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Review

Gas chromatographic enantioseparation of derivatized α-amino acids on chiral stationary phases—Past and present☆

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ABSTRACT

The historical development of the enantioseparation of derivatized α-amino acids by high-resolution capillary gas chromatography on chiral stationary phases derived from α-amino acid-derivatives and modified cyclodextrins is described. The pioneering work emerging from Emanuel Gil-Av and his associates at the Weizmann Institute of Science is reviewed. A bridge to more recent developments is spanned aimed at helping to select appropriate tools for contemporary chiral α-amino acid analysis by gas chromatography in different research areas.

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1. Introduction

In the evolution of the enantioseparation of α-amino acids by gas chromatography two approaches have been advanced. The *indirect* approach relies on the formation of diastereomeric derivatives via the reaction of the enantiomers with an enantiomerically pure chiral auxiliary and the subsequent separation of the stereoisomers on a conventional achiral stationary phase in the spirit of Pasteurs's resolution principles via diastereomers. This method as reviewed by Gil-Av and Nurok [1] requires the absence of kinetic resolution of the enantiomers and the absence of racemization of both reaction partners during the derivatization procedure as well as an unbiased detection of diastereomers. In the *direct* approach, enantiomers are separated via the noncovalent diastereomeric interaction with a nonracemic *chiral stationary phase* (CSP). The group of Emanuel Gil-Av at *The Weizmann Institute of Science* in Rehovot, Israel, played the pioneering role in the development of the *direct* separation of enantiomers by gas chromatography (GC) starting in the sixties of the previous century. According to Gil-Av (*cf.* Fig. 1), '*the handedness needed for resolution is provided by an optically active environment in the phase, and not introduced covalently into the molecules to be separated*' [2]. The rapid and reversible formation of diastereomeric association complexes of distinct stabilities generates different retention factors of the enantiomers leading to chromatographic resolution. In the present account only the *direct* approach will be reported. The discoveries of Gil-Av, Feibush and Charles-Sigler in gas chromatography stimulated similar developments in enantioselective liquid chromatography, supercritical fluid chromatography and in various electromigration methods. In the course of the historical development of enantioselective *high-resolution capillary gas chromatography* (HRC-GC) it became soon evident that high efficiency, sensitivity, reproducibility, precision, speed of separation and straightforward detection led to important applications of chiral α-amino acid analysis in different research areas. The universal flame ionization detector (FID) is linear over five-orders of magnitude and detection sensitivity can further be increased to the picogram level by electron-capture detection (ECD) and by element-specific detection. Yet, as the GC technique is inherently restricted to compounds which can be transformed into the gas phase without decomposition, α-amino acids have to be transformed into volatile derivatives, *e.g.*, into *N*-perfluoroacyl-*O*-alkyl esters or *N*-alkoxycarbonyl-*O*-alkyl esters. Due to the high separation power of HRC-GC, contaminants and impurities are separated from the analytes and the simultaneous analysis of multicomponent mixtures of proteinogenic α-amino acids is straightforward. The work started by Gil-Av et al. also paved the way to ancillary techniques in the chiral analysis of α-amino acids such as enantioselective multidimensional gas chromatography (enantio-MDGC), *i.e.*, in-series-coupled column operation and coupling methods such as enantioselective gas chromatography–mass spectrometry (enantio-GC–MS). Employing the selected ion monitoring mode, trace amounts of enantiomers can be detected by GC–MS(SIM).

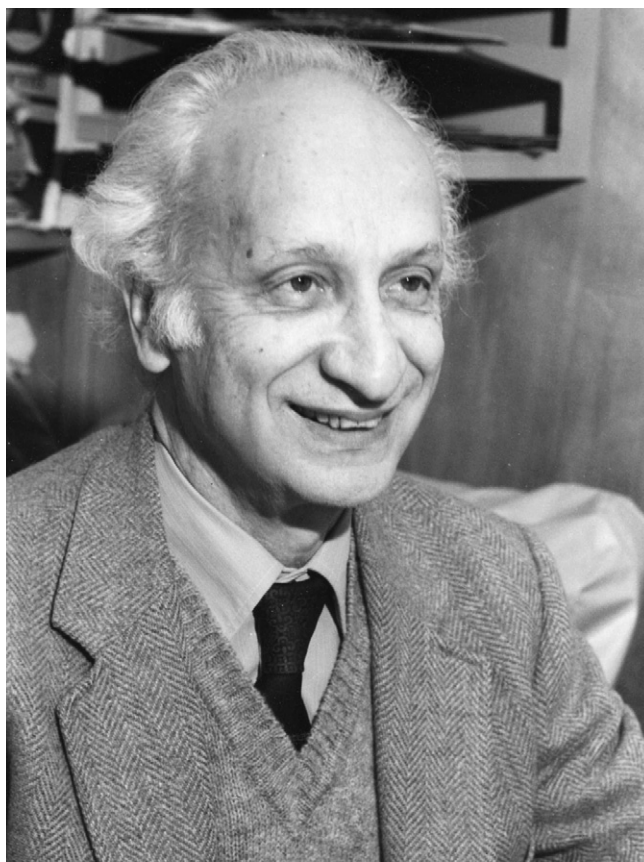
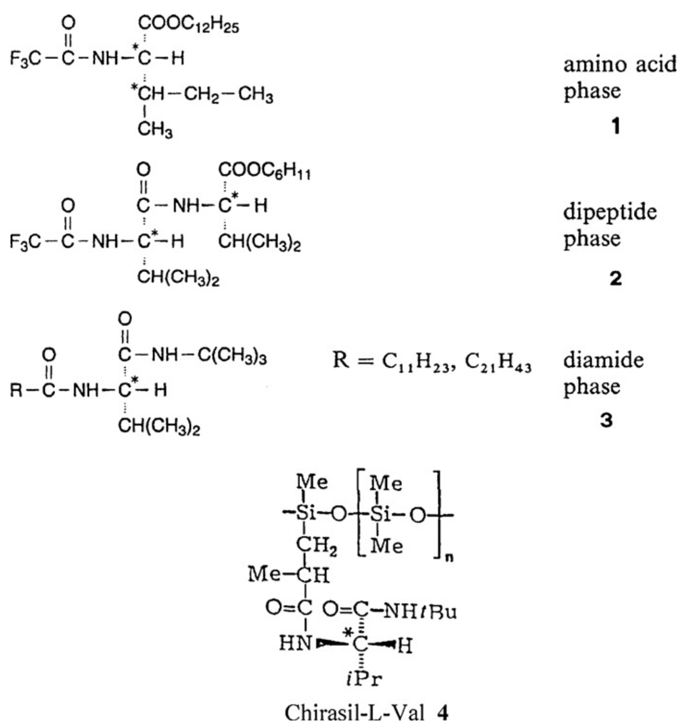


Fig. 1. Emanuel Gil-Av (1916–1996).

2. Early development of CSPs derived from α-amino acid derivatives in hydrogen-bonding-GC

At the outset of the development of enantioselective GC, Gil-Av remarked [2]: "*When we started this work in 1964, this topic was in a "state of frustration". Nobody believed that it could be done. In fact, people were convinced that there could not possibly be a large enough difference in the interaction between* D- *and* L-*solute with an asymmetric solvent. This was the feeling people had, even those known as unorthodox thinkers. This view had also some experimental basis, because a number of communications has been published, in which it was claimed that such resolutions could be effected, but nobody was able to reproduce these results, and some of them were shown to be definitely wrong. When we started our work with B. Feibush – who had the courage to accept this problem for his Ph.D. thesis* [3] *– we based ourselves on the following two ideas. First of all, nature can do it, enzymes differentiate between enantiomers. Therefore we thought: let us have a system which has some of the properties, at least in a rudimentary fashion, of an enzyme. In other words, we decided to try phases with –CO- and –NH-functions grouped around an asymmetric center, capable of forming hydrogen bonds with suitable solutes, i.e. derivatives of α-amino acids. Secondly, we reasoned that we had to amplify the effect, because we expected it to be very small. This meant the use of long capillaries. As stainless steel is expensive, and we expected the need for many columns we decided to start by building a (glass) capillary drawing machine, and by acquiring the delicate skills of drawing long capillaries and coating them. After a period of frustration . . . a period lasting about one year . . . the idea worked*".

The first direct enantioseparation of a derivatized α-amino acid on a chiral stationary phase (CSP) comprising an involatile α-amino acid derivative by gas chromatography was described in 1966 by Gil-Av, Feibush and Charles-Sigler. [4]. Detailed accounts on this fundamental work carried out at the Weizmann Institute of Science, Israel, have been advanced [5,6]. Gil-Av et al. proceeded as follows: a home-made 100 m Pyrex-glass capillary column was coated with a 20% solution of *N*-trifluoroacetyl(TFA)-*O*-*n*-dodecyl (lauryl) ester of L-isoleucine **1** (*cf.* Scheme 1) in diethylether by the plug method. After conditioning overnight and raising the temperature to 80 °C, the column exhibited 140 000 theoretical plates for 2-heptylacetate and trials were performed to resolve racemic *N*-trifluoroacetyl-*O*-alkyl esters of various proteinogenic α-amino acids. The 2-propanol, *n*-butanol and cyclopentanol esters of *N*-trifluoroacetylated (TFA) alanine were partially resolved. More



Scheme 1. Hydrogen-bonding CSPs for the GC enantioseparation of derivatized α-amino acids.

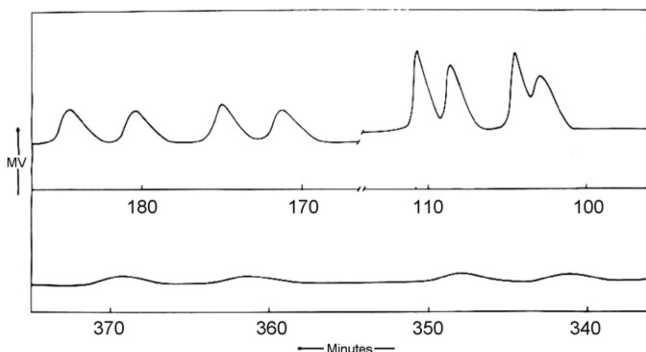


Fig. 2. Combination of the indirect and direct approach for stereoisomeric differentiation. Gas chromatogram of each of the four stereoisomers of the *N*-TFA-alanine-*O*-2-butanol esters (at 102.9, 104.5, 108.6, 110.7 min), *N*-TFA-valine (at 171.5, 175.0, 180.5, 184.7 min) and *N*-TFA-leucine (at 341.2, 348.0, 360.8, 369.5 min) on a 100 m Pyrex glass capillary column coated with *N*-TFA-L-isoleucine-*O*-*n*-dodecanyl (lauryl) ester.
From Ref. [4] with permission.

intriguingly, the diastereomeric *N*-TFA-α-amino acid esters of chiral 2-butanol, which were previously applied for the indirect method [1], gave rise to *four* chromatographic peaks on the CSP, corresponding to two enantiomeric pairs DD/LL (*like descriptors*) and DL/LD (*unlike descriptors*) (*cf.* Fig. 2). Incidentally, the separation of diastereomeric esters of proteinogenic and non-proteinogenic amino acids by capillary GC on chiral and non-chiral stationary phases was later adopted in its own right by others [7]. The high efficiency of the glass capillary columns, not attainable with packed columns, was essential for the first success of the experiment of Gil-Av and associates. Retention times were rather long due to the low column temperature applied in order to avoid bleeding of the moderately volatile CSP.

As stated by Gil-Av [2], "*we were very sceptical, when we saw the first set of four peaks (alanine), became hopeful when the second set of peaks appeared (valine), and were convinced that we had solved the problem, when the leucine peaks emerged one after the other after six hours of tense waiting.*" In order to prove that indeed enantiomers were separated, control experiments were carried out by Gil-Av [2]. Thus only two unresolved peaks were observed for the *like* and *unlike* diastereomers on an achiral stationary phase [4] and mixtures of the D- and L-amino acid derivatives in varying nonracemic ratios gave peak areas corresponding to the proportions of the enantiomeric mixture investigated [2]. In retrospect it should be noted that this control experiment actually laid the basis for the most important application of enantioselective chromatography, *i.e.*, for the correct quantitation of enantiomers in nonracemic mixtures via the enantiomeric ratio *er* (or enantiomeric fraction *EF*), enantiomeric excess *ee* and enantiomeric composition *ec* (for definitions see Ref. [8]) in lieu of the chiroptical methodology (via the optical purity *op* by polarimetry or circular dichroism). When the configuration of the CSP was reversed from L- to D-isoleucine, the expected inversion of the elution order was observed for a nonracemic sample. The strategy to employ oppositely configurated CSPs represents an important validation tool in enantiomeric analysis as the enantiomeric ratio *er* is not biased by peak reversal. These experiments unequivocally proved that incongruent mirror-image isomers, *i.e.*, enantiomers, had been separated for the first time by enantioselective GC [2]. The authors recognized the importance of their discovery [2]: "*The achievement of resolution of optical isomers is one of the most striking demonstrations of the efficiency of gas liquid partition chromatography. The columns described should have interesting analytical applications and might also serve as a tool for the study of stereospecific interactions*".

