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Indirect separation of chiral proteinogenic α-amino acids using the fluorescence active (1*R*,2*R*)-N-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide) as chiral derivatizing agent A comparison

O.P. Kleidernigg, W. Lindner*

Institute of Pharmaceutical Chemistry, Karl-Franzens University of Graz, A-8010 Graz, Austria

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Abstract

Enantiomerically pure *trans*-1,2-diaminocyclohexane (DACH) based (1*R*,2*R*)- and (1*S*,2*S*)-N-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide ((*R*,*R*)- and (*S*,*S*)-CDITC) was designed as a new fluorescence tagging chiral derivatizing agent (CDA) for the separation of amino acids and chiral amines. The derivatization of amino acids with CDITC yielded highly fluorescent diastereomeric thioureas. These derivatives were separated on a non-chiral RP-HPLC system employing C_{18} as stationary phase. The respective optimum separation condition were evaluated and the optimum pH was found to be 4.30. The α-value achieved ranged from 1.14 to 3.16 with peak resolutions ranging between 0.59 and 13.94. The fluorescent labeled amino acids were detected at λ_{ex}=333 nm, λ_{em}=430 nm. These fluorescence properties almost matched the nitrogen laser line of 335 nm making laser-induced fluorescence detection (LIF) possible. The HPLC separations were compared to quasi capillary electrochromatographic (CEC) measurements of the same diastereomers using polyvinyl pyrolidone (PVP) as pseudo-stationary phase. All 19 chiral proteinogenic amino acids were separated. The selectivity values ranged between 1.026 and 1.232 with R_s values from 1.37 to 16.25. Detection was achieved by a diode array detector. The effect of PVP concentration, pH, temperature and organic modifier on the separations was evaluated. The optimum CEC separation conditions were found to be 20 m*M* sodium citrate (pH 3), 0.5% (w/v) PVP, 9% 2-propanol and 1% *tert*.-butylmethyl ether. © 1998 Elsevier Science B.V.

Keywords: Derivatization, LC; Derivatization, electrophoresis; Enantiomer separation; Diastereomer separation; Amino acids

1. Introduction

Numerous projects related to peptide synthesis and peptide analysis [1–3] also necessitate highly sensitive analytical methods for the determination of amino acids [4,5]. During the last two decades, the analytical methodologies in this area have been considerably strengthened due to the rapid advances of liquid chromatography and the development of fluorogenic reagents for LC detection. Furthermore, capillary electrophoresis (CE) and laser induced fluorescence (LIF) detection of fluorescent labeled

*Corresponding author. Present address: University of Vienna, Institute of Analytical Chemistry, Währingerstr. 38, A-1090 Wien, Austria.

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amino acids [6–8] set a new milestone in the field of investigations and the highly sensitive analysis of amino acids and peptides.

Two fundamentally different strategies for the separation of enantiomers of α- β- and γ-amino acids have been established. The first so called 'direct mode' uses a chiral stationary phase (CSP) in HPLC [9–11] or chiral additives as e.g. β-cyclodextrins in CE [12–14]. Dealing with the direct method the amino acids may be N-terminally derivatized with achiral tags [9,15] in order to overcome the lack of detection sensitivity or the absence of suitable interaction sites. The enantioseparation of underivatized amino acids may be accomplished by chiral ligand-exchange chromatography (CLEC) [16,17] or by special selectors eg. crown ether based CSPs [18,19] or additives.

The second 'indirect mode' employs precolumn derivatization with chiral derivatizing agents (CDAs) — which may also be called chiral selectors (SO)-forming diastereomers with amino acids, the selectands (SAs), followed by separation on non-chiral stationary phases [20–23] or involving sodium dodecyl sulfate (SDS) in micellar electrokinetic chromatography (MEKC) [2,24,25].

$$(R,S)\text{SA}+(R)\text{SO}\rightarrow(R)\text{SA}-(R)\text{SO}+(S)\text{SA}-(R)\text{SO}$$

mixture of diastereomers

Recently Schützner et al. [26] reported on a new pseudo-stationary phase to be used in capillary electrochromatography (CEC) namely polyvinyl pyrolidone (PVP).

In recent years various chiral fluorescent tagging reagents for the derivatization of diverse proteinogenic and non-proteinogenic amino acids have been developed [27,28]. The most widely reported fluorescence active CDAs are 4-(3-isothiocyanato-2-pyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (NBD-PyNCS) [1,5,29,30] and 4-(3-isothiocyanato-pyrrolidin-1-yl) - 7 - (N, N - dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS) [29]. A chloroformyl-based fluorescent, chiral reagent is (+) and (−)-1-(9-fluorenyl)-ethyl chloroformate ((+)- and (−)-FLEC) [14,31–33]. Exceptions in this field are the achiral ortho-phthaldialdehyde (OPA) [23,25,34] and naphathalene-2,3-dicarboxaldehyde (NDA) [35] used together with chiral thiols thus introducing the 'chiral handle' within the diastereomers.

The aim of this work was the diastereoseparation of the 19 chiral proteinogenic α-amino acids employing the new fluorescence tagging CDA [36] (1*R*,2*R*)- and (1*S*,2*S*)-N-[(2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide ((*R*,*R*)- and (*S*,*S*)-CDITC) by HPLC and CE and comparing the results with the emphasis on finding optimum separation conditions.

