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Filtered stillage from the distillation of ethanol made by yeast fermentation of sugarcane molasses, citrus waste, and sweet whey was analyzed by gas chromatography/mass spectroscopy and by high-performance liquid chromatography. Nearly all of the major peaks representing low molecular weight organic components were identified. The major components in cane stillage were, in decreasing order of concentration, lactic acid, glycerol, ethanol, and acetic acid. In citrus stillage they were lactic acid, glycerol, *myo*-inositol, acetic acid, *chiro*-inositol, and proline. Finally, in whey stillage the major components were lactose, lactic acid, glycerol, acetic acid, glucose, arabinitol, and ribitol.

INTRODUCTION

The use of ethanol as an automotive fuel has caused its production by fermentation of maize and sugarcane, the first predominantly in the United States and the second in Brazil, to markedly increase in recent years. This has led to the production of large amounts of stillage, known elsewhere as vinaça and vinasse, as a byproduct of the distillation process that concentrates ethanol. This aqueous material, rich in organics, protein, and salts, is often used for animal feed after concentration, a costly and energy-intensive step that yields a product that is still relatively low in value.

Substantial effort has been expended to determine the composition of various types of stillage (Wu et al., 1981, 1984, 1989; Wu and Sexson, 1984; Wu and Bagby, 1987; Celestine-Myrtil and Parfait, 1988; Wu, 1989, 1990). In general, however, these studies have not yielded extensive information on the composition of low molecular weight organic materials in stillage, even though valuable materials potentially could be extracted from it. We have determined the concentrations of such materials in stillage from maize (Dowd et al., 1993). This paper reports a similar study of stillages produced from the yeast fermentation of sugarcane molasses, citrus waste, and sweet whey. We used gas chromatography (GC) and high-performance liquid chromatography (HPLC) to determine concentrations of low molecular weight organics and electron ionization and chemical ionization mass spectroscopy (EIMS and CIMS), both in conjunction with GC, to identify them.

MATERIALS AND METHODS

Stillage Preparation. Raw stillage from sugarcane molasses and citrus waste was obtained from domestic ethanol producers. Sweet whey stillage was donated by Kraft General Foods (Evanston, IL). Each sample was centrifuged at 10000*g* for 30 min to remove solids. Supernatants were filtered twice through 22-μm cutoff filters to yield nonviscous samples of different colors (cane stillage was black, citrus stillage brown, and whey stillage pale yellow). Portions were concentrated to syrups by rotary vacuum evaporation. Filtered 700-μL samples of cane and citrus stillage typically yielded about 100 mg of concentrated syrup; the same-sized sample of whey stillage yielded about 200 mg.

Proximate Analysis. Bulk and filtered samples were analyzed by Woodson-Tenent Laboratories (Des Moines, IA) using standard ASTM procedures. Moisture was measured by evaporation in a forced-draft oven. Protein was measured by Kjeldahl analysis and fat by acid hydrolysis. Carbohydrate was found by difference.

Derivatization Reactions. Stillage syrups (15–20 mg) were mixed with 500 μL of pyridine, 450 μL of hexamethyldisilazane (HMDS), and 50 μL of trifluoroacetic acid from Pierce (Rockford, IL) (Sweeley et al., 1963; Brobst and Lott, 1966), shaken for 30 s, and then held at 70 °C for at least 1 h to yield a single liquid phase containing volatile trimethylsilyl (TMS) derivatives. Higher amounts of syrup often gave two liquid phases. Derivatized protein and fibrous material tended to settle to the bottom of the reaction vial. Samples for GC injections were taken from the clear liquid. Standards (0.2–0.3 mg) were derivatized as above, except for a minimum of 30 min or until a single clear solution was formed. Amino acids and inositols generally required longer periods. *myo*-Inositol, which was relatively highly concentrated in citrus stillage, yielded several peaks in addition to the expected hexa-TMS-*myo*-inositol peak. We could reproduce these peaks, of 540 molecular mass corresponding to penta-TMS-derivatized products, by increasing the *myo*-inositol concentration in our preparation of the standard.

Standards. Most standards were purchased from laboratory supply firms. *allo*-, (−)*chiro*, *muco*-, and *neo*-inositols were gifts from Prof. L. Anderson (University of Wisconsin—Madison). Available sugar acid lactones were incubated in slightly basic conditions (pH 8 phosphate buffer) to produce an equilibrium mixture with the free acid that could be detected by GC.