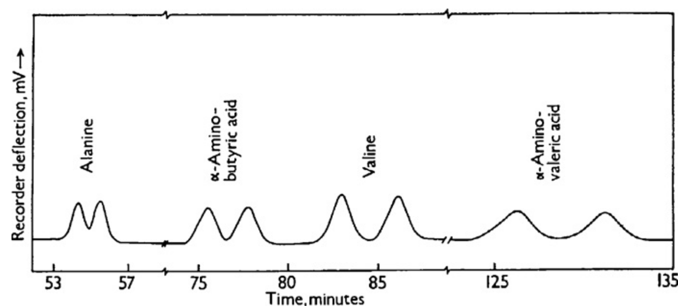


Fig. 3. Gas chromatogram of the enantiomers of four racemic *N*-TFA α-amino acid *O*-2-propyl esters on a 72 m Pyrex glass capillary column coated with *N*-TFA-L-phenylalanine-*O*-cyclohexyl ester at 90 °C [9].

Gil-Av and his associates subsequently studied the structural features influencing resolution hoping that the results would permit extrapolation to other enantioselective solute–solvent systems and thus help to find suitable CSPs also for other classes of racemic compounds. Soon Gil-Av et al. presented a major report on their work entitled the "*Separation of Enantiomers by Gas–Liquid Chromatography with an Optically Active Stationary Phase*" at the Sixth International Symposium on Gas Chromatography held in September 1966, in Rome, Italy [9]. In this contribution, Gil-Av et al. demonstrated the resolution of various enantiomeric pairs of *N*-TFA-*O*-alkyl and *O*-cyclopentyl esters of α-amino acids using the CSPs *N*-TFA-D- or L-isoleucine-*O*-*n*-dodecyl (lauryl) ester and *N*-TFA-L-phenylalanine-*O*-cyclohexyl ester [9]. An example is depicted in Fig. 3.

In all instances it was found that the derivatives of L-α-amino acids eluted after the corresponding D-enantiomers on columns coated with a stationary phase having the L-configuration (and *vice versa* for the D-phase). The participants of the Symposium immediately recognized the importance of Gil-Av's presentation [9]. This is best shown by the remark of B. L. Karger (Northeastern University, Boston, Massachusetts) during the discussion session: "*I would like to congratulate Dr Gil-Av on succeeding in a problem that*

Table 1
Gas chromatographic enantioseparation factors of *N*-TFA α-amino acid *O*-methyl esters on octakis(3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin (Lipodex E) **6**.

Amino acid	Separation factor, α	Temp. [°C]
Ala	1.10	130
Abu	1.107	150
Val	1.148	130
Ile	1.139	130
αIle	1.158	130
Leu	1.126	130
Thr	1.059	130
αThr	1.119	130
Ser	1.153	130
Pro	1.173	130
3,4-DehydroPro	1.152	130
Asp	1.163	160
Cys	1.042	160
Met	1.053	160
Glu	1.065	160
Phe	1.038	170
Orn	1.112	180
Lys	1.184	180
His	1.012	190
Trp	1.014	190
Pipecolic acid	1.086	150
β-Abu	1.118	150
β-iso-Abu	1.075	140
β-Phe	1.023	175
2,3-Diamino-propionic acid	1.043	180
tert-Leu	1.152	120
2-Me-phe	1.000	160
2-*n*-Propyl-ala	1.035	120
Isovaline	1.119	120
2-Me-val	1.051	140
2-Me-ser	1.057	120
2-Me-thr	1.091	140
N-Me-Leu	1.084	120
N-Me-Phe	1.022	180
β-Me-Asp	1.242/1.171	150
Tyr	1.024	175

From Ref. [76] with permission.

several workers have tried over the years. I have no doubt that this work will have great analytical implications" (see p. 256 in Ref. [9]). In the ongoing discussion it was clarified that the second stereogenic center of the *sec*-butyl groups in the isoleucine stationary phase did not contribute to chiral discrimination. Gil-Av also mentioned that the difference in the free energies of solvation $\Delta(\Delta G)$ of the diastereomeric associates leading to resolution amounted to only 0.006–0.030 kcal/mol at the ambient temperature of the measurement. The separation was thought to be due to hydrogen-bonding between NH···F and NH···O=C– functions whereby the latter contribution was considered to be more important [9].

The further development of the direct enantioseparation by GC by α-amino acid diamide and dipeptide CSPs has thoroughly been reviewed [2,10–13]. In order to increase the various steric and polar interactions between the diastereomeric associates, both the derivatization strategy of the α-amino acids to be resolved and the structure of the CSP were varied systematically (*cf.* Table 1 in Ref. [14]). The 'second generation' CSPs **2**–**3** (*cf.* Scheme 1) introduced by Gil-Av and Feibush exhibited better enantioselectivities than the CSP **1** due to the presence of an additional amido-functionality capable of forming altogether three hydrogen bonds in the bridged diastereomeric association complex containing the derivatized α-amino acid to be resolved. Mechanistic details on chirality recognition mediated by enantioselective hydrogen-bonding interactions are discussed by Feibush [15]. The first dipeptide phase tested, *N*-TFA-L-valyl-L-valine-*O*-cyclohexyl ester **2** was also used in a 2 m packed column for the enantioseparation of racemic *N*-TFA-alanine-*O*-*tert*-butyl ester [16]. It represented the first instance of the use of an enantioselective packed column for the semipreparative enantioseparation by GC (*cf.* Fig. 4). Moreover, a chiroptical detector producing opposite optical rotatory dispersion (ORD) curves was employed for the first time in chiral chromatography for unequivocally discriminating the separated enantiomers [16].

Subsequent studies of Gil-Av et al. [17] on the role of the configuration of the *N*- and *C*-terminal α-amino acids of the *N*-TFA-Val-Val-*O*-cyclohexyl ester dipeptides (*N*-Val-*C*-Val) on the order of elution of the *N*-TFA-*O*-alkyl α-amino acid ester enantiomers revealed that peak reversal took place, as expected, on the enantiomeric dipeptides L-Val-L-Val and D-Val-D-Val. However, when the dipeptide L-Val-L-Val was compared with the diastereomeric dipeptides D-Val-L-Val and L-Val-D-Val, peak reversal only occurred on the former in which the configuration of the *N*-terminal α-amino acid was inverted. This was confirmed by the finding that Gly-L-Val possessed significantly inferior enantioseparating properties as compared to L-Val-Gly. It was also found that for *N*-Val-*C*-Val dipeptides containing one α-amino acid in the racemic DL form gave good enantioselectivity when the racemic composition was present in the *C*-terminal α-amino acid (L-Val-DL-Val) but no enantioselectivity when it resided in the *N*-terminal α-amino acid (DL-Val-L-Val) [18]. These results were in agreement with the provision that the *C*-terminal α-amino acid in the dipeptide phase did not contribute to enantioselectivity, however provided a second amide bond required for hydrogen bonding. Therefore Binyamin Feibush substituted the *C*-terminal α-amino acid by an achiral *tert*-butyl group to obtain the novel α-amino acid diamide selector *N*-dodecanoyl-L-valine-*tert*-butylamide **3** (*cf.* Scheme 1) [19]. The elimination of the non-essential polar side group in **2** by replacing it with an apolar alkyl group in **3** [15] led to a unique selector which has been used almost exclusively in the ongoing further development of α-amino acid-based CSPs in enantioselective GC.

The ability of Gil-Av et al. to separate minute quantities of proteinogenic α-amino acid enantiomers coincided with intensive efforts of the Manned Spacecraft Center of NASA to demonstrate the presence of α-amino acids in interstellar space, in meteorites and lunar samples. The NASA invited Gil-Av to Texas, USA, to determine organogenic compounds in lunar samples retrieved by the crew of Apollo 11 from the surface of the Sea of Tranquility. At this time, the results turned out to be negative at the nanogram detectability range [20]. In fact, the search for *racemic* α-amino acids was considered since enantiomerically pure specimens, whenever present on moon, were expected to have undergone radio-racemization.

The historical encounters between scientists engaged in the new field of chiral separation went on as follows. In the laboratory of Albert Zlatkis at the University of Houston, Texas, Emanuel Gil-Av met Ernst Bayer from the University of Tübingen, Germany, who held a Robert A. Welch professorship for his work on enrichment of rare metals such as gold by selective chelating agents [21]. As Bayer was also an established peptide chemist embarking on Merrifield synthetic approaches, he supplied various di- and tripeptides to Gil-Av as potential CSPs in an ensuing co-operation. Co-workers of Bayer from Tübingen, Wolfgang Parr and Wilfried A. König, conducted experiments at the University of Houston [22,23]. König later worked in the area of enantioselective GC at the University of Tübingen [24] and later at the University of Hamburg, Germany [13,25] thereby significantly contributing to the emerging field. König and Nicholson detailed the deactivation procedures for home-made glass capillaries prior to coating with dipeptide CSPs [24]. Derivatization strategies of α-amino acids were discussed by Gil-Av et al. [26] and Parr et al. [27] (*vide infra*). Recommended were *N*-pentafluoropropionyl(PFP)-*O*-2-propyl esters of α-amino acids as suitable derivatives (*vide infra*). Another cooperation of the Weizmann Institute of Science and the University of Tübingen between Emanuel Gil-Av and Volker Schurig dealt with the first use of chiral and nonracemic metal coordination compounds as novel CSPs for the enantioseparation of chiral compounds devoid of hydrogen-

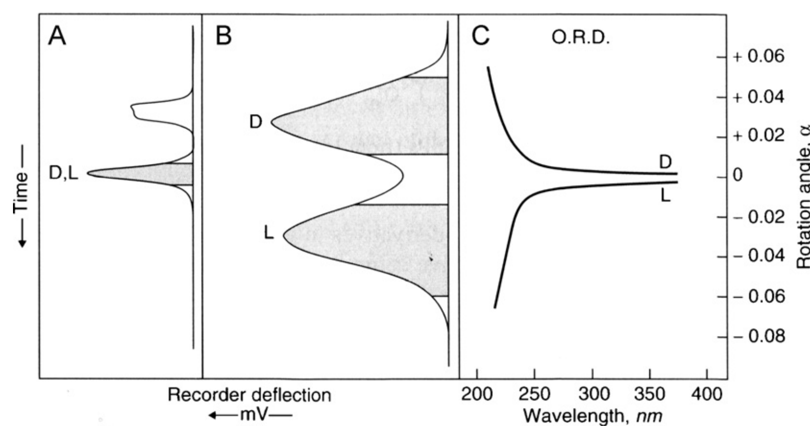


Fig. 4. (a) Gas chromatogram of (impure) racemic *N*-TFA-L,D-alanine-*O*-*tert*-butyl ester (shaded area; white area: impurity) on a 4 m × 6 mm (i.d.) column containing 20% achiral SE-30 on Chromosorb W at 125 °C: no resolution occurs. (b) Gas chromatogram of the collected fraction (shaded area) corresponding to the second peak in (a) on a 2 m × 1 mm (i.d.) column containing 5% *N*-TFA-L-valyl-L-valine-*O*-cyclohexyl ester **2** on Chromosorb W at 100 °C: partial resolution occurs. (c) Optical rotatory dispersion (ORD) diagram of the two collected fractions corresponding to the shaded areas in (b) showing opposite rotation angles.
From Ref. [16] with permission.

bonding properties such as an alkene [28] and oxiranes, thiiranes and aziridines as well as flavours and pheromones [29]. Before the advent of cyclodextrins as versatile CSPs (*vide infra*), complexation GC represented an important complementary approach to enantioseparations by GC without hydrogen-bonding interactions [30]. However, given the overwhelming success of hydrogen-bonding CSPs, complexation GC has never been tried for the enantiomeric analysis of derivatized α-amino acids.

