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Unexpected effects of mobile phase solvents and additives on retention and resolution of *N*-acyl-D,L-leucine applying *Cinchonane*-based chiral ion exchangers

Dániel Tanács[a], Tímea Orosz[a], István Ilisz[a,*], Antal Péter[a], Wolfgang Lindner[b,*]

[a] Institute of Pharmaceutical Analysis, Interdisciplinary Excellence Centre, University of Szeged, H-6720 Szeged, Somogyi u. 4, Hungary
[b] Department of Analytical Chemistry, University of Vienna, Währinger Strasse 38, 1090 Vienna, Austria

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ABSTRACT

Chiral ion exchangers based on quinine (QN) and quinidine (QD), namely Chiralpak QN-AX and QD-AX as anionic and ZWIX(+) and ZWIX(-) as zwitterionic ion exchanger chiral stationary phases (CSPs) have been investigated with respect to their retention and chiral resolution characteristics. For the evaluation of the effects of the composition of the polar organic bulk solvents of the mobile phase (MP) and those of the organic acid and base additives acting as displacers necessary for a liquid chromatographic ion-exchange process, racemic *N*-(3,5-dinitrobenzoyl)leucine and other related analytes were applied. The main aim was to evaluate the impact of the MP variations on the observed, and thus the apparent enantioselectivity (α_{app}), and the retention factor. Significant differences were found using either polar protic methanol (MeOH) or polar non-protic acetonitrile (MeCN) solvents in combination with the acid and base additives as counter- and co-ions. It became clear, that the charged sites of both the chiral selectors of the CSPs and the analytes get specifically solvated, accompanied by the adsorption of all MP components on the CSP, thereby building a stagnant "stationary phase layer" with a composition different from the bulk MP. Via a systematic change of the MP composition, trends of resulting α_{app} and retention factors have been identified and discussed.

In a detailed set of experiments, the effect of the concentration of the acid component in the MP containing MeOH or MeCN was specifically investigated, with the acid considered to be a displacer in anion-exchange type chromatographic systems.

Surprisingly, all four chiral columns retained and resolved the tested *N*-acyl-Leu analytes with α_{app} values up to 21 within a retention factor window of 0.03 and 10 with pure MeOH as eluent. However, using pure MeCN as eluent, an almost infinite-long retention of the acidic analyte was noticed in all cases. We suggest that the rather different thickness of the solvation shells generated by MeOH or MeCN around the charged/chargeable sites of the chiral selector determines eventually the strength of the electrostatic selector–selectand interactions.

As a control experiment we included the non-chiral *N*-acylglycine derivatives as analyte in all cases to support the interpretations with respect to the contribution of the enantioselective and non-enantioselective retention factor increments as a part of the observed α_{app}.

1. Introduction

Various liquid chromatographic enantiomer and diastereomer separation concepts have reached a high analytical and preparative standard over the years as exemplified by diverse dedicated review articles [1–9]. Beside the focus on relevant applications, investigations and interpretations related to the underlying enantioselective molecular recognition mechanisms and adsorption models have also been undertaken and described in detail [10–13].

A generalized view, where the enantiomer resolution is based on the formation of intermediate molecule associates between the chiral selector (SO) moiety and the individual enantiomers of the chiral analytes, the selectands [(*R*)-SA and (*S*)-SA] does not pay the necessary attention to the actual situation of a wetted

* Corresponding authors.
E-mail addresses: ilisz.istvan@szte.hu (I. Ilisz), wolfgang.lindner@univie.ac.at (W. Lindner).

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chiral stationary phase (CSP), where the chemically modified surface of the silica particles is not fully homogenous. What we actually observe as a chromatogram is the result of all occurring stereospecific and non-stereospecific interactions of SO–SA molecule associates, and additional interactions of SA with the imperfectly derivatized and wetted silica surface, including, *e.g.*, the remaining free silanol groups. In addition to these considerations, the conformational flexibility of the chiral motif around the binding sites of the SO moiety has to be taken into consideration which, in turn, will depend on the solvent environment of the wetted CSP being in equilibrium with all components of the MP.

The observed (*apparent*) retention factor k_{app} of each individual enantiomer (*R*)-SA (1) and (*S*)-SA (2) is the sum of enantioselective (*es*) and non-enantioselective (*ns*) adsorption phenomena (including partition, when applicable), which can largely differ in their magnitude [14–16]. The apparent retention factors can be written as $k_{app1} = k_{es1} + k_{ns1}$ for the first-eluting enantiomer and $k_{app2} = k_{es2} + k_{ns2}$ for the second-eluting enantiomer of a chiral analyte, where the nonselective retention factors (k_{ns1} and k_{ns2}) are identical.

The apparent enantioselectivity factor α_{app} is therefore:

$$\propto_{app} = \frac{k_{app2}}{k_{app1}} \quad (1)$$

In liquid chromatography an integral part of the observed phenomena relates to the chemical structure of the SO motif, including all the functional groups capable for hydrogen bonding, electrostatic interaction, etc., and its conformational flexibility and thus to the solvated CSP, which is directly associated with all mobile phase components and their physico-chemical characteristics [17,18]. The SO–SA interactions encompass essentially electrostatic, hydrogen bonding, π–π and van der Waals (hydrophobic) forces, which are quite different in their magnitude. In a simplified view, solvents act as *quasi* competitors (displacers) to the overall active SO–(*R*)-SA and SO–(*S*)-SA interactions. As a consequence, the liquid-chromatographic process can be formulated by a stepwise adsorption, desorption, and elution of the solvated analytes from the solvated CSP. First, there is a de-solvation event of the individually solvated molecular entities (SO and SAs) during the formation of the two SO–(*R*)-SA and SO–(*S*)-SA associates accompanied by their solvation, followed by a re-solvation of the individual entities (the SO and the SAs) as a consequence of the dissociation of the diastereomeric associates. The chemical characteristics of the SO and SA as well as the type of solvents, acids, bases, salts or neutral additives and their composition in the MP may play a significant role in building up an environment on the surface, which we assign in the following as the solvated CSP layer. The solvated CSP may be considered as a thin "layer" on the modified surface, which may give rise to any additional non-stereoselective partition-type retention causing increments of the retention of the SAs. As already mentioned, the type of the solvents and the resulting solvation status of all interaction sites of the SO and SAs will also affect the composition of the various conformers of the SO moiety as well which, naturally, depends on its chemical structure. For small or polymeric type SO molecules it may be quite different, but it is a dynamic process, whereby induced fit phenomena of SO upon the approaching and complex formations with the SA has to be considered [19].

In the present paper, the contributions of diverse MP compositions on four *Cinchona* alkaloid-based CSPs are evaluated. Namely, utilization of Chiralpak QN-AX and QD-AX CSPs employed as chiral anion exchangers [20], and zwitterionic ion exchangers ZWIX(+) and ZWIX(-) CSPs used as anion-exchanger type "chiral columns" [21] for the resolution of three acidic racemic *N*-acyl type leucine (Leu) derivatives are discussed. In these experiments, *N*-tagged glycine (Gly) derivatives served as non-chiral reference compounds for the discussion of the molecule-dependent retention and stereoselectivity characteristics. Fig. 1A and 1B schematically depict the most prominent interactions of the employed SOs, as elucidated earlier by diverse studies [18,20–29]. In such representations, neither conformational nor solvation effects are usually considered. However, because of solvation issues discussed above, we intended to depict, at least schematically, the status of solvated CSPs and SAs, but omitting the eventually present co- and counterions in the stagnant mobile phase layer close to the CSP surface and within the pores (Fig. 2). As shown previously, these types of ion exchangers show excellent enantioselectivity, particularly for *N*-(3,5-dinitrobenzoyl) (DNB) derivatized amino acids [19–23]. Between the π-acidic DNB-group and the π-basic chinoline residue of the QN/QD selector moiety, strong intermolecular π–π interaction can be formed in cooperation with dominant hydrogen supported Coulomb attraction and strong hydrogen bonding of the amide groups of the SOs and SAs (Fig. 1A and 1B), thus leading to relatively large α_{app} values.

