Low Molecular Weight Organic Composition of Ethanol Stillage from Corn

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ABSTRACT

Filtered stillage from the distillation of ethanol made by yeast fermentation of hydrolyzed corn starch was subjected to gas chromatography-mass spectroscopy and to high-performance liquid chromatography. Nearly all the major chromatographic peaks were identified and quantified. Low molecular weight organics in the soluble part of corn stillage were lactic acid, glycerol, and alanine, as well as smaller amounts of ethanol, and various nonnitrogenous and nitrogenous acids, polyhydroxy alcohols, sugars, and glucosides.

The production of ethanol by yeast fermentation has again become a major industry, spurred by the increasing use of ethanol in automotive fuels. The major feedstocks used for this are D-glucose and maltose from grain or tuber starch as well as sucrose from sugarcane or sugar beet. In either case, distillation of ethanol from the fermentation broth leaves a mainly aqueous bottoms-fraction rich in organics, proteins, and salts. This material, known variously as stillage, vinhaça, or vinasse, is produced in enormous amounts. In the United States, for example, the distillation of ethanol made from corn yields roughly 10^8 m^3 of stillage per year.

In the United States, stillage usually does not go to waste; instead, most of it is concentrated and added to ruminant feed. The high cost of concentrating stillage, coupled with its relatively low nutrient value, suggests that it may have a more cost-effective use as a source of high-value organic materials. However, previous studies of stillage composition of corn (Sebree et al 1983, Rasco et al 1987, Wu 1989) and sugarcane (Bittencourt et al 1978, Bolsanello and Vieira 1980, Machado and Sant'Anna 1987, Celestine-Myrtil and Parfait 1988) did not yield detailed information on the identities and relative amounts of low molecular weight organic components in stillage. This study, specifically with corn stillage, was conducted to close that gap. Highly sensitive gas chromatography (GC) methods, mass spectroscopy (MS), and high-performance liquid chromatography (HPLC) were used for identification and analysis.

MATERIALS AND METHODS

Stillage Preparation

Bulk stillage, the distillation bottoms left after the production of ethanol by yeast fermentation of hydrolyzed cornstarch and corn-steep liquor, was obtained from Archer-Daniels-Midland (Cedar Rapids, IA). It was centrifuged at 10,000 × *g* for 30 min to remove solids. The supernatant was filtered twice through 0.22-μm cutoff filters to yield a clear yellow liquid that was concentrated approximately 14-fold into a syrup by means of a rotary vacuum evaporator. Some concentrated samples required resolubilization by addition of twice their volume of deionized water, but this led to no change in their chromatographic properties.

Proximate Analysis

Proximate analysis was conducted by Woodson-Tenent Laboratories (Des Moines, IA) using standard techniques. Moisture was measured by evaporation of the filtered sample in a forced-draft oven. Protein was determined by Kjeldahl analysis and fat by acid hydrolysis.

Derivatization Reactions

Stillage samples were derivatized to form volatile trimethylsilyl (TMS) derivatives with either hexamethyldisilazane (HMDS) (Sweeley et al 1963, Brobst and Lott 1966) or bis(trimethylsilyl)acetamide (BSA) (Klebe et al 1966), both purchased from Pierce (Rockford, IL). For concentrated samples, approximately 20 mg of syrup was added to a reaction vial, followed by 500 μl of pyridine, 450 μl of HMDS, and 50 μl of trifluoroacetic acid (TFA) used as a catalyst. This mixture was heated to 70°C for 30–60 min with periodic shaking. For a 20-μl sample of resolubilized stillage, 480 μl of pyridine, 450 μl of HMDS, and 50 μl of TFA formed the reaction mixture. Standards of 0.3–0.7 mg, obtained from Sigma Chemical (St. Louis, MO), Fisher Scientific (Fairlawn, NJ), or Aldrich Chemical (Milwaukee, WI), were used to identify chromatographic peaks. They were derivatized with 500 μl of pyridine, 450 μl of HMDS, and 50 μl of TFA. In a second derivatization technique, 500 μl of pyridine-stillage mixture was reacted with 500 μl of BSA, shaken for 30 sec, and held for 30–60 min at 70°C. Initial peaks in the resulting chromatograms often required 10 min to be eluted, significantly diminishing the usefulness of this method for MS analysis.