3. Polysiloxane-linked α-amino acid chiral stationary phases—Chirasil-Val

In order to improve the temperature stability of the CSPs, Ôi et al. linked the selectors of Gil-Av et al. to a triazine ring [31]. However, a real breakthrough in enantioselective GC was only achieved in 1977 when Frank, Nicholson and Bayer of the University of Tübingen attached the valine diamide selector of Feibush [19] onto a copolymer consisting of carboxy-alkylmethylsiloxane and dimethylsiloxane units [32–35]. By this approach the very useful coating properties of silicones for producing high-resolution and high-efficiency capillary columns are not only maintained but combined with the inherent enantioselectivity of the chiral diamide selector. The thermally stable and involatile *chira*l poly*sil*oxane containing the *val*ine diamide (see Scheme 1) was termed *Chirasil-Val* **4** (*cf.* Scheme 1). The simultaneous enantiomeric separation of all proteinogenic α-amino acids as *N*(*O*,*S*)-pentafluoropropionyl-*O*-2-propyl esters on a deactivated 20 m × 250 μm i.d. glass capillary column coated with Chirasil-L-Val **4** is depicted in Fig. 5 [34–37]. On Chirasil-L-Val, the L-configurated α-amino acid enantiomers are eluted as the second peak. The GC trace shows some overlap of the α-amino acids *allo*-isoleucine/leucine/glycine and phenylalanine and glutamic acid and not all enantiomeric pairs are separated as well as others. Among the problematic α-amino acids are proline (its secondary amino group is devoid of an amide hydrogen after derivatization) and histidine (an additional derivatization step is required). The trifluoroacetyl- and pentafluoropropionyl ester derivatives of arginine are most sensitive against humidity and active sites in the GC system [13,34]. The derivatives should be stored at −20 °C in anhydrous solvents (*e.g.*, dichloromethane). Duran glass capillaries are preferentially used because deactivation of the inside capillary surface is more straightforward than that of fused silica capillaries [38]. Efficient enantioseparations were also obtained with *N*(*O*,*S*)-trifluoroacetyl-*O*-*n*-propyl esters and *N*(*O*,*S*)-pentafluoro-*O*-2-propyl esters of α-amino acids (*cf.* Fig. 6) [39]. If compared to the rather archaic type of peaks in the first trials of Gil-Av et al. (*cf.* Figs. 2 and 3), this high-resolution enantioseparating approach is truly remarkable. Chirasil-Val **4** has been obtained in the L- *and* D-configuration [40] allowing the switching of the elution order of derivatized L,D-α-amino acid analytes [40–44]. Peak inversion represent an important tool in the validation of the determination of enantiomeric compositions (*vide infra*). In a variant of Chirasil-Val, Abe and Ohtani substituted the *tert*-butyl amide group

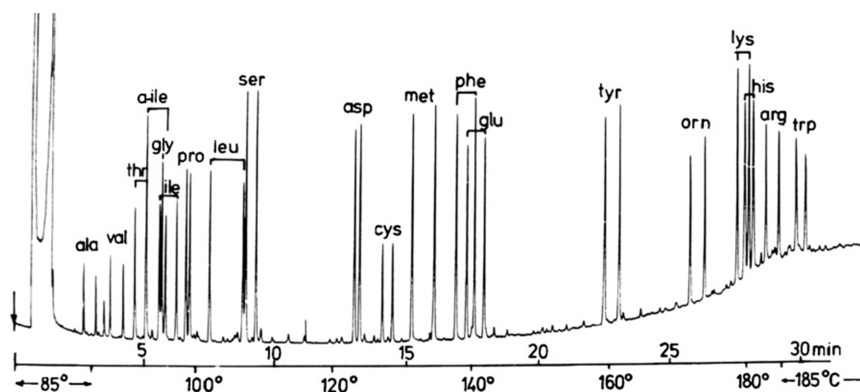


Fig. 5. Enantioseparation of *N*(*O*,*S*)-pentafluoropropionyl α-amino acid *O*-2-propyl esters (histidine as $N^{imidazol}$-ethoxycarbonyl) by HRC-GC on a 20 m × 270 μm i.d. Duran-50-glass capillary coated with Chirasil-L-Val **4** at 85 °C (3 min), 3.8°/min to 185 °C. The first eluted peak corresponds to the D-enantiomer.
From Refs. [34,35] with permission.

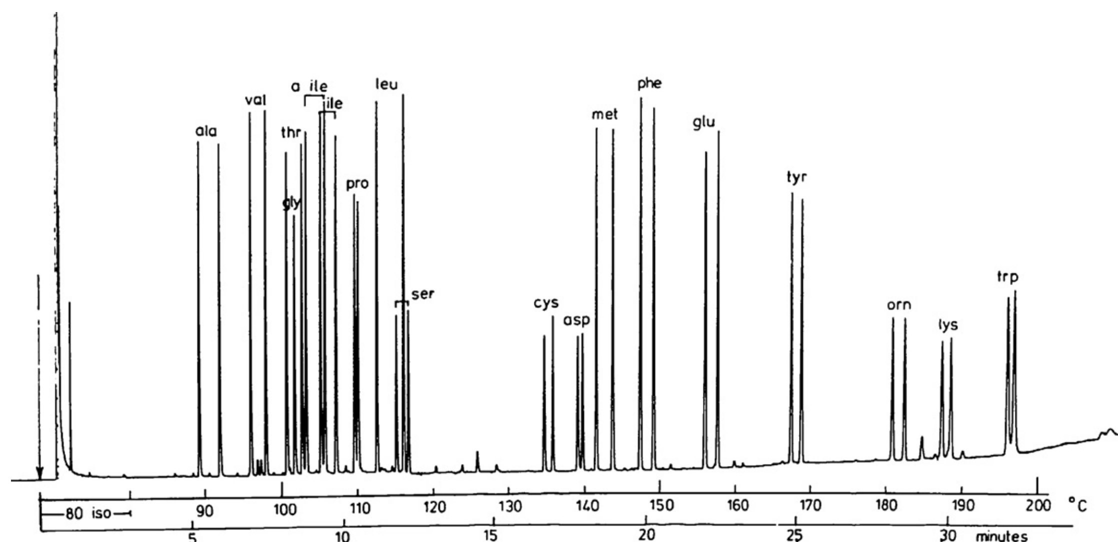


Fig. 6. Enantioseparation of *N*(*O*,*S*)-TFA α-amino acid *O*-*n*-propyl esters by HRC-GC on a 20 m × 250 μm i.d. glass capillary coated with Chirasil-L-Val **4**. The first eluted peak corresponds to the D-enantiomer.
From Ref. [39] with permission.

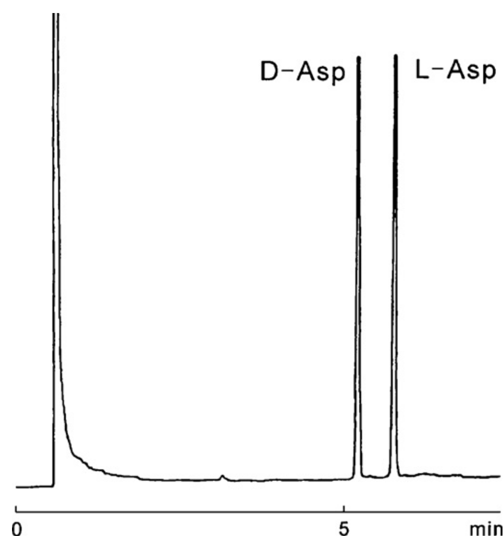


Fig. 7. Enantioseparation of *N*-TFA-aspartic acid-*O*-2-propyl ester by HRC-GC on a 12 m × 250 μm fused silica capillary coated with *S*-(−)-*tert*-leucine-cyclooctylamide anchored to poly(dimethylsiloxane) at 130 °C, isothermal.
From Ref. [45] with permission.

by a cycloalkyl amide group (*e.g.*, cyclooctylamide) and valine by *tert*-leucine (*cf.* Fig. 7) [45].

The pre-treatment of borosilicate glass columns prior to coating has been described in detail [34–36] and the immobilization property of Chirasil-Val **4** on the glass surfaces was also studied [46]. In later work, Chirasil-Val-coated borosilicate glass capillary columns were replaced by flexible fused silica capillary columns which are commercially available from Agilent, USA (formerly by Varian, Inc., USA, taking over the original supplier Chrompack, Middelburg, The Netherlands). A direct straightforward access to polymeric Chirasil-Val-type CSPs is based on the modification of cyanoalkyl-substituted polysiloxanes (XE-60, OV-225) [47,48]. Koppenhoefer et al. modified the chiral backbone in Chirasil-Val by variation of the loading and polarity of the CSP and by the introduction of rigid spacers [49–51]. In Chirasil-Val-C_{11} a long undecamethylene spacer separates the valine diamide selector from the polymeric backbone [52]. In Chirasil-Val **4** the chiral moieties are statistically distributed about the polymer chain. A more ordered Chirasil-type CSP has been obtained by block condensation of 1,5-*bis*-(diethylamino)-hexamethyl-trisiloxane and 2′,2′,2′-trifluoroethyl-(3-dichloromethylsilyl)-2-methylpropionate followed by nucleophilic displacement of the functionalized polysiloxane with chiral amines and amino acids [53–56]. The immobilization of the CSPs by thermal [54] and radical-induced cross-linking [56] has been studied and the extent of radical-induced racemization was determined.

A highly ordered supramolecular structure has been prepared by linking chiral L-valine-*tert*-butylamide moieties to the eight hydroxyl groups of a resorcine[4]arene basked-type structure obtained from resorcinol and 1-undecanal. The calixarene was subsequently chemically linked via four spacer units to a poly(dimethylsiloxane) to afford Chirasil-Calixval [57]. However, the ordered cyclic arrangement of the chiral selectors juxtaposed in close proximity to each other did not improve enantioselectivity as compared to Chirasil-Val **4** and aromatic α-amino acid derivatives did not exhibit any beneficial effect due to supramolecular inclusion. The synthesis of thiacalix[4]arenes with pendant chiral amines and their application as CSP for the enantioseparation of derivatized α-amino acids, alcohols and amines has also been described [58].

4. Chiral stationary phases derived from modified cyclodextrins in (*inter alia*) inclusion-GC

At the outset of the development of enantioselective *inclusion-type* CSPs based on cyclodextrins, a most difficult enantioseparation task was selected which was subsequently extended to a highly successful tool for α-amino acid analysis by enantioselective GC. Since chiral unfunctionalized hydrocarbons are not prone to hydrogen-bonding and metal coordination, they resisted the enantioseparation by Chirasil-Val- and Chirasil-Metal-type CSPs. Gil-Av therefore suggested to consider cyclodextrins as inclusion-type CSPs in enantioselective GC. Since native cyclodextrins have high melting points and are not soluble in organic solvents, they have to be derivatized (alkylated and/or acylated). Young Hwan Kim working with Gil-Av on the enantioseparation of helicenes on nucleosides and nucleotides by liquid chromatography LC [59], came in 1981 from the Weizmann

Institute of Science to the University of Tübingen to conduct enantioselective GC trials using permethylated β-cyclodextrin mixed with poly(dimethylsiloxane) in stainless steel capillaries for the resolution of the unfunctionalized chiral hydrocarbon 2,2,3-trimethylheptane. Unfortunately, the attempt failed at this time although the original selector as used today constitutes one of the most versatile selector system for the separation of enantiomers by GC [60,61] including also the original target, namely the quantitative enantioseparation of chiral unfunctionalized hydrocarbons [62]!

Following the first case of enantioseparation of α- and β-pinene on a packed column coated with native α-cyclodextrin dissolved in formamide in 1983 [63], this methodology was followed up in 1987 by the enantioseparation of various chiral compounds on modified cyclodextrins (alkylated and/or acylated) coated on high-resolution glass capillary columns [64,65]. Whereas König et al. [61,66] and Armstrong et al. [67] employed *n*-pentylated/acylated cyclodextrins (α,β,γ) as *undiluted* liquid stationary phases, Schurig and Nowotny adopted former strategies in complexation gas chromatography [30] and *diluted* alkylated cyclodextrins in moderately polar polysiloxanes (OV-1701) [65,68]. Permethylated β-cyclodextrin was also bonded to a poly(dimethylsiloxane) matrix to provide Chirasil-Dex **5** (*cf.* Scheme 2), commercialized by Agilent, USA (formerly by Varian, Inc., USA, and the original supplier Chrompack, Middelburg, The Netherlands) as a versatile polymeric CSP [69–72]. In the currently state of the art, a host of alkylated/acylated cyclodextrins are either physically *diluted* in polysiloxanes or *bonded* to polysiloxane backbones and then coated on fused silica capillary columns for use in enantioselective HRC-GC [73–75].

