Composition of Corn Steep Water during Steeping

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Corn wet-milling generates large volumes of corn steep water, which is composed of kernel extractives, microbes (principally lactobacilli), numerous products of fermentation processes, and dissolved SO_2. In order to understand better the process of steeping and the potential uses of corn steep water, its composition was analyzed and compared at various times during the steeping from four different industrial processes. Of particular interest were the analyses of corn steep water for carbohydrates, amino acids, polypeptides, fatty acids, and other organic compounds, hydrolytic enzymes, heavy metals, and inorganic ions. This study, together with an earlier analysis of corn steep water for *myo*-inositol phosphates (Hull and Montgomery, 1995), provides a basis for informed judgment of the nutritional and biotechnological value of corn steep water, an abundant byproduct of the corn wet-milling industry.

Keywords: *Corn steep water; compositional analysis; steeping*

INTRODUCTION

The steeping of corn is a necessary prerequisite to the fractionation of corn components in the wet-milling process. It involves the countercurrent flow of water, initially containing some SO_2, and dried corn in a number, around 10, of steeping tanks at 50−55 °C over a period of approximately 30 h. The details of the process vary among different industries. It follows that the fresh, dried corn enters the tank that contains the steep water from exposure to the partially steeped corn in the previous tanks.

The volume of steep water produced in the corn wet-milling industry is large. It is primarily handled by evaporation to a concentrated thick liquor that is a complex mixture of carbohydrates, amino acids, peptides, organic compounds, heavy metals, inorganic ions, and *myo*-inositol phosphates.

The steeping of corn in the wet-milling industry results in the extraction of water-soluble components. Microorganisms, especially lactobacilli, are detected in the process and contribute to the fermentation of the corn extract throughout the remainder of the steeping. The steeping process culminates with a concentration step to give corn steep liquor, officially known as condensed fermented corn extractives.

Analyses were conducted to determine the identity and concentration of components present in the corn steep at early, middle, and late times of steeping prior to the final step of concentration. The results of this study provide insights into the processes occurring during steeping and the potential products that might be isolated from corn steep water.

MATERIALS AND METHODS

Materials. The sources of materials were as follows: the ion exchange resins, AG 50W-X8(H^+) and AG3(OH^-), BioRad (Hercules, CA); the CarboPac HPLC column, Dionex Corp. (Sunnyvale, CA); the sodium hydroxide solution (50%) for HPAEC-PAD, Fisher Scientific (Pittsburgh, PA); the OV-1 and DB-5 capillary gas chromatographic columns, J&W Scientific (Folsom, CA); the Apizym kit, Bio Merieux SA (Paris, France); the amylase substrate, 4,6-ethylidene(G7)-*p*-nitrophenyl(G1)-α-D-maltoheptaoside, Boehringer Mannheim, (Indianapolis, IN); the sodium and lithium high-performance columns for total and free amino acid analysis, respectively, Beckman (Palo Alto, CA); the C-18 reversed-phase HPLC column, Vydac (Hesperia, CA); a panel of saturated fatty acid methyl ester standards, Sigma (St. Louis, MO); sugar standards, Pfanstiel (Waukegan, IL). All other chemicals were of the highest purity available.

Table 1. Corn Steep Descriptions

stream[a]	pH	dry wt (g/L)	total carbohydrate (g/L)[b]	total amino acids (g/L)[c]	total lipid (g/L)
1 e	3.5	27.5	5.6	8.0	0.022
1 m	4.3	68.9	10.8	21.6	0.021
1 l	4.1	112.2	17.2	33.1	0.036
2 m	3.9	81.8	14.8	52.1	0.078
2 l	3.7	126.1	8.8	51.5	0.209
4 e	3.6	25.9	8.2	9.3	0
4 m	3.9	40.2	8.8	16.6	0.046
4 l	4.2	79.0	16.8	23.7	0.028
15 e	3.5	24.2	4.8	9.5	nd[d]
15 m	3.8	63.4	9.0	19.0	nd
15 l	3.9	149.1	8.9	62.6	nd

[a] e, early; m, middle; l, late. [b] Expressed as glucose equivalent. [c] From the acid hydrolysis of each stream and includes both free amino acids, peptides, and protein. [d] ND = not determined.

Collection of Steep Waters. Samples of corn steep water at early (1−3 h), middle (14−17 h), and late (27−30 h) stages in the steeping (Table 1) were collected in sterile glass containers from four industries which were regionally separate and had significantly different milling capacities. All the corn was of a normal dent variety. The samples were stored at −20 °C until analyzed.

Methods. *Preparation of Steep Water for Analysis.* The samples were either clarified by centrifugation at 1000*g* for 15 min and/or filtered with a No. 1 Whatman filter or a 0.2 μm filter. The clear supernatant was stored at −20 °C until analyzed. In preparation for carbohydrate or organic acid analyses, the steep water was subjected to ion exchange treatment to separate the organic acid (anionic fraction) and the carbohydrate (neutral fraction). Clarified corn steep water was first passed over a cation exchange column (AG50, H^+, 1.5 cm × 3.0 cm). The column was washed with water (25−30 mL) and the washings, after adjustment to pH 8, were then passed over an anion exchange column (AG3, OH^-, 1.5 cm ×

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3.0 cm). The column was washed with water (25−30 mL) and the neutral fraction, containing the carbohydrate, was recovered; its volume was measured to determine the dilution factor. The components of corn steep water that bound to the anion exchange resin (hereafter referred to as the anionic fraction) were eluted with 1.5 M pyridinium acetate (30 mL), and the eluant was lyophilized.

Dry Weight Determinations. Triplicate 1 mL aliquots of clarified corn steep water were each lyophilized in preweighed containers, followed by exhaustive drying at room temperature over P_2O_5 under vacuum to constant weight.

