EXTRACTION OF CAROTENOIDS FROM CORN MILLING COPRODUCTS

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MASTER OF SCIENCE

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ABSTRACT

Two experiments were completed to develop methods for extracting xanthophylls from corn industry co-products, post fermentation (PF) corn oil and corn gluten meal (CGM). A solid phase extraction (SPE) method was used to fractionate a xanthophyll-rich portion of PF corn oil by varying conditioning and eluting solvents used with a diol SPE column. Conditioning with dichloromethane yielded highest xanthophyll fractionation, 86.5%. The elution solvent selected did not impact fractionation based on a two-way ANOVA. Supercritical fluid extraction of xanthohpylls from CGM was modeled using a Box-Behnken design, varying temperature, pressure, and co-solvent ratio. The optimum conditions were determined to be 40 °C, 6820 psi, and 15% co-solvent, which would extract 85.4 µg lutein/g CGM, 2.6 times more lutein than an ethanol and chloroform: dichloromethane solvent extraction. Co-solvent was the most influential extraction parameter and increasing it further could yield higher xanthophyll recovery. With further studies, this work has industrial potential.

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1. GENERAL INTRODUCTION

Corn is the most abundant crop grown and processed in the United States. It is produced so extensively because it is the feedstock for many large industries including food products, corn ethanol, sweeteners, oils, and animal feed. Two of the largest corn products, ethanol and sweeteners, utilize only 60-70% of the kernel leaving 30-40% of the kernel components to go to lower value residuals (Moreau et al., 2010).

Several coproducts derived from this unused part of the corn kernel are created when corn is processed by wet milling or dry-grind milling. These coproducts are important to the corn processing companies because by producing them, additional revenue can be generated. Extracting additional value from these coproducts has been part of a research effort put forth to further increase plant income and create more diverse bio refineries. Two examples of corn industry coproducts that contain underutilized value are corn gluten meal and post fermentation corn oil (PF corn oil).

Corn gluten meal (CGM) is a coproduct of the wet grind milling process. It is produced when the starch of the corn kernel is separated from the gluten by centrifugation. This high protein meal (60%) is dried down to <10% moisture and sold as poultry feed. The yellow color of corn is caused by compounds called carotenoids, which are structurally bound within the gluten of the kernel. Because they are tightly associated with the gluten, a majority of the carotenoids end up in the CGM. Poultry farmers find the carotenoid content of the meal to be beneficial because it gives a yellow color to the chicken flesh and egg yolks. However, farmers of other livestock have found that the yellow color in their livestock meat is perceived by customers as low quality. Therefore, although carotenoids are beneficial components in chicken feed, overall the carotenoid content in CGM reduces its versatility in the animal feed market.

Another corn milling coproduct affected by its carotenoid content is PF corn oil. This orange colored oil is a coproduct of the dry-grind milling process. The main product of this process is fuel ethanol, which is produced when whole corn kernels are ground, cooked, treated with enzymes, and then fermented with yeast. After fermentation, the residual material contains lipids, fiber, protein, and unfermented starch from the corn. This beer is centrifuged to create solid and liquid fractions. Since about 2009, some dry-grind millers have begun to centrifuge the liquid stream a second time to fractionate and sell the oil present (Winsness and Cantrell, 2009). This oil is PF corn oil, which is sold from the plants as feedstock for biodiesel production as a way to further increase plant revenue.

Though carotenoids can reduce the quality of the coproducts, when extracted they have potential value as preservatives in food products or, if purified, as pharmaceuticals. Both PF corn oil and CGM contain other compounds capable of food preservation including tocochromanols, and phytosterols, however, the presence of carotenoids is of special interest, because in addition to being food preservers, carotenoids also are valued as natural food colorants.

Consumer demand for natural food colorants has increased over the past three decades because of public concern with the safety of synthetic food additives (Downham and Collins, 2000). Carotenoids, present in many fruits and vegetables, can be extracted to provide a yellow, orange or red pigment to food products. This carotenoid pigment is what food producers are looking for to serve as an alternative to synthetic colorants.

Collecting the carotenoids present in the corn milling coproducts could bring additional revenue to the corn processing companies. Increasing revenue is crucial because the annual per capita consumption of the main product from corn wet mills, high fructose corn syrup, has dropped from 37 lbs/yr in 2000 to 26 lbs/yr in 2015 (USDA, 2015). Additionally, at the dry grind

milling plants, ethanol prices fluctuate with oil prices, so having additional income from the coproducts allows millers to extract more value from the corn and increase margins (Rausch and Belyea, 2006). By increasing the marketability of the coproducts at these corn plants they will be able to survive in the changing market.

The goal of this project was to develop methods to extract the carotenoids from PF corn oil and CGM with the intention of producing more profitable co-products such as natural food colorants. Carotenoids were fractionated from the PF corn oil using a solid phase extraction method that increased carotenoid concentration in the final fraction by ten times. Supercritical fluid extraction was successfully used to extract the carotenoids from CGM better than an ethanol and chloroform: dichloromethane solvent extraction. Further work is needed to understand if these methods are viable on an industrial scale.

2. LITERATURE REVIEW

2.1. Introduction

The dry-grind and wet milling processing of corn will be explained including the primary coproducts of interest, post fermentation corn oil and corn gluten meal. The importance of carotenoids and the quantities present in different corn products will be discussed. Finally, the extraction methods available for removing the carotenoids from the corn coproducts will be reviewed.

2.2. Corn Structure and Components

As mentioned before, corn is the feedstock for many different industries including food products, corn ethanol, sweeteners, oils, and animal feed. To supply these industries, over 90 million acres of U.S. land are planted with corn each year, producing 13.6 billion bushels of corn. Long term projections indicate that corn production will remain at least this high for the foreseeable future (Westcott and Hansen, 2016).

Corn is an incredibly versatile grain, which accounts for its success in industries. To understand corn's utility, it is helpful to know the composition of the corn kernel. The corn kernel is comprised of four basic parts (Figure 1): the tip cap, pericarp, endosperm, and germ (Watson, 1987). Each of these components have unique value in the corn market.

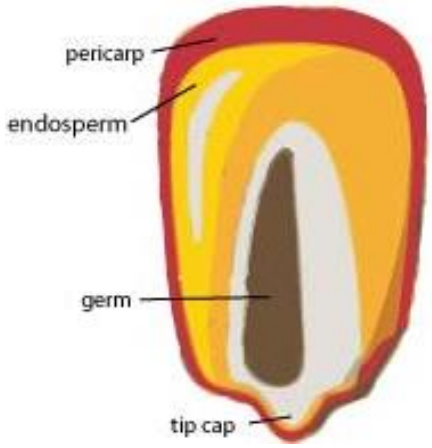

Figure 1. Components within the corn kernel (Georgia Corn Commission, 1993)

The tip cap is a fractionated portion of the pedicel, which attaches the kernels to the corn cob. The pedicel is crucial during the growth of the crop because it is the passageway for nutrients into the kernel. When the developed kernel is removed from the cob after harvesting, the pedicel fractures leaving the conical tip cap attached to the kernel. This tip cap makes up just 1% of the dry kernel weight (Watson, 1987).

The pericarp, also called the bran or fiber, covers the entire kernel and merges with the tip cap at the bottom of the kernel (Kiesselbach, 1949). It is the outer fibrous layer, which protects the internal components of the kernel. The pericarp makes up 5-6% of the total kernel dry weight. This layer is semi-permeable, which allows water to pass to the endosperm and germ within the kernel (Watson, 1987).

The endosperm lies just beneath the pericarp and consists of starch and protein. It makes up 82-84% of the corn kernel and contains 86-89% starch. The small starch granules are fixed within a thick protein matrix (Watson, 1987). The proteins present in the endosperm are primarily of a class called zein, which is a mix of different prolamine proteins that are soluble in aqueous alcohol (70%). The majority of the remaining proteins present in corn are glutelins

(soluble in dilute acids or bases), but they are distributed throughout the endosperm and the germ (Shukla and Cheryan, 2001).

The germ sits below and is surrounded by the endosperm. It contains the majority of the oil in the corn kernel, which amounts to 3-5% of the kernel (Watson, 1987). In total, the make-up of the kernel is around 65% starch, 8% protein, 15% moisture, 1 % ash, and 10% fiber (Shukla and Cheryan, 2001).

2.3. Corn Milling Coproducts

2.3.1. Dry-grind Milling

2.3.1.1. Ethanol Production

The production of corn ethanol is extensive in the United States because we aim to be energy independent and use more renewable fuel sources. More than 95% of vehicles on the road today are fueled with gasoline that contains up to 10% ethanol. The Renewable Fuel Standard legislation adopted by Congress in 2007 mandates production of 36 billion gallons of renewable fuels per year by the year 2022 of which corn ethanol will account for 15 billion gallons. These figures indicate that dry-grind corn ethanol production, the vast majority of current ethanol production in the US, will remain a large industry in the United States.

In dry grind milling operations, 65% of the kernel (starch) is used to produce corn ethanol, leaving 35% of the kernel for other uses. Post fermentation corn oil and dry distillers grains with solubles (DDGS) are coproducts sold from the plant which take advantage of the unfermented portion of the kernel.

Corn ethanol can be produced by either a dry-grind or wet milling process, but more than 80% of the facilities operate a dry-grind process because it is simpler and requires less capital (Kim et al., 2008a). Most dry-grind ethanol facilities follow the same operation principles,

though some plants vary slightly. The goal of all ethanol production facilities is to break down the corn starch into sugars, and ferment the sugars with yeast to produce ethanol.

Corn that is brought to the plant is stored in grain bins. The corn is then conveyed to hammer mills where the entire kernel is ground to a particle diameter of 1-mm (Kim et al., 2008a). Following milling, the corn undergoes a cooking step with water to gelatinize the starch. The particles are then steeped with enzymes that break down the starch into smaller sugar units (Winkler et al., 2007). The first enzymatic step is called liquefaction. The enzyme, α-amylase, is added to reduce the degree of polymerization in the starch and the viscosity of the mixture. Glucoamylase is added to complete the starch breakdown into individual glucose units that yeast ferment (Kim et al., 2008b).

The yeast consume the sugars and produce ethanol and carbon dioxide during fermentation. The ferment is distilled to separate and capture the ethanol produced during fermentation (Kim et al., 2008a; Winkler et al., 2007). After distillation, the ethanol still contains 5% water, which cannot be further separated by simple distillation. A molecular sieve is used to remove the remaining water. The molecular sieve is a bed of small beads that attract both water and ethanol molecules. The beads have pore openings large enough to allow water molecules in, but small enough to prevent ethanol molecules from entering. As the ethanol/water vapor passes through the molecular sieve, the water is absorbed in the beads and pure ethanol exits.

The fermented mash remaining at the bottom of the distillation column is called whole stillage and contains the yeast solids, protein, fiber, unfermented carbohydrates and oil from the corn kernel. This stream is centrifuged, which results in the separation of thin stillage (liquid fraction) and wet cake (solid fraction). The thin stillage undergoes an evaporation step and the

resulting product is a syrup called condensed distiller's solubles (CDS) (Kim et al., 2008a). The CDS is typically added back to the wet cake and dried to make dried distillers grains with solubles (DDGS).

2.3.2. Post Fermentation Corn Oil

Since about 2009, some dry grind corn ethanol producers have realized the high level of oil in the DDGS (12%) and have begun to capture a fraction of the oil before adding the CDS to the wet cake (Winsness and Cantrell, 2009). This oil, high in both free fatty acids and antioxidants (Moreau et al., 2010), is extracted one of two ways. The first, and most simple method, is by heating the CDS to promote oil separation, then centrifuging the oil from the stream. The second method uses the CDS to rinse the free oil from the wet cake, then it follows the first methods steps of heating and centrifuging. The first method recovers about 1.0 lb oil per bushel of corn while the second method captures 50% more oil per bushel than the first (Winsness and Cantrell, 2009). Both oils are termed post fermentation corn oil (Winkler-Moser and Breyer, 2011) and are typically transported by railcar or tank truck to biodiesel plants. Sometimes the oil is sold as a feed ingredient.

The remaining oil that was not captured during centrifugation remains in the CDS and is added to the wet cake. The reduced-oil CDS is sprayed onto the wet cake while in a drum dryer producing DDGS with a slightly lower oil content, 6-8%, than typical DDGS. Moisture content is reduced in the dryer to increase shelf life of the DDGS. Select plants provide "Modified Distiller's Grains," which have a higher moisture content (30-40% wb) than the DDGS and a shorter shelf life of 3-7 days. Both DDGS and MDGS are sold from the ethanol plant as an animal feed. The DDGS can sell for $130-250 per ton and PF corn oil for around $0.27-$0.50 per pound, though these prices change as other competing product values change.

2.4. Wet Milling Production

When corn is processed by wet milling, the primary products are corn sweeteners, but there is processing flexibility to produce ethanol or other products. The corn sweeteners industry has seen many changes since its initial commercialization in 1866. By 1968, efforts had shifted away from the initially produced corn sugars and instead focused on 42% fructose corn syrup, the first high fructose corn syrup (HFCS) available. After this syrup was available, demand for sugar in general was boosted causing an increase in sucrose (table sugar) price. This drove the market for the less expensive alternative, HFCS, causing a nearly exponential growth in sales (Hebeda, 1987). Sales and consumption of different HFCS blends grew until reaching a peak in 2000 (USDA, 2015), when concerns were raised about the supposed link between HFCS and obesity. Since then, there has been a steady decline in HFCS production to 8.5 million short tons produced annually in 2015, which mirrors production levels of the 1980s. Though production has decreased, corn syrups still account for 37% of all sugar calories consumed in the U.S. (USDA, 2015) and a large reason that these wet milling plants are able to handle the changing market is because of the value of coproducts produced.

Wet milling is different from dry-grind milling because it separates the components of the kernel to be used for multiple end products, e.g. syrups, oils, feed. The corn is heated and soaked in a water-sulfur dioxide solution to soften the kernel and ease component separation. A series of milling, centrifuging, and washing steps separates the germ from the corn slurry. Fiber is then removed by screening, which leaves the corn slurry containing primarily the endosperm of the kernel. The endosperm contains the majority of the starch and 75% of the protein. Starch is separated from the protein, or gluten, by centrifuging. The starch moves forward in the process to become corn syrups (May, 1987).

The protein fraction from the corn is dewatered in a centrifuging step to 12% solids. Next it is dried to 10% moisture then sold for animal feed (CGM) (May, 1987). The final product, CGM, contains 60-70% protein, 12-15% starch, and 3-7% oil (Di Gioia et al., 1999). The CGM is valued to the wet milling companies because it is a highly digestible, protein-rich animal feed, sold for $555/ton, (Anonymous, 2016).

2.5. Carotenoid Background

2.5.1. General Carotenoid Chemistry

The yellow color of corn is caused by the presence of carotenoids (Wright, 1987). Carotenoids are compounds responsible for the natural yellow, orange, and red pigment present in a variety of plants, animals, fruits, and vegetables. Carotenoids are important phytochemicals and have been studied extensively for their health benefits. They are also valued as a source for natural food colorants.

Carotenoids exist in two structural forms (Figure 2): polyunsaturated hydrocarbons and oxygenated hydrocarbons, more commonly labeled as carotenes and xanthophylls, respectively (Güçlü-Üstündağ and Temelli, 2004). Although both xanthophylls and carotenes provide color to biological materials and have value in the nutraceutical market, they are different in structure and activity.

