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Computerized optimization of the high-performance liquid chromatographic enantioseparation of a mixture of 4-dinitrophenyl amino acids on a quinine carbamate-type chiral stationary phase using DRYLAB

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Abstract

A method is proposed for the sensitive chiral analysis of amino acid enantiomers by high-performance liquid chromatography (HPLC). Thus the enantiomers of a mixture of seven racemic amino acids were resolved as their DNP derivatives from each other and from the peak of the hydrolyzed reagent, employing a quinine carbamate-based chiral anion exchange-type chiral stationary phase (CSP) and aqueous buffered mobile phases. However, the initial isocratic chromatogram yielded many peak overlaps although the corresponding enantiomers were well resolved. Therefore, the separation of the complex mixture had to be optimized; we utilized the commercial computer method development software DRYLAB. First, the influence of the manifold mobile phase parameters and chromatographic conditions (pH, type and content of organic modifier, buffer concentration, temperature, type of co-ion, etc.) on retention and resolution was studied by isocratic elution. Furthermore, with such optimized conditions linear and multi-segmented organic modifier and buffer salt gradients, respectively, were simulated with the computer program and experimentally verified. Average errors of prediction of retention times lay between 2 and 8%. Finally, a highly improved HPLC gradient method resulted in almost all components being baseline separated and equally spaced and accelerated by a factor of more than 3 compared to the initial run.

Keywords: Enantiomer separation; Amino acids; 4-Dinitrophenylamino acids

1. Introduction

Nowadays, separation of enantiomers in liquid chromatography is preferentially performed by direct high-performance liquid chromatographic (HPLC) approaches employing chiral stationary phases (CSPs), either with or without achiral derivatization of the analytes [1]. However, although the selection of an appropriate CSP is the essential prerequisite for a successful separation of the given pairs of enantiomers, the resources of CSPs have not been exhausted in many instances. Thus the potential of several CSPs might be even better exploited if the chromatographic conditions are optimized more systematically.

However, this, in fact, would be a time-consuming and troublesome task since HPLC in general and enantioseparation by HPLC in particular are multifactorial processes. Besides selection of the adequate CSP a large number of other experimental

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conditions may possibly influence (non-)stereoselective retention and resolution factors significantly. With increasing number of (perhaps interrelated) factors, selection of optimal experimental conditions becomes more and more complicated. This problem is often intuitively treated with a trial and error strategy by varying only a few variables on the basis of experience or, most often, with the "one factor at a time" procedure. In both cases, a large number of experimental runs are required; nevertheless, the optimum is very often not met.

To assist chromatographers in this endeavour, a variety of HPLC method development schemes have been described [2–8]. Some of them have been commercialized as computer simulation software packages to facilitate the laborious task of method development, to reduce the number of experiments, to spare time and materials and thus costs. Additionally, the "best separation" concerning sufficient resolution and short analysis time, as well as rugged method may easier be obtained. Among these packages the most popular is DRYLAB. The theory of this software package and some basic considerations for the application have been well documented [9–14]. This computer simulation program aims at a significant reduction of experimental runs. It has been successfully applied to the optimization of reversed-phase (RP) HPLC separations of complex mixtures in isocratic as well as gradient elution mode [15–20]. Nevertheless, no application was found in the literature that involved DRYLAB for the optimization of direct chromatographic separations of enantiomers upon chiral stationary phases.

Recently, we reported the preparation of quinine and quinidine carbamate based CSPs and their application as chiral anion-exchangers for the separation of chiral acids, including N-derivatized amino acids, using aqueous buffered mobile phases [21]. A concept for the sensitive analysis of amino acids' enantiomeric composition combining such chiral anion-exchangers and achiral derivatization with Sanger's reagent (2,4-dinitrofluorobenzene, DNFB) was presented [22] and was adopted for this study. For this experiment, seven pairs of N-(2,4-dinitrophenyl)amino acid (DNP-AA) enantiomers, resulting from the derivatization of a mixture of racemic alanine (Ala), valine (Val), leucine (Leu), threonine (Thr), proline (Pro), phenylalanine (Phe) and tryptophan (Trp) (see Fig. 1 and Table 1) with Sanger's reagent, should be resolved from each other and from the peak of the hydrolyzed derivatizing agent (2,4-dinitrophenol, DNP-OH, which sums up to fifteen components) using a quinine carbamate type CSP (see Fig. 1) with an aqueous buffered mobile phase. The goal was to elute as many bands as possible, with satisfactory resolution and almost equal band spacing, within reasonable run time and as little organic modifier consumption as possible.

Thus one faces two separation problems. First, the chiral separation of the corresponding DNP-amino acid enantiomers and second the achiral separation of the different amino acid derivatives.

Chiral separation, in this case, is easily achieved by the utilized CSP, a weak chiral anion-exchanger, which possesses high stereodiscriminating capability for DNP-amino acid enantiomers [21,22]. As already emphasized, it is operated with aqueous buffered mobile phases. At the usual working pH the tertiary amine of the quinuclidine ring is protonated. Thus strong non-directed, long ranged ionic type interactions between the selector (SO), the quinine carbamate type chiral auxiliary and the ionized selectands (SA), the DNP-amino acids, turned out as primary binding and/or retention mechanism (see Fig. 1). Additional more specific intermolecular SO–SA interactions may occur as $\pi-\pi$ interaction of the π-acidic dinitrophenyl group with the π-basic quinoline ring. These SO–SA binding forces, overall, differ markedly in strength with respect to the corresponding enantiomers. Thus reversible diastereomeric SO–SA complexes of different stability or association constant are generated to which the difference in retention time of the both corresponding optical antipodes may be attributed. Spectroscopic investigations led to a SO–SA interaction model as illustrated in Fig. 1. The primary ion-exchange type retention mechanism is considerably accompanied by a hydrophobic component or non-specific adsorption to the lipophilized silica surface in the way of common reversed-phase chromatography yielding a mixed mode retention mechanism. However, this hydrophobic adsorption component is, at the usual working pH, of minor importance related to ion-exchange.

