JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preparative Continuous Annular Chromatography (P-CAC) Enables the Large-Scale Fractionation of Fructans

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Fructans (fructo-oligosaccharides and inulin) are of increasing physiological and nutritional interest due to their health-promoting effects. Fructans originally extracted from chicory roots were separated by continuous annular and fixed-bed conventional gel chromatography. Both columns were packed with Toyopearl HW 40 (S) and eluted with deionized water. A multicomponent fractionation was established to obtain single oligosaccharides in a low molecular weight range up to a chain length of five and fractions containing an overall size distribution in the high molecular weight range up to a chain length of 90 monosaccharide units. The productivity and resolution of the continuous annular size exclusion chromatograph (40 cm bed height) were investigated and compared with those of the fixed-bed counterpart (2 × 100 cm bed height). The eluting fractions were analyzed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The productivity of the annular system was found to be 25-fold higher than the conventional system. Thus, annular chromatography exemplified for the fractionation of fructans is a powerful method for the large-scale and continuous fractionation of oligomeric and polymeric carbohydrates.

KEYWORDS: Fructan; gel filtration; annular chromatography; HPAEC−PAD

INTRODUCTION

Fructans (inulin and oligofructose) are oligo- and polymeric fructose containing plant storage carbohydrates. Two kinds of fructans naturally exist, the GF_n-type and the F_n-type. The former are nonreducing carbohydrates composed of one glucose unit per molecule and are prolonged by several fructose moieties. The F_n-type fructans contain fructose as the only monosaccharide building unit and have a reducing end. They belong to the linear 1-kestose series, which is deduced from isokestose (β-2,1-bonds). The fructosyl residues are linked in β-6,2 bonds (*1*) if they are branched.

Chicory roots contain predominant fructans of the GF_n-type, only a small part of which consists of the F_n-type containing only β2-1 linkages between the fructose moieties. Furthermore, an additional third type of fructan was found: The core structure from this type is levanbiose [β-D-fructofuranosyl-(β2-6)-D-furctofuranose], which is possibly prolonged at the nonreducing residue by fructose in the β2-1 position (*2*).

The ratio of GF_n-type to F_n-type and their degree of polymerization depend on ripening and the storage of the chicory roots (*3*). Fructans are major storage carbohydrates in higher plants. A naturally high fructan accumulation in plants is often accompanied by frost hardening (*4*, *5*).

Inulin-type fructans are classified as prebiotics. They are resistant against human digestion and beneficially affect the host by selectively stimulating the growth of a limited number of bacterial species (bifidobacteria and lactobacilli) in the colon (*6*−*8*). It has been reported that short-chain fructans reduce the occurrence of colon tumors in mice by modulation of the colonic ecosystem and by decreased production of secondary bile acids. Oligofructans decrease serum triglycerides in rats. This is due to a reduction of the de novo fatty acid synthesis in liver (*9*). The systemic physiological effects on carbohydrate and lipid metabolism and mineral absorption and the potential application in risk reduction for some diseases are reviewed elsewhere (*9*−*11*).

The average daily intake of fructans for humans is estimated to be between 3 and 11 g in Europe and between 1 and 3 g in the United States. It is desirable to increase the intake to 15 g/day (*11*). The higher intake of fructans could be achieved on the one hand by increased consumption of plants with a naturally high fructan content such as wheat, barley, artichoke tubers, onions, and bananas or, on the other hand, by the supplementation of fructans as a food ingredient.

It could be shown that the kinetics of the physiological effect of naturally occurring fructans in man depends on the chain length of the molecules. Roberfroid et al. found a chain length dependent prebiotic effect of inulin (*10*). Especially long-chain inulin [degree of polymerization (DP) > 20] is hardly fermented in the proximal colon, enabling supply of prebiotic oligosac-

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10.1021/jf011576q CCC: $22.00
Published on Web 07/23/2002

charides to the more distal part of the colon with the possible benefit of a stronger anticarcinogenic effect. Therefore, a shift to longer fermentation time is related to a shift of location in the gut. Due to the location-dependent pattern of the microflora it is obvious that different microflora-dependent physiological effects will be found (e.g., pattern of short-chain fatty acids).

