

A comprehensive chemoselective and enantioselective 2D-HPLC set-up for fast enantiomer analysis of a multicomponent mixture of derivatized amino acids

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Abstract A feasibility study on the fast enantioselective two-dimensional HPLC separation of racemic amino acid derivatives is presented. The method involves the on-line coupling of a narrow-bore C18 RP column in the first dimension to a short enantioselective column based on nonporous 1.5 μm particles modified with quinidine carbamate as chiral selector in the second dimension. Conceptually, the system was designed to enable both time-controlled repeated transfer of fractions of the eluate and detector-controlled transfer of selected fractions from column 1 to column 2. To avoid volume overloading of the second chiral column, a narrow-bore reversed phase column was installed in the first dimension. Due to the fast (less than 1.5 minutes) enantiomer separation that occurs in the second dimension, the overall analysis time for the two-dimensional separation of a mixture of nine racemic 3,5-dinitrobenzoyl amino acids was optimized at 16 minutes.

Keywords Comprehensive two-dimensional high-performance liquid chromatography · Reversed phase separation · Enantioselective separation · 3,5-Dinitrobenzoyl amino acids · Chiral stationary phase · *O*-9-(*tert*-butylcarbamoyl) quinidine · Nonporous silica · Sub-2- μm particles

Introduction

Over the last two to three decades, high-performance liquid chromatography has developed into one of the most important methods for chiral separation and enantiomer excess analysis (see for example [1–4]). Most HPLC enantiomer separations are performed with columns packed with a chiral stationary phase (CSP) operated with an achiral mobile phase, which is either a normal phase, a polar organic phase or a reversed phase (hydroorganic phase) eluent. One common problem encountered with this approach is the intrinsically limited chemical selectivity of CSPs, especially when considered in combination with the rather limited efficiencies of enantioselective columns. This means that complex mixtures of diverse pairs of enantiomers cannot be analyzed in one run due to peak overlapping. A solution to this dilemma is to set up a one- or two-dimensional analytical scheme by combining a nonenantioselective column that provides sufficient target chemoselectivity with an enantioselective one (the two columns are coupled in series in the 1D set-up).

Simple mixtures can be separated directly on such a serially coupled column system [5]. For more complex mixtures, the transfer of fractions of interest from the first to the second column in a heart-cut type technique has been preferred: parts of the eluate pre-separated from other

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compounds in the first dimension according to their size, polarity, hydrophobic character, or ion exchange capability are transferred to the second column, where enantiomer separation takes place [6–10]. For bioanalytical applications, such a coupled column technique may be used as a sample preparation technique [11, 12].

The main drawback of approaches like these is the long total analysis time often needed, particularly when several “heart-cuts” must be separated on a second chiral column. In an on-line mode, the mobile phases employed must harmonize to allow on-line fraction transfer and to permit on-column peak (fraction) compression on the second column, if necessary.

With the introduction of quinine carbamate or quinidine carbamate chiral selectors by Lindner and coworkers [13–15] (Fig. 1), which were then bound to nonporous silica particles 1.5 μm in size (based on MICRA NPS 1.5 μm) [16], the analysis time required for the chiral separation (e.g., of 3,5-dinitrobenzoyl amino acids) could be reduced to a few minutes or even less than a minute. This enables a comprehensive two-dimensional separation where eluate fractions (peak fractions) from an RP-type first column are transferred onto quinine-type CSPs, since they work at RP conditions in an anion exchange mode. Short analysis times and orthogonal selectivity in the second dimension — prerequisites for comprehensive two-dimensional separations [17–20] — are realised by employing these types of stationary phases and by applying nonporous particle columns that are known to permit very fast separations in the second dimension. In this paper, we describe the development and application of such 2D-HPLC systems, and discuss some advantages, limitations, and caveats that should be considered when attempting fast comprehensive two-dimensional on-line HPLC.