These isothiocyanate based CDAs form highly fluorescent derivatives with the amino functions of amino acids (Fig. 1). The derivatives' fluorescence properties ($\lambda_{ex}=333$ nm, $\lambda_{em}=430$ nm) within a pH range of 1–7.5 almost match the nitrogen laser line of 335 nm but also a line of He/Cd laser.

The resulting diastereomeric thioureas were separated on a non chiral RP-HPLC system using a C_{18} stationary phase. These results were critically compared to a CE method employing PVP [26], a linear polymer, as pseudo-stationary phase.

The optimum separation conditions for the HPLC method are described in detail. The effect of pH, temperature and organic modifiers on the CE separations of the α-amino acid diastereomers is discussed.

2. Experimental

2.1. Chemicals and reagents

All solvents used were of HPLC grade. Water for CE and HPLC use was double distilled and deionized by a Millipore Milli-Q Plus system. Mobile phases were filtered through a Nalgene nylon membrane filter (0.2 μm) (Nalge, New York, USA) and degassed before use. The pH of the mobile phases refers to the apparent pH (pH_a) and was measured with an Orion pH meter, model 520A. Sodium hydroxide and sodium citrate buffer solutions in a pH range between 3 and 6 (each 20 m*M*) were obtained from Fluka (Buchs, Switzerland). Racemic and enantiomerically pure α-amino acids were purchased from Sigma (St. Louis, USA). PVP with an average molecular weight of 25 000 was available at the Institute of Pharmaceutical Chemistry, University of Graz, Graz, Austria. The fluorescent active CDAs (*R*,*R*)- and (*S*,*S*)-CDITC) were synthesized as de-

scribed elsewhere [36,37]. All other chemicals were of analytical reagent grade.

2.2. Apparatus

2.2.1. HPLC

All HPLC measurements carried out in this work were performed with a Hewlett-Packard HP 1050 compact system with UV detector, a Jasco FP 920 intelligent fluorescence detector with a standard flow cell and a HP ^{2D}HPLC CHEMSTATION version A.03.02.. The column used was a Beckman ODS (150×4.6 mm I.D., 5 μm). The various mobile phase compositions are summarized in detail in Table 1.

2.2.2. CE

All CE separations were accomplished employing a Hewlett-Packard HP ^{3D}CE compact system (Hewlett-Packard, Vienna, Austria) with diode array detection (DAD) and HP ^{3D}CE CHEMSTATION. The separation capillary was unmodified fused-silica of 62 cm total length (with 75 mm I.D.) and 54 cm effective length. Injection was achieved by pressure application of 30 mbar for 3 s. The running buffer compositions used are described in detail in Table 1.

Table 1
Liquid chromatographic and electrophoretic data of (*R,R*)-CDITC derivatized (*R,S*)-α-amino acids

Amino acids	RP-HPLC						CE				
	Conditions	k'^{a}_1	k'^{a}_2	α^{b}	R_s^{c}	e.o.[d]	μ_1^{e}	μ_2^{e}	α^{f}	R_s^{c}	e.o.[d]
Gly	A	1.81	–	–	–	–	3.50	–	–	–	–
Ala	A	1.85	3.59	1.94	5.57	*R*<*S*	3.96	3.78	1.048	2.99	*R*<*S*
Val	A	3.37	8.64	2.56	9.60	*R*<*S*	3.73	3.49	1.069	6.53	*R*<*S*
Leu	A	4.85	15.32	3.16	13.94	*R*<*S*	3.79	3.56	1.065	5.49	*R*<*S*
Ileu	A	4.80	14.70	3.06	–	*R*<*S*	3.97	3.87	1.026	1.37	*R*<*S*
Ser	A	1.43	2.11	1.48	2.08	*R*<*S*	3.62	3.35	1.081	5.42	*R*<*S*
Thr	A	1.90	4.14	2.17	5.33	*R*<*S*	4.11	3.84	1.070	5.99	*R*<*S*
Cys[g]	B	1.11	1.27	1.14	0.59	*S*<*R*	5.07	4.77	1.063	5.68	*S*<*R*
Met	A	3.12	7.04	2.26	7.84	*S*<*R*	3.70	3.59	1.031	2.71	*S*<*R*
Arg[g]	C	6.31	8.43	1.34	1.95	*R*<*S*	6.25	5.84	1.070	2.59	*R*<*S*
Lys[g]	A	1.88	2.47	1.32	1.39	*R*<*S*	4.48	3.97	1.128	6.26	*R*<*S*
Asp	D	8.63	13.5	1.57	5.48	*R*<*S*	5.43	4.82	1.127	8.97	*R*<*S*
Asn	A	1.29	1.86	1.44	1.53	*R*<*S*	5.56	4.78	1.163	10.62	*R*<*S*
Glu	D	9.14	14.18	1.55	5.18	*R*<*S*	5.41	4.80	1.127	8.85	*R*<*S*
Gln	A	1.22	1.53	1.37	1.55	*R*<*S*	3.25	2.98	1.091	5.96	*R*<*S*
Phe	E	2.92	6.03	2.07	6.24	*R*<*S*	3.67	3.36	1.092	8.48	*R*<*S*
Pro	C	4.30	11.66	2.71	11.41	*R*<*S*	4.04	3.84	1.052	10.60	*R*<*S*
Tyr	–	–	–	–	–	–	3.72	3.17	1.174	16.25	*R*<*S*
Trp	A	5.21	15.29	2.91	11.98	*R*<*S*	3.98	3.23	1.232	12.65	*R*<*S*
His	A	1.57	1.90	1.21	1.02	*R*<*S*	6.09	5.66	1.076	8.68	*R*<*S*

