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Developing Food Quality Standards for Distiller's Dried Grains – Evaluating Composition Quality and Safety

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DEVELOPING FOOD QUALITY STANDARDS FOR DISTILLER'S DRIED GRAINS – EVALUATING COMPOSITION, QUALITY AND SAFETY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

˚C	Degree Centigrade
µm	micrometers
%	Percentage
AACC	American Association of Cereal Chemists
AACCI	American Association of Cereal Chemists International
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
ADON	Acetyl deoxynivalenol
ADF	Acid detergent fiber
AF	Aflatoxins
ANOVA	Analysis of variance
APF	All-purpose flour
ASE	Accelerated Soxhlet extraction
Aw	Water activity
CDS	Condensed distiller's solubles
CFR	Code of Federal Regulations
CG	Corn germ
CHO	Carbohydrates
CO_2	Carbon dioxide
CV	Coefficient of variance
db	Dry basis
DG	Wet distiller's grains without solubles

DGD	Distiller's dried grains distillate
DGS	Wet distiller's grains with solubles
DON	Deoxynivalenol
DPPH	2,2-diphenyl-1-picrylhydrazyl
DWG	Distiller's wet grains
ESCL	Agricultural experimental station chemical laboratories, University of Missouri-Columbia, MI
EU	European Union
Solvent-treated dried products	Food grade distiller's dried grains
FM	Fumonisins
FPE	Ferulate phytosterol esters
FrD	Freeze-dried form
FRAP	Ferric reducing antioxidant power
g	grams
GRAS	Generally regarded as safe
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
kg	kilograms
L	liters
LC-MS	Liquid chromatography-mass spectrophotometry
mins	minutes
NDF	Neutral detergent fiber
NIRS	Near Infrared Spectroscopy

ppb	Parts per billion
ppm	Parts per million
PSD	Particle size distribution
psi	Pounds per square inch
RCF	Relative centrifugal force
RPM	Revolutions per minute
SD	Standard deviation
SSL	Sodium stearoyl lactylate
sec	seconds
Tg	Glass transition temperature
TDF	Total dietary fiber
TPA	Total phenolic acid
TPC	Total phenolic content
wb	Wet basis
USDA	United States Department of Agriculture
USFDA	United States Food and Drug Administration

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ABSTRACT

DEVELOPING FOOD QUALITY STANDARDS FOR DISTILLER'S DRIED GRAINS – EVALUATING COMPOSITION, QUALITY AND SAFETY

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Introduction: Distillers Grains represent a major co-product of corn ethanol production. At the production rate of 44 million tons/year and a price of $95/ton, this co-product has potential as a food ingredient (solvent-treated dried products) owing to its high protein content (38%) and high fiber content (40%TDF). The aim of the research was to determine the composition, quality and safety of several initial moisture types of distiller's grains – distiller's grains without solubles (DG) and distiller's grains with solubles (DGS) in wet and freeze-dried form (FrD) from 2017 and 2018.

Method: Processing methods were optimized for raw DG & DGS by employing food grade solvents namely: a) Ethanol b) Ethyl acetate + Ethanol and c) Hexane + Ethanol on the wet and FrD raw material to reduce pigments, odor, and oils to improve compositional quality and shelf stability. The resulting solvent-treated dried product was ground to 0.5mm and heat-sterilized. Linear models were generated, and analysis of variance was used to compare proximate composition, total phenolic content (TPC) and mycotoxin content of raw DG & DGS - wet, FrD form and their corresponding solvent-treated dried products. The mycotoxins were determined through controlled spiking studies and using rapid mycotoxin detection test kits and LC-MS technique. Particle size distribution (PSD) was

determined using a series of stacked sieves (40, 60, 80, 100 & 200 mesh) and correlated with solvent-treated dried product color parameters.

Results: Raw DG and DGS - wet and FrD from 2017 and 2018 were significantly different from each other in terms of composition. Use of defatting solvents - Hexane and ethyl acetate reduced the fat content from ~11% to ≤1% for solvent-treated dried products. Maximum retention with 150-180µm particle size (PS) range was seen for finished products obtained using wet and FrD DG whereas 250-400µm PS range was seen for finished products obtained using wet and FrD DGS. The mycotoxin content was found to be below the FDA guidance levels of 20ppb (aflatoxins) and 2ppm (fumonisins) for solvent-treated dried products. Solvent treatment of wet & FrD DG & DGS yielded TPC ranges of 250-400mgGAE/100gm and 30-250mgGAE/100gm, respectively.

Significance: Processing treatments enhanced the food functionality traits of solvent-treated dried products. This food grade product was found to be odorless, tasteless, color neutral, gluten-free with minimal oil content and consistent PS. A material specification sheet was developed to highlight the characteristics of a food grade distillers dried grains product.

CHAPTER 1. INTRODUCTION

Distiller's Grains are co-products obtained from ethanol production using corn as the principle substrate. One bushel of corn (56 lbs) yields 2.7 gallons of ethanol and 17.5 pounds of distiller's grains (Service, 2015). As the ethanol industries continues to grow, so have the large quantities of residues generated from the industry. Current projections estimate 60 million tons of distiller's grains is produced annually. As feed markets become saturated, new avenues and markets need to be explored. Based on the average price of distiller's grains collected from the U.S. market by Data Transmission Network (DTN), Burnsville MN, the price per unit of protein for distiller's grains is approximately $5.48 in contrast to $6.57 for soybean meal. Distiller's grains are generally used as a livestock feed. As feed markets become saturated, new markets must be explored. In recent years, the use of industrial grade distiller's grains in the food industry has been developing slowly. In view of the saturated feed market and low prices, the potential for enhanced use as a food ingredient is significant. Distiller's grains raw material is a rich ethanol plant fraction containing about 38% protein and 40% dietary fiber. Transitioning from industrial grade distiller's grains to food grade product requires stringent control over its quality characteristics and nutritional composition.

To determine the impact on quality and composition, it is necessary to understand how distiller's grains are produced. As per the definition, distiller's grains material is produced as a co-product in the ethanol production. Since corn is the principal substrate used for ethanol production, it is beneficial to know the location of the nutrients present inside a corn kernel (Figure 1).

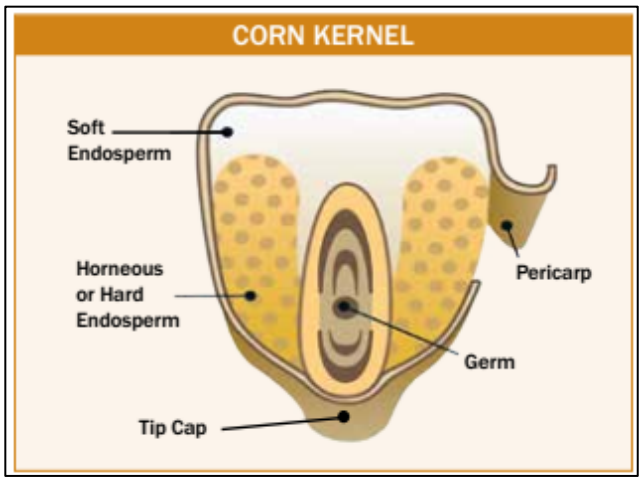


Figure 1: Parts of corn kernel (Image source: https://corn.org/wp-content/uploads/2009/11/CornRefiningProcess.pdf)

Corn kernels are the fruits of the maize grain. As seen in Figure 1, the outermost layer is called the pericarp and makes up about 5.3% of the corn kernel's dry weight. The pericarp protects the kernel from mold growth and abrasion and maintains the moisture level and nutrient value within the kernel (Encyclopedia 2019). It contains fibers such as cellulose, hemicellulose and lignin that can be produced later as a corn gluten feed (Encyclopedia 2019). The endosperm is the largest component and makes up about 82% of the kernel's dry weight. It is divided into soft endosperm and hard endosperm. It contains starch that is primarily converted to ethanol during fermentation process. The endosperm also contains proteins and provides energy for the germinating embryo (germ). The germ makes up about 11.9% of kernel's dry weight. It supplies the necessary enzymes and micronutrients required for the growth of the plant. The germ also accounts for about 25% of the corn oils and is considered to be the valuable part of the kernel (Encyclopedia 2019). The last part of the kernel is called the tip cap which makes about 0.8% of the kernel's dry weight. It is a pathway for chemicals, nutrients and water to enter the kernel.

Based on how the corn kernels are utilized, production of ethanol from corn can be accomplished in two ways namely wet milling and dry milling. During the wet milling process, the corn kernels are fractionated into different components namely: starch, germ, cake, fiber, gluten meal, crude oil and solubles. (Figure 2) (Gulati et al., 1996; Nyendu 2011). The wet milling process requires large scale investments for capital, resources, technology etc. However, the dry milling process is easier since it is a relatively simple process that utilizes the entire corn kernel for ethanol processing yielding high-value end products with low capital and energy costs. Hence the dry grinding method is more popular in the ethanol industry (Rosentrater et al., 2005).

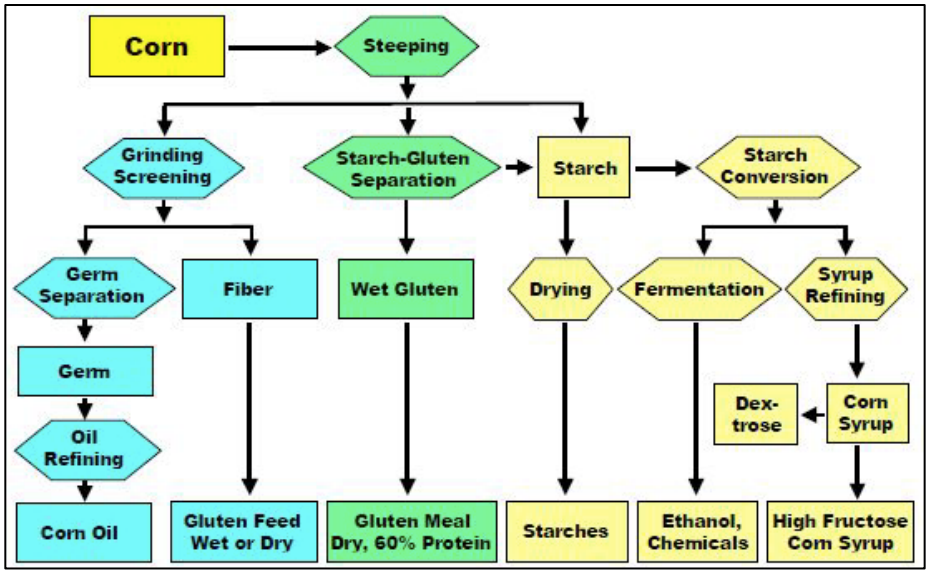


Figure 2: Wet grind corn-to-ethanol manufacturing process (Image source: www.mobt3ath.com/uplode/book/book-16193.pdf)

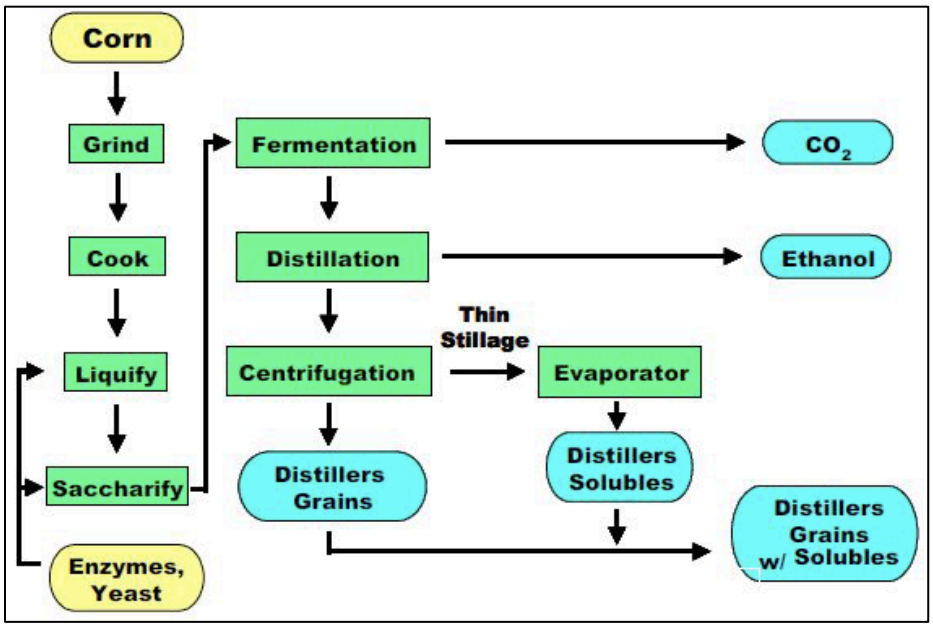


Figure 3: Dry grind corn-to-ethanol manufacturing process (Image source: www.mobt3ath.com/uplode/book/book-16193.pdf)

Dry mill ethanol production method consists of several key steps highlighted in Figure 3, namely grinding, cooking, liquefying, saccharifying, fermenting and distilling. In this process, the corn is ground into fine particle size and cooked in water at 320^{o}F to form a "mash". This mash is first treated with enzymes alpha-amylase enzyme to convert starch into glucose dimers during the liquefaction step. It is further treated with amyloglucosidase enzyme to hydrolyze the dimers into glucose monomers during the saccharification step. These simple sugars then undergo fermentation during which the yeast breaks down the sugars into ethanol and carbon dioxide. After the fermentation step, ethanol is distilled off leaving behind a fibrous slurry called as wet cake/ wet syrup. This slurry is then typically centrifuged and dried to remove the excess water before it is disposed. This material being disposed contains the remaining protein and fiber and is referred to as distiller's grains without solubles (DG). Often the solubles are condensed after centrifugation and then added back to the DG before drying. The resulting product

is referred to as dried distiller's grains with solubles (DGS) (RFA 2015). The solubles are added back to the DG to reduce product losses.

The starting material corn contains approximately, 60% starch, 4% corn oil, 8% protein, 11% fiber and 17% is moisture. During the corn to ethanol conversion process, the major portion of starch is converted to ethanol due to which the resulting co-product distiller's grains appears to have on an average increased level of 38% protein and 40% fiber on a dry weight basis. Along with these nutrients, oil/crude fat also becomes concentrated in the co-product and can be found in the range of 9-12%. Increase in the fat percentage affects the concentration of oil-soluble nutrients namely antioxidants such as total phenolic acids. As the nutritional value of the co-product is increased, a food grade product can be perceived as a functional ingredient in the food industry.

Problem Statement:

The distiller's grains quality and composition is different for different distiller's grains co-products produced in the ethanol plants across United States. This can be attributed to several factors such as the environmental conditions conducive for the growth of corn, quality of the corn kernel, any modifications in the dry or wet milling process, extent and efficiency of the fermentation, drying conditions of distiller's grains, quantity of solubles blended back with distiller's grains etc. (Kaiser 2008). These factors raise concerns for the safe use of distiller's grains in food matrices. Furthermore, the corn plant and the co-product DG can both be contaminated with mycotoxins namely aflatoxins and fumonisins. These toxins can be potentially life-threatening and are therefore being regulated by the government agencies such as USFDA, USDA, and EU. The USFDA has developed a mycotoxin regulatory guidance document which states the toxin action level

of 20 ppb for Aflatoxins and 2ppm for total fumonisins (FB1, FB2 and FB3) (USFDA website). Over the years, the literature has provided the quality and safety information on distiller's grains with solubles (DGS). Limited research information can be found for the nutritional aspects of distiller's grains without solubles (DG). DG and DGS contain high moisture in the range 55-70%. As a consequence of high moisture content, they are prone to spoilage within hours. One of the ways to prevent such spoilage is to reduce the moisture content of distiller's grains. There are variety of conventional drying techniques available but can tend to be disadvantageous for distiller's grains drying. For example, for high moisture DG, drying time and temperature is critical. If the material is dried too quickly at high temperatures (180-240F), then the excess heat can damage the grains. The grains may become brittle thus splitting apart and exposing the nutrients within. Also, if the grains are arranged in a compact manner it may cause uneven heating or drying. These challenges could be overcome by a 'vacuum freeze-drying technique'. In this method, the moisture is removed from the product by the process of 'sublimation' in presence of vacuum. Sublimation is a process in which the frozen water molecules (solid state) are directly converted to gaseous state without passing through the liquid state. The technique offers several advantages over conventional drying: prolongs shelf-life of the product, preserves the quality and nutritional value, prevents spoilage, maintains freshness during storage and deodorizes the product. The resulting freeze-dried product has moisture content of less than 5%. In recent years, food companies have preferred modern drying techniques over conventional techniques to closely monitor the quality and safety aspects of the consumer food products.

Since this research was carried out at the South Dakota State University, Brookings SD, the literature review on ethanol plants was narrowed down to the Mid-western states. Also, for the ease of convenience and transportation, the DG and DGS raw materials were procured in bulk quantities from the ethanol plants in the vicinity of Brookings city.

Research Objectives:

1. To determine the effects of treatment with defatting solvents namely: 1) Ethanol, 2) Hexane + Ethanol, 3) Ethyl Acetate + Ethanol on DG & DGS raw materials and their corresponding solvent-treated dried products.
2. To evaluate and compare the physical and chemical composition of DG & DGS raw materials obtained from the year 2017 and 2018 - 'wet form' & 'freeze-dried form' and their corresponding solvent-treated dried products.
3. To determine effectiveness of processing techniques on aflatoxins and fumonisins in controlled spiking studies using rapid detection methods and chromatography techniques.
4. To develop a material specification sheet for a food grade product.

Research Hypotheses:

1. H0: Freeze-drying of raw DG and DGS will not significantly increase the crude protein content and total dietary fiber content of their corresponding solvent-treated dried products.

   H1: Freeze-drying of raw DG and DGS will significantly increase crude protein content and total dietary fiber content of their corresponding solvent-treated dried products.

2. H0: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will not significantly decrease the crude fat content of their corresponding solvent-treated dried products.

   H1: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will significantly decrease the crude fat content of their corresponding solvent-treated dried products.

3. H0: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will not produce a significant difference in color (L, a and b) i.e. increase the 'L' scores (brightness), decrease the 'a' scores (redness) and decrease the 'b' scores (yellowness) of their corresponding solvent-treated dried products.

   H1: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will produce a significant difference in the color (L, a and b) i.e. increase the 'L' scores (brightness), decrease the 'a' scores (redness) and decrease the 'b' scores (yellowness) of their corresponding solvent-treated dried products.

4. H0: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will not significantly decrease the toxicological content (aflatoxins and fumonisins) of their corresponding solvent-treated dried products.

   H1: Solvent extraction for raw DG and DGS in wet and freeze-dried forms will significantly decrease the toxicological content (aflatoxins and fumonisins) of the corresponding solvent-treated products.

5. H0: The overall raw material processing technique will not reduce the fumonisin content below the USFDA permissible limit of 2 ppm after the material was spiked with a high dose of 50 ppm fumonisin mixture.

   H1: The overall raw material processing technique will reduce the fumonisin content below the USFDA permissible limit of 2 ppm after the raw material was spiked with a high dose of 50 ppm fumonisin mixture.

CHAPTER 2. LITERATURE REVIEW

Studies on contamination of Distiller's grains with mycotoxins:

The quality of distiller's grains produced as a co-product depends on the quality of the corn used as a substrate. Corn quality depends on its growth and harvesting conditions. The corn being supplied by the farmers to the ethanol manufacturers is the potential and a major ethanol plant fractions for contamination with mycotoxins. Mycotoxins are chemical compounds produced in crops as secondary metabolites by specific molds (from fungus kingdom). Corn related mycotoxins can occur during plant growth, maturity, harvesting, storage and grain processing under certain environmental conditions (Richard 2007). The resulting co-product, namely distiller's grains, may also be contaminated with a high concentration of mycotoxins that were initially present in the corn used for fermentation (Bothast et al., 1992). The toxin levels were reported to be three times as high as the level in the grain ethanol plant fractions (Bennett, 1996). Over the years, scientists have raised concerns about mycotoxins levels in DGS, since it is primarily used as an animal feed. This in turn raises questions on safety, for use of DG in food products. Approximately 300-400 mycotoxins have been identified in the literature and some among them may also pose a threat to animals and humans (Bennett et al., 1999). There are a few other mycotoxins such as T-2 toxin, ergot alkaloids, patulin and citrinin which also are considered harmful to the growth of corn. But there are no specific guidelines established by the FDA for these toxins. Table 1 provides a summary of primary mycotoxins that are detrimental to the growth of corn.

