**Further proof to the utility of polysaccharide-based chiral selectors in
combination with superficially porous silica particles as effective chiral
stationary phases for separation of enantiomers in high-performance liquid
chromatography**

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13   **Abstract**

14   The first ever report on the preparation of chiral stationary phases (CSP) based on
15   superficially porous silica (SPS) particles for the separation of enantiomers in HPLC
16   demonstrated clear advantages of such materials. Higher enantioselectivity at the
17   comparable content of a chiral selector, limited dependence of plate height on the mobile
18   phase flow rate and higher plate numbers and resolution calculated per unit time (i.e.
19   higher speed of separation) were observed with columns made with superficially porous
20   CSP in comparison to columns made with fully-porous silica-based CSP. However, later
21   studies reported diverging conclusions. In this report further evidences are described
22   supporting the findings of our first study about the superior performance of
23   polysaccharide-based chiral selectors in combination with SPS when compared to
24   traditional CSPs based on fully porous silica (FPS) particles.

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26   *Keywords:*

27   Enantioseparations, chiral HPLC, chiral stationary phases based on superficially porous
28   silica, polysaccharide-based chiral stationary phases

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1. Introduction

The advantages of superficially porous silica (SPS) over fully porous silica (FPS) for achiral liquid phase chromatographic separations have been extensively documented [1-8]. These advantages include significantly higher chromatographic efficiency, a marked shift to higher optimal flow rates and limited dependence of column performance on mobile phase linear velocity. The higher efficiency of columns made with SPS particles is probably due to the more uniform particle-size distribution of SPS particles compared to their fully porous analogues [1, 6] resulting in higher radial homogeneity of columns packed with SPS [7, 8] and to the shorter diffusion path available to analytes [1-5]. These same characteristics are responsible for the limited dependence of column performance on the mobile phase linear velocity, minimizing both the eddy diffusion term (A term) and also the mass-transfer contribution (C term) to the height equivalent to a theoretical plate (HETP) as detailed in the van Deemter equation. Realizing the minimum HETP value at higher flow rate and maintaining such low HETP at even higher mobile phase flow rates makes SPS columns suitable for high-speed separations [1].

On the other hand, columns made with SPS particles have been shown to demonstrate lower retention and lower loadability, both caused by the lower surface area typical to such particles. Consequently, such sorbents can be loaded with lower amounts of chiral selector compared to FPS materials, which typically have higher surface area.

Since polysaccharide esters and phenylcarbamates are recognized to be the most successful chiral selectors for the separation of enantiomers in liquid phase techniques such as high-performance liquid chromatography (HPLC) [9, 10], supercritical fluid chromatography (SFC) [11, 12], nano-chromatography [13, 14] and capillary

electrochromatography (CEC) [15-17], their combination with SPS seems logical for the preparation of highly efficient chiral stationary phases (CSP).

In our first study on the utility of SPS for the preparation of CSPs for chiral HPLC certain promise of these materials has been demonstrated [18]. In particular, higher enantioselectivity was observed with SPS-based CSPs compared to FPS-based CSPs at comparable content of (polysaccharide derivative) chiral selector. Also, higher plate numbers and resolution calculated per unit time (i.e. speed of analysis) with only limited dependence of HETP on the mobile phase flow rate were observed. A polysaccharide-derivative chiral selector, namely, cellulose tris(4-chloro-3-methylphenylcarbamate) was used in our first study [18]. Later, the successful use of SPS in combination with polysaccharide-based chiral selectors was also demonstrated in other techniques such as nano-liquid chromatography and CEC [19, 20]. However, in some recent publications our earlier results were somehow misinterpreted [21, 22] and polysaccharide-based chiral selectors were declared to be at least less promising if not even incompatible with SPS for the preparation of highly efficient chiral stationary phases for use in chiral HPLC [23, 24]. To the contrary, this paper brings further proof demonstrating that polysaccharide-based chiral selectors are at least as effective in combination with SPS as they are in combination with FPS.

2. Experimental

2.1 Materials

The chiral test compounds benzoin, *trans*-stilbene oxide and Tröger's base (Fig. 1A-C) were commercially available from Sigma-Aldrich (St. Louis, MI, USA). Etzoline

(Fig. 1D) was provided by Prof. Gottfried Blaschke (Institute of Pharmaceutical and Medicinal Chemistry, University of Münster, Münster, Germany). HPLC-grade n-hexane, methanol and 2-propanol were supplied by Karl Roth (Karlsruhe, Germany). SPS particles with 2.8 μm nominal particle diameter and 30 nm nominal pore size, as well as with 3.6 μm nominal particle diameter and 20 nm nominal pore size were provided by Phenomenex Inc. (Torrance, CA, USA). The 2.8 μm SPS particles were coated with cellulose tris(3,5-dimethylphenylcarbamate, CDMPC) made of Avicel cellulose (Fluka, Buchs, Switzerland) as described earlier [25] while 3.6 μm SPS particles were coated with amylose tris(3,5-dimethylphenylcarbamate, ADMPC) made of semisynthetic amylose. The synthesis of amylose and the chiral selector based on it will be described elsewhere. The structures of chiral selectors are shown in Fig. 2.

