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Title: Managing the column equilibration time in hydrophilic interaction chromatography.

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Abstract: For a wide variety of hydrophilic interaction chromatography stationary phases, a repeatable partial equilibration was demonstrated in gradient elution after purging with as little as 12 column volumes of mobile phase. Relative standard deviations of retention time of on average ~0.15% could be obtained after 1 or 2 conditioning (blank) runs. The equilibration period must be kept strictly constant, otherwise selectivity changes occur, but this is not problematic on modern instruments. Partial equilibration was largely independent of stationary phase or gradient slope. Alternatively, full column equilibration is favoured for stationary phases that do not trap extensive water layers, and for materials with a wider pore size that have a lower surface area. Temperatures somewhat above ambient also shorten the equilibration time. Some stationary phases under optimum conditions can achieve full column equilibration using purging with ~12 column volumes, which is useful for rapid set-up of isocratic separations or for conventional gradient analysis.

***Cover letter**

***Response to Reviewer Comments**

***Highlights (for review)**

- Repeatable partial gradient elution equilibrium shown for different HILIC columns.
- Rapid partial equilibration independent of stationary phase and gradient slope.
- Full equilibration time depends on water layer thickness on stationary phase.
- Full equilibration faster at elevated temperature and increased column pore size.
- Full equilibration achieved on some columns with passing only 12 column volumes.

Managing the column equilibration time in hydrophilic interaction chromatography.

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Abstract

For a wide variety of hydrophilic interaction chromatography stationary phases, a repeatable partial equilibration was demonstrated in gradient elution after purging with as little as 12 column volumes of mobile phase. Relative standard deviations of retention time of on average ~0.15% could be obtained after 1 or 2 conditioning (blank) runs. The equilibration period must be kept strictly constant, otherwise selectivity changes occur, but this is not problematic on modern instruments. Partial equilibration was largely independent of stationary phase or gradient slope. Alternatively, full column equilibration is favoured for stationary phases that do not trap extensive water layers, and for materials with a wider pore size that have a lower surface area. Temperatures somewhat above ambient also shorten the equilibration time. Some stationary phases under optimum conditions can achieve full column equilibration using purging with ~12 column volumes, which is useful for rapid set-up of isocratic separations or for conventional gradient analysis.

1. Introduction.

Hydrophilic interaction chromatography (HILIC) is now an established method of LC separation that is particularly suited to the analysis of polar and ionised solutes which are difficult to retain by classical reversed-phase (RP) techniques [1]. It has been widely applied in pharmaceutical [2], environmental [3] and clinical analysis [4, 5], and for metabolite profiling [6]. For solutes amenable to either separation mechanism, HILIC has advantages over RP, including low back pressures, and often greater sensitivity of detection with coupled mass spectrometry, both attributable to the high concentrations of acetonitrile (ACN) generally used in the mobile phase [7]. Investigations into the mechanism of HILIC separations continue [8] . A perceived disadvantage of HILIC is the apparently long column equilibration time. In a study of gradient analysis of antibiotic solutes, about 40 column volumes of mobile phase were necessary to achieve full equilibrium for silica, urea and amide stationary phases [5]. However, considerably larger volumes were necessary for some commercial zwitterionic phases [9]. These have thick polymeric layers of stationary phase that can trap extensive amounts of water into which analytes can partition [10]. However, even on a zwitterionic phase, a reproducible partial equilibrium was shown after the passage of as little as ~10 column volumes of mobile phase [9]. The gradient program must be kept strictly constant in this procedure as retention and selectivity can vary as a function of the equilibration time. Nevertheless, the reproducibility of the gradient program does not appear to be a problem on modern HPLC systems. The relative standard deviation (rsd) of 6 consecutive gradient separations of acidic, basic and neutral solutes on a ZIC-cHILIC column varied from about 0.02 to 0.3 % after as few as two conditioning runs had been performed. These preliminary findings were reflected in similar results for a bare silica column in a different study [5] and agreed with those of Bell and co-workers [11]. Thus, partial equilibrium seems to be a viable approach in HILIC, as it has also previously been shown to be for RP chromatography [12, 13], where the emphasis was on applications in 2-dimensional RP- HPLC. This technique requires a very rapid orthogonal second dimension separation and equilibration cycle to deal with the fractions that issue from the first dimension column. There has been interest in coupling HILIC and RP, due to the orthogonal nature of the selectivity of the techniques [14] .

We aimed to ascertain whether this somewhat surprising result for partial equilibration applied also to a wider variety of HILIC stationary phases in gradient analysis (including totally and superficially porous materials). We studied partial equilibration from both steep

and shallow gradients to ascertain if the gradient profile had any effect on partial equilibration. We also explored the factors that might influence the establishment of full column equilibration, as this is required for isocratic analysis, and for applications where regulatory control might not permit the use of the partial equilibration approach. Thus, we have studied factors such as the nature of the stationary phase, temperature and pore size of the material. A number of new types of column were included in the study that had not been investigated previously, including Torus (Waters) columns that are primarily designed for use in SFC, but might potentially have application also in HILIC. As Torus columns are synthesised on the same hybrid silica substrate, they provide the opportunity for comparison of the performance of a charged (diethylamino, DEA) and neutral (Diol) phase. The effect of the pore size of the base silica on equilibration was investigated through a suite of silica columns obtained from the same manufacturer (AMT).

2. Experimental

Experiments were performed using a 1290 ultra-high performance liquid chromatograph (UHPLC, Agilent, Waldbronn, Germany) comprising a binary pump, autosampler and photodiode array UV detector (0.6 μL flow cell). The physical properties of the columns (all 10cm x 0.21 cm ID) are shown in Table 1.

ACN (gradient UV grade), formic acid and ammonium formate (AF) were purchased from Fisher (Loughborough, U.K.). All test solutes were obtained from Sigma-Aldrich (Poole, U.K.). Standards were prepared at concentrations of 20-50 mg/L and injected dissolved in the buffered 95% ACN mobile phase. Buffered mobile phases were prepared by adjusting the pH of the aqueous portion before addition of ACN ($_w^w$ pH); solvent volumes were metered by weight employing the liquid density. The pump was purged with 50 mLs of liquid at each change of mobile phase. Flow rate for equilibration and analysis was 0.5 mL/min. except where stated. For the isocratic experiments, columns were pre-equilibrated in the appropriate mobile phase (5 mM AF pH 4.4 in 60 % ACN) for at least 2 hours at 0.5 mL/min. prior to use with the analysis solvent (exclusively 5 mM AF pH 4.4 in 95% ACN). This experiment replicates the change from a strong solvent at the end of a typical gradient to the initial weak gradient solvent. The equilibration volumes mentioned represent volume passed through the system between the time of the solvent switch and the beginning of the chromatographic run indicated. The void volume of the columns was measured using toluene. For gradient elution, columns were equilibrated with a number of conditioning gradient runs (detailed below) where the data was not used other than to

establish whether partial equilibration had occurred, by comparing gradient retention times with those obtained after two hours of further equilibration.

