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DETERMINATION OF ORGANIC ACIDS IN SUGAR CANE PROCESS JUICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: IMPROVED RESOLUTION USING DUAL AMINEX HPX-87H CATION-EXCHANGE COLUMNS EQUILIBRATED TO DIFFERENT TEMPERATURES

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SUMMARY

The application of high-performance liquid chromatography to the quantitative analysis of organic acids in sugar cane process juice is described. Separation of these acids is achieved on Aminex HPX-87H cation-exchange columns. Resolution is improved by connecting two columns in series and equilibrating them at different temperatures. The acids in the sample are first isolated on DEAE-Sephadex A-25 anion-exchange resin equilibrated with 0.5 *M* isobutyric acid. They are then eluted with 0.5 *M* sulphuric acid and injected directly onto the HPLC column after filtration through a 0.45-μm membrane. This method allows the simultaneous determination of oxalic, *cis*-aconitic, citric, phosphoric, malic, *trans*-aconitic, succinic, glycolic, lactic, formic and acetic acids. Isobutyric acid is also separated on this system and provides a useful guide to the loading capacity of the anion-exchange resin.

INTRODUCTION

The Aminex HPX-series of strong cation-exchange resins were reviewed in 1980[1]. These resins are prepared from a sulphonated polystyrene–divinylbenzene copolymer and are available in prepacked columns. The separation mechanisms and applications of these columns have been described[2]. The separation of organic acids on the HPX-87 column (with hydrogen counterion) was said to involve both ion exclusion and reversed-phase partition mechanisms. More recently, gel permeation has also been implicated[3].

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TABLE I

SAMPLE TYPE, CLEAN-UP PROCEDURE, COLUMN CONDITIONS AND DETECTION METHODS USED FOR ORGANIC ACID SEPARATIONS ON AMINEX HPX-87H (1981–1986)

?, not specified; →, denotes "connected in series to"; RT, room temperature; TCA, tricarboxylic acid cycle; PDA-UV, photodiode-array detection.

Year	*Ref.*	*Sample type*	*Clean-up of sample extracts*	*Temp. (°C)*
1981	4	Keto-acid standards	Filtered, 0.22 μm	?
1981	5	Urine	Filtered, 0.3 μm	RT
1981	6	Sugar processing	Filtered ?	?
1981	7	Glucose oxidation	Diluted, filtered, 0.22 μm	65
1981	8	Cultured buttermilk	Deproteinized, centrifuged	65
1981	9	Dairy products	Deproteinized, centrifuged, filtered, 0.2 μm	65
1982	10	Urine	Filtered, 0.3 μm	RT
1982	11	Silage	Centrifuged, filtered, 0.2 μm	30
1982	12	Bacterial cultures	Ether extraction, re-extraction 0.1 *N* NaOH	RT RT
1982	13	Strawberries	Centrifuged, H^+ resin, filtered, 0.45 μm	65
1982	14	Apple, pear	Diluted, filtered, 0.45 μm	25
1983	15	Algae, ascorbic	H^+ resin, Sep-Pak C_{18}, filtered, 0.2 μm	30
1983	16	Atmospheric, acetic	Charcoal tubes, desorbed 0.1 *N* NaOH, diluted	RT
1983	17	Cheese	Ion-exchange	15 18
1983	18	Fermented vegetables	Centrifuged, filtered, 0.22 μm	40
1983	19	Apple juice	Diluted, filtered, 0.45 μm	80
1984	20	Standards	None	60
1984	21	Urine	Filtered, 0.22 μm	RT
1984	22	Ruminal fermentation	Centrifuged	25
1984	23	Silage	Filtered, 0.2 μm	41
1984	24	Bacterial cultures	Ether extraction, re-extraction, 0.1 *N* NaOH	50
1984	25	Bacterial cultures	Centrifuged, filtered, 0.45 μm	85 85
1984	26	Wood degradation	Reverse osmosis, filtered, 0.2 μm	70
1984	27	Ground beef	Deproteinized, filtered, 0.45 μm	60
1984	28	Plant acid standards	None	RT
1984	29	Potatoes	Filtered, 0.45 μm	RT
1984	30	Food lactic	Filtered or centrifuged, EDTA added, diluted	?
1984	31	Food citric	Filtered or centrifuged, EDTA added, diluted	?
1984	32	Food acetic	Filtered or centrifuged, EDTA added, diluted	?
1984	33	Sugar degradation	Ion-exchange	60

