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Characterization of Spent Grain from Irish Whiskey Distilleries for Biorefinery Feedstock Potential to Produce High-Value Chemicals and Biopolymers

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Abstract: Distiller's spent grain (DSG) is a byproduct generated in large quantities during the mashing process, particularly in the production of alcoholic beverages such as whiskey. This study aimed to characterize DSG from nine different distilleries as a potential biorefinery feedstock for the synthesis of high-value bioproducts. Key components, including protein (12.38–26.32%), cellulose (11.75–32.75%), hemicellulose (6.97–19.47%), lignin (8.44–15.71%), and total phenolics (1.42 to 3.97 mg GAE/g), were analyzed to evaluate their variability and suitability for industrial applications. The results reveal that DSG composition varies significantly across distilleries due to differences in processing techniques, even though the starting grain composition had minimal influence. Statistical analysis highlighted the variability of water- and ethanol-soluble extractives (17.34–31.77%) and their potential impact on product consistency. This compositional variability highlights the importance of understanding DSG's structural properties to optimize its use as a lignocellulosic biomass feedstock. This study emphasizes the potential for utilizing DSG in the production of nanocellulose, bioplastics, phenolic resins, and other sustainable materials, thereby contributing to the circular economy. By linking compositional insights to specific applications, this work establishes a foundation for tailored utilization of DSG in biopolymer production and chemical synthesis. These findings provide valuable insights for biorefinery operations, addressing both sustainability challenges and the economic potential of industrial byproducts.

Keywords: distillery spent grain; cellulose; hemicellulose; lignin; bioproducts; biorefinery

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1. Introduction

The increasing demand for renewable alternatives to replace fossil resources has driven research into exploring alternative feedstocks for the synthesis of fuels and chemicals. Lignocellulosic biomass has been identified as a promising feedstock with great potential to serve as a carbon-neutral source of fuels and chemicals due to its abundance and composition [1]. Lignocellulosic biomass is typically defined as plant-derived materials, such as agricultural residues (e.g., barley straw, corn stover), byproducts of industrial processes (e.g., brewer's spent grain (BSG), distiller's spent grain (DSG), sugarcane bagasse), or dedicated energy crops (e.g., Miscanthus) [2]. All lignocellulosic plant sources share the same fundamental building blocks, with the main structural components—cellulose, hemicellulose, lignin, and protein—varying depending on the source [3].

Malted barley is a common ingredient in most whiskey, although other unmalted cereal grains can also be included in certain variants. For example, in Ireland, Irish pot still whiskey is made from a mash containing a minimum of 30% malted barley, a minimum of 30% unmalted barley, and other unmalted cereals, such as rye and oat (up to 5%), depending

on the distillery's process. Meanwhile, the mash for Irish Malt Whiskey contains 100% malted barley. Furthermore, Irish Grain Whiskey is produced from malted barley (not exceeding 30%) and can include whole unmalted cereals, such as maize, wheat, or barley. On the other hand, Irish Blended Whiskey is a combination of Grain Whiskey with added Malt Whiskey and/or pot still whiskey [4].

Distiller's spent grain (DSG) is the wet, unhydrolyzed solid fraction left after the separation of wort from the mash grain. It is primarily composed of kernel husk, pericarp, and seed coat, making it a rich source of carbohydrates, proteins, lignin, phenolic compounds, lipids, and minerals. Large quantities of DSG are generated during mashing, with additional forms, such as distiller's dried grains with solubles (DDGS), also available. Other waste streams from whiskey production include spent lees and pot ale [5].

Figure 1 illustrates the process leading to the generation of DSG from malted barley during whiskey production. Briefly, ground malt is mixed with water to produce a mash, which is then heated. During a resting period, enzymes transform the protein and starch in the mash into fermentable sugars and amino acids. After draining the mash, a liquid filtrate (wort) and a solid residue (DSG) are obtained [5]. The wort is subsequently fermented, distilled, aged for a minimum of three years in wooden casks, and bottled as whiskey.

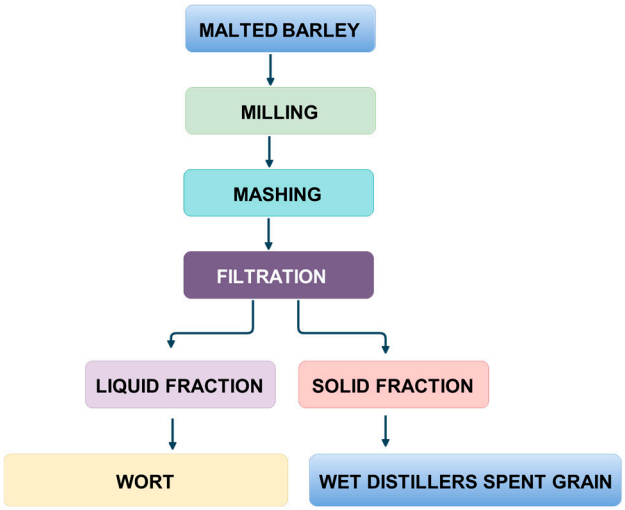


Figure 1. Illustration of DSG generation process in a distillery.

