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Micro-HPLC and standard-size HPLC for the separation of peptide stereoisomers employing an ion-exchange principle

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

Standard-size (4 mm ID) and micro-HPLC columns (0.5 mm ID) packed with a quinine-based ion-exchange type chiral stationary phase are comparatively evaluated for the separation of peptide enantiomers with up to six amino acid residues. The results show that downscaling the separation system in order to gain the advantages of miniaturized HPLC is possible without sacrificing separation power. Further, five different N-terminal protections (3,5-dinitrobenzoyl, 2,4-dinitrophenyl, 3,5-dinitrobenzyloxycarbonyl, carbazole-9-carbonyl, and 9-fluorenylmethoxycarbonyl) of the analytes are investigated regarding their effect on enantioselectivity. A comparison between a hydro-organic and a polar-organic mobile phase is also reported. The enantiomers of the peptides containing one to four amino acid residues were baseline resolved, while for the penta- and hexamers only partial separations were possible. In addition, all four stereoisomers of alanylalanine could be baseline separated.

Keywords: Enantiomer separation; Peptides; Micro-HPLC; Mobile phase composition; *Tert*-butylcarbamoylquinine; Chiral stationary phase

1. Introduction

Amino acids and peptides are one major group of chiral molecules, which are extensively investigated in enantiomer separation research. HPLC is, besides CE, the most common separation technique employed. Most methods described so far for the enantiomer separation of amino acids and peptides use conventional standard-size HPLC (with columns having an ID of 4.6 mm or 4.0 mm), however some papers deal with the application of micro-HPLC employing columns with an ID smaller than 1.0 mm [1–7]. The principles employed in these studies included ligand-exchange [3,7] or cyclodextrin bonded [4] chiral stationary phases (CSPs), addition of a chiral selector to the mobile phase [1,2,6] and indirect

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enantioseparation by pre-column derivatization with a chiral reagent [5].

As has been the topic of several reviews [8–11], micro-HPLC has a number of advantages over standard-size HPLC (2–4.6 mm ID). The smaller inner diameter of the micro-HPLC columns leads to a general reduction of all volume-based system characteristics, whereby the decrease is proportional to the square of the decrease in column diameter. Consequently, flow rates are in the range of μl/min (0.5–1.0 mm ID) or even nl/min ($\leqslant$0.1 mm ID), resulting in a drastically reduced consumption of mobile phase with the obvious economic consequences. Column miniaturization also reduces the amount of stationary phase that is required, which is a special advantage in enantioseparation applications using CSPs, since these are quite expensive. A general advantage of micro-HPLC is the reduction of the sample amount that is required for analysis, which often can be a limiting factor for biological samples, e.g., in pharmacokinetic studies. In addition, micro-HPLC offers a higher eluted peak concentration and consequently a higher sensitivity for concentration-based detection methods due to the reduction of peak volume. However, this advantage can be seldom fully exploited due to a smaller detection cell volume or a shorter detection path. Other benefits of HPLC miniaturization include more efficient heat transfer, easier interfacing with mass spectrometry, simpler hyphenation with other microseparation techniques and the possibility of using the same column in micro-HPLC, CEC and SFC.

However, one must not overlook that micro-HPLC also has some drawbacks compared to its standard-size parent technology. Suitable apparatus that deliver appropriate micro-flow rates, have nanolitre injectors and detection flow cells are nowadays commercially available but more expensive than wide-bore analogues. Moreover, instrument miniaturization also can cause reduced system robustness. Finally, to exploit all the claimed advantages the system set-up needs to be optimized to avoid extra-column band broadening effects.

In the present paper we describe in a comparative way the enantiomer separation of alanine peptides consisting of up to six amino acid residues in their N-protected form employing five different protection groups having marked influence on the overall retention and enantioselectivity characteristics. These studies were carried out on a standard-size (4 mm ID) and micro (0.5 mm ID) column system containing a CSP relying on an ion-exchange principle (Fig. 1) [12,13]. Further, preliminary reproducibility data were acquired for the micro-HPLC column.

2. Experimental

2.1. Materials

(*R*)- and (*S*)-alanine were purchased from Sigma-Aldrich (Gillingham, UK) and the (all-*R*)- and (all-*S*)-enantiomers of the di-, tri-, tetra-, penta- and hexa-alanine peptides from Bachem (Bubendorf, Switzerland). 2,4-Dinitrofluorobenzene (Sanger's reagent) was obtained from Sigma-Aldrich, while *N*-(9-fluorenylmethoxycarbonyloxy)succinimide and carbazole-9-carbonyl chloride were from Fluka (Buchs, Switzerland). 3,5-Dinitrobenzoyloxy succinimide and 3,5-dinitrobenzyloxycarbonyloxy succinimide were prepared from the corresponding acid chlorides and hydroxysuccinimide according to standard protocols. All other reagents used were of analytical grade.

The preparation of the chiral selector, *tert*-butylcarbamoylquinine, and the corresponding CSP by subsequent coupling of the selector to thiol-modified silica gel (Fig. 1) has been described

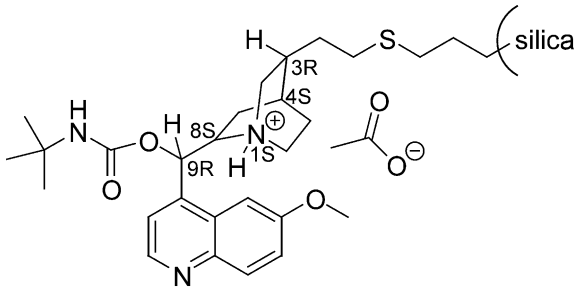

Fig. 1. Structure of *tert*-butylcarbamoylquinine CSP in its protonated form.

elsewhere [13]. The 5 μm CSP particles, commercialized as chiral AX-QN1 (Bischoff Chromatography, Leonberg, Germany), were slurry-packed into stainless steel columns of the dimensions 150 × 4.0 mm ID (Austrian Research Centers, Seibersdorf, Austria) for standard-size HPLC and 150 × 0.5 mm ID (Agilent Technologies, Waldbronn, Germany) for micro-HPLC, respectively.