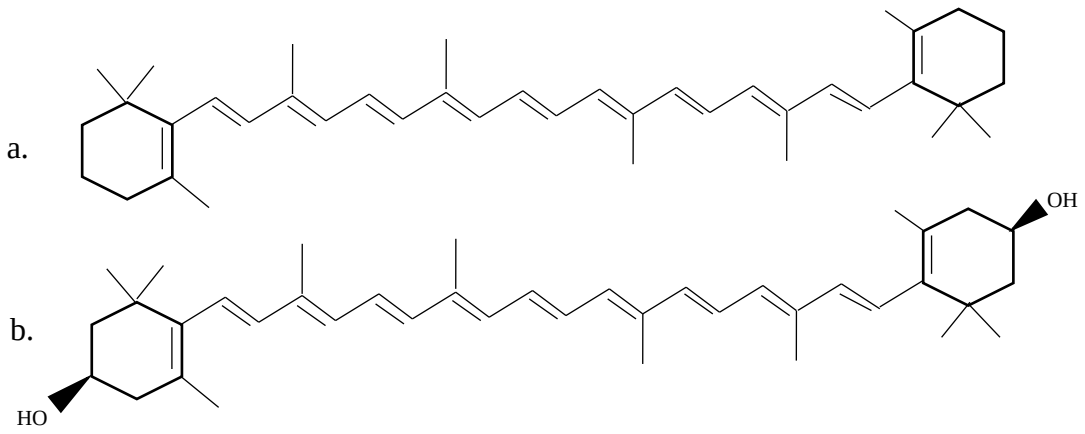

Figure 2. Structure of β-carotene (a) and zeaxanthin (b), examples of carotenes and xanthophylls, respectively

Carotenes consist of long polyunsaturated hydrocarbon chains, making them nonpolar. They are soluble in organic solvents such as petroleum ether and hexane (Craft and Soares, 1992). These compounds are precursors to vitamin A which means, when β-carotene, for example, is cleaved in half by the enzyme carotene deoxygenase, it becomes a molecule which contains vitamin A activity (Mukhopadhyay, 2000a). Vitamin A activity is important because it protects the body from free radical cell damage that can cause the growth and replication of abnormal cells resulting in cancerous tumors (Güçlü-Üstündağ and Temelli, 2004).

Xanthophylls are oxygenated carotenoids, which makes them more polar than carotenes (Shen et al., 2009). They are soluble in semi polar solvents such as ethanol and methanol. This type of carotenoid has no provitamin A activity because of the hydroxyl groups present on either one or both ends of the xanthophyll structure. Lutein and zeaxanthin are two specific types of xanthophylls. Both of these xanthophylls are the only carotenoids found in the macular of the retina (Luo and Wang, 2012) so they have been studied extensively for their ability to lower the occurrence of cataracts and macular degeneration in the human eye (Seddon et al., 1994).

One concern with all carotenoids, xanthophylls and carotenes, is that the conjugated double bonds in their structure makes them susceptible to oxidation in the presence of heat, light, unsaturated fats, peroxides, and some metals (Weber, 1987). Additionally, heat, light, acids, and refluxing in an organic solvent can cause the carotenoids to isomerize from the natural trans state, to the cis state resulting in reduced color intensity and Vitamin A activity (Güçlü-Üstündağ and Temelli, 2004). Carotenoid degradation is an important aspect to consider when developing an extraction method to maximize carotenoid extraction.

2.5.2. Carotenoids as Natural Food Colorants

Carotenoids are valuable to the food industry because they can be used as natural food colorants to provide a range of pigment, from yellow to red. Food color has a huge impact of consumer perception of quality. In fact, colorants have been added to our food since the 1500s in Egypt. Synthetic food colorants began being developed in the late 19th century. The list of available synthetic colorants grew to 700, but by 1906 only 7 of the original compounds developed were allowed into food products by the FDA. Safety risks highlighted from several tragedies associated with the synthetic colorants were the primary concern (Downham and Collins, 2000). To take the place of the synthetic additives, natural colorants, such as carotenoids, grew in popularity.

Carotenoids have been used for centuries to add color to food. Typical sources of these have been saffron, tomatoes, and most popular, annatto. Marigold is another source for carotenoids and this plant has high concentration of lutein similarly to corn. In addition to being used as a food color additive, the lutein from marigolds has been used as a feed additive to pigment broiler chicken skin (Delgado-Vargas and Paredes-Lopez, 2003). The carotenoids, specifically xanthophylls, present in corn coproducts could be used in similar applications.

2.5.3. Carotenoids in Corn

Approximately 95% of the carotenoids present in corn are located in the endosperm of the kernel. The remaining carotenoids are found in the germ (4%) and the bran (1%). The amount of these carotenoids present will fluctuate greatly depending on the corn hybrid. Carotenoid contents in different corn hybrids can vary from 8.5 mg/kg to 72.0 mg/kg (Weber, 1987). Additionally, harvesting, post harvesting, and processing conditions can have a significant impact on carotenoid content. For example, during one year of storage, carotenoid concentration in a sample of corn maintained at 25°C was found to decrease by half (Quackenbush, 1963).

There are five major carotenoids in yellow dent corn. The xanthophylls present are lutein, zeaxanthin, and β-cryptoxanthin and the carotenes are α-carotene and β-carotene. Xanthophylls make up the majority of the carotenoids present in corn and lutein is typically found in the highest quantity. Moros et al. (2002) reported lutein was the most abundant xanthophyll present with a concentration nearly three times that of zeaxanthin

Table 1. Moreau et al. (2007) reported nearly the opposite with zeaxanthin concentration being twice as high as lutein. In both cases, β-cryptoxanthin was found in very small quantities. The differences in content are likely caused by differences in hybrid or extraction and analysis methods. However, as a rule in corn, xanthophyll concentration is higher than carotene concentration.

Table 1. Content of carotenoids in whole corn

	Carotenoid Content in whole corn (µg/g corn)	
Component	Ground corn [a]	Ground corn [b]
Lutein	14.5 a.	2.6
Zeaxanthin	5.2 a.	4.6
β-cryptoxanthin	0.39 a.	2.2
β-carotene	No data	1.2

[a] (Moros et al., 2002)
[b] (Moreau et al., 2007) reported in µg/g oil – converted to µg/g corn

2.5.4. Carotenoids in Corn Coproducts

A large portion of the carotenoids present in corn are retained and concentrated through processing and end up in the corn coproducts. Though carotenoids are present in both CGM and PF corn oil, the milling methods to obtain the products are so unique that the location and quantity of the carotenoids in the two products are likewise quite different. In dry grind milling, the entire kernel is crushed together, which causes the oil in the germ to disperse through the carotenoid rich endosperm. The fat soluble carotenoids migrate to the oil phase. However, during wet milling, the germ is separated from the kernel, therefore the carotenoids remain in the endosperm. They then subsequently end up in high concentration in the CGM due to their association with protein.

2.5.5. Carotenoids in Post Fermentation Corn Oil

The carotenoids present in the PF corn oil are the same as those found in unprocessed corn: β-carotene, lutein, zeaxanthin, and β-cryptoxanthin. The levels of carotenoids in PF corn oil was reported to be as high as approximately 400 µg/g (Table 2). The results again represent the impact hybrid and processing conditions can have on variability in carotenoid content, especially in the case of β-carotene and β-cryptoxanthin.

Table 2. Carotenoid content in Post Fermentation corn oil

	Content in PF oil (µg/g oil)	
Component	Study 1 [a]	Study 2 [b,c]
Lutein	75.7	85.8-92.8
Zeaxanthin	45.6	55.6-88.3
β-cryptoxanthin	7.4	102.6-169.8
β-carotene	0.86	35.3-56.5

[a](Winkler-Moser and Breyer, 2011)
[b] Range of numbers is presented because they are the values from multiple plants
[c] (Moreau et al., 2011)

The concentration of all carotenoids in PF corn oil is much higher than the amount found in traditional corn germ oil, which is produced and used for cooking oil (Figure 3). This is because corn germ oil is the oil extracted from the separated corn germ and only 4% of the total carotenoids found in corn reside in the germ. But, when the entire kernel of corn is ground and extracted with an alcohol solvent, the carotenoid content of the oil is more comparable to the PF corn oil (Figure 3).

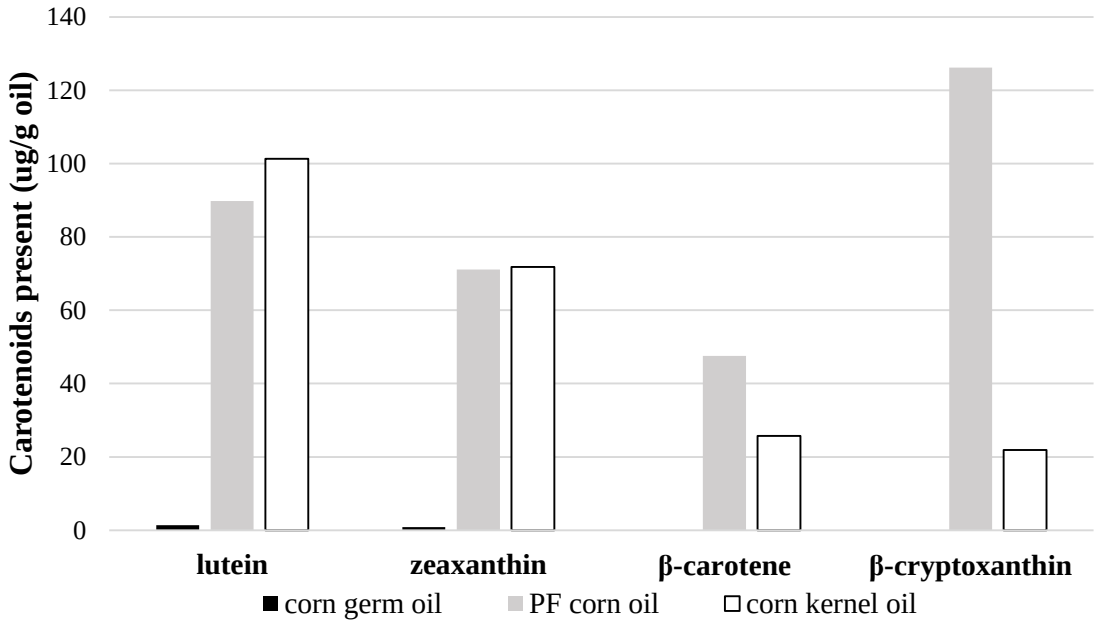

Figure 3. Comparison of carotenoid content in different corn oils (Moreau et al., 2010)

2.5.6. Carotenoids in Corn Gluten Meal

Corn gluten meal is known for its bright yellow color, which is caused by the carotenoids tightly associated with the proteins present in the CGM. The largest portion, ~95%, of carotenoids present in CGM are of the xanthophyll subclass (Blessin et al., 1963). In total, CGM has been reported to contain 290-520 µg/g xanthophylls depending on corn variety (Wright, 1987). Two specific xanthophylls, lutein and zeaxanthin, make up the majority of the

xanthophylls present, though lutein content tends to be higher than the zeaxanthin content (Table 3).

Table 3. Carotenoid content in samples of corn gluten meal

	Corn Gluten Meal		
Component	Carotenoid concentration (µg/g)		
	Study 1[a]	Study 2[b]	Study 3[c]
Lutein	106.9 ± 1.41	91 ± 0	113.5
Zeaxanthin	34.3 ± 0.56	49 ± 1	140.1
β-cryptoxanthin	4.8 ± 0.09	3 ± 0	No data
β-carotene	No data	15 ± 0	No data

[a] (Moros et al., 2002)
[b] (Saez et al., 2015)
[c] (Lu et al., 2005)

2.6. Extraction Methods

2.6.1. General Approaches for Extracting Carotenoids

The carotenoids present in corn end up in large quantities in the industry coproducts such as PF corn oil and CGM. Extracting the lutein and zeaxanthin from the coproducts would allow them to be used as components in food colorants or pharmaceutical applications. Appropriate extraction methods for recovering the carotenoids present in PF corn oil and CGM need to be established. Possible extraction methods for carotenoid extraction from solid or liquid matrices include solvent extraction and supercritical fluid extraction. Additionally, solid phase extraction can be used to remove carotenoids from liquid samples.

Solvent extraction is the most widely used, method because it is a simple process that has been scaled up industrially in the past (Mattea et al., 2009). Unfortunately, solvent extraction requires large amounts of organic solvents, typically hexane, which can bar the use of the product in the *natural* food colorant market. Additionally, carotenoids can degrade when extracted with heated solvents.

Solid phase extraction uses solvents and a solid media to separate components from a liquid matrix like PF corn oil. This method uses less solvent than solvent extraction and has the selectivity to separate very similar compounds from each other. Finally, supercritical fluid extraction takes advantage of the unique properties that materials possess in the supercritical state such as high diffusivity, increased density, and low viscosity. Some supercritical fluids, such as carbon dioxide or propane, are strong solvents when they are compressed and heated. Supercritical extraction is advantageous because it minimizes the use of organic solvents.

2.6.2. Solid Phase Extraction

During solid phase extraction (SPE), a packing material in a column (stationary phase) is used to bind analytes present in the sample by different interactions. Solvents with varying properties are used to condition the column to bind the components of interest. Once the sample has been passed through the column, the bound analyte is rinsed off the packing material and collected.

Solid phase extraction (SPE) can be a very selective and effective method to separate components that are quite similar from each other. It is also a cost effective and versatile method (Shen et al., 2009). There are three types of SPE: ion exchange, reversed phase, and normal phase. Ion exchange SPE separates charged compounds from aqueous solutions. Reversed phase and normal phase SPE both operate on the same principles as adsorption by separating compounds based on polarity differences (Supelco, 1998).

In reversed phase SPE, a nonpolar stationary phase is used to adsorb nonpolar components from a moderately polar to polar sample matrix, typically aqueous. As the sample is passed through the column matrix, less polar compounds present in the sample will be retained on the solid phase due primarily to the carbon-hydrogen hydrophobic bond interactions with the groups on the adsorbent. The interactions can be broken when a less polar solvent is passed

through the sorbent. Typical sorbents in reversed phase extraction are alkyl- or aryl- bonded silica.

Normal phase SPE contains a polar stationary phase, which selects slightly polar to polar components from a less polar sample matrix (Supelco, 1998). The adsorption media in this case are polar bonded silica. Often times the sample matrix used with normal phase SPE is oil-based. Polar compounds present in the oil matrix will be attracted to the hydrophilic groups on the adsorbent material by hydrogen bonding, and dipole interactions. These interactions are disrupted by increasingly polar solvents.

Many advances have been made in SPE recently because it is a popular sample preparatory step for some HPLC methods (Hennion, 1999). Scientists have developed sorbents beyond the classic silica allowing researchers to separate isomers from each other. Silica is still the backbone of many adsorbent materials, but silica on its own has been known to cause irreversible binding (Mateos and García-Mesa, 2006). This becomes a problem when desorbing compounds of interest. Bonded silica like diol-silica and NH2 solve this issue by containing chains, which form slightly weaker interactions that are capable of being reversed.

To develop an SPE extraction method appropriate for the sample matrix and analytes of interest, an understanding of the properties of both is required. In order to maximize compound separation, the interactions between the sorbent, matrix, and analyte must be considered (Hennion, 1999). Normal phase SPE is likely the best for isolating carotenoids from PF corn oil. This is because the PF corn oil sample matrix is nonpolar and our analytes of interest, the xanthophylls called lutein and zeaxanthin, each contain at least one polar group on their structure. These polar groups will interact with the polar groups on the sorbent, while the bulk oil

will pass through the column first. Selecting the proper normal phase SPE column and conditioning and rinsing solvents require modification to identify the best SPE method.

2.6.3. Supercritical Fluid Extraction

2.6.3.1. Principle of Supercritical Fluid Extraction

A supercritical fluid is a liquid or gas that has been compressed and heated beyond a critical temperature and pressure. This critical pressure and temperature are unique properties to each different fluid. Beyond this critical point (Figure 4), the fluid exists in a supercritical fluid state where it exhibits properties of both liquids and gases. The fluid's density and diffusivity increase, while its viscosity decreases.

