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Comparative study of zwitterionic Cinchonane-based ion-exchangers for liquid chromatographic enantioseparations of free fluorinated tryptophanes: mechanistic and thermodynamic insights under green mobile phase conditions

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ABSTRACT

In recent years, growing awareness of environmental sustainability has driven the search for eco-friendly alternatives in analytical chemistry. In liquid chromatography, this includes replacing traditional organic solvents like acetonitrile (MeCN) with greener options that comply with environmental standards while maintaining chromatographic performance. In this study, we describe in a comparative manner the performance of four *Cinchona* alkaloid-based ion-exchangers for the enantioresolution of fluorinated tryptophan analogs, applying dimethyl carbonate (DMC) as a green alternative to MeCN. Quinine- and quinidine-based zwitterionic chiral stationary phases (CSPs), namely ZWIX(+), ZWIX(–), ZWIX(+A), and ZWIX(–A) were utilized with binary mobile phase systems consisting of methanol (MeOH), MeCN, and DMC. To the best of our knowledge, this is the first report of the *Cinchona* alkaloid-based ion-exchangers applied with DMC-based mobile phase systems.

In polar ionic mode using MeOH/MeCN and MeOH/DMC mobile phases similar retention characteristics were obtained on all the *Cinchona*-based columns. Under the same eluent conditions, higher retention factors were observed in the presence of DMC, and in both systems, increasing the proportion of the less polar component (MeCN or DMC) in the mobile phase resulted in a significant increase in retention for all investigated tryptophan analogs, due to stronger interactions with the stationary phase. The stoichiometric displacement model described the dependence of retention on the counterion concentration with good accuracy in both mobile phase systems, and the more negative slopes observed in the case of DMC indicate the formation of stronger ionic interactions. Unlike in the MeCN-based system, the DMC-based eluents showed a general decrease in both enantioselectivity and resolution for most analytes as the DMC content increased on all four columns. Given the substantial difference in polarity between MeCN and DMC, notable variations in enantioselectivity were anticipated. Surprisingly, only modest deviations were detected when comparing mobile phases containing MeCN to those with DMC. Despite the similar chromatographic properties observed for the two solvents, the thermodynamic studies revealed significant differences between the two mobile phase systems. For MeCN-based eluents, retention times slightly decreased with temperature on most CSPs, in contrast, with DMC-based eluents, retention times increased for all analytes on all CSPs as temperature rose. Interesetingly, enantioselectivity changed differently on the studied CSPs; enthalpically driven separations were observed in the MeCN-containing systems on all zwitterionic CSPS, whereas several entropically driven separations were observed in the DMC-containing systems on the ZWIX(–) CSP. The elution order of the enantiomers remained unchanged, regardless of replacing MeCN with the greener solvent DMC or varying the eluent composition and temperature.

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1. Introduction

In recent years, the principles of green chemistry have gained increasing importance across many areas of analytical chemistry [1–3]. Liquid chromatography (LC) has progressed towards more sustainable practices [4–6], with advances such as low-flow systems, small particles and miniaturized columns reducing solvent use and vaste. Capillary electrophoresis and supercritical fluid chromatography (SFC) may also provide effective alternatives to LC. Unconventional solvents have been uitilzed to green the chromatographic separations [7]. Besides more traditional solvents like ethanol [8] or azeotropic ethanol [9], ionic liquids [10] have been considered to reduce environmental impact. In case of LC-based separations, propylene carbonate (PC) [11,12], and dimethyl carbonate (DMC) [13–17] have recently been suggested as novel green solvents. DMC is a moderately polar, aprotic solvent, miscible with most of the organic solvents, but has limited solubility in water. It is listed among the most environmentally friendly solvents, it belongs to the same class as water and alcohols [18] and it may be applied as a mobile phase constituent in LC to substitute organic solvents with higher environmental impact, as e.g., acetonitrile (MeCN). DMC has already been tested for enantioselective separations in LC [19–22]; however, so far only with polysaccharide-based columns, but not for ion-exchange-type chiral stationary phases (CSPs).

In this contribution we integrate ionizable *Cinchona* alkaloids, such as quinine (QN) and quinidine (QD), and their derivatives, as chiral ion-exchanger-type CSPs, while applying DMC as a solvent component of the mobile phase. QN and QD are multi-chiral-center molecules with a rigid polycyclic framework containing multiple functional groups, providing numerous sites for non-covalent interactions, including hydrogen bonding, π–π stacking, and ion pairing [23]. *Cinchona* alkaloid-based CSPs are notable for their unique structural and stereochemical features, providing high enantioselectivity across a wide range of analytes, especially those bearing mono- or zwitterionic functionalities, particularly natural and unnatural amino acids [24–27].

Within the last few years, fluorinated drugs have become increasingly prominent, with over 30 % of marketed pharmaceuticals containing at least one fluorine atom [28]. Fluorine's small size, high electronegativity, and hydrogen bonding modulation can significantly enhance a drug's ADME (adsorption, distribution, metabolism, excretion) profile, leading to improved stability, permeability, and bioavailability. Nearly 300 fluoropharmaceuticals have now been registered worldwide [29]. In parallel, the development of peptide drugs has made tremendous progress over the last few decades due to advances in modification chemistry and analytical technologies [30]. Fluorinated amino acids represent one of the most promising and rapidly developing areas of modified amino acids [31]. Their growth underscores the importance of enantioselective separations, as the biological activity of peptide-based drugs often depends on the absolute configuration of constituent amino acids. Nevertheless, enantioselective LC studies of fluorinated amino acids remain very limited. Ligand-exchange micellar capillary chromatography has been utilized for fluorine-substituted phenylalanine analogs [32], whereas the resolution of stereoisomers of non-proteinogenic polyfluorinated amino acids and peptides has been accomplished using a polysaccharide (PS) based column [33]. Additionally, PS-based CSPs have been employed to separate five fluorinated cyclic β^3-amino acid analogs with -NHBoc and -CO_2Et protecting groups [34]. More recently, macrocyclic glycopeptide and cyclofructan-6 CSPs [35], alongside *Cinchona* alkaloid-based zwitterionic CSPs [36], have demonstrated promising efficacy in the enantioseparation of fluorinated β-phenylalanine derivatives, while teicoplanin- and teicoplanin aglycon-based CSPs have also been investigated for the enantioseparation of fluorinated tryptophan analogs, demonstrating promising selectivity and resolution capabilities in this challenging compound class [37].

This study focuses on the direct enantioseparation of fluorinated tryptophan analogs with the zwitterionic *Cinchona* alkaloid-based CSPs used in the ion-exchange mode. A central objective of this work was to evaluate the potential of aprotic DMC as an alternative to the likewise aprotic but more polar MeCN, when used as a mobile-phase constituent in combination with the protic solvent methanol (MeOH). To the best of our knowledge, this represents the first systematic study investigating the effect of DMC on ion-exchange CSPs, demonstrating that its application can advance the rational understanding, design, and optimization of mobile phases, as well as enable practical applications in analytical method development.

Comparative enantioseparations were carried out in polar ionic mode using MeOH/MeCN and MeOH/DMC mobile phases and four zwitterionic *Cinchona*-derived CSPs, namely, ZWIX(+), ZWIX(–), ZWIX(+A), and ZWIX(–A). The analytical performance of DMC was systematically assessed, highlighting its benefits and limitations in chiral separations. To gain a deeper insight into the enantiorecognition and retention processes of the zwitterionic chiral selectors in symbiosis with the chiral zwitterionic analytes, the effects of key chromatographic parameters, including mobile phase composition, additive type, selector structure, and temperature, were thoroughly investigated. The known absolute configurations of the enantiomers, along with the structural features of both analytes and selectors, allowed for the identification and rationalization of general trends in chiral recognition.

2. Materials and methods

2.1. Chemicals and materials

Racemic and L-Trp were obtained from Sigma-Aldrich (St. Louis, MO, USA), while racemic and L-5-fluorotryptophan (5-FTrp) and 6-fluorotryptophan (6-FTrp) were purchased from Apollo Scientific (Whitefield Rd, Bradbury, Stockport, UK). All the details of the preparation of racemic and enantiopure form of 4,5-difluorotryptophan (4,5-diFTrp), 4,6-difluorotryptophan (4,6-diFTrp), 5,6-difluorotryptophan (5,6-diFTrp), 5,7-difluorotryptophan (5,7-diFTrp), and 4,5,6,7-tetrafluorotryptophan (4,5,6,7-tetraFTrp) were described earlier [37]. The structures of the samples are depicted in Fig. 1.