Experimental

Apparatus

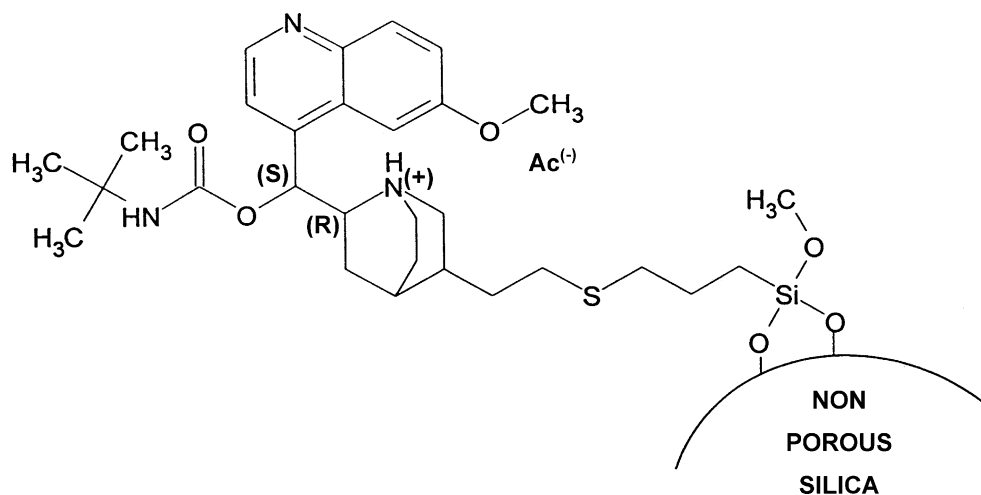
The experimental set-up is depicted schematically in Fig. 2. The system was designed with small extra column volumes and small fraction volume handling in mind. Two HPLC pumps (PU-980, Jasco, Tokyo, Japan) were used to deliver the eluents with high precision at flow rates ranging from a few $\mu\text{L min}^{-1}$ to 2 mL min^{-1} . A UV detector (WellChrom K 2000, Knauer, Berlin, Germany) equipped with a capillary electrophoresis cell served as an “on-capillary” monitor detector and was positioned after the narrow-bore reversed phase column in the first dimension. The second UV detector was a Jasco UV-975 equipped with a 4- μL cell.

The set-up contained three valves. Injection was performed with a Rheodyne (Cotati, CA, USA) model 7520 micro injection valve with an internal sample loop (0.5 μL). Valve 1 and valve 2 were motor-driven six-port two-position valves from Vici (Schenkon, Switzerland) with small dead volumes.

Chromatograms were recorded using the chromatography software Chromeleon from Gynkotek (Germering, Germany).

Dummy columns were included to induce flow resistances similar to those of the small-bore column and the nonporous particle column, respectively, and to protect the separation columns from sudden pressure drops when the valves were switched for fraction transfer and/or flow interruption. To minimize the extra column volume, the microcolumn was directly connected to the injection valve without any further tubing. The column outlet was connected to transfer valve 2 via an 18-cm-long fused

Fig. 1 Structure of the *tert*-butyl quinidine carbamate selector bound to the surfaces of nonporous silica particles



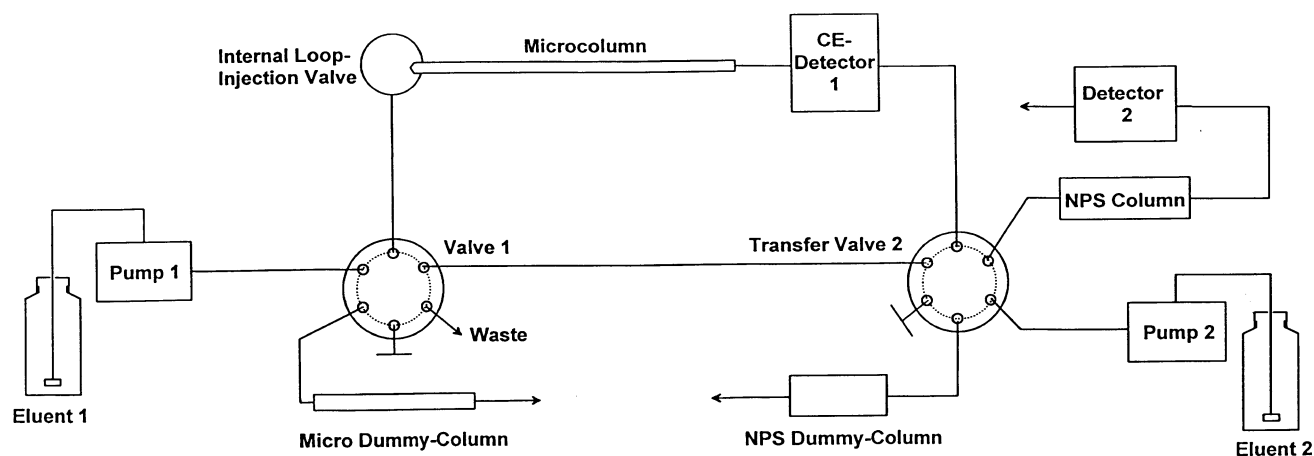


Fig. 2 Schematic of the two-dimensional column coupling system

silica capillary with an inner diameter of 75 μm . Other connection tubing was from PEEK and had an inner diameter of 130 μm (Upchurch, Oak Harbor, WA, USA).