Tagging the amino acids with the DNP-label is necessary with regard to chiral separability of the

Table 1
Racemic DNP-amino acids (DNP-AAs) under investigation and their chromatographic data corresponding to the preliminary experimental run under isocratic conditions[a]

No.	AA	R[b]	k'[c]	α[d]	R_S[e]	N[f]
1	L-Ala	Methyl-	27.34			3927
				1.28	3.45	
2	D-Ala		35.08			2834
3	L-Val	Isopropyl-	21.84			3134
				1.47	5.11	
4	D-Val		31.99			3181
5	L-Leu	Isobutyl-	23.87			3191
				1.44	4.97	
6	D-Leu		34.35			3387
7	L-Thr	1-Hydroxyethyl-	19.75			3028
				1.58	6.23	
8	D-Thr		31.22			3541
9	L-Pro	See Fig. 1	23.09			2716
				1.57	5.90	
10	D-Pro		36.33			3191
11	L-Phe	Benzyl-	43.84			2997
				1.31	3.78	
12	D-Phe		57.54			3532
13	L-Trp	3-Indolyl-	81.81			3091
				1.44	5.01	
14	D-Trp		118.15			3069
15	DNP-OH		36.44			4800

[a] CSP: see Fig. 1 and Experimental; mobile phase: MeOH–ammonium acetate (80:20, v/v), molarity of the final mixture is 20 mM; pH_a 6.0; T=25°C.
[b] See Fig. 1.
[c] $k'=(t_R-1.83)/1.83$.
[d] $\alpha=k_{(D)}'/k_{(L)}'$.
[e] $R_S=1.18\ (t_{R\,(D)}-t_{R\,(L)})/(w_{1/2\,(D)}+w_{1/2\,(L)})$.
[f] $N=5.54\ (t_R/w_{1/2})^2$.

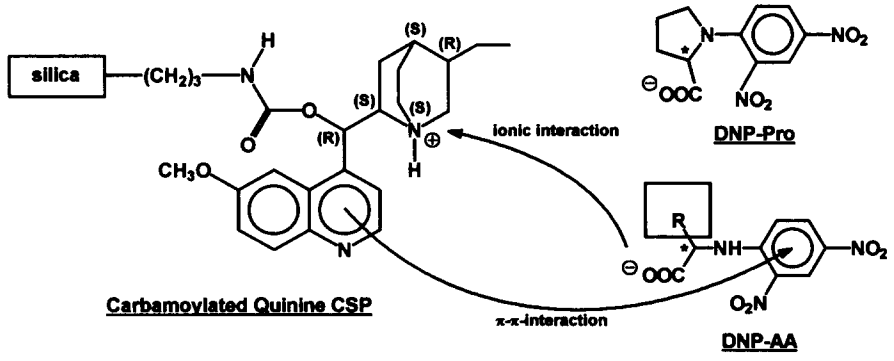

Fig. 1. Structure of the chiral stationary phase (CSP) as well as of the analytes (see also Table 1) and proposed sites of specific intermolecular interaction between the selector (SO) and the selectand (SA).

analytes on the quinine carbamate based chiral anion-exchanger. However, this derivatization strategy obviously resembles the chromatographic behaviour of the different AA derivatives with respect to achiral separation on the CSP. While these cinchona alkaloid based CSPs are highly selective for chiral separations of N-derivatized amino acids, their achiral selectivity and efficiency is significantly less pronounced. Thus, for a mixture of racemic DNP-AAs the separation of the different amino acid derivatives is probably the major challenge for the given task.

On the basis of the aforementioned mixed mode retention mechanism (anion-exchange with a hydrophobic adsorption component) it is obvious that a large number of experimental parameters might have a significant impact on non-stereoselective and stereoselective retention data. Optimization without a dedicated strategy or computer aid would require a lot of effort. Thus we decided to test the practicality and applicability of DRYLAB to predict rapidly and reliably the retention of the individual enantiomers of DNP-amino acids on the chiral anion-exchange type CSP as a function of chromatographic conditions in isocratic and gradient elution mode and whether it has potential to optimize the separations. The results should be discussed with special emphasis on accuracy of the predictions of this computer simulation program for the given application.

Thus several experiments have been performed sequentially. After a preliminary isocratic run the influence of mobile phase parameters such as pH, type and content of organic modifier, buffer concentration, temperature, type of co-ion on retention and separation was studied by isocratic elution. These experiments, which were executed in the order as listed in Table 2, were followed by gradient elution optimization experiments. First, linear and multi-segmented organic modifier gradients were performed and, finally, linear and multi-segmented salt gradients.

2. Experimental

2.1. Equipment and software

DRYLAB/Windows (Vers. 1.62) from LC Resources (Walnut Creek, CA, USA) was obtained by the Institute for Applied Chromatography (Berlin, Germany).

