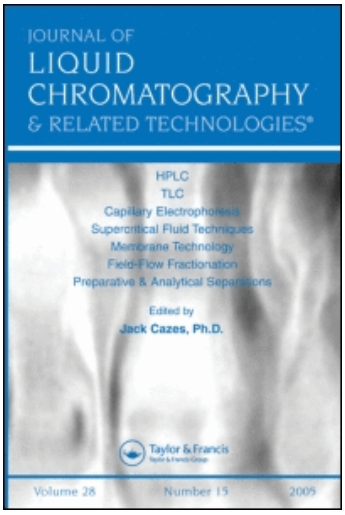

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Corradini, Claudio , Cristalli, Antonio and Corradini, Danilo(1993) 'High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection of Nutritionally Significant Carbohydrates', Journal of Liquid Chromatography & Related Technologies, 16: 16, 3471 — 3485
To link to this Article: DOI: 10.1080/10826079308019702
URL: http://dx.doi.org/10.1080/10826079308019702

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(16), 3471-3485 (1993)

HIGH PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION OF NUTRITIONALLY SIGNIFICANT CARBOHYDRATES

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ABSTRACT

The separation of nutritionally significant saccharides in fruit juices was investigated by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The effect of sodium hydroxide concentration in the mobile phase on the retention and selectivity was investigated on three different pellicular anion-exchange columns (all from Dionex). The sugar alcohol sorbitol and the sugars glucose, fructose, and sucrose were well resolved in less then 10 minutes on the CarboPac PA 100 column by using simple isocratic elution with 150 mM sodium hydroxide solution, without column regeneration between runs. Under these conditions the relative standard deviations of the retention times were better than 0.98%. HPAEC-PAD was successfully applied to the determination of the major sugars in a fruit concentrate and in several varieties of commercial fruit juices by

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an internal standard method without any sample pretreatment.

INTRODUCTION

The increasing interest in the characterization of nutritionally significant carbohydrates in food products has created new demands for the development of rapid, sensitive, and specific analytical methodology for the identification and quantification of this class of chemical compounds in fresh and processed food. Traditional approaches include gas-liquid chromatography (GLC) and various high-performance liquid chromatography (HPLC) methods. GLC has been the method of choice where high sensitivity is required. However, this technique necessitates laborious and time-consuming derivatization procedures (1) that may result in unreacted sample or the production of side products, making quantitation difficult. Bonded-amino phases (2-3), cation-exchange resins in various ionic forms (4-5), and octadecylsilica packings (6) have been widely used for the separation of underivatized carbohydrates by HPLC with either low-wavelength UV or refractive index detection. However, these modes of carbohydrate HPLC can suffer several drawbacks such as instability and short lifetime of the bonded-amino phases, lack of sensitivity of direct detection, and long analysis times.

Recently, high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) has been introduced for the highly selective separation of carbohydrates at the nanomol level (7-9). HPAEC coupled with PAD under alkaline conditions enables complete single step separation of neutral carbohydrates based upon their molecular size, saccharide composition and glycosidic linkages (10). Most neutral carbohydrates are in fact weak acids with pk_a values in the range 12-14 and, at high pH, they can be separated as anions by anion-exchange mecchanism.

This paper presents the results of a study of the chromatographic conditions for the separation and determination of the sugar alcohol sorbitol and the sugars glucose, fructose, and sucrose by HPAEC-PAD. These saccharides are the major sugars in fruit juices and their identification and dosage can be used to determine authenticity and nutritional content of these beverages.

In order to select the optimum column and chromatographic conditions to perform rapid and reproducible separations and quantifications of the above saccharides, the effect of mobile phase composition on retention time and selectivity was examined using three different commercial pellicular anion-exchange columns.

The application of this method to the identification and dosage of the major sugars in a fruit concentrate and some varieties of fruit juices from different commercial sources is also described.

EXPERIMENTAL

Materials

All saccharide standards were obtained from Sigma (St. Louis, MO, USA), with the exception of DL-arabinose that was purchased from Fluka (Buch, Switzerland). Fifty percent (w/w) sodium hydroxide solution was purchased from J.T. Baker (Deventer, Netherlands). Fruit juices from three different commercial sources (A, B, and C) were purchased from a local market, apple concentrate (sorce D) was kindly provided by Trentofrutta (Trento, Italy).