Chiral selectors were coated onto silica by dissolving appropriate amounts of CDMPC or ADMPC in tetrahydrofuran or dichloromethane, respectively, by adding the weighed amount of silica to this solution, by sonicating the suspension for a few minutes and finally by evaporating the solvent to dryness at room temperature using a rotary evaporator under reduced pressure. The obtained powder was further dried under reduced pressure at 60 °C for 1 hr and used for packing of HPLC columns. A slurry of the packing material was prepared in n-hexane/2-propanol mixture 9/1 (v/v), decanted two times and then packed at 600 bar in stainless-steel HPLC columns of 150x4.6 mm dimensions in the case of CDMPC-based CSP and of 100x4.6 mm dimensions in the case of ADMPC-based CSP. The empty column hardware was provided by Phenomenex Inc.

2.2. Instruments

HPLC separations of test compounds dissolved in the mobile phase at the concentration 0.2 mg/ml were performed on an Agilent 1290 Infinity series UHPLC system equipped with G4220A binary pump, G4226A automated liquid sampler, G1316C thermostated column compartment and G1315C diode array detector with 2 microliter detector cell. The rotary evaporator used in this study was Rotavapor R-210/R-215 from BUCHI Labortechnik GmbH (Essen, Germany) with temperature control and an ultrasonic bath Sonorex RK-100 was from Bandelin (Berlin, Germany). Elemental analysis (N, C) was performed using an Elemental Combustion System CHNS-O, Model ECS4010 by Costech Analytical Technologies Inc., (Valencia, CA, USA).

3. Results and Discussions

3.1. Comparison of columns made with chiral selector supported on SPS and FPS particles

Due to the presence of their nonporous core SPS particles are typically more dense compared to FPS particles. Therefore, the amount of CSP, and hence of chiral selector (coated at same % w/w) is higher in SPS-based columns compared to FPS based columns of the same dimension [18]. The impact of the higher content of chiral selector in the column packed with SPS-based CSP compared to FPS-based CSP on the separation of enantiomers of Tröger's base is shown in Fig. 3. The enantiomers of this analyte are only marginally resolved on the FPS-based column in contrasts to almost baseline resolved on the column packed with SPS-based CSP, while both CSPs were prepared with the same percentage content (w/w) of chiral selector. This significant difference in resolution was observed along with higher retention time but actually lower

retention factor ($k=0.83$) on the FPS-based CSP compared to the SPS-based CSP (observed retention factors for the latter were $k_1=0.95$ and $k_2=1.15$). The reason why retention times and retention factors do not correlate between these two types of chiral columns is the difference in column void volume/time: the column packed with SPS particles has significantly lower void volume compared to the column packed with FPS particles (again due to the presence of solid cores in SPS particles).

3.2. Enantiomer resolving ability of SPS-based CSPs

Due to the inherent lower surface area of SPS particles compared to FPS particles laying out (coating) a large amount of chiral selector on their surface is counterproductive leading to significant changes in particle morphology, in particular in average pore diameter, in total pore volume and in surface area (data to be published later). On the other hand, as mentioned in the previous subsection (and demonstrated in reference [18]), coating a large amount of chiral selector on the surface of SPS particles may not be necessary since adequate resolution can be achieved even with lower % coating. The results observed for several chiral analytes under normal-phase and polar organic mobile phase conditions with SPS-based CSP coated with only 2% (w/w) of chiral selector shown in Fig. 4 prove this point. Very good baseline separation of enantiomers was obtained with plate counts in the range of 20 000-140 000 plate/m for all analytes. The lower plate numbers observed for etozoline and in part for Tröger's base enantiomers most probably originate from the structural properties of these chiral compounds rather than from the overall performance of coated SPS particles. Thus, the correct approach is to coat a significantly lower amount/percentage of the chiral selector onto SPS (see Figs.

3b and 4c). Perhaps this essential aspect of preparing coated SPS-based chiral sorbents was overlooked in other studies leading to the wrong conclusion that polysaccharide-based chiral selectors can be problematic in combination with SPS. Thus, preserving the benefits of two technologies requires fine-tuning and making some compromises.

3.3 Plate number dependence on mobile-phase flow rate and instrumental requirements

The limited dependence of the plate count (column efficiency) on the mobile phase flow rate in the case of SPS-based CSPs has been reported in our earlier study [18] and confirmed later by other authors [21, 22]. In this study the dependence of column efficiency on the mobile-phase flow rate was studied at two different settings of the data collection frequency by the UV-detector. A dramatic effect was observed for all studied analytes and mobile phases. The results for the enantiomers of *trans*-stilbene oxide are shown as an example in Fig. 5. The trend was essentially the same for all other analytes. The separation of enantiomers of *trans*-stilbene oxide at two different flow-rates and with data recorded at two different acquisition speeds is shown in Fig. 6. As these results indicate, the separation results at lower mobile-phase flow rate can be adequately described at both 5 and 80 Hz detection speed, while fast separations are inadequately recorded at low detection speed (5 Hz). This observation is in line with published results for achiral and chiral [22] separations performed with SPS-based packing materials in HPLC. High data acquisition speeds are required for properly reflecting the resolving power of SPS-based LC columns.

