Journal of Chromatography, 207 (1981) 99–114
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,490

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PECTIC ENZYMES*

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SUMMARY

Technical pectic multienzyme preparations, Pectinex Ultra and Rohament P, were chromatographed on an analytical scale using medium pressure liquid chromatography on a glycol methacrylate rigid macroreticular gel, Spheron 1000 and its ion-exchange derivatives. A combination of isocratic and linear-gradient elution (with gradients in ionic strength or pH) was employed and fractions were monitored by measurements of absorbance (A_{285} and A_{254}), conductivity, pH and enzyme activity. Conditions for rapid separations of pectic enzymes are elaborated.

The results indicate the possibilities of separating, the technically undesirable pectin-esterase activity from the other enzyme activities, and of a more detailed biochemical investigation of these enzymes, important for the food industry.

INTRODUCTION

Microorganisms grown in a medium containing pectin produce a mixture of enzymes which catalyze its degradation, via the de-esterification of units of D-galactopyranuric acid and the hydrolysis or β-elimination of glycosidic α-1,4-bonds in D-galacturonan. The composition of the enzyme mixture and the content of individual enzymes are determined by the type of microorganism and the conditions of cultivation. High yields of pectic enzymes are obtained from microscopic fungi, which are therefore often used as sources of commercial and technical preparations of pectinases.

Various methods have been used[1] for the separation of microbial pectic enzymes from cultivation media or commercial preparations, such as ion-exchange chromatography on CM-cellulose[2], DEAE-cellulose[3,4], cellulose phosphate[5], affinity chromatography on pectic acid cross-linked with epichlorohydrin[6] and on its amino

* Presented at the *6th International Symposium "Advances and Application of Chromatography in Industry", Bratislava, September 16–19, 1980.*

0021-9673/81/0000–0000/$02.50

derivative[7] and gel chromatography on Bio-Gel[8]. These methods generally lead to the separation of one enzyme from a mixture of other enzymes, and all enzymes present in the starting material can be separated only by chromatography on DEAE-cellulose[3,4].

Modern development of high-performance liquid chromatography (HPLC) of biopolymers[9–11] has permitted the application of this rapid method to the separation of pectic enzymes. In earlier papers[12–15] we have demonstrated the possibility of using the matrix of the hydrophilic polymer Spheron® for the preparation of macroreticular ion exchangers. These are suitable for rapid chromatography of enzymes[16] and other proteins[12], simple sugars[17], oligosaccharides[18], nucleoside phosphates[19] and oligonucleotides[20]. We also demonstrated (ref. 16, Fig. 5) the possibility of chromatographing the Czechoslovak technical pectic enzyme Leozym on DEAE-Spheron 300 prepared in the laboratory.

The aim of this paper is to examine the possibilities of HPLC using various commercial derivatives of Spheron 1000 for rapid separation of mixtures of pectic enzymes in preparations of microbial origin, on an analytical scale. The work is oriented towards commercial preparations used in the foodstuffs industry for canning, which contain the pectic enzymes *endo*-D-galacturonanase and *exo*-D-galacturonanase [poly(1–4)-α-D-galactosiduronate glycanohydrolase, E.C. 3.2.1.15 and E.C. 3.2.1.67], pectin-lyase [poly(methyl-D-galactosiduronate) lyase, E.C. 4.2.2.10] and pectin-esterase (pectin pectyl-hydrolase, E.C. 3.1.1.11).

EXPERIMENTAL

Materials

Enzymes. Pectinex Ultra (Ferment, Basel, Switzerland) and two batches of the preparation Rohament P (Rohm and Haas, Darmstadt, G.F.R.) were employed.

Substrates. Citrus pectin, degree of esterification (DE) 65.1%, was purified from Genu Pektin Type B, Slow Set (Københavns Pektinfabrik, Copenhagen, Denmark) by washing with 60% ethanol containing 5% hydrochloric acid and then with 60% and 96% ethanol. Pectic acid was prepared from citrus pectin by repeated alkaline de-esterification with 0.1 *M* sodium hydroxide and subsequent precipitation at pH 2.5. The highly esterified pectin (DE 93.8%) was prepared by esterification of pectic acid with 1 *M* sulphuric acid solution in methanol[21]. Digalacturonic acid was isolated from the partial acid hydrolysate of pectic acid by gel chromatography on Sephadex G-25 (Fine)[22].

Chromatographic materials. Spheron 1000, particle size 25–40 μm, was obtained from Lachema (Brno, Czechoslovakia). Its characteristics are given in ref. 10 (Table I) and in ref. 23 (Table 6.3A); *cf.*, also ref. 13 (Tables I and II). The ion exchangers employed, based on the matrix Spheron 1000, were also from Lachema and their characterization is given in the monograph[23] (Table 5.6B). Nominal capacities: weakly acidic carboxylic cation exchanger Spheron C-1000 (25–40 μm), 1.85 mequiv./g; medium acidic cation exchanger Spheron Phosphate 1000 (40–63 μm), 3.1 mequiv./g; strongly acidic sulphonated cation exchanger Spheron S-1000 (25–40 μm), 1.72 mequiv./g; weakly basic anion exchanger Spheron DEAE-1000 (25–40 μm), 1.5 mequiv./g; strongly basic quaternized anion exchanger Spheron TEAE-1000 (25–40 μm), 1.4 mequiv./g. Other pure chemicals were obtained from Lachema.

Chromatographic methods

The preliminary treatment of Spheron ion exchangers, the chromatographic methods and the equipment used were described in detail in refs. 10, 15 and 16. In this paper a combination of isocratic elution and a linear gradient was employed. The concentrations of the buffers mentioned relate to the counter ions. The chromatographic column from the amino acid analyzer (8 mm I.D.) was packed with 20 cm of the ion exchanger using the slurry method, with occasional pressure pulses of 15–20 atm. The ion exchanger was equilibrated with the first elution buffer. The sample was dissolved in 0.3 ml of the first elution buffer. The eluent (flow-rate *ca.* 3 ml/min) flowed through a tandem system of two flow-through photocells (absorbance recorded at 254 nm and 285 nm) into a fraction collector, the fractions being changed at 90-sec intervals. The pressure in individual experiments was between 4 and 15 atm. The chromatography was carried out at room temperature (20–25°C). The conductivity, pH and enzyme activity of each fraction were measured. The fractions were kept in closed test-tubes, with 2–3 drops of toluene, in an ice-box at 4°C until required for the activity measurements.

