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Journal of Chromatography A 817 (1998) 297–306

JOURNAL OF CHROMATOGRAPHY A

Quality control of pentosane polysulfate by capillary zone electrophoresis using indirect detection

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Abstract

Pentosane polysulfate sodium salt (PPS) is a mixture of multiply charged anionic polysaccharides, used for urological treatment. Several constituents of the polysaccharide can be characterized by a highly reproducible fingerprint. In comparison with earlier approaches the separation efficiency has been further improved using an anionic benzene-1,2,4-tricarboxylic acid buffer (8.7 mmol l^{-1}, pH=4.9) with indirect UV detection (λ=217 nm) and a special capillary pretreatment (1 *M* NaOH for 10 h at 25°C applying −20 kV). The method has been optimized with regard to buffer concentration and pH. The robustness was tested on several capillaries. PPS was separated from all major synthetic impurities such as sulfate, chloride and acetate. Twelve PPS batches from two manufacturers were measured and compared.

Keywords: Buffer composition; Quality control; Indirect detection; Polysaccharides; Pentosane polysulfate

1. Introduction

Pentosane polysulfate sodium salt (PPS) consists of sulfated, linear polysaccharides of about 12 to 18 1→4 conjugated β-D-xylopyranose units (M_r=4000–6000, Fig. 1), which has a D-gluconic acid at approximately every tenth unit [1]. PPS is semi-synthetically manufactured from phytogenic substances. The drug PPS is used as an anticoagulant, preventing the formation of blood clots, and for treatment of hematomes, hemorrhoids, frostbites and burns [2]. A new therapeutic aim is the soothing of interstitial cystitis, which is a painful disease caused by an inflammation of the mucous membrane in the bladder [3].

Till now, quality control has been done by gel chromatography. Drawbacks are long analysis times of more than 6 h and poor separation efficiency. The principal impurities found by this method are sodium sulfate and sodium acetate. Hexosane polysulfate, methanol free and bounded pyridine and chloride are found in lower concentrations by several analytical methods [3].

CE has become a powerful and attractive technique to analyze a wide variety of molecules. Recently, the applications of CE for the investigation of carbohydrates have been recognized with increased interest [4–7]. Carbohydrates can be classified in mono-, oligo-, and polysaccharides and moreover in neutral or acidic molecules. A specific feature of this class of molecules is the lack of chromophores. Low-mass saccharides can be separated after loading by complexion using borate buffer [8]. They can be detected directly due to their aldehyde moiety with sufficient absorption coefficient [9–12]. Several methods are known for the analysis of oligo- and polysaccharides. Mostly the carbohydrates are cleaved or are derivatized with an

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0021-9673/98/$19.00
PII: S0021-9673(98)00421-X

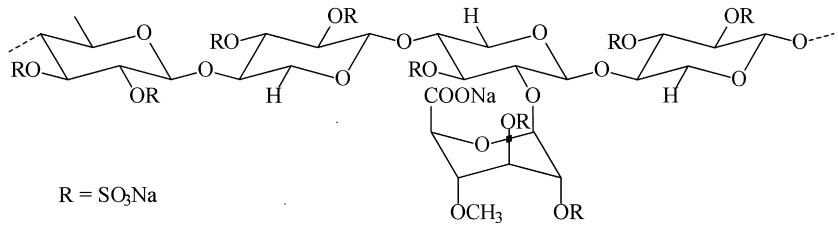

Fig. 1. Basic structure of PPS.

anionic or neutral chromophore [13]. Now the detection can be done directly. Furthermore a characterization by indirect detection [14] is established. For CE separation a couple of different techniques are used: capillary zone electrophoresis (CZE) [11,12,15–19], capillary gel electrophoresis (CGE) [20], capillary electrochromatography (CEC) [21] and micellar electrokinetic capillary chromatography (MEKC) [12,22,23].

A powerful standard separation method for highly charged large carbohydrates (well-known representatives are heparins, carrageenin, pectin and sulfated saccharides like dextran sulfate) is not available so far [24]. Experiments to analyze large heparins directly are already done [25]. However, the resolution of the fingerprint is mediocre.