2.2. Derivatization procedures

The following N-derivatives of alanine amino acid and peptide enantiomers were prepared: 3,5-dinitrobenzoyl (DNB), 2,4-dinitrophenyl (DNP), 3,5-dinitrobenzyloxycarbonyl (DNZ), carbazole-9-carbonyl (CC), and 9-fluorenylmethoxycarbonyl (FMOC). Thus, 5 μmol of the amino acids and peptides, respectively, were dissolved in carbonate buffer (sodium hydrogencarbonate (0.1 M)–sodium carbonate (0.1 M), 2:1, v/v) for the preparation of the DNB, DNP, DNZ and FMOC derivatives and in borate buffer (0.1 M $Na_2B_4O_7$) in the case of the CC derivatives.

DNB derivatization was carried out by adding 300 μl of a solution of 3,5-dinitrobenzoyloxy succinimide in acetonitrile (1.4%, w/v). The reaction was allowed to proceed at 50 °C over night.

DNP and FMOC derivatives were prepared by adding solutions of 2,4-dinitrofluorobenzene in acetonitrile (Sanger's reagent; 2.5%, w/v; 400 μl) and *N*-(9-fluorenylmethoxycarbonyl) succinimide in acetonitrile (2.5%, w/v; 300 μl), respectively, to the given aqueous peptide enantiomer solutions and reaction for 2 h at room temperature.

DNZ derivatives were obtained by adding 250 μl of a solution of 3,5-dinitrobenzyloxycarbonyloxy succinimide in 1,4-dioxane (2.5%, w/v). The mixture was then shaken vigorously at room temperature for 3 h.

For the preparation of the CC derivatives 300 μl of a freshly prepared solution of CC chloride in acetonitrile (0.5%, w/v) were added to the aqueous peptide solution. The resulting mixture was vigorously shaken. After reaction for 2 h at room temperature the reaction mixture was extracted twice with *n*-heptane in order to remove excess of reagent.

2.3. HPLC methods

The standard-size HPLC separations were carried out using an Agilent HP1090 series high-performance liquid chromatograph (Agilent Technologies) equipped with a 13 μl flow cell (10 mm optical path length), while the micro-HPLC separations were performed on an Agilent 1100 series capillary LC system with a 20 μl flow controller, 50 μm ID capillaries as connecting tubing and a 500 nl flow cell (10 mm optical path length). The hydro-organic mobile phase consisted of methanol–aqueous ammonium acetate (0.5 M) (80:20, v/v) and the apparent pH (pH*) was adjusted to 6.0 with glacial acetic acid. The polar-organic mobile phase consisted of acetonitrile–methanol (80:20, v/v) and contained 400 mM acetic acid and 4 mM triethylamine. The flow rate of the standard-size HPLC system was set to 1 ml/min, that of the micro-HPLC to 10 μl/min. All derivatization mixtures were diluted 5 times with mobile phase prior to injection, resulting in concentrations of approximately 1 mM. For the standard-size HPLC studies an aliquot of 50 μl was injected, while for the micro-HPLC investigations the injection volume was 250 nl. In both systems the columns were thermostated at 25 °C and the chromatograms were recorded by UV detection at 254 nm.

3. Results and discussion

Over the last years a diversified set of cinchona alkaloid based chiral selectors with various modifications has been developed and used for the chromatographic separation of amino acid [12–15] and peptide [16] enantiomers utilizing an ion-exchange principle and employing standard-size HPLC. The separation mechanism relies on various interactions between the chiral selector and the analyte, which have been described in detail previously [12,13] and include ionic attraction between the protonated quinuclidine nitrogen of the selector and the deprotonated carboxylic group of the analyte as well as hydrogen bonds between the selector's carbamate group and the analyte's amide group, π–π-interactions between

the aromatic systems of selector and analyte and steric interactions.

One of these selectors, namely *tert*-butylcarbamoylquinine (Fig. 1), yielded high enantioselectivities for N-protected amino acids in HPLC [13,14]. In a recent paper we also showed its usability as chiral ion-pairing agent added to the background electrolyte for the capillary electrophoresis separation of peptides with up to six amino acid residues [17].

In the present study we investigated the applicability of a micro-HPLC system equipped with a 0.5 mm ID column containing the 5 μm quinine-based CSP for peptide stereoisomer separations in order to evaluate pros and cons of downscaling of the well established method. Especially the application of this enantiodiscrimination method to biological samples would greatly profit by or even necessitate miniaturization of the separation system due to limited sample amounts. Besides comparing standard-size HPLC and micro-HPLC two different mobile phase conditions were also studied. While peptide enantiomer separations by HPLC have so far been restricted to di- and tripeptides, we report enantioselective separations of peptides containing up to six amino acid residues. Additionally, the separation of all four stereoisomers of alanylalanine was studied in order to obtain selectivity information for enantiomers as well as diastereomers.

For these studies alanine peptides with one to six amino acid residues, namely (all-*R*)– and (all-*S*)–Ala_n with *n* ranging from 1 to 6, were used as model substances to show the stereoselective potential of the CSP for peptide stereoisomers. It was necessary to derivatize the N-terminal amino group of the peptides in order to eliminate their zwitterionic nature and to give them an acidic character to be retained on the chiral anion exchanger. In addition, the protection group takes part in the overall molecular recognition mechanism of the chiral selector and provides the chromophore necessary for sensitive UV detection. For this purpose five different protection groups were employed. The structures of the peptide analytes and the N-protection groups are shown in Fig. 2.