In the first part of this study, we aimed to elucidate the individual contribution of the two polar solvents, namely, protic methanol (MeOH) and non-protic acetonitrile (MeCN), together with a constant amount of formic acid (FA) and triethylamine (TEA) as mobile

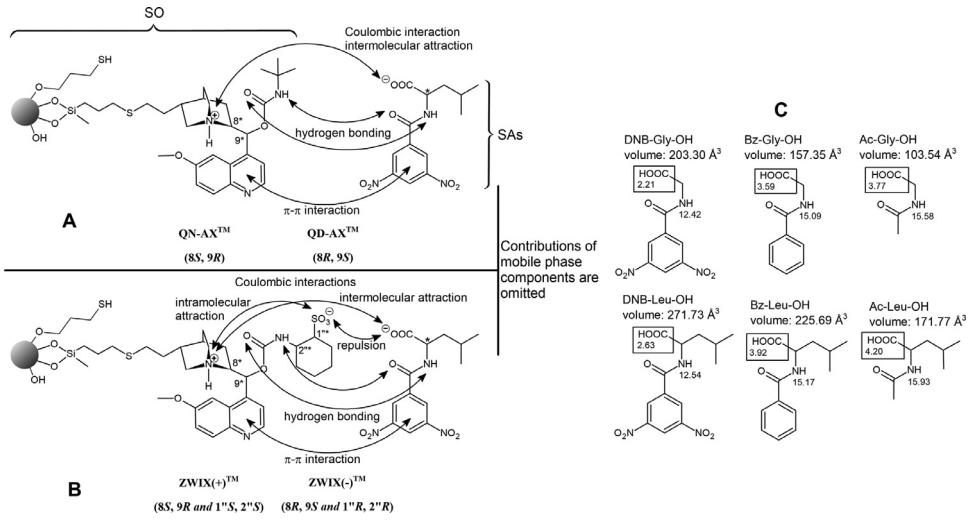

Fig. 1. Molecular structures of chiral selectors (SOs), scheme of intermolecular interactions between the chiral selectors (SOs) and the chiral selectands (SAs): **A**, Scheme for QN-AX and QD-AX type CSPs; **B**, Scheme for ZWIX(+) and ZWIX(-) type CSPs; **C**, Molecular structure of analytes including their molecular volume (Å^3) and pK$_a$ values.

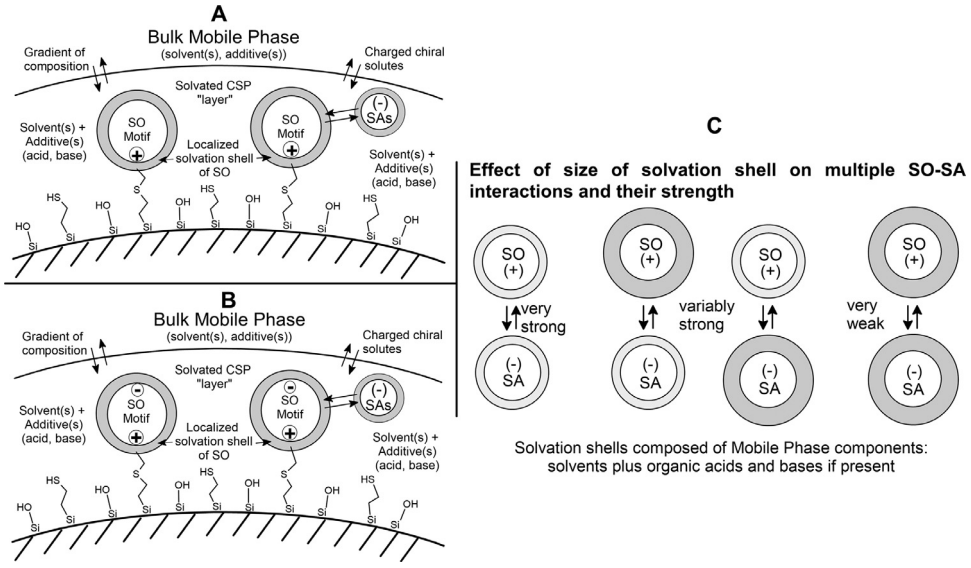

Fig. 2. Scheme of the status of solvated SOs and of solvated SAs. **A**, Solvation layer developing on QN-AX and QD-AX type CSPs; **B**, Solvation layers developing on ZWIX(+) and ZWIX(-) type CSPs; **C**, Effect of size of solvation shell on multiple SO–SA interactions.

phase additives. The composition of freely mixable MeOH/MeCN was gradually changed from 100/0 to 0/100 (*v*/*v*), while the concentration of the MP additives was kept constant. The questions to be answered were twofold: to which extent do the two polar solvents and their composition have an effect on (i) the overall retention of the individual enantiomers of the investigated acidic analytes, and on (ii) the apparent enantioselectivity (α_{app}). In the second part of this paper, we discuss the effect of various amounts of acid and base additives applied in 100% MeOH or 100% MeCN as bulk solvent with focusing on (i) the observed retention characteristics and (ii) the apparent enantioselectivities. Mechanistically, it was assumed that the well-established stoichiometric displacement model [27,30,31] remains applicable for the description of the retention characteristics of the investigated acidic analytes on the studied CSPs under the applied conditions.

2. Experimental

2.1. Chemicals and reagents

N-(3,5-Dinitrobenzoyl)glycine (DNB-Gly), *N*-(3,5-dinitrobenzoyl)-D,L- and L-alanine (DNB-Ala), *N*-(3,5-dinitrobenzoyl)-D,L- and L-leucine (DNB-Leu) were home-made according to a standard protocol. *N*-Benzoylglycine (Bz-Gly), *N*-benzoyl-D,L-leucine (Bz-Leu), *N*-acetylglycine (*N*-Ac-Gly), and *N*-acetyl-D,L- and L-leucine (*N*-Ac-Leu) were from TCI (Eschborn, Germany) (for structures see Fig. 1C).

MeCN, MeOH, tetrahydrofuran (THF) of HPLC grade, and TEA, FA, acetic acid (AcOH) of analytical reagent grade were purchased from VWR International (Radnor, PA, USA). Ultrapure water was obtained from Ultrapure Water System, Puranity TU UV/UF (VWR International bvba, Leuven, Belgium).

2.2. Apparatus and chromatography

Chromatographic measurements were performed on a 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany) consisting of a solvent degasser, a pump, an autosampler, a column thermostat, a multi-wavelength UV–Vis detector, and a corona-charged aerosol detector from ESA Biosciences, Inc. (Chelmsford, MA, USA). Data acquisition and analysis were carried out with ChemStation chromatographic data software from Agilent Technologies.

The commercially available *Cinchona* alkaloid-based Chiralpak ZWIX(+)^{TM} and ZWIX(-)^{TM} columns (150 × 3.0 mm I.D., 3-μm particle size) and Chiralpak QN-AX and QD-AX (150 × 3.0 mm I.D., 5-μm particle size) were gifts from Chiral Technologies Europe (Illkirch, France). Dead-time (t_0) of the columns was measured by injection of acetone dissolved in methanol.

3. Results and discussions

3.1. Gradual exchange of MeOH and MeCN as bulk solvent at constant MP additive compositions

As a standard protocol for the elution and enantiomer separation of *N*-acyl-amino acids on the chiral anion exchanger QN-AX and QD-AX and also the ZWIX(+) and ZWIX(-) columns, it is common to use organic acids and bases (forming organic salts) as additives, which act as counter-ions and displacers, respectively, dissolved in the MP. It is expected that the concentration of the counter-ion, in the form of a deprotonated acid, regulates the retention, but should not take much part in the overall SO–SA molecule recognition mechanism as symbolized in Fig. 1A and 1B. Such insensitivity towards the type of polar organic solvents was largely assumed.

For the zwitterionic CSPs, here considered as anion exchangers, a similar concept holds, that is, one has to account for an additional, more or less strong, intramolecular counter-ion effect *via* the stoichiometric amount of the deprotonated sulfonic acid moiety of the zwitterionic selector motif (see Fig. 1A and 1B).