General HPLC conditions. Column: Hypersil ODS (125×4 mm I.D., 5 μm); flow-rate: 1 ml/min; detection fluorescence: $\lambda_{ex}=333$ nm, $\lambda_{em}=430$ nm; mobile phase compositions: (A) MeOH–20 m*M* ammonium acetate (55:45, v/v) apparent pH 4.28; (B) MeOH–20 m*M* ammonium acetate (55:45, v/v) apparent pH 3.70; (C) MeOH–20 m*M* ammonium acetate (45:55, v/v) apparent pH 4.28; (D) MeOH–20 m*M* ammonium acetate (40:60, v/v) apparent pH 4.22; (E) MeOH–20 m*M* ammonium acetate (55:45, v/v) apparent pH 5.62.
General CE conditions: capillary: fused-silica, $L_{total}=62$ cm and $L_{effective}=54$ cm (75 mm I.D.); applied voltage: 30 kV; detection, UV 254 nm; mobile phase composition: 20 m*M* sodium citrate (pH 3), 0.5% (w/v) PVP, 10% 2-propanol, 1% TBME; temperature: 20°C.
[a] $k'=t_R-t_0/t_0$.
[b] $\alpha=k'_2/k'_1$.
[c] $R_s=1.18\times t_2-t_1/w_{(0.5)1}+w_{(0.5)2}$.
[d] Elution order.
[e] $\mu=L_{tot.}\times L_{eff.}/t_R\times V=[cm^2/min\times V]\times 10^{-3}$.
[f] $\alpha=\mu_1/\mu_2$.
[g] *bis* derived diastereomers.

The CE capillary was preconditioned at the beginning of each day. This was performed by flushing the capillary (ca. 930 mbar) with water for 3 min, 1.0 *M* NaOH for 10 min, water for 5 min and running buffer for 10 min. The capillary was further conditioned prior to each run by flushing with 0.1 *M* NaOH for 1 min, then with water for 3 min and finally with buffer for 7 min before sample introduction.

2.3. Derivatization procedure

(*R*,*R*)- and (*S*,*S*)-CDITC reagent solutions were prepared by dissolving the reagent in acetonitrile (6 mg/ml). Racemic and enantiomerically pure amino acids were dissolved in water (1 mg/ml). The derivatization was carried out in a standard 1.5-ml autosampler vial by mixing 100 μl amino acid stock solution with 5 μl 2.5% Na_2CO_3 solution, 100 μl water–acetonitrile (1:1) and 100 ml reagent solution. The reaction mixture was vortexed (30 s) and then stored in an oven at 60°C for 2 h. The cooled reaction solution was diluted and neutralized with 200 μl slightly acidic mobile phase or running buffer. This solution was directly injected onto the RP-HPLC column or CE capillary.

3. Results and discussion

3.1. Purity of the reagent

The risks involved in using CDAs for separation of enantiomers and determination of enantiomeric purity includes racemisation of any stereogenic center during the derivatization and the limited enantiomeric purity of the CDA [38].

The determination of the enantiomeric purity of (*R*,*R*)- and (*S*,*S*)-CDITC has been reported recently [15]. It was found to be greater than 99.9% enantiomeric excess (ee) in both cases.

The proposed reagents enantiomers exhibit excellent chemical and configurational stability, not only in solid form, but also in solution. Neither significant chemical degradation nor racemisation was observed in acetonitrile solution after storage for 2 weeks at ambient temperature.

3.2. Peculiarities associated with precolumn derivatization

As with any isothiocyanate based CDA [22,30,39] CDITC reacts chemoselectively with primary and secondary amino functions of amines, amino alcohols, amino acids and peptides forming the corresponding diastereomeric thioureas. This is considered an advantage over other fluorescence tagging reagents such as OPA [23] and NDA [35], which react only with primary amines making a fluorescence derivatization of secondary amino function of e.g. proline impossible. The derivatization kinetics of (*R*,*S*)-Leu with (*R*,*R*)-CDITC are displayed in Fig. 1. The derivatization conditions are described in Section 2.3.

Isothiocyanate type reagents require basic reaction conditions [40] for the derivatization of amines. In this work 2.5% aqueous Na_2CO_3 solution was employed as base component. The reaction medium

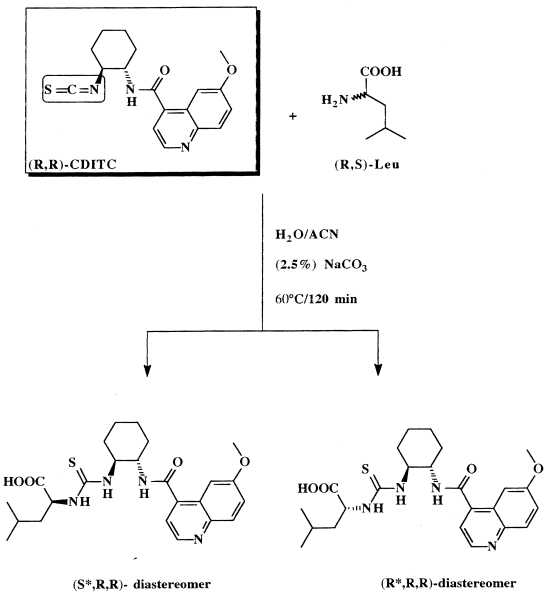

Fig. 1. Derivatization of (*R*,*S*)-leucine employing (*R*,*R*)-CDITC as CDA and formation of the corresponding diastereomeric thioureas.

was acetonitrile–water but methanol or THF water mixtures were equally suitable. The opportunity of also using aqueous reaction media with CDITC should be of particular interest in the field of amino acid and peptide analysis or tagging where aqueous media are very common.