Analytical Procedures. An initial analysis of total carbohydrate followed the phenol−sulfuric colorimetric procedure (Dubois et al., 1956), using glucose as the standard sugar. Total protein was determined by either the Lowry method (1951) or the BCA method (Smith et al., 1985). Instrumentation used in analyses were as follows: carbohydrate analyses, HPAEC-PAD using a BioLC system (Dionex) equipped with computer-based data acquisition and instrument control, a solvent degas module, a gradient pump module, a CarboPac HPLC column, and a pulsed amperometric detector; analyses for lactic and glycolic acid, fatty acids, carbohydrate, and lipophilic components of steep water, GLC with a Hewlett-Packard 5890 series II gas chromatograph coupled to either a flame ionization detector for quantitative data or to a Hewlett-Packard 5971A mass selective detector for qualitative data. Identification of components was proposed from mass spectral comparisons with a Wiley mass spectral library; amino acid analyses, HPLC with a Beckman 6300 amino acid analyzer; polypeptide analysis, RP-HPLC with an Altex 110 HPLC system complete with two pump modules, a Model 420 program controller, a gradient mixer, a Rheodyne injection valve, and a Hitachi Model 100-40 spectrophotometer. Peptides were detected at 210 nm and chromatograms were recorded on an HP 3380A integrator.

Analysis of Steep Water for Carbohydrates. Identification and quantitation of the mono-, di-, and trisaccharide composition of the neutral fraction of corn steep water was accomplished by HPAEC-PAD (Townsend and Hardy, 1991; Hardy and Townsend, 1994). The gradient utilized for the separation of mono- and oligosaccharides was the following: T_0 = 20 mM NaOH, T_{20} = 20 mM NaOH, T_{25} = 100 mM NaOH, and T_{55} = 100 mM NaOH−500 mM NaOAc. A standard mixture of monosaccharides and maltodextrins up to the 7-mer was used to calibrate the column and generate response factors.

GLC of TMS Derivatives of Carbohydrates. GLC analysis of carbohydrates generally followed the procedure of Chaplin (1982) with some modification. TMS derivatives of sugars were prepared by the addition of 200 μL of bis(trimethylsilyl)-acetamide−10% trimethylchlorosilane to rigorously dried samples in a sealed tube. The reaction mixture was heated at 40 °C for 30 min. A portion (1−2 μL) of the reaction mixture was analyzed by GLC using an OV-1 capillary column (0.2 mm × 30 m) with flame ionization detection using the following temperature program: initial temperature of 130 °C for 3 min after which a temperature gradient of 5 °C/min was applied. The column was held at a final temperature of 300 °C for 5 min to remove any strongly bound contaminants. The carrier gas was helium, and its flow rate was set in order to maintain a split flow of 1 mL/min through the column. Injection and detector ports were maintained at 250 °C.

Analysis of Corn Steep Water for Hydrolytic Enzymes. The presence of phosphatases, proteases, and glycohydrolases, except α-amylase, was ascertained using the Apizym kit, a qualitative solid-phase screening assay (Humble et al., 1977). α-Amylase activity was measured colorimetrically with 4,6-ethylidene(G7)-*p*-nitrophenyl(G1)-α-D-maltoheptaoside as substrate (Kruse-Jarres et al., 1989).

Amino Acid Analysis of Corn Steep Water. Total amino acids were determined by HPLC, after hydrolysis of an aliquot of clarified steep water with 6 N HCl for 24 h (Moore and Stein, 1963), with a sodium high-performance column (0.46 × 12 cm). Free amino acids were determined, after deproteinization of the sample by ultrafiltration (Gressner, 1973), with a lithium high-performance column (0.46 × 10 cm). Cysteine and methionine were determined after oxidative hydrolysis of the clarified steep water with performic acid (Glazer et al., 1987; Liu and Boykins, 1989).

Polypeptide Analysis of Corn Steep Water. HPLC analysis of the polypeptide content of steep water was accomplished using C-18 RP-HPLC and detection at 210 nm. Samples of clarified corn steep water were directly analyzed on a C-18 reversed-phase high-performance column (0.46 × 25 cm) using a programmed gradient elution: T_0 = 0.1% TFA; T_5= 0.1% TFA; T_{25} = 70% CH_3CN−0.1% TFA. A tetrapeptide, Leu-Val-Trp-Ser, was used to calibrate the column, and the peptides in steep water were quantitated by comparison of peak area relative to a peak area generated from a known quantity of this tetrapeptide. The purity of selected polypeptides was ascertained by CZE (McCormick, 1988). CZE was performed on a Beckman P/ACE 5010 with a fused-silica capillary column (50 μm × 50 cm) eluted with 50 mM sodium phosphate, pH 2.5, and UV detection at 214 nm.

Lactic and Glycolic Acid Analysis of Corn Steep Water. The anionic fraction of corn steep water was analyzed for lactic and glycolic acid content by analysis of their TMS derivatives using GLC analysis. TMS derivatives were prepared by heating a rigorously dried sample with 200 μL of a mixture of bis(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane, and pyridine (100:1:25, v/v/v) at 80 °C for 1 h in a sealed tube and analyzed by GLC using a capillary column (DB-5, 0.25 mm × 30 m, J&W Scientific). Tetradecane added just prior to derivatization was utilized as an internal standard. The temperature was programmed initially for 3 min at 60 °C and then to 300 °C at 5 °C/min. Injection and detection temperatures of the GLC were set at 270 and 300 °C, respectively. Column head pressure was set at 5 psi. Mass calibration range was 45−650 (*m*/*e*). L-Lactic acid was determined using an original Behring Diagnostics procedure (document N00074, Behring Diagnostics, Somerville, NJ) based upon the L-lactate dehydrogenase assay (Hohorst, 1963). The modified assay, which measures the production of NADH formed when L-lactate is oxidized to pyruvate, consists of the addition of glutamate and alanine aminotransferase to the lactate dehydrogenase reaction system, thus forcing the reaction to completion by removing pyruvate from the mixture.

Heavy Metal Analysis of Corn Steep Water. Heavy metal analysis was by atomic absorption spectroscopy.

Inorganic Ion Analysis of Corn Steep Water. Potassium, sodium and chloride were analyzed using specific ion-selective electrodes (Tietz, 1983). Inorganic phosphate was determined by the method of Fiske and Subbarow (1925).

