Three versus five micrometer chlorinated polysaccharide-based packings in chiral capillary electrochromatography: performance evaluation

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ABSTRACT: In this study, a test set of 44 nonacidic compounds was analyzed on four 3 μm chlorinated polysaccharide-based chiral stationary phases with cellulose tris (3-chloro-4-methylphenylcarbamate) (Lux Cellulose-2®, LC2), amylose tris (5-chloro-2-methylphenylcarbamate) (Lux Amylose-2®, LA2), cellulose tris (4-chloro-3-methylphenylcarbamate) (Lux Cellulose-4®, LC4) and cellulose tris (3,5-dichlorophenylcarbamate) (Sepapak-5®, Sp5) as selectors. The analysis times, retention factors, efficiencies and enantioselectivities were compared with the results obtained on their 5 μm analogs. All 3 μm packings, except for LA2, individually separated more compounds than their 5 μm analogs. When the cumulative success rates on the 3 and 5 μm packings were considered, it was observed that they were similar for both particle sizes; the combination of three or four 5 μm columns separated one compound more from the considered test set than that of the same number of 3 μm columns. Furthermore, it was observed that the 3 and 5 μm packings showed some complementarity. Only four compounds were not separated on any of the columns, while the use of only either the 3 or 5 μm columns resulted in 10 and nine not-separated compounds, respectively. The analyses on 5 μm LC2 and Sp5 were faster than on their 3 μm analogs. For LC4 the 3 μm packing showed the shortest analysis times and diverse analysis times for both particle sizes were obtained on LA2. Furthermore, three out of four 3 μm packings, that is, LC2, LC4, and Sp5, were found to be more efficient than their 5 μm analogs. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: 3 vs. Sum packings; chiral electrochromatography; chlorinated polysaccharide-based chiral stationary phases

Introduction

During recent years capillary electrochromatography (CEC) has become a more frequently used microseparation technique. It combines useful features of both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). It is a hybrid technique because the separation is based on both chromatographic partition and electrophoretic migration. The high numbers of theoretical plates which in principle can be obtained with CEC, because of the flat electrosomotic flow profile (limited band broadening), should be an advantage when a minor peak must be detected in the presence of a major, like in impurity profiling experiments (Hendrickx et al., 2011b). The application domains of CEC are quite similar to those of HPLC and CE, and include amongst others, the determination of drug impurities, active-component assays and chiral separations, of which the latter field is very actively and extensively investigated (Altria et al., 1998; He et al., 2009; Hendrickx et al., 2011b; Liu et al., 2012; Lv et al., 2011; Wu et al., 2011). Moreover, compounds of different natures – including biomolecules – can be analyzed by CEC and analytes of interest can be determined at therapeutic levels in small sample volumes (Fu et al., 2003; Hendrickx et al., 2011b; Quaglia et al., 2002).

Generally, it is time-consuming to develop chiral separation methods because the separation of a compound in a given system is rather unpredictable. A rational and generic screening strategy, which allows – in a limited number of experiments – evaluation the enantioselectivity of some systems towards the chiral compound of interest, is therefore an efficient tool for chiral method development. Such strategies are developed based on diverse sets of test compounds and have already been defined for different techniques, like HPLC (in normal-phase, reversed-phase and polar organic solvent modes), supercritical fluid chromatography, CE and CEC (Ates et al., 2008; De Klerck et al., 2012a, b; Eelink et al., 2005; Hendrickx et al., 2010, 2011a; Mangelings et al., 2003, 2005a–c, 2006; Matthijs et al., 2006a, b; Matthijs and Vander Heyden, 2006; Perrin et al., 2002a, b; Rathore and Horváth, 2001; Tachibana and Ohnishi, 2001; Wang et al., 2012; Younes et al., 2011a, b; Zhang et al., 2004). A generic chiral separation strategy starts with a screening step, in which a number of

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Abbreviations used: ACN, acetonitrile; AT, analysis time; CEC, capillary electrochromatography; CSP, chiral stationary phases.
columns and given experimental conditions (e.g. mobile phase composition), that is, chromatographic systems, are suggested to be evaluated for any compound to be separated. Depending on the result of this screening step, optimization steps are proposed. These aim at obtaining a baseline separation for partially separated enantiomers, decreasing the analysis time and/or improving the quality of the separation when a baseline separation has already been achieved, or inducing enantioselectivity when none was seen in the screening. Screening and optimization steps are defined based on experimental data and the literature (Hendrickx et al., 2011b).