Testing methods

The activity of *endo*-D-galacturonanase and *exo*-D-galacturonanase was followed using the method of Somogyi[24] by determining the increase in the number of reducing groups in the reaction mixture composed of 0.8 ml of substrate and 0.2 ml of the enzyme. A 0.5% solution of pectic acid in 0.1 *M* acetate buffer, pH 4.2, was used as substrate for the determination of the activity of D-galacturonanases. For *exo*-D-galacturonanase, a 1 m*M* solution of digalacturonic acid in 0.1 *M* acetate buffer, pH 4.5, was employed.

The activity of pectin-esterase was determined by titration (0.1 *M* sodium hydroxide) of the carboxyl groups set free during reaction for 60 min. A 0.5% pectin solution (DE 65.1%) in 0.1 *M* acetate buffer, pH 4.4, was used as substrate. The reaction mixture contained 5 ml of substrate and 1 ml of enzyme solution.

The activity of pectin-lyase was determined from the increase in absorbance at 235 nm (ref. 25), using a 0.5% solution of highly esterified pectin (DE 93.8%) in 0.1 *M* acetate buffer, pH 5.6, as substrate. The reaction mixture contained 2.5 ml of the substrate and 0.5 ml of the enzyme solution.

RESULTS

The possibility of using HPLC on Spheron derivatives for the separation of technical pectic enzymes was tested with two commercial preparations differing in the content of individual pectic enzymes. Pectinex Ultra is a multienzyme preparation from a culture of *Aspergillus niger*, having a high content of *endo*-D-galacturonanase (N), pectin-esterase (S) and pectin-lyase (L). Chromatography on cellulose phosphate indicated the presence of two *endo*-D-galacturonanases[5]. The activity of *exo*-D-galacturonanase (X), which generally occurs as a minor component of microbial materials, is substantially lower in this material. Rohament P has a high content of *endo*-D-galacturonanase (N), while the activity of other pectic enzymes (S, L, X) in it is low. Therefore some idea of the separation possibilities of pectic enzymes on Spheron derivatives can be obtained on the basis of the results with Pectinex Ultra.

Most of the chromatography on unmodified Spheron 1000 and all its ion-exchanging derivatives was carried out with this preparation, which was also used to determine suitable conditions (buffer composition, pH, gradient) for separation of the enzymes. Rohament P was used for comparison and, in the case of the chromatography on a carboxylated cation exchanger, also for investigating the effect of column loading on the quality of the separation.

Technical pectic enzymes were chromatographed mainly on ion-exchange derivatives. However, in order to elucidate the effect of the matrix on the separation process, chromatography on Spheron 1000 alone, which was not ionogenically modified, was also performed. With Pectinex Ultra, experiments were carried out on Spheron 1000 and weakly, medium and strongly acidic cation exchangers and weakly and strongly basic anion exchangers. With Rohament P, separation experiments on non-modified Spheron 1000 were carried out and the possibility of eluting the column not only with aqueous solutions but also with a solution of *tert.*-butanol was also tested. Chromatography on a weakly and medium acidic cation exchanger was also carried out with two batches of Rohament P.

The chromatography of Pectinex on non-modified Spheron 1000 is illustrated in Fig. 1. Since this macroporous material has an exclusion limit of 1,000,000 daltons and the maximum of the pore-size distribution curve is at diameter 370 Å (ref. 13), we do not consider that the separation is a consequence of gel permeation but of hydrophobic chromatography on the matrix of this macroreticular polymer (*cf.*, ref. 26). However, we emphasize that the conditions used are not favourable for hydrophobic separation and were employed only for comparative purposes with ion-exchange chromatography. While all the esterase activity, S, remains in the hold-up volume and the peak of the pectin-lyase activity, L, is only slightly retarded, the *endo*-polygalacturonanase activity, N, appears in two peaks: one moves with the hold-up volume while the second is more retarded. Since two forms of this enzyme have already been described[5], their separation could have taken place here (in this case the material of the second peak, N, has more hydrophobic properties). However, without further characterization of the fractions the result may be interpreted assuming that the capacity of the column was insufficient for the retention of this most abundant enzyme and that a part of the material N was eluted with the void volume.

Pectinex Ultra gave two peaks of *endo*-polygalacturonanase activity, N, even on a weakly acidic cation exchanger (Fig. 2) at pH 3.5. The first peak is little and the second considerably retarded. However, pectin-lyase is strongly bound on the cation exchanger, while the asymmetry of the peaks may also indicate multiple forms of this enzyme. Replacement of formic acid by acetic acid in buffers of pH 3.5 was not appropriate since the required high concentration disturbed the enzymatic tests. Chromatography at pH 8 (concentration gradient of sodium acetate, not shown) led to the separation of pectin-lyase from the mixture of other enzymes.

The use of a medium acidic cation exchanger having a bivalent functional group for the chromatography of Pectinex Ultra is illustrated in Fig. 3. Although this type of ion exchanger has been found to be very suitable for the chromatography of proteins[12], under the given experimental conditions the separation was not satisfactory. We believe that a further systematic study of a larger number of milder gradients could result in an improvement of the separation even on this ion exchanger.