Gas Chromatography

Samples were analyzed with a Perkin-Elmer gas chromatograph (model Sigma 1, Norwalk, CT) using a 30-m × 0.25-mm i.d. DB-5 fused-silica capillary column and 1-m × 0.25-mm i.d. deactivated silica precolumn (J&W Scientific, Folsom, CA). The injector and flame ionization detector were held at 270°C, the split ratio was 1:100, and the He flow rate was 80 ml/min. Sample sizes were 1–4 μl. Because it was not possible to separate all the components with one column temperature program, two different ones were used. In the first, the temperature was held at 50°C for 10 min, followed by a 2.5°C/min gradient to 150°C, maintained for 10 min, rapidly increased to 250°C, and held for 15 min to clear the column. In the second program, the temperature was held at 150°C for 10 min, followed by a 2.5°C/min gradient to 250°C, and maintained for 10 min. The first program separated components containing three to five carbon atoms, and the second was used for the separation of components containing five to twelve carbon atoms.

Mass Spectroscopy

Both electron ionization (EI) and chemical (ammonia) ionization (CI) MS were performed on stillage samples with a Finnigan mass spectrometer (Model 4000, San Jose, CA) coupled to a capillary gas chromatograph containing a DB-5 column and following the same temperature programs described above. The sample was not split; all of it went to the mass spectrometer. Computerized libraries of mass spectra were searched to help identify fragmentation patterns.

HPLC

Filtered, but unconcentrated and underivatized, stillage was analyzed by HPLC to determine volatile components that were difficult to isolate by gas chromatography. A Waters (Milford, MA) model 712 WISP auto-injector, model 501 pump, and model R401 differential refractometer were used with a 300-mm long × 7.8-mm i.d. Bio-Rad (Richmond, CA) Aminex HPX-87H strong-acid cation column and Micro-Guard cation H+ pre-column. The column temperature was controlled at 65°C and a flow rate of 0.8 ml of aqueous 0.012*N* H_2SO_4 per minute was maintained. Sample size was 20 μl.

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RESULTS

Proximate Analysis

The filtered stillage sample was 95.05% moisture. Protein was 2.38%, crude fiber 0.3%, ash 1.12%, carbohydrate 1.38%, and fat 0.07%. The materials identified and quantified in this article comprise essentially all of the carbohydrate and a small part of the protein fractions, making up about 3% of the total. The pH of the mixture was 3.95.

Gas Chromatography

Figures 1 and 2 are gas chromatograms of filtered and concentrated stillage derivatized with HMDS, using the 50→150°C