Table 1. Primary mycotoxins detrimental to corn growth

Fungus species	Mycotoxins produced	Special Features	Images
Aspergillus Flavus	Aflatoxins B1, B2, G1, G2, M1 and M2	Aflatoxin B1 is classifies as group 1- Human carcinogen by the Intl' Agency for Research on Cancer (IARC)	
Aspergillus Ochraceus	Ochratoxin	Suspected to be Human Carcinogen by IARC	
Fusarium Verticilliodes	Fumonisin B1 B2 and B3	Fumonisin B1 is classified as group 2B- Human Carcinogen by IARC	
Fusarium Graminearum	Zearalenone Deoxynivalenol (Vomitoxin)	Zearalenone can mimic the actions of hormone estrogen	
Fusarium sporotrichioides	T-2 toxin (Trichothecenes)	Its production is greatest with increased humidity and temperature (6-24^{o}C)	

Zhang et al. (2009) studied the occurrence and concentration of mycotoxins such as aflatoxins, deoxynivalenol, fumonisins, T-2 toxin and zearalenone in 235 DGS samples collected from 20 representative ethanol plants spread over the midwestern region of United States. The results showed that aflatoxins, deoxynivalenol and fumonisins levels were found to be lower than the USFDA guidance limits. The T-2 toxin and zearalenone were also found to be less than the detection limit but no USFDA guidance levels are available for these toxins.

Busman et al. (2010) reported the application of liquid chromatography coupled with electron spray ionization mass spectrometry to determine the fumonisins levels in ethanol by- products produced from corn. During sample preparation, the authors spiked DG and DGS samples with known amounts of Fumonisin B1. The chromatogram in Figure 4 was obtained for DG sample which shows that appearance was similar to the unprocessed corn sample fumonisin content.

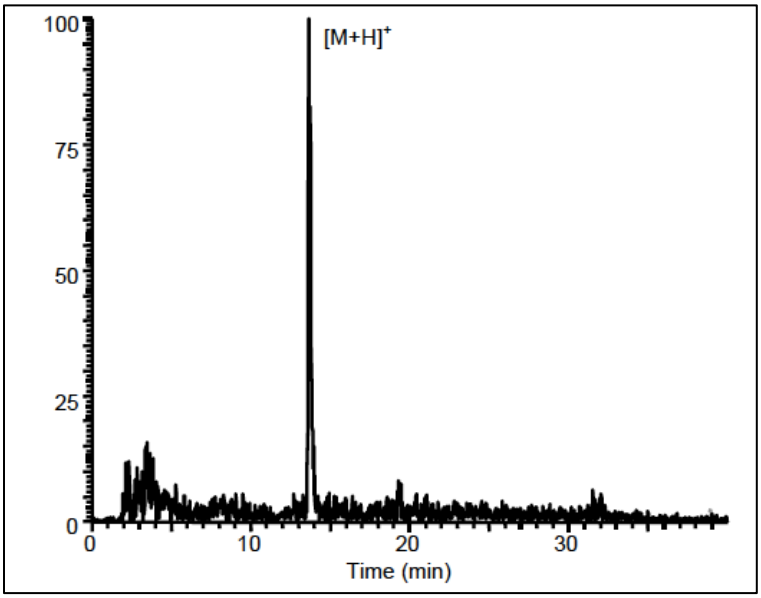


Figure 4: Extracted ion chromatogram for detection of Fumonisin B1 from a DG sample
(Source of information: Busman et al. (2010))

Khatibi et al. (2014) reported on screening of five fusarium species mycotoxins namely-deoxynivalenol (DON), 15- acetyldeoxynivalenol (15-ADON), 3-acteyldeoxynivalenol (3-ADON), nivalenol (NIV) and zearalenone (ZON). One hundred and forty-one DGS samples from the year 2011 were collected from 78 ethanol plants located in 12 states in USA. The mycotoxin levels found in the samples are shown in table 2.

Table 2. Mycotoxins levels found in DGS samples from ethanol plants across USA

DGS Samples from ethanol plants in different states in US	**Mycotoxin**	**Levels found**
OH	DON	Highest levels with a mean of 9.51μg/g
NY, MI, IN, NE, WI	DON	Mean levels > 1 and < 4μg/g
26% (36/141) DGS samples	DON	1-5μg/g
2% (3/141) DGS samples	DON	>5 and <10μg/g
3% (4/141) DGS samples	DON	>10μg/g
141 DGS samples	DON	<0.5-14.62μg/g
141 DGS samples	15-ADON	<0.1-7.55μg/g
141 DGS samples	ZON	<0.1-2.12μg/g
141 DGS samples	3-ADON and NIV	No contamination

Source of information: Khatibi et al. (2014)

Depending on the ethanol plant from which the distiller's grains material is produced, the mycotoxin concentrations vary. In this research project, the raw material was procured from the ethanol plants in the state of South Dakota. It was necessary to generate a toxicology profile for the raw materials used as it was processed in a food grade product. To determine the impact of the processing steps on the mass balance of the toxins, the solvent-treated dried product was also inspected for mycotoxins.

Incorporation of distiller's grains in baked products:

Numerous studies have been conducted relating to the use of distiller's grains as a functional ingredient in the baked food products.

Rosentrater and Krishnan (2006), have reported on the challenges faced while incorporating distiller's grains in food products. The authors have summarized in their article the application of distiller's grains obtained from wheat, barley, corn and rye in a variety of food products such as chocolate chip cookies, sugar cookies, spice cookies, pasta, muesli, yogurt, whole desserts, granola bars, spaghetti and extruded products from early 1970s to 2005. Results showed that there was a significant impact of distiller's grains addition on the sensory qualities of the products.

Liu et al. (2011), formulated corn bread fortified with 0, 5, 10, 15, 20, 25 and 30gm/100gm of corn flour. The different levels of fortifications were evaluated for properties such as moisture, odor, texture, water activity, batter rheology and appearance. It was found that 20-25gm/100gm was the maximum amount of distiller's grains that could be incorporated. Beyond 25gm, the color darkened, and textural properties declined.

Saunders et al. (2014), evaluated the effects of substituting corn DGS and a dough conditioner namely sodium stearoyl lactylate (SSL) with all-purpose flour and bread flour. The DGS levels used in formulations were 0%, 25%, and 50% for flour substitution and 0%, 0.15% and 0.3% for SSL on a flour weight basis. The substitution levels for SSL were within the limit of <0.5% regulated by the USFDA under the list of food additives in 21CFR section (USFDA 2018). The results showed that as the substitution levels of DGS increased; protein, ash, moisture and Hunter-a values also increased. Substitution

with SSL improved the bread quality, dough strength, rate of hydration, mixing tolerance, crumb volume, loaf volume and shelf-life. Level of substitution of less than 25% of DGS was found to be desirable. The critical point to be noted is that the DGS used in the formulations was used ‘as is’ and no process of pretreatment was done to render the product as food grade quality.

The following studies were conducted South Dakota State University campus with focus on incorporation of DG in baked products. Arra et al., (2011) processed DGS into solvent-treated dried product through exhaustive washing with ethanol and water followed by drying and sterilization before incorporation into flat breads of Indian origin (Chapathi and Naan). Results showed that the nutrient composition for fortified breads increased as compared to control wheat flour breads. The sensory analysis for DG fortified food products were found to be acceptable. Studies evaluating the effect of DGS in barbari and tortillas, two Latin American flat breads, were conducted by Pourafshar et al., 2014 and 2015, respectively. The distiller’s grains used in these baked products was used ‘as is’ and not washed with food grade solvents. Results of these experiments concluded that doughs supplemented with distiller’s grains produced breads that were significantly higher in protein, fiber and ash than controls. However, these differences in composition negatively affected the texture of tortillas causing a decrease in extensibility and firmness of final products. While statistically significant differences were seen both in the color and textural properties between the control and distiller’s grains supplemented tortillas, no sensory analysis was done to determine whether tortillas supplemented with distiller’s grains could be considered acceptable based on consumer opinion (Pourafshar and Rosentrater 2015). Similar results were found in the study on

barbari breads. No significant difference was seen between center thickness, extensibility, or density of DGS supplemented and control breads, however statistical differences were seen in edge thickness, firmness and color (L*a*b*).

Most recently, Alrayyes et al. (2018) have investigated the nutritional properties of food grade distiller's dried grains product supplemented pita bread. The preliminary findings of this research have found significant increases in protein and total dietary fiber (TDF) in breads supplemented with food grade distiller's dried grains product. A significant effect on the glycemic index of persons who consumed the pita bread including food grade distiller's dried grains product as compared to control breads has also been found. Ongoing research by Konst et al. (2019) investigates the effects of oat flour and food grade distiller's dried grains product supplemented steamed noodles on the glycemic index. This is a similar study to Alrayyes et al. using a different food product and supplementing oat flour instead of chick pea flour. The results obtained so far show a decrease in the glycemic response with oat flour noodles.

Studies on properties and composition of distiller's grains:

Ranhotra, et al. (1982) determined the nutritional composition of five distiller's spent grains samples as described in table 3. The results showed that protein content and the crude fiber content ranged from 26.9-34.9% and 29.1-35.8%, respectively in Table 4. The samples also had appreciable amounts of potassium, magnesium, phosphorus, zinc, copper, iron, chromium, thiamin, riboflavin and niacin (Table 4).

Table 3 . Test samples of distiller's spent grains

Sample[a]	Source	Mash composition (%)			
		Corn	Rye	Barley	Milo
A	Distillery X	93.1	5.3	1.6	-
B	Distillery X	95.9	3.0	1.1	-
C	Distillery X	98.5	-	1.5	-
D	Distillery Y	-	-	-	100
E	Distillery Z	75.0	13.0	12.0	-

[a] Ground to particle size of 1mm; Source of information: Ranhotra, et al. (1982)

Table 4. Proximate analysis, mineral content and vitamin content for spent grains samples- A, B, C, D and E.

	Sample				
	A	**B**	**C**	**D**	**E**
Proximate composition, %					
Protein (N*6.25)	27.6	27.6	27.7	34.9	26.9
Fat (ether extract)	10.6	10.3	11.5	6.3	6.5
Ash	4.36	4.45	4.45	4.64	4.55
Fiber (crude)	7.9	7.4	7.5	8.5	7.0
Fiber (dietary)	35.6	35.8	34.1	29.1	32.4
Moisture	6.8	7.13	6.64	6.85	6.76
Minerals, %					
Sodium	0.06	0.13	0.06	0.12	0.03
Potassium	1.07	1.07	1.02	0.85	0.98
Calcium	0.068	0.071	0.076	0.083	0.049
Phosphorus	0.82	0.85	0.89	0.79	0.86
Magnesium	0.325	0.328	0.336	0.315	0.328
Minerals, ppm					
Chromium	4.62	4.48	4.50	4.74	4.43
Zinc	59.4	68.6	60.4	69.8	68.6
Copper	20.9	25.3	23.7	15.0	19.9
Iron	9.3	10.0	11.0	29.9	7.7
Vitamins, mg/100g					
Thiamin	0.19	0.19	0.18	0.60	0.61
Riboflavin	0.39	0.43	0.49	0.36	0.62
Niacin	6.66	6.57	6.22	10.37	7.17
Pyridoxine	1.20	0.97	0.90	0.75	1.05
Folic acid	0.018	0.016	0.019	0.038	0.027
Pantothenic acid	0.22	0.24	0.20	0.71	0.94

Source of information: Ranhotra, et al. (1982)

Mcguire (1986) evaluated the quality of distiller's' dried grains by near infrared (NIR) analysis. He used barley distiller's grains to develop a calibration set for NIR. The regression coefficients were obtained for % crude protein, % crude fiber, % neutral detergent fiber (NDF), % acid detergent fiber (ADF) moisture content and amino acids. The results were compared to standard chemical methods for the same parameters (Table 5).

Table 5. Results of analysis of verification for DG samples from chemical methods and NIR analysis

	Protein		Crude Fiber		Neutral Detergent Fiber		Acid Detergent Fiber	
Sample ID	Kjeldahl (%)	NIR (%)	Lab (%)	NIR (%)	Lab (%)	NIR (%)	Lab (%)	NIR (%)
1109-5	25.0	27.9	19.2	17.4	65.4	66.3	28.6	24.2
1123-2	28.7	27.8	15.6	14.0	69.7	62.6	29.8	26.5
0105-1	30.4	30.5	15.9	14.8	70.0	61.6	28.7	27.6
0315-1	26.3	25.8	16.3	17.4	68.8	68.2	27.6	27.6
0825-1	26.8	27.9	15.7	16.5	...	...	...	...
1015-1	28.3	29.1	14.6	14.6	...	...	22.2	26.2
1101-1	28.0	27.7	15.5	16.4	66.1	60.3	26.5	25.0
0713-1	25.1	25.6	20.5	19.8	57.5	65.6	27.4	29.5
0810-1	25.0	26.0	20.5	20.2	64.2	65.0	27.6	30.1
1109-1	13.5	14.0	24.6	23.6	78.9	84.1	32.4	35.2
1118-1	17.9	16.2						
0301-3	19.1	20.3						
0315-11	18.5	21.1						
0609-1	22.9	24.7						
$\bar{x}$	24.0	24.6	17.8	17.1	66.5	67.6	27.9	26.2
Range	13.5–30.4	14.0–30.5	14.6–24.6	14.0–23.6	57.5–78.9	60.3–84.1	22.2–32.4	24.2–35.2
Correlation coefficient (lab vs. NIR)	0.968*[a]		0.89**		0.627		0.66*	
β	0.831		1.20		0.55		0.50	
SE (β)	0.108		0.212		0.281		0.213	
SE (dif)	1.41		1.44		4.75		2.05	
F reg	59.0**		31.9**		3.89		5.42*	

[a]*, ** Significant at the 0.05 and 0.01 probability levels, respectively.

Source of information: Mcguire (1986)

Spiehs et al. (2002) conducted a study to evaluate the nutrient content and variability of DGS from less than 5-year-old ethanol plants located in the states of Minnesota and South Dakota. A total of 118 DGS samples were collected from ten plants (8 MN & 2 SD). The results were compared within and between the ethanol plant samples and to the reference chemical methods used for testing proximate analysis, amino acid composition and mineral analysis. There were significant differences observed between the results that were attributed to the quality of the corn substrate used, fermentation process times and

the sampling time period. These studies suggested that due to the existing variability in corn DGS, a complete chemical profile should be developed annually to account for the differences.

Ganesan et al. (2007) characterized the glass transition temperature (T_g) for different initial moisture types of distiller's grains namely, unmodified distiller's grains, reduced fat distiller's grains, de-waxed distiller's grains and distiller's grains without solubles using differential scanning calorimetry. It was found that the T_g was near 20^{o}C for all the samples. The authors predicted that the thermal properties are affected by the chemical composition of the distiller's grains. The DSC profiles were attributed to the amorphous nature of the distiller's grains proteins, that are thermodynamically unstable in nature and have a tendency to crystallize to gain stability. This research formed a basis for linking the T_g with the physical and flowability properties of distiller's grains samples.

Winkler and Rennick (2007) determined the amounts of phytosterols, tocopherols, ferulate phytosterol esters (FPE) and tocotrienols present in oil extracted from distiller's grains (DG) and in distiller's grains with solubles (DGS). DG corn oil was extracted by Soxhlet extraction, Accelerated Soxhlet Extraction (ASE) and using hexane and ethanol as solvents and also by super critical carbon dioxide (CO_2) treatment. As seen in table 6, the results showed that the phytosterols, tocopherols and FPE were found to be in similar amounts for DG corn oil when extracted using hexane (Soxhlet and ASE) and super critical CO_2 treatment. Phytosterol composition and FPE were found to be in similar amounts among other methods.

Table 6. Oil, phytosterol and ferulate phytosterol esters contents in extracts of corn DG

		phytosterol composition[b] (mg/g of extract)									ferulate phytosterol esters	
solvent, method	% oil[c] (w/w)	Camp	Campst	Stigm	Sito	Sitost	Aven	Stigsten	total[d] (mg/g of extract)	total (mg/g of DDGS)	mg/g of extract[e]	mg/g of DDG[e]
hexane, Soxhlet	12.67 ± 0.16 c	2.53 (15.6)	1.10 (6.8)	0.82 (5.0)	8.05 (49.6)	2.70 (16.6)	0.73 (4.5)	0.31 (1.9)	16.2 ± 0.65 b	2.05 ± 0.09 b	3.99 ± 0.04 a	0.51 ± 0.06 b
hexane, ASE	11.15 ± 2.19 c	2.69 (15.4)	1.19 (6.8)	0.89 (5.1)	8.58 (49.1)	2.94 (16.9)	0.81 (4.6)	0.34 (2.0)	17.3 ± 0.26 a	1.92 ± 0.33 d	3.97 ± 0.39 a	0.44 ± 0.04 d
ethanol, Soxhlet	32.73 ± 1.90 a	1.36 (15.4)	0.59 (6.6)	0.50 (5.6)	4.30 (48.6)	1.44 (16.3)	0.45 (5.1)	0.22 (2.5)	8.87 ± 0.30 e	2.91 ± 0.22 a	1.62 ± 0.06 d	0.53 ± 0.02 a
ethanol, ASE	17.55 ± 0.09 b	1.75 (15.3)	0.82 (7.2)	0.62 (5.4)	5.50 (48.3)	2.03 (17.8)	0.49 (4.3)	0.19 (1.6)	11.4 ± 0.78 d	2.00 ± 0.14 c	1.98 ± 0.09 c	0.35 ± 0.01 e
CO_2	12.50 ± 0.26 c	2.46 (15.6)	1.06 (6.7)	0.83 (5.2)	7.85 (49.7)	2.63 (16.6)	0.72 (4.6)	0.25 (1.6)	15.8 ± 0.67 c	1.97 ± 0.11 c	3.75 ± 0.47 b	0.47 ± 0.07 c

[a] Abbreviations: Camp, campesterol; Campst, campestanol; Stig, stigmasterol; Sito, β-sitosterol; Sitost, sitostanol; Aven, Δ5-avenasterol; Stigsten, Δ7-stigmastenol. See text for other abbreviations. [b] Values in parantheses are the relative percent for each phytosterol. [c] Values are the average ± standard deviation for three extracts.[d] Values are the average ± standard deviation for three extracts, each analyzed in duplicate ($n = 6$). Within each column, means followed by different letters are significantly different ($p < 0.05$) by Duncan's multiple-range test. [e] Values are the average ± standard deviation for three extracts, each analyzed in triplicate ($n = 9$). Within each column, means followed by different letters are significantly different ($p < 0.05$) by Duncan's multiple-range test.

Source of information: Winkler and Rennick (2007)

Liu (2008) investigated the particle size distribution (PSD) of DGS and its relationships to nutrient composition and surface color to the original DGS sample and its sieved fractions. The results showed that the distribution of nutrients in the sized fractions was highly heterogenous. Hence, the data could be used to fractionate DGS for compositional enrichment based on PSD. This study formed a baseline for the quality and consistency aspects of DGS.

Winkler-Moser and Kristine (2009) determined the concentrations of phytosterols, steryl frulates, tocopherols, tocotrienols and carotenoids in distiller's grains oil and DG distillate (DGD). DGD was collected using molecular distillation of the oil extracted from distiller's grains that is free from fatty acids. This was accomplished by using high temperatures of 100, 120 and 250ºC and a pressure of <1mTorr. As seen in table 7, concentration of phytosterols and steryl ferulates was 5.75 times greater in DGD than distiller's grains corn oil. Concentration of total carotenoids in DGD was found to be approximately half the amount found in distiller's grains corn oil. However, the composition of all these phytonutreints was found to be similar in both DGD and distiller's grains corn oil.

Table 7. Phytochemicals in corn distiller's grains oil and DGD

Component	Amount	
	Distiller's grains oil	**DGD**
Phytosterols (mg/g)[a]	16.2 (0.7)	93.2 (3.2)
Steryl ferulates (mg/g)[b]	4.0 (0.0)	10.4 (0.3)
α-Tocoherol (μg/g)	194 (5)	273 (7)
α- Tocotrienol (μg/g)	155 (3)	241 (8)
γ- Tocopherol (μg/g)	948 (17)	910 (23)
γ- Tocotrienol (μg/g)	453 (3)	429 (12)
δ- Tocopherol (μg/g)	51 (2)	41 (2)
β- carotene (μg/g)	4.5 (0.1)	0.97(0.0)
Lutein (μg/g)	34.6 (1.8)	11.9 (0.9)
Zeaxanthin (μg/g)	23.3 (0.0)	14.2 (1.3)
β- Cryptoxanthin (μg/g)	3.9 (0.1)	4.0 (0.1)

Results shown are the average of triplicate measurments with the standard deviation in parentheses
Legends:
DGD: dried distillers grain oil distillate
[a]Total phytosterols including free sterols, steryl-fatty acid esters and steryl ferulate esters as determined by saponifiction and GC analysis
[b]Steryl ferulates as determined by HPLC analysis, which contribute to the total phytosterol content shown in the row above.
Source of information: Winkler-Moser and Kristine (2009)

In another study, Winkler-Moser, Kristine and Breyer (2011) investigated the use of oils extracted from corn germ (CG) and distiller's grains with solubles (DGS) from the ethanol dry-grind process. The oils were extracted using hexane solvent. These oils were analyzed by High Performance Liquid Chromatography (HPLC) method for the content and composition of tocopherols, tocotrienols, carotenoids, phytosterols and steryl ferulates. As seen in Table 8, the composition of tocols and total carotenoids was found to be similar in CG oil and DGS oil, with the exception that beta-carotene was not detected in CG oil. CG oil had higher concentration of tocopherols than DGS oil since the germ portion of the corn kernel contributed to the high levels of the tocopherols. DGS oil showed higher tocotrienol content than CG oil. This could be attributed to the removal of endosperm frations rich in tocotrienols during corn germ fractionation. DGS had a higher carotenoid content than CG oil since the carotenoid rich endosperm fraction was removed

during the corn germ fractionation. As seen in Table 9, the composition of CG oil and DGS oil were similar except for the absence of 24-methylene cycloartanol and citrostadienol in CG oil. DGS had higher levels of phytosterols than CG oil due to the presence of phytosterols and ferulate phytosterol esters from bran and pericarp. DGS oil had 5.9 times higher concentration of steryl ferulates than CG oil.

