Special issue paper

Biomedical Chromatography

Received: 28 June 2013, Revised: 16 September 2013, Accepted: 20 September 2013 Published online in Wiley Online Library: 18 October 2013

(wileyonlinelibrary.com) DOI 10.1002/bmc.3071

Three versus five micrometer chlorinated polysaccharide-based packings in chiral capillary electrochromatography: efficiency and precision evaluation

Ans Hendrickx, Katrijn De Klerck, Debby Mangelings, Lies Clincke and Yvan Vander Heyden*

ABSTRACT: In an earlier part of this study (performance evaluation) it was observed, for home-made capillary electrochromatography (CEC) columns, that smaller particle diameters do not always generate higher efficiencies. This phenomenon was further examined in this study, evaluating Van Deemter curves. Naphthalene and *trans*-stilbene oxide were analyzed on four 3 μm and four 5 μm chlorinated polysaccharide-based chiral stationary phases (CSPs) applying voltages ranging from 5 to 30 kV. Neither the 3 nor the 5 μm packings generated systematically the highest efficiencies. The varying column efficiencies were optimized by evaluating nine packing procedures for both 3 and 5 μm CSPs. Again it was observed that smaller particle-size packings were not necessarily beneficial for the efficiency of the CEC analysis. This observation was statistically evaluated. A variability study evaluated different precision estimates related to column packing and replicate measurement conditions. The best columns with the highest efficiencies (for chiral separations) and good precision, that is, the lowest RSD values, were generated by the packing procedure in which an MeOH-slurry and a water rinsing step of 8 h were applied. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: chiral electrochromatography; chlorinated polysaccharide-based selectors; 3 vs 5μm packings

Introduction

Capillary electrochromatography is an analytical technique which is receiving increasing interest, especially in the field of chiral separations. In theory, such a technique with an electrodriven flow results in all analyte molecules appearing in sharp peaks, keeping band broadening small. When band broadening is limited, high peak efficiencies, which are important for complex samples, are achieved. Chiral separations also benefit from these increased efficiencies because better separations can be obtained with higher resolutions (Hendrickx *et al*., 2011a).

Theoretically, it is expected that smaller particle diameters increase the column efficiency (Eeltink, 2005; McDonald and Neue, 2009; Rathore and Horváth, 2001). This phenomenon is illustrated by the Van Deemter equation and curve. The equation assembles the key factors that contribute to on-column band broadening and thus affect the efficiency. The equation describes the plate height H (height of a theoretical plate) as the sum of the contributions of three principal processes, that is, eddy diffusion, longitudinal diffusion and mass transfer:

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{f_1(k) d_p^2}{D_m} u + \frac{f_2(k) d_p^2}{D_s} u = A + \frac{B}{u} + C_m u + C_s u \quad (1)$$

where H is the plate height, λ the packing characterization factor, which is related to the particle shape and about 1 for monodisperse spherical particles, d_p the particle diameter, γ, $f_1(k)$ and $f_2(k)$ constants, u the linear velocity, D_m the diffusion coefficient of the mobile phase and D_s the diffusion coefficient of the stationary phase.

The A term is the eddy diffusion term and describes the dispersion of analytes in the spaces between the particles owing to changes in velocity (direction and magnitude) of the mobile phase. The B term is the longitudinal diffusion in the mobile phase, owing to Brownian motion, that is, the random drift of particles suspended in a fluid, and to the laminar flow profile between the particles. The C term represents the lack of mass equilibrium in the mobile phase (C_m) and stationary phase (C_s) and comprises the $C_m u + C_s u$ term in the Van Deemter equation. Equation 1 shows that, by using a smaller particle diameter, the A and C terms and consequently the plate height will decrease. Since the efficiency N is equal to the effective length (packed length) of the column divided by the plate height, a smaller plate height implies a better efficiency (Eeltink, 2005; McDonald and Neue, 2009; Rathore and Horváth, 2001).

In earlier research on the performance evaluation, it was studied whether 3 μm particle size packings indeed perform better than their 5 μm counterparts by comparing the obtained retention times (t_R), retention factors (k), efficiencies (N), and

* Correspondence to: Vander Y. Heyden, Analytical Chemistry and Pharmaceutical Technology, Center for Pharmaceutical Research, Vrije Universiteit Brussel-VUB, Brussels, Belgium. Email: yvanvdh@vub.ac.be

Analytical Chemistry and Pharmaceutical Technology, Center for Pharmaceutical Research, Vrije Universiteit Brussel-VUB, Brussels, Belgium

Abbreviations used: ACN, acetonitrile

Copyright © 2013 John Wiley & Sons, Ltd.

resolutions (R_s) (Hendrickx *et al*., 2013). Four chlorinated polysaccharide-based CSPs, that is, amylose *tris* (5-chloro-2-methylphenylcarbamate) (Lux Amylose-2; LA2), cellulose *tris* (4-chloro-3-methylphenylcarbamate) (Lux Cellulose-4; LC4), cellulose *tris* (3-chloro-4-methylphenylcarbamate) (Lux Cellulose-2; LC2), and cellulose *tris* (3,5-dichlorophenylcarbamate) (Sepapak-5; Sp5), each of 3 and 5 μm particle size, were used to analyze a test set of 44 structurally and chemically diverse nonacidic compounds. All but one 3 μm packings were able to separate more compounds than their 5 μm counterparts. It was also observed that, although individually the 3 μm CSPs usually were more enantioselective than the 5 μm CSPs, a combination of their results produced a similar cumulative number of separated compounds on both particle sizes because of a complementary behavior of the 3 and 5 μm columns. Some separations were obtained on one particle-size column, but not on the other. Furthermore, on the 3 μm CSPs fewer baseline separations were obtained. Because of these unexpected results, it was decided to evaluate the efficiencies of both 3 and 5 μm packings more thoroughly by constructing Van Deemter curves.

To assess which particle diameter allows the highest efficiencies to be obtained, two compounds, naphthalene and *trans*-stilbene oxide, were analyzed at 13 different applied voltages on both the 3 and 5 μm chlorinated CSPs, that is, LA2, LC4, LC2 and Sp5. *Trans*-stilbene oxide was selected as a chiral compound that can be rather easily separated. Its information reflects the chiral interaction with the stationary phase. Naphthalene is a rather apolar achiral compound, whose retention also reflects nonenantioselective interactions with the stationary phase.

Furthermore, the influence of the packing method on the efficiency was evaluated by testing 18 different columns (nine different packing procedures, two particle sizes) at 13 different applied voltages. The three columns that allowed obtaining the highest efficiencies out of the 18 columns were then produced six times and their retention times, peak areas, efficiencies and resolutions were determined to evaluate the between-column variance, injection variability, method repeatability (intra-day variance), inter-day variance and time-dependent intermediate precision.

Materials and methods

Chemicals and reagents

The achiral compound naphthalene (Fluka, Buchs, Switzerland) and the chiral compound *trans*-stilbene oxide (Aldrich, Steinheim, Germany) were used to produce the Van Deemter curves. Both substances were dissolved in ultrapure water–acetonitrile (ACN) (30:70, v/v) at a concentration of 0.5 mg/mL. Ultrapure water was prepared in-house by an Arium Pro UV instrument (Sartorius, Vilvoorde, Belgium). A 5 mM disodium hydrogen phosphate (Na_2HPO_4) (Merck) solution in ultrapure water, adjusted to pH 11.5 with 0.1 M sodium hydroxide (Merck) was the mobile phase electrolyte. The electrolyte was mixed with ACN (HPLC-grade, Fisher, Leicestershire, UK) in a 30:70 (v/v) ratio. All mobile phases were filtered through a 0.2 μm filter and degassed on an ultrasonic bath before use. All solutions were kept at 4°C.

Packing of the capillary columns

The fused silica capillaries (100 μm i.d. × 375 μm o.d.; Composite Metal Services, Hallow, Worcestershire, UK) were packed based on the slurry packing method of Hendrickx *et al*. (2010, 2011b). The stationary phase (50 mg) was suspended in 1 mL slurry solvent (see Table 1), sonicated for 5 min, and then transferred into the slurry reservoir by means of a syringe. All CSPs were kindly donated by Professor B. Chankvetadze, Tbilisi State University, Georgia.

At one end of the fused-silica capillary a temporary frit was produced; the other end was connected to the reservoir containing the slurry of the packing material. An external air-driven pressure pump from Haskel (Burbank, CA, USA) of about 600 bar was applied to the reservoir to push the particles into the column for a length of about 35 cm (total/effective lengths of 33.5/25.0 cm). The slurry reservoir was shaken mechanically to prevent precipitation of the particles. After filling the column it was rinsed with a flow-splitted L-6000 HPLC pump (Merck-Hitachi, Tokyo, Japan) or an air-driven pump with solvents and rinsing times specified in Table 1. 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. The excess stationary phase was then removed by rinsing (HPLC pump) the capillary in a reversed direction with the solvent specified in Table 1 and finally the detection window was burned closely behind the outlet frit with the capillary burner at a low temperature for 20 s. Before new columns were used, they were preconditioned by applying 5, 10, 15, 20 and 25 kV for 10 min each.