In the present study the salt of TEA and FA well-soluble in MeOH and MeCN has been employed to fulfill the operational modus of ion exchangers. In such a non-aqueous medium the organic salt (ionpair) formation takes place via a proton transfer reaction between the acid to the non-protonated base. At first glance, the acid-type analytes (SAs), in our case the DNB-Leu-OH and the others (Fig. 1C), will thus get retained *via* a hydrogen supported ion-pair formation between the protonated basic site of the SO (*i.e.*, the quinuclidine scaffold of the QN and QD moiety) and the deprotonated SA. The strength of this electrostatically driven interaction depends on the pK_a values of the acidic and basic sites and the size of solvated charged sites, respectively. In this context it should be noted that the negatively charged deprotonated state molecules are differently solvated by the protic MeOH and the aprotic MeCN

than in water resulting in a significant shift in pK_a values [32]. These aspects will be taken into consideration during the discussion of the experimental results. Elution is enforced by the amount of counter-ions solvated in the MP depending on their characteristics (pK_a, size, polarity). All association and dissociation events of ion-exchanger type reactions are in equilibrium; that is, a higher concentration of a particular displacer (counter-ion) should result in a reduced retention following the stoichiometric displacement model [33]. As expected, the concentration of the acid and the base determines the concentration of the counter-ion. In the case of using only the free acid (FA or AcOH) or free acid in excess to TEA in the MP, an additional displacement effect by the acid in excess may be expected (see later). Nevertheless, free FA and AcOH need to be classified as protic solvent components in combination with MeOH or MeCN and in this way they may also contribute to the overall chromatographic results.

Assuming that the acid additive in the MP is in excess to both the ion-paired TEA and the quinuclidine moieties of the SOs, it can get adsorbed onto the surface of the solvated CSP and it is in equilibrium with the stagnant mobile phase within the pores. Due to the proton transfer reaction of the free acid in the MP, specifically, in the solvated CSP layer, towards the protonizable site of the SO, it will also act as a displacer thus enforcing elution of the ionically bound SAs. Assuming a proton transfer equilibrium, the free acid exists in a higher concentration in both the stagnant MP and the solvated CSP layer. Consequently, it should lead to a reduction of the retention factor, similar to that formulated for the common stoichiometric displacement model shown previously for weak acidic SAs [34]. This way we envisioned to get a handle on the underlying mobile phase inducing retention and molecular recognition principles.

Based on the discussion detailed above, displacement (ion exchange) may be affected by both the solvent composition of the solvation shell and its thickness, where the charged sites of the SO and SA will depend on the organic solvent type of the MP. Naturally, this applies for all ionizable molecules of the system. However, as the MP contains either free FA or FA plus TEA as components, these entries may additionally take part in the formation and composition of the "entire solvation shell" of the solvated CSP due to the diverse, but overlaid adsorption equilibria. In any case, the strength of the long-range electrostatic forces between the SO(+) and SA(−) sites will in essence be affected by the thickness of the solvation shell (Fig. 2C). It is assumed that the charged sites of both the SO and the SAs are fully solvated and their status will be directly correlated to the retention factors particularly affected by Coulomb interactions. However, since we have to consider also additional SO–SA non-stereoselective and stereoselective interactions in enantioselective ion-exchange chromatography, several effects occurring simultaneously will be involved.

The whole set of chromatographic data, generated in these broadly concerted experiments of the solvent exchange series for the DNB-Leu and DNB-Gly as well as for Ac-Leu and Ac-Gly analytes, are summarized for the QN-AX, QD-AX, ZWIX(+), and ZWIX(-) columns in a comparable way in Tables S1–S4 as Supporting Information. In addition to the MeCN-based experiments, we investigated the effect of the polar non-protic THF as solvent in various combinations with MeOH in a comparable fashion (Tables S1D and S2D).

Intentionally, we also tried to replace MeOH with the much more polar H_2O in combination with various amounts of MeCN to explore the differences of the type of protic solvents in the context of the present study (the results are summarized in Tables S1E and S2E). Based on these data, we attempted to draw conclusions with focus (i) on the interpretation of the observed strong shifts of retention factors and (ii) on the shift of the observed apparent enantioselectivity α_{app} values. The outcome of these diverse experiments will be discussed in more detail in the following.

3.1.1. Behavior of QN-AX and QD-AX columns employing polar organic MP variants

It should be noted here, that QN-AXE and QD-AXE columns behave pseudo-enantiomerically to each other, although they are actually in diastereomeric relation, which implies that the elution order of the resolved analytes switch accordingly [35]. The experimental data where the MeOH/MeCN ratio was gradually changed from 100/0 to 0/100 (*v*/*v*) and the MP additives FA/TEA from 50/25 to 25/25 and 50/0, are illustrated in Fig. 3 and are summarized in the Tables of Supplementary Information. We can clearly notice a characteristic U-shaped profile of all retention factors of DNB-Gly and DNB-Leu enantiomers with a more pronounced shaping of the more strongly retained DNB-Leu enantiomer. This trend, however, is not paralleled with the experimental α_{app} values, which are always the highest with pure MeOH and decrease with increasing MeCN ratios in a more or less linear fashion. The magnitudes are divergent for the QN-AX and the diastereomeric QD-AX columns. Namely, for the QD-AX column, a decrease of α_{app} from about 20 for MeOH to about 16 for MeCN is significantly less pronounced than that for the QN-AX column, where a change from 18 to 9 can be noticed. Note, that in the latter cases FA was present in an excess.

Inspecting more closely the data depicted in Fig. 3, several additional significant observations can be drawn. At the minima of the U-shaped curves at around 40/60 MeOH/MeCN eluent composition, the solvation shells of the charged sites seem to be the largest assuming the electrostatically driven SO–SA interaction being the most dominant one. Let us emphasizehere, that the actual composition of MeOH and MeCN in the solvation shells, in particular, in the case of the CSP, might be different from the bulk composition. This trend is more pronounced for the second-eluting enantiomer and applies essentially for all three MP additive compositions, including the FA/TEA (50/0 *v*/*v*) case. As a further proof of this concept, we undertook a sort of titration experiment by selecting the solvent composition MeOH/MeCN (60/40 *v*/*v*) and adding only different amounts of FA (from 25 to 100 mM) to the mobile phase in order to reveal, whether or not the displacement model raised earlier holds for free FA as displacer. The obtained results (listed in Table S5, and depicted in Fig. S1) fit perfectly to the model. Under these conditions, free FA in the MP acts as a perfect displacer, but it is not completely clear whether the varied amounts of free FA are adsorbed *via* secondary equilibria in the solvation layer of the solvated CSP. Additional considerations will be discussed later.

An entirely different and even more pronounced trend is noticed, when exchanging MeCN with the polar non-protic THF as bulk solvent, as depicted in Fig. S2. Respective data are summarized in Table S1D. Both retention factors and α_{app} values reduced strongly and continuously with increasing THF contents. In particular, α_{app} reduced to one third, which is much more pronounced than that found with MeCN as solvent. THF obviously solvates well all charged sites of the ion-exchange type CSP and the SA, but also disrupts the additional SO–SA interactions more strongly than MeOH or MeCN does.

Replacing the protic MeOH with the even stronger polar water and carrying out a similar solvent-exchange protocol as in the other experiments towards 100% MeCN with pure water as solvent, an infinitively high retention of the analytes was obtained. Consequently, we could start the solvent-exchange experiments only with a mixture of H_2O/MeCN (70/30 *v*/*v*). The data are summarized in Tables S1E and S2E and Fig. S3. As an outcome of this investigation, it became evident that in the overall retention characteristics the hydrophobic, multiple van der Waals-type SO–SA interaction increments have to be taken into account in addition to the