The chromatography of Pectinex Ultra on a strongly acidic cation exchanger

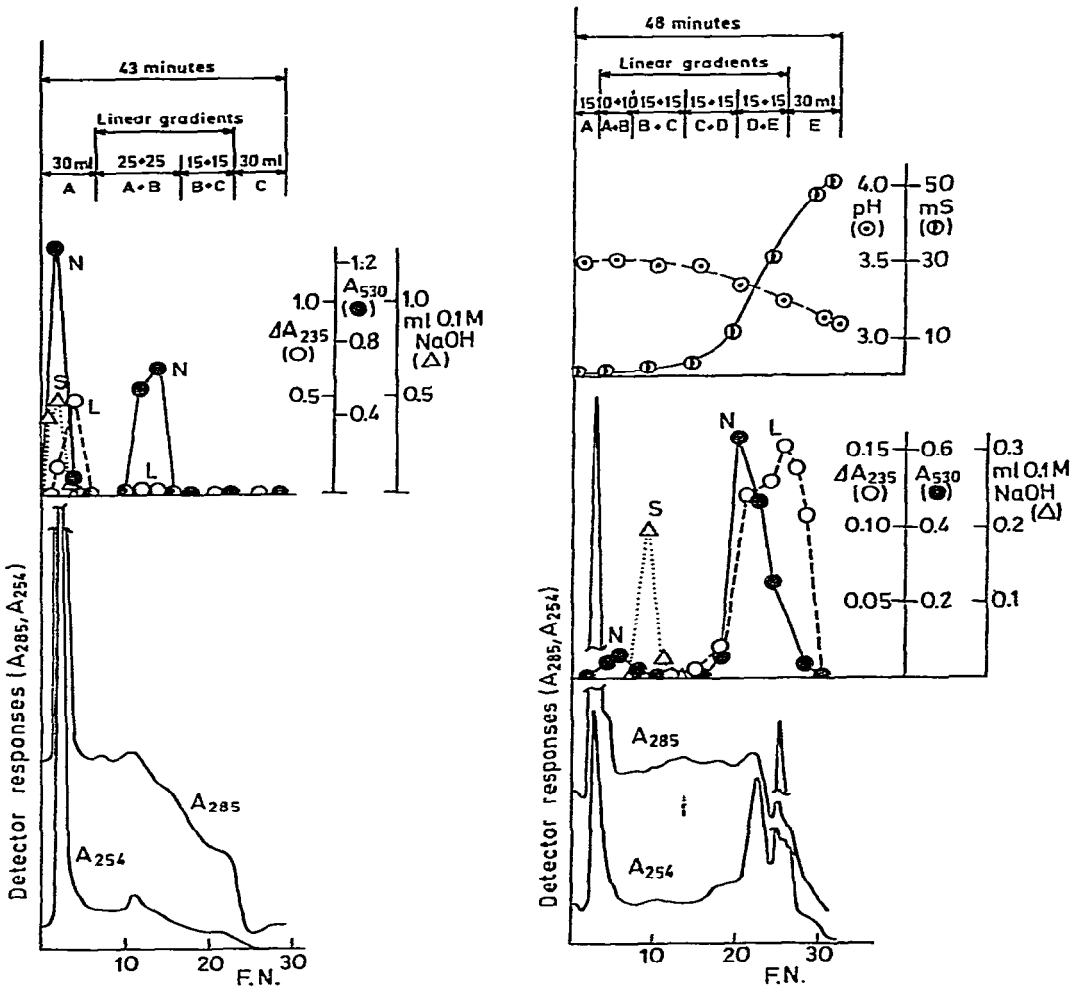

Fig. 1. Chromatography of 25 mg of Pectinex Ultra on unmodified Spheron 1000. Fractions 4.8 ml per 90 sec; pressure 0.4 MPa. Eluents: A, 0.05 *M* sodium hydroxide + acetic acid, pH 3.5. B, 1 *M* sodium chloride. C, 2 *M* sodium chloride. Enzymatic activities: L = pectin-lyase; N = *endo*-D-galacturonanase; S = pectin-esterase. For methods of determination and calculation of results see text. F.N. = fraction number.

Fig. 2. Chromatography of 25 mg of Pectinex Ultra on carboxylated cation exchanger Spheron C-1000. Fractions and pressure as in Fig. 1. The buffers were prepared from sodium hydroxide solutions of the indicated concentration, adjusted with formic acid to pH 3.5: A, 0.05 *M*; B, 0.1 *M*;C, 0.2 *M*; D, buffer C 1 *M* in sodium chloride; E, 2 *M* sodium chloride. Enzymatic activities as in Fig. 1. The pH values were determined using a glass electrode. mS = Electrical conductivity of the effluent in milliSiemens, as an illustration of the ionic strength value. F.N. = fraction number.

gave a satisfactory separation (Fig. 4). The process of separation of pectin-lyase activity, L, and esterase activity, S, into two peaks can be evaluated the same way as the two peaks in Fig. 1. However, this explanation cannot be used for the separation of *endo*-polygalacturonanases, N, because both peaks were retarded to different

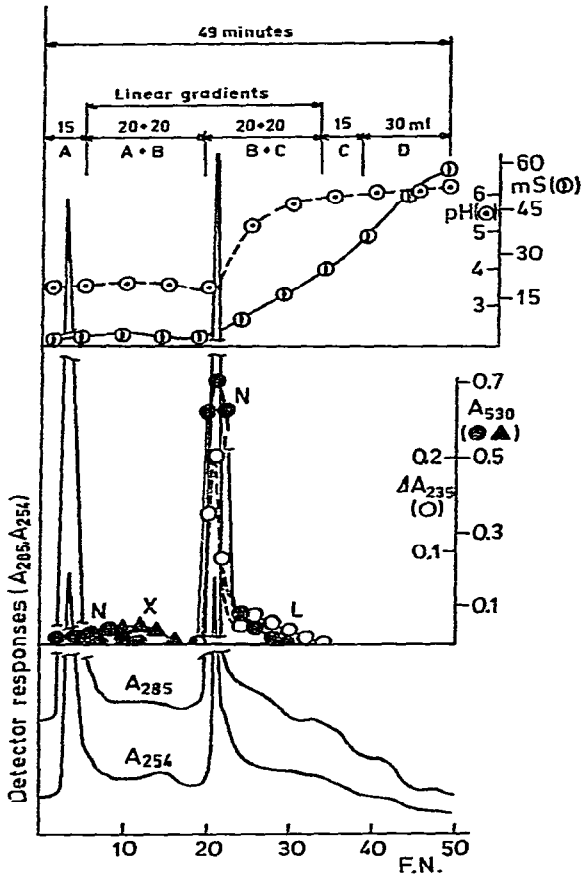

Fig. 3. Chromatography of 25 mg of Pectinex Ultra on Spheron Phosphate 1000. Fractions 2.8 ml per 1 min; pressure 0.3 MPa. The buffers were prepared from ammonium hydroxide of the indicated concentration, which was adjusted to the given pH value with formic acid in the case of buffer A, and with acetic acid in the case of the other buffers: A, 0.05 *M* (pH 3.5); B, 0.3 *M* (pH 6); C, 1 *M* (pH 8), 0.5 *M* in sodium chloride. Enzymatic activities as in Fig. 1, except X = *exo*-D-galacturonanase. F.N. = fraction number.

extents; the largest peak N was the most strongly bound on the strongly acidic cation exchanger.