However, it is crucial that the derivatization proceeds quantitatively under mild conditions in order to prevent any kinetic resolution effects caused by different reaction rates of the enantiomers of the selectands. Therefore it is recommended to use a significant excess of the CDA and to drive the reaction to completion. In this contribution at least a 2.5-fold molar excess of the CDA was used. In a course of time study shown in Fig. 2 using (*S*)-valine as analyte it is demonstrated that the derivatization is complete within 120 min under mild conditions (60°C). At all derivatization conditions tested no racemization of any stereogenic center, either of the CDA or of the analyte, was observed.

At too-basic conditions (pH>10) a slight degradation of the isothiocyanate to the corresponding amine was observed. Since the kinetics of the latter reaction may be faster then the derivatization kinetics it is recommended to add another portion of the CDA after e.g. 60 min to the reaction solution to yield quantitative derivatization. Once the diastereomeric thioureas are formed no base induced degradation was observed. Derivatized samples were stored at 4°C in the dark, and may be used for analysis within 6 days.

3.3. HPLC

3.3.1. Optimum separation conditions

In order to establish optimum RP-HPLC conditions for the separation of the diastereomeric amino acid derivatives (thioureas) several mobile phase compositions were tested. A mixture of 20 m*M* ammonium acetate (only aqueous phase) and methanol delivered the best results. The different methanol–ammonium acetate ratios for the individual measurements are summarized in Table 1.

pH effects on the indirect chiral separation were studied over the apparent pH range of 3.50–4.90. This study showed clearly that the best selectivity values were obtained at a pH value of about 4.30 as demonstrated in Fig. 3 for (*S*,*S*)-CDITC derived (*R*,*S*)-alanine. Other amino acids tested delivered the same results. The high α-value of 1.94 at pH 4.30 may possibly indicate intramolecular interactions and binding forces including steric ones. In particular coulomb interactions between the carboxylic acid functionality and the basic nitrogen within the quinoline ring may be taken into consideration. This hypothesis may be supported by CE results mentioned below, where the (*R*,*S*)-leucine diastereomers using (*R*,*R*)-CDITC as CDA did not migrate either from cathode to anode or from anode to cathode at a pH of 4.2. The latter pH may lie in the range of the isoelectric point (p*I*) value, where all charges are intramolecularly neutralized and thus making migration in an electric field impossible. However, the

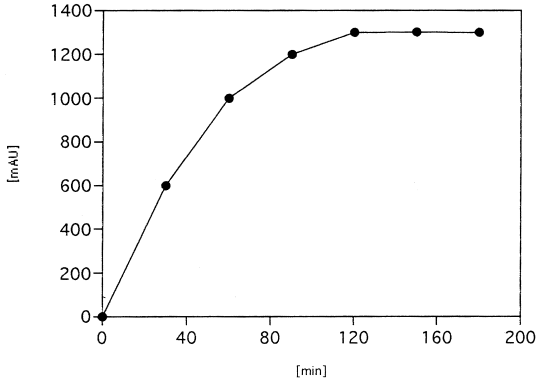

Fig. 2. Time course study of the derivatization of (*R*,*S*)-valine using (*R*,*R*)-CDITC as CDA.

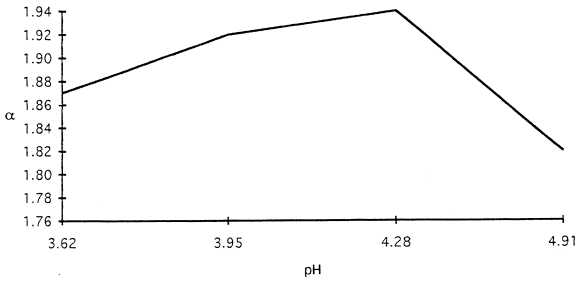

Fig. 3. Effect of pH on the selectivity of (*R*,*R*)-CDITC derived (*R*,*S*)-alanine. Column: ODS 150×4.6 mm I.D. 5 μm; Mobile phase: MeOH–20 m*M* ammonium acetate (55:45, v/v) apparent pH 3.62–4.91 (adjusted with glacial acetic acid). Flow-rate: 1 ml/min; detection λ_{ex}=333 nm, λ_{em}=430 nm.

intramolecular 'zwitterion' formation and neutralization — also described by Lindner et al. [21] for amino alcohols derived as tartaric acid mono esters — may be considered as an important binding increment. The diastereoselectivity expressed as α-value of a pair of diastereomeric derivatives is much lower without the additional ring formation. In fact, Fig. 3 displays that the α-value of the alanine diastereomers is altered by pH. The pK_a value of the chinoline nitrogen may be in the range between 4.5 and 5.0 derived from values of quinine [41].

