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Evaluation of new mixed-mode UHPLC stationary phases and the importance of stationary phase choice when using low ionic-strength mobile phase additives

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ABSTRACT

In this study, the selectivity, retention properties, peak shape and loading capacity for bases were practically evaluated using two UHPLC mixed-mode hybrid CSH stationary phases modified by C18 or Phenyl group. The data were compared with the data obtained on other UHPLC hybrid stationary phases (BEH C18, BEH C8, BEH Phenyl and BEH Shield RP18) at both basic and acidic conditions using conventional HPLC buffers (50 mM ammonium formate/acetate) as well as low ionic-strength additives such as, e.g. 0.1–0.01% formic/acetic acid and 1 mM solution of ammonium formate/acetate, which are widely used in LC–MS applications.

Ten pharmaceutically important compounds encompassing acids, bases and neutral were included into the study. Due to properties of CSH sorbent (which possess positively charged surface besides RP group), much improved peak shapes and weaker retention was obtained for bases even at very low concentration of acidic additives. Such conditions are ideally suited for LC–MS analysis of bases, where typical RP chromatographic separation (retention and good selectivity at basic pH) and LS–MS conditions (efficient ionization at acidic pH) are not in agreement. On the other hand, acids were more strongly retained and for some compounds the peak shape was influenced negatively due to ion-exchange mechanism. Further, the behavior of acidic, basic and neutral solutes is discussed using various additives at both basic and acidic pH for all above stated columns. The robustness of retention times after pH change from basic to acidic was also evaluated. The new CSH stationary phases represent an interesting selectivity tool preferably for separation of basic compounds.

1. Introduction

In early beginning of ultra-high performance liquid chromatography (UHPLC) in 2004 there were only few stationary phases available. Initially they covered mostly reverse-phase separations including C18, C8 and Phenyl modified stationary phases. Recently, the range of applicability of UHPLC stationary phases has been widely extended including normal phase, ion-exchange and hydrophilic interaction chromatography (HILIC) applications as well [1]. The attention has been attracted to mixed-mode stationary phases, which allow for multiple retention process to occur simultaneously due to surface modification. Such modification enables to obtain further selectivity and to add dimensionality in 2D separations [2]. Typical mixed-mode stationary phases contain C18 reverse chain and simultaneously strong anion exchange (SAX) and/or weak anion exchange (WAX) group. Such sorbents are also widely used for SPE technique [3,4].

In reversed-phase (RP) chromatography the retention of ionizable analytes is influenced by the ionic properties of the packing caused by surface silanol groups, besides of hydrophobic interactions and hydrogen-bond interactions [5,6]. Positively charged analytes interact with negatively charged surface silanols via an ion-exchange mechanism, which results in an enhanced retention. Conversely, negatively charged analytes are subjected to ion-exclusion effect [5]. In order to suppress ion-exchange mechanism of silanol groups following approaches might be applied: (1) lowering pH of the mobile phase to suppress silanol ionization, (2) an addition of tertiary amines, that preferentially bind to charge silanol groups, (3) an increase in ionic strength of mobile phase, (4) an addition of more retentive buffer cations (e.g. potassium) or finally (5) use a column with low silanol activity [6].

When using the first approach, lowering pH of the mobile phase, the protonation of bases will be also increased. In fact, there is an important divergence in the development of appropriate conditions for LC–MS analysis of basic compounds. For a good retention of bases on RP stationary phase basic pH is required in order to obtain non-ionized base, which will be well retained on non-polar stationary phase. On the other hand, an efficient ionization of bases in an ion source of mass spectrometer is obtained at acidic conditions

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(protonization of bases). Several volatile additives, including formic acid, acetic acid, ammonium formate and ammonium acetate at low concentrations (<0.5% or <5 mM), are used for the purpose of ionization enhancement in mass spectrometry. Often a volatile additive is used rather as an ionization additive than a buffering one. Low concentration of volatile additives are required due to reduced signal suppression effects [7,8]. When using weakly acidic low ionic-strength additives, the ability to reduce the ion-exchange activity of residual silanol groups is substantially decreased, therefore wide or tailing peaks of basic compounds are often observed. D.V. McCalley described a serious loss in column efficiency for ionized basic drugs and peptides, when working with weakly acidic mobile phases of low ionic-strength suitable for mass spectrometry [9]. The loss in efficiency was attributed to overloading of C18 stationary phase.