Different molecular weight oligosaccharide fractions are required in gram amounts for in vitro and in vivo investigation of these effects. Gel chromatography is a suitable method for this separation (*12*). Short-chain and long-chain fructans, respectively, have been recently produced by enzymatic hydrolysis with endoinulinase (*13*) or by enzymatic synthesis using sucrose as substrate and 1,2-β-fructan-1-fructosyltransferase (*5*). Traditional batch chromatography for the separation is limited by low throughput and low productivity relative to other separation processes. The separation of recombinant proteins by annular chromatography in comparison to fixed-bed batch chromatography was recently described (*14*, *15*). In this study, inulin-type fructan mixtures from natural sources were separated with continuous liquid annular chromatography into oligomers with different chain lengths. The aim of this study was to compare the fractionation of inulin by discontinuous batch chromatography with annular chromatography by using the same gel filtration stationary phases. With respect to the resolving power and the productivity both techniques were investigated.

PRINCIPLE OF PREPARATIVE CONTINUOUS ANNULAR CHROMATOGRAPHY (P-CAC)

Continuous annular chromatography introduced by Martin (*16*) is a potential and promising technique for the preparative separation of biomolecules and pharmaceuticals in the food and pharmaceutical industries. In contrast to simulated moving bed (*17*) based purification, where only two fractions—raffinate and extract—can be separated, P-CAC allows the recovery of more than two fractions (*18*). Giddings demonstrated theoretically how the rotating annular column can be superior to a fixed column of the same volume for process-scale applications (*19*). He recognized that many industrial packed tower operations exhibit nonuniformities in flow at large diameters, resulting in an increased plate height and loss of resolution. By using a rotating column with the same total cross-sectional area and bed height as a fixed column, but with an annulus size small enough so that flow nonuniformities do not occur, a process can be scaled up without loss of resolution. This means that all other parameters being equal, the geometry of a rotating bed with a small annulus should have a larger number of stages, resulting in better resolution. He also pointed out that process control might be easier in continuous operation, because it results from the column geometry and not from careful timing of feed injection and product withdrawal as in a simulated continuous operation. Due to its really continuous character the P-CAC technology features a very high throughput compared to traditional batch chromatographic separations.

Figure 1 shows a schematic drawing of a P-CAC apparatus. The apparatus consists of two concentric cylinders that form an annulus into which the stationary phase is packed. The annular bed slowly rotates about its vertical axis. The feed mixture to be separated is introduced continuously at the top of the moving bed by a stationary inlet while the rest of the annulus is flooded with elution buffer. As time progresses, helical component bands develop from the feed point, with slopes dependent upon elution velocity, rotational speed, and the distribution coefficient of the component between the fluid and adsorbent phase. At steady state, the component bands form regular helices between the feedsector at the top of the bed and the individual fixed exit points at the bottom of the annular bed, where the separated components are continuously recovered. As long as conditions remain constant, the angular displacement of each component band from the feed point also remains constant.

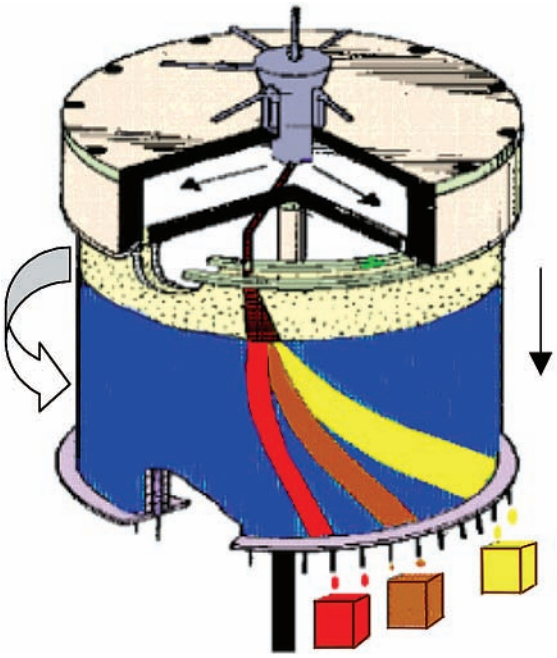

Figure 1. Schematic diagram of the principle of preparative continuous annular chromatography (P-CAC). The arrows indicate the eluent flow direction and the direction of the solid phase movement, respectively. For details, see the Introduction.