3. Results and Discussion.

3.1 Partial column equilibration in gradient runs.

We first studied a gradient run starting with 5 mM AF pH 4.4 in 95% ACN and progressing to 60 % ACN-buffer over a period of 7 min. (5 % increase in aqueous concentration/min.). Note that in all gradient experiments, the AF concentration was maintained at overall 5 mM throughout, by inclusion of the salt in both of the mobile phase reservoirs. The column was pre-equilibrated prior to the gradient run in the starting solvent for at least 2 hours. 95% ACN (5% water) was chosen as the weak solvent as water-lean mobile phases are likely to offer a worst case for equilibration problems [11, 15]. The sample was injected; after the end of the gradient, the solvent was immediately switched back to 95 % ACN- buffer and equilibrated for the passage of different numbers of column volumes corresponding to times of 5.6, 10.6, 15.6 and 30.6 min. (the cycle time of the autosampler was 0.6 min.) at which time a further sample injection was made. A similar series of experiments was performed using a shallower gradient where the weak solvent was the same but the "strong solvent" was 88% ACN- buffer where a 1 % increase in aqueous concentration/min. was used over the 7 min. run, i.e. a much shallower increase in solvent strength. Shallower gradients could also be produced using longer gradient times, which we would expect to have a similar effect on partial equilibration times. Fig. 1a, b and c show the effect of the number of equilibration column volumes on the retention of a neutral (uridine, peak 1), an acidic (4-OH benzoic acid, peak 2) and a basic solute (nortriptyline, peak 3) on BEH amide, silica, and zwitterionic columns respectively. The equilibration times were held constant, corresponding to slightly different numbers of column volumes in each case. Clearly, the selectivity of the separation varies for each column dependent on the number of column volumes passed, so it is necessary to maintain the equilibration period strictly constant to achieve repeatable retention. Thus the amide column (Fig. 1a) elutes the solutes in the order 2, 3, 1 after purging with 12.2 column volumes of the starting mobile phase, but as 3, 2, 1 after 23 column volumes. Consistent selectivity of the separation (full equilibration) is apparently obtained after purging somewhere between 23 to 33.9 volumes for the silica column Fig. 1b), but requires considerably more purging for the zwitterionic column (Fig. 1c-more than 70 volumes-noting also data for full isocratic equilibration below). For each column, the basic solute moves to shorter retention times as the equilibration period increases whereas the neutral and acidic solutes move to

longer retention. A system peak appeared (only) for the ZIC-HILIC column (Fig. 1c) at around 5 minutes which increased in area as the number of equilibration volumes of 95 % ACN-buffer increases. This result could be due to the release of formate buffer anions (detectable at 254 nm) which have preferentially accumulated in the aqueous rich zone in 60 % ACN. As the number of column volumes of 95 % ACN increases, there will be a greater mismatch between 60 % ACN (the solvent concentration mostly in the column at the end of the gradient) and the actual concentration of ACN in the mobile phase at the start of the gradient in the partially equilibrated column. In contrast, a column only marginally equilibrated will contain more residues of the strong solvent, lessening the degree of mismatch. The system peak could be detected even at 13 column volumes on the ZIC-HILC column by use of detection at 215 nm (a wavelength more sensitive to formate anions) instead of 254 nm (data not shown). This peak did not appear using the shallower gradient on the same column (1% water increase/min.), presumably due to the smaller solvent mismatch at the start of the gradient. The system peak was very small at 254 nm for the silica and amide columns, which are known to have much less extensive stationary phase water layers than commercial zwitterionic phases [10].

Table 2 indicates that for all 3 columns using either a steep or a shallow gradient, good repeatability of gradient retention time was shown even for purging with as little as 12-13 column volumes of mobile phase. A number of “conditioning runs” or “blank gradients” where data was not collected were performed to stabilise the system-only 0-2 such runs were necessary. The rsd of retention times of 6 subsequent injections was in the range of ~0.03 to 0.36 % but many results show a rsd of < 0.1 %. The overall mean of the rsd for the three columns and three solutes over the four different equilibration cycles (~12-~70 column volumes) was 0.14 % for the steep gradient condition. Higher precision could be obtained by increasing the number of conditioning runs (results not shown). The overall mean for the shallow gradients was slightly better with overall mean of the rsd values 0.08 %. In general, little difference in repeatability was shown between the different stationary phases, between the different solutes or between the gradient steepness. Thus considering for example the steep gradient results, these were as repeatable on the zwitterionic column (which required the most column volumes to achieve full equilibration) as on the silica column (which required the least).

While repeatability can be achieved in gradient analysis by partial equilibration, it is of interest to investigate parameters than influence full equilibration of the column. Full equilibration is necessary for isocratic analysis, and offers increased robustness and method transferability in gradient work.

3.2 Effect of stationary phase, pore size and temperature on retention and selectivity.

It is conceivable that factors such as temperature, and pore size of the stationary phase could influence equilibration. We first studied the influence of these parameters on retention and selectivity, as their influence (particularly of pore size) has not been extensively reported previously. Fig. 2 shows the effect of temperature on the separation of the 3 probe compounds on the BEH amide column at full equilibrium. At 30 ^{o}C, nortriptyline is retained the least of the 3 solutes consistent with its low hydrophilicity (log D_{ow}~+1.0) [7, 16]. Bare silica columns such as Halo (Fig. 3) demonstrated in contrast highest retention of nortriptyline, consistent with strong ionic retention on silanols which are more abundant than on bonded phases. As the column temperature of the amide phase was increased from 20 to 60 ^{o}C, the retention times of uridine and 4-hydroxybenzoic acid decreased, as is typical in LC [17]. However, the retention of nortriptyline slightly *increased* with temperature. This unusual behaviour is obscure, although it is possible that increased temperature disrupts the adsorbed water layer somewhat, reducing the screening effect on silanols ~~may be attributable to decreased screening of silanols by water molecules or buffer cations as the temperature is raised~~ [18] .

Fig. 3 shows the influence of pore size over the range 90 to 1000 Å on retention on a series of Halo bare silica columns at 30 ^{o}C. Direct comparison of the 90, 160 and 1000 Å phases is possible as they all have particle diameter 2.7 μm and shell thickness 0.5 μm and decreasing surface are (Table 1). ~~, although t~~The 400 Å column has somewhat different physical characteristics with particle diameter 3.4 μm and shell thickness ~~(~~0.2 μm. ~~Table 1).~~ It seems logical that retention should decrease broadly in line with decreasing surface area (which decreases with increasing pore diameter), as is indeed shown in Fig. 3. Low retention and large pore size are particularly advantageous for the separation of large molecules, which can show hindered diffusion/ exclusion, or strong ~~irreversible~~ adsorption on smaller pore size, high surface area materials [19] .