Recently, a chiral separation strategy for nonacidic compounds in CEC was updated (Hendrickx et al., 2010, 2011a). The final strategy included stationary phases containing cellulose tris (3,5-dimethylphenylcarbamate) (5 μm ODRH), amylose tris (3,5-dimethylphenylcarbamate) (5 μm ADRH), amylose tris (5-chloro-2-methylphenylcarbamate) (5 μm LA2) and cellulose tris (4-chloro-3-methylphenylcarbamate) (5 μm LC4) selectors, coated onto 5 μm silica particles. At the studied conditions, these chiral stationary phases (CSPs) were found most enantioselective and complementary out of eight tested, that is, 5 μm ODRH, 5 μm ADRH, 5 μm ASRH (amylose tris [(5)-α-methyl-benzylcarbamate]), 5 μm OJRH [cellulose tris (4-methylbenzoate)], 5 μm LC2 [cellulose tris (3-chloro-4-methylphenylcarbamate)], 5 μm LA2, 5 μm LC4 and 5 μm Sp5 [cellulose tris (3,5-dichlorophenylcarbamate)]. With the above combination of chlorinated and nonchlorinated CSPs, improved success rates were obtained compared with the screening step solely based on nonchlorinated CSPs, developed earlier by Mangelings et al. (2005a).

In this study, the 3 μm analogs of LC2, LA2, LC4 and Sp5 were evaluated in CEC for their performance. The conditions of the earlier defined screening step for the CSPs with 5 μm silica particles were applied on 44 chemically and structurally diverse nonacidic (basic, neutral, and amphoteric) compounds (Hendrickx et al., 2010). A comparison based on enantioselectivity, analysis time and efficiency was made between these 3 and 5 μm packings with chlorinated selectors.

Materials and methods

Chemicals and reagents

Acebutolol HCl, alprenolol HCl, atropine, cinnarizine, chlorothalidone, flutoxetine HCl, labetalol HCl, naldolol, pindolol, praziquantel, promethazine HCl, sulpiride and tetramisole HCl were obtained from Sigma (St Louis, MO, USA); propranolol, HCl and trans-stilbene oxide were obtained from Aldrich (Steinheim, Germany); methadone was obtained from Federa (Brussels, Belgium); ephedrine HCl and verapamil HCl were obtained from Fluka (Buchs, Switzerland); lorazepam was obtained from Wyeth (Collegeville, PA, USA); ambucetamide and nebivolol were obtained from Janssen Pharmaceutica (Beerse, Belgium); nitrendipine and nimodipine were obtained from Bayer (Leverkusen, Germany); tertatolol HCl was obtained from Servier (Neuilly-Sur-Seine, France); bupropranol HCl was obtained from Schwarz Pharma (Monheim, Germany); oxprenolol was obtained from Ciba Geigy (Basel, Switzerland); mianserin was obtained from Organon (Oss, The Netherlands); carteolol HCl was obtained from Madaus (Köln, Germany); carazolol was obtained from Klinke Pharma (München, Germany); dimethindene maleate and bopindolol hydrogennmaleate were obtained from Novartis (Basel, Switzerland); esmolol HCl was obtained from Duphar (Amsterdam, The Netherlands); felodipine was obtained from Astra-Zeneca (Södertälje, Sweden). Betaxolol HCl, bisoprolol, carbinoxamine maleate, celiprolol HCl, chlorpheniramine maleate, metazapin HCl, metoprolol, oxazepam, propiomazine and sotalol were gifts from unknown sources. As dead-time marker, thiourea from Merck (Darmstadt, Germany), was used.