Table 8. Content of tocols and carotenoids at 110^{o}C for oils extracted from corn germ (CG) and distillers dried grains with solubles (DGS)

Component	**CG**	**DGS**
Total tocopherols (μg/g)	1433.6	1104.2
Alpha-tocoherol	213.8	295.6
Gamma- tocopherol	1185.4	760.8
Delta- tocopherol	34.3	47.8
Total tocotrienols (μg/g)	235.6	1762.3
Alpha-tocotrienol	21.9	471.9
Gamma-tocotrienol	165.6	1210.0
Delta-tocotrienol	48.1	80.3
Total carotenoids (μg/g)	1.33	75.02
Lutein	0.37	46.69
Zeaxanthin	0.4	24.16
Beta- cryptoxanthin	0.56	3.31
Beta- carotene	ND	0.86

Legends:
ND: Not detected
Source of information: Winkler-Moser, Kristine and Breyer (2011)

Table 9. Content and composition of phytosterols in oils extracted from corn germ (CG) and distillers dried grains with solubles (DGS)

	CG		DGS	
Component	**mg/g**	**%[b]**	**mg/g**	**%**
Total phytosterols	14.9		21.7	
Campesterol	3.08	20.7	2.97	13.7
Campestenol	0.25	1.7	1.35	6.2
Stigmasterol	0.98	6.6	1.10	5.1
Sitosterol	9.04	60.9	10.3	47.5
Sitostanol	0.66	4.4	3.72	17.2
Avenasterol	0.54	3.7	0.93	4.3
Cycloartenol	0.28	1.9	0.71	3.2
24-Methylene cycloartanol	ND[c]	0	0.30	1.4
Citrostadienol	ND	0	0.31	1.4
Steryl ferulates (mg/g)	0.58		3.42	

Legends:
[b]The relative weight percentage of total phytosterols
[c]ND: Not detected
Source of information: Winkler-Moser, Kristine and Breyer (2011)

In another study, Luthria, Liu and Memon (2012), determined the total phenolic acid (TPA) content and antioxidant capacity of ground corn and corresponding DGS obtained from 3 ethanol plants located in Iowa state in USA. Five phenolic acids were identified in corn and DGS namely: vanillic, caffeic, p-coumaric, ferulic and sinapic acids. The TPA was assayed by two methods: ultrasonic assisted base hydrolysis and HPLC-LC-ESI-MS. The extracts for the antioxidant assay were prepared using as a pressurized liquid extractor and were then analyzed using ferric reducing antioxidant power (FRAP) assay. The FRAP assay uses Trolox as a standard and is based on the formation of O-Phenanthroline-Fe (2+) complex and its disruption in the presence of chelating agents (Wikipedia 2017). The comparative results showed that DGS and corn had same phenolic acid composition, however, DGS contained 3.40 folds and 2.58 folds higher concentration of phenolic content and antioxidant content respectively than corn. As seen in table 10, out of the five acids, ferulic and p-coumaric acid made up 80% of the TPA

present in ground corn and DGS. Both ground corn and DGS had similar TPA compositions across the three plants. The TPA content per gram basis was 3.4 times higher in DGS than ground corn. The FRAP value for DGS increased 2.68-2.72 times higher than ground corn. This indicated a significant increase in DGS antioxidant capacity over ground corn. The abbreviation used in the table 'DDGS' is same as 'DGS'.

Table 10. Contents (mg/g dry matter) of phenolic acids and FRAP values (μmol Trolox equivalents/g sample) in ground corn and DGS from three bioethanol processing plants

Plant no.	Sample type	Vanillic	Caffeic	*p*-coumaric	Ferulic	Sinapic	Total	FRAP
Sample type								
	Ground corn	0.13b	0.12b	0.15b	1.25b	0.10b	1.75b	44.6b
	DDGS	0.22a	0.14a	0.72a	4.59a	0.33a	5.99a	114.4a
Plant no.								
P1		0.17b	0.12b	0.58a	2.38b	0.14b	3.40b	90.0a
P2		0.16b	0.12b	0.39b	1.96c	0.14b	2.76c	76.5b
P3		0.19a	0.14a	0.35c	4.43a	0.35a	5.46a	72.0b
Plant no. × sample type								
P1	Ground corn	0.12e	0.11c	0.17d	0.99e	0.08d	1.47e	49.0c
P2	Ground corn	0.12e	0.11c	0.12e	0.92e	0.08d	1.35e	46.1 cd
P3	Ground corn	0.15d	0.12c	0.17d	1.84d	0.15c	2.43d	38.7d
P1	DDGS	0.22b	0.13b	0.99a	3.77b	0.21b	5.32b	131.1a
P2	DDGS	0.19c	0.13b	0.65b	2.99c	0.21b	4.18c	106.9b
P3	DDGS	0.23a	0.15a	0.53c	7.02a	0.56a	8.49a	105.3b
Mean		0.17	0.13	0.44	2.92	0.21	3.87	79.5

Source of information: Luthria, Liu and Memon (2012)

In a similar study, Luthria, Memon and Liu (2014) analyzed the nine fractions mentioned in table 11 by two methods: ultrasonic assisted base hydrolysis and HPLC-LC-ESI-MS while the antioxidant activity was measured by FRAP assay. As seen in table 12, five phenolic acids were identified except for vanillic acid which was absent in fractions 2 and 3. Compared to ground corn, TPC concentration tripled post fermentaion due to the conversion of starch to ethanol and the removal of CO_2. DWG contained maximum TPC for all the three plants because this fraction included all pericarp, rich in phenolic acids. Overall, it was observed that pre-fermentation fractions showed lower antioxidant activity as comapred to post fermentation fractions.

Table 11. Nine fractions collected at different steps from 3 dry-grind ethanol plants

Pre-fermentation step	Post fermentation step
1. ground corn	4. fermented mash
2. cooked slurry	5. whole stillage
3. liquified slurry	6. thin stillage
	7. condensed distillers solubles (CDS)
	8. distillers wet grains (DWG)
	9. Distillers dried grains with solubles (DGS)

Source of information: Luthria, Liu and Memon (2014)

Table 12. Individual and total phenolic acid content as well as FRAP value in various step samples collected from three commercial dry-grind ethanol processing plants.

Plant	Fractions	Vanillic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Total acid	FRAP
3-plant average	1 Ground corn	0.143e	0.116g	0.142g	1.225e	0.102e	1.728g	44.371h
	2 Cooked slurry	0.000f	0.128f	0.494e	1.964d	0.313d	2.899e	67.732g
	3 Liquefied mash	0.000f	0.130f	0.514e	2.015d	0.332d	2.990e	66.394g
	4 Fermented mash	0.394c	0.164c	1.259c	4.429c	0.715b	6.962c	166.446d
	5 Whole stillage	0.426b	0.168b	1.547b	5.317b	0.802a	8.259b	178.354c
	6 Thin stillage	0.435b	0.179a	0.402f	0.712f	0.446c	2.174f	225.166b
	7 Condensed solubles	0.487a	0.169b	0.358f	0.670f	0.428c	2.111f	240.926a
	8 Distillers wet grains	0.392c	0.155d	2.240a	7.851a	0.816a	11.456a	123.974e
	9 DDGS	0.307d	0.144e	0.725d	4.574c	0.323d	6.074d	110.958f
Plant A	9 fraction average	0.299b	0.157a	1.069a	3.574a	0.481b	5.579a	153.712a
Plant B		0.213c	0.136b	0.709c	2.514b	0.377c	3.949c	139.723b
Plant C		0.351a	0.158a	0.782b	3.498a	0.567a	5.356b	114.672c

1. Means of four replicate results for each data point of individual plant samples.
2. Individual and total phenolic acids content are expressed as mg g^{-1} (dry matter basis).
3. FRAP (ferric reducing antioxidant power) is expressed as µmol Trolox equivalent g^{-1} dry sample.
4. Column means for the three-plant average having different letters differed significantly at $P < 0.05$.
5. Column means for the nine fraction average having different letters differed significantly at $P < 0.05$. Standard deviations, although not shown, were 0.9–5.3% of the means for vanillic, 0.00–4.0% for caffeic, 0.6–8.6% for *p*-coumaric, 0.8–6.9% for ferulic, 1.0–6.9% for sinapic, 0.8–5.1% for total and 0.9–13.4% for FRAP.

Source of information: Luthria, Liu and Memon (2014)

Saunders et al. (2013) investigated the potential effects of bleaching techniques on the removal of color pigments and reduced lipid values on the DGS to produce a food grade ingredient. Ethanol was used as an extracting solvent. The extraction time and number of extractions linearly increased in the protein content and inversely decreased the lipid content. The extraction process caused the L value (brightness) to increase, a value to decrease with no significant impact on b value. The higher the L brightness value, the greater was the consumer acceptability.

Krishnan (2015) filed a patent application that described the methods and process that could be used to process industrial grade distiller's grains to a food grade distiller's dried grains product. The results showed that the processing techniques helped to minimize the distiller's grains variability and insured uniform food functionality traits.

Shin et al. (2018) obtained 16 DGS samples from different ethanol plants in US. The samples were analyzed for xanthophylls, vitamin E, ferulic acid content and antioxidant activity. The results were compared between corn and DGS. The analysis was performed by DPPH assay and HPLC. As seen in table 13, lutein was found to be 4.4 to 9.3 times higher than zeaxanthin in DGS samples versus corn. Average DGS and total xanthophyll concentration were found to be similar to that of the corn samples. Average total ferulic acid was found to be three times higher in DGS samples than in corn. Free ferulic acid in DGS and corn correlated with antioxidant activity. Total ferulic acid content could not be correlated since almost all the ferulic acid found in corn was in bound form. DGS samples had five times more antioxidant capacity than corn sample.

Table 13. Comparison of xanthophylls, ferulic acid and antioxidant activity values between corn and 16 DGS samples

Item	**Xanthophylls, μg/kg dry matter**			**Ferulic acid, mg/g dry matter**		**Antioxidant activity, mmol tocopherol equiv./kg**
	Lutein	**Zeaxanthin**	**Total**	**Free ferulic acid**	**Total ferulic acid**	
Corn sample	385	63	448	0.001	2.053	8.09
Mean value for 16 DGS samples	409-845	45-145	440-954	0.026-0.058	6.78-8.13	38.07

Source of information: Shin et.al (2018)

All of these research studies have created a platform for further exploration of distiller's grains and its role as a functional ingredient in food applications.

CHAPTER 3. MATERIALS AND METHODOLOGY

Materials:

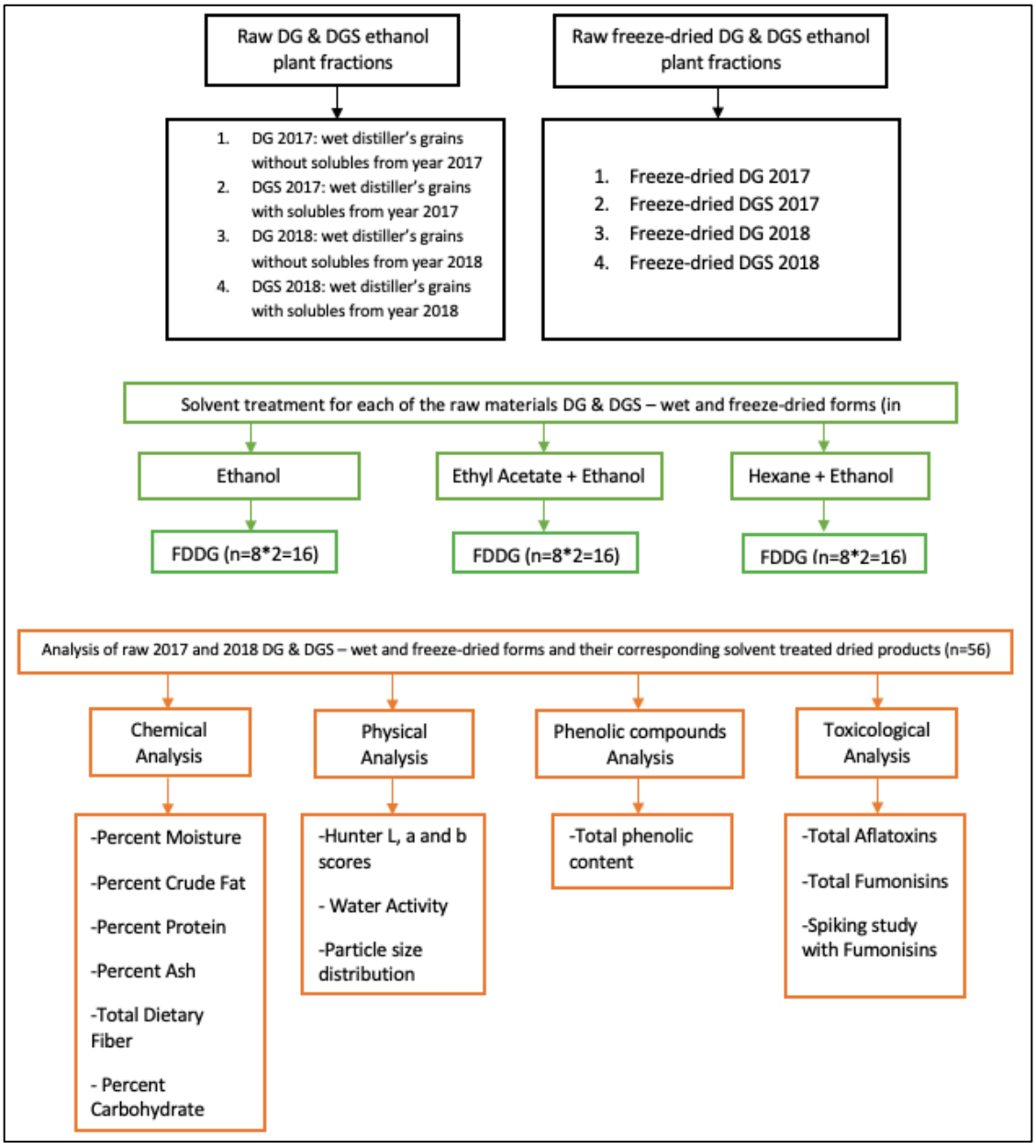
Table 14 explains the initial moisture types of raw material obtained from two ethanol plants in the state of South Dakota. This material was used 'as is or the wet form' and in the 'freeze-dried' (moisture < 5%) form to conduct the experiments. Absolute ethanol was purchased in bulk quantities from Chemistry department at South Dakota State University, Brookings SD. Hexane and Ethyl acetate solvents used were of analytical grade and purchased through Fisher Scientific. All the other reagents used during analysis were of analytical grade.

Table 14. Procurement of starting material

Starting material	Plant location	Collection Year	Moisture content
Distiller's grains with solubles (DG)	Watertown, SD	2017	~50%
Distiller's grains without solubles (DGS)	Wentworth, SD	2017	~65%
Distiller's grains with solubles (DG)	Wentworth, SD	2018	~55%
Distiller's grains without solubles (DGS)	Wentworth, SD	2018	~45%

For ease of explanation and understanding this research, the food grade distiller's dried grains product was termed as 'solvent-treated dried products' or 'finished products'. Depending on the ethanol plant fractions, if wet distiller's grains without solubles (DG) was used as the starting material, then the corresponding solvent-treated dried product was abbreviated as FDG product. Similarly, if wet distiller's grains with solubles (DG) DGS was used as the starting material, then the corresponding solvent-treated dried product was abbreviated as FDGS product. Furthermore, raw DG and DGS ethanol plant fractions were freeze-dried and were termed as freeze-dried DG and freeze-dried DGS. Similarly, their corresponding solvent-treated dried product was termed as FDG obtained using freeze-dried DG and FDGS obtained using freeze-dried DGS.

Figure 5: Experimental outline describing four DG and DGS samples in their wet and freeze-dried forms from the years 2017 & 2018 subjected to 3 solvent systems and multiple assays (N=48)



Experimental Design:

Treatment	Raw material description	Solvents
1	DG 2017	Ethanol
2		Ethyl acetate + Ethanol
3		Hexane + Ethanol
4	DGS 2017	Ethanol
5		Ethyl acetate + Ethanol
6		Hexane + Ethanol
7	Freeze-dried DG 2017	Ethanol
8		Ethyl acetate + Ethanol
9		Hexane + Ethanol
10	Freeze-dried DGS 2017	Ethanol
11		Ethyl acetate + Ethanol
12		Hexane + Ethanol
13	DG 2018	Ethanol
14		Ethyl acetate + Ethanol
15		Hexane + Ethanol
16	DG 2018	Ethanol
17		Ethyl acetate + Ethanol
18		Hexane + Ethanol
19	Freeze-dried DGS 2018	Ethanol
20		Ethyl acetate + Ethanol
21		Hexane + Ethanol
22	Freeze-dried DGS 2018	Ethanol
23		Ethyl acetate + Ethanol
24		Hexane + Ethanol

Legends: DG: wet distiller's grains with solubles, DGS: wet distiller's grains without solubles

Each treatment was performed in duplicates, thus (8*3*2) = 48 trials

(8*3*2) = Raw ethanol plant fractions- wet and freeze-dried forms from 2017 and 2018 treated in duplicates with three solvents namely-

a) Ethanol, b) Ethyl acetate + Ethanol, c) Hexane + Ethanol

Thus, the total number of trials were 48.

Processing of raw DG and DGS samples – wet and freeze-dried forms into solvent-treated dried products:

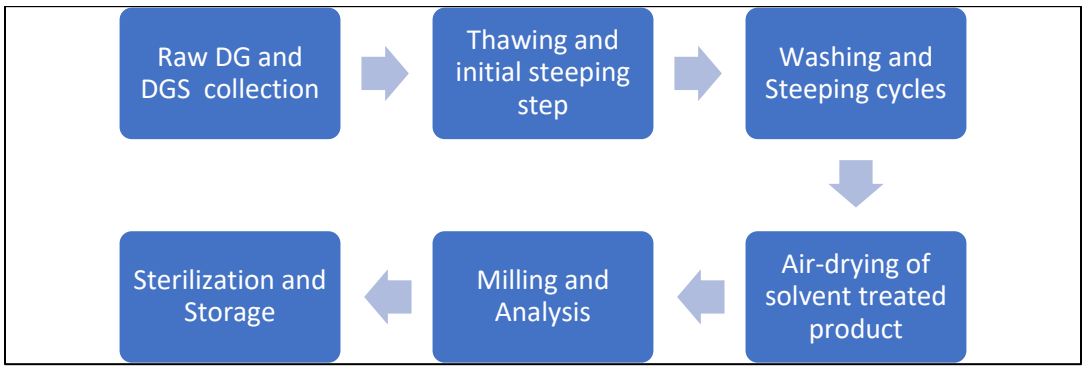


Figure 6: Flow chart for processing of solvent-treated dried products from wet DG & DGS raw material

Procedure:

Figure 6 shows the flowchart employed for raw DG and DGS ethanol plant fractions obtained from the ethanol plants. The raw material was stored in the freezer in labelled ziploc bags until further use. It was then thawed at room temperature for 30 minutes. 1000 gm of this raw material was steeped in 2000mL of each of 3 solvents for 2 hours with intermittent stirring (cycle 0). After initial steeping in the selected solvent, DG was washed with 700mL of Ethanol through #170 sieve by pressing the material with hands. The residual ethanol was discarded. DG was again steeped in 1000mL of Ethanol for 1 hour with intermittent stirring and washed similarly as in cycle 0. The washing and steeping cycles were repeated five times (cycle 1- cycle 5). After the final wash (cycle 5), DG was spread on aluminum foil-lined trays and air dried overnight (figure 8). The solvent-treated dried product produced was milled using a 0.5mm sieve in a Retsch mill (figure 10). Comparative analysis was performed for wet forms of raw DG and DGS ethanol plant fractions and their respective solvent-treated dried products. Ground solvent-treated dried was stored in labelled mason jars and sterilized in an autoclave

(figure 11) at 121ºC for 15 minutes at 15 psi. The sterilized solvent-treated dried product jars were then stored in the freezer (-18ºC) until further use.