A promising separation was achieved on a weakly basic anion exchanger (Fig. 5). Here the *endo*-polygalacturonanase activity, N, occurs in three peaks of which the first small peak (in the hold-up volume) may be due to exceeding the column capacity. The change from pH 7 to 5 (which would favour higher stability of *endo*-D-galacturonanase[4]) and the adjustment of gradients (Fig. 6) led to increased retardation of *endo*-polygalacturonanase, N, while the esterase, S, peak moved into the hold-up volume; this may be of great importance — see the Discussion. However, at this pH value of the elution buffer, the pH of the effluent varied. At a still lower pH (4.2–4.5, not illustrated) and by using acetate buffers, all the enzymes were eluted in a single fraction.

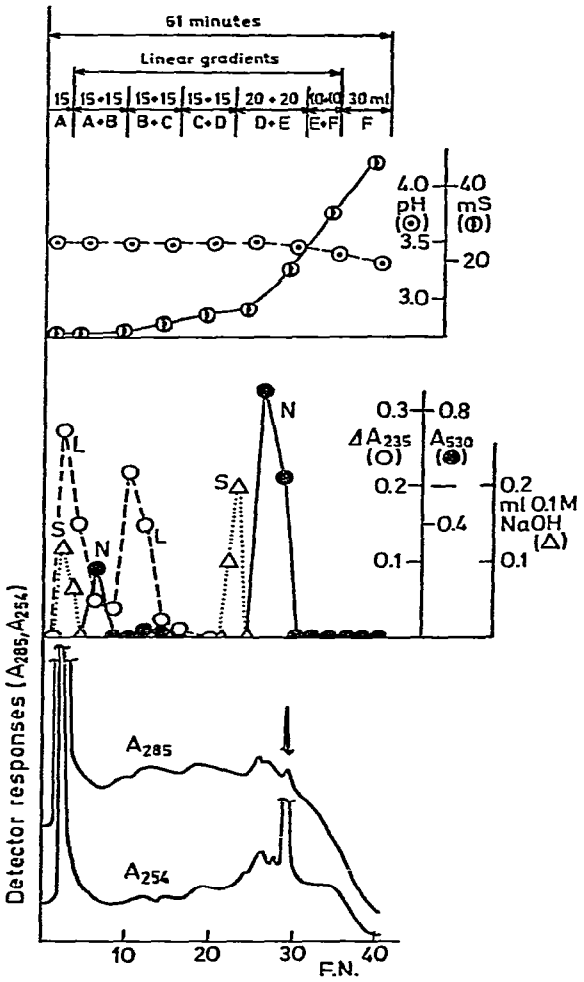

Fig. 4. Chromatography of 25 mg Pectinex Ultra on sulphonated cation exchanger Spheron S-1000. Fractions 4.7 ml per 90 sec; pressure 0.7 MPa. The buffers were prepared from sodium hydroxide of the indicated concentration, adjusted to pH 3.5 with formic acid: A, 0.05 *M*; B, 0.1 *M*; C, 0.2 *M*; D, 0.3 *M*; E, 0.5 *M*, 1 *M* in sodium chloride; F, 2 *M* sodium chloride. Enzymatic activities as in Fig. 1. F.N. = fraction number.

The importance of the adjustment of the gradient in ion-exchange chromatography, using buffers having the same pH value, is evident from the comparison of Fig. 7 with Fig. 5. In this experiment (Fig. 7) the separation of the activities of *endo*-polygalacturonanase, N (two peaks, both retarded), esterase, S, and pectin-lyase, L, was achieved. None of the analysed enzymes occurred in the hold-up volume, so that under these conditions the capacity of the column was not exceeded.

On a strongly basic anion exchanger (Fig. 8) the elution order was similar to that on the weakly basic anion exchanger (Fig. 7), but the separation was not improved. This may be due not only to the stronger basicity of the quaternized functional

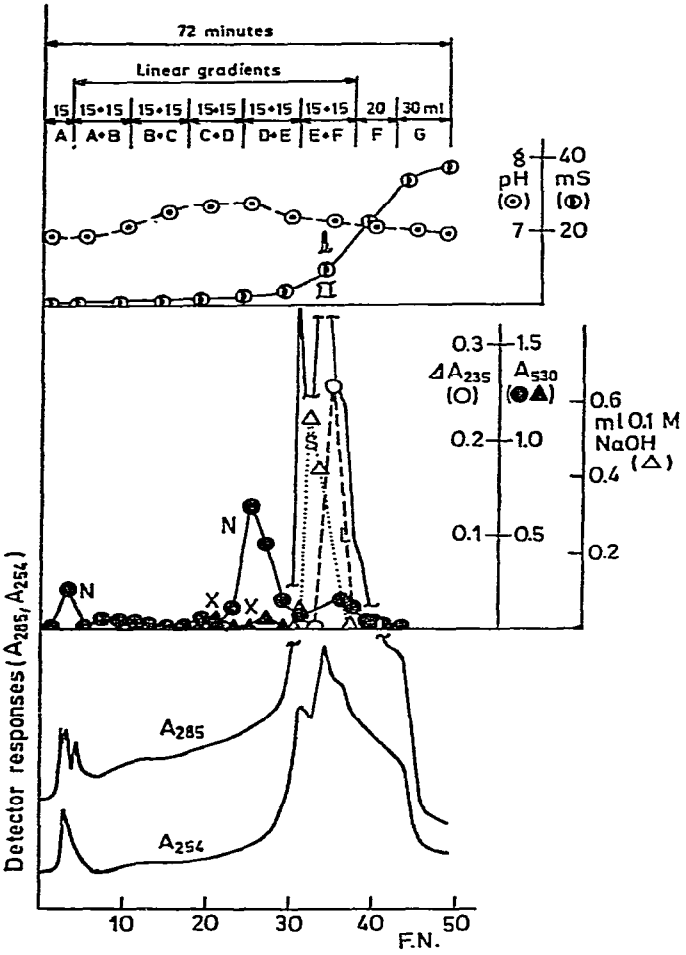

Fig. 5. Chromatography of 25 mg of Pectinex Ultra on the anion exchanger Spheron DEAE-1000, eluting with buffers of pH 7. Fractions 4.4 ml per 90 sec; pressure 0.4 MPa. The buffers were prepared from hydrochloric acid of the given concentration, which was adjusted with Tris to pH 7: A, 0.005 *M*; B, 0.05 *M*; C, 0.1 *M*; D, 0.2 *M*; E, 0.4 *M*; F, buffer E with 1 *M* sodium chloride; G, 0.2 *M* sodium chloride. Enzymatic activities as in Figs. 1 and 3. F.N. = fraction number.

groups, but also to the imperfect nature of the ion exchanger, which was developed as the last of the series of ion exchangers with classical functional groups.