All samples were dissolved in ultrapure water–acetonitrile (ACN; HPLC-grade, Fisher, Leicestershire, UK; 30:70, v/v) at a concentration of 0.5 mM. The electrolyte solution of the mobile phase was a 5 mM disodium hydrogen phosphate (Na2HPO4; Merck) solution in ultrapure water (prepared in-house using an Arium Pro UV instrument; Sartorius, Vilvoorde, Belgium) and adjusted to pH 11.5 using 0.1 M sodium hydroxide (Merck). This electrolyte solution was mixed with ACN in a 30:70 (v/v) ratio. All mobile phases were filtered with a 0.2 μM filter and degassed on an ultrasonic bath. All solutions were kept at 4 °C.

Packaging of the capillary columns

A slurry packing method was used to fill 33.5 cm (25 cm effective length) fused-silica capillaries (100 μm i.d. × 375 μm o.d.; Composite Metal Services, Hallow, Worcestershire, UK; Matthijs et al., 2006b). Stationary-phase particles (50 mg) were suspended in 1 mL ACN, sonicated for 5 min and transferred into a slurry reservoir by means of a syringe. At one end of the fused silica capillary, a temporary frit was made while the other end was connected to the reservoir containing the CSP slurry. An external air-driven pressure pump (Haskel, Burbank, CA, USA) of 600 bar was connected to the reservoir to force the particles into the column for a length of about 30 cm. A mechanical shaker was put on the reservoir to prevent particle precipitation during the filling process. The packed column was rinsed with ACN for about 20 min, after which the inlet and outlet frits were burned, 25 cm from each other, with a capillary burner (Capital HPLC, Broxburn, West Lothian, Scotland) by local heating at a low temperature for 40 s. After removing the temporary frit, the excess stationary phase was rinsed away by flushing the capillary in a reverse direction with ACN. A detection window was burned closely behind the outlet frit with the capillary burner at a low temperature for 15 s. Then the capillary was rinsed with the analyzing mobile phase for at least 1.5 h at a 100 bar pressure using a flow-splitted L-6000 HPLC pump (Merck-Hitachi, Tokyo, Japan). Finally the capillary was preconditioned with the mobile phase by applying 5, 10, 15, 20 and 25 kV during 10 min each. All CSPs were kindly donated by Professor B. Chankvetadze, Tbilisi State University, Georgia.

Capillary electrophromatography

An Agilent Technologies CE system® (Waldbornn, Germany), with a UV–vis diode array detector set at 214 nm was used for the CEC experiments. The temperature of the capillary column (25 °C) was controlled by an air thermostated system and the samples were kept at room temperature.

The samples were electrokinetically introduced in the capillary column by applying 10 kV during 20 s. A mobile phase plug was injected behind the sample at 5 kV for 5 s to prevent back-migration of the sample upon application of the electrical field. A voltage of 10 kV in normal polarity was applied to elute the samples. A maximum run time of 60 min was set. Gas bubble formation was prevented by applying a pressure of 5.5 bar on both vials during analysis. The vial content was replaced every 60 min to avoid buffer depletion (Mangelings et al., 2003).
Data processing

Retention times (t₁₀), resolutions (Rₛ) and theoretical plate numbers (N) were collected using Agilent ChemStation Software (Agilent Technologies 1994, 1995–2006). The unretained compound thiourea (dead time marker) was injected six times to determine the average t₀ value. In this paper, the analysis time (AT) is either the retention time of a nonseparated compound or that of the last eluting enantiomer when the compound is resolved. The retention factors (k) were then calculated. In case of a (chiral) separation, the retention factor of the first eluting peak of a pair was selected for evaluation. The retention factor value should not be too high; values above 20 indicate an excessive retention. In CEC negative k-values are expected for positively charged compounds when eluting in normal-polarity mode. In these cases, compounds will migrate faster than the neutral marker owing to their own electrophoretic mobility towards the cathode. However, in our case study it is desirable to avoid ionized analytes because chiral polysaccharide-based selectors are neutral selectors and will only interact limitedly with charged species (Tachibana and Ohnishi, 2001).