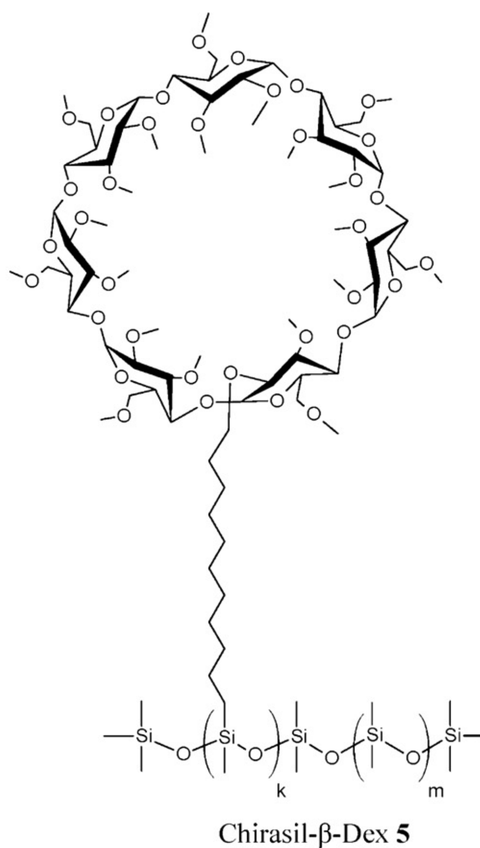
Despite its large molecular cavity, octakis(3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin (Lipodex E) **6** (*cf.* Scheme 2) introduced by König et al. [76] and distributed by Macherey Nagel, Düren, Germany, represents a versatile CSP for the enantioseparation of a multitude of chiral compounds including derivatized α-amino acids [61,76,77]. In the table, the enantioseparation factors α of 36 *N*-TFA-*O*-methyl α-amino acid esters is listed [76]. A gas chromatogram of 11 specimens is depicted in Fig. 8. Whereas the L-configurated enantiomers are always eluted as the second peak, an exception was found for cyclic proline and 3,4-dehydroproline where the L-configurated enantiomers are eluted as the first peak whereby both α-amino acids display a much larger enantioseparation factor α on Lipodex E **6** as compared to Chirasil-Val **4**. In the original work of König et al., undiluted Lipodex E **6** was coated on Pyrex glass capillaries [76,77]. In order to improve the chromatographic performance of undiluted octakis(3-*O*-butanoyl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin (Lipodex E) **6** [76], the selector was (i) dissolved in poly(0.1–0.3%-vinyl-methylsiloxane) PS 255 (*cf.* Scheme 2) or OV-1701 [78] and (ii) chemically linked to poly(dimethylsiloxane) with batches of 20, 40 and 60% (w/w) via an octamethylene spacer yielding a polymeric CSP referred to as Chirasil-γ-Dex **7** (*cf.* Scheme 2) (not commercially available) [78]. By thermal treatment, Chirasil-γ-Dex **7** can be immobilized on fused silica tubings (250 μm i.d.) [78]. A comparison of the selectors Chirasil-Val **4** and Chirasil-γ-Dex **7** for the gas chromatographic enantioseparation of proteinogenic amino acid derivatives has been provided by Schurig et al. [36]. On Chirasil-γ-Dex **7** the *N*-TFA-*O*-ethyl esters of proline and aspartic acid are well enantioseparated in contrast to phenylalanine, tyrosine and notably tryptophan (*cf.* Fig. 9). The mono-*N*-TFA-*O*-ethyl esters of arginine and histidine are not eluted from the column due to surface effects of the capillary. All L-configurated amino acids are eluted as the second peak on Chirasil-γ-Dex **7** except for proline, 3,4-dehydroproline, threonine and *allo*-threonine where the opposite elution order is observed [36]. Chirasil-γ-Dex **7** also enantioseparates the *N*-TFA-*O*-methyl esters of β- and γ-amino acids and of *N*-methyl-α-amino acids [36].

N-TFA-*O*-methyl esters of alanine, valine, aspartic acid, methionine, proline and phenylalanine were also enantioseparated on heptakis(2,6-di-*O*-methyl-3-*O*-*n*-pentyl)-β-cyclodextrin diluted in polysiloxane OV-1701 (1:1) and that of proline, aspartic acid, glutamic acid and phenylalanine on octakis(2,6-di-*O*-methyl-3-*O*-*n*-pentyl)-γ-cyclodextrin dissolved in polysiloxane OV-1701 (1:1) [79]. *N*-TFA-*O*-2-propyl esters of proline, serine, alanine, phenylalanine and cysteine have been enantioseparated on Chirasil-β-Dex **5** and the enantioseparation factors α (1.02–1.06) were always higher as compared to permethylated β-cyclodextrin diluted in polysiloxane OV-1701 [80]. The enantioseparation of different *N*-TFA-*O*-alkyl esters (methyl, ethyl, *n*-propyl, 2-propyl, *n*-butyl, *n*-pentyl, 3-pentyl) of alanine, valine, leucine, 2-aminopentanoic acid and proline on Chirasil-β-Dex **5** were compared with that on heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin diluted in OV-1701 (1:1, w/w) [81]. In a recent comparison of the influence of the cavity size of the cyclodextrin selector on the enantioselectivity toward *N*-TFA-*O*-alkyl esters (methyl, ethyl, 2-propyl) of 19 α-amino acids, a comparison was performed between permethylated Chirasil-α-Dex, Chirasil-β-Dex **5** and permethylated Chirasil-γ-Dex [82]. Whereas permethylated Chirasil-γ-Dex provided only few baseline enantioseparations, permethylated Chirasil-α-Dex and Chirasil-β-Dex **5** exhibited complementary behaviour which may indicate the need of employing the two selectors in one CSP as proposed previously [36]. By mixing the two versatile CD selectors octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin (Lipodex G) [83] and heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in the polysiloxane PS 086, a comprehensive enantioselectivity spectrum for racemic unfunctionalized alkanes, racemic derivatized α-amino acids, lactones, diols, secondary alcohols, ketones and terpenes has been achieved [84].

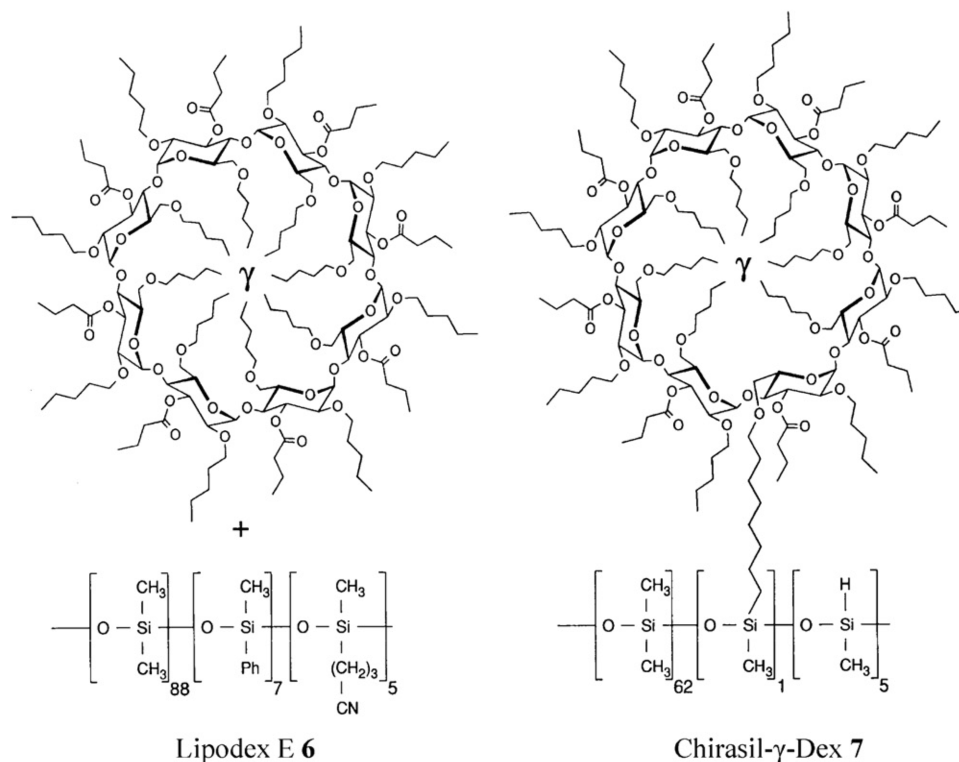
The possibility to enantioseparate derivatized α-amino acids on modified cyclodextrins represents a complementary tool for the validation of enantioselective GC of α-amino acids. The advantage of cyclodextrin CSPs **5**–**7** rests on their high configurational stability as compared to amino acid CPS **1**–**4** which are prone to racemization at high temperatures [36]. A disadvantage of the CSPs **5**–**7** is associated with the fact that the selector is only available in the natural D-form of the glucose buildings blocks preventing the possibility to switch the elution order in contrast to the availability of the L- and D-configurated CSPs **1**–**4**. However, it has been shown that *N*-TFA α-amino acid *O*-methyl esters can also be enantioseparated by GC on acetylated/*tert*-butyldimethylsilylated *linear* dextrins ("acyclodextrins"), including even the simple derivatized glucose monomer [85]. Maltooligosaccharides are readily accessible in both enantiomeric forms (D and L) allowing for peak switching scenarios in enantioseparations.

For the determination of extraterrestrial homochirality, enantioselective GC columns coated with three commercially available CSPs, *i.e.*, Chirasil-Val **4** [32–34], Chirasil-Dex **5** [70,72] and octakis(2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl)-γ-cyclodextrin (G-TA) [86], respectively, are integrated in the COSAC (*cometary sampling and composition*) campaign as part of the payload of the Rosetta mission of ESA. Started in 2004 and on the way to the comet 67P/Churyumov-Gerasimenko, it is landing there in 2014 (*cf.* Fig. 10) [87–90]. Chiral organic molecules like amino acids were detected via laboratory simulations dealing with UV photo- and thermal processing of pre-cometary ice analogs [91]. Photolysis of racemic leucine with circularly polarized synchrotron radiation has been described (ee = 5.2 ± 0.5% for D-leucine) [92].

For future space experiments it will be advantageous to provide mixed CSPs (*vide infra*) consisting of a combination of hydrogen-bonding and inclusion type of selectors in a single column.



Chirasil-β-Dex **5**



Lipodex E **6** Chirasil-γ-Dex **7**

Scheme 2. Cyclodextrin-derived CSPs for the GC enantioseparation of derivatized α-amino acids.

5. Mixed chiral stationary phases comprising diamides and modified cyclodextrins in enantioselective GC

When two different chiral and nonracemic selectors are utilized, their individual contributions to chiral recognition may lead to enhancement ('matched case') or to compensation ('mismatched case') of enantioselectivity [93]. Whereas cyclodextrins are available only in the all-D form, α-amino acid diamide selectors, *e.g.*, Chirasil-Val, can be used in the D′- and in the L′-form [40]. Thus, in principle two 'diastereomeric' systems (DD′ and DL′) may be

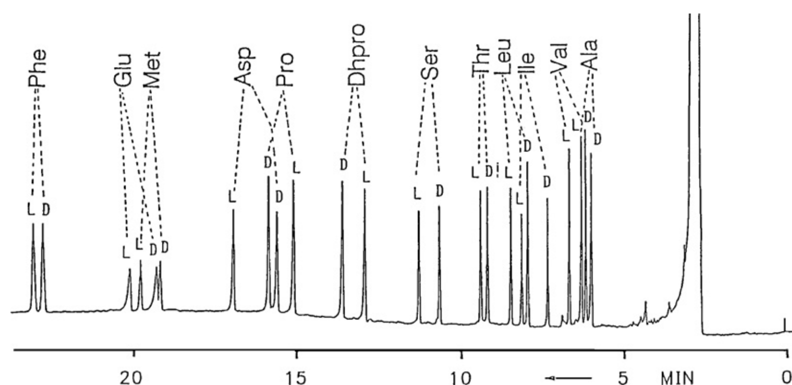


Fig. 8. Enantioseparation of *N*-TFA α-amino acid *O*-methyl esters by HRC-GC on a 50 m × 250 μm i.d. Pyrex glass capillary coated with octakis(3-*O*-butanoyl-2,6-di-*O*-pentyl)-γ-cyclodextrin (Lipodex E) **6** at 120 °C, temperature program 3 °C/min to 160 °C, carrier gas 1 bar dihydrogen.
From Ref. [76] with permission.

employed for optimization. In the absence of cooperative effects, the enantioselectivity obtained on a mixed binary chiral selector system is smaller than that of a single chiral selector system containing the more enantioselective selector. Therefore, at a first glance it may be unfavorable, as inferred by Pirkle and Welch [94], to combine different selectors in one CSP. Yet for practical purposes, the combination of chiral selectors with complementary enantioselectivities toward different classes of chiral compounds in one CSP may result in a broader enantioselectivity spectrum as those provided by either of the single selector CSP. In addition, for chiral analysis of α-amino acids the complementary behaviour of diamide and cyclodextrin selectors can be combined. A comprehensive quantitative analysis of mixed chiral selector systems has been advanced recently [93].