3.1. Separation of alanine peptide stereoisomers with five different protection groups using micro-HPLC and standard-size HPLC

The (all-*R*)-/(all-*S*)-enantiomer separations of the alanine peptide series derivatized with five different N-protection groups (Fig. 2) were carried out on the standard-size HPLC system and the micro-HPLC. In addition, the separation of all four stereoisomers of the alanine dipeptide was also investigated in dependence of the N-terminal protection group. Like for amino acid enantiomer separations [12,13,15], it was supposed that structural variations of the N-terminal label, which may represent a potential binding motif with complimentary interaction sites of the selector, is an effective way to manipulate and fine-tune stereoselectivity. The separations were performed employing two different mobile phases, a hydro-organic one as well as a polar-organic one in order to evaluate the contribution of water to the overall retention characteristics and the counterbalance of organic acids and bases to adjust retention based on the ion-exchange mechanism.

In Tables 1–3 the results obtained with the standard-size HPLC system using a column with 4.0 mm ID and the miniaturized system employing a 0.5 mm ID column are compared. As expected, the variations of retention factors and selectivities with increasing peptide length are congruent for the two systems as completely identical CSPs are utilized. For example, the enantioselectivities obtained for the DNB-derivatized amino acid and dipeptide were 7.61/6.87 (standard-HPLC/micro-HPLC) and 5.69/5.01, respectively, while a pronounced drop of selectivity was noticed when proceeding to the tripeptide, for which an α-value of 1.56/1.45 was achieved. Overall, the miniaturization of the HPLC system is well possible without a significant loss in enantiomer resolution with only minor alterations of resolution values that can mainly be attributed to differences in the performances of the columns.

Fig. 3 shows representative chromatograms obtained with standard-size HPLC and corresponding micro-HPLC and highlights one of the advantages of micro-HPLC, namely the pronounced reduction in the required sample amount.

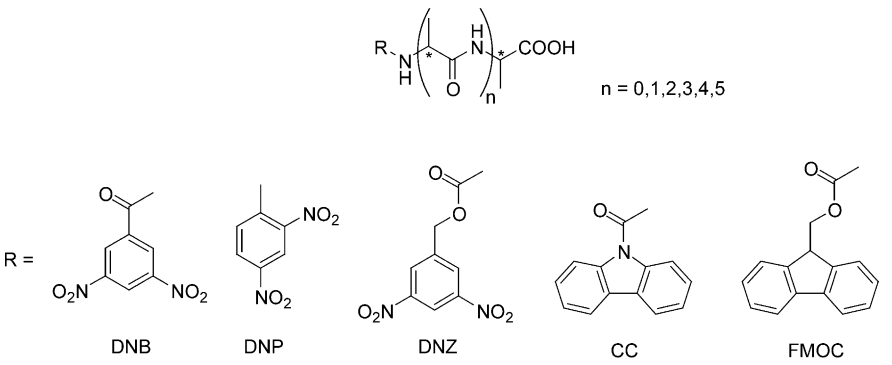

Fig. 2. Structure of investigated analytes with different N-protection groups R.

Although the micro-HPLC detector that had a detection cell volume by a factor of 26 smaller but same optical path length has a higher noise level than its standard-size counterpart, a 50 ng amount of sample (25 ng each enantiomer) yielded nearly as large peaks as a 10 μg amount of sample injected in standard-size HPLC, which corresponds to a factor of 200 lower amount injected onto the column. To conclude, if enantioselectivity is high enough ($\approx \alpha > 1.1$), miniaturization of the investigated separation system is possible without a significant change of enantiomer separation characteristics, while the advantages of smaller columns can be gained.

From Tables 1–3 it is clearly seen that the choice of protection group has a very pronounced influence on enantiodiscrimination. This is particularly highlighted by the case of DNP-Ala, where the elution order is even reversed when compared with the other derivatives as well as with the other species in the DNP-series, i.e., the peptide congeners. All *N*-carbonyl type derivatives (DNB, DNZ, CC and FMOC) exhibit the same enantiomer elution order and a similar tendency in enantioselectivity decrease with growing peptide length. This finding points towards a similar binding mechanism of these derivatives, which differ mainly by the strength of π–π-interactions with the quinoline ring of the chiral selector (DNB ≫ DNZ > CC ∼ FMOC) and/or the conformational flexibility, which in turn influences the magnitude of enantioselectivity. For example, the following α-values were obtained for the alanine tripeptide: 1.56/1.45 (standard-HPLC/micro-HPLC) with the DNB-label, 1.23/1.23 with the DNZ-label, 1.12/1.11 with the CC-label and 1.15/1.14 with the FMOC-label. DNP-Ala, on the other hand, lacks the *N*-carbonyl group, which leads to a different enantiodiscrimination process; contrary to all other N-derivatives the (*S*)-enantiomer is eluted first. The DNP-derivatized peptides possess—like the *N*-carbonyl derivatives of the amino acids—the amide group at the C-terminus, which enables the hydrogen-bonding mediated selector–analyte interactions again with stronger retention for the (all-*S*)-enantiomers. The enantioselectivities obtained for the DNP-derivatives from the dipeptide onwards are at a similar level as those of the CC and FMOC derivatives. Overall, the DNB-protection groups yields the highest enantioselectivities with especially high discrimination for the amino acid and dipeptide enantiomers. Baseline resolutions are obtained also for tri- and tetrapeptide enantiomers, while the penta- and hexa-alanine analogues are only partially resolved and require further optimization of separation conditions (Tables 1 and 2).

