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# Comparative study on the use of *ortho*-phthalaldehyde, naphthalene-2,3-dicarboxaldehyde and anthracene-2,3-dicarboxaldehyde reagents for α-amino acids followed by the enantiomer separation of the formed isoindolin-1-one derivatives using quinine-type chiral stationary phases

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**Abstract**

Cinchona alkaloid based chiral stationary phases (CSPs) were evaluated and compared for the enantiomer separation of a set of α-amino acid derivatives as selectands (SA), using *ortho*-phthalaldehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA) and anthracene-2,3-dicarboxaldehyde (ADA) as reagents in the presence of acetonitrile. Protocols have been developed for the derivatization of most common amino acids in the absence of the usual thiol components (2-mercaptoethanol, mercaptosulphonic acid, sodium sulfite) under acidic and neutral conditions providing the corresponding isoindolin-1-one (phthalimidine) derivatives. They are stable for hours at various reaction conditions compared to thiol or sulfide modified isoindoles resulted by the OPA-thiol reaction type. Among the derivatizing agents, ADA afforded the highest retention factors ($k$) and for the majority of the analytes also resolution ($R_S$) and enantioselectivity ($\alpha$) values (i.e. for tryptophan $k_1 = 23$, $R_S = 4.93$ and $\alpha = 1.43$). Structure variation of the CSPs and selector (SO), respectively indicates that steric arrangement around the binding cleft plays a major role in the enantiodiscriminating events. To provide more detailed information about the derivatization reaction itself, the proposed mechanism for the formation of the OPA derivative (isoindolin-1-one) was further evaluated by deuterium labeling and LC–MS analysis.
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*Keywords:* LC; Chiral stationary phases; Enantiomer separation; Quinine carbamate; *ortho*-Phthalaldehyde; Naphthalene-2,3-dicarboxaldehyde; Anthracene-2,3-dicarboxaldehyde; Isoindolin-1-one; Phthalimidine

## 1. Introduction

The large group of primary amino compounds like α-amino acids and their esters, amino alcohols, alkyl- and aryl-amines, heterocyclic amines etc. react with *ortho*-phthalaldehyde (OPA) (1) via the condensation of the amino functionality (2) with the aromatic *ortho* dicarboxaldehyde group to yield the corresponding *N*-substituted isoindolin-1-one (phthalimidine) derivative (4) [1–6] (see Fig. 1a). Since the first contribution of phthalimidine synthesis developed by Thiele and Schneider [1], several modifications of the reaction conditions have been attempted in order to increase the yield of this very simple one-step reaction towards 100% [2–6]. Consequently, this reaction concept could also be of interest in analytical chemistry for the indirect determination of amines.

According to Grigg [4], the reaction can be carried out in different solvents like acetonitrile, dimethylformamide, methanol even at low temperature, in the presence of

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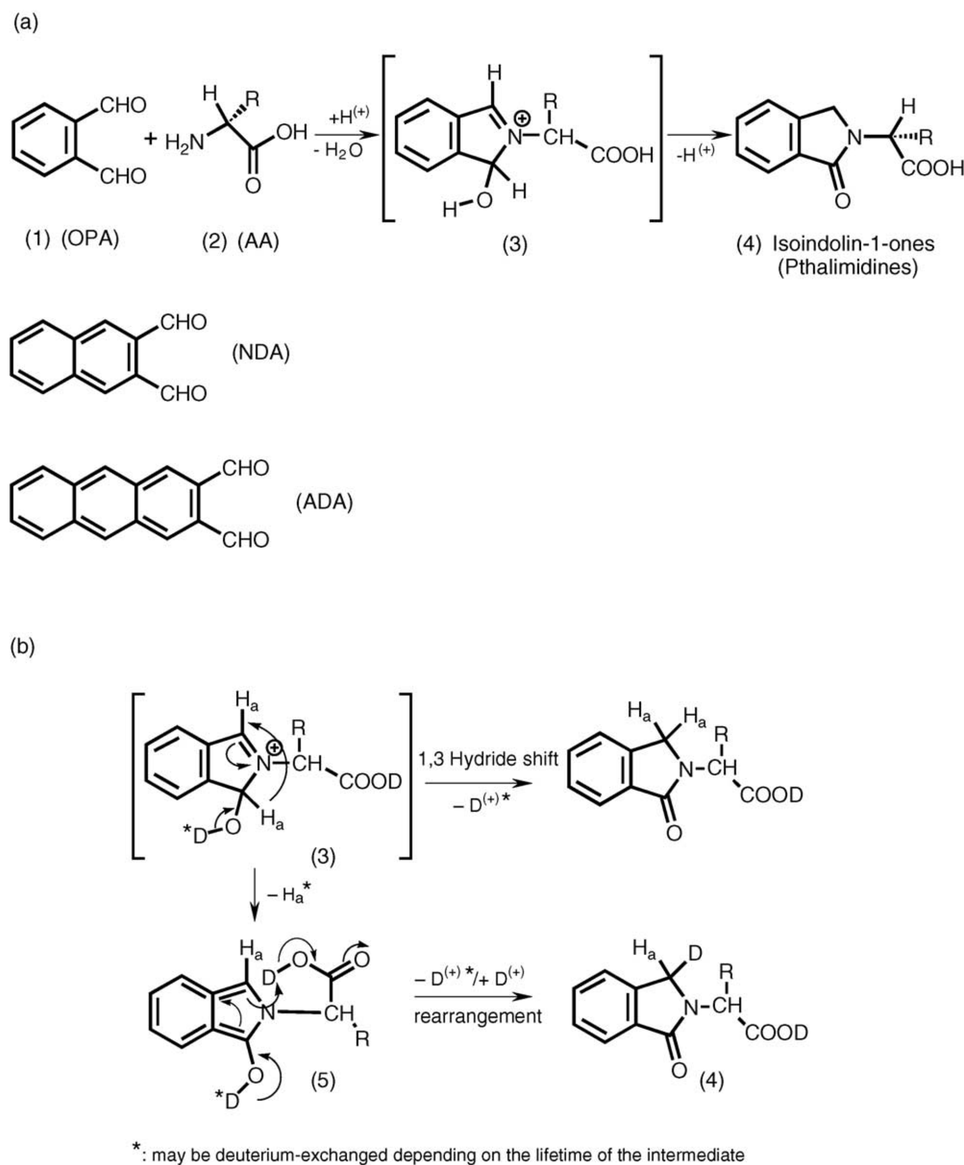


Fig. 1. (a) Reaction mechanism for the formation of isoindolin-1-ones; (b) H/D exchange conditions of labile protons/deuterons.

a catalytic amount of acetic acid. It was supposed that two reaction mechanisms lead to the formation of the isoindolin-1-ones: a 1,3-hydride shift facilitated by the hydroxy group of the hydroxy-iminium (3) congener and/or a deprotonation–protonation process via the isoindolinol (5) skeleton that could also bring forth an isoindolin-1-one (Fig. 1b).