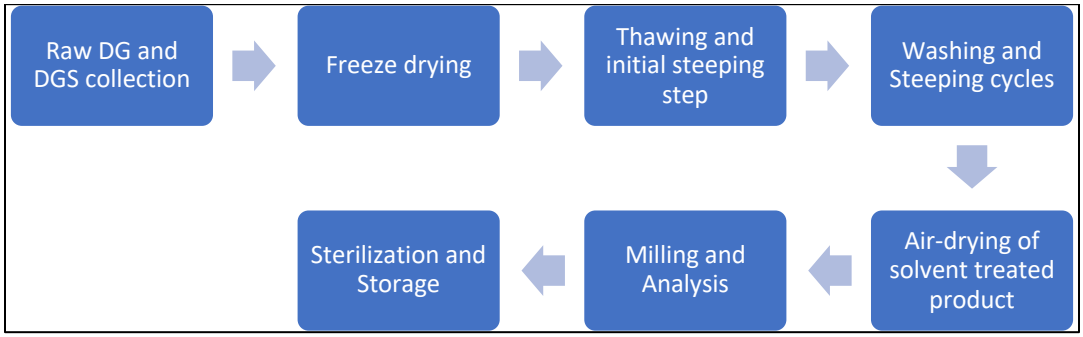


Figure 7: Flow chart for processing of solvent-treated dried products from 'freeze-dried' DG and DGS raw material

Procedure:

Figure 7 shows the flowchart employed for raw DG and DGS ethanol plant fractions obtained from the ethanol plants. The raw material was stored in the freezer in labelled ziploc bags until further use. It was then thawed at room temperature for 30 minutes. The wet material was spread on six freeze dryer trays lined with aluminum foil. The trays were kept in the freeze dryer (figure 9) and the material was allowed to dry using vacuum and heat functions for 3-4 days. Initial moisture content of the wet material was recorded, and the final moisture content of the freeze-dried material was expected to be below 5%. 1000 gm of this freeze-dried material was steeped in 2000mL of each of the 3 solvents for 2 hours with intermittent stirring (cycle 0). After initial steeping in the selected solvent, DG was washed with 700mL of Ethanol through #170 sieve by pressing the material with hands. The residual ethanol was discarded. DG was again steeped in 1000mL of Ethanol for 1 hour with intermittent stirring and washed similarly as in cycle 0.

The washing and steeping cycles were repeated five times (cycle 1- cycle 5). After the final wash (cycle 5), DG was spread on aluminum foil-lined trays and air- dried overnight (figure 8). The solvent-treated dried product produced was milled using a 0.5mm sieve in a Retsch mill (figure 10). Comparative analysis was performed for freeze-dried forms of raw DG and DGS ethanol plant fractions and their respective solvent-treated dried products. Ground solvent-treated dried product was stored in labelled mason jars and sterilized in an autoclave (figure 11) at 121°C for 15 minutes at 15 psi. The sterilized solvent-treated dried product jars were stored in the freezer (-18°C) until further use.

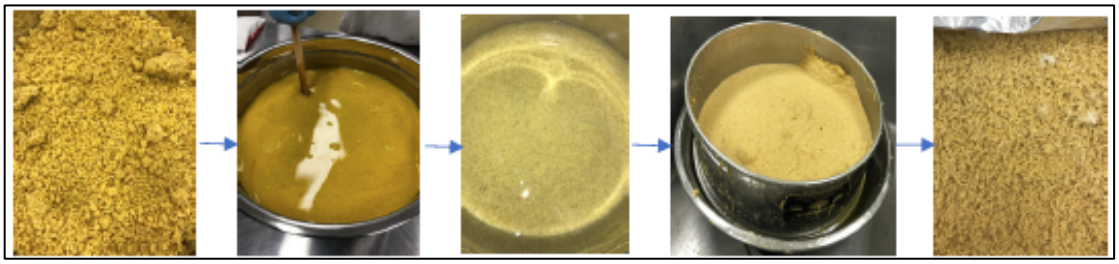


Figure 8: Common processing steps involved in a typical procedure

Figure 9: Bulk freeze dryer (Make: Virtis Inc.)

Figure 10: Retsch mill (Make: Retsch Brinkmann, high speed rotor mill)

Figure 11: Autoclave (Make: Amerex instruments Inc., Model: Hirayama HA-300MW, Image ethanol plant fractions: http://www.amerexinst.com)

For ethyl acetate + ethanol and hexane + ethanol treatments, the total volume of ethanol consumed from cycle 0 to cycle 5 was 8500 mL. For ethanol only treatments, the total volume consumed is 10,500 mL. The turbidity and the yellowness of the ethanol used for steeping decreased by the end of cycle 5, which indicates that the solvent-treated dried product was sufficiently washed. As per the following calculation:

1 gallon = 3.785 L = 3785 mL

Therefore, 8500 mL = 8.5 L = 2.245 gallons and

10,500 mL = 10.5 L = 2.774 gallons

Hence, to process 1 kg of solvent-treated dried product, 2.245 gallons of ethanol was consumed for ethyl acetate + ethanol and hexane + ethanol treatments whereas 2.774 gallons were consumed for ethanol only treatments.

This quantity was kept fixed based on the solvent-treated dried product washing procedures performed previously. In order to achieve a desirable L, a and b value for the solvent-treated dried product, minimum of 2.2 gallons of ethanol was required for processing. This volume may vary depending on the initial moisture type of distiller's grains used as a starting material. To obtain higher L values, more volumes of ethanol can be used for exhaustive washing and steeping cycles. There was a direct impact on the proximate composition of the corresponding solvent-treated dried products based on the raw material ethanol plant fractions (table 15) and the volume of ethanol used in solvent extraction (table 16).

Table 15. Solvent consumption volume for initial steeping step for all trials

Raw material description	Solvents used for initial steeping step (in mL)		
	Ethanol	**Ethyl acetate**	**Hexane**
Wet DG without solubles (DG 2017)	2000	2000	2000
Wet DG with solubles (DGS 2017)	2000	2000	2000
Wet DG without solubles (DG 2018)	2000	2000	2000
Wet DG with solubles (DGS 2018)	2000	2000	2000
Freeze dried DG 2017	2000	2000	2000
Freeze dried DGS 2017	2000	2000	2000
Freeze dried DG 2018	2000	2000	2000
Freeze dried DGS 2018	2000	2000	2000
Total volume (in mL) for duplicate trials	16000*2= 32,000	16000*2= 32,000	16000*2= 32,000

Table 16. Ethanol consumption for washing and steeping cycles for a typical procedure

Number of cycles	Ethanol volume (in mL)	
	Steeping volume	**Washing volume**
0	Material from first steeping step	700
1	1000	700
2	1000	700
3	1000	700
4	1000	700
5	1000	700
Total volume (in mL)	5000	3500

Table 17 shows the amount of raw DG and DGS ethanol plant fractions required in their wet form and freeze-dried form to conduct trials with the three solvents. The amount was calculated for all trails in duplicates.

Table 17. Quantity of raw material required for all trials

Raw material description	Quantity calculation
Wet DG without solubles (DG 2017) Wet DG with solubles (DGS 2017) Wet DG without solubles (DG 2018) Wet DG with solubles (DGS 2018)	For each ethanol plant fractions, 1000 gm * 3 solvents * 2 (in duplicates) = 6000 gm
DG 2017 to be used for freeze drying DGS 2017 to be used for freeze drying DG 2018 to be used for freeze drying DGS 2018 to be used for freeze drying	For each ethanol plant fractions, 4000 gm * 5 (to produce sufficient quantity of freeze-dried material) = 20,000 gm
Freeze-dried DG 2017 Freeze-dried DGS 2017 Freeze-dried DG 2018 Freeze-dried DGS 2018	For each ethanol plant fractions, 1000 gm * 3 solvents * 2 (in duplicates) = 6000 gm

The raw material ethanol plant fractions DG & DGS - wet and freeze-dried forms and their corresponding solvent-treated dried products were analyzed in duplicates for the following parameters:

Proximate analysis:

Fat Analysis:

Percent crude fat content was determined in ANKOM Fat extractor (Model: XT115, figure 12) using petroleum ether as a fat extracting solvent. This is a modified method approved by the American Oil Chemists' Society as an Official Procedure, Am 5-04 (Society 2005). The method measured the loss in the weight of the sample after fat extraction. The extraction process occurred at 90^{o}C. The fat values obtained were expressed on dry weight basis since the samples were pre-dried at 103^{o}C for 3 hours prior to extraction process. The following formula was used to calculate the fat percentage:

% Crude oil = ((W2-W3)/W1) *100

Where, W1 = Original weight of sample

W2 = Weight of pre-extraction dried sample and filter bag

W3 = Weight of dried sample and filter bag after extraction

Figure 12: ANKOM Fat extractor (Model: XT115)

Protein analysis:

The samples were analyzed for protein using the AOAC official method 990.03 Protein (crude) in Animal feed. N/protein analyzer rapid MAX N exceed (Elementar, Germany) was used for determination as seen in figure 13. This analyzer works on the principle of Dumas method. In this method, combustion of known mass of sample (approx. 150 mg) occurs at high temperature (900°C) in presence of oxygen leading to release of CO_2, N_2, H_2O. The resulting nitrogen content obtained for each sample was multiplied by a conversion factor of 6.25 (Council 2012) to calculate percent crude protein. The values were expressed on a dry weight basis through moisture correction for all samples.

Figure 13: N/protein analyzer rapid MAX N exceed

Ash analysis:

Ash content was determined using dry oxidation method (Method. 08-03, AACC, 2000) in a muffle furnace (Company: Model: Box furnace, 51800 series) as seen in figure 14. The samples were incinerated at 525°C for 12 hours in muffle furnace to estimate inorganic mineral content. The values were expressed on a dry weight basis through moisture correction for all samples.

Figure 14: Muffle furnace by Cole Parmer (Model: Box furnace, 51800 series)

Total Dietary Fiber (TDF) analysis:

Fiber content was analyzed by enzymatic gravimetric method employing AOAC method 991.43 for Total Soluble, and Insoluble Dietary fiber in Foods' and AACC method 32-07.01 for Determination of Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products. The Megazyme assay test kit for total dietary fiber was used to determine TDF in raw DG and DGS ethanol plant fractions- wet and freeze-dried forms. The outline for the TDF assay procedure was followed as seen in figure 15. The values were expressed as dry weight basis through moisture correction for all samples. Of the 24 replicated trials, one replicate of each trial (i.e. solvent-treated dried product) was analyzed by ANKOM technology using the ANKOM automated TDF Fiber analyzer instrument (figure 16). This instrument automates the AOAC 991.43 and AACC 32.07.01 methods and also uses the Megazyme assay test kits. The results were provided on a dry weight basis. The following formula was used to calculate TDF (image ethanol plant fractions: www.megazyme.com):

$$\text{Dietary Fiber (\%)} = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

where:

R_1 = residue weight 1 from m_1; R_2 = residue weight 2 from m_2
m_1 = sample weight 1; m_2 = sample weight 2
A = ash weight from R_1; p = protein weight from R_2 and

$$B = \text{blank} = \frac{BR_1 + BR_2}{2} - BP - BA$$

where:

BR = blank residue; BP = blank protein from BR_1
BA = blank ash from BR_2.

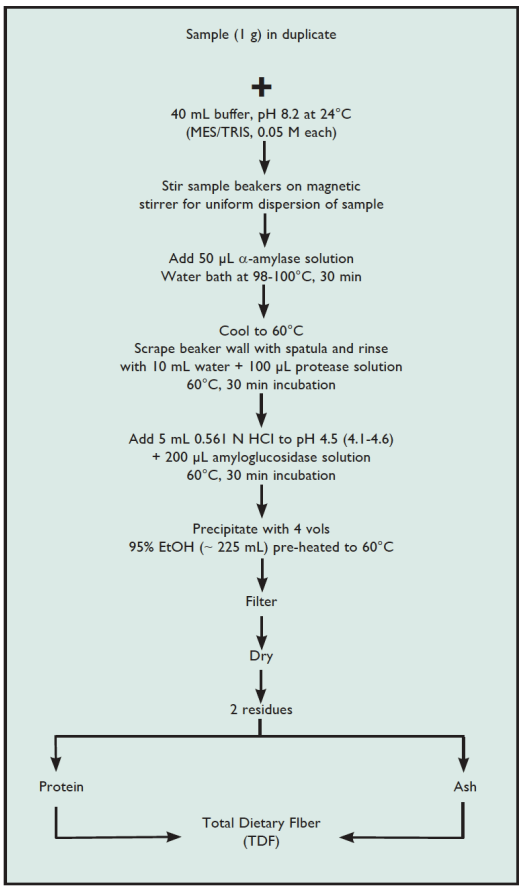


Figure 15: Analytical scheme showing enzyme incubation and ethanol precipitation for determination of total dietary fiber content (Ethanol plant fractions: www.megazyme.com)

Figure 16: ANKOM TDF fiber analyzer (Image ethanol plant fractions: https://www.ankom.com/product-catalog/ankom-tdf-fiber-analyzer

Carbohydrates (CHO) determination:

The carbohydrates in the samples was calculated by difference [100%-(protein%, + fat%+ ash%, + moisture%)].

Amino acid composition:

Selected samples of raw DG and DGS in the wet form and their corresponding ethanol washed solvent-treated dried products were sent to Agricultural Experimental Station Chemical laboratories (ESCL), University of Missouri-Columbia, MI for individual amino acid determination. The complete amino acid profile was determined using AOAC official method 982.30 E (a, b, c), chapter 45.3.05, 2006. The results obtained were used for calculating the amino acid scores based on the essential amino acids.

$$\text{Amino acid score (\%)} = \frac{\text{mg of amino acid in 1 gm of test protein}}{\text{mg of amino acid in 1 gm of reference protein}} * 100$$

The scoring pattern recommended by FAO/WHO ((WHO 1973) was used to determine the score based on the above calculation. The results were provided on a dry weight basis.

Physical analysis:

Color L, a, b values determination:

A Konica Minolta colorimeter was used to evaluate the color profiles of all the samples using the Hunter L, a, b scale for color (figure 17). On this scale L refers to the "brightness" of the sample and is scored from 0 being pure black to 100 being pure white. Parameters a and b are scored on positive and negative scales with negative and positive a signifying green and red respectively, and negative and positive b indicating blue and yellow. To measure the L, a, b values a chromameter (figure 18) was used. The

instrument was calibrated using a white tile before measuring the L, a, b values for the samples.

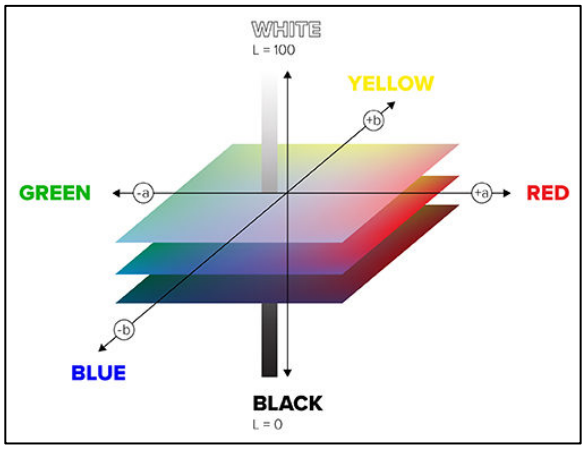


Figure 17: Hunter L,a,b Color Scale (Image ethanol plant fractions: https://cindyallen.wordpress.com/2015/02/19/learning-to-calibrate-the-gift-of-the-seer/

Figure 18: Konica Minolta CR-400 chromameter (Image ethanol plant fractions: https://www5.konicaminolta.eu/en/measuring-instruments/products/colour-measurement/chroma-meters/cr-400-410/introduction.html

Water activity measurement:

Water activity was measured using the Aqualab water activity meter (figure 19). Before running the raw DG and DGS samples in wet form, the instrument was calibrated using LiCl 8.57 molal in water reference standard with an Aw= 0.500. To run the raw DG and DGS samples in freeze-dried form and the solvent-treated dried products, the instrument was calibrated using LiCl 13.41 molal in water reference standard with an Aw= 0.250.

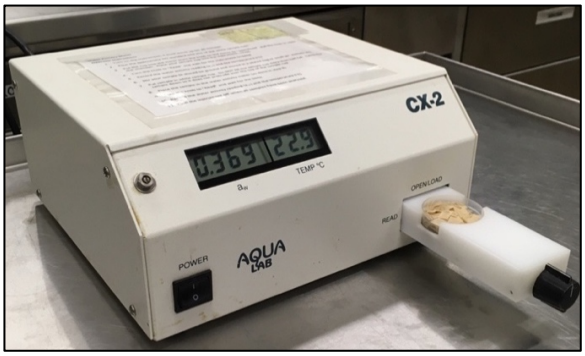


Figure 19: Aqualab water activity meter

Particle Size Distribution (PSD):

100 gm of representative sample was weighed. Sieves of the Ro-tap machine (figure 20) were stacked in the following order from top to bottom: #40, #60, #80, #100, #200 and pan. The particle size was ranged in 'microns (μ)' and the particles retained were expressed as 'percentage (%)' since the initial sample weight used was 100 gm. Weighed sample was poured on the first sieve of #40. Sieve analysis was performed for 5 minutes and fractions on each sieve were re-weighed. The color L, a, b values were also determined for each sieve fraction. The color values were correlated to the particle size of the fractions retained on each sieve. Sieve analysis was performed for raw DG and DGS ethanol plant fractions in freeze dried form and solvent-treated dried products. Sieve analysis was not performed for raw DG and DGS ethanol plant fractions that were in wet form.

Figure 20: Ro-tap sieve shaker for particle size distribution

Moisture analysis:

Approximately 1 gm of sample was weighed in a tin cup and kept in Fisher Isotemp™ oven (Fisher Scientific, Pittsburgh, PA, figure 21) forced-air convection for 3 hours at 103°C. Moisture content was determined by the loss of water as per AACCI method 44-15.02. The percent moisture content was calculated using the following formula:

% Moisture content= [100* (W2-W3)]/ W1

where:

W1= original weight of the sample

W2= Initial weight of the cup + sample

W3= final weight of the cup + sample

Figure 21: Fisherbrand™ Isotemp™ Forced Convection Oven

Phenolic compounds analysis:

Total phenolic content (TPC) determination:

The TPC was determined for all samples based on the Singleton method (Singleton, Orthofer and Lamuela-Raventos 1999). The method was modified for the sample preparation step and the volumes of the reagents used for the colorimetric reaction to occur in each sample test tube (Velioglu 1998) (Singleton, Orthofer and Lamuela-Raventos 1999). A standard calibration curve was developed using 0.1mg/mL gallic acid

as the phenolic compound standard stock solution (Makkar 2003). The concentrations used to plot the std. curve were in the range of 0.000-0.005µg/mL. The curve was plotted with concentration (µg of gallic acid) on x-axis versus absorbance on y-axis. The equation of line was determined to calculate the concentration of unknown samples. The quantity of sample used for analysis was based on previous TPC trials to determine the absorbance values that lie within the range of the std. curve. For either 2gm or 5gm of the sample a fixed volume of 50mL methanol was used to extract the phenolic compounds from the sample while shaking on the orbital shaker (figure 22) for 2 hours at 145-150 RPM at room temperature. After 2 hours, the samples were allowed to settle for about 30 mins and 1.5mL of supernatant turbid methanol layer was pipetted in a microcentrifuge tube. The tubes were then centrifuged at 10,000RPM for 5 minutes. The turbidity settled at the bottom as a pellet in the tube.

Each test tube contained a total of 4000µL (4mL) of the reagents added in the following sequence: 100-150µL of sample extract from the microcentrifuge tube + 850-900µL of deionized water + 500µL of 10% Folin-Ciocalteau phenolic reagent (Ainsworth and Kelly 2007) (Kukula-Koch and et.al 2013) (Velioglu 1998) mixture was vortexed and was left undisturbed for 5 mins. Next, 2500µL of 20% sodium carbonate solution (Makkar 2003) (Singleton, Orthofer and Lamuela-Raventos 1999) was added and the mixture was vortexed again. All the test tubes were prepared in a similar manner and were kept in the dark for 2 hours. The absorbance was then measured at 765nm for gallic acid std. using Genesys 20 UV-vis spectrophotometer (figure 23). The values were expressed as ‘mg gallic acid equivalence (GAE)/ 100gm of sample’ on a dry weight basis through moisture correction for all samples.

