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PREPARATIVE ISOLATION OF ^{14}C-LABELLED SACCHAROSE FROM A PARTLY PURIFIED FRACTION OF *CHLORELLA VULGARIS*

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SUMMARY

A liquid chromatographic method for the preparative isolation of ^{14}C-labelled saccharose from a partly purified fraction of *Chlorella vulgaris* is described. From nine systems examined for the separation on the strong cation exchanger Ostion LGKS, the cation exchanger in the Li^+ form with 60% ethanol as the eluent was chosen. It was found that the adsorption isotherm is linear up to a concentration close to the saturated solution of saccharose in the sample. In spite of its simplicity, the determination of the parameters of the preparative procedure by calculation from the results acquired with an analytical column proved to be satisfactory.

INTRODUCTION

Liquid chromatography is a useful technique for the separation of carbohydrates. Microparticulate silica with bonded organic phases, mostly amino phases[1–9], has often been used for analytical applications. Reversed-phase chromatography using mostly C_{18} phase doped with a primary amine[10–14] has also been reported for the determination of carbohydrates in various materials.

Strong cation exchangers are used more often than anion exchangers[15–20]. The most common are styrene–divinylbenzene (DVB) copolymers. For the separation of sugars, ion-exchange resins with a percentage of cross-linking from 4%[21–23] to 8% DVB are recommended.

Among the variety of the metal forms of cation exchangers. Ca^{2+} (refs. 22 and 24–28), Pb^{2+} (refs. 25, 26 and 29), Ag^+ (refs. 23 and 25), Li^+, Na^+ (refs. 30–32) or mixtures of two cations (refs. 21 and 22) are the most commonly used. The use of water as a mobile phase is very advantageous[21,22,25,26]. Ethanol[33–35], acetonitrile[30], triethylamine[36], etc., are also often used as eluents.

Our hypothesis concerning the separation of sugars on a strongly acidic cation exchanger was based on the work of Goulding[37]. In his opinion, for systems using water as the mobile phase, the structure of carbohydrates and the size and shape of the hydration sphere of the cation attached to the ion-exchange resin are the decisive parameters. These two factors determine the stability of the complex formed by the

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exchange of the sugar hydroxyl groups with the water molecules in the hydration sphere of the cation (so-called ligand exchange). If aqueous ethanol is used as the eluent, the distribution of water and ethanol between the two phases also plays an important role.

The purpose of this study was to develop a method for the isolation of saccharose from a sample obtained by partial fractionation of *Chorella vulgaris*. In addition to saccharose, other saccharides and phospholipid residues could be expected in this sample.

To find suitable conditions for the isolation of saccharose, the following nine systems were examined: three ionic forms of the strong cation exchanger Ostion LGKS (Pb^{2+}, Ca^{2+}, Li^{+}) and three eluents (water, 80% ethanol, 60% ethanol). The following experiments were carried out:

(a) retention volumes and performances of analytical columns for standard samples of glucose, fructose, saccharose and maltotriose were determined in the above-mentioned systems;

(b) a real sample containing ^{14}C-labelled saccharose was separated by using the same systems;

(c) the most suitable system was chosen and the measurements needed for the calculation of the conversion from an analytical to a preparative column were carried out;

(d) the accuracy of the calculation was verified on the preparative column.

A study comparing different chromatographic methods [high-performance liquid (HPLC), thin-layer and paper chromatography] for the separation of mono- and disaccharides was published recently[38]. The HPLC method was shown to be superior to the others for both analytical and preparative separations. The experiments carried out in our Institute confirmed this conclusion.

EXPERIMENTAL

Chemicals

Lead dinitrate, lithium chloride, calcium chloride, lithium hydroxide, calcium hydroxide and hydrochloric acid were analytical reagent grade products from Lachema (Brno, Czechoslovakia), D-(+)-glucose (USP), D-(−)-fructose, maltotriose (93%) and N,N, N′,N′-tetramethyldiaminomethane (99%) (TMDAM) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Saccharose was purchased from Sigma (St. Louis, MO, U.S.A.) and acetonitrile for HPLC from Fluka (Buchs, Switzerland). Ethanol (96%) was obtained from Zlíchov Distillery (Prague, Czechoslovakia). Water was doubly distilled. Samples of a partly purified fraction of *Chlorella vulgaris* were obtained from ÚVVVR (Prague, Czechoslovakia).