Determination of Lipophilic Components of Corn Steep Water. Corn steep water, after appropriate dilution, was centrifuged at 7500*g*, 5 °C for 30 min. An aliquot of the corn steep supernatant together with an equal volume of dichloromethane and a solution of NaCl (6.5 g/mL) was transferred into a 100 mL round-bottomed flask and stirred in an ice−water bath for 2 h. The resultant two-phase suspension was centrifuged at 4200*g* for 20 min at 5 °C to break the emulsion. The dichloromethane extract was evaporated to a small volume under reduced pressure and analyzed by GLC−MS for identification of components. GLC−MS analysis was performed using a DB-5 capillary column (0.25 mm i.d. × 30 m). The temperature program was as follows: T_0 = 60 °C, T_3 = 60 °C, T_{41} = 250 °C, T_{44} = 280 °C, and T_{47} = 280 °C. Injection and detection temperatures of the GLC were set at 230 °C and transfer line held at 280 °C, respectively, with the column head pressure at 5 psi; flow rate was 24 cm/s.

Fatty Acid Composition of Corn Steep Water. The fatty acids contained in the dichloromethane extract were identified and quantitated after saponification as their methyl esters by GLC analysis. A known proportion of the dichloromethane extract from each corn steep water sample was saponified by the addition of ethanol (0.5 mL) and 10 N KOH (2.5 mL) in a 13 × 100 screw-cap glass test tube. The sealed tube was heated for 45 min at 60 °C. After cooling, water (3 mL) was added and the samples were extracted three times with *n*-heptane (1 mL) to remove nonsaponifiable lipids. The aqueous phase was then acidified to pH 2. Heptane was again used to extract the solution recovering any free fatty acids formed. Methyl

Table 2. Simple Carbohydrate of Corn Steep Water[a]

	monosaccharides (g/L)					di- and trisaccharides (g/L)				
stream	Ara	Gal	Glc	Fru	Xyl	trehalose	sucrose	melibiose	raffinose	maltose
1 e	0.09	0.28	3.27	1.97	–[b]	0.03	0.07	0.02	0.10	0.01
1 m	0.24	0.81	6.92	5.06	–	0.12	0.25	0.09	0.49	0.07
1 l	0.60	1.65	7.30	5.38	0.07	0.31	0.50	0.31	0.80	0.21
2 m	0.27	1.04	7.23	4.29	0.03	0.18	0.28	0.19	0.53	0.09
2 l	0.63	1.74	0.40	–	0.12	0.34	0.63	0.27	0.61	0.09
4 e	0.08	0.21	2.15	0.99	–	0.04	–	–	0.13	–
4 m	0.12	0.31	2.84	1.01	–	0.05	0.14	0.03	0.19	0.03
4 l	0.26	0.83	6.23	3.06	0.02	0.16	0.47	0.21	0.12	0.15
15 e	0.11	0.21	2.03	1.50	–	–	0.04	<0.01	0.07	–
15 m	0.25	0.48	4.29	2.11	–	–	0.18	0.04	0.20	–
15 l	0.67	1.42	1.93	1.47	0.12	–	0.45	0.20	0.37	0.06

[a] Mono-, di, and trisaccharides were identified and quantitated using HPAEC-PAD. [b] –, not detected.

esters of the free fatty acids were produced by evaporating the heptane completely and redissolving the sample in 0.5 mL of 12% BF_3–methanol solution. The mixture was heated at 90 °C for 10 min in a sealed tube to ensure complete reaction. The methyl esters were extracted three times with *n*-heptane (1 mL), concentrated under a stream of nitrogen, and analyzed by GLC–MS. GLC–MS analysis was performed using a DB-5 capillary column (0.25 mm i.d. × 30 m). The temperature program was as follows: $T_0 = 120$ °C, $T_1 = 120$ °C, $T_{46} = 300$ °C, and $T_{51} = 300$ °C. Injection and detection temperatures of the GLC were set at 270 and 300 °C, respectively, with the column head pressure at 5 psi. Flow rate was 24 cm/s. A solution of saturated fatty acid methyl esters was used to calibrate the GLC column.

RESULTS AND DISCUSSION

The steeping process does not start with pure water, rather with the combined washings from the preparation of starch, gluten, and the other fractions of corn. Consequently the analysis of the early steeps give constituents that were in the feed water and those in the extraction of corn that had been through the process of much steeping, since the corn and the steep water are moving countercurrently. This countercurrent movement adds further to the difficulty in interpreting the analyses since as the corn is exposed to an earlier steep tank it takes with it the components of the steep water in which it is saturated. For example; the corn in tank 3 will be saturated with the steep water of tank 3 so that when tank 3 corn is exposed to the steep water in tank 2, the components of steep water 3 will be back extracted into steep water 2 as well as the new components of the corn not yet extracted. This is particularly relevant in the early steeps.

Carbohydrates of Corn Steep Water. It was noted (Table 1) that the dry weight of materials in the steep water increases as the steeping progresses.

The carbohydrates in each of the steeps were analyzed quantitatively by HPAEC-PAD and as their TMS derivatives by GLC (Table 2). Complex carbohydrates were hydrolyzed with 2N TFA or 0.1N HCl and the component sugars identified (Table 3). Glucose and fructose are the predominant monosaccharides, with smaller amounts of galactose, arabinose, and occasionally xylose. The oligosaccharides, present in smaller amounts than the monosaccharides, were sucrose, raffinose, melibiose, trehalose, and maltose (Table 2). There were little, if any, of the higher oligosaccharides in any of the steeps as determined by HPAEC-PAD (data not shown), an analytical procedure that separates the maltodextrin series up to a 30-mer (Koizumi et al., 1989). However, acid hydrolysis of some of the steeps produced increases in arabinose, xylose, and/or glucose (Table 3) that exceeded the amount of these sugars expected from hydrolysis of the di- and trisaccharides present (Table 2). These results suggest that polysaccharides, composed of arabinose, xylose, and/or glucose, are present in the stream.

Table 3. Complex Carbohydrate of Corn Steep Water[a]

		monosaccharide (g/L)			% of oligomeric as
stream	sugar	total[b]	free[c]	oligomeric[d]	polysaccharide[e]
2 l	Ara	0.75	0.63	0.12	100
	Xyl	0.12	0.12	0.00	
	Gal	1.60	1.74	0.00	
	Glc	1.30	0.40	0.90	0
4 l	Ara	0.33	0.26	0.07	100
	Xyl	0.067	0.016	0.051	100
	Gal	0.89	0.83	0.06	0
	Glc	10.70	6.23	4.47	84
15 l	Ara	0.72	0.67	0.05	100
	Xyl	0.24	0.12	0.12	100
	Gal	1.53	1.42	0.11	0
	Glc	2.54	1.93	0.61	14

[a] Complex carbohydrate of selected corn steeps was determined from the difference between the carbohydrate occurring as mono-, di, and trisaccharides and that from the total acid hydrolysate of the sample. [b] Determined by HPAEC-PAD after 2N TFA hydrolysis. [c] Determined by HPAEC-PAD before 2N TFA hydrolysis. [d] Total – free (i.e., oligo- and polysaccharides). [e] [Oligomeric – (sum of sugar expected from di- and trisaccharides)]/oligomeric × 100%.