3.4 Fast separations

The most important advantage of SPS-based chromatographic materials is realized in fast separations. Due to their limited dependence of column efficiency on the mobile phase linear velocity, SPS-based sorbents maintain most of their performance even at the very high mobile phase flow-rates typical to very fast separations (see Figs. 5 and 6). To illustrate this advantage, several baseline separations are shown in Figure 7 with analyses times as low as 15-25 seconds. It seems worth mentioning that plate counts remain over 100 000 plate/m even at very high mobile phase flow rates. Figures 5-6 also show optimal reduced plate heights of 2 or below 2 for some analytes. For example, the 100x4.6 mm column prepared with 2% (w/w) amylose tris(3,5-dimethylphenylcarbamate) coated on SPS with 3.6 μm nominal particle size and 20 nm nominal pore size, generated over 13 600 plates (see Fig. 6B) at the flow rate 0.5ml/min for *trans*-stilbene oxide. This corresponds to an HETP = 7.3 μm or $h = 2.0$.

Another interesting point worth making is that adequate resolution could be achieved in spite of the minimal time spent by enantiomers on column: out of a total analysis time of about 15 seconds (see Fig. 7) analytes spent less than 5 seconds on column while the rest of the time was required for analyte transfer to/from the column from/to other instrument components. Further shortening transfer times (as on a **lab-on-chip** device) may enable baseline separation of enantiomers even at sub-second scale.

4. Conclusions

The results of this study indicate that a combination of polysaccharide based chiral selectors with superficially porous silica is a very useful approach for the preparation of

highly efficient chiral stationary phases for HPLC enantioseparations. When working with adequate instrumentation the decrease in column performance at higher flow rates is minimal and highly efficient separation of enantiomers can be achieved with the analysis time in the range of 15-30 seconds.

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Legends to the figures:

Fig. 1 Structures of benzoin (A), etozoline (B), *trans*-stilbene oxide (C) and Tröger's base (D).

Fig. 2 Structure of cellulose tris(3,5-dimethylphenylcarbamate, CDMPC) (a) and amylose tris(3,5-dimethylphenylcarbamate, ADMPC) (b).

Fig. 3 Separation of the enantiomers of Tröger's base on a chiral column packed with packing material prepared by coating of 5% (w/w) cellulose tris(3,5-dimethylphenylcarbamate on FPS (a) and SPS (b). FPS was with 3 µm nominal particle size and 30 nm nominal pore size while SPS was of 2.8 µm nominal particle size and 30 nm nominal pore size. The mobile phase was n-hexane/2-propanol 80/20 (v/v) with the flow rate 0.5 ml/min. Column dimensions were 150 x 4.6 mm.

Fig. 4 Separation of enantiomers of *trans*-stilbene oxide (4A), benzoin (4B), Tröger's base (4C) and etozoline (4D) on the chiral column made with CSP prepared by coating of 2% (w/w) amylose tris(3,5-dimethylphenylcarbamate on SPS with 3.6 µm nominal particle size and 20 nm nominal pore size. The mobile phases were n-hexane/2-propanol 90/10 (v/v) in cases A and B and methanol in cases C and D with the flow rate 0.5 ml/min.

Fig. 5 Dependence of plate height on mobile phase flow rate for the enantiomers of *trans*-stilbene oxide recorded at 5 Hz (1 and 2) and 80 Hz (3 and 4) detector frequency. The column and the mobile phase were as indicated in the legend to Fig. 4A.

Fig. 6 Separation of enantiomers of *trans*-stilbene oxide at the mobile phase flow rates 0.5 ml/min (A, B) and 5.0 ml/min (C, D) recorded at 5 Hz (A and C) and 80 Hz (B and D) detector frequency. The column and the mobile phase were as indicated in the legend to Fig. 4A.

Fig. 7 Fast separation of enantiomers of *trans*-stilbene oxide (4A), benzoin (4B), Tröger's base (4C) and etozoline (4D) on the chiral column packed with packing material prepared by coating of 2% (w/w) amylose tris(3,5-dimethylphenylcarbamate on SPS with 3.6 μ m nominal particle size and 20 nm nominal pore size. The mobile phases were n-hexane/2-propanol 90/10 (v/v) in cases A and B and methanol in the cases C and D with the flow rate 5 ml/min.

(A) Benzoin

(B) Etozoline

(C) *trans*-Stilbene oxide

(D) Tröger's base

Figure 2

(a)

(b)

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7