Rohament P was chromatographed on non-modified Spheron 1000 (Fig. 9), first under conditions similar to those employed in the chromatography of Pectinex Ultra (Fig. 1). Whereas in the experiment in Fig. 1 all esterase activity, S, was eluted within the hold-up volume, in Fig. 9 it is considerably retarded. In both experiments (Figs. 1 and 9) the lyase activity, L, is retarded slightly more than the first peak of *endo*-polygalacturonanase activity, N, which is eluted within the hold-up volume. The lyase activity, L, does not belong to a single form of enzyme either in Rohament P (Fig. 9) or in Pectinex Ultra (Fig. 1). The experiment in Fig. 9 was also used to investigate the

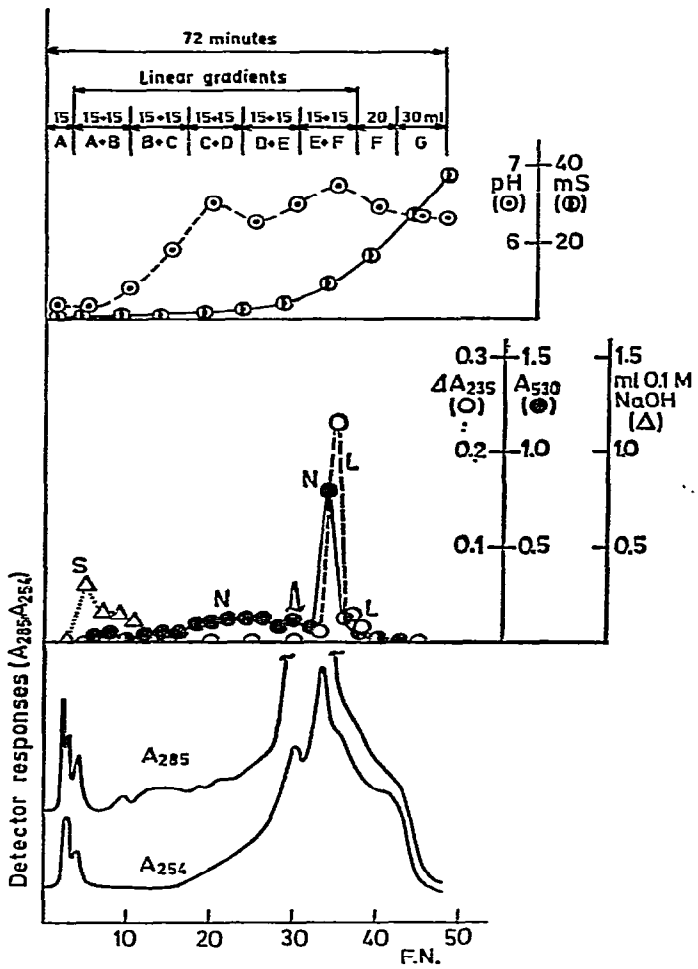

Fig. 6. Chromatography of 25 mg of Pectinex Ultra on Spheron DEAE-1000, on elution with buffers of pH 5. The buffers were prepared as in Fig. 5, but were adjusted to pH 5. Enzymatic activities as in Fig. 1. F.N. = fraction number.

elution of the column with organic solvents, with the aim of removing non-protein or coloured substances that are often present in technical enzyme preparations. Spheron and its ion-exchange derivatives are fairly resistant to organic solvents, but because of the photometric detection they must first be extracted with such solvents (prior to use) in order to eliminate contaminants. No enzyme activity was found in the peaks eluted with eluents containing *tert.*-butanol, even though it was shown in other experiments that some enzymes are stable for short periods in these dilute solutions of *tert.*-butanol. Aqueous solutions of *tert.*-butanol eluted only inactive components of the original preparations, which contained strong chromophores. This indicates the possibility of regenerating the packings by elimination of coloured material.

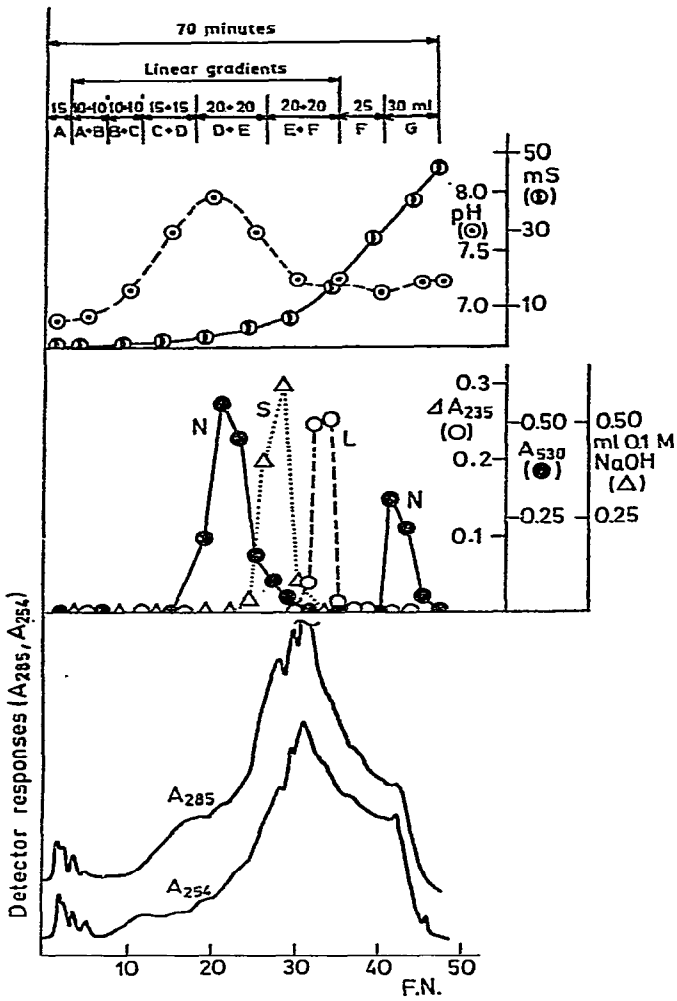

Fig. 7. Chromatography of 25 mg of Pectinex Ultra on Spheron DEAE-1000 with an adjusted gradient elution with buffers of pH 7. The buffers were prepared as in Fig. 5 and the labelling of enzymatic activities is also the same. Fractions 5.1 ml per 90 sec; pressure 0.6 MPa. F.N. = fraction number.

