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Liquid chromatographic–mass spectrometric separation of oligoalanine peptide stereoisomers: influence of absolute configuration on enantioselectivity and two-dimensional separation of diastereomers and enantiomers

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Abstract

This contribution describes the chromatographic separation of peptide stereoisomers. Thereby, one focus is laid on the influence of the absolute configurations of peptide enantiomer pairs on their enantioselective separation. Three different N-terminal protecting groups and three different chiral stationary phases (CSPs) based on cinchona alkaloid derivatives were employed and oligoalanine di-, tri- and tetra-peptides were used as model set. The absolute configurations of the individual enantiomeric pairs were found to profoundly influence both the elution order and the enantioselectivity. The stereoselective molecular recognition mechanism was observed to be dependent on the combination of configuration and the chosen protecting group and CSP. As the CSPs on their own exhibited insufficient diastereoselectivity, a two-dimensional liquid chromatography–mass spectrometry (LC–MS) system was developed for the separation of both diastereomers and enantiomers of peptides in the second part of this study. Diastereomers were separated by reversed phase (RP) and the resulting enantiomeric pair fractions were transferred to a CSP for enantioseparation. All eight stereoisomers of a tripeptide (Ala–Ala–Ala) and 9 out of 10 stereoisomers of a tetrapeptide (Ala–Ala–Ala–Ala) could be successfully resolved.

Keywords: Enantiomer separation; Configuration; Diastereomers; Two-dimensional liquid chromatography–mass spectrometry; Peptides

1. Introduction

Stereoisomeric forms of peptides are formed by linking (*R*)- and/or (*S*)-forms of chiral amino acids. The stereochemistry of peptides is important for their biological function and thus needs to be taken into account in peptide analysis. The assessment of the stereochemical properties of larger peptides is complicated by the large number of possible stereoisomers that may exist. A peptide containing n amino acids, of which m are glycines (which is the only natural nonchiral amino acid) and o is the sum of threonines and isoleucines (which both possess two chiral centres) in the peptide molecule, can occur in $2^{(n-m+o)}$ stereoisomeric forms. For a specific peptide stereoisomer only one other stereoisomer exists, whose relationship to the former is that of an enantiomer, while all other $2^{(n-m+o)}-2$ stereoisomers are diastereomeric towards the selected peptide stereoisomer, which has consequences also for peptide analysis and purification.

Concerning the separation of peptide stereoisomers, the separation of enantiomeric forms requires the utilization of a chiral auxiliary, often called a chiral selector. This auxiliary may take the form of a chiral stationary phase (CSP) or a chiral mobile phase additive in chromatography or constitute a chiral buffer (electrolyte) additive in electrophoresis in so-called "direct enantiomer separations"; alternatively it may serve as a chiral derivatizing agent in "indirect enantiomer separations" [1]. On the contrary, the separation of peptide diastereomers does not require a chiral auxiliary and can be achieved with an achiral separation system by exploiting the different physico-chemical properties of the diastereomers. High-performance liquid chromatography (HPLC)

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has been and still is the classical and most widely used separation method utilized for analyzing the stereochemical composition of peptides, although the use of capillary electrophoresis has experienced a considerable growth over the last years [2,3].

The $2^{(n-m+o)}$ stereoisomeric forms of a peptide can be divided into $2^{(n-m+o-1)}$ enantiomeric pairs. As most natural peptides possess an (all-L)-configuration, the majority of studies concerning the separation of peptide enantiomers has focussed on the (all-D)-/(all-L)-pair. However, other pairs of enantiomers with non-natural configurations are also worthwhile studying, since they can be of importance in synthetic chemistry or in the development of proteolysis-resistant peptides that may be e.g. interesting drug candidates. Moreover, the exploration of the capability of a chiral selector to successfully separate enantiomeric pairs with different absolute configurations will yield information on its application spectrum and provide insights into the molecular recognition mechanism responsible for the selector's stereoselectivity.

Recently, we have shown the usefulness of cinchona alkaloid based chiral selectors for the separation of peptide enantiomers by HPLC [4–6]. These studies described various aspects of the enantiomer separations of (all-*R*)/(all-*S*)-oligoalanines with up to 10 amino acid residues, including the impact of different selector modifications on the enantiomer discrimination process [4] and the influence of the N-terminal protecting group on enantioselectivity [5,6]. In the present report, we focus on the influence different absolute configurations of model oligoalanine peptides have on enantiomer discrimination. Specifically, we have investigated how the introduction of an inverted chiral centre into a homochiral (i.e. all stereocentres have the same configuration) peptide chain at different positions will affect the chromatographic enantioseparation process. The results obtained from these separations should provide extended insights into the chiral recognition mechanism of the cinchona alkaloid derived CSPs.

Most reports describing studies of the separation of peptide stereoisomers focus on the separation of either diastereomers or enantiomers but not on both types of stereoisomers together. These approaches may be viewed as having an inherent deficit, as the presence of both types of stereoisomers can never be excluded. For example, during solid phase peptide synthesis, isomeric byproducts due to racemization of one or more amino acids may be formed, which are difficult to separate from the main product. Therefore, the development of methods that are capable of addressing both the enantiomeric as well as the diastereomeric composition of peptide samples seems to be useful and necessary. However, it must be noted in this context that many publications on the topic of peptide enantiomer separations also incorporate the separation of diastereomers "en passant". For example, a chiral stationary phase often possesses not only enantioselectivity for a certain analyte but also sufficient diastereoselectivity to resolve, e.g. the two enantiomeric pairs of a dipeptide.

For normal phase systems, Hara and Dobashi [7] described the separation of all four stereoisomers of benzyloxycarbonyl leucylleucine methyl ester already in 1979, while Pirkle et al. [8] reported the separation of the stereoisomers of several dipeptides including a discussion on the chiral recognition mechanism for the two pairs of enantiomers in the late 1980s [9]. Other normal-phase studies used Chiralcel OD and Pirkle-type columns [10,11], a molecularly imprinted polymer [12], poly(L-leucine) beads [13], and a synthetic macrobicyclic receptor [14]. Ligand-exchange chromatography has also been utilized for the simultaneous separation of dipeptide enantiomers and diastereomers in several cases [15–17]. Various CSPs employed in the reversed phase (RP) mode have also been successfully used for the concomitant separation of peptide diastereomers besides the primary enantioseparation: such separations have been reported for CSPs based on crown ethers [18–20], cyclodextrins [21–23], macrocylic antibiotics [24–26], and a quinine derivative [5].

The possibility of separating both enantiomers and diastereomers on a chiral stationary phase will become more and more difficult and finally impossible for increasing peptide lengths, as the number of stereoisomers to be separated increases rapidly with the growing number of amino acid residues. Therefore, it comes as no surprise that successful separations of all stereoisomers of a peptide have so far only been reported for samples containing two stereocentres, i.e. dipeptides or tripeptides incorporating one glycine. Consequently, it is necessary to employ an alternative, more sophisticated approach to obtain a complete separation of all stereoisomers of larger peptide analytes. For achieving the necessary enlargement of peak capacity and increase in stereoselectivity two-dimensional chromatography using complementary stationary phases seems to be a promising approach. Thus, we developed a separation system that combines a reversed phase column as first dimension, on which the diastereomers were separated, and a chiral stationary phase as second dimension, on which the pairs of enantiomers were further resolved into the single stereoisomers.

Thus, the presented investigations had two goals. (1) The influence of the absolute configuration of a specific peptide stereoisomer on the power of cinchona alkaloid derived CSPs to separate it from its enantiomer was to be evaluated. Comparing the enantiomer separation results obtained for homochiral peptides with up to four amino acid residues containing an inverted stereocentre at different positions within their chain should also yield information on the molecular recognition process of the chiral selector. (2) The series of peptide stereoisomers used in those experiments were subsequently employed as analytes in the development of a two-dimensional liquid chromatography–mass spectrometry (LC–MS) system for the separation of both diastereomers and enantiomers.