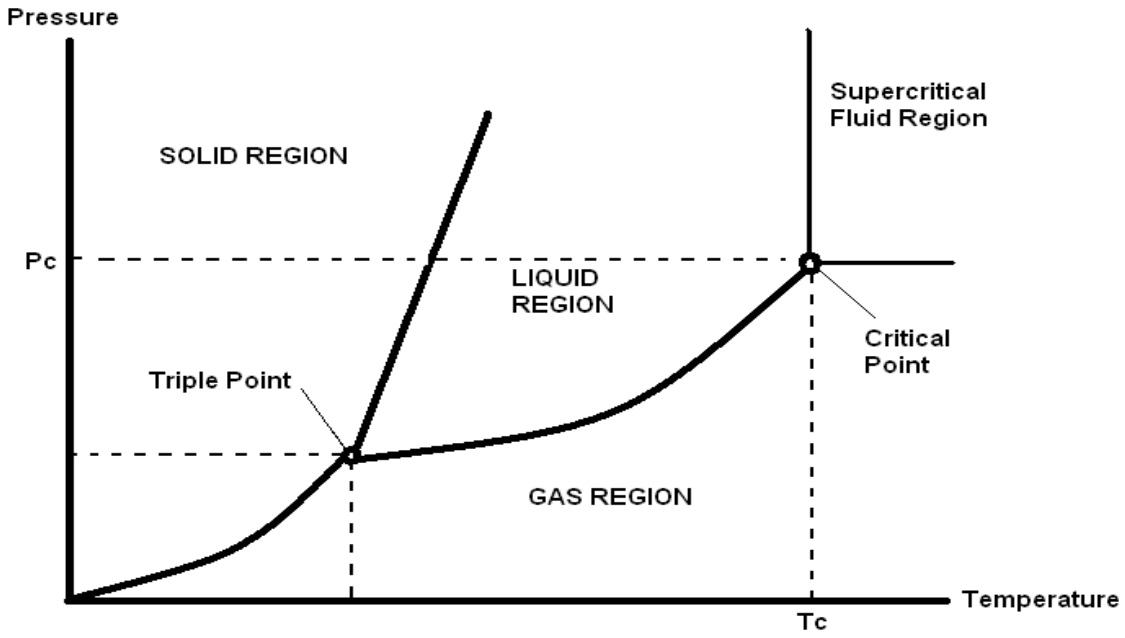

Figure 4. A typical phase diagram indicating the different states of matter at given pressure and temperature settings (Barron, 2012)

Supercritical fluids can extract components of interest from solid matrices by taking advantage of the fluid's solvent-like properties. One of the most popular fluids used for SCF extraction is CO_2 because of its low cost, inert nature, and availability. Extracts obtained with supercritical carbon dioxide (SC-CO_2) maintain no solvent odor or taste and are generally

recognized as safe (GRAS) by the FDA (Mukhopadhyay, 2000b). Moreover, extracting with SC-CO_2 is considered environmentally friendly. This is because the commercial CO_2 used for extracting is already produced as by-product from industrial processes like fermentation so using it with SC-CO_2 extraction does not cause a net increase in CO_2 present in the atmosphere and the CO_2 can be recycled in the extraction unit and reused (Mukhopadhyay, 2000b). Another advantage to CO_2 is its relatively low critical temperature of 31˚C. The low critical temperature is beneficial for low industry costs but even more important for extracting thermally labile components like carotenoids. On the other hand, the critical pressure of 1070 psi is higher than most other commonly used solvents such as propane (616 psi) and ethylene (730 psi) (Mukhopadhyay, 2000b). Despite the high critical pressure, SC-CO_2 has favorable properties for use as an extraction solvent.

Supercritical CO_2 is a nonpolar solvent that can replace one of the most commonly used nonpolar solvents, hexane. The solubility of a compound in supercritical CO_2 is dependent on the compound's polarity, molecular weight, and structure. Lower molecular weight and low polarity components are extracted easily at low pressures in SC-CO_2 because they best match the polarity of the SC-CO_2. Moderate to highly polar compounds are almost insoluble in supercritical CO_2. The pressure and temperature of the fluid can be adjusted to better solvate certain compounds. When adjusting these parameters is not enough, a solvent modifier can be added during extraction to adjust the overall polarity of the solvent. Methanol and ethanol are often used as solvent modifiers to increase extraction of polar compounds. SC-CO_2 extraction, especially when coupled with a modifying solvent, can be as successful as solvent extraction.

SCF extraction has gained popularity in the last three decades because when carbon dioxide is used as the solvent, the extracts obtained are considered natural and contaminant free

(Güçlü-Üstündağ and Temelli, 2004). It is used already on a large scale for decaffeination of tea and coffees as well as for refining of cooking oils (Mukhopadhyay, 2000b). SC-CO_2 extraction has also been the subject of much research and development over the years for extraction of various compound from samples derived from nature. Though SCF extraction can be performed on various sample types, the basic system for all extractions is the same.

The four primary components included in an SCF extraction system are a high pressure pump, heater, extraction chamber, and separation chamber. The fluid is heated and pressurized before being pumped into the extraction chamber. This chamber is able to withstand extreme pressure conditions. Following extraction, the extract-laden fluid exits the pressurized chamber and undergoes the separation step where a reduction in pressure causes precipitation of the extract. The solvent, free of any extract, can then reenter the pump to be pressurized for reuse. Some systems have a more complex separation chamber especially if the goal is to separate more than one component in the extract. The separation chamber can be held at different pressures and/or temperatures in order to facilitate the precipitation of only certain components in the extract. All simple systems will contain at least the four main components discussed previously.

2.6.3.2. Extracting Carotenoids from Corn Gluten Meal

During the wet milling process, the carotenoids present in corn concentrate in the protein fraction of the corn that will end up in the final product, i.e. corn gluten meal (CGM), which is sold as animal feed. The carotenoids deposit in the animal's flesh, pigmenting the meat yellow. This is beneficial in some animals' diets, such as poultry, because it imparts a desirable yellow hue in the meat and brightens the color of egg yolks (Wright, 1987). Unfortunately, xanthophyll levels can vary substantially in CGM which can cause inconsistent color and makes formulating diets difficult (Muralidhara, 1997). If CGM is fed to fish or other livestock, the resulting yellowed flesh and fat can reduce the market value of the animal products. The carotenoids

present do not actually affect the flavor or shelf life of the product, but buyers will shy away from meat with yellow pigmentation as they perceive it to be of lower quality (Lovell, 1984). These concerns have led animal feed researchers, especially those in aquaculture, to investigate different ways to remove the xanthophylls from the CGM.

Many researchers (Li and Han, 2009; Lu et al., 2005; Park et al., 1997; Saez et al., 2015; Sessa et al., 2003) have investigated CGM decolorization so that incorporating the high protein meal into animal's diets does not affect the color of the animal. Extracting the carotenoids from corn gluten meal can happen in a number of ways, but selecting an effective method has been especially challenging because the proteins in the gluten meal form a complex with the carotenoids. The proteins present trap the carotenoids in a hydrophobic pocket of their helical structure. This complex has been studied since the 1970s (Zagalsky, 1976) and a model of the protein was proposed in 2004 (Lawton et al., 2004). This model clarified that the protein structure must be disrupted to access the carotenoids. Maintaining the high protein level in the meal is also important though, because CGM is valued for its protein content. An extraction of carotenoids from CGM will not be effective unless it is able to open up the protein structure to access the carotenoids without solubilizing and extracting the proteins.

Solvent extraction of carotenoids from CGM has typically been performed with organic alcohols because the alcohol's polar nature will open up the alcohol soluble protein group, zein (Sessa et al., 2003). Park et al. (1997) performed solvent extraction using ethanol and butanol to remove carotenoids from CGM. Using ethanol, 97% of the carotenoids present were removed by the 4^{th} extraction compared to 94% by the 2^{nd} extraction using butanol. This group reported a solvent odor remaining in the butanol extracted CGM, which is a concern for animal feed. Park

et al. (1997) was concerned with just removing the carotenoids from the CGM, but other researchers were also interested in purifying the carotenoids for further use.

A number of patents (Cook et al., 1993; Muralidhara, 1997; Muralidhara and Cornuelle, 1998) concerning purifying CGM with some form of solvent extraction have been filed. One patent (Cook et al., 1993) established a method for extracting and purifying the zein protein from CGM as it can be used in things like plastics, coatings, and food products. During this process, the pigments are removed after the enzyme and alkaline treated CGM is washed with 95% ethanol. Previous work on purification of zein from CGM used techniques that would restrict the use of the zein in food products. For example, some (Mason and Palmer, 1934) used toxic hydrocarbon solvents while others (Carter and Reck, 1970) left impurities in the zein after the process. The work done by Cook et al. (1993) used a GRAS solvent—ethanol—but it generated a xanthophyll extract with a rubbery, paste-like consistency. This paste product was difficult to incorporate into food matrices and thus additional steps such as saponifying and purifying of the alcohol extracted CGM were completed (Muralidhara, 1997; Muralidhara and Cornuelle, 1998). These final steps in the process are important because they convert the extract from a crude oleoresin to a purified powder suitable for use in food products or pharmaceuticals. Though these methods were successful in developing a purified powder, the xanthophyll recovery was only 36% (Muralidhara, 1997) and 47% (Muralidhara and Cornuelle, 1998) indicating that the protein-carotenoid complex was likely not disrupted adequately to facilitate extraction.

The protein-carotenoid complex issue was addressed by pretreating the CGM with protease enzymes to break apart the zein structure with the intent of recovering more xanthophyll (Li and Han, 2009; Lu et al., 2005). The parameters of the enzymatic treatment, including enzyme concentration, solids loading, and hydrolyzing time, were varied to determine an

optimum pretreatment for maximizing xanthophyll extraction. The commercial proteases (manufactured by Novozymes) used in these two studies varied in their optimum operating pH (Alcalase, pH 8 (Li and Han, 2009) and Neutrase, pH 6.5 (Lu et al., 2005)), but the operating temperature was the same for both (37°C). In both experiments, carotenoid extraction from CGM increased (Table 4), though in the study using Neutrase (Lu et al., 2005) the increase was much more significant. However, disrupting the protein complex clearly increased carotenoid removal (Lu et al., 2005; Li and Han, 2009).

Table 4. Comparison of carotenoid yield from solvent extracted corn gluten meal (CGM) and enzyme pretreated CGM then extracted with solvent

Compound	**Pigment yield (µg/g)**			
	Neutrase treatment [a]		Alcalase treatment [b]	
	Without enzyme-treatment	Enzyme-treated	Without enzyme-treatment	Enzyme-treated
Lutein	74.1	113.5	35.63	41.42
Zeaxanthin	77.1	140.1	14.14	15.38
β-Cryptoxanthin	No data	No data	1.43	1.44
Total carotenoids	393.6	599.1	No data	No data

[a] (Lu et al., 2005)
[b] (Li and Han, 2009)

Though the previously discussed studies establish that solvent extraction of CGM can be quite effective, these methods all use large quantities of solvent that must later be removed from both the CGM and the extract by means of evaporation, which is a large energy expense for industrial plants. In addition, if the goal is to use the extract in food applications, solvent extraction can limit the use of the extract in certain food systems.

There have been other experimental approaches tested for decoloring the CGM or the zein present in CGM including activated carbon treatment (Sessa et al., 2003) and bleaching with soy flour, a source of carotenoid destroying lipoxygenases (Saez et al., 2015). These methods have been, in general, less than satisfactory. Activated carbon treatment was applied to

commercial zein dissolved in 80% ethanol, which produced zein with a green hue. In the same study (Sessa et al., 2003), zein was extracted from CGM with 65% ethanol. The supernatant was discarded and the remaining solids were then extracted twice with 82.5% ethanol, centrifuged and supernatant collected between each extraction. The supernatant was mixed with activated carbon, then filtered and spray dried. Sessa et al. (2003) found that though the powder remaining after the spray drying step was white, the recovery of zein protein was low – 25% (Sessa et al., 2003). They concluded the low zein recovery occurred because the activated carbon retained some of the zein. In addition to the low zein recovery, the colored extract was not available to be collected because it was trapped in the activated carbon. In another study Saez et al. (2015), bleaching the CGM by mixing it in a slurry with soy flour. More than 60% of the pigment present was removed, but again, this method did not allow for collection of the pigment. Collecting and selling the extract from CGM can be instrumental in offsetting the cost of the extraction, thus it is an important consideration when decolorizing CGM.

2.6.3.3. Supercritical CO_2 Extraction of Carotenoids from CGM

To overcome the issue of large solvent usage and ensure that the xanthophyll rich extract is available for collection, supercritical carbon dioxide extraction (SC-CO_2) has been studied for its ability to recover lipid components from a wide range of natural sources including corn germ (Christianson et al., 1984), sage (Reverchon, 1996), and fennel (Simándi et al., 1999). Even more relevant, it has been used in carotenoid extraction studies from various matrices such as carrot (Barth et al., 1995), marjoram (Vági et al., 2002) and microalgae (Mendes et al., 1995). This method is as effective as solvent extraction for capturing lipids as well as carotenoids and the extracts produced by SC-CO_2 are considered natural (Mukhopadhyay, 2000a).

Though using SC-CO_2 extraction for carotenoids is ideal because of the low temperature conditions, some carotenoids have a relatively low solubility in SC-CO_2 (Mattea et al., 2009). β-

carotene, and other nonpolar hydrocarbon carotenes, are soluble in CO_2 at a wide range of temperatures (14-80˚C) and pressures (725-26,000 psi), but carotenes only make up a small fraction of the total carotenoids present in corn (Gast et al., 2005). The xanthophylls, lutein and zeaxanthin are in higher concentration in corn, and they have limited solubility in SC-CO_2. Additionally, carotenoid-protein complexes present specifically in CGM reduce the ability of SC-CO_2 to interact with the xanthophylls for extraction. The solution to this issue is to use solvent modifiers, such as ethanol, which can be added in small quantities during extraction to manipulate the polarity of the fluid to better match the xanthophyll polarity. Modifiers can also open up the protein structure to better access the xanthophylls (Sessa et al., 2003).

A SC-CO_2 extraction of CGM as a means to reduce flavor compounds present in the meal has also been evaluated (Wu et al., 1994). Although the goal of this study did not include removing carotenoids, lipid extraction was measured and because carotenes have lipid solubility maximizing lipid extraction will likely increase carotene extraction as well. Xanthophylls on the other hand, are only slightly oil soluble, thus maximizing lipid extraction may not increase xanthophyll recovery. Wu et al. (1994) found that by reducing the particle size from 637 µm to 105 µm, fat extraction increased by 12% with CO_2 pressure of 9,800 psi and temperature of 80°C. Sessa et al. (2003) measured fat extraction of zein, using SC-CO_2 zein extracted with or without a solvent modifier (absolute ethanol). The extraction with solvent modifier increased fat extraction an additional 43.4% when extraction conditions were 10,000 psi, 70°C, 15% modifier. These authors also reported higher removal of yellow pigment from the zein.

The incomplete removal of yellow pigment was evidence of both the insolubility of xanthophylls in CO_2 even with an ethanol solvent modifier, and the inability of the protein-carotenoid complex to be completely broken apart (Sessa et al., 2003). The purpose of the

solvent modifier is to increase the polarity of the CO_2 and to open up the structure of the alcohol soluble zein protein. Zein is actually a mixture of a variety of peptides of different size and solubility, the two in largest quantities being α-zein and β-zein. α-Zein is soluble in 95% aqueous ethanol and β-zein is insoluble in 95% ethanol, but soluble in 60% ethanol (Shukla and Cheryan, 2001). When extracting xanthophylls from zein – containing systems, aqueous ethanol will likely be a more effective modifier. Sessa et al. (2003) used absolute ethanol, which could explain the incomplete decoloring of the zein.