MeOH, MeCN, formic acid (FA), and acetic acid (AcOH) of LC-MS grade, DMC (99 %), ammonia (NH_3), ethylamine (EA), diethylamine (DEA), triethylamine (TEA), and triethanolamine (TEOA) of analytical reagent grade were obtained from VWR International (Radnor, PA, USA). All of the sample stock solutions were made with MeOH (1 mg mL^{-1}), and then diluted with eluent.

2.2. Apparatus and chromatography

The applied Waters® ACQUITY UPLC® H-Class PLUS UHPLC system with Empower 3 software (Waters Incorporation, Milford, MA, USA) consisted of a quaternary solvent manager, a sample manager FTN-H, a column manager, a PDA and a QDa mass spectrometer detectors. *Cinchona*-alkaloid-based zwitterionic columns, namely the QN-based (8*S*, 9*R*) ZWIX(+) and the QD-based (8*R*, 9*S*) ZWIX(-) were obtained from Chiral Technologies Europe (Illkirch, France). Details of the preparation of ZWIX(+A) and ZWIX(–A) were previously published [38, 39].

The structures of the chiral selectors are depicted in Fig. 2. All of the columns had the same physical size, 150 mm length, and 3.0 mm internal diameter, filled with the chemically modifed 3 μm porous particles. The measurements were carried out in isocratic mode, at a 0.5 mL min^{-1} flow rate and column temperature of 20 °C, unless stated otherwise. The hold-up times were measured by injecting acetone diluted with eluent.

3. Results and discussions

The mechanism of chiral recognition in the case of *Cinchona* alkaloid-based CSPs is multifaceted, often involving a synergistic combination of

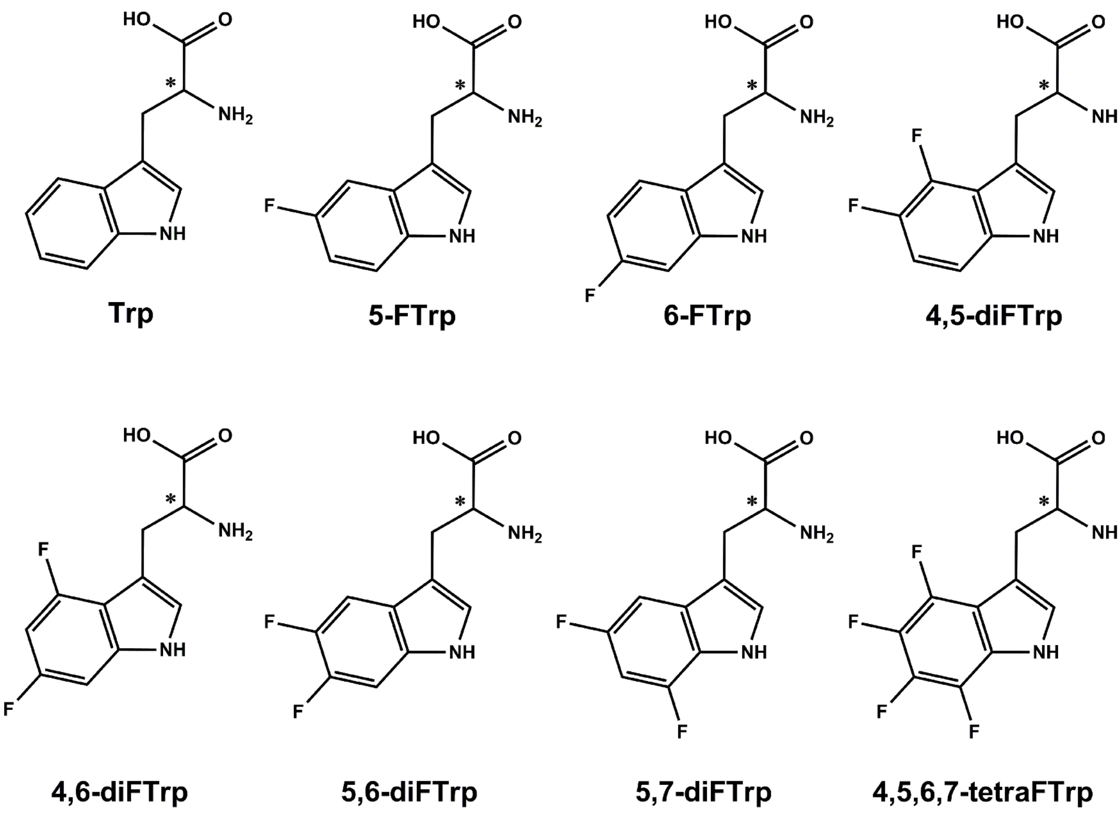
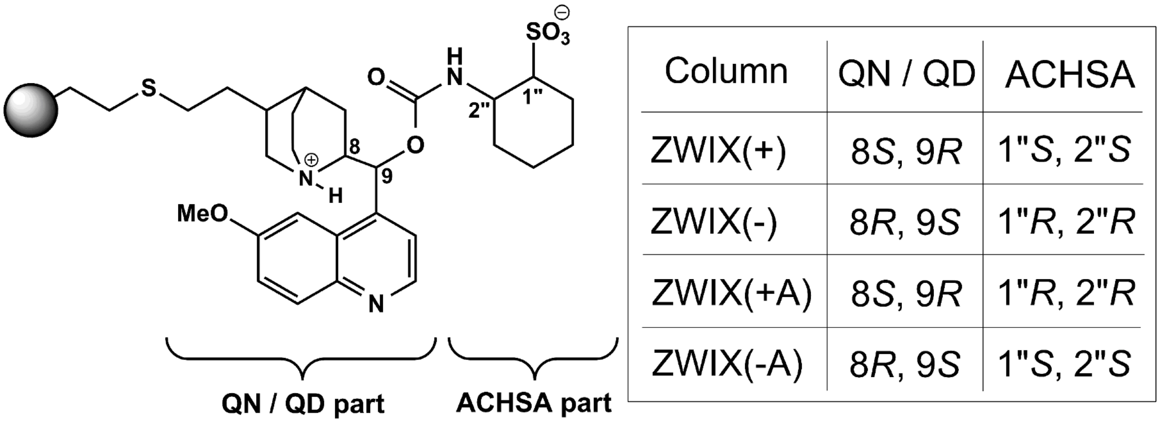


Fig. 1. Structure of the studied analytes.
Trp: tryptophan; **5-FTrp**: 5-fluorotryptophan; **6-FTrp**: 6-fluorotryptophan; **4,5-diFTrp**: 4,5-di-fluorotryptophan; **4,6-diFTrp**: 4,6-di-fluorotryptophan; **5,6-diFTrp**: 5,6-di-fluorotryptophan; **5,7-diFTrp**: 5,7-di-fluorotryptophan; **4,5,6,7-tetraFTrp**: 4,5,6,7-tetra-fluorotryptophan.



Column	QN / QD	ACHSA
ZWIX(+)	8*S*, 9*R*	1"*S*, 2"*S*
ZWIX(-)	8*R*, 9*S*	1"*R*, 2"*R*
ZWIX(+A)	8*S*, 9*R*	1"*R*, 2"*R*
ZWIX(-A)	8*R*, 9*S*	1"*S*, 2"*S*

Fig. 2. Structure of ZWIX selectors.

steric complementarity and differential interaction strengths with the enantiomers of the analyte, which can easily be tuned by the mobile phase composition [24,25]. Furthermore, the modularity of *Cinchona* alkaloid structures allows for chemical modifications that fine-tune the selectivity and retention behavior of these CSPs, thus enhancing their applicability in different chromatographic modes.

Based on this concept, utilizing QN or QD and aminocyclohexane sulfonic acid [(*R*,*R*)- and (*S*,*S*)-ACHSA], four CSPs, namely ZWIX(+) and ZWIX(–), and ZWIX(+A) and ZWIX(–A) were involved in this study.