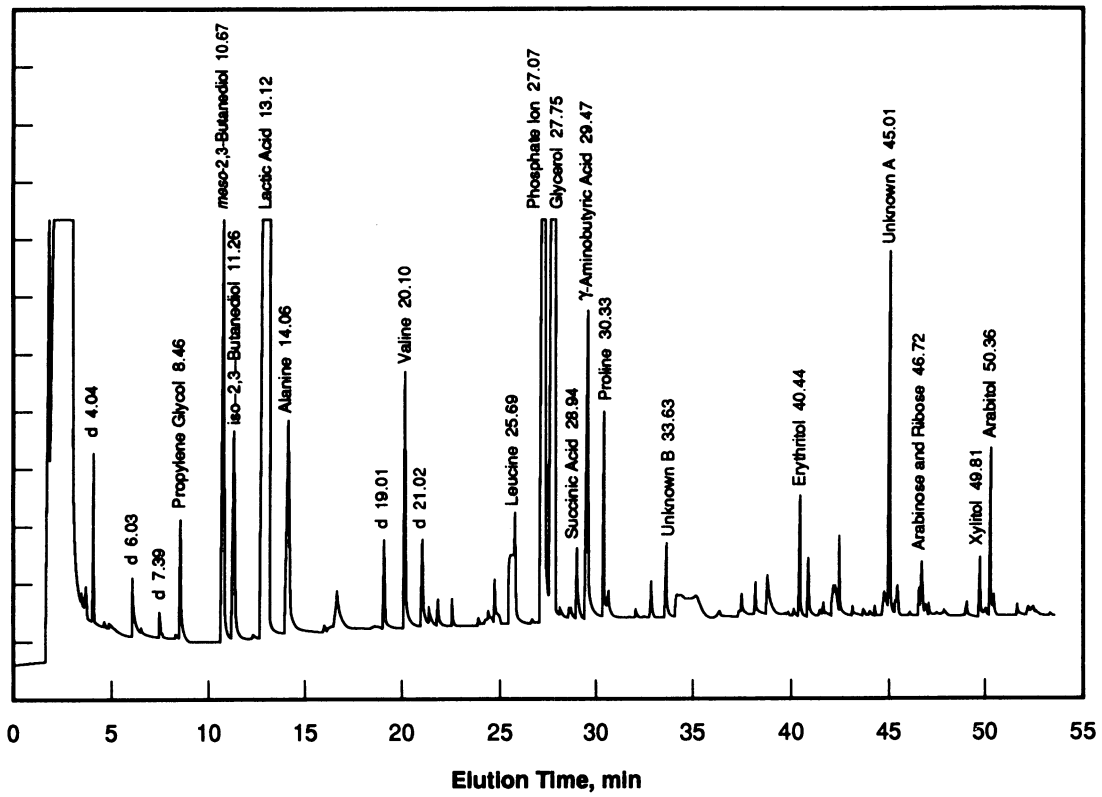

Fig. 1. Gas chromatogram of trimethylsilyl-derivatized, filtered, and concentrated stillage on a 30-m × 0.25-mm i.d. DB-5 capillary column using a 50→150°C temperature program and 80-ml/min He flow rate. d = by-product of the derivatization procedure.

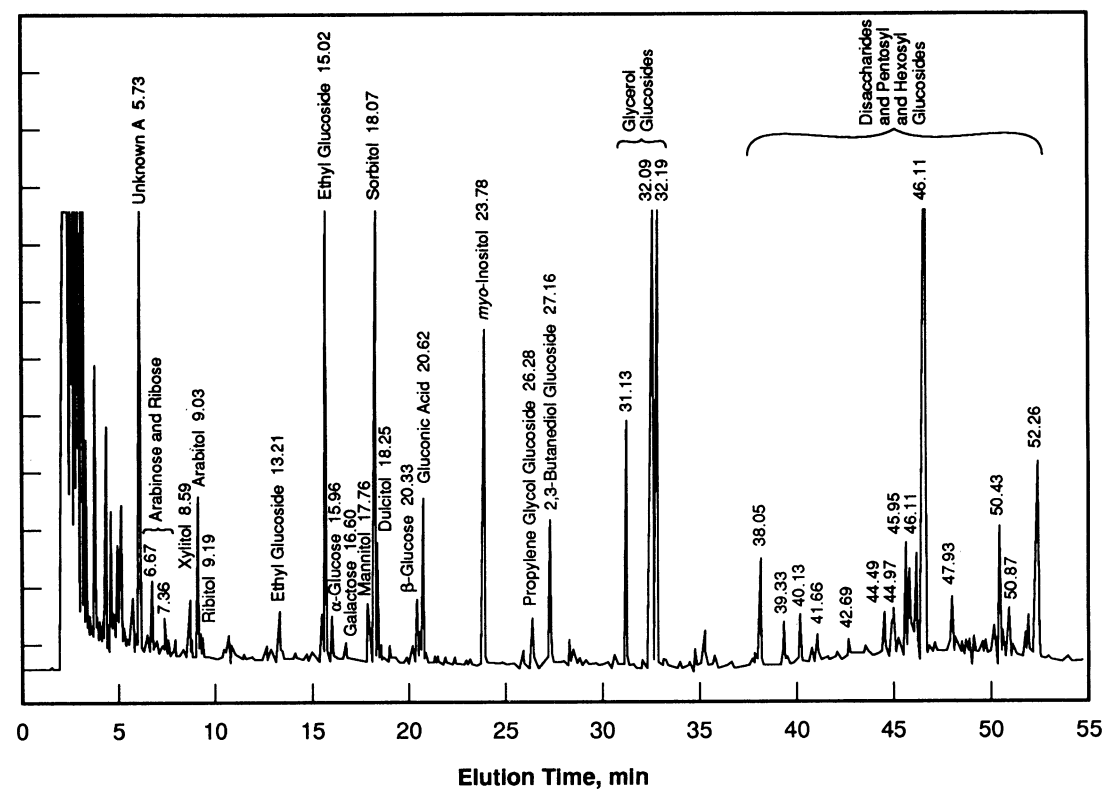

Fig. 2. Gas chromatogram of trimethylsilyl-derivatized, filtered, and concentrated stillage on a 30-m × 0.25-mm i.d. DB-5 capillary column using a 150→250°C temperature program and an 80 ml/min He flow rate.