Gas Chromatography. Derivatized samples were separated with a Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph using a 30-m × 0.25-mm i.d. × 0.1-μm film thickness DB-5 fused-silica capillary column (J&W Scientific, Folsom, CA) with a flame ionization detector. Injector and detector were held at 270 °C, the split ratio was 1:100, and the He flow rate was 80 mL/min. Injection volumes were between 1 and 4 μL. Three temperature programs were used: (1) 50 °C for 10 min followed by a 2.5 °C/min rise to 150 °C, held for 10 min, followed by a 20 °C/min rise to 250 °C; (2) 150 °C for 10 min, followed by a 2.5 °C/min rise to 250 °C; (3) 80 °C for 4 min, followed by a 2.5 °C/min rise to 280°C. In each case the final temperature was held for 15 min.

Mass Spectroscopy. EIMS was with a Finnigan (San Jose, CA) Magnum ion-trap mass spectrometer using each temperature program with a 30-m long × 0.25-mm i.d. × 0.25-μm film thickness DB-5ms column with 50 mL/min He flow rate. Samples were diluted 1:10 to 1:20 with pyridine, the split ratio was 1:50, and 1 μL of sample was injected. CIMS with CH_4 or NH_3 was conducted with a Finnigan 4000 quadrapole mass spectrometer with the third temperature program and the column above, but with undiluted samples. The He flow rate was 80 mL/min, there

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Table 1. Proximate Analyses (Percent) of Raw and Filtered Stillages

	cane		citrus		whey		corn[a]	
	raw	filtered	raw	filtered	raw	filtered	raw	filtered
moisture	89.64	91.17	91.10	92.57	82.03	85.55	93.96	95.05
protein	2.92	2.08	2.20	1.50	4.49	2.41	2.54	2.38
fiber	0.2	0.2	0.6	0.3	0.3	0.3	0.2	0.3
fat	0.41	0.24	0.47	0.21	0.27	0.05	0.13	0.07
ash	3.61	3.86	1.09	1.27	6.93	6.93	2.36	1.12
carbohydrate	3.42	2.65	5.14	4.45	6.28	5.06	1.01	1.38
pH	4.4		4.0		5.0		4.0	

[a] Dowd et al. (1993).