3.1. Effect of the nature of the mobile phase additives

ZWIX columns are primarily utilized with MeOH as the bulk solvent, mixed most frequently with MeCN. Due to their zwitterionic nature, and ion pairing-based retention mechanism, the most effective separations require both a base and an acid additive in the mobile phase, e.g., NH_3 or TEA as the base and FA or AcOH as the acid, essentially acting as counterions [24,25]. Applying the ZWIX(–) column and MeOH/MeCN (50:50, *v/v*) mobile phase, first, we studied the effect of different additives to select the ones used in later measurements. The studied basic additives were NH_3, EA, DEA, TEA, and TEOA, the acidic additives were FA and AcOH. In all cases, the bases were utilized in 25 mM, and the acids in 50 mM concentration forming the respective organic salts dissolved in the organic mobile phase.

When comparing the effects of different acids in the chromatographic system (Fig. 3), FA was found to be a more effective additive in

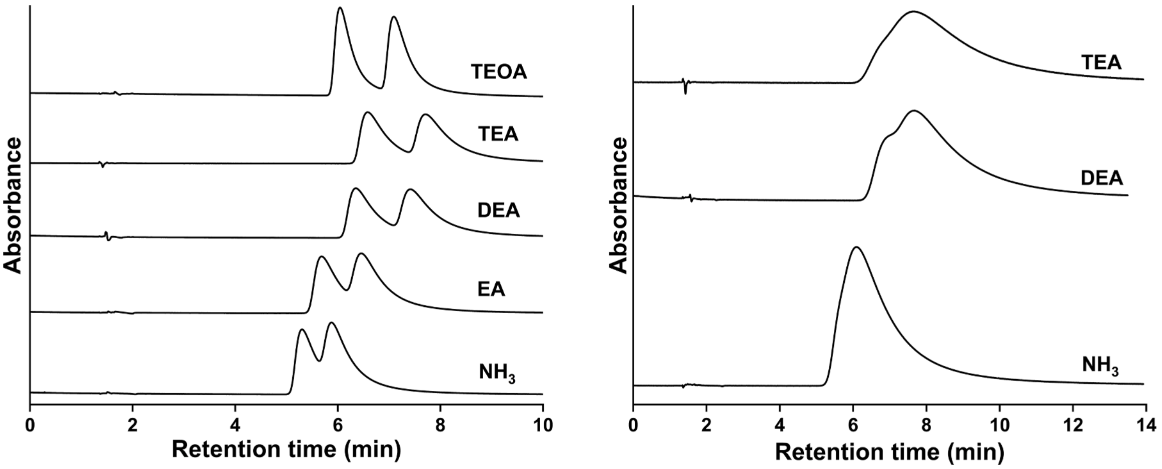


Fig. 3. Effect of additives on retentions and selectivities.
Chromatographic conditions: column, ZWIX(–); mobile phase, MeOH/MeCN 50/50 containing 25 mM base and **A**: 50 mM FA, **B**: 50 mM AcOH; detection: 280 nm; flow rate, 0.6 mL min^{-1}; temperature, 20 °C; analyte, 6-FTrp.

promoting the separation of the enantiomeric analytes, whereas AcOH did not result in any observable enantioseparation under the same conditions. A mild increase in enantioselectivity was observed upon the application of basic modifiers in the order of $NH_3 < EA < DEA < TEA$; however, this improvement in selectivity was accompanied by a concomitant increase in retention times. Among the basic additives tested, DEA, TEA, and TEOA all yielded separations approaching baseline resolution with the employed mobile phase compositions. Notably, TEOA provided the highest enantioselectivities and resolutions, while also resulting in shorter retention times compared to DEA and TEA, indicating a more favorable balance between resolution and analysis time.

TEOA has a markedly lower pK_a than DEA or TEA (7.76, 10.84, 10.75, respectively [40]), thus, it is a weaker base. In addition to basicity, the three protic OH groups and the larger molecular size of TEOA are likely to influence the solvation environment and intermolecular interactions within the mobile phase. The OH groups can form hydrogen bonds with the solvent and partially compete with the analytes for interaction with the CSP, while the bulkier structure may restrict the accessibility of analytes to some binding sites. Together, these effects increase the effective elution strength of the mobile phase, resulting in shorter retention times, while improving enantioselectivity and peak shapes. Due to the better peak shapes, higher selectivities, and shorter retention times, TEOA (25 mM) and FA (50 mM) were choosen as additives for the later experiments.

3.2. *Study of the MeOH/MeCN mobile phase system*

The binary solvent system composed of MeOH and MeCN was selected as the bulk eluent based on prior experimental findings and literature data [24,25], which indicated favorable chromatographic behavior for the target analytes under these conditions. This systematic approach follows similar concept that we studied earlier in detail for the chromatographic behavior of *N*-Acyl-DL-leucines as acidic analytes [41]. In contrast to previous studies on derivatized amino acids with only a single ionizable carboxyl group, the free amino acids examined here possess both amino and carboxyl groups, allowing simultaneous ion-pairing at positive and negative sites of the analyte and chiral selector. This dual interaction can produce additive effects, contributing to enhanced enantioselectivity compared to single ion-pairing systems.

To systematically investigate the influence of solvent composition on retention and separation efficiency, the volumetric ratio of MeOH to MeCN was varied within the range of 100:0 to 30:70 (*v/v*). Detailed chromatographic performance data obtained using the ZWIX(+) and ZWIX(–) CSPs are presented in Fig. 4, while the corresponding results for the ZWIX(+A) and ZWIX(–A) CSPs can be found in Figure S1. The retention profiles were very similar in all four columns; increasing the proportion of MeCN in the mixture led to substantially prolonged retention times on all four columns, which is a typical behavior for the *Cinchona* alkaloid-based CSPs [24,25,41], and may be attributed to changes in the solvation and partitioning behavior of the analytes as the polar but non-protic MeCN increasingly replaces the polar but protic MeOH in the mobile phase. The retention times of the analytes increase gradually with the increasing proportion of MeCN in the mobile phase up to a 50/50 (*v/v*) MeOH/MeCN composition. Beyond this point, retention increases more markedly, likely due to the further decreased solubility of the doubly charged analytes at higher MeCN concentrations. At MeCN concentrations exceeding 70 % (*v/v*) in the mobile phase, the retention factors of the analytes increased to an extent that rendered the separations impractical for routine analysis. This marked increase in retention is likely due to the stronger ionic interactions between the charged sites of the analytes and those of the chiral selector at high MeCN content. In general, ZWIX(+A) and ZWIX(–A) exhibited somewhat lower retention factors than the corresponding ZWIX(+) and ZWIX(–) columns, but the trends are very similar. (It should be noted here, that the selector loading of the ZWIX (+A) and ZWIX(–A) are ca. 15 % lower compared to the ZWIX (+) and ZWIX(–) columns).

In contrast to the similar retention characteristics, a significant difference was observed in the enantioselectivity and resolution capability of the zwitterionic CSPs. Although QD- and QN-based CSPs are considered to be in a pseudoenantiomeric relationship (as discussed in Section 3.6), they are in fact diastereomers; therefore, their functional groups are arranged in a non-equivalent three-dimensional environment. Consequently, changes in the mobile phase composition do not influence their interaction patterns in a symmetric manner. When utilizing the ZWIX(–) CSP, with increasing MeCN content, all analytes exhibited improved selectivity and resolution suggesting that the modified solvation conditions enhance the preorganized interaction geometry between the analytes and the QD and ACHSA-based stationary phase under these conditions (Fig. 4). Only the tetrafluorinated Trp analog was an exception, showing no marked change in selectivity. In contrast, on the other CSPs, a deviation from this trend was observed. Specifically, on ZWIX(+), only 4,5-diFTrp, 4,6-diFTrp, and tetraFTrp exhibited increasing selectivity and resolution with increasing MeCN content. Interestingly, the same trends were observed on the ZWIX(+A) column. In the case of ZWIX(–A), enantioselectivity increased only for 4,5-diFTrp and 4,6-diFTrp with increasing MeCN content. The distinct behavior of Trp and its fluorinated derivatives suggests that the number and