While from a scientific viewpoint it may be worthwhile and challenging as well as interesting in terms of molecular recognition to develop separation methods for the resolution of enantiomers of longer peptides (e.g. with more than four amino acid residues), the probability for their practical relevance is diminished with increasing number of amino acid residues. In contrary, a practically more realistic scenario is the separation

Table 1
Comparison of standard-size HPLC and micro-HPLC for hydro-organic mobile phase conditions

Protection group	Peptide	Standard-size HPLC			Micro-HPLC			Elution order
		k_1	α	R_s[a]	k_1	α	R_s[a]	
3,5-Dinitrobenzoyl (DNB)	Ala	4.11	7.61	19.81	4.01	6.87	20.26	(*R*) < (*S*)
	Ala_2	3.34	5.69	15.57	2.95	5.01	16.19	(all-*R*) < (all-*S*)
	Ala_3	2.05	1.56	3.78	1.76	1.45	3.54	(all-*R*) < (all-*S*)
	Ala_4	1.68	1.32	2.12	1.43	1.23	1.82	(all-*R*) < (all-*S*)
	Ala_5	1.46	1.21	1.28	1.22	1.14	1.01	(all-*R*) < (all-*S*)
	Ala_6	1.33	1.14	0.75	1.12	1.06	0.40	(all-*R*) < (all-*S*)
2,4-Dinitrophenyl (DNP)	Ala	8.33	1.19	2.57	10.67	1.18	2.43	(*S*) < (*R*)
	Ala_2	6.21	1.52	4.92	6.71	1.54	5.30	(all-*R*) < (all-*S*)
	Ala_3	4.31	1.08	0.69	4.20	1.06	0.63	(all-*R*) < (all-*S*)
	Ala_4	2.99	1.09	0.76	2.70	1.08	0.69	(all-*R*) < (all-*S*)
	Ala_5	2.60	1.07	0.58	2.29	1.05	0.45	(all-*R*) < (all-*S*)
	Ala_6	2.41	1.06	0.24	1.97	1.09	0.69	(all-*R*) < (all-*S*)
3,5-Dinitrobenzyloxycarbonyl (DNZ)	Ala	3.81	1.80	6.98	4.10	1.78	7.28	(*R*) < (*S*)
	Ala_2	2.72	1.66	5.06	2.66	1.65	5.57	(all-*R*) < (all-*S*)
	Ala_3	1.77	1.23	1.71	1.61	1.23	1.94	(all-*R*) < (all-*S*)
	Ala_4	1.47	1.18	1.27	1.29	1.15	1.19	(all-*R*) < (all-*S*)
	Ala_5	1.30	1.16	0.81	1.14	1.13	0.88	(all-*R*) < (all-*S*)
	Ala_6	–[b]	–[b]	–[b]	–[b]	–[b]	–[b]	–
Carbazol-9-carbonyl (CC)	Ala	7.96	1.26	3.08	8.60	1.24	2.88	(*R*) < (*S*)
	Ala_2	4.39	1.29	2.86	4.03	1.29	2.95	(all-*R*) < (all-*S*)
	Ala_3	2.84	1.12	1.08	2.34	1.11	1.01	(all-*R*) < (all-*S*)
	Ala_4	2.13	1.11	0.92	1.66	1.10	0.81	(all-*R*) < (all-*S*)
	Ala_5	1.79	1.12	0.80	1.38	1.09	0.68	(all-*R*) < (all-*S*)
	Ala_6	1.19	1.19	1.20	1.38	1.00	0.00	(all-*R*) < (all-*S*)
9-Fluorenylmethoxycarbonyl (FMOC)	Ala	4.63	1.42	4.14	4.40	1.43	4.39	(*R*) < (*S*)
	Ala_2	3.25	1.39	3.33	2.82	1.39	3.37	(all-*R*) < (all-*S*)
	Ala_3	2.06	1.15	1.24	1.62	1.14	1.24	(all-*R*) < (all-*S*)
	Ala_4	1.66	1.15	1.13	1.27	1.14	1.07	(all-*R*) < (all-*S*)
	Ala_5	1.47	1.14	0.55	1.11	1.11	–[c]	(all-*R*) < (all-*S*)
	Ala_6	1.55	1.03	0.18	–[b]	–[b]	–[b]	(all-*R*) < (all-*S*)

[a] Resolution $R_s = 1.18(t_2 - t_1)/(w_{b,1} + w_{b,2})$.
[b] Coelution with reagent peak.
[c] Could not be calculated.

of diastereomers or epimers e.g., of dipeptides, for example when one of the stereogenic centers is inverted due to a racemization process. As can be seen from Fig. 4 and Table 3, the quinine carbamate based chiral anion exchanger exhibits besides high enantioselectivity also reasonable diastereoselectivity which is exemplified for the four Ala–Ala dipeptide stereoisomers (at this point, it is also noted that the separation conditions are not fully optimized in view of diastereoselectivity). Again the N-protection group plays a major role for the molecular recognition mechanism and thus for enantioselectivity and diastereoselectivity as well (compare Fig. 4a–d). The elution order of the four stereoisomers of Ala–Ala (Table 3) shows an interesting picture: In any case the (*R*,*R*)-enantiomer is eluted before the (*S*,*S*)-enantiomer. Regarding the (*R*,*S*)/(*S*,*R*) enantiomeric pairs, it is the same for DNB, DNZ and FMOC with the (*R*,*S*)-isomers eluting before the (*S*,*R*) ones (Fig. 4a–c), while the elution order of this enantiomeric pair is reversed for the DNP-

Table 2
Comparison of standard-size HPLC and micro-HPLC for polar-organic mobile phase conditions