3. Effect of stationary phase and temperature on full column equilibration.

Figure 4 shows the progress of full column equilibration and its profound effect on retention of the probe solutes on the ZIC-HILIC column at 45 ^{o}C when changing from strong to equilibration with weak solvent in isocratic mode. The column was pre-equilibrated for at least 2 hours with 60 % ACN-buffer followed by changing the solute to (isocratic) 95 % ACN-buffer and recording the chromatogram after increasing periods of

equilibration. The baseline disturbance after purge of 53 column volumes acts as a marker of the onset of full equilibration, as proposed by Bell and co-workers [11, 15], which occurs after the passage of 66 volumes. Fig. 5a shows the effect of temperature on the number of column volumes required to achieve full equilibration of the ZIC-HILIC column. Full equilibration was deemed to occur when retention times were within +/- 1% of the retention time measured after several hours of equilibration [9]. At 30 ^{o}C, full equilibration was achieved with passage of ~80-90 column volumes (~34-39 min., Fig. 5a). This slow equilibration is attributable to the polymeric structure of the stationary phase ligands, and their ability to trap thick layers of water [10] . Nevertheless, this value is somewhat smaller than that reported for a similar column previously [9], which we showed to be due to insufficient purging of the pump between change of solvents –we now recommended purging with 50 mL (see experimental). Fig 5a shows that equilibration volume reduced progressively from 20 to 45 ^{o}C, at which temperature 50-65 column volumes are required. However, further increase in temperature to 60 ^{o}C had no beneficial effect. In addition, higher temperatures may adversely affect column stability

Fig. 5b shows the effect of temperature on equilibration of the BEH amide column. Clearly, at all temperatures, the amide column equilibrates much faster than the ZIC-HILIC column, which can be attributed to a less extensive water layer. Once again, equilibration volumes reduce when increasing column temperature from 20 to 45 ^{o}C, but 60 ^{o}C showed no additional benefit. At 45 ^{o}C, only ~25 column volumes were needed to achieve full equilibration (equivalent to ~ 12 min. equilibration time).

Similar variations in equilibration volumes with temperature were again obtained for the Halo silica 90 Å column (Fig. 5c). Nevertheless, the minimum number of column volumes (~40) required for full equilibration (at 45 ^{o}C) was higher than for the amide column. Data for Halo was not collected at 60 ^{o}C, for fear of damaging the stationary phase, which is more likely due to the absence of protective bonding ligands.

Fig. 5d shows that the smallest equilibration volume was obtained for the Torus columns. The DEA phase gave consistently low equilibration column volumes over the range 20-45 ^{o}C of ~16, corresponding to an equilibration time of ~ 8 min. This column gave low retention of nortriptyline due to repulsion from its protonated amine groups, but high retention of 4-hydroxybenzoic acid due to ionic retention (k = 8.1 at 30 ^{o}C using 95% ACN-buffer), indicating potential applications in the separation of acidic solutes. The diol column showed the usual pattern of decreasing equilibration volumes with increasing temperature up to 45 ^{o}C, when only 13 column volumes corresponding to about 6 minutes were required. The diol column gave lower average retention than the DEA phase

(maximum *k* of 2.7 was for 4-hydroxybenzoic acid with 95% ACN-buffer at 30 ^{o}C). This may be a disadvantage of this column for the analysis of moderately hydrophilic solutes as used in this study. The low retention of diol columns in HILIC has been noted previously [18]. Although Fig. 5d indicates that the (charged) DEA column equilibrates faster than the (neutral) diol phase at lower temperatures, further comparisons of different stationary phases bonded on the same substrate (as is true with these two stationary phases) are necessary to explore the effect of column charge on equilibration.

3.4 Effect of pore size on full column equilibration.

Fig. 6 shows the effect of the pore size of a series of Halo silica columns on the equilibration volume required at a constant temperature of 30 ^{o}C when changing the mobile phase from 60 % ACN-buffer to 95 % ACN-buffer. The number of volumes reduces from ~50 for the 90 Å to as little as 13-25 for the 1000 Å phase. This decrease seems more likely to be due to a reduced surface area of larger pore phases than any effect of hindered access of (small) water molecules to smaller pores. Unfortunately, we were not able to speed up the equilibration process further on these wider pore phases by increasing the temperature above 30 ^{o}C (results not shown).

4. Conclusions

It was demonstrated that partial equilibration could be obtained by purging with as little as ~12 column volumes (corresponding to ~ 6 min.) of the initial mobile phase in gradient elution. While the column is not fully equilibrated, the precision of retention times is good with % rsd typically in the range 0.03-0.3% after 0-2 conditioning runs. Similar repeatability was obtained with columns that could achieve relatively rapid full equilibration (e.g bare silica) as those requiring more extensive equilibration (e.g. polymeric bonded zwitterionic). Results were also comparable when using steep or shallow mobile phase gradients. Due to variations in selectivity and retention with equilibration time, the gradient programme must be kept strictly constant in a series of runs, which does not appear to be problematic on modern instruments.

Isocratic elution requires full equilibration as selectivity and retention change with equilibration time. Isocratic analysis of a batch of samples requires only a single initial full equilibration period and therefor long equilibration times might therefore be regarded as relatively unproblematic. For the purposes of method transferability and robustness in gradient analysis, full equilibration of the column is still of interest. Full equilibration is

facilitated on columns which trap less extensive water layers, by the use of temperatures above ambient (e.g. ~45 ^{o}C) or by columns with large pore size (smaller surface area/less extensive water layer). The latter finding suggests a good outlook for the separation of large molecules by HILIC. Under optimum conditions, some columns required little more than ~12 volumes of mobile phase (~ 6 min.) to achieve full equilibration. Elevated temperature was also found to give interesting retention and selectivity changes. Of course, full equilibration times can be shortened by increasing the flow rate between runs, which is not difficult due to the low viscosity mobile phases used in HILIC, and thus the low back pressures encountered.

5. Acknowledgements

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6. Legend to Figures

Fig. 1. Selectivity and retention of in gradient elution under partial equilibration conditions after conditioning runs (see Table 2). Initial purge: 2 hours with mobile phase 5 mM AF pH 4.4 in 95 % ACN, 0.5 mL/min. then gradient to 60 % ACN buffer in 7.0 mins. (5 % aqueous increase/min), 0.5 mL/min. Injection volume 1 μL. Column temperature 30 ^{o}C. UV detection at 254 nm. Peak identities 1 = uridine; 2 = 4-OH benzoic acid; 3 = nortriptyline. Columns a) BEH amide; b) Cortecs silica; c) ZIC-HILIC.

Fig. 2. Effect of temperature on retention and selectivity of BEH amide column at full equilibration. Mobile phase isocratic 5 mM AF pH 4.4 in 95 % ACN 0.5mL/min. UV detection at 215 nm. Peak identities and other conditions as Fig. 1.

Fig. 3. Effect of pore size of a series of Halo bare silica columns on retention and selectivity. Mobile phase and other conditions as Fig. 2.

Fig. 4. Progress of full equilibration of ZIC-HILIC column (following initial purge) after passing different numbers of column volumes of analysis mobile phase. Initial purge mobile phase: 2 hours with 5 mM AF pH 4.4 in 60 % ACN 0.5 mL/min. Analysis mobile phase 5 mM AF pH 4.4 in 95% ACN at 0.5 mL/min. Column temperature 45 ^{o}C. Other conditions as Fig. 2.

Fig. 5. Effect of temperature on column volumes required to achieve full equilibration. Conditions as Fig. 4. Columns a) ZIC-HILIC b) BEH amide c) Halo (silica) 90Å d) Torus Diol and Torus DEA.

Fig. 6. Halo silica. Effect of pore size on number of column volumes to achieve full equilibration at 30 ^{o}C. Other conditions as Fig. 4.

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large biomolecule high performance liquid chromatography and polymer size exclusion chromatography, J. Chromatogr. A, 1489 (2017) 75-85.