Flow (ml/min)	*Eluent ($N\ H_2SO_4$)*	*Guard column*	*Detection*
0.8	0.0045	No	UV, 200 nm → amperometric
0.8	0.0045	No	UV, 200 nm → amperometric
0.4	0.002	No?	RI → conductivity
0.8	0.006	No	UV, 210 nm
0.7	0.009	No	UV, 220 nm
0.7	0.009	No	UV, 220, 275 nm
0.8	0.0072	No	UV, 200 nm
0.6	0.013	Yes	UV, 210 nm
0.5	0.007*	Yes	UV, 210 nm
	0.012*		
0.8	0.013	No	UV, 210 nm
0.6	0.075	Yes	UV, 207 nm
0.7	**	Yes	Amperometric
0.6	0.02	No	UV, 210 nm
0.8	0.01	No	UV, 210 nm
0.8	0.03		
0.5	0.013	Yes	RI
0.6	0.006	No	UV, 207 nm
0.7	0.01	Yes	RI
0.7	0.016	Yes	UV, 210 nm
0.7	0.013	No	RI
0.6	0.05	Yes	UV, 210 nm
0.6	0.006*	No	UV, 210 nm
0.8	0.0045	No	UV, 210 nm
0.8	0.002		
0.6	0.01	Yes	RI
0.8	0.048	Yes	UV, 210 nm
0.6	0.01	No	UV, 205, 220 nm?
0.7	0.018	No	UV, 210, 220, 230, 260 nm
0.7	0.009	Yes	UV, 210 nm
0.5	0.009	Yes	UV, 210 nm
0.7	0.009	Yes	UV, 210 nm
0.6	0.01	Yes	RI

(*Continued on p. 268*)

TABLE I (*continued*)

Year	*Ref.*	*Sample type*	*Clean-up of sample extracts*	*Temp. (°C)*
1984	34	Sugar processing	Sep-Pak C_{18}, filtered, 0.45 μm	?
1985	35	TCA standards	None	55
1985	36	Urine	Filtered, 0.3 μm	RT
1985	37	Plasma	Anion-exchange	RT
1985	38	Semen	Ultrafiltration, diluted	40
1985	39	Cardiotonic solutions	Ion-exchange	RT
1985	40	Acetic odour	Trapped in 0.02 *M* $Na_2B_4O_7$	?
1985	41	Insect haemolymph	Deproteinized, ion-exchange	41 33
1985	42	Wood degradation	Filtered, 0.45 μm	55
1985	43	Polysaccharides	Filtered	35
1985	3	Sugar acids, lactones	H^+ resin, deproteinized, filtered, 0.2 μm	35
1985	44	Sweet potatoes	Deproteinized, diluted, filtered, 0.45 μm	75
1985	45	Wine	Filtered, 0.45 μm	65 25
1986	46	Sugar acids, lactones	None	25
1986	47	Sorghum	Diluted, filtered, 0.2 μm	25

Many publications have appeared describing the use of HPX-87H columns for organic acid separations[4–47]. The application, sample clean-up, column conditions and detection methods used by these authors are summarized in Table I.

Despite the wide variety of applications, many authors experienced similar problems with co-elution and identification, and the quantification of poorly resolved peaks. Acid profiles are often complicated by the presence of non-acidic components due to the convenience of direct injection of samples onto the column.

In sample preparation prior to high-performance liquid chromatography (HPLC) acids can be separated from neutral and basic compounds by ion-exchange procedures[17,33,37,39,41]. This simplifies the profiles obtained and makes both identification and quantification easier.

Problems of co-elution and quantification have been overcome by some authors by using separate analyses at different eluent concentrations and temperatures[12,17,25,41,45]. Other authors have used multicolumn systems to improve analytical results.

Buchanan and Thoene[10,36] connected a C_{18} column in series with a HPX-87H column to improve resolution and aid identification of urinary acids, while Wilson[39] used a C_{18} guard column to protect a HPX-87H column and improve resolution of lactic acid in cardiotonic solutions. Loh *et al.*[25] and Oefner *et al.*[38] found no improvement in resolution by connection of two HPX-87H columns in series to double column length.

