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Liquid chromatographic resolution of natural and racemic *Cinchona* alkaloid analogues using strong cation- and zwitterion ion-exchange type stationary phases. Qualitative evaluation of stationary phase characteristics and mobile phase effects on stereoselectivity and retention

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ABSTRACT

Liquid chromatographic (LC) and subcritical fluid chromatographic (SFC) resolution of the basic natural and synthetic *Cinchona* alkaloid analogues has been studied. Focus has been placed on the employment of four enantiomerically structured chiral strong cation-exchangers and four chiral diastereoisomeric *Cinchona* alkaloid and cyclohexyl aminosulfonic acid-based zwitterionic ion-exchangers. Except for the novel, recently synthesized racemic quinine the other investigated pairs of basic analytes are diastereomeric, but often called "pseudoenantiomeric" compounds of quinine and quinidine, cinchonidine and cinchonine, 9–*epi*–quinine and 9–*epi*–quinidine. As expected, the elution order of the resolved racemic quinine was reversed for all the eight investigated enantiomeric and (pseudo)enantiomeric pairs of chiral stationary phases, whereas this was not necessarily the case for the diastereomeric pairs of the *Cinchona* alkaloid related analytes. Varying the type and composition of the protic (methanol) and non-protic (acetonitrile) but polar bulk solvents in combination with organic salt additives in the mobile phase the overall retention and stereoselectivity characteristics could be triggered, leading to well performing LC and SFC systems.

Thus the retention behavior of the basic analytes on both the chiral cation-exchangers and the diastereomeric zwitterionic ion-exchangers, used as cation-exchangers, could be described by the stoichiometric displacement model related to the counter-ion effect of the mobile phase additives. In addition, it became obvious that the non-protic acetonitrile compared to methanol as bulk solvent lead to a significant increase in retention, which can be associated with an increased electrostatic interaction of the charged sites due to a smaller solvation shell of the solvated cationic and anionic species. Based on the chromatographic results of the systematically selected chiral analytes and stationary phases attempts were undertaken to interpret qualitatively the observed stereoselectivity phenomena.

1. Introduction

The liquid chromatographic separation and analysis of the major *Cinchona* alkaloids *per se* and in extracts of *Cinchona* bark have been a subject of scientific research for a long time and led to a number of publications summarized in a review by D. McCalley [1]. It is evident that HPLC in both the normal phase (NP) and reversed phase (RP) mode became the method of choice. Recently, a method using supercritical fluid chromatography (SFC) has also been published in context with the analysis of *Cinchona* bark extracts [2]. In this work non-chiral RP type and dedicated SFC type stationary phases have been investigated in terms of their selectivity spectrum to resolve the major isomers of natural *Cinchona* alkaloids with reasonable resolution values. In expanding the opportunities to separate not only natural *Cinchona* alkaloids but also some synthetic derivatives, Hoffmann presented a method using a novel strong chiral cation-exchanger (cSCX) with an unexpected selectivity [3]. The chemical structures of the major natural *Cinchona* alkaloids, which encompass the three pairs quinine (QN) and quinidine (QD), dihydroquinine (DHQN) and dihydroquinidine (DHQD),

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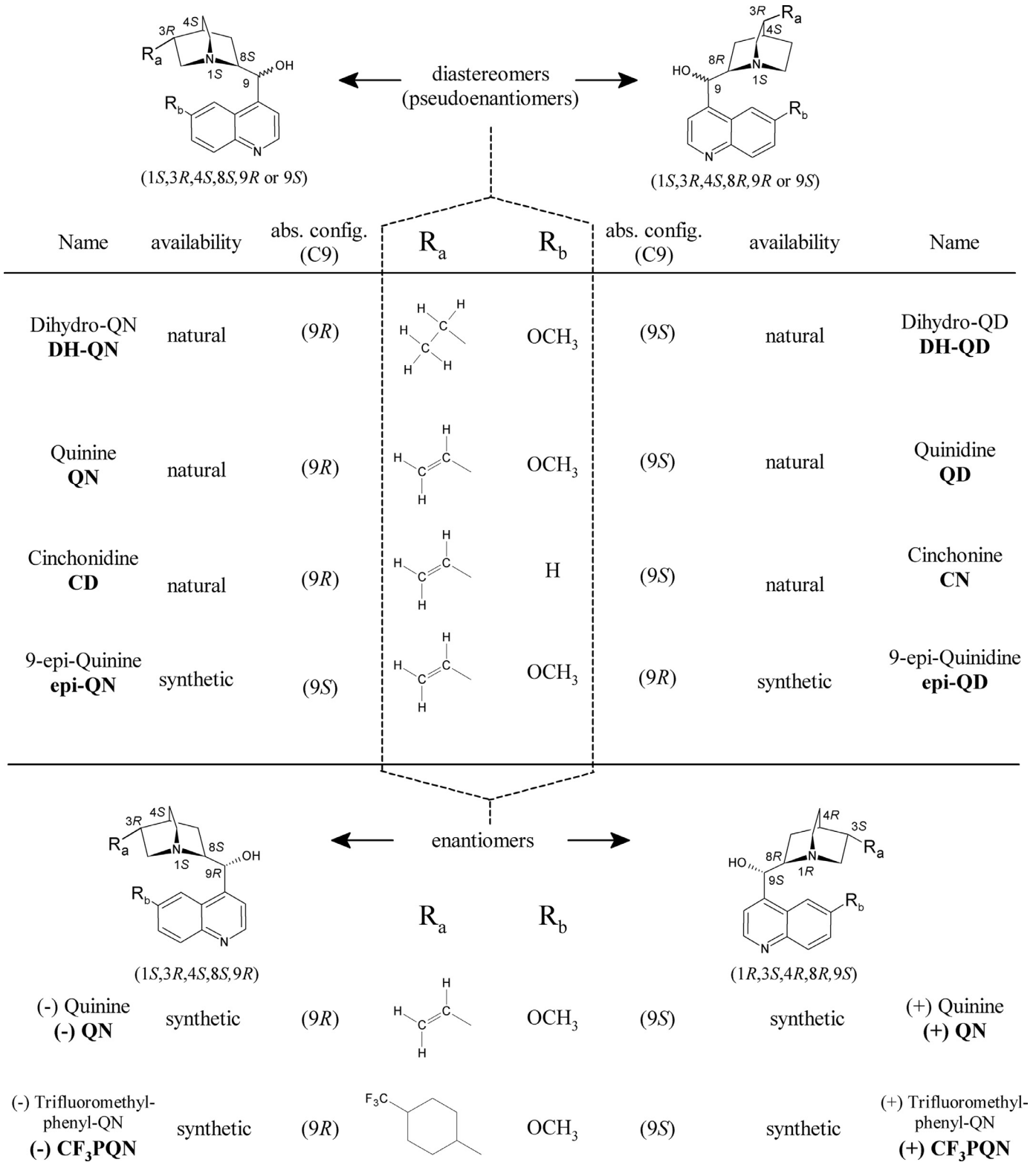

Fig. 1. Structure of natural and synthetic *Cinchona* alkaloids.
QN, quinine, QD, quinidine; *rac*.QN, racemic quinine; *rac*.CF_3PQN, racemic trifluoromethylphenyl quinine; CN, cinchonine, CD, cinchonidine; DH-QN, dihydroquinine, DH-QD, dihydroquinidine; *epi*–QN, 9–*epi*–quinine, *epi*–QD, 9–*epi*–quinidine.

cinchonidine (CD) and cinchonine (CN) as well as the synthetic 9–*epi*–quinine (*epi*–QN) and 9–*epi*–quinidine (*epi*–QD) pair are depicted in Fig. 1. These four pairs are diastereomeric to each other, but frequently termed also pseudo-enantiomers, and are characterized by the configuration of 5 stereogenic centers. The configuration of the C-8 and C-9 carbon atoms switch in the case of quinine to quinidine, whereas the 1*S*,3*R* and 4*S* stereogenic centers of the quinuclidine ring remain constant for QN/QD, DHQN/DHQD, CD/CN, and for *epi*–QN/*epi*–QD (Fig. 1). In nature, the genuine enantiomers of (−)QN, of (−)DHQN and of (−)CD, do not exist, which

qualified them as highly interesting targets for total synthesis concepts. These challenges could recently be mastered for (−)QN and (+)QN by Lee and Chen [4], and for racemic (±)QN by the Maulide group [5,] via two different synthesis strategies and diastereocontrolled reaction cascades. The protocol developed by Maulide [5] allowed also the straightforward synthesis of racemic QN derivatives (see CF_3PQN in Fig. 1), but not that of QD derivatives. In contrast, the synthesis way of Chen [4] opens up conceptually the production of (−)QN and (+)QD including the unnatural enantiomers, namely (+)QN and (−)QD. The synthesis of unnatural *epi*–QN/*epi*–QD was also published in detail [6]. Recently, a bio-inspired synthesis concept of (+)CD and its enantiomer the natural (−)CD was described as well [7].