The structural components of DSG, such as cellulose, hemicellulose, lignin, and proteins, make it a valuable lignocellulosic feedstock for developing high-value bioproducts and chemicals [6]. Cellulose, which constitutes up to 25.4% of DSG, is a key precursor for producing nanocellulose, widely used in bio-based polymers, composites, and sustainable packaging [7]. Lignin (27.8%) offers diverse applications, including adhesives, polyurethanes, and phenolic resins, due to its unique aromatic structure, while hemicellulose (28.4%) and proteins (26.9–34.9%) expand the potential for creating biodegradable films, bioethanol, and other bioproducts [7,8]. Additionally, the high organic content and oil (10%) in DSG further enhance its suitability for diverse biorefinery operations [7,9]. Brewer's spent grain, a similar by-product, has already been successfully valorized for bioplastics and nanocellulose production, underscoring the feasibility of converting DSG into sustainable materials. Despite its primary use as animal feed due to its high protein content, DSG presents environmental challenges because of its high moisture content, which makes it prone to spoilage. Optimizing its use in biorefineries and addressing these management challenges could significantly contribute to a low-carbon, circular bioeconomy [6].

Data on the global volume of spent grain generated from distilleries are scarce in the literature. However, DSG is reported to account for about 85% of the waste generated in distilleries. According to Mohana et al. [10], approximately 2.5–3.0 kg of wet DSG is generated for every 1 L of whiskey produced, amounting to about 2400 tons of spent grain

per million liters of alcohol generated annually [11]. DSG is very similar to brewer's spent grain (BSG), a byproduct of beer production. DSG and BSG are generated during the initial stage, prior to fermentation, in whiskey and beer production, respectively. However, they differ in composition due to the differences in the types of grain used in the mash bill between breweries and distilleries.

Recently, there has been considerable research interest in the processing of spent grain into value-added products, with a significant number of studies focusing on BSG. For example, BSG has been used to produce butanol and methane [12], arabinoxylan [13], single-cell protein [14], lignin [15], protein [16], bioethanol [17], nanocellulose [18], phenolic compounds [19], and food products [20]. The various studies on brewer's spent grain primarily focus on its utilization in food and feed applications [6,21–24].

However, there is an inadequacy of studies on the composition and utilization of DSG. This lack of robust data on DSG is one of the major barriers to its exploitation as a viable lignocellulosic biomass feedstock for the synthesis of high-value products [25]. Therefore, the availability of scientific data will provide deeper insights into its components and aid in the development of tailored processes to maximize DSG's potential as a biorefinery feedstock. In addition to the lack of studies on DSG composition, there is also limited data on the compositional variability of DSG from different origins. Since DSG sources are highly diverse, significant variations in their composition make it challenging to standardize their utilization in large-scale biorefinery operations. Variations in the chemical composition of the distiller's spent grain have also been reported across different batches from the same distilleries and within the same production plants [26,27]. This high compositional variation presents additional barriers to DSG utilization in various applications.

This variation can be mitigated by understanding the sources and extent of variation among the different DSG components [28]. A comprehensive analysis of the composition of DSG obtained from various sources is, therefore, vital for its industrial applications. Such an analysis would provide insights into the compositional variability of DSG generated by different distilleries and further help optimize conversion processes and tailor product development for the maximum yield of value-added products from distillery waste. This approach would benefit the distillery industry by increasing economic viability through waste utilization and contribute to realizing an environmentally sustainable whiskey production process.

To the best of our knowledge, studies on the composition analysis of distillery spent grain from multiple sources are very scarce. In this study, we investigate the composition of distiller's spent grains from nine different distilleries, focusing on variability in their structural composition and identifying the principal components of DSG as a potential lignocellulosic biomass feedstock for synthesizing value-added bioproducts and chemicals. The rationale for using DSG from various sources is to simulate real-life scenarios, as sourcing DSG from a single distillery for biorefinery operations may be challenging. Additionally, this study provides comparative data on DSG from various sources. Further, the utilization of food industry waste, such as DSG, in biopolymer production aligns with UN Sustainable Development Goal 12, which focuses on responsible consumption and production. This approach promotes sustainable resource use and waste reduction, addressing the environmental challenges associated with distillery waste while supporting a circular bioeconomy.

2. Materials and Methods

2.1. Sample Preparation

Fresh distiller's spent grain (DSG) samples were collected from nine different distilleries across Ireland. The mash bills for the distilleries' DSG are presented in Table 1. Four samples were obtained from a mash bill composed of 100% malted barley (D1, D5, D7, and D8), two samples from a process using 60% malted barley and 40% unmalted barley (D3 and D6), one sample from a process using 40% malted barley and 60% unmalted barley

(D4), and two samples (D9 and D2) from a mix of malted barley, unmalted barley, rye, and oat in different proportions.

Table 1. The composition of the spent grains mash bill.

Distillery Code	Sample Code	Malted Barley (%)	Unmalted Barley (%)	Oat (%)	Rye (%)	Whiskey Type
Distillery 1	D1	100	-	-	-	Malt Irish Whiskey
Distillery 2	D2	60	35	5	-	Single pot still whiskey
Distillery 3	D3	60	40	-	-	Single pot still whiskey
Distillery 4	D4	40	60	-	-	Single pot still whiskey
Distillery 5	D5	100	-	-	-	Malt Irish Whiskey
Distillery 6	D6	60	40	-	-	Single pot still whiskey
Distillery 7	D7	100	-	-	-	Malt Irish Whiskey
Distillery 8	D8	100	-			Malt Irish Whiskey
Distillery 9	D9	40	55	3.25	1.75	Single pot still whiskey

Fresh DSG samples were collected immediately after production in sterile bags as single-batch samples from each distillery. The samples were dried at 60 °C for 24 h until a constant weight was achieved. The dried samples were then milled using a laboratory grinder, sieved to pass through a 0.250 mm sieve, and stored for further analysis.

All analytical-grade reagents and standards, including sulfuric acid (95–98%), Folin–Ciocalteu reagent, sodium carbonate, gallic acid, sugar standards (D(+) glucose, D(+) xylose, D(+) galactose, L(+) arabinose), sodium hydroxide, hexane, absolute ethanol, acetone, and calcium carbonate, were procured from Merck, Ireland.