The different characteristics of some enzymes in the analysed preparations were demonstrated by the chromatography of Pectinex Ultra (Fig. 2) and Rohament P (Fig. 10) on a weakly acidic cation exchanger under the same conditions. For example, while pectin-lyase activity, L, is eluted last in Fig. 2, it is eluted first in Fig. 10. In contrast, the esterase activity, S, is eluted more easily from Pectinex Ultra (Fig. 2). A five-fold decrease in the column load did not lead to an improvement in the separation (Fig. 11) under identical conditions, from which it may be concluded that even a loading of 25 mg of pectic enzyme did not limit the separation ability of the column.

The chromatography of Rohament P on a medium acidic cation exchanger (Fig. 12) can be compared with the chromatography of Pectinex Ultra (Fig. 3). The

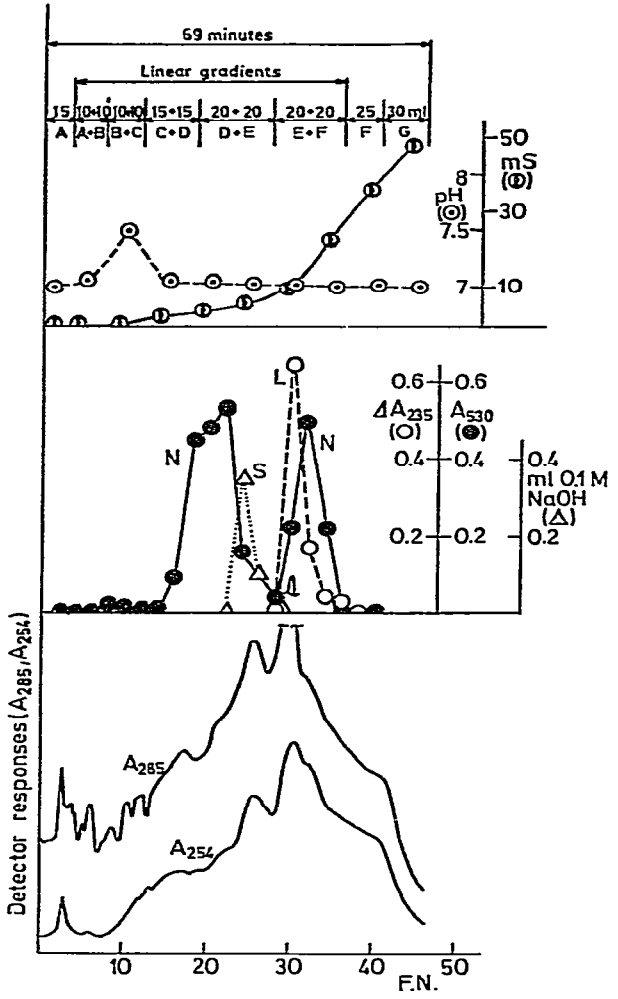

Fig. 8. Chromatography of 25 mg of Pectinex Ultra on strongly basic anion exchanger Spheron TEAE-1000. Fractions 4.6 ml per 90 sec; pressure 0.6 MPa. The buffers of pH 7 were identical to those in Fig. 5, and the labelling of enzymatic activities is also the same. F.N. = fraction number.

results achieved were similar, *i.e.*, both preparations contained small amounts of *exo*-polygalacturonanase, X. Similar comments to those on Fig. 3 can be made on the suitability of the phosphate ion exchangers for the separation.

DISCUSSION

The method described represents an advance upon previous methods of analysis of mixed commercial and technical preparations of pectic enzymes. Up to now the determination of the activity of individual enzymes in these preparations has been considerably complicated by the simultaneous effect of several enzymes on the same substrate, as a consequence of which the effect of the determined enzyme is

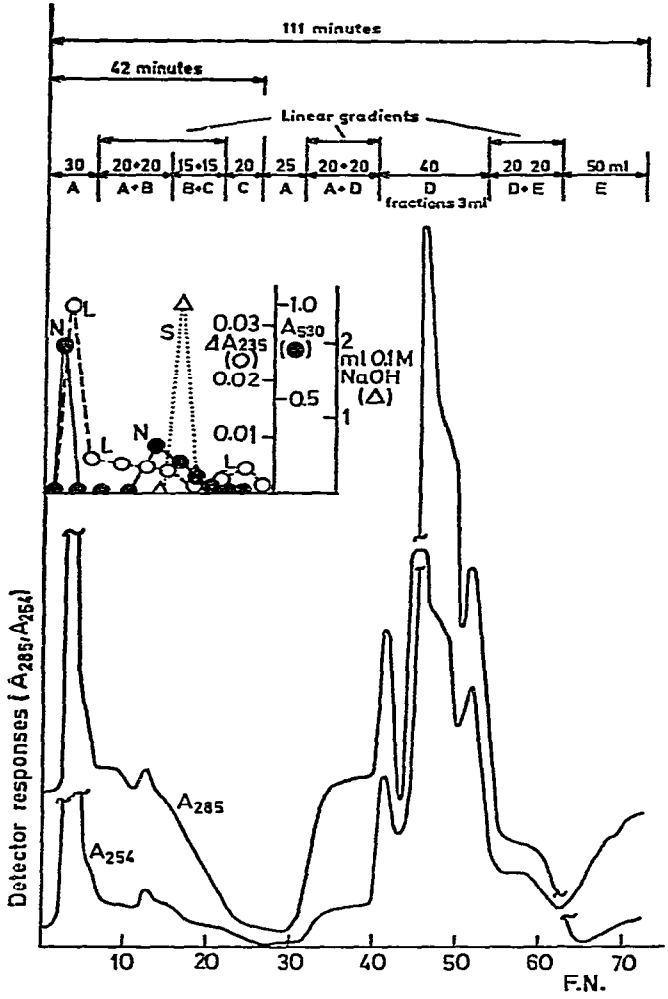

Fig. 9. Chromatography of 25 mg of Rohament P on unmodified Spheron 1000. Fractions 4.5 ml per 90 sec; pressure 0.5 MPa until the elution stage A+D. During the elution D microscopic bubbles appeared in the effluent, the fraction volume dropped to 3 ml and the pressure increased to 1 MPa. During the elution D+E the fraction volume and the pressure returned to normal. Eluents: A, 0.05 *M* sodium hydroxide adjusted to pH 3.5 with acetic acid; B, 1 *M* sodium chloride; C, 2 *M* sodium chloride; D, 50% *tert.*-butanol in buffer A; E, pure water. Enzymatic activities as in Fig. 1. F.N. = fraction number.