The aim of this study is to investigate the general approaches of separation for high anionic loaded polysaccharides and to develop and optimize a fast and accurate and reproducible method for quality control of pentosane polysulfate.

2. Experimental

2.1. CE system

The method has been performed on a SpectraPhoresis 1000 instrument (TSP, Fremont, CA, USA) with the software CE-1000, Version 3.0.1 controlled by a personal computer featuring OS/2 Warp 3 operating system. A fused-silica capillary (I.D.=47 μm) obtained from Polymicro Technologies (Phoenix, AZ, USA) has been used. The total length was 42 cm, the effective length was 34.0 cm. The wavelength of the UV detector was set to 217 nm with a rise time of 0.5 s.

The applied voltage has been −20 kV (capillary inlet at cathode) resulting in a current of 14.4 μA. Sample injection has been done hydrodynamically (50 mbar) for 5 s by vacuum. The temperature has been held constant at 25°C.

Integration was done by a laboratory-written integration program.

Before the first use the capillary was conditioned with freshly prepared 1 *M* NaOH for 10 h at 25°C and equilibrated with running buffer for 120 min at 25°C applying 20 kV.

The capillary was rinsed between each run for 2 min with running buffer at 25°C.

2.2. Chemicals

2.2.1. Running buffer

367.8 mg (1.75 mmol) of benzene-1,2,4-tricarboxylic acid (BTC) were dissolved in 50 ml Milli-Q (millipore, Darmstadt, Germany) water. The pH was adjusted to 4.9 by approximately 41 ml of freshly prepared 0.1 *M* sodium hydroxide solution. The solution was made up to 200.0 ml, resulting in a final concentration of 8.75 mmol l^{-1} BTC buffer.

2.2.2. Sample solution

Approximately 10 mg (2 mg PPS were made up to 10 ml with Millipore water. A concentration of 1 g l^{-1} was obtained.

The same concentrations were used for all the other samples, like xylane sulfate and hexosane polysulfate.

2.2.3. Spiking of the sample solution

One crystal (approximately 0.1 mg) or one drop (~20 μL) of the reference substance was dissolved in 2.0 ml sample solution. Reference substances were sodium sulfate, sodium chloride and sodium acetate.

All samples of pentosane polysulfate, hexosane

polysulfate and xylane sulfate were kindly provided by the BENE Pharmaceuticals, Munich, Germany. All reagents were of analytical reagent grade. They were purchased from Merck, Darmstadt, Germany. Water was of HPLC-grade (Milli-Q, Millipore).

3. Results and discussion

Often polysaccharides are fragmented using enzymes or strong acid. Mono- or disaccharides are obtained, which can been detected directly after borate complexion. However, the linkage information is lost using this approach. In order to avoid this problem, a method is developed by Evangelista and Chen, using different grades of depolymerization of the polycarbohydrates. After a trisodium 8-aminopyrene-1,3,6-trisulfonic acid (APTS) derivatization of the fragments, single polymers can be identified by reconnecting the hydrolyzation parts [26–30].

The most frequently used method to analyze carbohydrates of high relative molar mass without degradation is to derivatize the terminal semi-acetal by reductive amination [31,32] or condensation [33,34] with an anionic or neutral chromophore. However, only neutral or weakly acidic carbohydrates can be separated this way. Furthermore, using pretreatments like fragmentation or derivatization causes additional error sources. For example, usually the yield of derivatization reactions of polysaccharides is low and changing. Thus the distribution pattern after derivatization is unpredictably different to the original polymer. These properties of derivatization reactions are unfavorable for quality control.

Another approach is the characterization of polysaccharides by indirect detection. In this technique the background electrolyte (BGE) of the buffer system has a high extinction coefficient. Sample molecules displace the BGE by electromigration. The reduction of absorption results in negative peaks. Using indirect detection the analyte is dissolved in water and can be injected without modification. Thus this approach is favorable for quality control.

Indirect detection has been tested several times for low-molar-mass carbohydrates [15–19]. In some works poor peak resolutions, poor sensitivities and the detection of all impurities have been reported.

The central issue is the choice of the right BGE. A check list of necessary properties is suggested. Decisive aspects are listed in Table 1.