and 150→250° C programs, respectively. The identities of virtually all labeled peaks were confirmed by a three-step procedure: 1) rough identification by comparison of the EI mass spectrum of each peak with those stored in an on-line library of spectra and by analysis of the molecular weights of the fragmentation products; 2) comparison of the peak's molecular weight measured by CI with that of the molecular weight of the TMS derivative of the suspected substance; 3) comparison of the GC retention time of the derivatized standard with that of the peak in question. There were two exceptions to this procedure. First, authentic samples of many glucosides were not commercially available, so unseparated mixtures were made by roughly following the procedure of Helferich and Schäfer (1941), using a mixture of D-glucose and ethanol, propylene glycol, 2,3-butanediol, or glycerol. However, strong-acid cation exchange resin was used rather than dry hydrogen chloride as a catalyst. Second, the amino acids found (alanine, valine, leucine, and proline, along with γ-aminobutyric acid) gave mixed TMS-TFA derivatives. The TMS groups derivatized their carboxyl moieties and the trifluoroacetyl groups derivatized their amino moieties. When BSA was substituted for HMDS and TFA was eliminated as a catalyst, the GC retention times of the five compounds changed to reflect the fact that both amino and carboxyl moieties were now derivatized with TMS groups.

Table I presents the relative retention times and concentrations of the various components using the chromatographic response parameters found in Table II. Components in the two tables are arranged within classes in order of increasing GC retention time and, in general, in order of increasing molecular weight. The response parameters form an intercept (positive for all components except ethanol) and a slope for each component because a straight line of weight versus detector area was always obtained upon standardization. There was almost no difference in relative retention times from run to run, so standard deviations are not shown. Stillage samples vary with the fermentation and distillation processes used and with the composition of the corn-steep liquor used as fermentation supplement; therefore, it is not expected that any single sample, as measured here, is indicative of all others. However, the identities of the major components and their relative magnitudes should be roughly the same from sample to sample.

The major components chromatographed with the 50→150° C program are, in decreasing order of concentration: lactic acid,

TABLE I
Retention Times and Concentrations of the Trimethylsilyl (TMS) Derivatives of Soluble Components of Corn Stillage

Component	Retention Times			Concentrations[d] (mg/ml)
	HPLC[a]	GC[b] (50→150° C)	GC[c] (150→250° C)	
Nonnitrogenous acids				
Acetic acid	1.134			0.77 (0.04)
Propionic acid	1.346			Trace
Lactic acid	0.955	0.470		10.4 (3.1)
Succinic acid		1.046		0.070 (0.026)
Unknown B		1.215		0.063 (0.019)
Gluconic acid			1.293	0.278 (0.062)
Amino and other nitrogenous acids				
Alanine[e]		0.509		4.08 (1.75)
Valine[e]		0.759		0.587 (0.260)
Leucine[e]		0.915		0.220 (0.060)
γ-Aminobutyric acid[e]		1.062		0.615 (0.104)
Proline[e]		1.096		0.444 (0.072)
Unknown A		1.625	0.378	0.661 (0.290)
Alcohols				
Ethanol	1.598			1.28 (0.22)
Propylene glycol	1.269	0.306		0.105 (0.017)
meso-2,3-Butanediol	1.408	0.386		0.337 (0.072)
dl-2,3-Butanediol		0.409		0.167 (0.030)
Glycerol	1.000	1.000		5.80 (1.51)
Erythritol		1.461	0.268	0.079 (0.024)
Xylitol		1.798	0.538	0.039 (0.012)
Arabitol		1.818	0.565	0.099 (0.034)
Mannitol			1.113	0.036 (0.017)
Sorbitol	0.765		1.132	0.305 (0.111)
Dulcitol			1.143	0.082 (0.029)
myo-Inositol			1.491	0.460 (0.206)
Sugars				
α-Glucose	0.660		1.000	0.036 (0.020)
β-Glucose	0.660		1.274	0.034 (0.023)
Arabinose/ribose				Trace
Disaccharides	0.567			
Trisaccharides	0.515			
Tetrasaccharides and larger	0.488			
Glucosides				
Ethyl glucoside (1)			0.828	0.039 (0.012)
Ethyl glucoside (2)			0.973	0.553 (0.018)
Propylene glycol glucoside			1.647	0.034 (0.012)
2,3-Butanediol glucoside			1.703	0.127 (0.034)
Glycerol glucoside (1)			1.951	0.190 (0.058)
Glycerol glucoside (2)			2.031	0.636 (0.254)
Glycerol glucoside (3)			2.049	0.357 (0.113)
Phosphoric acid		0.977		1.08 (0.48)