It is a common practice to chromatographically resolve diastereoisomers on non-chiral stationary phase (*e.g.*, on RP), because they have different physicochemical properties [1,2,8]. For the resolution of enantiomers, in turn, the so-called chiral stationary phases (CSPs) or other innovative separation methods are required [9–16]. Naturally, diastereomers should also be separable on CSPs. However, this may give a different spectrum of diastereoselectivity and thus the elution order of the diastereomers may vary compared to non-chiral stationary phases.

In consideration of previous investigations of separating the main natural and synthetic diastereomeric *Cinchona* alkaloids and some derivatives using a cSCX [3], in this study we aimed at expanding this concept substantially using (i) two pairs of enantiomeric cSCX type CSPs, and (ii) two pairs of chiral, but still diastereomeric zwitterionic *Cinchona*- and aminocyclohexanesulfonic acid (ACHSA)-based CSPs as depicted in Fig. 2. The focus of the present systematic investigations was on studying the stereoselectivity aspects to find correlations between molecular structure and chromatographic resolution, which includes diastereoselectivity and enantioselectivity aspects as function of the mobile phase composition and the CSPs employed. Specifically, we wanted to learn the type and composition of bulk organic solvents and of the salt additive needed for the operation of ion-exchangers. The latter strongly influences the retention characteristics of the analytes on the various “chiral columns”. In addition, elution orders may be affected as a consequence of the underlying (ion pairing, ion-exchange) interaction principles of the protonated, positively charged basic analytes and the deprotonated sulfonic acid moiety of the chiral selectors. For the zwitterionic ZWIX type selectors (Fig. 2) a fixed intramolecular counter-ion effect needs also to be considered in this concept due to the positively charged quinuclidine residue (site) within the zwitterionic selector moieties. For more details on the working principles of ZWIX type selectors and CSPs for the chromatography of chiral acids, bases and ampholytes we refer to recent studies of our groups [5,17–25].

2. Materials and methods

2.1. Chemicals and reagents

Commercially available quinine (−)QN, quinidine (+)QD, and 10,11-dihydroquinine (−)DH-QN were purchased from Buchler (Braunschweig, Germany), while 10,11-dihydroquinidine (+)DH-QD, cinchonine (CN), and cinchonidine (CD) were from Sigma-Aldrich (Vienna, Austria). C9-epiquinine (−)*epi*–QN and C9-epiquinidine (+)*epi*–QD were synthesized according to the literature [7] (Fig. 1). Racemic quinine, 1:1 mixture of (−)QN and (+)QN as well as of racemic trifluoromethylphenyl quninine (*rac*.CF_3PQN) were a generous gift of N. Maulide and was synthesized according to [5].

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were purchased from VWR International (Arlington Heights, IL, USA). The base additive diethylamine (DEA), the acid additive formic acid (FA) and ammonium acetate (NH_4OAc), all analytical reagent grades, were from VWR. Liquid CO_2 was from Messer (Budapest, Hungary).

2.2. Apparatus and chromatography

Liquid chromatography was performed on two chromatographic systems. Waters Breeze apparatus consisted of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, a column thermostat and, for data acquisition, the Empower 2 data manager software (Waters Corporation, Milford, MA, USA). 1100 Agilent system contained a solvent degasser, a pump, an autosampler, a column thermostat, a multi-wavelength UV–Vis detector, and a Chemstation chromatographic data software (Agilent Technologies, Waldbronn, Germany). Experiments in polar ionic mode (PIM) were carried out in isocratic mode at a flow rate of 0.6 ml min^{-1} and column temperature 25 °C.

For subcritical liquid chromatography (SFC) the Waters Acquity Ultra Performance Convergence Chromatography™ (UPC^2, Waters Corporation) system was applied with a binary solvent pump, an autosampler with injection system, a backpressure regulator, a column oven, and a PDA detector. For system control and data acquisition the Empower 2 software was used. SFC was performed in isocratic mode at a flow rate of 2.0 ml min^{-1} and column temperature 40 °C. The outlet pressure was maintained at 150 bar. The mobile phase consisted of CO_2 and MeOH in different ratios (v/v) and contained different additives (acids, bases, salts).

Stock solutions of different *Cinchona* alkaloids were prepared in 1.0 mg ml^{-1} concentration and further diluted when necessary. Dead-time of columns (t_0) in LC mode was determined by injecting methanolic solution of acetone and in SFC mode at a first negative signal by injecting MeOH (see footnotes to Tables). In both chromatographic techniques analytes were detected by UV absorption at 215–230 nm.

The synthesis of all investigated chiral stationary phases (and the chiral columns thereof) investigated in this study depicted in Fig. 2, has already been described previously. Specifically, the development of DCL-(*R,R*)- and DCL-(*S,S*)- as well as DML-(*R,R*)- and DML-(*S,S*) (150 × 4.0 mm I.D., 5-µm particle size)-CSPs is found in the paper by Wolrab et al. [26]; the development of ZWIX(+) and ZWIX(−) columns was described in the paper by Hoffmann et al. [17] and Zhang [27]; and the development of the ZWIX(−A) and ZWIX(+*A*) (150 × 3.0 mm I.D., 3-µm particle size) columns was disclosed in the paper by Grecsó et al. [14]. The commercially available Chiralpak ZWIX(+) and ZWIX(−) columns (150 × 3.0 mm I.D., 3-µm particle size) were provided by Chiral Technologies Europe (Illkirch, France).

3. Results and discussion

3.1. Selection of the CSPs

The common structured core of all eight investigated CSPs employed as chiral strong cation-exchangers as depicted in Fig. 2 is characterized by the chiral *trans*-aminocyclohexanesulfonic acid moiety either in the (*R,R*) or in the (*S,S*) configuration. These enantiomeric units are linked via an amide and the functionally related carbamoyl group, respectively, to non-chiral or chiral subunits, which provide additional interaction sites of the entire selector (SO) motifs with the *Cinchona*-type analytes, the selectands (SAs). This concept led for the genuine cation-exchanger to two pairs of enantiomeric CSPs and columns (Fig. 2). Besides the stereochemical differentiation, they differ also in the π-electron density of the aryl group.

The second group of four chiral ion-exchangers belong formally to the so-called zwitterionic type CSPs due to the formal fusion of molecules containing the basic quinuclidine residue

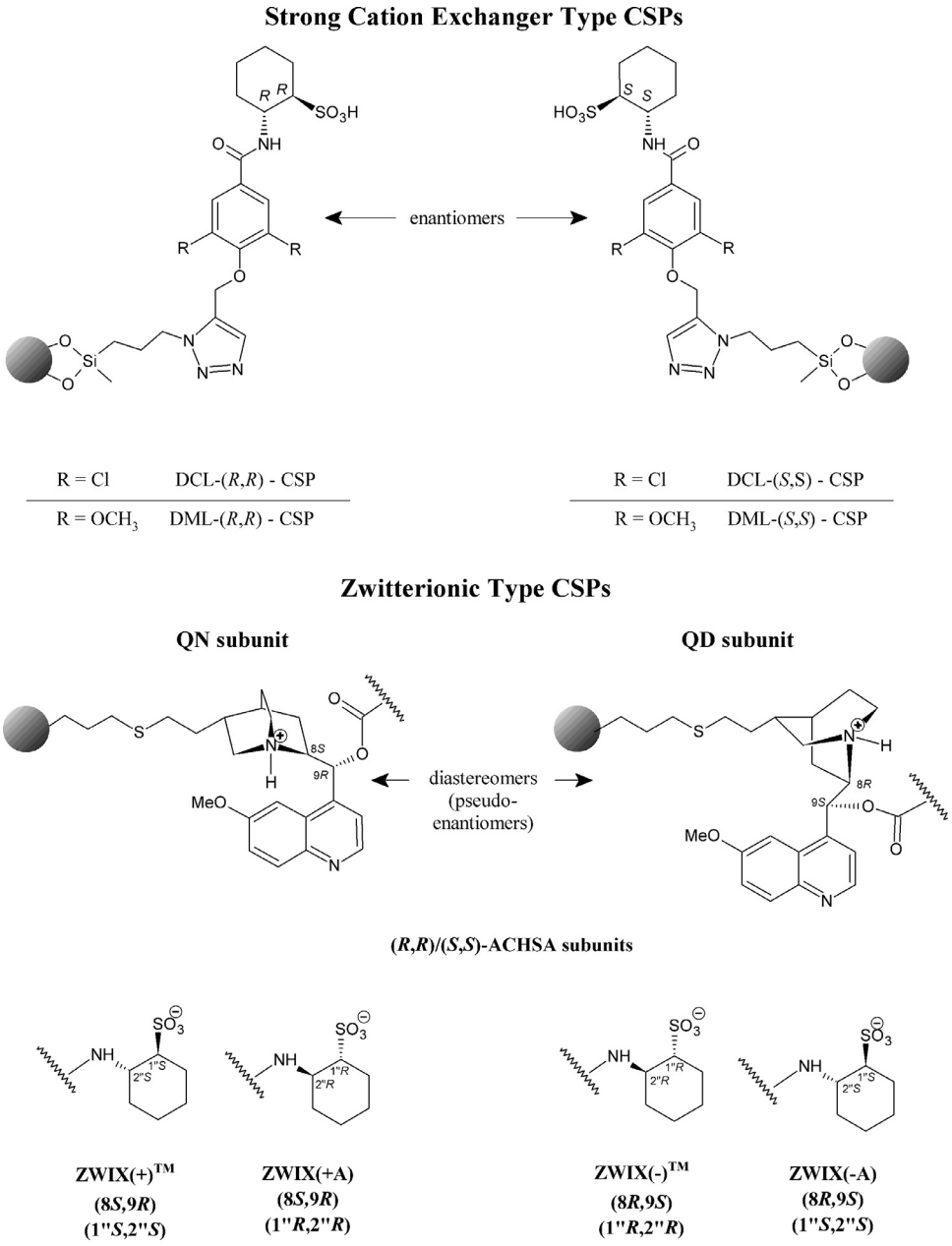

Fig. 2. Structure of strong cation-exchanger and zwitterionic *Cinchona* alkaloid-based chiral selectors.

