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Journal of Chromatography A, 763 (1997) 331–335

JOURNAL OF CHROMATOGRAPHY A

Short communication

# Coupled size-exclusion chromatography–anion-exchange chromatography in the analysis of poly- and oligosaccharides

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**Abstract**

The use of anion-exchange chromatography (AEX) in the analysis of oligosaccharides is hampered by the fact that more complicated samples result in a jungle of peaks, which are hard to interpret. In two-dimensional chromatography with a combination of size-exclusion chromatography and AEX, the interpretation is facilitated by the use of hydrodynamic volume data along with AEX data. Therefore, the highly branched and linear gluco-oligosaccharides may be easily distinguished, as well as linear gluco-oligosaccharides with different bonds. Two-dimensional chromatography also provides a simple and reliable method to avoid interferences from buffer components or from major peaks or even from overwhelming concentrations of larger polysaccharides.

*Keywords:* Saccharides; Oligosaccharides; Polysaccharides

## 1. Introduction

High-performance anion-exchange chromatography combined with pulsed amperometric detection has proved to be a very valuable tool in the analysis of mono- and oligosaccharides. With regard to the application of this technique, more than hundred references dealing with the separation of carbohydrate oligomers could be found. Very few of these are, however, concerned with the separation of glucose oligomers.

Koizumi et al. [1] studied the basic relationship between the retention of various (1–2)-, (1–3)-, (1–4)- and (1–6)-linked homogeneous D-gluco-oligosaccharides. They showed that the type and conformation of the linkage between monomeric units is the primary factor determining the retention, besides the degree of polymerization (DP). They later used this method to estimate the chain length of different amylopectins [2] after enzymatic hydrolysis. Ammeraal et al. [3] widened the applicability of this method to slightly branched glucosyl- and maltosyloligosaccharides. The methods of analysis of starch hydrolysis products in ruminal digesta have been published by Bahrsuhn and Kotarski [4].

In spite of these works, some basic problems still remain in the application of this technique. In the study of the early stages of various hydrolytic reactions, most of the polymeric material will still have a high molecular mass. As the limited solubility of various larger dextrins requires the use of alkaline solvents, most of the polymeric material will also, unavoidably, be dissolved in the injection solution and will stick to the column. As the detector sensitivity decreases with an increasing DP, the determination of longer oligomers will require injection of a more concentrated solution, which will have a negative effect on column performance. The more complicated sample matrix will affect the stability of retention times or require sample clean-up, which may have a negative effect on recoveries of various dextrins [4]. Also, the use of mixtures of


*PII* S0021-9673(96)00996-X

different enzymes will result in a mixture of reaction products that is hard to resolve and interpret.

In order to overcome some of these problems, the application of two-dimensional chromatography is reported in this study. The first step is aqueous size-exclusion chromatography (SEC) with an alkaline eluent, which can separate the sample components according to their hydrodynamic volume. As this provides some data on molecules, it also releases interesting components from high-molecular-mass polymeric material and from the buffer's components. From consecutive injections of samples, the fractions that cover the retention range of interesting components are then fed into a second chromatographic step consisting of anion-exchange chromatography with pulsed amperometric detection (PAD).

## 2. Experimental

### 2.1. Instrumentation

The SEC chromatographic system (Fig. 1) used in this study consisted of an M-6000A pump, an M-712 automatic injector and μHydrogel 250 and 120 columns (300×7.8 mm) at 40°C. The eluent was 10 m*M* NaOH at a flow-rate of 0.5 ml/min. The volume of the injected samples was 100 μl and the samples were dissolved in either 1 or 0.1 *M* NaOH on the basis of sample dissolution. When the system was used as a SEC system for the determination of molecular masses, a dual angle laser light scattering detector (PDI 2000, Precision detectors, Adherst, MA, USA) was used as the detector with ~5000 mg/l sample concentration with 100 μl injections for the determination of molecular masses of branched amylopectin fractions. For the determination of detector responses, the same samples (with a concentration of ~500 mg/l) were injected as 100 μl injection for the determination of response for refractive index detection and as 10 μl injections to determine the response for PAD.

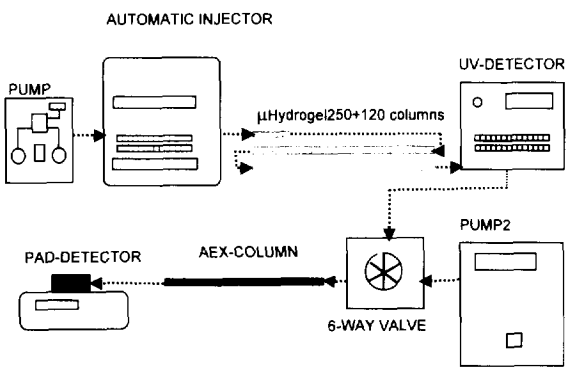


Fig. 1. Instrumentation for two-dimensional chromatography.