The separation behavior of the P-CAC can be simulated from the theoretical parameters derived from small batch chromatography experiments. The theoretical background of annular chromatography is described in detail, elsewhere (*16*, *18*, *19*).

MATERIALS AND METHODS

Samples. Fructans (Raftiline ST) were from Orafti (Tienen, Belgium). Inulin was extracted from chicory roots by hot water, purified by filtration, and partially hydrolyzed by using an endo-inulinase. After sterile filtration, the product was spray-dried. The total number of monosaccharide units ranges mainly between 2 and 60. Most of the oligosaccharide structures (>97%) were terminated by a glucose unit (GF_n-type). The sucrose content in the sample used was <8%. Fructans (250 mg) were dissolved in 1 mL of deionized water at 70 °C with thorough stirring. Prior to gel filtration the solution was microfiltered by using a 0.2 μm filter (Whatman, Göttingen, Germany). For reference, maltodextrins (Glucidex 12, Roquette Freres, Lestrem, France) and galacto-oligosaccharides (Borculo, Zwolle, Netherlands) were used.

Discontinuous Gel Filtration. The oligosaccharides were separated by gel filtration on Toyopearl HW 40 (S) (Tosoh Biosep, Stuttgart, Germany) jacketed columns [2 × (5 × 110 cm)] connected in series and equilibrated with deionized water. The total gel volume was 4317 mL. During operation, the columns were maintained at 55 °C. Fructans (250 g/L) were applied to the column in a sample volume of 5 mL. The flow rate was 1.65 mL/min. Dextrans (T1 and T5; Pharmacia, Uppsala, Sweden) were used as reference oligomers to calibrate the system for an estimation of the DP, because these oligosaccharides are elongated by β1-6 linkages in linear fashion without branches leading to clearly resolved chromatograms. The concentration of T1 and T5 dextran oligosaccharides has a maximum at 1000 and 5000 Da, respectively. The oligosaccharides used were monitored by refractive index (RI) detection (Knauer, Berlin, Germany).

Preparative Continuous Annular Chromatography. On the basis of the chromatogram and the operating parameters from the fixed-bed chromatography, the separation parameters for the runs in the lab-P-CAC (Prior Separation Technology GmbH, Götzis, Austria) were chosen. The configuration of the lab-P-CAC used during the studies was the following: The outer cylinder of the annular column had an inner diameter of 15 cm. Together with the inner cylinder, which had a diameter of 13 cm, the annulus width was 1 cm. In addition, the thermostatic jacket of the inner cylinder was used to keep the temperature of the column constant at 60 °C during operation (Lauda RE 107, Königshofen, Germany). At the bottom of the unit the two cylinders are attached to a stainless steel plate, which contains 90 exit holes covered by a nylon filter (14 μm). The bottom plate of the column is connected to a stationary slip ring via a glide ring device. The glide ring itself contains 90 exit ports each connected to a short section of flexible tubing. The exit ports are evenly distributed at 90 intervals along the column annulus. During the experiment the feed stream was pumped to the top of the gel bed through a fixed feed nozzle, the tip of which was located within a layer of glass beads (150−250 μm), while the eluent (deionized water) was pumped to the column through a central inlet port, thus flooding the entire annulus. The column used for the studies was packed with the same size exclusion resin [Toyopearl HW 40 (S)] as used for fixed-bed gel chromatography. The final bed height of the annular column was 40 cm. The total gel volume of the column was therefore 1760 mL.