As stated previously, many have optimized xanthophyll extraction from samples using SC-CO_2. Optimizing the SC-CO_2 procedure is an essential step because the solubility of the analytes in the CO_2 is controlled by its density. Processing parameters, which can have a significant impact on the recovery of xanthophylls include CO_2 pressure, temperature, and flow rate, along with amount, if any, of solvent modifier added. Both Ciftci et al. (2012) and Vági et al. (2002) found pressure to the most significant factor affecting SC-CO_2 recovery of xanthophylls from dry distillers grains with solubles and marjoram, respectively,.

SC-CO_2 extraction on DDGS without solvent modifier was somewhat effective (Ciftci et al., 2012). By varying pressure from 5100 psi to 7100 psi and temperature from 50°C to 70°C, this group was able to model and determine optimum extraction conditions. They measured carotenoid content of the extract and found the highest extraction of 108 mg/kg and 107 mg/kg total carotenoids at 7100 psi and both 60 and 70°C, respectively. The SC-CO_2 method extracted 20 mg/kg more carotenoid than the solvent extraction of DDGS using petroleum ether. SC-CO_2 extraction of lutein from marjoram was explored (Vági et al., 2002). In this study, best conditions for lutein recovery with no solvent modifier were observed at 50°C and 6526 psi, and at 60°C and 5801 psi. The recovery of lutein with SC-CO_2 extraction, 56.5 mg/kg, was lower but

comparable with hexane solvent extraction recovery 69.2 mg/kg. Lutein recovery with ethanol extraction, however, was nearly double that of SC-CO_2, 95.4 mg/kg. Temperature is important when working with carotenoids because they are thermally labile, but in both studies, the highest temperatures gave the highest carotenoid recovery. This is because the higher temperature increases the solubility of the carotenoids in the CO_2 and, as long as 80°C is not exceeded, carotenoid degradation was avoided (Careri et al., 2001).

Neither of the previously discussed studies used a solvent modifier to manipulate the CO_2 polarity. Work done by Careri et al. (2001) did use varying amounts of modifier to optimize extraction of specific carotenoids (zeaxanthin, β-cryptoxanthin and β-carotene) from *Spirulina pacifica* algae. The procedure conditions varied were temperature (40°C-80°C), pressure (2175 psi-5076 psi), dynamic extraction time (40-100 min), and modifier percentage (5-15%). The following conditions were found to maximize zeaxanthin extraction: 80°C, 5076 psi, 70 min dynamic extraction, and 15% v/v modifier. SC-CO_2 extract recovery of zeaxanthin from the algae was nearly equal to that of solvent extraction. Their results indicate that the CO_2 pressure and percentage of modifier added had the largest effect on the zeaxanthin extraction.

2.7. Conclusion

Post fermentation corn oil and corn gluten meal as sources of valuable carotenoids has been reported. These carotenoids could be used as food additives to supply yellow pigment and protect from oxidation, which could result in customer appeal and increased shelf life. In order to add them into food products, extraction procedures that are effective need to be analyzed and tested. Solid phase extraction of carotenoids from PF corn oil has potential because it can be highly selective and cut down on solvent usage. Supercritical CO_2 extraction of CGM is feasible because past studies have shown it can be effective for when solvating carotenoids especially in the range of the following conditions: 5,076-7,100 psi, 50-80 °C, and 5-15% solvent modifier

(Careri et al., 2001; Ciftci et al., 2012; Vági et al., 2002). Additionally, the clean extracts that can be obtained by SC-CO_2 will increase their marketability.

3. PROBLEM STATEMENT

3.1. Summary of Literature Review

To seek additional value in co-products derived from the corn industry, our group initially looked at the dry distillers grains with solubles (DDGS) obtained from dry-grind corn ethanol plants. Research had shown that DDGS contain 11-14% crude oil (Kim et al., 2008a; Moreau et al., 2011), which contains approximately 60-80 µg/g carotenoids,

Table 5 (Winkler-Moser and Breyer, 2011; Winkler-Moser and Vaughn, 2009). Carotenoids, known for their yellow, orange and red pigment are important components because they possess value as natural colorants and as nutraceutical additives. Concentrating these carotenoids from the DDGS oil was of interest.

Table 5. Carotenoid content in dry distillers grains with solubles oil and Post Fermentation corn oil from different plants and studies

Carotenoid content in DDGS oil (µg/g)		**Carotenoid content in PF Corn oil (µg/g)**		
Conventional dry grind plant	Raw starch ethanol plant	Raw starch ethanol plant	Conventional dry grind plant	Conventional dry grind plant
60.0[a]	80.0[b]	70.0[b]	100.0[b]	300[c]

[a] (Winkler-Moser and Vaughn, 2009)
[b] (Winkler-Moser and Breyer, 2011)
[c] (Moreau et al., 2010)

Many corn ethanol plants centrifugally remove a fraction of the corn oil present in the stillage remaining after distillation. The oil is sold to biodiesel production plants and occasionally used in feed applications. This corn oil, termed post fermentation (PF) corn oil, is a readily available source of the same carotenoids found in DDGS, thereby saving the step of extracting the oil from the DDGS. The levels of carotenoids found in PF corn oil are 70 – 300 µg/g, depending on processing conditions at the plant (Moreau et al., 2011; Winkler-Moser and Breyer, 2011).

In order to concentrate the carotenoids from the PF corn oil, solid phase extraction (SPE) was identified as a viable option. A conditioned polar packing material in the column would allow many of the antioxidants, which have polar groups on their structure, to adsorb to the packing material. The majority of the nonpolar oil would then flow through the column with reduced carotenoid content. By then rinsing the packing material with different solvents, the carotenoids could be collected and analyzed.

Another source of carotenoids exists in the wet corn milling industry. Wet corn mills are different from dry mills because they separate the kernel to provide a variety of products from the different parts of corn, namely corn oil, corn syrup, and animal feed. The corn protein, or gluten, is separated from the starch, is dried and sold as a high protein animal feed with the caveat that its high carotenoid content can cause a yellowing of the animal tissue. In some animal markets, e.g. aquaculture, meat with a yellow pigment does not sell well in the United States. Decolorizing the corn gluten meal (CGM) could be beneficial to the animal feed market, but collecting the carotenoids that are removed could be even more valuable.

The major protein class present in the gluten meal is called zein and some research has been done to decolorize and purify the zein from the gluten meal to be used in polymers (Sessa et al., 2003). Most previous decolorizing work has used solvent extraction, which can remain in trace amounts in the final product. There is opportunity to decolorize the gluten meal in its entirety using SC-CO_2 extraction. SC-CO_2 extraction is an appropriate extraction method because it occurs at a lower temperature and in a reduced oxygen environment to preserve the thermally labile and oxygen-sensitive carotenoids better than other extraction methods. Past experiments extracting carotenoids from zein using SC-CO_2 have shown that carotenoids reside structurally wrapped in the proteins and therefore it is necessary to disrupt the protein structure

in order to extract the compounds (Sessa et al., 2003). Additionally, a solvent modifier such as ethanol must be used to increase the polarity of the CO_2 so that it is able to extract the carotenoids. The carotenoids can then be collected and analyzed to determine extraction recovery.

The carotenoids found in the corn milling byproducts are growing in value to the food and pharmaceutical market, especially because they are obtained from a natural source. Their presence in the corn products does not add value to the product and in the case of CGM, it actually reduces the product's marketability. In order to understand if collecting these carotenoids from post fermentation corn oil and corn gluten meal is technically feasible, we need to develop extraction methods. To complete this project, the following objectives were addressed:

3.2. Objectives

Objective 1: To develop a solid phase extraction method using diol silica cartridges to extract carotenoids from post fermentation corn oil by varying the following parameters: column conditioning solvent and rinsing solvent.

Objective 2: To develop a SC-CO_2 extraction method for optimizing extraction efficiency of carotenoids from CGM by varying the extraction temperature, pressure, and amount of co-solvent added. Determine if protein is lost during SC-CO_2 extraction and if so, at what temperature and pressure settings the loss is least significant.

3.3. Hypothesis

Objective 1 hypothesis: The amount of antioxidants that adsorb to the SPE column and are subsequently rinsed off will be affected by the conditioning solvent and rinsing solvent used.

Objective 2 hypothesis: Extraction efficiency of supercritical carbon dioxide extraction of xanthophylls from CGM will vary based on the temperature, pressure, and co-solvent addition.

4. PAPER 1: EXTRACTION OF XANTHOPHYLLS FROM POST-FERMENTATION OIL PRODUCED AT DRY MILL CORN ETHANOL PLANTS

4.1. Abstract

Post fermentation corn oil obtained from dry-grind corn mills was separated using normal phase solid phase extraction (SPE) to yield a xanthophyll-enriched fraction. To optimize this xanthophyll fractionation, the conditioning and rinsing solvents used to prepare and rinse the diol packing material were varied. The solvents used were dichloromethane, isopropanol, and methanol, which represent a range of polarities. The effectiveness of the different solvent combinations was studied to determine a best practice. Conditioning the column with dichloromethane before sample application and then rinsing the xanthophylls from the column with methanol was the most effective method and it led to recovery of 90.5 ± 3.4 % of the xanthophylls present in the oil.

4.2. Introduction

Post fermentation (PF) corn oil is a coproduct from the dry-grind corn ethanol process (Winkler-Moser and Breyer, 2011). Since 2009, many dry-grind corn plants have begun to fractionate this oil from the thin stillage by heating and centrifuging it in order to generate additional revenue from the coproducts (Winsness and Cantrell, 2009). This oil is most often sold as biodiesel feedstock for $0.27-$0.50 per pound. PF corn oil is different from traditional corn oil because of its high (120-200 µg/g) carotenoid content and the associated orange color (Moreau et al., 2011; Winkler-Moser and Breyer, 2011). The compounds present are primarily the oxygenated hydrocarbon carotenoids called xanthophylls, the largest fraction of which are two specific xanthophylls, lutein and zeaxanthin. Xanthophylls are marketable as natural yellow

food additives. High concentration xanthophyll extracts could generate additional income for dry-grind corn ethanol production plants.

Solid phase extraction (SPE) is a simple, cost effective mechanism of separating very similar components from a liquid medium. It can be performed in reversed phase or normal phase to separate compounds based on their differences in polarity. Normal phase SPE separates polar compounds from oil matrices while reversed phase separates nonpolar compounds from polar matrices. A variety of different sorbents are available to best suit the sample matrices and analytes of interest (Supelco, 1998). In addition to selecting the best sorbent, the conditioning, loading and subsequent rinsing solvents must be chosen to optimize retention and subsequent elution of the analyte.

SPE has been used to separate carotenoids from various sample matrices including human blood serum, breast milk, and olive oil (Mateos and García-Mesa, 2006; Shen et al., 2009). Shen et al. (2009) evaluated different SPE sorbent materials, both reversed phase and normal phase, to maximize carotenoid extraction from breast milk and blood serum. The group worked with samples containing a mix of both xanthophylls, which are more hydrophilic, and carotenes, which are more hydrophobic. Although the reversed phase SPE sorbents, C_{18} and C_{30} bonded silica isolated the carotenoid mix best, the normal phase sorbent, diol, isolated 100% of lutein present when the sample was dissolved in hexane. In a another study concerning SPE of olive oil, diol silica was used to isolate 99% of pigments while C_{18} bonded silica only led to recovery of 82% because the C_{18} sorbent had poor retention capacity causing the pigments to be desorb from the solid phase too easily (Mateos and García-Mesa, 2006). The diol sorbent material was developed to solve the irreversible binding problem associated with the classic SPE sorbent, silica. Diol contains alkyl chains with polar functional groups at its surface, which will

interact with compounds containing polar groups (Supelco, 1998). Lutein and zeaxanthin, though hydrocarbon chains, contain hydroxyl groups on both ends of their structure, increasing their polarity and interaction with sorbents such as diol.

The objective of this work was to maximize separation of xanthophylls from post fermentation corn oil using solid phase extraction by varying the column conditioning and rinsing solvents. We hypothesize that we can increase the carotenoids extracted from the oil by varying these solvents. This research would provide a means to extract these carotenoids from the oil, which could result in a more valuable use for the post fermentation corn oil.

4.3. Materials and Methods

4.3.1. Chemicals and Materials

4.3.1.1. Samples

PF corn oil sample was collected from a local corn ethanol producer, Hankinson Renewable Energy (Hankinson, ND). Oil was stored in the refrigerator (4 °C) until used for testing. During refrigeration, a white precipitate settled to the bottom of the oil container. This precipitate in PF corn oil has previously been reported to be made up of primarily triacylglycerol (78%), free fatty acids (14%), and steryl and wax esters (8.6%) (Moreau et al., 2010). When SPE experiments began, PF corn oil samples were taken from the oil in the container without disturbing the precipitate.

4.3.1.2. Materials

Supelco Discovery DSC-Diol SPE tubes (1 g, 6 mL) (Sigma – Aldrich Co., St, Louis, MO) were kept in their packaging or stored in desiccators if packages were opened until the experiment. Diol is a polar packing material and used in normal phase for SPE testing.

All organic solvents used were HPLC-grade; hexane, methanol, dichloromethane, isopropanol, and methyl tert butyl ether (MTBE) were purchased from VWR (Radnor, PA).

Lutein (>93% purity) and zeaxanthin (>98% purity) standards were obtained from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Standard solutions were made by dissolving the pure compounds into Methanol:MTBE (25/75 v/v) and stored at -20°C. The HPLC column used was YMC C30 carotenoid column (3 µm, 250 mm x 4.6 mm i.d.) (VWR, Inc.).

4.3.2. Experimental Design

To optimize the retention of xanthophylls from PF corn oil on the diol packing material, the solvents used for conditioning and rinsing column were varied. The solvents selected to test with these steps were isopropanol, dichloromethane, and methanol. These solvents were selected because they lie in a polarity range similar to those of xanthophylls and they have been successful in SPE of carotenoids in the past (Mateos and García-Mesa, 2006). Polarity indices for solvents range from 0.0 (hexane; nonpolar) to 9.0 (water; polar), and represent the relative polarity of a solvent (Table 6).

Table 6. Polarity index of common solvents

Solvent	Polarity index
Hexane	0.0
Dichloromethane	3.1
Isopropanol	3.9
Methanol	5.1
Water	9.0

All solvent combinations were tested using a 3^2 full factorial experimental design (Table 7). Following a method adapted from Mateos et al. (2006), cartridges were conditioned with 4 mL of a solvent according to the experimental design (Table 7), then conditioned with 4 mL of hexane before adding the sample (1 g oil dissolved in 4 mL of hexane). The sample was then eluted from the column using successive rinses with 3 mL of hexane followed by 6 mL of another solvent according to the experimental design (Table 7). Each treatment was performed

in triplicate for a total of 27 experiments. All test tubes containing oil fractions were purged with nitrogen until dry, then weighed. The final elution fraction was diluted with HPLC diluent, (methanol: MTBE, 25/75 v/v) to a final volume of 5 mL, then stored at -20°C until HPLC analysis.

Table 7. Treatments for Post Fermentation corn oil solid phase extraction experiment indicating solvent conditioning and rinsing steps

Treatment #	Conditioning solvent	Eluting solvent
1	Dichloromethane	Dichloromethane
2	Dichloromethane	Isopropanol
3	Dichloromethane	Methanol
4	Isopropanol	Dichloromethane
5	Isopropanol	Isopropanol
6	Isopropanol	Methanol
7	Methanol	Dichloromethane
8	Methanol	Isopropanol
9	Methanol	Methanol

4.3.3. Carotenoid Separation by HPLC

The final elution oil fraction were analyzed using a method adapted from Gupta et al. (2015) with some modifications. The HPLC column used was YMC C30 carotenoid column (YMC Co., Kyoto, Japan) (3 µm, 250 mm x 4.6 mm i.d.) purchased from VWR, Inc. Reversed phase HPLC using a Waters 2795 HPLC (Waters, Corp,; Milford, MA) equipped with a photodiode array detector (PDA) was used to determine carotenoid concentration. During HPLC runs, the column was maintained at 25 °C. Final elution fraction samples of 10 µl were injected on the HPLC column. A gradient method was developed for sample separation using 98% methanol (v/v with water) and MTBE. The elution gradient is shown in Table 8. For each step in the HPLC method, a linear gradient change occurred over the time allotted until reaching the next solvent level.

