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Rapid NMR Method for the Quantification of Organic Compounds in Thin Stillage

Kornsulee Ratanapariyanuch,[†] Jianheng Shen,[‡] Yunhua Jia,[‡] Robert T. Tyler,[†] Youn Young Shim,*[,‡] and Martin J. T. Reaney*[,‡]

[†]Department of Food and Bioproduct Sciences and [‡]Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, Saskatchewan S7N 5A8 Canada

ABSTRACT: Thin stillage contains organic and inorganic compounds, some of which may be valuable fermentation coproducts. This study describes a thorough analysis of the major solutes present in thin stillage as revealed by NMR and HPLC. The concentration of charged and neutral organic compounds in thin stillage was determined by excitation sculpting NMR methods (double pulse field gradient spin echo). Compounds identified by NMR included isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, glycerophosphorylcholine, betaine, glycerol, and 2-phenylethanol. The concentrations of lactic and acetic acid determined with NMR were comparable to those determined using HPLC. HPLC and NMR were complementary, as more compounds were identified using both methods. NMR analysis revealed that stillage contained the nitrogenous organic compounds betaine and glycerophosphorylcholine, which contributed as much as 24% of the nitrogen present in the stillage. These compounds were not observed by HPLC analysis.

KEYWORDS: thin stillage, ethanol, 2-phenylethanol, glycerophosphorylcholine, HPLC, NMR

INTRODUCTION

Thin stillage is a liquid byproduct that remains after microbial ethanol fermentation of carbohydrates by yeast and subsequent distillation of the fermented mash.[1] Alcohol is openly produced from cereal grain. For example, wheat is the preferred raw material in western Canada.[2] Thin stillage contains salts, carbohydrates, proteins, and organic compounds. Ojowi et al.[3] conducted proximate analysis of samples of thin stillage from wheat-based ethanol and reported the dry matter, crude protein, ether extract, neutral detergent fiber, acid detergent fiber, crude fiber, ash, calcium, phosphorus, and magnesium contents. In addition, the stillage also contained the transition elements cobalt, zinc, manganese, and iron. Barley- and wheat-based thin stillage also were reported to contain a range of mineral constituents including calcium, phosphorus, magnesium, copper, iron, manganese, sodium, potassium, and zinc.[1]

The composition of organic compounds present in stillage has also been reported. Dowd et al.[4,5] used gas chromatography—mass spectroscopy and high-performance liquid chromatography (HPLC) to analyze the components of filtered stillage from sugarcane molasses, citrus waste, sweet whey, and corn. They found that the major components in cane stillage were lactic acid, glycerol, ethanol, and acetic acid, in decreasing order of concentration. In citrus stillage, the major components were lactic acid, glycerol, *myo*-inositol, acetic acid, *chiro*-inositol, and proline. Whey stillage included lactose, lactic acid, glycerol, acetic acid, glucose, arabinitol, and ribitol. Previous research used chromatographic methods to identify stillage components, whereas the current study describes the use of quantitative nuclear magnetic resonance (NMR) techniques. The authors are not aware of any other instances of the use of NMR to quantify organic stillage components.

NMR is a spectroscopic tool with the ability to analyze a large range of organic materials.[6−10] Although NMR equipment may be costly, each analysis is typically inexpensive as sample preparation may be simple and rapid.[9] Often, little or no sample pretreatment is required.[6] Due to its ability to detect numerous tissue metabolites, proton NMR spectroscopy has become a well-established method as a noninvasive technique of studying complex biological solutions. Quantitation of the NMR-observable metabolites can provide considerable biochemical information.[7] NMR analysis of dilute aqueous solutions has become a routine practice.[10] However, if large amounts of water are present in a sample, solvent suppression is required for NMR analysis to improve the dynamic range of the detector and to improve the spectra of metabolites that overlap with the broad baseline caused by the strong water resonance.[10]

The use of ^{1}H NMR with water suppression to identify and quantify the organic compounds in wheat-based thin stillage was one focus of this research. It was anticipated that the use of NMR would simplify the identification and quantification of organic constituents present in thin stillage. The ion content and properties of wheat-based thin stillage were also studied. The compositional and solution property data acquired should be useful for future studies of thin stillage.

MATERIALS AND METHODS

Sample Preparation. Wheat-based thin stillage was provided by Pound-Maker Agventures Ltd. (Lanigan, SK, Canada). Samples were obtained on four collection dates spaced 10 days apart (batches 1−4). Prior to analysis of any physical or chemical properties, or chemical constituents, samples were centrifuged at 1053*g* for 20 min at 4 °C in an

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Avanti centrifuge to separate particulate matter (Beckman Coulter Canada Inc., Mississauga, ON, Canada).