Packing and Operating Procedure of P-CAC. A 50% resin slurry was prepared. The column was packed by pumping the slurry into the column through a feed nozzle using a peristaltic pump (Ismatec MCP, ISM404, Wertheim, Germany) and simultaneously compressing the resin bed by pumping eluent to the column through the central inlet port. The gel bed was compressed for 45 min at a pressure rate of 3.5 bar. The bed was then manually overlaid with a 3 cm high layer of glass beads.

For the preparation of the feed sample 100 g of fructans (Raftiline ST) was dissolved in 1 L of deionized water at 60 °C. The solution was microfiltered by using a 0.22 μm filter (Whatmannn,Göttingen, Germany) and then used as a feed solution for the P-CAC experiments.

Feed and eluent were applied to the column by piston rotation pumps (Kronlab VP120, Kronlab, Sinsheim, Germany). Feed and eluent solutions were preheated to 60 °C in an external water bath (Haake, Pfaffenhofen, Germany). The operating parameters for the P-CAC were as follows: eluent flow rate, 15 mL/min; feed flow rate, 0.2 mL/min; column rotation rate, 120°/h. After 3 h, equilibration and steady state time of continuous operation samples were collected for every outlet and collected for 5 h. Therefore, a total of 90 different fractions, each ~50 mL, were obtained for each run. Each collected sample represented the effluent of a 4° sector. The refractive index for each fraction was measured, giving a rough estimation for the carbohydrate concentration within the sample. Aliquots of 1 mL were then used for high-pH anion-exchange chromatography (HPAEC) analyses.

HPAEC with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC analyses were performed on a DX-300 Bio-LC-system (Dionex, Idstein, Germany) with a pulsed electrochemical detector (PED 2, Dionex). Aliquots of 25 μL of samples were loaded on a CarboPac PA-100 (Dionex) pellicular anion-exchange column (4 × 250 mm) equipped with a guard column (4 × 50 mm) and separated at a flow rate of 1 mL/min. The temperature was 21 °C. The concentration of the oligosaccharide fractions applied was 1−2 g/L. The oligosaccharides were analyzed using modified gradient conditions as described previously (*20*).

RESULTS AND DISCUSSION

In **Figure 2** is shown the HPAEC elution profile of the total starting material of fructans. The main peaks correspond to the oligomers of the GF_n-type, the adjacent small signals between DP 3 and 8 and between DP 13 and 18 correspond to structures containing only fructose as monosaccharide unit according to the determination of the HPAEC elution order already made for chicory fructans (*2*).

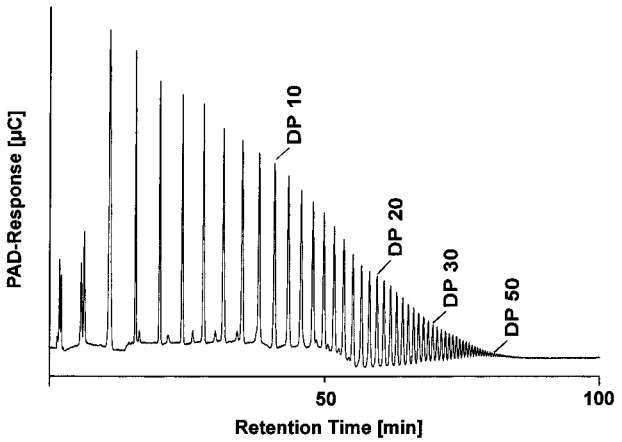

Figure 2. HPAEC elution chromatogram of the total fructan fraction. Abbreviations: DP, degree of polymerization; PAD, pulsed amperometric detection; μC, microcolumb.