Table 8. High performance liquid chromatography gradient method for carotenoid separation

Flow Rate (mL/min)	Time (min)	Solvent A (%)	Solvent B (%)
1.4	0	80	20
1.4	2	60	40
1	12	0	100
1.4	13	80	20
1.4	20	80	20

The original method included a third mobile phase of methanol/water (95/5, v/v), but using this solvent caused a pressure spike on our system exceeding system limits (Gupta et al., 2015). Removing this third solvent from the method still allowed for clear lutein and zeaxanthin peaks and reduced the chance of column damage. Lutein and zeaxanthin standards were made up in five concentrations between 0.01 µg/mL and 300 µg/mL. Carotenoid concentrations were measured at an absorbance of 450 nm on the PDA. Lutein and zeaxanthin were quantified using a 5-point external standard curve.

4.4. Results

4.4.1. Total Xanthophyll Content in PF Corn Oil

Four raw oil samples taken from the bulk PF corn oil container were dissolved in HPLC diluent and evaluated via HPLC to determine the total concentration of carotenoids present. The total lutein concentration was 97.8 ± 15.0 µg/g oil and total zeaxanthin concentration was 84.5 ± 12.2 µg/g oil. The xanthophyll content of these samples varied by 14.4 – 15.3%. The concentration of lutein in the PF corn oil was higher, though comparable to the concentration reported in previous studies, which was 80.8 – 92.8 µg/g oil (Moreau et al., 2010) and 75.7 µg/g oil (Winkler-Moser and Breyer, 2011). Zeaxanthin concentration in the current study was approximately two times higher than the concentration reported in a previous study of PF corn oil, which was 45.6 µg/g oil (Winkler-Moser and Breyer, 2011). Moreau et al. (2010) reported

zeaxanthin concentration of 55.6 – 88.3 µg/g in PF corn oil from 3 plants, which is more comparable to the values found in the current study.

4.4.2. SPE of Xanthophylls from PF Corn Oil

In all experiments, the total mass recovered from the SPE columns was at least 90% of the total oil initially applied to the column and in a majority of the treatments, more than 97% of the oil was recovered in elution fractions (Table 9). High recovery indicated that diol columns did not cause irreversible binding of the oil sample as was reported for silica columns have been reported to do (Mateos and García-Mesa, 2006). This is an important consideration for industrial application because it demonstrates that oil and xanthophyll loss would be negligible. There were three fractions collected for each treatment, the final being the xanthophyll-enriched fraction. The mean oil mass eluted in the first fraction was 64.3 ± 6.8% of input oil while the second fraction had an oil mass elution of 23.5 ± 6.7%. Both of these oil fractions had low xanthophyll concentration, but could still be used as biodiesel feedstock. The oil eluted in the final xanthophyll-enriched fraction was 8.8 ± 2.6% of the input oil.

Table 9. Mass eluted in the three fractions during solid phase extraction

Treatment	**Mass recovery in each elution fraction (% of total oil applied to SPE column)**			
	First fraction	Second fraction	Third fraction	Total Mass recovered
D-D	60.1 ± 2.6%	27.3 ± 1.1%	10.0 ± 0.3%	97.3 ± 1.8%
D-I	62.9 ± 4.7%	24.7 ± 4.4%	9.7 ± 0.7%	97.4 ± 0.8%
D-M	71.5 ± 15%	16.6 ± 14.5%	9.6 ± 0.4%	97.7 ± 0.1%
I-D	61.1 ± 4.2%	27.0 ± 4.2%	7.5 ± 0.4%	95.6 ± 0.1%
I-I	56.9 ± 8.5%	24.5 ± 1.6%	9.0 ± 0.9%	90.4 ± 9.1%
I-M	64.4 ± 3.0%	24.7 ± 3.3%	8.3 ± 0.8%	97.5 ± 0.4%
M-D	66.6 ± 2.9%	24.6 ± 3.4%	6.4 ± 0.6%	97.7 ± 1.3%
M-I	67.2 ± 5.7%	22.3 ± 5.3%	7.3 ± 0.05%	96.9 ± 0.6%
M-M	65.7 ± 3.6%	19.6 ± 10.8%	11.4 ± 7.4%	96.8 ± 0.2%

Figure 5 shows the percentage of total xanthophylls fractionated into the final elution fraction of the SPE treatments. There was high variability between treatments which can be seen by the error bars in Figure 5. Variability within the treatments could have been caused by heterogeneous concentrations of xanthophylls in the oil applied to the solid phase cartridge, which as mentioned previously, varied by 14.4-15.3%. The repeatability of an SPE method for removing polar compounds from olive oil was satisfactory with the coefficient of variation of 4.4 – 6.4% (Grigoriadou et al., 2007). In the current study, the coefficient of variance between the three replicates of each treatment varied between 3% and 38%, and in 6 of the 9 treatments the COV was above the satisfactory level of 15%. One potential cause for the variability could be water adsorption by the SPE columns. The PF corn oil is separated from the dry-grind milling process by heating and centrifuging only, thus it could contain trace amounts of water. The diol packing material uses hydrogen bonding and dipole interactions to adsorb any compounds with hydroxyl groups or other polar groups. Therefore, if the available diol sites on the packing material were occupied by water adsorbed from the air or from the PF corn oil, the xanthophyll fractionation could decrease.

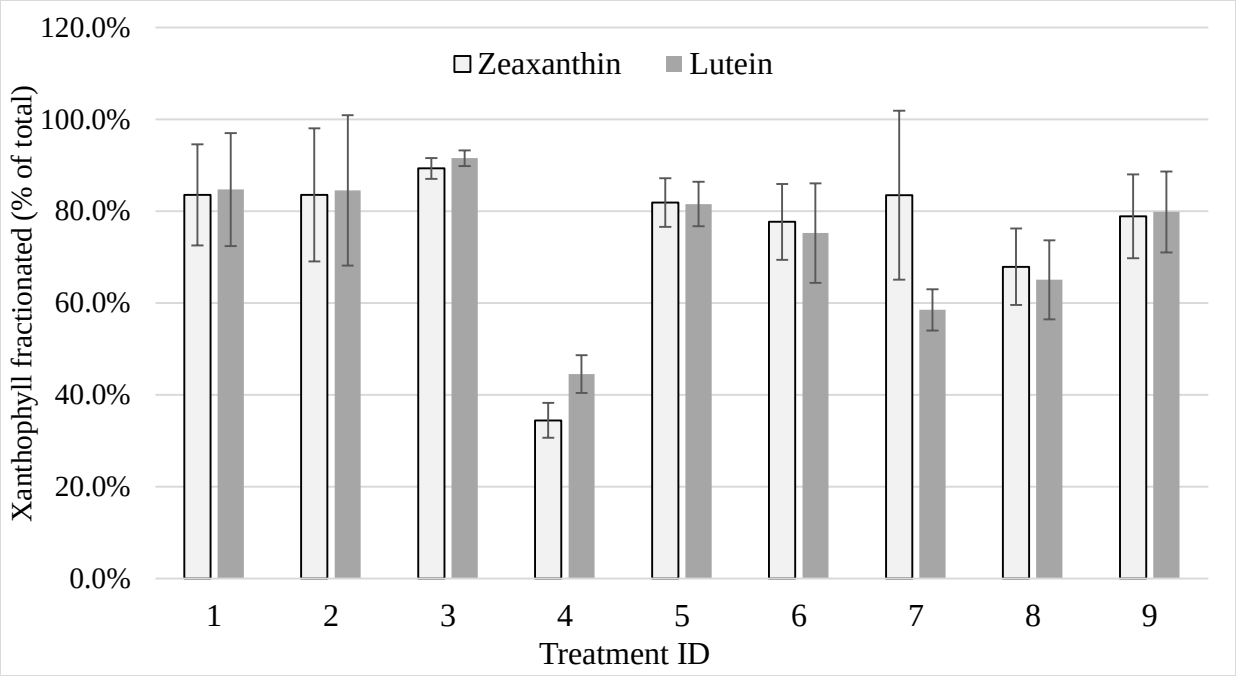

Figure 5. Fraction of xanthophyll separated from Post Fermentation corn oil using solid phase extraction and nine solvent treatment combinations. Treatment ID indicates condition solvent-elution solvent (D = dichloromethane, I = isopropanol, M = methanol)

At the $\alpha = 0.05$ significance level, varying the conditioning solvent caused significant ($p = 0.05$) differences in the treatment means (Table 10). In addition, there was no significant difference between the treatment means when the eluting solvent was varied ($p = 0.089$) and that no interaction between the condition and elution solvent existed ($p = 0.187$). Because the elution solvent did not have an impact on the fractionation of xanthophylls, it was clear that solvents in the polarity index range from 3.1 to 5.1 adequately desorbed the xanthophylls from the diol material for collection.

Table 10. Two-way ANOVA of xanthophyll extracted (% of total) from Post Fermentation corn oil by SPE using varying condition and elution solvent

Source	**DF**	**Adj SS**	**Adj MS**	**F-value**	**P-value**
Condition	2	0.1852	0.09259	3.59	0.05
Elution	2	0.1445	0.07223	2.80	0.089
C*E	4	0.1799	0.04497	1.74	0.187
Error	17	0.4384	0.02579		
Total	25	0.9751			

A Tukey's post-hoc test was performed to determine which conditioning solvent caused significant differences in the treatment means. The test indicated the data could be statistically divided into two groupings based on their means (Table 11), and the treatments conditioned with DCM were significantly different than those that were conditioned with isopropanol. When the column was conditioned with DCM, more xanthophylls were fractionated from the oil than when the column was conditioned with isopropanol. It has been reported previously that lutein is very soluble in DCM and quite insoluble in hexane, which the oil was dissolved in upon application to the column (Craft and Soares, 1992). This could have caused lutein to precipitate from the oil/hexane mixture and preferentially bind to the DCM conditioned sorbent.

Table 11. Statistical differences according to Tukey Pairwise Comparison

Condition solvent	**Mean total xanthophyll fractionated (%)**	**Grouping [a]**
DCM	86.2%	A
Methanol	71.9%	A B
Isopropanol	64.2%	B

[a] All means with same grouping letter were considered not significantly different (α= 0.05)

Thought the Tukey's test indicated that at the $\alpha = 0.05$ confidence level, elution solvent did not impact ($p = 0.089$) xanthophyll fractionation from the oil. It is notable however, that when the column was conditioned with DCM and then eluted with methanol, 91.5% of the xanthophylls were fractionated from the oil and the lowest COV, 3.5%, was observed. Though the treatment was not considered statistically different from the others, it fractionated the most xanthophylls and was far more repeatable than the other treatments.

For all treatments, the xanthophyll concentration was approximately ten times higher in the final fraction than in the untreated oil (97.8 ± 15.0 μg lutein/g oil and 84.5± 12.2 μg

zeaxanthin/g oil). In the best performing treatment where DCM was used to condition and then methanol was used to rinse, the concentration was increased to 937.0 μg lutein and 789.2 μg zeaxanthin per gram of oil.

4.5. Conclusion

The results of the present study indicate that SPE using diol column material is an effective method for fractionating xanthophylls from the PF corn oil produced at dry-grind corn ethanol plants. After a multiple comparisons of treatment means, it was clear that it is best to condition the diol column with DCM followed by elution with methanol, isopropanol, or DCM, without affecting the xanthophyll recovery. However, when methanol was used for elution after conditioning the column with DCM, the recovery was increased and the variability was reduced. This research could be applied to sample preparatory procedures, which many labs use to increase the reliability of HPLC measurements. More importantly, if this procedure was properly scaled up, it could provide dry-grind corn ethanol plants with a simple extraction procedure to fractionate and market the xanthophylls present in the PF corn oil. One of the main concerns with scale-up would be identifying the cause of the large variation between replicates. Large variation will cause issues for establishing production abilities. Further studies are needed using solvents that are generally recognized as safe by the FDA, which would fractionate the oil as well as the toxic alternatives, DCM and methanol. This step would allow the xanthophyll fraction to be incorporated into food systems.

5. PAPER 2: SUPERCRITCAL FLUID EXTRACTION OF XANTHOPHYLLS FROM CORN GLUTEN MEAL

5.1. Abstract

Supercritical CO_2 extraction of xanthophylls from corn gluten meal (CGM) was optimized by varying the extraction temperature (40 – 80 °C), pressure (5,500 – 7,500 psi), and fraction of ethanol co-solvent added (5 – 15 % by volume of total solvent). A response surface model was developed, which indicated that the amount of co-solvent added significantly impacts the extraction yield, while temperature and pressure have minor main term effects. Pressure, however, did have a quadratic effect on recovery and temperature had an interactive effect with co-solvent amount. The optimal extraction conditions determined with the model were 40 °C, 6820 psi, and 15% co-solvent. At these optimal conditions, the xanthophyll recovery from CGM was 2.6 times better than CGM extracted with ethanol and chloroform: dichloromethane (2:1). The model developed suggests that adding more co-solvent during extraction could further increase xanthophyll recovery.

5.2. Introduction

Corn gluten meal (CGM) is a high protein (60%) co-product from corn wet milling plants that is sold as animal feed for around $555/ton (Anonymous, 2016). The carotenoids present in corn are structurally wrapped within the corn gluten so they end up in high concentration (224-550 mg/kg) in the gluten meal (Park et al., 1997). The carotenoid content poses problems for the marketability of CGM because it can cause yellow pigment in the meat or fat of the animals that consume it such as fish, poultry, and cattle (Lovell, 1984; Mason and Palmer, 1934; Park et al., 1997; Saez et al., 2015; Sessa et al., 2003).

Carotenoids are categorized into two groups, xanthophylls and carotenes, based on differences in their structure. The carotenes are polyunsaturated hydrocarbons and xanthophylls are structurally similar, but more highly oxygenated. The majority (56.7 – 89.5%) of the carotenoids present in CGM are xanthophylls, which are valuable sources of natural yellow-red food colorants (Weber, 1987). Natural colorants have been growing in popularity since 1904 (Delgado-Vargas and Paredes-Lopez, 2003) and especially in the past decade as companies like General Mills have committed to remove synthetic colorants from all of their products (Hunt, 2015). Additionally xanthophylls are valuable as pharmaceuticals because they delay the onset of macular degeneration (Seddon et al., 1994). Extracting these xanthophylls could bring more revenue to the corn wet milling facilities and potentially increase the local market for CGM.

Supercritical fluid extraction is one way of isolating the xanthophylls from CGM. Supercritical fluids exhibit properties of both liquids and gasses, which make them ideal as extraction solvents. The fluid has diffusivity like a gas but a density more similar to that of a liquid. CO_2 is a popular supercritical fluid because of its availability, moderate critical point, and ability to produce natural extracts. Supercritical carbon dioxide (SC-CO_2) extraction is currently used at the industrial scale for processes such as decaffeination of teas and coffees, spice extraction, and oil deodorization (Mukhopadhyay, 2000b). Lab scale SC-CO_2 extraction has also been studied for the recovery of minor lipid components, like carotenoids, from materials including carrots (Barth et al., 1995), wheat germ (Ge et al., 2002), and dry distiller's grains with solubles (Ciftci et al., 2012).

SC-CO_2 extraction of xanthophylls from CGM is preferred over solvent extraction for a number of reasons. It has been reported that SC-CO_2 extracts xanthophylls as effectively as the traditional solvent extraction methods (Careri et al., 2001; Marsili and Callahan, 1993). In

addition, xanthophylls tend to isomerize when heated and refluxed in organic solvents which leads to reduced color intensity (Güçlü-Üstündağ and Temelli, 2004). SC-CO_2 extraction can provide solvent-free extracts eliminating the need to evaporate large solvent volumes. Further, SC-CO_2 extraction can be quite selective in extracting components of interest by simply adjusting the fluid's temperature and pressure (Mukhopadhyay, 2000b).