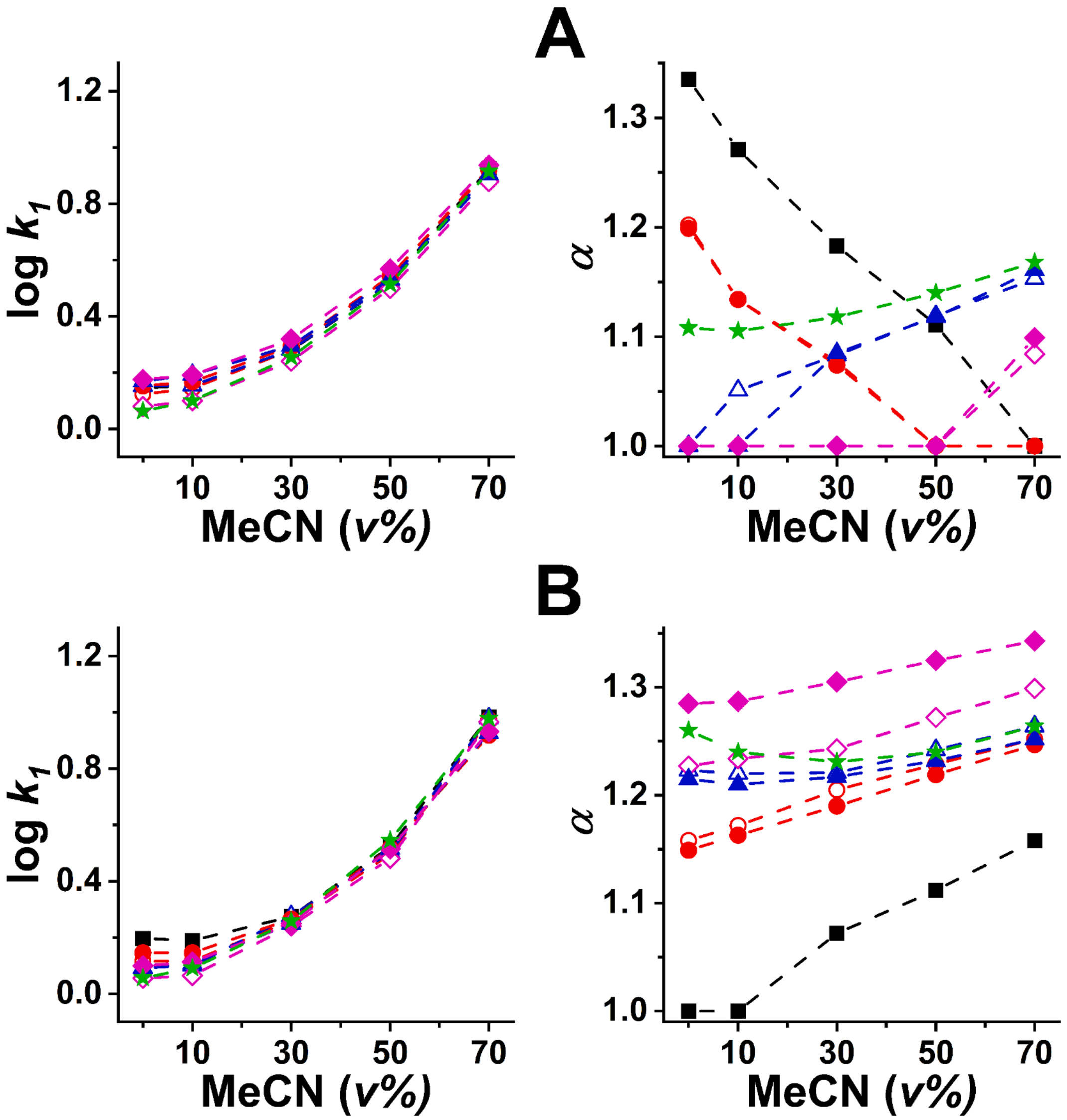


Fig. 4. Effect of MeCN ratio on the chromatographic parameters.
Chromatographic conditions: columns, **A**, ZWIX(+), **B**, ZWIX(–); mobile phase, MeOH/MeCN = 100/0 – 30/70 (*v*/*v*) containing 25 mM TEOA and 50 mM FA; detection, 280 nm; flow rate, 0.6 mL min^{-1}; temperature, 20 °C; symbols for analytes, Trp, ■; 5-FTrp, ●; 6-FTrp, ○; 4,5-diFTrp, ▲; 4,6-diFTrp, △; 5,6di-FTrp, ◆; 5,7-diFTrp, ◇; 4,5,6,7-tetraFTrp, ★.

positioning of electron-withdrawing substituents on the aromatic core influence enantioselectivity, likely through modulation of *π*–*π* interactions. However, no general rule can be established to describe their effects on the enantiorecognition. Overall, baseline separation ($R_S \geq 1.5$) was achieved for 1 analyte on the ZWIX(+) column, 2 analytes on ZWIX(–A), 5 analytes on ZWIX(+A), and 7 analytes on ZWIX(–), highlighting the outstanding resolution capability of the QD-based ZWIX(–) column in the case of the studied fluorinated Trp analogs.

3.3. Study of the MeOH/DMC mobile phase system

MeCN is a widely utilized solvent, paticularly in reversed phase LC, both in academic research and industrial applications, due to its favorable physicochemical properties such as low viscosity, low UV cut-off, high elution strength, and miscibility with water and most organic solvents. Despite these advantages, MeCN is associated with several environmental and safety concerns, including toxicity, flammability, and classification as a volatile organic compound (VOC). Its production is energy-intensive, and improper disposal can pose additional ecological risks, making it a critical focus in the field of green chemistry. The moderately polar and non-protic DMC has emerged as a promising green alternative to conventional organic solvents such as MeCN, owing to its low toxicity, high biodegradability, and favorable safety profile. In addition to its benign environmental characteristics, DMC offers sufficient elution strength for LC-based separations [13–16]. It mixes with MeOH and ethanol at any ratio, however, it is important to note that DMC mixes with water only up to around 10 *v*/*v* % [42], which can strongly limit its usage.

To enable a comparative evaluation of the effects of DMC vs. MeCN on the retention, enantioselectivity, and resolutions, the same chromatographic conditions as in the previously presented case were applied. To the best of our knowledge, this study represents the first investigation of the effect of DMC on ion-exchanger-based CSPs, in particular on zwitterionic ion-exchangers applied for the resolution of free amino acids. The experiments were carried out on the same four studied CSPs, allowing for a detailed assessment of the role and mechanism of action of DMC in relation to the distinct characteristics of each CSP. The results with MeOH/DMC eluent on ZWIX(+) and ZWIX(–) are presented in Fig. 5, while the results obtained with ZWIX(+A) and ZWIX(–A) are illustrated in Figure S2. In all cases, retention times of the ampholytic analytes gradually increased with the rising proportion of DMC in the eluent, a trend similarly observed with MeCN, but to a different extent. Comparing the retention factors, on all four columns higher retention factors were observed when using DMC instead of MeCN under the same eluent composition. DMC exhibits a relatively apolar character with a *logP* value of $\sim$0.5 [10], whereas MeCN and MeOH show *logP* values of -0.34 and -0.69, respectively [43], while their solvent polarity indices are 3.1, 5.1, and 5.8, respectively [44]. As mentioned above, *Cinchona*-based zwitterionic CSPs typically exhibit increased retention with decreasing mobile phase polarity under polar ionic conditions. Since DMC is less polar than MeCN, the higher retention factors observed when MeCN is replaced by DMC can be qualitatively attributed to the reduced polarity of the eluent, which also affects the solvation of all charged sites in the system. Consequently, possible changes in ionic interactions will be addressed in detail, with quantitative data provided in Section 3.4.

The polarity of the two solvents (MeCN vs. DMC) differs considerably, and thus, pronounced changes in the enantioselectivities were expected. However, in comparison with MeCN-containing mobile phases, relatively minor differences were observed. In the DMC-based eluent systems, a general decrease in both enantioselectivity and resolution was observed for most of the ampholytic analytes with increasing DMC content on all four zwitterionic columns. As exceptions, 5-FTrp, 6-FTrp, and 5,7-diFTrp can be mentioned, showing a slight increase in values of α and R_S in the case of ZWIX(–A) column, while resolutions were varied according to a maximum curve on the ZWIX(–) column as the DMC ratio increased.