In the long process of steeping at an elevated temperature, there are many reactions occurring during the extraction processes. The steeping is under slightly acidic conditions, pH 3.5–4.3 (Table 1), which is due in part to the initial presence of SO_2 and later due to lactic acid, a major fermentation product (Wright, 1987). In addition, several enzymes are present in the steeps, principally the glycohydrolases, α-amylase, β-D-galactosidase, β-D-hexosaminidase, and α-D-glucosidase. β-D-Galactosidase and β-D-hexosaminidase are present throughout the steeping, whereas α-D-glucosidase and acid phosphatase are only in the latter steeps.

It is noted in this complex system that, in general, as the steeping proceeds so do the amounts of arabinose, glucose, galactose, and xylose increase, most likely due to acidic and/or enzymatic hydrolysis of cell wall hemicelluloses (Wilkie, 1979) extracted from the corn during steeping. Of note is the observation that in the two steeps in which glucose and fructose concentrations decreased as the steeping progressed (streams from industries 2 and 15, Table 2), the di- and trisaccharides containing glucose and/or fructose increased. The decreased glucose and fructose concentration in steeps from industries 2 and 15 may be due to their utilization by lactobacilli in fermentative processes and/or to less hydrolysis of the di- and trisaccharides of these particular steep waters.

Table 4. Total and Free[a] Amino Acid Composition of Corn Steep Water[b]

	amino acid (g/L)																
stream	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Met	Cys	Ile	Leu	Tyr	Phe	His	Lys	Arg
1 e	0.5	0.3	0.4	1.2	0.7	0.3	0.7	0.4	0.2	0.2	0.4	1.4	0.2	0.4	0.2	0.3	0.2
	0.4	*0.2*	*0.2*	*0.3*	*0.5*	*0.1*	*0.5*	*0.3*	*0.1*	*0.0*	*0.3*	*1.2*	*0.1*	*0.4*	*<0.1*	*0.2*	*0.1*
1 m	1.3	0.9	1.1	3.0	2.0	1.0	2.0	1.2	0.5	0.6	1.2	3.5	0.3	1.0	0.5	1.0	0.5
	1.0	*0.4*	*0.5*	*0.5*	*1.2*	*0.3*	*1.4*	*0.7*	*0.4*	*<0.1*	*0.8*	*2.9*	*<0.1*	*0.9*	*<0.1*	*0.7*	*0.1*
1 l	2.3	1.3	1.8	4.9	3.0	1.6	2.9	1.8	0.7	1.0	1.7	4.7	0.6	1.4	0.8	1.5	1.1
	2.0	*0.6*	*0.8*	*1.4*	*2.0*	*0.4*	*2.1*	*1.1*	*0.7*	*<0.1*	*1.0*	*4.1*	*0.1*	*1.1*	*0.2*	*1.1*	*0.4*
2 m	3.8	1.9	2.5	8.0	4.4	2.1	3.9	2.6	1.0	1.3	2.4	7.1	2.0	2.1	1.6	2.0	3.4
	3.1	*0.8*	*1.1*	*2.9*	*2.6*	*0.5*	*2.6*	*1.5*	*0.9*	*<0.1*	*1.4*	*6.0*	*1.6*	*2.0*	*0.6*	*1.6*	*2.7*
2 l	3.0	2.0	2.6	8.4	4.7	2.3	4.0	2.6	1.0	1.3	2.4	6.7	1.3	1.6	1.8	2.2	3.6
	2.4	*0.8*	*1.1*	*2.2*	*2.3*	*0.5*	*2.6*	*1.2*	*0.7*	*<0.1*	*1.2*	*5.0*	*0.6*	*1.3*	*0.4*	*1.3*	*2.3*
4 e	0.6	0.3	0.4	1.4	0.7	0.4	0.6	0.5	0.2	0.2	0.5	1.5	0.4	0.4	0.3	0.3	0.6
	0.6	*0.2*	*0.2*	*0.5*	*0.4*	*0.1*	*0.4*	*0.3*	*0.2*	*0.0*	*0.3*	*1.2*	*0.4*	*0.5*	*0.1*	*0.3*	*0.4*
4 m	1.2	0.6	0.8	2.5	1.3	0.7	1.1	0.8	0.4	0.4	0.8	2.4	0.7	0.7	0.6	0.6	1.0
	1.0	*0.3*	*0.3*	*0.9*	*0.8*	*0.2*	*0.7*	*0.5*	*0.3*	*<0.1*	*0.4*	*2.0*	*0.6*	*0.7*	*0.2*	*0.5*	*0.8*
4 l	1.8	0.9	1.2	3.4	2.0	1.0	1.6	1.3	0.4	0.6	1.1	3.2	0.9	1.0	0.8	1.0	1.5
	1.9	*0.4*	*0.6*	*1.5*	*1.5*	*0.3*	*1.2*	*0.8*	*0.5*	*<0.1*	*0.8*	*3.2*	*0.9*	*1.0*	*0.4*	*0.9*	*1.4*
15 e	0.6	0.3	0.4	1.4	0.8	0.4	0.7	0.5	0.2	0.2	0.5	1.5	0.4	0.5	0.3	0.3	0.5
	0.5	*0.2*	*0.2*	*0.5*	*0.4*	*0.1*	*0.4*	*0.3*	*0.2*	*0.0*	*0.3*	*1.3*	*0.4*	*0.5*	*0.1*	*0.3*	*0.4*
15 m	1.1	0.7	0.9	2.8	1.5	0.8	1.7	1.0	0.4	0.4	0.9	2.7	0.8	0.9	0.4	0.8	1.2
	0.5	*0.2*	*0.3*	*0.7*	*0.6*	*0.2*	*0.9*	*0.4*	*0.2*	*<0.1*	*0.4*	*1.5*	*0.4*	*0.5*	*<0.1*	*0.4*	*0.5*
15 l	3.9	2.5	3.1	9.6	5.3	2.9	6.1	3.3	1.0	1.3	2.9	7.3	2.3	2.1	1.6	2.9	4.5
	2.7	*1.0*	*1.3*	*3.2*	*2.8*	*0.6*	*4.4*	*1.7*	*0.8*	*<0.1*	*1.5*	*5.5*	*1.5*	*1.7*	*<0.1*	*1.7*	*2.8*

[a] The free amino acids are italicized. [b] Total amino acid composition was determined, after acid hydrolysis, using a Beckman 6300 amino acid analyzer. Free amino acid was determined after deproteinization by ultrafiltration.