[a] Relative to high-performance liquid chromatography (HPLC) retention time of glycerol (10.54 min).
[b] Relative to gas chromatography (GC) retention time of TMS glycerol (27.75 min).
[c] Relative to GC retention time of TMS α-glucose (15.96 min).
[d] Standard deviations in parentheses are based on three or four determinations.
[e] Mixed TMS-trifluoroacetic acid derivative.

glycerol, alanine, phosphoric acid, an unknown (A), γ-aminobutyric acid, valine, two forms of 2,3-butanediol, proline, and leucine. Minor components include propylene glycol, arabitol, erythritol, succinic acid, another unknown (B), xylitol, and various characteristic products of the derivatization process. The major components with the 150→250°C program are three glucosides of glycerol, unknown A, two forms of ethyl glucoside, *myo*-inositol, sorbitol, gluconic acid, and an unidentified disaccharide (not maltose). Minor components are a glucoside of 2,3-butanediol, arabitol, dulcitol, erythritol, both anomers of glucose, xylitol, mannitol, a glucoside of propylene glycol, two anomeric forms each of arabinose and ribose, either β-galactose or β-mannose, and a number of species of 10–12 carbon atoms that appear to be glucosides and hexose disaccharides. Erythritol, unknown A, arabinose, ribose, xylitol, and arabitol are eluted near the end of the chromatogram generated by the first program and toward the beginning of that generated by the second one. The order of sugar retention times agrees with that of the TMS derivatives of the same materials chromatographed by Sweeley et al (1963).

Standards of ethylene glycol, 1,3-butanediol, *trans*-aconitic acid, fumaric acid, glyoxylic acid, malic acid, syringic acid, methyl malonate, ethyl malonate, aspartic acid, glutamic acid, isoleucine, methyl α-galactoside, methyl β-galactoside, methyl α-glucoside, methyl β-glucoside, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, threitol, fructose, xylose, cellobiose, maltose, and maltitol were derivatized with HMDS and submitted to GC but did not correspond to peaks that appeared when stillage was chromatographed. No mass-spectra characteristic of aldehydes were found. Some peaks were by-products of the derivatization procedure and are so labeled. Unlabeled peaks were too small to yield mass spectra.

A substantial, but unavailing, effort was made to identify unknown A, much the largest GC peak to remain unidentified. CI showed that the TMS-derivatized material has a molecular weight of 322. EI suggested the material is similar to the TMS ester of 3-(*p*-TMS hydroxyphenyl) propanoic acid ($TMSOC_6H_4CH_2CH_2COOTMS$, $C_{15}H_{26}O_3Si_2$, mol. wt. 310). However, several major peaks could not be explained by the EI fragmentation of that material or of *p*-TMS-cinnamic acid, nor of 322-Da relatives of either of them. The pattern of M, M+1, M+2, M+3, and M+4 (mol. wt. 323, 324, 325, 326, and 327, respectively) peak areas from CI was more consistent with that of a molecular composition of $C_{17}H_{27}O_4Si$ than that of $C_{16}H_{27}O_3Si_2$, except that in both cases the peak area of the M form was higher than justified by these compositions. A closer approach to the peak area pattern was afforded by the compositions $C_{15}H_{23}N_2O_4Si$ and $C_{14}H_{23}N_2O_3Si_2$. Again, the form with one Si atom was more consistent with the peak area pattern.