Protection group	Peptide	Standard-size HPLC			Micro-HPLC			Elution order
		k_1	α	R_s[a]	k_1	α	R_s[a]	
3,5-Dinitrobenzoyl (DNB)	Ala	6.31	6.19	28.06	5.81	5.62	23.61	(*R*) < (*S*)
	Ala_2	4.90	4.07	19.90	4.39	3.60	16.68	(all-*R*) < (all-*S*)
	Ala_3	3.36	1.45	5.16	2.99	1.34	3.77	(all-*R*) < (all-*S*)
	Ala_4	2.98	1.24	2.76	2.62	1.15	1.67	(all-*R*) < (all-*S*)
	Ala_5	2.75	1.07	0.75	2.40	1.00	0.00	(all-*R*) < (all-*S*)
	Ala_6	2.77	1.00	0.00	2.43	1.00	0.00	–
2,4-Dinitrophenyl (DNP)	Ala	17.09	1.15	3.01	16.88	1.16	2.84	(*S*) < (*R*)
	Ala_2	6.05	1.39	5.34	5.34	1.32	4.08	(all-*R*) < (all-*S*)
	Ala_3	4.43	1.12	1.71	3.89	1.06	0.75	(all-*R*) < (all-*S*)
	Ala_4	3.47	1.12	1.50	3.01	1.07	0.85	(all-*R*) < (all-*S*)
	Ala_5	3.10	1.08	0.94	2.73	1.00	0.00	(all-*R*) < (all-*S*)
	Ala_6	2.85	1.06	0.62	2.49	1.00	0.00	(all-*R*) < (all-*S*)
3,5-Dinitrobenzyloxycarbonyl (DNZ)	Ala	3.97	1.70	8.47	3.41	1.66	7.29	(*R*) < (*S*)
	Ala_2	3.85	1.55	6.50	3.25	1.52	5.60	(all-*R*) < (all-*S*)
	Ala_3	2.62	1.22	2.54	2.22	1.19	2.13	(all-*R*) < (all-*S*)
	Ala_4	2.23	1.16	1.73	1.88	1.13	1.38	(all-*R*) < (all-*S*)
	Ala_5	2.00	1.09	0.84	1.73	1.04	–[b]	(all-*R*) < (all-*S*)
	Ala_6	–[c]	–[c]	–[c]	1.59	1.00	0.00	–
Carbazol-9-carbonyl (CC)	Ala	10.44	1.13	2.27	9.70	1.10	1.52	(*R*) < (*S*)
	Ala_2	5.14	1.20	2.93	4.48	1.15	1.96	(all-*R*) < (all-*S*)
	Ala_3	4.04	1.10	1.36	3.45	1.06	0.65	(all-*R*) < (all-*S*)
	Ala_4	3.16	1.08	0.95	2.68	1.01	0.15	(all-*R*) < (all-*S*)
	Ala_5	2.66	1.05	0.56	2.25	1.00	0.00	(all-*R*) < (all-*S*)
	Ala_6	–[c]	–[c]	–[c]	2.00	1.00	0.00	–
9-Fluorenylmethoxycarbonyl (FMOC)	Ala	3.33	1.34	4.06	3.67	1.25	3.25	(*R*) < (*S*)
	Ala_2	3.52	1.39	4.60	3.54	1.22	2.71	(all-*R*) < (all-*S*)
	Ala_3	2.70	1.17	1.91	2.50	1.06	0.66	(all-*R*) < (all-*S*)
	Ala_4	2.34	1.16	1.66	2.10	1.04	0.49	(all-*R*) < (all-*S*)
	Ala_5	2.12	1.12	0.89	1.87	1.00	0.00	–
	Ala_6	–[c]	–[c]	–[c]	1.71	1.00	0.00	–

[a] Resolution $R_s = 1.18(t_2 - t_1)/(w_{b,1} + w_{b,2})$.
[b] Could not be calculated.
[c] Coelution with reagent peak.

and CC-derivatized samples (Fig. 4d). Differences also exist for the elution orders of the diastereomers. The α-values are larger for the (*R*,*R*)/(*S*,*S*)-pairs than of the (*R*,*S*)/(*S*,*R*)-pairs throughout except for the DNP-derivative (Table 3). The diastereoselectivity appears to be highest for DNB- and DNP-derivatives with the hydro-organic mobile phase (Table 3) which allows full baseline resolution of all four stereoisomers (Fig. 5).

3.2. Comparison of hydro-organic and polar-organic mobile phases

From initial experiments with a hydro-organic mobile phase it became evident that peptide enantiomers with more than four amino acid residues would be difficult to be resolved. Since selector–analyte interactions are widely of electrostatic nature in the given separation system, non-aqueous (polar-organic) conditions and less polar

Table 3
Standard-size HPLC versus micro-HPLC for the separation of all four stereoisomers of RS–Ala–RS–Ala derivatized with different protection groups and using two different mobile phases