The HPLC system consisted of L-6200 intelligent pump, L-4250 UV–Vis detector, AS-2000A autosampler, D-6000 interface, D-6000 chromatography

Table 2
Variables optimized under isocratic conditions, employed calibration runs and DRYLAB predicted optimum of the given variable

Step	Variable	Calibration runs	DRYLAB predicted optimum
1	pH_a [a]	3 (5.0; 6.0; 7.0)	5.5
2	Buffer concentration[b]	3 (25; 50; 100 m*M*;)	100 m*M*
3	Modifier (MeOH)[c]	2 (70%; 90%)	70%
4	Modifier (ACN)[d]	3 (45%; 65%; 80%)	45%
5	Type of modifier[e]	MeOH; ACN	ACN
6	Ternary eluent[f]	3 (1%; 5%; 10% THF in ACN)	1% THF in ACN
7	Temperature[g]	2 (10°C; 45°C)	25°C
8	Type of co-ion[h]	NH_4^+; $NH(C_2H_5)_3^+$	$NH(C_2H_5)_3^+$

The molarity values given below refer to those of the final mixture.
[a] MeOH–20 m*M* ammonium acetate (90:10, v/v); T=25°C.
[b] MeOH–ammonium acetate (90:10, v/v); pH_a 5.5; T=25°C.
[c] MeOH–100 m*M* ammonium acetate; pH_a 5.5; T=25°C.
[d] ACN–110 m*M* ammonium acetate; pH_a 5.5; T=25°C.
[e] No additional calibration run used.
[f] 110 m*M* Ammonium acetate–ACN (55:45, v/v); pH_a 5.5; T=25°C.
[g] 110 m*M* Ammonium acetate–(ACN–THF, 99:1) (55:45, v/v); pH_a 5.5.
[h] 110 m*M* Acetate buffer–(ACN–THF, 99:1) (55:45, v/v); pH_a 5.5; T=25°C.

data station software from Merck (Darmstadt, Germany) and a column thermostat from W.O. Electronics (Langenzersdorf, Austria). The pH of the mobile phases refers to the apparent pH of the mixture (pH_a) and was measured with an Orion pH meter, Model 520A. The molarity values in the Tables refer to those of the final mixtures.

The measurement of the dwell volume (V_D=2.3 ml) was performed according to the instruction manual of the software. The column dead time (t_0= 1.83 min) was determined by injection of mobile phase containing 0.1% (v/v) acetone employing the same chromatographic conditions as in the preliminary experimental run (see Table 1).

2.2. Materials

The synthesis of the chiral column (CSP) (see Fig. 1) as well as its application as chiral anion exchanger is described elsewhere [21]. Spherical silica, Kromasil 100 Å-5 μm from EKA Nobel (Bohus, Sweden), was used as the porous silica material for the immobilization of the quinine carbamate. The column dimensions were 150×4.6 mm I.D.

The racemic and optically pure amino acids under investigation were purchased from Sigma. The reagent, 2,4-dinitrofluorobenzene (DNFB) (Sanger's reagent), was obtained from Aldrich (Steinheim, Germany). An aqueous solution of a mixture of the various amino acids (10–25 μg/ml) was derivatized with Sanger's reagent. Thus to a 100-μl aliquot of the aqueous sample 100 μl of carbonate buffer (0.1 *M* sodium bicarbonate adjusted to pH 9.5 with 0.1 *M* sodium carbonate) was added. A 50-μl volume of a solution containing DNFB reagent (5% in acetonitrile, v/v) was added and the solution vortex-mixed. The sample was then heated for 1 h at 50°C to ensure complete derivatization. Afterwards, the reaction mixture was acidified with 250 μl of 1% acetic acid and diluted with 500 μl acetonitrile–water (50:50, v/v) and an aliquot of 20 μl was injected onto the chiral column.

Methanol (MeOH) and acetonitrile (ACN) for the preparation of the mobile phases were HPLC grade (J.T. Baker). Ammonium acetate was obtained from Merck (Darmstadt, Germany). HPLC grade water (purified by a Milli-Q-Plus filtration unit from Millipore) was used to prepare the mobile phases. Triethylamine and glacial acetic acid, which were used to prepare the triethylammonium acetate buffer, as well as tetrahydrofuran (THF) were of analytical grade.

The pH of the mobile phases (apparent pH, pH_a) was adjusted to the respective value by adding glacial acetic acid to the modifier–buffer mixture. Mobile phases were filtered through a Nalgene Nylon membrane filter (0.2 μm) (Nalge Company, New York, NY, USA) and degassed before use. All experiments were carried out with a flow-rate of 1.0 ml/min and UV detection at 390 nm.

3. Results and discussion

3.1. Initial run

As a first step in the optimization process, a preliminary isocratic experimental run (see Fig. 2)

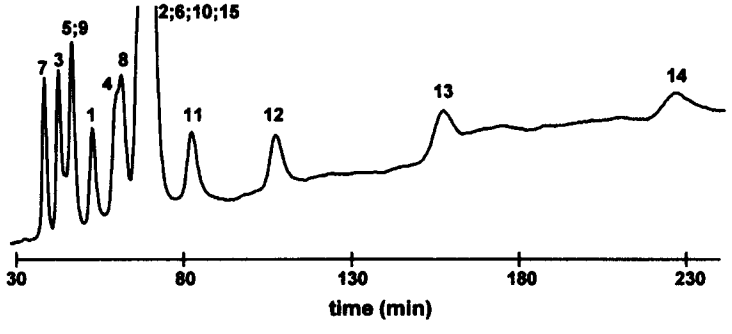

Fig. 2. Chromatogram of the initial experimental run. (Peak annotation and chromatographic conditions see Table 1).

was carried out with a mobile phase which was used in earlier studies [21]. The chromatographic retention data (k') of each component as well as the separation data (α, R_S) of the corresponding enantiomers are listed in Table 1. All pairs of enantiomers were well resolved and resolution values between 3.45 and 6.23 on the quinine carbamate based CSP were obtained with the non-optimized mobile phase of the initial run. However, the employed mobile phase for this experiment already approached the optimum state for the chiral separation of the individual pairs of DNP-AA enantiomers. Thus, the chiral separation of the analytes would need no further optimization.