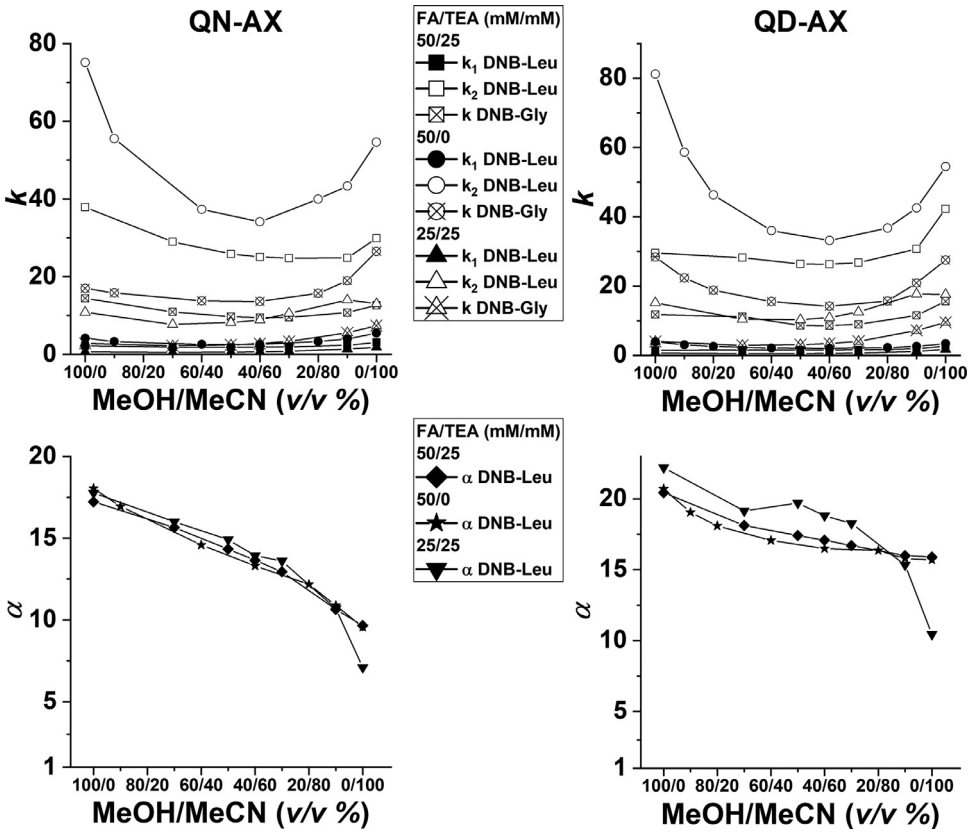

Fig. 3. Effect of MeOH/MeCN ratio on k and α values of DNB-Leu enantiomers and k values of DNB-Gly-on the QN-AX and on QD-AX type CSP. Chromatographic conditions: column, QN-AX and QD-AX; mobile phase, MeOH/MeCN (100/0 – 0/100 v/v) containing 50 mM FA and 25 mM TEA; 50 mM FA; and 25 mM FA and 25 mM TEA; flow rate, 0.6 ml min^{-1}; detection, 254 nm; temperature, 25 °C; elution sequence on QN-AX ($D<L$), on QD-AX ($L<D$).

electrostatically driven ion pair formation. Furthermore, it is obvious, that variation of the solvation shells of the SAs and SO caused by water hampers strongly the overall shape of the accessible binding grooves of the SO resulting in a reduction of α_{app}. The minima of the U-shaped curves is at around 30/70 H_2O/MeCN (v/v) (Fig. S3). Interestingly, at high water content, α_{app} is quite low on the QN-AX CSP and steadily increases when shifting towards 100% MeCN. In contrast, in the case of QD-AX CSP, we did not notice such a strong dependency of α_{app}. Values stay around 11–12, whereas for the QN-AX column, α_{app} shifted from 2.8 to about 10. Mechanistically, the stereodiscrimination can markedly be affected in one case by the presence of water as a strong hydrogen-bonding type solvent, whereas it is much less of an issue for the other case.

As a further control experiment, the impact of the type of the *N*-tagging groups of Leu and Gly on the overall retention and enantioselectivity characteristics was investigated. The results of analyzing Ac-Leu and Ac-Gly (instead of DNB-Leu and DNB-Gly thus avoiding the strong π-stacking retention-causing increment), are depicted in Fig. S4 and related chromatographic data are summarized in the Tables S1F and S2F for the QN-AX and QD-AX columns, respectively. What can immediately be seen are the *per se* strongly reduced retention factors for Ac-Leu and Ac-Gly accompanied with much smaller α_{app} values. In pure MeCN, retention is increased compared to that in pure MeOH, while α_{app} decreased strongly. What is worth mentioning is a still reasonable enantioselectivity. Obviously, it is not as high as for DNB-Leu, since the pronounced driving π–π-stacking increment is absent in these analytes [19].

3.1.2. Behavior of the ZWIX(+) and ZWIX(-) columns with polar organic MP variants

Conceptually similar to the QN-AX and QD-AX columns, a smaller set of experiments with the gradual exchange of MeOH with MeCN was also carried out, applying FA/TEA ratios of 50/25, 50/0, and 25/25 as the basis for a systematic comparison. Respective data are summarized in Tables S3A, S3B, S3C and S4A, S4B, S4C for ZWIX(+) and ZWIX(-) columns, respectively, and they are graphically depicted in Fig. 4. Although there is a structural similarity of the two series of chiral SOs because of the QN and QD motifs of SOs, the enantioselectivity will not be the same for DNB-Leu. The reason is that the *tert*-butyl-carbamoyl group of the QN-AX and QD-AX selectors represents an optimum structural scaffold around the enantioselective binding pocket, as it has been observed for the resolution of the DNB-Leu enantiomers, which could not be realized to the same extent with the ZWIX SOs [36,37]. Consequently, the α_{app} values are actually about half as high as those seen for the pure methanolic conditions (compare data of Table S1A and S3A as well as S2A and S4A and Figs. 3 and 4)

As expected, U-shaped retention factor curves have been observed, but the retention factors are significantly shorter for the pure methanolic conditions compared to the MeCN containing eluents, which may be a sign for a somewhat changed solvation status of the ZWIX SOs compared to those observed on the QN-AX and QD-AX columns. The retention characteristics in pure MeOH compared to those found under pure MeCN conditions favor the latter one for the ZWIX phases; however, the extent was unexpected. The overall enantioselectivity α_{app} values of the ZWIX(+) column drop significantly when shifting from MeOH towards MeCN, but obviously it goes first through a noticeable maximum when mixed solvents are used. The retention factors of DNB-Leu enantiomers are similar for the ZWIX(+) and QN-AX columns with pure MeCN at FA/TEA (50/25 v/v) conditions, whereas for the ZWIX(-) column, they are much lower than those on the comparable QD-AX column.

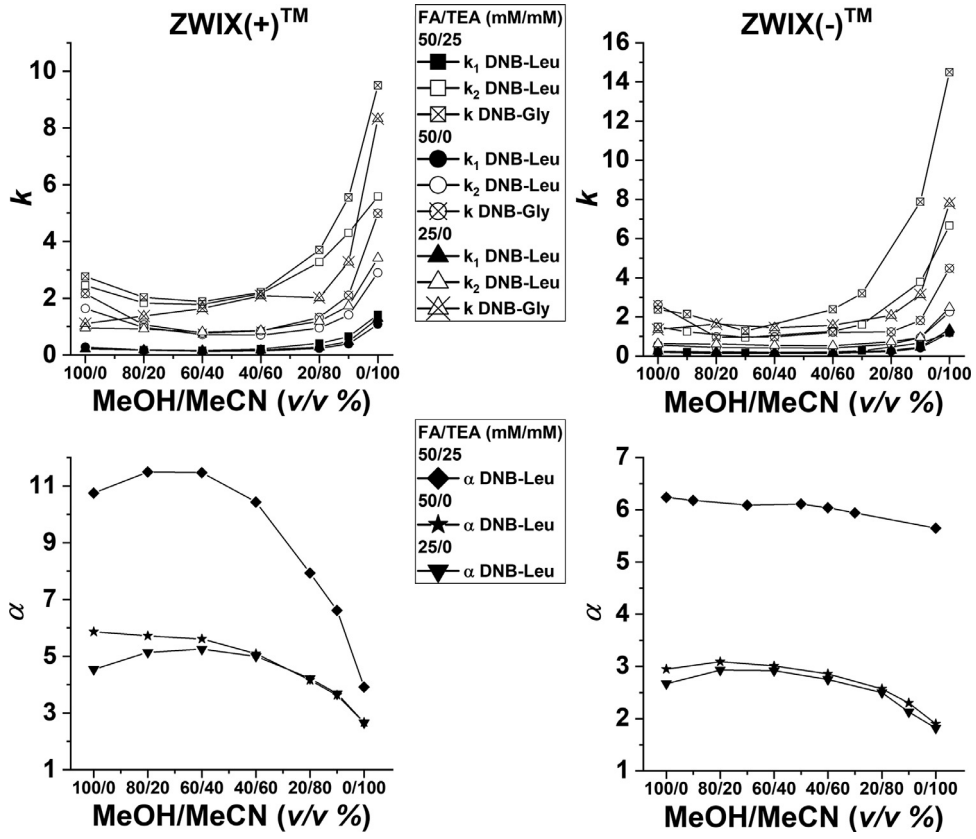

Fig. 4. Effect of MeOH/MeCN ratio on k and α values of DNB-Leu enantiomers and k values of DNB-Gly-on ZWIX(+)^{TM} and ZWIX(-)^{TM} CSPs. Chromatographic conditions: column, ZWIX(+)^{TM} and ZWIX(-)^{TM}; mobile phase, MeOH/MeCN (100/0 – 0/100 *v/v*) containing 50 mM FA and 25 mM TEA; 50 mM FA; and 25 mM FA; flow rate, 0.6 ml min^{-1}; detection, 254 nm; temperature, 25 °C; elution sequence on ZWIX(+)^{TM} (D<L), on ZWIX(-)^{TM} (L<D).