Columns and mobile phase

A narrow-bore column (glass-lined tubing, 150 \times 1 mm, SGE, Ringwood, VIC, Australia) served as the first-dimension analytical column. It was packed in-house with Nucleosil 5 μm C-18 particles (Macherey-Nagel, Düren, Germany). In the second dimension, a stainless steel column (33 \times 4.6 mm) packed by Bischoff Chromatography (Leonberg, Germany) with *tert*-butyl quinidine carbamate-modified nonporous silica particles (NPS-CSP) of size 1.5 μm (based on MICRA NPS 1.5 μm , Bischoff Chromatography) [16] was used. A mixture of ammonium acetate buffer, 50 mM (pH 5.5 in the first dimension, pH 4.5 in the second dimension), and acetonitrile (typically 75:25, v:v, in the first dimension, and 70:30, v:v, in the second dimension) was used as the mobile phase.

Chemicals

All solvents were HPLC-grade and purchased from Merck (Darmstadt, Germany). Racemic and enantiomerically pure amino acids were purchased from Sigma (Munich, Germany). Derivatization of the amino acids was performed with 3,5-dinitrobenzoyl chloride in dry acetonitrile according to a standard protocol [21].

Operation of the system

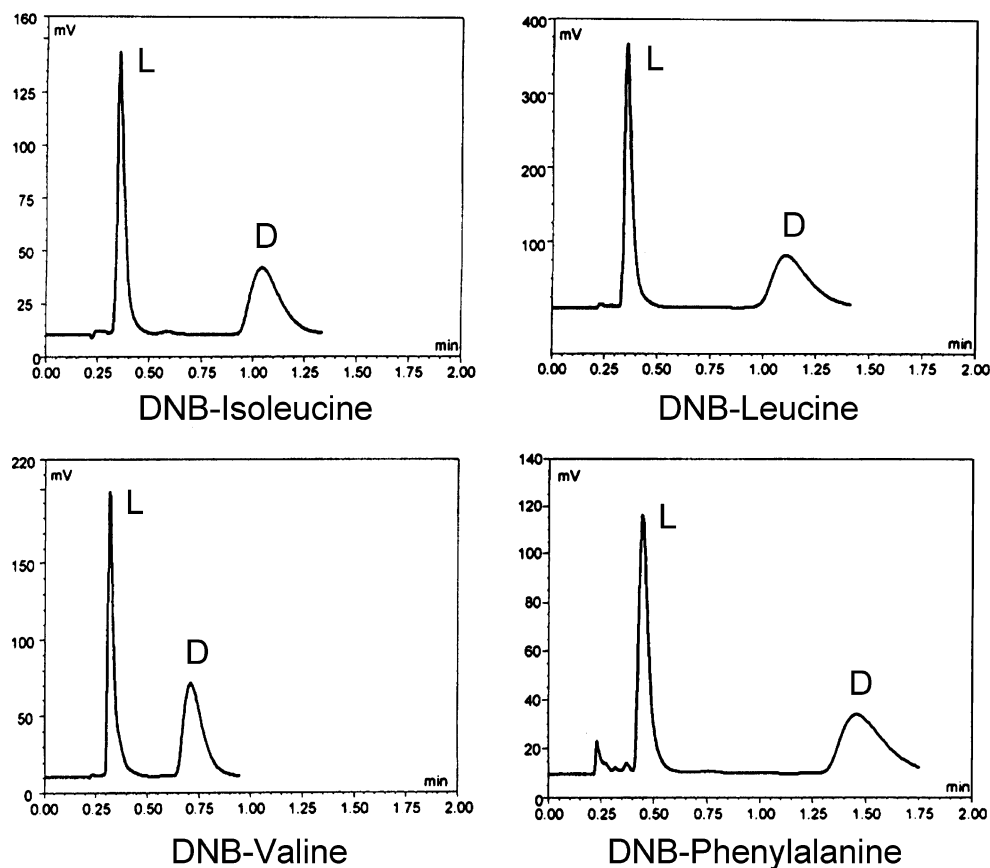
A flow diagram of the system is shown in Fig. 2. Fractions eluting from the first column can be directed to the second CSP-filled column via valve 2 or to waste via valve 1. If valve 1 is switched simultaneously with valve 2, the flow on the first column is interrupted and chromatographic

development on the first dimension is stopped while chiral separation on column 2 takes place. The development of the chromatogram on the first dimension and fraction transfer to column 2 is resumed when valve 1 and 2 are switched again. Both time-controlled repeated transfer of fractions of the total eluate and CE-detector-controlled transfer of selected fractions from column 1 to column 2 are possible with this set-up.