2. Experimental

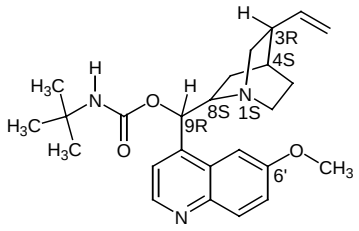
2.1. Materials

(*R*)- and (*S*)-Alanine were purchased from Sigma–Aldrich (Vienna, Austria). The four stereoisomers of dialanine as well as the (all-*R*)- and (all-*S*)-enantiomers of the tri- and tetraalanine peptides were obtained from Bachem (Bubendorf, Switzerland). All other stereoisomers of the tri- and tetraalanine peptides were synthesized according to standard protocols by piChem (Graz, Austria).

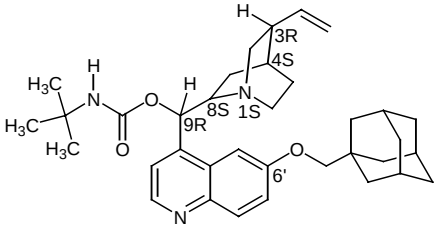
Acetic anhydride was from Loba (Fischamend, Austria), while *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide was from Fluka (Buchs, Switzerland). 3,5-Dinitrobenzoyloxy-succinimide was prepared from 3,5-dinitrobenzoyl chloride (Aldrich) and hydroxysuccinimide (Fluka) by Hünig base coupling. Ammonium acetate, acetic acid, sodium hydrogencarbonate and sodium carbonate were obtained from Fluka. HPLC grade solvents and doubly distilled water were used throughout.

The preparation of the *tert*-butylcarbamoylquinine (Fig. 1a) and 6′-(1-adamantylmethoxy)-9-*O*-*tert*-butylcarbamoylcinchonidine (Fig. 1b) chiral selectors is described in [27] and [28], respectively. The 1,4-bis(9-*O*-quinidinyl)phthalazine chiral selector (Fig. 1c) was prepared according to [29]. The corresponding chiral stationary phases were obtained by subsequent coupling of the selectors to thiol-modified silica gel (Kromasil 100–5 μm, Eka Chemicals, Bohus, Sweden) [27]. The 5 μm CSP particles were slurry-packed into 150 mm × 4.0 mm i.d. stainless steel columns (Austrian Research Centers, Seibersdorf, Austria).

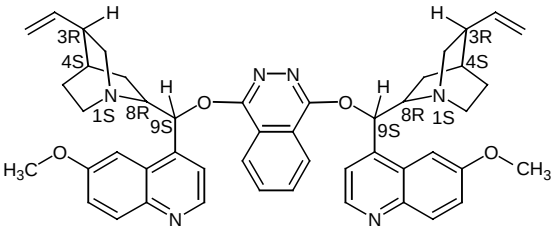
A 150 mm × 4.6 mm i.d. BetaBasic-18 column containing a C_{18} stationary phase with 3 μm particle size (Thermo Hypersil-Keystone, Runcorn, UK) was used for the RP separations.



(a) *tert*.-butylcarbamoylquinine



(b) 6'-(1-adamantylmethoxy)-9-*O*-*tert*.-butyl-carbamoylcinchonidine



(c) 1,4-bis(9-*O*-quinidinyl)phthalazine

Fig. 1. Selector structures.

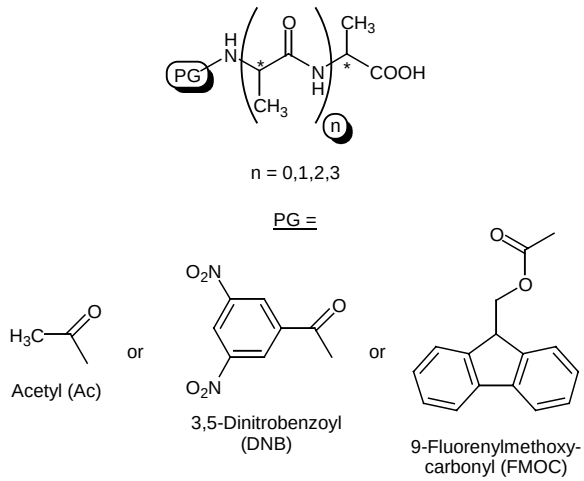


Fig. 2. Analyte structures (asterisks denote centres of chirality).

2.2. Derivatization procedures

Three different N-terminal derivatives of the alanine amino acid and peptide stereoisomers (Fig. 2) were prepared as follows: 4.5 μmol of the amino acid and peptide stereoisomers, respectively, were dissolved in 900 μl carbonate buffer (0.1 mol/l sodium hydrogencarbonate/0.1 mol/l sodium carbonate: 2/1 (v/v)). The acetyl protected samples were obtained by adding 5 μl of acetic anhydride and reaction for 6 h at room temperature. Addition of 300 μl of a saturated solution of 3,5-dinitrobenzoyloxy-succinimide in acetonitrile and reaction at 50 °C over night gave the 3,5-dinitrobenzoyl (DNB) derivatives. 9-Fluorenylmethoxycarbonyl (FMOC) protected analytes were prepared by adding 300 μl of a 2.5% (w/v) solution of *N*-(9-fluorenylmethoxycarbonyloxy)succinimide in acetonitrile and reaction for 2 h at room temperature.

2.3. Liquid chromatography–mass spectrometry separations

LC–MS separations were carried out using an HP1100 series high-performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PE Sciex API 365 triple quadrupole mass spectrometer (MDS Sciex,

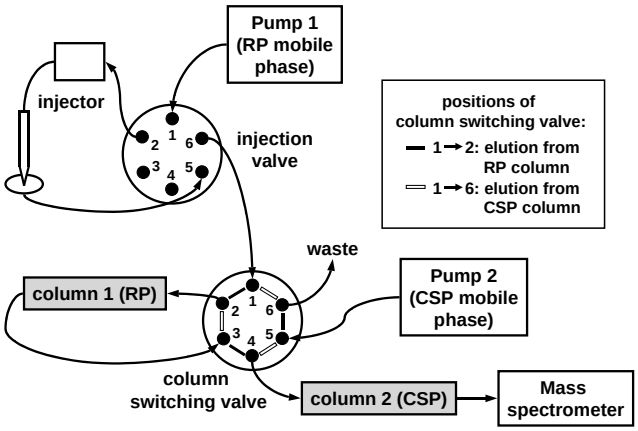


Fig. 3. 2D-LC–MS setup employing a column switching valve. RP: reversed phase (mobile phase: water/2-propanol (90/10 or 91/9 (v/v) + 0.5% (v/v) acetic acid), CSP: chiral stationary phase (mobile phase: 80% methanol, 20% 0.1 mol/l aqueous ammonium acetate (v/v), $pH_a = 6.0$).

Concord, Canada) equipped with an electrospray ion source. The LC effluent was split in an approximately 1:100 ratio before entering the mass spectrometer. MS detection was performed in the selected ion mode employing negative ionization with an ion-spray voltage of −5250 V. For enantiomer separations and RP separations only (one-dimensional separations) the outlet of the respective column (CSP or RP, respectively) was coupled directly with the inlet of the mass spectrometer via a transfer capillary. Two-dimensional separations (2D-LC–MS) were accomplished using the column switching valve of the HP1100 system, which was controlled by the chromatographic system's Chemstation software (Agilent Technologies), and a second pump (HP1050 series, Agilent Technologies) that was manually operated via its keypad. The 2D-LC–MS system setup is shown in Fig. 3.