Nevertheless, several DNP-amino acids coeluted in the preliminary run (see Fig. 2) since pK_a values and lipophilicity of a broad range of the derivatives are very similar and the achiral selectivity and efficiency of the CSP is low (with plate numbers of about 3000 per column). The hydrolyzed reagent (2,4-dinitrophenol, DNP-OH) itself represents an acid with similar pK_a value; thus it eluted on the chiral anion exchanger in the same time range as the DNP-amino acids. As the reagent and thus DNP-OH might be present in high excess, it might be possible that peaks coelute. Consequently, a sufficient separation of the reagent peak(s) from all other peaks is important. Furthermore, the run time is unreasonably high.

3.2. General procedures and criteria for estimation of the separation

The simulation software optionally provides several operational modes (isocratic, gradient LC as well as GC). The LC module of DRYLAB is based on reversed-phase retention mechanism approximating linear-solvent-strength theory [8,10]. In this respect, for ion-exchange separations a linear-solvent-strength model seems more approximate than for reversed-phase separations. However, it should still be applicable, but it seems likely that some of the divergences of the predicted results are related to assumptions of reversed-phase retention mechanism. Thus, the respective RP operation mode was chosen first, i.e., RP isocratic LC – pH, percentage organic modifier, ternary eluent, ionic strength, temperature – or RP gradient LC. For each variable studied a few (two or three) calibration runs were experimentally carried out and the report with retention times and peak areas were imported to the computer program. Peak tracking, i.e. the matching of bands for a given sample component among the various experimental runs, was performed automatically via the area ratio (which has to adopt a value about 1 if correctly assigned) and was verified by injecting also the pure racemates as references. Additionally, the simulation program was fed also with some column characteristics as medium plate number (in this case 3000), column dead volume, particle size diameter, pore diameter of the silica material, inner diameter of the column, column length and some apparatus settings as flow-rate, temperature, dwell volume because the optimization program can also be used to predict separations as a function of column conditions and of flow-rate. With this input data several simulated chromatograms could be generated. The quality of the simulated chromatograms is usually estimated by the resolution value for the critical peak pair ($R_{S\ crit.}$), the least resolved pair of peaks. The critical R_S at each value of a given variable is plotted by the computer and on the basis of this critical resolution map the optimum of the given variable is extracted. This optimized simulated run is experimentally verified and repeated twice to evaluate the experimental error.

Under isocratic conditions at least two components always co-eluted completely and $R_{S\ crit.}$ always adopted a value of 0. Hence, there was need for additional criteria to estimate the quality of the simulated separations. Thus, the best chromatogram was primarily assessed to be the one with the highest number of bands eluted. If equal numbers of bands were eluted, the decision was based on the critical resolution map which was obtained with co-eluted components dealing as single peak. When the simulated chromatograms were almost of equal quality, the faster run was preferred as optimum.

3.3. Isocratic elution

First, from a mechanistic point of view the influence of the manifold mobile phase parameters which affect retention and separation of the analytes was studied under isocratic elution. Those which have been optimized are listed in Table 2 together with the experimental calibration runs and the op-

timum that was predicted by the computer program for the respective variables. As we are dealing with mixed mode retention mechanisms, it contains parameters which influence ion-exchange processes, as well as "lipophilic adsorption" (sum parameter for all kind of Van der Waals type interactions) to the functionalized and lipophilized silica surface. The factors that seemed to have the greatest influence on retention and separation, i.e. pH and buffer concentration, were optimized first.

The mobile phase pH controls the dissociation degrees of several ionizable species (ion exchanger, analytes, counter-ions, co-ions) of the complex system with several interacting equilibrium processes and consequently the practical and/or actual capacity of the ion exchanger. Ionic interactions between SO and SAs are stated to be the essential prerequisite for the CSP to develop stereodiscriminating potential. Hence, the preferred working pH range of the weak chiral anion exchanger is expected to be between 5 and 7. Therefore, three experimental runs with pH values between 5 and 7 for calibration were carried out. Involving a fitting function which is based on the Henderson–Hasselbalch equation [11,12], DRYLAB is able to model solute retention and separation as a function of mobile phase pH. Several chromatograms were simulated to determine optimal pH with emphasis on the separation of a maximum number of bands eluted. The optimal pH value was found to be 5.5 for the given separation system. Predicted and experimental retention times of the various DNP-derivatives are illustrated in Table 3. As can be seen, correlation is fairly good with an average error of prediction of ±1.29 min or ±2.78%, whereas the experimental error of triplicate injections provided a coefficient of variation of only 0.32%.