partly suppressed. This is true of all pectic enzymes with the exception of *exo*-D-galacturonanase, X, the activity of which can be determined by means of a specific substrate. A further obstacle to the accurate determination of the enzymatic activity consisted in the high content of coloured and reducing contaminants, which considerably decrease the accuracy of the spectrophotometric determination of the activity of pectin-lyase and D-galacturonanases. As a consequence the measured values of the activities were usually subject to an error the magnitude of which was dependent

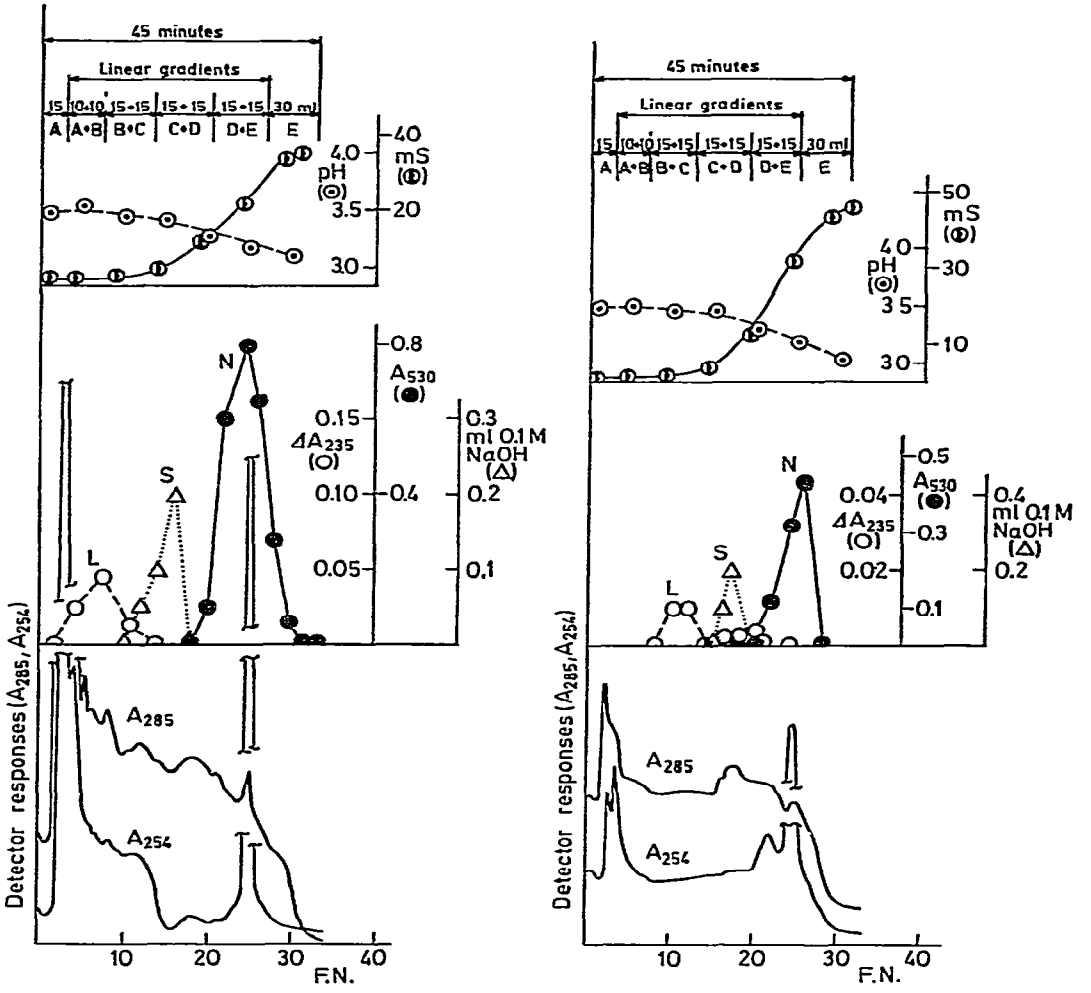

Fig. 10. Chromatography of 25 mg of Rohament P on carboxylated cation exchanger Spheron C-1000. Fractions 4.8 ml per 90 sec; pressure 0.4 MPa. The buffers were identical to those in Fig. 2. Enzymatic activities as in Fig. 1. F.N. = fraction number.

Fig. 11. Chromatography of 5 mg of Rohament P on Spheron C-1000. Fractions 4.7 ml per 90 sec. All other parameters as in Fig. 10. F.N. = fraction number.

on the relative proportions of the enzymes and the contaminants. HPLC on Spheron ion exchangers on an analytical scale, permitting a rapid separation of enzymes, enables the determination of the activity of isolated enzymes freed from contaminants and thus eliminates these drawbacks. The non-ionic reducing substances are often eluted within the hold-up volume. A further advantage of this method is the possibility of a rapid determination of the multiple forms of individual enzymes.

Experience gained from almost all the experiments carried out indicates that the evaluation of the effluent by both UV detectors is not sufficient even for a preliminary choice of separation conditions. Only a complete determination of all enzyme