The mobile phase for enantiomer separations was made up of 80% methanol and 20% 0.1 mol/l ammonium acetate (v/v). The apparent pH of this mixture was adjusted to 6.0 with acetic acid. The flow rate was set to 1 ml/min. The mobile phases for reversed phase separations consisted of mixtures of water and isopropanol, to which 0.5% (v/v) acetic acid were added. The RP separations were performed isocratically at a flow rate of 0.5 ml/min.

All derivatization reaction mixtures were diluted 5–10 times with mobile phase prior to injection. Aliquots of 50 μl were injected and the columns were thermostated at 25 °C.

3. Results and discussion

For the peptide stereoisomer separation studies presented herein a series of oligoalanine peptides containing two to four amino acid residues and alanine amino acid were used as model set. For enantiomer separations three different CSPs based on cinchona alkaloid derivatives (see Fig. 1) were employed. The tertiary amines of the cinchona alkaloids, that have pK_a-values in the range of 8.9–9.1 (calculated for an aqueous system by ACD/pK_a DB 7.0, Advanced Chemistry Development, Toronto, Canada), will be protonated at neutral or slightly acidic pH-values and these stationary phases will then function as weak anion-exchangers [4,30]. Thus, they are highly suitable for separating acidic analytes, such as negatively charged peptides. Besides the primary ion-pairing interaction, which is non-enantioselective, additional stereoselective hydrogen-bonding, π–π-stacking, and van der Waals interactions may come into effect [4]. The presence and magnitude of these interactions for the individual enantiomers determine the enantioselectivity value. Concerning retention, hydrophobic (non-enantioselective) increments also come into play, when these CSPs are used in hydro-organic, quasi reversed phase type, mobile phase conditions.

All analytes were protected at the N-terminus in order to avoid zwitter-ion formation, which would severely compromise or even hinder the anion-exchange process. The pK_a-values of the N-protected analytes were calculated by ACD/pK_a DB 7.0 to be in the range of 3.3–3.9 leading to practically complete deprotonation at the employed mobile phase pH_a of 6.0. Avoiding protonation of the N-terminus by using high-pH conditions is not feasible, as this would diminish the protonation of the chiral selector and consequently ion-exchange capacity. An additional benefit of using N-terminally protected analytes is the possibility of introducing additional interaction sites via the protecting group which may enhance the overall enantioselectivity.

3.1. Influence of absolute configuration of peptide stereoisomers on enantioselectivity

While discussions on the influence of the absolute configuration of the peptide analytes on enantioselectivity are seen quite frequently throughout the literature, they are almost all restricted to two enantiomeric pairs. However, Sänger-van de Griend et al. published a report on the separation of all eight pairs of enantiomers of the tetrapeptide Tyr–Arg–Phe–Phe–NH_2 by capillary electrophoresis using a cyclodextrin as chiral selector [31]. The resolution of different pairs of enantiomers was found to vary dramatically depending on the absolute configuration but no mechanistic explanation was given.

The absolute configurations of the peptide enantiomer pairs investigated in the present study are shown in Fig. 4. As the number of possible stereoisomers grows exponentially with peptide chain length and the present study was aimed at looking into the influence of different absolute configurations on enantioselectivity for different peptide chain lengths, the number of stereoisomers investigated had to be restricted to a subset, that nevertheless covered a sufficient amount of variation. The homochiral peptide isomers, having identical configurations at all stereocentres, were chosen as reference compounds and the enantiomer discrimination changes introduced by placing an inverted

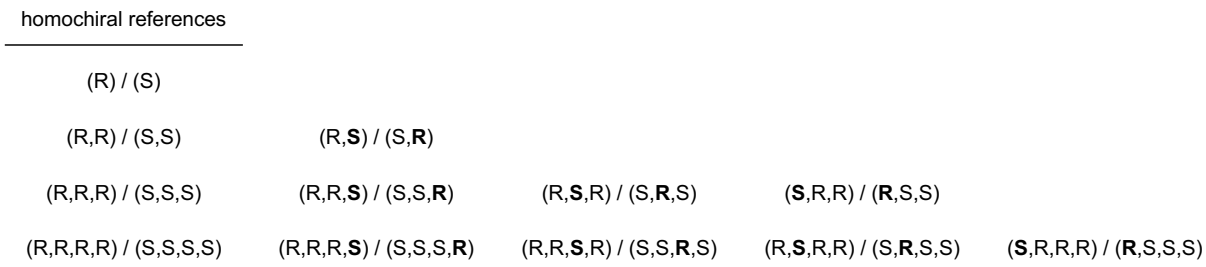


Fig. 4. Absolute configurations of di- to tetrapeptide enantiomeric pairs resulting from shifting an inverted stereocentre through a homochiral peptide chain.

stereocentre at different positions within the peptide chain were studied. Thus, an inverted stereocentre was "shifted" through the peptide chain from the C- to the N-terminus. Combined with the respective enantiomers this leads to a complete coverage of all possible stereoisomers of the amino acid, di- and tripeptide, while for the tetrapeptide 10 out of 16 possible stereoisomers were included (Fig. 4).

The analytes were derivatized at the N-terminus with three different protecting groups (Fig. 2), which were chosen according to the following reasons: 3,5-dinitrobenzoyl (DNB) was found to undergo strong π–π-interactions with cinchona alkaloid selectors, which led to a pronounced increase in enantioselectivity and rendered this group the best one for enantiomer separations of homochiral peptides [6]. On the other hand, acetyl is a N-terminal protecting group commonly occurring in natural peptides and the 9-fluorenylmethoxycarbonyl (FMOC) group is widely used in peptide synthesis. The use of different, structurally diverse N-protecting groups should elucidate whether possible changes in chiral discrimination between the various enantiomeric pairs with different absolute configurations were influenced by the choice of the protecting group.

In a combinatorial approach also three different CSPs were selected for the enantioseparations according to a previous selector optimization study [4]: one was derived from *tert*-butylcarbamoylquinine (*t*BuCQN, Fig. 1a), which has a bulky *tert*-butylcarbamoyl group attached to the 9-hydroxy group of quinine. This modification yielded the highest enantioselectivities among so-called "monomeric" quinine type selectors with an unaltered 6′-methoxy group. The second CSP used was based on 6′-(1-adamantylmethoxy)-9-*O*-*tert*-butylcarbamoylcinchonidine (6′-Adam-*t*BuCCD, Fig. 1b), which carries a second bulky moiety at the 6′-position, in addition to the 9-*O*-*tert*-butylcarbamoyl, thus inserting an additional space increment. This enhances the specificity of the binding cleft, making this CSP the one with the highest enantioselectivity among all "monomeric" ones. The third CSP was derived from 1,4-bis(9-*O*-quinidinyl)phthalazine (QD–Phthal–QD, Fig. 1c), a so-called "dimeric" selector, in which two quinidine moieties are bridged by a phthalazine moiety. In a previous study, this CSP exhibited the largest enantioselectivities for longer peptides among all selectors investigated [4]. Analogue to the usage of diverse protecting groups, different CSPs were employed to assess whether the choice of CSP had an influence on enantioselectivity changes effected by different absolute configurations of the peptide enantiomer pairs.

The results of the separations of the peptide enantiomer pairs with different absolute configurations, N-terminally protected with the three different groups and employing the three different CSPs detailed above are presented in Table 1. For the sake of comparison the hydro-organic mobile phase conditions were kept constant, although one has to note that the selector coverages of the CSPs are not identical which might have some influence on the retention factor but not so on the overall enantioselectivity values [32]. These mobile phase conditions were highly compatible with mass spectrometric detection, which was chosen for unambigious and sensitive detection of the analytes.