Sorbents

Ostion LGKS 0802, a strongly acidic cation exchanger, particle diameter (d_p) 12–15 μm (8% DVB), was purchased from Spolchemie (Ústí n/L., Czechoslovakia) and Separon SIX C_{18}, d_p = 5 μm, octadecylsilica from Laboratory Instruments (Prague, Czechoslovakia).

Apparatus

All experiments on the analytical scale were performed by using an apparatus consisting of a Model 302 pump (Gilson, France) with block 10, a liquid pulse absorber with a pressure indicator (ZPA Jinonice, Prague, Czechoslovakia), a Type 7125 six-way valve (Rheodyne, Cotati, CA, U.S.A.), an analytical stainless-steel column (250 × 4 mm I.D.) with a jacket connected to a circulating water bath, an RIDK 101 refractive index detector and a TZ 4200 recorder (all from Laboratory Instruments). Radioactive samples injected onto the analytical column were detected by using a radioactivity detector, described below.

Except for the Gilson pump, the preparative chromatography apparatus was constructed in our laboratory and consisted of the following:

(1) a sample inlet pump with a dead volume smaller than 5 μl (the minimum injected sample volume was 100 μl and the maximum volume was unlimited);

(2) a preparative column with a titanium insert (500 × 24.5 mm I.D.) with a water-jacket;

(3) a radioactivity detector constructed as a flow-through cell filled with a new, highly resistant inorganic scintillation material of the spinel type;

(4) The detector and the recorder were the same as used in the analytical apparatus. In both instances the columns were thermostated at 80°C by using an Ultrathermostat NBE (VEB Prïfgeräte, Werkh, Medingen, G.D.R.).

Sorbent treatment, column packing

Ostion was converted from the H^+ to the Na^+ form three times by washing with 1 *M* hydrochloric acid or 1 *M* sodium hydroxide solution. To convert the cation exchanger to the required form, 1 *M* lead dinitrate, 1 *M* calcium chloride or 1 *M* lithium chloride solution were used. For each form of the swollen cation exchanger the corresponding water content was determined in the exchanger phase by drying a known amount of the cation exchanger at 105°C to constant mass.

Columns were slurry packed by pumping a mixture of cation exchanger either with doubly distilled water of 80% aqueous ethanol (1:1).

Analytical experiments

The mobile phase flow-rate was 12 ml/h. An RIDK 101 refractive index detector connected to the column output was thermostated at 30°C. The concentrations of standard sugar solutions were 10 mg/ml. The concentration ratio for solutions of sugar mixtures was always equal to unity and the total concentration was 10 mg/ml. Sugars were always dissolved in the corresponding eluent and the sample volumes ranged from 5 to 20 μl.

The analogous experiments were carried out with three real samples of ^{14}C-labelled saccharose (the dry evaporation residue was dissolved in water and 80% and 60% aq. ethanol).

Determination of analytical column parameters

On the basis of the experiments performed with the described systems, the most suitable one was chosen (see Results). Using this system, we carried out several measurements enabling us to determine the basic column parameters, as follows:

(a) Determination of the column dead volume. Different volumes (5–500 μl) of 1 *M* lithium chloride in 60% ethanol were introduced onto the column.

(b) Determination of the optimum flow-rate. To measure the dependence of the reduced theoretical plate height, h, on the reduced velocity, v, a solution of 10 mg/ml saccharose in 60% aq. ethanol was used; the sample volume was always 10 μl.

(c) Determination of maximum sample volume introduced on to the column. The solution of the mixture of saccharose and glucose in 60% aq. ethanol was injected on to the thermostated column. The eluent flow-rate was 3.6 ml/h. The collected fractions were analysed by reversed-phase HPLC using a 150 × 3.3 mm I.D. column packed with Separon SIX C_{18}, 5 μm, and a mobile phase consisting of acetonitrile–water–TMDAM (91.6:7.9:0.5).