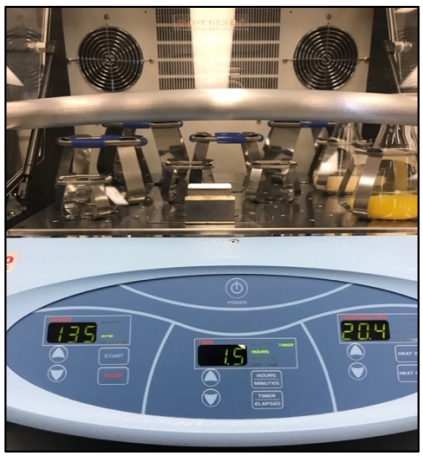


Figure 22: Fisher Scientific Orbital Shaker (Model: MaxQ4000)

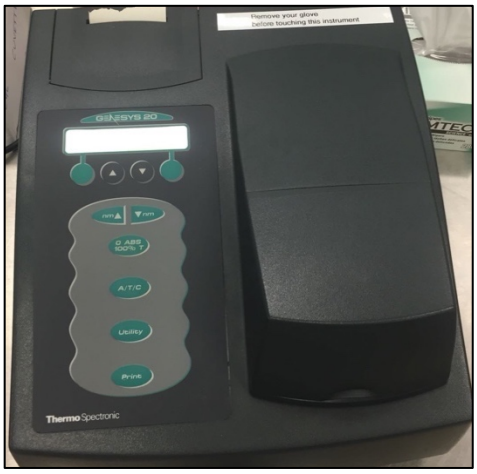


Figure 23: Genesys 20 UV-vis spectrophotometer

Toxicological Analysis:

Aflatoxins and Fumonisins determination:

The total aflatoxins and total fumonisins in all the samples were quantified using the QuickScan system by EnviroLogix (figure 24). This system measured the toxins using mycotoxin test strips specially designed for the QuickScan technology.

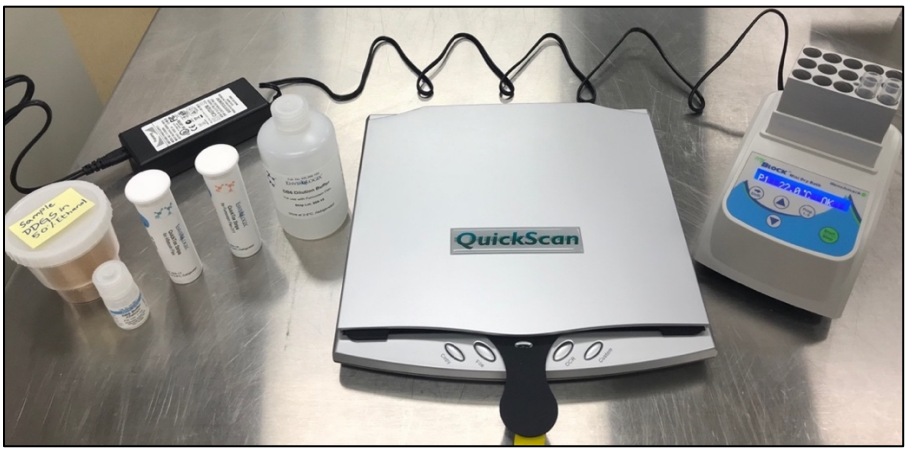


Figure 24: EnviroLogix QuickScan system setup

For analyzing aflatoxins, Aflatoxin flex AQ-309-BG QuickTox test kit was used along with AF MG2-DGS matrix with a base range of 0-30ppb. In case of fumonisins, Fumonisin flex AQ-311-BG QuickTox test kit was used along with FM MG2-DGS matrix with a base range of 1.5-7ppm. The test protocols provided by the EnviroLogix company were used. The values were expressed on 'as is' basis.

Fumonisin spiking study:

The following mycotoxin standard was purchased from Biopure, Romer Labs: Mycotoxin mix 3 (Fumonisin B1: 50.3µg/mL and Fumonisin B2: 50.1µg/mL) in 50/50 acetonitrile: water (1mL volume)

Calculation to determine spike levels in DG sample:

The sample used for spiking study was freeze-dried form of raw DG 2017 (unspiked sample). The guidance levels set by FDA for fumonisins is 2ppm. The standard solution contained 50µg/mL of Fumonisin B1 and B2 resp. Since 1ppm= 1µg/mL, the concentration can be expressed as 50ppm of Fumonisin B1 and B2 resp. In order to spike the raw freeze-dried DG at a level higher than the FDA permissible limit, the quantity

was calculated to be 20gms. Therefore, 50/20= 2.5ppm per gm of freeze-dried raw DG sample.

Procedure for spiking mycotoxin solution on freeze-dried raw DG sample:

The fume hood and all the apparatus used for the spiking experiment were sanitized prior to performing the experiment. The trash can was lined with an autoclavable bag to discard the contaminated material at the end of the experiment. The magnetic stirrer was covered with cling wrap. The separatory funnel was marked with graduations of one 115mL aliquot and five 50mL aliquots. A total volume of 365mL of absolute ethanol was used to wash 20gm of spiked freeze-dried DG. The experimental setup is seen as below in figure 25.

Figure 25: Experimental setup for mycotoxin spiking study

Half of the 115mL aliquot was poured through the separatory funnel in the beaker kept on the stirrer. A 3cc BD Leur-Lok syringe with detachable needle (1.5”, 20G) was used to withdraw 1mL of the mycotoxin solution from the vial. The liquid was dispensed in the beaker while stirring at 350RPM. To withdraw the residual liquid from the vial, ethanol was added to the vial using the syringe and then aspirated out again and poured in

the beaker. The syringe needle was capped and then discarded. After 2 mins, 20 gm of air-dried DG 2017 (passed through #20, unspiked sample) was poured in the beaker while stirring. The remainder of the aliquot was added. The stirring continued for 2 hours (0^{th} cycle). At the end of the cycle, the stirrer was stopped, and the DG was allowed to settle at the bottom. The supernatant ethanol layer was aspirated under vacuum. At this point, approx. 2 spoonsful of freeze-dried DG sample was collected in plastic container lined with aluminum foil and labeled as 'spiked sample'. The next aliquot of 50mL was added to the beaker through the separatory funnel. The DG solution was stirred for 1 hour (1^{st} cycle). At the end of first cycle, supernatant ethanol layer was aspirated, and the next aliquot was added. This step was repeated for four times, subjecting sample to a total of five washing cycles. At the end of 5^{th} cycle, all the DG sample from the beaker was collected in the second plastic container labeled as 'washed sample'. Both the containers were covered with perforated aluminum foil and left overnight under the fume hood for the ethanol to evaporate which resulted in a free-flowing solvent-treated dried product. The aspirated ethanol in the side arm flask was poured in a waste bottle and was allowed to evaporate. The samples were weighed and transferred to 50mL centrifuge tubes. The samples were analyzed by Romer Labs for 5 toxins namely: Aflatoxins (AF), Fumonisins (FM), Ochratoxin A, Deoxynivalenol (DON) and Zearalenone (ZON).

Statistical Analysis:

Statistical analyses on analytical data was performed using RStudio v. 1.1.463 (copyright 2009-2018 RStudio, Inc.) and Microsoft Excel v. office 365 tools (Microsoft Corp., Redmond, WA) software. Multiple linear regression models were generated for comparison of analytical data between the 2017 and 2018 raw DG & DGS ethanol plant

fractions - wet and freeze-dried forms and their corresponding solvent-treated dried products using P value of 0.05 to test significance of the results. A tabulated summary of the statistical data was provided for all the treatments.

The following variables were used as independent variables/ factors:

1. Year: 2017 versus 2018
2. Ethanol plant fractions: DG versus DGS
3. Initial moisture type: wet form versus freeze-dried form
4. Solvents: Ethanol, Ethyl acetate + Ethanol and Hexane + Ethanol
5. Rep: Replication for within the treatments

All the analytical values were considered to be dependent variables. Correlation plots were determined for all the dependent variables. Correlation analyses was also performed for the particle size distribution versus hunter L, a, b values for the fractions retained on each sieve. Bar plots were plotted using the R software to show the relation between the factors and the dependent variables.

CHAPTER 4. RESULTS AND DISCUSSION

In the first part of the study, raw starting materials from the ethanol plant, namely distiller's grains without solubles (DG) and distiller's grains with solubles (DGS), were subjected to drying treatments to bring down their initial moisture content (50%-60%) to a final level of less than 5%. Effects of drying were studied to determine their impact on compositional and functional traits.

The starting materials (DG & DGS) were also compared to determine if there were inherent differences between them in terms of composition that would have a bearing on the products down-stream.

Wet and dried DG and DGS were then subjected to solvent treatments namely - Ethanol, Hexane + Ethanol and Ethyl acetate + Ethanol. These treatments were primarily designed to remove lipids that also have a bearing on the product quality down-stream. Oils in the germ that are good solvents for grain pigments, may also inadvertently comingle with the endosperm constituents and these pigments may be removed in the defatting steps. While some pigment removal is useful for discoloration of the end product, excess color removal may be detrimental to retention of phytonutrients.

The ethanol plant fractions DG (distiller's grains without solubles) and DGS (distiller's grains with solubles) in wet and freeze-dried forms from 2017 and 2018 and their corresponding solvent-treated dried products were analyzed to determine proximate composition, color (L a b) values, fungal toxin (aflatoxins and fumonisins) content, water activity (Aw) and phenolic compounds (TPC). The results were compared between the raw ethanol plant fractions and their finished products to determine the changes resulting from solvent treatment. The results for solvent-treated dried products were also analyzed

within the individual treatments to determine reproducibility and consistency in terms of quality. The five factors (independent variables) used as a basis for analyzing the results were categorized as follows:

1. Year: 2017 versus 2018
2. Ethanol plant fractions: DG versus DGS
3. Initial moisture type: wet form versus freeze-dried form
4. Solvent treatment: Ethanol, Hexane + Ethanol and Ethyl acetate + Ethanol
5. Rep: Replication for within the treatments

Tables 18 provides a summary on the Analyses of variance (ANOVA) for the physical & chemical properties of the raw materials based on year, ethanol plant fractions and initial moisture type as factors. For raw materials, solvent treatment and replication were not considered as factors since these were the starting materials on which the treatments were going to be performed in replicates. As per the table, % moisture content, water activity and fumonisin levels of raw materials were significantly affected by initial moisture type (wet and freeze-dried forms) as a factor. None of the independent factors affected the % crude protein content of the raw materials. Percent crude fat content were significantly affected by year and initial moisture type (wet and freeze-dried forms) as independent factors. Percent TDF content and color L values were significantly affected by ethanol plant fractions (DG and DGS) as an independent factor. Percent ash content and color a value (redness) was significantly affected by year and ethanol plant fractions (DG and DGS) as factors. Color b values (yellowness) and total phenolic content (TPC) were significantly affected by ethanol plant fractions (DG and DGS) and initial moisture type (wet and freeze-dried forms) as independent factors.

Table 19 provides a summary on the Analyses of variance (ANOVA) for the physical & chemical properties of solvent-treated dried products based on solvent treatment, year, ethanol fractions, initial moisture type and replication as independent factors. As seen in table 19, % moisture content and water activity were significantly affected by year as an independent factor. Percent crude fat content, %TDF, color L values and color a values were significantly affected by year, ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried forms) as independent factors. Percent crude fat was significantly affected by year, ethanol plant fractions (DG & DGS) and solvent treatment as independent factors. Percent ash content, total phenolic content (TPC) and % yield was significantly affected by ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried forms) as independent factors. Color b values and fumonisin content were significantly affected by year and initial moisture type (wet and freeze-dried forms) as independent factors.

Table 18. Analyses of variance for physical & chemical properties of raw materials – wet and freeze-dried forms from 2017 and 2018

Constituents	Df	Mean square	F value	Significance level
% Moisture content				
Year: 2017 & 2018	1	30	0.721	N.S.
Ethanol plant fractions: DG & DGS	1	3	0.068	N.S.
Initial moisture type: wet & freeze-dried forms	1	9415	225.194	***
% Crude protein content				
Year: 2017 & 2018	1	16.646	1.407	N.S.
Ethanol plant fractions: DG & DGS	1	2.496	0.211	N.S.
Initial moisture type: wet & freeze-dried forms	1	0.137	0.012	N.S.
% Crude fat content				
Year: 2017 & 2018	1	4.040	9.036	*
Ethanol plant fractions: DG & DGS	1	1.664	3.722	N.S.
Initial moisture type: wet & freeze-dried forms	1	17.057	38.151	***
% Total dietary fiber content				
Year: 2017 & 2018	1	10.51	1.097	N.S.
Ethanol plant fractions: DG & DGS	1	261.18	27.263	**
Initial moisture type: wet & freeze-dried forms	1	1.54	0.161	N.S.
% Ash content				
Year: 2017 & 2018	1	0.50	6.289	*
Ethanol plant fractions: DG & DGS	1	35.94	454.780	***
Initial moisture type: wet & freeze-dried forms	1	0.03	0.388	N.S.
Color L value				
Year: 2017 & 2018	1	5.0	2.251	N.S.
Ethanol plant fractions: DG & DGS	1	420.3	188.574	***
Initial moisture type: wet & freeze-dried forms	1	6.6	2.964	N.S.
Color a value				
Year: 2017 & 2018	1	7.535	9.189	*
Ethanol plant fractions: DG & DGS	1	22.231	27.111	***
Initial moisture type: wet & freeze-dried forms	1	1.311	1.599	N.S.
Color b value				
Year: 2017 & 2018	1	0.02	0.035	N.S.
Ethanol plant fractions: DG & DGS	1	55.50	100.026	***
Initial moisture type: wet & freeze-dried forms	1	13.91	25.074	***
Water activity				
Year: 2017 & 2018	1	0.0057	0.609	N.S.
Ethanol plant fractions: DG & DGS	1	0.0298	3.180	N.S.
Initial moisture type: wet & freeze-dried form	1	2.2335	238.729	***
Total phenolic content				
Year: 2017 & 2018	1	143	0.395	N.S.
Ethanol plant fractions: DG & DGS	1	60197	166.363	***
Initial moisture type: wet & freeze-dried forms	1	44627	123.333	***
Fumonisin content				
Year: 2017 & 2018	1	0.01562	6.734	*
Ethanol plant fractions: DG & DGS	1	0.00490	2.112	N.S.
Initial moisture type: wet & freeze-dried forms	1	0.30803	132.743	***

Significant. codes: '***' 0.001 '**' 0.01 '*' 0.05, N.S.: Not significant, DG: Distiller's grains without solubles, DGS: Distiller's grains with solubles, Df: Degrees of freedom

Table 19. Analyses of variance for physical & chemical properties of solvent-treated dried products

Constituents	Factors	Df	Mean square	F value	Significance level
	Year: 2017 & 2018	1	84.43	50.141	***
	Ethanol plant fractions: DG & DGS	1	0.93	0.552	N.S.
% Moisture	Initial moisture type: Wet & Freeze-dried forms	1	1.95	1.159	N.S.
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.84	0.499	N.S.
	Rep: 1&2	1	0.36	0.212	N.S.
	Year: 2017 & 2018	1	21.911	33.999	***
% Crude	Ethanol plant fractions: DG & DGS	1	22.619	35.098	***
protein	Initial moisture type: Wet & Freeze-dried forms	1	18.813	29.192	***
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.086	0.133	N.S.
	Rep: 1&2	1	1.684	2.613	N.S.
	Year: 2017 & 2018	1	1.5301	20.466	***
	Ethanol plant fractions: DG & DGS	1	2.1294	28.483	***
% Crude fat	Initial moisture type: Wet & Freeze-dried forms	1	0.1789	2.392	N.S.
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	2.6888	35.964	***
	Rep: 1&2	1	0.0000	0.000	N.S.
	Year: 2017 & 2018	1	50.8	9.690	**
% Total	Ethanol plant fractions: DG & DGS	1	412.5	78.759	***
dietary fiber	Initial moisture type: Wet & Freeze-dried forms	1	187.6	35.818	***
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.3	0.058	N.S.
	Rep: 1&2	N.A.	N.A.	N.A.	N.A.
	Year: 2017 & 2018	1	0.08	0.723	N.S.
	Ethanol plant fractions: DG & DGS	1	104.70	975.949	***
% Ash	Initial moisture type: Wet & Freeze-dried forms	1	1.32	12.336	**
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.07	0.683	N.S.
	Rep: 1&2	1	0.04	0.375	N.S.
	Year: 2017 & 2018	1	69.17	8.287	**
Color L	Ethanol plant fractions: DG & DGS	1	72.28	8.659	**
value	Initial moisture type: Wet & Freeze-dried forms	1	73.16	8.765	**
	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	9.39	1.125	N.S.
	Rep: 1&2	1	13.29	1.593	N.S.
	Year: 2017 & 2018	1	3.927	11.798	**
	Ethanol plant fractions: DG & DGS	1	22.154	66.554	***
Color a	Initial moisture type: Wet & Freeze-dried forms	1	14.268	42.863	***
value	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.038	0.114	N.S.
	Rep: 1&2	1	0.089	0.268	N.S.
	Year: 2017 & 2018	1	6.1	7.328	**
	Ethanol plant fractions: DG & DGS	1	0.0	0.011	N.S.
Color b	Initial moisture type: Wet & Freeze-dried forms	1	391.1	473.178	***
value	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.8	0.972	N.S.
	Rep: 1&2	1	0.6	0.749	N.S.
	Year: 2017 & 2018	1	0.3223	56.250	***
	Ethanol plant fractions: DG & DGS	1	0.0001	0.010	N.S.
Water	Initial moisture type: Wet & Freeze-dried forms	1	0.0210	3.673	N.S.
activity	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.0016	0.278	N.S.
	Rep: 1&2	1	0.0000	0.003	N.S.
	Year: 2017 & 2018	1	274	0.289	N.S.
Total	Ethanol plant fractions: DG & DGS	1	49923	52.649	***
phenolic	Initial moisture type: Wet & Freeze-dried forms	1	383383	404.319	***
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	2678	2.824	N.S.
	Rep: 1&2	1	11	0.011	N.S.
	Year: 2017 & 2018	1	0.06235	15.297	***
	Ethanol plant fractions: DG & DGS	1	0.01577	3.869	N.S.
Fumonisin	Initial moisture type: Wet & Freeze-dried forms	1	0.04260	10.452	**
content	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.00033	0.082	N.S.
	Rep: 1&2	1	0.00013	0.033	N.S.
	Year: 2017 & 2018	1	30.9	3.159	N.S.
	Ethanol plant fractions: DG & DGS	1	45.0	4.599	*
% Yield	Initial moisture type: Wet & Freeze-dried forms	1	896.1	91.516	***
	Solvent: Ethanol, Hex + Ethanol, EA + Ethanol	2	0.1	0.013	N.S.
	Rep: 1&2	1	9.9	1.016	N.S.

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05; Df: Degrees of freedom; N.A.: Not Applicable; N.S.: Not significant, DG: Distiller's grains without solubles, DGS: Distiller's grains with solubles, Hex: Hexane solvent, EA: Ethyl acetate solvent

The Least significant difference (LSD) was determined for all the constituents using the 'LSD t test' in RStudio. This test ranked all the constituents for ease of comparison based on the independent factors. Tables 20 compares the physical & chemical properties of the raw materials based on year, ethanol plant fractions (DG and DGS) and initial moisture type (wet and freeze-dried) as factors. Table 21 compares the physical & chemical properties of the finished products based on solvent treatment, year, ethanol plant fractions (DG and DGS), initial moisture type (wet and freeze-dried) and replication as factors. As seen in table 19, since the samples of only one replication were sent out for total dietary fiber analysis, this chemical constituent was determined based on only four factors namely: solvent treatment, initial moisture type, ethanol plant fractions and year. Furthermore, all the results for the physical and chemical properties for finished products were not significantly different from each other with respect to replication. This indicated that the solvent treatments yielded consistent results in reproducible removal of pigments from the substrates (DG and DGS).