When the SEC system was used as the first step for two-dimensional chromatography, an M-440 UV detector, monitoring at 254 nm, was used to confirm the constancy of retention times. The eluent from the first chromatographic step was delivered through a pressure-activated automatic switching valve, as 200 μl injections into the second step consisting of an M-616 pump and a PA-100 anion-exchange column (300×4.6 mm) at 30°C. An M-464 pulsed amperometric detector (applied potentials: E1=0.10 V for 0.3 s, E2=0.6 V for 0.1 s and E3=−0.80 V for 0.3 s) was initially employed but it was later replaced by an ED40 pulsed amperometric detector (applied potentials: E1=0.05 V for 0.4 s, E2=0.75 V for 0.2 s and E3=−0.15 V for 0.4 s) (both the PA-100 column and the PED detector were from Dionex, Sunnyvale, CA, USA). The eluents for the second step were 100 m*M* NaOH (A) and 100 m*M* NaOH containing 400 m*M* sodium acetate (B) [5]. The eluent was changed from A to B over 60 min, using a linear gradient after a 10-min isocratic step. The other parts of the instrumentation were supplied by Waters (Milford, MA, USA) and the whole system was controlled and data were treated with a Millenium work-station. All of the eluents were sparged continuously with helium and further protected against carbonate contamination using ascarite tubes.

### 2.2. Samples

The barley samples used in this study were made by hydrolysing waxy barley starch (Primalco, Finland) with pullulanase (supplied by Mexazyme, Sydney, Australia). The hydrolyses were carried out at 50°C in 25 m*M* ammonium acetate buffer (pH 6) for various times. The highly branched amylopectin fraction was made as described by Bertoft and Spoof [5,6], with hydrolysis of the waxy maize starch using α-amylase, followed by precipitation with methanol–water (2.5:1, v/v). This precipitate containing the

branched dextrins with DP 200–20 was further fractionated by column chromatography into eight subfractions. Their DP were determined by column chromatography [7], by HPLC–SEC on μHydrogel 250 and 120 columns with pullulan and gluco-oligomers standards and finally by right angle laser light scattering chromatography.

The pullulan standards were from Showa Denko, Japan.

## 3. Results and discussion

The primary factor determining the reproducibility of the results is, of course, the constancy of the flow and thus the retention in the first chromatographic step. This is continuously monitored by calculating the retention of typical peaks and it never exceeded a R.S.D. of 0.3% during the six months of this study. As the detection is by UV, no calculation can be done between different kinds of samples, however, the measurement of flow-rate over the same period confirms the constancy of the flow-rate. The second critical point is the connection to the second chromatographic system. In this case, the eluent chosen for the first step will cause trace enrichment in the second step and the effect of large injection volumes is negligible. To determine the overall performance, pullulan ($M_w$=5800) standards ranging in concentration from 2000–20 mg/l were analyzed in triplicate with PAD. The calibration curve for the whole area of the peaks was linear ($r^2$=0.995), however, for individual peaks representing DP 33 and DP 36, according to Ammeraal et al. [3], the linearity was worse ($r^2$=0.97): This is mainly due to a decrease in the resolution with more concentrated samples (concentration for single peak is about 1/10 of the total concentration). The peak representing DP 48 in the area of the chromatogram containing the concentrations of single peaks was about 1/30 of the total sample concentration, thus, the resolution was not degraded with increasing sample concentration and the linearity was good ($r^2$=0.99). For all of the peaks, the retention time R.S.D. was less than 0.15%. To study the effect of the matrix, the samples were injected in 0.1 *M* NaOH, 1 *M* NaOH and in 100 mg/l sodium azide. None of these had any effect on the chromatographic retention in the SEC step and were eliminated by this SEC step from the AEX step. Neither did the addition of 1% starch or maltose affect the SEC separation and these were likewise eliminated from the second step.