Takahashi [5] has reported a novel procedure to afford phthalimidines in the presence of excess 2-mercaptoethanol (MET) and 1,2,3-1*H*-benzotriazole (Bt-H) as a so-called "dual synthetic auxiliaries" approach.

To shed light on the reaction mechanism, Allin [6] has established that by using acetonitrile in the absence of any external auxiliary, as reported by Takahashi [5], and also without the addition of acetic acid, as suggested by Grigg [4], the phthalimidine derivatives are formed by a mechanism involving neighbouring carboxylic group and easily exchangeable proton assistance of the analyte.

Besides the above cited reaction conditions and reaction mechanisms (for more detailed discussion see later), it is also well known that in the presence of thiol containing compounds, most commonly 2-mercaptoethanol, primary amines react with aromatic *ortho* dicarboxaldehydes in a different way, giving rise to the formation of a strongly fluorescent isoindole fluorophore [7–9]. This reaction formed the basis of the widely used and sensitive method of amino acid and peptide analysis by HPLC using fluorescence detection. However, a potential disadvantage of the method is that amino acid derivatives are usually quite unstable even at room temperature and with light protection, owing to the disintegration of the isoindole structures, therefore automated and standardized pre-column derivatization

is highly recommended to facilitate quantitative analysis.

The main goal of this study was to develop a reliable HPLC method for the enantiomer separation of the most common amino acids using aromatic *ortho*-dicarboxaldehyde derivatizing agents via formation of the corresponding isoindolin-1-one (phthalimidine) derivatives by adopting a known derivatization concept (see Fig. 1). The reaction should be quantitative, although not essentially in the course of enantiomeric excess (ee) analysis of individual amino acids, relatively fast and the final products should be stable. In this contribution we have extended the application of the *ortho*-dicarboxaldehyde reagent from the regularly used *ortho*-phthalaldehyde (OPA) to the corresponding naphthalene- and anthracene dicarboxaldehydes (NDA and ADA, respectively) in order to study also the structural influences. The formed phthalimidine derivatives of amino acids are bearing an acid functionality, an aromatic ring substituent, a carbonyl group and a chiral center which make them useful entities for direct enantiomer separation and enantioselective amino acid analysis, i.e. enantiomer excess determination.

In this context, cinchona alkaloid derived chiral stationary phases (CSPs) have been proven useful for the direct liquid chromatographic enantiomer separation of a wide range of chiral acids [10–14]. Besides the derivatization reaction per se and its mechanistic aspects, structural variations of the quinine-type chiral selectors and CSPs (depicted in Fig. 2) have been systematically investigated. Hydro-organic and polar-organic mobile phases were selected as further optimization criteria.

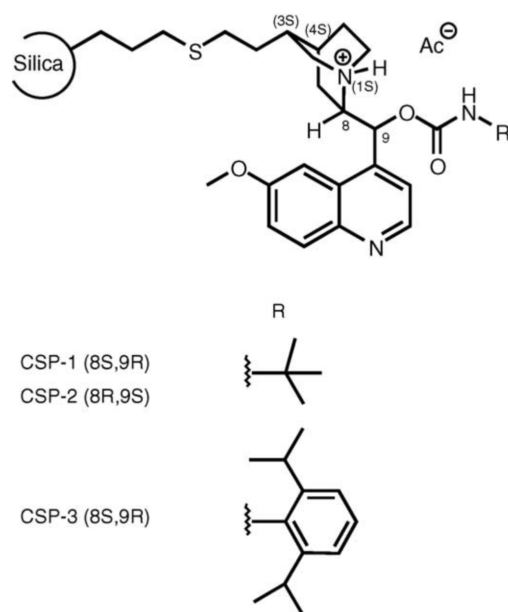


Fig. 2. Structures of chiral stationary phases: CSP-**1** with *tert*-butyl carbamoylated quinine; CSP-**2** with *tert*-butyl carbamoylated quinidine and CSP-**3** with 2,6-diisopropyl phenyl carbamoylated quinine as chiral selector.

Independent of the chromatographic application of the derivatization reaction, further insight into the reaction mechanism is desirable. To obtain additional data, we have used deuterium labelling [15] of the OPA-alanine derivative and LC–MS analysis of the reaction product.

## 2. Experimental

### 2.1. Materials

Amino acids (racemic and enantiomers) were obtained from different suppliers (Sigma-Aldrich (Vienna, Austria) and Fluka (Buchs, Switzerland)). For the chromatographic experiments, methanol (MeOH) was of HPLC grade and purchased from Merck (Darmstadt, Germany). Acetic acid, ammonium acetate and *ortho*-phthalaldehyde (OPA) were obtained from Fluka. Trifluoroacetic acid (TFA), naphthalene-2,3-dicarboxaldehyde (NDA) and ethyl acetate were supplied by Sigma-Aldrich. Dimethylsulfoxide (DMSO) was from Merck. All reagents used were of analytical grade. Anthracene-2,3-dicarboxaldehyde (ADA) was synthesized according to a previously described method [16]. The chemical purity of the samples was controlled by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in reversed-phase mode. Double distilled water was used for all measurements. Deuterium oxide ($D_2O$), $d_4$-acetic acid and $d_1$-TFA were from Aldrich.