Three CSPs, namely ZWIX(+), ZWIX(+A), and ZWIX(−A) exhibited similar changes in the enantioselectivities; somewhat higher enantioselectivities were obtained for Trp and the monofluorinated Trps, while lower α values for the tetrafluorinated Trp in the presence of DMC. It is worth noting that the difluorinated analogs could generally be separated

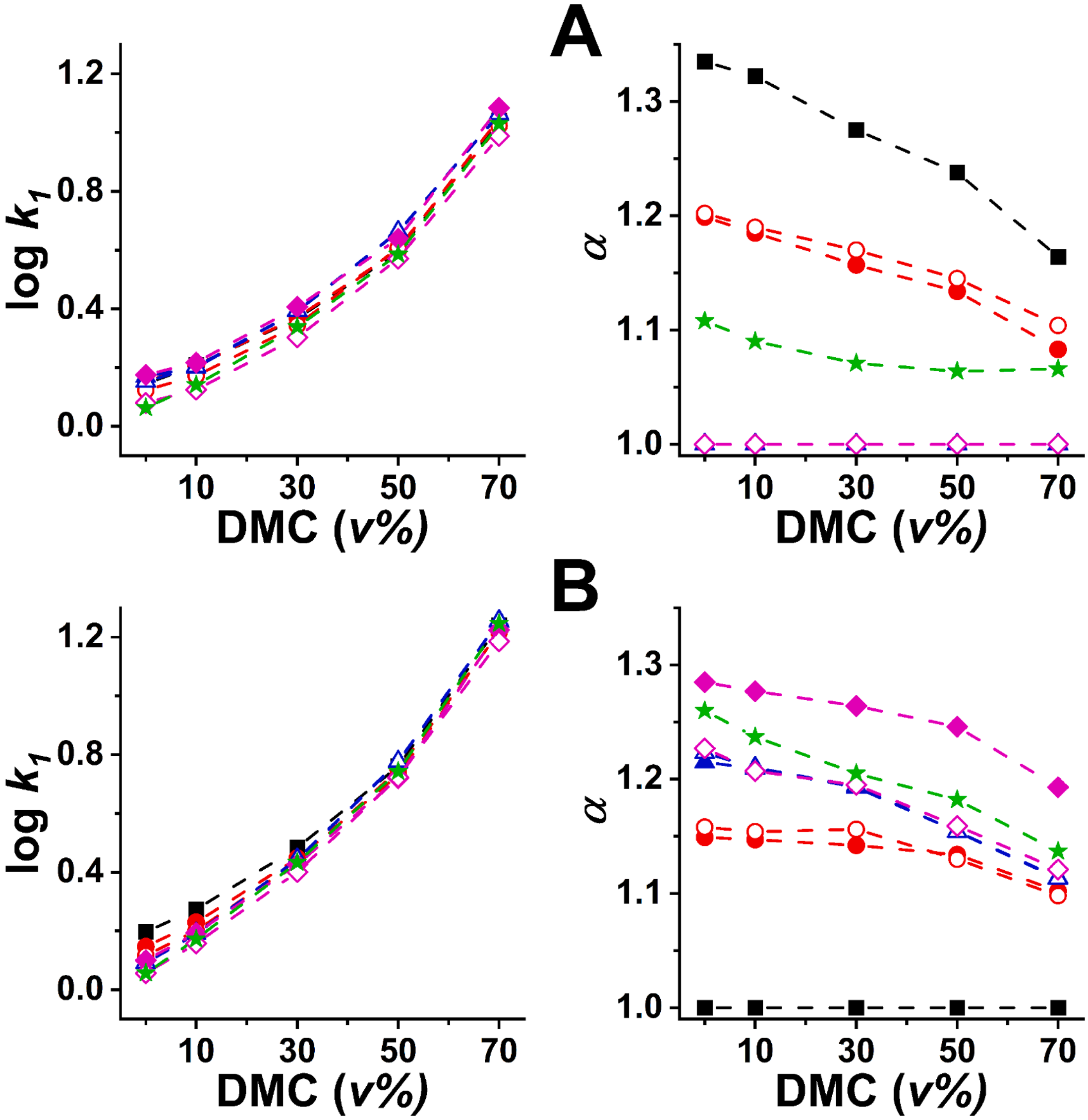


Fig. 5. Effect DMC ratio on the chromatographic parameters.
Chromatographic conditions: columns, **A**, ZWIX(+), **B**, ZWIX(–); mobile phase, MeOH/DMC = 100/0 – 30/70 (*v*/*v*) containing 25 mM TEOA and 50 mM FA; detection, 280 nm; flow rate, 0.5 mL min^{-1}; temperature, 20 °C; symbols for analytes, Trp, ■; 5-FTrp, ●; 6-FTrp, ○; 4,5-diFTrp, ▲; 4,6-diFTrp, △; 5,6di-FTrp, ◆; 5,7-diFTrp, ◇; 4,5,6,7-tetraFTrp, ★.

only with low selectivity, or they remained unresolved. The ZWIX(−) column exhibited slightly lower enantioselectivities with DMC, and higher sensitivity to the structural peculiarities of the analytes. Comparing the chromatographic efficiency of the double ion-pairing systems through the number of achieved baseline resolutions, the situation was the same as with the MeOH/MeCN mobile phase system; the ZWIX(–) column exhibited superior performance in the case of the studied fluorinated Trp analogs. These findings indicate that the ZWIX (–) stationary phase possesses a spatial configuration more conducive to chiral recognition and retention, as reflected in the generally higher retention times and improved separation performance. In comparing the two eluent systems, it becomes evident that subtle structural differences between selectors and analytes are sufficient to generate distinct interaction profiles, even if the overall changes seem modest.

Enantioselectivity on zwitterionic Cinchona-based CSPs originates from a combination of non-covalent interactions, dominated by ion pairing and complemented by hydrogen bonding, π–π interactions, and steric fitting. While ionic interactions mainly determine overall retention, enantioselectivity arises from subtle differences in the spatial arrangement and strength of these secondary interactions. Regarding hydrogen bonding, both MeCN and DMC act as hydrogen-bond acceptors but not as donors and exhibit comparable hydrogen-bond basicities (Kamlet–Taft $\beta \approx 0.31$ and 0.38, respectively), whereas MeOH shows substantially higher donor and acceptor ability ($\alpha \approx 0.93$, $\beta \approx 0.62$) and thus dominates the hydrogen-bonding network in the mobile phase [45]. Consequently, replacing MeCN with DMC is not expected to significantly alter hydrogen-bonding interactions relevant to chiral recognition. Similarly, differences in π–π interactions are expected to be minor and largely non-enantioselective under MeOH-rich conditions where ionic pairing governs retention. Therefore, the limited changes in enantioselectivity observed upon substituting MeCN with DMC are more plausibly attributed to differences in solvation, which may subtly influence steric complementarity and the spatial accessibility of interaction sites within the chiral recognition environment. Further studies are planned to clarify the role of such solvation effects.

3.4. Characterization of the ionic interactions in the MeOH/MeCN and MeOH/DMC mobile phase systems

The effectiveness of ZWIX CSPs arises from their zwitterionic character, which enables the formation of ionic interactions due to the presence of both positive and negative charges under optimal conditions. These ionic interactions occur not only with the analytes but also with other components of the mobile phase, particularly with the applied basic and acidic additives. The underlying mechanisms are described by the stoichiometric displacement model [46], according to which the logarithm of the retention factor exhibits a linear relationship with the concentration of counterions as described by Eq. 1., where K_Z describes the ion-exchange equilibrium and Z is the effective charge.

$$\log k = \log K_Z - Z \log c_{counterion}. \tag{1}$$

To quantitatively assess the role of ionic interactions, a series of experiments was conducted by varying the counterion concentration in both studied eluent systems employing the ZWIX(+) and the ZWIX(–) columns. The TEOA:FA concentration ratio was maintained at 1:2 throughout all experiments, with TEOA concentrations of 6.25, 12.5, 25, 50, and 100 mM. In all cases, irrespective of the eluent and CSP used, increasing the counterion concentration resulted in decreased retention times. The results fitted to the stoichiometric displacement model are presented in Fig. 6. The linear fits exhibited excellent correlation, with coefficients of determination (R^2) exceeding 0.99, supporting the validity of the stoichiometric displacement model also in the zwitterionic mode. The slopes of the linear regressions varied in the range from –0.311 to –0.360 for the MeOH/MeCN eluent, and –0.416 to – 0.477 for the MeOH/DMC mobile phase. The slopes determined for the MeOH/MeCN mobile phase are in good accordance with earlier results obtained in the same eluent systems [36,38]. The more negative slopes obtained with the MeOH/DMC mobile phases indicate stronger ionic interactions, probably due to the differences in the solvation properties of MeCN and DMC, which influence the solvation of all charged interaction sites within the system. It is interesting to note that the observed slopes within the same mobile phase were largely unaffected by the structures of the analytes studied. Furthermore, it is worth mentioning that only minor differences in slopes were observed for each enantiomer, i.e., the individual enantioselectivity characteristics remained almost constant when the counterion concentration was varied. As a result, it is possible to achieve reduced analysis times by increasing the counterion concentration, without inducing substantial loss or modification of enantioselectivity. This parameter therefore provides an additional tool for fine control of retention, beyond variations in eluent composition.