All percentages are based on the weight of individual grains in the mash bill.

2.2. DSG Proximate Composition Analysis

The moisture content, ash content, fat, extractives, total phenolics, crude protein content, structural carbohydrates, and lignin in the DSG were determined as described below.

2.2.1. Determination of Moisture Content

The moisture content in the fresh distiller's spent grain (DSG) was determined using convection oven drying methods as described by the NREL Laboratory Analytical Procedure for the determination of total solids and moisture in biomass [29]. Approximately 2 g of each fresh DSG sample was weighed into a pre-dried and pre-weighed pan. The pan containing the sample was dried in a convection oven at 105 °C for 24 h, cooled to room temperature in a desiccator, weighed, and returned to the oven until a constant weight was achieved. The moisture content was calculated as the difference in weight between the fresh and dried samples, with the weight of water determined as the difference in DSG weight before and after drying.

$$\text{Moisture content} = \frac{\text{Weight of water}}{\text{DSG Dry weight}} \times 100 \quad (1)$$

2.2.2. Ash Content

The ash content of the distiller's spent grain (DSG) was determined following the method described by Sluiter et al. [30]. Briefly, 2 g of oven-dried sample was placed in a pre-dried and pre-weighed crucible. The crucible with the sample was then placed in a muffle furnace equipped with a ramping program (SNOLTherm, Utena, Lithuania) at 575 °C for 12 h. Afterwards, the sample was removed from the furnace and cooled in a desiccator. The ash content was calculated as the difference in weight before and after ashing.

$$\text{Ash content} = \frac{\text{Weight of ash}}{\text{DSG Dry weight}} \times 100 \quad (2)$$

2.2.3. Fat Content Determination

The fat content of dried distiller's spent grain (DSG) samples was estimated after extraction with *n*-hexane in a Soxhlet system, as described by Wang et al. [31], with some modifications. Briefly, 5 g of milled dried DSG (DDSG) was placed into a tared extraction thimble, and the combined weight of the sample and thimble was recorded. The thimble was then inserted into the Soxhlet tube. Next, 150 mL of *n*-hexane was transferred into a pre-dried and pre-weighed 250 mL receiving round-bottom flask, which was then connected to the Soxhlet extractor. The heating mantle was set to 55 °C to provide reflux for 6 h, extracting the lipids into the solvent.

After extraction, the solvent was removed from the lipid extract using a rotary evaporator (Rotavapor® R-250, BÜCHI Labortechnik, Flawil, Switzerland) equipped with a vacuum. Following hexane recovery, the flask containing the extracted lipids was placed in a convection air oven set to 60 °C for 4 h to remove any residual hexane. This process was repeated by returning the flask to the oven and cooling it until a constant weight was achieved. The mass of the extracted lipid was determined as the difference in weight of the flask before and after extraction and drying. The fat content was calculated as the mass of the extracted lipid divided by the initial dry mass of the DSG sample (Equation (3)).

$$\text{Fat content} = \frac{\text{Weight of extracted fat}}{\text{DSG Dry weight}} \times 100 \quad (3)$$

2.2.4. Protein Content Determination

The protein content of the dried distiller's spent grain (DSG) sample was determined using the Kjeldahl method, as described by the Association of Official Analytical Chemists (AOAC) [32], with some modifications. Briefly, 1.5 g of milled raw dried DSG was mixed with 15 mL of 95–98% sulfuric acid (H_2SO_4) and 3 mL of 35% hydrogen peroxide (H_2O_2), and two copper catalyst tablets were added. The mixture was allowed to react for approximately 10 min before being placed in a KJELDATHERM digestion block unit (Gerhardt, Königswinter, Germany) at 410 °C for 3 h. A blank, containing all reagents but without the sample, was also prepared.

After the digestion process (indicated by a clear hydrolysate), the hydrolysates were transferred to a distillation unit (VAPODEST), where they were distilled into 25 mL of 4% boric acid containing two drops of Tashiro indicator. The distillate was titrated against 0.1 N HCl until the endpoint, indicated by a pink color, was achieved. The volume of acid used during titration was recorded. A blank titration was also performed using the blank hydrolysates. The percentage of Kjeldahl nitrogen and the crude protein content were calculated using Equations (4a) and (4b).

$$\text{Kjeldahl Nitrogen} = ((\text{Vs} - \text{VB}) \times \text{M} \times 14.01)/(\text{DSG Dry weight}) \times 100. \quad (4a)$$

$$\text{Crude Protein \%} = \text{\% Kjeldahl Nitrogen} \times 6.25. \quad (4b)$$

where Vs is the volume of acid used for the sample.

Vs is the volume of acid used for the blank.

M is the molarity of HCl.

2.2.5. Extraction and Determination of Total Phenolic Content

The ultrasound-assisted extraction of phenolic compounds from the distiller's spent grain (DSG) samples was performed using the method described by Chetrariu and Dabija [33], with some modifications. Briefly, 1 g of dried and milled DSG was mixed with 10 mL of 60% acetone and placed in an ultrasonic bath preheated to 50 °C for 30 min. After extraction, the mixture was centrifuged at 5000 rpm for 10 min, decanted, and the filtrate was immediately used for analysis. The volume of extract recovered after extraction was quantified and used for calculation.

The phenolic content was determined using the optimized method described by Chetrariu and Dabija [33], with modifications. Briefly, 200 µL of diluted DSG extract (1:10 dilution) obtained from the extraction was mixed with 1 mL of diluted Folin–Ciocalteu reagent (1:10 dilution). After 2 min, 1 mL of 7.5% (*w*/*v*) sodium carbonate was added to the mixture. The tube was left for 60 min at room temperature in the dark. The same procedure was repeated for the standard.