Melting points (uncorrected) were measured with a Kofler apparatus. IR spectra were recorded using a Bruker Tensor 27 ATR-FT-IR spectrometer (Bruker Optic GmbH, Vienna, Austria). Optical rotation was measured on a Perkin-Elmer 341 polarimeter at 25 °C in 10 cm cuvette. $^1$H NMR spectra were acquired on a Bruker DRX 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard. Fluorescence spectra were recorded on a Perkin-Elmer LS 50B luminescence spectrometer.

#### 2.1.1. Amino acid derivatization

First, the solvent of 100 μl of amino acid stock solution (5 mg/ml in water) was evaporated to dryness as non-aqueous reaction conditions are preferred. The residue was dissolved in 500 μl of OPA stock solution (10 mg/ml in acetonitrile) and NDA stock solution (5 mg/ml in acetonitrile), respectively, with a catalytic amount of TFA (2 μl) or DMSO (100 μl). ADA derivatives were prepared by adding 1 ml of ADA working solution (2.5 mg/ml in acetonitrile in the presence of 20 μl of DMSO) to the residue. Occasionally DMSO was necessary to improve solubility, its presence does not disturb the enantiomer separation protocols on the CSPs. The reagent stock solutions were prepared daily in light protected glassware. The sample mixtures were shaken for 90 min at room temperature and stored protected from light. The reaction was also carried out with the hydrochloric salt of alanine, phenylalanine and serine revealing similar results. The reagent was used in a 6–15-fold molar excess in case of OPA, 2–5-fold

molar excess for NDA and 2–4-fold molar excess for ADA relative to the analytes. Stoichiometric study was performed with alanine as model compound using 5:1, 10:1 and 20:1 molar ratio of OPA: alanine. The peak areas were quantified and compared to an external standard solution of the pure Ala derivative. From the repetitive experiments at even after 6 h reaction time the reaction yield was in all cases >95% with RSD < 3%. For other amino acids we have no such comparative data.

#### 2.1.2. Kinetic investigations

The rate of formation of OPA-alanine, OPA-phenylalanine and OPA-serine derivative, serving as model compounds for the derivatization protocol, was investigated at room temperature (25 °C) and 65 °C, respectively. The peak areas were quantified by removing aliquots of the reaction mixtures at 30-min intervals and analyzed by HPLC.

#### 2.1.3. Control of the racemization

During the reaction, the peak area of the individual enantiomers of enantiomerically highly enriched alanine, phenylalanine and leucine was investigated, using OPA as derivatizing agent.

#### 2.1.4. Synthesis of reference compounds

The racemic OPA-alanine and OPA-(*S*)-alanine was obtained according to Allin et al. [6]. In brief, the mixture of 0.17 g of the analyte, trifluoroacetic acid (TFA) (about 0.2 ml) and *ortho*-phthalaldehyde (0.30 g) in 30 ml of acetonitrile was refluxed for 3 h. The reaction mixture was filtered and the solvent allowed to cool to room temperature. The yellow crystals (0.34 g, 75%) were dried; m.p. 210–218 °C; $[\alpha]_{546}$ + 21 ($c$ = 1.0, DMSO); $\nu_{max}$ 2541, 1725, 1632, 1249 and 737 $cm^{-1}$; δH (400 MHz, $d_6$-DMSO) 1.51 (3H, d), 4.49 (1H, d), 4.54 (1H, d), 4.84 (1H, q), 7.58–7.7 (4H, m); $\lambda_{max}$ (DMSO) 222, 228, 270 and 277 nm; $\varepsilon_{228\ nm}$ 10,900; $\lambda_{excitation}$ 260 nm, $\lambda_{emission}$ 311 nm; $m/z$ 206,1 $(M + H)^+$.

The condensation product of anthracene-2,3-dicarboxaldehyde (ADA) with (*S*)-alanine as reference compound was synthesized according to Takahashi et al. [5]. First, ADA (0.06 g, 0.26 mmol) and mercaptoethanol (0.2 g) were mixed and dissolved in 80 ml of a 1:1 mixture of tetrahydrofurane and acetonitrile followed by the addition of (*S*)-alanine (0.1 g/ml in water), benzotriazole (0.05 g) and 1.0 ml of sodium borate buffer (0.05 M, pH 9.6). The reaction mixture was stirred at room temperature and light protected for 23 h. After evaporation of the solvent, the crude material was suspended in 50 ml of ethyl acetate and the precipitate was collected by filtration. The solid was washed with 30 ml of ethyl acetate and 30 ml of chloroform/ethanol 2:1, respectively. The precipitate was dissolved in 300 ml of methanol/chloroform/tetrahydrofurane 1:1:2 by pouring the solvent onto the filter paper. After evaporation of the solvent, yellow powder was obtained (0.06 g, 76%), m.p. > 350 °C; $[\alpha]_{546}$ + 40 ($c$ = 0.1, DMSO); $\nu_{max}$ 2600, 1750, 1620, 1587 and 747 $cm^{-1}$; δH (400 MHz, $d_6$-DMSO) 1.54 (3H, d), 4.71 (2H, s), 4.54 (1H, d), 4.90 (1H, q), 7.57–8.85 (8H, m); $\lambda_{max}$ (DMSO) 216, 261, 342, 360, 379 and 399 nm; $\varepsilon_{261\ nm}$ 41,000; $\lambda_{excitation}$ 270 nm, $\lambda_{emission}$ 432 nm.

