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Gas-Chromatographic Separation of Stereoisomers of Dipeptides

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ABSTRACT Synthetic dipeptides comprising mixtures of enantiomers, diastereomers, or sequential isomers were converted into their *N*-perfluoroacetyl dipeptide esters (perfluoroacetyl: trifluoroacetyl, pentafluoroacetyl, heptafluorobutyryl; ester: methyl, 1-propyl, 2-propyl, 2,2,2-trifluoroethyl) and analyzed by GC-MS on the chiral stationary phases Chirasil®-L-Val and Lipodex®-E using helium as carrier gas. Further, dipeptides were converted into their *N*-trifluoroacetyl dipeptide *S*-(+)-2-butyl esters and separated on achiral phenylmethyl polysiloxane column (HP-5 MS). Derivatization of dipeptides was performed at ambient temperature in order to avoid formation of the corresponding diketopiperazines. The best separation of stereoisomers was achieved with TFA and PFP methyl esters on Chirasil®-L-Val. *Chirality 18:551–557, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: dipeptide enantiomers; Chirasil®-Val; Lipodex®-E; diastereomeric esters; D-amino acids; gas chromatography–mass spectrometry

For the enantiomeric separation of free or derivatized protein L-amino acids and their corresponding D-enantiomers or epimers, an abundance of chromatographic methods have been described comprising thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), and capillary electrophoresis (CE).[1–4] Notably, most reports deal with the resolution of single or a few pairs of DL-amino acids. This is attributed to the fact that the 19 genetically encoded protein L-amino acids correspond to 19 D-enantiomers and that 38 enantiomers plus achiral Gly, that is, 39 compounds have to be resolved, ideally in a single chromatographic run. Notably, this chromatographic challenge has not yet been satisfactorily achieved using either direct or indirect methods.

The chromatographic problems increase very much if one considers the separation of stereoisomers of dipeptides resulting from the enantiomers of protein amino acids. If one considers all possible combinations of 19 chiral standard amino acids and the achiral glycine, in principle 1520 stereoisomers plus the achiral Gly-Gly can be formed.[5] Apart from the question whether there is a need to perform the complete chromatographic separation of such a mixture of stereoisomeric dipeptides, its realization is far beyond the analytical methodology currently available. Consequently, much less has been reported on attempts to separate multicomponent mixtures of stereoisomers of dipeptides comprising enantiomers, diastereoisomers, and sequential (positional) isomers.

Enantiomeric and diastereomeric free dipeptides were resolved on TLC plates coated with functionalized hydroxyproline using copper assisted ligand exchange chromatography.[6]

A number of underivatized stereoisomeric dipeptides were separated by HPLC on a chiral teicoplanin column[7] and use of a chiral Crown ether enabled the resolution of stereoisomers of underivatized synthetic dipeptides.[8] Notably, *N*-derivatized stereoisomeric tri- and tetra-alanines were resolved using a 2D liquid chromatographic approach combining an achiral reversed and cinchona-alkaloid-based chiral stationary phases.[9]

Various approaches for the resolution of stereoisomers of free or derivatized di- and tri-peptides employing capillary electrophoresis together with chiral crown ethers or functionalized cyclodextrins as chiral selectors or the addition of chiral additives such as β-cyclodextrins have also been described.[10]

With regard to gas chromatography, the majority of the reports are concerned with the nonchiral gas chromatographic separation of derivatized di- and oligo-peptides resulting from protein partial hydrolysates in order to establish series of overlapping amino acid positions. This approach is in particular used for sequencing peptide antibiotics.[11]

Some reports, however, deal with the direct or indirect chiral separation of di- and oligo-peptides by GC. Enantiomeric and diastereomeric dipeptides were resolved on a peptide-functionalized chiral *s*-triazine stationary phase[12] and stereoisomers of dipeptides were resolved on a laboratory-made Chirasil®-D-Val capillary column.[13,14]

The relatively few reports on the resolution of enantiomeric dipeptides are somewhat contradictory with regard to the abundance and importance of these peptides in microbiology, life sciences (including biomedical sciences), or geosciences, as exemplified in the following. The cell walls of Gram-positive and Gram-negative bacteria are