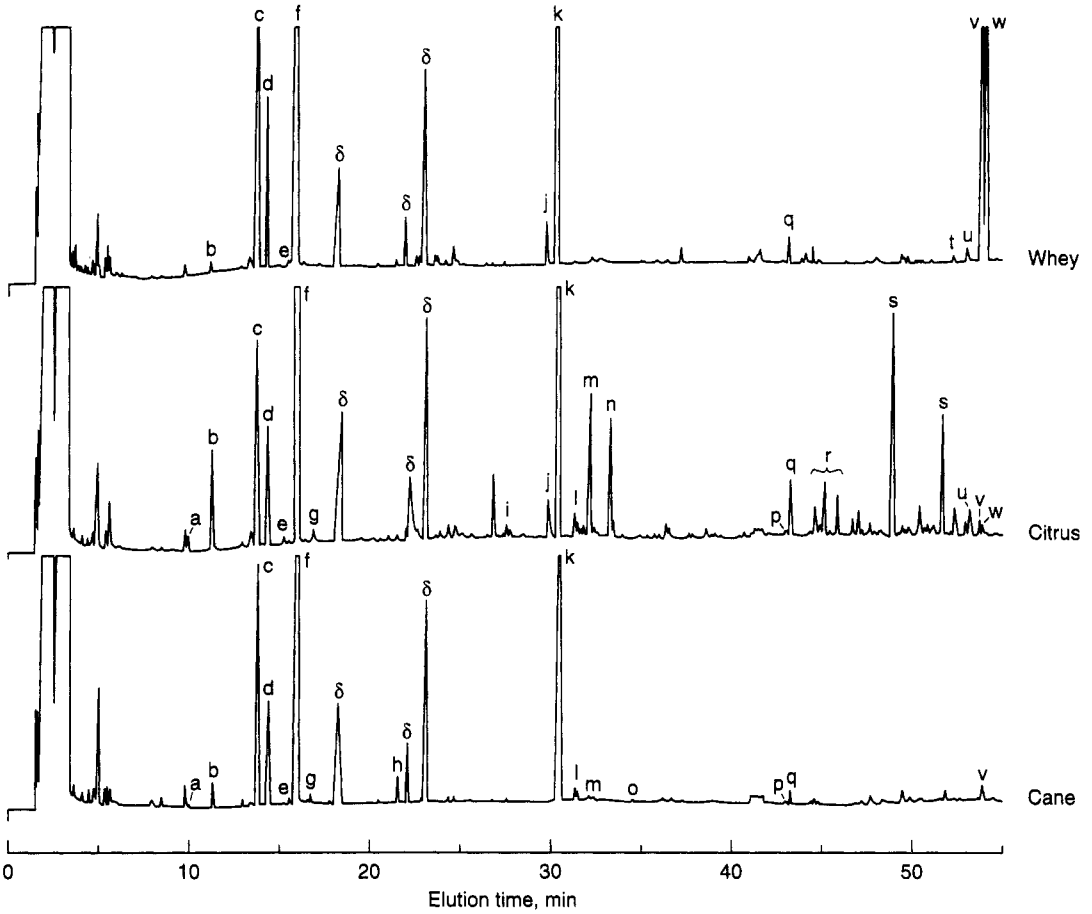

Figure 1. Capillary GC of TMS-derivatized cane, citrus, and whey stillages using a 50–150 °C program. (δ) Derivatization byproduct; (a) ethylene glycol; (b) propylene glycol; (c) *dl*-2,3-butanediol; (d) *meso*-2,3-butanediol; (e) 1,3-propanediol; (f) lactic acid; (g) alanine; (h) 3-hydroxypropanoic acid; (i) 4-hydroxybutyric acid; (j) phosphoric acid; (k) glycerol; (l) succinic acid; (m) γ-aminobutyric acid; (n) proline; (o) glyceric acid; (p) threitol; (q) erythritol; (r) C_4 sugar acids and C_5 deoxysugar alcohols; (s) rhamnose; (t) fucose; (u) xylitol; (v) arabinitol; (w) ribitol. Please note that the retention times of the two 2,3-butanediols were reversed by error in Dowd et al. (1993).

was no split, and 2 μL was injected. We identified GC peaks by a three-step procedure: (1) comparison of the EIMS fragmentation pattern of the peak with an on-line library of such patterns; (2) comparison of the peak's molecular weight by CIMS with the molecular weight of the TMS derivative of the putative substance; (3) comparison of the GC retention time of the derivatized standard with that of the peak in question.

HPLC. HPLC was conducted on underivatized and unconcentrated, but filtered, samples with an ISCO (Lincoln, NE) Model 2350 pump, a Bio-Rad (Richmond, CA) HPX-87H Aminex column and Micro-Guard cation H+ precolumn, and a Knauer (Berlin, Germany) differential refractometer. The eluent was room temperature 0.01 N H_2SO_4 at 1 mL/min. Injection volume was 20 μL.

Response Factors. GC and HPLC linear response factor equations were generated from five or six points of known composition over an appropriate range. Correlation coefficients were always >0.997 and typically were >0.999.

RESULTS AND DISCUSSION

Proximate analyses of raw and filtered cane, citrus, and whey stillages, as well as that of corn stillage (Dowd et al., 1993), are shown in Table 1. Whey stillage was much more concentrated than the others, as was evident when it was evaporated. It was especially high in inorganics and protein, but it was also highest in those materials analyzing as carbohydrates. All three stillages studied here were more concentrated and had much more carbohydrate than did corn stillage and are therefore potentially better candidates for extraction of low molecular weight components.

Derivatized samples of the three stillages were submitted to GC using 50–150 and 150–250 °C programs, to allow the most efficient separation of volatile components. Chromatograms are shown in Figures 1 and 2, with those from HPLC of nonderivatized samples shown in Figure