Several compounds like sorbic acid (pH=4.76), 5-sulphosalicylic acid (pH=3.0), benzene-1,2,4-tricarboxylic acid (trimellitic acid, BTC, pH=4.9) and benzene-1,2,4,5-tetracarboxylic acid (pyromellitic acid, PMA, pH=6.0) with these properties have been tested. It was possible to separate PPS in buffers of pH from 5.5 to 9 using normal polarity (capillary outlet at cathode) or in buffers of pH from 4.5 to 5.5 using reversed polarity.

In an earlier work a combined buffer system of sorbic acid as chromophore and borate as BGE (pH=8.82) was investigated. However, the separation was poor (Fig. 2, [40]). An improvement was desirable for quality control of PPS.

Further studies followed on single compound buffer systems close to the pK_a, in order to prevent disturbing system peaks. The best results were obtained using BTC (pK_a=5.12) at a pH of 4.9 and a concentration of 8.75 mmol l^{-1}. BTC and PMA are improvements to the frequently used benzene-1,2-dicarboxylic acid (phthalic acid) [39].

The pH of 4.9 shows a well resolved PPS finger-

Table 1
Properties necessary for choice of the right buffer system and chromophore for indirect detection

Good solubility in water
Carrying at least one anionic charge for a good electrostatic repulsion; more charges rise the ionic strength of the buffer system [13]
Electrophoretic mobility should be similar to the electrophoretic mobility of the analyte [14,35–39]
A good chromophore (high extinction coefficient) at a λ_{max}>210 nm
A suitable pK_a for the respective separation, typically close to the buffer pH
Commercially available in analytical grade quality
Stable in solution
Not expensive
Non-toxic

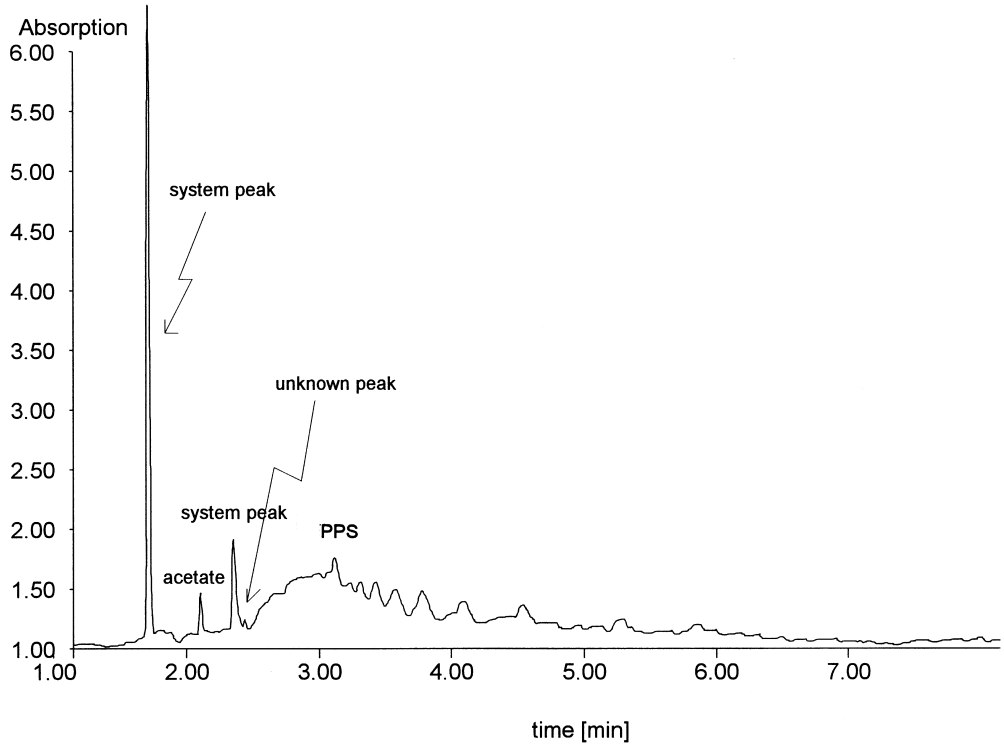

Fig. 2. PPS sample; peak sequence in subsequent order: system peak; acetate; system peak; unknown substance; PPS. The fingerprint is partly resolved. Conditions: buffer system: 3 mmol l^{-1} sorbic acid and 15 mmol l^{-1} borate at a pH of 8.82; separation voltage was 30 kV (capillary inlet at anode), wavelength was set to 254 nm; sample injection was done hydrodynamically (50 mbar) for 0.5 s. The temperature was held constant at 25°C.

print in a short separation time. Different concentrations of BTC were tested. Using a concentration lower than 7 mmol l^{-1} the baseline becomes unstable, above 10 mmol l^{-1} the signal-to-noise ratio is obviously reduced.