Unknown B gave an EI spectrum that corresponded to nothing in the MS library; CI showed a molecular weight of 351.

HPLC

Shown in Figure 3 is an HPLC trace of centrifuged and filtered, but unconcentrated and underivatized, stillage. Maltose, maltotriose, and maltotetraose were used as standards to tentatively assign the molecular weight of the components in the first-eluting peaks. Stillage components with the molecular weight of tetrasaccharides and higher elute at the void volume, and those with molecular weight of trisaccharides appear as the tailing shoulder of this peak. The next peak has the same retention time as maltose and almost surely encompasses the peaks representing components of 10–12 carbon atoms found by GC. Several small peaks associated with the large glucoside peaks found by GC precede large lactic acid and glycerol peaks and the much smaller peaks representing acetic acid, propylene glycol, propionic acid, *meso*-2,3-butanediol, and ethanol.

Standards of methanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol, *sec*-butanol, *tert*-butanol, formic acid, butyric acid, isobutyric acid, and valeric acid were submitted to HPLC, but

TABLE II
Response Factors for the Soluble Components of Corn Stillage

Component	Number of Points	Response Parameters[a]		Correlation Coefficient
		$a \times 10^6$	$b \times 10^9$	
High-performance liquid chromatography—refractive index detector				
Ethanol	6	−2020	1460	0.9993
Acetic Acid	6	1790	1070	0.9999
Gas chromatography—flame ionization detector[b]				
Lactic acid	7	127	0.732	0.9981
Succinic acid	7	169	0.998	0.9992
Mesaconic acid[c]	7	170	0.986	0.9990
Gluconic acid	4	86.6	2.12	0.9997
Alanine[d]	4	175	13.4	0.9939
Valine[d]	4	243	5.89	0.9971
Leucine[d]	5	173	1.26	0.9957
γ-Aminobutyric acid[d]	5	206	1.06	0.9949
Proline[d]	5	167	1.52	0.9919
p-Aminobenzoic acid[e]	6	43.6	2.96	0.9949
Propylene glycol	7	20.5	0.605	0.9983
2,3-Butanediol[f]	7	26.3	0.554	0.9975
Glycerol	7	90.5	0.568	0.9981
Erythritol	6	24.8	1.12	0.9999
Xylitol	5	13.8	1.02	0.9999
Arabitol	6	18.7	1.06	0.9968
Mannitol	5	8.03	0.905	0.9974
Sorbitol	5	7.00	1.02	0.9999
Dulcitol	5	7.86	1.15	0.9986
myo-Inositol	4	2.29	2.47	0.9999
Glucose[g]	6	60.4	1.08	0.9973
Phosphoric acid	5	74.1	0.785	0.9998

[a] Based on the linear equation: mg determined = $\alpha + (b \times$ detector area).
[b] Trimethylsilyl derivatives unless otherwise stated.
[c] For the calibration of Unknown B.
[d] Mixed trimethylsilyl-trifluoroacetic acid derivative.
[e] For the calibration of Unknown A.
[f] Based on a mixture of *dl*-butanediol and *meso*-2,3-butanediol.
[g] Based on a mixture of α and β-glucose.