With the aim to reduce the consequences of the ion-exchange effect of silanol groups many companies have introduced new stationary phases with decreased silanol activity. The greatest contribution was made by hybrid particle technologies. These materials contain organic moieties such as methyl or ethyl groups in their structures, which provides higher chemical and mechanical resistance as well as a significant reduction (by nearly one third) of number of silanol sites [10,11]. Despite all these improvements in column technology, serious peak deformation can still occur under certain experimental conditions. More favorable behavior was noted by D. V. McCalley et al on a mixed-mode RP/embedded cation-exchange stationary phase [12]. Even though the silanol groups also occur on such stationary phase, they may be shielded by embedded ionic groups. Therefore, an increase in loadability of bases is a substantial advantage.

New UHPLC mixed-mode stationary phases based on hybrid support were introduced in 2010 as a new family of CSH (charged surface hybrid) analytical columns [13]. So far, the properties of this so called "charged surface hybrid" sorbent have not been described yet in practical applications. The aim of this study was to evaluate the selectivity, retention properties, peak shape and loading capacity of basic compounds using a mixture of pharmaceutical compounds of different structures. Two UHPLC mixed-mode CSH stationary phases modified by C18 and Phenyl groups were evaluated. The obtained data were compared with the data from other UHPLC hybrid stationary phases belonging to the bridged ethyl hybrid (BEH) family (BEH C18, BEH C8, BEH Phenyl and BEH Shield RP18).

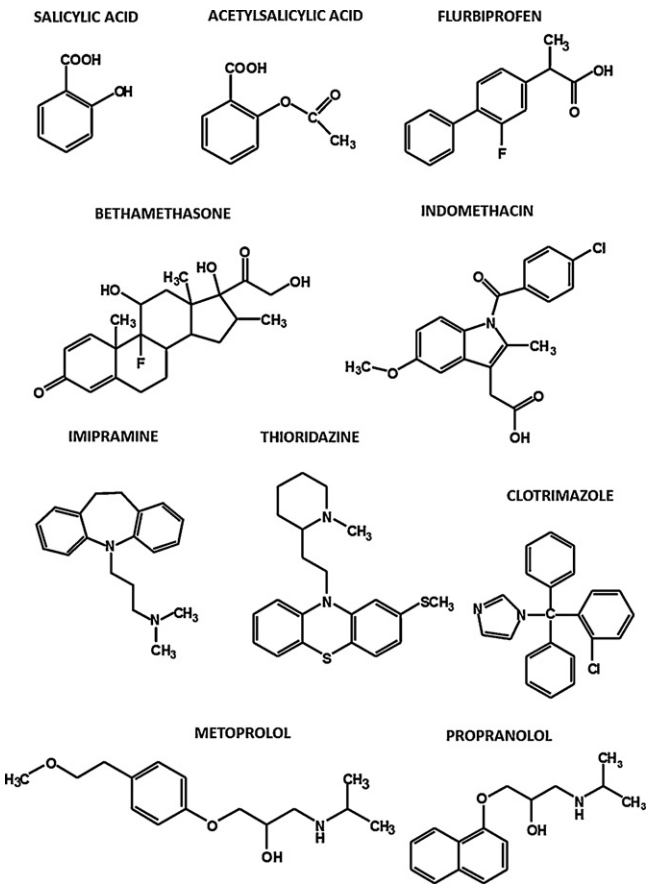

Fig. 1. Structures of compounds selected for this study.

2. Experimental

2.1. Chemicals and reagents

Working standards of metoprolol, salicylic acid, acetylsalicylic acid, propranolol, betamethasone, imipramine, clotrimazole, thioridazine, indomethacin and flurbiprofen were used for the purpose of this study. The structures are shown in Fig. 1. All compounds were

Table 1
The values of peak symmetry factor at selected chromatographic conditions. The numbers correspond as follows: (1) metoprolol, (2) acetylsalicylic acid, (3) salicylic acid, (4) propranolol, (5) betamethasone, (6) imipramine, (7) clotrimazole, (8) thioridazine, (9) flurbiprofen, (10) indomethacin.