From the summarized chromatographic data shown in Table 1 it is apparent that the (*R*,*R*)-CDITC diastereomers of 18 proteinogenic α-amino acids, were well separated on a standard ODS reversed-phase column. However, a separation of (*R*,*S*)-Tyr was not achieved due to derivatization by-products making peak identification and quantification uncertain and unclear. The phenolic OH function is prone to react with isothiocyanates under basic conditions, however the reaction conditions may be chosen to minimize this side reaction.

Using (*R*,*R*)-CDITC as derivatizing agent, the diastereomeric thioureas of the absolute (*R**,*R*,*R*) configuration were always eluted first, so far and from the stereochemical point of view no exception for this 'rule' was found. However, the reverse elution order of (*R*,*S*)-Cys and (*R*,*S*)-Met diastereomers is not based on conformational changes, but on the priority rules according to the Chan Ingold Prelog (CIP) nomenclature associated with amino acids containing a sulfur atom. Employing the older DL nomenclature, usually used for chiral amino acids, no change in the elution order would be observed. As expected, a reversal of elution order was observed for all diastereomers when employing (*S*,*S*)-CDITC instead of (*R*,*R*)-CDITC as CDA. In fact, this is an important advantage compared to CDAs which are based on naturally occurring chiral templates as, for instance, the carbohydrate based 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [42]. The same limitations may also be of significance for cellulose based chiral stationary phases [43]. Among the α-amino acids investigated the selectivity values ranged between 1.14 and 3.16, whereas the peak resolution displayed values ranged between 0.59 and 13.94. As indicated earlier a reasonable explanation may be the pronounced intramolecular folding and thus relatively rigid conformation of the diastereomeric thioureas. The separation of (*R*,*S*)-Pro employing (*R*,*R*)-CDITC as CDA is depicted in Fig. 4.

3.4. CE separation of diastereomers employing PVP as pseudo-stationary phase

3.4.1. Effect of PVP concentration on stereoselectivity

Schützner et al. [26] recently reported a new pseudo-stationary phase, namely linear PVP. Motivated by those results 0.5%-5.0% (w/v) PVP was added to the running buffer. The molecular mass of

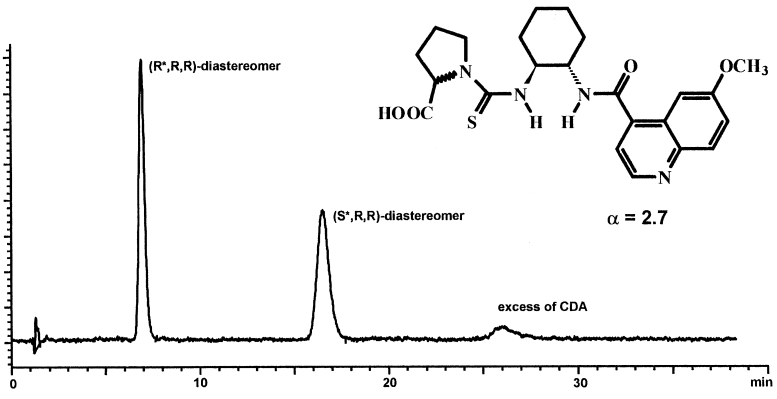

Fig. 4. Chromatogram of a RP-HPLC separation of the diastereomeric (*R*,*S*)-proline derivatives using (*R*,*R*)-CDITC as CDA. Column: ODS 150×4.6 mm I.D. 5 μm; Mobile phase: MeOH–20 m*M* ammonium acetate (45:55, v/v), apparent pH 4.28 (adjusted with glacial acetic acid). Flow-rate: 1 ml/min; detection λ_{ex}=333 nm, λ_{em}=430 nm.

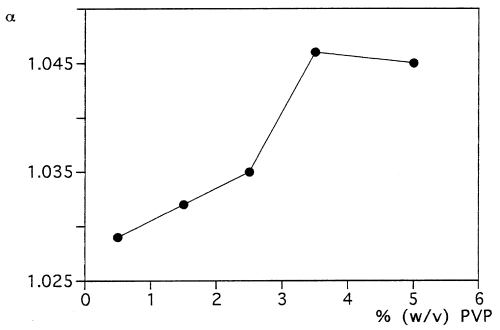

Fig. 5. Effect of the PVP concentration (% [w/v]) on the selectivity of CEC separations of (*R*,*R*)-CDITC derived (*R*,*S*)-leucine. Capillary: fused-silica $L_{total}=62$ cm and $L_{effective}=54$ cm (75 mm I.D.); applied voltage 30 kV; detection: UV 254 nm; mobile phase composition: 20 m*M* sodium citrate, 0.5–5% PVP, 10% 2-propanol, 1% TBME; apparent pH 3.2; temperature 20°C.

the polymer was 25 000. The results, summarized in Fig. 5, show that maximum selectivity of 1.046 and peak resolution of 4.08 was achieved by the addition of 3.5% PVP. The addition of 5% PVP decreased the α-value to 1.045 and the R_s value to 3.31.

Despite the enhanced α-value and R_s value, however, high PVP concentrations also created a system problem. Electropherograms (data not shown) involving PVP concentrations >1% (w/v) displayed an extreme baseline drift. This problem was greatly circumvented by adding only 0.5% (w/v) PVP to the running buffer. A further advantage of lower PVP concentrations was the shorter retention times of the diastereomeric analytes. However, PVP concentrations <1% (w/v) are indeed low but are a workable compromise in order to overcome baseline drifting as well as high UV background associated with higher PVP concentrations.