Besides the pH, the ionic strength of the buffer (which itself is influenced by the pH) belongs to the factors that affect the observed capacity of the ion exchanger. Higher concentrations of acetate as counter-ion elute the solutes much faster. Chiral resolution was not much affected by the buffer concentration; thus, the faster run (100 m*M*) was accepted as optimum. No further experimental verifi-

Table 3
Experimental versus DRYLAB predicted retention of the various racemic DNP-amino acids under isocratic conditions for predicted optimum pH and temperature, respectively

No.	AA	pH (5.5)[a]					Temperature (25°C)[b]				
		DRYLAB	Experimental		Difference		DRYLAB	Experimental		Difference	
		t_R	t_R	C.V.[c] (%) (n=3)	(min)	(%)	t_R	t_R	C.V.[c] (%) (n=3)	(min)	(%)
3	L-Val	29.59	28.63	0.65	−0.96	−3.35	20.56	19.93	0.00	−0.63	−3.15
5	L-leu	29.58	28.63	0.73	−0.95	−3.32	24.99	23.79	0.24	−1.20	−5.04
7	L-Thr	32.92	32.34	0.13	−0.58	−1.79	11.31	13.11	0.09	1.80	13.75
9	L-Pro	36.35	32.34	0.18	−4.01	−12.4	14.28	15.64	0.14	1.36	8.71
1	L-Ala	40.79	38.07	0.03	−2.72	−7.14	18.13	18.90	0.52	0.77	4.08
4	D-Val	41.52	40.99	0.16	−0.53	−1.29	27.08	26.04	0.09	−1.04	−3.99
6	D-Leu	41.53	40.99	0.19	−0.54	−1.32	32.61	30.37	0.07	−2.24	−7.37
8	D-Thr	52.39	52.08	0.51	−0.31	−0.60	17.05	18.29	0.11	1.24	6.79
10	D-Pro	52.44	52.08	0.63	−0.36	−0.69	20.75	21.47	0.07	0.72	3.36
2	D-Ala	52.41	52.08	0.43	−0.33	−0.63	22.54	22.43	0.31	−0.11	−0.48
15	DNP-OH	52.45	52.08	0.21	−0.37	−0.71	34.10	33.41	0.09	−0.69	−2.06
11	L-Phe	52.45	54.70	0.43	2.25	4.11	36.88	33.47	0.13	−3.41	−10.18
12	D-Phe	69.00	69.46	0.11	0.46	0.66	46.77	40.93	0.07	−5.84	−14.26
13	L-Trp	102.6	103.06	0.18	0.46	0.45	56.39	48.00	0.07	−8.39	−17.47
14	D-Trp	144.18	139.61	0.17	−4.57	−3.27	78.97	66.03	0.06	−12.94	−19.59
Average error				±0.32	±1.29	±2.78			±0.14	±2.83	±8.02

The molarity values given below refer to those of the final mixture.
[a] MeOH–20 m*M* ammonium acetate (90:10, v/v); T=25°C.
[b] 110 m*M* ammonium acetate–(ACN–THF, 99:1), (55:45, v/v); pH_a 5.5.
[c] Coefficient of variation for triplicate injections.

cation was necessary, because this experimental run was used as one of the calibration runs.

Also, the type and content of the organic modifier of the mobile phase significantly influences the relative retention behaviour of the various analytes. The effect of organic modifier content was evaluated for methanol and acetonitrile. Higher content of methanol accelerated the analysis with only little effect on the separation. With a relatively low content of acetonitrile (45%) (in combination with a high concentration of the buffer salt) the number of separated components could be increased, due to higher efficiency and sharper peaks compared to methanol as organic modifier. The addition of small amounts of THF (1%) forced a further improvement in efficiency and separation. For all three variables optimized (methanol, acetonitrile, ternary eluent) one of the calibration runs constituted already the optimum.

The various equilibria of the anion-exchange process and the distribution of the solutes between stationary and mobile phases are governed by temperature which may be varied to influence retention and separation. With calibration runs at 10°C and 45°C solutes' retention at each temperature was predicted involving linear Van't Hoff plots. Selectivity values (achiral) and enantioselectivity values (chiral) increase little with decreasing temperature but at the expense of efficiency. Thus, in contrast, mass transfer is accelerated at higher temperature which leads to smaller plate heights and larger plate numbers outlined as sharper peaks. Consequently, resolution values go through an optimum which is for the given separation around 25°C. Predicted and experimental retention times as well as their experimental error of 3 consecutive injections at this optimum temperature are listed in Table 3.

Finally, an additional experimental run was carried out using triethylammonium instead of ammonium as co-ion which yielded sharper peaks and higher plate numbers; an optimized isocratic method resulted. The respective experimental run is shown in Fig. 3. The number of eluted bands increased from 10 to 13 and 1 partially resolved (shoulder of component 1 at the descent of component 8). A slight decrease of enantioselectivity and resolution values of the corresponding enantiomers resulted at the end of the isocratic optimization procedure (deoptimization of the chiral separation on behalf of better resolution of the different amino acid derivatives), but this was of no practical relevance since corresponding enantiomers were in no case the critical peak pair. A few partially co-eluted bands in the middle part of the chromatogram still remained and component 11 completely overlapped with the reagent peak. The first half of the chromatogram in Fig. 3 is overcrowded with 12 components, whereas elution of the last 3 components might be accelerated.