Compound	1	2	3	4	5	6	7	8	9	10
0.1% formic acid in mobile phase										
BEH C18	1.30	1.15	1.39	1.75	1.42	1.79	1.97	1.87	1.34	1.34
BEH C8	1.48	1.71	2.30	1.50	1.47	1.51	1.48	1.51	1.72	1.71
CSH C18	1.17	1.13	1.85	1.20	1.16	1.15	1.12	1.16	1.07	1.08
CSH Phenyl	NA	1.19	2.23	1.29	1.14	1.26	1.26	1.21	1.12	1.12
BEH Phenyl	1.53	1.51	1.34	1.32	1.59	1.66	1.79	NA	NA	1.25
BEH Shield RP 18	1.87	1.10	NA	1.42	1.07	1.37	NA	1.25	1.04	1.03
10 mM ammonium acetate pH 3.0 in mobile phase										
BEH C18	1.19	1.09	1.22	1.36	1.22	1.36	1.38	1.45	1.30	1.30
BEH C8	1.41	1.62	1.52	1.35	1.36	1.41	NA	1.38	1.45	1.49
CSH C18	1.26	1.12	1.40	1.40	1.08	1.08	NA	1.18	1.07	1.08
CSH Phenyl	1.36	NA	1.58	NA	1.14	1.21	1.25	1.14	1.12	1.13
BEH Phenyl	NA	1.45	NA	1.48	1.35	1.50	1.50	1.13	1.13	1.29
BEH Shield RP 18	1.35	1.12	1.22	1.29	1.07	1.24	1.07	1.21	1.05	1.06
1 mM ammonium acetate pH 3.0 in mobile phase										
BEH C18	NA	1.14	NA	1.95	1.40	1.84	1.81	1.83	1.37	1.38
BEH C8	1.62	2.06	2.28	1.35	1.32	1.35	NA	NA	1.58	1.63
CSH C18	1.28	1.25	2.15	1.28	1.11	1.25	1.10	1.26	1.18	1.13
CSH Phenyl	1.30	1.22	2.86	1.22	1.29	1.29	1.02	1.16	1.16	1.15
BEH Shield RP 18	0.99	1.14	NA	1.41	1.18	NA	0.99	1.29	1.04	1.03

Table 2
The values of peak width at selected chromatographic conditions. The numbers correspond as follows: (1) metoprolol, (2) acetylsalicylic acid, (3) salicylic acid, (4) propranolol, (5) betamethasone, (6) imipramine, (7) clotrimazole, (8) thioridazine, (9) flurbiprofen, (10) indomethacin.

Compound	1	2	3	4	5	6	7	8	9	10
0.1% formic acid in mobile phase										
BEH C18	3.70	5.75	6.40	6.75	6.05	7.00	7.00	7.50	6.25	6.25
BEH C8	5.85	9.05	9.35	8.55	7.20	8.00	7.35	8.25	8.50	8.50
CSH C18	4.45	5.75	8.15	4.95	3.95	4.35	4.30	4.65	5.00	5.75
CSH Phenyl	6.05	5.65	10.35	5.95	4.25	4.65	4.75	7.80	4.80	5.10
BEH Phenyl	10.55	6.20	8.05	7.70	8.50	10.40	8.95	10.50	NA	9.25
BEH Shield RP 18	6.60	5.20	NA	4.95	4.20	4.10	NA	3.85	5.35	5.35
10 mM ammonium acetate pH 3.0 in mobile phase										
BEH C18	4.10	4.10	5.20	6.20	5.10	5.70	5.50	6.05	5.55	6.30
BEH C8	5.75	8.00	7.20	7.85	7.50	8.45	4.25	6.75	7.55	6.95
CSH C18	5.10	5.20	7.50	4.60	NA	NA	NA	4.70	5.05	5.60
CSH Phenyl	4.75	NA	9.25	NA	4.30	4.65	4.30	6.95	4.80	5.55
BEH Phenyl	NA	7.25	NA	6.85	6.45	6.60	6.40	NA	NA	9.10
BEH Shield RP 18	5.20	5.00	6.20	4.05	4.75	4.80	3.60	4.75	5.15	5.25
1 mM ammonium acetate pH 3.0 in mobile phase										
BEH C18	NA	6.40	NA	8.05	5.75	8.00	7.90	7.70	6.15	6.80
BEH C8	4.85	8.60	9.10	10.90	7.15	9.80	NA	NA	5.90	6.70
CSH C18	4.05	5.60	11.50	5.65	5.45	5.20	5.20	6.00	5.90	6.70
CSH Phenyl	2.80	4.90	12.55	4.85	5.75	4.80	5.00	9.55	4.90	5.60
BEH Shield RP 18	9.40	5.65	NA	4.90	4.00	NA	4.20	4.35	6.15	5.85