Columns for efficiency measurements were made with the conditions of procedure 1 in Table 1 because this packing procedure was used in previous studies (Hendrickx *et al*., 2010, 2011b). To evaluate the influence of the packing methods, different procedures were applied (Table 1). The solvent, in which the stationary phase slurry was suspended, was ACN, ACN–ultrapure water (50:50, v/v) or methanol (MeOH). After column filling with the stationary phase, the capillary was rinsed for 20 min with ACN (100 bar, HPLC pump), for 60 min with ACN–H_2O (50:50, v/v) (100 bar, HPLC pump), or for 8 h with ultrapure water (600 bar, air-driven pressure pump) (Eeltink, 2005; Mangelings *et al*., 2003).

From these packing methods, the three best procedures were selected to produce six capillaries for the variability study.

Table 1. Packing procedures

Packing procedure	Slurry solvent	Rinsing method
1	ACN	20 min ACN
2	MeOH	
3	ACN–H_2O (50:50, v/v)	
4	ACN	60 min ACN–ultrapure water (50:50, v/v)
5	MeOH	
6	ACN–H_2O (50:50, v/v)	
7	ACN	8h Ultrapure water
8	MeOH	
9	ACN–H_2O (50:50, v/v)	

Copyright © 2013 John Wiley & Sons, Ltd.

Capillary electrochromatography

An Agilent Technologies CE system (Waldbronn, Germany) equipped with a UV–vis diode array detector was used in this study and detection was performed at 214 nm. An air-thermostated system controlled the temperature of the capillary column (25°C) and the samples were kept at room temperature. The samples were electrokinetically injected by applying 10 kV during 20 s. To prevent sample back-migration after applying the electrical field, a mobile phase plug was injected (5 kV for 5 s) behind the sample. Different voltages (5–30 kV) were applied to elute both compounds in normal polarity mode. Bubble formation was prevented by applying a pressure of 5.5 bar on both vials during analysis. Buffer vials were replaced every 60 min to avoid buffer depletion (Mangelings *et al*., 2003). When mobile phases were changed, the column was rinsed for at least 1.5 h with a pressure around 100 bar using a flow-splitted HPLC pump.

Data processing

Agilent ChemStation for CE Systems (Agilent Technologies, 1994, 1995–2006) was used to collect and process retention times (t_R), resolutions (R_s), number of theoretical plates (N), peak areas and peak widths (W).

The efficiency N indicates numerically the column performance (separation power) and is given by:

$$N = \frac{5.54 \times t_R^2}{W_{1/2}^2} \quad (2)$$

The plate height (H; y-axis in Van Deemter curve) can easily be calculated starting from N because H also equals the length of the capillary up to the detection window (L_{eff}) divided by the plate height (Eeltink, 2005).

$$N = \frac{L_{eff}}{H} \quad (3)$$

The flow rate or linear velocity (u) is the volume of mobile phase passing through the capillary in unit time (Rathore and Horváth, 2001):

$$u = \frac{L_{eff}}{t_0} \quad (4)$$

with t_0 the migration time of a neutral and inert tracer, for example, thiourea.

The compound mobility (μ; x-axis in Van Deemter curve) is given by (Rathore and Horváth, 2001):

$$\mu = \frac{L_{eff}}{t_R} \quad (5)$$

The Van Deemter data points were fitted by a curve using the Solver function in Excel (Microsoft Corporation, 2010) and a Van Deemter model provided by G. Desmet, Department of Chemical Engineering, Vrije Universiteit Brussel, Brussels, Belgium. Standard deviations and relative standard deviations were calculated for the retention time, resolution, and peak area.

The between-column variance and the injection variability were estimated by injecting three times the same sample on six columns. The corresponding variances are estimated by the one-way ANOVA approach from $MS_{(between\text{-}columns)}$ and $MS_{(injection)}$.

For the method repeatability (intra-day variance) and the time-dependent intermediate precision estimations, one injection of three individually prepared samples was performed on one column for six consecutive days (18 injections). Again, the variances are estimated by a one-way ANOVA from $MS_{(inter\text{-}day)}$ and $MS_{(intra\text{-}day)}$.

Results and discussion

Van Deemter curves

In a preceding study, three out of four 3 μm CSPs separated more compounds of the test set than their corresponding 5 μm counterparts (Hendrickx *et al*., 2013). However, when considering the number of baseline separations, 5 μm LC2 and LA2 resolved more compounds than the equivalent 3 μm columns that is, four vs three, and 10 vs five compounds, respectively. For LC4 and Sp5, the number of baseline separations was the same for both 3 and 5 μm CSPs, that is, four and five compounds, respectively. In this second part of the study, these unexpected results were explained/confirmed by comparing the efficiencies obtained for both particle sizes. The possibility exists that a lower efficiency than expected of the 3 μm CSPs leads to lower resolution values and thus fewer baseline separations. For the chiral compound, *trans*-stilbene oxide, the N-value of the first eluting enantiomer was evaluated.

Two compounds, naphthalene and *trans*-stilbene oxide, were analyzed on four 3 μm and four 5 μm CSPs, that is, LC2, LA2, LC4 and Sp5, using a 70:30 (v/v) ACN–phosphate buffer pH 11.5 and analyzing voltages ranging from 5 to 30 kV (Tables 2 and 3). The Van Deemter curves were drawn in order to facilitate the comparison but also to determine which voltage needing to be applied to achieve the highest efficiencies (Fig. 1).

For the achiral compound, naphthalene, and the chiral compound, *trans*-stilbene oxide, rather similar results were obtained. The Van Deemter curves for the 3 μm CSPs LC2, and Sp5 were better (lower plate heights) compared with their corresponding 5 μm phases. Figure 1 shows that very similar Van Deemter curves for both compounds were obtained for both particle sizes of LC4. For LA2, the efficiencies obtained with the 5 μm particles for the analysis of both compounds were clearly better than the efficiencies for the 3 μm particles.

The most efficient column of all eight CSPs for the analysis of naphthalene was 3μmSp5, achieving theoretical plates around 17,900 (Table 2). The optimal compound mobilities for all four 5 μm CSPs, when analyzing naphthalene, were between 0.1 and 0.4 mm/s, corresponding to applied voltages from 9 to 19 kV. For the 3 μm CSPs, these optimal mobilities were approximately 0.2–0.6 mm/s with applied voltages ranging from 11 to 23 kV (Fig. 1 left).

For *trans*-stilbene oxide the highest efficiency was obtained when 11 kV was applied to the 3 μm Sp5 capillary, that is, about 25,000 plates (Table 3). This column also allowed obtaining the highest efficiencies for naphthalene. Its Van Deemter curve is almost horizontal, making it efficient in a broad range of applied voltages (9–30 kV). All four 5 μm packings achieved their optimum velocities for the analysis of *trans*-stilbene oxide when the compound mobility was between 0.1 and 0.3 mm/s, that is, for applied voltages in the range of 7–19 kV. For the 3 μm particle-size packings the optimal mobilities also were between 0.1 and 0.3 mm/s, and these mobilities were obtained with applied voltages between 9 and 17 kV (Fig. 1 right).

Copyright © 2013 John Wiley & Sons, Ltd.

Table 2. Results of naphthalene analyzed on four 3 μm and four 5 μm CSPs applying different analyzing voltages

Voltage (kV)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)
		5 μm LC2			*3 μm LC2*			*5 μm LA2*			*3 μm LA2*	
5	69.6	7766	32.2	51.9	8,237	30.4	70.4	5,952	42.0	26.6	6,126	40.8
7	43.2	10,633	23.5	25.9	9,867	25.3	53.1	9,945	25.1	21.6	6,248	40.0
9	25.5	13,340	18.7	19.7	10,567	23.7	36.7	12,948	19.3	17.3	7,031	35.6
11	17.4	12,706	19.7	16.3	10,568	23.7	25.3	13,961	17.9	14.3	7,342	34.1
13	12.1	11,373	22.0	13.7	11,370	22.0	18.0	13,539	18.5	11.9	7,343	34.0
15	10.5	11,059	22.6	11.6	11,505	21.7	13.1	11,555	21.6	10.2	7,435	33.6
17	9.5	9,996	25.0	10.5	12,051	20.7	10.6	10,655	23.5	8.6	7,088	35.3
19	8.3	10,094	24.8	9.4	12,462	20.1	9.6	9,801	25.5	7.7	7,137	35.0
21	7.2	9,292	26.9	8.4	12,908	19.4	8.5	9,876	25.3	7.1	7,251	34.5
23	6.4	8,743	28.6	7.4	12,676	19.7	7.4	9,394	26.6	6.3	7,029	35.6
25	5.6	7,946	31.5	6.5	12,026	20.8	6.4	8,468	29.5	5.4	6,567	38.1
27	4.9	7,235	34.6	5.7	11,402	21.9	5.7	8,348	29.9	4.9	6,422	38.9
30	4.4	6,058	41.3	5.0	11,211	22.3	4.8	7,933	31.5	4.0	6,300	39.7
		5 μm LC4			*3 μm LC4*			*5 μm Sp5*			*3 μm Sp5*	
5	64.6	7,306	34.2	55.0	6,499	38.5	71.3	9,230	27.1	39.9	8,690	28.8
7	45.8	9,705	25.8	40.3	9,626	26.0	51.7	9,843	25.4	31.4	10,526	23.8
9	32.4	11,189	22.3	31.2	11,197	22.3	36.5	12,523	20.0	25.1	12,260	20.4
11	24.8	12,275	20.4	24.9	12,552	19.9	23.7	14,047	17.8	20.5	14,098	17.7
13	19.6	12,808	19.5	19.8	13,181	19.0	16.0	12,977	19.3	18.7	15,109	16.5
15	15.7	12,561	19.9	15.5	12,651	19.8	11.5	10,803	23.1	16.3	16,044	15.6
17	13.2	13,157	19.0	13.2	12,594	19.9	10.8	10,082	24.8	15.5	17,412	14.4
19	10.9	12,876	19.4	11.7	12,850	19.5	9.8	9,865	25.3	13.0	17,933	13.9
21	9.1	11,429	21.9	10.3	13,016	19.2	8.8	9,668	25.9	10.1	17,806	14.0
23	7.4	10,695	23.4	9.0	12,715	19.7	8.0	9,220	27.1	7.8	14,603	17.1
25	6.0	10,100	24.8	8.0	12,006	20.8	7.1	9,193	27.2	6.1	10,694	23.4
27	5.0	8,375	29.9	6.9	10,826	23.1	6.3	9,457	26.4	5.5	9,363	26.7
30	3.1	6,667	37.5	5.8	8,997	27.8	5.8	9,322	26.8	5.1	9,212	27.1