Equipment

The experiments were performed on a Dionex (Sunnyvale, CA, USA) Model 4000i gradient pump module equipped with a Model PAD II pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode and a silver-silver chloride reference electrode. The following working pulse potentials and durations were used for detection of analytes: E_1 = 0.10 V (t_1 = 300 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = -0.60 V (t_3 = 300 ms). The response time of

the PAD was set to 1 second. The sample loop volume was 10 µl, and the eluent flow rate 0.8 ml/min. The Dionex Eluent Degas module was employed to sparge and pressurize the eluents with helium to degas and to prevent adsorption of carbon dioxide and subsequent production of carbonate which would act as diplacing ion and shorten retention times. The following pellicular anion exchange columns, all from Dionex, were used: an HPIC-AS6 (250 x 4 mm I.D.) equipped with a CarboPac PA guard column (25 x 3 mm I.D.), a CarboPac PA1 (250 x 4 mm I.D.) equipped with a CarboPac PA1 guard column (50 x 4 mm I.D.) and a CarboPac PA 100 (250 x 4 mm I.D.) equipped with a CarboPac PA 100 guard column (50 x 4 mm I.D.). Chromatographic data were collected and plotted using the Dionex Auto Ion 450 Chromatography Workstation. Eluents were prepared by suitable dilution of sodium hydroxide fifty percent solution with HPLC-grade water.

Standard Solutions and Sample Preparation

A stock solution of the analytes was prepared prior to use by dissolving 1.0 g of each standard saccharide in 500 ml of water. An internal standard aqueous solution containing 5.0 mg/ml of arabinose was also freshly prepared. Appropriate amounts of the stock solution were diluted with water to produce working standard solutions at five different concentrations

within the range 1-30 µg/ml. The appropriate volume of internal standard solution was added to each working solution to give a final concentration of 5.0 µg/ml of arabinose. Calibration graphs were plotted based on the linear regression analysis of the peak-area ratios. Fruit juice samples were prepared for analysis by a first 250-fold dilution with water followed by a further 25-fold dilution with water after the addition of the appropriate amount of internal standard solution to give a concentration of 5.0 µg/ml of arabinose. The apple concentrate was prepared for analysis by dissolving in water the appropriate amount of defrozen sample, conditioned at room temperature for 60 minutes. The solution was then subjected to the above procedure. All aqueous solutions were made with HPLC-grade water, filtered throuh a Type HA 0,22 µm single use membrane filter (Millipore, Bedford, MA, USA), and degassed prior to use by sparging with helium.

RESULTS AND DISCUSSION

Column and Mobile Phase Selection

The effects of mobile phase composition on the chromatographic retention of the sugar alcohol sorbitol, the monosaccharides glucose and fructose, and the disaccharide sucrose were examined using three different pellicular anion-exchange columns, marketed by Dionex as

HPIC-AS6, CarboPac PA 1 and CarboPac PA 100. The first two columns are usually devoted to the analysis of mono- and disaccharides, whereas the CarboPac PA 100 column has been recently introduced as a column specificaly tailored for oligosaccharide separations.

The three columns were evaluated by eluting the examined samples under isocratic conditions with mobile phases containing sodium hydroxide at concentration ranging from 100 to 300 mM. Since the low alkaline solutions compromise baseline stability and detector sensitivity (11-12), isocratic elution with mobile phases containing sodium hydroxide at concentration lower than 100 mM were not performed.

The retention behavior of the examined sugars was virtually identical on the three pellicular anion-exchange columns. As expected, the retention times of sugars linearly decreased with increasing sodium hydroxide concentration of the mobile phase. The sodium hydroxide concentration dependence of resolution between glucose and fructose was compared for all three pellicular anion-exchange columns. Figure 1 shows that all three columns exhibited increased resolution as the sodium hydroxide concentration decreased. However, with mobile phases containing sodium hydroxide at concentration lower than 150 mM, column regeneration between runs with stronger alkaline solution (300 mM