The change of the α_{app} values between ZWIX(+) and ZWIX(-) columns does not fully correlates with the change of α_{app} between the QN-AX and QD-AX columns, which again is a sign of noticeable differences of solvent (solvation) effects on the two pairs of chiral columns. It becomes clearly evident that the two MP additive compositions of either FA/TEA 50/25 (*v/v*) or 25/0 (*v/v*) do significantly diverge with regards to the α_{app} values, although the absolute amount of excess FA (25 mM) is similar. It applies for both ZWIX columns and gives a hint that possibly the FA/TEA salt also gets adsorbed into the solvated CSP layer thus giving rise to an additional modulation of the overall α_{app}. That is, the entire molecular recognition process becomes even more complex than anticipated.

Along this line it is astonishing that the retention factor of DNB-Gly is even higher than those for the better fitting (retained) DNB-Leu enantiomer, which is different from the results obtained on the QN-AX and QD-AX columns. There are two factors responsible for this behavior: (i) the longer retention of DNB-Gly is associated with the overall decrease of the solvent shell thickness of the binding SO and SA in addition to the higher pK_a of DNB-Gly (see Fig. 1C) and (ii) a geometrically (spatially) driven exclusion phenomenon towards the binding pocket in the course of the formation of the two diastereomeric SO-(*R*)-SA and SO-(*S*)-SA associates is undoubtedly involved. This phenomenon could already be partially in place for the more strongly retained DNB-Leu enantiomer, whereas a spatially driven repulsion will certainly be the case for the weakly retained DNB-Leu enantiomer. This observation is valid for both ZWIX columns. From a structural point of view, the ZWIX SOs provide less flexible of conformational freedom to adjust to the bonded SA molecules due to the relatively strong and intrinsic intramolecular Coulomb interaction (see Fig. 1A and 1B).

3.2. Further unexpected observations of the investigated chiral ion exchangers

In this subsection, the observed k_1, k_2, and α_{app} values of *N*-acylamino acids obtained with pure MeOH or MeCN as mobile phase solvents and FA or AcOH as acid and TEA as base additives will be discussed in the light of additional adsorption phenomena. The respective chromatographic results are summarized in Tables 1 and 2 for the QN-AX and the QD-AX CSPs and in Tables 3 and 4 for the ZWIX(+) and ZWIX(-) CSPs. It is considered that the benzoyl (Bz) group is less polar and less bulky than the DNB group, and the acetyl (Ac) group is even more polar and smaller in size with only a shallow hydrophobic increment for retention. The DNB group of strong π-acidity can undergo a strong face-to-face π–π-stacking with the π-basic quinoline ring of the QN and QD moiety devoid of the Bz- and Ac-tags. Nevertheless, for all these carbamate-type tags, the hydrogen bonding capacity with the SOs remains an important factor as hydrogen bonding is a directing force and acts as essential asset for chiral discrimination of the studied SAs.

3.2.1. Specific behavior of the QN-AX and QD-AX columns on MP changes

Inspecting the data presented in Tables 1 and 2, which refer to the QN-AX and QD-AX columns, respectively, it becomes obvious that in pure MeOH and MeCN as bulk solvents and for the five MP compositions significantly different effects become manifested. As already lined out, DNB-Gly elutes always much later than the weakly complexed and, consequently, weakly retained DNB-Leu enantiomers, but it elutes earlier than the more strongly retained DNB-L-Leu enantiomers. This also holds for Bz-Leu, but not for

Table 1
Comparison of chromatographic parameters, k_1, α, and R_S of racemic **DNB-Leu, Bz-Leu, Ac-Leu**, and **DNB-Gly, Bz-Gly, Ac-Gly** on **QN-AX** column operated with mobile phases of either MeOH or MeCN and formic acid (FA) and triethylamine (TEA) additives in different ratios.

Additives	DNB-Leu and DNB-Gly								Bz-Leu and Bz-Gly			
	Solvents +additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (0/100 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)			
FA/TEA mM/mM)	k_1D-DNB-LeuDNB-Gly	k_2L-DNB-Leu-	α_{app}	R_S	k_1D-DNB-LeuDNB-Gly	k_2L-DNB-Leu-	α_{app}	R_S	k_1Bz-LeuBz-Gly	k_2Bz-Leu-	α_{app}	R_S
(1) 50/0	4.16	75.1	18.0	43.0	5.49	54.6	9.95	35.7	1.06	2.80	2.64	9.85
	17.0	–	–	–	26.5	–	–	–	2.68	–	–	–
(2) 25/0	3.32	60.1	18.1	31.8	8.08	69.8	8.6	30.5	1.77	4.66	2.63	13.0
	22.5	–	–	–	39.7	–	–	–	4.63	–	–	–
(3) 0/0	0.43	8.92	21.0	18.5	>90	–	–	–	0.34	1.10	3.24	3.45
	1.70	–	–	–	>90	–	–	–	0.67	–	–	–
(3) 0/0	0.33§	0.57§	1.72§	1.62§	>90§	>90§	–	–				
	1.03§§	–	–	–	>90§§	–	–	–				
(4) 50/25	2.20	37.9	17.2	40.8	3.10	29.9	9.65	19.2	1.18	3.15	2.67	15.4
	14.4	–	–	–	12.6	–	–	–	2.68	–	–	–
(5) 25/25	0.61	10.8	17.8	25.1	1.84	13.0	7.09	20.2	0.36	1.05	2.93	9.16
	2.82	–	–	–	7.41	–	–	–	0.61	–	–	–

Chromatographic conditions: columns, **QN-AX**; mobile phase, MeOH/MeCN 100/0 and 0/100 (*v/v*) containing FA and TEA in various ratios; flow rate, 0.6 ml min^{-1}; detection, 230–254 nm; temperature, ambient; dead time, t_0, 3.31–3.55 min; ***k, α$_{app}$***, and ***R$_S$*** values of **Ac-Leu§** and **Ac-Gly§§,** respectively.

Table 2
Comparison of chromatographic parameters, k_1, α and R_S of racemic **DNB-Leu, Bz-Leu, Ac-Leu**, and **DNB-Gly, Bz-Gly, Ac-Gly** on **QD-AX** column operated with mobile phases of either MeOH or MeCN and formic acid (FA) and triethylamine (TEA) additives in different ratios.

Additives	DNB-Leu and DNB-Gly								Bz-Leu and Bz-Gly			
	Solvents +additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (0/100 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)			
FA/TEA mM/mM)	k_1L-DNB-LeuDNB-Gly	k_2D-DNB-Leu-	α_{app}	R_S	k_1L-DNB-LeuDNB-Gly	k_2D-DNB-Leu-	α_{app}	R_S	k_1Bz-LeuBz-Gly	k_2Bz-Leu-	α_{app}	R_S
(1) 50/0	3.92	81.2	20.7	39.2	3.42	54.5	15.7	32.7	0.67	2.14	3.20	13.0
	28.4	–	–	–	27.5	–	–	–	2.07	–	–	–
(2) 25/0	2.25	45.5	20.2	27.7	4.25	67.6	15.9	23.3	1.19	3.89	3.27	15.5
	24.3	–	–	–	50.6	–	–	–	3.71	–	–	–
(3) 0/0	0.53	10.8	20.6	24.9	>90	–	–	–	0.43	1.56	3.63	5.36
	2.31	–	–	–	>90	–	–	–	1.11	–	–	–
(3) 0/0	0.45§	0.73§	1.62§	1.85§	>90§	>90§	–	–				
	1.72§§	–	–	–	>90§§	–	–	–				
(4) 50/25	1.45	29.6	20.4	37.6	2.66	42.3	15.9	36.8	0.83	2.72	3.28	14.3
	11.8	–	–	–	15.6	–	–	–	2.36	–	–	–
(5) 25/25	0.68	15.2	22.2	33.6	1.69	17.6	10.4	25.8	0.40	1.35	3.38	10.5
	4.03	–	–	–	9.51	–	–	–	0.84	–	–	–

Chromatographic conditions: columns, **QD-AX**; mobile phase, MeOH/MeCN 100/0 or 0/100 (*v/v*) containing FA and TEA in various ratios; flow rate, 0.6 ml min^{-1}; detection, 230–254 nm; temperature, ambient; dead time, t_0, 3.36–3.47 min; ***k, α$_{app}$***, and ***R$_S$*** values of **Ac-Leu§** and **Ac-Gly§§,** respectively.