When using the DNB protecting group almost all enantiomeric pairs could be separated on all three CSPs. For the FMOC-derivatives enantiomer resolution could also be achieved in most cases but the use of acetyl as N-protecting group led to the co-elution of the enantiomers in several cases. As an overall picture, over 80% of all enantiomer separations were successful and all pairs of enantiomers could be separated with at least one protecting group/CSP combination. Along the line of the number of successful separations, the enantioselectivities obtained were generally largest for the DNB-protected samples. The FMOC-derivatives showed considerably reduced enantiomer discrimination but still superseded their acetyl analogues in general.

This behavior can be explained by different capabilities of the protecting groups of undergoing π–π-interactions with the selector units on the CSPs, which are favorable for achieving high enantioselectivities. While the DNB group is highly π-acidic and thus can strongly interact with the π-basic quinoline ring of the selector, the FMOC group is π-neutral and, therefore, π–π-interactions will be present only to a smaller extent for this group. On the other hand, the acetyl group is completely incapable of such interactions. A detailed discussion of this phenomenon can be found in [6]. The favorable π–π-interactions of the DNB-derivatized samples are especially pronounced for the amino acid and the dipeptide enantiomers, while they are largely lost for longer peptides [6,33]. This translates into a pronounced drop of enantioselectivity between the di- and the tripeptide enantiomers.

Concerning retention, the FMOC-protected analytes generally showed quite large values that were similar to those

Table 1
Investigation of the influence of different absolute configurations on the separation of oligoalanine peptide enantiomer pairs by LC–MS employing three different N-protecting groups and three different chiral stationary phases