(d) Determination of adsorption isotherm. At time $t = 0$, the pumping of saccharose solution (at a given concentration) in 60% aq. ethanol was started on the thermostated column with a flow-rate of 3.6 ml/h until the deviation of the detector stabilized.

Preparative column experiments

Model experiments were carried out by using a mixture of saccharose and glucose in a concentration ration of 10:1. When the radioactive sample was separated, both the radioactivity and the refractive index signals were recorded. All eluted peaks were collected and their radioactivity was measured, in addition to the radioactivity of the sample injected on the column.

RESULTS

The swelling parameters of the individual forms of the cation exchanger in water are given in Table I. As expected, a higher water content was found with the Li^+ form of the cation exchanger.

The results obtained with model solutions of sugars on the analytical column are summarized in Table II.

Table III shows the results measured with the radioactive solution. They are expressed as radiochemical purity of saccharose in the fraction separated on the column and analysed by HPLC. Both of these experiments and the HPLC analysis of the initial sample confirmed that glucose would be present as another component in the real sample.

To optimize the system, the following criteria were chosen: (A) results of the model experiments: (a) k' values, (b) column performance (N values) and (c) resolution (R_s values); and (B) experimental results measured with the sample of ^{14}C-

TABLE I

SWELLING CHARACTERISTICS OF THE CATION EXCHANGER OSTION LGKS 0802 IN THE Pb^{2+}, Ca^{2+} AND Li^+ FORMS IN WATER

Form of cation exchanger	*Density (g/ml)*	*Swelling (g H_2O/ml particles)*
Pb^{2+}	1.57	0.61
Ca^{2+}	1.32	0.65
Li^+	1.23	0.72

TABLE II

EXPERIMENTAL RESULTS OBTAINED WITH THE MODEL SUGAR SOLUTIONS ON THE ANALYTICAL COLUMN

Form of cation exchanger	*Eluent*	*Sugar**	*Amount of sample, Q (μg)*	*Retention volume, V_R (ml)*	*Capacity factor, k'***	*Number of plates, N*	*Resolution, R_S*		
							*G**	*F**	*S**
Pb^{2+}	Water	G	100	1.96	0.661	2100	–	2.20	1.35
		F	100	2.46	1.085	1660	2.20	–	–
		S	100	1.70	0.441	1320	1.35	–	–
		M	100	1.70	0.441	660	–	–	0
	80% Aq.	G	200	7.50	5.356	310	–	–	–
	ethanol	F	200	12.00	9.169	–	–	–	–
		S	200	7.50	5.356	–	–	–	–
		M	200	–	–	–	–	–	–
	60% Aq.	G	200	3.55	2.008	1030	–	3.3	0.96
	ethanol	F	200	5.20	3.407	1100	3.3	–	–
		S	200	2.92	1.474	230	0.96	–	–
		M	200	3.65	2.093	–	–	–	–
Ca^{2+}	Water	G	100	1.63	0.381	1470	–	1.92	1.44
		F	100	1.92	0.627	2000	1.42	–	3.60
		S	100	1.42	0.203	1740	1.44	3.60	–
		M	100	1.32	0.119	960	–	–	1.00
	80% Aq.	G	200	5.60	3.746	350	–	–	1.00
	ethanol	F	200	9.90	7.390	410	–	–	–
		S	200	4.80	3.068	100	1.00	–	–
		M	200	6.6	4.593	–	–	–	–
	60% Aq.	G	200	2.63	1.229	560	–	2.3	1.8
	ethanol	F	200	3.90	2.305	580	2.3	–	–
		S	200	1.92	0.627	300	1.8	–	–
		M	200	1.82	0.542	350	–	–	–
Li^{+}	Water	G	50	1.50	0.271	3460	–	1.00	2.40
		F	50	1.59	0.347	5600	1.00	–	–
		S	50	1.28	0.085	7400	3.40	–	–
		M	50	1.18	–	4700	–	–	1.40
	80% Aq.	G	200	5.50	3.661	3990	–	0	0
	ethanol	F	200	5.62	3.763	1600	0	–	0
		S	200	5.6/5.9	3.74/4.00	–	0	0	–
		M	200	5.8	3.915	520	0	0	0
	60% Aq.	G	100	2.50	1.119	3460	–	0	2.90
	ethanol	F	100	2.68	1.271	2350	0	–	–
		S	100	2.00	0.695	1540	2.90	–	–
		M	100	1.5/1.7	0.271/0.44	800/4400	3.00	–	1.77

* Sugars: G = glucose; F = fructose; S = saccharose; M = maltotriose.