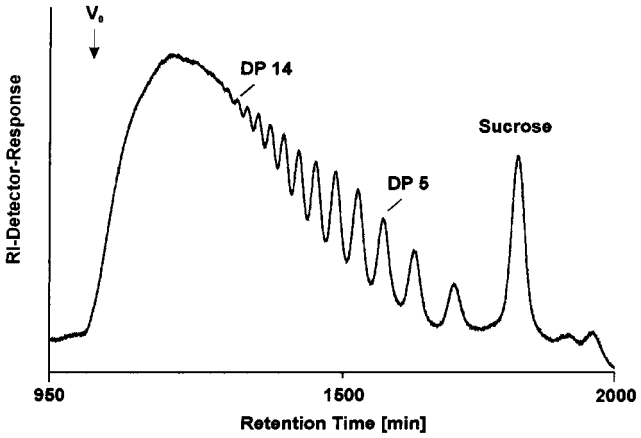

Figure 3. Fixed-bed gel chromatogram of the total fructan fraction on a Toyopearl HW 40 (S) column. The elution was monitored by refractive index (RI) detection. Abbreviations: DP, degree of polymerization; V_0, void volume.

Inulin-type fructans were first separated by conventional gel filtration on a Toyopearl HW 40 (S) column. The obtained retention times of distinct oligomers and substances that show no retention served as a starting point for the determination of suitable operating parameters for the annular column. The corresponding chromatogram is shown in **Figure 3**, and the predominant main signal is apparently at the elution position of sucrose. The relative mass response factors for each oligosaccharide are identical, and therefore this detection is suitable to show the quantitative overall size distribution of the oligosaccharides on the basis of their concentration in the analyzed sample. In contrast to RI detection, the electrochemical detection response of the HPAEC depends besides on other factors such as the chain length of the oligosaccharides. The higher the chain length, the lower the resulting PAD signal. Neighboring signals as a result of homooligomeric structures have similar PAD responses (*21*). For detailed quantitative analyses distinct structures must be isolated, allowing the determination of the respective response factor for each structure. The HPAEC analysis of the total fraction resulted in reverse peak area intensities in comparison to gel filtration. Up to a chain length of 14 monomers the oligomers were partly resolved (**Figure 3**) by gel filtration. Significant amounts of higher oligosaccharides were detected. In addition to the retention time resulting from the conventional Toyopearl HW 40 (S) column, HPAEC analyses have been used to determine the DP and the content

of positional isomers. HPAEC-PAD has been found to be a very efficient technique to separate either homooligomeric and positional and linkage isomers of oligosaccharides and glycopetides (*22*). The resolving capacity of the resin for gel filtration for uncharged and noncyclic carbohydrates ranges between 100 and 7000 Da. This was proven by applying oligomeric maltodextrins, dextrans, galacto- and fructans, and human milk oligosaccharides (*12*) and subsequent matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analyses of distinct fractions (*12*). Although oligosaccharides of 2500 Da molecular weight and above could not be resolved to a single peak for each different oligomer by Toyopearl gel filtration, fractions with an overall size distribution were obtained. The maximum of intensity shifted from chromatogram to chromatogram to the next higher oligosaccharide. This could be shown by HPAEC analyses of the subsequent eluted fractions. Each subsequent eluted fraction has shown slightly differences in their quantitative distribution of the oligomers. The relative PAD response has already been determined for inulin-type oligosaccharides from DP 2 to 17 (*21*); the differences in sensitivity from DP 2 to 8 decreased to a great extent, whereas from DP 11 to 17 the sensitivity decreased only slightly (*21*). On the basis of these results, it could be concluded for this study that the differences in PAD response for even higher chain length (DP > 17) are also slight, whereby neighboring signals in the high molecular weight range have nearly the same response.

If the column length, stationary phase, and eluent are given, four operating parameters for P-CAC can be varied: eluent flow rate, rotation rate, feed concentration, and feed flow rate. On the basis of the optimized fixed-bed chromatography conditions, the operating parameters for the P-CAC were chosen. There are two limitations for annular chromatography: maximal pressure (7 bar) and maximal temperature of the column gel material (60 °C).

Figure 4 shows the HPAEC chromatograms of three different fractions resulting after annular gel filtration. **Figure 4A** corresponds to the effluent taken at 272°: fructans with DPs between 3 and 9 were detected with a maximum intensity at DP 5. In addition, main peak signals with lower intensities corresponding to F_n-type oligosaccharides and/or with nonlinear structure were detected (DP 6 and 7). Nonlinear oligosaccharides are the result of side reactions of the fructosyltransferase, which occur during the synthesis of fructans in the chicory.