CSP[a]	Peptide	Configurations	Protecting group														
			Acetyl					3,5-Dinitrobenzoyl (DNB)					9-Fluorenylmethoxycarbonyl (FMOC)				
			k_1	k_2	α	R_s	Elution order	k_1	k_2	α	R_s	Elution order	k_1	k_2	α	R_s	Elution order
*t*BuCQN[b] (t_0 = 1.2 min)	A1a$_1$	(*R*) + (*S*)	2.80	3.30	1.18	0.54	(*R*) < (*S*)	8.73	61.22	7.01	21.17	(*R*) < (*S*)	11.18	16.44	1.47	4.89	(*R*) < (*S*)
	A1a$_2$	(*R*, *R*) + (*S*, *S*)	2.31	2.80	1.21	0.66	(*R*, *R*) < (*S*, *S*)	7.02	38.08	5.43	21.18	(*R*, *R*) < (*S*, *S*)	8.45	12.36	1.46	4.51	(*R*, *R*) < (*S*, *S*)
		(*R*, *S*) + (*S*, *R*)	2.62	2.83	1.08	0.56	(*R*, *S*) < (*S*, *R*)	9.38	25.37	2.70	15.10	(*R*, *S*) < (*S*, *R*)	9.97	11.28	1.13	1.41	(*R*, *S*) < (*S*, *R*)
	Ala$_3$	(*R*, *R*, *R*) + (*S*, *S*, *S*)	2.03	2.26	1.12	0.47	(*R*, *R*, *R*) < (*S*, *S*, *S*)	5.05	7.98	1.58	4.29	(*R*, *R*, *R*) < (*S*, *S*, *S*)	6.13	7.24	1.18	1.75	(*R*, *R*, *R*) < (*S*, *S*, *S*)
		(*R*, *R*, *S*) + (*S*, *S*, *R*)	2.36	2.36	1.00	0.00	–	5.83	9.79	1.68	7.09	(*R*, *R*, *S*) < (*S*, *S*, *R*)	7.14	7.65	1.07	0.68	(*R*, *R*, *S*) < (*S*, *S*, *R*)
		(*R*, *S*, *R*) + (*S*, *R*, *S*)	2.33	2.33	1.00	0.00	–	7.63	8.18	1.07	0.90	(*R*, *S*, *R*) < (*S*, *R*, *S*)	7.70	7.70	1.00	0.00	–
		(*S*, *R*, *R*) + (*R*, *S*, *S*)	2.23	2.38	1.07	0.43	(*S*, *R*, *R*) < (*R*, *S*, *S*)	6.69	8.61	1.29	3.32	(*R*, *S*, *S*) < (*S*, *R*, *R*)	6.99	7.63	1.09	1.03	(*S*, *R*, *R*) < (*R*, *S*, *S*)
	Ala$_4$	(*R*, *R*, *R*, *R*) + (*S*, *S*, *S*, *S*)	1.93	2.19	1.14	0.61	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)	4.68	6.29	1.34	2.61	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)	5.13	6.03	1.18	1.40	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)
		(*R*, *R*, *R*, *S*) + (*S*, *S*, *S*, *R*)	2.08	2.08	1.00	0.00	–	5.38	6.36	1.18	1.91	(*R*, *R*, *R*, *S*) < (*S*, *S*, *S*, *R*)	5.73	5.98	1.04	0.46	(*R*, *R*, *R*, *S*) < (*S*, *S*, *S*, *R*)
		(*R*, *R*, *S*, *R*) + (*S*, *S*, *R*, *S*)	2.06	2.06	1.00	0.00	–	5.32	6.43	1.21	1.81	(*R*, *R*, *S*, *R*) < (*S*, *S*, *R*, *S*)	6.11	6.11	1.00	0.00	–
		(*R*, *S*, *R*, *R*) + (*S*, *R*, *S*, *S*)	2.11	2.21	1.05	0.37	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)	6.03	6.97	1.16	1.27	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)	6.08	6.48	1.07	0.62	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)
		(*S*, *R*, *R*, *R*) + (*R*, *S*, *S*, *S*)	2.09	2.24	1.07	0.43	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)	6.16	6.16	1.00	0.00	–	5.91	6.33	1.07	0.64	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)
6′-Adam-*t*BuCCD[c] (t_0 = 1.1 min)	Ala$_1$	(*R*) + (*S*)	2.17	2.87	1.32	0.68	(*R*) < (*S*)	7.91	92.12	11.65	19.16	(*R*) < (*S*)	13.22	20.89	1.58	5.03	(*R*) < (*S*)
	Ala$_2$	(*R*, *R*) + (*S*, *S*)	1.81	2.55	1.41	1.71	(*R*, *R*) < (*S*, *S*)	5.90	94.24	15.97	24.55	(*R*, *R*) < (*S*, *S*)	8.26	17.59	2.13	7.86	(*R*, *R*) < (*S*, *S*)
		(*R*, *S*) + (*S*, *R*)	2.01	2.01	1.00	0.00	–	8.93	15.64	1.75	7.01	(*R*, *S*) < (*S*, *R*)	10.33	10.33	1.00	0.00	–
	Ala$_3$	(*R*, *R*, *R*) + (*S*, *S*, *S*)	1.56	1.99	1.27	0.65	(*R*, *R*, *R*) < (*S*, *S*, *S*)	4.31	11.54	2.68	9.33	(*R*, *R*, *R*) < (*S*, *S*, *S*)	5.95	8.26	1.39	3.28	(*R*, *R*, *R*) < (*S*, *S*, *S*)
		(*R*, *R*, *S*) + (*S*, *S*, *R*)	1.83	1.83	1.00	0.00	–	5.08	13.52	2.66	11.08	(*R*, *R*, *S*) < (*S*, *S*, *R*)	7.31	7.31	1.00	0.00	–
		(*R*, *S*, *R*) + (*S*, *R*, *S*)	1.75	1.75	1.00	0.00	–	5.93	5.93	1.00	0.00	–	7.45	7.45	1.00	0.00	–
		(*S*, *R*, *R*) + (*R*, *S*, *S*)	1.67	2.06	1.23	1.04	(*S*, *R*, *R*) < *R*, *S*, *S*)	5.79	6.86	1.19	1.76	(*S*, *R*, *R*) < (*R*, *S*, *S*)	6.48	8.85	1.36	3.84	(*S*, *R*, *R*) < (*R*, *S*, *S*)
	Ala$_4$	(*R*, *R*, *R*, *R*) + (*S*, *S*, *S*, *S*)	1.49	1.88	1.26	0.86	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)	3.84	7.95	2.07	5.22	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)	4.77	6.40	1.34	2.63	(*R*, *R*, *R*, *R*) < (*S*, *S*, *S*, *S*)
		(*R*, *R*, *R*, *S*) + (*S*, *S*, *S*, *R*)	1.62	1.62	1.00	0.00	–	4.39	7.29	1.66	4.78	(*R*, *R*, *R*, *S*) < (*S*, *S*, *S*, *R*)	5.63	5.63	1.00	0.00	–
		(*R*, *R*, *S*, *R*) + (*S*, *S*, *R*, *S*)	1.58	1.58	1.00	0.00	–	4.28	7.47	1.75	4.25	(*R*, *R*, *S*, *R*) < (*S*, *S*, *R*, *S*)	5.65	5.65	1.00	0.00	–
		(*R*, *S*, *R*, *R*) + (*S*, *R*, *S*, *S*)	1.64	1.85	1.13	0.66	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)	4.50	5.53	1.23	1.46	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)	5.52	6.70	1.21	1.84	(*R*, *S*, *R*, *R*) < (*S*, *R*, *S*, *S*)
		(*S*, *R*, *R*, *R*) + (*R*, *S*, *S*, *S*)	1.64	1.92	1.17	0.77	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)	4.50	5.31	1.18	1.46	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)	5.46	6.37	1.17	1.55	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)
QD–Phthal–QD[d] (t_0 = 1.1 min)	Ala$_1$	(*R*) + (*S*)	3.64	5.85	1.61	1.73	(*S*) < (*R*)	19.57	364.65	18.63	15.99	(*S*) < (*R*)	20.97	28.59	1.36	3.86	(*S*) < (*R*)
	Ala$_2$	(*R*, *R*) + (*S*, *S*)	2.98	4.79	1.61	1.89	(*S*, *S*) < (*R*, *R*)	14.90	281.79	18.91	16.58	(*S*, *S*) < (*R*, *R*)	15.88	19.68	1.24	1.84	(*S*, *S*) < (*R*, *R*)
		(*R*, *S*) + (*S*, *R*)	3.51	3.51	1.00	0.00	–	18.69	62.75	3.36	13.98	(*S*, *R*) < (*R*, *S*)	16.98	16.98	1.00	0.00	–
	Ala$_3$	(*R*, *R*, *R*) + (*S*, *S*, *S*)	2.63	3.31	1.26	0.99	(*S*, *S*, *S*) < (*R*, *R*, *R*)	10.94	46.72	4.27	14.48	(*S*, *S*, *S*) < (*R*, *R*, *R*)	10.94	17.26	1.58	4.55	(*S*, *S*, *S*) < (*R*, *R*, *R*)
		(*R*, *R*, *S*) + (*S*, *S*, *R*)	2.96	2.96	1.00	0.00	–	11.82	53.24	4.50	15.07	(*S*, *S*, *R*) < (*R*, *R*, *S*)	12.23	13.41	1.10	1.07	(*S*, *S*, *R*) < (*R*, *R*, *S*)
		(*R*, *S*, *R*) + (*S*, *R*, *S*)	2.74	2.95	1.08	0.46	(*S*, *R*, *S*) < (*R*, *S*, *R*)	12.95	26.04	2.01	8.37	(*S*, *R*, *S*) < (*R*, *S*, *R*)	12.28	15.23	1.24	2.99	(*S*, *R*, *S*) < (*R*, *S*, *R*)
		(*S*, *R*, *R*) + (*R*, *S*, *S*)	2.81	3.44	1.22	1.09	(*R*, *S*, *S*) < (*S*, *R*, *R*)	16.03	26.61	1.66	6.01	(*S*, *R*, *R*) < (*R*, *S*, *S*)	13.38	19.25	1.44	4.86	(*R*, *S*, *S*) < (*S*, *R*, *R*)
	Ala$_4$	(*R*, *R*, *R*, *R*) + (*S*, *S*, *S*, *S*)	2.35	2.87	1.22	0.83	(*S*, *S*, *S*, *S*) < (*R*, *R*, *R*, *R*)	10.36	27.16	2.62	9.12	(*S*, *S*, *S*, *S*) < (*R*, *R*, *R*, *R*)	9.09	11.65	1.28	2.52	(*S*, *S*, *S*, *S*) < (*R*, *R*, *R*, *R*)
		(*R*, *R*, *R*, *S*) + (*S*, *S*, *S*, *R*)	2.63	2.63	1.00	0.00	–	11.14	27.82	2.50	10.03	(*S*, *S*, *S*, *R*) < (*R*, *R*, *R*, *S*)	10.05	10.91	1.08	0.97	(*S*, *S*, *S*, *R*) < (*R*, *R*, *R*, *S*)
		(*R*, *R*, *S*, *R*) + (*S*, *S*, *R*, *S*)	2.39	2.68	1.12	0.79	(*S*, *S*, *R*, *S*) < (*R*, *R*, *S*, *R*)	10.40	36.77	3.54	11.96	(*S*, *S*, *R*, *S*) < (*R*, *R*, *S*, *R*)	10.27	10.75	1.05	0.46	(*S*, *S*, *R*, *S*) < (*R*, *R*, *S*, *R*)
		(*R*, *S*, *R*, *R*) + (*S*, *R*, *S*, *S*)	2.55	3.36	1.32	1.66	(*S*, *R*, *S*, *S*) < (*R*, *S*, *R*, *R*)	11.98	25.85	2.16	7.24	(*S*, *R*, *S*, *S*) < (*R*, *S*, *R*, *R*)	10.44	13.74	1.32	2.74	(*S*, *R*, *S*, *S*) < (*R*, *S*, *R*, *R*)
		(*S*, *R*, *R*, *R*) + (*R*, *S*, *S*, *S*)	2.65	3.11	1.18	1.03	(*R*, *S*, *S*, *S*) < (*S*, *R*, *R*, *R*)	15.68	18.91	1.21	2.04	(*S*, *R*, *R*, *R*) < (*R*, *S*, *S*, *S*)	10.77	15.91	1.48	5.24	(*R*, *S*, *S*, *S*) < (*S*, *R*, *R*, *R*)

Separation conditions: mobile phase. 80% methanol, 20% 0.1 mol/l ammonium acetate (v/v), $pH_a = 6.0$; flow rate: 1 ml/min, 25 °C; MS detection by selected ion monitoring of $[M–H]^-$ of the peptide isomers.

[a] Chiral stationary phase.

[b] *tert*-Butylcarbamoylquinine (Fig. 1a) based chiral stationary phase (selector coverage: 0.39 mmol/g).

[c] 6′-(1-Adamantylmethoxy)-9-*O*-*tert*-butylcarbamoylcinchonidine (Fig. 1b) based chiral stationary phase (selector coverage: 0.22 mmol/g).

