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Evaluation of Enantioselective Gas Chromatography for the Determination of Minute Deviations from Racemic Composition of α-Amino Acids with Emphasis on Tyrosine: Accuracy and Precision of the Method

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ABSTRACT Whereas the determination of high enantiomeric fractions (EF) of chiral compounds is very well established, the accurate determination of small deviations from racemic compositions has not yet received much attention despite its relevance to studies dealing with the origin of homochirality, where only small initial enantiomeric bias is expected. Racemic samples of representative α-amino acids were derivatized as *N*-(*O,S*)-trifluoroacetyl/ethylesters and analyzed by enantioselective gas chromatography (GC) on fused silica capillaries coated with the chiral stationary phases (CSPs) Chirasil-D-Val, Chirasil-L-Val, and Lipodex E with GC/FID and GC/MS detection. The validation (accuracy and precision) of the determination of the enantiomeric fraction EF of the D-enantiomer in racemic or near-racemic compositions for 10 DL-α-amino acids obtained from commercial sources has been carried out. Emphasis is given to DL-tyrosine, the enantiomers of which have recently been claimed to show different crystallization properties. Values of EF obtained from GC measurements using CSPs were compared with those from CE using chiral mobile phase additives. While the precision of the GC method is generally better than 0.08% for all DL-α-amino acids studied, accuracy (trueness) of determination of amino acids with polar side chains is poorer than expected from the precision as a result of systematic errors. The accuracy determined relied on measurements on two oppositely configurated CSPs. *Chirality 19:401–414, 2007.*

KEY WORDS: near racemic composition; minute enantiomeric excess; enantioselective gas chromatography; DL-α-amino acids; DL-tyrosine

INTRODUCTION

Enantioselective gas chromatography (GC)[1] has developed into an indispensable tool for the determination of enantiomeric composition (expressed throughout this publication as percent enantiomeric fraction of the D-enantiomer: EF = 100 [D]/[D + L], or percent enantiomeric excess of the D-enantiomer: ee = 100 [D − L]/[D + L]). It has previously been stated[2] that enantiomeric analysis should be capable of dealing with two important borderline cases: (i) the determination of minute amounts of enantiomeric impurities, e.g. in the evaluation of the enantioselectivity of enzymes and highly enantioselective asymmetric syntheses, kinetic resolutions and 'chirality pool' syntheses, or in the determination of the ee of α-amino acids in synthetic and natural peptides (ee > 99.9%) and (ii) the determination of small enantiomeric excesses, e.g. in experiments devoted to the amplification of enantiomeric bias under prebiotic conditions (ee ≥ 0%). There appear to be no validated studies available on the limit in determining minute deviations from a truly racemic composition by currently available techniques such as chiroptical methods, NMR spectroscopy employing chiral solvating agents (CSAs), chromatography using chiral mobile phase additives (CMPAs) and chiral stationary phases (CSPs), and capillary electrophoresis employing CMPAs.[3]

While 'single-molecule chirality' (ee = 100%)[4,5] and the excess of one enantiomer present in an uneven number of a few molecules of a racemate is not considered here, even the statistical fluctuation at 68% confidence level amounting to an excess of 2.5×10^{12} molecules of one

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enantiomer per mole of racemate (ee = 7.5×10^{-10}%) appears to be well below any analytical detectability. However, minute enantiomeric excesses can be identified indirectly by autocatalytic amplification reactions.[6,7] Thus a slight enantiomeric imbalance of D- or L-leucine (ee ~ 2%) obtained via photolysis with right or left circularly polarized light and used as initiator led to a high enantiomeric excess of the product in the autocatalytic Soai reaction.[8,9] Even few molecules of one enantiomer in an excess of the racemate were found to control the enantiomeric outcome in a random-chance stochastic process, leading to high product enantiomeric excess in the Soai transformation.[10] Consequently, Mislow proposed a new definition of absolute asymmetric synthesis as "the formation of enantiomerically enriched products from achiral precursors without the intervention of chiral chemical reagents or catalysts."[6] The Soai reaction has recently been proposed as a general enantioenrichment detection system.[11]

Quantitation of nearly racemic compositions is also important in the verification of enantiomeric enrichments caused by physical forces, should they in fact occur. The alignment of dipoles of prochiral molecules parallel or antiparallel to a magnetic field in conjunction with a chiral autocatalytic process has been suggested to cause (a still elusive) enantiomeric bias of a product in an absolute asymmetric reaction.[12] The parity violation energy difference (PVED) leads to a minute bias in the intrinsic energy of the enantiomers of a chiral compound and to a slight predominance in the abundance of one of the enantiomers.[13] Calculations of PVED[14] suggest that L-α-amino acids possess a lower intrinsic energy than their D-counterparts (in the order of 10^{-13}–10^{-14} J/mol) which can obviously not be detected by existing analytical methods. Nonetheless there have been claims of differences in the physicochemical properties of the D- and L-enantiomers of α-amino acids and these measured differences were tentatively ascribed to PVED, e.g. by Hodge et al.[15]. It has also been suggested that, as a result of an amplification process, PVED could manifest itself in differing crystallization characteristics of enantiomers and several attempts have been made to detect this postulated phenomenon.[16,17] Shinitzky et al.[18] also claimed to have observed a PVED in the crystallization of racemic tyrosine, whereby the D-enantiomer precipitated preferentially thus leaving the L-enantiomer enriched in the mother liquor, a finding which could not be reproduced and was hence disputed recently.[19] Nemoto et al. found differences in the rate of racemization of the enantiomers of α-amino acids in water when subjected to steep thermal gradients, whereby L-alanine was apparently less prone to racemization than D-alanine.[20] Recently Shinitzky et al. reported on differences in the measured circular dichroism (CD) spectra and the isothermal titration calorimetry traces of poly D- vs. poly-L-glutamic acid, as well as poly-D- vs. poly-L-lysine (24-mers), attributing the differences to the PVED between the enantiomers.[21] All these claims require reliable and precise analytical methods to quantify small enantiomeric excesses at near racemic composition, a fact which has been somewhat neglected in the pertinent publications.

The precise determination of measurable deviations from racemic compositions is also of paramount interest in the search for homochirality in extraterrestrial environments in ongoing space-related campaigns, i.e., the Rosetta mission,[22] or in forthcoming space explorations such as Chirons of Titan[23] and Exo-Mars.[24,25] Because the enantiomers of α-amino acids are prone to racemization over the course of time, the ee of extraterrestrial amino acids, had it in fact originally existed, could well be minute. When only trace amounts of proteinogenic α-amino acids are available for analysis as in the case of extraterrestrial materials brought to earth, the determination of minute deviations from true racemic composition is also hampered by the omnipresence of biogenic material in the laboratory environment. Armstrong et al.[26] concluded that in trace analysis contamination of a sample with even minute amounts of microscopic aerosol/dust tends to produce artificially elevated levels of L-α-amino acids. This problem is presumably still present in extraterrestrial analysis since the space laboratory can also be expected to be contaminated with terrestrial material containing predominantly L-α-amino acids. Finally, there is mounting evidence that commercial chiral compounds labeled as racemates are measurably nonracemic when they are obtained by racemization of optically active material. This is often the case for α-amino acids that are likely obtained by racemization of natural L-α-amino acids. Since the course of this process is asymptotic and truly racemic compositions are only attained after infinite time of racemization, a small but measurable ee of the L-α-amino acid originally present is expected. Thus, in situations where the use of a racemate instead of the oppositely configurated enantiomer as internal standard for quantitation by the method of enantiomer labeling[27] is required, the absence of a deviation from 1:1 enantiomeric composition of the internal racemic standard must be rigorously established.