3.5. Thermodynamic studies

In chiral liquid chromatographic separations, temperature is a critical parameter that can influence not only the retention behavior of analytes but also the efficiency and selectivity of enantiomeric resolution. Variations in column temperature may alter the intermolecular interactions between the chiral selector and the enantiomers, thereby affecting the thermodynamic equilibrium of the separation process. By performing systematic measurements under identical mobile phase conditions across a range of column temperatures, it becomes possible to investigate the underlying thermodynamic parameters, such as changes in enthalpy (ΔH°), entropy (ΔS°), and Gibbs free energy (ΔG°) that govern enantioselective recognition on a given CSP. Although van’t Hoff analysis is a widely used approach for evaluating the thermodynamic parameters of enantioselective separations, its applicability is subject to several limitations [47,48]. In the case of enantiomeric pairs, plotting the natural logarithm of the separation factor ($ln\ \alpha$) as a function of the reciprocal absolute temperature ($1/T$) enables the determination of thermodynamic parameters governing the enantioseparation process. This approach allows for the calculation of differential enthalpy ($\Delta\Delta H^\circ$) and entropy ($\Delta\Delta S^\circ$) without requiring knowledge of the phase ratio (β), as described in Eq. 2, where α is the selectivity, R is the universal gas constant, and T is the thermodynamic temperature.

$$\ln\alpha = -\frac{\Delta\left(\Delta H^0\right)}{RT} + \frac{\Delta\left(\Delta S^0\right)}{R}. \tag{2}$$

Thus, the van’t Hoff plot of ln α versus $1/T$ provides a practical means of evaluating the temperature dependence of chiral recognition in a system-independent manner. While the resulting thermodynamic values cannot be directly compared across different chromatographic systems in an absolute sense, they can still offer valuable insights when comparing structurally related compounds under identical experimental conditions, thereby aiding the interpretation of structure–selectivity relationships.

In this study, temperature-dependent measurements were conducted over the range of 5 to 50 °C using a MeOH/MeCN (70:30, v/v) eluent containing 25 mM TEOA and 50 mM FA, and from 20 to 50 °C using a MeOH/DMC (30:70, v/v) eluent with the same additive composition. Although the measurements were initially intended to be performed using identical solvent ratios, this was not feasible due to differences in the elution strength and in the thermodynamic characteristics of the MeCN- and DMC-based mobile phase systems. For the MeOH/DMC system, at a 70:30 ratio no significant change in enantioselectivity with changes in temperature was observed, so the experiments were instead performed at the higher DMC content (30:70). In contrast, a 30:70 MeOH/MeCN ratio resulted in excessively long retention times and pronounced peak broadening, making it unsuitable; therefore, the 70:30 composition was selected for the MeOH/MeCN measurements. In order to ensure the reliability of the thermodynamic evaluation, the lower temperature limit in the DMC-containing system had to be increased. This adjustment was required due to the higher viscosity of DMC

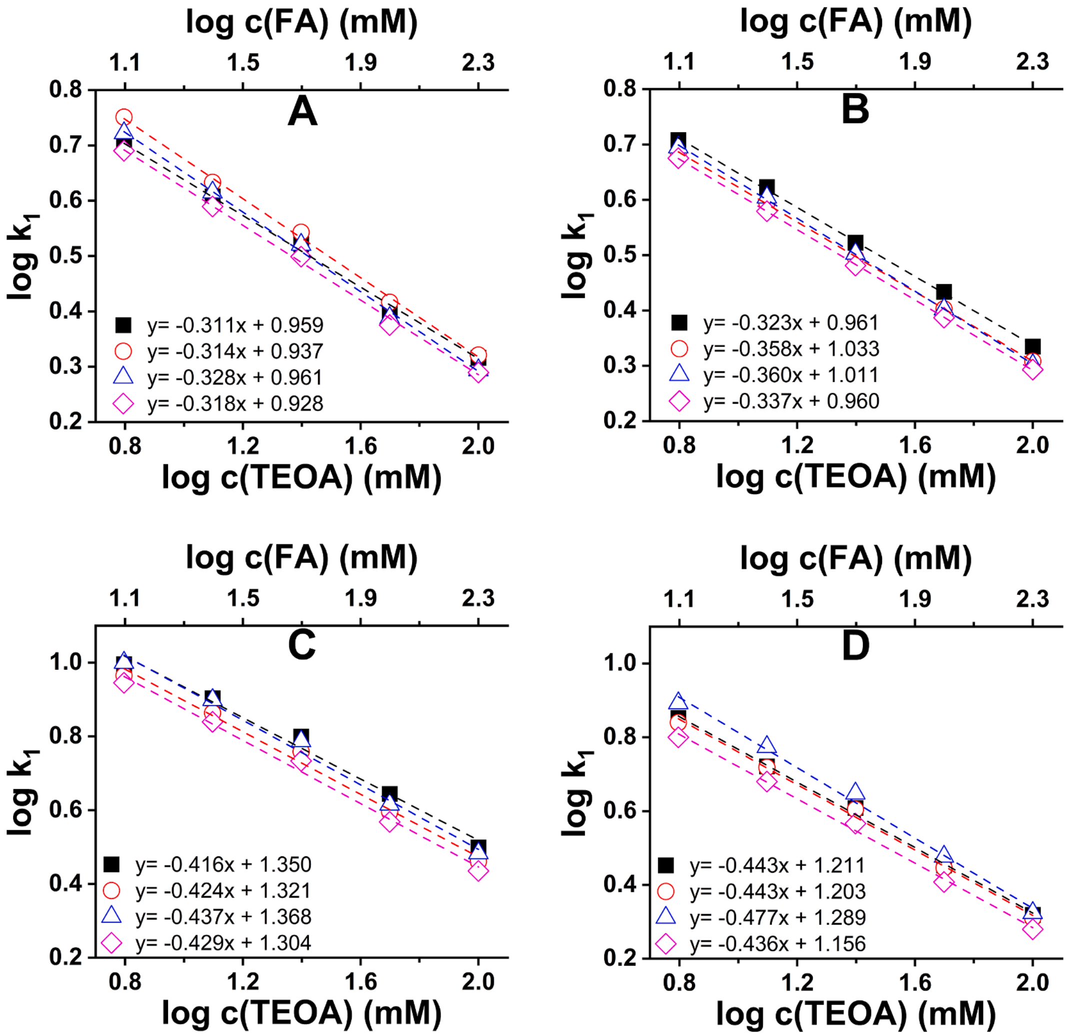


Fig. 6. Effect of the counterion concentration on the retention of the first eluting enantiomer (k_1).
Chromatographic conditions: columns, **A** and **C:** ZWIX(+), **B** and **D** ZWIX(–); mobile phase, **A** and **B:** MeOH/MeCN 50/50 (*v/v*), **C** and **D**: MeOH/DMC 50/50 (*v/v*), all containing 6.25 – 100 mM TEOA and 12.5 – 200 mM FA; detection, 280 nm; flow rate, 0.5 mL min^{-1}; temperature, 20 °C; symbols for analytes, Trp, ■; 6-FTrp, ○; 4,6-diFTrp, △; 5,7-diFTrp, ◇.

compared to MeCN, so as to keep the column backpressure within the operational safety limits of the instrument and below the pressure threshold calculated according to the approach of Asnin and Stepanova [48].