3.4. Gradient elution

Gradient elution technique seems to be the method of choice for the efficient separation of complex mixtures. From the optimized isocratic run it was obvious that a multi-segmented gradient would be necessary rather than a linear gradient. However,

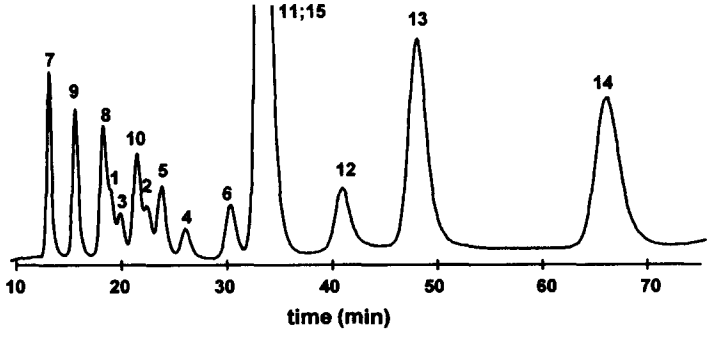

Fig. 3. Chromatogram at optimized experimental and isocratic conditions. (Mobile phase: 110 m*M* ammonium acetate–(ACN–THF, 99:1), (55:45, v/v); pH_a 5.5; T=25°C; peak annotation see Table 1). The given molarity value refers to that of the final mixture.

optimization of a multi-segmented gradient profile is more sophisticated and would probably need a large number of experiments, either performed experimentally or by computer simulation. With DRYLAB several linear and multi-segmented gradients can be simulated fairly quickly based on only two linear gradients which are carried out experimentally and utilized as calibration runs. In order to account for the mixed mode retention mechanism, gradient elution was performed with respect to organic modifier content and buffer salt concentration as well.

Thus initially linear gradients from 10 to 100% of organic modifier in 50 min and 150 min, respectively, were carried out (for composition of aqueous eluent A and organic modifier B see Table 4). About 30 different multi-segmented gradients were simulated on the computer by a trial-and-error strategy. The steepness and the gradient time as well as the position of nodes or breakpoints were varied without use of eluent. However, this procedure generated optimized conditions with acceptable resolution much faster than could be achieved experimentally. The best obtained simulated chromatogram and the responding multi-segmented gradient profile is shown in Fig. 4a. The critical peak pair is component 10 and 3 which coeluted. All other components were separated from each other in the simulated run, only bands 5 and 4 with low resolution. This simulated run was experimentally verified (see Fig. 4b). Even though compounds 10 and 3 were also the least resolved components in the experimental run, they are already partially resolved. A comparison between predicted and experimentally found retention times for the optimized multi-segmented organic modifier gradient is presented in Table 4 together with the experimental error of three repetitive injections. Despite some minor differences, agreement between experimental and computer predicted retention times (average error of prediction is ±2.23 min or ±5.81%) appears to be satisfactory.

Nevertheless, the resulting separation was only little improved compared to the optimized isocratic run. Since ion-exchange mechanisms dominate at the given working pH over conventional reversed-phase mechanisms, the retention of the solutes and/or elution strength is better balanced by a buffer salt gradient than an organic modifier gradient. Again, several linear and multi-segmented gradients were simulated based on two linear calibration runs (see Table 5). The best simulated chromatogram obtained together with the gradient profile is presented in Fig. 5a). All peaks are well separated, almost equally spaced, except peak pair 15 and 6 which overlap. Additionally, run time is acceptable. A further increase of speed would generally result in a larger number of overlapping peaks. Again, the experimental verification run (see Fig. 5b) compares rather well with the predicted separation and retention times agree within ±1.53 min or ±2.69% (average error of prediction) between simulated and experimental value. Only the three last eluting bands reveal higher errors in prediction. This can be attributed to the curvature of the gradient (gradient dispersion or mixing) at the juncture of a segmented gradient, as discussed in detail in Ref. [23]. Nevertheless, even a somewhat better chromatogram was obtained experimentally than with the simulation. Thus, the critical peak pair 15 and 6 was slightly resolved. The

Table 4
DRYLAB versus experimental retention times of DNP-amino acid enantiomers under organic modifier gradient elution[a]

No.	AA	DRYLAB	Experimental		Difference	
		t_R	t_R	C.V. (%)	(min)	(%)
7	L-Thr	22.24	24.87	0.14	2.63	10.58
9	L-Pro	26.56	27.91	0.00	1.35	4.84
8	D-Thr	30.03	29.81	0.17	−0.22	−0.73
1	L-Ala	32.38	31.89	0.48	−0.49	−1.53
10	D-Pro	32.85	32.83	0.15	−0.02	−0.07
3	L-Val	34.18	33.25	0.16	−0.93	−2.79
2	D-Ala	37.19	34.85	0.49	−2.34	−6.71
5	L-Leu	40.14	37.20	0.04	−2.94	−7.90
4	D-Val	41.70	38.03	0.14	−3.67	−9.65
15	DNP-OH	44.72	39.68	0.14	−5.04	−12.70
6	D-Leu	47.07	42.13	0.12	−4.94	−11.72
11	L-Phe	50.14	45.65	0.08	−4.49	−9.83
12	D-Phe	54.43	51.68	0.12	−2.75	−5.32
13	L-Trp	57.01	56.21	0.12	−0.80	−1.42
14	D-Trp	62.75	63.57	0.19	0.82	1.29
Average error				±0.17	±2.23	±5.81

Calibration run 1: 10–100% B in 50 min; Calibration run 2: 10–100% B in 150 min.

Gradient profile of optimized run: 25–46% B from 0 to 23.6 min; 46% B from 23.6 min to 47 min; 46–65% B from 47 min; to 61.5 min; 65% B from 61.5 min to 72.2 min; 65–100% B from 72.2 min to 78.3 min.

[a] A: 100 m*M* triethylammonium acetate in water; pH_a 5.5; B: 100 m*M* triethylammonium acetate in ACN–THF (99:1); pH_a 5.5.