with the aminocyclohexanesulfonic acid residue. In the present case this fused basic molecule scaffold relates to the diastereomeric *Cinchona* alkaloids, namely to quinine and quinidine, which often behave as pseudo-enantiomers of each other. This concept led to the four (two pairs) zwitterionic CSPs (and the related ion-exchanger type columns), which may behave pseudo-enantiomerically to each other, namely the ZWIX(+)/ZWIX(−) and ZWIX(+A)/ZWIX(−A) pairs.

The aims of our investigations, were (a) the elucidation of the retention characteristics of the eight chiral ion-exchangers (four genuine cation-exchangers and four zwitterionic ion-exchangers employed as cation-exchanger) as a function of mobile phase compositions, and (b) evaluation of the enantio- and diastereoselectivity of these structurally and functionally related CSPs for the carefully selected basic analytes of the *Cinchona* type compounds (Fig. 1). The comparisons were carried out in a systematic fashion whereby the focus of the discussion was placed on the stereoselectivity properties of the eight CSPs. The discussion is based on the assigned elution orders of all the pseudo-enantiomeric (still diastereomeric) quinine/quinidine type analytes and the truly enantiomeric racemic quinine analogs.

3.2. Chromatographic evaluation of the CSPs in LC and SFC

3.2.1. cSCX columns in LC modality

As known from previous studies [19], these chiral ion-exchanger columns can be conveniently operated in a non-aqueous mobile phase using MeOH as bulk solvent and an organic salt as counter-ion source (polar ionic mode, PIM). Based on initial experiments (data not shown) we selected conditions, which led to reasonable retention times of the basic analytes. Naturally, the retention times could have been further optimized *via* the counter-ion concentration in the mobile phase, but this was beyond the aim of the present study, which deals primarily with stereoselectivity

Table 1
Chromatographic data, k_1, k_2, α, R_S, plate numbers and elution sequence of *Cinchona* alkaloid analogs on strong cation-exchanger chiral stationary phases.

Compound	k_1	k_2	α	R_S	N_1	N_2	Elution order
				DCL-(*S,S*)			
QN/QD	6.74	9.17	1.36	5.16	5762	6297	(−)QN < (+)QD
rac.QN	6.73	7.09	1.05	0.73	4790	4600	(−)QN < (+)QN
rac.CF_3PQN	2.56	2.56	1.00	0.00	2722	–	–
DH-QN/DH-QD	10.35	13.87	1.34	5.44	6400	6505	(−)DH-QN < (+)DH-QD
epi-QN/*epi*-QD	9.07	9.07	1.00	0.00	1660	–	–
CD/CN	5.28	7.08	1.34	4.17	6305	7055	CD < CN
				DCL-(*R,R*)			
QN/QD	6.79	8.35	1.23	4.27	8521	8615	(−)QN < (+)QD
rac.QN	6.38	6.76	1.06	1.17	8163	8106	(+)QN < (−)QN
rac.CF_3PQN	2.31	2.40	1.04	0.20	915	1152	n.d.
DH-QN/DH-QD	10.31	12.37	1.20	3.96	8955	9345	(−)DH-QN < (+)DH-QD
epi-QN//*epi*-QD	8.06	8.79	1.09	1.68	7789	7117	(−)*epi*-QN < (+)*epi*-QD
CD/CN	5.77	5.77	1.00	0.00	8163	–	–
				DML-(*S,S*)			
QN/QD	1.76	2.45	1.39	4.22	4925	5851	(−)QN < (+)QD
rac.QN	1.76	1.99	1.13	1.42	5117	5043	(−)QN < (+)QN
rac.CF_3PQN	0.84	0.92	1.09	0.75	3483	3300	n.d.
DH-QN/DH-QD	2.65	3.63	1.37	4.39	4620	5420	(−)DH-QN < (+)DH-QD
epi-QN//*epi*-QD	2.35	2.35	1.00	0.00	2850	–	–
CD/CN	1.57	2.04	1.30	2.13	2972	4264	CD < CN
				DML-(*R,R*)			
QN/QD	4.46	5.53	1.24	3.94	7752	7372	(−)QN < (+)QD
rac.QN	4.07	4.48	1.10	1.72	7612	7787	(+)QN < (−)QN
rac.CF_3PQN	1.54	1.68	1.09	0.85	6377	6227	n.d.
DH-QN/DH-QD	6.70	7.97	1.19	3.51	8196	8267	(−)DH-QN < (+)DH-QD
epi-QN//*epi*-QD	5.74	6.26	1.09	1.32	1337	1498	(−)*epi*-QN < (+)*epi*-QD
CD/CN	3.65	3.83	1.05	0.65	4807	2859	CD < CN

Chromatographic conditios: columns, DCL-(*S,S*), DCL-(*R,R*), DML-(*S,S*), DML-(*R,R*); mobile phase, MeOH containing 37.5 mM NH_4OAc; flow rate, 0.6 ml min^{-1}; detection, 230 nm; *n.d.*, not determined; t_0: DCL-(*S,S*), 2.87 min; DCL-(*R,R*), 2.79 min; DML-(*S,S*), 2.99 min; DML-(*R,R*), 2.97 min.

aspects in connection with the resolution of the stereoisomeric *Cinchona* alkaloid analogues (Fig. 1), including the unique racemic (±)-quinine [(±)-QN] and the racemic trifluorinated (±)-quinine analogue [(±)-CF_3PQN]. As evidenced by the summarized chromatographic data in Tables 1 and 2, several unexpected characteristic features can be extracted.

a) The retention times of all six pairs of stereoisomers of the natural and synthetic *Cinchona* alkaloid analogues are significantly higher on the DCL-type cSCX columns compared to the DML-type cSCX columns (Table 1). Whether or not the different π-electron densities and H-bond formation abilities of the DML and DCL selector moieties contribute to the different retention characteristics cannot be answered here (Fig. 2). In accordance with previous observations [3], a marked increase in the retention times of the DHQN/DHQD pair compared to the QN/QD pair has also been noted. It is rather surprising as the very slightly increased hydrophobicity of the DHQN/DHQD analytes has such an effect for an ion-exchanger type system. To be more specific, the ion-exchanger (see Fig. 2) should be classified as a so-called mixed type stationary phase by combining ionic and hydrophobic interactions sites. For the present cases the enhanced retention seems to be originated from a cooperative effect of the slightly increased hydrophobicity and ionization of the dihydro compounds.
b) The novel racemic compounds (±)-QN and (±)-CF_3PQN can essentially be resolved on all four cSCX columns with the exception of CF_3PQN on the DCL(*S,S*)-column. The enantiomers of racemic (±)QN are baseline resolved on the enantiomeric DML-(*S,S*) and on the DML-(*R,R*) columns with the expected reversal of elution order, following the rule of reciprocity. In concrete, for the (*S,S*)-configurated SO the elution order is (−)QN before (+)QN, whereas for the (*R,R*)-configurated SO the elution order is reversed, thus (+)QN elutes before (−)QN.
c) It is somewhat unexpected to find the same elution order of the diastereomeric pairs of QN/QD, DHQN/DHQD, and of CD/CN on all four enantiomeric cSCX columns (except CD/CN on DCL-(*R,R*) column) being QN < QD, DHQN < DHQD, and CD < CN. An explanation for this behavior obviously has to deal with the constant configuration of the three chiral atoms (1*S*), (3*R*) and (4*S*) of the quinuclidine residue being part of all the diastereomeric analytes whereas the configuration of the C-8 and C-9 atoms switch from (8*S, 9R*) to (8*R*,9*S*) for QD/DHQD and CN. The configuration of the protonated and thus positively charged nitrogen (1*S*) remains for all the diastereomeric analytes constant, which indicates that the reversed configuration of the C-8 and C-9 atoms are the only variables. The electrostatically driven ion pairing of the negatively charged sites of the enantiomeric (*R,R*)- and (*S,S*)-selectors and of the constantly configurated and positively charged (1*S*) site of the diastereomeric SAs becomes dominant for the overall retention and for the elution order characteristics. The contributions of all the other more or less stereochemically driven SO–SA interaction increments remain uninterpretable at this point. It is a qualitative statement on the chromatographic observations, however, for a more detailed elucidation of the entire intermolecular SO–SA interactions more sophisticated techniques as e.g. dedicated NMR experiments of the diastereomeric SO–SA associates would be needed. However, this is beyond the aim of this study. Regarding the elution order, the stereochemically equal CD/CN pair behaves similarly to the QN/QD pair, only the overall retention decreases as a consequence of the missing methoxy group of the quinoline ring which leads to decreased hydrophobicity.