The refractive index response for glucose polymers/mg glucose can be regarded as being constant. In our studies, we [7] have found that the same is true for pullulan standards over the molecular mass range of 1 660 000 to 58 000. Thus, comparison of the PAD response divided by the response for refractive index detection in the SEC system, where no individual peaks are resolved (Fig. 2), will indicate directly the efficiency with which the molecules can be oxidized on the electrode surface of the pulsed amperometric detector. The response for pullulan standards decreases with increasing molecular mass (Table 1). The response for amylopectin fractions is similar to that for pullulan standards with similar molecular masses. For linear gluco-oligomers (DP 2–7), Koizumi et al. [1] have shown that the PAD response is almost the same per HCOH group.

They subsequently increased this range further, up to DP 13, with a slight decrease over the range from DP14 to 17. In the study of Ammeraal et al. [3], they found a much larger decrease in response per HCOH group as the DP increased. When these amylopectin fractions are chromatographed in two-dimensional chromatography, they show retention (Fig. 3) that is characteristic of the their estimated average chain-lengths ($EC_{av}$~12), although their elution volume and molecular masses calculated on the basis of calibration with pullulan standards correspond quite well with their absolute molecular masses (Table 1). Thus, the second chromatographic step seems to be controlled by the DP of single chains and not by the DP of the whole molecule. This is further confirmed by the fact that only the fractions with the smallest molecular masses showed any minor separation to different peaks, but all the others eluted as a hill without any resolvable peaks, even when they were chromatographed under optimized isocratic conditions. Thus, the sugar oligomers having significant branching may be very efficiently separated from linear oligomers with DP in the same range as in the SEC step they will elute without much resolution but they will have much less retention in the anion-exchange chromatography (AEX) step. As they will also elute as an unresolved hill and they have less

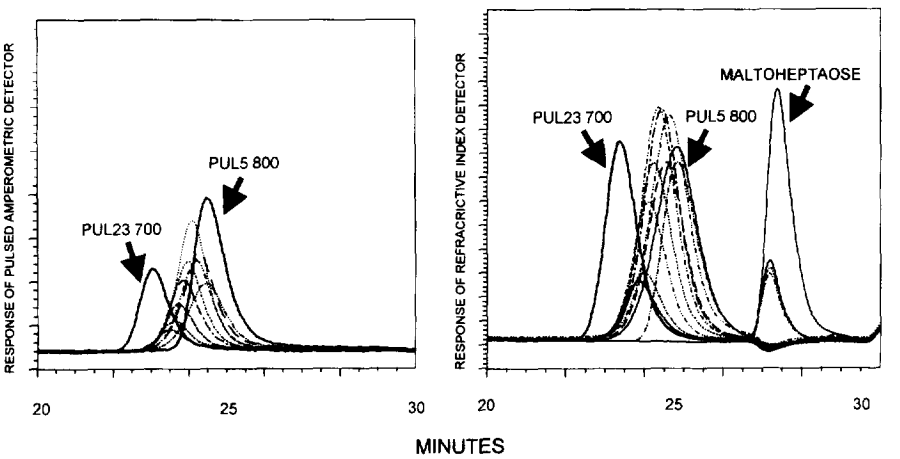


Fig. 2. Size-exclusion chromatogram of pullulan standards and a highly branched amylopectin fraction on column set μHydrogel 250 and 120, which is the column set used as the first step for two-dimensional chromatography. Refractive index detector and pulsed amperometric detection. Solid lines represent standards and the broken lines show various fragments. For more details see Section 2.

detector response than the linear oligomers eluting in the same region, they are also much more clearly distinguished after two-dimensional chromatography.

The application of two-dimensional chromatography on pullulan standard ($M_w$=5800) reveals that the different pullulan oligomers will have less retention on a PA-100 column than the linear maltooligosaccharides with the same DP and which elute in the same fraction (Fig. 4). This result is in agreement with the results of Ammeraal et al. [3], but is again more informative when connected with their retention data obtained in the first chromatographic step.

The system used provides a possible means of obtaining more information on glucose oligomers DP2...50, as the effect of different bonds between glucose units is apparent in the second step but not in the first step. Its application area can be widened with further studies using different but, alas, not commercially available oligomers with different

Table 1
Molecular masses and detector response [PAD and refractive index (RI) detection] for various α-amylase hydrolyzates of waxy maize

| Sample | $M_w$[a] | $M_n$[a] | $M_w$[b] | DP[c] | Response | PAD/RI |
|---|---|---|---|---|---|---|
| Fr1 | 20 000 | 18 400 | 13 500 | 110 | 19 | |
| Fr2 | 19 000 | 18 000 | 13 500 | 102 | 22 | |
| Fr3 | 16 200 | 15 700 | 11 800 | 85 | 20 | |
| Fr4 | 13 900 | 13 500 | 10 800 | 77 | 20 | |
| Fr5 | 11 800 | 11 500 | 9600 | 63 | 27 | |
| Fr6 | 10 900 | 10 600 | 8900 | 58 | 24 | |
| Fr7 | 10 400 | 10 000 | 8400 | 52 | 21 | |
| Fr8 | 9200 | 8700 | 7500 | 45 | 16 | |
| Pullulan P-5 | | 5800[d] | | | | 40 |
| Pullulan P-10 | | 12 200[d] | | | | 25 |
| Pullulan P-20 | | 23 700[d] | | | | 19 |