Flow (ml/min)	*Eluent (N H_2SO_4)*	*Guard column*	*Detection*
?	0.007	No	RI
0.5	0.01	Yes	UV, 210 nm
0.8	0.0072	No	PDA-UV, 190–350 nm
0.6	0.01	Yes	UV, 210 nm
0.6	0.01	Yes	RI
1.0	0.0045	C_{18} guard	UV, 210 nm
0.8	0.004	Yes	UV, 200 nm → conductivity
0.6	0.006	No	UV, 210 nm
0.5	0.004		
0.6	0.01	guard → guard	RI
0.6	0.016	Yes	UV, 210 nm
0.6	0.009	Yes	UV, 220 nm
0.8	0.0008	Yes	UV, 214 nm
0.8	0.013	Yes	RI
0.6	0.003		UV, 214 nm
0.6	0.01	Yes	UV, 220 nm
0.6	0.013	Yes	UV, 210 nm → RI

* The eluent contains acetonitrile.
** 0.1 N formic acid eluent was used instead of sulphuric acid.

This paper describes separations of organic acids found in sugar cane process juices that were achieved by chromatography on a dual-column system in which each column was equilibrated at a different temperature.

Little information has been published regarding separations of organic acids in cane and beet sugar products on Aminex HPX-87H resins. Charles[6] experimented with several samples including refinery molasses, using direct injection and a dual detection system (refractive index, RI, and conductivity). He found that useful acid profiles were obtained from samples containing low levels of sucrose although he did not quantify these. He detected the naturally occurring acids such as citric, aconitic and malic as well as formic, lactic, glycolic and glyceric acids produced by microbial and/or chemical degradation.

Clarke and Tsang[34] tested the column as part of a survey to evaluate the usefulness of HPLC as a routine control tool in factories and refineries. A comparative profile for fresh and deteriorated cane juice showed the transformation of sucrose to dextran, fructose and lactic acid.

De Bruijn *et al.*[33] investigated the formation of acids from the alkaline degradation of glucose and fructose in beet syrups and model solutions. The acids were first isolated from syrup samples by ion-exchange; then analyzed by HPLC directly and by gas–liquid chromatography (GLC) of the trimethylsilyl derivatives.The authors concluded that even though GLC provided superior resolution, HPLC had advantages of shorter sample preparation and analysis times, the simultaneous analy-

sis of formic and acetic acids, and better quantification. The HPLC profiles showed separation of oxalic, malic, citric, lactic, glycolic and the C_6-saccharinic acids (which elute as one broad peak).

Unfortunately, these publications give little information regarding quantification, and the experimental details are not always clear. The method outlined in this paper has enabled the quantitative measurement of the following acidic components of sugar cane process juice: oxalic, *cis*-aconitic, citric, phosphoric, malic, *trans*-aconitic, succinic, glycolic, lactic, formic and acetic acids. The retention times of 37 acids are also reported.

EXPERIMENTAL

Reagents

All commercial standards and reagents were analytical grade and, with the exception of glycolic acid, were used as supplied. Glycolic acid (85%, Aldrich, Milwaukee, WI, U.S.A.) was dissolved in distilled water, neutralized with sodium hydroxide and crystallized from ethanol.

Sample preparation

Juice samples collected from local sugar factories were preserved by addition of mercuric chloride (10 mg/l) and stored frozen. Before isolation by ion-exchange chromatography, the samples were carefully defrosted using a microwave oven and filtered through Whatman No. 54 paper under vacuum using Celite as a filter aid (Standard Supercell, Johns-Manville, Denver, CO, U.S.A.).

Ion-exchange chromatography

Ion-exchange chromatography was carried out in glass columns with Quickfit joints at each end, to facilitate the connection of two columns in series. Resin (*ca.* 2 ml) was contained in a section (70 × 6 mm I.D.) and a PTFE tap regulated flow. Columns containing Amberlite IR 120 (H^+) cation-exchange resin were coupled above columns containing DEAE-Sephadex A-25 anion-exchange resin equilibrated with 0.5 *M* isobutyric acid. Standards and samples (10.0–40.0 ml) were loaded onto the columns using a Technicon proportioning pump Model III fitted with tubing rated at 0.6 ml/min. Cation-exchange columns were washed with eight bed volumes of water and disconnected. The anion-exchange columns were then washed with a further eight bed volumes to ensure removal of all unretained components. The acids were eluted with 0.5 *M* sulphuric acid into 10.0 ml volumetric flasks and passed through 0.45-μm syringe filters before analysis by HPLC.