obtained from Sigma Aldrich (Prague, Czech Republic). Formic acid (98%, LC–MS grade, Fluka), acetic acid (>99%, LC–MS grade, Fluka), ammonium hydroxide (>25%, LC–MS grade, Fluka) and acetonitrile LC–MS grade were provided by Sigma Aldrich. Ultra-pure water was obtained with a Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and met the requirements of the European Pharmacopoeia.

2.2. Chromatography

The Acquity UPLC system (ACQ) (Waters, Prague, Czech Republic) was used for the purposes of this study. The system consisted of ACQ-binary solvent manager, ACQ-sample manager, ACQ-column manager and ACQ-PDA detector.

A mixture of pharmaceutical compounds was separated using gradient elution from initial conditions of 20/80 acetonitrile/water

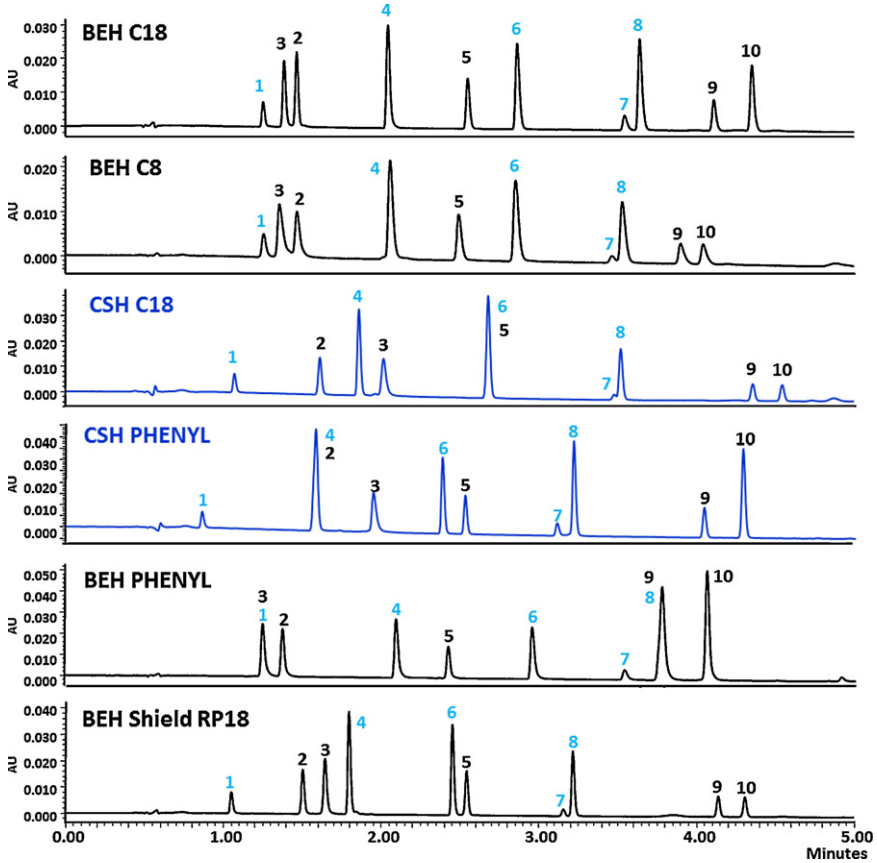

Fig. 2. A comparison of separation of the mixture on BEH and CSH analytical columns using gradient elution with mobile phase containing acetonitrile and 50 mM ammonium acetate pH 3.0. Peaks are eluted as follows: (1) metoprolol, (2) acetylsalicylic acid, (3) salicylic acid, (4) propranolol, (5) betamethasone, (6) imipramine, (7) clotrimazole, (8) thioridazine, (9) flurbiprofen, (10) indomethacin.