A methodological requirement common to all investigations aforementioned is the exact determination of the enantiomeric fraction EF (or enantiomeric excess ee) of chiral compounds at the near-racemic enantiomeric composition. In previous work, the determination of a small deviations from the true racemic composition has been performed by inadequate means, leading to an unreliable conclusion. Thus the claim of asymmetric synthesis in a rapidly spinning reaction vessel utilizing chiral gravitational fields,[28] disputed on theoretical grounds,[29,30] was solely based on few milli degrees of optical rotation whereby inherent polarimetric fluctuations were ignored. Polarimetry may only be considered for the screening of near-racemic mixtures when the compound in question exhibits an exceedingly large optical rotatory power. For example in the photochemical asymmetric synthesis of hexahelicene from stilbene precursors utilizing circularly polarized light,[31–33] the huge specific rotation of hexahelicene ($[\alpha]_D^{24}$ 3640 ($CHCl_3$)) left no doubt about the accomplishment of an enantiomeric bias even though the observed optical yields were very low (*P* about 1%).[34] However in the case of α-amino acids, which generally possess specific rotation values in water of <50 and these also being pH-dependent, measurement of the specific rotation is certainly insufficiently sensitive to detect small

deviations from a truly racemic composition. Shinitzky et al. used either polarimetry or a combination of UV absorption and radioactivity using tritiated L-tyrosine to measure the enantiomeric composition of tyrosine,[18] while Nemoto et al. separated diastereomers of alanine, formed by precolumn derivatization with a chiral reagent, by HPLC [20]. In both publications a relatively high standard deviation was reported and in addition no attempt was undertaken to validate the analytical methods used.

The earliest reported validation of the direct separation of enantiomers of α-amino acids including a racemic sample is due to Bonner et al.[35] These authors used both a packed column and a steel capillary coated with the chiral stationary phase *N*-lauroyl-L-valine-*tert*-butylamide to separate the enantiomers of leucine as their *N*-trifluoroacetyl/2-propylesters by GC. For a racemic sample of leucine an EF for D-leucine of (50.12 ± 0.17)% was determined. The advantage of the use of oppositely configurated CSPs for reliable determination of the enantiomeric composition was first demonstrated by Bonner and Blair.[36] However, analyzing L-leucine (as *N*-trifluoroacetyl/2-propylester) of high enantiomeric purity on D- and L-*N*-docosanoyl-L-valine-*tert*-butylamide as chiral selectors, the authors observed a discrepancy in the EF as measured on the enantiomeric CSPs, which was considerably larger than the standard deviation of the determination (EF 0.78% ± 0.04% D-leucine on the L-phase vs. EF 0.95% ± 0.03% D-leucine on the D-phase), indicating a small but significant systematic error, i.e. a ΔEF of 0.17%. In investigations aimed at high accuracy determination of the enantiomeric fraction of valine (*N*-trifluoroacetyl/methylester) in connection with questions of the influence of PVED in distillations, Trettin[37] analyzed valine samples containing 10% D-valine on the oppositely configurated CSPs Chirasil-D-Val[38] and Chirasil-L-Val[27]. Under optimum conditions of detection and integration, the measured values of the same sample on the enantiomeric CSPs (EF 10.076% ± 0.038% D-valine on the L-phase vs. EF 10.067% ± 0.021% D-valine on the D-phase), i.e. a ΔEF of 0.009% lay well within the precision of measurement (σ = 0.021%–0.038%). Although these samples were not racemates, more or less similar accuracy and precision can be expected for racemic valine.

Here we investigate the accuracy and precision of the determination of the enantiomeric fractions EF of representative DL-α-amino acids with an enantiomeric excess <1% by GC of their *N(O)*-trifluoroacetyl/ethylester derivatives on various CSPs. Special emphasis is placed on tyrosine, the compound used by Shinitzky et al. in crystallization experiments.[18] Some inherent sources of errors in the gas-chromatographic separation of enantiomers on CSPs are also discussed.[39]

In further studies, the merit and limit of enantioselective chromatography for determining small deviations from racemic compositions of other classes of chiral compounds should be explored. This applies for example to chiral persistent organic pollutants (POPs) that are released as synthetic racemates in the environment but are prone to enantioselective degradation in biota[40] allowing the specific fate of chiral pesticides in the environment to be traced. Some initial data have already been published in the literature.[41,42] Another study involves the unexpected accumulation of *S*-(+)-isoflurane (2-chloro-2-(difluoromethoxy)-1.1.1-trifluoroethane) with EF = 52% (mean) after anesthesia in humans with the racemate,[43] whereby the gas-chromatographic enantioseparation of synthetic racemic isoflurane on different cyclodextrin CSPs was carefully validated.[44] A similar validation for racemic desflurane (2-(difluoromethoxy)-1.1.1.2-tetrafluoroethane) was performed.[45]

MATERIALS AND METHODS

Materials

DL-α-amino acids were purchased from various sources: DL-tyrosine samples were from Serva Feinbiochemica (Heidelberg, Germany) and Fluka (Buchs, Switzerland), DL-ornithine and DL-lysine from Fluka, DL-leucine from Schuchardt (München, Germany), DL-alanine from Merck (Darmstadt, Germany) and Serva, DL-serine and DL-phenylalanine from Serva, and DL-aspartic acid, DL-proline, and DL-cysteine from Sigma-Aldrich (Taufkirchen. Germany). D- and L-tyrosine, (both puriss., ≥99.0%) were obtained from Fluka (Buchs, Switzerland). The recrystallization of DL-tyrosine (Fluka) is described later. Ethanol (abs.) was from Riedel-de-Haen (Seelze, Germany), acetyl chloride from Fluka and trifluoroacetic anhydride (~99%) from Sigma-Aldrich, and HPLC grade water from Fisher Scientific (Schwerte, Germany).

Recrystallization of DL-Tyrosine

DL-tyrosine (Fluka recrystallized). A 5.6 mmol/l solution of DL-tyrosine (Fluka) in HPLC grade water was heated at 100°C for 30 min, filtered through a membrane filter and kept for 10 days at room temperature plus a further 17 days at 4°C. A single crystal was removed with tweezers, washed with HPLC grade water, and dried at 110°C under a stream of dinitrogen. Approximately 5 mg of the crystal was derivatized as *N,O*-TFA/ethylester as described later.

DL-tyrosine (Fluka, 4× recrystallized). A 17.1 mmol/l solution of DL-tyrosine (Fluka) in HPLC water was heated at 100°C and kept at room temperature for 12 h and a further 4 days at 4°C. The crystals were filtered off and washed with HPLC grade water. This procedure was repeated three times. The finely divided crystals were lyophylized and ~5 mg were derivatized as *N,O*-TFA/ethylester as described later.

Derivatization

The DL-α-amino acids were sequentially esterified (200 μl of 15% acetyl chloride/ethanol (anhydr.)/110°C/30 min) and trifluoroacetylated (100 μl dichloromethane and ~50 μl trifluoroacetic anhydride/110°C/10 min). Excess reagent was removed by a stream of dinitrogen (at 110°C after esterification and at room temperature after acylation). Derivatized samples were taken up in toluene for analysis by GC.

Enantioselective Gas Chromatography

Gas-chromatographic measurements were carried out using a Hewlett-Packard 5890 gas chromatograph (Agilent Technologies, Waldbronn, Germany) with split injection (220°C, split flow = 50 ml/min) and flame ionization detection (240°C). Dihydrogen was used as carrier gas. Signals were processed with a Clarity integration system (DataApex, Prague, Czech Republic).

GCMS was performed with a Hewlett-Packard 6890/5973 MSD (Agilent Technologies, Waldbronn, Germany) operating in the selected ion mode (m/z 140 for alanine and m/z 288 for tyrosine) with EI ionization. The separation capillary was interfaced to the MS via an open-split coupling employing a 35 cm × 0.1 μm deactivated fused silica coupling capillary. Helium was used as carrier gas. Data acquisition and integration were performed with the Agilent Chemstation hard- and software.

All CSPs [Chirasil-L-Val,[27] Chirasil-D-Val,[38] and Lipodex E[46] (octakis (3-*O*-butanoyl-2,6-*O*-di-*n*-pentyl)-γ-cyclodextrin)] and all capillary columns were prepared in-house.

Duran 50 glass capillaries were leached with 30% HNO_3 overnight at 180°C, fused silica capillaries were leached with 1 mol/l KOH at room temperature followed by 30% HNO_3 at 210°C for 18 h. After rinsing with one column volume of 0.1% HNO_3, the leached glass or fused silica capillaries were dried at 280°C, dynamically coated with diphenyltetramethyldisilazane, evacuated for at least an hour, sealed under vacuum and heated to 280°C for 4 h. After washing with toluene, methanol, and diethylether, the capillaries were statically coated.[47] The capillary columns used are listed in Table 1.