Table 3
Comparison of chromatographic parameters, k_1, α and R_S of racemic **DNB-Leu, Bz-Leu, Ac-Leu**, and **DNB-Gly, Bz-Gly, Ac-Gly** on **ZWIX(+)** column operated with mobile phases of either MeOH or MeCN and formic acid (FA) and triethylamine (TEA) additives in different ratios.

Additives	DNB-Leu and DNB-Gly								Bz-Leu and Bz-Gly			
	Solvents +additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (0/100 *v/v*) + FA/TEA mM/mM)				Solvents + additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA mM/mM) or			
FA/TEA mM/mM)	k_1D-DNB-LeuDNB-Gly	k_2L-DNB-Leu-	α_{app}	R_S	k_1D-DNB-LeuDNB-Gly	k_2L-DNB-Leu-	α_{app}	R_S	k_1Bz-LeuBz-Gly	k_2Bz-Leu-	α_{app}	R_S
(1) 50/0	0.28	1.64	5.86	10.4	1.08	2.90	2.67	7.97	0.09	0.09	1.00	0.00
	2.16	–	–	–	4.99	–	–	–	0.21	–	–	–
(2) 25/0	0.21	0.95	4.54	6.35	1.29	3.42	2.66	3.90	0.13	0.13	1.00	0.00
	1.11	–	–	–	8.33	–	–	–	0.27	–	–	–
(3) 0/0	0.03	0.11	3.66	<0.20	>90	>90	–		1.32	1.34	n.d.	0.85
	0.05	–	–	–	*>90*	–	–		*1.98*	*–	–	–
(3) 0/0	0.01§	0.06§	6.00§	0.57§	>90§	>90§	–	–				
	0.41§§	–	–	–	>90§§	–	–	–				
(4) 50/25	0.23	2.47	10.8	8.38	1.42	5.58	3.92	10.1	0.13	0.25	1.89	1.11
	2.77	–	–	–	9.50	–	–	–	0.40	–	–	–
(5) 25/25	0.15	1.39	9.25	9.80	1.63	4.89	3.00	10.3	1.55	1.65	n.d.	1.82
	1.10	–	–	–	6.70	–	–	–	*1.78*	*–	–	–

Chromatographic conditions: columns, **ZWIX(+)™**; mobile phase, MeOH/MeCN 100/0 and 0/100 (*v/v*) containing FA and TEA in various ratios; flow rate, 0.6 ml min^{-1}; detection, 230–254 nm; temperature, ambient; dead time, t_0, 1.52–1.64 min; ***k, α$_{app}$*** and ***R$_S$*** values of **Ac-Leu§** and **Ac-Gly§§**; respectively; *retention time (min) as peak elutes before t_0; n.d., not determined.

Table 4
Comparison of chromatographic parameters, k_1, α and R_S of racemic **DNB-Leu, Bz-Leu, Ac-Leu**, and **DNB-Gly, Bz-Gly, Ac-Gly** on **ZWIX(-)** column operated with mobile phases of either MeOH or MeCN and formic acid (FA) and triethylamine (TEA) additives in different ratios.

Additives	DNB-Leu and DNB-Gly								Bz-Leu and Bz-Gly			
	Solvents +additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA (mM/mM)				Solvents + additives: MeOH/MeCN (0/100 *v/v*) + FA/TEA mM/mM)				Solvents + additives: MeOH/MeCN (100/0 *v/v*) + FA/TEA mM/mM)			
FA/TEA mM/mM)	k_1*L*-DNB-LeuDNB-Gly	k_2*D*-DNB-Leu-	α_{app}	R_S	k_1*L*-DNB-LeuDNB-Gly	k_2*D*-DNB-Leu-	α_{app}	R_S	k_1Bz-LeuBz-Gly	k_2Bz-Leu-	α_{app}	R_S
(1) 50/0	0.19	0.56	2.95	3.91	1.19	2.26	1.90	4.93	0.08	0.08	1.00	0.00
	2.62	–	–	–	4.48	–	–	–	0.21	–	–	–
(2) 25/0	0.24	0.63	2.67	4.08	1.36	2.48	1.82	2.80	0.11	0.11	1.00	0.00
	1.36	–	–	–	7.80	–	–	–	0.20	–	–	–
(3) 0/0	1.09	1.60	n.d.	2.36	>90	–	–	–	1.10	1.10	n.d.	0.00
	*0.33	*–	–	–	>90	–	–	–	*1.76*	*–	–	–
(3) 0/0	0.03§	0.09§	3.31§	0.54§	>90§	>90§	–	–				
	0.17§§	–	–	–	>90§§	–	–	–				
(4) 50/25	0.24	1.50	6.24	9.84	1.18	6.67	5.65	11.7	0.17	0.17	1.00	0.00
	2.37	–	–	–	14.5	–	–	–	0.38	–	–	–
(5) 25/25	0.10	0.72	7.20	6.70	1.40	4.48	3.20	9.60	1.51	1.54	n.d.	1.05
	1.16	–	–	–	10.6	–	–	–	*1.82*	*–	–	–

Chromatographic conditions: columns, **ZWIX(-)™**; mobile phase, MeOH/MeCN 100/0 and 0/100 (*v/v*) containing FA and TEA in various ratios; flow rate, 0.6 ml min^{-1}; detection, 230–254 nm; temperature, ambient; dead time, t_0, 1.52–1.64 min; ***k, α$_{app}$*** and ***R$_S$*** values of ***N*-Ac-Leu§** and ***N*-Ac-Gly§§**, respectively; *retention times (min) as peak elutes before t_0; n.d., not determined.

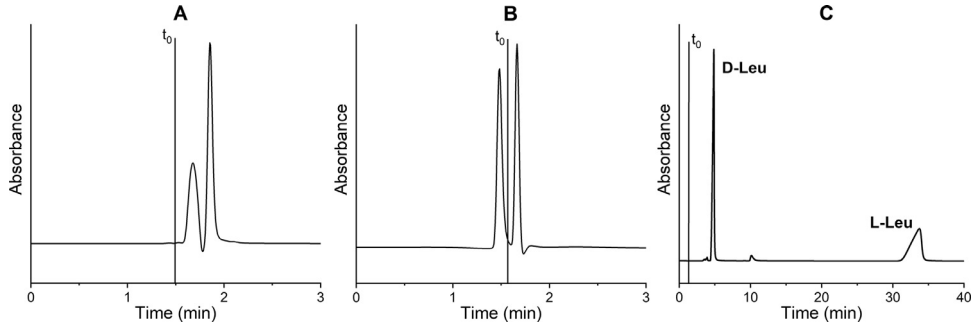

Fig. 5. Chromatograms of racemic-Bz-Leu (**A** and **B**) on ZWIX(+)™ and of racemic-DNB-Leu (**C**) on QN-AX CSP with 100% MeOH as bulk solvent. Chromatographic conditions: column, **A** and **B,** ZWIX(+)™ and **C**, QN-AX; mobile phase MeOH 100% containing FA/TEA at molar ratios **A,** 50/25 mM/mM, **B,** 25/25 (mM/mM) and **C**, containing no additives; flow rate, 0.6 ml min^{-1}; detection, 254 nm; temperature, 25 °C.