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composed of polysaccharide strands which are crosslinked by oligopeptides which contain D-Ala in most cases. For the biosynthesis of the resulting peptidoglycans the dipeptide D-Ala-D-Ala is essential. Conjugates with other D-amino acids are also abundant. Composition, stereochemistry, and variations of the chemical linkage of the crosslinking peptides, together with other molecular markers, have been used for a comprehensive chemotaxonomic classification of bacteria.[15] Indeed, we could detect free D-Ala-D-Ala in an ethanolic extract of cultures of the bacterium *Staphylococcus carnosus*.[16] Several dipeptides containing D-amino acids have been described in plants.[17] For example, presence of the dipeptides D-Ala-Gly, D-Ala-D-Ala, and D-Ala-L-Ala has been reported for rice plants[18] and D-Ala-D-Ala and L-Ala-L-Ala have been determined in native and processed tobacco leaves.[19] With regard to nutritional sciences, the food proteins digested in the intestinal tract are cleaved by proteases to oligo- and di-peptides which are transported through the intestinal mucosa by di- and tri-peptide transport systems. The oligo-peptide transporters involved show stereoselectivity and have been shown to transport di-peptides containing D-amino acid residues.[20] Although the occurrence of dipeptides in physiological fluids has been reported,[21] their stereochemistry has attracted little attention.

Since Gln and Cys in their free form are sparingly soluble amino acids, their better soluble conjugates with Ala or Gly are used in preparations for parenteral nutrition,[22] and the dipeptide ester L-Asp-L-Phe-OMe is used as a sweetener. Since these preparations represent peptide drugs or food additives, their enantiomeric purity has to be proven.

Marine sediments have been analyzed for the presence of dipeptides using TFA-di-peptide methyl esters and a Chirasil®-L-Val column. Although the majority of dipeptides was found to contain L-amino acids exclusively the presence of D-Ala-D-Ala together with trace amounts of other dipeptides containing D-amino acids has been reported.[23]

Notably, the search for dipeptides of the carbonaceous Murchison and Yamamoto meteorites using this approach revealed the presence of Gly-Gly and cyclo(Gly) in these extraterrestrial materials, but no evidence was found for the occurrence of other dipeptides.[24]

From this brief overview, it is evident that methods should be available which enable the detection and determination of the configuration of amino acids in peptides. Ideally, the method should separate all stereoisomers, that is, enantiomers, diastereoisomers, and sequential or structural isomers of mixtures of dipeptides, and allow their sensitive and reliable assignment by mass spectrometry in the selected ion monitoring mode. Further, the method should be applicable to biomaterials such as bacteria, plants, and physiological fluids and tissues, or agricultural and food materials, or geological samples. Here we report on the direct separation of derivatized dipeptide stereoisomers (diastereoisomers, enantiomers, and sequential isomers) on a commercially available Chirasil®-L-Val column[25] and a functionalized cyclodextrin Lipodex®-E column.[26] Further, we compare the data to an indirect approach using esterification of dipeptide stereoisomers with *S*-(+)-2-butanol and separation of the resulting diastereoisomers by GC on an achiral methylphenylpolysiloxane phase.