The number of theoretical plates or efficiency N is an indication of the column performance (separation power) and is calculated by:

\[ N = \frac{5.54 \times \tau_0^2}{W^2} \]  

(1)

In analogy with the retention factor, in case of a chiral separation, the number of theoretical plates of the first eluting peak of a pair was selected for evaluation. The resolution was calculated with peak widths at half height (Rathore and Horváth, 2001). In the further discussion, every compound with a resolution larger than zero, that is, for which enantioselectivity was observed, is denoted as ‘separated’; ‘no result’ or NR indicates a compound that was not eluted properly or gave results that were impossible/difficult to interpret. Analyses which led to the latter type of result were repeated three times before being denoted as ‘no result’.

Results and discussion

Comparison between 3 and 5 μm particle-size packings

Enantioselectivity and separation quality. The enantioselectivity of the four 3 μm CSPs toward a test set of 44 nonacidic compounds was evaluated and compared with that of their 5 μm analogs. To allow a comparison between both types of CSPs, all compounds were analyzed applying the experimental conditions used previously on the 5 μm CSPs, that is, a 70:30 (v/v) ACN–5 mM phosphate buffer pH 11.5 mobile phase, a temperature of 25 °C and an applied voltage of 10 kV. A mobile phase with a pH of 11.5 is expected to dissolve the silica structure of the CSP, but from earlier experiments we observed that degradation is much less than expected. Probably, the surface of the silica particles is shielded to some extent by the coated selector, and thus protected against degradation (Rathore and Horváth, 2001).

In Tables 1 and 2, and Figs 1–3, the results obtained with the equivalent 3 and 5 μm CSPs are presented. In some cases, no result was obtained for a given compound on either the 3 or the 5 μm equivalent. In these specific cases, no comparison could be made in the plot. Only the compounds that gave a result for both particle sizes could be included in the plots. In the following discussion, the success rates and percentages are expressed relative to the 44 compounds considered, unless stated otherwise. In Fig. 1, the enantioselectivity of the different columns is shown, while in Figs 2 and 3 a comparison is made between the resolutions and selectivity factors, respectively, obtained on the 3 and 5 μm phases.

In general, with the exception of LA2, the 3 μm packings separated more compounds than their 5 μm analogs (Tables 1 and 2 and Fig. 1). For the baseline separations, with the exception of LA2, similar numbers were found on both 3 and 5 μm CSPs. The smaller number of baseline separations and the deviating enantioselectivity for the 3 μm LA2 column might be partially owing to a lower efficiency (possibly resulting from a sub-optimal packing). The above observations are examined in a second part of this study by evaluating in more detail the efficiencies of both 3 and 5 μm particle-size CSPs by means of Van Deemter curves (Hendrickx et al., 2013).

The broadest enantioselectivity was seen on 5 μm LA2 which separated 25 analytes. The CSP 3 μm Sp5 followed closely with 23 separated compounds. Third and fourth most enantioselective were 3 μm LC4 (20 separations) and 3 μm LC2 (18 separations), respectively. The remaining CSPs separated 17 or fewer compounds.

Figure 2 shows the resolutions obtained on the 3 μm CSPs plotted against those obtained with their 5 μm analogs. These plots allow determination of whether the 3 or 5 μm has the best separation tendency. When the majority of data points lie either above or below the solid black line (equal resolution), one can conclude that either the 5 or 3 μm column, respectively, gives the best separation. In accordance with the above, for LC2, LC4 and Sp5, the majority of data points were below the line, meaning that the 3 μm columns, besides being more enantioselective, also provided better separations. The opposite is seen for LA2. When the selectivity factors on both columns are plotted (Fig. 3) figures rather similar to Rₛ are obtained.

This indicates that the enantioselectivity of a system can be evaluated not only the α values but also from the resolutions:

\[ α = \frac{k_2}{k_1} \]

(2)

with k₂ and k₁ the retention factor of the last and first eluting peak, respectively.