t_R, Retention time; N, number of theoretical plates; H, plate height.

Copyright © 2013 John Wiley & Sons, Ltd.

Table 3. Results of *trans*-stilbene oxide (TSO) analyzed on four 3 μm and four 5 μm CSPs applying different analyzing voltages

Voltage	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s
	5 μm LC2					*3 μm LC2*					*5 μm LA2*					*3 μm LA2*				
5	39.6	14,191	17.6	1.18	5.91	48.8	15,805	15.8	1.17	3.88	59.0	10,078	24.8	1.17	4.73	48.6	6,899	36.2	1.18	5.20
7	30.9	14,368	17.4	1.19	5.02	35.7	18,335	13.6	1.18	4.30	39.2	11,182	22.4	1.19	4.69	34.6	8,001	31.2	1.18	5.14
9	24.7	15,041	16.6	1.18	5.09	28.2	19,963	12.5	1.17	4.44	31.4	11,114	22.5	1.19	4.67	24.2	8,335	30.0	1.18	4.25
11	20.1	14,956	16.7	1.18	5.03	22.6	21,339	11.7	1.17	4.51	26.7	11,252	22.2	1.18	4.66	20.1	8,987	27.8	1.19	4.23
13	16.9	14,613	17.1	1.17	5.60	18.3	21,581	11.6	1.17	4.31	22.8	10,987	22.8	1.17	4.79	19.6	10,331	24.2	1.18	4.64
15	13.6	12,931	19.3	1.18	4.54	15.3	19,448	12.9	1.17	4.34	19.8	11,002	22.7	1.18	4.57	17.4	11,257	22.2	1.19	4.26
17	12.6	12,583	19.9	1.18	4.59	13.8	20,883	12.0	1.17	4.42	17.5	11,406	21.9	1.18	4.57	15.1	10,349	24.2	1.18	4.35
19	11.4	12,538	19.9	1.17	4.46	12.4	20,872	12.0	1.17	4.28	15.4	11,551	21.6	1.17	4.50	14.1	9,698	25.8	1.15	4.29
21	9.9	10,506	23.8	1.18	4.18	10.8	20,605	12.1	1.17	4.31	14.0	11,312	22.1	1.17	4.42	11.0	9,533	26.2	1.18	4.07
23	9.3	10,445	23.9	1.17	4.11	10.0	20,299	12.3	1.17	4.25	12.4	10,912	22.9	1.17	4.28	10.5	9,276	27.0	1.18	4.11
25	8.4	9,546	26.2	1.17	3.90	9.1	20,091	12.4	1.16	4.18	11.0	10,890	23.0	1.17	4.23	9.4	9,260	27.0	1.17	4.03
27	7.5	8,448	29.6	1.17	3.63	8.2	19,678	12.7	1.16	4.04	9.8	10,484	23.8	1.17	4.08	8.4	9,115	27.4	1.17	3.94
30	6.3	7,411	33.7	1.17	3.33	7.0	18,377	13.6	1.16	3.82	8.4	10,206	24.5	1.16	3.88	7.2	8,879	28.2	1.17	3.79
	5 μm LC4					*3 μm LC4*					*5 μm Sp5*					*3 μm Sp5*				
5	46.2	18,779	13.3	1.28	8.52	59.3	13,371	18.7	1.29	7.22	58.3	14,001	17.9	1.10	2.76	50.4	12,218	20.5	1.08	2.21
7	31.3	21,388	11.7	1.28	9.31	45.7	16,668	15.0	1.29	7.99	44.0	14,289	17.5	1.10	2.80	47.4	15,860	15.8	1.08	2.49
9	24.4	20,563	12.2	1.29	9.37	36.1	18,420	13.6	1.28	8.25	35.2	15,468	16.2	1.10	2.84	39.0	20,617	12.1	1.08	2.67
11	21.1	20,143	12.4	1.29	9.28	28.2	19,186	13.0	1.27	8.27	27.0	15,764	15.9	1.09	2.77	28.2	24,983	10.0	1.07	2.76
13	19.1	20,208	12.4	1.29	9.22	21.8	17,018	14.7	1.27	7.68	20.2	13,768	18.2	1.10	2.56	18.8	22,357	11.2	1.07	2.58
15	18.1	20,212	12.4	1.29	9.35	19.6	16,768	14.9	1.28	7.94	18.5	13,967	17.9	1.10	2.57	15.6	20,652	12.1	1.08	2.65
17	16.3	19,794	12.6	1.28	9.12	17.5	17,589	14.2	1.27	7.86	16.5	12,729	19.6	1.09	2.51	14.6	22,069	11.3	1.08	2.70
19	14.3	18,315	13.7	1.28	8.55	14.7	15,731	15.9	1.26	7.19	14.6	12,586	19.9	1.09	2.47	13.1	23,735	10.5	1.07	2.70
21	12.6	16,537	15.1	1.28	8.17	12.9	16,394	15.2	1.27	7.54	13.1	11,907	21.0	1.09	2.42	11.4	22,590	11.1	1.07	2.64
23	10.4	15,444	16.2	1.33	7.12	11.7	15,695	15.9	1.26	7.29	11.8	11,990	20.9	1.09	2.38	10.2	21,686	11.5	1.07	2.59
25	9.6	15,001	16.7	1.29	7.09	10.5	14,836	16.9	1.26	6.99	10.6	11,337	22.1	1.09	2.29	9.3	21,952	11.4	1.07	2.55
27	8.9	14,624	17.1	1.28	6.88	9.3	13,821	18.1	1.26	6.70	9.4	10,816	23.1	1.09	2.19	8.4	21,157	11.8	1.07	2.49
30	7.4	13,500	18.5	1.28	6.72	8.0	12,934	19.3	1.25	6.44	7.9	9,990	25.0	1.09	2.07	7.2	21,487	11.6	1.07	2.42

t_R, Retention time; N, number of theoretical plates; H, plate height; α, selectivity; Rs, resolution.

Copyright © 2013 John Wiley & Sons, Ltd.