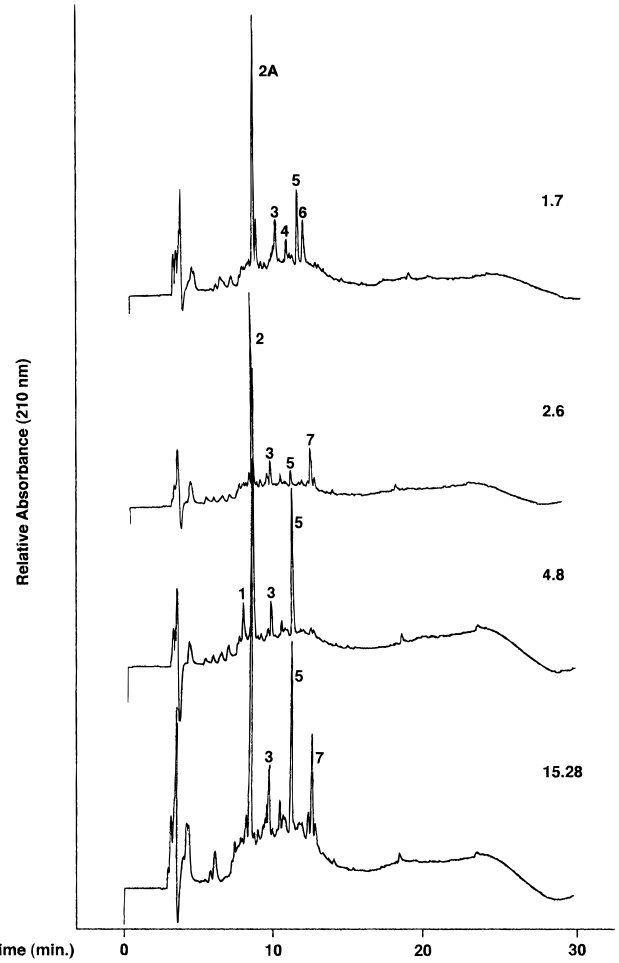

Figure 1. RP-HPLC analysis of the polypeptides of corn steep water. Late corn steep water from each industry was diluted as indicated and an aliquot (100 μL) analyzed for polypeptides by RP-HPLC. Peak numbers were assigned on the basis of increasing elution time.

The presence of trehalose in the steep water is consistent with the carbohydrate composition of several fermentation processes (Hull et al., 1995).

Table 5. Polypeptide Analysis of Corn Steep Water[a]

	polypeptide (μmol/L[b])								
stream	1	2	2a	3	4	5	6	7	total
1 e	–[c]	–	3.3	0.5	0.6	0.5	0.6	0.2	5.8
1 m	–	–	5.2	0.9	0.9	0.1	1.6	0.4	9.1
1 l	–	–	11.2	2.1	1.8	1.6	1.6	0.3	18.7
2 m	–	5.0	–	1.1	–	2.1	–	0.4	8.5
2 l	–	6.8	–	3.1	–	0.4	–	1.2	11.5
4 e	1.1	2.7	–	0.4	0.9	0.6	–	0.4	6.0
4 m	1.6	3.2	–	1.9	1.5	1.0	–	0.1	9.3
4 l	2.1	4.4	–	1.5	1.1	1.8	–	0.2	11.2
15 e	–	3.0	–	1.0	1.5	0.2	–	–	5.7
15 m	–	6.1	–	0.5	0.6	2.2	–	–	9.4
15 l	–	10.7	–	2.0	1.0	4.8	–	2.0	20.5

[a] The peptides separated by RP-HPLC (Figure 1) were quantitated relative to a tetrapeptide. Peaks were numbered on the basis of their order of elution. [b] Relative to the tetrapeptide, Leu-Val-Trp-Ser, added as an internal standard. [c] Not present.

Amino Acids, Peptides, and Non-Protein Nitrogenous Components of Corn Steep Water. Total amino acid analysis (Table 4) of corn steep water reveals a composition rich in glutamic acid/glutamine, leucine, proline, and aspartic acid/asparagine with low levels of lysine, cysteine, and methionine, a composition characteristic of the various corn albumin, globulin, glutelin and zein proteins (Wilson, 1987). These results, consistent with amino acid analyses of corn steep liquor (Wright, 1987), provide evidence for the notion that a majority of the amino acid content in steep water originates from the corn rather than from fermentation products. Comparison between industries shows a wide variation in dry weight composition, and within industries the amino acid content increases with steeping time, an exception being industry 2.

From the free amino acid content (Table 4) of corn steep water, it is clear that polypeptides are present. RP-HPLC analysis (Figure 1) shows that the polypeptides in the four steeps were surprisingly similar, with a major peptide (5) common to the steeps. However, a significant qualitative difference in polypeptide content was seen between industry 1 and all the others. The total polypeptide content of corn steep water increased during steeping (Table 5), and each polypeptide in turn

Table 6. Non-Protein Nitrogenous Components of Corn Steep Water[a]

	component (g/L)						
stream	phosphoserine	taurine	phosphoethanolamine	citrulline	γ-aminobutyric acid	ethanolamine	ornithine
1 e	0.3	<0.1	<0.1	0.1	0.3	0.0	0.3
1 m	0.4	0.0	<0.1	0.1	1.0	<0.1	1.3
1 l	0.5	<0.1	<0.1	0.2	0.8	<0.1	1.7
2 m	0.5	<0.1	0.1	0.1	0.9	<0.1	<0.1
2 l	0.4	<0.1	0.0	<0.1	0.8	0.0	<0.1
4 e	0.2	<0.1	<0.1	<0.1	0.2	0.0	<0.1
4 m	0.2	<0.1	<0.1	<0.1	0.3	<0.1	<0.1
4 l	0.2	<0.1	<0.1	<0.1	0.6	<0.1	<0.1
15 e	0.2	<0.1	<0.1	<0.1	0.2	<0.1	<0.1
15 m	0.1	0.0	<0.1	<0.1	0.2	<0.1	<0.1
15 l	0.2	0.0	0.1	0.1	1.1	<0.1	<0.1

[a] Non-protein nitrogenous components were determined on a lithium high-performance column using a Beckman 6300 amino acid analyzer.

