*Journal of Chromatography*, 235 (1982) 229–236
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,315

# CHROMATOGRAPHY OF CELLODEXTRINS AND ENZYMATIC HYDROLYSATES OF CELLULOSE ON ION-EXCHANGE DERIVATIVES OF SPHERON

Z. HOSTOMSKÁ-CHYTILOVÁ and O. MIKEŠ*
*Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 (Czechoslovakia)*
P. VRÁTNÝ
*Institute of Agriculture, 160 21 Prague 6 (Czechoslovakia)*
and
M. SMRŽ
*Research Institute of Pure Chemicals, Lachema, 621 33 Brno (Czechoslovakia)*
(Received September 1st, 1981)

## SUMMARY

Cellodextrins (cellohexaose to cellobiose) and glucose were separated on derivatives of Spheron®. The optimum conditions found (TEAE-Spheron 1000; 0.025 *M* sodium borate, pH 7.5 or 8.0) are suitable for the chromatography of cellodextrins prepared from acid hydrolysates of cellulose. The same conditions can be used for the separation of the enzymatic hydrolysates of cellulose. At higher buffer concentrations (0.1–0.35 *M*) and at higher pH (8.5–8.8) lower surgars can be chromatographed together with cellodextrins. The enzymatic hydrolysate of cellulose, prepared by means of cellulases from *Trichoderma viride*, was most conveniently chromatographed on DEAE-Spheron 300 in 0.25 *M* sodium borate, pH 7.5, when the separation of glucose and cellobiose was achieved. For simultaneous detection of glucose, cellobiose and further cellodextrins, a two-column system was developed, composed of DEAE-Spheron and TEAE-Spheron.

## INTRODUCTION

By use of enzymatic methods or partial acid hydrolysis, cellulose can be split into cellodextrins. These are linear oligomers consisting of two to seven glucose units linked by $\beta$-1,4-glucoside bonds. The final product of their hydrolysis is glucose. Study of the quantitative course of the enzymatic hydrolysis and of the catalytic effect of individual enzymes led to the development of numerous techniques for the separation of the hydrolysis products, one of which is liquid chromatography on various types of ion exchangers.

The separation of cellodextrins may be achieved on cation exchangers in various forms by elution with water[1] or aqueous solutions of organic solvents[2], or on

0021-9673/82/0000–0000/$02.75 

anion exchangers eluted with borate buffers[3]. Anion-exchange derivatives of Spheron have already been used for the chromatography of monosaccharides[4] and of mixtures of mono- and oligo-saccharides[5].

In this paper the liquid chromatography of sugars and their borate complexes on Spheron derivatives has been investigated for the separation of cellodextrins, for the study of the course of their hydrolysis and as a method for monitoring the enzymatic hydrolysis of cellulose.

## EXPERIMENTAL

### *Materials*

Medium basic anion exchangers DEAE-Spheron 300 (capacity 0.6 mequiv./g and 2.2 mequiv./g) were laboratory products[6] (particle size 20–40 μm). A partly quaternized derivative (*cf.*, Type 2, capacity 2.2 mequiv./g; ref. 7) was prepared from DEAE-Spheron 300 (particle size 20–40 μm). This derivative had half of the total number of diethylaminoethyl groups quaternized. Fully quaternized TEAE-Spheron 1000 (capacity 1.4 mequiv./g, particle size 25–40 μm; and 2 mequiv./g, 13–18 μm) and sulphonated S-Spheron cation exchanger (1.42 mequiv./g, 13–18 μm) were experimental laboratory products from Lachema (Brno, Czechoslovakia).

Cellohexaose, cellopentaose, cellotetraose, cellotriose and cellobiose* were prepared in the pure form according to Miller *et al.*[8]. Monosaccharides and all other analytical grade chemicals used were commercial products of Lachema.

The crude preparation of cellulases was prepared from a liquid cultivation medium of *Trichoderma viride* (Research Institute of the Food Industry, Prague, Czechoslovakia) by desalting on Bio-Gel P-6 and freeze-drying.

### *Methods*

Chromatography was carried out on a sugar analyzer 71 000 (Developmental Workshops of the Czechoslovak Academy of Sciences) described in ref. 4. The preparation of buffers, solutions of saccharides, detection reagent and the adjustment of ion exchangers has also been described[4]. 100-μl Samples, containing mostly 30 μg of individual saccharides, were injected.