Figure 1

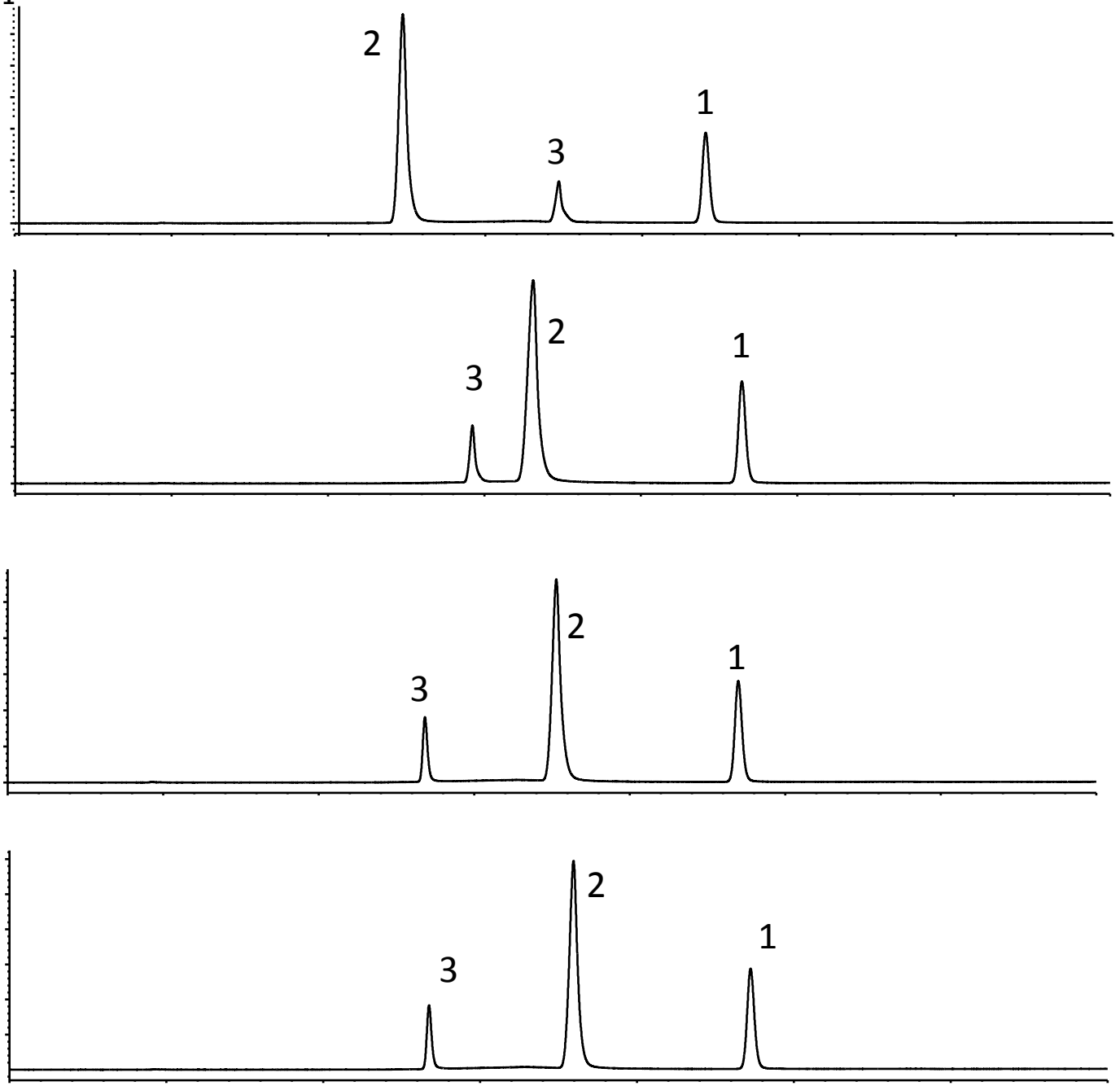


Fig.1 a

12.2 col. vols.

23.0 col. vols.

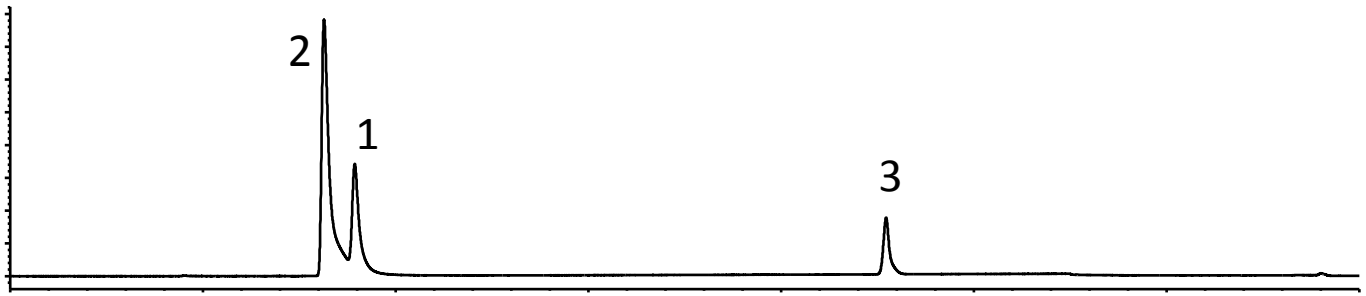
33.9 col vols.

66.5 col. vols.

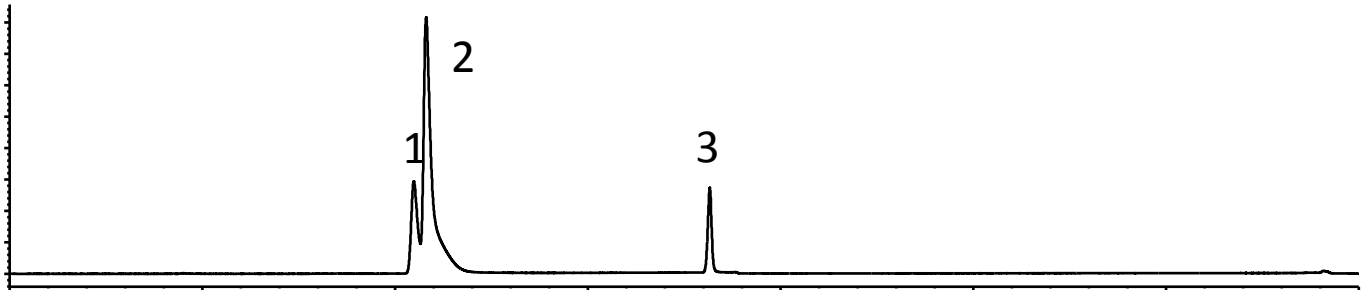
BEH amide 5% water gradients

3.5 min.