Table 2
Chromatographic data, k_1, k_2, α, R_S, plate numbers and elution sequence of *Cinchona* alkaloid analogs on zwitterionic chiral stationary phases in PI mode.

Compound	k_1	k_2	α	R_S	N_1	N_2	Elution order
				ZWIX(−)			
QN/QD	0.76	1.03	1.36	2.52	4333	2931	(−)QN < (+)QD
rac.QN	0.74	0.92	1.25	1.17	4270	3694	(−)QN < (+)QN
rac.CF_3PQN	0.52	0.60	1.16	0.67	4617	3233	n.d.
DH-QN/DH-QD	0.74	1.02	1.38	2.70	4343	3397	(−)DH-QN < (+)DH-QD
epi-QN/*epi*-QD	0.62	0.83	1.34	1.87	1844	2588	(−)*epi*-QN < (+)*epi*-QD
CD/CN	0.84	0.84	1.00	0.00	3218	–	–
CD/CN*	3.36	3.86	1.15	3.36	14726	10080	CN < CD
				ZWIX(−A)			
QN/QD	1.37	1.42	1.04	0.54	3101	2562	(+)QD < (−)QN
rac.QN	1.42	1.60	1.12	1.44	6694	7132	(−)QN < (+)QN
rac.CF_3PQN	1.35	1.52	1.12	1.07	6757	6520	n.d.
DH-QN/DH-QD	1.39	1.47	1.06	0.68	2106	3091	(+)DH-QD < (−)DH-QN
epi-QN/*epi*-QD	0.96	1.02	1.06	0.61	4036	3150	(+)*epi*-QD < (−)*epi*-QN
CD/CN	1.28	1.85	1.44	3.79	3237	6573	CN < CD
				ZWIX(+)			
QN/QD	1.21	1.45	1.20	2.32	5285	5795	(+)QD < (−)QN
rac.QN	1.12	1.43	1.28	2.36	6995	6396	(+)QN < (−)QN
rac.CF_3PQN	0.80	0.98	1.23	1.65	6490	5985	n.d.
DH-QN/DH-QD	1.20	1.44	1.20	2.46	6843	6239	(+)DH-QD < (−)DH-QN
epi-QN/*epi*-QD	1.14	1.14	1.00	0.00	2216	–	–
epi-QN/*epi*-QD*	4.54	4.54	1.00	0.00	7630	–	–
CD/CN	1.37	1.47	1.07	0.87	6076	6899	CD < CN
				ZWIX(+A)			
QN/QD	1.60	2.03	1.27	3.24	7419	7191	(+)QD < (−)QN
rac.QN	1.71	2.02	1.18	2.44	8071	7877	(+)QN < (-)QN
rac.CF_3PQN	1.50	1.84	1.22	2.72	7597	7331	n.d.
DH-QN/DH-QD	1.62	2.09	1.29	3.65	8145	7502	(+)DH-QD < (−)DH-QN
epi-QN/*epi*-QD	1.18	1.48	1.26	2.80	7332	6792	(−)*epi*-QN < (+)*epi*-QD
CD/CN	1.83	2.05	1.12	1.55	6326	7073	CN < CD

Chromatographic conditions: column, ZWIX(−), ZWIX(+), ZWIX(−A) and ZWIX(+A); mobile phase, MeOH/MeCN (50/50 v/v) containing 25 mM DEA and 50 mM FA, *MeOH/MeCN (10/90 v/v) containing 25 mM DEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 215–230 nm; temperature, 25 °C; *n.d.*, not determined; t_0: ZWIX(−)™ 1.51 min, ZWIX(−A) 1.53 min, ZWIX(+)™ 1.55 min, ZWIX(+A) 1.56 min.

d) The behavior of the diastereomeric 9–*epi*–QN/9–*epi*–QD pair compared to that of the QN/QD pair is again unexpected. Retention times increase in all cases, which indicates that most probably the electrostatic interactions of the charged quinuclidine and the sulfonic acid sites become stronger. It has to be attributed to the specific stereochemistry of the C-9 atom in relation to the chiral C-8 atom and thus to the different sterical direction of OH-group (compared to QN and QD), and as a result the overall conformation of the analytes is significantly altered. As a consequence, it can be hypothesized that the positively charged quinuclidine site gets better accessible by the negatively charged sulfonic acid sites of the SOs. Both the DCL-(*R,R*) and the DML-(*R,R*) SOs (columns) recognize stereoselectively the diastereomers of 9–*epi*–QN and 9–*epi*–QD, whereas the enantiomeric DCL-(*S,S*) and DML-(*S,S*) SOs don't provide diastereoselectivity for these analytes.

In the next set of studies, we extended the structural modification of the cSCX SOs with integrating positively charged sites into the selector design in favor of chiral QN and QD residues thus leading to ampholytic (zwitterionic) CSPs, which will be discussed in the forthcoming section.

3.2.2. Chiral ZWIX columns in LC modality

The intrinsic characteristics of (chiral) zwitterionic stationary phases is their flexibility to be used as (chiral) cation-exchanger for the resolution of chiral bases, as (chiral) anion-exchanger for the separation of chiral acids and as (chiral) zwitterionic ion-exchanger for the resolution of the enantiomers of ampholytic analytes. There are plenty of examples for these three application modes, for the resolution of chiral acids [17–19], chiral bases [20] and chiral ampholytes, *e.g.*, free amino acids and small peptides [21–25].

Ion-exchangers need inorganic or organic counter-ions in the mobile phase to adjust the retention. These are protonated bases for cation-exchangers, while deprotonated acids for anion-exchangers. However, in principle, free bases and acids could also be used assuming proton transfer events. For the investigated zwitterionic ion-exchangers an intramolecular ion pairing effect can be postulated due to the positively charged quinuclidine residue (site) within the zwitterionic QN/QD type selector moieties. This will essentially influence the amount of counter-ions needed in the mobile phase to adjust retention times. However, for the present study, only organic acids and bases are employed as counter-ions. They work for both sites of the ampholytic selector characterized by the acidic and basic functional groups (Fig. 2).

In continuation of the experiments with the cSCX columns, the main goal of this part of the project was to elucidate the retention and molecular recognition capacities of the four rationally designed ZWIX phases and columns (Fig. 2) in a comparative way for the six natural and synthetic *Cinchona* alkaloid analogues. Table 2 lists a representative set of chromatographic results, which will be interpreted in detail in the following.

The two pairs of ZWIX(−) and ZWIX(+) as well as ZWIX(−A) and ZWIX(+A) SOs are diastereomers, but could also be considered as pseudo-enantiomeric phases, as has frequently been proven for the resolution of the enantiomers of free amino acids [2,20]. In such cases a reversal of elution order will be obvious. Consequently, this concept was similarly valid for the resolution of

racemic (±)-quinine as found for the enantiomeric cSCX type CSPs and columns, but in a slightly different manner.

Inspecting the set of data in Table 2, it becomes obvious that the retention times are significantly lower. This might be attributed on one hand to the increased counter-ion concentration, and on the other hand to a repulsion effect caused by the anion-exchanger site of the zwitterionic phases. The type of bulk solvent composition has also a strong effect (see data marked with asterisks). Decreasing the amount of the protic MeOH in a mixture with MeCN leads to a strong increase of retention time, which can be attributed to a decreased thickness of the solvent sphere (solvation shell) around the ionized sites of the SO and of the selectand (SA, analyte) moiety. This is a known phenomenon which can directly be translated to the strength of the effective electrostatic interaction of the positively and negatively charged sites in their solvated status. The non-protic MeCN solvates less strongly than a protic solvent thus the radius of solvated ion changes. The significance of this behavior is visualized in Fig. 3. Keeping the organic salt and acid concentration constant in pure polar, but non-protic MeCN, the retention is roughly ten times higher than that in pure polar, but protic MeOH as bulk solvent, following a non-linear curve. As expected, the composition of the bulk solvent has an effect on the overall stereoselectivity and finally also on the efficiency of the chiral columns. Selected data are depicted in Fig. 3 and summarized in Table S1 for the ZWIX(+) and ZWIX(−) columns and for a set of four *Cinchona* alkaloid type analytes (Issues related to molecular recognition and elution order of the stereoisomers will be discussed later.).

3.2.3. Effect of the counter-ion concentration in the mobile phase

For basic analytes, it is generally accepted that an ion pairing process occurs with the sulfonic acid site of the chiral selector of the cSCX columns. Protonated bases as counter-ions in the mobile phase are needed to elute the analyte, whereby their concentration is directly correlated to the retention factor and can be evaluated by a stoichiometric displacement model [28].