HPLC separation and standardization

The liquid chromatograph consisted of a Waters (Milford, MA, U.S.A.) U6K injector and M6000A pump, a Shodex SE-51 refractive index monitor and a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A integrator. The system was operated in a constant temperature room (20°C).

The separation was carried out on two Aminex HPX-87H cation-exchange columns (300 × 7.8 mm I.D.) connected in series and protected by a guard column (40 × 4.6 mm I.D.) packed with Aminex HPX-85H resin (Bio-Rad, Richmond, CA,

U.S.A.). The columns were enclosed in aluminium jackets which were heated by water circulated from two separate baths. The first and second columns were operated at 35 ± 0.5°C and 85 ± 0.5°C (35/85°C) respectively. The eluent was 5 m*M* sulphuric acid. It was filtered through a 0.45-μm membrane, degassed under vacuum before use and stirred on a hot plate at about 70°C. Flow-rate was 0.5 ml/min and injection volume was 20 μl.

The effect of temperature on the resolution of oxalic, *cis*-aconitic, citric, phosphoric, malic, *trans*-aconitic, succinic, glycolic, lactic, formic and acetic acids was investigated by equilibrating the columns separately at various temperatures. Injections were made at first and second temperature combinations of 35/85, 85/35, 35/60, 60/35, 35/35 and 70/70°C.

The system was calibrated using the external standard method. Standard curves were constructed by passing four solutions containing various amounts (*ca.* 50–700 μg/ml) of the above mentioned acids through the ion-exchange step. Five injections of each eluate were then made onto the HPLC column to determine linearity and precision.

Several other acids (*ca.* 0.05%) were injected individually, to determine retention times and possible interference with the acids quantified.

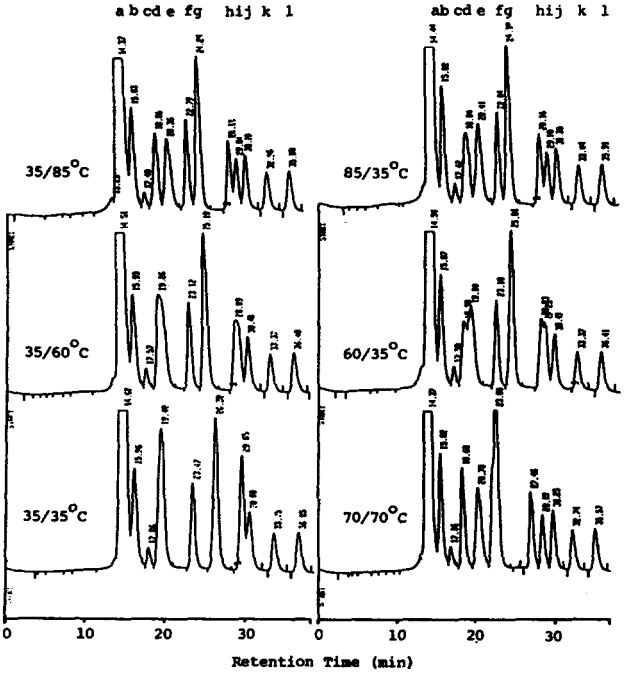

Fig. 1. HPLC profiles showing the effect of temperature on the retention times of (a) void volume peak; (b) oxalic; (c) *cis*-aconitic; (d) citric; (e) phosphoric; (f) malic; (g) *trans*-aconitic; (h) succinic; (i) glycolic; (j) lactic; (k) formic and (l) acetic acid.

RESULTS AND DISCUSSION

HPLC separation and standardization

Fig. 1 shows how the resolution of the acids present in sugar cane process juice can be improved, by connecting two Aminex HPX-87H columns in series and equilibrating them at different temperatures.

When both columns are equilibrated to the same temperature, not all the acids are separated satisfactorily. At low temperatures of 35/35°C, the acid pairs citric–phosphoric (19.48 min) and succinic–glycolic (29.65 min) co-elute, but there is good separation between malic (23.47 min) and *trans*-aconitic (26.30 min) acids. At high temperatures of 70/70°C, malic and *trans*-aconitic (23.05) acids co-elute but the other acids are well separated. A combination of high and low temperatures (35/60°C) also