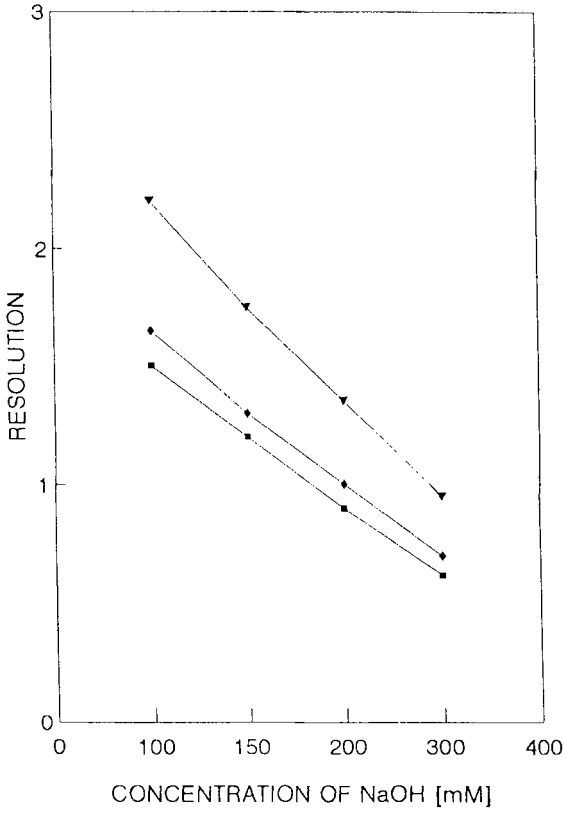

FIGURE 1 Resolution between glucose and fructose as a function of the concentration of sodium hydroxyde in the mobile phase. Columns, (■) HPIC-AS6; (◆) CarboPac PA1; (▲) CarboPac PA 100; flow rate, 0.8 ml/min; detector, PAD II; attenuation, 1000 nA; room temperature.

sodium hydroxide) was required in order to obtain reproducible retention times.

In order to avoid column regeneration between runs and the consequent equilibration to the initial conditions, which are both time-consuming operations,

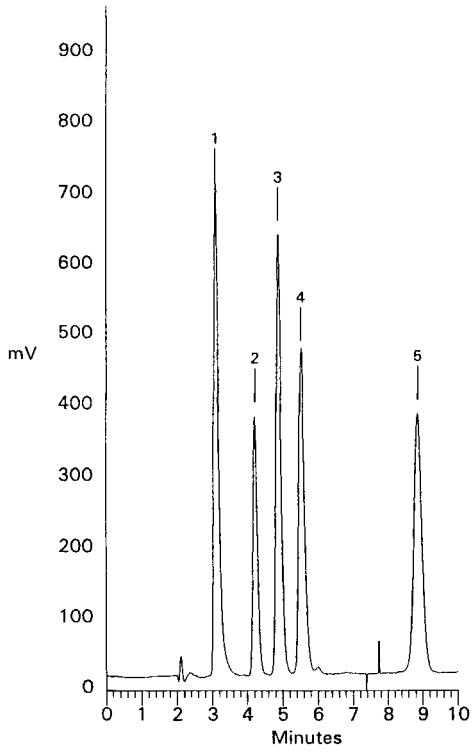

FIGURE 2 Separation of (1) sorbitol, (2) arabinose, (3) glucose, (4) fructose, and (5) sucrose. Column; CarboPac PA 100 (250 x 4 mm I. D.); eluent, 150 mM sodium hydroxyde. Other conditions as in Figure 1.

the qualitative and quantitative analysis of the above sugars were performed on the CarboPac PA 100 column with 150 mM sodium hydroxide as the mobile phase.

Qualitative and Quantitative Analysis

The quantification of sugars was performed by an internal standard method. Arabinose was selected as the

TABLE 1

Reproducibility of Retention Times*

Compound	Retention Time** (minutes)	S.D.	R.S.D. (%)
Sorbitol	3.1	0.0227	0.73
Arabinose	4.25	0.0371	0.87
Glucose	4.92	0.0409	0.83
Fructose	5.57	0.0428	0.77
Sucrose	8.81	0.0856	0.98

* Chromatographic conditions: as in figure 2
** Mean value of twentyfour repeated injections

internal standard because it is not naturally present in fruit juices, is completely resolved in the chromatogram from the other sugars and is eluted near the peaks of interest. A typical chromatogram of sorbitol, glucose, fructose, and sucrose with the internal standard arabinose is shown in Figure 2. The reproducibility of the retention times of the above sugars was investigated by repeated injection (n = 24) of an equimolar sample solution containing the internal standard. Results are summarized in Table 1 and show that the relative standard deviations were better than 0.98 % for the five compounds. The calibration graphs for the three sugars and the sugar alcohol sorbitol obtained by the peak-area ratio method showed excellent linearity over the concentration range 1 -30 ug/ml

TABLE 2

Results of the Quantitative Determination of the Major Sugars in Some Commercial Fruit Juices and One Apple Concentrate.

Sample	Source	Sorbitol g/l	R.S.D. %	Glucose g/l	R.S.D. %	Fructose g/l	R.S.D. %	Sucrose g/l	R.S.D. %
Apple	A	2.85	0.82	23.78	0.71	56.02	0.72	12.79	0.92
Apple	B	2.43	0.79	25.21	0.75	58.92	0.78	13.05	0.97
Apple	C	2.57	0.86	25.58	0.82	57.6	0.82	13.25	0.97
Mandarine	A	ND	=	42.93	0.86	44.53	0.69	64.95	0.95
Grapefruit	A	ND	=	38.06	0.89	36.02	0.73	11.89	0.92
Grapefruit	B	ND	=	37.32	0.76	35.04	0.79	10.51	0.98
Grapefruit	C	ND	=	35.61	0.84	36.71	0.83	11.42	0.96
Orange	A	ND	=	33.11	0.79	32.72	0.82	31.33	0.93
Orange	B	ND	=	31.87	0.72	32.11	0.76	30.87	0.97
Orange	C	ND	=	32.54	0.86	31.97	0.82	31.27	0.89
Apple Conc.	D	* 15.92	0.73	* 148.58	0.76	* 383.72	0.78	* 116.12	0.96

ND = No Detected
* = Expressed as g/Kg

with correlation coefficients better than 0.9998, and nearly passed through the origin.