[d] 1,4-bis-(9-*O*-Quinidinyl)phthalazine (Fig. 1c) based chiral stationary phase (selector coverage: 0.16 mmol/g).

of the DNB-derivatives or higher, even in cases where no enantioseparation occurred. On the contrary, the acetyl-derivatized peptide enantiomers showed only rather weak retention. One may speculate that aromatic moieties within the protecting group do not only offer the possibility for additional enantioselective interactions with the CSP but also lead to a generally enhanced but non-enantioselective binding to the stationary phase.

Consistent with the findings of a previous study [4], the selectivities for the separation of the various pairs of enantiomers were generally smallest on the *t*BuCQN-derived CSP. The use of the 6′-Adam-*t*BuCCD-based CSP increased the enantioselectivities, however, the highest enantiomer discrimination for the investigated peptide enantiomer pairs was found for the CSP derived from QD–Phthal–QD. A detailed discussion of the underlying mechanisms can be found elsewhere [4].

The comparison of the elution orders of the different pairs of enantiomers and the selectivities with which they were separated may allow to gain insights into the molecular recognition mechanisms that effect enantiomer discrimination. Thereby, one has to take into account that the cinchonine structures of the QD–Phthal–QD selector have inverted configurations at the C_8 and C_9 atoms compared to the cinchonidine moieties in the other two selectors ((8*R*, 9*S*) versus (8*S*, 9*R*)). This leads to a "pseudo-enantiomeric" behavior [4,27], which means that CSPs based on cinchonine will show an inverted enantiomer discrimination pattern and hence an inverted elution order of the enantiomers compared to cinchonidine-derived CSPs. Consequently, equivalent molecular recognition mechanisms will show up as *reversed* elution orders for the cinchonine and cinchonidine CSPs.

For the amino acid and dipeptide enantiomers the elution orders are consistent, regardless of the N-protecting group and the CSP (Table 1). For the *t*BuCQN and 6′-Adam-*t*BuCCD CSPs the (*R*)-, (*R*, *R*)- and (*R*, *S*)-enantiomers elute before their (*S*)-, (*S*, *S*)- and (*S*, *R*)-configured congeners, respectively, while elution orders of (*S*) before (*R*), (*S*, *S*) before (*R*, *R*), and (*S*, *R*) before (*R*, *S*) were observed with the QD–Phthal–QD CSP. These findings strongly suggest congruent chiral discrimination mechanisms in all cases (see above). For the dipeptide enantiomer pairs higher selectivities were achieved for the homochiral pair ((*R*, *R*)/(*S*, *S*)) than for the heterochiral pair ((*R*, *S*)/(*S*, *R*)) throughout.

The last finding is generally found also for the tri- and tetrapeptide samples: for the tripeptide enantiomer pairs the homochiral pair was separated with the highest selectivity in seven out of nine cases (referring to 3×3 possible combinations of protecting group and CSP) and with the second-highest selectivity in the remaining two (see Table 1). The homochiral tetrapeptide enantiomer pair showed the largest discrimination in all cases with the exception of the separations on the CSP derived from QD–Phthal–QD. Thus, in a wide majority of cases, the homochiral peptide enantiomers are the most strongly discriminated species among all enantiomeric pairs that were investigated. Elution orders for the homochiral samples were consistent throughout all experiments, being (all-*R*) before (all-*S*) for the cinchonidine-based CSPs and (all-*S*) before (all-*R*) for the cinchonine-derived one.

Turning to the other, heterochiral, enantiomeric pairs, they offer a less uniform picture. While the (*R*, *R*, *S*)/(*S*, *S*, *R*)- and (*R*, *R*, *R*, *S*)/(*S*, *S*, *S*, *R*)-pairs as well as the (*R*, *S*, *R*)/(*S*, *R*, *S*)- and (*R*, *R*, *S*, *R*)/(*S*, *S*, *R*, *S*)-pairs plus the (*R*, *S*, *R*, *R*)/(*S*, *R*, *S*, *S*)-pair showed a consistent elution order behavior with all protecting groups and CSPs, this was not the case for the (*S*, *R*, *R*)/(*R*, *S*, *S*)- and (*S*, *R*, *R*, *R*)/(*R*, *S*, *S*, *S*)-pairs. Therefore, it was not possible to develop a generally valid model in which the elution order can be predicted from the absolute configuration of a specific amino acid or a certain configurational pattern.

Concerning the variation of enantioselectivities with changing absolute configurations, it is difficult to extract any general trends, as the behavior is rather irregular across the set of analytes and CSPs. Nevertheless, it seems that peptides in which adjacent amino acid residues have the same configuration, e.g. (*S*, *R*, *R*, *R*)/(*R*, *S*, *S*, *S*), experience larger enantiomer discrimination than isomers having an alternating configuration pattern, e.g. (*R*, *S*, *R*)/(*S*, *R*, *S*).

Combining the findings discussed above, it becomes clear that the molecular recognition mechanism is more complicated than one might expect. The relative configuration of the enantiomer bound more strongly to the CSP, i.e. the one that has a larger retention, depends on the combination of a number of factors that certainly include, but may not be limited to, the protecting group, the CSP and the peptide's absolute configuration. Although certain predictions concerning the elution order of peptide enantiomers on cinchona alkaloid derived CSPs might be possible, e.g. a consistent behavior was found for the amino acid and dipeptide enantiomer pairs, this investigation has shown that in doing so one needs to take the entire stereochemistry of the peptide into account.

3.2. Two-dimensional separation of peptide diastereomers and enantiomers

The separation of both enantiomers and diastereomers of longer peptides containing more than two stereocentres requires a highly selective analytical system due to the large number of possible components (cf. Section 1). At least one part of such a system must have enantiomer discrimination abilities but its diastereoselective properties must also be sufficient for all stereoisomers contained in the peptide sample.

The peptide stereoisomers used in the separations described above were also used for this investigation. As protecting group, DNB was chosen, as it yielded the highest enantioselectivities. In a previous study, it was found to be possible to separate all four stereoisomers of the dialanine peptide on the *t*BuCQN derived CSP [5]. Therefore, only the tri- and tetraalanine stereoisomers (all eight and 10 out of a possible total of 16, respectively; see Fig. 4) were stud-

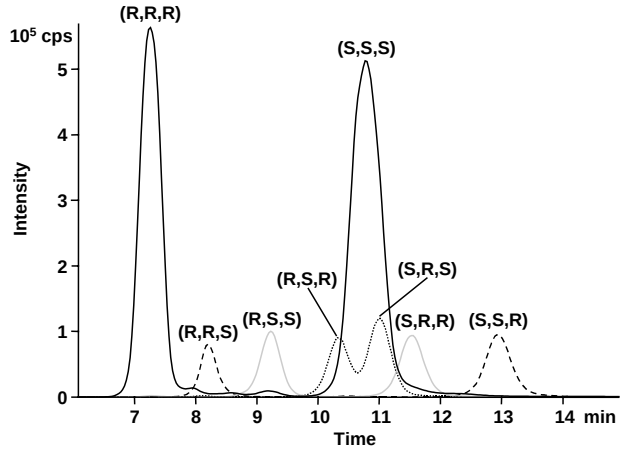


Fig. 5. Overlaid LC–MS chromatograms of enantiomeric pairs of 3,5-dinitrobenzoyl trialanine stereoisomers on a *tert*-butylcarbamoylquinine based chiral stationary phase obtained by selected ion monitoring of $[M–H]^-$ (m/z = 424.1). Mobile phase: 80% methanol, 20% 0.1 mol/l aqueous ammonium acetate (v/v), pH_a = 6.0, 25 °C.