Physicochemical Analysis. The osmotic potential of centrifuged thin stillage samples was determined by freezing point depression.[11] Samples were frozen on a mixture of ice and NaCl. The freezing point and supercooling point of the samples were recorded using a digital thermometer. Osmotic potentials of the samples were calculated from the freezing point and the supercooling point. The viscosity of centrifuged thin stillage samples was measured at 25 °C using a Shell cup (Norcross Corp., Newton, MA). The Shell cup method utilizes a cup with a precision-drilled hole in the base. The cup is first immersed in the fluid of interest and the time required for the cup to drain after it is raised from the liquid is measured. Sample viscosity was determined using the Shell cup conversion chart that relates viscosity and the time required for the cup to drain. The density of centrifuged thin stillage was determined at 25 °C using a hydrometer with a range of 1.000−1.250 g/cm^3. The pH of each sample was measured at 21 °C using a pH-meter (VWR Scientific, Mississauga, ON, Canada). The electrode was calibrated before measurements using buffer solutions (pH 4.00, 7.00, and 10.00) from VWR Scientific. Total nitrogen content was determined according to the Kjeldahl method (Kjeltec Autoanalyzer, Foss Tecator, Hillerod, Denmark). Samples were digested by heating with concentrated H_2SO_4 in a heating/digestion block using a package of Kjeldahl digestion mixture 200 (VWR International) as catalyst. After digestion, samples were distilled using a steam distillation unit (Büchi Analytical Inc., New Castle, DE) with 30% (w/v) NaOH. Boric acid (4%) was used to trap ammonia from the distillation. The distillate was titrated with 0.2 N HCl using an N-point indicator (Titristar N point indicator, EMD Chemicals Inc., Gibbstown, NJ). The protein content was calculated from the percentage of total nitrogen by multiplication by the conversion factor of 5.7 for wheat grain.[12] The preceding method was modified from AOAC method 981.10.[13] Moisture content was determined according to a modified version of AOAC method 925.23.[13] Three grams of sample was placed into predried aluminum pans. The samples were dried in a vacuum oven at 100 °C for 5 h until the weight was constant. All of the experiments were conducted in triplicate, and the data are presented as the mean value ± standard deviation (SD).

Isolation of Glycerophosphorylcholine. Thin stillage was lyophilized and then extracted with methanol. The resulting methanol solution was concentrated under vacuum and subjected to preparative thin layer chromatography (PTLC, 20 cm × 20 cm). PTLC plates were developed twice in 30% methanol in dichloromethane as the developing solvent. Five apparent bands, A, B, C, D, and E, in order of decreasing polarity, were obtained (data not shown). Each apparent band was scraped into a sintered glass funnel and washed with methanol. The methanol solution was evaporated under vacuum, and the resulting solids or syrups were prepared for ^{1}H NMR analysis. On the basis of ^{1}H NMR analysis, the least polar compound (band E) was pure glycerol. The less polar bands contained mixtures of compounds. Apparent bands D and C both contained lactic acid. Band B was a mixture containing betaine (BTN), a small amount of lactic acid, and trace amounts of sugars. The most polar band, A, was a mixture containing predominantly glycerophosphorylcholine (GPC).

NMR Analysis. The components of centrifuged thin stillage were determined by ^{1}H NMR analysis. The strong singlet peaks of water present in the NMR spectra were mitigated using double pulse field gradient spin echo (DPFGSE)[14] as embodied in Bruker Xwin-NMR software (Bruker, Mississauga, ON, Canada). Prior to analysis, samples were passed through a 0.45 μM pore size PTFE Acrodisc filter (Pall Corp., Ann Arbor, MI) and 0.5 mL of sample was mixed with 0.05 mL of deuterium oxide (D_2O, 99.8%) to provide a locking signal and 0.05 mL of dimethylformamide (DMF) (762.5 mg of DMF in 25 mL of stillage) as internal standard. NMR proton saturation of water using a frequency of 4.7 ppm was used to greatly decrease the water peak in the spectrum. In this research, eight scans were used in generating each spectrum. Isopropanol, ethanol, glycerol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, BTN, and 2-phenylethanol (2-PE) were used as standards. ^{1}H NMR (1D) spectra were recorded at 500 MHz with a Bruker AM500 spectrometer, and two-dimensional (2D) correlation spectroscopy (COSY) was used to identify and confirm the identification of the compounds present in thin stillage. Homonuclear shift correlation (2D ^{1}H/^{1}H COSY) experiments were recorded using Xwin-NMR software. The NMR results were compared with results from HPLC analysis of ethanol, lactic acid, and acetic acid.

Nonprotein Nitrogen. The presence of GPC and BTN contributed to the nitrogen content of stillage. These compounds both would be considered as nonprotein nitrogen (NPN) sources and should be removed from the total nitrogen present before protein content is calculated. BTN is 12% nitrogen, whereas GPC is 5.4% nitrogen. The contribution of these compounds to stillage total nitrogen (N_T) was calculated using NMR data.