Determination of Sugars in Fruit Juices and Concentrates

The present method was employed to analyze the sugar content of one fruit concentrate and four varieties of fruit juices from different commercial sources. The fruit juices were directly injected onto the column without any sample pretreatment, except that they were diluted with water to a concentration range that would ensure no significant loss of resolution due to overloading of the pellicular anion-exchange column. The diluted samples were then filtered through a 0.22 μm

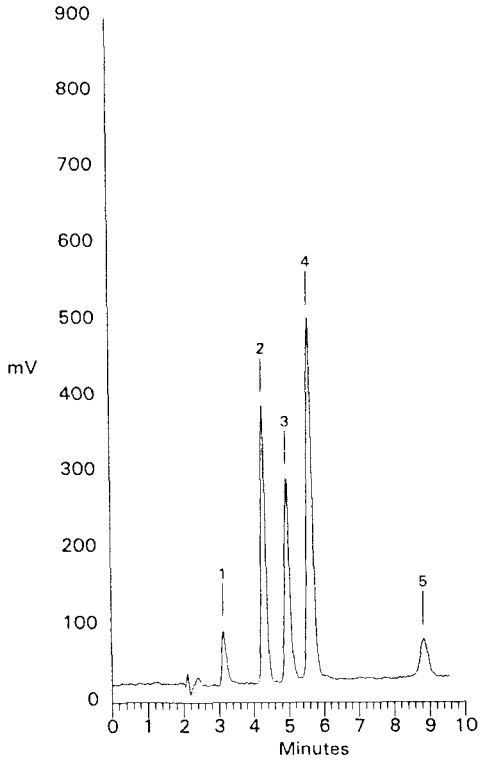

FIGURE 3 Separation of sugars in a commercial apple juice. Peak identification and chromatographic conditions as in Figure 2.

single use membrane filter after the addition of the internal standard solution.

Results of five replicate determinations are summarized in Table 2 and a typical chromatogram is shown in Figure 3.

In order to determine the accuracy of the method, recovery studies were carried out. Known amounts of sorbitol, glucose, fructose, and sucrose were added to a

TABLE 3

Recovery Study of the Analyzed Sugars Added to a Commercial Apple Juice.

Compound	Amount in Juice g/l	Amount Added g/l	Found g/l	Recovery %	R.S.D. %
Sorbitol	2.57	15	17.08	97.2	0.78
		30	31.85	97.8	0.69
		45	48.61	102.2	1,12
Glucose	24.58	15	40.75	102.9	0.87
		30	54.15	99.2	1.21
		45	68.84	98.9	0.98
Fructose	57.61	15	73.35	101.6	1.42
		30	85.61	97.2	1.21
		45	105.35	102.7	0.89
Sucrose	13.25	15	27.44	97.1	0.92
		30	44.05	101.8	1.31
		45	56.56	97.1	1.78

variety of commercial fruit juices and the resulting spiked samples were subjected to the entire analytical method. Three different amounts of each sugar were added to the samples. All samples were injected five times and an average of the response area ratio was the basis for the found concentrations. The recoveries were calculated based on the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples. Results and relative standard deviations for all samples were of the same order as those reported in Table 3 for a commercial apple juice. It can be seen that the average recoveries lied between 97.1 and 102.9, indicating that the method

has an adequate degree of accuracy for the determination of the major sugars in fruit juices.

CONCLUSIONS

High-performance anion-exchange chromatography with pulsed amperometric detection appears to be a useful and versatile procedure for the rapid and direct determination of sugars in fruit juices and concentrates. The developed method is specific (only sugars and the sugar alcohol sorbitol were detected), is reproducible, the quantification is linear over a wide range of concentrations, and the results of the recovery studies show good accuracy. Furthermore, the method is rapid, simple, economical and no sample pretreatment is required. For fruit juices and concentrates only sample dilution and filtration through a single use membrane filter is needed and analysis time is reduced to minutes.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Fabio Cereghini (Trentofrutta, Trento, Italy) for the kind gift of the apple concentrate.

This work was supported by the C.N.R. (Consiglio Nazionale delle Ricerche), Rome, Progetto Strategico "Characterization, Control, and Analysis of Food".

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Received: February 22, 1993
Accepted: March 3, 1993