	Mobile phase	Sample	k_1	k_2	k_3	k_4	$R_{S,1/2}$	$R_{S,2/3}$	$R_{S,3/4}$	$\alpha_{RR/SS}$[a]	$\alpha_{RS/SR}$[b]	Elution order
Standard-HPLC	Hydro-organic (methanol–ammonium acetate 80:20 v/v)	DNB–RS–Ala–RS–Ala	3.40	4.41	13.70	19.44	2.64	10.71	3.34	5.71	3.11	$(R,R)<(R,S)<(S,R)<(S,S)$
		DNP–RS–Ala–RS–Ala	5.37	6.23	9.07	14.91	1.66	3.65	7.00	1.57	2.39[c]	$(R,R)<(S,R)<(S,S)<(R,S)$
		DNZ–RS–Ala–RS–Ala	2.34	2.76	3.58	4.02	1.61	2.62	1.18	1.72	1.30	$(R,R)<(R,S)<(S,R)<(S,S)$
		CC–RS–Ala–RS–Ala	3.67	4.87	4.87	4.87	2.46	0.00	0.00	1.33	1.00	$(R,R)<(S,S),(S,R),(R,S)$
		FMOC–RS–Ala–RS–Ala	2.68	3.10	3.47	3.84	1.51	1.35	1.10	1.43	1.12	$(R,R)<(R,S)<(S,R)<(S,S)$
	Polar-organic (acetonitrile–methanol 80:20 v/v, 400 mM acetic acid, 4 mM triethylamine)	DNB–RS–Ala–RS–Ala	4.91	5.83	20.16	20.88	2.72	18.73	0.59	4.1	3.58	$(R,R)<(R,S)<(S,S)<(S,R)$
		DNP–RS–Ala–RS–Ala	6.07	7.94	8.47	24.01	4.25	1.10	17.51	1.39	3.02[c]	$(R,R)<(S,R)<(S,S)<(R,S)$
		DNZ–RS–Ala–RS–Ala	3.87	4.49	6.04	6.98	2.20	4.46	2.24	1.56	1.55	$(R,R)<(R,S)<(S,S)<(S,R)$
		CC–RS–Ala–RS–Ala	5.15	6.19	6.90	7.35	2.88	1.72	1.01	1.20	1.07[c]	$(R,R)<(S,S)<(S,R)<(R,S)$
		FMOC–RS–Ala–RS–Ala	3.53	4.32	4.89	5.20	2.88	1.59	0.72	1.39	1.20	$(R,R)<(R,S)<(S,S)<(S,R)$
Micro-HPLC	Hydroorganic (methanol–ammonium acetate 80:20 v/v)	DNB–RS–Ala–RS–Ala	2.76	3.55	10.36	14.49	2.79	12.10	4.20	5.24	2.92	$(R,R)<(R,S)<(S,R)<(S,S)$
		DNP–RS–Ala–RS–Ala	6.72	8.27	10.39	19.83	2.54	3.01	8.48	1.55	2.40[c]	$(R,R)<(S,R)<(S,S)<(R,S)$
		DNZ–RS–Ala–RS–Ala	2.66	3.09	4.17	4.38	1.67	3.49	0.55	1.65	1.35	$(R,R)<(R,S)<(S,R)<(S,S)$
		CC–RS–Ala–RS–Ala	4.06	5.30	5.47	5.53	3.86	–[d]	–[d]	1.31	1.01[c]	$(R,R)<(S,S)<(S,R)<(R,S)$
		FMOC–RS–Ala–RS–Ala	2.81	3.15	3.66	3.85	1.22	1.55	0.57	1.37	1.16	$(R,R)<(R,S)<(S,R)<(S,S)$
	Purely organic (acetonitrile–methanol 80:20 v/v, 400 mM acetic acid, 4 mM triethylamine)	DNB–RS–Ala–RS–Ala	4.43	5.09	16.19	17.43	2.04	15.45	1.11	3.65	3.42	$(R,R)<(R,S)<(S,S)<(S,R)$
		DNP–RS–Ala–RS–Ala	5.33	7.06	7.06	19.64	3.89	0.00	14.80	1.33	2.78[c]	$(R,R)<(S,R)<(S,S)<(R,S)$
		DNZ–RS–Ala–RS–Ala	3.29	3.75	4.98	5.74	1.83	3.94	2.01	1.52	1.53	$(R,R)<(R,S)<(S,S)<(S,R)$
		CC–RS–Ala–RS–Ala	4.50	5.15	5.93	6.25	1.95	1.90	0.66	1.14	1.05[c]	$(R,R)<(S,S),(S,R)<(R,S)$
		FMOC–RS–Ala–RS–Ala	3.55	4.07	4.31	5.07	1.72	0.68	2.17	1.21	1.25	$(R,R)<(R,S)<(S,S)<(S,R)$

[a] $\alpha_{RR/SS} = k_{SS}/k_{RR}$.
[b] $\alpha_{RS/SR} = k_{SR}/k_{RS}$.
[c] $\alpha_{SR/RS} = k_{RS}/k_{SR}$.
[d] not determined.

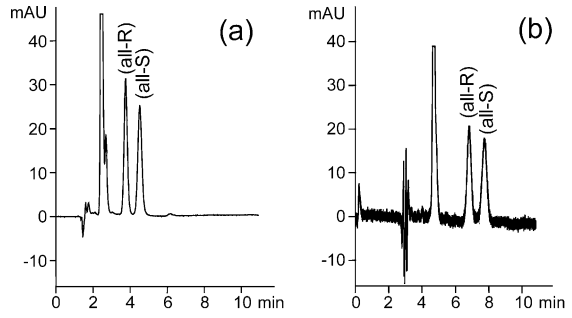

Fig. 3. Chromatograms of DNB-Ala_4 enantiomer separations acquired on standard-size HPLC and micro-HPLC. Experimental conditions: *tert*-butylcarbamoylquinine CSP; mobile phase: methanol–aqueous ammonium acetate (0.5M) (80:20, v/v), pH* 6.0; 25 °C. (a) Standard-size HPLC: 150 × 4.0 mm ID; flow rate: 1 ml/min; injection volume: 50 μl (10 μg). (b) Micro-HPLC: 150 × 0.5 mm ID; flow rate: 10 μl/min; injection volume: 250 nl (50 ng).

media compared to hydro-organic mode, respectively, were conceived as possibly better eluents, as they favour electrostatic interactions.

The comparison of the enantiomer separation results obtained with the hydro-organic and the polar-organic mobile phases (Table 1 versus Tables 2 and 3) reveals that the chiral discrimination mechanism is unaffected by the choice of the mobile phase, since both the enantiomer elution orders as well as the orders of magnitude of enantioselectivity remain the same. In general, the presently selected polar-organic conditions increase retention despite a high concentration of counterions (Fig. 6a), while yielding slightly lower selectivities at the same time (Fig. 6b), both adverse effects. In a number of cases, especially for the amino acid and shorter peptides, the slightly lower enantioselectivities are, however, largely compensated by the higher efficiencies obtained with the polar-organic mode, resulting in similar resolutions. The change of the mobile phase composition also reverses the elution order of the (*S*,*R*)- and (*S*,*S*)-stereoisomers of Ala–Ala derivatized with DNB, DNZ and FMOC (Table 3).