3.4.2. Effect of pH on separation

In this work pH effects were studied in a range between 3 and 5, for (*R*,*S*)-leucine derived as (*R*,*R*)-CDITC thiourea employing 20 m*M* sodium citrate buffer as background electrolyte and 2.5% (w/v) PVP as pseudo-stationary phase.

Due to the still basic nitrogen within the reagent's quinoline group (pK_a ca. 4.8–5.0) the CDITC and the respective derivatives were prone to act as cations in CE at a pH of 3 or lower. As expected good results were obtained by adjusting the pH below 4. As displayed in Table 2 the best selectivity values were achieved with a pH value of 3. The direction of the current during this experiment was adjusted as normally to run from anode (+) to cathode (−). At a pH of 4.2 the amino acid derivatives showed no or very little migration, which may indicate their zwitterionic status close to the p*I* value. Measurements at pH 5 showed a separation of the diastereomers with the inverted current direction from (−) to (+).

Table 2
Influence of pH on the selectivity and resolution of (*R*,*R*)-CDITC derivatized (*R*,*S*)-leucine

pH	Direction of current	μ_1	μ_2	α	R_s	e.o.
3.2	+	4.59	4.45	1.032	1.89	*R*<*S*
4.2	+	−	−	−	−	−
4.2	−	−	−	−	−	−
5.2	+	−	−	−	−	−
5.2	−	3.26	3.21	1.017	1.02	*R*<*S*

Conditions: buffer; 20 m*M* sodium citrate buffer containing 2.5% (w/v) PVP; capillary: 62 cm×75 mm I.D. (effective length 54 cm); applied voltage: 30 kV; temperature: 20°C; detection: UV 254 nm.
[a] As in Table 1.
[b] Elution order.

Interestingly, no evidence for inversion of elution order of the (*R**,*R*,*R*)- and (*S**,*R*,*R*)-diastereomeric thioureas of leucine was observed, when changing the pH from 3.2 to 5.2 and inverting the direction of current.

3.4.3. Effect of temperature

The temperature study ranging from 15°C to 30°C on the diastereomeric separation of (*R*,*R*)-CDITC derived (*R*,*S*)-leucine revealed the expected selectivity enhancement with decreasing temperature. The expected peak resolution improvement with increasing temperature was also observed. For exact condition see Fig. 6.

Since diastereomers have different physico–chemical properties they also differ in their energy content. The Gibbs–Helmholz equation displays the relationship between enantiomeric- and/or diastereomeric separation and temperature. Accordingly and in first approximation the enthalpy should be temperature dependent.

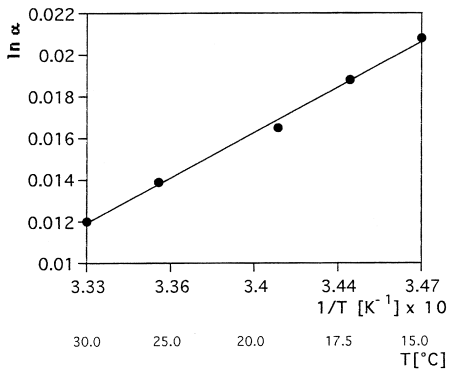

Fig. 6. Van't Hoff plot displaying the linear relationship of temperature on the selectivity of (*R*,*R*)-CDITC derived (*R*,*S*)-leucine. Capillary: fused-silica, $L_{total}=62$ cm and $L_{effective}=54$ cm (75 mm I.D.); applied voltage: 30 kV; detection: UV 254 nm; mobile phase composition: 20 m*M* sodium citrate, 0.5% (w/v) PVP, 9% 2-propanol, 1% TBME; apparent pH 3.2; temperature range: 15–30°C. Correlation coefficient 0.998. Curve equation: $y=-0.195+62.156x$.

$$\frac{\Delta\Delta G}{RT}=\frac{\Delta\Delta H}{RT}+\frac{\Delta\Delta S}{R}$$

$$\ln\alpha=-\frac{\Delta\Delta G}{RT}$$

If the latter approximation is correct the data points of ln α versus $1/T$ should follow a linear relationship. The curve displays a slope of $-\Delta\Delta H/R$ and an intercept of $\Delta S/R$. The present temperature study followed this rule and a linear relationship of ln α versus $1/T$ was observed. This Van't Hoff plot is shown in Fig. 6, the correlation coefficient was 0.998 and the equation appeared to be $y=62.156x-0.195$.

However, at a temperature of 12.6°C the diastereomeric thioureas of (*R*,*S*)-leucine were separated with an α-value of 1.019 with a resolution value of 0.51 without any organic modifier or pseudo-stationary phase additive in the background electrolyte.

3.4.4. Effect of organic modifier

In previous work on CE [13] and MEKC [25] of amino compounds including amino acids, it was demonstrated that the addition of organic modifiers enhanced enantio- and/or diastereo-separation.