*Enzymatic hydrolysis of cellulose.* Ten milligrams of chromatography paper Whatman No. 1 (Whatman, Springfield Mill, Maidstone, Great Britain), ground in a ball mill, indicated as W1, were incubated with 1.5 mg of lyophilized cellulase preparation (from *T. viride*) in 1.5 ml of 0.05 *M* citrate buffer, pH 4.8, at 40°C for 10, 45 and 90 min.

*Enzymatic hydrolysis of cellodextrins.* Cellopentaose (410 μg) was incubated with 48 μg of lyophilized cellulases (*T. viride*) in 60 μl of 0.05 *M* of citrate buffer, pH 4.8, at 40°C for 0.2, 0.45, 2.0 and 5.0 min.

* These cellodextrins and the monomer will be indicated as $G_6$, $G_5$, $G_4$, $G_3$, $G_2$ and $G_1$ (glucose). Substances $G_2$–$G_6$ are sometimes named cellooligosaccharides.

## RESULTS

### *Effect of pH*

The separation of a mixture of cellodextrins $G_2$–$G_6$ on TEAE-Spheron 1000 in the pH 7–9 region was studied at constant molarity of the borate buffer, 0.025 *M*. The dependence of the retention volumes and of the resolution of neighbouring pairs of cellodextrins on pH is shown in Fig. 1.

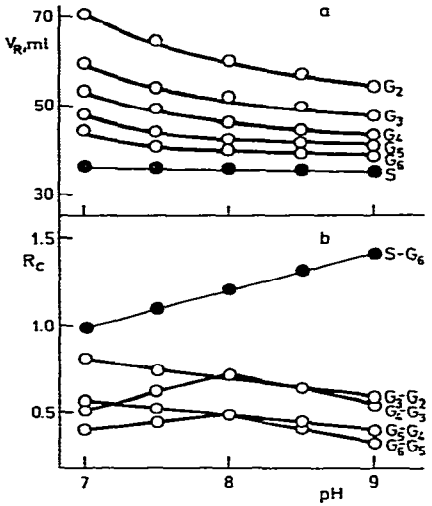


Fig. 1. Dependence of retention volumes, $V_R$, of cellodextrins $G_2$–$G_6$ and saccharose (S) (a), and of resolution, $R_C$ (b), on the pH values of 0.025 *M* borate buffers during chromatography on TEAE-Spheron 1000 (2.0 mequiv./g, 13–18 $\mu$m). Column: 200 × 8 mm I.D.; temperature 60 C. Flow-rate: 50 ml/h (flow of orcinol–$H_2SO_4$ through detector 1.93 ml/min plus 0.42 ml/min of effluent). The retention volumes are not corrected with respect to the volume of the detection system. The resolution values are corrected with respect to the spreading of the peaks in the detection system.

Saccharose (which is very weakly retained on the ion exchanger) was always added to the mixture of cellodextrins. In the pH 7–9 region it was found that with increasing pH the retention volumes of all cellodextrins are decreased. $G_6$ is least strongly retained, and with decreasing number of glucose units the retention volumes of cellodextrins increase.

The calculated values for the resolution of neighbouring pairs are dependent on pH. The pairs $G_6$–$G_5$, $G_4$–$G_3$ have optimum resolution at pH 8, while for $G_5$–$G_4$ and $G_3$–$G_2$ the resolution decreases with increasing pH. For further experiments the values pH 7.5 and 8 were selected.

From the calculation of the number of theoretical plates of a given column, $n_c$, it was found that the $n_c$ values for $G_6$, $G_5$ do not vary greatly with pH, and those for $G_4$, $G_3$ and $G_2$ slightly decrease with increasing pH.

### *Ionic strength effect*

Chromatography of a mixture of $G_2$–$G_6$ was carried out at constant pH 7.5 on a TEAE-Spheron column in the borate form. After equilibration, elution was carried

ont with water and borate buffers (0.005 *M*, 0.01 *M*, 0.017 *M*, 0.02 *M*, 0.25 *M* and 0.03 *M*). The dependence of the elution volumes, $V_R$, and the resolution, $R_C$, of cellodextrins on molarity is shown in Fig. 2.