Three different approaches of mixed binary selectors systems in enantioselective GC have been described. (i) The two CSPs Chirasil-Calixval [57] and Chirasil-β-Dex **5** [70,72] were bonded to poly(dimethylsiloxane) to furnish Chirasil-Calixval-Dex [95,96]. (ii) The two CSPs Chirasil-Val-C_{11} and Chirasil-β-Dex-C_{11} were bonded to poly(dimethylsiloxane) to furnish Chirasil-DexVal-C_{11} [52]. On the mixed CSP, both unfunctionalized 1,2-dialkylcycloalkanes and α-amino acids (as *N*-TFA-*O*-ethyl esters) can be enantioseparated simultaneously. This type of mixed selectors system might advantageously be used for classes of compounds, *e.g.*, derivatized β-amino acids [97], for which homologous members are only enantioseparated on either on of the CSP, Chirasil-Val **4** *or* Chirasil-β-Dex **5**, respectively. (iii) Octakis(3-*O*-butanoyl-2,6-di-*O*-pentyl)-γ-cyclodextrin (Lipodex E) [76] **6** was dissolved in the CSP Chirasil-Val-C11 to furnish Chirasil-Val(γ-Dex) [98]. The mixed phase (one CSP doped into another CSP) showed a greatly improved enantioselectivity toward proline and aspartic acid (as *N*-TFA-*O*-ethyl or *O*-methyl esters) compared to Chirasil-Val **4**. Furthermore, the presence of Lipodex E [76] extended the scope of enantioseparations achievable on Chirasil-Val **4** toward underivatized alcohols and many other racemic compounds.

A viable strategy to combine the enantioselectivities of hydrogen-bonding and inclusion type selectors as a single CSP consists of linking L-valine moieties directly to the permethylated β-cyclodextrin selector in similarity to Chirasil-Calixval [57]. Promising results were obtained for a selector that carries a sin-

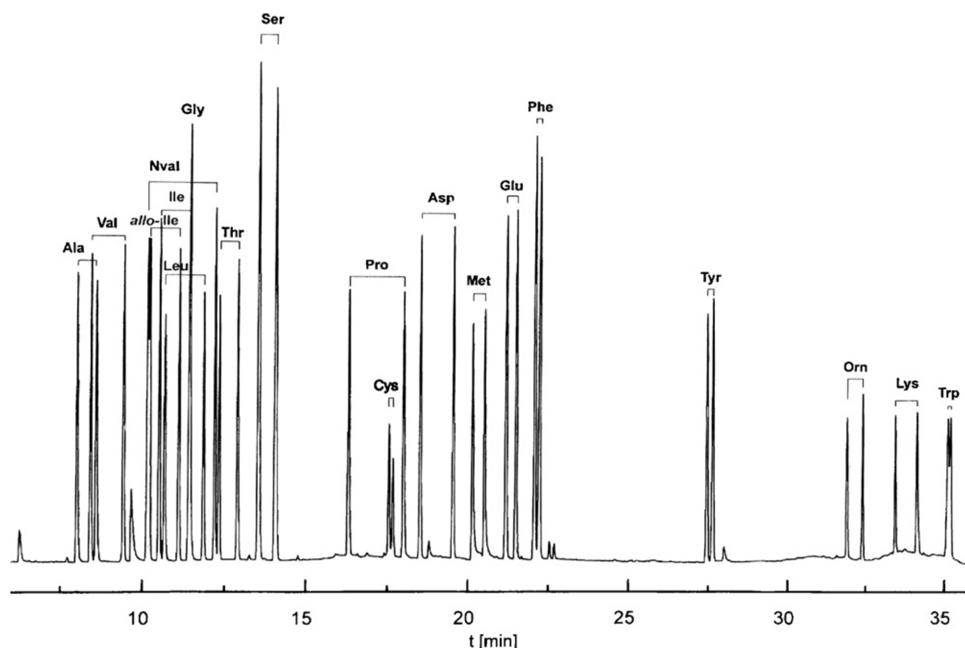


Fig. 9. Enantioseparation of *N*-TFA α-amino acid *O*-ethyl esters by HRC-GC on a 25 m × 250 μm i.d. fused silica capillary coated with Chirasil-γ-Dex **7** at 65 °C (2 min) isothermal, 3.5 °C/min to 180 °C, carrier gas 60 kPa dihydrogen. Arginine and also histidine (which was not subjected to further derivatization at the imidazolylic nitrogen) are not eluted from the column. All L-amino acids are eluted as second peak except for proline and threonine for which the opposite is true [36].

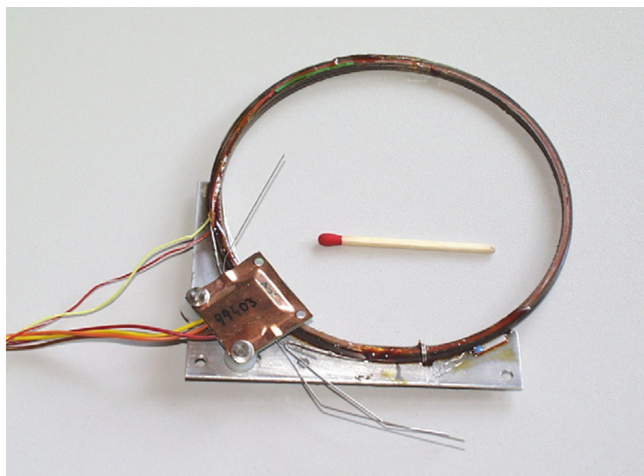


Fig. 10. Prototype of a fused silica capillary column connected to a micro-machined thermal conductivity detector of the COSAC gas chromatography experiment [90]. Courtesy, Prof. U. Meierhenrich.

gle L-valine diamide moiety in the C6-position of permethylated β-cyclodextrin for enantioseparations by GC [99].

6. Thermodynamic parameters of enantioseparation by GC—the isoenantioselective temperature T_{iso}

It is tempting to correlate the absolute configuration of the enantiomers of derivatized α-amino acids (L or D) with their order of elution from a given CSP. Indeed, on the L-valine diamide CSPs **3** and **4**, the L enantiomers of proteinogenic α-amino acids were found to elute as the second peak. By coincidence, this applies also to the cyclodextrin CSPs **6** and **7** with the exception of proline which assumes an opposite elution order. However, it is important to note that a temperature-dependent reversal of the elution order may occur in enantioselective gas chromatography as the result of enthalpy–entropy compensation within the extended temperature-range of operation [100].

The enantioseparation by GC requires a *fast* and *reversible* 1:1 selector-selectand-interaction which is governed by thermodynamics (and not by kinetics). Different stabilities of the transient diastereomeric associates AL and AD, formed between the non-racemic selector A and the selectand enantiomers L and D, is the prerequisite of enantioseparation by GC. Gil-Av and Feibush defined the enantioseparation factor α (referred to as the relative retention r in their work) as the ratio of the retention factors k_i of the enantiomers [101]

$$\alpha = \frac{k_L}{k_D}$$

Thus, the ratio α quantifies the enantioseparation imparted by an *undiluted* chiral selector A on the selectand enantiomers L and D [101] (in case of a *diluted* chiral selector A, a different methodology based on the retention-increment method should be employed [102]). The enantioselectivity, as defined by the Gibbs energy difference $-\Delta_{L,D}(\Delta G)$ between the diastereomeric association complexes is accessible from the enantioseparation factor α via the Gibbs–Helmholtz equation (the symbols L,D merely refer to enantiomers irrespective of the real elution order observed, *vide infra*)

$$-\Delta_{L,D}(\Delta G) = -\Delta_{L,D}(\Delta H) + T\Delta_{L,D}(\Delta S) = RT \ln \alpha$$

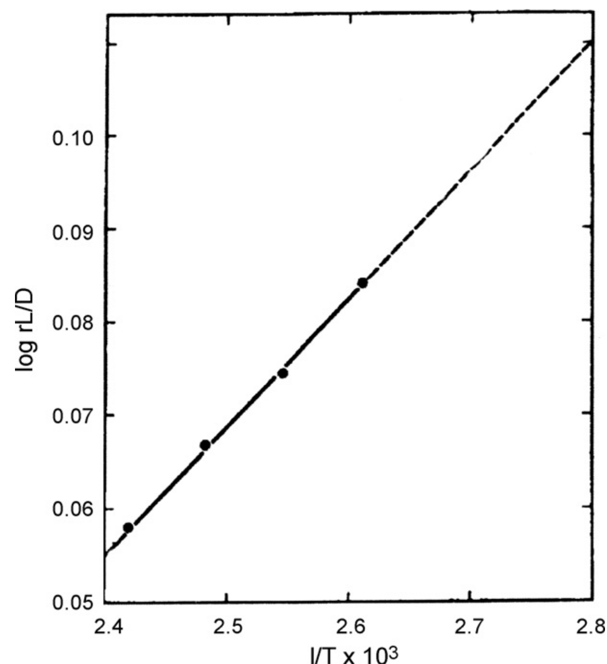


Fig. 11. Plot of the decadic logarithm of the enantioseparation factor α (referred to as log $r_{L/D}$) of racemic *N*-TFA-L,D-alanine-*O*-*tert*-butyl ester on the dipeptide *N*-TFA-L-valyl-L-valine-*O*-isopropyl ester vs. the inverse of the absolute temperature. From Ref. [101] with permission.

where R is the gas constant and T is the absolute temperature. From the Van't Hoff relation

$$\ln \alpha = \frac{-\Delta_{L,D}(\Delta H)}{RT} + \frac{\Delta_{L,D}(\Delta S)}{R}$$

the enthalpy difference $\Delta_{L,D}(\Delta H)$ and entropy difference $\Delta_{L,D}(\Delta S)$ for the diastereomeric association complexes can be obtained. From the linear plot of log α vs. $1/T$ (*cf.* Fig. 11), Gil-Av and Feibush determined $\Delta_{L,D}(\Delta H) = -0.63$ kcal/mol and $\Delta_{L,D}(\Delta S) = -1.22$ cal/mol K between 110 and 140 °C for racemic *N*-TFA-alanine-*O*-*tert*-butyl ester enantioseparated on the dipeptide *N*-TFA-L-valyl-L-valine-*O*-2-propyl ester [101]. As $\Delta_{L,D}(\Delta H)$ and $\Delta_{L,D}(\Delta S)$ have the same (negative) sign for a 1:1 association process (the more stable associate is also more ordered) the enantioseparation should vanish at the *isoenantioselective temperature* T_{iso} due to enthalpy/entropy compensation according to the Gibbs–Helmholtz equation

$$T_{iso} = \frac{\Delta_{L,D}(\Delta H)}{\Delta_{L,D}(\Delta S)} \quad \text{for} \quad \Delta_{L,D}(\Delta G) = 0 \quad \text{and} \quad \alpha = 1$$

At T_{iso} peak coalescence occurs and the elution order of the enantiomers above and below T_{iso} is opposite, causing a temperature-dependent peak reversal.

The existence of the isoenantioselective temperature T_{iso} in enantioselective gas chromatography was predicted by Koppenhoefer and Bayer [103] and by Schurig and Link [104]. In 1989 Gil-Av et al. observed the first case of enthalpy/entropy compensation in a hydrogen-bonding GC system [105]. The Van't-Hoff plots for *N*-TFA-*tert*-butylamides of alanine, 2-aminobutyric acid, leucine and valine enantioseparated on *N*-docosanoyl-L-leucine-(1,1,3,3-tetramethylbutyl)amide (*cf.* Fig. 12) show a linear branch between 110 and 220 °C which crosses the $\ln \alpha = 0$ line at T_{iso} (130–150 °C) causing an inversion of the elution order in agreement with the Gibbs–Helmholtz equation [105]. The nonlinear Van't Hoff plot above T_{iso} was ascribed to the fact that the thermodynamic parameters may not remain constant at higher temperatures and/or by the occurrence of multimodal enantioseparation mechanisms [105].

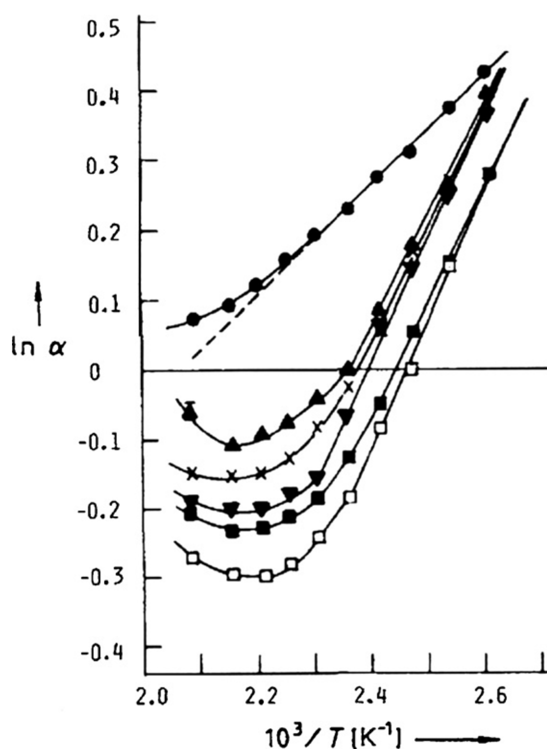


Fig. 12. Plot of the natural logarithm of the enantioseparation factor α of racemic *N*-TFA-*tert*-butylamides of ● = proline, ▲ = alanine, **x** = 2-aminobutyric acid, ▼ = leucine, ■ = valine and □ = *N*-acetylvaline *tert*-butylamide on *N*-docosanoyl-L-leucine-(1,1,3,3-tetramethylbutyl)amide vs. the inverse of the absolute temperature. From Ref. [105] with permission.