Fig. 3A depicts a typical electropherogram of 1 g l^{-1} PPS dissolved in water separated by a BGE buffer system. PPS is shown as a broad group of up to 35 single characteristic peaks. Each signal of the group peak represent a polymer of different chain-lengths and branching. Two contaminants (3 and 3.5 min) are identified as chloride and sulfate by spiking. The acetate impurity migrates later. It is depicted in Fig. 4.

However, in some case the fingerprint resolution was inferior (Fig. 3B). The reason for these varying resolutions was unclear but separations obtained using buffers with a pH between 4 and 7 have often been reported poorly reproducible, namely with respect to migration times.

In another project the problem of varying migration time reproducibility has been overcome by a special capillary conditioning [41]. Thus from now on the capillaries have been conditioned with 1 *M* NaOH for 10 h at 25°C. The resolutions of the fingerprints have been constantly improved as depicted in Fig. 3A.

The CE method was optimized for a voltage of −20 kV, a concentration of 8.75 mmol l^{-1} and pH of 4.9–5.0. About 50 injections over two months on several capillaries were done to guarantee the reproducibility of the fingerprint of PPS.

The intra-day precision of the migration time corresponds to 1.5% R.S.D. ($n=10$). The R.S.D. of inter-day investigations is higher (about 10%), probably due to variations of the electroosmotic flow (EOF). Despite of considerable R.S.D. in the migration times comparing series of different PPS batches, the fingerprint electropherograms can readily be overlaid and compared (see Figs. 5 and 6).