HPLC Analysis. Ethanol, lactic acid, glycerol, glucose, dextrin, maltose monohydrate, and acetic acid concentrations were analyzed according to Narendranath et al.[15] The isocratic solvent was delivered by a single HPLC pump at a flow rate of 1 mL/min of 5 mM sulfuric acid connected to an HPX-87H column (Bio-Rad Laboratories, Mississauga, ON, Canada) and a refractive index detector (Waters Chromatographic Division, Milford, MA). HPLC was monitored with Waters Millennium32 software.

Ion Content. Thin stillage samples were centrifuged at 7690*g* for 20 min at 4 °C and filtered through a 0.2 μm filter membrane. The anion (chloride, sulfate, nitrate, hydroxide, bicarbonate, and carbonate) and cation (calcium, magnesium, sodium, and potassium) contents of thin stillage were analyzed using ion chromatography and inductively coupled plasma atomic emission spectroscopy services of the Saskatchewan Research Council (Saskatoon, SK, Canada).

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. The physical and chemical properties of four batches of centrifuged thin stillage were analyzed (Table 1). Each of the four batches had similar properties. The osmotic potential and viscosity of thin stillage ranged from −0.97 ± 0.09 to −0.94 ± 0.01 MPa and from 1.61 ± 0.03 to 1.72 ± 0.14 cP centipoise (cP), respectively. The density of all batches at 25 °C was 1.01 ± 0.03 g/cm^3. Differences in physical and chemical properties among batches of thin stillage

Table 1. Physical and Chemical Characteristics of Four Batches of Thin Stillage[a]

characteristic	batch 1	batch 2	batch 3	batch 4
osmotic potential (MPa)	−0.96 ± 0.01	−0.97 ± 0.09	−0.95 ± 0.08	−0.94 ± 0.01
viscosity (cP)	1.71 ± 0.12	1.61 ± 0.03	1.72 ± 0.14	1.61 ± 0.05
density (g/cm^3)	1.01 ± 0.03	1.01 ± 0.01	1.01 ± 0.04	1.01 ± 0.03
pH	3.85 ± 0.01	3.97 ± 0.09	3.76 ± 0.01	3.78 ± 0.10

[a] Values are the means of triplicate determinations with standard deviations (SD) of a single sample of each batch.

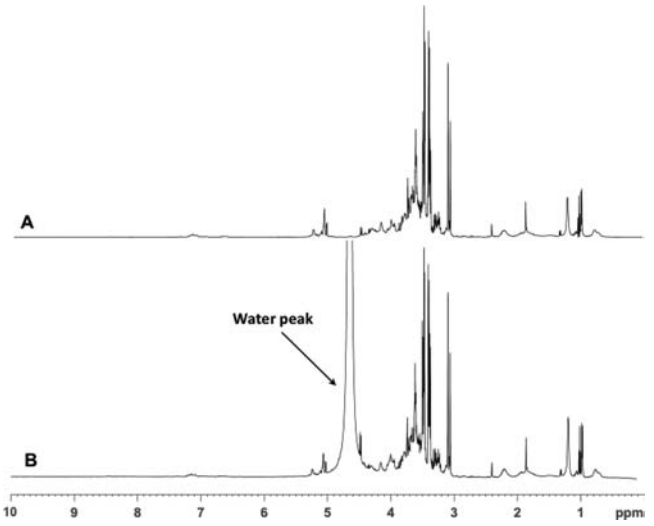

Figure 1. ^{1}H NMR spectra of thin stillage (batch 1) recorded at 500 MHz: (A) with water suppression; (B) without water suppression.

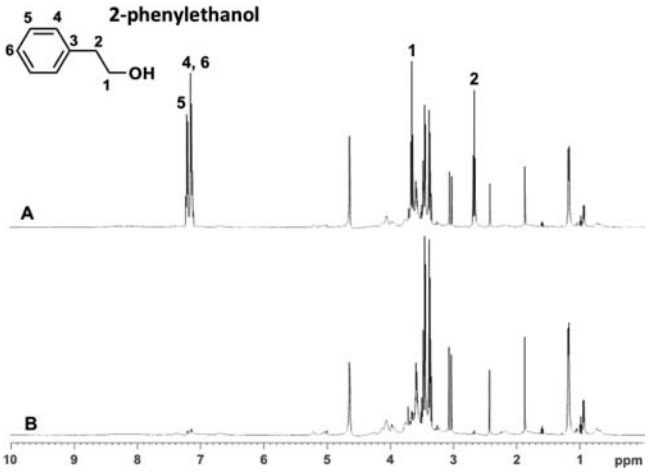

Figure 2. ^{1}H NMR spectra of thin stillage (batch 1) recorded at 500 MHz: (A) with added 2-phenylethanol (2-PE); (B) without added 2-PE.