Enantioselective Capillary Electrophoresis

Capillary-electrophoretic measurements were carried out using a Lauerlabs Prince instrument (Kassel, Germany) and UV var detector (200 nm) (Chrompack, Middelburg, Netherlands). Signals were processed with an Agilent CE 3D-CE Chem Station (Agilent Technologies, Waldbronn, Germany). A 70.5 cm (effective length 50 cm) × 50 μm (I.D.) uncoated fused silica capillary was used. Underivatized DL-tyrosine (Serva, 1 mg/ml in 0.1 mol/l HCl) was introduced by pressure-injection (2 sec, 50 m bar). The voltage was +25 kV (48–52 μA). The buffer consisted of 50 mmol/l phosphate (KH_2PO_4/H_3PO_4) at pH 2.5, plus either 1% (=4.3 mmol/l) of highly sulfated γ-cyclodextrin (HS-γ-CD, Michigan Diagnostics, LLC, Troy, MI) or 30% (=254 mmol/l) 2-hydroxypropyl-α-cyclodextrin (2-HP-α-CD, Fluka Chemie, Buchs, Switzerland). Between runs, the capillary was rinsed for 5 min with NaOH solution (0.1 mol/l) followed by backround electrolyt at 1 bar[48].

Abiotic Synthesis of Racemic Tyrosine

Acetylglycine[49]. One mol (75 g) glycine was dissolved in 300 ml water and treated in portions with 2 mol (198 ml) acetanhydride. The warmed-up mixture was stirred for 30 min, concentrated to half of its volume with a rotary evaporator and crystallized overnight by cooling. The precipitate was filtered-off and was dried using a rotary evaporator. A white powdered product was formed.

Yield: 82 g (0.7 mol, 70% of theory).

NMR (ppm): ^{1}H: 1.84 (s, CH_3); 3.70 (d, CH_2, J = 5.96); 8.13 (s, NH); 12.48 (s, COOH).

^{13}C: 22.56 (CH_3); 40.92 (CH_2); 170.09 (CO); 171.77 (COOH).

mp 207–208°C.

Azlactone [4-(4′-methoxybenzylidene)-2-methyloxazolin-5-one][50]. 0.8 mol (110 g) 4-methoxybenzaldehyde (anisaldehyde), 0.32 mol (32.7 g) sodium acetate, and 2.6 mol (246 ml) acetanhydride were mixed with 0.9 mol (108.3 g) acetylglycine and melted for 1 h at 100°C followed by stirring for 3 h at 70°C. Afterwards the mixture was poured into 1.5 l of −30°C cold methanol. The precipitated orange material was immediately collected on a frit and two times washed with −10°C cold methanol/water (9:1 v/v) and dried with H_2SO_4 and then with KOH.

Yield: 123 g (0.57 mol, 70% of theory).

NMR (ppm): ^{1}H: 2.14 (s, CH_3); 3.79 (s, OMe); 7.01 (s, CH); 6.88 (m, C_6H_4, J = 8.93); 7.99 (m, C_6H_4, J = 8.85).

^{13}C: 15.12 (CH_3); 54.96 (OMe); 113.94 (C_6H_4), 133.76 (C_6H_4); 129.81 (CH); 167.58 (COOH); 164.34 (C—OMe); 161.55 (C—CH_3); 130.81 (C=CH) 125.534 (C_6H_4—C).

mp 112–114°C.

N-*acetyl-4′-methoxyphenylalanine by ring-opening and hydrogenation.* Azlactone (30 mmol, 6.6 g) was stirred overnight in an aqueous NaOH (1.2 g in 30 ml

TABLE 1. Enantioselective capillary columns used in this investigation (all capillaries were prepared in-house)

Symbol	Stat. phase	Length diameter, material	Film thickness (μm)
A	Chirasil-D-Val	20 m × 0.25 mm, FS (deact.)	0.13
B	Chirasil- D-Val	25 m × 0.25 mm, FS (deact.)	0.13
C	Chirasil-L-Val	20 m × 0.30 mm, glass (deact.)	0.15
D	Chirasil-L-Val	20 m × 0.25 mm, FS (deact.)	0.13
E	Lipodex E	20 m × 0.25 mm, FS (deact.)	0.13
F	Lipodex E	22 m × 0.25 mm, FS (deact.)	0.13
G	Lipodex E	20 m × 0.25 mm, FS (deact.)	0.13

Chirasil-D-Val: dimethylpolysiloxane-linked *n*-propanoyl-D-valine *tert*-butyl amide; Chirasil-L-Val: dimethylpolysiloxane-linked *n*-propanoyl-L-valine *tert*-butyl amide; Lipodex E: 30% Octakis-(3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin in PS255 (dimethylpolysiloxane).

H_2O) and the clear solution was extracted three times with diethylether. The aqueous solution was hydrogenated with Raney-nickel at 25 bar dihydrogen at 30°C in an autoclave. Dihydrogen (22.5 mmol) were consumed. After filtration of the catalyst, the mixture was three times extracted with diethylether and acidified with 2 *N*-hydrochloric acid. The precipitate was filtered off and dried.

Yield: 4.8 g (20.2 mmol, 69% of theory).

NMR (ppm): ^{1}H: 1.77 (s, CH_3); 2.70–2.99 (m, CH_2); 4.31–4.33 (m, CH); 3.70 (s, OMe); 6.82 (m, C_6H_4, J = 8.48); 7.12 (m, C_6H_4, J = 8.48); 8.15 (d, NH, J = 38.16).

^{13}C: 22.63 (CH_3); 37.39 (CH); 52.20 (CH_2); 55.26 (OMe); 113.89 (C_6H_4); 130.37 (C_6H_4); 129.83 (C_6H_4—C); 158.18 (C—OMe); 169.54 ($COCH_3$); 173.55 (COOH).

mp 118–120°C.

Cleavage of the methyl group to give tyrosine HCl (4′-hydroxyphenylalanine HCl). Hydrogenated product (4.8 g; 20.2 mmol) was boiled in 50 ml concentrated hydrochloric acid and 5 g sodium iodide overnight. The nearly colorless solution was extracted three times each with ethylacetate and diethylether and concentrated to dryness. The residue was extracted three times with methanol, concentrated to one third of its volume, and precipitated with diethylether. The precipitate formed was collected and dried. It contains 10% phenylalanine hydrochloride as an impurity formed upon the previous hydrogenation with Raney-nickel as determined by GC.

Yield: 3.0 g (13.8 mmol, 68% of theory).

NMR (ppm): ^{1}H: 3.01 (d, CH_3, J = 5.15); 4.052 (m, CH); 8.353 (s, NH); 9.427 (s, OH); 6.72 (d, C_6H_4, J = 7.64); 7.05 (d, C_6H_4, J = 7.72).

^{13}C: 34.84 (CH_2); 53.36 (CH); 115.34 (C_6H_4); 130.47 (C_6H_4); 124.57 (C_6H_4—CH_2); 156.57 (C—OH); 170.36 (COOH).

All NMR-spectra were taken in DMSO.

RESULTS AND DISCUSSION

Choice of the Enantioselective Gas-Chromatographic Method

In principle, two approaches can be utilized for the analysis of the enantiomeric composition of DL-α-amino acids by GC: (i) derivatization with a chiral auxiliary and separation of diastereomers on an achiral stationary phase or (ii) derivatization with achiral reagents and separation of the enantiomers on a chiral stationary phase. The indirect method of separation of diastereomers has several shortcomings, which limit its use for the determination of small enantiomeric excesses. These include the requirement of quantitative enantiomeric purity of the derivatizing agent, complete absence of racemization of both reaction partners in the course of derivatization, identical reaction kinetics of both enantiomers with the chiral auxiliary, and an unbiased detector response to diastereomers. On the other hand, with the direct chromatographic separation of enantiomers on a CSP, the above factors are irrelevant except for the possibility of racemization of the analyte which, however, is less stringent since slight racemization (known from derivatization of enantiopure α-amino acids to be less than 0.1%[51]) does not measurably bias the results of a nearly racemic sample. Thus achiral derivatization of α-amino acids followed by separation on a CSP would appear to be the only viable gas chromatographic method.