Ac-Leu. Ac-Gly is retained significantly more strongly than Ac-Leu, although it has short retention times without FA and TEA additives [MP(3)]. The π–π-stacking of the DNB-tag is significantly stronger in the protic MeOH than in the non-protic MeCN, which is considered to weaken intermolecular π–π interactions. In other words, MeCN could be classified as a specific disrupter in the case of π–π-stacking, which implies that it solvates the π-binding sites to a certain extent. On the other hand, the polar but non-protic MeCN strengthens hydrogen-bonding events in contrast to protic MeOH. In the present situation, the carbamate groups of SO and SA may play a more prominent role in the enantioselective intermolecular interactions. In addition, we should consider at this point FA as a protic solvent, which may solvate the acidic site of the analytes. Hydrogen bonding sites of SO and SA may also be getting solvated this way. For the given MP compositions [(1)-(5)], we have thus a cascade of competing but overlaid effects on the stereoselective and intermolecular SO–SA binding, which hampers the interpretation of the diverse MP composition effects.

Unexpectedly, we observed that with MeOH as solvent [MP(3)] the DNB-Leu enantiomers could be eluted from a weak chiral ion exchanger without any counter-ion as displacer present in the MP but with roughly the same α_{app} as with counter-ion present in the MP, as depicted in Fig. 5C. In essence, all envisioned specific association and dissociation processes in the course of the stereoselective formation of the SO–SA complex in MeOH are taking place more or less simultaneously. They occur in an equilibrium fashion and the elution of the analytes from the CSP within a reasonable time frame with preservation of the α_{app} values is apparently possible. Under these conditions, MeOH acts actually as an effective displacer and weakens all active binding scenarios between SO and SA. For this observation, one may also recall enantioselective push-and-pull effects between the solvated SO and SA partners, similar to those in our previous study about the resolution of zwitterionic analytes on ZWIX phases without the use of any buffer system [28]. The peak of the late-eluting enantiomer with a non-Gaussian shape hints that the adsorption/desorption steps are somewhat divergent from each other resulting in a strongly fronting peak (Fig. 5C). Although no loading experiments of DNB-Leu has yet been made for such anion-exchange chromatographic systems, this observation could be of value for preparative applications as it enables an easy work-up of the collected salt-free peak fractions.

Discussing further the chromatographic results in context to the three MP compositions differing only in the absolute amount of FA in MeOH (in the absence of TEA), we noticed a strong effect of the acid on the overall retention, but not on enantioselectivity characteristics. With MeOH as bulk solvent, the retentions of DNB-Gly are much higher in the presence of FA [MP(1) and (2)] than without FA [MP(3)] on both anion-exchanger CSPs (Tables 1 and 2). Moreover, the retention factors of both DNB-Leu enantiomers increase sharply with the increased FA content, which is not plausible at this point. However, the α_{app} values remain roughly constant

between 18 and 21 (Tables 1 and 2). Using 100% MeCN as eluent, a striking difference is noticed in the absence of FA with extremely large (infinitive) retention factors of all analytes. For MP(2) the retention factors of DNB-Gly and D,L-DNB-Leu are higher than those for MP(1).

Inspecting now the results obtained in the presence of TEA in addition to FA in 100% MeOH as eluent, where MP(4) contains a 25 mM excess of FA. At first glance, these could be compared with MP(2) assuming a stoichiometric salt formation between FA and TEA. In MP(4), the retention factors are definitively lower than in MP(2) without TEA. MP(5) may also be comparable with MP(3) in terms of excess of FA. The retention factors under MP(5) conditions are somewhat higher than for MP(3) but, again, α_{app} remains similar to all other MP variants. At this point, it should be highlighted that the investigated CSPs are heterogeneous in their composition. Furthermore, a good part of the remaining slightly acidic silanol groups will be present, which eventually will adsorb some of the basic TEA when exposed to such type of MPs. Therefore, MP(5) may still be considered slightly acidic due to a slight excess of FA. The polar silica surface is known to adsorb polar solvents (*e.g.*, water) strongly, that is, the adsorption of the polar FA may also be possible. Even the less polar AcOH may be adsorbed onto the remaining silanol groups of the silica surface, which has been chemically modified according to the investigated CSPs.

In 100% MeCN as bulk solvent, a similar trend can be seen, although the α_{app} values are about half as high as those found with 100% MeOH, which is again partially assigned to the strong "bond breaking" effect of MeCN in the case of the strong $\pi-\pi$-stacking of the DNB and the quinuclidine groups. As a control experiment, we investigated the behavior of Bz-Leu and Bz-Gly under 100% MeOH conditions, as outlined in Tables 1 and 2. Although the retention factors and the α_{app} values are much lower, the same trend could be found for all five MP variants. Finally, Ac-Leu and Ac-Gly employing 100% MeOH [MP(3)] were also studied. In accordance with the DNB-Leu experiment, Ac-Gly and Ac-Leu enantiomers are retained and enantioseparated reasonably.

We argued earlier that an increase in retention may be associated (i) with a decrease in both the solvation shell of the SA molecules and the solvated SO moieties and (ii) with an adsorption of the excess acid onto the surface of silica and thus into the solvated CSP layer. We also argued that the free acid can act as a polar displacer in the course of the SO–SA interactions according to the stoichiometric displacement model, which was perfectly attested for a MeOH/MeCN bulk solvent mixture of 40/60 (see Fig. S1).

In polar aprotic MeCN, the retention factor decreases as the amount of displacer (in this case FA) increases, while for the polar protic MeOH the situation is reversed, which appears to be a contradiction, and we have to deal with overlaid effects, which are different in their directions. FA is now considered to be a polar protic solvent being in competition with MeOH as solvating agent leading, in essence, to a reduction of both the size of the solvation shell and the thickness of the layer of the solvated CSP. The result is an increase in retention, which is striking when compared to zero FA MP(3) conditions. In other words, the FA seems to get adsorbed onto the CSP in using both MeOH and MeCN as bulk solvents.

For both solvent situations, the α_{app} remains unaffected by the amount of FA and, therefore, it contributes in the same proportion to the retention factors k_{app1} and k_{app2} of the enantiomers. Hence, if we put these results in context to the observed results of the MeOH/MeCN (40/60 *v/v*) solvent combination, we notice that the α_{app} is around 12, which is significantly lower than with pure MeOH (about 18), but higher than with pure MeCN (about 9) used as bulk solvents (Table 1).

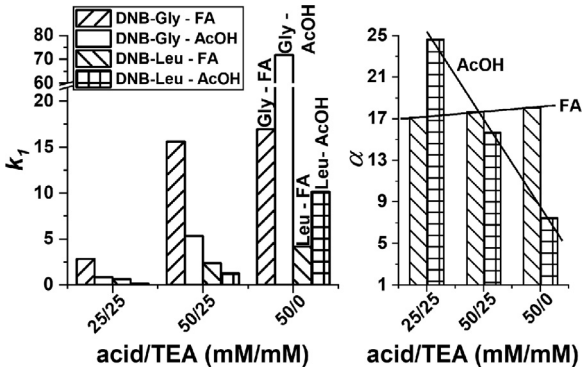

Fig. 6. Chromatographic data: retention factor (k) of DNB-Gly, and k_1 and α values of DNB-Leu on QN-AX type CSP with 100% MeOH as bulk solvent containing FA/TEA or AcOH/TEA at different molar ratios. Chromatographic conditions: analyte, DNB-Gly and DNB-Leu; column, QN-AX; mobile phase, MeOH containing FA/TEA or AcOH/TEA at molar ratios 25/25, 50/25 and 50/0 (mM/mM); flow rate, 0.6 ml min^{-1}; detection, 254 nm; temperature, 25 °C.

To stay consistent to the definition of α_{app} for the resolution of DNB-Leu in MeOH as bulk solvent containing MP additives, it can only happen when the sum of the MP effects is contributing to the non-stereoselective and the stereoselective SO–SA interactions in a proportional manner. A number of overlaid and partially competing effects generated by the MP, which essentially are reflected in the solvation shells of the SO and SA moieties, need to be considered when stereoselective SO–SA interaction mechanisms, driven by molecular structure, are discussed. Most of the time, such aspects are missing in the literature, although some striking results have recently been published supporting this view [38,39]. In principle, only minute amounts of MP additives can eventually change the α_{app} values significantly, because of their selective adsorption and distribution in the solvated CSP layer. Conformational changes of the given SO motifs may even result in a reversal of the elution order as shown for some exclusive cases [40].