** In calculating the capacity factor $k' = (V_R - V_0)/V_0$, a V_0 value of 1.18 ml was substituted for the column dead volume.

labelled saccharose, especially the resolution of radioactive impurities. Examination of Tables II and III reveals that, from the viewpoint of criteria Ab, Ac and B, all the systems using 80% ethanol as the mobile phase can be considered to be unsuitable. There is still another disadvantage concerning the Li^{+} and Pb^{2+} form of the sorbent in the presence of 80% aq. ethanol. Saccharose splits into glucose and fructose on

TABLE III

RADIOCHEMICAL PURITY OF SACCHAROSE IN THE ANALYSED FRACTION COLLECTED FROM THE ANALYTICAL COLUMN

Form of cation exchanger	*Eluent*		
	Water	*80% Aq. ethanol*	*60% Aq. ethanol*
Pb^{2+}	100	0	100
Ca^{2+}	100	100	99.4
Li^{+}	96.5	85	100

the column at 80°C. From the viewpoint of criterion Aa, the systems Li^+ form–water and Ca^{2+} form–water were eliminated. The system Pb^{2+} form–60% aq. ethanol was eliminated for a low N of saccharose and a poor resolution of saccharose and glucose. Having compared the three remaining systems (Pb^{2+} form–water, Ca^{2+} form–60% aq. ethanol and Li^+ form–60% aq. ethanol), the system Li^+ form–60% ethanol was chosen as it gave the highest k' and N values for saccharose and the best resolution of saccharose and glucose. In connection with the choice of this system, we obtained the content of Li^+ ions in the separated fractions analysed; both in the effluent and in the saccharose fraction a value of 0.6 μg/ml was found.

Before we determined the preparative column parameters, we examined some parameters of the chosen chromatographic system on the analytical column.

First we determined the dead volume of the column, V_0. A higher concentration and larger volumes of the marker solution (1 M lithium chloride) were chosen to suppress the electrolyte sorption. From the nine experiments performed, differing in the amounts of lithium chloride injected on to the column, an average value of $V_0 = 1.18$ ml with a relative error of 4.2% was determined.

The dependence of the reduced plate height, $h = L/Nd_p$, on the reduced velocity, $v = ud_p/D_m$ (L = column length; N = number of theoretical plates; D_m = diffusion coefficient of solute in the mobile phase, $D_m = 1.31 \cdot 10^{-5}$ cm^{-2}/s[39,40]; u = linear velocity of eluent) was then examined.

TABLE IV

RESULTS OF EXPERIMENTS PERFORMED TO DETERMINE THE RELATIONSHIP BETWEEN h AND v

Eluent flow-rate, F (ml/min)	*Reduced velocity, v*	*Retention volume, V_R (ml)*	*Exponentially modified Gaussian peak parameters*		*Number of plates, N*	*Reduced plate height, h*
			$\tilde{A}$ (ml)	*$\tilde{B}$ (ml)*		
0.02	0.768	1.86	0.0600	0.0675	5332	3.473
0.04	1.536	1.86	0.600	0.0840	4786	3.869
0.06	2.303	1.91	0.0640	0.0804	4394	4.215
0.08	3.071	1.89	0.0720	0.0856	3200	5.787
0.12	4.607	1.96	0.0842	0.0980	2345	7.848
0.20	7.678	1.98	0.1042	0.1280	1347	13.748

The experimental results are summarized in Table IV, where, in addition to N values calculated from the chromatographic peak, the parameters of the exponentially modified Gaussian-shaped peak $\tilde{A}$, $\tilde{B}$[41] read to 10% of the peak height are also given.