TABLE II

PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.) OF STANDARDS

Acid	*Standard solution no.*	*Concentration (μg/ml)*	*R.S.D. (%)*
Oxalic	1	203	3.37
	2	106	4.99
	3	390	1.20
Citric	1	318	0.30
	2	115	2.13
	3	160	1.89
Phosphoric	1	52	6.01
	2	208	0.74
	3	417	0.32
	4	573	2.02
Malic	1	67	2.75
	2	101	1.75
	3	305	1.14
Aconitic*	1	390	1.02
	2	506	1.07
	3	603	1.67
Succinic	1	45	2.62
	2	102	1.59
	3	307	1.52
Glycolic	1	56	3.50
	2	85	2.50
	3	254	1.09
Lactic	1	46	8.29
	2	98	1.89
	3	276	1.28
Formic	1	59	5.98
	2	121	3.15
	3	359	1.55
Acetic	1	51	7.03
	2	103	1.80
	3	313	1.85
	4	714	1.28

* *cis*- and *trans*-aconitic acid areas were combined for standardization.

fails to separate all the acids. Satisfactory separation is achieved however, when the second column temperature is increased to 85°C and these temperatures (35/85°C) were subsequently used for standardization and analysis of samples. Reversing the column temperatures (60/35°C, 85/35°C) shows only slight differences in resolution. This highlights the stability and uniformity of these columns, since the first column was purchased two years before the second column and had been used for a variety of other applications.

TABLE III

RETENTION TIMES OF ORGANIC ACIDS AT FIRST AND SECOND COLUMN TEMPERATURES OF 35 AND 85°C RESPECTIVELY

Acid	*Retention time*
Oxalic	15.94
cis-Aconitic	17.38
2,5-Diketogluconic	17.48
Tartronic	18.72
Citric	19.08
2-Ketogluconic	19.11
Isocitric	19.36
Maleic	19.52
α-Ketoglutaric	19.66
Glucuronic	19.90*
5-Ketogluconic	20.05
Galacturonic	20.20
Phosphoric	20.26
Tartaric	20.41
Gluconic	21.36
Pyruvic	22.33
Glyoxylic	22.80
Malic	22.90
Citramalic	23.37
Malonic	23.67
Quinic	23.93
trans-Aconitic	24.26
Glucuronolactone	24.87*
Gluconolactone	24.88*
Glyceric	25.78
Shikimic	28.08
Succinic	28.23
Glycolic	29.16
Lactic	30.35
Itaconic	31.03
Formic	33.09
Fumaric	34.46
Acetic	35.95
Levulinic	38.66
Pyroglutamic	42.23
Propionic	42.23
Mesaconic	42.40
Isobutyric	48.39
Gallic	61.75

* Broad peaks due to acid–lactone equilibration.

Standard curves for all acids are linear within the concentration ranges given in Table II. Relative standard deviation (RSD) values (Table II) were obtained over two weeks with several eluent changes and the RSD generally increases with decreasing acid concentration. The high values obtained for lactic, formic and acetic acid at about 50 μg/ml reflects the limitations of the integration at this level when peaks are small and broad.

Detection down to about 50 μg/ml is made possible by using a sensitive refractive index monitor (Shodex SE-51). Even though many authors use UV detection for organic acids, this was not suitable for cane juice analysis. Of the naturally occurring organic acids in sugar cane juice, aconitic acid is the major constituent. It has a high molar absorptivity within the wavelength range 200–220 nm most commonly used for detection, and selective enhancement of the detector response for this component was not desirable.

For quantification, the areas of *cis*- and *trans*-aconitic acids are combined for convenience since the *cis*-isomer is formed gradually from *trans*-aconitic acid even when the solution is stored at 4°C. When required they can be quantified separately but must be freshly prepared and analyzed immediately. Samples of cane process juice contained only small amounts of the *cis*-isomer (less than 5% of the *trans*-isomer area) and so it was not measured separately.

The retention times of 37 acids are given in Table III. A comparison of the acids quantified with other acids listed shows that interference is possible for some of these acids (if present) under the conditions of this procedure.