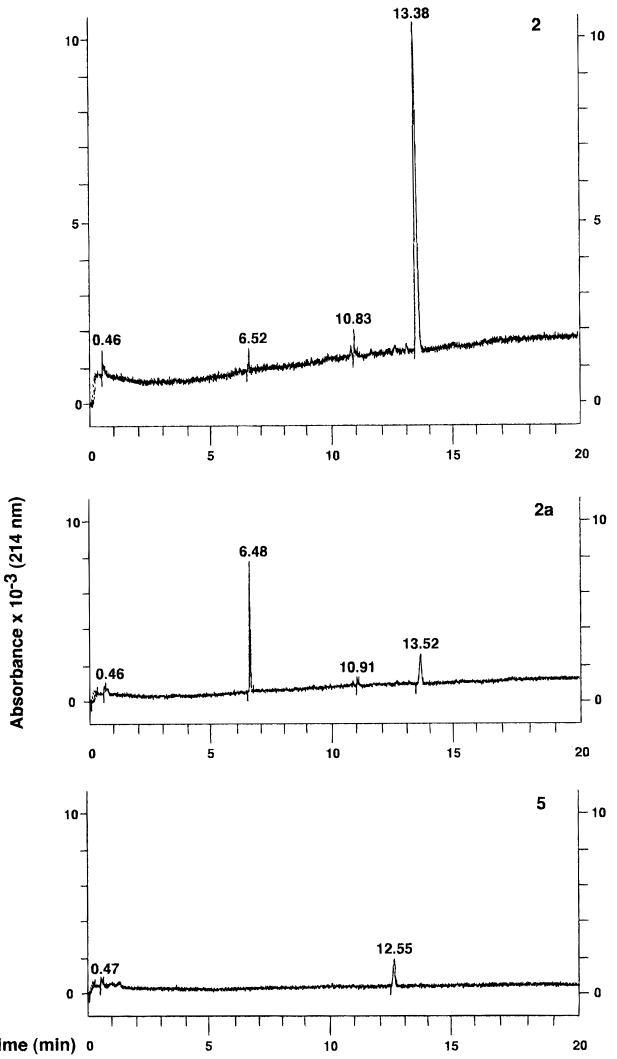

Figure 2. CZE of purified peptides from corn steep water. Purified peptides dissolved in water at the indicated concentrations, 2 (1405 μg/mL), 2a (662 μg/mL), and 5 (154 μg/mL), from late corn steep water were analyzed (5 nL injected) by CZE.

showed an increase during steeping, except in industry 2 in which peptide 5 decreased. Enzymatic activities provided no evidence for proteases during steeping; however, the length of steeping time (up to 30 h) coupled with the higher temperature (50−55 °C) and the presence of microorganisms could contribute to the enhancement of proteolytic activity during steeping.

The major polypeptide of industry 1 (2a) was not chromatographically identical with the major peptide

Table 7. Lactic and Glycolic Acid in Corn Steep Water[a]

	glycolic acid (g/L)[b]	lactic acid (g/L)		
stream		total[b]	L-isomer[c]	D-isomer[d]
1 e	0.3	4.0	1.6	2.4
1 m	0.4	18.4	6.1	12.3
1 l	0.4	28.2	8.9	19.2
2 m	0.2	18.1	5.9	12.3
2 l	0.3	33.0	14.2	18.8
4 e	0.4	6.7	2.3	4.4
4 m	0.3	14.5	5.7	8.8
4 l	0.2	22.2	10.9	11.3
15 e	0.3	7.5	1.8	5.7
15 m	0.5	27.5	8.7	18.8
15 l	0.4	46.6	18.8	27.8

[a] Lactic and glycolic acids were separated and identified by GLC−MS and quantitated by GLC-FID. [b] Determined by GLC analysis as described in Methods. [c] Determined using L-lactate dehydrogenase assay (Hohorst, 1963). [d] Determined by difference of the above determinations.

(2) of the other three industries examined. Compositional analysis of the major peptides confirmed a difference between 2 and 2a in their isoleucine, leucine, and tyrosine content. Upon analysis of the purified peptides by capillary electrophoresis, it was apparent that 2 and 5 were relatively pure (90−95%) whereas 2a consisted of two major components, one of which was chromatographically similar if not identical to peptide 2 (Figure 2). Gel filtration analysis (data not shown) demonstrated that the major peptides 2 and 5 were included into Bio-gel P6, suggesting that the polypeptides are no larger than 6 kDa. It seems likely that these qualitative differences in polypeptide content are reflective of the different processing strategies of each industry.

A number of other non-protein nitrogenous components were found in corn steep water (Table 6). Especially noteworthy was the presence of significant amounts of phosphoserine and γ-aminobutyric acid in most of the corn steeps analyzed.

Lactic and Glycolic Acid of Corn Steep Water. Lactic acid is the most prominent organic constituent of corn steep water (Wright, 1987), an observation consistent with our analysis of the anionic fraction from four different industries (Table 7). Glycolic acid was also present at 20−100-fold lower concentrations than the lactic acid. The L-lactic acid content was compared to the total lactic acid, from which the D-lactic acid isomer was estimated. The ratio of D to L isomer found in each stream should be indicative of the type of lactobacilli present in the corn steep, but it is noted that the ratio of the two isomers changes as the steeping proceeds. Also, significant lactate is present in the early