Derivatized proline, containing a secondary amide moiety does not show peak inversion (*cf.* Fig. 11).

At the same time, published side-by-side in *Angew. Chem. Int. Ed.*, Schurig et al. found another evidence of a temperature-dependent reversal of the elution order for a chiral spiroketal on a nonracemic nickel(II)-chelate in complexation GC characterized by a very low value for T_{iso} [106]. It is intriguing to realize that above T_{iso} preferential recognition of one enantiomer is not caused by stronger binding to the selector but is due to the higher degree of disorder of the association complex [106]. A temperature-induced inversion of the elution order in enantioselective GC has also been observed for *N*-ethoxycarbonyl propylamides (ECPA) [107] of α-amino acids on Chirasil-L-Val-C_{11} [102]. The change of the elution order and the increase of the enantioseparation factor α when the temperature is raised above T_{iso} is vividly evident from Fig. 13 for the enantioseparation of alanine and valine ECPA derivatives on Chirasil-L-Val-C_{11} [102]. It was concluded that in enantioselective GC of α-amino acids, temperature-dependent studies are required in order to optimize the enantioseparation of single racemates by isothermal operation or of mixtures of racemates in temperature-programmed runs [102]. The mere consideration of the elution order of enantiomers and the value of the enantioseparation factor α at a given temperature, without considering their dependence on the temperature, can lead to pitfalls in the determination of absolute configurations and in the elucidation of enantiorecognition mechanisms. This holds true also for cyclodextrin-derived CSPs.

Indeed, *N*-TFA-valine-*O*-*n*-butyl ester showed a temperature-dependent inversion of the elution order on Chirasil-β-Dex **5** [81]. Temperature-induced inversions of the elution order have also been observed for *N*-TFA α-amino acid *O*-ethyl esters on Chirasil-β-Dex **5** [102]. Thus the derivatives of valine and leucine showed an inversion of the elution order below and above T_{iso} = 70 °C. Only a single peak was observed at the coalescence temperature T_{iso}. For the isoleucine derivative, the isoenantioselective temperature was as low as T_{iso} = 30 °C [102].

Most gas chromatographic enantioseparations of derivatized α-amino acids on CSPs **1**–**7** are governed by the enthalpy term of the Gibbs–Helmholtz equation. Consequently, enantioselectivity increases by reducing the elution temperature. As involatile racemates usually require high elution temperatures, it is recommended to use short columns (1–5 m × 250 μm i.d.). The loss of efficiency due to the smaller theoretical plate number *N* of short columns is often compensated by the gain of enantioselectivity due to the increased enantioseparation factor α at the low elution temperature. An extensive thermodynamic study of the enantiomers of *N*-TFA-nipecotic acid *O*-alkyl esters (derived from a cyclic β-amino acid) on Chirasil-β-Dex **5** did not show a reversal of the elution order up to 140 °C [108].

The high resolution power of capillary GC enables the quantitative enantioseparation even for racemates with enantioseparation factors of less than α = 1.02, corresponding to $\Delta_{L,D}(\Delta G)$ as little as −0.015 kcal/mol at 100 °C. Therefore, attempts to rationalize chirality recognition by molecular modeling studies are discouraged below α = 1.5.

7. Selected applications

While the first trials to directly enantioseparate racemic compounds by GC on chiral stationary phases was rather a matter of scientific curiosity and academic challenge [2], the unique possibility to determine precise enantiomeric compositions of α-amino acids by the new method was subsequently utilized by Gil-Av and others for many applications. Thus, the absolute configuration of α-amino acid components (L or D) have been investigated in peptides, biological fluids, extraterrestrial material, sediments and soil [2,11,13,20,37,109–111]. Enantioselective GC employing Chirasil-Val **4** and Chirasil-β-Dex **5** has also widely been used by Pizzarello et al. to determine molecular asymmetry in interstellar materials such as carbonaceous chondrites (*e.g.*, the Murcheson and Murray meteorites) [112,113] (*cf.* Refs. in [114]). The configuration of α-amino acids in hydrolysates of peptide antibiotics have been determined via comparative retention time measurements on packed columns by the Gil-Av method [115–117], on the polymeric phase XE-60-L-valine-(*R*)-α-phenylethylamide polymer of König et al. [118] and on Chirasil-Val **4** [42,119–122]. It was shown on a Chirasil-Val-coated glass capillary column that isovaline in natural peptide antibiotics possesses either the D- or L-configuration or contains even both enantiomers in the same peptide [119–122]. The previous assignment of L-isovaline in peptide antibiotics [116,117] has been revised to D-isovaline [119]. Furthermore, D-α-amino acids have been determined by enantioselective GC in bacterial cell walls and in peptide antibiotics [119–122] and in many foodstuff and beverages as well as vegetables, fruits and plant saps and syrups [123–131]. The L-configuration of ornithine in a heptapeptide isolated from a ferrichrome-type siderophore has been assigned by GC on Chirasil-Val **4** [132]. Likewise, in insect chemistry, the L-configuration of all amino acids identified in the venom of the common wasp *Paravespula vulgaris* was established by GC on Chirasil-Val **4** [133]. 2,4- and 2,3-diamino-3-methylbutanoic acid, which contain two stereogenic centers, have been detected in roots of *Lotus tenuis* and their absolute configuration was assigned by GC on Chirasil-Val **4** [134,135].

An important application of enantioselective GC constitutes the determination of enantiomeric excesses (*ee*) in asymmetric synthesis and asymmetric catalysis [136]. In the chiral rhodium(I)-complex-catalyzed homogeneous hydrogenation [137] of the

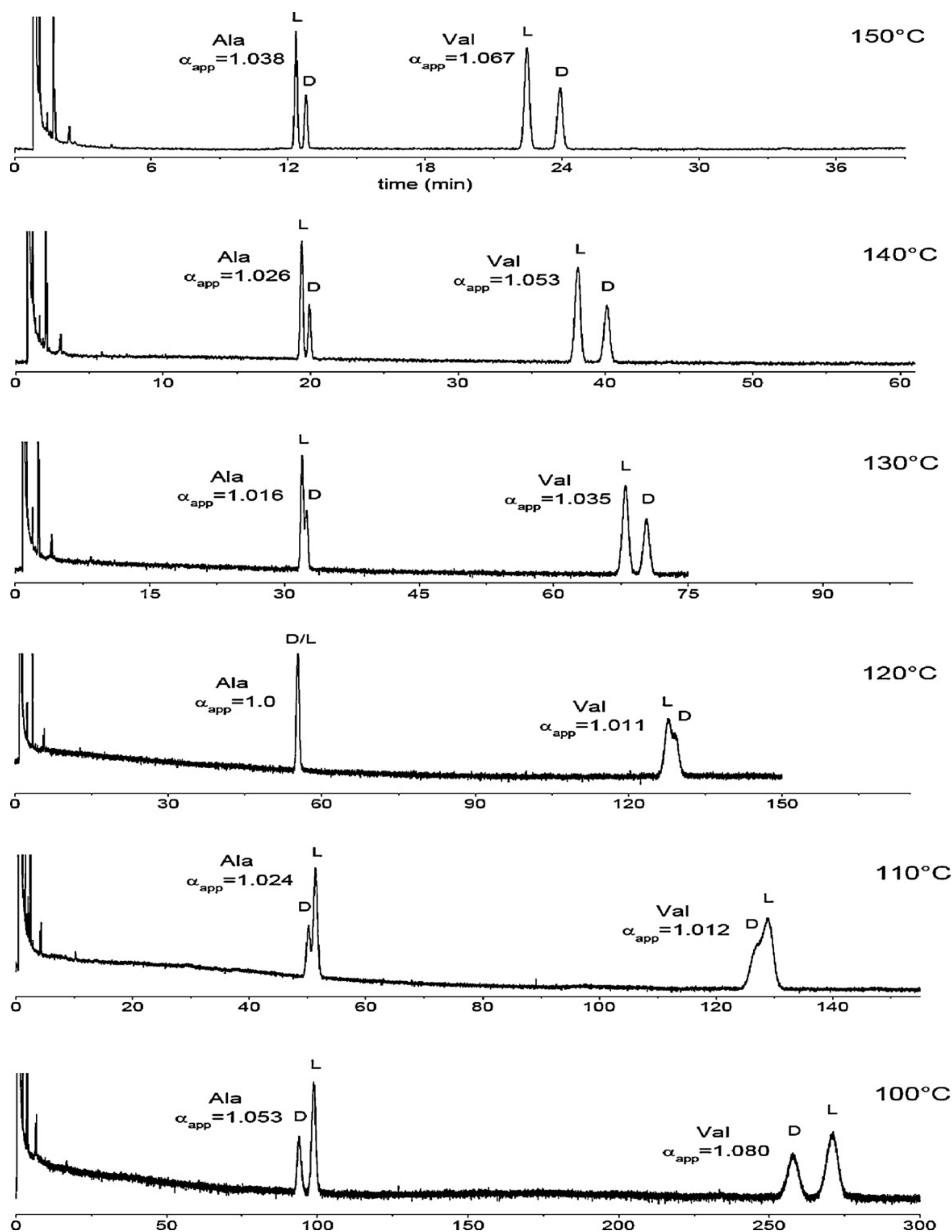


Fig. 13. Temperature-dependent reversal of the elution order of the enantiomer of alanine-ECPA and valine-ECPA (ECPA = *N*-ethoxycarbonyl *n*-propylamide [107]) on Chirasil-L-Val-C_{11}; the α-amino acids are enriched with the L-enantiomer. Isoenantioselective temperatures, T_{iso}, for alanine-ECPA and valine-ECPA are 120 and 114 °C, respectively. Column: fused silica, 20 m × 250 μm i.d. × 0.25 μm (polymer thickness); carrier gas: H_2; head pressure: 50 kPa (120–170 °C) and 100 kPa (100–110 °C); detector: FID. Time scale in minutes.
From Ref. [102] with permission.

corresponding dehydro-α-amino acids to optically active *N*-acetyl-alanine and *N*-acetyl-phenylalanine, the CSP L-*N*-behenyl-valine *tert*-butylamide, coated on Chromosorb, has been used [138]. L-α-Amino acids belong to the 'chirality pool' and utilized preferentially as versatile educts for the transformation to optically active target molecules. In the formation of *R*-oxiranes from *S*(L)-α-amino acids via *R*-α-chloro-carboxylic acids, the enantiomeric purity of the educts have been checked by GC on Chirasil-Val **4** [139]. The enantiomeric purity of the chiral reagents *R*- and *S*-1-amino-2-methoxymethylpyrrolidine (RAMP and SAMP) obtained from *R*- and *S*-proline were determined by enantioselective GC on Chirasil-Val **4** [140] and on an XE-60-L-valine-(*S*)-α-phenylethylamide polymer of König et al. [141]. Many applications are related to the topic of *α-amino acids and the asymmetry of life* [142], in

astrobiology [143,144] and investigations related to the origin of homochirality involving the validation of minute but important deviations from the true racemic composition (*vide infra*) [145].

8. Enantioselective gas chromatography/mass spectrometry (enantio-GC–MS)

When high sensitivity and analyte identification is required, the coupling of gas chromatography with mass spectrometry (GC–MS) is the method of choice. Thus Frank et al. determined L-DOPA [146] and *N*-acetyl-L-cysteine [147] in body fluids. Mass spectrometric detection and formation of D-α-amino acids in processed plant-derived beverages was performed by enantioselective GC on Chirasil-Val [148]. The mass spectrometric assignment of absolute configuration (L and D) of *N*(*O*,*S*)-perfluoroacyl-amino acid *O*-alkyl esters in pyoverdins (iron chelating chromopeptides of *Pseudomonas*) has been performed after GC enantioseparation on Chirasil-L-Val [149].

GC–MS in the selected ion monitoring mode (GC–MS(SIM)) [92] increases detection limits at the picogram range [150]. The mass fragmentogram of the *N*-hexafluorobutyryl-*O*-isobutyl ester of alanine in a hydrolysate of *streptococci* cells revealed the presence of D-alanine (10% enantiomeric composition, D/D + L) [150]. Analysis of D-amino acids as *N*-trifluoroacetyl-*O*-2-propyl esters in rumen bacteria isolated from sheep was performed on a fused silica capillary column coated with Chirasil-L-Val **4** [151]. Likewise, the determination of D-amino acids as *N*(*O*)-pentafluoropropionyl-*O*-2-propyl esters in body fluids of higher vertebrates on Chirasil-Val **4** by GC–MS(SIM) established that D-amino acids are widespread in mammals [152,153]. Nowadays, D-amino acids are not any more considered as unnatural specimens as they are part of physiological fluids and are fulfilling discrete biological functions. Furthermore, many peptides are known to contain D-amino acids as the result of post-translational, enzymatic modifications [154,155]. Their identification through enantioselective GC is therefore of paramount importance.