Results and discussion

Quinidine carbamate-type CSPs and columns, in general, are especially suitable for the separation of mixtures of *N*-3,5-dinitrobenzoyl (DNB) amino acid enantiomers in the reversed phase mode [13–15], and this can be accomplished with the currently employed NPS-based CSP in very short analysis times. Besides providing a chromophoric label for UV-detection, the DNB group promotes enantioselectivity through its ability to participate in stereoselective π - π interactions with the quinoline of the quinidine carbamate selector. Four chromatograms illustrating the high enantioselectivity yielded by the NPS-CSP column in the separation of different DNB-amino acids in under two minutes are shown in Fig. 3. The chromatograms in Fig. 3 also demonstrate how similar the retention times are for the enantiomers of different amino acids, thus reflecting high enantioselectivity but low chemical selectivity. All first-eluting *L*-enantiomers of the DNB-amino acids have practically the same retention times and would coelute when injected as a mixture onto the CSP in a conventional 1-D setup. The later eluting *D*-enantiomers of the amino acids show some retention time differences, as the enantioselectivity values α vary. Due to the relatively low plate numbers, the differences would, however, not be sufficient to resolve a mixture of several amino acids on this column. To further underline this problem, the retention

Fig. 3 Chiral separation of four DNB-amino acids on the NPS-CSP column. Mobile phase: ammonium acetate buffer (50 mM, pH 4.5); acetonitrile, 70:30 (v:v); flow rate: 1 mL min⁻¹; detection: UV, 254 nm



data for all nine DNB-labeled amino acids investigated in our studies are summarized in Table 1. This intrinsic limitation necessitates the use of a two-dimensional technique in which the various DNB-amino acids are separated in the first dimension on a reversed phase column according to their hydrophobicities, and then they undergo enantioselective separation on the quinidine carbamate-modified NPS column in the second dimension.

Fraction transfer can be realized in different ways in comprehensive two-dimensional HPLC. One approach is

Table 1 Retention data for different DNB-amino acid enantiomer pairs on the NPS-CSP column

	k (L)	k (D)	α	R_s
DNB-glutamic acid	0.61	1.43	2.4	1.9
DNB-serine	0.17	0.57	3.3	1.6
DNB-alanine	0.26	0.91	3.5	2.4
DNB-valine	0.39	2.09	5.3	3.6
DNB-methionine	0.52	2.83	5.4	3.9
DNB-isoleucine	0.52	3.52	6.8	4.3
DNB-leucine	0.52	3.78	7.3	4.0
DNB-phenylalanine	0.96	5.35	5.6	4.6
DNB-tryptophan	0.91	4.30	4.7	3.7

k (L): retention factor of the L-enantiomer; k (D): retention factor of the D-enantiomer; α : separation factor; R_s : resolution. Conditions as in Fig. 3

based on alternately filling two loops of an eight-port transfer valve [22]. In this way, the loop volume, and hence the fraction volume injected onto the second column, can easily be adapted to the maximum sample volume that can be applied to the second column. However, by chopping the peaks eluting from the first column into several lines (fractions) and then transferring them onto the second column, quantification of the resulting chromatograms may prove problematic when using common peak integration software.

Alternatively, a stop/flow transfer technique can be applied for the separation and transfer of fractions from column 1 to column 2 [23]. Using an on-line installed detector followed by a sample loop valve, the entire eluate fraction of the peak can be flexibly and conveniently transferred in a monitor-controlled fashion. Quantification is thus simplified. However, drawbacks of this transfer variant include an extension of the total analysis time and the need to adapt the peak volumes eluting from the first column to the maximum tolerable injection volume of the second column by coordinating the column dimensions and flow rates [23, 24]. Interestingly, multiple interruptions during chromatographic development on the first column do not appreciably affect the efficiency and peak resolution observed [23]. The exact enantiomer composition determined on the second CSP column was found to be

independent of which type of transfer is used to move fractions from column 1 to column 2.