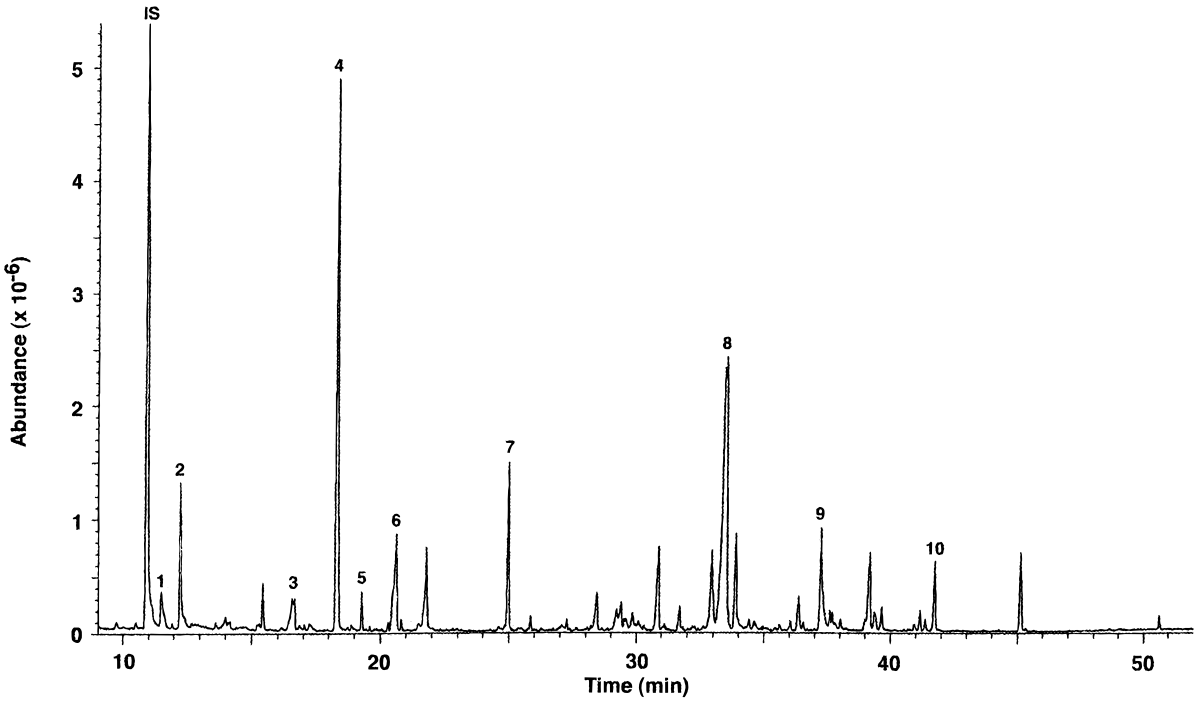

Figure 3. GLC−MS Analysis of the organic components of corn steep water. A dichloromethane extract of stream 2.6 was prepared and analyzed by GLC−MS. Numbered peaks were tentatively identified by mass spectra: (1) 2-methoxyphenol; (2) phenyl ethyl alcohol; (3) phenylacetic acid; (4) 2,4,6-trimethyl-1,3-benzenediamine; (5) 2,6-dimethoxyphenol; (6) benzamide; (7) 1,4-dihydroxyphenanthrene; (8) palmitic acid; (9) linoleic acid; (10) ergotamine.

Table 8. Fatty Acid Composition of Corn Steep Water[a]

	fatty acid (mg/L)							
stream	lauric	myristic	palmitic	stearic	elaidic	palmitoleic	oleic	linoleic
1 e	0.0	0.0	0.2	0.2	0.1	0.1	0.2	0.1
1 m	0.0	0.0	0.1	0.1	<0.1	<0.1	0.1	<0.1
1 l	0.0	0.0	0.2	0.2	<0.1	<0.1	0.2	0.2
2 m	<0.1	0.3	0.8	0.2	0.1	0.8	0.6	1.0
2 l	0.0	0.2	0.9	0.2	0.2	0.7	0.7	1.9
4 e	0.0	0.0	0.2	0.1	0.0	0.1	0.1	0.1
4 m	0.0	<0.1	0.6	0.2	0.0	0.0	0.2	0.5
4 l	0.0	0.1	2.6	0.4	0.0	0.2	0.7	2.7

[a] Fatty acids were extracted from the corn steep water, identified as their methyl esters by GLC−MS, and quantitated using flame ionization detection.

steeps when SO_2 is highest and lactobacilli are not functional . Whether the lactate soaks into the corn in the latter steep tanks and is fractionally extracted out in the early steep is open to further study.

Lipophilic Components of Corn Steep Water. Lipophilic components of corn steep water extracted by dichloromethane were quantitated and found to comprise less than 0.1% of the total dry weight (Table 1). GLC analysis of the lipophilic components of one particular stream revealed a complex mixture with several major components which, tentatively identified by mass spectral analysis, are phenolic acids, amines, alcohols, and fatty acids (Figure 3).

Fatty Acids of Corn Steep Water. The fatty acids extracted from corn steep water by dichloromethane were low in abundance (Table 8). It was interesting to note that the linoleic content in all three industrial steeps studied showed a dramatic increase during steeping. Since this is the most abundant fatty acid component of corn oil (Reiners and Gooding, 1970), this would suggest that corn oil is being extracted during the steeping process. Since corn oil is present only in the corn germ, the measurement of the increase in linoleic acid content during steeping could be an indicator of the amount of "cracked" corn in the steeping.

Table 9. Inorganic Content of Corn Steep Water[a,b]

	inorganic component							
stream	Fe[c]	Cu[c]	Ni[c]	Pb[d]	K^{+}[e]	P_i[e]	Na^{+}[e]	Cl^{-}[e]
1 e	0.9	0.1	<0.1	0.0	0.6	0.1	0.2	0.2
1 m	10.2	0.9	0.3	0.0	2.3	0.3	0.3	0.6
1 l	20.6	1.6	0.6	0.0	6.2	0.6	0.2	1.0
2 m	12.4	0.8	0.4	9.0	4.1	0.4	0.2	0.6
2 l	19.8	1.6	0.8	36.0	7.4	0.7	0.3	1.0
4 e	1.3	0.1	<0.1	0.0	0.8	0.1	0.1	0.2
4 m	3.6	0.2	0.1	0.0	1.6	0.2	0.1	0.2
4 l	10.2	0.6	0.4	0.0	4.3	0.4	0.2	0.5
15 e	0.8	<0.1	0.1	0.0	0.7	0.1	0.0	0.0
15 m	6.3	0.4	0.4	0.0	2.0	0.3	0.0	0.6
15 l	22.8	1.3	0.9	0.0	7.7	0.8	0.0	1.2

[a] The heavy metals were determined by atomic absorption spectroscopy. The inorganic ions were determined using ion-selective electrochemistry. [b] Cd and Cr were not found above background levels. [c] Expressed in milligrams per liter. [d] Expressed in micrograms per liter. [e] Expressed in grams per liter.