Accordingly, we investigated the validity of the model for the zwitterionic ZWIX columns as well by varying the amount of the base in the mobile phase. Under the given conditions we see a clear linear relationship between the log values of the retention factors and the log of the base (actually, the protonated base) concentration in the mobile phase, as shown in Fig. 4. (The corresponding chromatographic data are summarized in Table S2). This information represents the ratios of effective charges of the analytes and of the counter-ion in the bulk phase. The linear correlation (characterized with correlation coefficients higher than 0.979 for all the investigated columns and analytes) justifies the validity of the stoichiometric displacement model. Carefully inspecting the values for the slopes (Fig. 4), it becomes obvious that they are not entirely equal, but relatively similar with some exceptions. For pure MeOH as bulk solvent the slopes of *epi*–QN/*epi*–QD differ significantly, compared to the other three sets of analytes. This trend of deviation of *epi*–QN/*epi*–QD becomes even more pronounced for the 1:1 mixture of MeOH and MeCN bulk solvents.

The experimental findings corroborate also a significant effect of the solvation shell of the ionized sites. At this point, it should be emphasized that the solvent compositions of the solvation shell must not necessarily be the same as that of the bulk solvent mixture of the mobile phase. Therefore, interpretation of the experimental findings is difficult and needs further studies, which are currently ongoing.

3.2.4. Chiral ZWIX columns in SFC modality

As it has been investigated earlier, chiral ion-exchangers can in principle be operated in hydro-organic, in polar organic, and in SFC modes using liquid CO_2 with adapted bulk solvent compositions, but always containing appropriate amounts of organic salts in the mobile phase acting as counter-ions [29]. Based on these information, the two ZWIX(+) and ZWIX(−) columns have been investigated in SFC modality using liquid CO_2 mixed with various amounts of MeOH. From the inspection of the data summarized in Table 3, it is evident that, as expected, the retention factors of the six pairs of stereoisomeric *Cinchona* alkaloid analogues are strongly dependent on the amount of MeOH in the liquid CO_2/MeOH mixtures. On the basis of these preliminary findings, the comparison of the retention factors of the investigated analytes (visualized in Fig. 3 and listed in Table 3) gives a surprising result. Namely, liquid CO_2 at 150 bar and at 40 °C, compared to MeCN as a bulk solvent applied in a mixture with 10 v% MeOH in LC modality, appears to be less “polar” than the non-protic, but polar MeCN. (For the compared ion-exchange systems the counter-ion concentrations were identical).

This rather preliminary statement and observation are in conjunction with ion-exchange chromatography operated under supercritical and subcritical conditions, which is a subject of ongoing investigations [30]. Liquid CO_2 behaves as a somewhat polar solvent with hydrogen bonding properties, which makes it fully mixable with the polar, protic MeOH at pressurized conditions. With higher MeOH content in the mobile phase, the stereoselectivity becomes slightly higher for the resolution of QN/QD. A similar trend was observed under LC conditions in the PI mode (with the exception of ZWIX(+)). The listed selectivity values were measured at 40 °C, whereas selectivities for the LC modality were measured at 25 °C, which may bias a direct comparison of the stereoselectivity data to some extent.

3.2.5. Stereoselectivity and elution order

In comparison to the cSCX columns, it became evident that the enantiomers of racemic quinine (±)QN and (±)CF_3PQN can be well resolved on ZWIX columns in almost all cases, but with greater α-values (see Tables 1 and 2). The elution order of the QN enantiomers on ZWIX(−) and ZWIX(−A) is (−)QN<(+)QN [(−)QN is the natural alkaloid]. However, it is reversed on ZWIX(+) and ZWIX(+A) and becomes (+)QN<(−)QN. Therefore, these two sets of chiral columns behave pseudo-enantiomerically to each other. For the combination of QD with the (*S,S*)-ACHSA, which refers to the ZWIX(-A) selector, one observes the same elution order as for genuine (*S,S*)-ACHSA-based cSCX. The same trend, but a reversed elution order have been observed for the (*R,R*)-ACHSA related chiral columns, namely for ZWIX(+A) and the DCL-(*R,R*) and DML-(*R,R*) columns.

For a better visualization of the enantioselectivity and diastereoselectivity of the eight chiral selectors for the five pairs of *Cinchona* alkaloid stereoisomers, related chromatographic information has been summarized in Table 4. All data are based on comparable LC mode measurements, and in two cases, for the ZWIX(+) and the ZWIX(−) columns, the SFC modality has also been investigated. For the separation of enantiomers, consistencies have been noticed many times, but for the elution order (and resolution) of the diastereomers, including the epimers, several inconsistencies have also been observed. It is obvious that the QN and QD subunit in combination with the (*R,R*)- and (*S,S*)-ACHSA core, dedicated for a chiral cation-exchanger type CSP, may have a marked influence on the overall molecular recognition, leading to unpredicted elution sequence of the diastereomeric analytes. In principle, this is not surprising for the presented examples, since it gives evidence for the combinatorial effect of “chiral information” stemming from two different chiral subunits fused together via a carbamoyl linker. This observation indicates that the stereoselectivity driven by the absolute configuration, *e.g.*, of the (*S,S*)-ACHSA unit, is not disturbed by its fusion with the QD subunit. In contrast, its

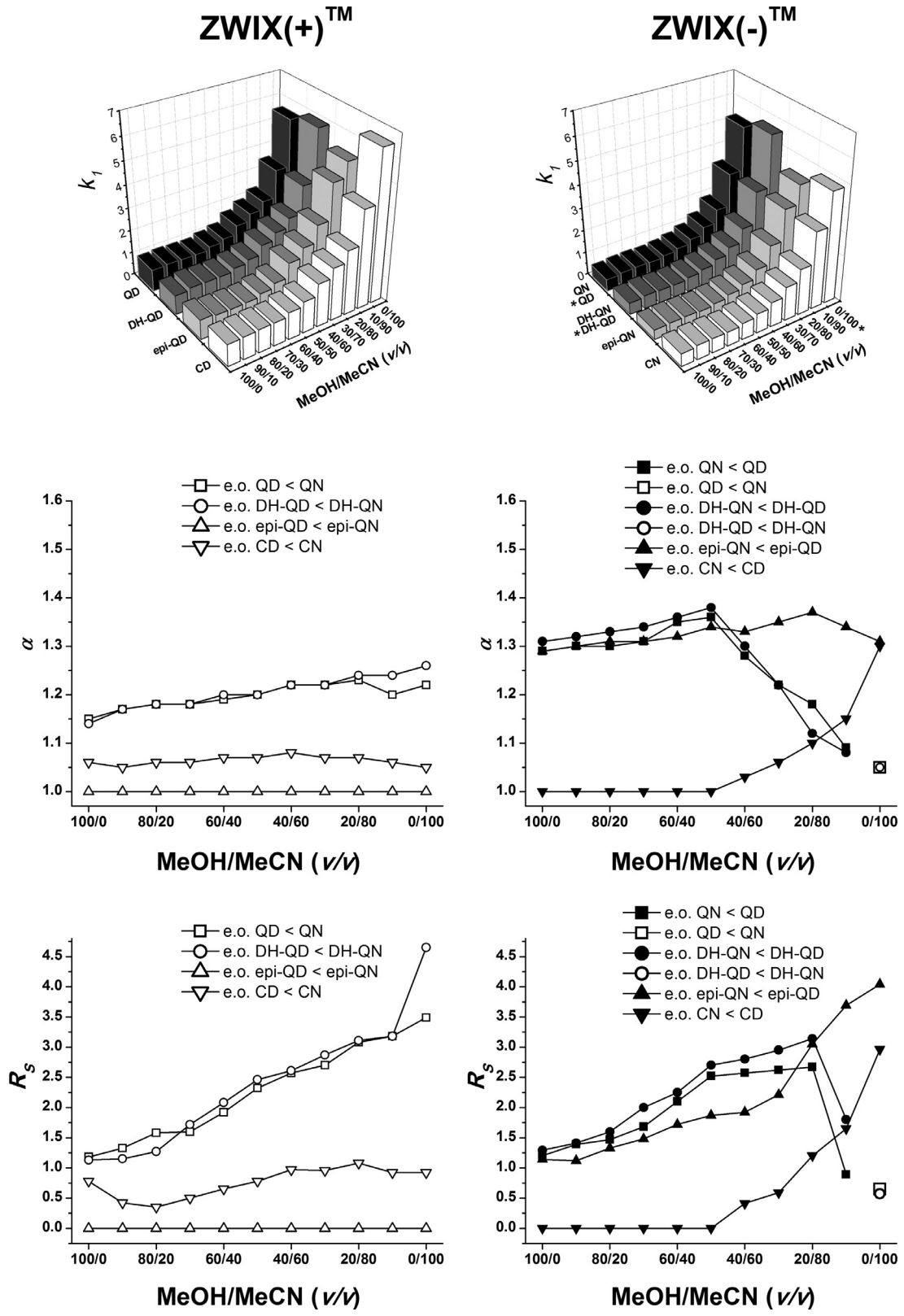

Fig. 3. Influence of mobile phase composition on k_1, α and R_S of *Cinchona* alkaloid analogs.
Chromatographic conditions: column, ZWIX(−), ZWIX(+); mobile phase, MeOH/MeCN (100/0–0/100 v/v) containing 25 mM DEA and 50 mM FA; flow rate, 0.6 ml min^{-1}; detection, 215–230 nm; temperature, 25 °C; symbols, ■, QN, □, QD; ●, DH-QN, ○, DH-QD; ▲, *epi*–QN, △, *epi*–QD; ▼, CN, ∇, CD; e.o., elution order; * reversal elution order.