In the present study, we used an instrumental setup based on the stop/flow transfer technique (Fig. 2) [24]. As an example, Fig. 4a shows the fractionation of the peak resulting from the injection of racemic DNB-leucine onto the first column, whereas the corresponding enantioseparations of the four fractions transferred to the second column are shown in Fig. 4b. The “plateaus” in the first dimension chromatogram are caused by the cessation of flow on column 1 during the development of the chromatograms on column 2. It is interesting to note that, although the peaks are still baseline-separated, the resolution of the two DNB-leucine enantiomers observed in Fig. 4b has significantly decreased ($R_s=1.5$ for the second fraction) compared to that shown in Fig. 3 ($R_s=4.0$), where the racemate solution was injected directly onto the NPS-CSP column. This can be attributed to band-broadening effects, which may occur in this preliminary two-dimensional experiment because the eluent strength was higher in the first than in the second dimension (note: if mobile phase 1 is weaker than mobile phase 2, which could easily be accomplished by reducing the buffer concentration and/or by reducing the acetonitrile content in eluent 1, then the resolution should be independent of the injection volume so long as column overloading does not occur). Additionally, in order to shorten the analysis time, a relatively high flow rate ($120 \mu\text{L min}^{-1}$) was selected on the RP column. This led to the transfer of fraction volumes of around $60 \mu\text{L}$, which caused volume overloading on the NPS-CSP column (see below).

Nonporous particle columns offer high speed of analysis but they are sensitive to mass and volume overloading due

to the absence of pores, which also yields a small accessible surface area and mobile phase volume [25–28]. Because of this, the fraction volume that can be injected onto such a NPS-CSP column with an acceptable loss of resolution was experimentally determined. The dependence of the resolution of the peak pair DNB-L-valine and DNB-D-valine on the injection volume is summarized in Table 2. The results show that the resolution decreases with increasing injection volume. However, the separation still exhibits acceptable resolution when a volume of $20 \mu\text{L}$ is injected. To restrict the fraction transfer volumes to this volume, a narrow-bore reversed phase column with an inner diameter of 1 mm was used in the first dimension, which could be run at a flow rate of $40 \mu\text{L min}^{-1}$. Thus, the fraction volume does not exceed the limit of $20 \mu\text{L}$ for a transfer period of 0.5 minutes.

As stated above, one drawback of the stop/flow transfer technique is a substantial lengthening of the analysis time. In order to quantify pairs of enantiomers and to get a total analysis time that is as short as possible, it may seem desirable to place only one fraction from each DNB-amino acid peak eluting from the first column onto the second NPS-CSP column. When directing the eluate to the waste container via valve 2 and valve 1, the separation in the first dimension can be continued while the enantiomer separation takes place in the second dimension. However, this is only possible if the enantiomeric ratio does not change during separation on the first nonchiral and thus non-enantioselective RP 18 column. This condition may not always be fulfilled: several authors (e.g., [29–31]) have reported on separations of nonracemic mixtures of enantiomers in so-called totally achiral chromatographic systems. This, however, is only possible if the chiral analytes