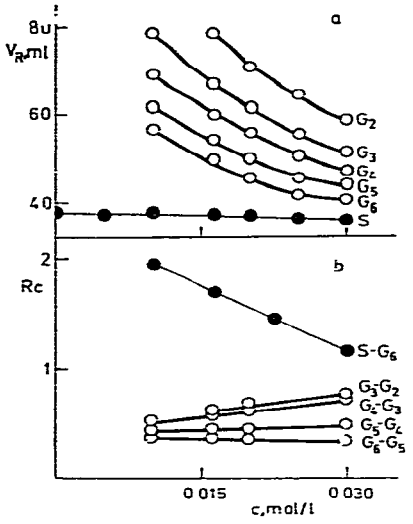


Fig. 2. Dependence of retention volumes of cellodextrins and saccharose (a) and of resolution (b) on the borate concentration in the elution buffer, pH 7.5, during chromatography on TEAE-Spheron 1000 (13–18 μm, 2 mequiv./g). Conditions as in Fig. 1.

None of the cellodextrins could be eluted with water or with the 0.005 *M* borate buffer. Only saccharose was eluted. On chromatography with the 0.01 *M* buffer only $G_2$ was retained, while higher cellodextrins were eluted with the indicated $V_R$ values. With increasing molarity of the borate buffers, the elution volumes of all cellodextrins present were decreased.

In the buffer molarity range investigated it was found that for neighbouring pairs of cellodextrins the resolutions usually slightly increased with increasing borate concentration. From the values of the theoretical plates, $n_c$, it was found that for $G_6$ and $G_5$ the $n_c$ values do not change with increasing molarity and for $G_4$, $G_3$ and $G_2$ the $n_c$ values increase.

For further experiments the concentration 0.025 *M* was selected.

*Elution of monosaccharides and cellodextrins*

The chromatography of cellodextrins was investigated in the presence of monosaccharides. It was evident that TEAE-Spheron 1000 is a suitable support for the separation of more complex mixtures of saccharides. Optimum conditions for the separation of $G_2$–$G_6$ and of mannose, arabinose, galactose, xylose and glucose by step-wise elution were as follows: elution with 0.025 *M* borate buffer of pH 7.5 on a column equilibrated with the same buffer, followed by 50 ml of 0.1 *M* borate buffer, pH 8.5, and 50 ml of 0.25 *M* borate buffer, pH 8.8. Elution of monosaccharides was only achieved when the 0.35 *M* borate buffer, pH 8.8, was applied. Glucose was eluted after 4 h.

The possibilities of substitution of step-wise elution by linear gradient elution with buffers of increasing pH and molarity are illustrated in Fig. 3. This figure shows a record of the chromatography of a mixture of monosaccharides and cellodextrins where isocratic elution of cellodextrins is followed by a steep linear gradient. Elution of glucose was achieved with 0.25 *M* buffer, pH 8.8, after 3.5 h. For the conditions see the caption to Fig. 1.

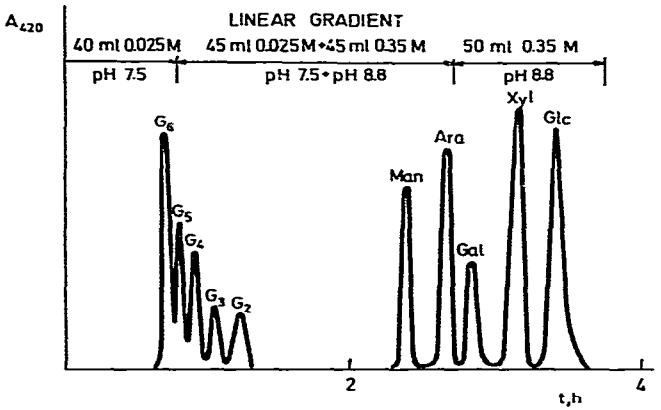


Fig. 3. Chromatography of a mixture of cellodextrins $G_2$–$G_6$ and five monosaccharides, arabinose (Ara), xylose (Xyl), galactose (Gal), mannose (Man) and glucose (Glc), on TEAE-Spheron 1000 (13–18 μm, 2.0 mequiv./g). The column was equilibrated with 0.025 *M* borate buffer, pH 7.5. Elution was carried out first isocratically with 0.025 *M* borate buffer, pH 7.5, then a linear gradient was applied (45 ml of 0.025 *M* borate buffer, pH 7.5, plus 45 ml of 0.35 *M* borate buffer, pH 8.8). Chromatography was terminated by elution with 0.35 *M* borate buffer, pH 8.8. For other parameters see Fig. 1.