The total polyphenol concentration was determined using a calibration curve of the standard prepared at concentrations ranging from 10 to 120 µg/mL, with an $R^2$2 value of 0.9964 and the equation 0.0072x + 0.0423. Absorbance was measured at a wavelength of 760 nm using a UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

The total polyphenol content was calculated and expressed as gallic acid equivalents (GAEs) per gram of DSG.

2.3. DSG Structural Composition Analysis

2.3.1. Quantification of Water and Ethanol Extractives in Biomass

Extractives are defined as extraneous components that can be removed from insoluble cell wall material using water or neutral organic solvents. The ethanol- and water-extractive content of raw dried distiller's spent grain (DDSG) was determined using an ultrasound-assisted extraction process, following the methods described by Matei et al. [34], with modifications.

Briefly, ethanol extractives were removed by mixing 3 g of dried DSG with 30 mL of absolute ethanol in a 50 mL centrifuge tube. The mixture was placed in an ultrasonic water bath operating at a frequency of 37 kHz (Fisher Scientific, Leicestershire, UK) and preheated to 45 °C for 30 min. After 30 min, the sample was cooled, centrifuged at 5000 rpm for 10 min, and the supernatant was decanted. The solid residue was further rinsed twice with 20 mL of absolute ethanol, centrifuged, and decanted each time.

For the removal of water extractives, 20 mL of ultrapure water was added to the ethanol-extracted solid residue. The mixture was placed in an ultrasonic water bath preheated to 45 °C for 30 min, followed by centrifugation at 5000 rpm for 10 min. The solid residue was then rinsed twice with 20 mL of ultrapure water, centrifuged at 5000 rpm for 10 min, and the supernatant was decanted. The remaining solid residue was freeze-dried and weighed.

The ethanol- and water-soluble extractives were calculated as the difference in the sample's weight before and after extraction.

2.3.2. Sample Preparation for Lignocellulosic Composition Analysis

Due to the high extractives and protein content of distiller's spent grain (DSG), a sequential extraction process was employed to remove extractives and protein prior to composition analysis. Ten grams (10 g) of milled and dried DSG samples were subjected to the ethanol- and water-soluble extractives removal process using the method described in Section 2.3.1. The solid residue obtained, referred to as the extractive-free sample, was freeze-dried and stored for further use.

The extractive-free sample was subjected to protein extraction using the method described by Ayim et al. [35], with some modifications. Briefly, 5 g of extractive-free DSG residue was mixed with 50 mL of 0.1 M NaOH and placed in an ultrasonic water bath operating at a frequency of 37 kHz (Fisher Scientific, Leicestershire, UK), preheated to 60 °C for 1 h. After ultrasonic bath protein extraction, the sample was cooled, and centrifuged at 5000 rpm for 15 min, and the supernatant was decanted and collected into a separate Duran glass bottle.

The solid residue was then washed with ultrapure water, centrifuged, and the supernatant was decanted into the same Duran glass bottle. This process was repeated twice. Afterwards, the residue was washed with 0.001% acetic acid (*v*/*v*) to neutralize the alkaline pH, centrifuged, and decanted. Further washing with ultrapure water was performed to remove any residual acid, and the pH was checked intermittently until it was close to neutral. The solid residue was then freeze-dried and stored for subsequent use.

2.3.3. Determination of Structural Carbohydrates and Lignin

The lignin and carbohydrate content of extractive- and protein-free distiller's spent grain (DSG) was determined using the National Renewable Energy Laboratory (NREL) protocol for the determination of lignin and carbohydrates in biomass [36]. Briefly, 300 mg of extractive- and protein-free DSG was mixed with 3 mL of 72% sulfuric acid and kept for 1 h in a water bath preheated to 30 °C, with stirring every 5–10 min without removing the sample from the bath. After acid hydrolysis, the samples were removed from the water bath and diluted to a 4% concentration by adding 84 mL of deionized water. The mixture was then autoclaved at 121 °C for 1 h. After autoclaving, the samples were cooled and vacuum-filtered using pre-weighed and pre-dried filtering crucibles to separate the filtrate from the solid residue. The solid residue was dried in an oven at 105 °C until a constant weight was achieved, and this was measured as acid-insoluble residue (AIR).

The crucible containing the acid-insoluble residue was placed in a muffle furnace equipped with a ramping program at 575 °C (SNOLTherm, Utena, Lithuania). After ashing, the crucible was cooled, and the weight of the ash was recorded. The acid-insoluble lignin (AIL) was calculated by subtracting the weight of the ash from the weight of the acid-insoluble residue (AIR). Acid-soluble lignin (ASL) was determined by diluting 100 µL of the filtrate with ultrapure water (1:20 dilution). The absorbance of the diluted filtrate was measured at a wavelength of 280 nm using a UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) [37]. The total lignin content on an extractive-free basis was calculated as the sum of acid-soluble lignin (ASL) and acid-insoluble lignin (AIL) using Equation (5).