### 2.2. Instrumentation and HPLC conditions for chiral separations

Chromatographic experiments were performed using a Hitachi-Merck HPLC system which consisted of L-6200 intelligent pump, L-4250 variable wavelength UV–vis detector, AS-2000A autosampler, D-6000 interface, HSM 7000 chromatography data station software from Merck and a JASCO OR-990 optical rotation detector (ORD) (Hg–Xe lamp, wavelength range 350–900 nm) (JASCO, Groß-Umstadt, Germany). The chiral stationary phases CSP **1–3** and respective columns, ProntoSIL Chiral AX QN-1; ProntoSIL Chiral AX QD-1 and ProntoSIL Chiral AX QN-2 (150 mm × 4.0 mm I.D., 5 μm particle size) (Fig. 2) were from Bischoff Chromatography (Leonberg, Germany). The HPLC columns were kept at a constant temperature of 25 °C using a W.O. Electronics column thermostat (Langenzersdorf, Austria). A flow rate of 1 ml/min and a UV detection wavelength of 250 nm were used throughout the study. The hydro-organic mobile phase consisted of methanol-ammonium acetate buffer (0.1 M) 80:20 (v/v) and the apparent $pH_a$ was adjusted to 6.0 with glacial acetic acid. The polar-organic mobile phase contained methanol–acetic acid 96:4 (v/v). Mobile phases were filtered through a 0.45 μm Nylon membrane filter and degassed by sonication prior to use. The injected volume for OPA and NDA derivatives was 20 μl, for ADA samples it was 40 μl. The void volumes of the respective CSPs were determined using acetone. Blank solutions were injected onto the column before all measurements.

### 2.3. Deuterium labeling and HPLC–MS analysis

OPA derivatives of racemic alanine were formed under different reaction conditions in the presence or absence of various deuterium sources. In all cases, rac-alanine was dissolved in a large excess of $D_2O$ and hydrogen/deuterium exchange was allowed to take place for 1 h before the sample was evaporated to dryness under a stream of nitrogen.

The following reaction conditions were evaluated: (i) using $D_2O$ stock solution of rac-alanine without external acid, (ii) using $D_2O$ stock solution of rac-alanine and $d_4$-acetic acid for acid catalysis, (iii) $d_4$-acetic acid or $d_1$-trifluoroacetic acid for acid catalysis in the absence of $D_2O$ and (iv) no external deuterium source (deuterium exchanged rac-alanine only). In this case, DMSO was added to the reaction solution to facilitate dissolution of the free amino acid.

After 90 min of reaction time, samples were diluted 1:100 with (non-deuterated) water to achieve back-exchange of the carboxylic proton (deuteron).

HPLC–MS analysis was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn,

Germany), which was connected to a PESciex API 365 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a pneumatically assisted electrospray ion source.

CSP-**1** was used as a stationary phase in combination with a mobile phase consisting of methanol–acetic acid, 96:4 (v/v). The flow rate was maintained 1.0 ml/min.

Prior to the introduction into the mass spectrometer, the HPLC flow was split approximately 1:100 by a restriction capillary connected to a mixing tee. MS instrumental settings were carefully optimized to tolerate the high content of acetic acid in the mobile phase. Detection was performed in positive ionization mode using a scan range of 200–210 *m/z* with 500 ms scan time.

The amount of deuterium incorporated into the rac-OPA-Ala derivative was calculated from the signal intensities of the isotope peaks (*m/z* 206.2 for $d_0$-OPA-Ala, $[M+H]^+$, and *m/z* 207.2 for $d_1$-OPA-Ala, $[M+H]^+$) and corrected for natural $^{13}C$ abundance (1.11% of $^{12}C$), background noise in the chromatogram and deuterium content of the respective reaction solutions. From multiple analyses per sample, an accuracy of ±2% deuterium incorporated was obtained.

## 3. Results and discussion

In primary focus we have studied the analytical aspects and consequences of the phthalimidine-type reaction of α-amino acids and the separation of the corresponding enantiomers employing enantioselective liquid chromatography. First, the chemical structures of the derivatives of (*R*)-and (*S*)-alanine and (*R*,*S*)-alanine (see also Fig. 1) were confirmed by analysis of standard isoindolin-1-one (phthalimidine) derivatives employing nuclear magnetic resonance (NMR), UV–vis, Fourier transform infrared (FT-IR), fluorescence and mass spectrometry (MS) (for data see Section 2). In the course of these experiments it was seen that the derivatization reaction catalyzed by addition of TFA yielded >95% judged for Ala under the mild and non-aqueous conditions used. Side reactions occur but they arise exclusively from the reagents, which are particularly sensitive to the light (data not shown).

Kinetic studies revealed that under these conditions the isoindolin-1-one derivatives of amino acids are formed at 25 and 65 °C within 90 min or shorter time and the final products are completely stable in the solution for at least 12 h. It was also carefully controlled whether or not this derivatization reaction may yield any racemization of the α-carbon of the amino acid. Different (*R*)- and (*S*)-amino acids of known ee values (between 0.05 and 0.2% ee) have been investigated and it was found that no racemization occurs under the given reaction and analysis conditions (data not shown).

In parallel to this study of the chemical reaction per se the enantiomer separation of the isoindolin-1-one derivatives of the amino acids were investigated employing the weak anion exchanger type *tert*-butylcarbamoyl quinine-based chromatographic selectors (see Fig. 2 and Section 2). These CSPs contain several binding sites for stereoselective intermolecular interactions within the intermediate complex formed between the enantiomers of the analyte (selectands, SAs) and chiral selector (SO), respectively. At slightly acidic working pH, the tertiary nitrogen of the quinuclidine moiety of the SO is protonated, providing a strong electrostatic interaction with the deprotonated acidic SAs. Retention and enantiomer separation of acidic analytes are predominantly based on an anion-exchange mechanism, which proved to be the primary driving force for the SO–SA association [6]. In addition, simultaneously acting $\pi$–$\pi$ interactions between the aromatic moiety of the SO and SAs, hydrogen bonding and dipole-dipole interaction between the carbamate functionality of the SO and the carbonyl oxygen atom of the isoindolin-1-ones, as well as van der Waals and attractive and/or repulsive steric interactions may play an important role in the overall stereodiscrimination event.