9. Enantioselective gas chromatography/thermal conversion-isotope ratio mass spectrometry (enantio-GC–TC-IRMS)

The authenticity of natural compounds can be evaluated by the determination of their enantiomeric purity. However, fortification of genuine biological matrices with artificial single enantiomers cannot be recognized by this means. Mosandl et al. therefore used enantioselective *gas chromatography/thermal conversion-isotope ratio mass spectrometry* (enantio-GC–TC-IRMS) to differentiate between true natural and synthetic enantiomers by virtue of subtle differences of their isotopic ratios due to kinetic isotopic effects operating in various synthetic pathways [156]. Thus, the enantiomers eluted from the GC column are burned in a pyrolysis interface and the $^{18}O/^{16}O$ and/or $^{13}C/^{12}C$ ratio of the carbon dioxide formed is then determined by mass spectrometry. This method has been used to differentiate genuine extraterrestrial amino acids from terrestrial contaminants [2] by determining the isotopic signature of amino acids found in the Murchison and Murray meteorites. An example is provided by the determination of the $^{13}C/^{12}C$ ratio and $^{2}H/^{1}H$ ratio of nonracemic isovaline (as *N*-TFA-*O*-2-propyl ester) enantioseparated on Chirasil-L-Val **4** and Chirasil-β-Dex **5**, respectively [157,158]. The analysis of the isotopic pattern of amino acids from fungal peptaibiotics using Chirasil-Val **4** was performed to assess their potential for meteorite contamination [159].

10. Racemization studies of α-amino acids

It was assumed for a long time that the enantiomeric purity of cell constituents in long-lived organisms would decrease with age due to the possibility of molecular racemization. However, only in 1975 the first supporting evidence was provided by demonstrating the age dependent accumulation of D-aspartic acid in enamel [160]. The racemization of α-amino acids (and the epimerization of peptides) requires modern methods of enantiomeric analysis and it resulted in a widely discussed topic in the literature [161–167]. The time-dependent racemization of α-amino acids is of great interest for (i) dating of fossils and archeological artifacts by determining the D/L ratio of α-amino acids (amino stratigraphy), especially those present in metabolically inert matrices such as tooth enamel or in collagen, *e.g.*, aspartic acid (*cf.* Fig. 7) (which is one of the fastest racemizing α-amino acid [45,167]) and (ii) for the search of the origins of homochirality as a pre-condition of life [142–144,168]. A pioneering archeometric investigation by Gil-Av et al. dealt with the determination of the L,D-aspartic acid ratio in parchment from the famous Dead Sea Scrolls, which provided information concerning the date of the degradation of collagen to gelatin [169]. It should be mentioned, however, that processing of collagen may already cause severe racemization [170].

It has been suggested to use enantioselective GC/MS for the study of radio-racemization of α-amino acids by ionizing radiation in outer-space missions [168]. Hereby CSPs based on cyclodextrins **5**–**7** are to be preferred as Chirasil-Val **4** is itself is prone to radio-racemization. By exposing DL-alanine and DL-valine crystals to temperatures ranging from 300 to 100 K, racemization could not be detected using a Chirasil-Val-coated capillary column thereby failing to verify the predicted D–L phase transition induced by the parity violating energy difference (PVED) [171].

The use of Chirasil-Val **4** has been described for studying α-amino acid racemization during refining of processed foodstuffs such as soy-flour and casein [172]. An attempt to correlate the amount of D-amino acids with the bottling age of wines inferred earlier [173] has been reinvestigated recently [174]. It has been suggested that the presence of D-α-amino acids in processed food can serve as an indication for 'overprocessed food' and for 'good manufacturing practice' [175].

Meienhofer advocated the use of Chirasil-Val **4** to quantify potential racemization during coupling of α-amino acids in peptide synthesis [176]. The racemization of chiral peptide nucleic acid (PNA) monomers during solid-phase synthesis and the effect of the coupling conditions on the enantiomeric purity have been investigated by GC–MS on a Chirasil-Val-coated capillary column [177].

Previously, the degree of racemization during peptide synthesis has already been estimated after hydrolysis and the important differentiation between the inversion of configuration of free and bonded α-amino acids was made [178–181]. Thus the racemization of α-amino acids during the acid-catalyzed hydrolysis of peptides can bias the true enantiomeric composition of the building blocks of a peptide [11,37]. This problem can be overcome by performing the hydrolysis in a fully deuterated medium, *e.g.*, in 6 N $^{2}H_2O/^{2}HCl$ [178–181]. Racemization of α-amino acids during acidic hydrolysis involves deprotonation/protonation at the α-C position. If hydrolysis is carried out in a fully deuterated environment, the racemate formed is deuterium-labelled at the α-C position with a consequent shift upward of one mass unit of all fragments containing this moiety. The proportion of D-amino acids originally present in the peptide is thus represented by the relative amount of the unlabelled form. However, three prerequisites must be fulfilled [181]: (i) the ion selected for monitoring $(I)^+$ includes the α-H, (ii) the ion $[I-1]^+$ should be of low intensity (less than 5%) relative to the monitored ion $[I+1]^+$ since incorporation of deuterium would lead to its being

detected together with the unlabelled ion $(I)^+$ resulting in a positive error and (iii) the interference from neighboring ions $[I-1]^+$ must be taken into account. Liardon et al. accounted for these issues by deriving a series of equations [182]. As a practical example, sample preparation is performed as follows [183,184]: 100 nmol of a mixture of α-amino acids (in peptides or proteinogenic matrices) is hydrolyzed with 300 μl 6 N ^{2}HCl in ^2H_2O for 24 h at 110 °C. After evaporation to dryness by a stream of dinitrogen, the sample is first esterified with 350 μl 2 N $^2HCl/CH_3O^2H$ for 15 min at 110 °C. After cooling the vial is opened and the reagent is evaporated with a gentle stream of dinitrogen at ambient temperature. The residue is dissolved in 250 μl trifluoroacetic anhydride and the vial is tightly closed and heated for 10 min to 130 °C. The acylation reagent containing the amino acid derivatives is decanted off and the excess of acylation agent is evaporated. The residue containing the amino acid derivatives is dissolved in 150 μl toluene and analyzed by enantioselective GC–MS(SIM) using a deactivated glass capillary column (or fused silica column) coated with a suitable CSP (*e.g.*, Chirasil-Val **4**, Lipodex E **6** or Chirasil-γ-Dex **7**) (*cf.* Table 2 in [183]). The reliable determination of enantiomeric purities of L-α-amino acids in peptides up to 99.9% is thus possible [182] and the method has also been used to determine the rate constants of configurational inversion at the stereogenic center (enantiomerization) of α-amino acids under acid hydrolysis conditions (110 °C, 6 N DCl) [182]. Using selected ions and deuterated derivatization reagents, a simpler version [185] of the method described previously [182] was proposed and its automation implemented [186]. In a comprehensive effort, the method was validated for all proteinogenic and several non-proteinogenic α-amino acids [185]. Deuteration and enantiomer labelling (*vide infra*) would represent further refinements of the method.

11. The method of enantiomer labelling

The amount of an L-amino acid in a sample can be extrapolated from the change of the enantiomeric ratio after addition of a *known amount* of the oppositely configurated D-amino acid (or the DL-racemate) used as an ideal *internal standard*. This method, termed *enantiomer labelling* [187–191], is intriguing because enantiomers possess identical (non-chiroptical) properties in an achiral environment and therefore the enantiomeric composition is not influenced by sample manipulation (isolation, derivatization, fractionation) or by chromatographic manipulations (dilution, injection, detection). Not even thermal or catalytic decomposition, losses, or incomplete isolation pose any effect on the analytical result. The method relies on the absence of self-recognition between enantiomers undergoing molecular association in concentrated nonracemic mixtures (the *EE*-effect) [192] and the linearity of detector response in respect to the concentration of both enantiomers. The amount of a particular amino acid (X_a) present in the sample is calculated from the ratio of the peak areas of the L-enantiomer (A_L) and the enantiomeric D-standard (A_D) multiplied by the amount of the D-standard added (m_D) [188–190]: $X_a = m_D \cdot (A_L/A_D)$. The method can also be used for amino acid samples and standards possessing incomplete enantiomeric purities including racemic compositions. Substance specific calibration factors need not be considered by the enantiomer labelling method [181]. The method of enantiomer labelling requires the precise knowledge of the enantiomeric ratios of the sample and the standard, preferentially being obtained by the same enantioselective GC method. The calculation of the amino acid concentration in a sample after addition of the standard is performed as follows [181]:

$$X_a = m_D \cdot \left[\frac{(A_L - A_D \cdot C_D + A_L \cdot C_L - A_D \cdot C_L \cdot C_D)}{(A_D - A_L \cdot C_L + A_D \cdot C_D - A_L \cdot C_L \cdot C_D)} \right]$$

where A_L = peak area of the L enantiomer after addition of the standard, A_D = peak area of the D enantiomer after addition of the standard, C_L = enantiomeric ratio D/L of the sample, C_D = enantiomeric ratio L/D of the standard, m_D = amount of standard amino acid added, X_a = amount of amino acid being determined.

Enantiomer labelling of α-amino acids in small volumes of blood and the combination of enantiomer labelling and nitrogen-selective detection, respectively, were described by Frank et al. [193,194]. The combination of enantiomeric labelling and isotopic labelling has also been carried out with GC–MS-SIM [195,196].

Problems of the method could be related to acid-catalyzed background racemization of peptides/proteins that have to be considered in blanks. Moreover, many natural products and physiological fluids contain relatively high amounts of D-amino acids rendering the methods in some cases unreliable.

12. Precision and accuracy in enantioselective gas chromatography of α-amino acid derivatives

Precision, accuracy and practical hints in enantioselective GC have been mentioned in former accounts [13,136]. In a quantitative GC analysis of leucine enantiomers of varying enantiomeric compositions (involving two indirect methods and one direct method), Bonner et al. inferred that each method provides comparable accuracy (0.03–0.70% absolute error) and precision (0.03–0.80% standard deviation). The same authors presented evidence for the absence of racemization during derivatization of leucine to *N*-TFA-*O*-alkyl esters [197]. Since these previous investigations were performed under non-optimal GC conditions, a higher precision and accuracy has been achieved subsequently by using capillary columns coated with Chirasil-L-Val **4** and Chirasil-D-Val [40].

For a given enantioselective GC system, the required validation comprises the determination of the following parameters for each α-amino acid: precision (repeatability), intermediate precision, linearity and accuracy, limit of quantitation (LOQ) and limit of detection (LOD) [181,185]. Enantioselective α-amino acid analyses are especially important in two borderline cases: (i) determination of high enantiomeric purities and (ii) minute deviations from the true racemic composition.

(i) The enantiomeric purity of commercially available α-amino acids has been determined as *N*-TFA-*O*-methyl esters by GC on Chirasil-Val **4** down to 0.01% before and after recrystallization [198]. Samples containing L-alanine in excess were measured on capillaries coated with Chirasil-L-Val, and samples with D in excess on Chirasil-D-Val, respectively, in order to make sure that the minor peak is eluted in front of the major peak (which usually involves a tailing edge) [198]. The trace enantiomeric analysis of leucine (as *N*-TFA-*O*-methyl ester) determined on Chirasil-L-Val and Chirasil-D-Val is depicted in Fig. 14. The use of oppositely configurated CSPs is important to validate the obtained result and to eliminate errors which may arise from the presence of impurities accidentally co-eluting with the sample peaks [30,40]. Since cyclodextrin CSPs **5**–**7** are based on D-glucose, the important peak switching strategy cannot be applied. The determination of an enantiomeric ratio of 1:10 000 requires a linear range of the detection and integrating system. The flame ionization detector possesses a large linear range of 1:10^7 [13]. It is generally accepted that enantioselective GC is capable of reliably determining 0.1% of the unnatural enantiomer (*ee* = 99.8%). For certain α-amino acids such as cysteine this value may increase to 0.3% for certain matrices [181].