Divergence from the stoichiometric displacement model discussed above can also be seen for the MP additive composition FA plus TEA with MP(4) and MP(5). We noticed a similar, but strong increase of the retention factor with an excess of free FA. It becomes evident that the presence of both TEA and FA/TEA salts has a moderate effect on the overall retention factors in comparison to the situation of FA/TEA 0/0 and 25/25 (mM/mM). There is only a slight increase in the presence of the FA/TEA salt, which indicates that it may not have a very high concentration in the solvated CSP.

Last but not least, we cross-evaluated a limited set of experiments with respect to the chromatographic effect generated by using FA as acid component in exchanging it with the less acidic and less polar acetic acid (AcOH) as MP additive. Here only the QN-AX CSP combination is investigated. The results are summarized in Table S6 and should be compared to data with FA listed in Table S5. In Fig. 6 the differences caused by FA vs AcOH in the absence and presence of TEA as a base additive are illustrated. Inspecting first the situation FA/TEA and AcOH/TEA with 50/0, 50/25 and 25/25 (mM/mM) compositions, we identify surprising effects. DNB-Gly is retained with AcOH about four-times more strongly compared to FA for the 50/0 (*v/v*) composite. A similar trend applies for the DNB-Leu enantiomers as well, although α_{app} drops for the AcOH condition to 7.5 compared to 18 for FA. Obviously, in the presence of AcOH, the solvation shells are smaller in size resulting in a stronger Coulomb attraction and increased retention factors. On the other hand, the adsorbed AcOH becomes more strongly involved in the observed molecular recognition events. Because DNB-Gly is less lipophilic than the DNB-Leu enantiomers and AcOH is more lipophilic than FA, this observation makes sense. However,

considering the complex formation of DNB-Leu enantiomer having a stronger binding ability with the CSP, the AcOH effect becomes contra-productive with respect to the observed enantioselectivity.

Turning our attention to the eluents containing TEA in ratios of 50/25 and 25/25 (mM/mM), it is clear that the TEA reduces the effect of the AcOH more as it does for the FA resulting in shorter retention factors. These findings are congruent with the different properties of the combined acid and TEA additives.

3.2.2. Specific behavior of the ZWIX(+) and ZWIX(-) columns on MP changes

In a similar fashion as discussed above, all experimental results obtained in the case of the ZWIX(+) and ZWIX(-) columns are summarized in Tables 3 and 4. The retention factors of all analytes in MeOH are rather small, whereas in MeCN higher retention factors were obtained due to the lower solvation strength of MeCN. Inspecting the validity of the stoichiometric displacement model for both zwitterionic columns, a clear discrepancy was found in the case of MeOH. Applying FA as displacer in 100% MeOH as eluent, the retention increases with increased FA concentration. In MeCN as solvent, this is not the case. The contribution of the intramolecular ion-pairing effect (see Fig. 1A and 1B) is obviously quite different in MeOH and MeCN. At this point we have to mention the unexpected and yet not fully understood chromatograms describing the resolution of rac.-Bz-Leu on ZWIX(+) column using either MP(4) or MP(5) (see Fig. 5A and 5B). With 100% MeOH and 50 mM/25 mM FA/TEA (*i.e.*, in an excess of FA), the weakly bound enantiomer of Bz-Leu eluted after t_0, while without excess FA (25 mM/25 mM FA/TEA), it elutes before t_0 (compare Fig. 5A and 5B). This stereoisomer is *quasi* repelled from a full entrance into the pores of the solvated CSP, which is due to the peculiar composition of the stagnant MP in the pores. To elucidate this phenomenon, further studies are currently undertaken. Similar to the case of the anion exchangers, the retention factors of the acidic analytes in pure MeCN are extremely high, which definitively results from the poor solvation of both the SAs and the SO unit, thus enforcing a very strong SO–SA Coulombic attraction. Following the same trend as we have seen for the QN-/QD-AX columns with MeCN as bulk solvent, a drop of α_{app} of about a factor of two compared to methanolic conditions was measured, which has to be attributed to the different spatial environment around the carbamoyl group (compare Fig. 1A and 1B) of the SO and SA moieties.

4. Conclusions

Systematic liquid chromatographic experiments have been carried out with two sets of weak chiral anion and zwitterionic ion exchangers based on immobilized *Cinchona* alkaloid quinine (QN) and quinidine (QD) type chiral selector (SO) motifs and respective chiral stationary phases (CSPs). These were applied in combination with the use of polar organic mobile phase (MP) in varying compositions and *N*-acyl-leucine and *N*-acyl-glycine as chiral and non-chiral weakly acidic analytes (selectands, SAs).

In our approach, we employed formic acid (FA) or acetic acid (AcOH) as acid MP additive acting as displacer or counter-ion to control the ion exchange-based elution process of the acid-type analytes, and triethylamine (TEA) as base for the formation of organic salts with the acids acting as positively charged co-ions. Free acids without the addition of a base in the non-aqueous MP could also serve as displacer in fulfilling the concept of liquid chromatographic “ion exchange” systems.

As core results of all experiments we can collect the following observations:

(a) MeOH, as a protic polar solvent, enables well the solvation of the polar carboxylic acid group of an acid-type analyte but also of the given SO unit and thus of the entire CSP. This includes the respective salts of the acids with both TEA and the quinuclidine group of the SOs. For the given probes, the carbamoyl groups classified as hydrogen donating and accepting sites, may also be solvated. The retention factors are reasonable, but they encompass a wide window.

(b) MeCN, as a non-protic solvent, lacks a solvation power of the polar and chargeable sites of SA and SO resulting in strong Coulomb attraction between the SO(+) and SAs(−) leading to extremely long retention times both on the QN/QD-AX and the ZWIX(+)/(−) type ion exchangers.

(c) Via mixed MeOH/MeCN bulk solvent compositions, the retention factors can be adjusted well, but enantioselectivities are compromised. They are generally lower in MeCN compared to those in MeOH due to the weakened $\pi-\pi$ stacking-type interactions between the respective sites of SO and SA. The composition of the solvation shell of the CSP may be different in comparison to that of the bulk MP, because of the selective adsorption phenomena eventually related also to the immobilization chemistry of the SO onto the silica surface and the structured heterogeneity of a CSP.

(d) As expected, the common counter-ion driven stoichiometric displacement model applicable for ion-exchange chromatography is commonly in place. It applies also in some cases for the use of a free acid dissolved in MeOH thus also acting as a displacer. However, there have been remarkable exceptions discussed accordingly in this contribution.

(e) The highly polar FA is apparently well adsorbed onto the surface of the CSP, thus changing its overall property. As a consequence, retention of the analytes can increase with the concentration of FA in the MP leading to a strong divergence of the stoichiometric displacement model. Nevertheless, in the case of free FA as MP additive in MeOH, retention increased but α_{app} kept constant. Similar experiments with MeCN as bulk solvent will not be possible (see entry b).

(f) With pure MeOH as bulk solvent without additives in the MP, it is possible to retain and resolve the probed *N*-acyl-Leu acids on chiral ion exchangers due to diverse overlaid as well as competing SO–SA association and dissociation events during the chromatographic process.

(g) When exchanging free FA with less-polar AcOH, another unexpected but still plausible effect was noticed. AcOH solvates the SA moieties apparently to a lesser extent and due to the reduced size of the solvation shell, the retention factor increases strongly for DNB-Gly and DNB-Leu enantiomers. In contrast to the three different FA conditions for which the α_{app} stays roughly constant, the use of AcOH brings about a marked change, which can surpass the α_{app} values of FA. The more lipophilic AcOH and its competition with MeOH as solvent will certainly change the composition of the solvated CSP layer which, to some extent, may modulate the conformational flexibility of the SO unit.

Although the MP components are non-chiral, they contribute *quasi* directly to the observed enantioselectivity of a solvated CSP. The solvents and the additives of the MP will affect the conformation of the SOs and, consequently, the accessibility of the stereoselective binding sites (groves) approached by the chiral analytes. Establishing the highest α_{app} values in LC for a given CSP is not easy, since one always deals with a number of overlaid adsorption and solvent effects, which are difficult to de-convolute.

Declaration of Competing Interest

Authors declare no conflict of interest.

CRediT authorship contribution statement

Dániel Tanács: Investigation, Writing – original draft, Visualization. **Tímea Orosz:** Investigation. **István Ilisz:** Conceptualization, Writing – original draft, Writing – review & editing. **Antal Péter:** Conceptualization, Writing – review & editing. **Wolfgang Lindner:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462212.

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