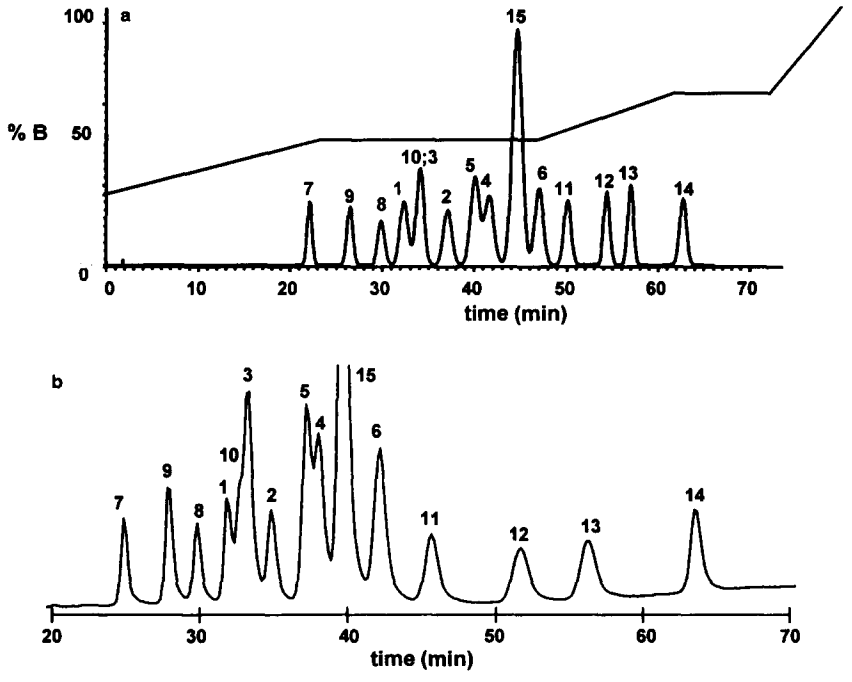

Fig. 4. Chromatogram of DRYLAB simulated (a) versus experimental (b) conditions using an optimized multi-segmented organic modifier gradient. (Gradient profile and other experimental conditions see Table 4; peak annotation see Table 1).

final separation shown in Fig. 5b appears to be suitable with acceptable resolution between all peaks (except peaks 15 and 6).

3.5. Discussion of the results and findings

Comparison of the elution order of the different amino acid derivatives between the two experimental isocratic runs presented in Table 3 (with very comparable experimental conditions but with respect to the type of modifier) reveals that some components changed positions with the different solvents or modifiers, e.g. L-Val (3), L-Leu (5) elute with methanol as modifier prior to L-Thr (7), L-Pro (9), L-Ala (1) whereas with acetonitrile or acetonitrile–tetrahydrofuran as modifier they elute after L-Thr (7), L-Pro (9), D-Thr (8), L-Ala (1) and so forth. This, however, may be attributed to the different solvatochromic properties [8,24–26] of the organic modifiers.

In the course of the optimization study the multicomponent separation was improved by each step and/or operation mode. Thus the number of bands eluted increased from 10 to 14 or, if counting the partially resolved peaks separately, all are separated from one another. Simultaneously, the run time was significantly reduced from 250 min for the initial run to 75 min for the optimized salt gradient run. The saving of time was associated with a considerable reduction of eluent and organic modifier consumption. Similarly, the time saved for method development was significant. For the isocratic method development, 17 calibration runs (repetitive injections not counted) and 2 verification runs, each repeated twice, have been performed experimentally. Four calibration runs together with 2 experimental verification runs (each repeated twice) were necessary for the gradient elution mode. Without computer aid many more experiments would have been necessary and one may probably assume that it would have been very uncertain to attain an equally good sepa-

Table 5
DRYLAB versus experimental retention times of DNP-amino acid enantiomers under buffer salt gradient elution[a]

No.	AA	DRYLAB	Experimental		Difference	
		t_R	t_R	C.V. ($n=3$) (%)	(min)	(%)
7	L-Thr	17.67	17.84	0.32	0.17	0.94
9	L-Pro	21.28	21.59	0.29	0.31	1.43
8	D-Thr	25.23	25.19	0.28	−0.04	−0.17
1	L-Ala	26.39	26.33	0.38	−0.06	−0.24
3	L-Val	27.77	27.99	0.33	0.22	0.78
10	D-Pro	29.73	29.75	0.28	0.02	0.06
2	D-Ala	31.43	31.33	0.32	−0.10	−0.33
5	L-Leu	33.93	33.76	0.33	−0.17	−0.51
4	D-Val	36.90	36.61	0.37	−0.29	−0.80
15	DNP-OH	42.03	41.39	0.31	−0.64	−1.55
6	D-Leu	42.16	41.58	0.22	−0.58	−1.40
11	L-Phe	45.81	46.99	0.44	1.18	2.51
12	D-Phe	50.80	54.69	0.35	3.89	7.11
13	L-Trp	55.13	59.33	0.26	4.20	7.08
14	D-Trp	60.47	71.55	0.16	11.08	15.48
Average error				±0.31	±1.53	±2.69

Calibration run 1: 0–100% B in 50 min; Calibration run 2: 0–100% B in 150 min.
Gradient profile of optimized run: 17% B from 0 min to 16.2 min; 17–25% B from 16.2 min to 41.1 min; 25–100% B from 41.1 min to 50.4 min; 100% B from 50.4 min to 80 min.
[a] Triethylammonium acetate–(ACN–THF, 99:1), (55:45 v/v); pH_a 5.5; A: 55 m*M*; B: 275 m*M*.