The carbohydrates in the filtrate were determined as cellulose (glucose content) and hemicellulose (the total sum of galactose, arabinose, and xylose) using Ultra High-Performance Liquid Chromatography (UHPLC, 1290, Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD and RI detector set at 35 °C. Ten milliliters (10 mL) of the filtrate was neutralized with calcium carbonate, centrifuged, and filtered using a 0.2 µm PTFE syringe filter (Sigma Aldrich, Darmstadt, Germany). The sugars were separated using a Pb-based carbohydrate analysis column (Aminex HPX-87P, BioRad, Richmond, CA, USA) at 80 °C with ultrapure water as the mobile phase at a flow rate of 0.6 mL/min. The concentration of each sugar was calculated from the area of the peaks using the equation of the line from the calibration curves of glucose, xylose, arabinose, and galactose standards. The percentage of sugars was calculated as the sum of glucose, xylose, arabinose, and galactose.

$$\% \text{ Lignin (extractives and protein-free basis)} = (\% \text{ AIL} + \% \text{ ASL}) \quad (5)$$

where
% Extractives = percent extractives in the raw DSG sample.
% Protein = percent protein in the raw DSG sample.
% AIL = percent acid-insoluble lignin in the extractive- and protein-free DSG.
% ASL = percent acid-soluble lignin in the extractive- and protein-free DSG.

2.4. Principal Component Analysis

Principal component analysis (PCA) was carried out to derive crucial insights into DSG samples collected from nine distinct distilleries. The characterization data such as the protein, cellulose, hemicellulose, lignin, and extractive content of the DSG samples from each distillery were utilized for conducting PCA. PCA was performed in OriginPro (Version 2024, OriginLab Corporation, Northampton, MA, USA).

2.5. Statistical Analysis

A one-way ANOVA was performed to compare the means of the DSG compositions from the nine distilleries, followed by a Tukey pairwise comparison using Minitab statistical software Version 19 (Minitab Inc., State College, PA, USA). The analysis was conducted

with an overall significance level of 95%. All experiments were performed in duplicate unless otherwise stated. Results are reported as mean ± standard deviation.

3. Results and Discussion

3.1. Composition Analysis

The results of the proximate and structural composition analysis of DSG obtained from nine different distilleries are presented in Tables 2 and 3, respectively. The moisture content of fresh DSG ranged from 73.22 to 80.17%, ash content from 2.01 to 4.11%, extractives from 17.33 to 31.77%, crude protein from 12.38 to 26.32%, cellulose from 11.75 to 32.75%, hemicellulose from 6.97 to 19.47%, and lignin from 8.44 to 15.71%, while the total phenolic content ranged from 1.42 to 3.91 mg GAE/g. The results show that the samples varied widely in their composition and were significantly different ($p < 0.05$), except for moisture and ash content. Several studies have also reported variations in spent grain composition. A comparison of spent grain composition from various studies is presented in Table 4.

Table 2. Proximate composition of DSG.

Sample Code	Moisture Content of Fresh DSG (%)	Fat (%)	Protein (%)	Ash (%)	Total Phenolics MgGAE/g DSG
D1	80.17 ± 1.57 ^{a}	13.63 ± 0.47 ^{b}	17.93 ± 1.06 ^{c}	3.28 ± 0.10 ^{a}	2.25 ± 0.17 ^{ab}
D2	75.54 ± 0.593 ^{b}	13.54 ± 0.20 ^{b}	18.28 ± 0.67 ^{bc}	3.95 ± 0.32 ^{a}	2.60 ± 0.05 ^{ab}
D3	75.02 ± 0.163 ^{b}	10.98 ± 0.57 ^{c}	24.73 ± 0.03 ^{a}	3.53 ± 0.51 ^{a}	2.61 ± 1.15 ^{ab}
D4	73.22 ± 0.192 ^{b}	10.01 ± 0.02 ^{cd}	18.46 ± 0.05 ^{bc}	3.69 ± 0.06 ^{a}	3.91 ± 0.01 ^{a}
D5	75.7 ± 0.367 ^{b}	8.96 ± 0.023 ^{d}	20.32 ± 0.65 ^{b}	3.78 ± 0.13 ^{a}	3.37 ± 0.46 ^{a}
D6	75.01 ± 0.085 ^{b}	13.49 ± 0.29 ^{b}	25.91 ± 0.01 ^{a}	4.11 ± 0.06 ^{a}	1.42 ± 0.15 ^{b}
D7	73.49 ± 0.645 ^{b}	14.92 ± 0.11 ^{a}	18.25 ± 0.21 ^{bc}	3.64 ± 0.47 ^{a}	3.74 ± 0.44 ^{a}
D8	74.71 ± 0.760 ^{b}	13.01 ± 0.06 ^{b}	12.38 ± 0.98 ^{d}	2.01 ± 0.09 ^{b}	2.27 ± 0.01 ^{ab}
D9	74.95 ± 0.487 ^{b}	10.17 ± 0.45 ^{cd}	26.32 ± 0.16 ^{a}	3.21 ± 0.11 ^{a}	3.29 ± 0.13 ^{a}

Means followed by different superscript letters within the same column indicate significant differences ($p < 0.05$).

Table 3. Composition analysis of DSG from the 9 different distilleries.

Distillery	Protein	Water/Ethanol Extractives	Cellulose	Hemicellulose	Lignin
D1	17.93 ± 1.06 ^{c}	24.77 ± 0.61 ^{bc}	20.2 ± 0.20 ^{cd}	17.07 ± 3.03 ^{ab}	14.04 ± 0.15 ^{a}
D2	18.28 ± 0.67 ^{bc}	31.14 ± 2.98 ^{a}	16.2 ± 1.93 ^{e}	16.07 ± 2.79 ^{abc}	12.89 ± 0.71 ^{abc}
D3	24.73 ± 0.03 ^{a}	17.33 ± 0.41 ^{d}	20.6 ± 0.12 ^{c}	19.47 ± 0.49 ^{a}	15.71 ± 0.65 ^{a}
D4	18.46 ± 0.05 ^{bc}	30.28 ± 0.13 ^{a}	16.5 ± 0.09 ^{de}	13.27 ± 0.01 ^{bc}	10.44 ± 0.27 ^{bcd}
D5	20.32 ± 0.65 ^{b}	24.74 ± 0.52 ^{bc}	22.6 ± 2.05 ^{bc}	12.31 ± 0.71 ^{bcd}	9.76 ± 1.39 ^{cd}
D6	25.91 ± 0 ^{a}	30.63 ± 1.54 ^{a}	11.8 ± 0.39 ^{f}	11.07 ± 0.41 ^{cd}	9.95 ± 1.01 ^{cd}
D7	18.25 ± 0.21 ^{bc}	29.16 ± 1.46 ^{ab}	19.2 ± 0.31 ^{cde}	15.41 ± 0.48 ^{abc}	13.45 ± 1.42 ^{ab}
D8	12.38 ± 0.98 ^{d}	31.77 ± 0.27 ^{a}	32.7 ± 0.49 ^{a}	6.97 ± 0.15 ^{d}	8.44 ± 0.51 ^{d}
D9	26.32 ± 0.16 ^{a}	20.32 ± 0.96 ^{cd}	25.48 ± 0.80 ^{b}	12.18 ± 0.27 ^{bcd}	13.99 ± 0.91 ^{a}