Ion-exchange chromatography

DEAE-Sephadex A-25 anion-exchange resin is used to isolate the acids from process juice before HPLC analysis. Equilibration with 0.5 *M* isobutyric acid provides a means of monitoring the ion-exchange capacity of the resin. Isobutyric acid has a retention time of 48.3 min (Fig. 2) and its absence from the HPLC chromato-

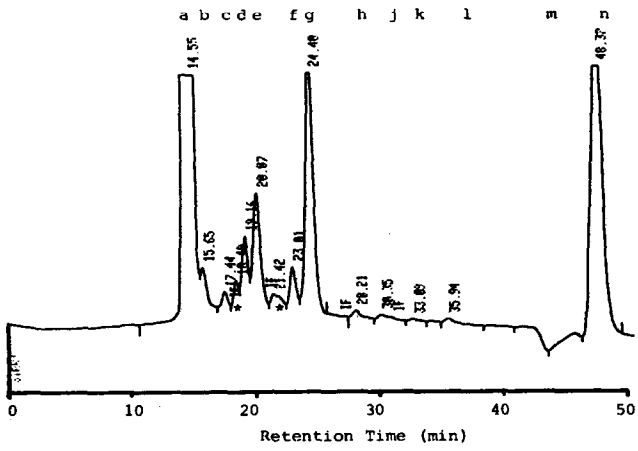

Fig. 2. HPLC profile of sugar cane juice. Peaks identified as in Fig. 1; (m) negative air peaks; (n) isobutyric acid. ★: Unidentified acids.

gram indicates the resin capacity has been exceeded. Acids can be concentrated on the resin efficiently and further concentration steps are not required if a sensitive refractive index monitor is used. Removal of sucrose is essential when using refractive index detection since it interferes with the acid profile[33]. Elution of the acids with 0.5 *M* sulphuric acid allows quantitative recovery of the acids from the resin and it is compatible with the HPLC eluent.

HPLC analysis of sugar cane process juice

Juice extraction from sugar cane gives the typical organic acid profile shown in Fig. 2. Slight variations in the organic acids do occur with variety and maturity of the cane when harvested, while the phosphoric acid level is mainly affected by fertilizer practices and natural soil concentrations. Plant acids dominate the profile with *trans*-aconitic acid, citric and malic comprising 0.52%, 0.13% and 0.06% of the dissolved solids, respectively. Smaller amounts of oxalic, *cis*-aconitic and succinic acids are also present.

Fig. 3 shows the effect of microbial contamination on clarified juice. Clarification of cane juice involves heating and addition of lime to precipitate impurities, such as protein and calcium phosphate complexes. (Note the removal of phosphoric acid in the chromatogram.) Microbial infection is indicated by the large lactic acid peak (30.24 min) which represents 0.27% of the dissolved solids and a greater than ten-fold increase over normal background levels.

Fig. 3 also shows the effect of increasing the HPLC eluent strength to 7.5 m*M* sulphuric acid. Oxalic acid is well resolved from the void volume and the unknown peak at 24.0 min is just resolved from *trans*-aconitic acid. Unfortunately, *cis*-aconitic now co-elutes with the unknown peak at 18.3 min. Careful adjustment of the eluent strength, as well as temperature, is therefore necessary to optimize resolution of the acids of interest.

Column life

The columns have proved to be stable with no loss of resolution after 12

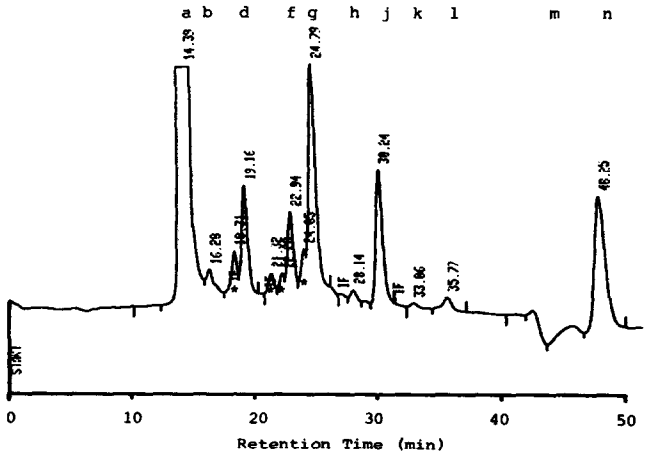

Fig. 3. HPLC profile of infected clarifier juice. Peaks identified as in Fig. 2. ★: Unidentified acids.

months even though the second column temperature of 85°C is maintained continuously five to seven days a week while in operation. The maximum temperature recommended by the manufacturer[48] is 65°C. Guard columns should be tested before use for retention of phosphoric acid. It was found that the shape of the phosphoric acid peak can be reduced to a short broad peak or the acid removed completely by some guard columns. However, the peak shapes and areas of the other acids were not affected and the problem was overcome by washing the guard column with phosphoric acid before use.

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