The following characteristics are considered important for the accurate determination of the enantiomeric fraction EF of the D-enantiomer in a nearly racemic DL-α-amino acid by GC: (i) sufficiently high resolution factors R_s permitting overloading of the column to enhance the S/N ratio without peak overlap, (ii) involatility of the chiral stationary phase at elevated temperatures, since bleeding increases the baseline noise, which affects the scatter of results and leads to higher standard deviations, (iii) complete inertness of the CSP and (deactivated) capillary inside surface toward the chosen derivatives, and (iv) the availability of the oppositely configurated CSP to verify the results by reversing the order of elution for enantiomers.

The enantioseparation of derivatized DL-α-amino acids by GC on several CSPs has been described. Of these, Chirasil-Val [27,38] and Lipodex E (octakis(3-*O*-butanoyl-2,6-*O*-di-*n*-pentyl)-γ-cyclodextrin)[46] have found the widest application.[52] In terms of enantioselectivity, these stationary phases are more or less equivalent, with Chirasil-Val in the advantage for the aromatic α-amino acids phenylalanine, tyrosine and tryptophan, and Lipodex E superior for proline. Lipodex E, especially when diluted in a dimethylpolysiloxane gum (e.g. SE 30 or PS 255), generally shows a somewhat lower bleeding than Chirasil-Val and it is not prone to configurational change during long-term usage. On the other hand, in contrast to Chirasil-Val, Lipodex E is unfortunately not available in the oppositely configurated all-L-form. This aspect is a distinct disadvantage. In the future, this shortcoming may be overcome by the use of derivatized linear dextrins, which are capable of resolving DL-α-amino acid derivatives and are more readily available in both all-D and all-L-forms.[53]

Choice of Derivatives

The gas-chromatographic enantioseparation of α-amino acids requires derivatization.[54] In principle several different types of derivatives of α-amino acids can be employed on either Chirasil-Val or Lipodex E, or both [52]. For the purpose of precise measurement of the enantiomeric composition of single DL-α-amino acids, maximum resolution of those α-amino acids with poor enantioselectivity values together with maximum stability of the derivatives under GC conditions will determine the choice of derivatives. In addition the volatility of the derivatives can play a role since the higher the elution temperature, the higher is the bleed of the CSP and correspondingly the detector noise, making accurate integration of peaks more difficult. According to our experience the *N*-perfluoroacyl/alkylester derivatives represent the optimal choice on account of their comparatively high volatility and the enantioselectivity obtainable with the available CSPs.

When the enantiomeric composition of α-amino acids must be determined in the presence of other α-amino acids, the question of separation of individual amino acids from each other must also be considered when choosing

TABLE 2. Comparison of the EF of the D-enantiomer of DL-tyrosine (Serva) as determined by GC (FID) of the *N,O*-TFA/ethylester derivative using three CSPs and by CE (UV detection) of underivatized tyrosine using two CMPAs

	DL-tyrosine (Serva)			
	EF	σ	*n*	R_s
GC				
Chirasil-D-Val (A)	49.72	0.10	10	6.39
Chirasil-L-Val (C)	49.84	0.07	10	4.34
Lipodex E (F)	50.90	1.08	6	2.35
CE				
2-HP-α-CD	49.67	0.36	4	3.39
HS-γ-CD	49.57	0.15	10	7.21

Mean: (49.94 ± 0.55)%.
Mean (excluding Lipodex E): (49.70 ± 0.11)%.

the derivatization strategy.[52] Although *N*-pentafluoropropionyl (PFP) derivatives possess a somewhat higher hydrolytic stability, the *N*-trifluoroacetyl (TFA) derivatives are preferred because the enantioselectivity towards TFA derivatives is always higher and the purity of commercial TFAA is superior to that of PFPA, which can contain mixed TFA/PFP anhydride, thus leading to satellite peaks, which can interfere by coelution with the principal derivatives.[52] With regard to the ester component, on Chirasil-Val, the best separation of all α-amino acid enantiomers were obtained in the form of their *n*-propyl esters, while the ethyl esters are preferable on 30% Lipodex E/70% PS 255.[52] The choice of derivatives is thus closely related to the choice of the CSP. In the present investigation involving only single DL-α-amino acids, the *N(O,S)*-TFA/ethylester derivatives were used throughout on all CSPs.

Accuracy of Measurement

In this work, accuracy, i.e. the degree of coincidence of a measured mean value with that considered to be the true value, was evaluated by employing different enantioselective methods and different chiral selectors for the analysis of DL-tyrosine (Serva) in the following hierarchical sequence: (i) comparison of enantioselective CE (using underivatized tyrosine) vs. enantioselective GC (using derivatized tyrosine), (ii) comparison of enantioselective CE employing the CMPAs 2-HP-α-CD and HS-γ-CD, (iii) comparison of enantioselective GC employing the CSPs Lipodex E vs. Chirasil-Val, and (iv) comparison of enantioselective GC employing the oppositely configurated CSPs Chirasil-L-Val vs. Chirasil-D-Val. The results are listed in Table 2.

Typical chromatograms and electropherograms featuring the enantioseparation of DL-tyrosine (Fluka) are shown in Figure 1. In enantioselective CE, on-column detection is performed in the presence of the chiral selector employed as CMPA during the separation. Therefore, the peak areas have to be correlated with the retention times.[55] Although, the extinction coefficient of enantiomers can be different in the presence of a chiral selector,[56] it is assumed that this effect is negligible for tyrosine and cyclodextrin selectors upon UV-detection. Indeed, the comparison of the data for CE vs. GC shows a very good agreement except for Lipodex E. The large standard deviation and the discrepancy in the absolute value of EF measured on Lipodex E is most likely caused by the low resolution factor of the enantiomers of the *N,O*-TFA ethylester of tyrosine on the cyclodextrin selector by GC (cf. Table 2). The slightly higher standard deviation in CE as compared with GC may be the result of UV-detection and/or the lower S/N ratio (cf. Fig. 1). The small standard deviation of the mean enantiomeric fraction (49.70 ± 0.11)% obtained by measurement with these four different methods (excluding Lipodex E) is an indication that accuracy is high. The deviation from the expected EF = 50.0% margin suggests that the commercial DL-tyrosine sample (Serva) is not truly racemic but in fact contains a slight excess of the L-enantiomer (*vide infra*). Therefore, additional specimens of 'racemic' DL-tyrosine were screened. Thus, a DL-tyrosine sample obtained from Fluka was measured (i) as received (ii) after one recrystallization (Fluka, 1× recryst.), and (iii) after four recrystallization steps in HPLC grade water (Fluka, 4× recryst.). Here, measurement was restricted to the oppositely configurated CSPs Chirasil-L-Val and Chirasil-D-Val since these proved to yield results of acceptable accuracy (*vide infra*).

DL-tyrosine (Fluka) was measured on Chirasil-D-Val (EF = 49.50% ± 0.05%, $n = 6$) and on Chirasil-L-Val (EF = 49.56% ± 0.05%, $n = 12$) (Table 3). The agreement of the values obtained on the oppositely configurated CSPs is high (ΔEF = 0.06%). Here also a preponderance of the L-enantiomer is found. Therefore, the sample was recrystallized slowly over a period of several weeks from a dilute (5.6 mmol/l) solution (Fluka, 1× recryst.) and a single crystal was investigated. The EF of the D-enantiomer increased to 49.86% when measured on Chirasil-L-Val and 49.74% when measured on Chirasil-D-Val. Finally, the sample was recrystallized four times from a solution containing 17.1 mmol/l (Fluka, 4× recryst.) and the EF of the D-enantiomer in these crystals was not further increased, i.e., EF = 49.83% ± 0.05%, $n = 6$ on Chirasil-D-Val and EF = 49.91% ± 0.05%, $n = 12$ on Chirasil-L-Val. The difference in the measured ΔEF on the oppositely configurated CSPs was generally ∼0.08%. According to these measurements, DL-tyrosine recrystallized four times still does not display the expected EF = 50.0%. At this point it cannot be decided whether this is indicative of an inherent nonracemic commercial sample or to a systematic error of the measurement.