were small and might have been caused by variations in the raw material used in the production of each batch of ethanol. The presence of organic acids, minerals, and carbohydrates decreased the osmotic potential and increased the viscosity and density of the thin stillage relative to pure water. The viscosity and density of water at 25 °C are 0.8903 cP[16] and 0.997048 g/cm^3[3,17] respectively. Thin stillage used in this research was acidic (pH 3.76 ± 0.01−3.97 ± 0.09; Table 1), due to the presence of organic acids. Thin stillage has been reported to contain organic acids such as lactic acid and acetic acid.[4] In agreement with the literature, lactic acid was the main organic acid identified in this study. This result was consistent with the work of Narendranath et al.,[15] who stated that lactic acid accumulation lowers pH. Jones and Ingledew reported that the range of pH of thin stillage from wheat and corn was 3.6−4.7.[18]

Organic Compounds Present in Thin Stillage. A thorough analysis of thin stillage requires the identification and quantification of the organic compounds present. Narendranath et al.[15] used chromatographic methods to identify stillage components, whereas the current study employs quantitative NMR spectroscopy for this purpose. Although NMR analysis of dilute aqueous solutions has become a routine practice,[10] the authors are not aware of prior instances of the utilization of NMR to quantify organic components in thin stillage. Analysis of constituents of aqueous solutions using NMR may require the use of extra preparative steps. When using proton NMR, for example, the strongest signal arises from the protons of water. The Fourier transform algorithm, used to interpret the spectrum, normalizes the whole spectrum intensity to the strongest signal in the spectrum. Therefore, the presence of large amounts of solvent (water) will reduce the sensitivity of the spectrometer to constituents at lower concentrations. Although it is possible to concentrate the dilute solution and replace the water with deuterated water, it is also possible to utilize electronic methods (suppression pulse sequences) to decrease the intensity of a solvent peak. The proton signals of water present in NMR spectra of centrifuged and filtered thin stillage were too large to allow for accurate analysis of stillage organic constituents. Therefore, a water suppression pulse sequence was employed to suppress the water peak. This method had two important consequences. First, it increased the signal-to-noise ratio of the whole spectrum, and, second, it allowed the observation and quantitative analysis of peaks that were close to the water signal. From the ^{1}H NMR spectrum of thin stillage, it was observed that the area of the water peak (4.7 ppm) prevented observations of peaks between approximately 3.7 and 5.7 ppm when water suppression was not performed (Figure 1).

The thin stillage NMR spectrum indicated the presence of several organic components. By adding standard compounds to thin stillage, observing the impact on ^{1}H NMR signals, and using ^{1}H NMR and the COSY techniques (2D NMR spectrum), it was possible to conclusively identify each compound in thin stillage. Pure standards of isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, BTN, glycerol, and 2-PE were added to particulate-free thin stillage. The ^{1}H NMR spectra and 2D NMR spectra of thin stillage prior to and following the addition of each of the organic chemicals were recorded. Proton spectra of thin stillage, with and without added 2-PE, are presented to confirm the presence of a compound in thin stillage with the NMR method (Figure 2). The spectra used to confirm the presence of the remaining nine compounds are not presented in this paper. In the conduct of this research, we identified a proton singlet at 3.03 ppm that could not be correlated with any published constituent of stillage. This last compound was isolated by PTLC. The compound was identified as GPC. To our knowledge, this is the first report of large quantities of GPC being present in thin stillage. The identification of this compound provides strong evidence of the benefits of NMR analysis of thin stillage and other solutions.

Identity Confirmed by ^{1}H NMR and 2D NMR. The computer-estimated chemical shifts of 2-PE protons are presented in Figure 2. As shown, the ^{1}H NMR of 2-PE has peaks with chemical shifts of 2.77, 3.66, 4.58, 7.27, 7.29, and 7.40 ppm. When 2-PE was added to thin stillage, peaks at these chemical shifts were increased. In addition, 2D NMR spectra could be used to show the interaction of protons on adjacent carbons to further confirm the identification of a compound. The COSY spectrum of thin stillage (Figure 3) revealed the presence of 2-PE with the correlated peaks observed at *x*, *y* coordinates of 7.29, 3.66, and 2.77 ppm. Furthermore, ^{1}H NMR spectra of thin stillage taken after the addition of isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, BTN, and

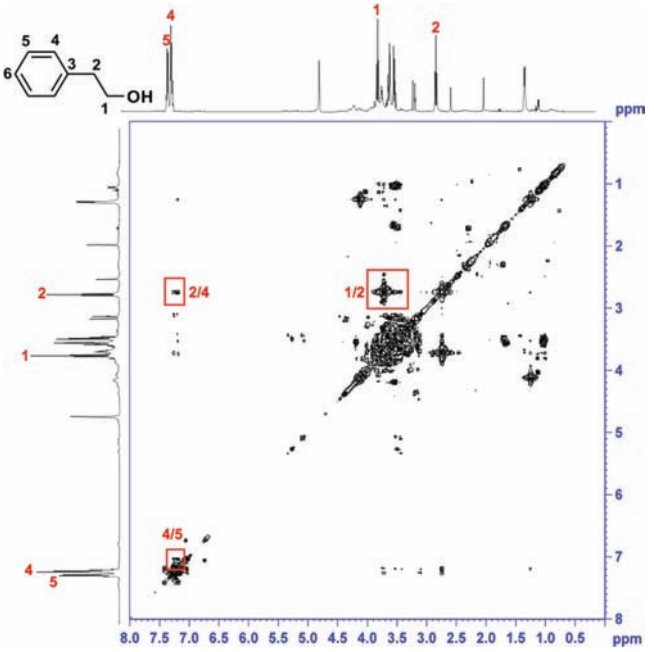

Figure 3. 2D ^{1}H NMR spectrum of thin stillage with added 2-phenylethanol.