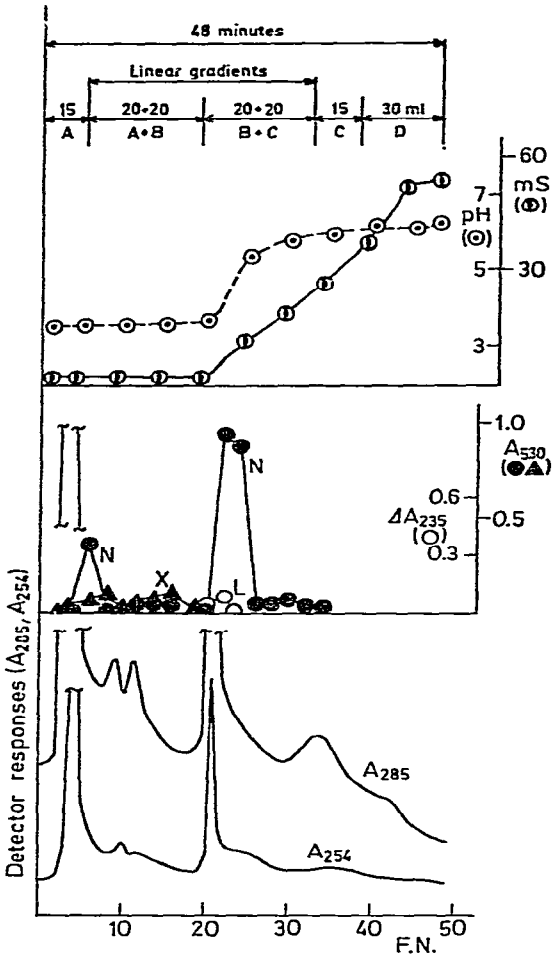

Fig. 12. Chromatography of 25 mg of Rohament P on Spheron Phosphate 1000. Fractions 2.9 ml per 1 min; pressure 0.4 MPa. Buffers A–D as in Fig. 3. Enzymatic activities as in Figs. 1 and 3. F.N. = fraction number.

activities in individual fractions afforded sufficient information for the adjustment of the gradients. Sometimes the peaks of activity occurred in regions where the UV-detectors recorded only a low absorbance, or peaks of high UV-absorbance were devoid of activity. The zones of the proteins are also evidently overlapped by the zones of other coloured contaminants.

The best separation of pectic enzymes was achieved on the anion exchanger Spheron DEAE-1000 with a concentration gradient of HCl–Tris buffer, pH 7.0 (Fig. 7), where both forms of *endo*-D-galacturonanase, N, were separated from Pectinex Ultra in addition to other individual pectic activities. The pectic enzymes from *Aspergillus niger* are acidic proteins with isoelectric points between p*I* 4.8 and 5.4 (ref. 27). At pH 7 they were retained on Spheron DEAE in 0.005 *M* buffer and were gradually eluted at higher concentrations of the buffer, beginning with 0.2 *M*,

in order of increasing acidity, *i.e.*, *endo*-D-galacturonanase I (N), pectin-esterase (S), pectin-lyase (L) and *endo*-D-galacturonanase II (N). The simple gradient which was used in ref. 16 (Fig. 5) for the chromatography of the technical pectic enzyme Leozym does not suffice for a good separation of all required enzymes.

The enzymes of the preparation Rohament behaved similarly on DEAE-Spheron (not illustrated). For their separation also the cation exchanger Spheron C-1000 (Fig. 10) was very suitable; the enzymes were eluted with sufficiently different elution volumes when the concentration gradient of the formate buffer, pH 3.5, was used, in the order pectin-lyase (L), pectin-esterase (S) and *endo*-D-galacturonanase (N). The results of the separation of two different batches of Rohament P on Spheron C-1000 also indicated the differences in the composition and the proportion of the enzymes of various production batches, two fractions with pectin-lyase activity (not illustrated) being found in one batch.

In the chromatography of Pectinex Ultra on the cation exchanger Spheron C-1000 in the formate buffer, pH 3.5 (concentration gradient 0.05–0.2 *M*), pectin-esterase, S, was very well separated from the mixture of enzymes, and was eluted before the other enzymes, with a small admixture of *endo*-polygalacturonanase, N (Fig. 2). On a preparative scale such a method can help to remove pectin-esterase from pectic multi-enzyme preparations. Enzyme of this specificity splits methanol from pectins and therefore it is undesirable in the foodstuff industry branches producing fruit juices and similar products. A similar separation was also achieved on the anion exchanger Spheron DEAE-1000, if the pH of the elution buffer was decreased to 5 (Fig. 6). On the strongly acidic cation exchanger Spheron S-1000 a good separation of the enzymes in the formate buffer, pH 3.5, was obtained (Fig. 4). Individual enzymes were eluted gradually from the cation exchanger, in the order of increasing basicity: *endo*-D-galacturonanase II (N), pectin-lyase (L), pectin-esterase (S) and *endo*-D-galacturonanase I (N).

The polymorphism of the pectic enzymes present in technical pectic preparations is clearly not limited to the two forms of *endo*-D-galacturonanase (N), I, II (Figs. 1–9 and 12). A more detailed inspection of Figs. 1–4 and 9 demonstrates (with the restrictions given in Results) the existence of several forms of lyases, L, separable on ion exchangers. The results of the experiments illustrated in Figs. 1 and 9 indicate that the esterases, S, present in Pectinex Ultra and Rohament P are different. Even the minor component, *exo*-polygalacturonanase, X, of the preparation Rohament P seems to be a composite, when chromatographed on Spheron Phosphate 1000 (Fig. 12). Various reasons for this polymorphism are: existence of genetically conditioned isoenzymes; effect of the limited proteolysis in the cultivation medium; topographic changes in conformers with deviations in the isoelectric points, etc. Chromatography on Spheron 1000 and its ion-exchange derivatives allows the possibility of studying these questions in greater detail. The aim of this paper was to study the chromatographic details not the biochemistry, however it has demonstrated that this method could be employed for such a biochemical study.

CONCLUSIONS

The experiments described in this paper have demonstrated the ability of Spheron ion exchangers in rapid chromatographic separation of pectic enzymes on

an analytical scale. The chemical stability of Spheron and its derivatives[12,13], the resistance to the effect of technical enzymes[16] and microbial infection, the independence of the bed volume on pH and ionic strength, the easy regeneration and possibility of repeated use indicate the potential of these ion exchangers for separations on a laboratory or technical scale. Preliminary experiments with technical protease have already been carried out (ref. 16, Fig. 2), where it was demonstrated that for reversible sorption of the technical enzyme the bed of the Spheron ion exchanger should have been a broad and low layer on a fritted filter, and desorption results in a peak similar in form to a chromatographic one, although not as perfect as those obtained on chromatographic columns.

The flow-rate for chromatography on Spheron and its ion-exchange derivatives was usually *ca.* 5 ml/cm^2·min, corresponding to *ca.* 30 l/min at a column diameter of 80 cm (using the medium pressures given in this paper). We believe that HPLC of technical enzymes can be carried out even on a semi-pilot plant scale, with short elution times.

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