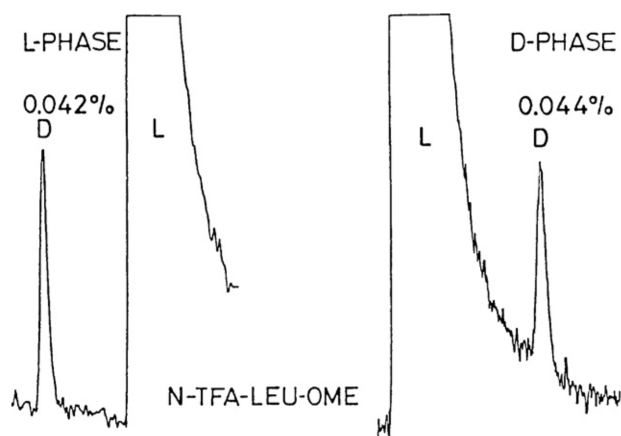
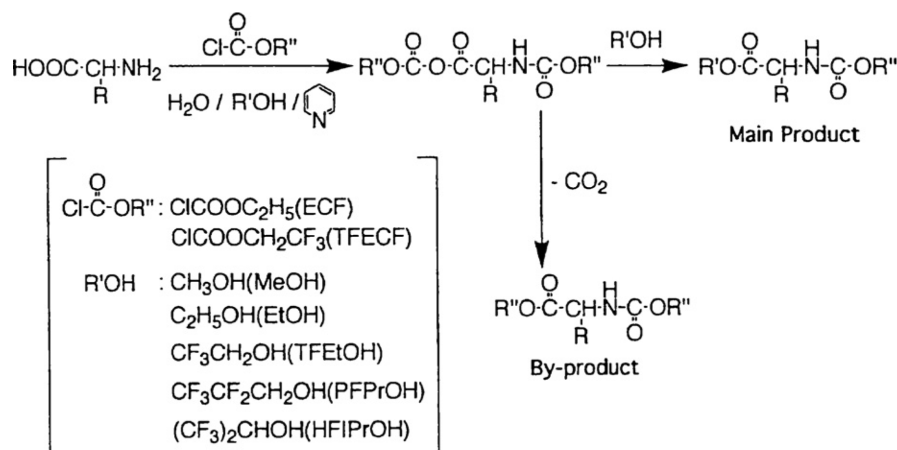


Fig. 14. Trace enantiomeric analysis of leucine (as the *N*-TFA-*O*-methyl ester) on Chirasil-L-Val and Chirasil-D-Val (20 m × 250 μm i.d. glass capillary, 95 °C, 0.3 bar H_2).
Courtesy Prof. B. Koppenhoefer, Habilitation Thesis, 1989, University Tübingen, p. 148.

(ii) The racemic composition of a chiral compound represents an ideal probe for the accuracy of integration devices because an unequivocal 1:1 ratio should be measured as statistical fluctuations of enantiomeric composition are well beyond detectability. In a comprehensive analytical study relevant to experiments mimicking the amplification of homochirality under prebiotic conditions, it was implied that the limit in determining minute deviations from the true racemic composition of target compounds by enantioselective gas chromatography, *e.g.*, of DL-α-amino acids, is generally in the range of 50.0 ± 0.1% [145]. This corresponds to an apparent *ee* of 0.2% (*ee* = (L − D)/(L + D). In order to attain such accuracy *and* precision, a number of requirements must be met, among them an enantioseparation with a resolution factor $R_s > 1.5$, the exclusion of co-eluting impurities, the absence of decomposition of the sample (or its derivative) during the gas 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, *e.g.*, Chirasil-L-Val **4** and Chirasil-D-Val [114]. Claims in the literature of dramatic differences in the growth and dissolution properties of D- and L-tyrosine crystals due to the parity violating energy difference (PVED) could not be confirmed in own crystallization experiments and using a validated GC method for *N*,*O*-TFA-*O*-ethyl esters of tyrosine enantioseparated on Chirasil-L-Val **4** and Chirasil-D-Val [199]. Careful validation employing different CSPs is also recommended in studies on enantioselective circularly polarized irradiation of racemic α-amino acids [92].

13. Derivatization strategies

Derivatization of α-amino acids is required to increase volatility, speed of analysis and good peak shapes, as well as to provide suitable functions for detection, hyphenation and improved chirality recognition. The derivatization strategy should also assist the simultaneous enantioseparation of α-amino acids without extensive peak overlapping [36]. At the outset of enantioselective GC (*cf.* Figs. 3–5), Gil-Av et al. used a two-step derivatization strategy for α-amino acids [4,9,15] consisting of the formation of *N*-perfluoroacyl-*O*-alkyl esters [26,27] which proceeds without racemization at ambient temperature [197]. This two-step derivatization strategy has also frequently been used for achiral GC–MS analyses of α-amino acids [200–207]. For Chirasil-Val **4** [32,34] and related selectors [45], *N*-trifluoroacetyl-*O*-methyl (or *O*-*n*-pentyl and *O*-2-propyl) esters and *N*-pentafluoropropionyl-*O*-2-propyl esters of α-amino acids are routinely employed. One of the inventors of Chirasil-Val **4** used *N*-TFA-*O*-*n*-propyl esters but switched later to *N*-TFA-*O*-ethyl esters of α-amino acids [38] as commercially available trifluoroacetic acid is significantly cheaper and purer than pentafluoropropionic acid. The resolution factors R_s of eight representative α-amino acid derivatives as a function of the *N*-perfluoroacyl group (*i.e.*, trifluoroacetyl (TFA), pentafluoropropionyl (PFP) and heptafluorobutyryl (HFB)) and ester alkyl group (*i.e.*, *n*-propyl, 2-propyl, 2-butyl and isoamyl) on Chirasil-Val **4** have been determined [208]. Enantioselectivity decreases with increasing size of the substituting groups which is more pronounced for the perfluoroacyl group [208–210] as compared to the alkyl group [208]. As expected, TFA-derivatives of α-amino acids exhibit lower retention times [210]. On Chirasil-γ-Dex **7** enantioselectivity is reduced with bulkier ester alkyl groups [36]. The derivatization protocol (described in Ref. [36]) requires a multistep process of about 1 h involving removal of water from the sample, esterification using an alcohol with heating, removal of excess alcohol, acylation with heating and removal of excess acylating reagent. These processes take about 1 h in total. Makida et al. reported a unique method for the derivatization of α-amino acids into *N*(*O*)-isobutyloxycarbonyl methyl esters for quantitative analysis [211]. By this method the first step, the removal of water, was eliminated but the subsequent steps were not straightforward [211]. Derivatization of α-amino acids with 1,3-dichloro-tetrafluoroacetone followed by treatment with pentafluoropropionic acid anhydride has also been described [212]. A very fast one-step derivatization procedure of the carboxylic group and all other reactive groups of α-amino acids has been developed by Hušek [213–219]. The use of alkyl chloroformates as derivatizing reagents leads to *N*(*O*)-alkoxycarbonyl-*O*-alkyl esters of α-amino acids (*cf.* Scheme 1) whereby the intermediate mixed anhydride is decarboxylated to the alkyl ester. The alkyl chloroformate approach bears a number of advantages [220]: (i) the rapid one-step reaction can be carried out in aqueous solution without heating, (b) the cost of reagent is negligible, (iii) the derivatized amino acids can easily separated from the mixture using an organic solvent thus reducing chemical contamination and (iv) the method can easily be automated [221]. In the chiral domain, derivatization with pentafluoropropyl chloroformate has been used for the determination of D- and L-α-amino acids on Chirasil-Val **4** [222]. The GC–MS analysis of biomarkers related to folate and cobalamin status in human serum after dimercaptopropanesulfonate reduction and heptafluorobutyl chloroformate derivatization has been described [223]. GC–MS analysis of fluorinated and non-fluorinated chloroformate and anhydride derivatives of amino acid enantiomers on two different chiral selectors was compared for the direct quantification of free L- and D-amino acids in human serum and urine in a single analytical run [224]. The best sensitivity was achieved with pentafluoropropionic anhydride/heptafluorobutanol derivatives separated on Chirasil-L-Val. However, the occurrence of racemization during derivatization precluded accurate quantification of amino acid enantiomers [224]. Derivatization with methyl chloroformate/methanol and separation on a modified γ-cyclodextrin did not exhibit racemization and yielded ten baseline separated racemates of proteinogenic amino acids with resolution factors $R_s > 2.4$ [224]. The combination of methyl chloroformate and heptafluoro-l-butanol allowed the enantioseparation of 14 amino acids in connection with space exploration efforts [225,226]. In a study of aspartic acid racemization for age dating of collagen, isopropyl chloroformate has been used as derivatizing agent for the amino acid which was then resolved by GC on a modified γ-cyclodextrin [167]. The enantioseparation of α-amino acids, derivatized with ethyl chloroformate,



Scheme 3. Reaction scheme of α-amino acids with alkyl chloroformates in a reaction mixture containing different alcohols. From Ref. [229] with permission.

has also been advanced by *comprehensive two-dimensional GC*. A Chirasil-L-Val-column was used in the first dimension and a polar achiral stationary phase was employed in the second dimension [227]. Wang et al. and Abe et al., respectively, advocated an extension of the alkyl chloroformate approach of Hušek [213–219] by using different alcohols in the reaction medium in order to obtain a variety of different α-amino acid esters (*cf.* Scheme 3) [228,229].

Abe et al. described 2,2,2-trifluoroethyl chloroformate as a rapid derivatizing reagent of α-amino acids for fast enantioseparation by GC [230] and *N*-alkyloxycarbonyl isobutylamides as readily accessible diamides for enantioseparation by GC [231]. The GC enantioseparation of α-amino acids on Chirasil-Val **4** as *N*-alkoxycarbonyl alkylamide derivatives showed improved enantioseparation factors α preferentially for proline [107]. *N*-Pivaloyl methyl esters of α-amino acids including proline were enantioseparated on L-leucine-*tert*-butyl amide linked to 3-carboxypropyl-poly(dimethylsiloxane) [232]. The four stereoisomers of threonine were separated on Chirasil-Val **4** as *N*,*O*-bis-isobutoxycarbonyl-*O*-2,2,2-trifluoroethyl ester derivatives [233].

For the search of extraterrestrial homochirality in forthcoming space missions, the GC–MS analysis of α-amino acid enantiomers, derivatization would benefit from a combined acylation and esterification by using a mixture of perfluorinated alcohols and perfluorinated anhydrides. Thus, α-amino acids were converted to their *N*(*O*,*S*)-perfluoroacyl perfluoroalkyl esters in a single-step procedure, using different combinations of the derivatization reagents trifluoroacetic anhydride (TFAA)/2,2,2-trifluoro-1-ethanol (FFE), TFAA/2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB), and heptafluorobutyric anhydride (HFBA)/HFB [234]. The derivatives obtained were analyzed on Chirasil-L-Val **4** and on a modified γ-cyclodextrin which showed different and complementary enantioselectivity [234]. This one-step may require further scrutiny due to possible side reactions. Silylation of amino acids constitutes another derivatization method. In the achiral domain, three derivatization agents used in GC analysis of α-amino acids were compared: *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), and isobutyl chloroformate (iBuCF) [235]. *tert*-Butyldimethylsilylation (TBDMS) has been used for the derivatization of amino acids for achiral GC–MS analyses [236–240]. In a quest for a straightforward derivatization procedure for the prospected investigation of the enantiomers of α-amino acids on Martian samples, a one-step *tert*-butyldimethylsilylation method has been suggested [241]. A comparison with the conventional two-step procedure (*N*-pentafluoropropionyl-*O*-alkyl esters) is included in the study employing the CSPs Chirasil-L-Val **4** and Lipodex E **6** [241].

An automated gas chromatographic system for enantiomeric α-amino acid analyses has been developed by Gerhardt et al. [186,189,242–245]. Freeze-dried body fluid, tissue and food proteins were hydrolyzed and the hydrolysates were automatically derivatized and analyzed on Chirasil-Val **4** and on Chirasil-γ-Dex **7** [245].

14. Miniaturization

Unless enantiomers are analyzed in complex matrices, the discrimination of a single enantiomeric pair represents a binary separation task for which the whole separation window of a long column (25 m × 250 μm i.d.) is not required. The loss of efficiency of a short column can be compensated by a gain in enantioselectivity at the reduced elution temperature as first demonstrated by Lindström [246]. Since small inside diameter capillary columns (50 μm i.d.) are difficult to coat with CSPs and are hence not commercially available, a good compromise is the use of short 2–5 m × 250 μm i.d. columns containing Chirasil-Val **4** or Chirasil-Dex **5**. Comprehensive studies on column miniaturization in enantioselective GC, although not yet available for fast α-amino acid analysis, have been advanced [247,248].

15. Data retrieval for enantioselective GC

The wealth of information on enantioselective gas chromatography has been documented in the Chirbase/GC data bank [249,250]. The data bank "ChirBase/GC" contains method information for over 24 000 enantioseparations of more than 8000 chiral molecules. The data bank contains experimental conditions, as well as the structure, the substructure and structural similarities. "ChirBase/GC" has been developed by the group of Koppenhoefer et al. (University of Tübingen, Germany) up to the year 2000 [251] http://www.acdlabs.com/products/adh/chrom/chirbase/.

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