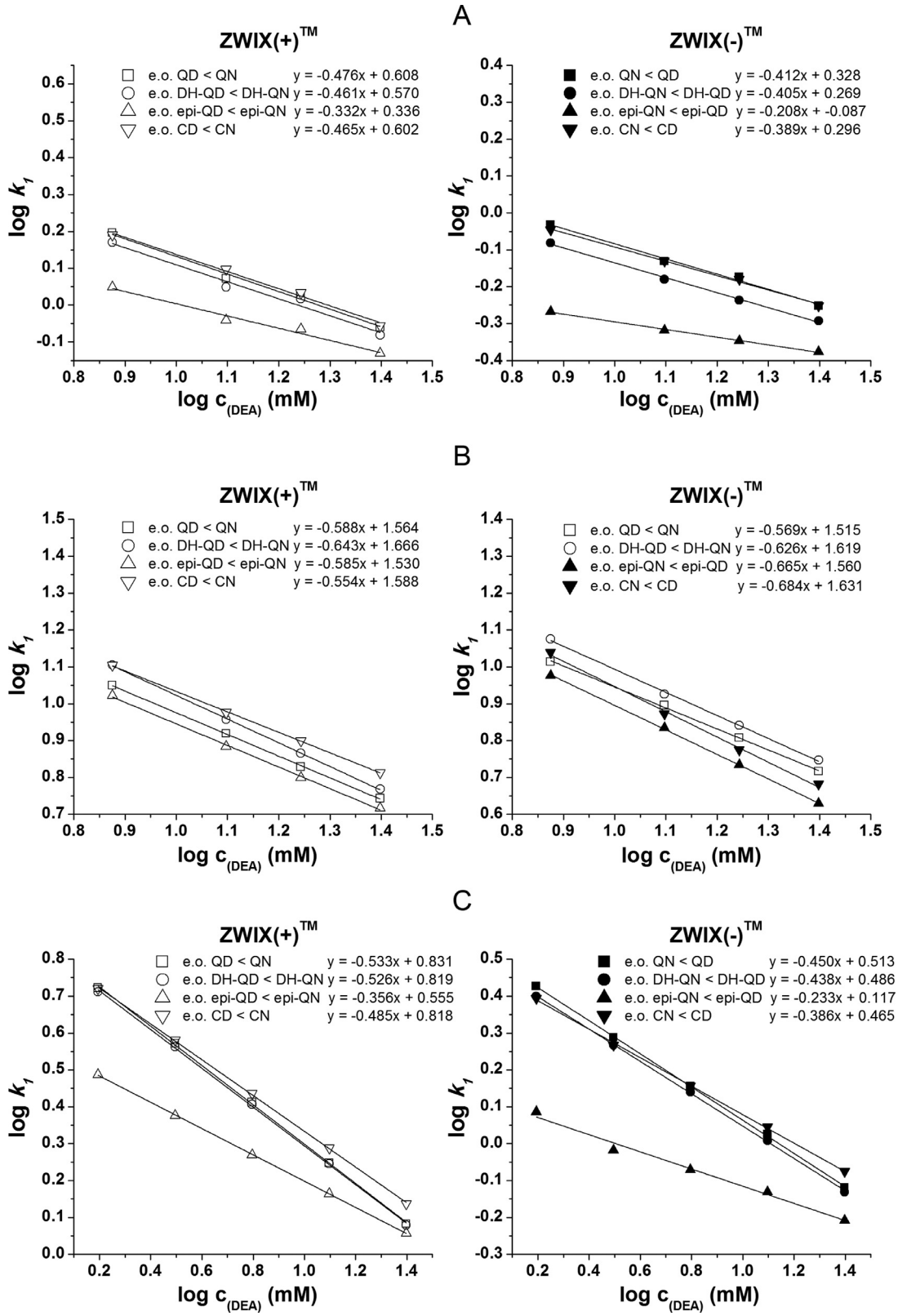

Fig. 4. Influence of the counter-ion concentration on the retention of the first-eluting enantiomer (k_1) for *Cinchona* alkaloid analogs on ZWIX(+) and ZWIX(−) Chromatographic conditions: column, ZWIX(+) and ZWIX(−); mobile phase, (A) MeOH (100 v%) containing 7.5/15, 12.5/25, 17.5/35 and 25/50 mM/mM DEA/FA, respectively; (B) MeCN (100 v%) containing 7.5/15, 12.5/25, 17.5/35 and 25/50 mM/mM DEA/FA, respectively; (C) MeOH/MeCN (50/50 v/v) containing 1.56/3.13, 3.13/6.25, 6.25/12.5, 12.5/25, 25/50 mM/mM DEA/FA, respectively; flow rate, 0.6 ml min^{-1}; detection, 215–230 nm; temperature, 25 °C; symbols, ■, QN, □, QD; ●, DH-QN, ○, DH-QD; ▲, *epi*–QN, △, *epi*–QD; ▼, CN, ▽, CD; e.o., elution order.

Table 3
Chromatographic data, k_1, k_2, α, R_S, plate numbers and elution sequence of *Cinchona* alkaloid analogs on zwitterionic chiral stationary phases at SFC condition.

Compound	k_1	k_2	α	R_S	N_1	N_2	Elution order
				ZWIX(−)			
QN/QD*	1.83	2.36	1.29	2.74	4238	3678	(−)QN < (+)QD
QN/QD	7.99	9.91	1.24	4.00	6499	7310	(−)QN < (+)QD
rac.QN*	1.83	2.24	1.22	1.90	3284	3654	(−)QN < (+)QN
rac.QN	7.99	9.03	1.13	2.60	7917	9211	(−)QN < (+)QN
rac.CF_3PQN*	2.13	2.33	1.09	0.97	7538	8606	n.d.
rac.CF_3PQN	10.80	11.36	1.05	0.84	5750	5524	n.d.
DH-QN/DH-QD*	1.97	2.59	1.31	3.06	3786	5210	(−)DH-QN < (+)DH-QD
DH-QN/DH-QD	7.24	9.14	1.26	4.01	5341	6901	(−)DH-QN < (+)DH-QD
epi-QN/*epi*-QD*	7.18	9.86	1.37	3.87	3003	3041	(−)*epi*-QN < (+)*epi*-QD
epi-QN/*epi*-QD	33.21	42.17	1.27	4.29	5768	6724	(−)*epi*-QN < (+)*epi*-QD
CD/CN*	2.25	2.25	1.00	0.00	984	–	–
CD/CN	9.09	9.46	1.04	0.77	6606	5842	CD < CN
				ZWIX(+)			
QN/QD*	2.52	2.73	1.08	1.02	4250	4244	(+)QD < (−)QN
QN/QD	10.27	10.27	1.00	0.00	6645	–	–
rac.QN*	2.30	2.73	1.19	2.27	5323	5079	(+)QN < (−)QN
rac.QN	9.16	10.23	1.12	2.65	10724	10475	(+)QN < (−)QN
rac.CF_3PQN*	2.54	2.95	1.16	1.47	12485	13573	n.d.
rac.CF_3PQN	12.33	13.16	1.07	1.25	7639	7338	n.d.
DH-QN/DH-QD*	2.39	2.58	1.08	1.01	12208	14226	(+)DH-QD < (−)DH-QN
DH-QN/DH-QD	9.41	9.41	1.00	0.00	2061	–	–
epi-QN/*epi*-QD*	10.93	10.93	1.00	0.00	5020	–	–
epi-QN/*epi*-QD	45.26	45.26	1.00	0.00	4813	–	–
CD/CN*	2.77	3.11	1.12	1.38	4718	3267	CD < CN
CD/CN	10.61	11.81	1.11	2.47	9401	9875	CD < CN

Chromatographic conditions: column, ZWIX(−) and ZWIX(+); mobile phase, CO_2/MeOH (90/10 v/v) or *(70/30 v/v) all containing 25 mM DEA and 50 mM FA; flow rate, 2.0 ml min^{-1}; detection, 215–230 nm; temperature, 40 °C; back pressure, 150 bar; *n.d.*, not determined; t_0: ZWIX(−) 0.44 min, ZWIX(+) 0.43 min.