Means followed by different superscript letters within the same column indicate significant differences ($p < 0.05$). The protein and extractives were determined from raw samples. Cellulose, hemicellulose, and lignin were determined from protein- and extractive-free samples and reported on a raw basis.

Due to its high moisture content, fresh DSG is very bulky and susceptible to rapid deterioration within five days, posing storage challenges. Therefore, fresh DSG must be stabilized to improve storage potential and facilitate its utilization. Drying is one of the most common methods employed to reduce moisture content, and bulk weight, and improve stability. However, drying is costly as it requires significant energy to evaporate water. Mechanical water removal processes, such as pressing, which require far less energy, can be employed before drying [38]. In mechanical pressing, fresh DSG is placed under a mechanical pressing device to expel some of the water and reduce the initial moisture content to about 31–43%, depending on the pressing duration and applied pressure [38].

The most common drying methods include oven drying, infrared drying, and freeze drying [39]. Drying has been found to influence the chemical composition of DSG by making dried samples more nutrient-dense than fresh DSG. For example, the drying of Indian almonds was reported to improve protein availability, attributed to the ability of drying to make foods more nutrient-dense [39]. Other preservation methods include freezing, ensiling, and the use of various additives and preservative chemicals such as lime, sodium formate, calcium propionate, formic acid, propionic acid, and acetic acid [40].

Extractives are non-structural biomass components that are soluble in water and organic solvents. Water-soluble materials may include inorganic compounds, non-structural sugars, and nitrogenous materials. Water- and ethanol-soluble materials also include chlorophyll, lipids, waxes, and other minor components. In this study, the water- and ethanol-soluble extractive content ranged from 17.33% to 31.77%. Spent grain samples with a higher percentage of malted barley showed higher extractive content (24.77–31.77%), while samples with a lower percentage of malted barley exhibited lower extractive content.

Similarly, high extractive values (34.8%) were reported by Kim et al. [41] for corn-based distillers' grains with solubles obtained from ethanol plants. In their study, Kim et al. [41] also noted that DSG contains more water-soluble extractives than organic solvent-soluble extractives, whereas Cervantes-Ramirez et al. [42] reported 24.1% water- and ethanol-soluble extractives, with a higher proportion of ethanol-soluble extractives.

The protein content of DSG ranges from 12.38% to 26.32%, with an average protein content of 20.29% across the nine distilleries. The protein content of six samples (D1, D2, D4, D5, D7, and D8) was significantly different from each other, while three samples (D3, D6, and D9) showed no significant differences. The results indicate that a substantial variation (67%) exists in the protein content of DSG among the distilleries, regardless of similarities in the starting grains. A similar variation in protein content was reported by Crane d'Heysselaer et al. [43] for brewer's spent grain.

The highest protein content (26.32%) was obtained from DSG composed of 40% malted barley, 55% unmalted barley, 1.75% rye, and 3.25% oat (D9). The lowest protein content (12.38%) was observed in DSG from Distillery 8, which used 100% malted barley. The protein content values obtained in this study align with those reported by Santos et al. [44], who found values of 24.2%, 21.8%, and 26.4% for oven-dried, freeze-dried, and frozen brewer's spent grain (BSG) samples, respectively. Similarly, Castro and Colpini [45] reported a protein content of 18.14 $\pm$ 0.25% for BSG obtained from a single brewery in Brazil.

Previous studies have found that DSG contains approximately 15% to 25% protein, making it a valuable source of animal feed [46]. The proteins in DSG are similar to those found in the original barley grain, including globulins, albumins, glutelins, and hordeins. However, the protein concentration in fresh distillers' grain is often higher than that of the original barley (9.65%) and barley malt (8.52%) because most of the starch is removed during the mashing stage, leaving a spent grain with a higher protein concentration and lower starch content [47].

Jaeger et al. [47] reported that protein accounts for approximately 19–30% of brewer's spent grain, whereas Waters et al. [48] reported a protein composition of 22.13%, and Xiros et al. [49] reported a value of 14.2%. Although the protein content of DSG in this study is not as high as that of protein-rich legumes such as soybeans, the protein content of DSG is appreciable as a waste material. It could be explored for various industrial applications and has the potential to serve as a cost-effective source of plant protein, offering benefits for nutrition, sustainability, and the economy.