In the case of eluent systems containing MeCN, retention times of the analytes generally decreased slightly with increasing temperature with some exceptions for the ZWIX(–) column, where the retention times remained largely unchanged. Enantioselectivities also slightly decreased or remained unchanged with increasing temperature (Table S1-S2). In contrast, for eluent systems containing DMC, a marked difference in the retention behavior was observed; retention times increased for all analytes on all examined CSPs as the temperature rose. Interesetingly, enantioselectivity changed differently on the studied CSPs; α slightly decreased or did not change significantly on the ZWIX(+), ZWIX(+A) and ZWIX(–A) CSPs, while it increased for several analytes on the ZWIX (–) CSP. To provide a quantitative description thermodynamic parameters were calculated based on Eq. 2. Table 1 presents the thermodynamic data obtained for the ZWIX(+) and ZWIX(–) CSPs, whereas Table 2 summarizes the corresponding results for ZWIX(+A) and ZWIX (–A). Since our primary objective was to compare the results obtained in the two different eluent systems, results are reported only when the variation in enantioselectivity could be clearly evaluated in both systems. This approach ensures that the observed differences are attributable to the chromatographic conditions rather than to experimental uncertainty or system-specific effects. As shown by the data in Tables 1 and 2, three of the four CSPs examined exhibited rather similar behavior also in thermodynamic terms. The $\Delta\Delta G$ values are remarkably consistent between the two eluent systems, with only minor to moderate differences in the contributions of the enthalpic and entropic terms. In all cases, however, the separation process is driven by enthalpy as seen in the calculated Q values [$Q = \Delta(\Delta H^{o}) / (298\ \mathrm{K} \times \Delta(\Delta S^{o}))$], where $Q > 1$ corresponds to enthalpy-dominated processes, and $Q < 1$ to entropy-dominated ones. Interestingly, the ZWIX(–) CSP showed a pronounced deviation from this trend. The determined $\Delta\Delta H$ and $\Delta\Delta S$ values (Table 1) were consistently much more negative in the DMC-containing systems than in those containing MeCN. This could be related to stronger specific interactions or a more ordered solvation environment in the presence of DMC, which may enhance the enthalpic contribution to the enantirecognition process. As the Q values indicate (Table 1) in the DMC-containing systems more entropy-driven separations were realized.

3.6. *Enantiomeric elution order (EEO)*

It is noteworthy that QN and QD share identical configurations at

Table 1

Thermodynamic parameters, $\Delta(\Delta H^0)$, $\Delta(\Delta S^0)$, $T\times\Delta(\Delta S^0)$, $\Delta(\Delta G^0)$, correlation coefficients (R^2) and Q values of fluorinated tryptophan analogs on ZWIX(+) and ZWIX(-) columns.

Analyte	Eluent	$-\Delta(\Delta H^o)$ (kJ/mol)	$-\Delta(\Delta S^o)$ (J/(mol*K)	Corr. coeff. (R^2)	$-T\times\Delta(\Delta S^o)_{298\ K}$ (kJ/mol)	$-\Delta(\Delta G^o)_{298\ K}$ (kJ/mol)	Q
ZWIX(+)							
1 Trp	A	1.41	3.40	0.999	1.01	0.40	1.39
	B	1.17	2.63	0.999	0.78	0.39	1.50
2 5-FTrp	A	0.41	0.73	0.989	0.22	0.20	1.90
	B	0.59	1.22	0.993	0.36	0.22	1.61
3 6-FTrp	A	0.65	1.60	0.999	0.48	0.18	1.37
	B	0.65	1.42	0.984	0.45	0.22	1.53
ZWIX(–)							
2 5-FTrp	A	0.43	0.25	0.985	0.07	0.35	5.75
	B	-0.65	-3.08	0.999	-0.92	0.26	0.71
4 4,5-diFTrp	A	0.40	0.37	0.991	0.11	0.29	3.60
	B	-1.11	-4.65	0.995	-1.38	0.28	0.80
5 4,6-diFTrp	A	0.44	0.21	0.998	0.06	0.38	7.07
	B	-1.10	-4.64	0.995	-1.38	0.28	0.80
7 5,7-diFTrp	A	0.97	1.60	0.990	0.48	0.49	2.04
	B	-0.52	-2.74	0.992	-0.82	0.29	0.64
8 4,5,6,7-tetraFTrp	A	0.71	0.43	0.999	0.13	0.58	5.51
	B	-0.76	-3.72	0.974	-1.11	0.34	0.69

Chromatographic conditions: mobile phase, A: MeOH/MeCN = 70/30 (*v/v*) containing 25 mM TEOA and 50 mM FA, B: MeOH/DMC = 30/70 (*v/v*) containing 25 mM TEOA and 50 mM FA; flow rate, 0.5 ml min^{-1}; detection, 280 nm; $Q = \Delta(\Delta H^\circ)/298\ K\ x\ \Delta(\Delta S^\circ)$; temperature, A: 5 °C – 50 °C, B: 20 °C – 50 °C

Table 2

Thermodynamic parameters, $\Delta(\Delta H^0)$, $\Delta(\Delta S^0)$, $T\times\Delta(\Delta S^0)$, $\Delta(\Delta G^0)$, correlation coefficients (R^2) and Q values of fluorinated tryptophan analogs on ZWIX(+A) and ZWIX(–A) columns.

Analyte	Eluent	$-\Delta(\Delta H^o)$ (kJ/mol)	$-\Delta(\Delta S^o)$ (J/(mol*K)	Corr. coeff. (R^2)	$-T\times\Delta(\Delta S^o)_{298\ K}$ (kJ/mol)	$-\Delta(\Delta G^o)_{298\ K}$ (kJ/mol)	Q
ZWIX(+A)							
1 Trp	A	1.12	2.01	0.999	0.60	0.52	1.87
	B	0.91	1.68	0.996	0.50	0.41	1.81
4 4,5-diFTrp	A	1.37	3.69	0.999	1.10	0.27	1.25
	B	1.40	3.73	0.992	1.11	0.29	1.26
5 4,6-diFTrp	A	1.38	3.71	0.999	1.10	0.28	1.25
	B	1.40	3.73	0.994	1.11	0.29	1.26
8 4,5,6,7-tetraFTrp	A	1.44	3.80	0.999	1.13	0.31	1.27
	B	0.87	2.16	0.997	0.64	0.23	1.36
ZWIX(–A)							
1 Trp	A	0.75	1.15	0.968	0.34	0.40	2.18
	B	1.07	2.15	0.998	0.64	0.43	1.67
4 4,5-diFTrp	A	1.63	4.18	0.999	1.25	0.38	1.31
	B	0.97	2.18	0.974	0.65	0.32	1.50
5 4,6-diFTrp	A	1.65	4.23	0.998	1.26	0.39	1.31
	B	1.19	2.90	0.999	0.86	0.32	1.38
8 4,5,6,7-tetraFTrp	A	1.84	4.84	0.998	1.44	0.40	1.28
	B	1.18	2.98	0.997	0.89	0.29	1.33

Chromatographic conditions: mobile phase, A: MeOH/MeCN = 70/30 (*v/v*) containing 25 mM TEOA and 50 mM FA, B: MeOH/DMC = 30/70 (*v/v*) containing 25 mM TEOA and 50 mM FA; flow rate, 0.5 ml min^{-1}; detection, 280 nm; $Q = \Delta(\Delta H^\circ)/298\ K\ x\ \Delta(\Delta S^\circ)$; temperature, A: 5 °C – 50 °C, B: 20 °C – 50 °C

three of their five chiral centers, whereas the configurations at the 8th and 9th carbon atoms – known to be crucial for chiral recognition – are opposite. Consequently, the two molecules exhibit a so-called pseudoenantiomeric relationship. Owing to this structural feature (depicted in Fig. 2), the four zwitterionic CSPs enables the reversal of the elution order simply by interchanging the pseudoenantiomeric CSPs, without requiring substantial modification of the chromatographic conditions. This provides a distinct analytical advantage, particularly when accurate quantification of low-concentration components is required. A few illustrative chromatograms for the reversed EEO on the pseudoenantiomeric CSPs are shown in Figure S3. Interestingly, identical EEO were observed on the ZWIX(+) and ZWIX(+A) columns, as well as on the ZWIX(–) and ZWIX(–A) columns. These observations indicate that alteration of the cation-exchanging ACHSA subunit site alone is insufficient to induce inversion of the elution order for the Trp analogs examined. The configurations of C8 and C9 in QN and QD are the main drivers of the EEO. Importantly, the EEO was identical in eluents containing MeCN or DMC, demonstrating that replacing MeCN with the greener solvent DMC did not significantly affect the enantioselective molecular recognition mechanism of these four zwitterionic CSPs. Representative chromatograms employing identical mobile phase conditions are shown in Fig. 7, wherein the sole variable was the replacement of MeCN with DMC at the same volumetric ratio. It is noteworthy that neither variations in the eluent composition nor changes in temperature altered the EEO, which clearly indicates the minor influence of the non-protic solvents DMC and MeCN on the overall spatial arrangement of the solvated chiral selector units.