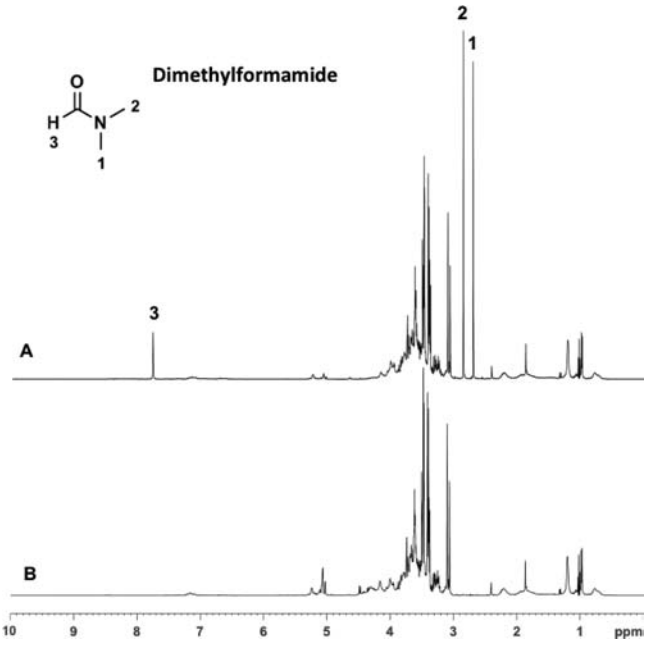

Figure 4. ^{1}H NMR spectra of thin stillage (batch 1) recorded at 500 MHz: (A) with added dimethylformamide (DMF); (B) without added DMF.

glycerol showed increased peak areas with chemical shifts of 1.02 (isopropanol), 1.1 (ethanol), 1.2 (lactic acid), 1.5−1.8 (1,3 propanediol), 2.05−2.09 (acetic acid), 2.47 (succinic acid), 3.03 (GPC), 3.07 (BTN), 3.3−3.6 (glycerol), and 7.06−7.36 ppm (2-PE) when compared with spectra of particulate-free thin stillage. In addition, 2D NMR spectra confirmed the results from the ^{1}H NMR spectra. Therefore, it was demonstrated that the thin stillage contained isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, BTN, glycerol, and 2-PE. According to Lovitt et al., isopropanol found in thin stillage might be a fermentation product of thermophilic anaerobic bacteria (*Clostridium thermohydrosulfuricum*).[19] The residual ethanol in thin stillage might be due to an incomplete distillation process.[20] Lactic acid and acetic acid found in thin stillage were produced by lactic acid-producing and acetic acid-producing bacteria, which are common resident microbes in fuel alcohol plants.[21] In addition, Narendranath et al.[15] stated that acetic acid is also an end product from yeast (*Saccharomyces cerevisiae*) fermentation. 1,3-Propanediol in thin stillage might be a fermentation product from *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter agglomerans*, and/or *Clostridium butyricum* using glycerol or another carbohydrate as a nutrient source.[22−25] Succinic acid is the second most abundant end product of alcohol fermentation synthesized and secreted by yeast via the Krebs cycle.[26] GPC might be synthesized from deacylation of phosphatidylcholine in *S. cerevisiae*.[27] Glycerol is produced by yeast by the reduction of dihydroxyacetone phosphate to glycerol phosphate followed by dephosphorylation to glycerol.[26] BTN is a compound found in stillage as a coproduct from ethanol fermentation.[28] According to Paananen et al., BTN is found in the roots, seeds and stems of plants.[29] Furthermore, Seibel and Walsh reported that BTN accumulated in wheat through phosphatidylcholine hydrolysis.[30] 2-PE is produced by yeast (*S. cerevisiae*) through the Ehrlich pathway via bioconversion of L-phenylalanine.[31] In this pathway, L-phenylalanine is transaminated to phenylpyruvate, which is then decarboxylated to phenylacetaldehyde and finally reduced to 2-PE.[31] 2-PE is found at concentrations of 10−35 mg/L in all fermented products.[32] In addition, 2-PE was a residue from distillation of thin stillage according to Schrader et al. and Savina et al.[31,32]

The ^{1}H NMR technique also was used to quantify the constituents of thin stillage. DMF was chosen as the internal standard because the reported chemical shifts of DMF in D_2O (CH = 7.92, CH_3 = 3.01 and 2.85[33]) did not coincide with the chemical shifts of thin stillage constituents and the DMF spectral peaks are all singlets that have little impact on the baseline. Additionally, singlet peaks do not interfere with observations of proton correlation in COSY spectra. ^{1}H NMR spectra of thin stillage, with and without added DMF, are shown in Figure 4.