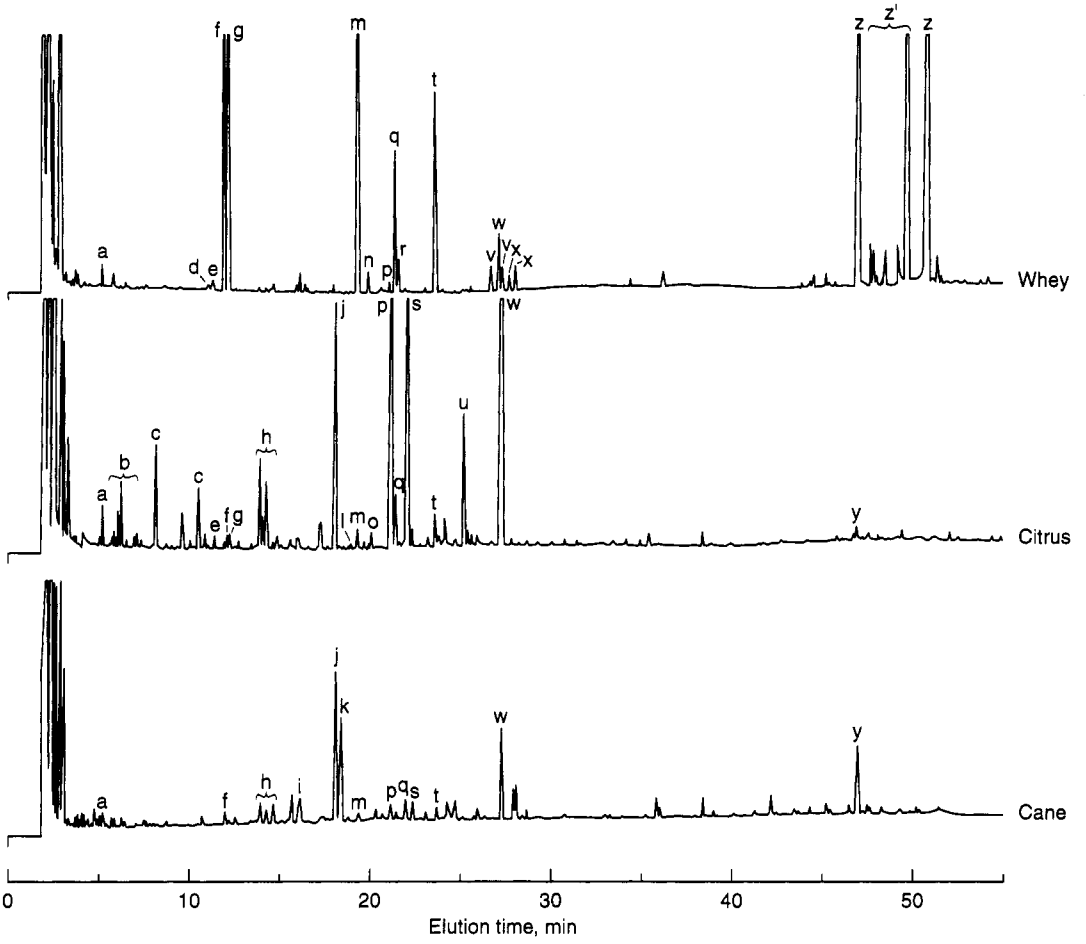

Figure 2. Capillary GC of TMS-derivatized cane, citrus, and whey stillages using a 150–250 °C program. (a) Erythritol; (b) C_4 sugar acids and C_5 deoxysugar alcohols; (c) rhamnose; (d) fucose; (e) xylitol; (f) arabinitol; (g) ribitol; (h) C_5 sugar acids and C_6 deoxysugar alcohols; (i) shikimic acid; (j) quinic acid; (k) C_6 deoxysugar acid; (l) *allo*-inositol; (m) α-glucose; (n) galactose; (o) *muco*-inositol; (p) mannitol; (q) sorbitol; (r) galactitol; (s) *chiro*-inositol; (t) β-glucose; (u) *scyllo*-inositol, (v) *N*-acetylgalactosamine; (w) *myo*-inositol; (x) *N*-acetylglucosamine; (y) sucrose; (z) lactose; (z′) unknown disaccharides.

3. As with derivatized corn stillage (Dowd et al., 1993), a number of GC peaks that eluted early represented characteristic byproducts of the derivatization process, while a number of early-eluting HPLC peaks are of poorly separated oligosaccharides. In neither case were these materials further considered. In general, the relative retention orders of sugars and inositols from the capillary DB-5 column were the same as reported for packed SE-30 columns (Sweeley et al., 1963; Brobst and Lott, 1966; Sasaki et al., 1987).