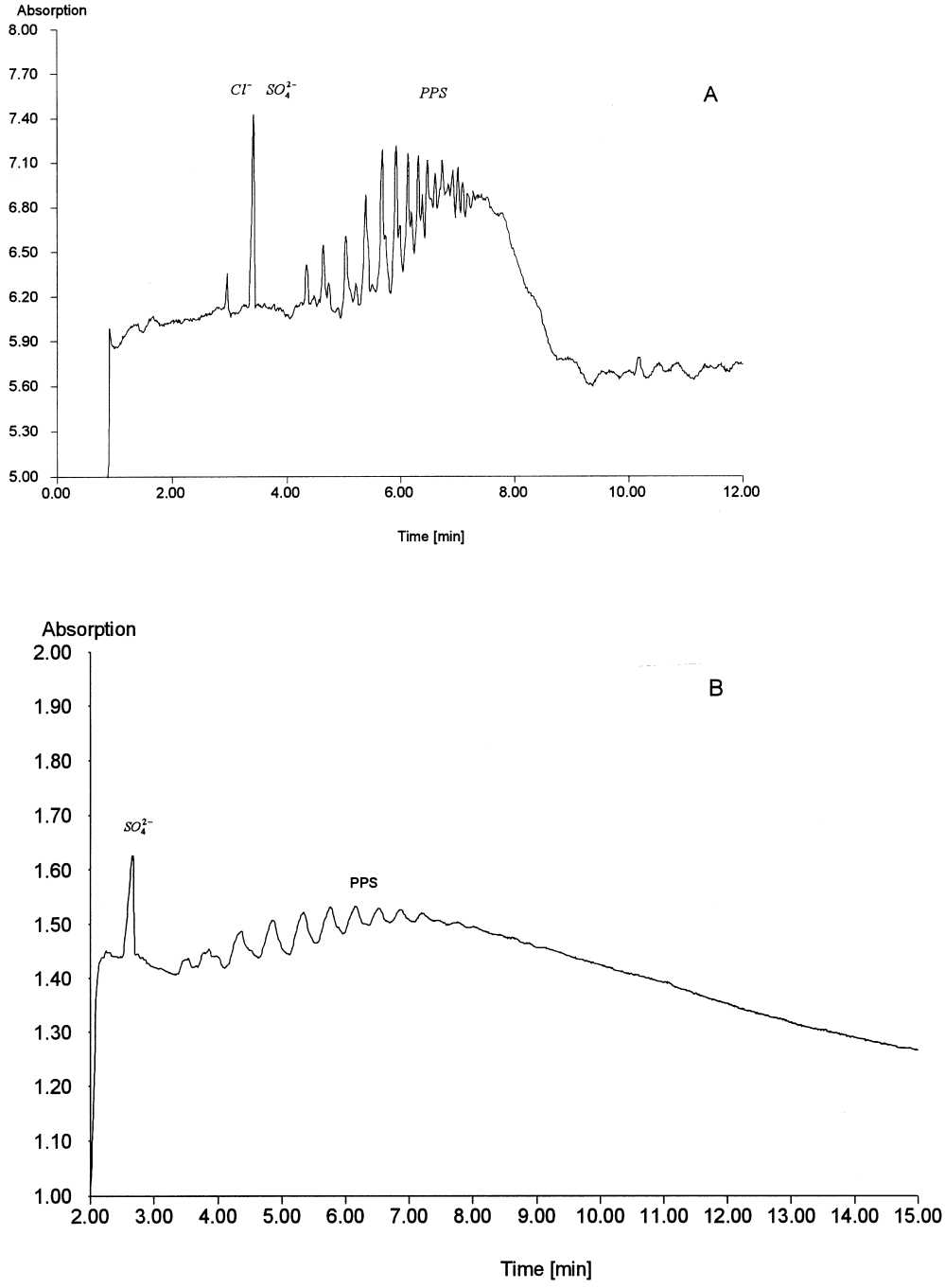

Fig. 3. (A) PPS sample; peak sequence in subsequent order: chloride (3 min), sulfate (3.5 min), PPS (4.2 to 9 min). PPS shows a broad group peak with a characteristic fingerprint, much better resolved compared to Fig. 2. Up to 35 single peaks are detected. For method see Experimental. (B) Same conditions but inferior fingerprint resolution. Capillary preconditioning 0.1 *M* NaOH for 30 min at 25°C.