Therefore, of the two mobile phases that have been evaluated, the hydro-organic one seems better suited for the separation of the N-protected

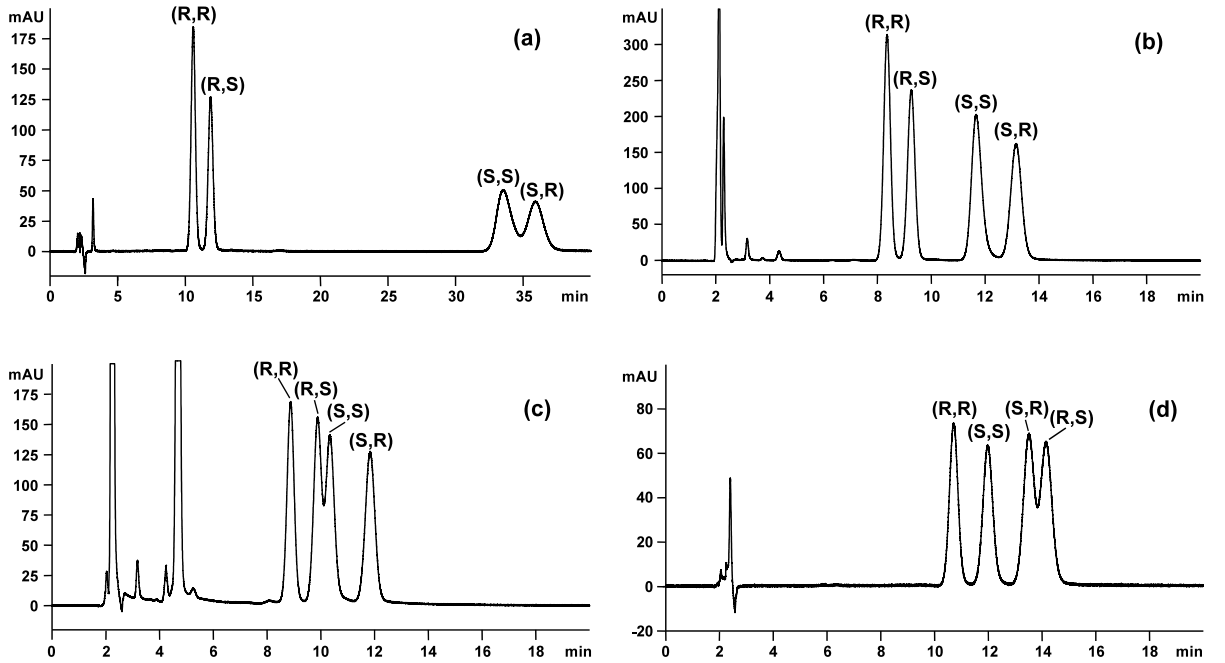

Fig. 4. Separation of all four stereoisomers of RS–Ala–RS–Ala N-protected with (a) DNB-group; (b) DNZ-group; (c) FMOC-group and (d) CC-group. Experimental conditions: micro-HPLC; *tert*-butylcarbamoylquinine CSP (150 × 0.5 mm); mobile phase: acetonitrile–methanol (80:20 v/v), 400 mM acetic acid, 4 mM triethylamine; flow rate 10 μl/min; injection volume: 250 nl; 25 °C.

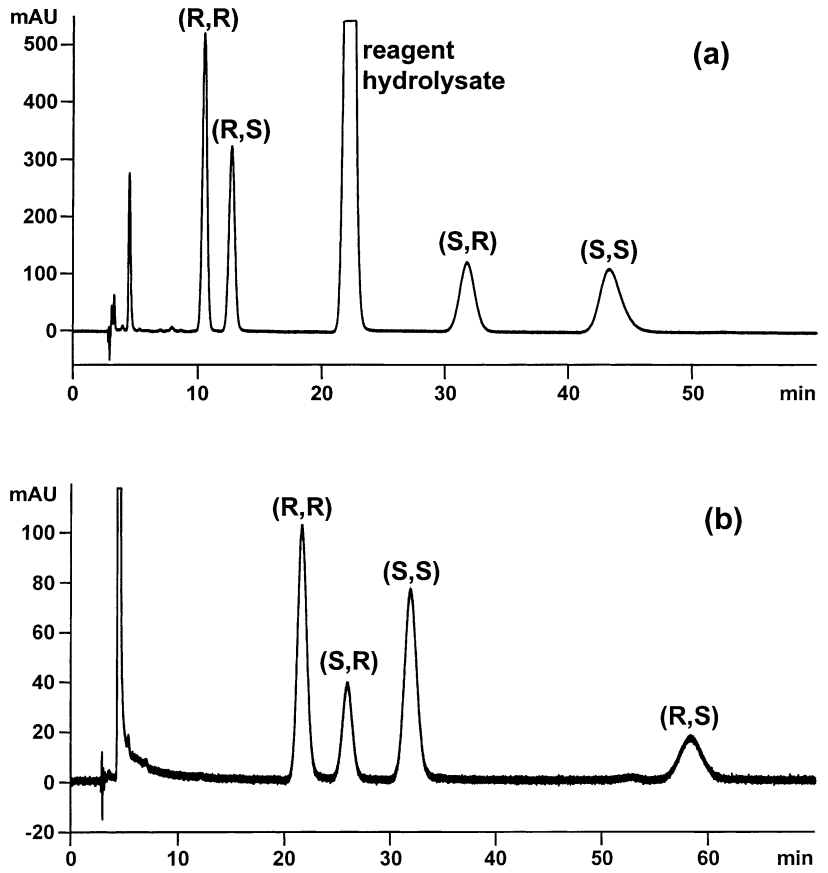

Fig. 5. Full baseline separation of all four stereoisomers of (a) DNB–RS–Ala–RS–Ala and (b) DNP–RS–Ala–RS–Ala. Experimental conditions: micro-HPLC; *tert*-butylcarbamoylquinine CSP (150 × 0.5 mm); mobile phase: methanol–aqueous ammonium acetate (0.5 M) (80:20, v/v), pH* 6.0; flow rate 10 μl/min; injection volume: 250 nl; 25 °C.

alanine peptide enantiomers, especially for the longer species.