In addition, a difference between the two particle-size columns at the level of separation quality was also observed. Moreover, a number of compounds show enantioselectivity on one particle size of the CSP-type, while for the other column they did not. The reason for this difference in enantioselectivity between the two particle-size columns could possibly be found when considering also the retention factors or retention times. When on one column the retention times become too short, the resolution may be lost. This is a loss of separation owing to a too fast elution.

The 3 μm LC2, 3 μm LC4 and 3 μm Sp5, and the 5 μm LA2 CSPs separated the highest numbers of compounds relative to their equivalent counterparts. Moreover, on the LC2, LC4 and Sp5 phases, higher N values (higher efficiencies) generally were obtained with the 3 μm columns (Fig. 4).

For the LA2 phase, the highest efficiencies mostly were seen on the 5 μm column. The smaller efficiencies obtained with 3 μm LA2, may result from material differences on a sub-optimal column packing procedure.

For LC2, nine compounds, partially separated on the 3 μm column, were not on the 5 μm column, that is, atropine, betaxolol, bopindolol, dimethindene, esmolol, flouoxetine, promethazine, sotalol (NR) and tertatolol (Tables 1 and 2 and Fig. 2). Evaluation
of their analysis times (Fig. 5) and retention factors revealed that on the 3 μm column both parameters were higher. On the other hand, praziquantel and sulpiride only showed enantioselectivity on the 5 μm particles.

The 5 μm LA2 CSP separated 11 compounds that were not separated on the 3 μm column. Here the difference in analysis times was less clear (Fig. 5). A number of the test compounds eluted fastest on the 3 μm column, the others on the 5 μm column. Lorazepam, nimodipine and oxazepam were only separated by the 3 μm particle-based CSP.

The 3 μm LC4 column separated six compounds, that is, bupranolol, cefprozil, dimethindene, promethazine, methadone, and metoprolol, which were not separated by its 5 μm analog, despite the lower AT and retention factor values on the 3 μm column.
The 5 μm LC4 column separated five compounds that were not separated by 3 μm LC4, that is, acebutolol, ambucetamide, cinnarizine, meptazinol and praziquantel. For the final CSP, Sp5, the 3 μm column separated nine extra compounds. These compounds all had higher analysis times on the 3 μm CSP (Fig. 5). Mebeverine and sulpiride were only separated by 5 μm Sp5.

In general, a difference in enantioselectivity between the two analog columns with different particle size CSPs was seen. Three out of four 3 μm columns (LC2, LC4 and Sp5) were more enantioselective than their 5 μm counterparts. The observations for the column efficiencies are in agreement with the obtained resolutions; higher efficiencies were seen on 3 μm LC2, LC4 and Sp5.
Cumulative success rates on 3 or 5 μm packings. After evaluation of the enantioselectivity and complementarity on the 3 μm CSPs, the column sequence which gave the highest cumulative number of separated compounds was 3 μm Sp5 > 3 μm LC2 > 3 μm LA2 > 3 μm LC4 (Fig. 6). This sequence was determined as follows. The column with the highest number of separations (broadest enantioselectivity) was selected first, followed by that with the highest number of additionally separated compounds (maximal complementarity). Twenty-three compounds (52%) were separated on 3 μm Sp5. On 3 μm LC2, 3 μm LA2 and 3 μm LC4, an additional eight, six and seven compounds, respectively, were separated. Therefore LC2 was selected as the second column. The third and fourth columns were selected similarly. Figure 5 illustrates that the inclusion of the 3 μm LC4 in the column sequence does not increase the number of separated compounds. However, the selection of three 3 μm CSPs could only baseline separate nine compounds at the considered conditions, whereas all four CSPs were able to baseline separate 11 compounds. When defining the screening approach of a separation

**Figure 1.** Number of partially (dark grey) and baseline separated (light grey) compounds on eight columns. The values above the stacked bars are the total numbers of separations. Experimental conditions: 70:30 (v/v) ACN–5 mM phosphate buffer pH 11.5. Capillary temperature: 25 °C; Analyzing voltage: 10 kV.