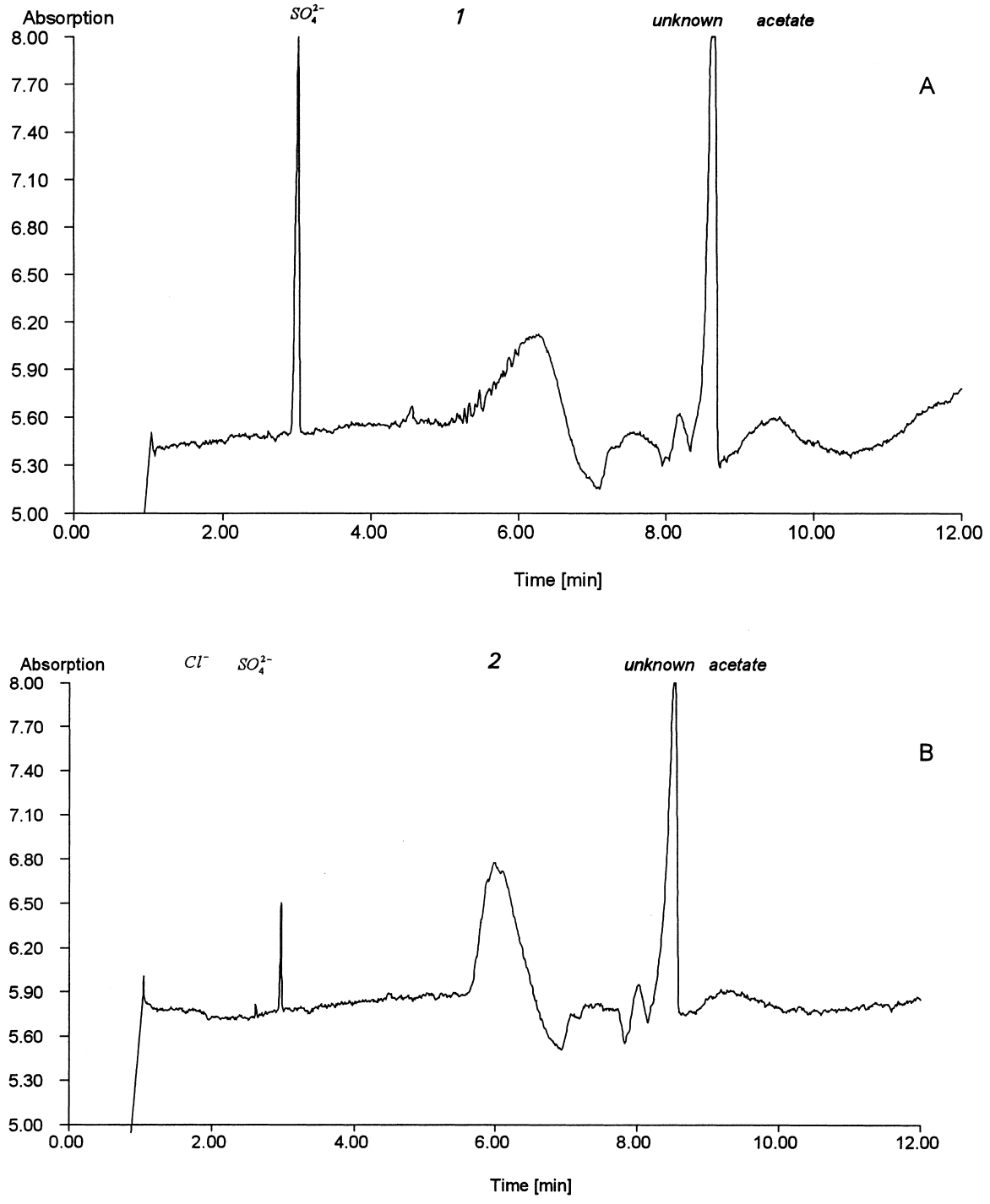

Fig. 4. (A) Hexosane polysulfate sample; peak sequence: sulfate (3 min), hexosane polysulfate (peak 1, 5 to 7 min), unknown (8.2 min), unknown (8.7 min), acetate (9.2 min); (B) Xylane sulfate sample; peak sequence: chloride (2.7 min), sulfate (3 min), xylane sulfate (peak 2, 6 min), unknown (8 min), unknown (8.5 min), acetate (9.2 min); conditions same as given in Section 2.