Incidentally, the results obtained with recrystallized samples of DL-tyrosine confirm our earlier finding[19] that crystallization of DL-tyrosine yields more truly racemic crystals and does not lead to an enantiomeric bias, in contrast to a report of Shinitzky et al.[18]. These authors claim that in water the solubility of D-tyrosine is lower than L-tyrosine, with the consequence that from aqueous solutions of DL-tyrosine, the D-enantiomorph precipitates preferentially, leaving the solution enriched with the L-enantiomer. This phenomenon they attributed to a parity violating energetic difference (PVED) between enantiomorphous D- and L-crystals of tyrosine. No such effect is observed by the present carefully validated gas-chromatographic meas-

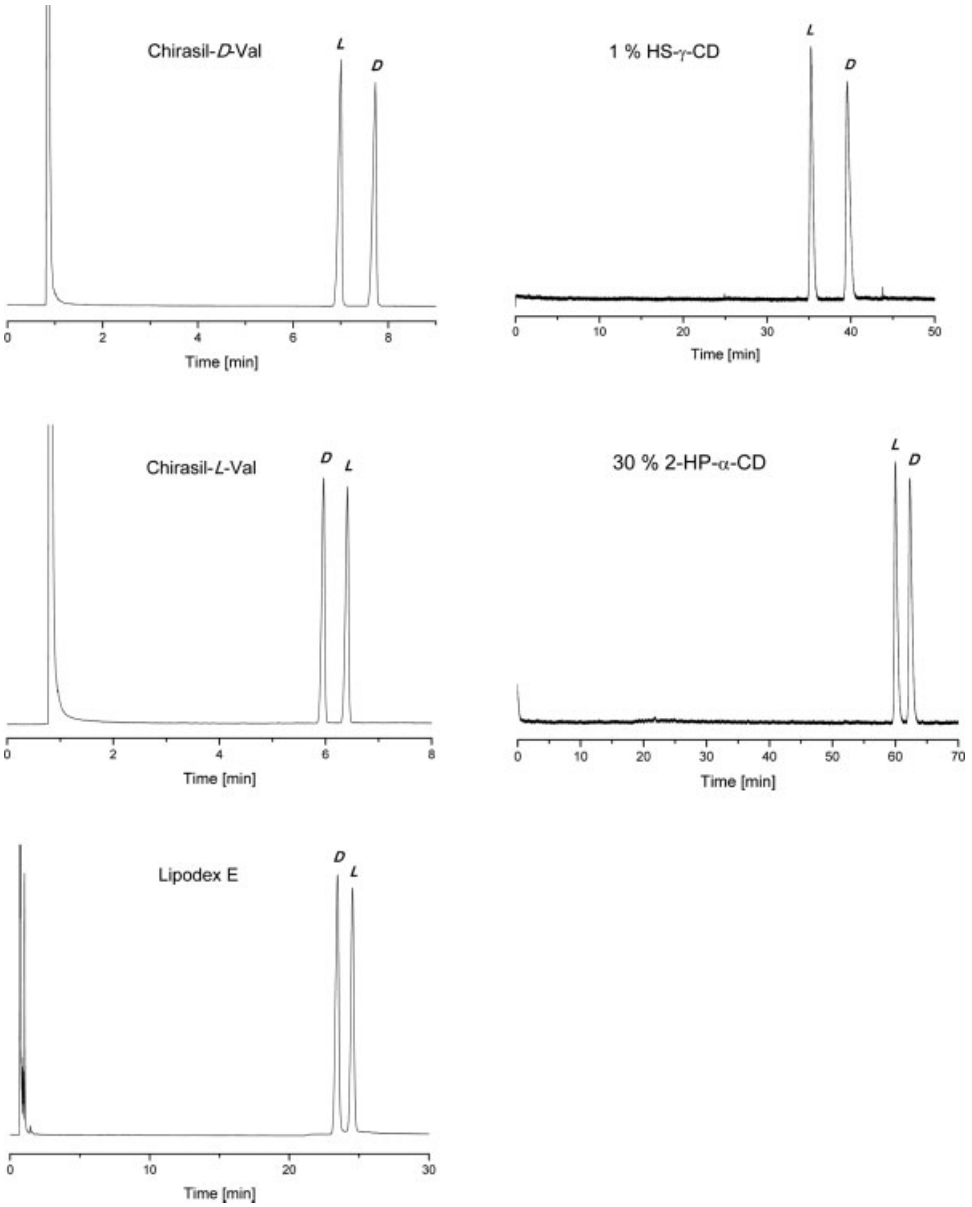

Fig. 1. Chromatograms and electropherograms of DL-tyrosine (Fluka). Left: GC of the *N,O*-TFA/ethylester derivatives on three different CSPs. Right: CE of underivatized tyrosine on two different CMPAs.

urements of DL-tyrosine (Fluka, 1× recryst. and 4× recryst.).

In validation studies, accuracy should be determined with certified standards. To our knowledge, DL-α-amino acids with a certified exact enantiomeric composition are not available. As proteinaceous DL-α-amino acids are usually produced by racemization of the L-enantiomers obtained from natural sources, an excess of the L-enantiomer is indeed to be expected if the racemization has not gone to completion. Considering the asymptotic course of the reaction, a truly racemic mixture is attained only after an infinite reaction time. For this reason it cannot be assumed that commercial samples of DL-α-amino acids are indeed truly racemic [above the statistical bias (*vide supra*)]. Unfortunately, no differentiation can be made at this point between an enantiomeric bias of a DL-α-amino acid produced by racemization of single enantiomers and a systematic error of the gas-chromatographically determined EF values. However, a truly racemic mixture can be expected to result from an abiotic synthesis in an achiral environment employing strictly achiral educts. To avoid enantiomeric bias via traces of chiral contamination from the laboratory environment,[26] a large-scale synthesis of DL-tyrosine hydrochloride according to Figure 2 was carried out.

Although not chemically pure as the yellow-orange sample contained ~5% DL-phenylalanine (Fig. 3), the DL-tyrosine thus obtained is expected to be truly racemic within

TABLE 3. Measurement of the EF of the D-enantiomer of four DL-tyrosine samples (Fluka, Fluka 1× and 4× recrystallized and synthetic), determined as the *N,O*-TFA/ethylester derivatives by GC (FID) on Chirasil-D- and L-Val

	DL-tyrosine (Fluka)			DL-tyrosine (Fluka, 1× recryst.)			DL-tyrosine (Fluka, 4× recryst.)			DL-tyrosine (synthetic)		
	EF	σ	*n*	EF	σ	*n*	EF	σ	*n*	EF	σ	*n*
Chirasil-D-Val												
(A)	49.50	0.05	1 × 6	49.74	0.03	3 × 5	49.83	0.05	1 × 6	49.90	0.03	3 × 4
(B)							49.77	0.08	1 × 10	50.10	0.07	1 × 10
Chirasil-L-Val												
(D)	49.56	0.05	1 × 12	49.86	0.07	3 × 5	49.91	0.05	1 × 12			
(D)							49.92	0.06	1 × 10	50.03	0.06	1 × 10
(C)							49.76	0.05	1 × 10	50.00	0.06	6 × 3

the boundaries of statistic stochastic distribution (*vide supra*). For example, six separately derivatized (*N,O*-TFA/ethylester) samples (b)–(g) of this material were measured in triplicate on Chirasil-L-Val (column C). The mean of all measurements was (50.00 ± 0.06)% (Table 4) as expected for a truly racemic composition.

However, the data also show that due to fluctuations of unknown origin, a multitude of measurements is necessary to arrive at this result. This is illustrated by measurements repeated later by a different analyst. Thus, a singly derivatized sample of synthetic DL-tyrosine (a) was measured on Chirasil-L-Val yielding EF = 50.03% ± 0.06%, n = 10 and on Chirasil-D-Val affording EF = 50.10% ± 0.07%, n = 10. Surprisingly, the same sample (a) gave a large EF = 54.09% ± 0.42%, n = 10 on Lipodex E (Table 4). This low accuracy may be caused by the lower resolution factor (R_s = 2.35 according to Table 2) or by an unknown systematic error. It serves as a stern warning not to rely on just one measurement, which in the present case would imply an enantiomeric bias in a sample which is in fact racemic!