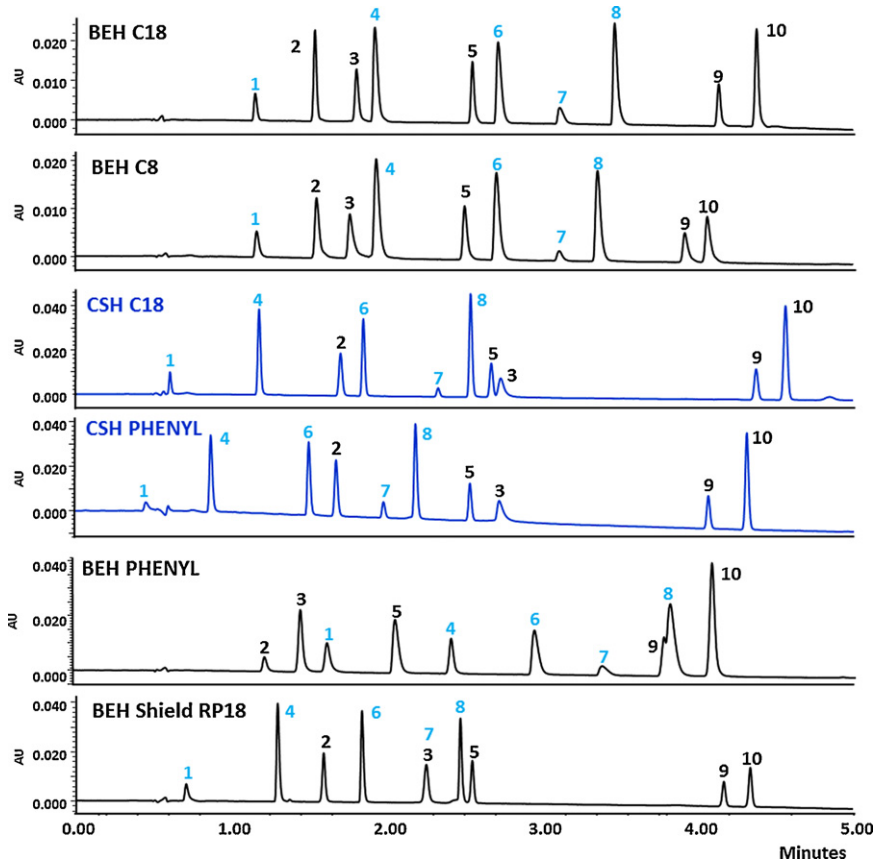

Fig. 3. A comparison of separation of the mixture on BEH and CSH analytical columns using gradient elution with mobile phase containing acetonitrile and 0.1% formic acid (pH 2.65). Peaks are eluted as follows: (1) metoprolol, (2) acetylsalicylic acid, (3) salicylic acid, (4) propranolol, (5) betamethasone, (6) imipramine, (7) clotrimazole, (8) thioridazine, (9) flurbiprofen, (10) indomethacin.

to 80/20 of acetonitrile/water within 5 min at flow rate 0.5 ml/min linearly. A water component included consecutively following additives: 0.1% formic acid (pH = 2.62), 0.01% formic acid (pH = 3.21), 0.1% acetic acid (pH = 3.09), 0.01% acetic acid (pH = 3.65), 0.1% ammonia (pH = 10.93), 50 mM ammonium formate pH 3.0, 50 mM ammonium acetate pH 10, 1 mM ammonium formate pH 3.0 and 1 mM ammonium acetate pH 10.0. The analytes were detected at 272 nm. The injection volume was 2 μL.

Analytical columns included into this study were following: Acquity BEH C18, Acquity BEH Shield RP 18, Acquity BEH Phenyl and Acquity BEH C8 from the group of BEH sorbents. The group of CSH sorbents was represented by Acquity CSH C18 and Acquity CSH Phenyl. All the columns were filled with 1.7 μm particles in 100 mm × 2.1 mm dimensions. They were all obtained from Waters (Prague, Czech Republic). During the separation the columns were kept at 30 °C.

2.3. Preparation of standard solutions and samples

Stock solutions of standards of selected pharmaceuticals were prepared in methanol at a concentration of 1 mg/ml. They were used to obtain a solution of 10 μg/ml after dilution with water. The solution was directly injected into the UHPLC system.