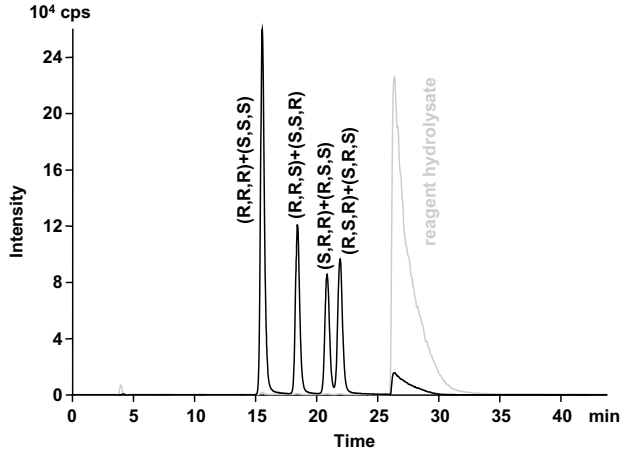


Fig. 6. Reversed-phase separation of all four enantiomeric pairs of 3,5-dinitrobenzoyl trialanine. The black trace depicts the selected ion monitoring of $[M–H]^-$ of the peptide isomers (m/z = 424.1), while the gray trace shows the selected ion monitoring of $[M–H]^-$ of the reagent hydrolysate (m/z = 211.0). Mobile phase: 90/10 water/2-propanol (v/v) + 0.5% (v/v) acetic acid, 25 °C.

ied. At first, we tried to find a mobile phase that would allow the separation of both diastereomers and enantiomers on one of the CSPs. However, this approach was not successful, as peak overlapping occurred between the different enantiomeric pairs (Fig. 5), because the diastereoselectivity of the CSPs was not sufficient. Consequently, we decided to develop a two-dimensional HPLC setup consisting of a reversed phase column for diastereomer separations in the first dimension, followed in the second dimension by a CSP, to which the peaks of the first column were transferred online. Peak tracking and identification was carried out by mass spectrometric detection.

3.2.1. Reversed phase separation of peptide diastereomers

When developing a reversed phase method for separating peptide diastereomers, one must take into account whether they are protected or not, as the presence of a protecting group (which in the current case is necessary for successful enantiomer separation) will certainly have a large influence on the overall hydrophobicity of the entire peptide molecule. Therefore, the various studies that describe the separation of native peptide diastereomers will not be useful orientation points for the development of a diastereoselective separation method for protected peptides. Although a few reports have described the reversed phase separation of N-terminally protected peptide diastereomers [34–36], two of them have focussed on the separation of only two stereoisomers. However, the complete diastereomer separation of *Z*-Ala–Val–Gly–Phe (resolution of four stereoisomers) has been reported [34]. In the present case, four (tripeptide) and five (tetrapeptide) diastereomerically related pairs of enantiomers, respectively, needed to be separated.

Various mobile phases (standard peptide gradient (water/acetonitrile plus trifluoroacetic acid), different mixtures of methanol and water containing acetic acid) were tested for the separation of the tripeptide diastereomers, however, complete baseline separation of all four pairs of enantiomers could not be achieved (data not shown). Finally, a mixture of 90% water and 10% 2-propanol (v/v) containing 0.5% acetic acid (v/v) proved to be able to successfully separate all diastereomers (Fig. 6, Table 2). Moreover, all the peptide stereoisomers were cleanly separated from the reagent hydrolysate originating from the derivatization reaction. For the tetrapeptide stereoisomers a complete separation of all five pairs of enantiomers could not be achieved. Nevertheless, using a slightly modified mobile phase (a mixture of 91% water and 9% 2-propanol (v/v), containing 0.5% acetic acid (v/v)) it was possible to obtain three fractions containing 2 + 2 + 1 pairs of enantiomers, which were all well separated from the reagent hydrolysate (Table 2). This partial

Table 2
Separation of 3,5-dinitrobenzoyl protected peptide diastereomers on a C_{18} reversed phase column

Peptide	Configurations	t_R (min)
Ala_3[a]	(*R*, *R*, *R*)/(*S*, *S*, *S*)	15.54
	(*R*, *R*, *S*)/(*S*, *S*, *R*)	18.42
	(*S*, *R*, *R*)/(*R*, *S*, *S*)	20.84
	(*R*, *S*, *R*)/(*S*, *R*, *S*)	21.90
	Reagent hydrolysate	26.37
Ala_4[b]	(*R*, *R*, *R*, *R*)/(*S*, *S*, *S*, *S*)	19.75
	(*R*, *R*, *R*, *S*)/(*S*, *S*, *S*, *R*)	19.75
	(*R*, *S*, *R*, *R*)/(*S*, *R*, *S*, *S*)	23.41
	(*S*, *R*, *R*, *R*)/(*R*, *S*, *S*, *S*)	23.41
	(*R*, *R*, *S*, *R*)/(*S*, *S*, *R*, *S*)	25.52
	Reagent hydrolysate	31.53

[a] Mobile phase: 90/10 water/2-propanol (v/v) + 0.5% (v/v) acetic acid, flow rate: 0.5 ml/min.

[b] Mobile phase: 91/9 water/2-propanol (v/v) + 0.5% (v/v) acetic acid, flow rate: 0.5 ml/min.

separation of the diastereomers was deemed to be sufficient as the (modest) diastereoselectivities of the CSPs should allow the concurrent separation of two pairs of enantiomers.

The elution orders of the peptide diastereomers on the reversed phase can be explained by the relative orientations of the methyl side chains of the amino acid residues towards each other [37]. Thereby, the side chains of two adjacent amino acid residues having the same absolute configurations, e.g. (*R*, *R*), will point towards different sides of the molecule, while they are on the same side for unlike configurations, e.g. (*R*, *S*). If the side chains point towards the same side, the hydrophobic surface will be increased and a higher retention can be expected. Taking the tripeptide as example, the homochiral stereoisomers have the lowest hydrophobicity, as the side chains alternate around the peptide backbone. Thus, this enantiomeric pair should elute first, which is exactly what was observed experimentally. The (*R*, *R*, *S*)/(*S*, *S*, *R*) and (*S*, *R*, *R*)/(*R*, *S*, *S*) pairs have intermediate hydrophobicity with two adjacent side chains oriented towards the same side of the molecule, while the (*R*, *S*, *R*)/(*S*, *R*, *S*) pair has maximum hydrophobicity with all methyl groups on the same side. An analogous elution pattern was observed for the tetrapeptide isomers.

3.2.2. 2D-LC–MS separations by coupling a reversed phase and a chiral stationary phase column

After completing the optimization of the reversed phase separation of the peptide diastereomers, the RP column was coupled to one of the three CSPs used in the enantiomer separations described above. As a first step, it was evaluated whether the enantiomer separations could also be achieved by using the mobile phase of the RP separation. However, retention turned out to be much too high and the analytes did not elute from the column. The reason for this behavior is that the overall retention mechanism on the cinchona alkaloid CSPs is a mixture of ion-exchange and reversed phase retention increments. While the replacement of ammonium acetate used previously for balancing the ion-pairing between stationary phase and peptide analytes by acetic acid is possible and leads to a similarly effective elution force [38], the elution strength concerning the hydrophobic retention part was obviously much too low, since the RP mobile phase contained 90% water. Therefore, two different mobile phases were employed in the two separation dimensions: The water/2-propanol/acetic acid mixture described in the previous section was used for the RP column, while the methanol/aqueous ammonium acetate mixture used in the enantioseparations discussed above was applied for the separations on the CSPs.