In regard to a recent claim by Nemoto et al.[20] of differences in the rate of racemization of D- and L- alanine in a hydrothermal environment, which they attributed to the PVED, it seemed appropriate to validate the determination of the EF of the aliphatic α-amino acid DL-alanine by GC-FID and by GC-MS. Samples of DL-alanine (from Merck and Serva, respectively) were investigated as *N*-TFA/ethylesters on both Chirasil-D-Val and Chirasil-L-Val and on Lipodex E. Two samples (a) and (b) from each of the two suppliers were measured four times by GC-FID and five times by GC-MS (only Chirasil-L-Val) in the SIM mode. The results are listed in Table 5.

Despite the absence of labile groups of derivatized DL-alanine, the values of EF determined on Chirasil-D- and L-Val and Lipodex E differ by up to 0.16%. This deviation must be ascribed to an unexplained systematic error. In contrast to DL-tyrosine (Table 4), the EF of DL-alanine determined on Lipodex E is compatible with that measured on Chirasil-Val (Table 5).

Precision of Measurements

Precision is used here as a term for repeatability or reproducibility estimated by the standard deviation around the mean as result of random (not systematic) errors when measuring replicate samples. Precision is affected by numerous parameters, among others the injector type, injector design and injection technique employed, electronics of detection, purity and regulation of the burning gases for the FID, quality of the integrator, in particular the resolution of the A/D converter, integration parameters used, and in cases of low enantioselectivity, the resolution of the two enantiomeric peaks. As far as possible for a routine GC laboratory, the earlier factors were addressed and optimized in the present investigation. In Tables 2–4 the high precision as judged from the low standard deviations observed in the present enantioselective GC system is already evident for DL-tyrosine (except for Lipodex E)

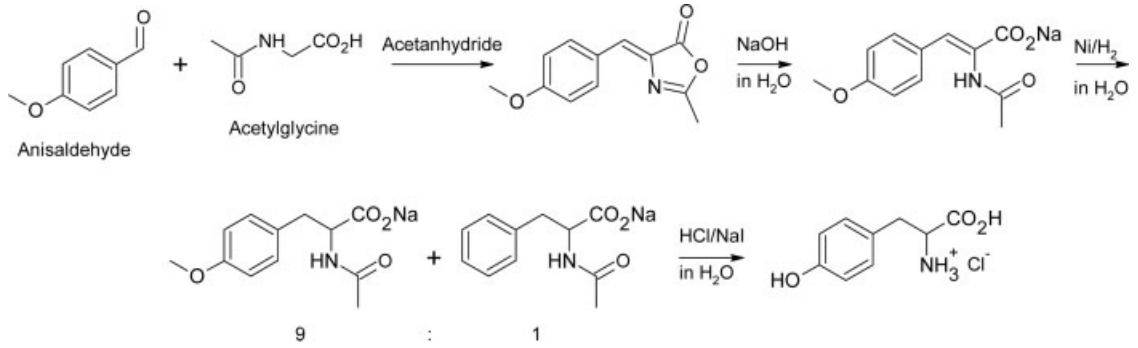

Fig. 2. Abiotic synthetic pathway to racemic DL-tyrosine hydrochloride.

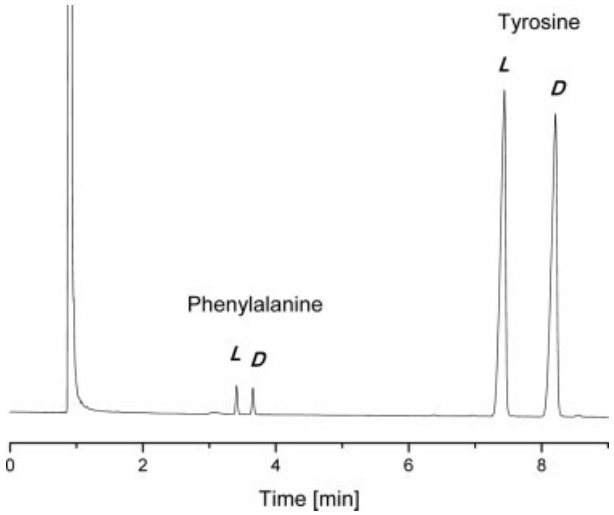

Fig. 3. Gas chromatogram of synthetic (abiotic) DL-tyrosine as *N,O*-TFA/ethylester on Chirasil-D-Val (column B).

and in Table 5 for DL-alanine. To widen the scope of this investigation, a number of other commercially available DL-α-amino acids (as *N(O)*-TFA/ethylesters) were scrutinized by enantioselective GC with FID detection on both Chirasil-D-Val and Chirasil-L-Val.

Alanine and leucine and proline represent aliphatic or alicyclic amino acids, ornithine and lysine represent basic amino acids, aspartic acid an acidic amino acid and phenylalanine and tyrosine aromatic amino acids. Serine, tyrosine, and cysteine represent amino acids, the TFA esters of, which are hydrolytically labile. The precision ($n = 10$) is in general $\leq$0.08% (Table 6). The results indicate that apart from cysteine all samples are close to racemic. In terms of accuracy, the agreement of the data obtained on the oppositely configurated CSPs is very good in most instances, but not for all amino acids. The exceptional precision for ornithine is shown in Table 7, which depicts the individual data of 10 measurements. Here again there is slight bias of the true value for EF between Chirasil-Val and Lipodex E whereas precision is comparable.

Influence of the Amount Injected upon Precision

The precision of a gas-chromatographic measurement is dependent upon the amount of sample reaching the detector. With ample sample available (>30 ng at the detector), a standard deviation (with $n = 10$) of ~0.05% was generally attained. This value was typical for the majority of DL-α-amino acids (cf. Table 6). An exception was proline

TABLE 4. Individual values of the enantiomeric fraction of the D-enantiomer EF_i, means of EF and standard deviation σ of synthetic DL-tyrosine (as *N,O*-TFA/ethylester) obtained by up to seven separate derivatizations (a)–(g) and employing the CSPs Chirasil-D-Val (column A and B), Chirasil-L-Val (column C and D), and Lipodex E (column E)

Chirasil-D-Val (B/A)					Chirasil-L-Val (D/C)					Lipodex E (E)				
Sample	Run	EF_i	EF	σ	Sample	Run	EF_i	EF	σ	Sample	Run	EF_i	EF	σ
a	1	49.95			a	1	50.02			a	1	53.43		
	2	50.20				2	49.94				2	54.07		
	3	50.08				3	50.03				3	54.11		
	4	50.16				4	50.11				4	54.35		
	5	50.11				5	50.06				5	53.90		
	6	50.14	50.10	0.07		6	50.06	50.03	0.06		6	54.08	54.09	0.42
	7	50.06				7	49.99				7	54.04		
	8	50.07				8	50.02				8	54.31		
	9	50.11				9	50.07				9	54.98		
	10	50.13				10	49.95				10	53.66		
b	1	49.90			b	1	49.85							
	2	49.97				2	50.00							
	3	49.86				3	49.96							
	4	49.88			c	1	50.03							
c	1	49.89				2	50.02							
	2	49.88				3	50.02							
	3	49.92	49.90	0.03	d	1	50.03							
	4	49.89				2	50.03							
d	1	49.89				3	50.01	50.00	0.06					
	2	49.94			e	1	50.02							
	3	49.90				2	50.01							
	4	49.92				3	50.05							
					f	1	50.03							
						2	50.04							
						3	50.03							
					g	1	49.94							
						2	50.00							
						3	49.92							

TABLE 5. Enantiomeric fraction EF of the D-enantiomer of two samples of DL-alanine (as *N*-TFA/ethylester) as determined by GC (FID) on Chirasil-D-Val (A), Chirasil-L-Val (D), and Lipodex E (E) and by GC/MS (SIM: m/z 140) on Chirasil-L-Val (D)

	DL-alanine (Merck)			DL-alanine (Serva)		
	EF	σ	*n*	EF	σ	*n*
GC						
Chirasil-D-Val (A)	49.93	0.04	2 × 4	49.91	0.03	2 × 4
Chirasil-L-Val (D)	50.00	0.03	2 × 4	49.99	0.04	2 × 4
Lipodex E (E)	49.88	0.02	2 × 4	49.89	0.01	1 × 10
GC/MS						
Chirasil-L-Val (D)	50.03	0.05	2 × 5	50.04	0.08	2 × 5

measured on Chirasil-Val. Here, because of the relatively low resolution of the enantiomers, an increase in the amount injected induced overlap of the peaks, while a decrease in the amount injected increased the effects of baseline noise, both effects contributing to the poorer standard deviation. As expected, the standard deviation increases with decreasing amounts of sample reaching the detector. For a more detailed study of the influence of the amount injected on precision, nearly racemic DL-tyrosine (Fluka, 4× recryst.) was selected. Thus, a series of dilutions of a derivatized sample was measured on Chirasil-L-Val with both flame-ionization and MS-SIM detection (Table 8). Experimental details are given in the legend of Table 8.