Comparison between NMR and HPLC Analyses. ^{1}H NMR results were compared with results from HPLC, the traditional method of quantifying and identifying organic compounds present in thin stillage. A typical HPLC chromatogram is provided in Figure 5. ^{1}H NMR and HPLC data are presented in Table 2. Stepping in the baseline of the HPLC chromatogram was the result of changes in the refractive index detector amplification setting made at 2, 10, and 25 min. NMR lacked sufficient resolution to accurately distinguish carbohydrates and glycerol that were readily observed by HPLC because the NMR proton frequencies of carbohydrates and glycerol overlapped. Variations were observed among stillage batches in the concentrations of thin stillage constituents by both analytical methods. The concentrations of lactic acid and acetic acid from HPLC confirmed the ^{1}H NMR results. Therefore, the NMR technique can be used to quantify some compounds present in thin stillage. However, HPLC and NMR determinations of ethanol concentration were not comparable. This might be explained by slow ethanol production in samples before analysis was completed.

HPLC results also identified the residual carbohydrates present in thin stillage after fermentation (maltotetraose and longer

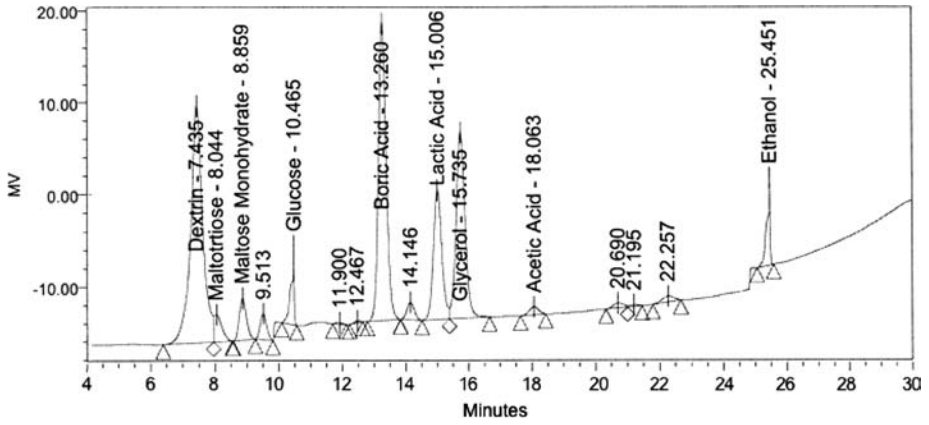

Figure 5. HPLC chromatogram of thin stillage.

Table 2. Organic Components of Four Batches of Thin Stillage Analyzed by ^{1}H NMR and HPLC

	amount of the compound[a] (g/L)							
	batch 1		batch 2		batch 3		batch 4	
component	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC
dextrin	ND[b]	11.65 ± 0.05	ND	9.31 ± 0.31	ND	10.7 ± 0.21	ND	8.47 ± 0.04
maltotriose	ND	0.73 ± 0.01	ND	1.10 ± 0.81	ND	0.53 ± 0.01	ND	0.14 ± 0.02
maltose monohydrate	ND	1.05 ± 0.01	ND	0.31 ± 0.01	ND	0.97 ± 0.03	ND	0.03 ± 0.00
glycerol	ND	7.87 ± 0.03	ND	6.21 ± 0.21	ND	6.92 ± 0.04	ND	2.39 ± 0.01
isopropanol	0.34 ± 0.01	ND	0.35 ± 0.01	ND	0.33 ± 0.01	ND	0.31 ± 0.01	ND
ethanol	0.24 ± 0.01	1.31 ± 0.21	0.49 ± 0.00	0.59 ± 0.01	0.25 ± 0.02	0.23 ± 0.10	0.22 ± 0.01	1.20 ± 0.17
lactic acid	5.89 ± 0.03	6.52 ± 0.06	6.32 ± 0.26	7.41 ± 0.23	4.28 ± 0.01	5.07 ± 0.04	5.71 ± 0.31	6.55 ± 0.01
1,3-propanediol	0.41 ± 0.02	ND	0.97 ± 0.03	ND	0.19 ± 0.02	ND	3.30 ± 0.19	ND
acetic acid	0.87 ± 0.04	0.65 ± 0.03	1.22 ± 0.01	1.14 ± 0.07	0.71 ± 0.01	0.56 ± 0.00	2.28 ± 0.08	2.72 ± 0.82
succinic acid	0.90 ± 0.03	ND	0.72 ± 0.02	ND	0.63 ± 0.01	ND	0.93 ± 0.05	ND
glycerophosphorylcholine	1.11 ± 0.12	ND	0.95 ± 0.04	ND	0.91 ± 0.02	ND	0.99 ± 0.05	ND
betaine	1.03 ± 0.01	ND	0.85 ± 0.03	ND	0.82 ± 0.02	ND	0.80 ± 0.05	ND
2-phenylethanol	0.37 ± 0.01	ND	0.26 ± 0.02	ND	0.26 ± 0.02	ND	0.26 ± 0.02	ND