Cellulose is a major component of spent distillers' grains (DSG), originating from the cereals used in mashing. In this study, cellulose content ranged from 11.75% to 32.75%. The highest cellulose content (32.75%) was obtained from Distillery 9, which used 100% malted barley, while the lowest cellulose content (11.75%) was observed in Distillery 6, which used 60% malted barley and 40% unmalted barley. The cellulose content differed significantly, irrespective of similarities in the starting grain composition. However, operational technicalities between distilleries (e.g., craft vs. large-scale distilleries) may account for these

differences. The cellulose content in DSG can vary between batches from the same distillery and across different distilleries [26,44]. Factors such as the extent of starch removal during mashing and wort filtration can also influence cellulose content.

The range of cellulose content observed in this study compares favorably with previously reported values: 16.8% by Mussatto [46], 19.21% by Ravindran et al. [50], 13.8% by Cervantes-Ramirez et al. [42], and 21.84% by Outeiriño et al. [51]. However, the cellulose content of DSG is lower than that of some lignocellulosic biomass sources, such as sugarcane bagasse (40–50%) and barley straw (31.09%) [52]. Nevertheless, for a waste product, DSG presents an affordable cellulose source for synthesizing bioproducts.

Hemicellulose is the second most abundant polysaccharide in DSG after cellulose. In this study, hemicellulose content ranged from 6.97% to 19.47%. Significant differences in hemicellulose composition were observed among DSG samples from the various distilleries, despite similarities in the starting grain. Xylose was the most abundant hemicellulose backbone in DSG (70–80%), followed by arabinose (20–30%) and a small amount of galactose. These findings are consistent with values reported by Mishra et al. [53], who documented a 9.8% hemicellulose content in brewer's spent grain. Similarly, Castro and Colpini [45] reported a hemicellulose content of 9.44% in brewer's spent grain from a single brewery. However, other studies have reported higher hemicellulose content for spent grain from similar sources. For example, Mussatto et al. [54,55], Crane d'Heysselaer et al. [43], Ravindran et al. [50], and Lynch et al. [56] reported hemicellulose content values of 28.4%, 22.7%, 26.94%, and 26.4%, respectively.

Table 4. Comparison of spent grain composition from various studies.

	Composition %						
	Protein	**Cellulose**	**Hemicellulose**	**Lignin**	**Ash**	**Extractives**	**Reference**
DSG	12.38–26.32	11.75–32.75	6.97–19.47	8.44–15.71	2.01–4.11	17.33–31.77	This work
BSG	15.25	16.8	28.4	27.8	4.6	5.82	[54]
BSG	20.3	11.1	22.7	13.8	4.7	35.5	[43]
BSG	NR	19.21	26.94	30.48	NR	NR	[50]
BSG	14.2	12	40	11.5	NR	NR	[49]
BSG	28	13.2	16.75	NR	4.5	34.8	[43]
BSG	NR	21.84	30.43	25.26	4.41	NR	[51]
BSG	22.54–30.24	NR	NR	NR	3.30–4.29	NR	[26]

The variation in hemicellulose content is attributed to factors such as grain type and variety, processing conditions, and malting techniques.

Lignin is one of the main components of plant cell walls, a natural phenolic, and the second most abundant biopolymer in plants after cellulose [57]. In this study, the lignin content of DSG ranged from 8.44% to 15.71% across the nine samples. The results show that most samples were significantly different ($p < 0.05$) in lignin content, irrespective of the starting grain type. The highest lignin content (15.71%) was observed in DSG from Distillery 3 (single pot whiskey), while the lowest lignin content (8.44%) was found in DSG from Distillery 8 (single malt). The lignin content of lignocellulose biomass is influenced by the biomass type. In this study, the lignin in DSG showed a higher proportion of acid-insoluble lignin compared to acid-soluble lignin. However, no specific relationship could be established between the starting grain type and the lignin content, as similar grains exhibited variations in lignin levels.

Lignin has diverse application potentials, including uses in energy production, construction (e.g., adhesives, binders, cement), bioremediation, polymer synthesis (e.g., polyurethane, polylactic acid, polyethylene, polypropylene), biomedicine (e.g., drug carriers, hydrogels, delivery systems), and the food industry (e.g., antioxidants, antimicrobials), among others [58].

DSG is also a rich source of phenolic compounds, which are known for their antioxidant, antiradical, anticarcinogenic, and anti-apoptotic properties [6]. These phenolics are predominantly located in the barley husk, where they accumulate in the cell walls. They are often bound to sugar residues, which reduces their antioxidant efficacy as free hydroxyl groups are required to stabilize free radicals [59]. The primary phenolic compounds in DSG are hydroxycinnamic acids, with ferulic acid being the most prevalent. Ferulic acid is strongly correlated with the antioxidant capacity of cereals [60].

In this study, the total phenolic content (TPC) of DSG ranged from 1.42 to 3.91 mg GAE/g DSG. The phenolic content did not vary significantly across the samples. These results align with findings by Chetrariu and Dabija [33], who reported TPC values ranging from 0.62 to 1.76 mg GAE/g SG DW for Ultra-Turrax extraction and 0.57 to 2.11 mg GAE/g SG DW for ultrasound-assisted extraction of spent grain from malt whiskey. Similarly, Carciochi et al. [61] used 72% (*v*/*v*) aqueous ethanol at 80 °C to extract phenolic compounds from brewer's spent grain derived from pale barley malt and reported TPC values of 4.11, 3.91, and 3.62 mg GAE/g for ultrasound extraction, microwave-assisted extraction, and batch extraction, respectively. Conclusively, DSG represents a cost-effective source of valuable phenolic compounds.

3.2. Principal Component Analysis

Principal component analysis (PCA) is an effective technique used in pattern recognition to identify complex datasets with interrelated variables. It is a statistical approach that derives eigenvalues and eigenvectors from the correlation matrix of the dependent variables. The principal component (PC) is formed by creating a linear combination of the original variables, achieved by multiplying the variables by their corresponding eigenvector [62].