The resistance of fructans to sodium hydroxide eluent during HPAEC-PAD was high. This was proven by isolated low molecular weight fructans and subsequent analyses. The oligosaccharide structures remain intact during HPAEC in contrast to MALDI-MS analyses, when these oligosaccharides tend to fragment resulting in false oligosaccharide distributions. Fructans were found to be acid labile, especially at elevated temperature (personal communication, Orafti), and most of the matrices used in MALDI-MS are acids.

Figure 4B depicts the analysis of the fraction taken at 212°. Although the fraction volume was equal to that of 272°, the distribution of peaks between DP 15 and DP 50 was much more heterogeneous than this fraction. Signals could not be observed in the low molecular weight range or at the retention time up to DP 50 and above. In the last chromatogram of this series (176°, **Figure 4C**) oligosaccharides between DP 25 and 90 were observed. The resolution depends on the gradient conditions to a lesser extent than in the lower molecular weight range. In contrast to the total fructan fraction (**Figure 2**) the high molecular weight oligosaccharides were separated well. This is due to the weak PAD response of the higher oligosaccharides

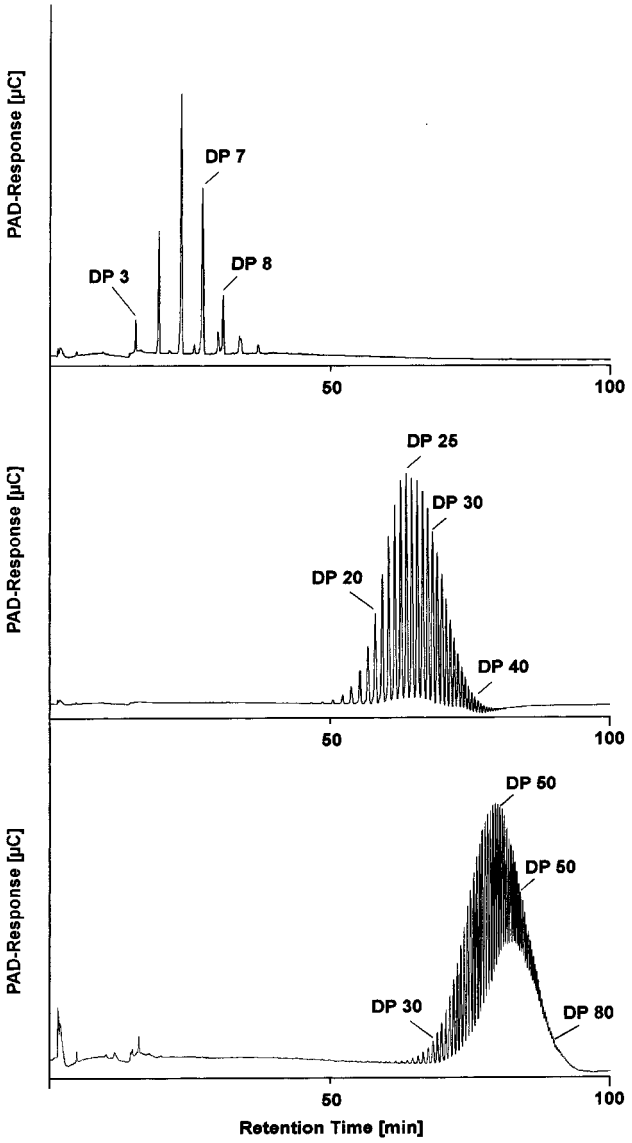

Figure 4. HPAE chromatograms of fructan fractions separated by annular gel chromatography on a Toyopearl HW 40 (S) column taken at different degrees at outlets: (A, top) 272°; (B, middle) 212°; (C, bottom) 176°. Abbreviations: DP, degree of polymerization; PAD, pulsed amperometric detection; μC, microcolumb.