*Chromatography of enzymatic hydrolysates of cellodextrins*

The optimum conditions found for chromatography of cellodextrins may be used in the analysis of the effect of cellulolytic enzymes on individual cellodextrins. As an example, the hydrolysis of $G_5$ with the cellulolytic system of the *T. viride* strain was selected. The reaction was carried out as mentioned in *Methods*. 30-μl Aliquots were taken from the mixture and injected directly into the column (see Fig. 4).

*Chromatography of enzymatic hydrolysates of cellulose*

From the results of the chromatography of monosaccharides (Fig. 3) it follows that the strongly basic group of TEAE-Spheron enables the elution of glucose only by disturbance of the isocratic conditions through the introduction of a new buffer of 0.25 *M* and pH 8.8. To enable the isocratic elution of all hydrolysis products, the basicity of the functional group must be decreased. For the monitoring of the main products of the hydrolysis of cellulose, $G_1$ and $G_2$, a suitable support from the series of DEAE-Spherons[6] was sought. DEAE-Spheron of capacity 0.6 mequiv./g and particle size 20–40 μm was selected. With this support a 0.025 *M* buffer (pH 7.5) could be used which had already been found to be suitable in connection with TEAE-Spheron. The chromatography of the enzymatic hydrolysate is shown in Fig. 5.

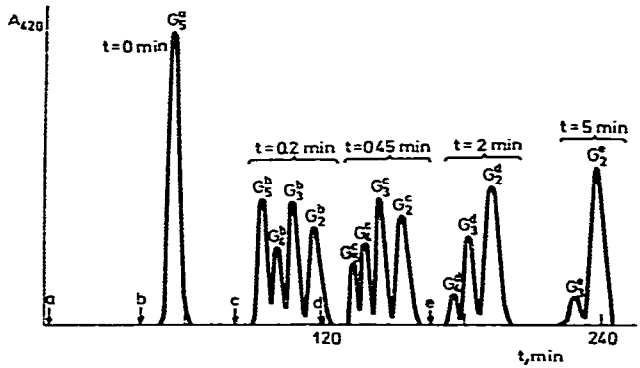


Fig. 4. Formation of lower cellodextrins by action of the cellulolytic enzymes from *T. viride* on $G_5$. The column of TEAE-Spheron 1000 was equilibrated with 0.025 *M* borate buffer, pH 7.5, which was also used for isocratic elution. Conditions as in Fig. 1. The arrow and the letters a, b, c, d and e indicate the points of injection of the hydrolysates. a, Cellopentaose was not incubated with cellulases; b, time of incubation was 0.2 min; c, 0.45 min; d, 2 min; e, 5 min. The total time of chromatography was 78 min. The effluent persisted for 27 min in the detector. New samples were injected before the preceding analysis was terminated, approximately at 45-min intervals.

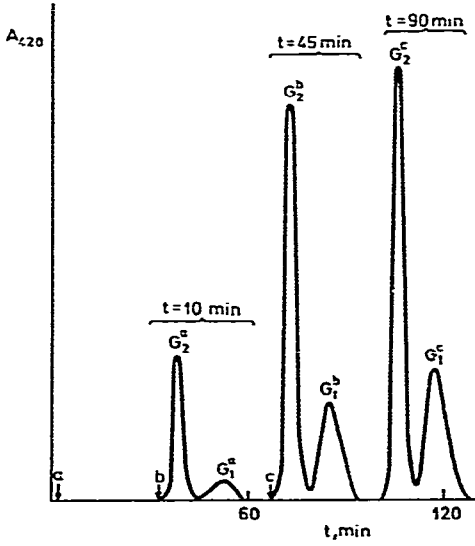


Fig. 5. Chromatography of the hydrolysate of the ground paper W1 with the cellulolytic enzymes of *T. viride* carried out on a column of DEAE-Spheron 300 (0.6 mequiv./g, 20–40 μm), 250 × 8 mm I.D., at 60°C; flow-rate 50 ml/h. The column was equilibrated and the elution carried out with 0.025 *M* borate buffer, pH 7.5. For the preparation of the hydrolysate see *Methods*. 100-μl Samples hydrolysed for 10 min, 45 min and 90 min were injected. The total time of analysis was about 60 min, of which the eluent spent 27 min in the detector, and further samples were injected every 30 min. For the labelling of peaks see Fig. 4.