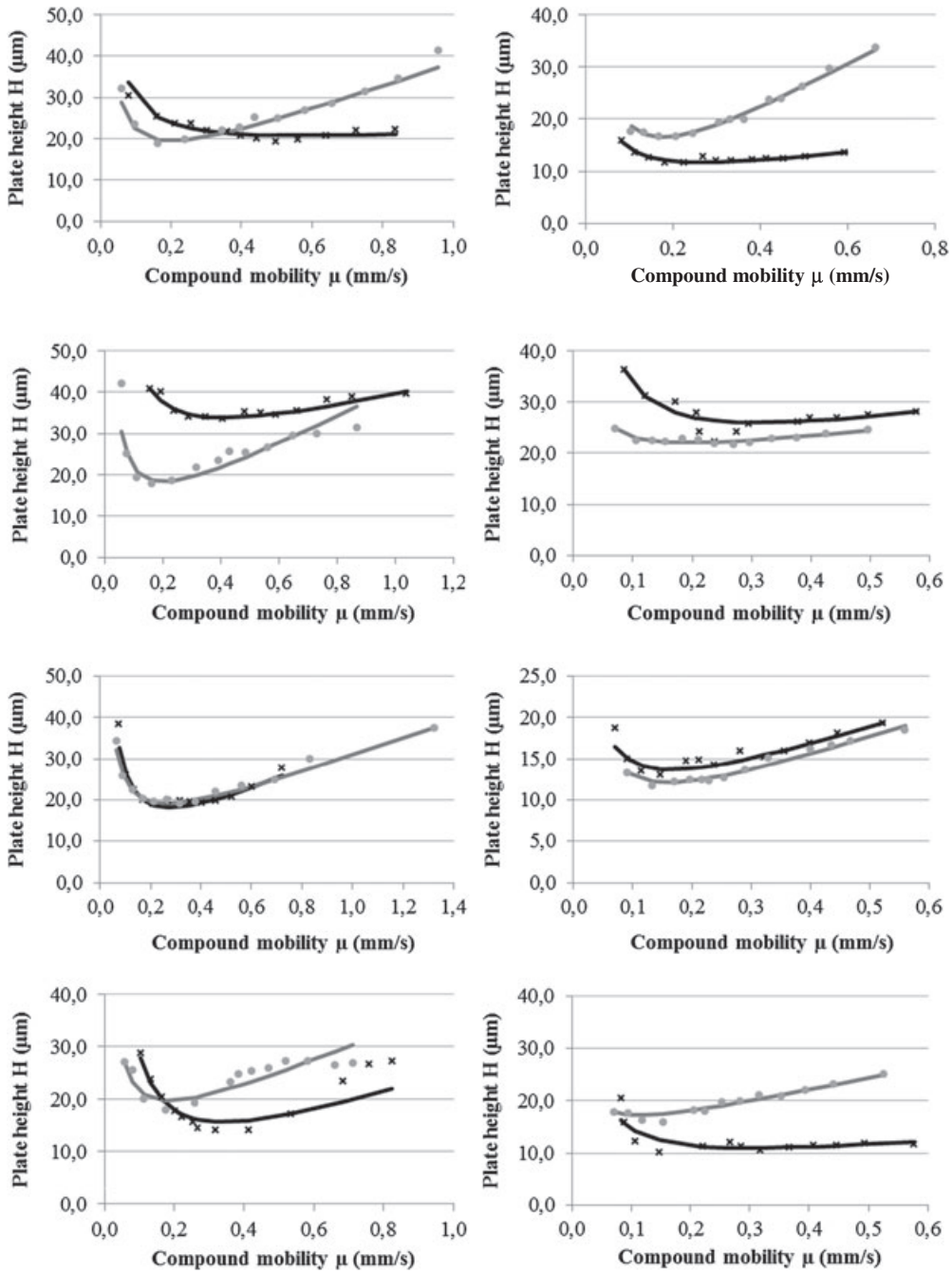

Figure 1. Van Deemter curves for naphthalene (left) and *trans*-stilbene oxide (right) analyzed on 3 μm (×) and 5 μm (●) CSPs. Experimental conditions: 70:30 (v/v) ACN–phosphate buffer pH 11.5; analyzing voltages, 5–30 kV.

Furthermore, note that, for 3 μm LC2 for both compounds, and for 3 μm Sp5 for *trans*-stilbene oxide, it is possible to work at higher analyzing voltages (higher compound mobility) because the efficiency remains practically constant (an almost horizontal C-term). Higher applied voltages may be beneficial because they are associated with shorter retention times (Table 2). This behavior was, for instance, not seen on 5 μm LC2 where the efficiency dropped rapidly when the applied voltage was increased.

Although we prefer to work with the parameter R_s, because it gives an indication of the quality of a separation, the separation factor α, describing the enantioselectivity, is also briefly discussed. When looking at one type of CSP (Table 3), the selectivities were found to be practically constant, and independent of the applied voltage, while the obtained resolution values varied much more. For LC4 the highest selectivities and resolutions were seen; the lowest α and R_s values were obtained with Sp5. The Sp5 columns thus are the most efficient columns, but certainly do not yield the best enantioseparations.

In summary, for both compounds, neither the 3 nor the 5 μm packings systematically revealed the best efficiencies. This is in contradiction to what is expected from theory, that is, smaller particle diameters should reduce band broadening and thus lead to higher efficiencies, but it confirms the results from Hendrickx *et al*. (2013). This could be due to a nonideal column packing procedure or it might be related to the CSP material

Copyright © 2013 John Wiley & Sons, Ltd.

properties. The former phenomenon is investigated in the next section by evaluating different packing procedures. Furthermore, it was also seen for the chiral separations that the most efficient column does not necessarily lead to the best chiral separation. This is a consequence of the fact that a CSP has both enantioselective and nonenantioselective interactions with the chromatographed compounds.

Improving column efficiency by adapting the packing procedure

In an attempt to study and optimize the varying column efficiencies, as seen above, several packing procedures (Table 1) were tested for both 3 and 5 μm silica particles using LA2 as CSP. LA2 was chosen because in previous studies this CSP showed the highest enantioselectivity for the analysis of nonacidic compounds and moreover the 5 μm columns behaved better than the 3 μm ones (Hendrickx *et al*., 2010).

First three different solvents, that is, ACN, MeOH and ACN–ultrapure water 50:50 (v/v), used to produce the stationary phase slurries were selected. Second, the step after filling the column, that is, the column rinsing, was varied. The capillaries were rinsed for 20 min with ACN, for 60 min with ACN–Ultrapure water 50:50 (v/v), or for 8 h with Ultrapure water. By constructing the Van Deemter curves, it was possible to visualize which packing procedures generated the highest efficiencies (Fig. 2).

When on the 18 different columns naphthalene was analyzed, packing procedures 1 (= original procedure) and 5 generated 5 μm particle-size columns that were more efficient than their corresponding 3 μm equivalents. The results for procedure 1 (Table 4) confirm those in Table 4. For packing procedures 7–9, rather similar efficiencies were observed for both 3 and 5 μm CSPs. For all other procedures (2, 3, 4 and 6), the obtained numbers of theoretical plates for the 3 μm columns were higher at a given voltage (Fig. 2 and Table 4).

When the 18 columns were evaluated with *trans*-stilbene oxide, different results were seen for naphthalene. Four out of nine packing procedures, that is, 3, 6 and 7, provided more efficient 5 μm columns (Fig. 2 and Table 4). For packing procedures 1, 4, 8 and 9, similarly efficient 3 and 5 μm columns were obtained, and for methods 2 and 5, the 3 μm CSP was most efficient. The results for procedure 1 again confirmed what was observed earlier (Table 3). The columns for which higher efficiencies were observed, were, as theoretically expected, since only one type of chiral selector was used, also capable of producing the best separations (highest resolutions). The difference is more striking than that described above. The selectivity factor was not influenced by the varying voltage. Moreover, the obtained values were similar for all columns, regardless of the packing procedure, the column efficiency or the particle size.

For *trans*-stilbene oxide, the 5 μm LA2 column produced by method 6 achieved the highest resolution, that is, up to 5.74 (Table 4). The obtained resolutions for the 5 μm columns packed using procedures 1, 3, 4, 6 and 7 were higher than their corresponding 3 μm columns. For procedures 2 and 5 the 3 μm CSPs performed better (Table 4). On the columns packed by procedures 8 and 9 similar resolutions were seen for both particle sizes. For naphthalene, procedure 8 and 5 μm columns and also procedure 2 provided high efficiencies over a broad range of voltages (between 13 and 25 kV; Table 5, Fig. 2). These packing procedures also allowed the highest efficiencies (between 9 and 25 kV) to be obtained for *trans*-stilbene oxide.

In summary, smaller particle-size packings are not necessarily more efficient. This is an important observation because it implies that smaller particles do not necessarily generate higher efficiencies, meaning that higher resolutions *a priori* are not

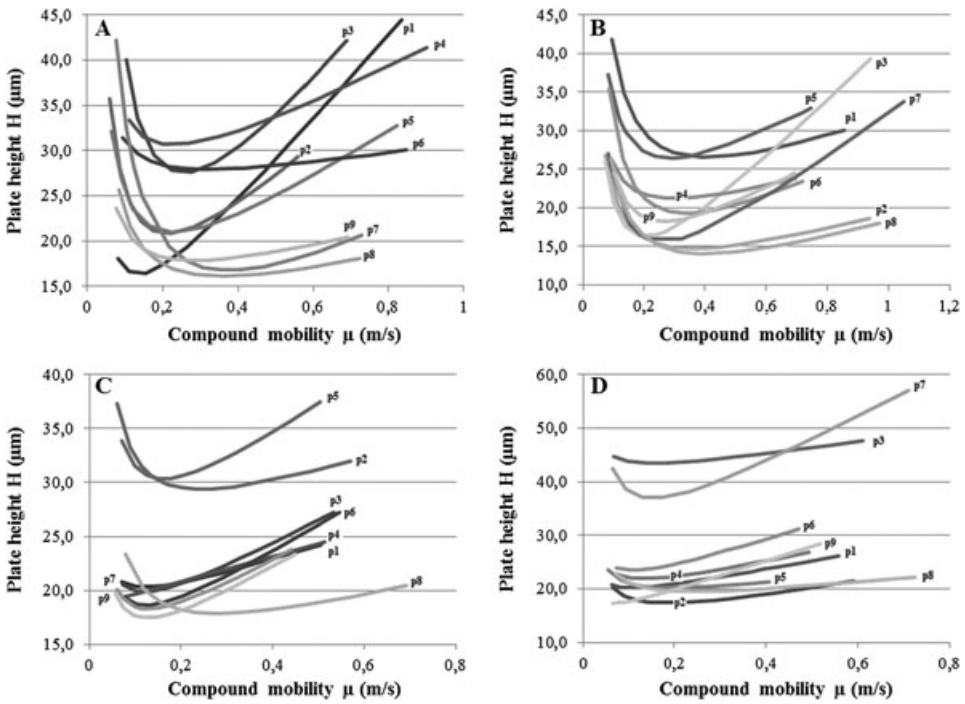

Figure 2. Van Deemter curves for naphthalene (A, 5 μm; B, 3 μm) and for *trans*-stilbene oxide (C, 5 μm; D, 3 μm). p1–p9 for procedures 1–9, respectively. Experimental conditions: 70:30 (v/v) ACN–phosphate buffer pH 11.5; analyzing voltages: 5–30 kV. Stationary phase: LA2. p1–p8 stand for packing procedures 1–8, respectively.