There are two challenges with extracting xanthophylls from CGM using SC-CO_2. First, xanthophylls are only slightly soluble in CO_2 due to differences in polarity (Jay et al., 1991; Mattea et al., 2009) and second, the xanthophylls are structurally enveloped within the proteins in CGM (Lawton et al., 2004). To increase the polarity of the CO_2 and to open up the protein structure, a solvent modifier like ethanol can be used (Jay et al., 1991). Addition of the ethanol modifier has been shown to nearly double the extract yield from CGM (Sessa et al., 2003). Higher pressure levels of up to 10,000 psi resulted in the best extraction yield of lipids from corn sources (Sessa et al., 2003; Wu et al., 1994). However, lower pressure levels were reported to optimize SC-CO_2 extraction of xanthophylls from other sources including algae (5,076 psi), DDGS (7193 psi), and marjoram (6527 psi). Optimum extraction temperatures for the same xanthophyll extraction studies were reported to be 50 °C (marjoram), 70 °C (DDGS), and 80 °C (algae) and optimum ethanol co-solvent fraction was reported in one study to be 15% (algae) (Careri et al., 2001; Ciftci et al., 2012; Vági et al., 2002). Typically, extraction temperature should not exceed 80 °C to avoid xanthophyll degradation (Careri et al., 2001). In order to quantify potential xanthophyll recovery from a corn wet milling facility, the most favorable SC-CO_2 extraction settings must be determined.

The objective of this experiment was to develop a model to quantify the relationship between supercritical fluid extraction parameters and extraction of xanthophylls from CGM.

Parameters included in the study were extraction temperature, pressure, and ethanol (as co-solvent):CO_2 ratio. Response surface modeling was used to understand the interactions between the factors and to develop a visual model to display each factor's impact on CGM extraction. Lutein and zeaxanthin extraction were used as response variables.

5.3. Materials and Methods

5.3.1. Samples

Corn gluten meal (5.8% moisture db) was obtained from a local corn wet milling facility (Cargill, Inc.; Wahpeton, ND). Material was stored in sealed buckets at ambient temperature until used for testing.

5.3.2. Chemicals

Aqueous ethanol (95%) was purchased from the NDSU chemistry stock room (Fargo, ND). Dichloromethane (DCM), chloroform, methanol, and methyl-tert butyl ether (MTBE) were HPLC-grade and obtained from VWR (Radnor, PA). HPLC-grade CO_2 provided with a dip tube in the cylinder was purchased from Praxair Distribution, Inc. (Fargo, ND). Lutein (>93% purity) and zeaxanthin (>98% purity) standards were obtained from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Lutein and zeaxanthin standards were made by dissolving the compounds in a methanol:MTBE (25/75 v/v) solution and were stored at -20°C.

5.3.3. CGM Solvent Extraction

CGM was extracted using traditional solvent extraction to provide a comparison for the SC-CO_2 extraction experiment. CGM was solvent-extracted following a modified method adapted from Lu et al. (2005). Three CGM samples were placed in amber vials to reduce light degradation of carotenoids. For each sample, approximately 1.5 g (wet weight) of CGM was extracted sequentially 5 times with the solvents and volumes indicated in Table 12. During extractions, the samples were constantly agitated on a magnetic stir plate for one hour and after

each extraction the supernatant was collected. Chloroform and dichloromethane were used during the final three extractions because xanthophylls are readily soluble in these solvents and only moderately soluble in ethanol (Craft and Soares, 1992).

Table 12. Solvents used for reference corn gluten meal extraction

Extraction #	Solvent Used
First	25 mL 95% ethanol
Second	25 mL 95% ethanol
Third	20 mL 95% ethanol and 3 mL chloroform: DCM (2:1)
Fourth	20 mL 95% ethanol and 3 mL chloroform: DCM (2:1)
Fifth	20 mL 95% ethanol and 3 mL chloroform: DCM (2:1)

The solvent was removed from the combined extracts by rotary evaporation at 40 °C. The residues were dissolved in acetone to precipitate any zein that may have been co-extracted. The extract was then vacuum filtered and the cake on the filter was washed with acetone until the filtrate was clear. The filtrate was again dried by rotary evaporation at 40 °C. Each extract was diluted to a final volume of 5 mL with HPLC diluent (methanol:MTBE 25/75 v/v). Samples were transferred to 2-mL, amber-colored HPLC vials and capped. HPLC vials were stored at -20 °C until HPLC analysis.

5.3.4. SC-CO_2 Extraction of CGM

5.3.4.1. Supercritical Fluid Extraction Unit

The ISCO supercritical fluid extractor used for extractions uses two pumps: the main solvent pump and modifier pump. The main pump has a capacity of 260 mL and maximum pressure of 7,500 psi. The modifier pump can hold 100 mL solvent and pressurize to 10,000 psi. The pumps are run by a controller, which controls the pressure, flow rate, and amount of

modifier added during extractions. The extraction chamber contains two extraction cells, each of which is able to hold a maximum of approximately 6 g of CGM.

5.3.4.2. Static Extraction Time Determination

The SC-CO_2 extraction can either be static or dynamic. Part of the SC-CO_2 extraction method development was to determine the time allotted for static extraction. An extraction is static when the solvent is stagnant in the chamber. In contrast, dynamic extraction occurs when fresh CO_2 and ethanol are continuously pumped through the sample matrix. Static extraction before dynamic extraction allows for better penetration of the SC-CO_2 into the sample and reduces solvent channeling (Careri et al., 2001).

An initial experiment was done to determine the best static extraction time. Four runs were performed; three of which varied the length of static extraction from 0, 5, or 10 minutes and the final used alternate 5 minutes static and 5 minutes dynamic for a total of 35 minutes. The fluid pressure was set to 5,000 psi and temperature to 40 °C and no solvent modifier was used during this experiment. After the static extraction, dynamic extraction began for a total extraction time of 35 min. The extracts from this preliminary experiment were collected and dissolved to a final volume of 5 mL with HPLC diluent (methanol: MTBE, 25/75 v/v) prior to analysis by HPLC.

5.3.4.3. SC-CO_2 Extraction Experimental Design

To study the effects and interaction of temperature, pressure, and co-solvent addition on xanthophyll extraction from CGM, a Box-Behnken experimental design was used to develop a response surface model. Each parameter was varied between low, medium, and high levels (Table 13). All treatment conditions as well as the randomized run order resulted in 15 total runs (Table 14). The first, fifth, and tenth run were center point replicates run to calculate variability.

Table 13. Factor levels for corn gluten meal response surface model experiment

Factors	**Levels**		
	Low	Medium	High
Temperature (°C)	40	60	80
Pressure (psi)	5500	6500	7500
Ethanol co-solvent (% v/v CO_2)	5%	10%	15%

Table 14. Experimental conditions for response surface model experimental design. Developed in Minitab (version 17) (Minitab Inc.; State College, PA)

StdOrder	**RunOrder**	**Temperature (°C)**	**Pressure (psi)**	**Co-solvent (% v/v)**
1	13	40	5500	10
2	6	80	5500	10
3	8	40	7500	10
4	9	80	7500	10
5	4	40	6500	5
6	7	80	6500	5
7	12	40	6500	15
8	15	80	6500	15
9	11	60	5500	5
10	2	60	7500	5
11	14	60	5500	15
12	3	60	7500	15
13	5	60	6500	10
14	10	60	6500	10
15	1	60	6500	10

For all SC-CO_2 extractions, approximately 2.5 g (wet weight) of CGM was added to one of the extraction vials equipped with stainless steel frits on either end to prevent loss of solids. The other extraction cell was left empty and was isolated from solvent flow due to technical limitations. The extraction chamber temperature was set per the experimental design (Table 14). The CO_2 and 95% ethanol pumps were set such that the pump adjusts the flow rate of the co-solvent based on the flow rate of the main solvent pump (CO_2). The modifier mode ensures 5, 10, or 15% of the solvent flowing was the co-solvent (95% ethanol). Once the pumps

were filled and pressurized, the valves were opened and a CO_2:ethanol blend flowed into the extraction chamber. The outlet valve from the extraction cell was closed to allow for an initial static extraction after which dynamic extraction began. The solvent flowed through the sample chamber at approximately 2 mL/min. Solvent flow rate could only be controlled by hand valves, which regulate back pressure in the extraction chamber causing flow rate variation. For each sample, a total of 40 mL solvent/gram of CGM was used. The solvent: CGM ratio was constant for each sample (40:1), but the ratio of co-solvent:CO_2 varied based on experimental design (Table 14).

The extracts were collected in amber vials attached to the extraction chamber, purged with nitrogen until dry to remove the ethanol. Samples were then diluted to a final volume of 5 mL with HPLC diluent (methanol:MTBE 25/75 v/v) and stored at -20 °C. The extraction unit was not rinsed between each extraction because the carryover was found to be less than 1%.

5.3.5. Protein Content Analysis

CGM is a valuable commodity because of its high protein content. The protein content of the extracted CGM was measured by the AOAC official crude protein analysis method #2001.11(AOAC, 2010) to ensure the SC-CO_2 extraction removed minimal protein.

5.3.6. Carotenoid Separation by HPLC

CGM extracts were analyzed using a method adapted from Gupta et al. (2015) with some modifications. The HPLC column used was YMC C30 carotenoid column (YMC Co., Kyoto, Japan) (3 µm, 250 mm x 4.6 mm i.d.) purchased from VWR, Inc. Reversed phase HPLC using a Waters 2795 HPLC (Waters, Corp,; Milford, MA) equipped with a photodiode array detector (PDA) was used to determine carotenoid concentration. During HPLC runs, the column was maintained at 25 °C. Oil fraction samples of 10 µl were injected on the HPLC column. A gradient method was developed for sample separation using 98% methanol (v/v with water) and

MTBE. The elution gradient is shown in Table 15. For each step in the HPLC method, a linear gradient change occurred over the time allotted until the next solvent level was reached.

Table 15. High performance liquid chromatography gradient method for carotenoid separation

Flow Rate (mL/min)	Time (min)	Solvent A (%)	Solvent B (%)
1.4	0	80	20
1.4	2	60	40
1	12	0	100
1.4	13	80	20
1.4	20	80	20

The original method included a third mobile phase of methanol/water (95/5, v/v) but using this solvent caused a pressure spike on our system exceeding system limits (Gupta et al., 2015). Removing this third solvent from the method still allowed for clear lutein and zeaxanthin peaks and reduced the chance of column damage. Lutein and zeaxanthin standards were made for concentrations of 0.01 µg/mL to 300 µg/mL. Carotenoid concentrations were measured at an absorbance of 450 nm on the PDA. Lutein and zeaxanthin were quantified using a 5-point external standard curve.

5.3.7. Data Modeling

A Box Behnken design was used to understand the impact and interactions that temperature, pressure, and co-solvent ratio have on the extraction of xanthophylls from CGM. Each factor contained three levels to be tested: high, medium, and low. Three replicates were run at the design center (60 °C, 6500 psi, and 10% co-solvent) to quantify experimental error within the system. The base model contains 10 parameters including the overall mean, 3 main factor effects, 3 quadratic effects, and 3 two-factor interactions, seen in the equation below

$$y= \beta_0+\beta_1 x_1+\beta_2 x_2+\beta_3 x_3+\beta_{11} x_1^2+\beta_{22} x_2^2+\beta_{33} x_3^2+\beta_{12} x_1 x_2+\beta_{13} x_1 x_3+\beta_{23} x_2 x_3 \quad (1)$$

Where:

y = lutein extracted (µg/g CGM)

x_1 = temperature (°C)

x_2= pressure (psi)

x_3= co-solvent ratio (%)

β_i, $\beta_{i,j}$ = model coefficients for constant, linear, quadratic, and interaction terms

Once the response data from the experiment was collected, the model coefficients were estimated by the method of least squares using Minitab software. Analysis of variance (ANOVA) tables were created to determine the significance of each term in the model and to develop the model that best fit the data based on R^2 and R^2_{adj}.

5.4. Results

5.4.1. Solvent Extraction

To compare the extraction ability of SC-CO_2 to traditional solvent extraction, the CGM was extracted with solvent 5 times. Solvent extraction of CGM yielded 33.0 ± 1.6 µg lutein /g CGM and 23.7 ± 1.0 µg zeaxanthin /g CGM. After the 5 extractions the CGM was near white which indicated a majority of the carotenoids had been removed. A previous study using the same extraction method on CGM samples yielded at least 2 times more xanthophylls with 74.1 µg lutein /g CGM and 77.1 µg zeaxanthin /g CGM (Lu et al., 2005), though the source of CGM may be the reason for carotenoid differences. In that study, the xanthophyll concentration was quantified by positive-ion mass spectrometry interfaced with HPLC. They used a C8 HPLC column type while in the present study a C30 column was used because Gupta et al. (2015) reported that columns with stationary phase ligand lengths of less than C30 were not as able to resolve carotenoids and their isomers.

5.4.2. Static Extraction Time

Table 16 shows the results of the initial experiment to determine proper length of static extraction. The results showed that by holding the solvent static in the extraction chamber for 5

minutes prior to dynamic extraction, xanthophyll recovery was 27.2% higher than an extraction with no static extraction. When the static extraction was increased to 10 minutes, the extraction yield increased by another 14.3%, or 45.4% higher than the baseline dynamic extraction (0 minute static). Alternating between static and dynamic extraction did not improve xanthophyll extraction as well as the 5 and 10 minute static extraction and it also requires more labor and causes wear on the equipment. During this initial testing, the other extraction parameters were set at lower levels (pressure at 5000 psi, temperature at 40 °C, modifier at 0%, and extraction length at 35 minutes), which accounts for the low overall xanthophyll recovery. Nevertheless, the experiment indicated that the best initial static extraction time was 10 minutes and this extraction sequence was used for all subsequent experiments.

Table 16. Effect of static extraction length on xanthophyll recovery

Static extraction time (minutes)	Total extraction time (minutes)	Xanthophyll extracted (µg/g CGM)
0	35	4.4
5	35	5.6
10	35	6.4
5 static, 5 dynamic-alternate	35	5.4

5.4.3. SC-CO_2 Extraction of Xanthophylls from CGM

The results of the SC-CO_2 extraction of CGM show varying results (Table 17). The yield of lutein was plotted against the yield of zeaxanthin revealing a strong linear relationship between the two (R^2= 0.991) (Figure 6). This indicates the process parameters have essentially the same impact on lutein and zeaxanthin yield, and that extraction parameters cannot be adjusted within the design space to select one compound over the other. Lutein and zeaxanthin are stereoisomers only varying by the direction of the alcohol groups on the ends of their structure so it is expected that by optimizing the extraction of lutein, zeaxanthin extraction is also

enhanced. Because of this, lutein recovery was modeled to represent the impact that the process parameters have on total xanthophyll recovery.

The three replicates performed at the center of the design space (6,500 psi, 60 °C, 15% co-solvent) averaged 55.7 ± 8.4 µg lutein and 26.2 ± 3.1 µg zeaxanthin extracted per gram of CGM. Xanthophyll recoveries vary by 12% and 15%, which does not demonstrate ideal repeatability of the experiments.