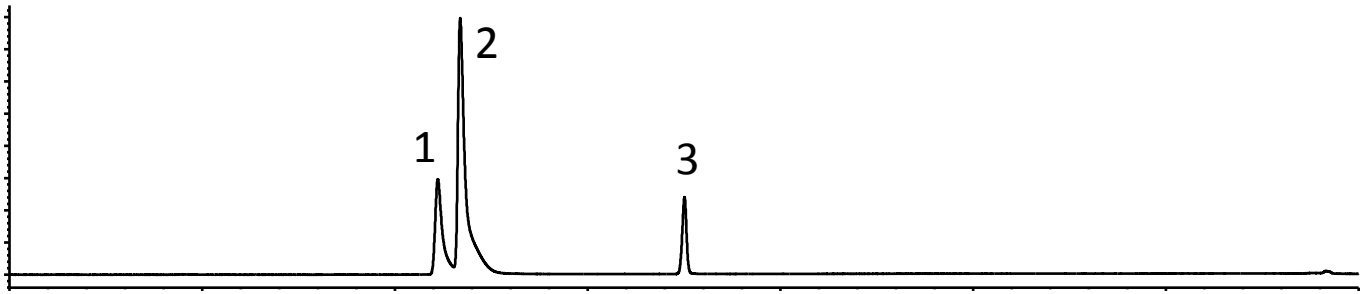
Fig. 1 b



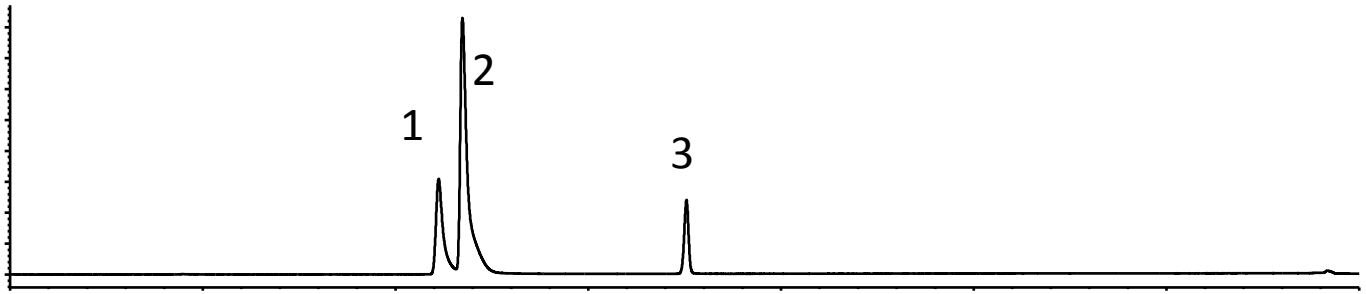
12.2 col. vols.



23.0 col. vols.



33.9 col. vols.



66.5 col. vols.

3.5 min.

Cortecs 5% water gradients

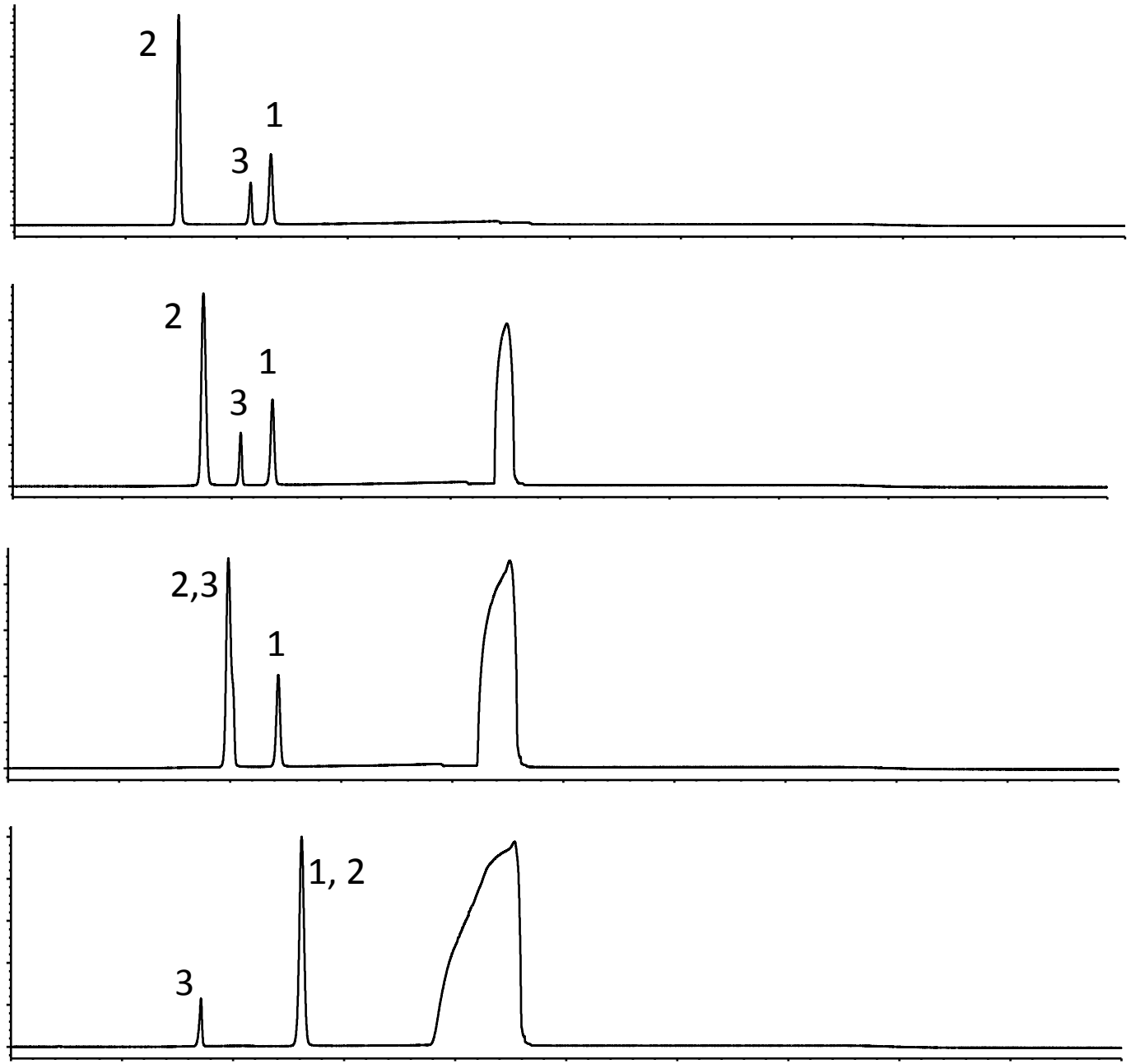


Fig. 1c

13.0 col. vols.

24.7 col. vols.

36.3 col. vols.

71.2 col. vols.

ZIC-HILIC 5 % water gradients

10.0 min.