Copyright © 2013 John Wiley & Sons, Ltd.

Table 4. Results of naphthalene and *trans*-stilbene oxide analyzed on 3 and 5 μm LA2 columns manufactured using nine different packing procedures

		Naphthalene						*Trans*-Stilbene oxide									
		5 μm			3 μm			5 μm					3 μm				
	Voltage (kV)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s
Method 1	5	51.5	11,453	21.8	44.0	5,967	41.9	58.7	12,039	20.8	1.17	5.02	65.5	11,552	21.6	1.18	4.59
	7	37.9	15,064	16.6	31.1	7,266	34.4	40.1	12,218	20.5	1.18	4.72	50.2	12,311	20.3	1.17	4.29
	9	26.9	16,232	15.4	24.0	7,929	31.5	30.9	12,412	20.1	1.18	4.76	41.6	12,988	19.2	1.16	4.33
	11	20.5	16,588	15.1	19.4	8,835	28.3	25.3	11,875	21.1	1.18	4.68	34.1	12,789	19.5	1.17	4.25
	13	15.8	13,501	18.5	16.4	8,964	27.9	22.2	12,116	20.6	1.17	4.96	29.9	12,054	20.7	1.17	4.53
	15	12.2	11,071	22.6	14.0	8,699	28.7	18.5	12,079	20.7	1.17	4.64	25.1	12,003	20.8	1.17	4.21
	17	10.3	9,058	27.6	10.7	9,458	26.4	16.2	11,940	20.9	1.17	4.61	19.2	11,866	21.1	1.16	4.18
	19	8.9	8,321	30.0	8.6	9,302	26.9	14.6	11,381	22.0	1.17	4.76	13.1	11,578	21.6	1.17	4.33
	21	7.8	7,512	33.3	7.4	9,105	27.5	12.6	11,534	21.7	1.17	4.44	11.2	11,588	21.6	1.17	4.01
	23	6.9	7,298	34.3	6.6	9,122	27.4	11.3	11,002	22.7	1.17	4.32	10.5	10,534	23.7	1.17	3.89
	25	6.1	6,699	37.3	5.9	8,756	28.6	10.3	10,836	23.1	1.17	4.26	9.8	9,886	25.3	1.17	3.83
	27	5.7	6,884	36.3	5.3	8,643	28.9	9.5	10,667	23.4	1.16	4.15	8.6	9,877	25.3	1.18	3.72
	30	5.0	6,857	36.5	4.9	8,312	30.1	8.2	10,313	24.2	1.16	4.04	7.5	9,564	26.1	1.17	3.61
Method 2	5	69.0	8,502	29.4	57.0	10,543	23.7	69.2	6,672	37.5	1.18	4.81	63.4	11,721	21.3	1.17	5.44
	7	48.4	8,918	28.0	36.3	11,605	21.5	47.0	7,071	35.4	1.17	4.66	47.0	13,107	19.1	1.18	5.53
	9	36.8	10,309	24.3	27.8	13,358	18.7	36.7	7,894	31.7	1.18	3.90	36.5	13,972	17.9	1.18	5.52
	11	29.2	10,966	22.8	22.2	15,244	16.4	31.4	8,141	30.7	1.18	3.96	29.1	14,302	17.5	1.17	5.42
	13	23.9	11,817	21.2	17.8	16,818	14.9	27.8	8,560	29.2	1.17	3.99	19.5	14,885	16.8	1.17	5.36
	15	18.8	12,930	19.3	14.2	16,806	14.9	24.4	8,881	28.1	1.17	4.00	19.1	13,563	18.4	1.18	5.21
	17	14.6	11,739	21.3	12.0	16,796	14.9	21.1	9,094	27.5	1.17	3.94	16.7	13,840	18.1	1.18	5.22
	19	11.7	11,116	22.5	10.5	16,879	14.8	17.8	8,715	28.7	1.16	3.80	14.6	13,968	17.9	1.18	5.16
	21	9.5	9,817	25.5	9.3	17,160	14.6	15.6	8,053	31.0	1.17	3.69	12.9	14,047	17.8	1.17	5.08
	23	8.6	8,848	28.3	8.1	16,895	14.8	14.6	7,890	31.7	1.17	3.66	11.3	13,834	18.1	1.17	4.95
	25	8.1	8,837	28.3	6.2	15,655	16.0	13.3	8,130	30.8	1.17	3.62	9.9	13,109	19.1	1.17	4.77
	27	7.9	8,732	28.6	5.5	14,765	16.9	8.6	8,055	31.0	1.18	3.59	8.3	12,327	20.3	1.17	4.57
	30	7.4	8,854	28.2	4.4	12,581	19.9	7.3	7,988	31.3	1.17	3.43	7.0	11,686	21.4	1.16	4.39
Method 3	5	40.6	7,552	33.1	58.1	10,414	24.0	57.4	12,333	20.3	1.16	5.15	62.4	5,583	44.8	1.19	4.50
	7	31.1	7,945	31.5	41.9	11,180	22.4	40.4	12,301	20.3	1.17	5.68	42.5	5,709	43.8	1.18	3.70
	9	23.5	8,213	30.4	30.5	11,138	22.4	30.3	12,703	19.7	1.17	4.56	29.7	7,327	34.1	1.18	3.51
	11	18.7	8,851	28.2	22.3	10,955	22.8	24.1	12,328	20.3	1.16	4.46	22.9	6,611	37.8	1.18	3.37
	13	15.1	8,937	28.0	16.9	11,191	22.3	20.0	13,640	18.3	1.16	4.58	19.2	5,416	46.2	1.18	3.27
	15	12.3	8,730	28.6	15.5	14,783	16.9	16.4	11,298	22.1	1.16	4.23	16.7	5,720	43.7	1.18	3.33
	17	10.2	8,377	29.8	14.0	15,448	16.2	14.2	10,617	23.5	1.17	4.14	14.5	5,634	44.4	1.17	3.25
	19	8.6	7,677	32.6	10.6	16,571	15.1	12.8	11,506	21.7	1.16	4.22	12.3	5,154	48.5	1.17	3.08
	21	7.2	6,978	35.8	7.2	8,564	29.2	11.5	10,298	24.3	1.16	4.03	11.0	5,497	45.5	1.17	3.16
	23	7.3	6,653	37.6	6.4	8,062	31.0	10.4	10,215	24.5	1.16	3.96	9.7	5,480	45.6	1.17	3.46

(Continues)

Copyright © 2013 John Wiley & Sons, Ltd.

Table 4. (Continued)