Table 17. Results from response surface model (RSM) supercritical CO_2 extraction of corn gluten meal including extract amount, actual lutein and zeaxanthin extracted, and the predicted lutein yield based on the RSM model developed

Design factor levels			**Actual yield**			**Model predicted yield**
Temp (°C)	Pressure (psi)	Co-solvent (% v/v)	Extract (%)	Zeaxanthin extracted (µg/g)	Lutein extracted (µg/g)	Lutein extracted (µg/g)
40	5500	10	2.9%	19.2	33.5	40.6
80	5500	10	3.2%	20.3	38.8	48.0
40	7500	10	3.4%	26.9	55.3	49.6
80	7500	10	3.8%	24.4	50.2	57.0
40	6500	5	2.3%	8.4	9.2	19.6
80	6500	5	2.9%	18.3	37.0	40.3
40	6500	15	3.9%	32.5	72.4	84.7
80	6500	15	4.0%	31.3	73.5	78.8
60	5500	5	2.5%	13.6	19.9	18.4
60	7500	5	2.3%	10.5	14.4	27.4
60	5500	15	4.3%	33.4	72.0	70.2
60	7500	15	4.5%	31.3	69.5	79.2
60	6500	10	3.8%	24.5	49.6	55.8
60	6500	10	3.8%	24.4	52.1	55.8
60	6500	10	4.5%	29.8	65.3	55.8

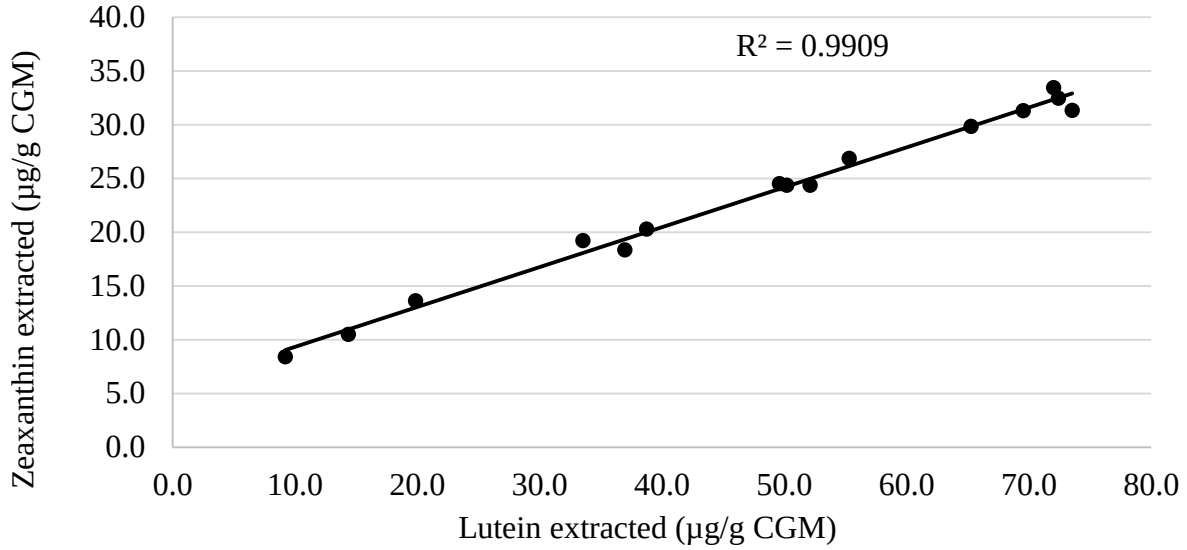

Figure 6. Lutein vs zeaxanthin extracted. Linear relationship indicates the extraction parameters cannot be adjusted to select one compound over the other

Using the raw data, a model was developed (Equation 2) with Minitab 17 (Minitab Inc.; State College, PA) to determine the relationship between the extraction parameters and the response variable, i.e. lutein yield from CGM. Again the variable parameters included temperature, pressure and amount of co-solvent were used. The model-building began with the original full quadratic equation and statistically insignificant terms were sequentially removed to determine their effect on the model and to get the best fit based on R^2_{adj}. The quadratic term associated with pressure and the temperature-modifier interaction term both had a lower significance level than desired (0.126 and 0.136, respectively), but were kept in the model because they positively impacted the R^2_{adj} values and removing them reduced the overall fit. The final model developed (Equation 2) fit the data adequately with an R^2 of 90.9% and an R^2_{adj} of 85. The model passed the lack-of-fit test (p=0.62), which tells how well the model explains the variation in the data. The actual yield of lutein along with the predicted yield based on the model (Equation 2) shows some variability. The predicted values do not differ significantly from the actual measured values and do not vary substantially in any specific region of the design space.

$$Y= -372+.0847\ T+.0955\ P+9.17\ C-0.000007\ P^2-0.0665\ T*C \quad (2)$$

Where:

Y = Lutein extracted (µg/g oil)

T = Temperature (°C)

P = Pressure (psi)

C = amount co-solvent added (% v/v CO_2)

The most significant ($p < 0.001$) extraction parameter impacting lutein recovery was co-solvent addition (Table 18). This parameter has a positive relationship with lutein recovery, which confirms both the importance of disrupting the zein-carotenoid structure and that 95% ethanol can be effective in this role. The fact that the model co-solvent term is positive and the quadratic term is insignificant suggests that adding more than 15% co-solvent could increase the yield of lutein from the CGM above what is shown here. In a study that used SC-CO_2 to extract carotenoids from pumpkins, the group varied the amount of co-solvent between 0, 10, and 30% (Seo et al., 2005). They found that the extraction using 30% modifier did increase the extract recovery slightly**,** however, they concluded that at that level of co-solvent addition, the total solvent nullifies the advantages that SC-CO_2 extraction has over traditional solvent extraction. SC-CO_2 extraction is attractive because it reduces use of large quantities of flammable solvents like ethanol. Further studies of the current experiment need to be performed to determine if increasing modifier concentration further enhances extraction recovery of xanthophylls.

Table 18. ANOVA for response surface model of lutein extracted from corn gluten meal by supercritical CO_2 when varying extraction pressure, temperature, and added co-solvent

Source	DF	Adj SS	Adj MS	F	P
Regression	5	5913.28	1182.66	17.92	0.000
Linear	3	5547.93	1849.31	28.01	0.000
Temperature (T)	1	105.11	105.11	1.59	0.239
Pressure (P)	1	79.76	79.76	1.21	0.300
Co-solvent (C)	1	5363.07	5363.07	81.24	0.000
Square	1	188.23	188.23	2.85	0.126
P*P	1	188.23	188.23	2.85	0.126
Interaction	1	177.12	177.12	2.68	0.136
T*C	1	177.12	177.12	2.68	0.136
Error	9	594.11	66.01		
Lack-of-fit	7	452.15	64.59	0.91	0.615
Pure Error	2	141.96	70.98		
Total	14	6507.39			

The main effects from pressure and temperature were not significant, but they were kept in the model because the second-order term for pressure and interaction term for temperature and modifier were included. The low impact of the linear temperature term is further illustrated in the contour plot of the model (Figure 7). The plot shows that the temperature interaction is only relevant at low modifier concentrations, where the extraction efficiency is low. At those modifier concentrations, increasing temperature does increase extraction yields but not enough to match those attained at higher modifier concentrations. Temperature had little practical impact when modifier is above 12%.

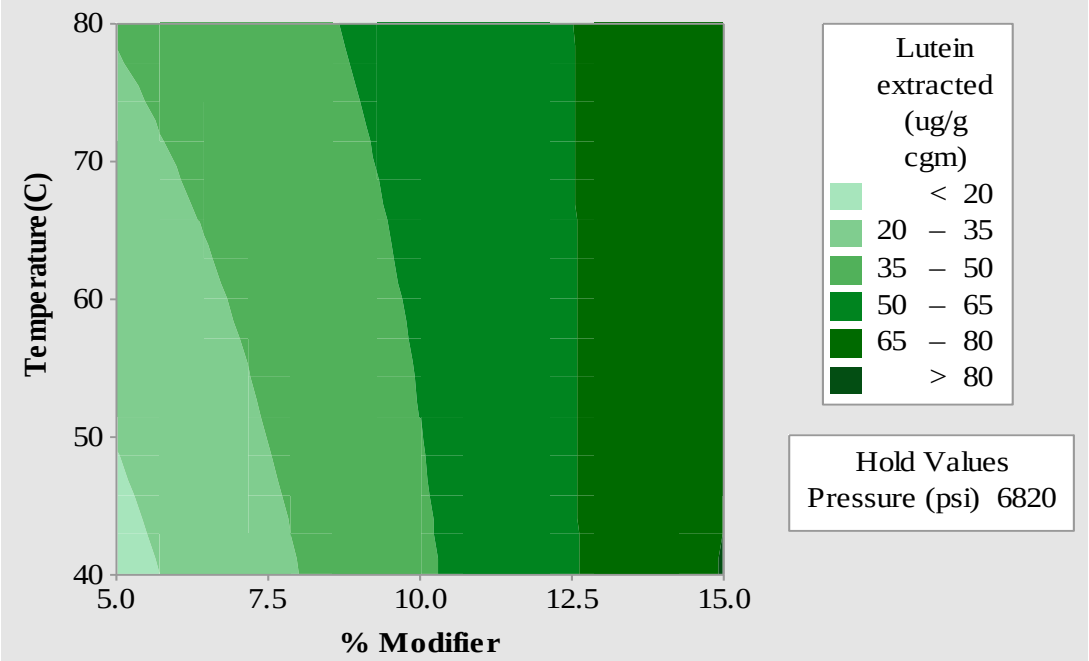

Figure 7. Contour plot of lutein yields while varying temperature and % co-solvent and holding pressure constant at its optimum value, 6820 psi

It is surprising that the main effects of temperature and pressure were not significant because other studies indicated that these parameters were important factors (Careri et al., 2001; Yi et al., 2009). Careri et al. (2001) optimized the extraction of xanthophylls from algae with an RSM that used a pressure range of 1,116-5,583 psi, which was lower than in this study and co-solvent range of 5-15%. They found the amount of modifier added along with pressure level had a significant impact on the recovery of xanthophylls and that by increasing the pressure and modifier, xanthophyll recovery from the algae was increased. The optimum conditions for the extraction were 80 °C, 5,076 psi, and 15% co-solvent (Careri et al., 2001). The extraction of lycopene from tomato skins was optimized by varying the temperature (40-100 °C), pressure (2,900-5,800 psi) and solvent flow rate (1-2 mL/min). Again in this experiment the pressure range studied was lower and no co-solvent was used, but the temperature and pressure were both significant model terms and lycopene yield was more dependent on temperature than pressure (Yi et al., 2009). In contrast to lutein, lycopene is a non-polar carotenoid. This could account for

the difference in model parameter significance because lycopene is completely soluble in SC-CO_2 while lutein and zeaxanthin require the CO_2 modifier to increase the polarity of the solvent and the extraction of these carotenoids.

In the present study, temperature and pressure were hypothesized to significantly impact xanthophyll extraction because these parameters affect the density of the supercritical fluid. An increase in the fluid temperature or decrease in fluid pressure will cause a decrease in density. Additionally, by increasing temperature, the solubility of the xanthophylls can be increased, which should result in higher yields. Lutein solubility in SC-CO_2, based on the CO_2 density, supported that lutein was not soluble until CO_2 density was 600 g/L, which occurred at 40 °C and 1,400 psi (Gómez-Prieto et al., 2007). In the same study, the solubility continued to increase as temperature and pressure were increased to the maximum values tested, 60 °C and 3,750 psi, respectively. Adjusting the density of the CO_2 and the solubility of the solute enhanced the interaction between the extract and the fluid. In the present study, the change in fluid density did not have a large influence on the lutein yield from CGM.

In the model (Equation 2) pressure is quadratic and does not interact with the other terms, i.e. temperature and co-solvent. Pressure was plotted against the response variable (lutein extracted), while holding the other two terms constant to determine the impact on the response variable (Figure 8). As pressure does not interact with the other terms, the impact it had on the response was equivalent at all temperature and co-solvent levels. It is clear that the maximum lutein recovery occurs between 6,500 – 7,500 psi (Figure 8). By taking the partial derivative of the model with respect to P, the exact optimum pressure was determined to be 6,820 psi. Over the entire pressure range of 5,500 – 7,500 psi, the lutein recovery changed by 13 µg/g, which could be substantial. At an average wet mill that grinds 100,000 bushels of corn and produces

135,000 kg of CGM daily, the difference between operating at 5,500 or 6,820 psi could be nearly 2 kg in lutein extract. In further work, an economic analysis is needed to understand the relationship between energy costs for pressurizing and price of xanthophyll extract to determine the optimum extraction pressure.

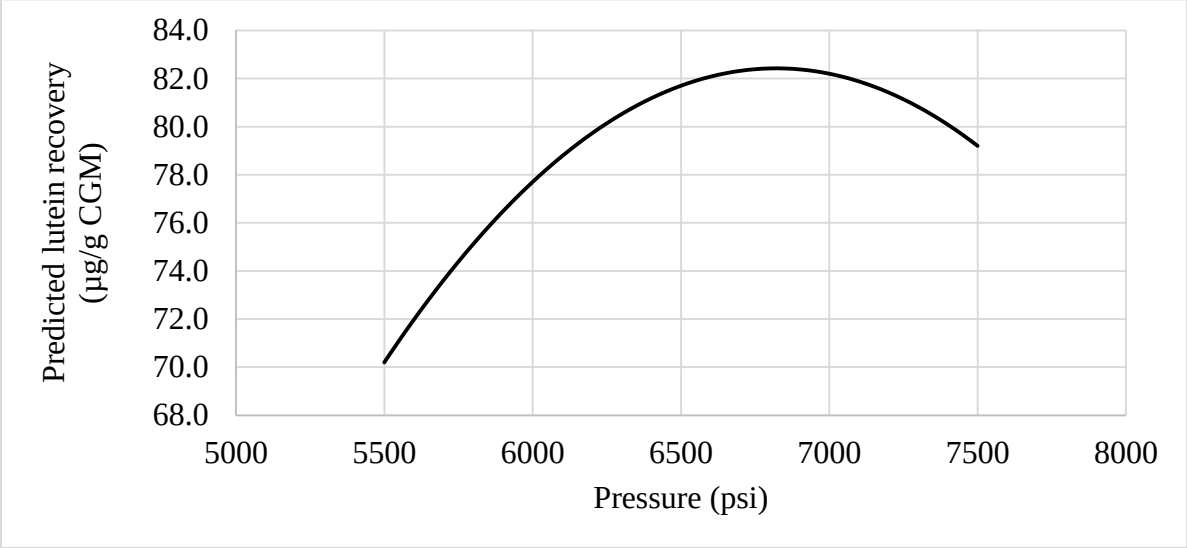

Figure 8. Impact of pressure on lutein recovery while the temperature and co-solvent are held constant at 60 °C and 15%

The model contour plot (Figure 7) shows that at low modifier levels, temperature has some interaction with modifier, but at high modifier levels, temperature had a minor impact. When pressure and modifier are set at optimum levels, 6,820 psi and 15% respectively, the response only changed by 7% over the whole temperature range. But, given the R^2_{adj} (85.8%) of the model, it is not reasonable to make a practical distinction between the response at 40 °C and 80 °C, therefore the optimum temperature is considered 40 °C, which is beneficial for industrial energy savings.

At the optimum values for all extraction parameters (40 °C, 6,820 psi, 15% co-solvent), the model estimates 85.4 µg lutein/g of CGM will be extracted which is 2.6 times more than the amount extracted with ethanol and chloroform: dichloromethane (2:1) (33.0 ± 1.6 µg lutein/g

CGM). In a previous study which used the enzyme Neutrase to pretreat the CGM before solvent extraction, considerably more lutein, 113.5 µg, was extracted from each gram of CGM (Lu, 2005). In the same study, when enzyme pretreatment was not used, 74.1 µg lutein/g CGM was extracted from the CGM using solvent. Compared to this study, SC-CO_2 extraction with ethanol as a modifier does extract more xanthophyll than SC-CO_2 extraction with no modifier, though not as well as when the CGM is pretreated with enzyme. These findings indicate that the enzyme pretreatment may denature zein proteins more effectively than 95% aqueous ethanol thereby allowing for a more complete extraction.