MATERIALS AND METHODS

Chemicals

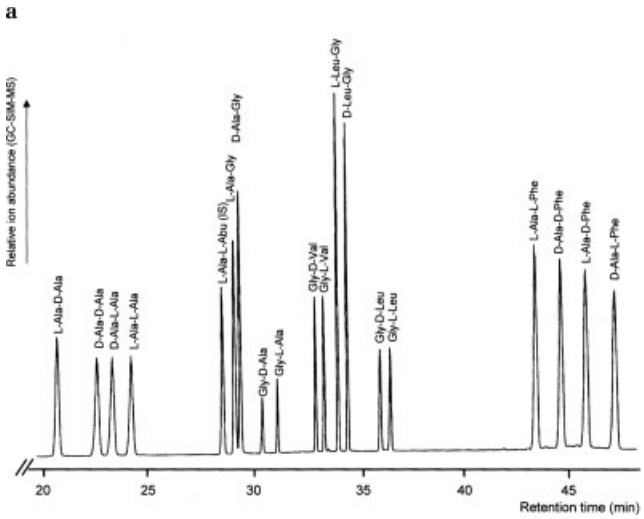
Methanol (MeOH) and dichloromethane (DCM) were purchased from Carl Roth (Karlsruhe, Germany); 1-propanol (1-PrOH), 2-propanol (2-PrOH), were from Riedel-de Haën, (Seelze, Germany); ethanol (EtOH) and acetyl chloride (AcCl) were from Merck (Darmstadt, Germany), and *S*-(+)-2-butanol was from Fluka Chemie (Buchs, Switzerland) and had a chemical purity of 99.3%; no *R*-(−)-2-butanol was detected under the derivatization conditions. Trifluoroacetic acid anhydride (TFAA) and pentafluoropropionic acid anhydride (PFPAA) were from Fluka; heptafluorobutyric acid anhydride (HFBAA) and 2,2,2-trifluoroethanol were from Sigma (Deisenhofen, Germany). Synthetic dipeptides were purchased from various suppliers. Bachem Biochemika, (Heidelberg, Germany): D-Ala-D-Ala; L-Ala-L-Abu; D-Ala-Gly, L-Ala-Gly, D-Ala-D-Phe; D-Ala-L-Phe; L-Ala-D-Phe; L-Ala-L-Phe; Gly-DL-Ala; Gly-DL-Leu; Gly-L-Leu; Gly-DL-Val; Gly-L-Va, and DL-Leu-DL-Phe. Sigma: DL-Ala-DL-Ala; Gly-D-Ala; D-Leu-Gly; L-Leu-Gly; L-Asp-L-PheOMe. Serva Feinbiochemika (Heidelberg): D-Ala-L-Ala; L-Ala-D-Ala; L-Ala-L-Ala.

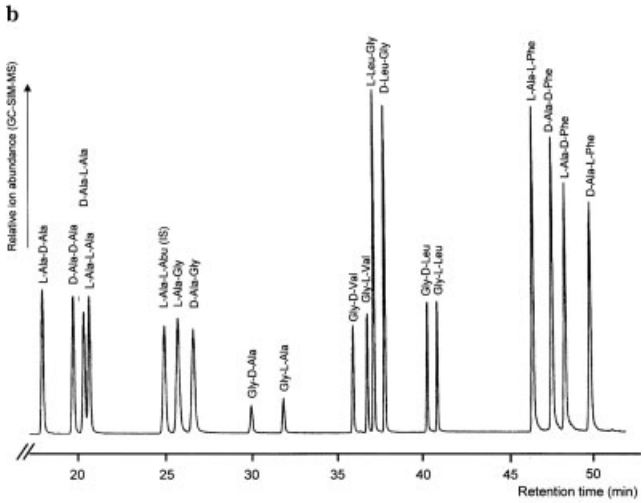
Amino acids are abbreviated with the common three-letter code; the prefix DL refers to racemic amino acids, L and D to individual enantiomers. In cases where the elution order of dipeptides is given, stereochemically defined stereoisomers were used for the assignment of the elution order of peptides.

Preparation of Standard Solutions and Derivatization Procedures for Dipeptides

Standard solutions (1 mM) were prepared by dissolving appropriate amounts of dipeptides in 0.1 M HCl. Aliquots were removed or mixed if required, then evaporated to dryness in a stream of nitrogen and derivatized as described in the following.

Dipeptides were converted into peptide esters at ambient temperature using treatment with a mixture of 500 μL AcCl in the appropriate alcohol (1:9, v/v) for 3 h (esterification with MeOH) or for 17 h (esterification with 1-PrOH, 2-PrOH, EtOH, trifluoroethanol, or S-(+)-2-butanol).[5,27,28] Multicomponent mixtures of dipeptides (Figs. 1a and 2) or individual dipeptides (Fig. 3) were analyzed. After evaporation of reagents with a stream of nitrogen, TFAA, PFPAA, or HFBAA (50 μL) in DCM (300 μL) was added for the *N*-perfluoroacyl dipeptide esters. After 15 min reaction time at room temperature, reagents were removed with a stream of nitrogen, the remaining dipeptide derivatives were dissolved in DCM (100 μL), and then 0.5 μL aliquots were injected into the GC injector at a splitting ratio of 1:30 and analyzed using the SIM mode as described below.