eluted between 80 and 90 min. The occurrence of such oligosaccharides in this molecular weight range could be shown by HPAEC only by removing the lower oligosaccharides. The maximum DP of the fructan oligosaccharides used in this study was a chain length of 90 as determined by HPAEC-PAD. The total number of peaks increases with decreasing retention on Toyopearl HW 40 (S) gel. This is due to the fractionation range and the void volume of 7000 Da as determined by dextran oligomers. Fructans with a chain length of 90 monosaccharides have an average molecular weight of 14611 Da. Nevertheless, the subsequently eluted fractions in this high molecular weight range differ with regard to the maximum of intensity.

In the past, continuous annular chromatography has been used to separate sugars mainly by using ligand-exchange resins (Ca^{2+}-form) (*23*−*25*) into three or four compounds, respectively. Fructose, glucose, and sucrose are very well suited for scale-up and optimization studies; however, for multicomponent separation of carbohydrates it is advisable to use nearly homooligomeric oligosaccharides such as inulin-type fructans,

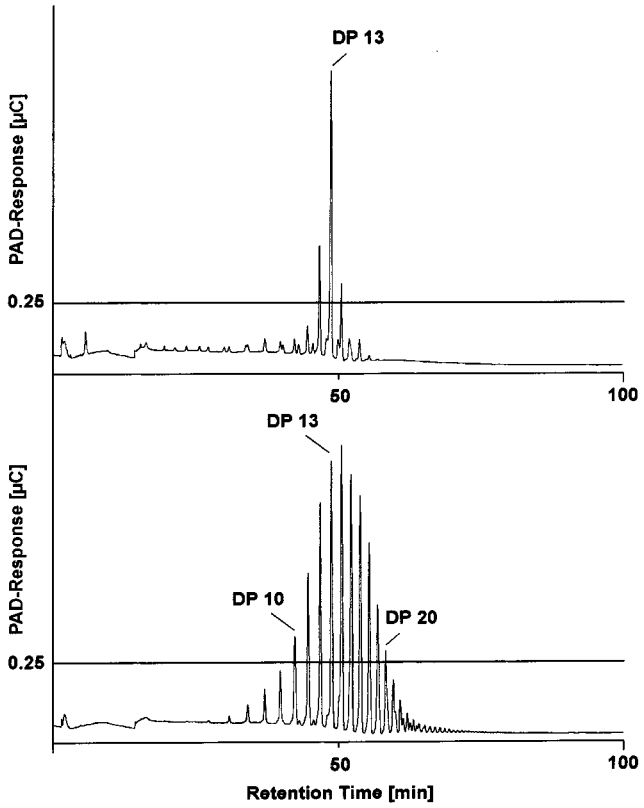

Figure 5. HPAE chromatograms of fructan fractions separated by (A, top) fixed-bed conventional gel chromatography and (B, bottom) annular gel chromatography on a Toyopearl HW 40 (S) column. Abbreviations: DP, degree of polymerization; PAD, pulsed amperometric detection; μC, microcolumb.

showing the potency of the resolving performance in comparison to discontinuous chromatography.

The resolutions of continuous and discontinuous gel chromatographies were compared by collecting the same volume of fractions and analyzing them by HPAEC. Those chromatograms showing the highest peak intensity for a distinct degree of oligomerization have been compared on the basis of the number and intensities of the adjacent peaks. All signals >0.25 μColumb were considered. As an example, **Figure 5** shows the HPAE chromatograms of the respective fractions. The fraction from discontinuous gel chromatography displays 2 neighboring signals, corresponding to DP 12 and 14, whereas the corresponding fraction from the continuous separation shows 10 signals. The same holds for further fractions with the exception of the high molecular weight area (DP 40 and above), where only slight differences were observed. The fixed-bed chromatographic separation of fructans offers a higher resolution than the continuous column. The resolution of these substances with the fixed-bed column was sufficient to isolate single oligomers up to a chain length of 10 monomers. Higher oligosaccharides resulting from both annular and fixed-bed chromatography were obtained only in fractions with an overall size distribution. Considering a 5-fold longer fixed-bed column in comparison to the annular system, a higher resolving capacity for the discontinuous gel chromatography is not surprising.