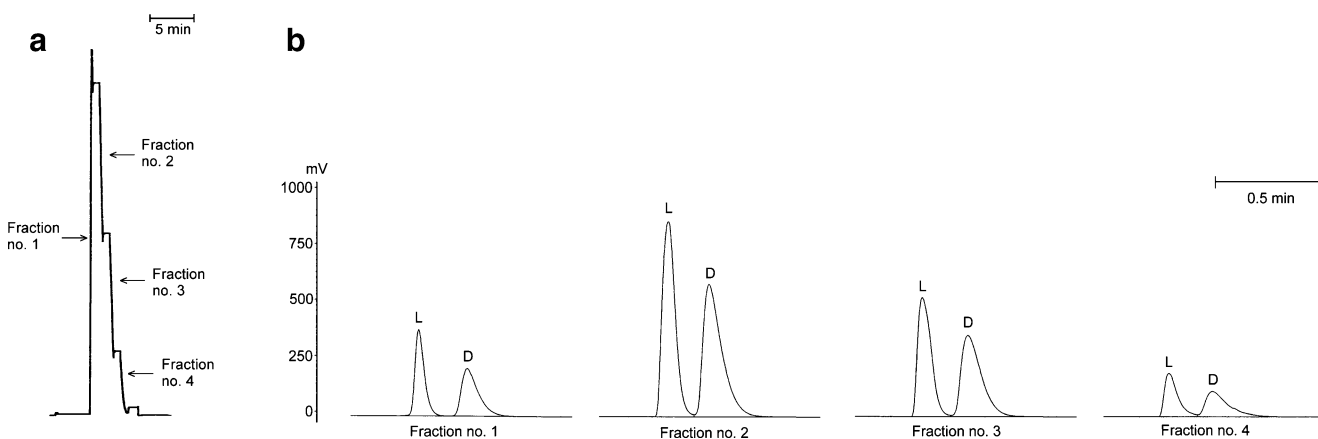


Fig. 4a–b Comprehensive two-dimensional chromatography of a racemic mixture of DNB-D- and DNB-L-leucine. **a** DNB-DL-leucine peak taken from the chromatogram of the first dimension. **b** Second-dimension enantioseparations of the four successively transferred fractions. Column 1: glass-lined tubing ($150 \text{ mm} \times 1 \text{ mm}$ i.d.), packed with Nucleosil RP-18, $5 \mu\text{m}$; mobile phase: ammonium acetate buffer

(50 mM , $\text{pH } 5.5$):acetonitrile, $75:25$ (v:v); flow rate: $120 \mu\text{L min}^{-1}$; detection: UV, 254 nm . Column 2: stainless steel ($33 \times 4.6 \text{ mm}$ i.d.), packed with *tert*-butyl quinidine carbamate-modified nonporous silica particles, $1.5 \mu\text{m}$; mobile phase: ammonium acetate buffer (50 mM , $\text{pH } 5.5$):acetonitrile, $81:19$ (v:v); flow rate: 1 mL min^{-1} . Components: *D*: 3,5-DNB-D-leucine; *L*: 3,5-DNB-L-leucine

Table 2 Dependence of the resolution of the peak pair DNB-D-valine and DNB-L-valine on the injection volume

Injection volume (μL)	Resolution
1	3.78
3	3.69
5	3.68
10	3.44
15	3.20
20	2.58

Conditions as in Fig. 3

tend to self-associate, thus forming diastereomeric adducts. Although this is a rare scenario, we checked to see whether it occurred in our system. In order to ensure that such a separation does not take place on our first-dimension C18 RP column, and to guarantee that one single fraction does indeed represent the composition of the enantiomers in the peak eluting from the RP column, we conducted a series of experiments with mixtures of a DNB-D- and a DNB-L-amino acid in various ratios, using the enantiomers of DNB-leucine as model compounds. Each mixture was injected onto the first-dimension column, the resulting peak was divided into four fractions, and each of them was transferred on-line onto the CSP-NPS column, using the stop/flow setup described above. The enantiomeric excess (e.e.) was calculated for each fraction from the peak areas of the two enantiomers in the second-dimension chromatograms (Table 3). The experiments were performed in

septuplicate, and the standard deviations were determined. From the data shown, it can be deduced that no separation of the enantiomers occurs on the RP column, at least up to e.e. ~94%, since the calculated e.e. values remain essentially constant in all fractions obtained from each mixture. Although there is a slight trend in these values for mixtures b and c, the standard deviations show that these trends are not significant and should rather be attributed to variability during peak integration. We were not able to investigate mixtures with e.e. > 94%, as the limit of detection of the system was then approached for the minor enantiomer. Note that a simple way to improve this situation would be to overload the second-dimension column and elute the trace enantiomer as the first peak; i.e., use a quinidine carbamate-based column for the analysis of D-amino acid derivatives and a corresponding quinine carbamate column for the L-amino acid derivatives. In this case, more accurate quantitative results may be achieved if, for example, standard addition is used for calibration rather than the area percent ratio for the e.e. determination.

To increase the speed of the determination of the enantiomeric composition, we therefore considered it adequate to transfer only one fraction of each peak from the first dimension to the second, without stopping the flow onto the RP column. In the case of the DNB-isoleucine peak, which shows a shoulder in the first-dimension chromatogram, indicating a partial separation from DNB-allo-isoleucine, two fractions were transferred. In Fig. 5, the

Table 3 Enantiomeric excess (e.e.) values for different D/L mixtures of DNB-leucine enantiomers, transferred in four fractions eluting from the first (achiral) column onto the second (chiral) column