[a] Values are the means of triplicate determinations with SD of a single sample of each batch. [b] Not determined.

components). HPLC measurements of glucose were not consistent within the same sample, and the data were excluded from Table 2. Dextrin is the product of a liquefaction step in the ethanol industry whereby gelatinized starch is hydrolyzed with α-amylase[34] by cleaving α-1,4 glycosidic linkages in amylose and amylopectin.[35] In addition, maltotriose and maltose can be generated during amylose liquefaction (1.5 h[34]).

HPLC is currently the standard method for stillage analysis, but proton NMR could be used to quantify many of the compounds present in thin stillage. Preparation of the samples for NMR analysis requires minimal technical effort. The analysis is rapid, requiring less than a minute per sample. The sample is not destroyed, and the proton NMR method can detect and identify small quantities of compounds even without the use of standards.[9] The advantages of NMR are worthy of consideration.

Protein and Moisture Content of Conventional Thin Stillage. The concentration of NPN (N_p) contributed by GPC and BTN was calculated by subtracting the GPC nitrogen (N_{GPC}) and BTN nitrogen (N_{BTN}) from total nitrogen (N_T) according to eq 1:

$$N_p\ (\%) = N_T - N_{GPC} - N_{BTN} \quad (1)$$

Using this method, nitrogen present in thin stillage that arose from GPC and BTN was subtracted from N_T. The resulting nitrogen content affords a better estimate of true protein nitrogen. Corrected protein (CP) was calculated according to eq 2, where 5.7 is the conversion factor for wheat grain:[12]

$$\mathrm{CP}\ (\%) = N_p \times 5.7 \quad (2)$$

Protein and moisture contents illustrated that thin stillage contained small amounts of nitrogen, possibly in the form of protein (from 0.39 ± 0.02 to 0.48 ± 0.03%, w/w) and <5% solids (w/w) (Table 3). Differences in protein and moisture contents between batches were slight. Thin stillage contained protein and peptides with more than three amino acids,[36] which may derive from yeast or the cereal grain used as raw material. The protein content of thin stillage, at approximately 19% of dry matter, is comparable to that reported by Mustafa et al. for soluble protein

Table 3. Protein and Moisture Contents of Thin Stillage[a]

characteristic (%, w/w)	batch 1	batch 2	batch 3	batch 4
total nitrogen[b]	0.11 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.10 ± 0.02
GPC nitrogen[c]	0.012 ± 0.001	0.010 ± 0.001	0.010 ± 0.001	0.011 ± 0.002
BTN nitrogen[c]	0.013 ± 0.001	0.011 ± 0.001	0.011 ± 0.002	0.010 ± 0.001
corrected protein[b,d]	0.48 ± 0.03	0.39 ± 0.03	0.39 ± 0.02	0.45 ± 0.01
moisture	96.70 ± 0.03	97.30 ± 0.01	97.10 ± 0.01	97.30 ± 0.02

[a] Values are the means of triplicate determinations with SD of a single sample of each batch. [b] Total nitrogen was determined by the Kjeldahl method. [c] GPC (glycerophosphorylcholine) and BTN (betaine) nitrogens were obtained from Table 2. [d] Corrected protein was calculated by eqs 1 and 2 and expressed as crude protein (% N × 5.7).

Table 4. Concentrations (Milligrams per Liter) of Ions in Four Batches of Thin Stillage[a]

ion	batch 1	batch 2	batch 3	batch 4
bicarbonate	<1.00 ± 0.01	<1.00 ± 0.01	<1.00 ± 0.01	<1.00 ± 0.01
calcium	51.5 ± 0.7	51.5 ± 0.7	53.5 ± 0.7	45.1 ± 0.3
carbonate	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.00	<1.00 ± 0.01
chloride	224.5 ± 0.8	227 ± 0.8	215.5 ± 0.7	237.0 ± 0.6
hydroxide	<1.00 ± 0.01	<1.00 ± 0.01	<1.00 ± 0.01	<1.00 ± 0.01
magnesium	205.2 ± 7.4	185.1 ± 7.3	175.7 ± 7.1	175.2 ± 7.2
potassium	530.1 ± 14.1	485.5 ± 7.3	475.2 ± 7.1	480.1 ± 7.9
sodium	140.0 ± 0.4	130.0 ± 0.3	130.0 ± 0.1	120.0 ± 0.3
sulfate	590.0 ± 0.2	460.1 ± 0.3	495.2 ± 0.7	405.1 ± 0.7
nitrate	8.1 ± 0.2	9.3 ± 0.2	8.2 ± 1.2	9.3 ± 0.2
total	1750.1 ± 14.2	1545.2 ± 7.2	1555.1 ± 7.2	1470.6 ± 28.2