### 3.1. Influence of the type of mobile phase on retention and enantiomer separation

Cinchona alkaloid derived CSPs have often been used in hydro-organic buffered mobile phases showing excellent enantiomer separation capability. Table 1. summarizes the separation results of the most common α-amino acids on CSP-**1** using *ortho*-phthalaldehyde as derivatizing agent. The non-polar amino acids with exception of β-phenylalanine (β-Phe), methionine (Met) and valine (Val) are baseline separated (resolution $R_S$ ranges between 1.52 and 1.92) within reasonable retention times. The presence of aromatic ring substituents in the SA increases the retention factor, i.e. $k_1 = 9.20$ for phenylalanine (Phe), 12.03 for phenylglycine (Phg) and 18.17 for tryptophan (Trp), emphasizing the importance of $\pi$–$\pi$ interactions within SO–SA complexes. As expected, for the bivalent acidic analytes, glutamic acid (Glu) and aspartic acid (Asp), significantly higher retention factors were obtained by more then a factor of 10, compared to glutamine (Gln) and asparagine (Asn), suggesting the dominant feature of the ion-pair formation. Interestingly, OPA-Glu was separated with $R_S$ value of 1.68, however, aspartic acid (Asp) was only partially resolved with definitely higher retention factors (i.e. $k_1$ was found to be 49.4 for OPA-Glu and 64.19 for OPA-Asp, respectively). These data reveal that the conformation and the spatial fit of the SA plays an important role in the overall binding and enantiodiscrimination process.

In case of histidine (His), many additional peaks were observed, indicating the formation of unstable side-products. Likewise, it was not possible to analyse the enantiomers of cysteine (Cys), as Cys did not react uniformly with OPA under the conditions used. Lysine (Lys) formed exclusively bis-derivatives (confirmed by MS). Since arginine (Arg) and its derivative is a cationic polar analyte, it was eluted too fast and no enantiomer separation was obtained throughout the study. However, it seems that Arg reacts with the derivatizing agent

Table 1
Enantiomer separation data of isoindolin-1-ones, using hydro-organic mobile phase

| Analyte | $k_1$ | $k_2$ | $R_s$ [a] | α | Optical rotation [b] | Elution order [c] |
|---|---|---|---|---|---|---|
| Ala | 5.46 | 6.12 | 1.76 | 1.12 | −/+ | R |
| Phe | 9.20 | 10.3 | 1.65 | 1.12 | +/− | R |
| β-Phe | 7.41 | – | – | 1.00 | +/− | n.d. |
| Leu | 6.43 | 7.30 | 1.92 | 1.14 | −/+ | R |
| ILeu | 6.77 | 7.64 | 1.92 | 1.13 | −/+ | R |
| NorLeu | 6.76 | 7.63 | 1.90 | 1.13 | −/+ | R |
| Met | 8.42 | 9.24 | 1.32 | 1.10 | +/− | R |
| Val | 5.91 | 6.18 | 0.63 | 1.05 | +/− | R |
| Phg | 12.03 | 13.19 | 1.52 | 1.10 | −/+ | R |
| Trp | 18.17 | 19.73 | 1.32 | 1.09 | +/− | R |
| Tyr | 9.42 | 10.6 | 1.65 | 1.13 | +/− | R |
| Ser | 5.21 | 5.63 | 1.13 | 1.08 | −/+ | R |
| Thr | 4.83 | 5.05 | 0.38 | 1.05 | −/+ | R |
| ABA | 5.44 | 6.08 | 1.64 | 1.12 | −/+ | R |
| β-ABA | 2.87 | 3.42 | 0.84 | 1.08 | −/+ | n.d. |
| Asp | 64.2 | 66.5 | 0.60 | 1.03 | n.d. | R |
| Asn | 5.03 | – | – | 1.00 | −/+ | n.d. |
| Glu | 49.4 | 55.4 | 1.68 | 1.12 | −/+ | R |
| Gln | 4.35 | 4.72 | 1.20 | 1.08 | −/+ | R |
| Arg | 0.18 | 0.37 | – | – | +/− | n.d. |
| His | n.e. | n.e. | – | – | n.d. | n.d. |
| Cys | n.r. | n.r. | – | – | n.d. | n.d. |
| Lys | 7.47 | 8.02 | 1.18 | 1.07 | −/+ | R |

Derivatizing reagent: OPA; chromatographic conditions: CSP: CSP-**1**; mobile phase: MeOH–ammonium acetate buffer (0.1 M, $pH_a$ 6.00) (80:20). Other conditions given in Section 2; n.d.: not determined; n.e.: not evaluated under conditions used due to the degradation of the derivative (see in text); n.r. not reacted uniformly with the reagent under conditions used.

[a] Resolution $R_s = 1.18\ (t_2 - t_1)/(w_{b,1} + w_{b,2})$.

[b] Optical rotation of the corresponding peaks.

[c] Elution order indicating the configuration of the first eluted enantiomer.

only at the primary amino group and most probably not at the guanidino group under the conditions applied.

Using polar-organic mobile phases, different interactions might come into force during the stereodiscriminating process, resulting in distinct enantioselectivity as well. However, comparing the enantiomer separation results obtained in hydro-organic and methanol containing mobile phases (Table 1 compared to Table 2), it can be stated, that the chiral discrimination mechanism is almost unaffected by the type of the mobile phases on CSP-**1**. In general, Table 2 shows that the methanol-based mobile phase provides the same elution order, but lower retention factors and resolution values for the selected analytes compared to the hydro-organic mobile phase, without significant changes in the enantioselectivity.

### *3.2. Effect of the homologue structure of the derivatizing agent on the enantiomer separation*

A homologue set of aromatic dicarboxaldehyde derivatizing agent (see Fig. 1a) was compared with respect to the retention factor ($k$), enantioselectivity ($\alpha$) and resolution $R_S$ of the selected amino acid derivatives. Table 2 shows that by increasing the number of the condensed aromatic rings, which is directly correlated with the hydrophobicity of the reagent, the retention factors of the derivatives increased significantly, by a factor of ∼2.5. These observations indicate that the larger the aromatic framework of the SA, the stronger the interactions, mainly via $\pi$–$\pi$ interactions, that may take place within SO–SA complexes. In addition, these $\pi$–$\pi$ interactions seem to play an incremental role in the enantiomer discrimination as well, as by using larger conjugated reagent, i.e. naphthalane-2,3-dicarboxaldehyde (NDA) and anthracene-2,3-dicarboxaldehyde (ADA), both enantioselectivity and resolution values improved (see Fig. 3). The most pronounced enhancement in the resolution was obtained for serine (Ser) on CSP-**1**, where the $R_S$ value of 0.73 was increased by a factor of 4 using ADA reagent instead of OPA.