To find the optimum (*i.e.*, the minimum) in the relationship between h and v, the Knox equation was chosen[42]:

$$h = \frac{B}{v} + Av^{1/3} + Cv \tag{1}$$

According to the calculations performed, the optimum was found to be in the region of $u \approx 1.06 \cdot 10^{-2}$ cm/s, which was, however, a very low velocity for the practical purposes. Therefore, we decided to use a velocity of 2.12 cm/s in our experiments. This higher velocity did not cause any pronounced increase in h.

The aim of our further experiments using the analytical column was to find the relationship between the resolution of saccharose and glucose on the amount of their mixture injected or its volume and concentration. As a criterion for the determination of a maximum volume and the concentration of the injected sample, the condition chosen was that the resolution of a sample having a concentration ratio of the two sugars of 1:1 should be higher than 2.5. As is evident from the results in Table V, a volume injected of 50 μl and a concentration of 10 mg/ml (of each sugar) conform to this condition. To determine the preparative column parameters, we chose the procedure described by Coq *et al.*[43].

TABLE V

EFFECT OF THE SAMPLED VOLUME V_i AND CONCENTRATION c ON THE RESOLUTION R_s OF THE SACCHAROSE–GLUCOSE PAIR ON THE ANALYTICAL COLUMN

Volume sampled, V_i (µl)	*Glucose*			*Saccharose*			R_S
	C (mg/ml)	*Q* (mg)*	*N*	*C (mg/ml)*	*Q* (mg)*	*N*	
25	10	0.25	4375	10	0.25	2700	3.45
50	10	0.50	2530	10	0.50	1280	2.53
100	10	1.00	1580	10	1.00	540	1.77
100	10	1.00	1520	10	1.00	570	1.72
250	10	2.50	363	10	2.50	182	0.75
25	20	0.50	3620	20	0.50	1834	2.82
50	10	0.50	3390	10	0.50	1210	2.54
100	5	0.50	1411	5	0.50	522	1.67
250	2	0.50	370	2	0.50	270	0.70
50	2	0.10	2735	2	0.10	1310	2.60
50	5	0.25	2690	5	0.25	1290	2.60
50	10	0.50	3160	10	0.50	1246	2.66
50	20	1.00	2617	20	1.00	1246	2.38
50	40	2.00	1490	40	2.00	1055	1.53

* Q = Amount sampled.

For the required amount of saccharose separated on the preparative column in one operation, Q_p = 40 mg, column productivity R_p = 10 mg/h (the subscript p denotes the preparative column) and considering the inner diameter of the preparative columns available in our laboratory (dc_p = 24.5 mm), a column length L_p = 50 cm, an eluent flow-rate F_p = 48 ml/h and a pressure drop P_p = 600 kPa were found by using the relationships given in ref. 43.

Provided that the maximum concentration of the separated compound in a sample injected on to the column is independent of the column size (here C_{max} = 10 mg/ml), the volume V = 4 ml corresponds to the chosen condition Q_p = 40 mg of saccharose. This volume of the injected saccharose–glucose mixture was chosen for the first experiment carried out on the preparative column using the parameters given above. The saccharose:glucose concentration ratio of 10:1 is close to the ratio expected in real samples.

The amount injected is satisfactory from the point of view of the resolution achieved, but from the point of view of the column productivity it is small because the distance between the peaks of saccharose and glucose is about 25 ml. Therefore, further experiments were carried out in which both the amount of mixture injected and the eluent flow-rate were increased. A chromatogram of the separation using the largest sample of saccharose (320 mg) is shown in Fig. 1. The fraction of saccharose collected and analysed by the quantitative HPLC method is indicated. The resolution achieved in this experiment and also the results of other experiments are summarized in Table VI.

The last experiment performed on the preparative column represented a separation of the radioactive sample (Fig. 2). The volume injected was 5 ml (*i.e.*, 30 mg of saccharose) because, in contrast to the model solutions, further impurities in addition to glucose were expected to be present in the real sample.