Figure 2

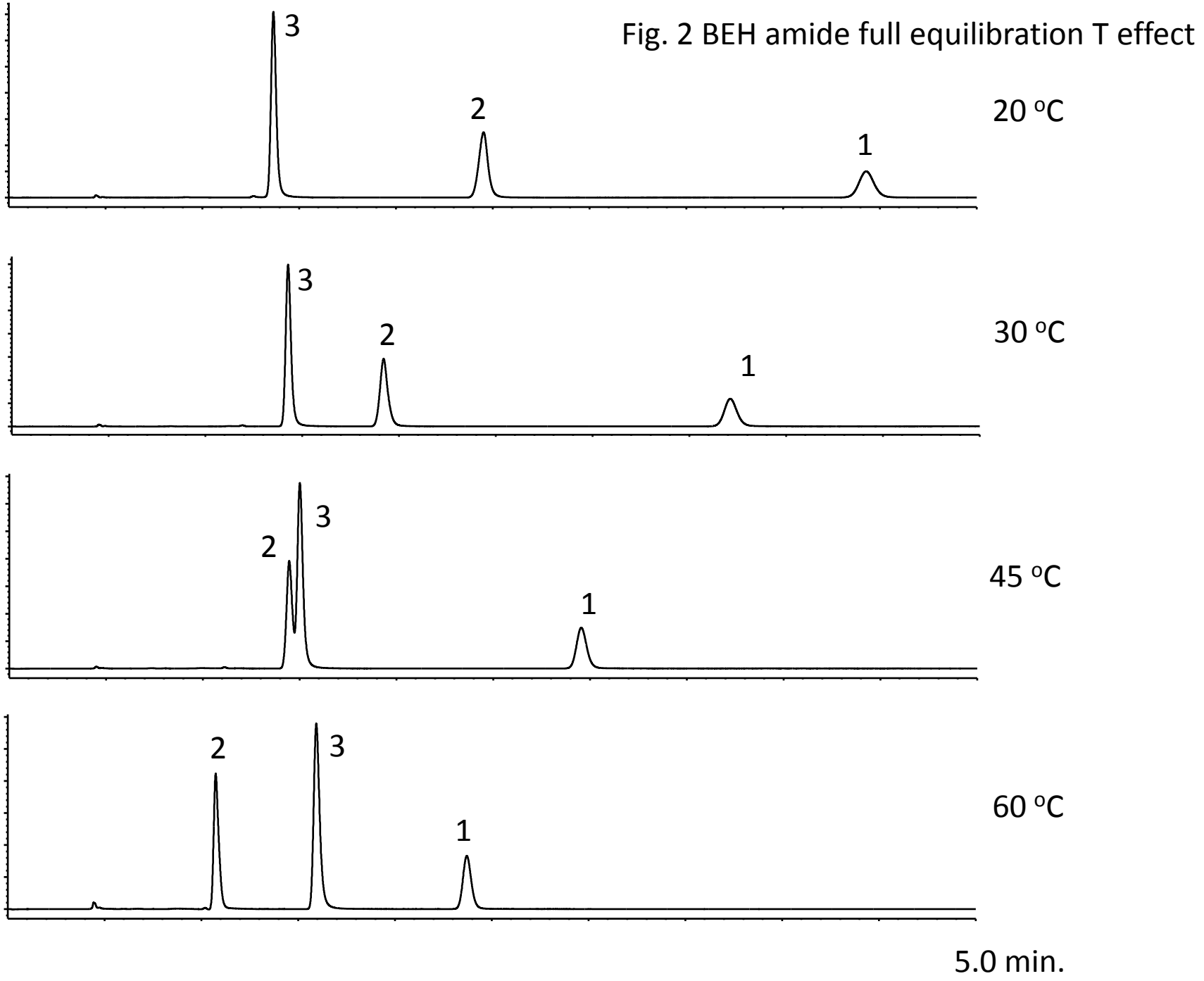


Fig. 2 BEH amide full equilibration T effect
3
2
1
20 °C
3
2
1
30 °C
2
3
1
45 °C
2
3
1
60 °C
5.0 min.