### *3.3. Influence of the absolute configuration of the selector*

The configuration of the $C_8$ and $C_9$ stereogenic centers of quinine-based SOs have a vital importance on enantiomer separation, as they have a large affect on the size and shape of the binding cavity, consequently also on the spatial orientation of the binding sites of the SOs. Using the opposite configuration of $C_8$ and $C_9$ of the *tert*-butylcarbamoyl quinine selector; the corresponding CSP-**2** shows a substantial effect on the enantiomer separation process. As expected, the quinine/quinidine pair exhibits reversed elution order, as the elution order of the SA on quinine-derived SOs is determined by the absolute configuration of the $C_9$ stereogenic center [12]. This property of the SO is especially advantageous when reversal elution order is desired, i.e. for enantiomeric excess assessment, since from the detection point of view, it is more favored when the enantiomeric impurity elutes before the major component.

Data reveal (see Table 2) that steric arrangement around the binding pocket of the SO plays a significant role in the binding mechanism and enantiomer separation as well. For Phe and Trp bearing aromatic functionalities, higher $k$ values were achieved on CSP-**2** (except for the ADA derivative), comparing to CSP-**1** and **3**, although the SO density on the CSPs are quite similar. The quinidine-based CSP-**2** was superior for the resolution of Leu, Met, Trp and Ser enantiomers, i.e. using NDA derivatizing agent, Leu was separated with an $R_S$ value of 2.95, while using ADA reagent, $R_S$ values of 2.43, 3.10, 4.93 and 3.70 were obtained, respectively. On the other hand, CSP-**2** was not suitable for the separation of β-Phe enantiomers under the conditions applied.

### *3.4. Influence of the carbamate moiety of the selector*

It is noteworthy that by structural modification in the carbamate moiety of the quinine type skeleton, the spatial arrangement of the interaction sites and consequently the enantiodiscriminating potential of the SO can also be easily modified. Table 2 shows that surprisingly, the 2,6-diisopropylphenyl-carbamoyl quinine-based CSP-**3** provides

Table 2
Comparison of enantiomer separation of α-amino acid derivatives using *ortho*-phthalaldehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA) and anthracene-2,3-dicarboxaldehyde (ADA) as reagents using polar-organic mobile phases

| CSP | Analyte | OPA | | | | NDA | | | | ADA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $k_1$ | $R_s$ | $\alpha$ | Elution order[a] | $k_1$ | $R_s$ | $\alpha$ | Elution order[a] | $k_1$ | $R_s$ | $\alpha$ | Elution order[a] |
| **1** | Ala | 3.23 | 1.47 | 1.14 | R | 5.32 | 2.45 | 1.2 | R | 8.52 | 2.66 | 1.21 | R |
| | Phe | 5.57 | 1.65 | 1.12 | R | 8.76 | 2.37 | 1.18 | R | 13.69 | 2.72 | 1.19 | R |
| | β-Phe | 1.38 | – | 1.00 | n.d. | 2.26 | – | 1.00 | n.d. | 3.61 | 0.50 | 1.03 | R |
| | Leu | 2.56 | 1.67 | 1.16 | R | 4.04 | 2.74 | 1.23 | R | 6.20 | 2.70 | 1.24 | R |
| | Met | 5.51 | 1.01 | 1.08 | R | 8.60 | 1.82 | 1.14 | R | 13.59 | 2.24 | 1.15 | R |
| | Val | 2.65 | 0.69 | 1.08 | R | 4.32 | 0.89 | 1.08 | R | 6.56 | 1.19 | 1.10 | R |
| | Trp | 7.94 | 0.53 | 1.05 | R | 13.06 | 1.18 | 1.08 | R | 21.72 | 1.50 | 1.10 | R |
| | Ser | 6.23 | 0.73 | 1.06 | R | 10.08 | 1.47 | 1.10 | R | 16.24 | 3.03 | 1.11 | R |
| | Arg | 0.08 | – | 1.00 | n.d. | 0.21 | – | 1.00 | n.d. | 0.30 | – | 1.00 | n.d. |
| **2** | Ala | 2.91 | 2.15 | 1.25 | S | 5.08 | 3.21 | 1.32 | S | 8.38 | 3.51 | 1.31 | S |
| | Phe | 5.98 | 1.68 | 1.14 | S | 9.34 | 2.64 | 1.22 | S | 15.01 | 2.53 | 1.21 | S |
| | β-Phe | 1.13 | – | 1.00 | n.d. | 1.84 | – | 1.00 | n.d. | 3.17 | – | 1.00 | n.d. |
| | Leu | 2.32 | 1.85 | 1.20 | S | 3.61 | 2.95 | 1.27 | S | 5.84 | 2.43 | 1.24 | S |
| | Met | 5.27 | 2.18 | 1.19 | S | 8.13 | 3.08 | 1.28 | S | 13.44 | 3.10 | 1.26 | S |
| | Val | 2.31 | 0.50 | 1.09 | S | 3.74 | 0.84 | 1.08 | S | 6.09 | 0.85 | 1.08 | S |
| | Trp | 8.64 | 3.81 | 1.35 | S | 14.42 | 4.89 | 1.43 | S | 23.00 | 4.93 | 1.43 | S |
| | Ser | 6.16 | 2.44 | 1.22 | S | 10.03 | 3.31 | 1.29 | S | 17.30 | 3.70 | 1.27 | S |
| | Arg | 0.06 | – | 1.00 | n.d. | 0.16 | – | 1.00 | n.d. | 0.27 | – | 1.00 | n.d. |
| **3** | Ala | 2.49 | 2.30 | 1.28 | R | 4.28 | 3.60 | 1.39 | R | 6.78 | 3.72 | 1.42 | R |
| | Phe | 4.19 | 2.08 | 1.18 | R | 7.12 | 2.42 | 1.23 | R | 11.13 | 2.80 | 1.26 | R |
| | β-Phe | 1.03 | – | 1.00 | n.d. | 1.74 | 0.80 | 1.09 | R | 2.75 | 0.73 | 1.10 | R |
| | Leu | 1.80 | 1.34 | 1.17 | R | 3.13 | 2.26 | 1.24 | R | 4.72 | 2.19 | 1.26 | R |
| | Met | 4.44 | 0.87 | 1.08 | R | 7.87 | 1.54 | 1.14 | R | 11.86 | 1.88 | 1.17 | R |
| | Val | 2.25 | – | 1.00 | n.d. | 3.78 | – | 1.00 | R | 5.89 | – | 1.00 | R |
| | Trp | 6.73 | 1.33 | 1.16 | R | 13.61 | 0.80 | 1.02 | R | 23.30 | 0.80 | 1.01 | R |
| | Ser | 4.68 | 1.49 | 1.13 | R | 7.81 | 2.22 | 1.21 | R | 12.32 | 2.55 | 1.25 | R |
| | Arg | 0.03 | – | 1.00 | n.d. | 0.11 | – | 1.00 | n.d. | 0.20 | – | 1.00 | n.d. |

