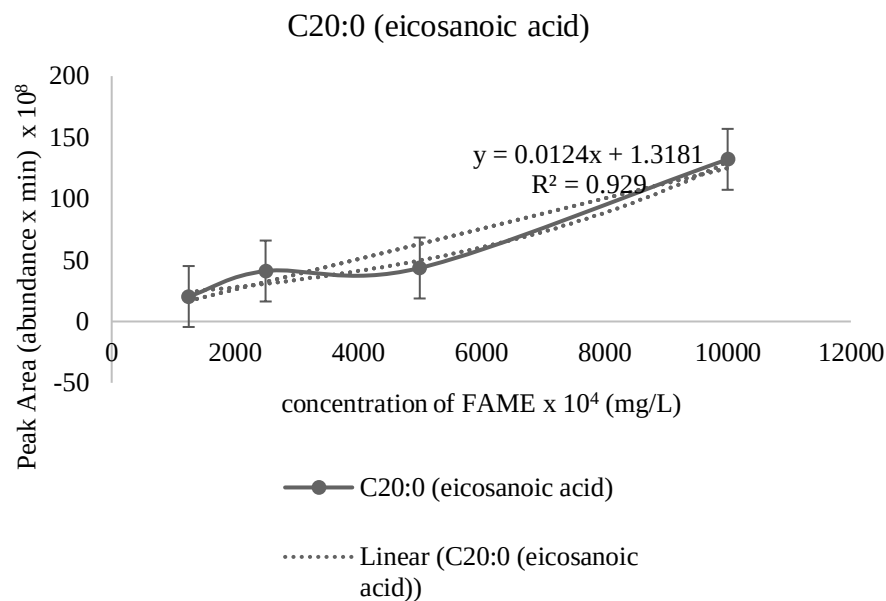


Figure A-11 Standard calibration curve for eicosanoic acid used for FAME calculations

Table A-1 FAME standard mixture with individual fatty acids and retention times

Retention times (min)	Fatty acid
7.91	C14:0 (Myristic acid)
10.09	C16:0 (palmitic acid)
11.87	C16:1 (Δ^9) (palmitoleic acid)
14.71	C18:0 (octadecanoic acid)
14.85	C18:1 (ω 9-cis) (oleic acid)
16.08	C18:1 (ω 9-trans) (elaidic acid)
16.26	C18:2 (linoleic acid)
18.35	C18:3 (gamma linolenic acid)
21.28	C20:0 (eicosanoic acid)
21.92	C22:1 (ω -9) (erucic acid)

Appendix B - Chromatograms

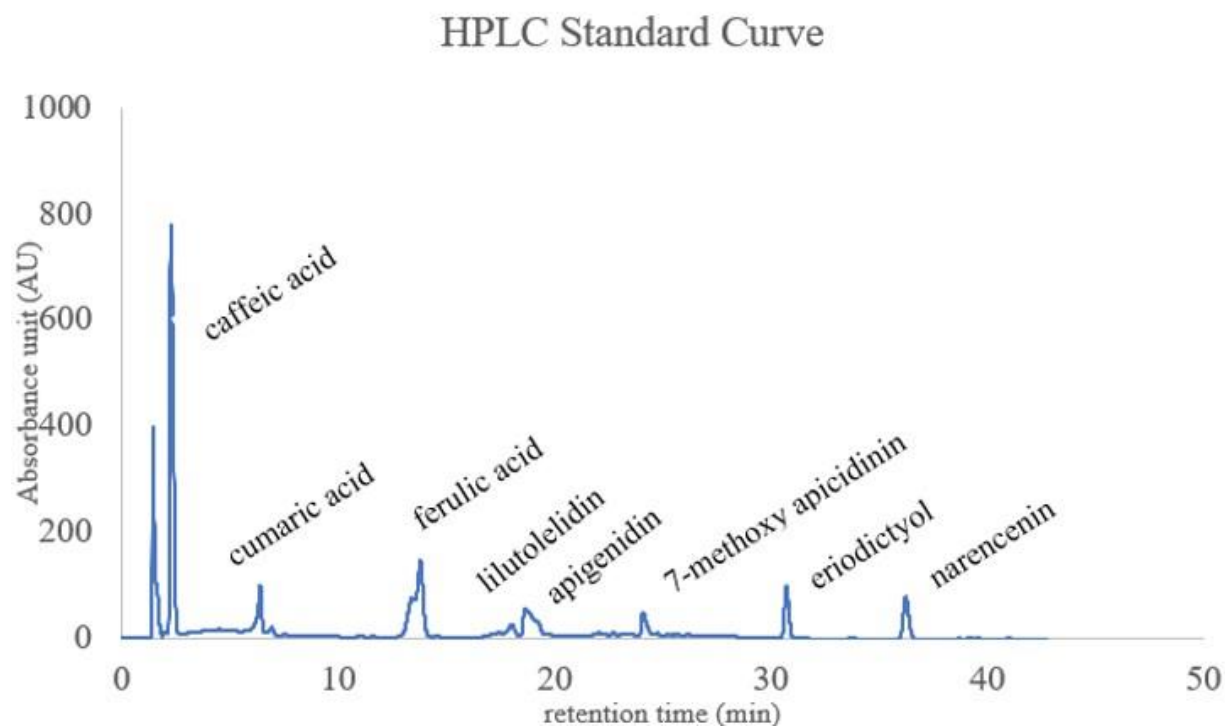


Figure B-12 HPLC reference chromatogram with retention times