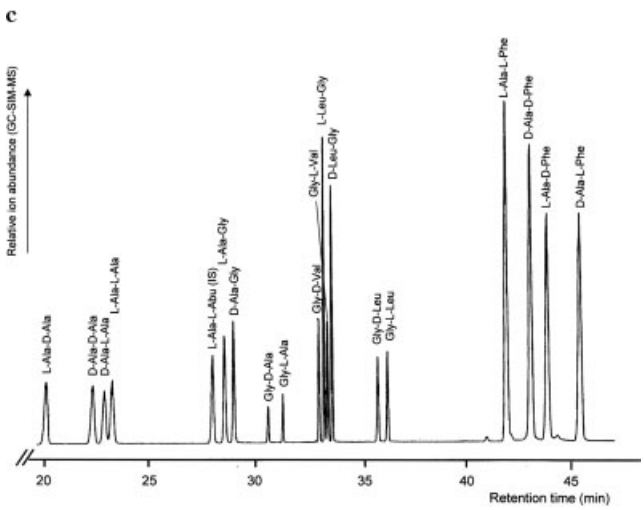


Fig. 1. GC-SIM-MS chromatograms of derivatized dipeptide stereoisomer standards on Chirasil®-L-Val column: (a) TFA/OMe; (b) PFP/OMe; (c) HFB/OMe. For chromatographic conditions, see Experimental.

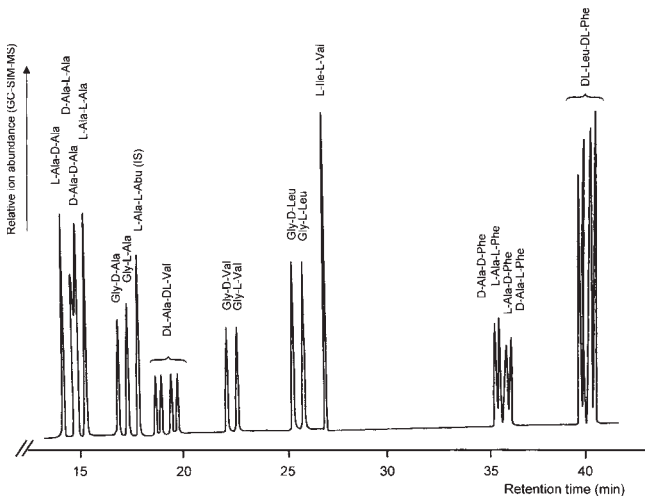


Fig. 2. GC-SIM-MS chromatogram of PFP-dipeptide-S(+)-2-butyl esters on achiral HP5MS column. For chromatographic conditions, see Experimental.

Instruments

Separations of derivatized dipeptides were performed on three instruments: A, B, and C. He was used as carrier gas and temperature and pressure programs were employed, as given in Table 1.

Instrument A. A Chirasil®-L-Val[25,28,29] fused silica capillary column (*N*-propionyl-L-valine *tert.* butyl amide polysiloxane), 25 m length × 0.25 mm I.D., film thickness 0.12 μm (from Varian Inc., Darmstadt, Germany) was installed in a Model A17 gas chromatograph coupled to a Model QP5000 mass spectrometer (Shimadzu, Kyoto, Japan).

Instrument B. A chiral Lipodex®-E[26,28,29] fused silica capillary column (octakis-(3-*O*-butyryl-2,6-di-*O*-*n*-pentyl)-γ-cyclodextrin), 25 m length × 0.25 mm I.D., film thickness