3. Results and discussion

3.1. Separation using conventional buffers

Typical mobile phases in LC applications employing UV or fluorescence detection utilize various buffers in common concentration range 10–200 mM. In our experiments 50 mM ammonium formate pH 3.0 and 50 mM ammonium acetate pH 10.0 were applied in gradient elution together with acetonitrile. The separations obtained with buffer at pH 3.0 demonstrated symmetrical peak shapes (symmetry factor < 1.5) on all tested columns, except for salicylic acid on CSH Phenyl and except for salicylic and acetylsalicylic acids on BEH C8 (Table 1). Narrow peaks were eluted, which is demonstrated by peak width values around 5–6 s or less in most cases (Table 2).

The best selectivity for mixture of 10 compounds of basic, acidic and neutral structures at pH 3.0 was provided by BEH C18 and BEH Shield RP 18 (Fig. 2). On both CSH columns stronger retention of acids and weaker retention of bases were observed due to combined hydrophobic and ion-exchange mechanism. For bases, there was the ion repulsion between positively charged CSH stationary phase and ionized base molecule therefore ion-exclusion occurred besides hydrophobic interaction, which decreased the retention. For acids, there was the ion attraction between positively charged surface of CSH stationary phase and acid molecule therefore ion-exchange mechanism occurred besides hydrophobic interaction, which increased the retention. Moreover in case of salicylic acid (peak 3 in Fig. 2) negative influence on a peak shape was observed. It is interesting to note, that the elution order on BEH columns, even though modified by different functional group, was quite similar, while on CSH columns peak order was changed due to earlier elution of bases (peak 4 and 6, Fig. 2).

At basic pH 10.0 with 50 mM ammonium acetate again, the separations demonstrated symmetrical peak shapes except for very lately eluted strongly retained peak of thioridazine on all columns. Apparently, the selectivity changed completely with peak elution order 2, 3, 9, 1, 10, 5, 4, 7, 6 and 8 quite similar on all columns (data