Both accuracy and precision deteriorate considerably with decreasing amounts of sample injected. In the GC-FID mode, the standard deviation of EF for DL-tyrosine increases from $\sim$<0.06 with >30 ng entering the column to 2.5% with 160 pg. In the GC-MS in the SIM mode with its higher sensitivity and selectivity, σ of $\sim$0.6% is still achieved with 20 pg DL-tyrosine entering the column. At the latter level, however, a highly significant deviation (6%) of the measured value from the true value (reduced accuracy) is observed, probably because of the increasing effect of decomposition of derivatized tyrosine on the capillary column.

Effect of Handedness of the CSP on the Enantiomeric Composition Values Determined

For all racemic DL-α-amino acids obtained from commercial sources, the samples were analyzed on both Chirasil-D-Val and Chirasil-L-Val (Tables 2 and 6). Unfortunately, it is not possible to differentiate between systematic errors of the enantioselective gas-chromatographic system and the presence of enantiomeric bias in the samples labeled as racemates. Theoretically the enantiomeric composition determined should be independent of the handedness of the CSP. However, the value determined could be falsified by (i) an impurity, coeluting with either of the enantiomers and thus spuriously increasing its peak area, (ii) decomposition of the α-amino acid derivative in the column, whereby the second eluted enantiomer, which spends a longer time in the column will be depleted preferentially, or (iii) by enantioselective degradation (= kinetic resolution) of the α-amino acid derivative on the CSP [3,39]. A special case (iv) of decomposition of derivatives may arise with substances bearing an *O*-TFA ester (e.g. serine or tyrosine), *S*-TFA thioester (e.g. cysteine), or the *N*-TFA-guanidino group (arginine). These TFA derivatives are hydrolytically instable, particularly in the presence of basic groups. Cleavage of trifluoroacetic acid from the first eluted fraction; however, deactivates the basic groups (which can be both on the capillary surface or within the stationary phase) for the subsequently eluted fraction, thus leading to a bias in favor of the second eluted peak. On the other hand if the derivative were prone to decomposition on the column under the influence of temperature [case (ii)], the second eluted enantiomer can be expected to be depleted since it is subjected to the temperature for a longer period. Reversing the handedness of the CSP can shed light on whether these scenarios are in fact operative for a particular separation. In the case of (iii), however, no change of the peak elution pattern will be observed since

TABLE 6. Enantiomeric fraction EF of the D-enantiomer, standard deviation σ, and resolution factor R_s of representative racemic DL-α-amino acids measured as *N(O, S)*-TFA/ethylester derivatives determined by GC (FID) on three CSPs (mean and σ of 10 injections)

	Chirasil-D-Val (1st peak: L-, 2nd peak: D-)				Chirasil-L-Val (1st peak: D-, 2nd peak: L-)				Lipodex E (1st peak: D-, 2nd peak: L-; proline vice versa)			
	Column	EF	σ	R_s	Column	EF	σ	R_s	Column	EF	σ	R_s
DL-alanine	A	49.91	0.04	13.56	D	49.99	0.04	7.43	E	49.89	0.01	2.75
DL-leucine	B	50.01	0.09	20.35	D	50.02	0.09	12.56	F	49.93	0.04	7.51
DL-ornithine	A	49.99	0.06	8.53	D	49.98	0.03	2.84	G	49.90	0.04	3.48
DL-lysine	A	49.90	0.08	4.45	D	49.90	0.02	3.51	G	49.66	0.08	6.43
DL-aspartic acid	B	50.03	0.03	2.87	D	49.95	0.05	2.41	G	49.98	0.04	3.15
DL-phenylalanine	A	50.01	0.04	7.43	D	49.98	0.02	3.48	F	49.82	0.11	1.74
DL-tyrosine (Fluka, 4× recryst.)	B	49.77	0.08	5.52	D	49.92	0.06	4.13	E	53.27	0.63	2.32
DL-tyrosine (synthetic)	B	50.10	0.07	5.65	D	50.03	0.06	3.92	E	54.09	0.42	2.77
DL-serine	B	50.10	0.04	4.08	D	49.97	0.04	4.01	G	49.54	0.32	2.93
DL-cysteine	A	48.58	0.17	6.65	D	47.77	0.10	3.28		–	–	–
DL-proline	B	50.50	0.08	1.31	D	49.67	0.44	1.11	G	49.99	0.09	11.02

TABLE 7. Individual values of the enantiomeric fraction of the D-enantiomer EF_i, means of EF and standard deviation σ of DL-ornithine (as *N,O*-TFA/ethylester) obtained by 10 separate derivatizations and employing the CSPs Chirasil-D-Val (column A), Chirasil-L-Val (column D), and Lipodex E (column G)

	Chirasil-D-Val (A)			Chirasil-L-Val (D)			Lipodex E (G)		
Run	EF_i	EF	σ	EF_i	EF	σ	EF_i	EF	σ
1	49.99			49.95			49.92		
2	50.03			49.98			49.94		
3	50.02			50.05			49.90		
4	50.09			49.99			49.86		
5	49.97	49.99	0.06	49.98	49.98	0.03	49.85	49.90	0.04
6	49.89			49.93			49.87		
7	49.89			49.98			49.91		
8	49.99			49.98			49.89		
9	50.02			49.99			49.90		
10	49.98			50.02			49.99		

two mutually dependent enantioselective processes are involved when the handedness of the CSP is inverted, i.e., both the chromatographic elution order and the sense of the kinetic resolution will be changed.[3,39]

According to Table 6, for the DL-α-amino acids alanine, leucine, ornithine, lysine, and phenylalanine, no significant difference (95% confidence limits) between the EF of the D-enantiomers on the oppositely configurated CSPs was detectable. For these amino acids, the matching probabilities of a *t*-test performed on the two EF data populations obtained from determination on Chirasil-D- and Chirasil-L-Val were in excess of 0.05. With all other DL-α-amino acids measured, the *t*-test indicated that the mean EF-values determined on oppositely configurated CSPs were significantly different. The magnitude of ΔEF, (i.e. the difference of the mean values for EF measured on Chirasil-D-Val and Chirasil-L-Val) is dependent upon the amino acid and was greatest in the case of cysteine (ΔEF = 0.19%). If it is assumed that the degree of deactivation of both columns is identical and that the discrepancy is not caused by coeluting components, one would expect that the correct value for % D correspond to the mean of the results from the two oppositely configurated CSPs. A test of this hypothesis is given in Table 9 (the values of which are derived from Table 6).

Inspection of the means of the values obtained on oppositely configurated CSPs indicates that this could be a viable way of improving the accuracy of determination the EF of racemic DL-amino acids. Unfortunately, in our hands, it was not possible to produce Chirasil-D-Val and Chirasil-L-Val columns with strictly identical properties. The Grob test,[57] as a measure of the inertness of the capillary surface of the two Chirasil-Val columns, indicated that the Chirasil-D-Val column used was in fact less inert than the Chirasil-L-Val column. This is expected to affect the values obtained for cysteine, serine, and tyrosine. The true value of EF of the D-enantiomer of these amino acids should thus lie closer to that obtained on the Chirasil-L-Val column. On the other hand, the enantioselectivity of the Chirasil-D-Val column was somewhat superior to that of the mirror-image CSP. Thus the true value for % D-proline can be expected to lie closer to that determined on Chirasil D-Val.