The complete 2D-LC–MS system setup is shown in Fig. 3. While the pump of the HP 1100 HPLC apparatus was used to deliver the mobile phase for the RP separation of the diastereomers, a second pump was employed for the delivery of the mobile phase for enantiomer separations. The two columns and the second pump were connected to the column switching valve of the HPLC system. According to the position of the valve, one of the two pumps was connected to its associated column and the analytes were eluted from that stationary phase, while the eluent delivered by the other pump was directed to waste. The enantiomeric pairs separated on the RP column (first dimension) were transferred onto the head of the CSP column (second dimension). Due to the extremely low elution strength of the RP mobile phase on the CSP, the analytes were focussed at the head of the CSP column. After completing the transfer of a fraction to the CSP column, the valve was switched and elution took place on the CSP (separation of the enantiomers) followed by mass spectrometric detection, while the RP column was put on hold. As soon as the enantiomer separation was completed, the valve was switched back and the next fraction from the RP column was transferred to the CSP column and so forth. This procedure was repeated until all RP fractions had been trapped sequentially on the CSP followed by the separation of the enantiomers. The switching times of the valve were determined from a one-dimensional RP run. Although this setup does not have maximum flexibility, e.g. the entire effluent of the first column is transferred to the second one, it was sufficient for the intended purpose, namely to demonstrate the principle of the separation method and show the advantages the combination of a RP and a CSP column has to offer.

For the separation of the eight tripeptide stereoisomers the *t*BuCQN based CSP was chosen as column for the second dimension, as it was shown to be capable of separating all enantiomeric pairs within relatively short run-times (see Table 1). The QD–Phthal–QD derived CSP would offer higher enantioselectivities, however, at the cost of long run-times. The total elution times of the 3,5-dinitrobenzoyl trialanine peptide stereoisomers separated on the 2D-LC–MS system are presented in Table 3. The chromatogram obtained by selected ion monitoring of the peptide anion (Fig. 7) shows that all eight stereoisomers could be well separated within 90 min. The high efficiencies of the single stereoisomer peaks that persist throughout the chromatogram are due to the focussing effect on the head of the CSP column (see above).

The QD–Phthal–QD based CSP was selected as column for the second dimension for the separation of the tetrapeptide stereoisomers, as it is superior in terms of both enantio- as well as diastereoselectivity compared to the other two CSPs (see Table 1). The separation results of the 10 stereoisomers are given in Table 3. Nine of the 10 isomers could be resolved, only the (*R*, *R*, *R*, *R*) and (*R*, *R*, *R*, *S*) diastereomers coeluted. The overall high degree of diastereoselectivity of the separation system is a combination of the diasteroselective properties of the RP and the CSP column. In this instance, the CSP not only separates the enantiomers but also improves the diastereomer separation offered by the RP column (cf. previous section).

Thus, the 2D-LC–MS setup combining a RP and a CSP column proved to be a very useful and highly effective tool for separating both diastereomers as well as enantiomers of peptides, even though the 2D-LC setup was not fully

Table 3
2D-LC–MS separations of 3,5-dinitrobenzoyl protected peptide stereoisomers employing a C_{18} reversed phase column in the first and a chiral stationary phase column in the second dimension

Peptide	Configuration	t (min)
Ala_3[a]	(*R*, *R*, *R*)	24.19
	(*S*, *S*, *S*)	27.50
	(*R*, *R*, *S*)	43.12
	(*S*, *S*, *R*)	47.60
	(*R*, *S*, *S*)	63.60
	(*S*, *R*, *R*)	65.80
	(*R*, *S*, *R*)	85.26
	(*S*, *R*, *S*)	85.93
	Reagent hydrolysate	115.01
Ala_4[b]	(*S*, *S*, *S*, *S*)	34.01
	(*S*, *S*, *S*, *R*)	34.99
	(*R*, *R*, *R*, *R*)	53.06
	(*R*, *R*, *R*, *S*)	53.06
	(*S*, *R*, *S*, *S*)	73.85
	(*S*, *R*, *R*, *R*)	77.74
	(*R*, *S*, *S*, *S*)	81.25
	(*R*, *S*, *R*, *R*)	89.03
	(*S*, *S*, *R*, *S*)	109.39
	(*R*, *R*, *S*, *R*)	137.73
	Reagent hydrolysate	175.85

[a] *tert*-Butylcarbamoylquinine chiral stationary phase as second dimension.
[b] 1,4-bis(9-*O*-Quinidinyl)phthalazine chiral stationary phase as second dimension.

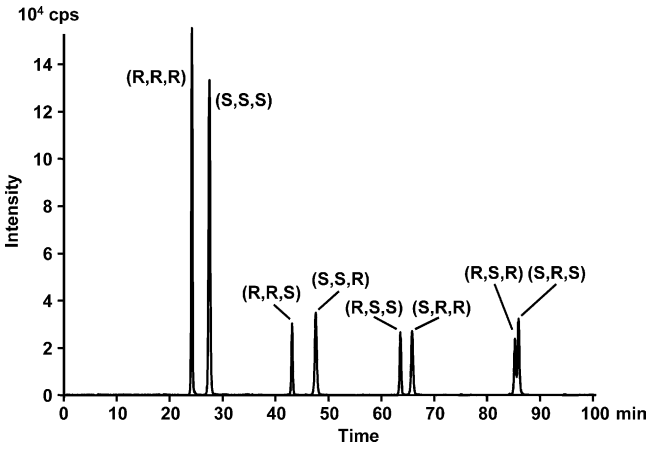


Fig. 7. 2D-LC–MS separation of all eight stereoisomers of 3,5-dinitrobenzoyl trialanine using a C_{18} reversed phase in the first dimension (mobile phase: 90/10 water/2-propanol (v/v) + 0.5% (v/v) acetic acid) and a *tert*-butylcarbamoylquinine based chiral stationary phase (mobile phase: 80% methanol, 20% 0.1 mol/l aqueous ammonium acetate (v/v), $pH_a = 6.0$) in the second dimension. The signal shown was obtained by selected ion monitoring of $[M–H]^-$ of the peptide isomers ($m/z = 424.1$).

optimized, as the focus of the present investigation was more on the proof of principle.

4. Conclusions

The development and application of one- and two-dimensional chromatographic systems for the separation of peptide stereoisomers (enantiomers and diastereomers) has been described. Thereby, cinchona alkaloid based chiral stationary phases have been employed for the resolution of enantiomers of N-terminally protected peptides. The separation results for various pairs of enantiomers of the same peptide having different absolute configurations were compared and evaluated in terms of the molecular recognition mechanism. The studied CSPs were found to be capable of successfully discriminating all investigated pairs of peptide enantiomers in the wide majority of all possible protecting group/CSP combinations. However, enantioselectivities varied markedly for the different pairs of enantiomers, with the homochiral one being separated with the largest selectivity in general. The investigation of the enantiomer elution orders provided insights into the molecular recognition mechanism. Some general trends concerning the impact of the absolute configuration of the peptide on the enanantiomer discrimination process could be established but the choice of the protecting group and the CSP are also of major importance for the actual stereoselective recognition process. As the molecular recognition mechanism is thus very complex and depends on a multitude of variables, it is difficult to make general predictions.

A system for the separation of both diastereomers as well as enantiomers was also developed. The final two-dimensional liquid chromatography–mass spectrometry setup combined the separation of the diastereomers on a reversed phase column, enantiomer separation on a CSP column and unambigious detection by mass spectrometry. All eight possible stereoisomer forms of the N-terminally protected Ala–Ala–Ala tripeptide were successfully separated with high efficiency, while for the Ala–Ala–Ala–Ala tetrapeptide 9 out of 10 studied stereoisomers could be resolved. These results demonstrate the highly discriminative nature of the presented system in terms of both dia- and enantioselectivity and the potential of the column switching setup. The combination of a RP and a CSP column greatly enhanced the peak capacity of the system, enabling not only the concomitant separation of enantiomers and diastereomers but also enlarging the overall diastereoselectivity.

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