Table 20. Comparison of physical & chemical properties of raw materials based on ethanol plant fractions, initial moisture type and year as factors

Factors	% Moisture	% CP	% CF	% TDF	% Ash	L	a	b	Aw	TPC	FM
	Mean values										
Ethanol plant fractions											
DG	26.54a	35.51a	10.11a	50.47a	1.82a	67.86a	4.14a	31.06a	0.525a	210.85a	0.156a
DGS	27.38a	34.72a	9.47a	39.05b	4.82b	57.61b	6.50b	27.33b	0.611a	333.53b	0.121a
Initial moisture type											
Wet form	51.22a	35.21a	10.82a	45.20a	4.46a	63.38a	5.03a	30.13a	0.942a	325.00a	0.278a
FrD form	2.70b	35.02a	8.75b	44.32a	3.28a	62.10a	5.61a	28.26b	0.194b	219.38b	0.000b
Year											
2017	28.33a	34.10a	9.29a	43.62a	3.50a	63.30a	4.63a	29.16a	0.587a	275.18a	0.108a
2018	25.59a	36.14a	10.29b	45.91a	3.14b	62.18a	6.00b	29.23a	0.549a	269.20b	0.170b
LSD	7.116	3.785	0.736	6.076	0.309	1.643	0.997	0.820	0.106	20.933	0.053
MSE	41.808	11.830	0.447	9.580	0.079	2.229	0.820	0.555	0.009	361.839	0.002

Legend:
%CF: Percent Crude Fat, %CP: Percent Crude Protein, Aw: Water activity
DG: Distiller's grains without solubles, DGS: Distiller's grains with solubles
FM: Total fumonisins measured in parts per million
FrD: freeze-dried form
LSD: least significant difference value, MSE: Mean square error value
TPC: Total Phenolic Content measured in mg Gallic acid equivalence/100 gm of sample
TDF: Total Dietary Fiber
Means with the same letter within columns are not significantly different from each other ($P \leq 0.05$)

Table 21. Comparison of physical & chemical properties of solvent-treated dried products based on solvent treatment, ethanol plant fractions, initial moisture type, year and replication as factors

Solvent treatment	% Moisture	% CP	% CF	% TDF	% Ash	L	a	b	Aw	TPC	FM	% Yield
						Mean values						
Ethanol	8.81a	37.98a	1.99a	40.09a	3.42a	79.80a	1.60a	24.03a	0.482a	142.53a	0.034a	89.66a
Hex + Ethanol	8.48a	37.85a	1.20b	40.43a	3.39a	80.39a	1.55a	23.87a	0.468a	167.31b	0.043a	89.83a
EA+ Ethanol	8.36a	37.85a	1.39b	40.43a	3.29a	81.32a	1.50a	23.58a	0.462a	148.47ab	0.040a	89.77a
LSD	0.927	0.573	0.195	2.404	0.234	2.063	0.412	0.649	0.054	21.987	0.046	2.234
Ethanol plant fractions												
DG	8.69a	38.58a	1.32a	44.46a	1.89a	81.73a	0.87a	23.84a	0.470a	120.52a	0.057a	90.73a
DGS	8.41a	37.21b	1.74b	36.17b	4.85b	79.28b	2.23b	23.81a	0.472a	185.02b	0.021a	88.79b
Initial moisture type												
Wet form	8.75a	38.52a	1.47a	43.11a	3.20a	81.74a	1.01a	20.97a	0.492a	63.40a	0.009a	85.44a
FrD form	8.35a	37.27b	1.59a	37.52b	3.54b	79.27b	2.10b	26.68b	0.450a	242.16b	0.068b	94.08b
Year												
2017	9.88a	37.22a	1.71a	38.86a	3.41a	79.30a	1.26a	23.47a	0.553a	155.15a	0.075a	88.95a
2018	7.22b	38.57b	1.35b	41.77b	3.33a	81.70b	1.84b	24.18b	0.389b	150.38a	0.003b	90.56a
Replication of treatments												
1	8.46a	37.08a	1.53a	N.A.	3.40a	79.98a	1.59a	23.71a	0.470a	152.29a	0.040a	90.21a
2	8.64a	37.71a	1.53a	N.A.	3.34a	81.03a	1.51a	23.94a	0.471a	153.24a	0.037a	89.30a
LSD	0.757	0.468	0.159	1.963	0.191	1.684	0.336	0.530	0.044	17.952	0.037	1.824
MSE	1.684	0.644	0.075	5.238	0.107	8.347	0.333	0.826	0.006	948.218	0.004	9.791

Legend:
%CF: Percent Crude Fat, %CP: Percent Crude Protein, Aw: Water activity
DG: Distiller's grains without solubles, DGS: Distiller's grains with solubles
Hex: Hexane solvent, EA: Ethyl acetate solvent
FM: Total fumonisins measured in parts per million
FrD: freeze-dried form, N.A.: Not Applicable
LSD: least significant difference value, MSE: Mean square error value
TPC: Total Phenolic Content measured in mg Gallic acid equivalence/100 gm of sample, TDF: Total Dietary Fiber
Means with the same letter within columns are not significantly different from each other ($P \leq 0.05$)

The rationale for using three initial moisture types of solvents - ethanol, hexane + ethanol and ethyl acetate + ethanol was to measure relative effects on the overall quality, physical and chemical properties of solvent-treated dried products. The extraction of chemical constituents depended on the polarity index of the three individual solvents- ethanol, hexane and ethyl acetate, Ethanol is a highly polar solvent, whereas ethyl acetate is moderately polar, and hexane is a highly non-polar solvent. These solvents selectively extracted lipid-soluble portions and water-soluble portions from the raw DG and DGS ethanol plant fractions.

The results for individual constituents are discussed below:

Proximate composition:

Moisture content:

As previously mentioned in table 14, the wet DG material from 2017 and 2018 had moisture content in the range of 55-65%, whereas the wet DGS (distiller's grains with solubles) had a moisture content in the range of 45-55%. The vacuum freeze drying significantly reduced the moisture content of raw materials to less than 5% moisture. Moisture reduction was noted in Table 18, wherein the initial moisture type - wet and freeze-dried forms as an independent factor had significant effects on the moisture content of raw material. Year as an independent factor had a significant effect on the moisture content of solvent-treated dried products as seen in table 19. This could be attributed to the difference in moisture content of the stating material procured from 2017 and 2018. Table 20 provided mean values for moisture content of raw materials based on ethanol plant fractions, initial moisture type and solvent as independent factors. The overall moisture content for solvent-treated dried products was found to be in the range of

7-10% as seen in table 21. However, the mean values were not significantly different from each other based on solvent treatment (Ethanol, Hexane + Ethanol and Ethyl acetate + Ethanol), ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried) as independent factors. Another observation to be noted is that even after processing the raw material with solvents, 7-10% moisture still remained in the finished product. This could be attributed to the bound water that is held tightly by the other nutrient constituents. The air- drying process in the last step of raw material processing may also have contributed to the removal of moisture and simultaneous evaporation of the volatile solvent.

Crude protein content:

Table 18 shows that none of the independent variables significantly affected % crude protein for raw materials. However, in terms of finished products (table 19), independent variables such as year, ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried forms) significantly affected the % crude protein content. Tables 20 and 21 show that the overall crude protein content for raw materials ranged from 35%-37% whereas a range of 37% to 38% crude protein content for solvent-treated dried products. As seen in table 21, the variation in % crude protein for solvent-treated dried products based on initial moisture type (wet and freeze-dried forms) as a factor could be attributed to the removal of moisture during raw material processing. This could have happened since the freeze-dried forms had low moisture in the range of 0.5-5% whereas the finished products had a slight increase in moisture content up to 11%.

Crude fat content:

Table 18 shows that the year (2017 & 2018) and initial moisture type (wet and freeze-dried forms) as independent factors significantly affected the % crude fat for raw materials. Table 19 shows that the % crude fat was significantly affected by year, ethanol plant fractions and solvent as factors. The raw DG and DGS ethanol plant fractions in wet and freeze-dried forms had fat content in the range of 8-11% (table 20) whereas after processing with defatting solvents, the fat content was reduced to 0.5-2.5% for all their corresponding solvent-treated dried products (table 21). This could be attributed to the initial steeping step with hexane and ethyl acetate which proved to be more effective than ethanol alone at extracting the fat-soluble portions. Additional washing with ethanol helped to further reduce the fat content. The overall extracting power for the defatting solvents could be ranked as in following order:

Hexane > Ethyl acetate > Ethanol

The initial steeping time with solvents such as hexane, ethyl acetate and ethanol were two hours. This time period proved to be sufficient to optimally extract oil-soluble pigments from the raw material. The crude fat present in DGS is mainly composed of unsaturated free fatty acids (FFA), of which linoleic (C18:2) and oleic (C18:1) make up approximately 50% and 25%, respectively of the total fatty acids content. The remaining FFA include stearic acid (C18:0), linolenic (C18:3) and palmitic acid (C16:0) (Fernando and Garcia 2012). The fat composition varies depending upon the addition or removal of solubles in the distillers dried grains. Moreau, Liu and Winkler (2011) indicated that the lipid portion of DGS also contains phytochemical constituents ‘phytosterols’ namely sitosterol, campesterol, sitostanol and campestanol that make up to ~2%. Other

phytoconstituents found to be present in DGS are ‘tocopherols’ and ‘tocotrienols’ namely γ-tocopherol and γ-tocotrienol which are the major isomers. The low-fat content of solvent-treated dried products indicates that there is a possible loss of these valuable phytonutrients during removal of oil.

Total dietary fiber (TDF) analysis:

Table 18 shows that ethanol plant fractions (DG and DGS) as an independent variable thus significantly affected the % TDF content for raw materials. Table 19 shows that the % TDF content was significantly affected by year (2017 & 2018), ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried forms). Overall, the raw materials had a % TDF content in the range of 39-50% (table 20) whereas for solvent-treated dried products % TDF ranged from 36-44% (table 21). It was observed that DG had a higher % TDF content when compared to DGS (%TDF). Hence the resulting solvent-treated dried products from DG as a starting material yielded higher values when compared to finished products from DGS.

TDF is made up of soluble and insoluble dietary fiber. Of the two fractions, the insoluble portion is the predominant type of fiber found in DGS (Martinez-Amezcua 2007). This dietary fiber includes non-starch polysaccharides resistant to digestion in small intestine and fermentable in the large intestine (AACC 2001). It is beneficial to have high dietary content as it promotes physiological effects such as laxation, blood cholesterol attenuation, blood sugar attenuation and aids in achieving a healthy weight through low calorie intake. The TDF components found in distiller’s grains are cellulose, hemicellulose, arabinose, xylose, xylan and lignin (Shurson and Urriola 2019).

Ash content:

As per table 18, the % ash content was significantly affected by year and ethanol plant fractions (DG & DGS) as independent factors for raw materials. However, as per table 19, % ash content was significantly affected by ethanol plant fractions and initial moisture type (wet and freeze-dried forms) as factors for solvent-treated dried products. As per table 20, raw DG had lower range of ash content (1-2%) while DGS had a higher range of ash content (4-5%). Ash content in solvent-treated dried products (table 21), had a lower range of 1-2% for finished products obtained using DG whereas for those products obtained from DGS had higher range of 4-5% ash content. This could be attributed to the presence of solubles in the DGS raw material. Solubles include reagents and processing aids used in ethanol plants that eventually find their way into the eluents. The ash content measures the concentration of total minerals present within the sample. As per K. Liu (2011) and Spiehs and Whitney (2002), the major minerals present in raw DGS are calcium (Ca), phosphorus (P), potassium (K), magnesium (Mg), sulphur (S), and sodium (Na). The minor elements found to be are zinc (Zn), magnese (Mn), copper (Cu), iron (Fe), aluminum (Al), and selenium (Se). As per the literature, concentration of major minerals is in the range of 0.05% - 1.15% whereas for minor minerals, it is 6 ppm - 149 ppm for raw DGS. It would be reasonable to assume that the high concentration of major minerals in DGS resulted in % ash values greater than DG % ash values.

Physical analysis:

Color L, a, b values:

The color scores were measured using Hunter L a b scale (figure 16). On this scale, L is scored from 0 (pure black) to 100 (pure white). Parameters a and b are scored on positive

and negative scales. For color a score, negative values indicate greenness and positive values indicate redness. For color b score, negative values indicate blueness and positive values indicate yellowness. For all raw materials, table 18 shows that ethanol plant fractions (DG & DGS) as an independent factor, significantly affected color L values; year & ethanol plant fractions as factors significantly affected a values; and ethanol plant fractions & initial moisture type (wet and freeze-dried forms) as factors significantly affected b values. However, for all solvent-treated dried products, table 19 shows that year, ethanol plant fractions & initial moisture type as factors significantly affected L values; year & ethanol plant fractions as factors significantly affected L and a values; and year & initial moisture type as factors significantly affected b values. Table 20 shows that for raw DG and DGS materials in wet and freeze-dried forms, the L values ranged from 57.61-67.86, color a value (redness) ranged from 4.16-6.50, and color b value (yellowness) ranged from 28.26-31.06. These wide range of values indicate that the freeze-drying of the raw materials in itself may have resulted in the color a value to increase, b value to decrease and did not significantly affect the L value.

For solvent-treated dried products (table 21), when wet form of DG and DGS were extracted directly with solvents (without first using freeze-drying step), L value ranged from 79.27-85.21, a value ranged from negative 0.36 (redness) to positive 2.10 (greenness) and b value ranged from 18.62-22.82 (yellowness). However, when freeze-dried DG and DGS raw materials were solvent-treated, the resulting finished products had lower L value for brightness with a range from 74.94-84.31, a value ranged from negative 0.48 (redness) to positive 4.04 (greenness) and b value with high yellowness in a range of 25.59-28.29. The differences in the color L, a, & b scores between raw materials

and their corresponding solvent-treated dried products could be attributed to the multiple washing and steeping cycles with ethanol during processing. This information was in agreement with Saunders et. al (2013) wherein increase number of extractions with ethanol led to increase in L value, and decrease in a and b value of the solvent-treated dried product. Another reason for high L value is that the finished product was milled using 0.5mm sieve. The milling effect caused an increase in the surface area of the particles thus improving the brightness of the product.

Water activity (Aw):

Table 18 shows that initial moisture type (wet and freeze-dried forms) as an independent variable, significantly affected Aw of all raw materials. In case of all solvent-treated dried products, year (2017 & 2018) as an independent factor, significantly affected water activity as noted in table 19. As seen in table 20, the Aw ranged from 0.525-0.942 for raw DG and DGS materials in wet and freeze-dried forms. The overall water activity ranged from 0.389-0.553 for finished products obtained from wet and freeze-dried forms of DG and DGS starting materials (table 21). Reduction in Aw for finished products could be directly attributed to the multiple steeping and washing cycles and also to the final air-drying method for solvent evaporation from finished products. Thus, solvent treatment proved to be beneficial in Aw reduction which is desirable in food ingredients. Low Aw values indicated that the product has a potential for reduced microbial growth and has a prolonged shelf-life.

Particle size distribution:

For performing particle size distribution analysis, the wet form of DG and DGS was not used due to high moisture content. Instead, the freeze-dried form of DG and DGS ethanol

plant fractions were used. The sieve fractions with their corresponding particle size ranges were: #40 (>400μm), #60 (400-250μm), #80 (250-180μm), #100 (180-150μm), #200 (150-75μm) and bottom pan (≤75μm). The bar plots in figure 26, 27, 28 and 29 used the label 'without solvent' which indicates the starting material namely: freeze-dried DG and DGS that were not treated with solvents. The purple bars for 'without solvent' take into consideration the mean values of raw freeze-dried DG and DGS that were retained on each sieve fraction (figure 26) and the mean values of color L, a, & b scores in figures 27, 28 and 29, respectively.

Figure 26 shows that for all solvent-treated dried products, maximum retention of the particles was observed over the #100 sieve for 150-180μm particle size range. For freeze-dried raw DG and DGS ethanol plant fractions, maximum retention of the particles was observed over the #40 sieve for particle size larger than 400μm. This could be attributed to the fact that the freeze-dried raw ethanol plant fractions were not milled through 0.5mm sieve before being processed into a solvent-treated dried product.

There was an inverse correlation seen between particles retained on each sieve fraction and their corresponding color L a b values. Figure 27 shows that as the particle size decreased, the brightness (L value) increased gradually for the solvent-treated products when compared to their freeze-dried raw ethanol plant fractions (without solvent). This could be attributed to the increase in the surface area of the particles for greater accessibility by solvents.

Figure 28 shows that as the particle size increased, color a value (or redness value) decreased gradually for freeze-dried raw DG and DGS ethanol plant fractions (without solvent) and all the solvent-treated products. This means that the redness in the particles

decreased leading to increase in the greenness as the color a value approached zero. For particles retained on the bottom pan (fine particles) negative color a values were observed.

Figure 29 shows that an inverted bell-shaped curve was observed for raw materials (without solvent) indicating that the b values were higher for the highest (>400µm) and lowest (<75µm) particle size ranges. Moderate b values were observed for particles ranging from 150-250µm. For solvent-treated products, a normal bell-shaped curve was observed indicating that the b values were lower for the highest (>400µm) and lowest (<75µm) particle size ranges and moderate b values were observed for particles ranging from 150-250µm. This indicates that there was a decrease in the yellowness of all the solvent-treated dried products.

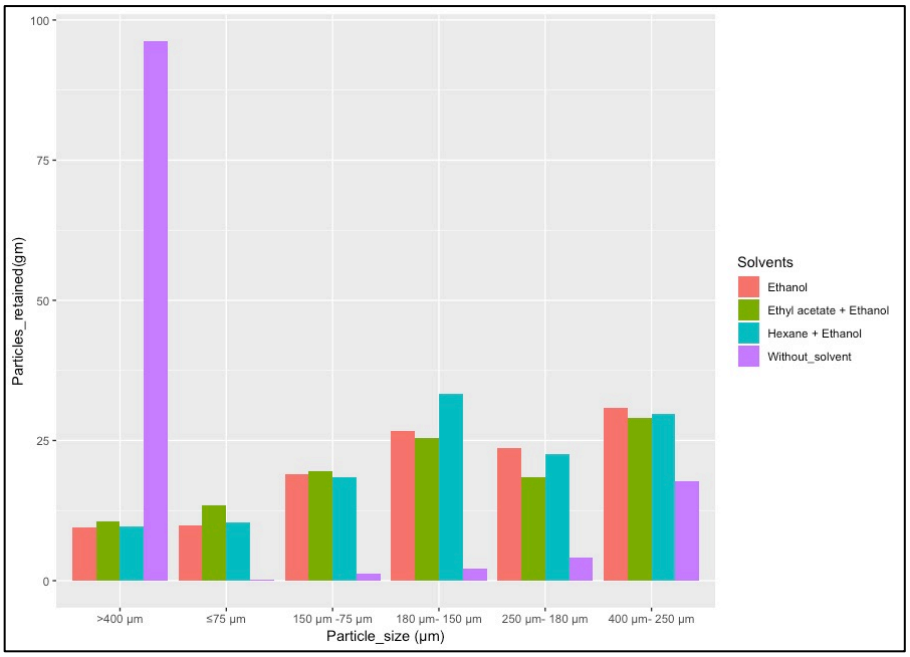


Figure 26: Bar plot comparing the particles retained on particle size sieve fractions and with the solvent used

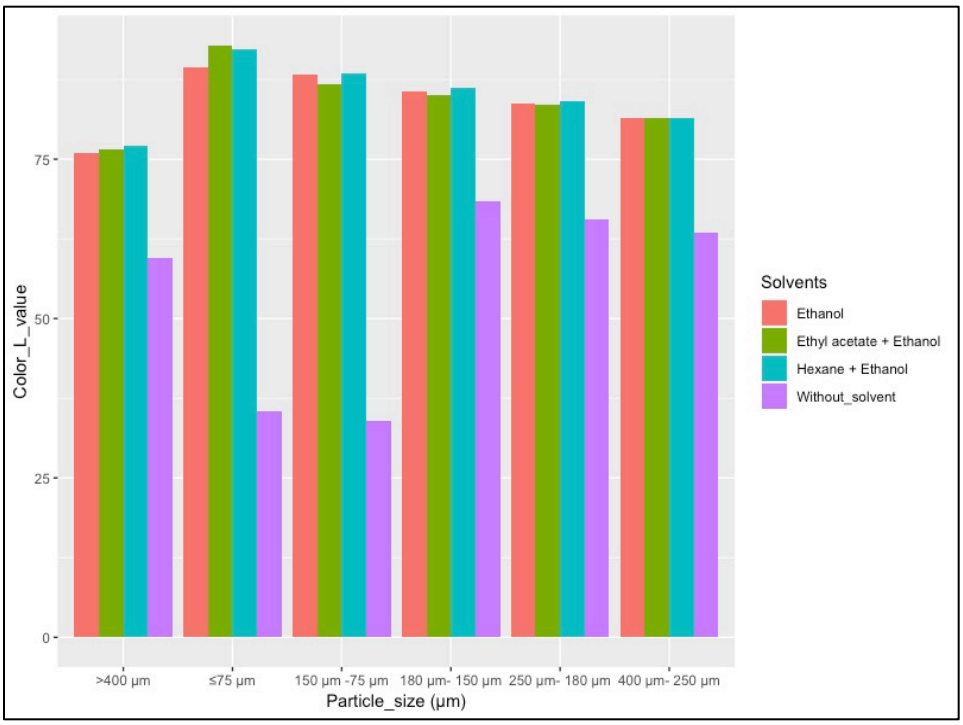


Figure 27: Bar plot comparing color L values with particle size and solvent treatment as factors