PCA was conducted using the data provided in Table 3, with the mean values of each variable from each distillery used for the analysis. The Scree plot and Biplot derived from the PCA are illustrated in Figures 2 and 3, respectively. The Scree plot revealed that the eigenvalues for only PC1 and PC2 were greater than 1. Consequently, only two principal components were extracted in this study. PC1 accounted for 56.28% of the variance, while PC2 accounted for 24.37% of the variance (Figure 3). Together, PC1 and PC2 explained approximately 80.65% of the variance in the dataset, which is generally considered satisfactory. The eigenvectors extracted from PCA are provided in Table 4.

It was observed that protein, hemicellulose, and lignin were the primary contributors to the variance in PC1, with positive loading values. The largest contributor to PC1 was lignin, followed by hemicellulose and protein. In contrast, the largest contributor to the variance in PC2 was cellulose, followed by lignin, both with positive loading values. However, extractive content was negatively associated with both PC1 and PC2, as indicated by negative loading values.

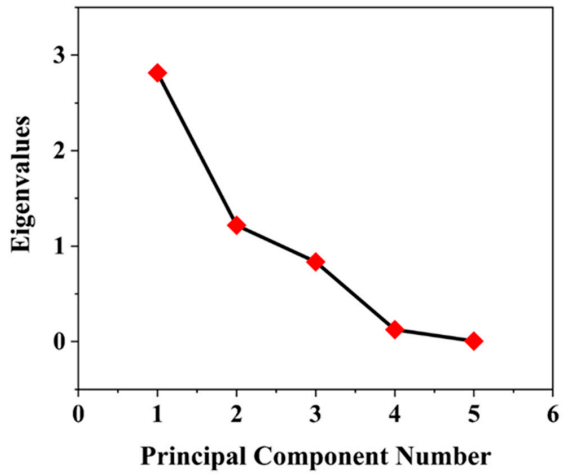


Figure 2. Scree plot.

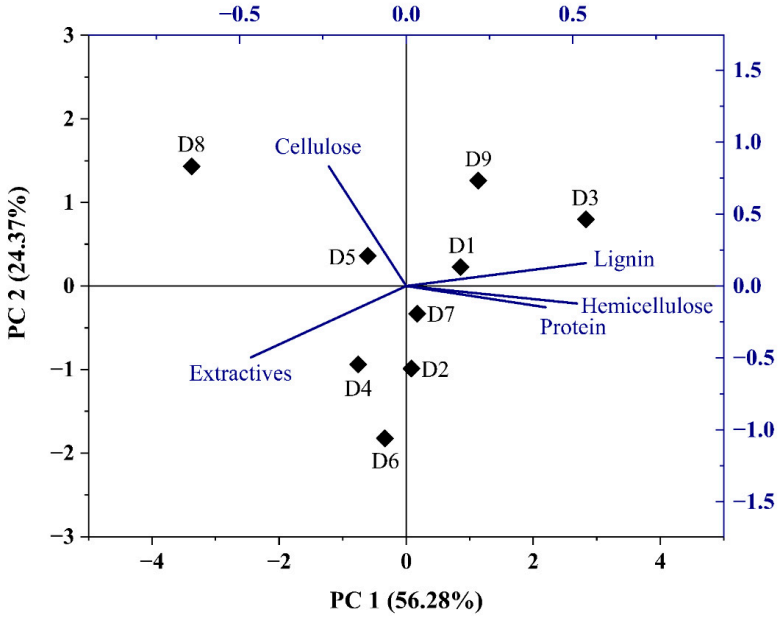


Figure 3. Biplot derived from PCA.

The positive loading values of cellulose, hemicellulose, lignin, and protein in either PC1 or PC2 demonstrate that the PCA model effectively extracted meaningful insights from the data, highlighting the similarities or differences among samples from various distilleries. From Figure 3, it was observed that no distinct clusters or groupings were formed among the distilleries. This finding indicates that the DSG samples obtained from the nine distilleries were different, with no significant similarities among them.

4. Conclusions

This study provides a detailed analysis of the composition of DSG obtained from nine distilleries, emphasizing its variability and potential as a valuable resource for biorefinery applications. The findings reveal significant differences in the proximate and structural composition of DSG, including moisture, protein, cellulose, hemicellulose, lignin, and extractive content, which can be attributed to variations in processing techniques and raw material inputs across distilleries. Despite these differences, this study highlights the consistent presence of key components like protein, cellulose, and lignin, underscoring DSG's suitability as a feedstock for the synthesis of high-value bioproducts. The high moisture content of fresh DSG poses storage and stability challenges, necessitating pretreatment processes such as drying and mechanical water removal. While drying improves nutrient density and stability, it is energy-intensive, highlighting the importance of optimizing cost-effective preservation methods. This study also underscores the potential of DSG as a rich source of phenolic compounds and plant-based proteins, which can be harnessed for diverse applications in food, biomedicine, biopolymers, and energy sectors. PCA further demonstrated that protein, lignin, cellulose, and hemicellulose are the primary contributors to the compositional variance among samples, with no discernible clustering of distilleries based on their DSG composition. This variability reinforces the importance of characterizing DSG from multiple sources to develop standardized and tailored approaches for its utilization in biorefinery operations. This study highlights the potential of DSG as a cost-effective and sustainable resource for the development of bioproducts, contributing to the circular bioeconomy. By addressing challenges related to variability and storage, this study lays a foundation for the broader industrial application of DSG, aligning with global efforts to promote sustainability, reduce waste, and create value from industrial byproducts. Future research could focus on optimizing processing techniques to enhance the recovery of valuable components and exploring innovative applications to maximize the economic and environmental benefits of DSG.

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