To assess the influence of the rotation rate for the resolution of fructan oligomers, two different parameters have been tested: 180 and 240°/h. Ninety fractions were collected at 4° intervals around the P-CAC annulus. The refractive index of each fraction was measured to estimate the concentration. We have analyzed aliquots of this fraction by HPAEC to compare the size distribution of the first with those of the second series. We could not find an influence of the rotation rate on the resolution, although reducing the rotation rate corresponds to an increased feed loading. This is due to the fact that we are not limited in the feed loading. A feed concentration of 100 g/L is much less than the maximum feed solubility at 60 °C. Higher feed concentrations cause the angular velocity profile to become unstable. Eluent breaks through the viscous feed layer and projects downstream, whereas the viscous feed sample is delayed. This results in a peak broadening and has been reported for gel permeation chromatography carried out at high feed loading (*24*).

Discontinuous conventional gel chromatography was compared with continuous annular chromatography on the basis of productivity criteria. Both of the systems used have not been optimized with regard to their maximal feed loading capacity. Nevertheless, the productivity of the annular gel filtration was ~25-fold higher than conventional gel filtration per unit volume of resin. At least 14 g of fructans was separated per 24 h into 24 different fractions. During continuous operation only monitoring of the eluted substances is required to prove stable separation conditions. For this a UV−vis on-line detection system is available for all 90 outlets.

The column length of the continuous annular column is so far limited to 60 cm in the system used. A longer column in the laboratory-scale P-CAC would be mechanically unstable. The advantage is a reduced contact time of the oligosaccharides to the stationary phase during separation in comparison to the fixed-bed conventional gel chromatography.

Transfer of a separation task from an existing approach from discontinuous batch to annular chromatography has to be fast, easy, and reliable. For batch chromatography several simulation tools have been designed to establish and optimize a given separation task on the basis of a few main parameters. These theories have been extended to annular chromatography to simulate the separation based on parameters derived from small batch chromatography experiments. The theoretical equations used in this work for transfer of the process to the P-CAC are outlined in the Introduction. For practical purposes the use of software is recommended for the determination of the P-CAC process parameter.

It could be shown that annular gel chromatography is a suitable technique for the continuous multicomponent separation of carbohydrates. Fructans were separated, yielding >20 different fractions at a gram scale showing the ability to separate multicomponent mixtures. In comparison to discontinuous gel chromatography a higher productivity was obtained, but the resolving performance is slightly better for conventional gel filtration under the conditions used. In addition to gel filtration, annular chromatography can be performed in ion-exchange (*25*), reversed phase, and affinity chromatography (*26*, *27*) modes. The choice of the operating parameters is straightforward if retention data are available from discontinuous chromatography.

The continuous annular chromatography experiments in this study, which were performed under isocratic conditions, show also the applicability for multicomponent oligosaccharide mixtures. The oligosaccharide fractions isolated in this study can be used in experiments to investigate the physiological effect of different chain lengths, for example, on the growth characteristic of various strains of bacteria by animal and in vitro studies.

In the expanding areas of biotechnology there are many opportunities where annular chromatography could be advantageously applied. In contrast to simulated moving bed chro-

matography, an alternative to the continuous working chromatographic method, this technique is not limited to isocratic applications. Stepwise elution and displacement chromatography are also feasible. By using a displacer in the eluent, which shows a higher affinity to the stationary phase than the feed components, the desired components are enriched, resulting in savings for eluent and their following preconcentration steps (*28*).

ACKNOWLEDGMENT

We thank Beate Mueller-Werner for excellent technical assistance in performing the HPAEC analyses.

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Received for review November 30, 2001. Revised manuscript received May 7, 2002. Accepted May 16, 2002. Financial support from the Bundesministerium für Bildung und Forschung under Grant 0311827A/B/C is gratefully acknowledged.

JF011576Q