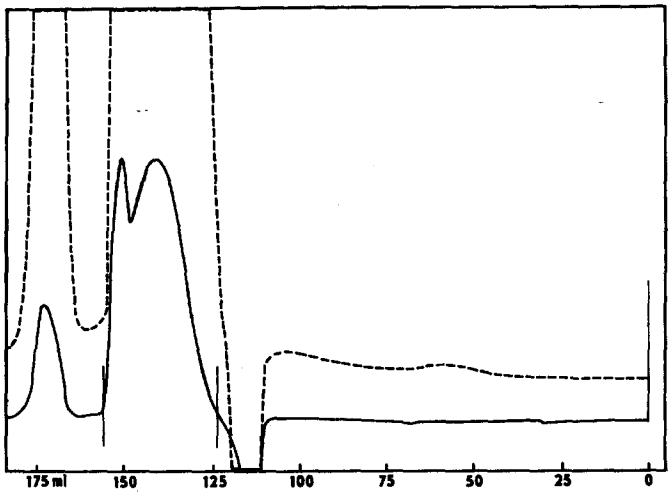

Fig. 1. Chromatogram from the preparative column. Sample, 320 mg of saccharose and 32 mg of glucose in a volume of 8 ml; eluent flow-rate, 48 ml/h. ———, First channel, 100 mV; – – – –, second channel, 10 mV.

TABLE VI

ISOLATION OF SACCHAROSE FROM THE MODEL SUGAR SOLUTIONS ON THE PREPARATIVE COLUMN

Eluent flow-rate, F (ml/h)	*Volume sampled, V_i (ml)*	*Amount of saccharose sampled, Q_S (mg)*	*Amount of glucose sampled, Q_G (mg)*	*Saccharose fraction*		R_S
				Volume, V_S (ml)	*Amount, Q_{SF} (mg)*	
48	8.0	80	8	22	76	1.88
	8.0	160	16	26	156	1.65
	8.0	320	32	35	318	1.05
96	8.0	160	16	26	141	1.58
	10.0	200	20	28	178	1.38

The adsorption isotherm of saccharose from the solution of 60% aq. ethanol on the cation exchanger in the Li^+ form is shown in Fig. 3. The concentration $\bar{C}$, *i.e.*, the amount of saccharose adsorbed on the column relative to the volume of sorbent particles and corresponding to the initial concentration of the solution C, was determined by using the calibration graph of the RIDK 101 detector and numerical integration of an output adsorption curve.

DISCUSSION

By using water as an eluent, the order of elution of sugars for all three ion forms of the cation exchanger were as follows: maltotriose < saccharose < glucose < fructose. If the individual forms of the cation exchanger were compared, the V_r

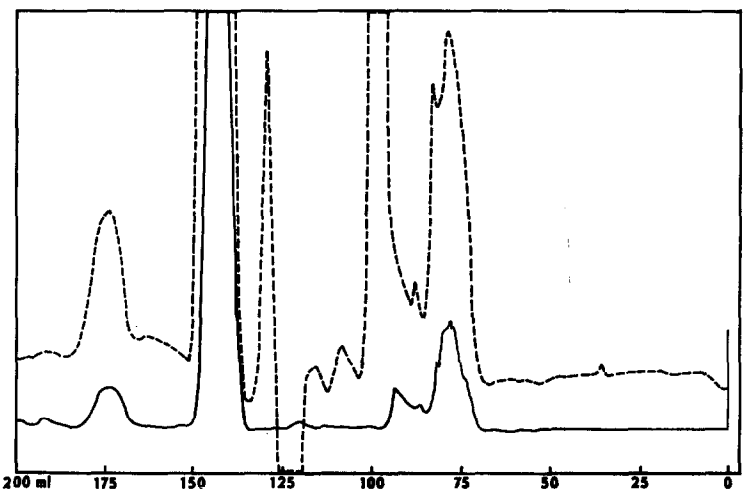

Fig. 2. Refractive index (broken lines) and radioactivity (solid lines) traces for ^{14}C-labelled saccharose from the preparative column. Sample, 5 ml of [^{14}C]saccharose (concentration *ca.* 6 mg/ml).