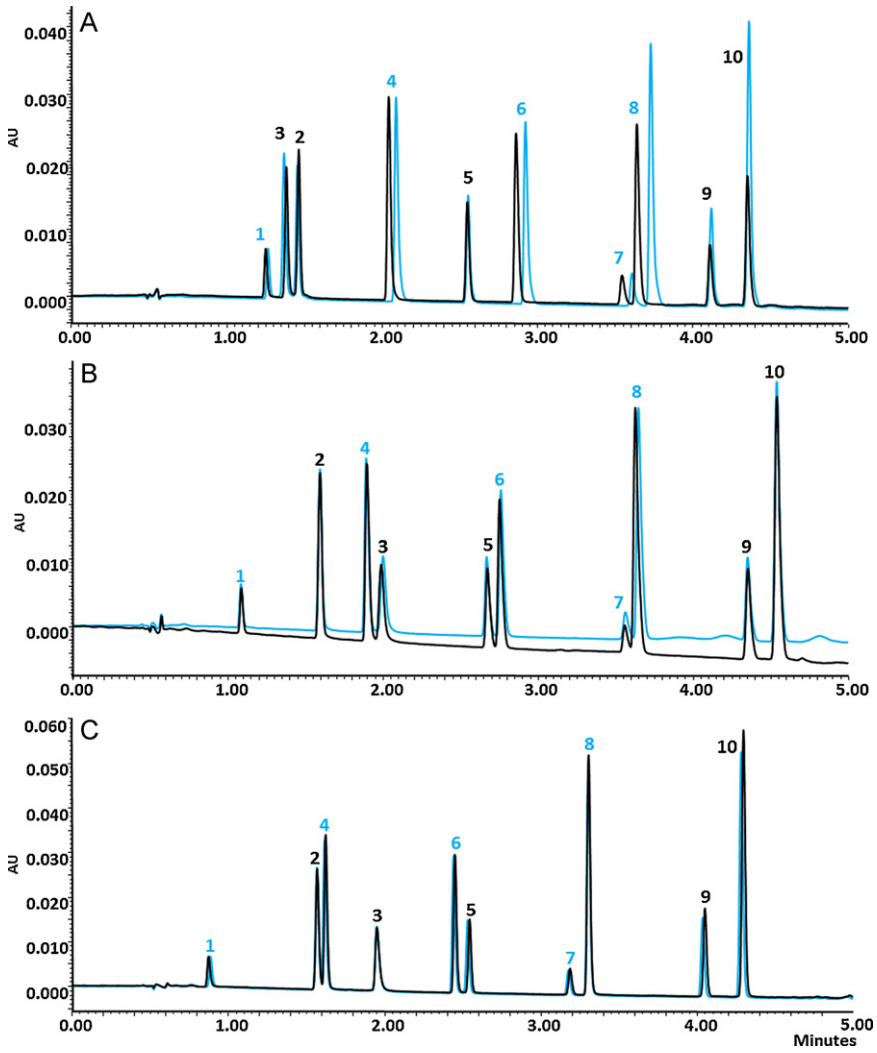

Fig. 4. An overlay of chromatograms obtained during the experiment with acetonitrile/10 mM ammonium acetate pH 3.0 measured before (black line) and after (blue line) the experiment with acetonitrile/10 mM ammonium acetate pH 10.0. (A) Acquity BEH C18, (B) Acquity CSH C18, (C) Acquity CSH Phenyl.

not shown) indicating early elution of charged acids due to lack of hydrophobic interactions on all columns (within about 2 min). No peak tailing of acidic compounds was observed as opposed the same conditions at pH 3.0. Late elution of basic compounds, which were much stronger retained in their uncharged form due to hydrophobic interactions, was observed on BEH C8, C18 and CSH C18, and was even more pronounced on CSH C18 and BEH C18 for lately eluted compounds (peak 6, 7 and 8). There was an inversion of peak 10 and 1 on both CSH columns compared to BEH columns indicating somewhat stronger retention of bases. Otherwise, the retention profiles of basic analytes were quite similar, probably due to their presence in uncharged form, which prevented from ion-exclusion interaction with positively charged ion-exchange group of CSH sorbent.

3.2. Separation using low-concentration mobile phase additives

In LC–MS applications volatile low ionic-strength additives are often used in order to prevent signal suppression. In our study the experiments were performed with formic acid (0.1% and 0.01%), acetic acid (0.1% and 0.01%), 1 mM ammonium formate pH 3.0 and 1 mM ammonium acetate pH 10.0.

When using low ionic-strength additives, such as formic acid 0.1%, of course the peak shape of bases at acidic pH becomes distorted. This situation is demonstrated in Table 1, where the values of peak symmetry for bases exceed the value 1.5 or are very close to it on conventional BEH columns including C18, C8 and Phenyl modification. On the other hand, on CSH columns the value of peak symmetry is still very low for all bases (around 1.1–1.2, see Table 1). Similar situation (no peak tailing) might be observed on BEH Shield RP 18 due to embedded polar group, which is shielding remaining free silanol groups. The free silanol group, even if their number is substantially decreased on the surface of BEH sorbent, are ionized and possess negative charge, which allows for the interaction with positively charged groups of basic molecules. This phenomenon is not observed on CSH columns due to different surface chemistry and on BEH Shield RP 18 due to shielding embedded polar group.

The peak width is subsequently still narrow (4–5 s) for bases on CSH and BEH Shield RP column, while it becomes broader on the other tested BEH columns (7–10 s), see Table 2. Ammonium formate pH 3.0 at 1.0 mM concentration provided similar results in terms of peak symmetry and peak width (see Tables 1 and 2). The selectivity of BEH and CSH columns becomes very different when using 0.1% formic acid (Fig. 3) or ammonium formate 1 mM pH 3.0 in mobile phase compared to conventional buffers (Fig. 2).

The difference is more pronounced for CSH columns, which provided better selectivity for our target mixture of compounds when using formic acid or low concentration buffer (1 mM) compared to conventional buffer (50 mM) at pH 3.0. Even if the concentration of additive is further decreased (formic acid 0.01%), the peak shape of basic compounds remains acceptable on CSH columns. The same applies if the additive is changed and formic acid is replaced by acetic acid. The example is shown for CSH C18 (S1). The selectivity in this case changes slightly until the pH value 3.65 (0.01% acetic acid), when more significant changes in selectivity are observed. For peak 3 (acetylsalicylic acid) and peak 7 (clotrimazole) significant shift in retention times are observed at any condition change. A comparison of selectivity of selected BEH columns and CSH columns is shown in (S2).