[a] Values are the means of triplicate determinations with SD of a single sample of each batch.

in barley-based thin stillage.[1] However, protein contents determined in thin stillage as measured by the Kjeldahl method are not a direct measurement of protein content but rather a measurement based on nitrogen content. This would introduce error as thin stillage contains substantial NPN. Both GPC and BTN are present in thin stillage, and both compounds contain nitrogen. Consequently, the true protein content of thin stillage should be adjusted to account for the presence of GPC and BTN and other NPN components. According to Licitra et al., true protein content in ruminant feeds was calculated by measuring protein content after chemical precipitation with tungstic acid or trichloroacetic acid.[37] These methods are believed to precipitate protein from the sample. The precipitant N content may then be determined using the Kjeldahl method. In addition, Mustafa et al. utilized sodium tungstate to precipitate protein from a thin stillage sample, and NPN was determined using the Kjeldahl method.[1,2] Mustafa et al. reported the NPN content as a percentage of protein plus NPN to be 38% for wheat-based thin stillage.[1] ^{1}H NMR allows the measurement of both GPC and BTN contents, and indirectly NPN may be estimated without the use of chemicals to precipitate protein. More generally, the proposed ^{1}H NMR method enables the measurement of specific NPN species.

Ion Content. The four batches of thin stillage (batches 1−4) had similar concentrations of the various ions (Table 4). The cation present in the highest concentration was potassium, followed consecutively by magnesium, sodium, and calcium. The anion present in the highest concentration was sulfate, followed consecutively by chloride and nitrate. The results showed that both cations and anions were present in thin stillage. Wilkie et al.[20] found that beet molasses stillage contained total sodium, phosphorus, potassium, and sulfur (SO_4^{-2}) in the ranges of 56−7340, 91−222, 5560−14500, and 1042−5800 mg/L, respectively. Mustafa et al.[1] found that barley-based thin stillage contained calcium, phosphorus, and magnesium (5.3, 11.3, and 5.4 g/kg, respectively). For copper, iron, manganese, sodium, potassium, and zinc, the amounts of these ions were 5.4, 493.2, 52.2, 0.6, 1.6, and 84.5 mg/kg, respectively, in barley-based thin stillage.[1] Moreover, wheat-based thin stillage contained calcium, phosphorus, and magnesium (4.2, 12.1, and 5.9 g/kg, respectively). They also found copper, iron, manganese, sodium, potassium, and zinc at 5.7, 419.1, 110.1, 0.2, 1.6, and 63.8 mg/kg, respectively, in wheat-based thin stillage. Ingledew reported that corn thin stillage had a mineral content of 3.64 g/L and that the major ions were phosphorus, sulfur, potassium, calcium, magnesium, copper, iron, manganese, and zinc.[36] Phosphorus levels ranged from 815 to 1762 mg/L, whereas potassium ranged from 705 to 2643 ppm and magnesium levels in all stillage samples examined were from 200 to 721 ppm. However, the levels of zinc, iron, copper, and manganese were low, and sodium was not measured. From this information, it was concluded that the levels of ions found in thin stillage depend upon the raw materials and process used in ethanol fermentation. When wheat-based thin stillage[1] is compared with the thin stillage used in this research, the calcium and magnesium contents of thin stillage used in this research were higher, and the sodium and potassium levels were lower.

In conclusion, thin stillage is composed of a number of compounds: (1) yeast metabolites, including glycerol, ethanol, succinic acid, GPC, and 2-PE; (2) bacterial metabolites, including isopropanol, acetic acid, lactic acid, and 1,3-propanediol; and (3) wheat metabolites, including BTN. In addition, inorganic cations and anions were found in thin stillage.

NMR analysis is complementary to the analysis of thin stillage by HPLC. This was particularly evident with the discovery that thin stillage contained substantial amounts of GPC, a component that was not apparent in HPLC analyses. NMR is nonselective (it detects organic compounds that contain protons and carbon), requires little sample preparation, and rapidly analyzes many relatively abundant compounds.

AUTHOR INFORMATION

Corresponding Author

*Phone: (306) 966-5050. Fax: (306) 955-5015. E-mail: (Y.Y.S.) younyoung.shim@usask.ca, (M.J.T.R.) martin.reaney@usask.ca.

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■ ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; DMF, dimethylformamide; BTN, betaine; GPC, glycerophosphorylcholine; 2-PE, 2-phenylethanol; COSY, correlation spectroscopy; NPN, nonprotein nitrogen.

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