**Figure 2.** Resolutions, obtained on the equivalent 3 and 5 μm packings for 44 basic, neutral, and amphoteric compounds; 70:30 (v/v) ACN–5 mM phosphate buffer pH 11.5. Capillary temperature, 25 °C; analyzing voltage, 10 kV.
strategy, a more economical alternative would be to examine the latter column only in a later optimization step of the strategy. In that case only a limited number of compounds, instead of the entire test set, would be examined on LC4. Cumulatively, the four CSPs separated 34 out of 44 compounds (77%) of which eleven (25%) were baseline separated. Compounds that could

Figure 3. Selectivity factors, obtained on the equivalent 3 and 5 µm packings for 44 basic, neutral and amphoteric compounds; 70:30 (v/v) ACN–5 mM phosphate buffer pH 11.5. Capillary temperature, 25 °C; analyzing voltage, 10 kV.

Figure 4. Efficiencies, expressed as $N$, the number of theoretical plates obtained on the equivalent 3 and 5 µm packings for 44 basic, neutral and amphoteric compounds; 70:30 (v/v) ACN–phosphate buffer pH 11.5. Capillary temperature, 25 °C; analyzing voltage, 10 kV.
not be separated on any CSP were carbinoxamine, chlorthalidone, cinnarizine, ephedrine, labetalol, meptazinol, nadolol, nitrendipine, propiomazine and sulpiride.

Of the four 5 μm columns tested, 5 μm LA2 showed the broadest enantioselectivity, separating 57% of the analyzed substances (25/44). With 5 μm LC4 and 5 μm Sp5 an enantioselectivity of approximately 37% each could be achieved, while LC2 separated 25% of the 44 compounds (Table 2 and Fig. 1). The 5 μm LA2 column was thus chosen to be the first column for the screening step when only considering 5 μm columns. Compared with 5 μm LA2, 5 μm LC2 added four additional separations, 5 μm LC4 added five and 5 μm Sp5 added seven. Thus 5 μm Sp5 was chosen as the second and 5 μm LC4 as the third column. The 5 μm LC2 gave no additional (baseline) separations compared with the first three. In summary, the preferred column sequence for the used dataset was 5 μm LA2 > 5 μm Sp5 > 5 μm LC4 > 5 μm LC2. Here again, one now might eliminate LC2 from the screening and include it in an optimization step of a separation strategy. Cumulatively, 80% of the compounds (35/44) could be separated, of which 13 were baseline separated (30%). Analytes that were not separated on any of the 5 μm CSPs were carbinoxamine, ephedrine, labetalol, lorazepam, nimodipine, nitrendipine, promethazine, sotalol and verapamil.

From the above results, both 3 and 5 μm CSPs seem to be comparable in their complementarity, that is, the 5 μm column combination separated only one compound more than the 3 μm CSPs. However, based on the tendencies in Figs 1–3, one would expect a higher number of separations on the 3 μm columns.

Complementarity between the 3 and 5 μm phases is also seen since only four compounds were not separated on any column, while the individual particle sizes did not separate 10 and nine substances, respectively. Summarizing, we have to conclude that the success-rate differences between the 3 and 5 μm columns are quite small when three or four CSPs are considered.

Figure 5. Analysis times, obtained on the equivalent 3 and 5 μm packings for 44 basic, neutral and amphoteric compounds; 70:30 (v/v) ACN–5 mM phosphate buffer pH 11.5. Capillary temperature, 25 °C; analyzing voltage, 10 kV.

Figure 6. Cumulative separation results of 44 compounds on 3 μm (black) and 5 μm (gray) chlorinated polysaccharide-based chiral stationary phases. (■) Number of partially and baseline-separated compounds; (●) number of baseline-separated compounds.
However, the column-manufacturing process using 3 μm particles is more tedious than with 5 μm particles because the smaller particles tend to precipitate faster in the slurry solvent and reservoir. Because of the similar cumulative success rates and the more difficult fabrication of 3 μm columns, the 5 μm columns are preferred from this study.