4. Conclusions

This study provides a comprehensive evaluation of the potential of DMC as a green solvent alternative to MeCN, particularly in enantioselective ion-exchange-based LC. To the best of our knowledge, this is the first systematic investigation addressing the effect of DMC on zwitterionic-type CSPs. In both the MeOH/MeCN and MeOH/DMC binary solvent systems, increasing the proportion of the non-protic, less polar component led to a significantly prolonged retention, consistent with the typical behavior of zwitterionic CSPs applied in polar ionic mode. While the overall retention profiles were comparable across the four CSPs, significant differences in enantioselectivity were observed.

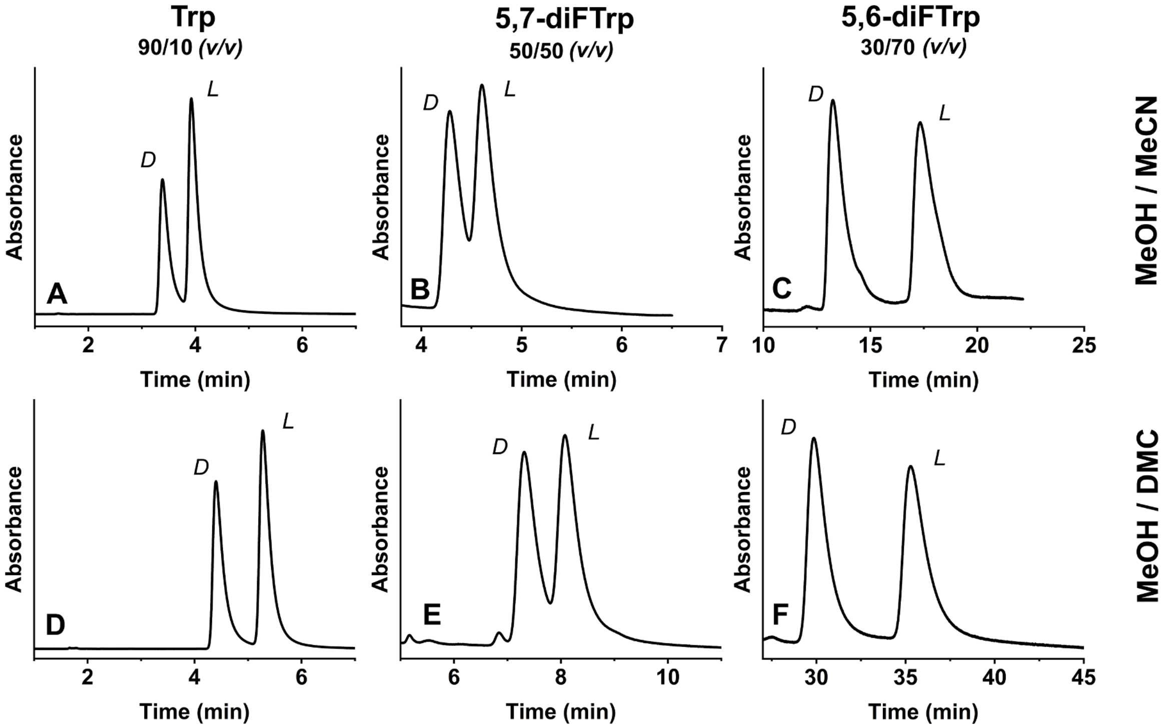


Fig. 7. Enantioemric elution orders in the MeOH/MeCN and MeOH/DMC mobile phase systems.
Chromatographic conditions: column, **A, D**: ZWIX(+), **B, E**: ZWIX(+A), **C, F**: ZWIX(–); mobile phase, **A–C**: MeOH/MeCN, **A:** 90/10 (*v/v*), **B**: 50/50 (*v/v*), **C**: 30/70 (*v/v*), all containing 25 mM TEOA and 50 mM FA, **D–F**: MeOH/DMC, **D**: 90/10 (*v/v*), **E**: 50/50 (*v/v*), **F**: 30/70 (*v/v*), all containing 25 mM TEOA and 50 mM FA; flow rate, 0.6 mL min^{-1}; detection, 280 nm; temperature, 20 °C.

The ZWIX(–) column exhibited superior separation efficiency and selectivity for most analytes, achieving baseline resolution for the highest number of compounds, which highlights the favorable spatial configuration of the quinidine-based selector for chiral recognition of the studied fluorinated tryptophan derivatives. Mechanistically, double ion-pairing events occur, involving the concentrations of the counter-ions used as mobile-phase additives and the solvent composition. These factors influence the solvation shells of all involved species and ionic sites, thereby determining the overall retention characteristics observed for the analytes.

Replacement of MeCN with DMC, as an unconventional alternative, resulted in similarly tunable, moderately higher retention factors and slightly reduced enantioselectivities, yet maintained the overall selectivity trends. The linear correlation between retention and counterion concentration confirmed the applicability of the stoichiometric displacement model, with more negative slopes observed in DMC-containing systems, indicating stronger ionic interactions due to altered solvation properties. Thermodynamic evaluation further demonstrated that enantioseparation on these CSPs is predominantly enthalpy-driven ($Q > 1$ in most cases). However, the ZWIX(–) CSP exhibited distinct behavior, with substantially more negative $\Delta\Delta H$ and $\Delta\Delta S$ values in DMC-based eluents, suggesting stronger specific interactions and/or a more ordered solvation environment. Notably, in DMC-containing systems, several separations shifted toward entropy-driven behavior ($Q < 1$), emphasizing the solvent's influence on the mechanism of chiral recognition. The thermodynamic analysis also confirmed that while the overall enantioselectivity was maintained, the relative contributions of the enthalpic and entropic terms can change considerably upon solvent replacement, reflecting the complex role of solvation in chiral recognition. Despite the change in solvent composition, the replacement of MeCN with DMC did not induce any drastic alterations in chromatographic behavior. The CSPs preserved their enantioselective recognition capability, demonstrating that the use of this greener solvent does not compromise chiral discrimination. Nevertheless, the thermodynamic analysis revealed that the relative contributions of the enthalpic and entropic terms to enantioseparation can vary significantly upon solvent exchange, highlighting the sensitive balance between solvation effects and molecular interactions in chiral recognition mechanisms. Replacing MeCN with the DMC, as well as changes in eluent composition and temperature, did not alter the elution order of the enantiomers, thereby supporting that the enantiomer recognition mechanism remained unchanged.

Overall, these findings confirm the strong impact of solvent strength (polarity and aprotic/protic character) of the mobile phase on both retention and enantioselectivity, thereby demonstrating the excellent performance and solvent sensitivity of the ZWIX(–) columns. The results also underline the feasibility and relevance of employing environmentally benign solvents, such as DMC, as sustainable alternatives in chiral chromatography, contributing to the ongoing advancement of green analytical chemistry.

CRediT authorship contribution statement

Dániel Tanács: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gábor Németi:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Róbert Berkecz:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Zsolt Bozsó:** Writing – original draft, Resources. **Gábor K. Tóth:** Writing – original draft, Resources. **Wolfgang Lindner:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Antal Péter:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **István Ilisz:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2026.466811.

Data availability

Data will be made available on request.

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