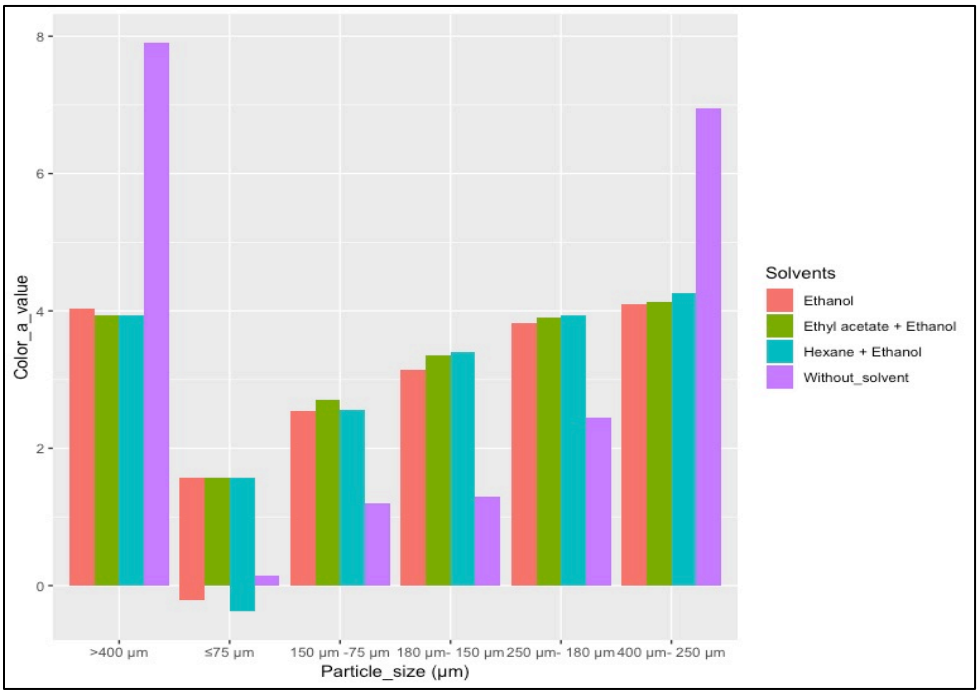


Figure 28: Bar plot comparing color a values with particle size and solvent treatment as factors

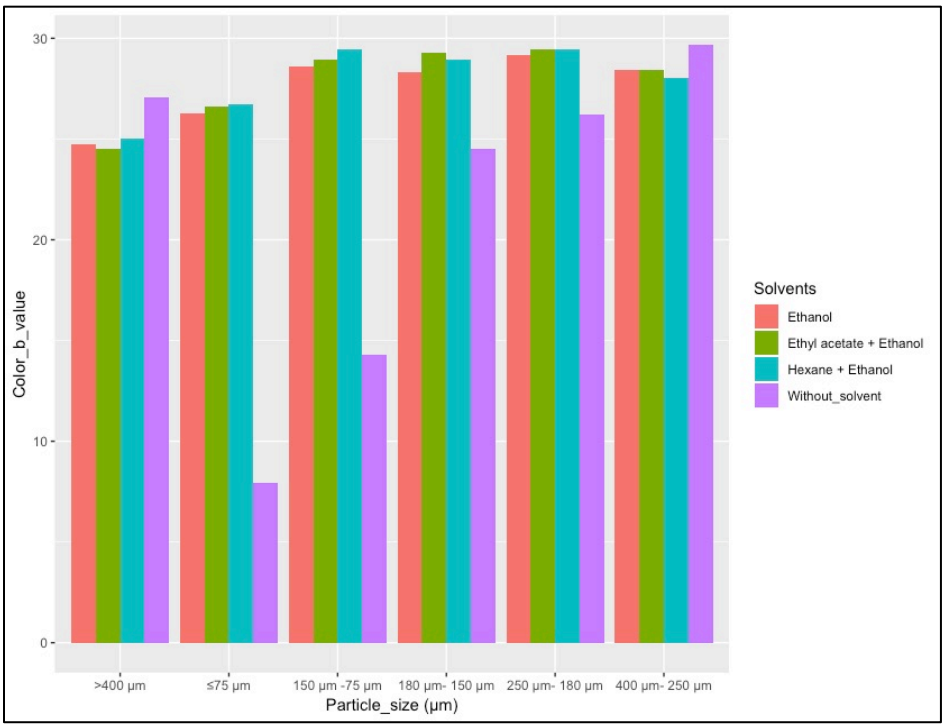


Figure 29: Bar plot comparing color b vlaues with particle size and solvent treatment as factors

Phenolic compounds analysis:

Total phenolic content (TPC) analysis:

Independent variables such as ethanol plant fractions (DG & DGS) and initial moisture type (wet and freeze-dried forms) significantly influenced the TPC content of all the raw materials and their corresponding solvent-treated dried products as seen in table 17 and 18, respectively.

Figure 30 provides the mean values of TPC content for wet form of DG and DGS raw materials and their corresponding solvent-treated dried products. As seen in the figure, the wet form of raw DG and DGS from 2017 and 2018 had a TPC range of 240 ~ 415 mg GAE/ 100gm sample. There was a large variation seen among the TPC values for all the solvent-treated dried products. For solvent-treated dried products obtained using wet

form of raw DG and DGS from 2017 and 2018, the TPC values ranged from 40 ~ 190 mg GAE/ 100gm sample.

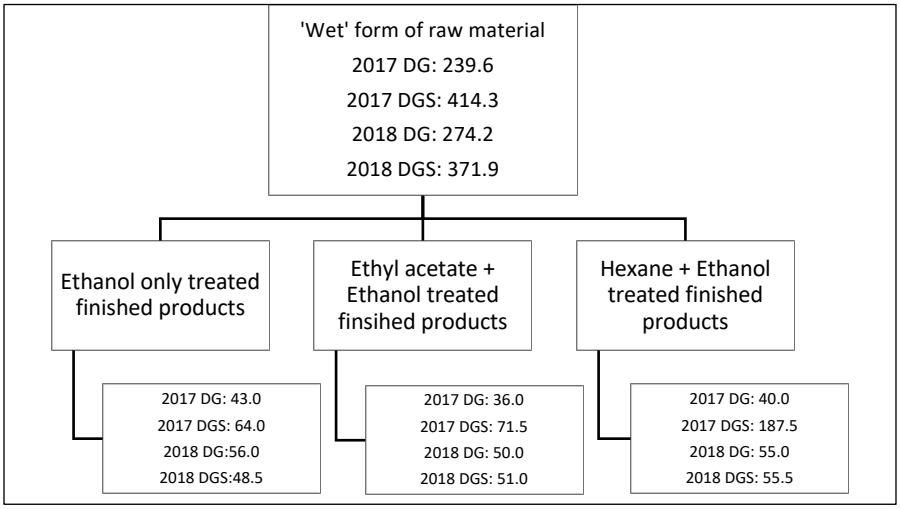


Figure 30: Process flowchart with mean TPC content (mg GAE/100gm of sample, dry wt. basis) for wet form of DG and DGS raw material and their corresponding solvent-treated dried products

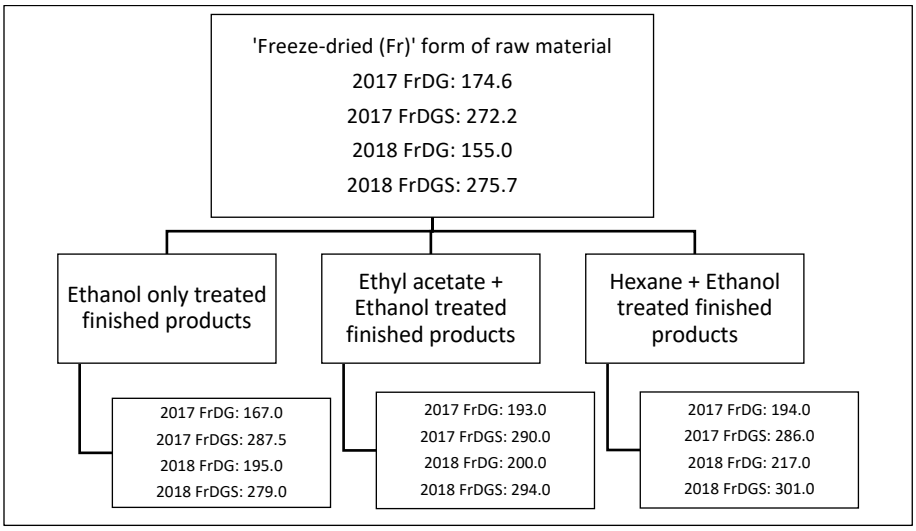


Figure 31: Process flowchart with mean TPC content (mg GAE/100gm of sample, dry wt. basis) for freeze-dried form of DG and DGS raw material and their corresponding solvent-treated dried products

Figure 31 indicates the mean values of TPC content for freeze-dried form of DG and DGS raw materials and their corresponding solvent-treated dried products. As seen in the

figure, the freeze-dried form of raw DG and DGS from 2017 and 2018 had a TPC range of 155 ~ 275 mg GAE/ 100gm sample. For solvent-treated dried products obtained using freeze-dried form of raw DG from 2017 and 2018, the TPC values ranged from 165 ~ 220 mg GAE/ 100gm sample. The TPC values increased further for solvent-treated dried products obtained using freeze-dried form of raw DGS from 2017 and 2018, ranging from 280 ~ 302 mg GAE/ 100gm sample.

In general, the trend observed was that solvent-treated dried products from freeze-dried form of raw DG and DGS ethanol plant fractions had higher TPC values as compared to solvent-treated dried products from wet form of raw DG and DGS ethanol plant fractions. This could be attributed to the freeze-drying step for the raw DG and DGS. Physical disruption of raw materials due to freezing and subsequent drying may have rendered the antioxidants more available for extraction and measurement.

Figure 32 shows the calibration curve plotted for 0.1mg/mL gallic acid standard. The equation of line presented in the graph was used to determine the TPC values for all the raw ethanol plant fractions and all of their corresponding solvent-treated dried products. Figure 33 shows the color intensities of the solutions prepared to plot the standard curve. It was observed that as the color intensity of the solution increased, the absorbance also increased. In the figure, the solutions arranged in the cuvettes from right to left are- reagent blank (0µg gallic acid), 4µg gallic acid, 8µg gallic acid, 12µg gallic acid, 16µg gallic acid and 20µg gallic acid. The reagent blank had the lowest intensity whereas 20µg gallic acid solution had the highest color intensity.

The TPC content determined in this research was based on Singleton, Orthofer and Lamuela-Raventos (1999) method and calculated the values for the total content of

phenolic acids. This method did not help to predict the composition of the phenolic acids present in the sample. However, the type of phenolic compounds expected to be present are ferulic acid, sinapic acid, p-coumaric acid, vanillic acid and caffeic acid as mentioned by Luthria, Liu and Memon (2012) when they studied DGS samples using HPLC techique for determination of phenolic compounds.

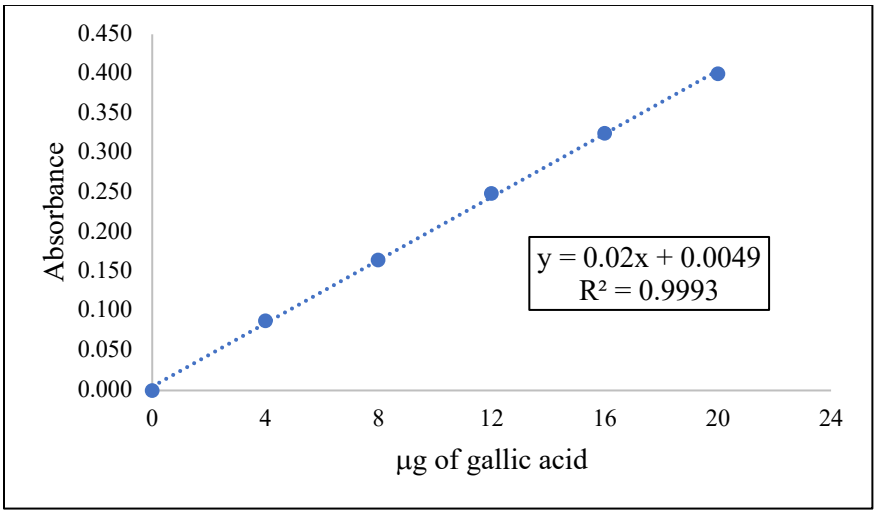


Figure 32: Standard calibration curve for 0.1mg/mL gallic acid solution used for determination of total phenolic content

Figure 33: Comparing color intensities of standard solutions used for plotting calibration curve

Toxicological content:

Total Aflatoxins:

Aflatoxins were not detected for 2017 and 2018 raw DG & DGS ethanol plant fractions in wet and freeze-dried forms and their corresponding solvent-treated dried products using the Envirologix aflatoxin test kit. Therefore, no statistical analysis was performed for this parameter. This also indicated that the aflatoxins were well within the limits prescribed by USFDA (USFDA n.d.) of less than 20ppb and also below the limit of detection set for the Envirologix quick scan system reader.

Total Fumonisins:

Tables 18 and 19 show that the fumonisin levels were significantly affected based on year and initial moisture type (wet and freeze-dried forms) as factors for all the raw materials and their corresponding solvent-treated dried products, respectively. The fumonisin levels for raw materials ranged from 0.108 ~ 0.278 ppm (table 20) whereas for solvent-treated dried products, it ranged from 0.009 ~ 0.075 ppm (table 21). Solvent treatment clearly had an effect in lowering the fumonisin levels. It should be noted that the fumonisin levels detected for raw materials and their corresponding solvent-treated dried products were well within the limits prescribed by USFDA (USFDA n.d.) of less than 2ppm. This means, the initial low levels in the starting material was an added advantage for processing the finished products.

Mycotoxin screening using US-Multitoxin LC-MS method:

Raw 2017 and 2018 DG & DGS ethanol plant fractions in wet form and their corresponding ethanol treated finished products were screened for five types of mycotoxins namely: Aflatoxins (AF), Fumonisins (FM), Ochratoxin A (Oc A),

Deoxynivalenol (DON) and Zearalenone (ZON). Furthermore, AF, FM and DON mycotoxins were screened for their subtypes namely: AFB1, AFB2, AFG1, AFG2; FMB1, FMB2, FMB3 and AcetylDON (ADON) respectively. The mycotoxins were screened using the US-Multitoxin LC-MS method. Based on the LC-MS method, each mycotoxin had a specific value set as the limit of detection as seen in Table 22. It was observed that DG 2017 had higher toxin levels for FMB1 (0.3 ppm), DON (0.7 ppm) and ZON (66.9 ppb). The processed DG 2017 yielded values less than the LOD for all toxins except for FMB1 with a value of 0.1 ppm. Next, DGS 2017 had higher toxin levels for FMB1 (0.3 ppm) and ZON (58.1 ppb). The FDGS 2017 yielded values lower than the LOD for all toxins except for FMB1 with a value of 0.2 ppm. DG 2018 was detected for FMB1 with 0.1 ppm value. The remaining DGS 2018, FDG 2018, and FDGS 2018 yielded values lower than the LOD for all toxins. The USFDA guidance level for Aflatoxins from corn kernels and its co-product DGS, is 20 ppb for human food consumption and livestock feed whereas for Fumonisins (FMB1, FMB2 and FMB3) is 2 ppm intended for human consumption. As per table 22, the 2017 and 2018 raw DG & DGS in wet form had negligible concentration of the AF and FM toxins. Even though these toxins were detected in the solvent-treated dried products using DG 2017 and DGS 2017, they were still below the USFDA guidance levels. The low toxins levels in the FDG could be attributed to ethanol solvent used during the treatment process. These low values indicate that the solvent-treated dried products are capable of significant reduction of toxin loads and have good implications for food safety and wholesomeness.

Table 22. Mycotoxin levels in raw DG & DGS ethanol plant fractions from 2017 and 2018 in wet form and their corresponding ethanol treated finished products using the US-Multitoxin LC-MS method

Mycotoxin	FDA limits	LOD	A	B	C	D	E	F	G	H
AF B1	20 ppb	1.3 ppb	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3
AF B2	20 ppb	1.2 ppb	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
AF G1	20 ppb	1.1 ppb	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1
AF G2	20 ppb	1.6 ppb	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6
FM B1	2 ppm	0.1 ppm	0.3	0.1	0.3	0.2	0.1	< 0.1	< 0.1	< 0.1
FM B2	2 ppm	0.1 ppm	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
FM B3	2 ppm	0.1 ppm	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Oc A	N.A.	1.1 ppb	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1	< 1.1
DON	N.A.	0.6 ppm	0.7	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
ADON	N.A.	0.8 ppm	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8
ZON	N.A.	51.7ppb	66.9	< 51.7	58.1	< 51.7	< 51.7	< 51.7	< 51.7	< 51.7

Legend:
A: wet DG 2017
B: Ethanol washed finished product using wet DG 2017
C: wet DGS 2017
D: Ethanol washed finished product using wet DGS 2017
E: wet DG 2018
F: Ethanol washed finished product using wet DG 2018
G: wet DGS 2018
H: Ethanol washed finished product using wet DGS 2018
ppm: parts per million, ppb: parts per billion
LOD: Limit of detection
N.A.: Not Applicable
AF B1, AF B2, AF G1, AF G2: Aflatoxins B1, B2, G1 and G2
FM B1, FM B2, FM B3: Fumonisins B1, B2 and B3
Oc A: Ochratoxin A
DON: Deoxynivalenol
ADON: Acetyldeoxynivalenol
ZON: Zearalenone

Fumonisin spiking study analysis:

Deliberate in-vitro spiking of DG with known concentrations of fumonisins was done to determine if protocols for food grade DG are also effective in significantly reducing toxins found in DG. The samples from fumonisin spiking experiment were screened for Fumonisins (FM), Ochratoxin A (Oc A), Deoxynivalenol (DON) and Zearalenone (ZON). Furthermore, AF, FM and DON mycotoxins were screened for their subtypes namely: AFB1, AFB2, AFG1, AFG2; FMB1, FMB2, FMB3 and AcetylDON (ADON) respectively. The mycotoxins were screened using the US-Multitoxin LC-MS method. Based on the LC-MS method, each mycotoxin had a specific value set as the limit of detection as seen in Table 23. The air-dried raw DG 2017 (unspiked sample, SD1) was screened for toxins to determine the concentration of toxins already present in the sample. As seen in table 23, unspiked sample had levels of FMB1 (1.6ppm), FMB2 (0.3ppm) and ZON (166.7ppb). The levels detected for FMB1 and FMB2 in unspiked sample were still below the USFDA guidance levels of 2ppm but were higher than the limit of detection set for the LC-MS instrument. When this sample was spiked with a known concentration of Fumonisin toxin mixture, the concentration of FMB1 and FMB2 increased to 2.7ppm and 1.1ppm, respectively as seen for spiked sample (SD2) in the table. The FMB1 level was higher than the FDA guidance level of 2ppm whereas for FMB2, the concentration detected was below the FDA level. This sample was further washed multiple times with ethanol as per the spiking study protocol. Finally, the washed sample (SD3) showed a level of 1.5ppm and 0.2ppm for FMB1 and FMB2, respectively. These levels detected were within the FDA limit. This indicates that the processing steps with solvent treatment is effective in diminishing the fumonisins from the raw material to produce a food grade

product. Overall, this experiment proved that the raw material processing method is robust, yielding a good quality product that is safe for human consumption.

Table 23. Mycotoxin levels unspiked, spiked and ethanol washed sample of raw air-dried DG 2017 using US-Multitoxin LC-MS method

Mycotoxin	FDA limits	LOD	SD1	SD2	SD3
AF B1	20 ppb	1.3 ppb	< 1.3	< 1.3	< 1.3
AF B2	20 ppb	1.2 ppb	< 1.2	< 1.2	< 1.2
AF G1	20 ppb	1.1 ppb	< 1.1	< 1.1	< 1.1
AF G2	20 ppb	1.6 ppb	< 1.6	< 1.6	< 1.6
FM B1	2 ppm	0.1 ppm	1.6	2.7	1.5
FM B2	2 ppm	0.1 ppm	0.3	1.1	0.2
FM B3	2 ppm	0.1 ppm	< 0.1	< 0.1	< 0.1
Oc A	N.A.	1.1 ppb	< 1.1	< 1.1	< 1.1
DON	N.A.	0.6 ppm	< 0.6	< 0.6	< 0.6
ADON	N.A.	0.8 ppm	< 0.8	< 0.8	< 0.8
ZON	N.A.	51.7ppb	166.7	136.1	106.7

Legend:
ppm: parts per million
ppb: parts per billion
LOD: Limit of detection
SD1: unspiked raw air-dried DG 2017 sample
SD2: spiked raw air-dried DG 2017 sample
SD3: raw air-dried DG 2017 sample - ethanol washed
N.A.: Not Applicable
AF B1, AF B2, AF G1, AF G2: Aflatoxins B1, B2, G1 and G2
FM B1, FM B2, FM B3: Fumonisins B1, B2 and B3
Oc A: Ochratoxin A
DON: Deoxynivalenol
ADON: Acetyldeoxynivalenol
ZON: Zearalenone

Percent yield:

The starting material used to make each batch of solvent-treated dried product was 1000gms. This value was considered to be 100%. Hence, no statistical analysis was performed for raw material % yield values. The recovery and losses were calculated once the raw ethanol plant fractions were processed to solvent-treated dried products. Percent yield values for all finished products were highly affected by the ethanol plant fractions and solvent treatment as factors (table 19). The yield values ranged from 88 to 95 % for all solvent-treated dried products (table 21). Overall, higher rates of recovery were observed for solvent-treated dried products obtained using freeze-dried form of raw DG & DGS ethanol plant fractions. This could be attributed to the use of freeze-dried material having a moisture content of less than 5% in comparison to using a wet material having moisture content of more than 55% to begin with as a starting material. The losses incurred during processing could be attributed to the manual pressing of the material during washing step. The sieve mesh size used during washing was #170 which is equivalent to 90 microns particle size. Repeated cycles of soaking and washing with ethanol caused the particle size to decrease gradually. The manual pressing during washing step caused the fine particles to pass through the sieve leading to losses.