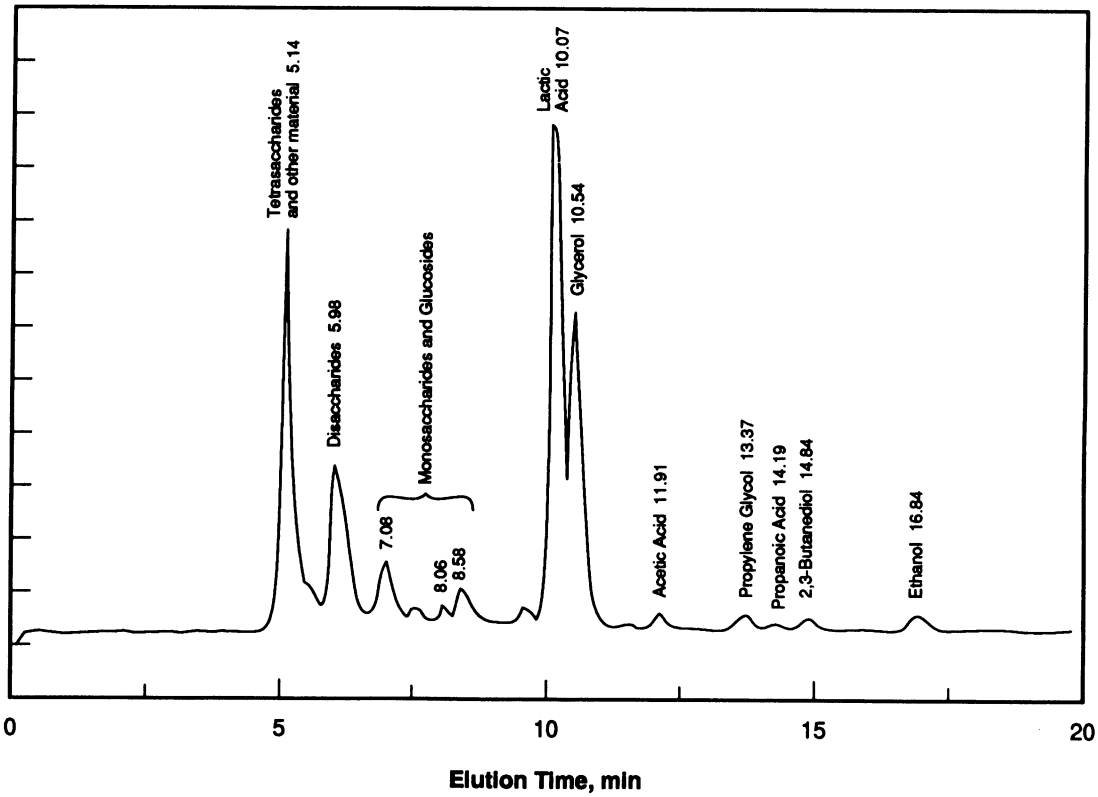

Fig. 3. High-performance liquid chromatography of filtered stillage on a 300-mm long × 7.8-mm i.d. HPX-87H strong-acid cation exchange column at 65°C with a flow rate of 0.8 ml of aqueous 0.012*N* H_2SO_4 per minute.

they did not correspond to peaks obtained when stillage was chromatographed.

DISCUSSION

Compared to other products of ethanolic yeast fermentations (Harrison and Graham 1970), filtered stillage contains an extensive, but still relatively limited, list of low molecular weight organic components. With the exception of small amounts of ethanol, acetic acid, and propionic acid, no highly volatile components remain in significant quantities. This, of course, has occurred because those materials have been removed during industrial distillation of ethanol (or during laboratory vacuum evaporation to concentrate the stillage). To some extent, this is also because the fermentation that produces industrial ethanol is a short one; many materials are not elaborated in concentrations high enough to be identified. Instead, what remains in stillage, in addition to ethanol, acetic acid, and propionic acid, is a mixture of higher-boiling or nonvolatile hydroxylated, dicarboxylic, amino, and other nitrogenous acids, polyhydric alcohols, and various sugars, sugar alcohols, and glucosides, along with proteins, fats, and salts.

The low molecular weight organic compounds in stillage either enter the process in the corn-steep liquor used as a fermentation supplement or are made by yeast during the fermentation. Corn-steep liquor has large amounts of lactic acid, γ-aminobutyric acid, choline, inositol, and free amino acids (Zabriskie et al 1980). The mainly anaerobic environment during the fermentation encourages the reduction of sugars to sugar alcohols, and the production by yeast of polyhydric alcohols, especially glycerol, is well known (Spencer and Sallans 1956, Spencer and Shu 1957, Spencer et al 1957). The presence of high concentrations of glucose in conjunction with significant amounts of ethanol, propylene glycol, glycerol, and 2,3-butanediol during the fermentation would lead to the formation of glucosides of each alcohol.

Only four amino acids (alanine, valine, leucine, and proline) are found in significant amounts in filtered corn stillage. Of these, proline is reported as being very slowly absorbed from the fermentation medium by brewer's yeast; alanine is absorbed after a lag; leucine and valine are absorbed gradually (Jones and Pierce 1964). Large amounts of others (e.g., glutamic acid) in corn-steep liquor (Zabriskie et al 1980) are more rapidly assimilated by yeast.

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