Figure 3

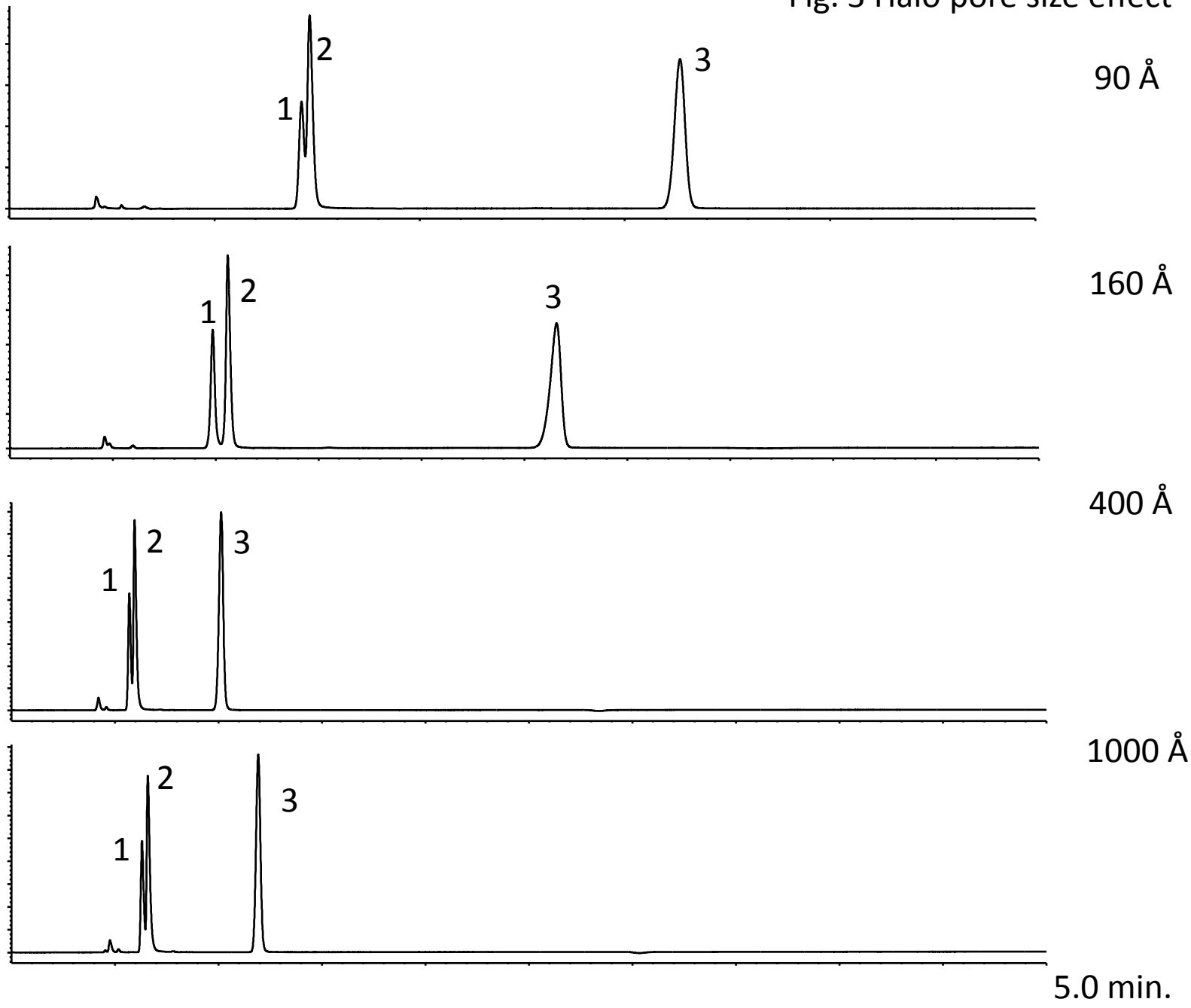


Figure 4

Fig. 4 ZIC –HILIC eq progress

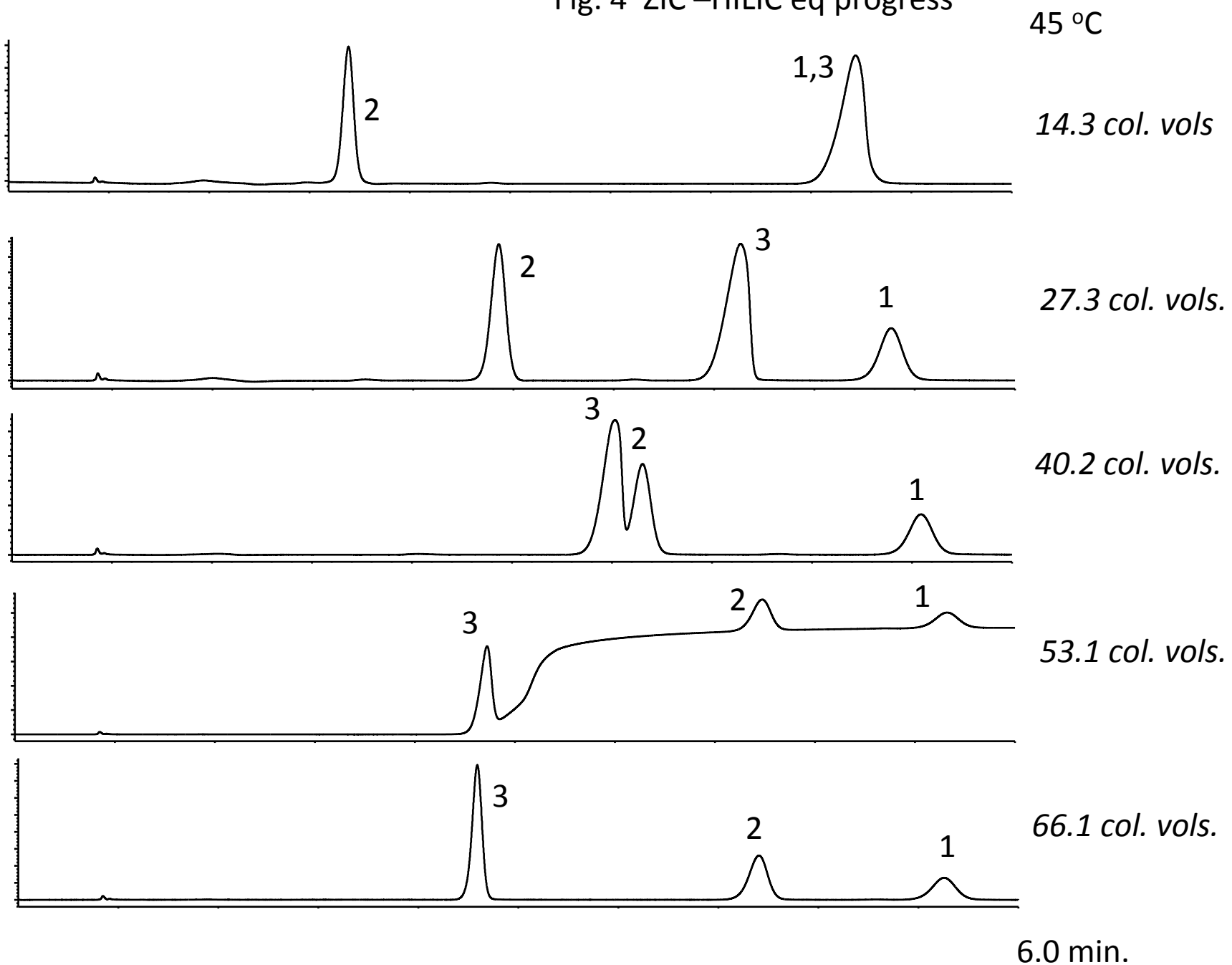


Figure 5

Fig. 5

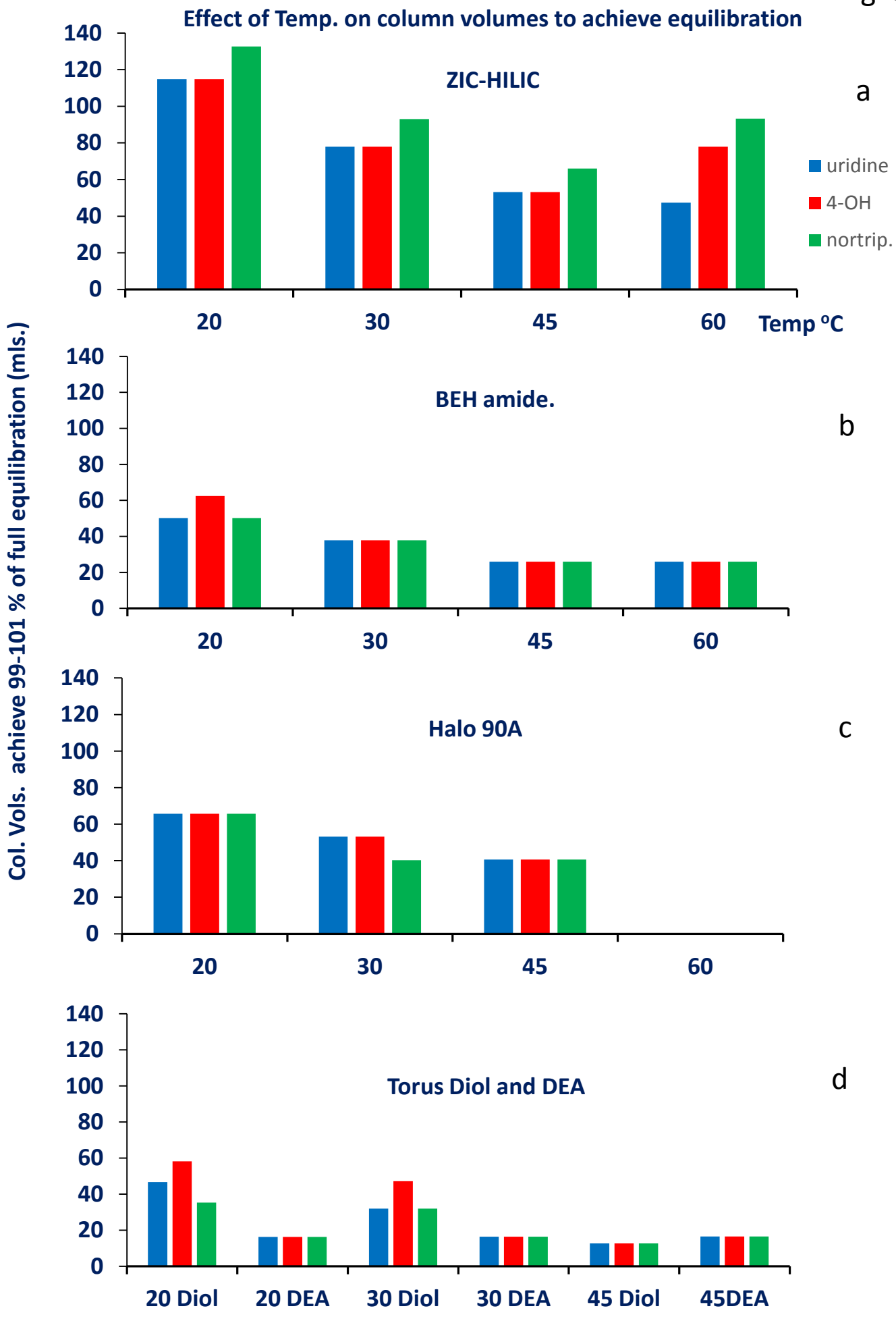


Figure 6

Fig. 6

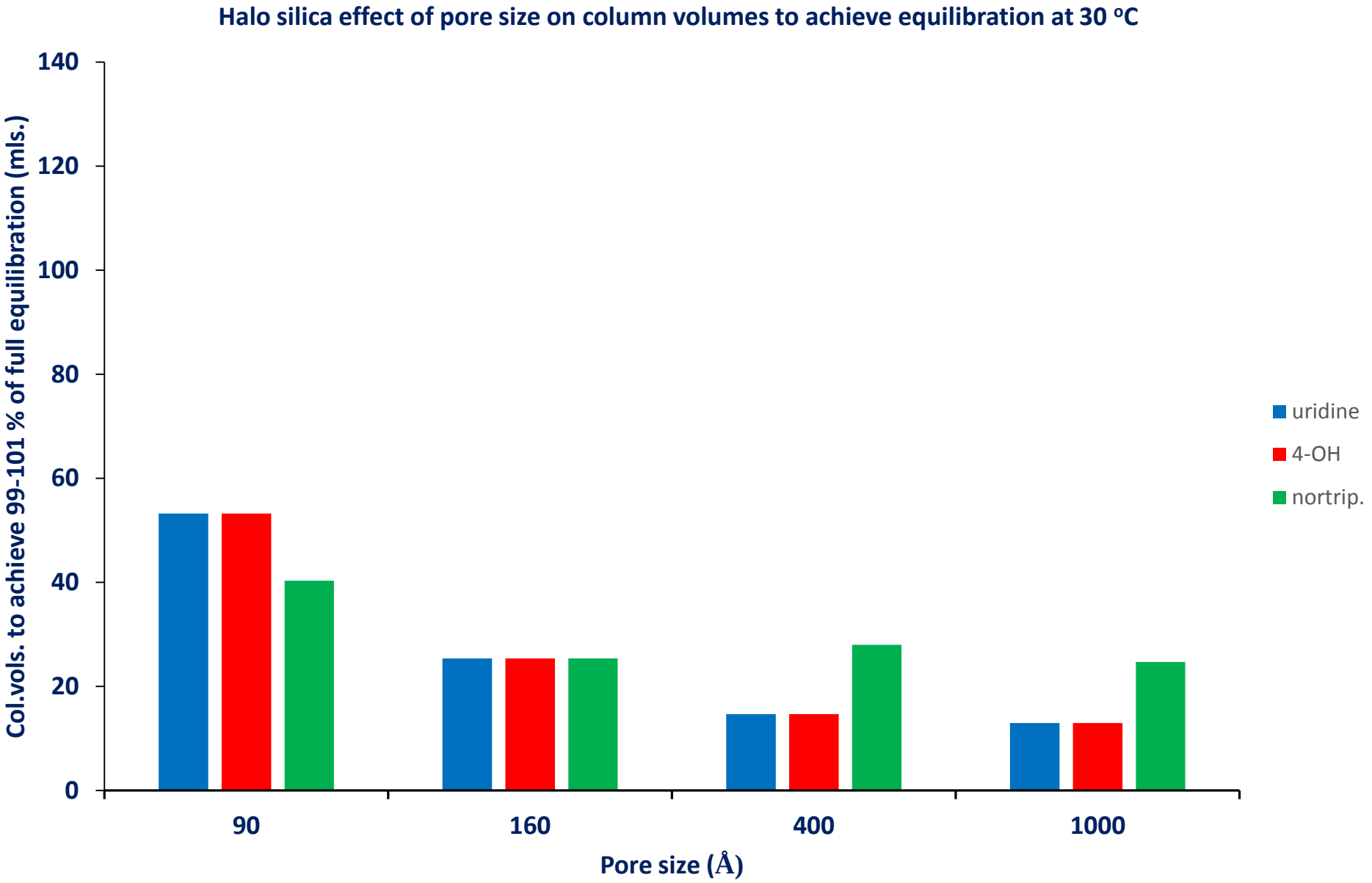


Table 1

Name	Manufacturer	Particle Size (μm)	Pore Size (Å)	Surface area (m^2/g)	Shell or Totally Porous particle	Shell thickness
Halo 90	AMT,Wilmington USA	2.7	90	135	Shell	0.5 μm
Halo 160	AMT	2.7	160	90	Shell	0.5 μm
Halo 400	AMT	3.4	400	15	Shell	0.2 μm
Halo 1000	AMT	2.7	1000	22	Shell	0.5 μm
Torus DEA (hybrid silica)	Waters, Milford USA	1.7	130	185	TPP	-
Torus Diol (hybrid silica)	Waters	1.7	130	185	TPP	-
BEH amide (hybrid silica)	Waters	1.7	130	185	TPP	-
Cortecs (SPP silica)	Waters	1.6	90	100	Shell	n/a
ZIC HILIC	Merck, Darmstadt, D	3.5	100	n/a	TPP	-

Table 1 Columns used in the study (all 10 cm x0.21 cm ID). n/a = not available.

Table 2

Column	Col. Vols.	grad %/min.	Cycles (n)	% rsd of t_R for 6 runs (following n cycles)		
				Nortrip	4-OH	uridine
BEH amide						
	12.2	5	2	0.31	0.18	0.09