Amino acid composition:

Table 24 and 25 provide information on the individual amino acid composition and amino acid scores for 2017 and 2018 wet DG & DGS raw materials with their corresponding ethanol treated finished products, respectively. Of the non-essential amino acids, taurine, lanthionine, hydroxylysine and ornithine had the lowest percentage. Of the essential amino acids, lysine was found to be the most limiting amino acid in the raw wet

form of DG & DGS and their corresponding ethanol treated finished products. However, as seen in table 24, the amino acid scores increased for the ethanol treated finished products as compared to their respective raw ethanol plant fractions used. The highest amino acid score was observed for leucine and the lowest was observed for lysine in all ethanol treated finished products. The increased scores could be attributed to processing the raw material with ethanol solvent. Furthermore, these amino acid scores can be used to determine the protein digestibility-corrected amino acid score (PDCAAS). PDCAAS is the method adopted by FAO/WHO that evaluates the quality of the protein based on amino acid requirements for human body and the ability of the body to digest the proteins ((t. f. Wikipedia, Protein digestibility corrected amino acid score (2019), Schaafsma (2000)).The PDCAAS formula is as follows:

PDCAAS (%) = (mg of limiting amino acid in 1g of test protein / mg of same amino acid in 1g of reference protein) * fecal true digestibility (%) * 100

This reference pattern is based on the essential amino acid requirements for the preschool-age child. A PDCAAS value of 1 is the highest and 0 is the lowest.

This PDCAAS method has been recently replaced in 2013 with Digestible Indispensable Amino Acid Score (DIAAS) by Food and Agriculture organization (FAO). The DIAAS method determines amino acid digestibility at the end of the small intestine thus providing a more accurate measure of amino acid concentration absorbed by the human body (t. f. Wikipedia 2018). K. Liu (2011) reported that the lowest lysine content was found in darker color DGS ethanol plant fractions, attributed to the Maillard reaction between reducing carbohydrates such as glucose and the ε-amino group of lysine causing signficant loss in amount of lysine during excessive heating.

Table 24. Amino acid composition for raw DG & DGS ethanol plant fractions from 2017 and 2018 in wet form and their corresponding ethanol treated finished products

Amino Acid	**DG 2017**	**T1**	**DGS 2017**	**T7**	**DG 2018**	**T26**	**DGS 2018**	**T32**
Unit				w/w%				
Taurine	0.08	0.09	0.08	0.09	0.13	0.14	0.11	0.15
Hydroxyproline	0.12	0.08	0.09	0.09	0.10	0.07	0.09	0.08
Aspartic acid	2.17	2.47	2.48	2.45	2.21	2.68	2.13	2.58
Threonine	1.26	1.42	1.43	1.39	1.35	1.60	1.31	1.53
Serine	1.44	1.60	1.63	1.59	1.51	1.83	1.48	1.78
Glutamic acid	5.49	6.84	6.60	6.42	5.56	7.30	4.87	6.96
Proline	2.77	3.17	3.01	2.92	2.85	3.45	2.64	3.26
Lanthionine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycine	1.26	1.41	1.61	1.54	1.25	1.50	1.32	1.50
Alanine	2.49	2.88	2.78	2.74	2.44	3.04	2.35	2.88
Cysteine	0.71	0.86	0.79	0.81	0.72	0.89	0.69	0.89
Valine	1.80	2.05	2.03	1.96	1.66	2.08	1.68	1.95
Methionine	0.72	0.85	0.74	0.73	0.71	0.84	0.64	0.79
Isoleucine	1.46	1.62	1.52	1.52	1.41	1.69	1.39	1.56
Leucine	4.42	5.07	4.63	4.45	4.29	5.37	4.02	4.97
Tyrosine	1.30	1.50	1.35	1.40	1.39	1.60	1.27	1.53
Phenylalanine	1.91	2.15	1.90	1.90	1.85	2.25	1.76	2.09
Hydroxylysine	0.08	0.04	0.02	0.05	0.08	0.06	0.16	0.06
Ornithine	0.03	0.02	0.04	0.04	0.00	0.02	0.02	0.02
Lysine	1.10	1.21	1.26	1.22	1.12	1.35	1.19	1.36
Histidine	0.99	1.11	1.05	1.13	0.88	1.10	0.91	1.09
Arginine	1.40	1.60	1.67	1.65	1.42	1.70	1.47	1.71
Tryptophan	0.26	0.32	0.30	0.31	0.29	0.32	0.27	0.32
Total amino acid content	33.21	38.31	36.98	36.34	33.17	40.83	31.70	39.02
Standard Deviation	0.1485	0.1556	0.0200	0.7990	0.2263	0.0283	0.0283	0.1344
Coefficient of Variation	0.4472	0.4061	0.0541	2.1991	0.6822	0.0693	0.0892	0.3444

Legend:
DG 2017: Distiller's grains without solubles from 2017
DGS 2017: Distiller's grains with solubles from 2017
DG 2018: Distiller's grains without solubles from 2018
DGS 2018: Distiller's grains with solubles from 2018
T1: ethanol treated finished product using DG 2017
T7: ethanol treated finished product using DGS 2017
T26: ethanol treated finished product using DG 2018
T32: ethanol treated finished product using DGS 2018

Table 25. Amino acid scores for raw DG & DGS ethanol plant fractions from 2017 and 2018 in wet form and their corresponding ethanol treated finished products

EAA (g/100g protein)	DG 2017 AAC	DG 2017 AAS	T1 AAC	T1 AAS	DGS 2017 AAC	DGS 2017 AAS	T7 AAC	T7 AAS	DG 2018 AAC	DG 2018 AAS	T26 AAC	T26 AAS	DGS 2018 AAC	DGS 2018 AAS	T32 AAC	T32 AAS	FAO/WHO Ref.pat.
Leucine	4.42	63.14	5.07	72.43	4.63	66.14	4.45	63.57	4.29	61.29	5.37	76.71	4.02	57.43	4.97	71.00	7.00
Isoleucine	1.46	36.50	1.62	40.50	1.52	38.00	1.52	38.00	1.40	35.25	1.69	42.25	1.39	34.75	1.56	39.00	4.00
Lysine	1.10	20.00	1.21	22.00	1.26	22.91	1.22	22.18	1.12	20.36	1.35	24.55	1.19	21.64	1.36	24.73	5.50
Methionine + Cysteine	1.43	40.86	1.71	48.86	1.53	43.71	1.54	44.00	1.43	40.86	1.73	49.43	1.33	38.00	1.68	48.00	3.50
Phenylalanine + Tyrosine	3.21	47.21	3.65	53.68	3.25	47.79	3.30	48.53	3.24	47.65	3.85	56.62	3.03	44.56	3.62	53.24	6.80
Threonine	1.26	31.50	1.42	35.50	1.43	35.75	1.39	34.75	1.35	33.75	1.60	40.00	1.31	32.75	1.53	38.25	4.00
Valine	1.80	36.00	2.05	41.00	2.03	40.60	1.96	39.20	1.66	33.20	2.08	41.60	1.68	33.60	1.95	39.00	5.00

Legend:
AAC: Amino acid composition
AAS: amino acid score
EAA: Essential amino acid
DG 2017: Distiller's grains without solubles from 2017
DGS 2017: Distiller's grains with solubles from 2017
DG 2018: Distiller's grains without solubles from 2018
DGS 2018: Distiller's grains with solubles from 2018
T1: ethanol treated finished product using DG 2017
T7: ethanol treated finished product using DGS 2017
T26: ethanol treated finished product using DG 2018
T32: ethanol treated finished product using DGS 2018
AA score was calculated based on the FAO/WHO reference pattern

Correlation plot:

A Pearson correlation matrix was generated between all the dependent variables as seen in figure 34. This plot discovers the various subsets of dependent variables that seem to be highly correlated within the subset. The columns and rows in this plot are sorted in the order suggested by the hierarchical clustering by the RStudio software. In this heat map, the blue boxes indicate that the dependent variables are positively correlated whereas the red boxes indicate that the variables are negatively correlated with each other. The white boxes indicate that the variables are lightly associated with each other. These box values define the degree of correlation (correlation 'r' value) established between the variables. The negative or the positive sign is determined by the covariance that exists between the two variables. Covariance is defined as the measure of joint variability between two random variables (t. f. Wikipedia 2019). Positive relationship means when the value of variable is increases, the value of the other variable also increases.

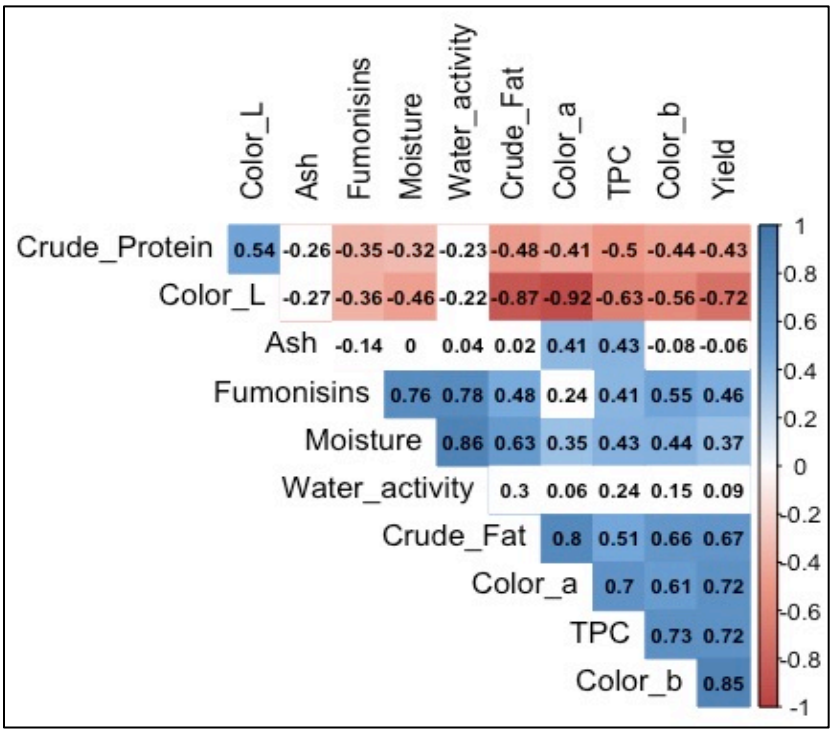


Figure 34: Correlation plot between all dependent variables

The following observations were seen in figure 34:

- Crude protein was negatively correlated with TPC content (-0.59), crude fat content (-0.40), ash content (-0.56) and color a value (-0.41). This indicated that as the protein content increased for all the solvent-treated products, there was a simultaneous decrease in the TPC content, crude fat content, ash content and color a value. However, a positive correlation of 0.47 between crude protein content and color L value indicated that as the protein content increased, the brightness of all the solvent-treated products also increased.
- Color L values were negatively correlated with moisture content (-0.56), water activity (-0.43), percent yield (-0.43), TPC content (-0.47), ash content (-0.43) and color a value (-0.68). This indicated that as the brightness of all the solvent-treated products increased, the other correlated dependent variables decreased.
- Fumonisins were positively correlated to moisture content (0.46), water activity (0.55), percent yield (0.42) and color b value (0.44). The growth of fumonisins is highly susceptible in the presence of high moisture content and high water activity of the product. It is reasonable to see that as the moisture content and water activity increases, the fumonisin content also increases. Furthermore, as the fumonisin content also increased with increase in percent yield and color b values.
- a positive correlation value of 0.92 between % moisture and water activity (Aw) indicated that as % moisture content increased, the water activity of the finished product also increased.
- Percent yield was highly correlated with color b value (0.80), TPC content (0.68) and color a value (0.49).

- a positive correlation value of 0.83 between TPC content and color b value indicated the TPC content for all solvent-treated products increased as the yellowness of the finished products increased. Color b value was also positively correlated with color a value (0.55) indicating that as the yellowness of the finished products increased, the color value (greenness) also increased.
- TPC content for all solvent-treated dried products was highly correlated with ash content (0.41) and color a value (0.68). This could be attributed to the presence of certain pigments in the finished products that were not extracted with solvents might have caused an increase in their TPC content along with ash content and color a value.
- Crude fat content was positively correlated to ash content at 0.48 correlation ‘r’ value.
- Ash content was highly correlated with color a value at 0.71 correlation ‘r’ value.

CHAPTER 5. SUMMARY AND CONCLUSION

Summary:

Distillers grains are a diverse co-product from the ethanol industry that has a wide range of composition. Consistency and predictability of nutritional composition will be beneficial for both livestock applications and food applications. The high protein and dietary fiber content make it an ideal candidate for optimization for food applications. The data generated in this research will be of interest to ethanol industry and regulatory authorities in relation to the quality, safety and efficacy of the product. The research attempted to minimize the quality variability in DG for use as a food ingredient. Three types of solvents - ethanol, hexane + ethanol, and ethyl acetate + ethanol were compared to show their effects on the overall quality, physical and chemical properties of solvent-treated dried products. While Hexane and ethyl acetate are well-known food grade solvents, Ethanol may be preferred as it is a product of the corn industry. The physical and chemical properties were compared between raw materials and their corresponding solvent-treated dried products based on five factors namely – a) ethanol plant fractions: DG & DGS, b) initial moisture type: wet & freeze-dried forms, c) year: 2017 & 2018, d) solvents: ethanol, hexane + ethanol & ethyl acetate + ethanol, e) rep: replicates 1 & 2. The study showed that the wet form of raw DG without solubles was a good starting material to achieve a desirable solvent-treated dried product with high ‘L’ value (brightness) of 85, low ‘a’ value (redness) of -0.11, ‘b’ value (yellowness) of 18, crude protein of 38%, crude fat of less than 2%, total dietary fiber content of 42% and ash content of less than 1.5%, Aw of less than 0.450 and negligible amounts of fungal toxins. The total phenolic content for the solvent-treated dried product obtained using raw DG in

wet form was less than 100mg GAE/100gm of sample. This means there was potential loss of phytonutrients while processing the wet form of raw DG into its corresponding solvent-treated dried product. If the number of washing and steeping cycles are increased, even finished product obtained using wet form of DGS would give desirable physical and chemical properties. The freeze-drying process significantly affected the color (L, a & b) values resulting in a darker yellow colored raw material when compared to the wet form of DG and DGS. This eventually affected the color L, a, & b scores of the corresponding solvent-treated dried product. Decrease in brightness of the finished product might not seem appealing and satisfactory in the western culture. Conversely, a high brightness score (L values above 80) of the product is perceived to be a desirable trait. The efficacy of solvent-treated dried product has been established in the literature by incorporating this food grade product in baked products. The particle size distribution (PSD) for a food grade distiller's grains product should be compatible with cereal flours in formulating DG-flour blends. In this research, for all the solvent-treated DG products, maximum retention of particles was observed on #100 mesh in the range of 150-180μm. From the literature, it is known that for all-purpose flour (APF), maximum retention of particles was also observed on #100 in the range of 150-180μm. This indicates that using the finished product particles retained on #100 sieve fraction (150-180μm particle size range) for blending with the APF will help in the homogenous distribution and food functionality (Krishnan and Rosentrater 2006). Lastly, the solvent-treated dried products obtained in this research were microbiologically sterile, since the material was heat processed under pressure and subsequently extracted using ethanol.

Conclusion:

From the experimental results obtained in this research, following conclusions were made regarding the research hypotheses statements:

- Hypothesis 1:

  There was not sufficient evidence to reject the null hypotheses. This indicated that the freeze-drying of raw DG and DGS did not significantly increase the crude protein content and the total dietary fiber content of their corresponding solvent-treated products.

- Hypothesis 2:

  There was sufficient evidence to reject the null hypotheses. The evidence suggested that the solvent-extraction of raw DG and DGS in wet-form and freeze-dried forms significantly decreased the crude fat content of the corresponding solvent-treated products.

- Hypothesis 3:

  There was sufficient evidence to reject the null hypotheses. The evidence suggested that the solvent-extraction of raw DG and DGS in wet-form and freeze-dried forms produced a significant difference in the color (L, a and b) scores i.e. the ‘L’ scores increased, while the ‘a’ and ‘b’ scores decreased for their corresponding solvent-treated products.

- Hypothesis 4:

  There was sufficient evidence to reject the null hypotheses. The evidence suggested that the solvent-extraction of raw DG and DGS in wet-form and freeze-

dried forms significantly decreased the toxicological content (aflatoxins and fumonisins) of their corresponding solvent-treated products.

- Hypothesis 5:

  There was sufficient evidence to reject the null hypotheses. The evidence suggested that the overall raw material processing technique reduced the fumonisin content below the USFDA permissible limit of 2 ppm after the raw material was spiked with a high dose of 50 ppm fumonisin mixture.

Material specification sheet:

Based on the literature review and the data collected in this research, a material specification sheet was prepared for food grade distiller's dried grains product.

Food Grade Dried Distiller's Grains Product Specification Sheet

Definition: Distiller's grains are co-products obtained from ethanol production using corn as the principle substrate. Distiller' Grains are excellent ethanol plant fraction that are rich in protein and dietary fiber content. A food grade dried distiller's grain product is obtained when distiller's grains are exhaustively washed with food grade solvents.

Physical description: An ideal food grade dried distiller's grains product is sterile, odorless, tasteless, color-neutral, gluten-free and free-flowing with a consistent particle size.

Packaging & Storage: The product should be stored in a sterile mason jar at -20^{o}C.

Proposed shelf-life: ~1 year

Physical and chemical properties:

Properties	**Composition range**
Crude protein	35% to 38%
Total dietary fiber	40% to 45%
Crude fat	0% to 2%
Moisture content	6% to 8%
Ash value	< 5%
Carbohydrates	< 15%
Bulk density	0.310 - 0.485 gm/mL
Water activity (Aw)	0.300 to 0.500
Total phenolic acid content	250 - 300 mg GAE/100gm of sample
Aflatoxin content	< 0.1ppb
Fumonisin content	< 0.1ppm
Hunter color L value	82.00 to 88.00
Hunter color a value	-0.10 to +2.00
Hunter color b value	19.00 to 25.00

Amino acid (AA) scores:

Essential amino acid (g/100gm protein)	Food grade product	% AA score	FAO/WHO Reference pattern
Leucine	4-5	57-71	7.00
Isoleucine	1.3-1.6	32.5-40	4.00
Lysine	1.1-1.2	20-22	5.50
Methionine + Cysteine	1.5-1.7	43-49	3.50
Phenylalanine+ Tyrosine	2-3	29-44	6.80
Threonine	1.2-1.4	30-35	4.00
Valine	1.5-2	30-40	5.00

Particle size distribution:

Mesh Size		% Particle Size Distribution
>400µm	40	~4.00
400µm-250µm, 0.0165”	60	~16.00
250µm-180µm, 0.0098”	80	~18.00
180µm-150µm, 0.007”	100	~25.00
150µm-75µm, 0.059”	200	~19.00
≤ 75µm, 0.0029”	Bottom pan	~16.00

Mineral composition:

Mineral	Mineral range
Boron	4 -5 ppm
Calcium	0.14 - 0.18%
Cobalt	0.06 - 0.6 ppm
Copper	6 -7 ppm
Iron	40 - 42 ppm
Magnesium	0.13 - 0.15%
Manganese	21- 23 ppm
Molybdenum	3 -7 ppm
Phosphorus	0.4-0.5%
Potassium	0.2 - 0.3%
Sodium	~0.02%
Sulfur	0.5 - 0.6%
Zinc	29 - 32 ppm

Future directions:

The processing steps for raw distiller's grains performed during this research involved manual treatments. Critical steps such as washing, and steeping can be semi-automated by scaling up the process using a large mixing vessel with a stirrer to continuously agitate the material during the steep process. For the washing step, a hydraulic press can be used to squeeze out solvent from the material before it comes into contact with the fresh solvent. The mixing vessel will have a sieve resting at the bottom on which the material is retained during steeping and washing steps. The solvent can be discarded using a spigot attached at the bottom of the vessel. This solvent can be further dried down and analyzed for physical and chemical properties on the residue that remains after solvent evaporation. By doing so, we could learn about constituents that are lost or removed during raw material processing.

The statistical information generated in this research can also be used for USFDA GRAS (generally regarded as safe status) application of solvent-treated dried product. The GRAS status will ultimately help to launch the functional ingredient in the food market.

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