3.3. The stability of retention times with the change of pH

Equilibration time in gradient elution is an important parameter in order to obtain repeatable separation and retention times. The column equilibration requires the passage of a certain volume of mobile phase – rather than the passage of a certain time. Typically 10–20 column volumes of mobile phase are required for column equilibration, except for mobile phases with a low organic content or which contain an ion-pair reagent. Moreover, the equilibration time was found to be dependent on many other factors, such as analyte structure or column history. The important shifts in retention times were described after the column exposure to basic pH and then the return to acidic pH [14,15].

In our experiments we verified the stability of retention times under the influence of pH change between 3.0 and 10.0. The first experiment was performed using gradient elution with acetonitrile/50 mM ammonium formate pH 3.0, which was followed by an experiment with gradient elution by acetonitrile/50 mM ammonium acetate pH 10.0. Three injections were performed at each conditions and column was always equilibrated properly (about 40 column volumes). Thereafter, the pH was switched back to acidic and the same experiment with acetonitrile/50 mM ammonium formate pH 3.0 was performed after an appropriate equilibration of 30 min (at flow-rate 0.5 ml/min this corresponds to 43 column volumes, which is substantially exceeding common requirements). However, on BEH columns the shift in retention times for all basic compounds was noted, while on CSH columns the retention times of basic compounds were perfectly repeatable. The example is shown for BEH C18, CSH C18 and CSH Phenyl in Fig. 4. This effect might be attributed to residual silanols on BEH sorbent, which become ionized at basic pH and subsequently the change in column charge state after the pH change and the equilibration is very slow [14]. Similar phenomenon was not observed on CSH stationary phase, as its surface is modified by positively charged functional group prior to attachment of C18 ligand [13].

3.4. Loading capacity for bases

An overload effect for basic compounds is a very well-known phenomenon, which has been widely described in the literature [15–17], especially when working with low ionic-strength mobile phases [9]. This effect was also tested on BEH and CSH stationary phase modified by C18 ligand. Four basic compounds from our mixture were individually injected at described gradient conditions, however at high concentration such as 100 ppm. As demonstrated in (S3) for all basic compounds from the group, CSH C18 had much higher loading capacity for bases than BEH C18. These results are in agreement with the results published previously for Primesep column (mixed mode RP/embedded cation-exchange group) by McCalley et al. [12].

4. Conclusions

New mixed-mode stationary phases based on CSH technology were tested and practically evaluated within this study. Positively charged surface of the support demonstrated many favorable properties:

- CSH stationary phases were convenient for analysis of basic compounds at acidic pH without significant influence of mobile phase additive. Low-ionic strength additives as well as conventional buffers might have been used with perfectly symmetrical peaks as a result. Such approach is highly convenient for LC–MS applications. Selectivity and peak shape for bases has not been substantially changed with the change of low ionic-strength additive concentration or type, which increased method robustness substantially. Moreover, CSH stationary phases provided better selectivity for target mixture of basic, neutral and acidic compounds with low-ionic strength additives compared to conventional buffers. Similar results have been observed on BEH Shield RP 18, therefore these stationary phases could be recommended to be used with low-ionic strength mobile phases. Other tested stationary phases often demonstrated very significant peak tailing for basic compounds with acidic low-ionic strength additives.
- CSH stationary phases demonstrated higher loading capacity for basic compounds compared to hybrid BEH stationary phase.
- The equilibration on CSH stationary phase was much faster compared to BEH sorbents under various conditions applied. Typical problem – retention time shift, when acidic and basic pH is changed on the column was not observed on CSH stationary phases, while it was observed on BEH stationary phases.
- CSH stationary phases provided completely different selectivity compared to currently available BEH stationary phases. With low ionic-strength additives the selectivity of CSH C18 column was somewhat similar to BEH Shield RP 18.

On the other hand, for some acidic compounds some problems might be encountered on CSH stationary phases. In our case, salicylic acid demonstrated an important peak tailing on both tested CSH stationary phases related to partial ionization and ion-exchange interactions at tested experimental conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.054.

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