Integration

At the level of precision aimed at here, the quality of integration also plays an important role. In this investigation the computer-based Clarity integration system with a 24 Bit A/D converter (DataApex, Prague, Czech Republic) was employed. The optimum values for the parameters peak width and threshold were determined experimentally. For each individual measurement, the peak start and end points were also checked visually and, where considered necessary, were corrected manually.

Linearity/Recovery of the Enantiomeric Fraction of Tyrosine at Near Racemic Composition

The response to enantiomers is strictly linear for an achiral detection system. In this work, linearity and recovery for tyrosine at near racemic composition was assessed

TABLE 8. Dependency of the measured EF of the D-enantiomer upon the amount of DL-tyrosine (Fluka, 4× recryst derivatized as *N,O*-TFA/ethylester and diluted after derivatization) entering the column (Chirasil-L-Val, column D)

Sample concentration		GC/FID			GC/MS		
mmol/l	ng/μl	ng entering column (split, $f = 55$)	EF	σ	ng entering column (splitless)	EF	σ
10	1812	32.95	49.88	0.06		49.09	0.18
1	181	3.29	50.04	0.56	181	49.95	0.15
0.5	91	1.65	50.80	1.41	91	49.57	0.04
0.1	18.1	0.33	49.98	1.89	18.1	48.96	0.22
0.05	9.1	0.16	51.81	2.37	9.1	49.36	0.07
0.01	1.81				1.81	49.30	0.12
0.005	0.91				0.91	48.83	0.68
0.001	0.18				0.18	49.38	1.14
0.0005	0.09				0.09	48.40	0.68
0.0001	0.02				0.02	44.00	0.61

GC(FID): 1 μl, split injection (split ratio 1:55), $n = 5$; GCMS: (EI, SIM, m/z 288) 1 μl, splitless injection, $n = 4$.

TABLE 9. Difference in the EF of the D-enantiomers of DL-α-amino acids measured on Chirasil-D- and L-Val (= ΔEF), mean of EF values, and *t*-test

	Chirasil-D-Val	Chirasil-L-Val	ΔEF (%)	Mean of EF (%)	*t*-Test
DL-alanine	49.91	49.99	−0.08	49.95	0.001
DL-leucine	50.01	50.02	−0.01	50.02	0.81
DL-ornithine	49.99	49.98	0.01	49.99	0.88
DL-lysine	49.90	49.90	0.00	49.90	0.92
DL-aspartic acid	50.03	49.95	0.08	49.99	7.0×10^{-4}
DL-phenylalanine	50.01	49.98	0.03	50.00	0.058
DL-tyrosine	49.77	49.92	−0.15	49.85	1.9×10^{-4}
DL-tyrosine (synth.)	50.10	50.03	0.07	50.07	0.012
DL-serine	50.10	49.97	0.13	50.04	1.3×10^{-6}
DL-cysteine	48.58	47.77	0.81	48.18	2.3×10^{-10}
DL-proline	50.50	49.67	0.83	50.09	1.8×10^{-4}

over the range of EF = 49.5%–50.5%. Thus, DL-tyrosine (Fluka, 4× recryst.) was fortified with known amounts (up to 1%) of both D- and L-tyrosine. The results are shown in Figure 4 and in Table 10.

Linearity of the determination is confirmed by the calibration line, the correlation coefficient of which is 0.9997. Assuming an EF of the racemic tyrosine (Fluka, 4× recryst.) of 50.00%, the rate of recovery lay between 99.94% and 99.98%.

According to the calibration equation, the EF of this tyrosine sample (unspiked) was 49.99%. In separate measurements of the EF of this unspiked tyrosine sample on the same column (D), however, values of 49.88 ± 0.06% (Table 8) and 49.92 ± 0.06% (Table 3) were determined. This discrepancy of up to 0.11% in the EF, which is outside the precision limits, appears to be a result of day to day fluctuation in the GC conditions (measurements were not made in one session). This must be considered as an inherent problem in the accurate determination of EF of sensitive amino acid derivatives by GC.

CONCLUSIONS

It is implied that the limit in determining minute deviations from the true racemic composition of target compounds, specifically DL-α-amino acids, e.g., in scenarios mentioned in the introduction, employing enantioselective GC is generally only in the range of EF = 50.0% ± 0.1%, corresponding to an ee = 0.2% (or 1.2×10^{21} molecules in one mole of racemate). To attain such accuracy and precision, a number of requirements must be met, among them enantiomeric separation with a resolution factor $R_s > 1.5$, absence of coeluting impurities and decomposition of the sample or its derivative during the chromatographic process, an elution-time independent detector response and a correct peak area integration. Measurement with a precision (reproducibility) equal or in excess of ±0.05% is feasible but the accuracy (trueness) of the value determined must be carefully validated by employing oppositely configurated CSPs, leading to the reversal of the elution order of the separated enantiomers. The EF of aliphatic, alicyclic, and aromatic α-amino acids without further polar groups of near-racemic composition may be determined by GC on Chirasil-Val and/or Lipodex E (whereby Lipodex E is recommended for proline while Chirasil-Val is recommended for phenylalanine, tyrosine, and tryptophan) with a standard deviation of ∼0.05% and a high degree of confidence that the true value lies within the range defined by the standard deviation. For amino acids with polar side chains, the precision of measurement need not necessarily decrease but the true value of the EF can easily lie outside the precision limits as a result of systematic errors. Such errors can be counteracted by the use of oppositely configurated CSPs. The mean of the values obtained from these CSPs can generally be considered as a better estimate of the correct value than measurement on one CSP only, provided the degree of deactivation of both columns is comparable. Column deactivation is best assessed by the standard Grob Test[57] for capillaries. The difference ΔEF is an indication of the confidence limits associated with the determination.

It must be stressed that in this study sample amounts in the milligram range were derivatized. In situations where only trace amounts of DL-α-amino acids are available, the precision of the measurement can be expected to deteriorate as shown in this work. More importantly, however, the probability of contamination with α-amino acids from the laboratory environment (airborne contaminants, contaminated reagents, and glassware) leading to spurious enrichment of the biogenic L-amino acid [23] must always be anticipated in trace analysis of EF.

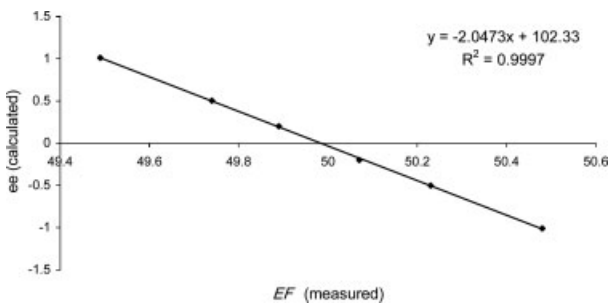

Fig. 4. Calibration diagram of DL-tyrosine (Fluka, 4× recryst.) fortified with up to 1% D-tyrosine (positive ee) or L-tyrosine (negative ee).

TABLE 10. Linearity of determination of EF of the D-enantiomer over a narrow concentration range close to racemic composition: Calculated and measured EF of DL-tyrosine (Fluka, 4× recryst.) fortified with either D- or L-tyrosine and rate of recovery as determined on Chirasil-L-Val (column D; *N,O*-TFA/ethylester derivatives)

Sample concentration			Calculated[a]	Measured ($n = 4$)		Rate of recovery
Parts DL (%)	Parts D (%)	Parts L (%)	EF	EF	σ	(%)
99.0		1.0	49.50	49.49	0.05	99.98
99.5		0.5	49.75	49.74	0.03	99.98
99.8		0.2	49.90	49.89	0.02	99.98
99.8	0.2		50.10	50.07	0.04	99.94
99.5	0.5		50.25	50.23	0.02	99.96
99.0	1.0		50.50	50.48	0.03	99.96

[a]DL-tyrosin (Fluka, 4× recryst), conc. 10.00 mmol/l, assumed EF = 50.00%; D-tyrosine: conc. 10.00 mmol/l, EF = 100.0%; L-tyrosine: conc. 10.00 mmol/l, EF = 0.0%.

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