Table 4
Elution sequence of *Cinchona* alkaloid analogs on zwitterionic ZWIX(−), ZWIX(−A), ZWIX(+), ZWIX(+A) and on chiral strong cation-exchanger DCl-(*S,S*), DML-(*S,S*), DCl-(*R,R*) and DML-(*R,R*) CSPs.

Chiral core	Sub-units	Columns	QN/QD	*rac*.QN	CD/CN	DH-QN/DH-QD	*epi*-QN/*epi*-QD
–	(*S,S*)-ACHSA	DCl-(*S,S*)	(−)QN < QD	(−)QN < (+)QN	CD < CN	(−)DH-QN < (+)DH-QD	–
–	(*S,S*)-ACHSA	DML-(*S,S*)	(−)QN < QD	(−)QN < (+)QN	CD < CN	(−)DH-QN < (+)DH-QD	–
QN	(*S,S*)-ACHSA	ZWIX(+)™	QD < (−)QN	(+)QN < (−)QN	CD < CN	(+)DH-QD < (-)DH-QN	–
**QN	(*S,S*)-ACHSA	ZWIX(+)™	QD < (−)QN	(+)QN < (−)QN	CD < CN	(+)DH-QD < (-)DH-QN	–
QD	(*S,S*)-ACHSA	ZWIX(−A)	QD < (−)QN	(−)QN < (+)QN	CN < CD	(+)DH-QD < (-)DH-QN	(+)*epi*–QD < (−)*epi*–QN
–	(*R,R*)-ACHSA	DCl-(*R,R*)	(−)QN < QD	(+)QN < (−)QN	–	(−)DH-QN < (+)DH-QD	(−)*epi*–QN < (+)*epi*–QD
–	(*R,R*)-ACHSA	DML-(*R,R*)	(−)QN < QD	(+)QN < (−)QN	CD < CN	(−)DH-QN < (+)DH-QD	(−)*epi*–QN < (+)*epi*–QD
QD	(*R,R*)-ACHSA	ZWIX(−)™	(−)QN < QD	(−)QN < (+)QN	*CN < CD	(−)DH-QN < (+)DH-QD	*(−)*epi*–QN < (+)*epi*–QD
**QD	(*R,R*)-ACHSA	ZWIX(−)™	(−)QN < QD	(−)QN < (+)QN	CD < CN	(−)DH-QN < (+)DH-QD	(−)*epi*–QN < (+)*epi*–QD
QN	(*R,R*)-ACHSA	ZWIX(+A)	QD < (−)QN	(+)QN < (−)QN	CN < CD	(+)DH-QD < (−)DH-QN	(−)*epi*–QN < (+)*epi*–QD

Chromatographic conditions: columns, ZWIX(−), ZWIX(−A), ZWIX(+), ZWIX(+A), DCl-(*S,S*), DML-(*S,S*), DCl-(*R,R*) and DML-(*R,R*); mobile phase, on zwitterionic phases, in PIM MeOH/MeCN (50/50 v/v) containing 25 mM DEA and 50 mM FA, *MeOH/MeCN (10/90 v/v) containing 25 mM DEA and 50 mM FA, **at SFC condition CO_2/MeOH (90/10 v/v) containing 25 mM DEA and 50 mM FA and on strong ion-exchanger phases, MeOH containing 37.5 mM NH_4OAc; flow rate, in PIM, 0.6 ml min^{-1}, in SFC, 2.0 ml min^{-1}; detection, 215–230 nm; temperature, in PIM, 25 °C, in SFC, 40 °C.

fusion with QN leads to a fully changed spatial arrangement of the molecular residues around the stereoselective binding grove.

For the resolution of the diastereomeric pairs QN/QD, DHQN/DHQD, CD/CN, and *epi*–QN/*epi*–QD, such a clear trend cannot be seen. On both (*R,R*)- and (*S,S*)-ACHSA-based cSCX columns the elution sequence QN < QD, DHQN < DHQD, and CD < CN remains, as a clear indication of the difficulty to interpret enantioselectivity versus diastereoselectivity. The situation becomes even more complicated when studying chiral selector motifs with multi-chiral centers, such as ZWIX(−)/ZWIX(−A) and ZWIX(+)/ZWIX(+A) CSPs. (See an example of the chromatographic resolution of CD/CN in Fig. S1.).

Unexpected reversals of elution order of the diastereomeric (pseudo-enantiomer) pairs can easily happen as a function of the diastereomeric chiral selector motifs *per se*, but also as a function of the bulk solvent composition of the mobile phase, as can be seen in Figs. 3 and 4.

In this context, it became particularly interesting that under LC conditions the elution orders of CN < CD changed in SFC modality to CD < CN on the ZWIX(−) column (Table 4). It is another strong indication for the role of solvation on the overall diastereoselectivity. As an essential part of this discussion, the conformational aspects of the selector moieties need also to be taken into account. It is also necessary for the so-called anti-open

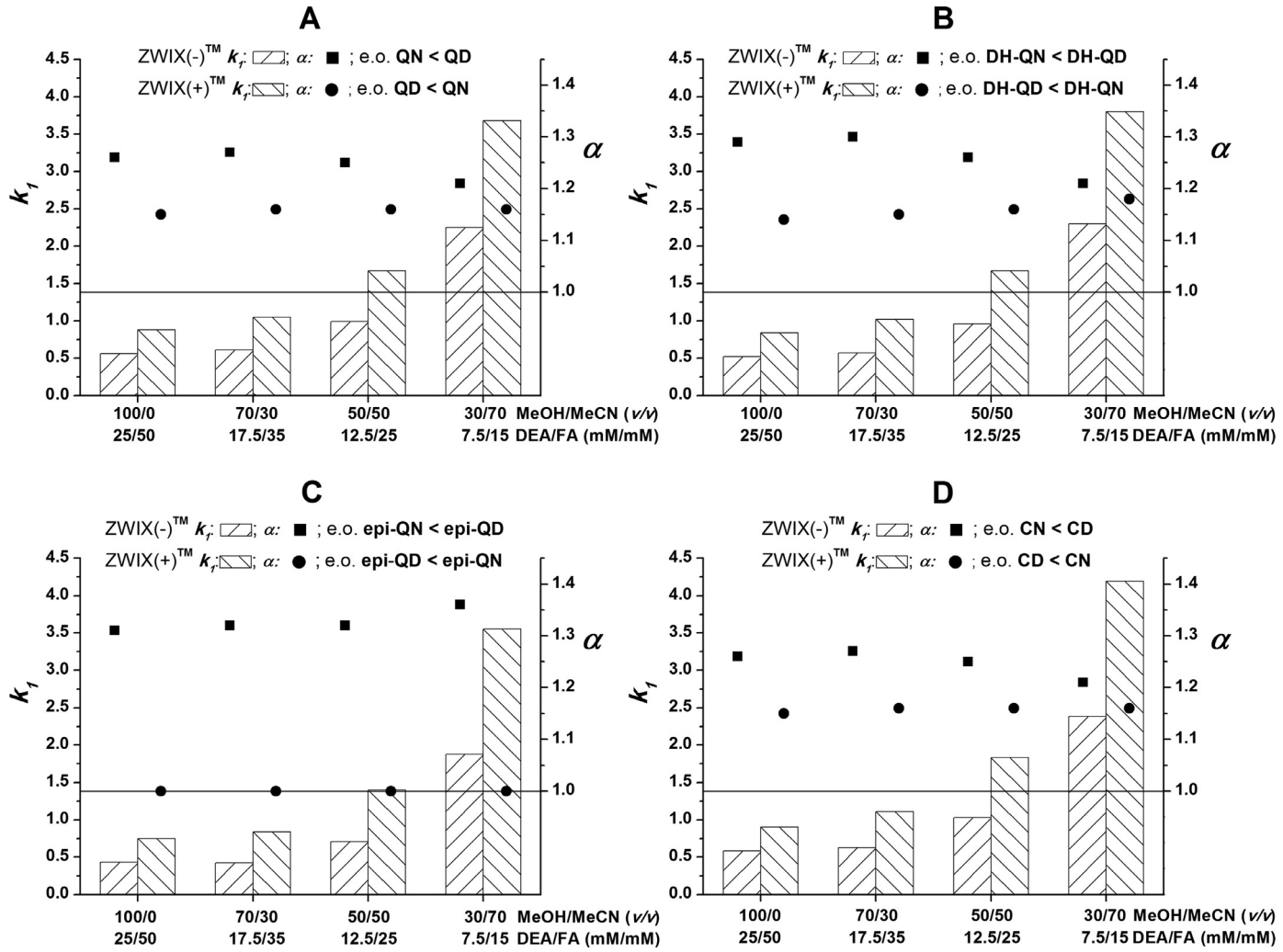

Fig. 5. Influence of mobile phase composition and the counter-ion content on the retention of the first-eluting enantiomer (k_1), separation factor (α) and resolution (R_S) for *Cinchona* alkaloid analogs on ZWIX(+) and ZWIX(−).
Analytes: **(A)** QN/QD; **(B)** DH-QN/DH-QD; **(C)** *epi*-QN/*epi*-QD; **(D)** CD/CN; chromatographic conditions: columns, ZWIX(+) and ZWIX(−); mobile phase, MeOH/MeCN (100/00 v/v) containing 25/50, mM/mM DEA/FA; MeOH/MeCN (70/30 v/v) containing 17.5/35 mM/mM DEA/FA; MeOH/MeCN (50/50 v/v) containing 12.5/25 mM/mM DEA/FA; MeOH/MeCN (30/70 v/v) containing 7.5/15 mM/mM DEA/FA; flow rate, 0.6 ml min^{-1}; detection, 215–230 nm; temperature, 25 °C; symbols, k_1: ▨ for ZWIX(+), k_1: ▨ for ZWIX(−); α: ● for ZWIX(+), α: ■ for ZWIX(−).

conformational behavior of the QN moiety under acidic conditions, the one having the highest probability [31,32]. However, minor conformers may also exist, which will certainly affect the overall stereoselectivity performance of such type of motifs as of the ZWIX selectors and phase.