		Naphthalene						*Trans*-Stilbene oxide									
		5 μm			3 μm			5 μm					3 μm				
	Voltage (kV)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s
	25	6.9	6,433	38.9	5.7	7,823	32.0	9.4	9,822	25.5	1.16	3.87	8.8	5,525	45.2	1.17	3.09
	27	6.5	6,214	40.2	5.2	7,599	32.9	8.7	9,585	26.1	1.16	3.83	7.7	5,326	46.9	1.17	3.00
	30	6.0	6,222	40.2	4.4	6,431	38.9	7.8	9,233	27.1	1.16	3.69	6.8	5,374	46.5	1.16	3.68
Method 4	5	37.4	7,251	34.5	44.9	9,652	25.9	54.4	13,546	18.5	1.17	4.90	55.3	11,038	22.6	1.17	4.62
	7	28.0	7,926	31.5	31.9	10,133	24.7	38.5	12,619	19.8	1.17	4.78	38.7	11,208	22.3	1.17	4.59
	9	20.9	8,227	30.4	24.5	11,152	22.4	30.1	12,515	20.0	1.18	4.75	29.6	11,423	21.9	1.16	4.60
	11	15.5	8,720	28.7	19.6	11,821	21.1	24.8	12,405	20.2	1.17	4.72	22.1	11,866	21.1	1.16	4.47
	13	12.0	7,962	31.4	15.5	11,275	22.2	20.9	11,989	20.9	1.17	4.86	21.0	11,154	22.4	1.16	4.49
	15	10.3	7,631	32.8	13.4	13,255	18.9	17.5	11,751	21.3	1.17	4.55	17.9	11,023	22.7	1.16	4.24
	17	8.5	6,898	36.2	11.4	11,328	22.1	15.4	11,547	21.7	1.17	4.49	15.9	10,850	23.0	1.16	4.12
	19	7.6	6,761	37.0	10.1	11,599	21.6	13.8	11,531	21.7	1.17	4.57	13.6	10,355	24.1	1.16	4.29
	21	6.9	6,656	37.6	9.0	11,658	21.4	12.1	11,210	22.3	1.17	4.34	12.0	10,264	24.4	1.16	4.02
	23	6.2	6,633	37.7	8.1	11,384	22.0	11.0	10,814	23.1	1.17	4.25	11.0	10,005	25.0	1.16	3.94
	25	5.7	6,566	38.1	7.3	11,180	22.4	10.1	10,703	23.4	1.17	4.18	9.8	9,876	25.3	1.16	3.88
	27	5.2	6,480	38.6	6.7	10,734	23.3	9.3	10,542	23.7	1.17	4.11	9.2	9,666	25.9	1.16	3.83
	30	4.6	6,326	39.5	6.0	10,520	23.8	8.1	10,705	23.4	1.16	4.05	8.4	9,305	26.9	1.16	3.67
Method 5	5	63.7	7,035	35.5	50.0	6,709	37.3	58.9	7,617	32.8	1.17	4.15	75.5	10,965	22.8	1.17	5.95
	7	46.2	9,318	26.8	36.0	7,491	33.4	42.5	7,463	33.5	1.17	3.87	50.1	10,699	23.4	1.17	5.95
	9	34.2	11,431	21.9	34.1	8,587	29.1	33.3	8,157	30.6	1.17	3.84	37.3	12,274	20.4	1.18	4.86
	11	25.0	12,676	19.7	30.0	9,403	26.6	27.5	7,962	31.4	1.17	3.81	30.8	12,303	20.3	1.18	4.90
	13	18.5	12,435	20.1	21.5	9,373	26.7	23.6	8,569	29.2	1.17	3.87	26.1	13,125	19.0	1.16	4.92
	15	13.4	11,340	22.0	17.5	9,405	26.6	20.2	7,918	31.6	1.17	3.74	22.1	12,437	20.1	1.18	4.87
	17	10.4	10,863	23.0	14.2	9,448	26.5	17.8	7,652	32.7	1.17	3.68	19.5	12,284	20.4	1.18	4.84
	19	8.7	9,132	27.4	12.6	8,702	28.7	16.0	7,989	31.3	1.17	3.69	17.3	12,563	19.9	1.17	4.77
	21	8.5	9,205	27.2	11.9	8,433	29.6	14.1	7,915	31.6	1.16	3.63	15.3	12,344	20.3	1.17	4.74
	23	7.7	9,514	26.3	8.7	8,685	28.8	12.9	7,928	31.5	1.16	3.59	13.8	11,738	21.3	1.16	4.63
	25	7.0	9,166	27.3	6.2	8,032	31.1	11.3	7,812	32.0	1.16	3.52	12.6	12,051	20.7	1.17	4.65
	27	6.0	8,576	29.2	5.8	7,752	32.2	9.6	7,019	35.6	1.16	3.35	11.6	11,722	21.3	1.17	4.55
	30	5.1	7,981	31.3	5.6	7,603	32.9	8.3	6,446	38.8	1.16	3.21	10.2	11,716	21.3	1.17	4.49
Method 6	5	44.3	7,711	32.4	49.8	7,414	33.7	55.7	12,910	19.4	1.17	4.86	57.2	10,455	23.9	1.17	4.18
	7	31.2	8,437	29.6	32.0	9,497	26.3	40.8	13,080	19.1	1.18	5.66	40.7	10,535	23.7	1.17	4.98
	9	24.8	9,071	27.6	24.4	10,589	23.6	31.2	13,427	18.6	1.18	5.55	33.6	10,644	23.5	1.17	4.87
	11	19.4	9,019	27.7	20.5	11,057	22.6	24.7	12,514	20.0	1.17	5.74	26.0	10,988	22.8	1.16	5.06
	13	16.1	8,493	29.4	17.7	14,233	17.6	20.3	13,171	19.0	1.17	5.53	21.6	10,355	24.1	1.16	4.85
	15	13.4	8,767	28.5	14.9	13,358	18.7	16.7	12,501	20.0	1.17	5.46	19.3	10,035	24.9	1.17	4.78
	17	10.8	8,938	28.0	12.8	13,353	18.7	14.4	11,771	21.2	1.16	5.40	17.1	9,766	25.6	1.17	4.72
	19	10.1	8,721	28.7	11.1	12,682	19.7	12.9	12,012	20.8	1.16	5.32	15.7	9,433	26.5	1.17	4.64

wileyonlinelibrary.com/journal/bmc Copyright © 2013 John Wiley & Sons, Ltd.

	21	8.6	8,805	28.4	9.8	12,706	19.7	11.1	10,526	23.8	1.16	5.19	13.4	9,025	27.7	1.17	4.51
	23	7.0	8,375	29.9	8.5	12,478	20.0	10.1	9951	25.1	1.16	5.09	12.1	8,899	28.1	1.17	4.41
	25	6.2	9,070	27.6	7.4	11,289	22.1	9.2	9,775	25.6	1.16	4.95	10.5	8,655	28.9	1.17	4.27
	27	5.6	7,608	32.9	6.5	11,072	22.6	8.6	9,690	25.8	1.16	4.80	9.1	8,403	29.8	1.17	4.12
	30	4.9	8,319	30.1	5.8	10,655	23.5	7.6	9,264	27.0	1.16	4.75	8.8	8,032	31.1	1.17	4.07
Method 7	5	53.7	6,055	41.3	50.8	6,762	37.0	70.8	9,233	27.1	1.18	4.30	64.4	5,898	42.4	1.18	3.54
	7	40.1	7,055	35.4	47.4	8,844	28.3	55.9	13,011	19.2	1.19	5.10	44.4	6,519	38.3	1.18	3.72
	9	33.8	7,432	33.6	27.3	12,689	19.7	40.9	14,641	17.1	1.19	4.99	32.3	6,740	37.1	1.18	3.71
	11	28.8	7,947	31.5	22.6	15,138	16.5	35.4	15,010	16.7	1.18	5.18	24.2	6,383	39.2	1.18	3.54
	13	19.3	12,914	19.4	20.3	18,473	13.5	27.9	13,597	18.4	1.18	4.97	17.9	6,559	38.1	1.18	3.39
	15	16.0	16,055	15.6	19.6	16,395	12.3	23.6	12,993	19.2	1.18	4.90	15.0	6,484	38.6	1.18	3.23
	17	13.5	16,979	14.7	13.0	16,123	15.5	20.7	13,052	19.2	1.17	4.84	13.1	6,303	39.7	1.18	3.23
	19	11.7	15,084	16.6	10.9	14,814	16.9	18.0	13,103	19.1	1.17	4.76	11.4	5,598	44.7	1.17	3.23
	21	10.3	14,631	17.1	6.6	11,770	21.2	15.9	12,344	20.3	1.17	4.63	10.0	5,366	46.6	1.17	3.16
	23	8.9	13,880	18.0	6.0	9,905	25.2	14.4	11,776	21.2	1.17	4.53	9.0	5,263	47.5	1.17	3.15
	25	7.7	12,955	19.3	5.4	9,658	25.9	12.9	11,550	21.6	1.17	4.39	8.0	5,103	49.0	1.17	3.07
	27	6.8	12,837	19.5	4.7	8,197	30.5	11.3	11,265	22.2	1.17	4.24	6.8	4,747	52.7	1.17	2.96
	30	5.7	12,294	20.3	4.0	7,107	35.2	9.4	10,608	23.6	1.17	4.19	5.9	4,773	52.4	1.17	2.91
Method 8	5	50.0	9,389	26.6	46.5	9,010	27.7	53.6	10,707	23.3	1.17	4.55	58.8	10,164	24.6	1.17	4.31
	7	36.5	11,557	21.6	34.5	11,648	21.5	34.9	11,878	21.0	1.17	4.74	42.5	11,827	21.1	1.17	4.66
	9	28.0	13,500	18.5	26.8	13,595	18.4	27.1	12,671	19.7	1.17	4.75	33.1	12,162	20.6	1.17	4.73
	11	22.4	14,387	17.4	21.4	15,643	16.0	21.7	13,676	18.3	1.16	4.83	26.8	12,659	19.7	1.17	4.76
	13	18.6	15,296	16.3	17.0	17,362	14.4	17.9	13,983	17.9	1.16	4.79	21.6	12,562	19.9	1.16	4.60
	15	14.7	15,284	16.4	13.2	17,116	14.6	15.0	14,105	17.7	1.16	4.69	17.8	12,820	19.5	1.16	4.67
	17	12.9	15,259	16.4	10.8	17,797	14.0	12.9	14,303	17.5	1.16	4.58	15.5	12,493	20.0	1.16	4.61
	19	11.5	15,354	16.3	9.4	17,273	14.5	11.2	13,588	18.4	1.16	4.49	13.6	12,450	20.1	1.16	4.46
	21	10.2	15,374	16.3	8.4	17,185	14.5	9.8	13,399	18.7	1.16	4.43	11.9	12,508	20.0	1.16	4.49
	23	9.1	15,186	16.5	7.4	17,140	14.6	8.7	13,328	18.8	1.16	4.28	10.8	12,613	19.8	1.16	4.47
	25	8.0	15,100	16.6	6.5	17,835	14.0	7.3	12,695	19.7	1.16	4.21	9.7	12,699	19.7	1.15	4.42
	27	7.3	14,724	17.0	5.6	16,276	15.4	6.2	12,606	19.8	1.16	4.49	7.3	11,988	20.9	1.16	4.35
	30	5.8	14,046	17.8	4.3	15,051	16.6	6.0	12,229	20.4	1.15	3.92	5.8	11,053	22.6	1.16	4.29
Method 9	5	54.6	10,463	23.9	42.0	10,197	24.5	74.4	11,695	21.4	1.17	4.45	64.5	13,430	18.6	1.15	4.38
	7	35.9	11,634	21.5	29.5	11,792	21.2	56.3	13,687	18.3	1.17	4.70	52.1	14,379	17.4	1.24	4.16
	9	28.1	12,427	20.1	22.7	12,995	19.2	42.9	14,133	17.7	1.17	4.68	46.5	15,366	16.3	1.17	4.22
	11	22.7	13,432	18.6	18.3	14,055	17.8	35.4	14,989	16.7	1.7	4.66	38.1	15,201	16.4	1.17	4.18
	13	18.9	13,739	18.2	15.3	15,039	16.6	28.6	14,838	16.8	1.17	4.52	25.1	14,058	17.8	1.14	5.03

(Continues)

Copyright © 2013 John Wiley & Sons, Ltd.