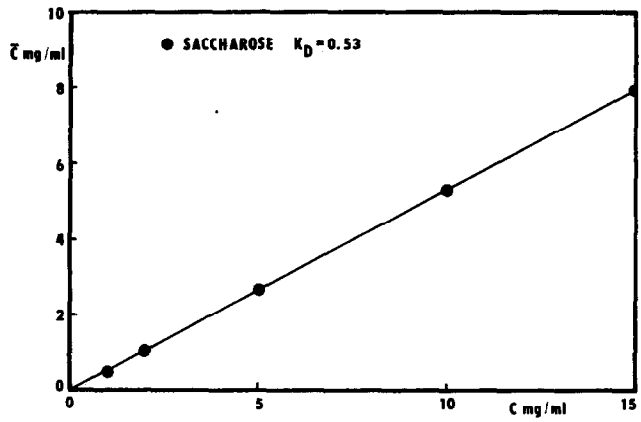

Fig. 3. Adsorption isotherm of saccharose on Ostion LGKS 0802 in the Li^+ form at 80°C with 60% aq. ethanol.

values of each sugar increased as follows: Li form < Ca form < Pb form. We assume that this trend corresponds to the increasing content of water from the Pb to the Li form (see Table I), probably caused by the increasing size of the hydration sphere.

If aqueous ethanol was used as the eluent, the V_r or k' values always increased. This increase was more pronounced with 80% than with 60% aq. ethanol. Disadvantages were a decrease in N (more pronounced with 80% aq. ethanol) and decomposition of saccharose into glucose and fructose in the case of Li and Pb forms using 80% aq. ethanol. The sequence of retention volumes given for water as the eluent was valid for individual sugars except maltotriose with the Li and Ca forms in 80% ethanol and the Pb form in 60% aq. ethanol (for 80% aq. ethanol no signal occurred even when a sample volume of 200 μl was injected and 12 ml of the eluent passed through the column).

In connection with these exceptions concerning maltotriose, we should mention the work of Havlíček and Samuelson[44], who measured the relationship between the distribution coefficient K_D and the number of D-xylose units, m, in oligosaccharides at various ethanol concentrations and at 75°C by using Dowex 50W-X8 in the Li^+ form. The result was a linear relationship between log K_D and m, where the log K_D intercept increased with increasing concentration of ethanol and the slope of the line changed from a negative to a positive value, which meant that the retention order reversed.

As far as the use of the Knox equation for the description of the relationship between the reduced plate height h and the reduced velocity v is concerned, the results of its verification can be used only for orientation purposes.

It was verified experimentally that the deformation of the saccharose peak for a sample amount of 320 μg (see Fig. 1) was caused by a non-linear response of the RIDK 101 detector if higher concentrations of saccharose were sampled on the column (the deviation passes a maximum twice). We believe that even if larger samples are used the shape of the saccharose peak and a good separation of the saccharose–glucose pair will not deteriorate significantly because the adsorption iso-

therm of saccharose keeps its linear course even if high solution concentrations are used (see Fig. 3).

CONCLUSION

Nine chromatographic systems using the strong cation-exchanger Ostion LGKS (Pb^{2+}, Ca^{2+} and Li^{+} forms) with water or 80% or 60% aq. ethanol as eluents were studied for the isolation of saccharose from a partly purified fraction of *Chlorella vulgaris*. Using the cation exchanger in the Pb^{2+} and Li^{+} forms with 80% ethanol at 80°C, saccharose split on the column into glucose and fructose. The order of the retention volumes of glucose, fructose, saccharose and maltotriose in the individual systems corresponded with the results of other workers[3-7]. The cation exchanger in the Li^{+} form with 60% aq. ethanol was the most suitable system for the isolation of saccharose.

In spite of its simplicity, the determination of the preparative column parameters for a required resolution R_s, processed amount Q and productivity R, based on the work of Coq *et al.*[43], proved to be completely satisfactory.

As a result of the linearity of the adsorption isotherm of saccharose over a relatively wide concentration range, both the shape of the saccharose peak after passing through the preparative column and the separation of the saccharose–glucose pair did not deteriorate significantly even when a large amount of the sample was injected on to the column.

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