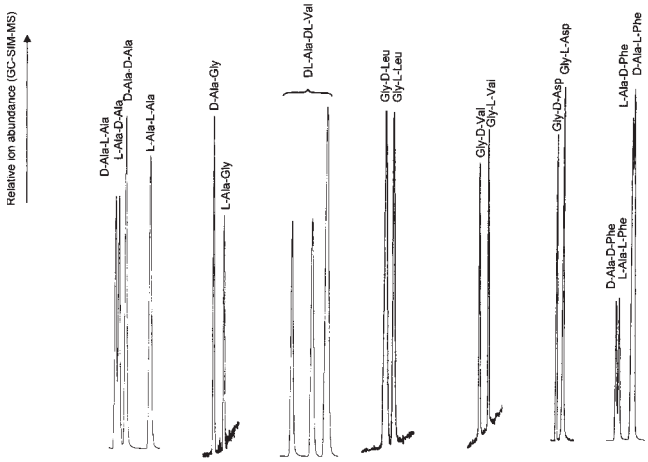


Fig. 3. GC-SIM-MS chromatograms of TFA dipeptide methyl esters on Lipodex® E column. For chromatographic conditons, see Experimental; for retention factors *k*, see Table 3.

TABLE 1. Temperature and pressure programs for columns: A (Chirasil®-L-Val/Shimadzu GC), B (Lipodex®-E/Hewlett-Packard GC), and C (HP-5 MS/Shimadzu GC)

	Increasing rate [°C/min]	Temperature [°C]	Time held [min]	Pressure rate [kPa/min]	Pressure [kPa]	Time held [min]
A	—	140	10	—	5.0	—
	5	190	33	1.0	7.0	33
B	—	140	5	—	5.0	0
	2	150	15	0.2	7.0	2
	1	210	5	0.3	11.0	0
C	—	135	5	1.6	15.0	8
	4	200	10	5.0	65.0	7

12 μm (from Macherey and Nagel, Düren, Germany) was installed in a Model HP6890 gas chromatograph coupled to a mass spectrometer (Hewlett Packard, Avondale, PA).

Instrument C. An achiral HP-5 MS fused silica capillary column (crosslinked 5% phenyl methylpolysiloxane), 30 m length × 0.25 mm I.D., film thickness 0.25 μm (from Hewlett Packard, Avondale, PA) was installed in a Model A17 gas chromatograph coupled to a Model QP5000 mass spectrometer. The temperatures of all GC injectors and GC-MS interfaces were 300°C.

For SIM detection of TFA dipeptide methyl esters the following mass fragments (m/z) were used: Ala-Ala (140, 141); Ala-Abu (140, 141, 225); Ala-Gly (140, 141), Ala-Val (140, 141, 158); Gly-Ala (126, 196); Gly-Val (126, 225), Leu-Gly (126, 140, 141, 183), Gly-Leu (126, 239), Ile-Leu (126, 154); Ile-Val (130, 154); Gly-Asp (102, 113, 126); Ala-Phe (140, 141, 162); Asp-Phe (131, 162, 161); Glu-Val (152, 180); Pro-Ala (166); Ala-Leu (140, 141); Leu-Phe (126, 154). Mass fragments of PFP and HFB methyl esters increase by 50 and 100 mass units, respectively.

RESULTS

In order to systematically test the elution behavior of the four stereoisomers of the dipeptide alanyl-alanine, a mixture of DL-Ala-DL-Ala, comprising the two enantiomeric pairs L-L/ D-D and L-D/D-L, representing four stereoisomers, was analyzed. The mixture was converted at room temperature into the *N*-acyl-alanyl-alanine esters (acyl: TFA, PFP, HFB; ester: methyl, ethyl, 1,1,1-trifluoroethyl, 1-propyl and 2-propyl) and retention factors *k* were determined on Chirasil®-L-Val (Table 2). The stereoisomers of all derivatives could be resolved, but it is of interest to note that the elution time of the esters is L-D < D-D < D-L < L-L with the exception of the trifluoroethyl esters showing the elution order L-D < D-L < D-D < L-L (see Table 1).