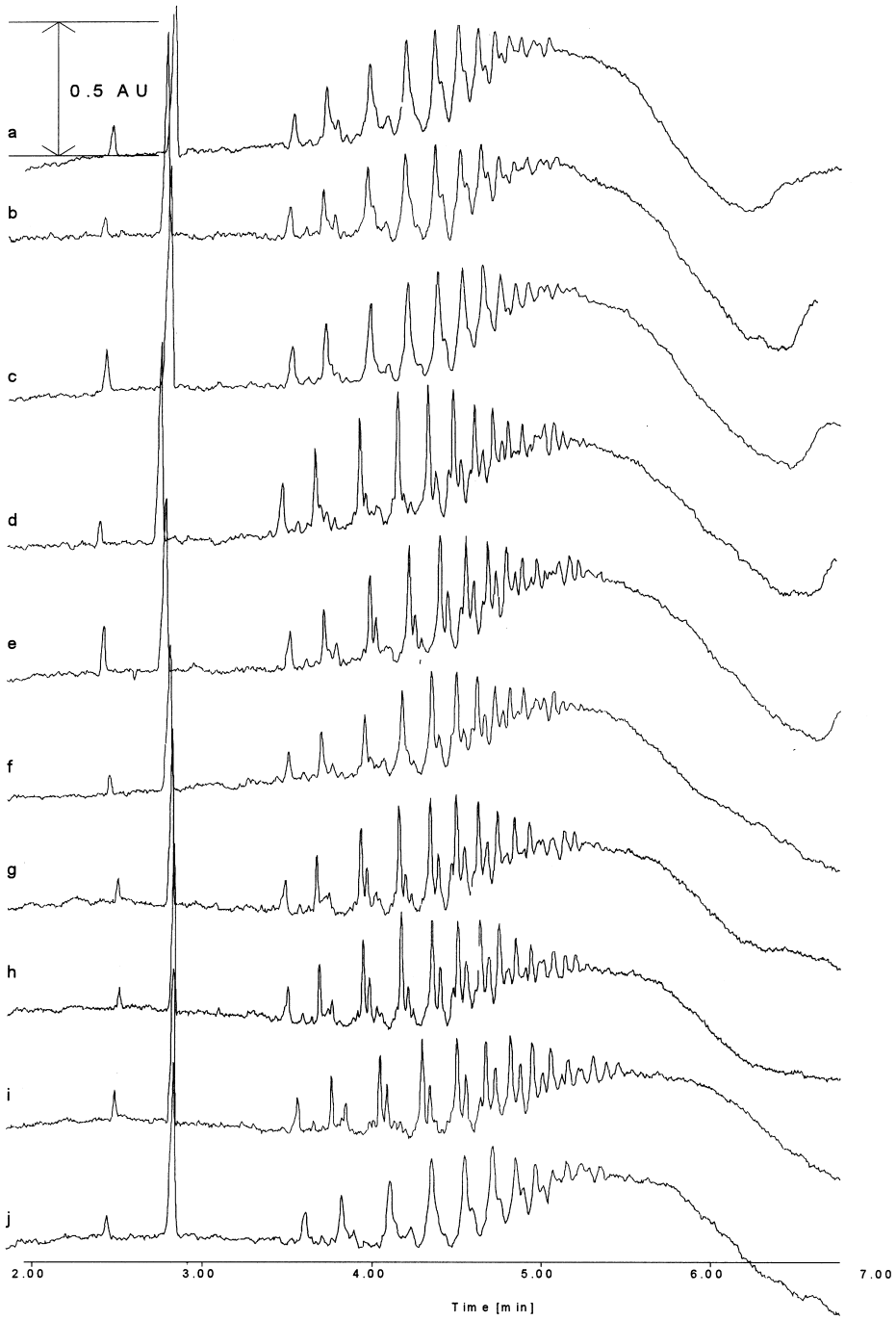

Fig. 5. Comparison of several PPS batches from one manufacturing company measured during four days. The migration times and resolutions of the peaks are slightly varying. However, the fingerprints can easily be compared in order to control product quality. The different batches show a very similar pattern, the amount of impurities such as chloride and sulfate is almost the same. For method see Section 2, for assignment of the peaks see Fig. 3.