At a medium sized corn wet mill plant that produces 135,000 kg, the SC-CO_2 extraction could amount to 11.5 kg of lutein extract per day. A comparable product to this extract is a marigold extract which is sold for $65-380/kg (Arisn ChemPharm Co., Ltd, China). CGM is currently sold for $0.60/kg so the lutein extract could be sold for a minimum of one hundred times the current selling price of CGM. This xanthophyll product would be marketable to food companies as natural food colorant though it would likely need to be processed further to ease its incorporation into food products (Mattea et al., 2009).

5.4.4. Protein Content

Protein content of both the untreated CGM and the SC-CO_2 – extracted CGM were measured to ensure no protein was co-extracted (Table 19). In all treatments, the overall protein content increased because some compounds were removed indicating negligible protein loss. Maintaining protein in CGM is important because the high protein makes it a valuable animal feed.

Table 19. Protein content of extracted corn gluten meal

RSM standard order	Crude Protein (% db)
1	71.2
2	72.0
3	71.0
4	71.6
5	71.9
6	71.8
7	71.4
8	72.1
9	71.1
10	70.6
11	72.7
12	72.6
13	70.5
14	72.0
15	71.8
Untreated CGM	69.0

5.5. Conclusion

Supercritical fluid extraction of xanthophylls from CGM was optimized at 6820 psi, 40 °C, and 15% co-solvent (95% ethanol) addition and the most significant model factor was the amount of added co-solvent. The extraction model developed suggests that adding co-solvent beyond 15% could continue to increase the xanthophyll yield. At the optimum SC-CO_2 processing conditions, xanthophyll extraction was 2.5 times higher than it was when the CGM was extracted with solvent. SC-CO_2 extraction with the ethanol modifier did not negatively impact the protein content so the CGM would still be potentially viable as animal feed. The decolored CGM could also be added to animal feed in higher blend ratios than before especially in the diets of animals that were affected by its yellow color, i.e., aquaculture. At the optimum level, a wet corn mill that grinds 100,000 bushels of corn daily could reasonably expect to produce 11.5 kg of lutein daily and the cost of extraction would likely be recovered by the value of the xanthophyll extract.

6. GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE WORK

Corn is the most abundant crop in the U.S. because of its role in food and fuel. Most corn-based primary products utilize the starch present in corn and the leftover protein, lipid, and fiber are typically made into co-products, which helps the plants increase profit margins. Carotenoids, which provide corn with its yellow hue, are not used in the main products and tend to become concentrated in some of the co-products. The carotenoids present in these co-products have the potential to generate additional revenue at these plants because the extract could be used in the growing natural food colorant market. This study was done to evaluate the extraction of xanthophylls from two different co-products from corn processing plants including post fermentation corn oil (PF corn oil) and corn gluten meal (CGM).

Xanthophylls present in PF corn oil were extracted by solid phase extraction using diol sorbent material. The fractionation method of the oil was optimized by varying the solvents used to condition and rinse the column. The conditioning solvent used impacted the recovery of the xanthophylls, but the eluting solvent used did not. DCM was the best choice for conditioning solvent as 86.2% of the xanthophylls were fractionated from the oil.

Further work on this project should involve understanding the variability between treatments because, in most of the SPE experimental treatments, variability was substantial. This caused problems in statistically analyzing for small differences between the treatments. First, an experiment should be done to understand if applying vacuum to the SPE manifold affects xanthophyll retention. The amount of vacuum applied in this study could have varied enough between replicates to cause inconsistent results. A procedure for removing the water from the PF corn oil should be developed to see if there is any water present and if it affects the xanthophyll fractionation from the oil. The HPLC analysis method should also be studied to validate that the column is being rinsed adequately and that xanthophylls are being properly separated from each

other. This would indicate whether the analysis is causing the variability. Beyond the repeatability issues, scale-up studies should be done to assess the feasibility of the process on an industry scale.

The SC-CO_2 extraction of xanthophylls present in the CGM was studied and optimized by varying the extraction, pressure, temperature, and amount of co-solvent (ethanol) in a response surface experimental design. The optimal conditions for maximizing xanthophyll recovery were determined to be 6820 psi, 40 °C, and 15% co-solvent. According to the model, the amount of modifier added was highly significant ($p < 0.001$) and positively related to xanthophyll yield which indicates that disrupting the zein-carotenoid complex present in the CGM greatly increases the solvent's ability to interact with the xanthophylls. The center point replicates in the design space gave a coefficient of variation of 15.1% for lutein and 11.9% for zeaxanthin therefore, understanding variation in this experiment is also necessary. Sources of variability could be derived from the HPLC analysis method or from differences between CGM subsamples. The particle size of the CGM was not consistent throughout the samples either. The samples were not milled because the heat produced during milling could cause xanthophyll degradation. Sieving the CGM samples before extraction could improve repeatability.

Validation experiments should be performed at the optimal extraction conditions to know how accurate the model is. Further studies should also be done to determine if, as the model suggests, adding higher modifier ratio will further increase xanthophyll extraction. It is also suggested that an experiment be done to determine the optimum water content in the modifier. Zein is soluble in 70-90% aqueous ethanol so adjusting the water content in the modifier might allow for a better protein denaturation and thus higher xanthophyll recovery. Additionally, lower extraction pressure settings should be investigated because they could impact industrial energy

savings. An economic analysis is needed for this extraction process to determine how viable it would be at a corn wet-mill and which extraction parameters have the most impact on cost.

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APPENDIX

Empower software

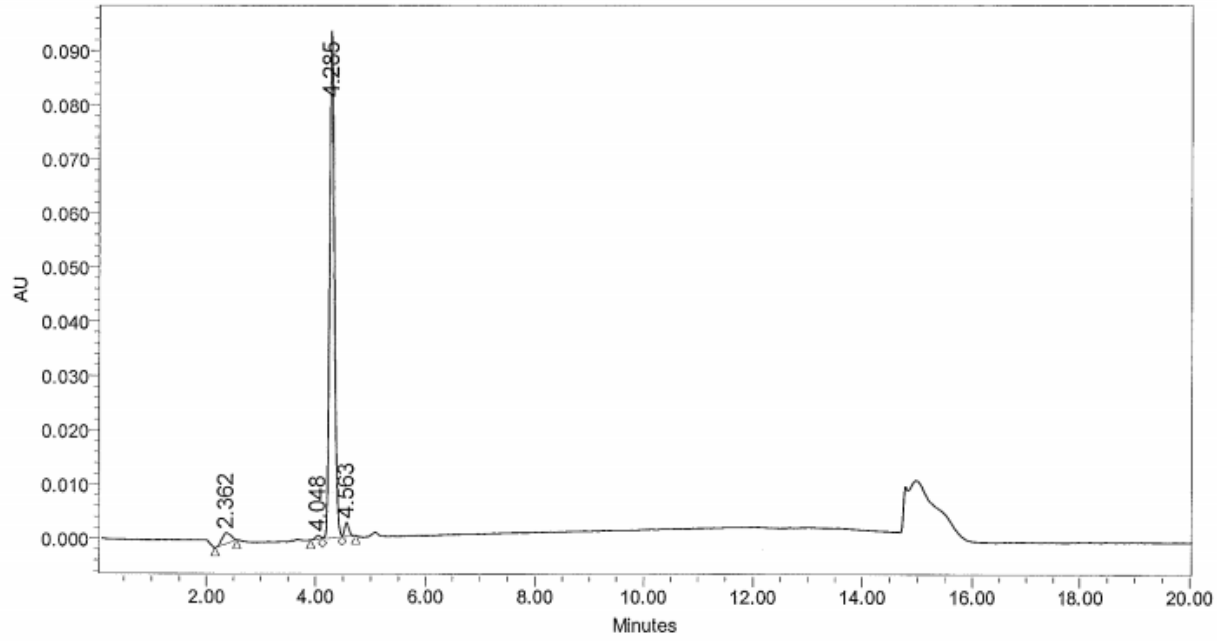
Default Individual Report

Reported by User: System

Project Name: Carotenoids

SAMPLE INFORMATION

Sample Name:	lutein 10ppm	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	3/14/2016 4:28:52 PM
Vial:	1:A,4	Acq. Method Set:	Carotenoid 2 mob phs Met Set
Injection #:	1	Date Processed:	3/14/2016 4:49:04 PM
Injection Volume:	10.00 ul	Processing Method:	Lutein and Zeaxanthin
Run Time:	20.0 Minutes	Channel Name:	Wvln Ch1
Sample Set Name:	CGM carotenoid rerun of RSM	Proc. Chnl. Descr.:	PDA 450.0 nm



	RT	Area	% Area	Height
1	2.362	22793	3.79	2027
2	4.048	4685	0.78	737
3	4.285	560637	93.12	93853
4	4.563	13932	2.31	2553

Figure A1. High performance liquid chromatogram of lutein standard eluting at 4.285 min (10 ppm)

Empower software

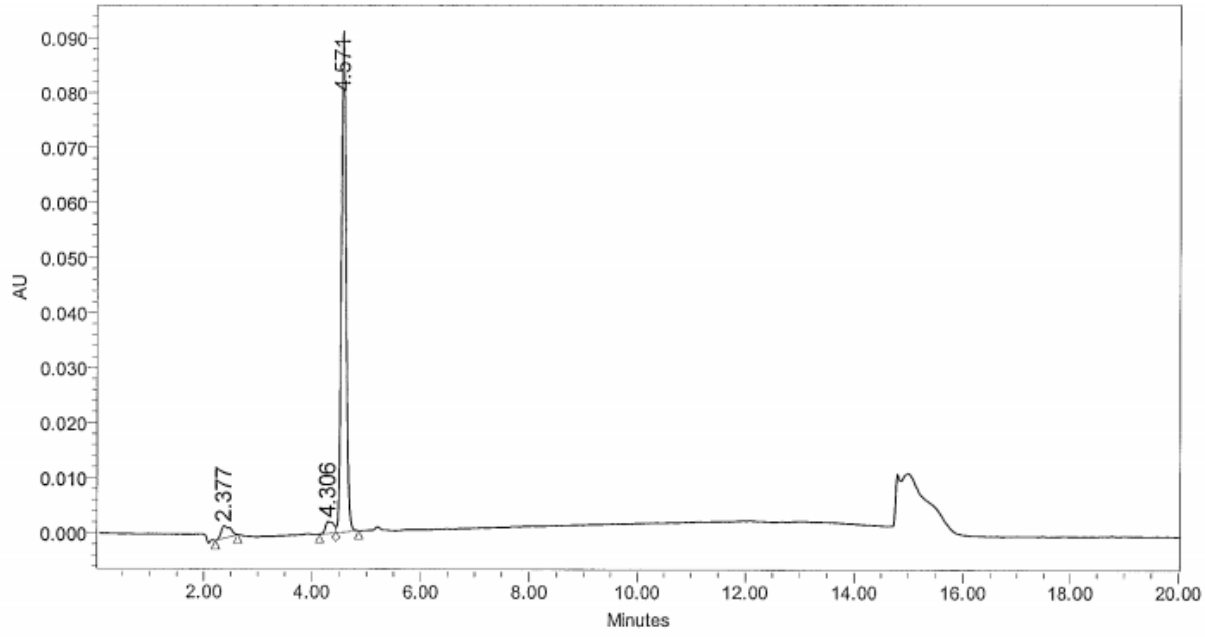
Default Individual Report

Reported by User: System

Project Name: Carotenoids

SAMPLE INFORMATION

Sample Name:	zea 10ppm	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	3/14/2016 4:49:29 PM
Vial:	1:A,5	Acq. Method Set:	Carotenoid 2 mob phs Met Set
Injection #:	1	Date Processed:	3/14/2016 5:09:40 PM
Injection Volume:	10.00 ul	Processing Method:	Lutein and Zeaxanthin
Run Time:	20.0 Minutes	Channel Name:	Wvln Ch1
Sample Set Name:	CGM carotenoid rerun of RSM	Proc. Chnl. Descr.:	PDA 450.0 nm



	RT	Area	% Area	Height
1	2.377	26765	4.42	2233
2	4.306	22222	3.67	2186
3	4.571	557222	91.92	91149

Figure A2. High performance liquid chromatogram of zeaxanthin standard eluting at 4.571 min (10 ppm).

Empower software

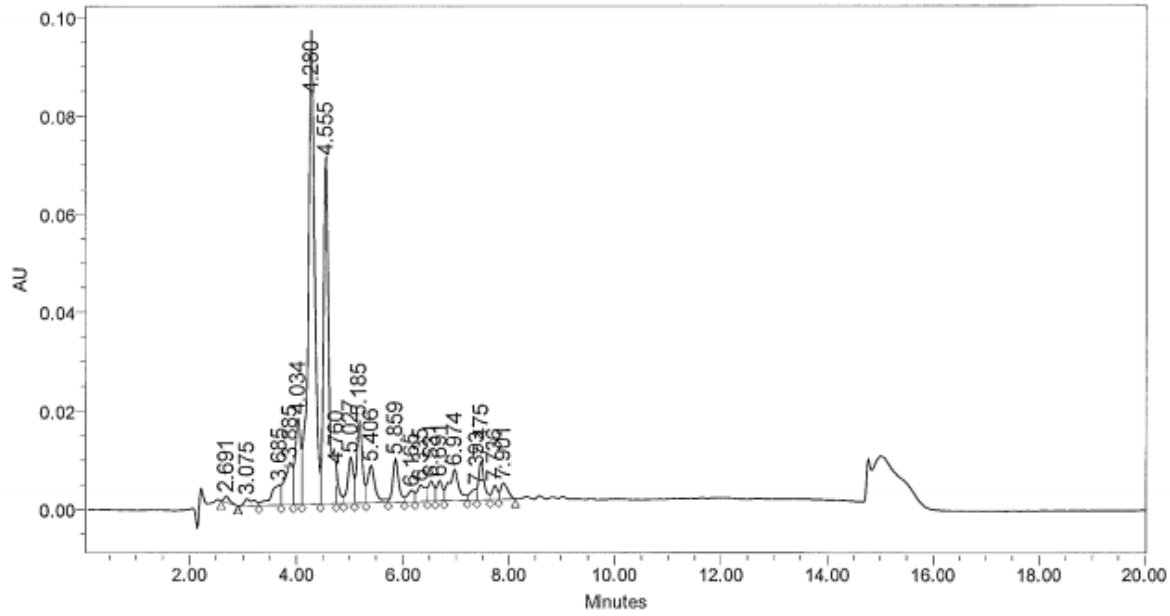
Default Individual Report

Reported by User: System

Project Name: Carotenoids

SAMPLE INFORMATION

Sample Name:	CGM #9 RSM	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	3/14/2016 10:19:09 PM
Vial:	1:C,6	Acq. Method Set:	Carotenoid 2 mob phs Met Set
Injection #:	1	Date Processed:	3/14/2016 10:39:18 PM
Injection Volume:	10.00 ul	Processing Method:	Lutein and Zeaxanthin
Run Time:	20.0 Minutes	Channel Name:	Wvln Ch1
Sample Set Name:	CGM carotenoid rerun of RSM	Proc. Chnl. Descr.:	PDA 450.0 nm



	RT	Area	% Area	Height
1	2.691	9342	0.41	1309
2	3.075	17771	0.77	1400
3	3.685	52475	2.28	4100
4	3.885	90401	3.93	8626
5	4.034	130819	5.69	17621
6	4.280	815531	35.49	95799
7	4.555	498102	21.67	70351
8	4.760	24471	1.06	7267
9	5.027	64579	2.81	9317

	RT	Area	% Area	Height
10	5.185	123193	5.36	16895
11	5.406	72636	3.16	7561
12	5.859	64538	2.81	8779
13	6.165	20455	0.89	2325
14	6.335	38243	1.66	3340
15	6.531	30821	1.34	4097
16	6.691	32230	1.40	4167
17	6.974	82710	3.60	6338
18	7.393	19247	0.84	2316

Figure A3. High performance liquid chromatogram of supercritical CO2 extracted corn gluten meal