The perfluoroacyl dipeptide esters of stereoisomers of individual dipeptides were investigated (Table 3) and finally the PFP-dipeptide methyl esters were selected. A standard mixture of stereoisomers (diastereoisomers and/or enantiomers) was prepared, with each comprising four stereoisomers (two enantiomeric pairs and two diastereoisomeric pairs) of DL-Ala-DL-Ala and DL-Ala-DL-Phe, the enantiomeric dipeptides DL-Ala-Gly, Gly-DL-Ala, Gly-DL-Val, DL-Leu-Gly, and Gly-DL-Leu, and the internal standard L-Ala-L-Abu. Peptide mixtures were converted into series of *N*-acyl dipeptide methyl esters (acyl: TFA, PFP, and HFP) and investigated by capillary gas chromatography on the chiral stationary phases Chirasil®-L-Val and Lipodex®-E. The resolution was investigated by applying the temperature and pressure programs of the GC-MS instruments employed (see Table 1). The retention factors of the derivatives eluted from the capillary columns are compiled in Table 3 and chromatograms demonstrating the separation of all stereoisomers within about 50 min are shown in Figures 1a–1c. The elution order of stereoisomers investigated in this work was assigned by a comparison of dipeptides with known stereochemistry (if available). Notably, the detector response of dipeptides with *N*-terminal

TABLE 2. Retention factors *k* of stereoisomers of *N*-perfluoroacyl-DL-Ala-DL-Ala esters on Chirasil®-L-Val

	TFA					PFP					HFB				
	Me	Et	CH_2CF_3	1-Prp	2-Prp	Me	Et	CH_2CF_3	1-Prp	2-Prp	Me	Et	CH_2CF_3	1-Prp	2-Prp
L-D	10.4	12.1	10.9	17.5	12.5	9.1	10.6	9.6	15.1	10.9	10.1	11.8	10.7	16.9	12.2
D-D	11.4	13.6	13.3	19.9	14.3	9.7	12.1	11.7	17.4	12.6	11.3	13.6	13.2	19.7	14.2
D-L	11.8	14.3	12.8	21.0	15.2	10.4	12.6	11.3	18.2	13.3	11.7	13.9	12.6	20.3	14.7
L-L	12.3	13.2	14.7	21.9	15.9	10.6	12.9	12.6	18.7	13.6	12.2	14.3	14.0	21.0	15.2

For temperature and pressure program, see Table 1; perfluoroacyl: trifluoracetyl (TFA), pentafluoropropionyl (PFP), heptafluorobutyryl (HFB), esters: Me, methyl; Et, ethyl; 1-Prp, 1-propyl; 2-Prp, 2-propyl; CH_2CF_3, 2,2,2-trifluoroethyl.

TABLE 3. Retention factors k of N-perfluoroacyl dipeptide methyl esters on (a) Chirasil®-L-Val (instrument A), (b) Lipodex® E (instrument B), and (c) of N-trifluoroacetyl dipeptide S-(+)-2-butyl esters on achiral HP-5 MS (instrument C). For temperature and pressure programs, see Table 1