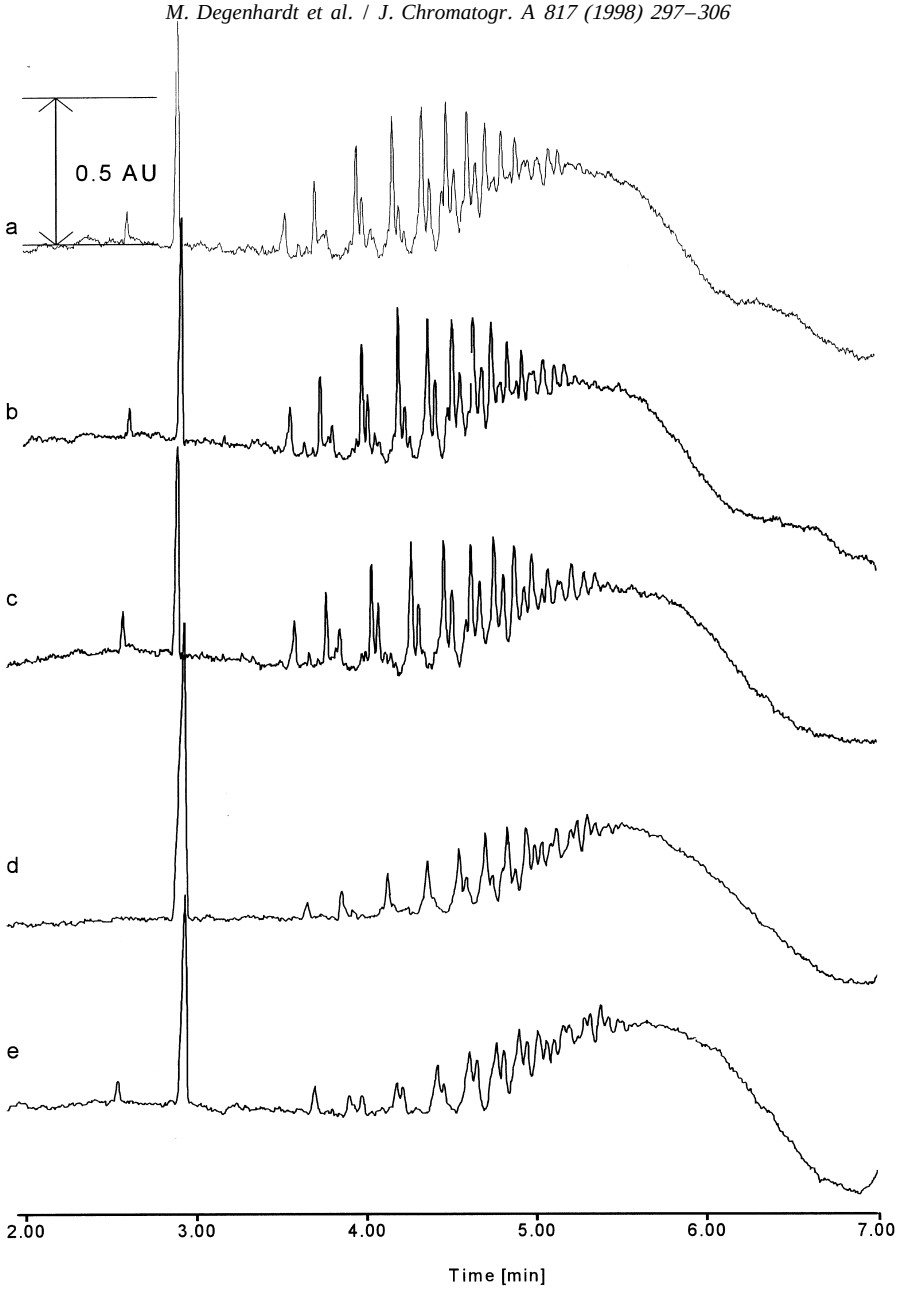

Fig. 6. Comparison of three different PPS samples of one company (a–c) and two batches of another manufacturing company (d, e). Fingerprints a–c are very similar (compare Fig. 5), d and e can well be distinguished. The samples (d) and (e) contain a smaller amount of PPS, the peak height in the fingerprint range is smaller.

Additional PPS impurities, hexosane polysulfate and xylane sulfate are depicted in Fig. 4. It is possible to identify these compounds in PPS samples. They migrate slightly slower than PPS. The shape of the hexosane polysulfate peak is very similar to the PPS signal, but its width is shorter and the fingerprint is less characteristic. Xylane sulfate is characterized by a sharper peak.

Several PPS batches of one manufacturing company are investigated and compared (Fig. 5). All

batches are produced by the same manufacturing process. They only vary in the batches of raw materials used during the manufacturing process and its production date. The PPS fingerprints show a remarkable correlation. The quantity of anionic impurities and the quality of these PPS batches are almost constant.

In order to check the performance of the method, two PPS samples from another manufacturing company were measured as well (Fig. 6d and e). The sample quality obviously differs. The height of the PPS main peaks is lower comparing to the other batches (Fig. 6a–c). This deviations are possibly caused by a different production process.

4. Conclusions

A well resolved and reproducible CZE-method is developed for the quality control of PPS. Sample derivatization has been avoided using indirect detection in comparison to common CZE methods for polysaccharides analysis. Moreover, it was not necessary to add polymeric reagents to the buffer system. This has been achieved using a BTC buffer system. This method should be useful for similar polymeric analytes as well.

In order to achieve a constantly good peak resolution a special capillary pretreatment has been introduced. This pretreatment is possibly beneficial to improve reproducibility and resolution when buffers with pH between 4 and 7 are used.

Acknowledgements

We would like to thank the company BENE for providing all samples and a financial support. Further, the authors would like to thank Mr. Stefan Günter and Mr. Bernhard Schirm for their skillful assistance.

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