3.3. Reproducibility of micro-column packing

The characteristics of three micro-HPLC columns steming from one batch of CSP were compared, in order to determine whether the column packing was reproducible enough to yield column-to-column repeatabilities good enough to become a commercial product. For this study the DNB-derivatives of the alanine peptide series (DNB-Ala_{1-6}) were used as test samples. The results of the comparison are presented in Table 4. The retention factors and selectivities obtained with the different columns are in good agreement with each other with RSDs for the selectivities below or around 1%. Thus, column-to-column reproducibility of the micro-columns is quite acceptable and can meet the requirements of validated assays.

4. Conclusions

The separation of alanine peptide stereoisomers was carried out employing a standard-size HPLC and a micro-HPLC system. Concerning the separation characteristics no significant differences exist between the two systems, therefore the advantages of micro-HPLC, especially reduction of sample amount, stationary and mobile phases,

Table 4
Evaluation of the reproducibility of the packing of micro-HPLC columns

Sample	Column 1				Column 2				Column 3				RSD (%)			
	t_1	k_1	α	R_S	t_1	k_1	α	R_S	t_1	k_1	α	R_S	t_1	k_1	α	R_S
DNB-Ala	14.53	4.19	6.92	20.40	15.07	4.20	6.90	20.10	14.02	4.01	6.87	20.26	3.62	2.59	0.36	0.74
DNB-Ala_2	11.36	3.06	5.03	16.24	11.79	3.06	4.97	16.21	11.06	2.95	5.01	16.19	3.21	2.10	0.61	0.16
DNB-Ala_3	7.83	1.80	1.43	3.46	8.11	1.80	1.43	3.43	7.73	1.76	1.45	3.54	2.50	1.29	0.80	1.64
DNB-Ala_4	6.82	1.43	1.23	1.82	7.06	1.44	1.23	1.81	6.79	1.43	1.23	1.82	2.17	0.40	0.00	0.32
DNB-Ala_5	6.28	1.24	1.14	0.96	6.49	1.24	1.12	0.85	6.20	1.22	1.14	1.01	2.35	0.94	1.02	8.71
DNB-Ala_6	5.99	1.14	1.05	0.41	6.20	1.14	1.04	0.39	5.93	1.12	1.06	0.40	2.36	1.02	0.95	2.50

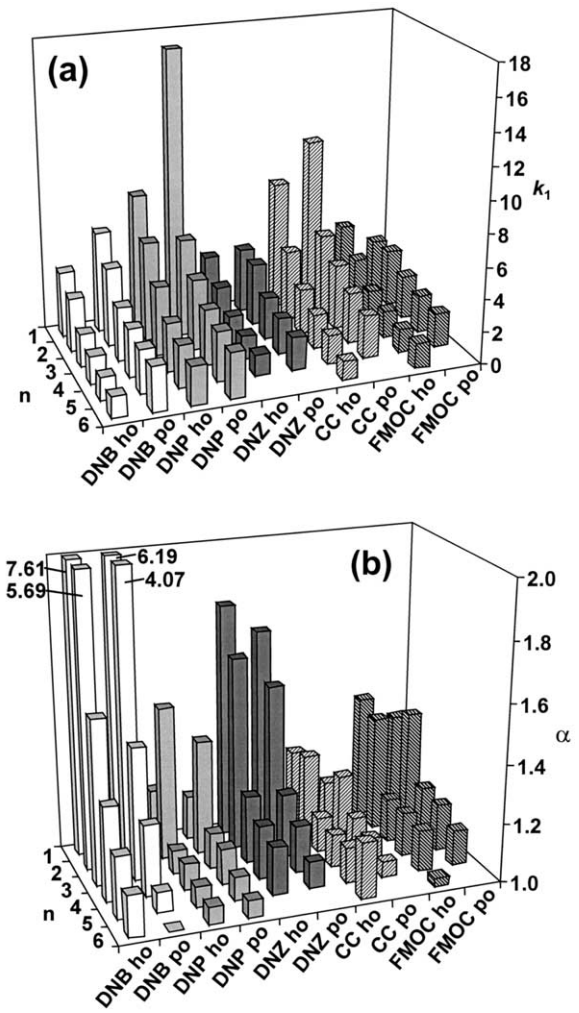

Fig. 6. Comparison of hydro-organic (ho) and polar-organic (po) conditions: (a) retention factor of the first eluted enantiomer k_1; (b) selectivity α (standard-HPLC). n, number of amino acid residues.

can be easily acquired. The reproducibility of the column packing, a vital parameter for the successful use of micro-HPLC, was shown to be satisfactory as well. It was found that the choice of the N-terminal protection group has a large influence on stereoselectivity: besides a possible change of the elution order of both enantio- and diastereomers the magnitude of enantioselectivity strongly depends on the type of derivative. The change from a hydro-organic mobile phase to a polar-organic one did not change the chiral recognition mechanism, but it had effects on overall retention time and selectivity.

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