A study of the effect of organic modifiers was also undertaken in order to compensate for the loss of diastereoselectivity by the reduced PVP concentration of 0.5% PVP as described above and depicted in Fig. 5. The effect was studied on the diastereomeric separation of (*R*,*R*)-CDITC derived (*R*,*S*)-leucine. The surprising results are displayed in Table 3. The addition of 9% 2-propanol and 1% *tert*.-butyl methyl ether (TBME) showed the greatest effect on diastereoselectivity resulting in an increase of α from 1.020 to 1.096-and a peak resolution of 5.71. Even without PVP as pseudo-stationary phase within the running buffer the selectivity value of 1.046 was as great as the selectivity with the highest PVP concentration in the background electrolyte. Addition of 2-propanol or acetonitrile but without TBME and PVP did not alter the separation much. Without further detailed studies it was very difficult to make predictions about the effect of the individual solvent components on stereoselectivity. Multivariant approaches will be necessary to facilitate reasonable optimization strategies for multi-component systems.

3.4.5. CE separations of the α-amino acid diastereomers employing PVP as pseudo-stationary phase

From the summarized chromatographic data shown in Table 1 it is apparent that the (*R*,*R*)-CDITC diastereomers of all 19 chiral, proteinogenic α-amino acids, were well separated by CE employing PVP as pseudo stationary phase. The α-values of the investigated α-amino acids ranged between 1.026 and 1.232, whereas the peak resolution displayed values ranging from 1.37 and 16.25. However, at the chosen

Table 3
Influence of different organic modifiers on the selectivity and resolution of (*R*,*R*)-CDITC derivatized (*R*,*S*)-leucine

Modifier	μ_1[a]	μ_2[a]	α[a]	R_s[a]
10% ACN	5.17	4.97	1.041	1.19
9% ACN–1% TBME	4.82	4.63	1.042	1.47
10% 2-Propanol	3.45	3.22	1.072	4.55
9% 2-Propanol–1% TBME	2.11	2.84	1.096	5.71
9% 2-Propanol–1% TBME[b]	6.72	6.42	1.046	0.83

Conditions: buffer; 20 m*M* sodium citrate buffer (pH 3) containing 0.5% (w/v) PVP; capillary: 62 cm×75 mm I.D. (effective length 54 cm); applied voltage: 30 kV; temperature: 20°C; detection: UV 254 nm.
[a] As in Table 1.
[b] Without PVP.

pH of 3 intramolecular neutralization of the ionizable groups may be limited and the noticed diastereoselectivity has to be attributed to intramolecular interactions [44] based on hydrophobic, hydrogen bonding, dipole–dipole forces in conjunction with solvophobic effects. This results in pronounced conformational changes and diastereomers with different size and/or charge distribution. Some preliminary NMR-based investigations on intramolecular interactions of a CDA belonging to the same family, have been undertaken by our group recently [45].

As observed, the amino acid derivatives were easily and more efficiently separated in presence of PVP as pseudo-stationary phase. Therefore intermolecular interactions between the diastereomers and polymer have also to be considered. The effect of PVP concentration on the stereoselectivity are displayed in Fig. 5. As demonstrated in Table 1 selectivity values of the aromatic amino acids were the greatest, followed by the acidic and basic amino acids. The relatively lowest α-values were observed with the group of neutral amino acids. However, the multi-component mobile phase system (sodium citrate, 0.5% (w/v) PVP, 9% 2-propanol and 1% TBME) made the prediction of the retention behavior of the amino acid CDITC diastereomers and thus selectivity difficult.

3.5. HPLC versus CE

Due to the relatively low plate numbers obtained using polymer additives in CE and the lower diastereoselectivity, the RP-HPLC separations were still superior to the CE separations. However, a particular amino acid mixture of six amino acids as CDITC derivatives (Fig. 7) were quite well separated by CE but not with HPLC either isocratically or by gradient elution. This indicates that the described CE and HPLC methods are complementary to each other thus supportive to solve a given analysis task. As depicted in Fig. 8 the separation of (*R*,*R*)-CDITC derivatized (*R*,*S*)-Ala via CE resulted in less resolution, selectivity and longer retention (migration) time than that obtained via RP-HPLC.

Referring to the elution order both separation techniques depend predominantly on the overall hydrophobicity of the diastereomeric species, which seems also to correlate with the overall size of the dissolved analytes manifested by the identical elution orders, and hydrophobic surface area, respectively. The retention mechanism of RP systems is mainly determined by hydrophobic interactions between the modified silica surface and the analyte. When introducing PVP to the CE running buffer we also have a hydrophobic pseudo-stationary phase within the separation system and thus the elution orders of the