Table 4. (Continued)

Voltage (kV)	Naphthalene						*Trans*-Stilbene oxide											
	5 μm			3 μm			5 μm						3 μm					
	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	t_R (min)	N	H (μm)	α	R_s	t_R (min)	N	H (μm)	α	R_s
15	16.0	13,861	18.0	12.9	14,312	17.5	24.1	14,023	17.8	1.17	4.28	17.7	12,254	20.4	1.17	4.11
17	13.9	14,059	17.8	11.1	13,141	19.0	21.1	13,529	18.5	1.17	4.34	16.0	10,507	23.8	1.17	4.33
19	12.2	13,644	18.3	9.8	12,082	20.7	18.6	13,476	18.6	1.17	4.27	14.1	10,083	24.8	1.16	4.16
21	10.9	13,155	19.0	8.9	11,609	21.5	13.2	13,166	19.0	1.2	3.51	12.3	10,263	24.4	1.16	4.20
23	9.7	13,084	19.1	8.1	11,368	22.0	14.7	12,442	20.1	1.16	3.93	11.0	10,168	24.6	1.16	4.13
25	8.3	12,451	20.1	7.3	10,695	23.4	13.2	12,078	20.7	1.16	4.33	9.9	9,702	25.8	1.16	4.02
27	7.2	12,362	20.2	6.3	10,506	23.8	11.3	11,618	21.5	1.19	3.68	9.5	9,984	25.0	1.17	4.81
30	7.0	12,229	20.4	6.0	10,229	24.4	9.3	10,928	22.9	1.17	3.56	8.0	10,524	23.8	1.16	4.03

t_R, Retention time; N, number of theoretical plates; H, plate height. Methods 1–9: further details can be found in Table 1.

certain. Moreover, when different types of CSPs are considered, there is no relationship between the efficiency and the quality of the separation. When considering one type of selector it is seen that the efficiency varies with the packing procedure but within one procedure it is different for the analysis of chiral and nonchiral compounds. For our case study we observe that for the achiral analysis of naphthalene the 3 μm columns provide mostly the best efficiencies while for the chiral separation of *trans*-stilbene oxide often the opposite is observed.

To assess the intra-day, inter-day, injection and column-to-column variabilities (for the retention time, peak area and resolution), the efficient packing procedures, that is, procedures 2 and 8, were chosen. In the variability studies only three types of columns were considered, that is, 3 and 5 μm LA2 columns produced by procedure 8, and 3 μm LA2 columns produced by procedure 2 (Fig. 2).

Variability study

The two most efficient packing procedures, that is, procedures 2 and 8, were used to fabricate six columns for each procedure. In procedure 2, an MeOH slurry was used and the packed capillary was rinsed with ACN for 20 min. In procedure 8, also an MeOH slurry was used, but the column rinsing was performed using water during 8 h (Table 1). An ACN–phosphate buffer pH 11.5, 70:30 (v/v) mobile phase and analyzing voltages of 13 and 17 kV were applied for packing procedures 2 and 8, respectively. These analyzing voltages were selected because they allowed the highest efficiencies to be achieved.

The naphthalene and of *trans*-stilbene oxide solutions were injected three times onto each column (18 injections per procedure) in order to estimate the injection and the between-column variances. The inter-day and intra-day variances were obtained by injecting three independently prepared samples of naphthalene and of *trans*-stilbene oxide onto one column for six consecutive days (again 18 injections in total). For the estimation of the different variances, one-way ANOVA tables were used.

In Table 5, the results of the analysis of variance are shown as percentage RSDs. These results are compared with values reported in the literature (Table 6) from different particulate-packed columns used in CEC (Bragg and Shamsi, 2011; Aturki *et al.*, 2010; Fonseca *et al.*, 2007). This comparison helps establish the obtained results relative to what has been found earlier for particulate-packed columns.

The response t_R for both compounds had, compared with the literature, good injection variabilities and intra-day variances for both packing procedures (≤ 6.0%). The values estimated for the between-column variance and the time-dependent intermediate precision were <10.0% for the 3 μm columns packed with procedure 8 and thus in agreement with the literature; the other columns performed slightly worse. For the peak area, good values were obtained for all variances (< 11.5%), except for the between-column variance, where values up to 17% were seen. The variances for the resolution of *trans*-stilbene oxide were good compared with the literature for the injection variability, the intra-day variance, and the time-dependent intermediate precision (< 9.5%). For the between-column variance, procedure 8 performed well but for procedure 2 values as high as 20.5% were obtained. This indicates that column packing still may introduce a large variability.

From Table 5, by comparing the injection variability and the intra-day and intermediate precision, it is seen that measuring

Copyright © 2013 John Wiley & Sons, Ltd.

Table 5. Results expressed as RSD (%) of the analysis of variance using different packing procedures

	Injection variability			Between-column variability			Intra-day variability			Time-dependent intermediate precision		
Naphthalene												
Procedure	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$
t_R	4.0	1.3	0.9	31.2	21.3	5.3	1.6	6.0	0.6	9.3	8.8	5.5
Peak area	2.7	7.4	4.0	14.6	8.2	11.5	5.8	8.1	11.3	8.0	5.8	10.5
TSO												
Procedure	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$	$2_{3\ \mu m}$	$8_{5\ \mu m}$	$8_{3\ \mu m}$
t_{R1}	3.8	1.0	0.8	31.5	21.2	9.9	1.5	5.7	0.8	9.4	10.0	8.5
Peak area$_1$	6.1	7.5	8.6	16.8	13.4	9.0	8.0	5.5	7.0	8.3	5.0	6.3
R_s	2.7	2.1	3.1	20.5	8.3	8.2	1.6	2.7	2.9	3.6	5.1	9.5

t_{R1}, Retention time of the first or only eluting peak; peak area$_1$, area under the curve of the first or only eluting peak; R_s, resolution; TSO, *trans*-stilbene oxide.
Procedure $2_{3\ \mu m}$, procedure 2 on 3 μm particles; procedure $8_{5\ \mu m}$ or $8_{3\ \mu m}$, procedure 8 on 5 or 3 μm particles, respectively.

Table 6. Literature results, expressed as RSD (%), of different samples and particulate-packed columns in capillary electrochromatography

Stationary phase	Responses	Injection variability	Intra-day variance	Time-dependent intermediate precision	Reference
Strong anion exchange C6 (fritless)	t_R	—	3.2–3.6	3.7	Bragg and Shamsi (2011)
	Peak area	—	8.3–8.5	8.4	
	R_s	—	5.6–5.8	5.8	
Strong anion exchange C6 (single-frit)	t_R	—	5.3–5.5	5.5	
	Peak area	—	8.3–8.5	8.3	
	R_s	—	6.1–6.3	6.1	
Cyano silica	t_R	—	1.3–2.2	1.8–3.3	Aturki *et al.* (2010)
	Peak area	—	5.8–9.9	10.7–16.3	
Hypersil SCX/C18	t_R	0.9–2.0	—	—	Fonseca *et al.* (2007)
	Peak area	5.5–15.0	—	—	

on different days and preparing different samples do not contribute much to the variability. It is mainly caused by the injection, which is not surprising since there is no loop injection. Thus for quantitative analysis, the use of an internal standard would be recommended. The between-column variance indicates that the packing procedure is still a major factor limiting the precision of results measured on different columns. Overall, packing procedure 8 allowed good variances to be obtainedfor most responses. For procedure 2, less satisfactory results were seen.

Summarizing, when a choice has to be made between the two selected packing procedures, that is, 2 and 8, of which the latter was applied on both particle sizes (3 and 5 μm), procedure 8 seems to be the best choice to produce columns with high efficiency and limited variability (good precision). Procedure 8 also proves to be a better approach to pack efficient columns compared with procedure 1, the original packing procedure (Fig. 2). The original packing procedure was thus successfully optimized, although one should keep in mind that further optimizations are necessary since the between-column variance remains large.

What is the significance of the obtained results?

The differences between the obtained variances for the retention time, peak area, efficiency and resolution on the various columns, packed by different procedures, were evaluated on their statistical significance by applying *F*-tests (Table 7). The *F*-values were calculated using the pooled variance of the repeated injections on procedures 2 and 8 and the variances of the different procedures, estimated with the values obtained at the optimal applied voltage. The variances of the procedures were then divided by the pooled variance to obtain the *F*-values, which were then compared with the critical *F*-value ($F_{8,5,\alpha=0.05} = 4.82$).