Heavy Metals and Inorganic Ions of Corn Steep Water. Iron is the most prevalent heavy metal present in corn steep water (Table 9). Because of the presence of phytate in corn (Sands et al., 1986) and its ready chelation with iron to form an insoluble complex, the level of iron bears significance to the recovery of phytate

and lower inositol phosphates present in corn steep water (Hull and Montgomery, 1995). Though chromium and cadmium were not detectable, copper and nickel were present at approximately 5−10% of the concentration of iron. In all but one industry lead was not detectable. Physiologically relevant ions such as potassium, sodium, inorganic phosphate, and chloride were detected in corn steep water at significant levels, potassium being the most prevalent (Table 9). The inorganic phosphate is in part a product of dephosphorylation of *myo*-inositol phosphates found in corn steep water (Hull and Montgomery, 1995), a hydrolytic process facilitated by the presence of phosphatases in corn steep water.

ABBREVIATIONS USED

BCA, bicinchoninic acid; CZE, capillary zone electrophoresis; GLC-FID, gas liquid chromatography with flame ionization detection; GLC−MS, gas liquid chromatography with mass spectral detection, HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; RP-HPLC, reversed-phase high-performance liquid chromatography; TMS, trimethylsilyl.

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LITERATURE CITED

Boyer, C. D.; Shannon, J. C. Carbohydrates of the kernel. In *Corn: Chemistry and Technology*; Watson S. A., Ramstad, P. E., Eds.; Am. Assoc. of Cereal Chemists: St. Paul, MN, 1987; pp 253−272.

Chaplin M. F. A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. *Anal. Biochem.* **1982**, *123*, 336−341.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

Glazer, A. N.; Delange, R. J.; Sigman, D. S. *Chemical Modification of Proteins, Selected Methods and Analytical Procedures*; Elsevier Biomedical Press: New York, 1987.

Gressner, A. M. Routine amino acid capillary column chromatography of microquantities in the picomole range-procedures and modifications of a commercial analyzer. *Anal. Biochem.* **1973**, *56*, 532−545.

Hardy, M. R.; Townsend R. R. High-pH anionic-exchange chromatography of glycoprotein-derived carbohydrates. *Methods Enzymol.* **1994**, *230*, 208−225.

Hohorst, H. J. L-(+)-lactate. Determination with lactic acid dehydrogenase and DPN. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1963; p 266.

Hull, S. R.; Montgomery, R. M. *myo*-Inositol phosphates in corn steep water. *J. Agric. Food Chem.* **1995**, 43, 1516−1523.

Hull, S. R.; Gray, J. S. S.; Koerner, T. A. W.; Montgomery, R. M. Trehalose as a common industrial fermentation byproduct. *Carbohydr. Res.* **1995**, *266*, 147−152.

Humble M. W.; King, A.; Phillips, I. API ZYM: A simple rapid system for the detection of bacterial enzymes. *J. Clin. Pathol.* **1977**, *30*, 275−277.

Inglett, G. E. Kernel structure, composition and quality. In *Corn: Culture, Processing, Products*; Inglett, G. E., Ed.; Avi Publishing Co.: Westport, CT, 1970; pp 123−137.

Koizumi, K.; Kubota, Y.; Tanimoto, T.; Okada, Y. High-performance anion-exchange chromatography of homogeneous D-gluco-oligosaccharides (polymerization degree greater than or equal to 50) with pulsed amperometric detection. *J. Chromatogr.* **1989** *464* (2), 365−373.

Kruse-Jarres, J. D.; Kaiser, C.; Hafkenscheid, J. C.; Hohenwallner, W.; Stein, W.; Bohner, J.; Klein, G.; Poppe, W.; Rauscher, E. Evaluation of a new α-amylase assay using 4,6-ethylidene-(G7)-1−4-nitrophenyl-(G1)-α-D-maltoheptaoside as substrate. *J. Clin. Chem. Clin. Biochem.* **1989**, *27-(2)*, 103−113.

Liu, T. Y.; Boykins, R. A. Hydrolysis of proteins and peptide in a hermetically sealed microcapillary tube: High recovery of labile amino acids. *Anal. Biochem.* **1989**, *182*, 383−387.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

McCormick, R. M. Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. *Anal. Chem.* **1988**, *60*, 2322−2328.

Moore, S.; Stein, W. H. Chromatographic determination of amino acids by the use of automatic recording equipment. *Methods Enzymol.* **1963**, *6*, 819−831.

Reiners, R. A.; Gooding, C. M. Corn Oil. in *Corn: Culture, Processing, Products*; Inglett, G. E., Ed.; Avi Publishing: Westport, CT, 1970; pp 241−261.

Sands, S. H.; Biskobing, S. J.; Olson, R. M. Commerical aspects of phytic acid: an overview. In *Phytic Acid: Chemistry and Applications*; Graf E., Ed.; Pilatus Press: Minneapolis, MN, 1986; pp 119−125.

Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.

Tietz, N. W., Ed. *Clinical Guide to Laboratory Tests;* W.B. Saunders Co.: Philadelphia, PA, 1983; pp 110, 398, 446.

Townsend, R. R.; Hardy M. R. Analysis of glycoprotein oligosaccharides using high-pH anion exchange chromatography. *Glycobiology* **1991**, *1*, 139−147.

Wilkie, K. C. B. The hemicelluloses of grasses and cereals. *Adv. Carbohydr. Chem. Biochem.* **1979**, *36*, 215−264.

Wilson, C. M. Proteins of the kernel. In *Corn: Chemistry and Technology*; Watson, S. A., Ramstad, P. E., Eds.; Amer. Assoc. of Cereal Chemists: St. Paul, MN, 1987; pp 273−310.

Wright, K. N. Nutritional properties and feeding value of corn and its by-products. In *Corn: Chemistry and Technology*; Watson, S. A., Ramstad, P. E., Eds.; Amer. Assoc. of Cereal Chemists: St. Paul, MN, 1987; pp 447−478.

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