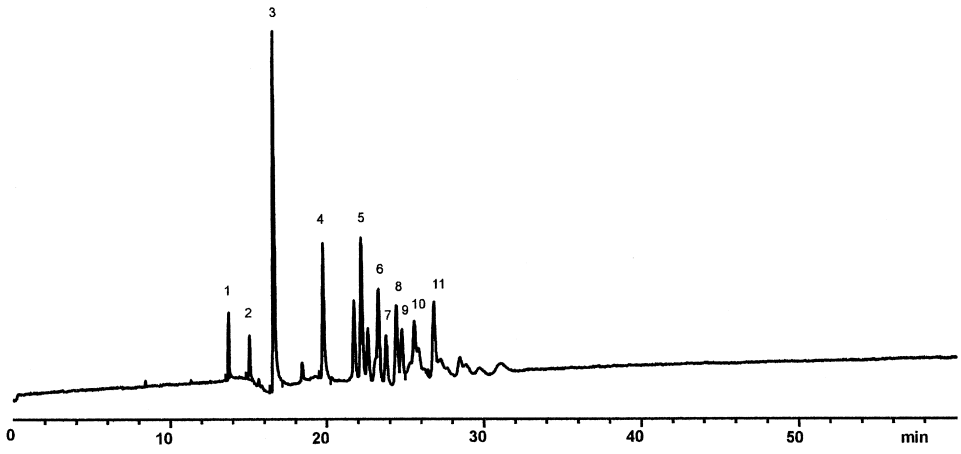

Fig. 7. Single run indirect separation of 6 (*R*,*S*)-amino acids (Val, Leu, Met, Pro, Phe, His) derived with (*R*,*R*)-CDITC. Capillary: fused silica, L_{total} = 62 cm and $L_{effective}$ = 54 cm (75 mm I.D.); applied voltage: 30 kV; detection: UV 254 nm; mobile phase composition: 20 m*M* sodium citrate, 0.5% (w/v) PVP, 9% 2-propanol, 1% TBME; apparent pH 3.2; temperature: 20°C. Peak identification: 1 = (*R*)-His, 2 = (*S*)-His, 3 = (*R*,*R*)-CDITC, 4 = (*R*)-Pro, 5 = (*S*)-Pro, 6 = (*R*)-Phe, 7 = (*R*)-Leu, 8 = (*R*)-Val, 9 = (*R*)-Met, 10 = (*S*)-Leu, 11 = (*S*)-Phe.

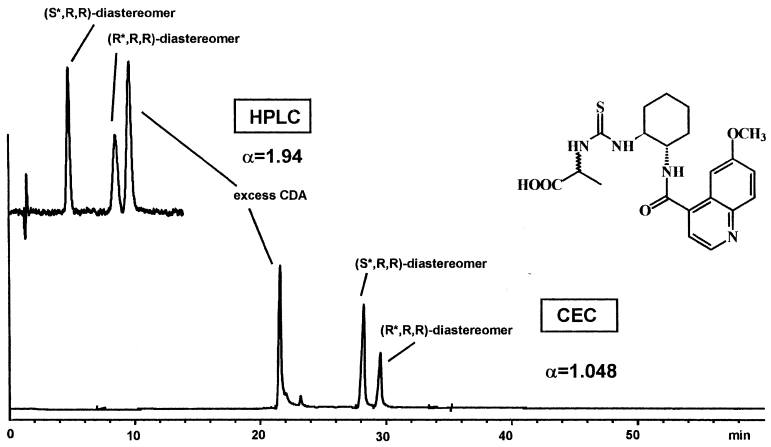

Fig. 8. RP-HPLC versus CEC: Indirect separation of (*R*,*S*)-alanine using (*R*,*R*)-CDITC as CDA. RP-HPLC: Column: ODS 150×4.6 mm I.D. 5 μm; Mobile phase: MeOH–20 m*M* ammonium acetate (55:45, v/v) apparent pH 4.28 (adjusted with glacial acetic acid). Flow-rate: 1 ml/min; detection: $\lambda_{ex}=333$ nm, $\lambda_{em}=430$ nm. CE: Capillary: fused-silica, $L_{total}=62$ cm and $L_{effective}=54$ cm (75 mm I.D.); applied voltage: 30 kV; detection: UV 254 nm; mobile phase composition: 20 m*M* sodium citrate, 0.5% (w/v) PVP, 9% 2-propanol, 1% TBME; apparent pH 3.2; temperature: 20°C.

identical analytes under similar conditions proved to be the same.

In this application CE is not superior to HPLC, the method development was easier for HPLC and the reproducibility data were superior. Another disadvantage of CE is that fluorescence detection is not very common and available yet, therefore detection limits of 20 p*M* at a signal-to-noise ratio of 3 were lower employing HPLC with a tunable fluorescence detector. However, LIF detection systems match the spectroscopic properties of the investigated CDA and the diastereomeric analytes, respectively, which will certainly be of high interest for further applications.

4. Conclusion

(*R*,*R*)- and (*S*,*S*)-CDITC as chiral derivatizing agents provide excellent separation of enantiomers of amino acids by formation of the corresponding diastereomeric thioureas. The derivatization reaction with primary and secondary amino functions of α-, β- and γ-amino acids proceeds chemoselectively without any racemization. The separation of the corresponding diastereomers can be easily achieved by RP-HPLC and CE. Selectivity values for amino acid diastereomers are superior to any other reported CDA [1,22,23,31]. The elution order of the diastereomers can be inverted with the use of the opposite enantiomer of the CDA. This feature can be an important issue in the field of chiral trace analysis where the enantiomeric/diastereomeric impurity should elute before the main enantiomer and/or diastereomer in order not to elute on the tailing edge of the main peak leading to inaccurate results. (*R*,*R*)- and (*S*,*S*)-CDITC have already been successfully employed to monitor the enantiomeric purity of (*R*)- and (*S*)-proline [15].

Another interesting aspect of the isothiocyanate based reagents is the fact that the reaction proceeds in both organic solvents as well as in aqueous solution. This is important in the field of amino acid and peptide analysis, where aqueous conditions are very common.

Both (*R*,*R*)- and (*S*,*S*)-CDITC and the diastereomeric derivatives show the excitation wavelength of 333 nm which almost matches the nitrogen laser emission line of 335 nm and one line of the He/Cd laser, making LIF possible.

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