## DISCUSSION

Saccharides can be separated on ion exchangers[9] either using the partition chromatography principle, *i.e.*, elution with aqueous solutions of organic solvents,

mainly ethanol[10] of various concentrations or water[11], or using ion-exchange chromatography with borate buffers of various pH and ionic strengths[12]. However, during the separation of some cellodextrins in the ethanol–water system precipitation may take place[13]. Moreover, the hydrolysates contain salts and proteins which also precipitate in aqueous–alcoholic solutions. The elimination of the precipitate affects the total content of cellodextrins in the sample. Chromatography of cellodextrins on cation exchangers with water as eluent is more advantageous than the use of organic solvents in that the sample is completely soluble in water, thus avoiding adjustment of the sample before chromatography[14].

We therefore tested the possibility of chromatographing cellodextrins on a cation-exchange derivative of S-Spheron 1000 (particle size 13–18 $\mu$m) converted into its calcium form. Elution was carried out with deionized water at 85°C. Under these conditions individual saccharides could not be separated. The mixtures applied were eluted within the hold-up volume as a single peak. The previous separation of cellodextrins by this method[1] was carried out on supports of the xerogel type in which the sugar molecules must diffuse into the gel matrix. The hybrid support used by us has a macroreticular structure[7] created by very densely cross-linked xerogel microspheres into which sugar molecules cannot penetrate. The macropores are of the aerogel type and they are so large that the size exclusion effect cannot occur; this is probably the reason why the separation did not take place.

Therefore a suitable support was sought from the group of Spheron anion-exchange derivatives. On DEAE-Spheron higher oligosaccharides cannot be separated[4]. However, we used this type of anion exchanger to study the hydrolysis of cellulose with cellulases, during which glucose and cellobiose were well separated (Fig. 5). On a derivative of DEAE-Spheron 300, Type II (ref. 7), quaternized to 50% (*cf.*, ref. 5), higher cellodextrins $G_3$–$G_6$ were poorly separated. On TEAE-Spheron 1000 (capacity 1.2 mequiv./g), a DEAE-derivative additionally fully quaternized with ethyl halide, $G_2$ and $G_3$ were well separated but the separation of $G_4$–$G_6$ was imperfect. The best results were achieved on TEAE-Spheron 1000 (capacity 2 mequiv./g), particle size 13–18 $\mu$m) which even gave good separations of mixtures of saccharides more complex than those mentioned in Fig. 3. For this support the optimum conditions for elution were determined (Figs. 1 and 2) and they were exploited for monitoring the hydrolysis course of cellodextrins (Fig. 4).

Chromatography on a strongly basic quaternized support does not permit simultaneous elution of cellodextrins and glucose under isocratic conditions. Higher cellodextrins are very weakly retained on DEAE-derivatives and are therefore not separated, while glucose is retained sufficiently. On TEAE-derivatives, cellodextrins are well separated, while glucose is retarded excessively. Therefore, we tested the separation of $G_1$–$G_6$ by means of a system of two columns (Fig. 6). Before the column with TEAE-Spheron (capacity 2 mequiv./g) another column was inserted, packed with DEAE-Spheron of lower capacity (0.6 mequiv./g) which should be selected optimally. On this column the group of cellodextrins were separated from glucose on isocratic elution. Their mutual separation was achieved on a second column with TEAE-Spheron using the same buffer. Switching between columns permitted consecutive detection of the separated cellodextrins and glucose after a single injection (Fig. 7).