We submitted the following standards after HMDS derivatization to GC or without derivatization to HPLC without finding peaks corresponding to those in Figures 1–3: (carbohydrates) D-allose, D-altrose, cellobiose, 2-deoxy-D-galactose, 2-deoxy-D-glucose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 2-deoxy-D-ribose, D-erythrose, D-erythrulose, D-fructose, gentiobiose, DL-glyceraldehyde, isomaltulose, laminarabiose, leucrose, D-lyxose, maltose, D-mannose, melibiose, D-psicose, D-ribose, sophorose, L-sorbose, D-talose, D-threose, D-xylose, D-xylulose; (carbohydrate derivatives) *N*-acetylmannosamine, fucitol, D-galactonic acid, D-galactonic acid γ-lactone, D-galactosamine, α-D-galactose 1-phosphate, D-galacturonic acid, D-gluconic acid, D-gluconic acid δ-lactone, D-glucosamine, α-D-glucose 1-phosphate, D-glucose 6-phosphate, D-glucuronic acid, D-glucuronic acid lactone, lactitol, maltitol, L-mannonic acid γ-lactone, D-mannosamine, methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside; (alcohols) methanol, 1-propanol, 2-propanol, 1-butanol, iso-, *sec*-, and *tert*-butyl alcohol; (polyalcohols) 1,3-butanediol, phloroglucinol (1,3,5-trihydroxybenzene), pyrogallol (1,2,3-trihydroxylbenzene); (organic acids) *trans*-aconitic, ascorbic, benzoic, *n*-butyric, isobutyric, caffeic, citric, citramalic, *m*-coumaric, *o*-coumaric, *p*-coumaric, 3,4-dimethylbenzoic, ethylmalonic, fumaric, ferulic, glycolic, glyoxylic, 2-hydroxybutyric, 3-hydroxybutyric, hexonic, lauric, L-malic, mesaconic, methylmalonic, palmitic, β-phenyllactic, protocatechuic, propanoic, stearic acid, syringic, L-tartaric, *n*-valeric, isovaleric, vanillic; (amino acids) L-aspartic acid, L-glutamic acid, L-isoleucine, L-leucine, L-serine, L-tyrosine, L-valine; (miscellaneous) α-glycerophosphate, β-glycerophosphate, phenol, umbelliferone, and vanillin. Furthermore, of the nine organic acids found in cane stillage by Celestine-Myrtil and Parfait (1987), we found only two, lactic acid and shikimic acid. We found no GC peaks with retention times corresponding to derivatized *trans*-aconitic, citric, fumaric, glycolic, and malic acids and no EIMS or CIMS evidence of them or of *cis*-aconitic and oxalic acids. There are two explanations for this: (1) Our cane stillage sample may have had concentrations of these materials below our detection limit (0.03 g/L), which was in most cases below the concentrations found by Celestine-Myrtil and Parfait. (2) Their method of peak identification, by comparing only HPLC retention times of peaks in their sample with those of standards, could have given incorrect conclusions.

Concentrations of identified components in all three stillages studied here, as well as those in corn stillage, all based on samples before concentration, are presented in Table 2. The major components in cane stillage were, in

Table 2. Concentrations (Grams per Liter) of the Soluble Components of Cane, Citrus, Whey, and Corn Stillages[a]