Mixture		Fraction number			
		1	2	3	4
a	e.e. (%)	93.9 (D)	93.9 (D)	93.8 (D)	93.9 (D)
	<i>s</i> (%)	0.2	0.1	0.1	0.2
b	e.e. (%)	77.5 (D)	77.2 (D)	77.0 (D)	76.9 (D)
	<i>s</i> (%)	0.3	0.3	0.4	0.5
c	e.e. (%)	58.2 (D)	58.2 (D)	57.9 (D)	57.9 (D)
	<i>s</i> (%)	0.7	0.4	0.4	0.5
d	e.e. (%)	0.2 (L)	0.5 (L)	0.0	0.7 (L)
	<i>s</i> (%)	0.8	0.4	0.5	0.5
e	e.e. (%)	58.4 (L)	58.1 (L)	58.4 (L)	58.5 (L)
	<i>s</i> (%)	0.8	0.5	0.9	0.6
f	e.e. (%)	78.3 (L)	77.0 (L)	77.4 (L)	77.9 (L)
	<i>s</i> (%)	0.5	0.4	0.3	0.7
g	e.e. (%)	93.8 (L)	93.7 (L)	93.8 (L)	93.9 (L)
	<i>s</i> (%)	0.3	0.3	0.3	0.2

The e.e. values were calculated according to the following formula, where *D* is the peak area of DNB-D-leucine in the second dimension chromatogram, and *L* is the corresponding peak area of DNB-L-leucine: $e.e. = \frac{|D-L|}{D+L} \times 100\%$.

The standard deviation *s* is the absolute standard deviation calculated from seven measurements of the enantiomer mixture (these are given in % because e.e. is in %). Conditions as in Fig. 4

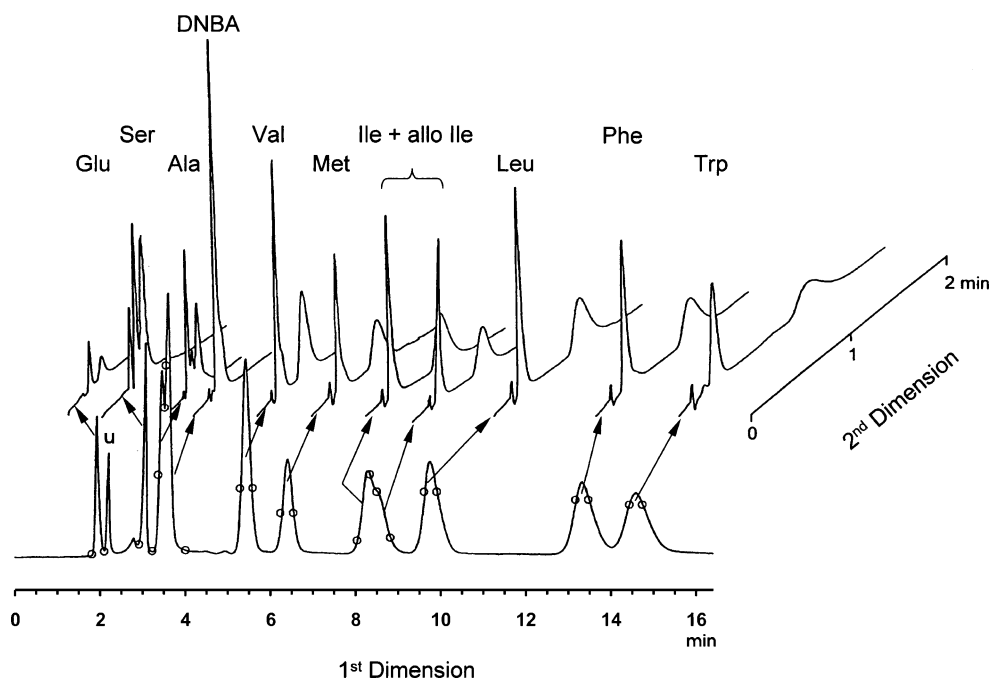


Fig. 5 Two-dimensional enantioselective separation of a mixture of nine racemic DNB-amino acids. Column 1: glass-lined tubing (150 mm×1 mm i.d.), packed with Nucleosil RP-18, 5 μ m; mobile phase: ammonium acetate buffer (50 mM, pH 5.5):acetonitrile, 75:25 (v:v); flow rate: 40 μ L min⁻¹; detection: UV, 254 nm. Column 2: stainless steel (33×4.6 mm i.d.), packed with *tert*-butyl quinidine carbamate-modified nonporous silica particles, 1.5 μ m; mobile phase: ammonium acetate buffer (50 mM, pH 4.5):acetonitrile, 70:30 (v:v);