Chromatographic conditions: mobile phase: MeOH–acetic acid (96:4). Other conditions given in Section 2; n.d.: not determined.
[a] Elution order indicating the configuration of the first eluted enantiomer.

relatively lower retention factors (except for ADA-Trp) compared to the *tert*-butylcarbamoyl congener (CSP-**1**). Most probably the steric congestion in the binding site of the 2,6-diisopropylphenyl carbamoyl moiety is responsible for this phenomenon. However, CSP-**3** turned out to be the most promising SO for Ala and Phe, i.e. using ADA reagent, $R_S$ values of 3.72 and 2.80 and enantioselectivity values of 1.42 and 1.26 were obtained, respectively. It provided a partial enantiomer separation of β-Phe using NDA and ADA derivatization reagent. Interestingly, for Trp, by increasing the bulkiness of the reagent, the $R_S$ and $\alpha$ values were significantly decreased from 1.33 (OPA-Trp) to 0.80 (ADA-Trp) and from 1.16 to 1.01, respectively. For Val, bearing a short branched side chain, surprisingly no enantiomer separation was obtained under chromatographic conditions used, indicating again the strict steric requirements within the SO–SA complexes.

### 3.5. Study on the reaction mechanism by deuterium labeling and LC–MS

Grigg et al. [4] have suggested two mechanisms that could lead to the formation of the isoindolin-1-one (phthalimidine) structure: The proposed 1,3-hydride shift involves the intramolecular transfer of a hydrogen originating from the reagent (OPA in this case), while the second mechanism proceeds via proton donation (labile proton) from the carboxylic group of the amino acid as depicted in Fig. 1b.

Mass spectrometry in combination with hydrogen/deuterium exchange (HDX-MS) is a convenient way to systematically analyze such a reaction mechanism. Replacing an easily exchangeable hydrogen atom with deuterium leads to a change in the molecular mass, which can easily be observed by MS. However, in our case, a multitude of exchange pathways involving exchangeable hydrogens might exist, including the carboxylic group of the amino acid and possible labile hydrogens of reaction intermediates. HDX kinetics are very much pH dependent and thus results have to be interpreted with care. Therefore, to overcome possible ambiguities when studying the OPA derivatization reaction, alanine as the model analyte was preincubated in $D_2O$ before reacting it with OPA, resulting in the quantitative exchange of the labile hydrogens. In addition, prior to LC–MS analysis, labile deuteriums were backexchanged by dilution into $H_2O$ to avoid any interference by remaining deuterium on the carboxylic group of the derivative. Since it was assumed that only one