	23.0	5	2	0.23	0.07	0.02
	33.9	5	2	0.15	0.13	0.06
	66.5	5	2	0.29	0.15	0.06
	12.2	1	1	0.08	0.10	0.05
	23.0	1	1	0.06	0.05	0.03
	33.9	1	1	0.06	0.06	0.06
	66.5	1	1	0.07	0.10	0.32
ZIC-HILIC						
	13	5	2	0.04	0.15	0.03
	24.7	5	1	0.07	0.18	0.06
	36.3	5	1	0.06	0.06	0.06
	71.2	5	2	0.36	0.10	0.10
	13	1	2	0.09	0.04	0.032
	24.7	1	2	0.14	0.07	0.05
	36.3	1	1	0.08	0.05	0.04
	71.2	1	1	0.08	0.19	0.19
Cortecs						
	12.2	5	1	0.19	0.27	0.18
	23.0	5	2	0.17	0.10	0.18
	33.9	5	0	0.06	0.12	0.22
	66.5	5	0	0.09	0.06	0.23
	12.2	1	1	0.12	0.11	0.09
	23.0	1	2	0.03	0.10	0.08
	33.9	1	1	0.03	0.04	0.07
	66.5	1	1	0.04	0.07	0.06

Table 2 % rsd of gradient elution runs on 3 different columns at two different values of gradient steepness.

Steep gradient: 5mM AF buffer pH 4.4 in 95% ACN to 60% ACN-buffer in 7min

Shallow gradient: 5% mM AF buffer pH 4.4 in 95% ACN to 88% ACN-buffer in 7min

Flow rate 0.5 mls/min. Temperature 30 ^{o}C

***Conflict of Interest**

The authors declare no conflict of interest in this work. They acknowledge that submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.