compound	cane	citrus	whey	corn[b]
aldehydes				
acetaldehyde	0.697 (0.040)[c]	0.343 (0.002)	1.42 (0.02)	
alcohols, polyols, and sugar alcohols				
ethanol	3.83 (0.04)	1.15 (0.04)	1.01 (0.20)	1.28 (0.22)
ethylene glycol	d[e]	d	d	
propylene glycol	0.084 (0.004)	0.211 (0.006)	0.123 (0.006)	0.105 (0.017)
2,3-butanediols[d]	0.568 (0.035)	0.516 (0.231)	2.14 (0.14)	0.504 (0.096)
1,3-propanediol	d	d	d	
glycerol	5.86 (0.45)	5.09 (0.23)	13.2 (1.1)	5.80 (1.51)
threitol	tr[f]	tr	tr	
erythritol	0.088 (0.001)	0.121 (0.011)	0.228 (0.014)	0.079 (0.024)
xylitol		0.082 (0.004)		0.039 (0.012)
arabinitol	0.064 (0.001)	0.065 (0.010)	2.75 (0.21)	0.099 (0.034)
ribitol	tr	d	2.29 (0.17)	tr
allo-inositol		d		
neo-inositol		tr		
muco-inositol		d		
mannitol	0.089 (0.009)	0.845 (0.135)	d	0.036 (0.017)
sorbitol	d	0.170 (0.023)	1.08 (0.08)	0.305 (0.111)
galactitol		tr	0.234 (0.014)	0.082 (0.029)
chiro-inositol	0.114 (0.006)	1.58 (0.26)		
epi-inositol		tr		
scyllo-inositol		0.308 (0.055)		
myo-inositol	0.236 (0.200)	2.88 (0.47)	0.408 (0.032)	0.460 (0.206)
sugars				
arabinose	tr		tr	tr
rhamose[d]	tr	0.536 (0.059)		
fucose			d	
galactose[d]		tr	0.221 (0.020)	tr
glucose[d]	d	0.262 (0.021)	6.16 (0.40)	0.070 (0.043)
sucrose	0.222 (0.017)	0.066 (0.005)		
lactose[d]			25.1 (1.9)	
sugar derivatives				
C_5 sugar acid (1)	d	d		
C_5 sugar acid (2)	d	d		
C_5 sugar acid (3)	d	d		
N-acetylgalactosamine			d	
N-acetylglucosamine			d	
amino acids				
alanine	tr	tr		4.08 (1.75)
γ-aminobutyric acid	d	1.01 (0.07)		0.615 (0.104)
proline		1.47 (0.28)		0.444 (0.072)
acids				
acetic acid	1.56 (0.10)	2.31 (0.04)	10.0 (0.21)	0.77 (0.04)
formic acid	0.582 (0.149)	0.045 (0.004)	tr	
lactic acid	7.74 (0.39)	9.76 (2.36)	15.4 (1.1)	10.4 (3.1)
3-hydroxypropanoic acid	tr			
4-hydroxybutyric acid		tr		
phosphoric acid	tr	0.226 (0.161)	0.587 (0.454)	1.08 (0.48)
succinic acid	d	d		0.070 (0.026)
4-hydroxybenzoic acid	tr			
glyceric acid	d	tr		
shikimic acid	d			
quinic acid	0.508 (0.020)	0.740 (0.094)		

[a] Based on response factor equations of the following form: mg determined = $a + (b \times$ detected area). [b] Dowd et al. (1993). [c] Standard errors based on three to four determinations. [d] Summation of individual isomer or anomer peaks. [e] d, detectable by flame ionization detector and ion-trap mass spectrometer but not quantifiable. [f] tr, detectable by ion-trap mass spectrometer only.

decreasing order of concentration, lactic acid, glycerol, ethanol, and acetic acid. In citrus stillage they were lactic acid, glycerol, *myo*-inositol, acetic acid, *chiro*-inositol, and proline. In whey stillage the major components were lactose, lactic acid, glycerol, acetic acid, glucose, arabinitol, and ribitol.

Despite the fact that most major components, with the exception of lactose, were similar in each stillage, the overall compositions of the three stillages were markedly different from each other and from that of corn stillage in the more minor components. For instance, whey stillage had substantial amounts of the sugar alcohols arabinitol, ribitol, sorbitol, and galactitol, while cane and citrus stillages had very low concentrations of these and the latter had significant amounts of mannitol. The only sugar alcohol found in more than very minor concentations in corn stillage was sorbitol. Citrus stillage had seven inositol isomers, four in nonmeasurable quantities, but with sizable amounts of *myo*-, *chiro*-, and *scyllo*-inositol, while the other stillages were essentially limited to *myo*-inositol. Corn stillage was notable for having a large amount of alanine and substantial quantities of γ-aminobutyric acid and proline; the latter two were found in citrus stillage, but no amino acid was found in anything but trace amounts in cane and whey stillages. Quinic acid was present in cane and citrus but not in whey and corn stillages.

Most of the materials that greatly varied from one stillage to the next clearly must come from the feeds to the ethanolic fermentations and then survive degradation. For instance, mannitol is a minor sugar in molasses, while formic acid is a degradation product found there and quinic acid comes from raw sugar (Meade and Chen, 1977).