3.3. *Enantiomer and diastereomer separations of a set of* Cinchona *alkaloid analogues*

In the section above, results of a set of several systematic investigations were summarized in Tables, Figures, and in the Supporting Material. On the basis of these data, it becomes obvious that the enantiomer resolution of the novel racemic quinine (±QN) and of its racemic analogues [(±)CF_3PQN] is straightforward and works essentially on all eight investigated cation-exchangers, whereby four of them have zwitterionic character (Tables 1 and 2). The expected reversal of elution order of the enantiomers is evident for the (*S,S*)-ACHSA- and (*R,R*)-ACHSA-based cSCX columns. The same sequence occurs for the QD-(*S,S*)-ACHSA type ZWIX(−A) and QN-(*R,R*)-ACHSA type ZWIX(+A) columns.

However, the combination of the chiral subunits of QN and (*S,S*)-ACHSA, which relate to the ZWIX(+) column, and the one of QD and (*R,R*)-ACHSA relating to ZWIX(−), show a reversed enantioselectivity, but still behaving pseudo-enantiomerically to each other.

The effects of bulk solvent composition and counter-ion concentration on the retention and diastereoselective characteristics of four *Cinchona* alkaloid analogues on the ZWIX(+) and ZWIX(−) columns are presented in Fig. 5 and in Table S3. The α values of four pairs stay relatively constant, whereas the retention data change progressively with the dilution of the organic salt and acid additives in the mobile phase.

Representative chromatograms for the resolution of racemic quinine on the ZWIX(+) and ZWIX(−) columns in LC and SFC mode are depicted in Fig. 6, corroborating the potential of zwitterionic ion-exchangers used as chiral cation-exchanger. Similar useful columns are the chiral strong cation-exchangers for the separation of the diverse natural and synthetic *Cinchona* alkaloid analogues exemplified by Fig. S2. Both peak symmetries and efficiencies of the columns are very reasonable. Additional chromatographic

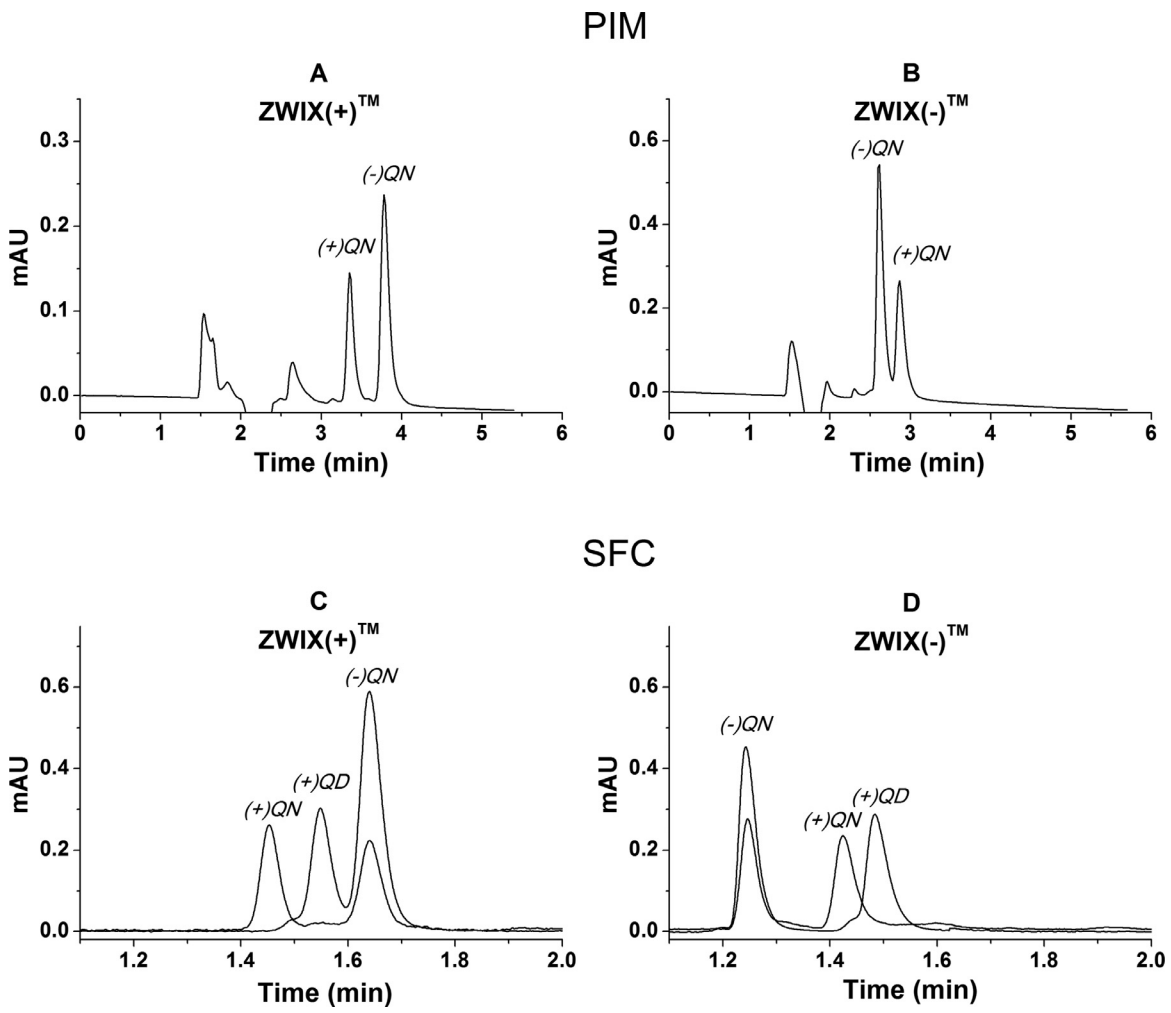

Fig. 6. Chromatograms indicating elution order for *rac*.QN stereoisomers on ZWIX(+) and ZWIX(−) CSPs in PIM and SFC techniques.
Chromatographic conditions: columns, ZWIX(+), ZWIX(−); mobile phase, **(A,B)** MeOH/MeCN (50/50 v/v) containing 25 mM DEA and 50 mM FA and, **(C,D)** CO_2/MeOH (70/30 v/v) containing 25 mM DEA and 50 mM FA; flow rate, in PIM, 0.6 ml min^{-1}, in SFC, 2.0 ml min^{-1}; detection, 215–230 nm; temperature, in PIM, 25 °C, in SFC, 40 °C.

results (as the basis of data interpretation) can be found in the Supplementary Information (Figs. S3–S9).

4. Conclusions

In this comprehensive study we have demonstrated the use of chiral strong cation-exchangers and chiral zwitterionic ion-exchangers for the separation of natural and synthetic *Cinchona* alkaloid analogues. Additional possibilities to trigger the stereoselective retention and separation characteristics, including the reversal of elution orders have been described. It is important to emphasize that these possibilities cannot be reached by non-chiral stationary phases employed in reversed phase, normal phase or SFC mode [1]. Usually, the elution sequence QD < QN, DHQD < DHQN, *epi*–QD < *epi*–QN, and CN < CD remains fixed, whereas with the employment of the eight, somewhat related ionic CSPs and "chiral columns" thereof, described here, the sequences can be reversed.

Particular interest has been focused on the observed enantioselectivity of these CSPs for the resolution of the very unique and only recently described racemic quinine samples [5]. All investigated chiral ion-exchange type columns are characterized by excellent efficiency and high flexibility to adjust the retention times of the basic analytes via the mobile phase composition, *e.g.*, by varying the type of bulk solvents used and the concentration of the organic salt additives. A central part of this study relates also to the evaluation and discussion of the molecular parameter and structural motifs of the chiral selectors influencing the retention characteristics in the light of molecular recognition phenomena.

This study demonstrates the principal use of appropriate chiral cation ion-exchangers and chiral zwitterionic phases working as cSCX type stationary phases for liquid chromatography of basic analytes with mobile phase conditions, compatible with LC–MS/MS applications. This includes also the straightforward application of SFC with liquid CO_2 in combination with protic solvents under subcritical conditions.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgments

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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.2019.460498.

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