To conclude, it can be stated that, although individually the 3 μm CSPs seem to be more enantioselective than the 5 μm CSPs, cumulatively both types result in a similar number of separated compounds (Fig. 6). Chlorthalidone, cinnarizine, meptazinol, nadolol, propiomazine and sulpiride were not separated by any of the 3 μm CSPs but could be separated on the 5 μm CSPs. On the other hand, the 5 μm CSPs were not able to separate lorazepam, nimodipine, sotalol and verapamil. Thus, as indicated higher, a selectivity difference exists between the column types.

**Analysis time.** As mentioned above, the AT is either the retention time of a nonseparated compound or that of the last eluting enantiomer. It is expected that 5 μm particle size columns generate faster analyses. The stationary-phase bed generally has both small mesopores and large through-pores. The small mesopores give rise to a large surface area beneficial for the resolution of (chiral) compounds. On the other hand, the distribution and size of the large pores influence the column efficiency and the mobile phase flow; the flow channels are wider and thus easier to pass through (less resistance). A large through-pore size thus offers high column permeability, allowing the mobile phase to flow faster. However, this is at the expense of efficiency since a wide through-pore size distribution results in an increase in eddy diffusion contribution in the van Deemter equation (Hendrickx et al., 2013; Eeltink et al., 2005; Wang et al., 2012).

Furthermore, a wider flow channel implies the compounds to be in a less close contact with the stationary phase, resulting in less retention or smaller retention times. Faster analyses in combination with a decreased interaction with the stationary phase are thus the expected results of a faster mobile phase flow.

When comparing the ATs obtained on the 3 and 5 μm columns (Tables 1 and 2, Fig. 5), it was observed that the retention times on 5 μm LC2, and 5 μm Sp5 tend to be shorter than on their 3 μm analogs and thus acted as expected. On LA2, the analyses on 3 and 5 μm particles did not require similar analysis times, but similar numbers of compounds eluted faster on the two columns. With LC4, the majority of compounds eluted faster on the 3 μm CSP, although for a limited number of compounds the opposite was true. Overall, 5 μm LC2 and 5 μm Sp5 produced the shortest ATs and 5 μm LC4 the longest (Fig. 5). The long retention times of 5 μm LC4 might be the result of nonenantioselective retention.

ATs above 20 min are less desirable, certainly in industry where large sets of molecules have to be analyzed in a limited time. On 3 and 5 μm LC2, 3 and 5 μm LA2, and 5 μm Sp5, most compounds eluted within 20 min. Nine, 11 and 24 compounds eluted after 20 min on 3 μm Sp5, 3 μm LC4 and 5 μm LC4, respectively. However, the ATs can be reduced by increasing the applied voltage (but with the risk of losing the separation) in an optimization step of the separation strategy (Hendrickx et al., 2010, 2011a).

**Conclusions**

Four 3 μm polysaccharide-based CSPs were evaluated for their enantioselectivity towards 44 compounds and their results were compared with those on the equivalent 5 μm CSPs using the same experimental conditions. Except for one, LA2, the 3 μm packings separated more compounds than their 5 μm counterparts. Although individually the 3 μm CSPs seemed to be more enantioselective than the 5 μm CSPs, their combination to maximize the number of cumulative separations resulted in a similar number of separated compounds on three CSPs per size. The reason is that the 3 and 5 μm phases show some complementarity.

When considering the analysis times, the 5 μm LC2 and 5 μm Sp5 CSPs provided faster analyses than their 3 μm counterparts. On LA2, the analysis times were diverse for both particle sizes, and in case of LC4 the 3 μm packing was the fastest.

As mentioned above, better enantioselectivities were observed on 3 μm LC2, LC4 and Sp5. This corresponds with their higher efficiencies compared with their 5 μm analogs. However, 5 μm LA2 was found to be more efficient than its 3 μm analog. This latter result is in contrast to what is expected theoretically and therefore will be investigated in a continuation of this study.

To conclude, although the 3 μm particle sizes are associated with a more difficult manufacturing process, which also may be the reason for the observed discrepancy on the LA2 columns, they seem to be beneficial for the enantioselectivity and efficiency. In a future study the column efficiency might thus be optimized by refining the column packing procedure.

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