$M_w$=weight-average molecular mass; $M_n$=number-average molecular mass.
[a] Right angle laser light scattering.
[b] SEC–HPLC.
[c] Column chromatography.
[d] Manufacturer's data.

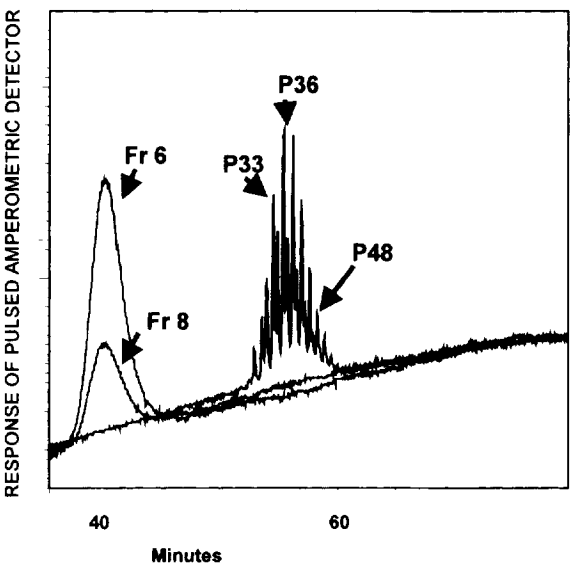


Fig. 3. Chromatograms of two amylopectin fractions and the pullulan standard from the fraction 24.0–24.4 min from the first chromatographic step. The samples were branched amylopectin fractions 5 ($M_w$=10 900) and 8 ($M_w$=9200) and pullulan ($M_w$ 5800). The steps chosen for amylopectin fractions were those giving the highest peak area. The number with P indicates DP of pullulans. Detection: PAD. For more details see text.

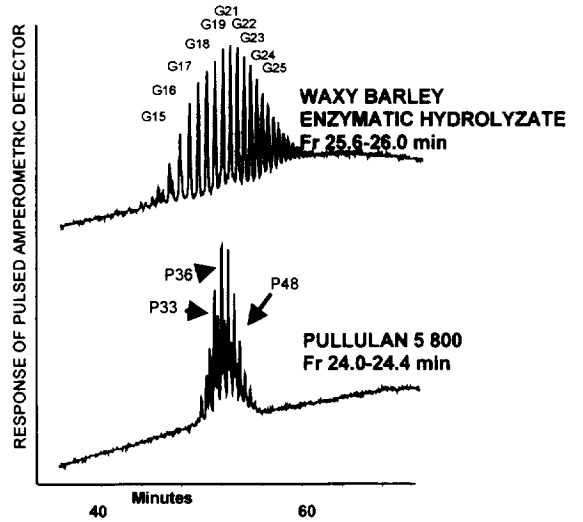


Fig. 4. Chromatogram of pullulan standard ($M_w$=5800) fraction 24.0–24.4 min) and barley linear dextrins from the fraction 25.6–26.0 of the first chromatographic step. Detection: PAD. For more detail see text.

bonds between the glucose units. The quantitative range of glucose oligomers will be increased by calculating the PAD response with the SEC system for fractions with different DPs. The use of this system to release the sample from unwanted polymeric components and buffer components is hampered by the fact that to cover the whole DP range from DP 4 to DP 50, ten successive injections from the same sample must be made. When the first step is not supposed to give any essential information, just one SEC column may be used, which will cut the number of successive injections in half and further employment of non-commercially available 4 mm columns will reduce the number of required injections to a mere two.

Further studies on the application of this system on different carbohydrate oligomers will be published later. The first applications of this method will be starches after severe thermal and mechanical treatment and enzymatic hydrolyzates of pentosans.

## Acknowledgments

The author thanks Jaana Lehtinen and Leena Öhrnberg for skilful technical assistance, Marianna Lauro for enzymatic hydrolyzates and Robin Maneliius, Åbo Akademi, Turku, for preparation of α-amylase hydrolyzates.

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