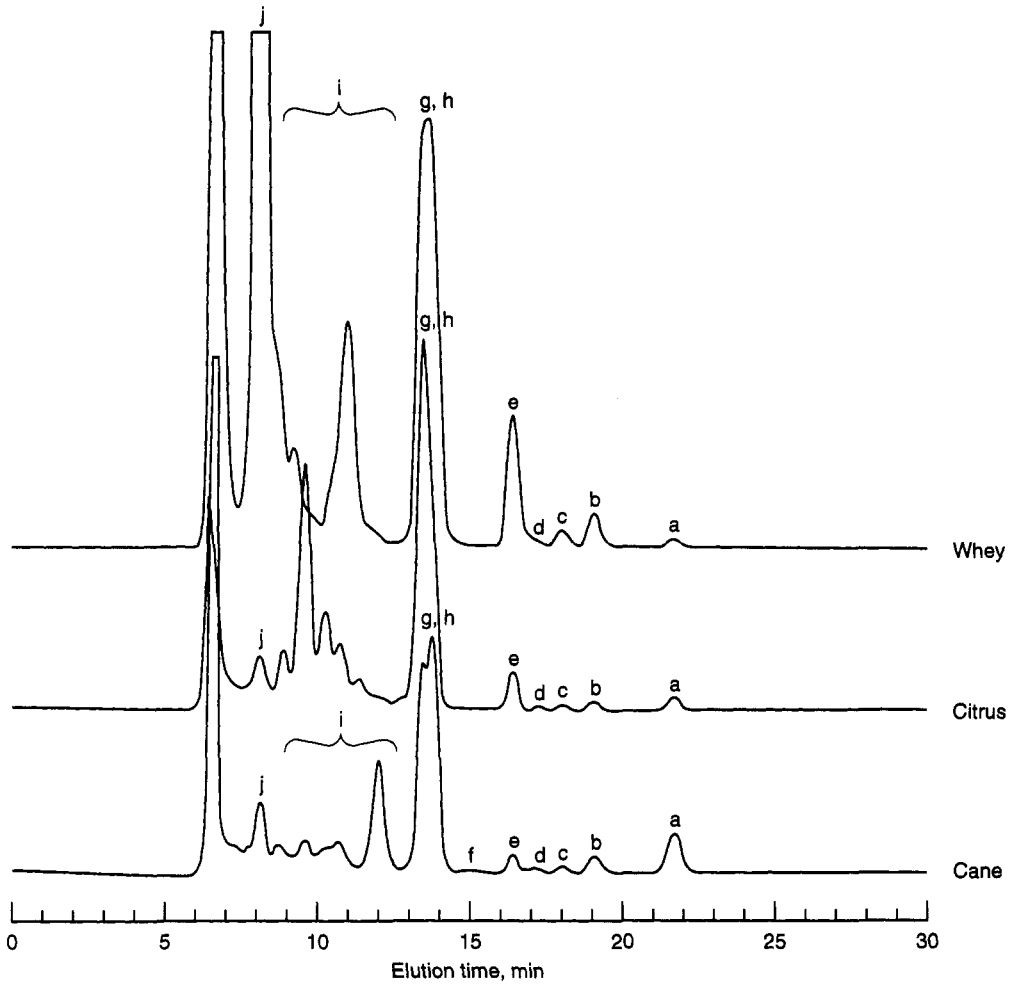

Figure 3. HPLC of cane, citrus, and whey stillages. (a) Ethanol; (b) 2,3-butanediol; (c) acetaldehyde; (d) propylene glycol; (e) acetic acid; (f) formic acid; (g) glycerol; (h) lactic acid; (i) C_5 and C_6 sugars and sugar alcohols; (j) disaccharides and larger oligosaccharides.

Proline, γ-aminobutyric acid, *myo*-inositol (but not the other inositols), and quinic acid have been reported as major or substantial components of citrus juice and peel, with rhamnose as a constituent of citrus pectic materials (Sinclair, 1984). Those materials that are found in substantial quantities in all of the stillages tested, such as ethanol, propylene glycol, 2,3-butanediol, acetic acid, and lactic acid, are almost surely produced in the main by the fermentations themselves. Acetaldehyde, found in all of the stillages (it was probably incorrectly identified by HPLC as propionic acid in corn stillage), probably is a fermentation product also. Finally, the very high concentrations of glycerol, sugar alcohols, and acetic acid in whey stillage suggest a different type of fermentation than used to produce the other stillages, while the high concentrations of glucose and lactose in whey stillage suggest that the fermentation was incomplete, leading to the possibility that other samples of whey stillage might have very different amounts of these two sugars. Other differences in relative concentrations of each component of the same stillage from one sample to the next should be smaller.

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