To establish whether or not the analogue (manufactured by the same procedure) 3 and 5 μm particle size columns provide significantly different responses, a *t*-test was used (Table 8). The *t*-values were calculated using the variances of the different procedures (parameter values obtained at the optimal applied voltage). The null hypothesis, stating that there is no significant difference between the obtained responses for the 3 and 5 μm columns packed by the same packing procedure, is accepted when the obtained probability value (*p*) is higher than the

Copyright © 2013 John Wiley & Sons, Ltd.

Table 7. *F*-Tests

	t_R	Peak area	N	R_s
Naphthalene				
s^2_{pooled} of the variances of the replicates on procedures 2 and 8	3.371	6842602	2478907	
$s^2_{procedures}$ of 3 μm columns packed with nine different procedures	4.939	103533782	11632055	
$s^2_{procedures}$ of 5 μm columns packed with nine different procedures	6.492	408065556	29845598	
F-Value$_{3\ \mu m}$ $\left(F = \frac{s^2_{procedures}}{s^2_{pooled}}\right)$	1.47	**15.13**	4.69	
F-value$_{\mathbf{5\ \mu m}}$ $\left(F = \frac{s^2_{procedures}}{s^2_{pooled}}\right)$	1.93	**59.64**	**12.04**	
trans-Stilbene oxide				
s^2_{pooled} of the variances of the replicates on procedures 2 and 8	6.224	126771	5190089	0.25
$s^2_{procedures}$ of 3 μm columns packed with nine different procedures	31.307	1980304	6299442	0.38
$s^2_{procedures}$ of 5 μm columns packed with nine different procedures	28.476	10281217	51328255	0.27
F-Value$_{3\ \mu m}$ $\left(F = \frac{s^2_{procedures}}{s^2_{pooled}}\right)$	**5.03**	**15.62**	1.21	1.56
F-Value$_{5\ \mu m}$ $\left(F = \frac{s^2_{procedures}}{s^2_{pooled}}\right)$	4.57	**81.10**	**9.89**	1.11

t_R, Retention time; N, efficiency; R_s, resolution. F-Value$_{3\ \mu m\ or\ 5\ \mu m}$ are the F-values for the 3 or 5 μm columns packed by applying packing procedures 1–9. s^2_{pooled} for procedures 2 and 8 was considered at an applied voltage of 13 and 17 kV, respectively. $s^2_{procedures}$ for naphthalene and *trans*-stilbene oxide was considered at an applied voltage of 15 and 9 kV, respectively. $F_{8,5,\alpha\ =\ 0.05} = 4.82$. Significant F-values are marked in bold.

Table 8. Two-tailed *t*-test

	t_R	Peak area	N	R_s
Naphthalene				
t-Value	0.801	1.047	2.231	
p-Value	0.446	0.326	0.056	
trans-Stilbene oxide				
t-Value	0.908	−0.174	−0.407	−0.478
p-Value	0.390	0.866	0.695	0.645

t_R, Retention time; N, efficiency; R_s, resolution. Null hypothesis H_0: $y_{3\ \mu m} = y_{5\ \mu m}$, with y a given response. $t_{8,\alpha\ =\ 0.05} = 2.306$.

critical *p*-value of 0.05 or if the *t*-value is lower than the critical *t*-value ($t_{8,\alpha\ =\ 0.05} = 2.306$).

Table 8 shows that for naphthalene significantly different results were obtained for the peak areas on the columns from procedures 2 and 8 compared with those on all columns. For the efficiency similar results were seen: the variability on the columns from the different procedures was larger than that on the procedure 2/8 columns. Similar observations were made for *trans*-stilbene oxide. Furthermore, for *trans*-stilbene oxide the packing procedure did not significantly affect the resolution value observed between the different packing procedures. Consequently, the differences observed in Table 5 were not found to be statistically significant.

The conclusions for the *t*-tests were the same for naphthalene and *trans*-stilbene oxide, that is, there were no significant differences between both particle-size columns, packed by the same procedure, for the considered responses (t_R, peak area, N and R_s). However, for naphthalene the result for N was on the limit of significance. The theoretical expectation that states that smaller particle-size packings are more efficient and therefore increase the separation quality thus is not applicable here, especially not for chiral separations. This is an important observation for defining generic chiral separation strategies, where the resolution is the main factor taken into consideration.

Taking into account the results of the repeatability study, packing procedure 8 seems the best method to pack efficient 3 and 5 μm columns intended for chiral separations. The problem to be solved or improved is the less good repeatability of packing the columns.

Conclusion

In an earlier study, the behavior of 3 μm particle-size CSPs was found to be not entirely as theoretically expected (Hendrickx *et al.*, 2013). As a result, this study was initiated to evaluate the efficiencies of four chlorinated polysaccharide-based chiral selectors coated onto 3 or 5 μm silica particles.

Naphthalene and *trans*-stilbene oxide were analyzed on four 3 μm and four 5 μm CSPs, that is, LC2, LA2, LC4 and Sp5, with voltages ranging between 5 and 30 kV. With the obtained data Van Deemter curves were produced in order to compare the efficiencies. On neither the 3 nor the 5 μm CSPs were the highest efficiencies systematically obtained. This is in contrast to the theory stating that smaller particle diameters reduce band broadening and thus induce higher efficiencies.

In an attempt to optimize the varying column efficiencies, nine different packing procedures, on both 3 and 5 μm LA2 CSPs, were evaluated. Finally, the two best column packing procedures were selected to perform a variability study. In conclusion, procedure 8 where a MeOH slurry is used followed by a rinsing for 8 h with water, allows columns to be produced with high efficiencies (for chiral separations) and the best precisions. Procedure 8, for the packing of both 3 and 5 μm

Copyright © 2013 John Wiley & Sons, Ltd.

particle-size columns, performed best with RSD values for injection variability(t_R, area, and R_s), intra-day variance (t_R, area, and R_s) and time-dependent intermediate precision (t_R, area and R_s) below 10.5%. These values were better than or similar to what was found in the literature.

Globally it is observed that for the achiral analysis the obtained columns with most procedures behaved as expected, that is, the smaller particles provide the best efficiencies. For the considered chiral separation the opposite was seen, that is, the 5 μm columns most often provided the highest efficiencies. Moreover, the first part of our study, where different chiral selectors were considered, showed that for a chiral separation the efficiencies are not very informative. Columns with clearly less good efficiencies may provide the best chiral separations.

It might be interesting to study the same objectives in other chromatographic modes, such as HPLC or SFC, where one can work with commercial CSPs where the column reproducibility issues that in CEC clearly play an important role are much smaller or negligible.

References

Aturki Z, D'Orazio G, Rocco A, Bortolotti F, Gottardo R, Tagliaro F and Fanali S. CEC-ESI ion trap MS of multiple drugs of abuse. *Electrophoresis* 2010; **31**: 1256–1263.

Bragg W and Shamsi SA. Development of a fritless packed column for capillary electrochromatography–mass spectrometry. *Journal of Chromatography A* 2011; **1218**: 8691–8700.

Eeltink B. Packed and monolithic capillary columns for LC. Ph.D thesis, Universiteit van Amsterdam, Amsterdam, 2005, pp. 2–6.

Fonseca FN, Tavares MFM and Horváth C. Capillary electrochromatography of selected phenolic compounds of *Chamomilla recutita*. *Journal of Chromatography A* 2007; **1154**: 390–399.

Hendrickx A, Mangelings D, Chankvetadze B and Vander HY. Comparative enantioseparations of pharmaceuticals in capillary electrochromatography on polysaccharide-based chiral stationary phases containing selectors with or without chlorinated derivatives. *Electrophoresis* 2010; **31**: 3207–3217.

Hendrickx A, Mangelings D and Vander Heyden Y. Capillary methods for drug analysis. *Journal of AOAC International* 2011a; **94**: 667–702.

Hendrickx A, Mangelings D, Chankvetadze B and Vander HY. Updating a chiral separation strategy for non-acidic drugs with capillary electrochromatography applicable for both chlorinated and non-chlorinated polysaccharide selectors. *Electrophoresis* 2011b; **32**: 2718–2726.

Hendrickx A, De Klerck K, Mangelings D, Clincke L and Vander Heyden Y. Three versus five μm chlorinated polysaccharide-based packings in chiral capillary electrochromatography: performance evaluation, 2013, accepted for publication in the Journal of Biomedical Chromatography.

Mangelings D, Hardies N, Maftouh M, Suteu C, Massart DL and Vander HY. Enantioseparations of basic and bifunctional pharmaceuticals by capillary electrochromatography using polysaccharide stationary phases. *Electrophoresis* 2003; **20**: 2567–2576.

McDonald PD and Neue UD. *The Quest for Ultra Performace in Liquid Chromatography. Origins of UPLC Technology*. Waters Corporation: Milford, MA, 2009; 11–12 and 28–30.

Rathore AS and Horváth C, Migration of charged sample components and electroosmotic flow in packed capillary columns. In *Capillary Electrochromatography*, Deyl Z, Svec F (eds). Journal of Chromatography Library, Vol. **62**. Elsevier Science: Amsterdam, 2001; 1–38.

Copyright © 2013 John Wiley & Sons, Ltd.