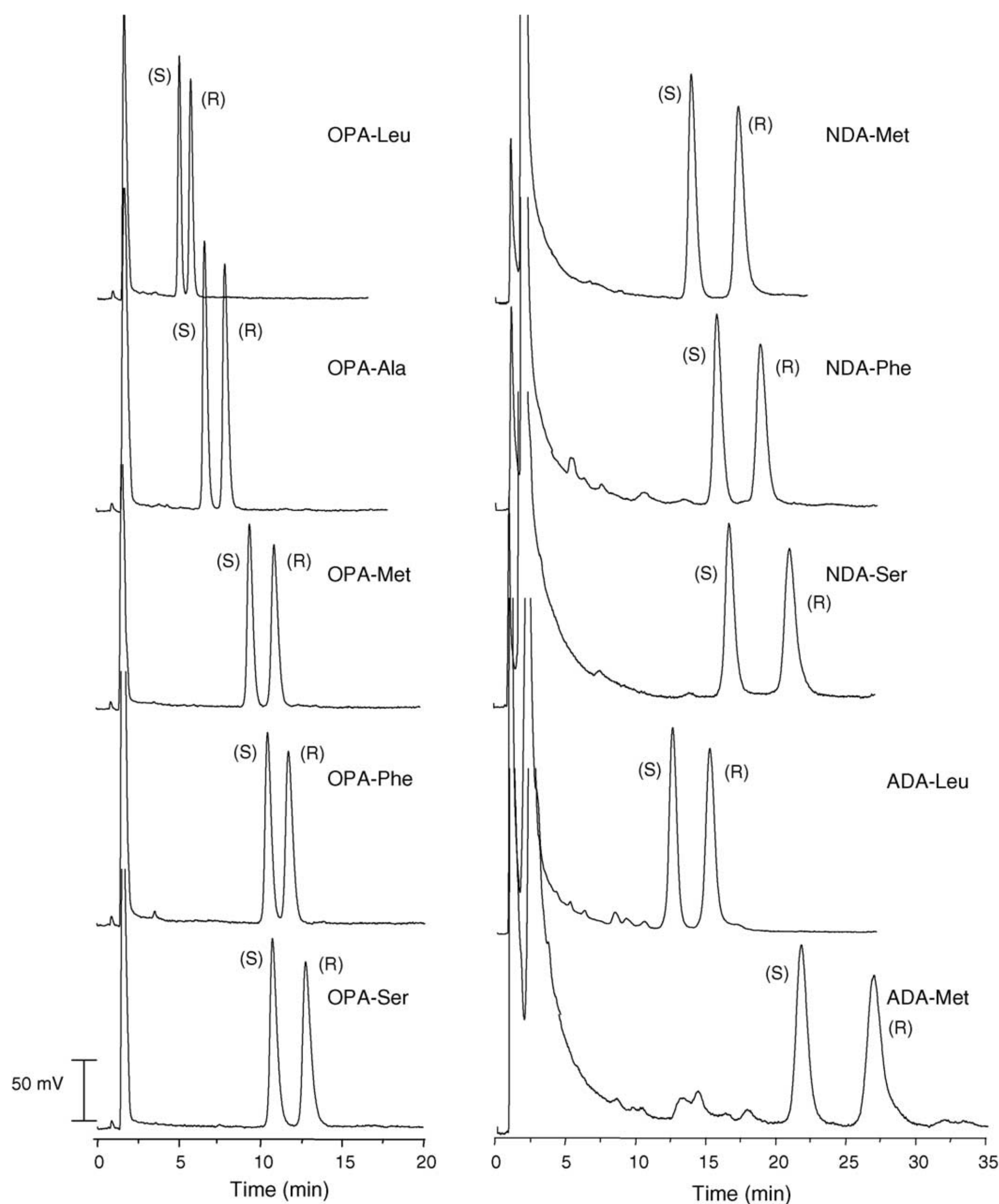


Fig. 3. Representative chromatograms of the enantiomer separation of isoindolin-1-one derivatives. CSP: CSP-**2**, mobile phase: MeOH–$CH_3COOH$; 96:4 (v/v), Other conditions given in Section 2.

deuterium will be built in, the mass shift of +1 made it necessary to apply corrections for the natural $^{13}C$ isotope content leading to a mixed distribution of $^{13}C_1$- and $^{2}H_1$-signals for the first isotope peak.

While the intramolecular hydride shift mechanism should not lead to the incorporation of deuterium into the reaction product (4), involvement of the (deuterium-exchanged) carboxylic group as part of the second mechanism should result in a noticeable deuterium content in the derivative (4). Several reaction conditions were compared regarding deuterium incorporation, which are summarized in Table 3. As can be seen, in absence of any other deuterium source except from the COOD group of alanine, only a small amount of

Table 3
Deuterium incorporation into the OPA-Ala derivative under various reaction conditions as determined by LC–MS[a]

| Solvent composition | Acid catalysis | Deuterium content (%) |
|---|---|---|
| ACN/DMSO | – | 10 |
| ACN | $d_1$-TFA | 73 |
| ACN | $d_4$-acetic acid | 75 |
| ACN/$D_2O$ | – | 86 |
| ACN/$D_2O$ | $d_4$-acetic acid | 85 |

[a] Alanine was always used in its COOD form; conditions are given in Section 2.

deuterated product was found (∼10%). However, water as a by-product of the reaction (one molecule of water formed per molecule of OPA-Ala) reduce the maximum percentage of deuteration to ∼50% due to the availability of exchangeable protons from $H_2O$. In contrast, in the presence of a large excess of $D_2O$ and/or catalytic amounts of deuterated acids, significantly elevated levels of deuterium were observed (73–85%). While the type of acid used did not influence severely the results, as it is only a question of the available proton/deuteron concentration, the acetonitrile/$D_2O$ solvent system caused a higher degree of deuteration than when acetonitrile alone was used as the reaction medium. Grigg et al. [4] observed ∼70% monodeuteration (determined by NMR) under their reaction conditions, which is in the range of the values obtained by us under comparable conditions.

From these results, it can be concluded that the presence of protic solvent or acids leads to a much higher degree of deuteration. It is likely that an interplay of multiple reaction pathways exists that is very much influenced by the solvent composition and pH. Aprotic conditions, without any acidic additives, apparently favor the 1,3-hydride shift pathway, causing less deuterium incorporation. The observed 10 % are probably stemming from the deuteron of the $D_2N$-Ala-OD used in these experiments whereby the acid proton may be the driving one. In a control experiment, this OPA reaction was also carried out with deutero-exchanged alaninamide in acetonitrile (data not shown). In this case no deuterium got incorporated (only 1,3-hydride shift) into the final product, which corroborates that there must be an easily exchangeable acidic proton/deuteron available in the reaction environment for its incorporation. These findings lead to the conclusion that both proposed mechanisms [4] are at play, depending on the reaction conditions used.

## 4. Conclusions

A new analytical method was developed based on the formation of isoindolin-1-one (phthalimidine) derivatives via a condensation reaction with the primary amine function of amino acids and the aromatic *ortho*-dicarboxaldehyde group of the derivatizing compound. The reaction is catalyzed by acidic conditions. The derivatives are stable and resistant against racemization even in the presence of TFA. The developed method could be an alternative possibility for the analysis of $\alpha$-amino acid and their hydrochloric salts. Preliminary experiments revealed that the UV absorption of the phthalimidine derivatives is reasonable and that the $\varepsilon$ values increase as the aromaticity of the reagent increases (from OPA to ADA). The derivatives also show fluorescence and the intensity follows the same order.

Weak anion-exchanger type quinine-derived chiral stationary phases were successfully used for the liquid chromatographic enantiomer separation of a set of $\alpha$-amino acid derivatives using *ortho*-phthalaldehyde, naphthalene-2,3-dicarboxaldehyde and anthracene-2,3-dicarboxaldehyde as derivatizing reagents in the absence of the commonly used sulfur-containing reducing agent. The effects of the homologue reagents on the chromatographic behavior were investigated in detail. It turned out that structure variation in the vicinity of the binding sites of the SO has a rather strong impact on the chiral recognition via the modification of the properties of the binding cleft. The configuration of the $C_8$ and $C_9$ stereogenic centers affects the elution order and the enantioselectivity fundamentally, indicating the significance of the steric arrangement around the binding sites.

Mechanistic studies by hydrogen/deuterium exchange-mass spectrometry revealed a strong dependence of the degree of deuteration of the reaction product on the reaction conditions.

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