		(a)			(b)		(c)	
		TFA	PFP	HFB	TFA	PFP	TFA	PFP
DL-Ala-DL-Ala	L-D	10.4	9.1	10.1	11.2	9.4	7.2	6.8
	D-D	11.4	10.1	11.3	12.6	9.8	7.4	7.2
	D-L	11.8	10.4	11.7	11.4	9.2	7.3	7.1
	L-L	12.3	10.6	12.2	14.3	11.3	7.8	7.5
L-Ala-L-Abu	L-L	14.6	12.9	14.1	13.8	11.1	9.4	8.9
DL-Ala-Gly	L	15.2	13.3	14.8	n.d.	14.8	8.9	8.3
	D	15.3	13.8	15.1	n.d.	14.4	9.2	8.6
DL-Ala-DL-Val[a]		n.d.	n.d.	n.d.	10.8	8.8	9.8	9.3
		n.d.	n.d.	n.d.	11.6	9.7	10.0	9.5
		n.d.	n.d.	n.d.	12.6	10.3	10.2	9.8
		n.d.	n.d.	n.d.	12.6	10.3	10.4	9.9
Gly-DL-Ala	L	15.9	15.7	16.0	24.0	15.8	n.d.	n.d.
	D	16.6	16.8	16.4	22.8	15.7	n.d.	n.d.
Gly-DL-Val	D	17.3	19.1	10.2	23.3	15.6	11.8	11.4
	L	17.6	19.5	17.6	22.2	15.8	12.1	11.7
DL-Leu-Gly	L	17.9	19.7	17.4	n.d.	n.d.	n.d.	n.d.
	D	18.2	18.4	17.7	n.d.	n.d.	n.d.	n.d.
Gly-DL-Leu	D	19.1	21.4	19.8	25.9	16.2	13.4	13.0
	L	19.3	16.2	19.1	25.9	16.3	13.7	13.2
L-Ile-L-Leu		n.d.	n.d.	n.d.	17.3	14.2	n.d.	n.d.
L-Ile-L-Val		n.d.	n.d.	n.d.	12.7	10.9	14.3	14.0
Gly-DL-Asp	D	n.d.	n.d.	n.d.	34.2	34.5	n.d.	n.d.
	L	n.d.	n.d.	n.d.	34.6	34.8	n.d.	n.d.
DL-Ala-DL-Phe	L-L	23.2	24.9	22.3	34.9	18.5	19.2	18.9
	D-D	23.8	25.6	22.9	34.6	18.4	19.1	18.8
	L-D	24.5	25.9	23.4	36.4	18.9	19.4	19.0
	D-L	25.3	26.8	24.2	36.2	18.9	19.6	19.1
L-Asp-L-Phe		n.d.	n.d.	n.d.	44.3	22.4	n.d.	n.d.
L-Glu-L-Val		n.d.	n.d.	n.d.	n.d.	16.7	n.d.	n.d.
L-Pro-L-Ala		n.d.	n.d.	n.d.	n.d.	18.3	n.d.	n.d.
L-Ala-L-Leu		n.d.	n.d.	n.d.	n.d.	12.9	n.d.	n.d.
DL-Leu-DL-Phe[a,b]		n.d.	n.d.	n.d.	n.d.	n.d.	21.5	20.9
		n.d.	n.d.	n.d.	n.d.	n.d.	21.6	21.1
		n.d.	n.d.	n.d.	n.d.	n.d.	21.7	21.2
		n.d.	n.d.	n.d.	n.d.	n.d.	21.8	21.4

not determined; (n.d.).
[a]elution order not assigned.
[b]chromatogram not shown; trifluoroacetyl (TFA); pentafluoropropionyl (PFP); heptafluorobutyryl (HFB).

Gly, that is, Gly-DL-Ala and Gly-DL-Leu, is about one-fourth of the sequential isomers, that is, DL-Ala-Gly and DL-Leu-Gly.

For comparison, this standard mixture of stereoisomers (to which the additional dipeptides DL-Ala-DL-Val, DL-Leu-DL-Phe, and L-Ile-L-Val were added) was converted into a mixture of TFA-dipeptide *S*-(+)-2-butyl esters and the derivatives analyzed by GC-MS on an achiral phenylmethylpolysiloxane capillary column. The resulting chromatogram is shown in Figure 2, where it can be seen that the resolution of stereoisomers is not as good as on Chirasil®-L-Val. However, the derivatives of DL-Leu-DL-Phe, which are not eluted from Chirasil®-L-Val, are resolved and eluted at 220°C. This temperature is not recommended for Chirasil®-L-Val. Since the Lipodex®-E column did not separate multicomponent standards of the TFA-dipeptide-methyl esters, selected dipeptides (DL-Ala-DL-Ala, DL-Ala-Gly, DL-Ala-DL-Val, Gly-DL-Leu, Gly-DL-Val, Gly-DL-Asp, and DL-Ala-DL-Phe were investigated in individual runs; sections of the resulting chromatograms are presented in Figure 3. As can be seen, the enantiomers of DL-Ala-Gly, Gly-DL-Leu, and Gly-DL-Val are baseline resolved. The stereoisomers of DL-Ala-DL-Ala and DL-Ala-DL-Phe are also resolved, albeit not as completely as on Chirasil®-Val. The stereoisomers of DL-Ala-DL-Val provide three peaks, indicating that the two stereoisomers elute together. The elution order of the stereoisomers of this dipeptide was not determined, however. For the retention factors of the derivatives presented in Figure 3 determined under the GC-MS conditions applied, see Table 3.

Retention factors of several dipeptides (L-Ala-L-Abu, L-Ile-L-Leu, L-Ile-L-Val, L-Asp-L-Phe, L-Glu-L-Val, L-Pro-L-Ala, and L-Ala-L-Leu) were determined on Lipodex® E and included in Table 3 in order to establish their elution order from this column under the chromatographic conditions applied.