The described method of chromatography of borate complexes of cellodextrins

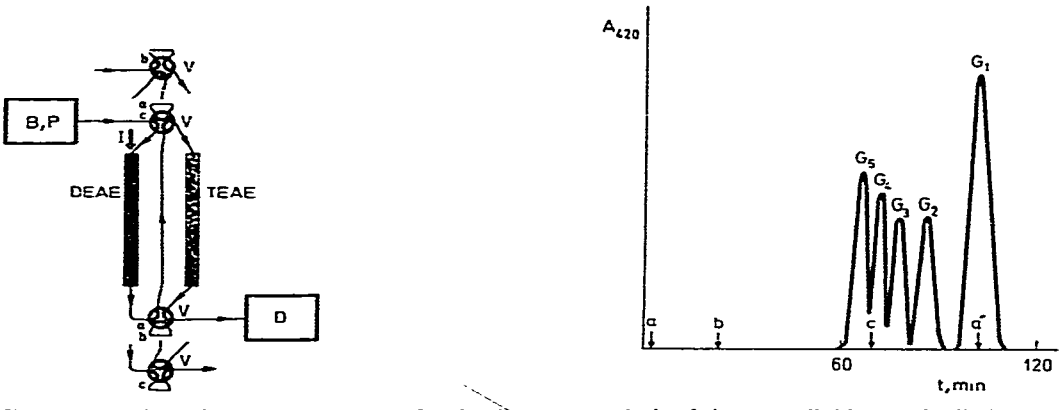


Fig. 6. Two-column ion-exchange system for simultaneous analysis of glucose, cellobiose and cellodextrins by isocratic elution. B, P = Buffer container and a laboratory piston-pump; V = six-port valve; a, b, c = individual positions of the valve; I = injector; DEAE, TEAE = ion-exchange columns of DEAE-Spheron 300 (250 × 8 mm), 20–40 μm, 0.6 mequiv./g, and TEAE-Spheron 1000 (200 × 8 mm), 13–18 μm, 2.0 mequiv./g; D = detection system. A three-step operation cycle is employed (*cf.* Fig. 7): after the introduction of the sample, valve position a connects the two columns for a short time (glucose is retained on the first, and cellodextrins pass into the second column). Position b excludes the first column and a separation of cellodextrins takes place in the second column. After detection of $G_5$ ($G_2$–$G_4$ are already in the detector system) the second column is excluded by position c and the first column is simultaneously connected to the detector until glucose ($G_1$) is eluted. Then the system is ready for a further cycle. Buffer: 0.025 *M* sodium borate, pH 7.5. Flow-rate: 50 ml/h. Temperature: 60°C.

Fig. 7. Chromatography of cellodextrins and glucose on the two-column system described in Fig. 6. Samples of 20 μg of cellodextrins $G_2$–$G_6$ and 40 μg of glucose ($G_1$) were injected into a DEAE column. The letters a–c correspond to the three positions of the valve in Fig. 6. A further sample for analysis can be injected into this isocratic elution at the position a' (arrow).

on an automatic analyzer for sugars permits easy identification and quantification. The isocratic elution permits repetition of chromatography without equilibration. Thus, the anion-exchange derivatives of Spheron represent suitable supports not only for the separation of monosaccharides[4] and lower oligosaccharides[5] but also for cellodextrins.

## REFERENCES

1 M. R. Ladisch, A. L. Huebner and G. T. Tsao, *J. Chromatogr.*, 147 (1978) 185.
2 E. Martinsson and O. Samuelson, *J. Chromatogr.*, 50 (1970) 429.
3 R. B. Kesler, *Anal. Chem.*, 39 (1967) 1416.
4 Z. Chytilová, O. Mikeš, J. Farkaš, P. Štrop and P. Vrátný, *J. Chromatogr.*, 153 (1978) 37.
5 P. Vrátný, O. Mikeš, J. Farkaš, P. Štrop, J. Čopiková and K. Nejepinská, *J. Chromatogr.*, 180 (1979) 39.
6 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 180 (1979) 17.
7 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339.
8 G. L. Miller, J. Dean and R. Blum, *Arch. Biochem. Biophys.*, 91 (1960) 21.
9 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 55.
10 O. Samuelson and B. Swenson, *Acta Chem. Scand.*, 16 (1962) 2056.
11 R. M. Saunders, *Carbohyd. Res.*, 7 (1968) 76.
12 J. X. Khym and O. P. Zill, *J. Amer. Chem. Soc.*, 74 (1952) 2090.
13 J. Havlicek and O. Samuelson, *Anal. Chem.*, 47 (1975) 1854.
14 M. R. Ladisch and G. T. Tsao, *J. Chromatogr.*, 166 (1978) 85.