ration as that presented in Fig. 5b). Moreover, from the practical point of view, it might have been also possible to obtain an equally suitable separation with less variables studied, but it was the purpose of this study to evaluate the influence of a very broad range of variables. However, especially for the optimization of multi-segmented gradient profiles the simulation software was of great help. To develop such a gradient profile (optimal number and position of nodes or breakpoints and best steepness of various steps) is a sophisticated task. Trial-and-error is the usually used method for this purpose. Thus a lot of linear and multi-segmented gradients have been simulated which to carry out experimentally would have been inefficient due to unreasonable time and eluent consumption. Since an infinite number of different gradient profiles exist, probably none of which would have led to the demanded baseline separation of the critical peak pair, the chromatographer faces the dilemma of when to stop simulations and to pick out the best simulated chromatogram. Recently, a procedure was proposed to automatically optimize gradient profiles and to select the most suitable one, which reflects the overall optimum profile [7].

The average errors of prediction for retention times were for the presented chiral anion-exchange type separation case study between 2.69 and 8.02%, depending on the respective operating mode. Compared to the average errors reported in the literature for diverse applications of DRYLAB for RP-HPLC separations (which lay e.g. between ±0.2 and ±1.0% [15,18,19] or between ±1 and ±8% [16,17]), it should be emphasized that the errors of prediction of retention times for the presented chiral application were well in the range of the reported ones for achiral RP applications. However, the software used in this study is based on reversed-phase retention data (applying the linear-solvent-strength theory). Thus it seems likely that some of the errors in prediction may result from the assumption of reversed-phase retention and applied to an ion-exchange system involving hydrophobic adsorption components including peculiarities associated with enantioseparation mechanism. Nevertheless, the retention data of the given separation system fit to the same fitting functions, although a different basic theory is underlying. This, in turn, allowed quite accurate predictions by DRYLAB which were suitable throughout this study.

There is one point which emerged as evident problem during this study; it is related to the univariate optimization strategy of the computer simulation program. Overall, and in a strict sense, it is impossible to vary only one parameter while holding all others constant (e.g. changing the modifier content varies the pH of the mobile phase, adjusting the pH varies the buffer concentration or ionic strength and so forth). Thus some errors of prediction are caused by the univariate approach itself that neglects parameter interactions and generally does not arrive in the overall or global optimum. If resolution between the bands is high enough, this, however, may be less of a problem. Especially for such complex separation systems as for the given case with very small resolution values, an univariate strategy may be problematic and multivariate meth-

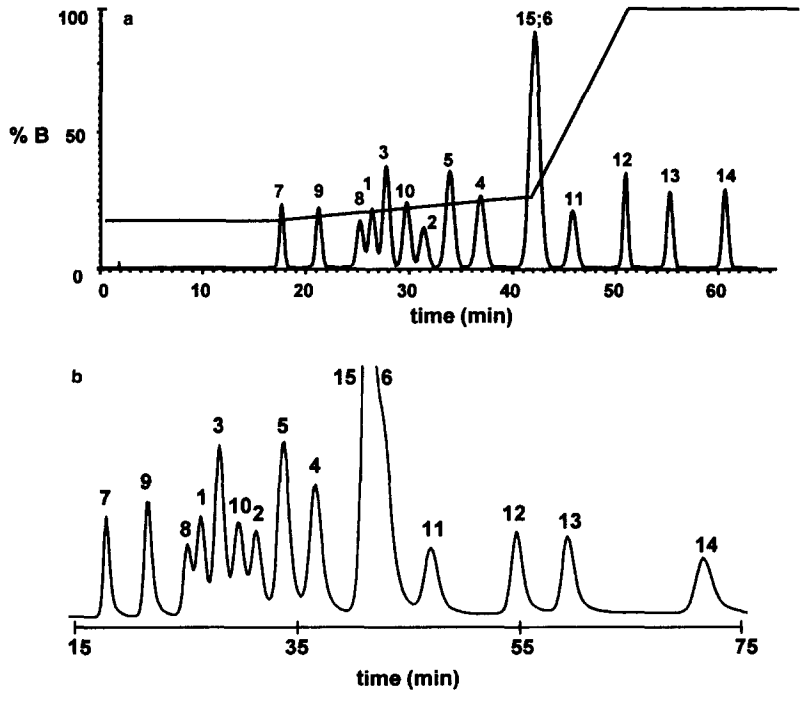

Fig. 5. Chromatogram of DRYLAB simulated (a) versus experimental (b) conditions using an optimized multi-segmented buffer salt gradient. (Gradient profile and other experimental conditions see Table 5; peak annotation see Table 1).

ods might overcome this dilemma and would be preferable [27,28].

4. Conclusion

Chiral separation of DNP-amino acid enantiomers employing a chiral anion-exchange type CSP (quinine carbamate based CSP) and aqueous buffered mobile phases was easily achieved in both isocratic and gradient elution modes. However, due to the low achiral efficiency of the chiral column complete resolution of all components with $R_S > 1.5$ could not be obtained, but a suitable separation resulted. The commercial DRYLAB software package offered great help for the optimization and method development throughout this study. The key advantage of this computer program is the very easy handling in combination with the fact that, based on accurate predictions, the number of actual experiments can be significantly reduced during method development studies. Several other features, e.g. ruggedness tests, in the course of validation issues might be also of interest. Thus, one can conclude that computer simulation software and tools including DRYLAB have significant input also in the field of enantioseparation method development employing CSPs. This may in particular be of interest for CSPs to be used with aqueous mobile phases as demonstrated for the ion exchanger type CSPs based on quinine carbamates as chiral selectors.

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