flow rate: 2 mL min⁻¹. Components: *Glu*, 3,5-DNB-glutamic acid; *u*, unknown; *Ser*, 3,5-DNB-serine; *Ala*, 3,5-DNB-alanine; *DNBA*, dinitrobenzoic acid; *Val*, 3,5-DNB-valine; *Met*, 3,5-DNB-methionine; *Ile + allo Ile*, 3,5-DNB-isoleucine + 3,5-DNB-allo-isoleucine; *Leu*, 3,5-DNB-leucine; *Phe*, 3,5-DNB-phenylalanine; *Trp*, 3,5-DNB-tryptophan (the open circles indicate the switching times at the beginning and end of a fraction, respectively)

chromatograms of a two-dimensional separation of a mixture of nine racemic DNB-amino acids are shown. The switching times are indicated on the first-dimension chromatogram (shown in the foreground) with a circle. The foreground chromatogram was generated with the monitor detector installed between the two columns. Chromatograms of the enantiomer separation on the NPS-CSP column are depicted behind each peak of the foreground chromatogram.

It is obvious that most of the pairs of enantiomers were separated in less than one minute on the second-dimension NPS-CSP column at a flow rate of 2 ml min⁻¹. This flow rate is fast enough to perform the separation of at least one fraction of a peak eluting from the first column without having to interrupt the first-dimension separation. In this way, the total analysis time can be kept below 16 minutes. Further, it should be emphasized that the results of such a 2D-HPLC enantiomer composition analysis can be conveniently verified by using the corresponding *tert*-butyl quinidine carbamate NPS-CSP in the second dimension, which elutes the enantiomers in reverse order [32].

At this point, we would like to return to the *Ile*/*allo-Ile* issue. This example clearly demonstrates the critical problem that may occur if there is insufficient selectivity

in the first-dimension separation, as is the case under the given conditions for this diastereomeric peak pair. As mentioned above, we noticed a peak shoulder but incomplete resolution in the first-dimension chromatogram (see Fig. 5). After transferring different slices of this peak onto column two (CSP), the peak ratios of the enantiomers did not corroborate with the expected 1:1 ratio. On the CSP, both of the DNB-labeled analytes had about the same α values and the same retention characteristics. In other words, one can barely distinguish between the enantiomers of isoleucine and allo-isoleucine on the CSP. This fact may have the consequence that a partially resolved peak of isoleucine and allo-isoleucine on the first column being transferred onto the second column may be mistaken for a distinct enantiomer ratio and thus lead to erroneous results. This was the case with our commercial *DL*-isoleucine sample, which did not actually consist solely of isoleucine enantiomers, but a mixture of isoleucine and diastereomeric allo-isoleucine. Due to the isobaric nature of these diastereomeric solutes, not even mass spectrometric detection can overcome this problem. Hence, the only way out of this dilemma is to provide adequate selectivity in the first dimension. In fact, RP chromatography has the ability to separate diastereomeric *Ile*/*allo-Ile* derivatives (see also

[32]). Thus, in a separate experiment the conditions were optimized (data not shown) to achieve baseline resolution for DNB-Ile and DNB-allo-Ile on the first-dimension RP column, which could only be accomplished, however, at the expense of a much longer retention time. Future work will focus on optimizing the chemoselectivity between the two isomeric forms of Ile and allo-Ile in the first dimension without compromising on run time. In fact, it seems that there is some physiological relevance to the selective analysis of allo-isoleucine, and the selectivity problem outlined above cannot be alleviated by mass spectrometric detection [32]. The same idea applies to DNB-Ser and DNB-Ala, for which the selectivity from the injection peak (the first peak in the second-dimension chromatogram) and from the 3,5-dinitrobenzoic acid (DNBA) peak (the middle peak in the second-dimension chromatogram, which originates from the hydrolysis of the derivatizing agent) is in need of further optimization, particularly if the method is intended for use in the enantiomeric purity determinations of highly enantioenriched samples. However, the results presented here clearly constitute a proof of principle for the viability of a fast comprehensive 2D-HPLC enantiomer composition assay.

Conclusion

Using the concept of a comprehensive on-line 2D-HPLC setup obtained by coupling an RP system in the first dimension to a fast-separation enantioselective (CSP) system in the second dimension, we were able to prove the feasibility of the enantioselective analysis of complex mixtures. The mobile phases used for both column systems were almost identical, which permitted easy on-line peak (fraction) transfer. This approach was ideally realised using the CSP described above, which tolerates hydroorganic mobile phases without sacrificing enantioselectivity.

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