DISCUSSION

From the data presented in Table 3 and chromatograms shown in Figure 1, it can be seen that for direct enantiomeric separation on Chirasil-L-Val, the *N*-trifluoroacetyl dipeptide methyl esters are highly suitable derivatives, although use of the other derivatives investigated is also feasible. Use of trifluoroethyl esters in place of alkyl esters provides no advantage. Esterification and acylation of peptides at ambient temperature[5] was confirmed to proceed without racemization of chiral amino acids and use of a mixture of AcCl and the respective alcohol for esterification was confirmed to be very convenient.[27]

Several TFA-dipeptide methyl esters had been previously investigated[5,13] on a homemade Chirasil®-D-Val[14] column having the opposite configuration of the chiral selector in comparison to the commercially available Chirasil®-L-Val column used in this work. Use of Chirasil®-D-Val results in a reversal of the elution order of enantiomers.[5,13] It is worth noting that the derivatives eluted much earlier and at much lower temperature using hydrogen as carrier gas from the homemade fused silica Chirasil®-D-Val column (25 m × 0.3 mm I.D., film thickness not given; 0.6 bar hydrogen) in comparison to the purchased Chirasil®-L-Val column employed in this work using helium in place of hydrogen as carrier gas. For example, TFA-DL-Ala-DL-Phe-OMe derivatives were eluted and baseline resolved within less than 4 min at 200°C from the former column,[5] whereas 40 min were required for the latter column used in our work (25 m × 0.25 mm I.D.; film thickness 0.25 μm, pressure program starting with 5 bar helium).

The highest temperature recommended by the manufacturer to be applied to the Chirasil®-L-Val column is 200°C. At this temperature the stereoisomers of DL-Ala-DL-Phe eluted within about 50 min. Stereoisomers of dipeptide derivatives

of a higher boiling point such as DL-Leu-DL-Phe are not eluted within reasonable time. This is a limitation of the approach described and confirms the established superiority of hydrogen over helium as the carrier gas. The use of hydrogen, probably together with the lower film thickness of the stationary phase, explains the lower elution temperature, shorter elution and analysis time, and higher separation factors reported. Owing to the higher temperature applicable to the achiral phenylmethylpolysiloxane phase, the stereoisomers of the TFA-DL-Leu-DL-Phe *S*-(+)-2-butyl esters are eluted within about 40 min at 200°C. Neither kinetic discrimination on the formation of diastereomeric esters nor discrimination of derivatives in the injector or different MS detector responses was observed, since peak areas of the enantiomers of racemic Gly-DL-Val and Gly-DL-Leu and of stereoisomers of DL-Ala-DL-Val were equal (see Fig. 2). Since Val is the sterically most hindered α-amino acid, kinetic discrimination is not expected for the other dipeptides investigated. The separation of the stereoisomers using this indirect approach in most cases is not as good as on Chirasil®-L-Val.

Comparison of the resolution of TFA-dipeptide methyl esters on Chirasil®-L-Val and Lipodex®-E shows that few stereoisomers are satisfactorily resolved on the latter stationary phase and that the majority of the dipeptides investigated are not as well resolved as on Chirasil®-L-Val column.

From a mechanistic point of view, the chiral recognition of dipeptide derivatives via hydrogen-bonding on the chiral diamide phases is more effective in comparison to discrimination via formation of γ-cyclodextrin inclusion compounds.

From an applied point of view, the methods described will make possible the stereoselective analysis of synthetic dipeptides, the search for and assignment of an abundance of stereoisomeric dipeptides expected to occur in biomaterials, such as bacteria,[15,16] plants,[17,18] and native or processed foodstuffs,[19,30] for monitoring the metabolization of dipeptide stereoisomers administered to living organisms, and the search for enantiomeric and epimeric dipeptides resulting from the change of stereogenic centers in the course of protein diagenesis proceeding in sediments or fossils. Use of GC-SIM-MS together with established or advanced[31] enantioselective columns will make the assignment of stereoisomers highly reliable.

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