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Gas chromatographic separation of stereoisomeric esters of α-amino acids and α-alkyl-α-amino acids on chiral stationary phases

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ABSTRACT

The separations of stereoisomers of (a) N-pentafluoropropionyl (PFP)-(*R,S*)-α-amino acid (AA) (*R,S*)-alkyl esters and (b) N-trifluoroacetyl (TFA)-(*R,S*)-α-alkyl-α-amino acid (AAA) (*R,S*)-α-alkyl esters were investigated by capillary gas chromatography on Chirasil-L-Val, and Chirasil-D-Val, respectively. The order of elution of the four possible stereoisomers (a) was found to be *R–S* < *R–R* < *S–R* < *S–S* for PFP-(*R,S*)-AA 2-butyl and 2-octyl esters, and *R–S* < *S–R* < *R–R* < *S–S* for PFP-(*R,S*)-AA 3-methyl-2-butyl esters (the first letter in the diastereomers refers to the configuration of the amino acid and the second to the configuration of the alcohol). For (b) it was found that the *S–S* diastereomers eluted first and the *R–S* diastereomers last (fourth) from the column. In the few instances in (b) in which all four stereoisomers were separated, with the TFA-(*R,S*)-α-butyl-Ala (*R*)-2-butyl esters the *S–R* diastereomer eluted second and the *R–R* diastereomer eluted third whereas with the TFA-(*R,S*)-α-butyl-Ala (*R*)-2-octyl esters and TFA-(*R,S*)-α-methylleucine (*R*)-2-octyl esters, the *R–R* diastereomers eluted second and the *S–R* diastereomers third. Further, the temperature dependence of the elution order of the four stereoisomers of PFP-(*R,S*)-Ala (*R,S*)-2-octyl esters on Chirasil-D-Val showed that the *S–R* diastereomer eluted first and the *R–R* diastereomer last (fourth) at all temperatures; however, at 125°C *S–S* eluted before *R–S* and at 150°C *S–S* and *R–S* eluted together, whereas at 175°C *R–S* eluted before *S–S*. The order of elution of stereoisomeric derivatives of AA and AAA from chiral stationary phases is rationalized by superimposed diastereomeric and enantiomeric resolution effects on the chiral stationary phase. That the chiral alcohol contributes to the resolution of stereoisomeric N-acyl-(*R,S*)-AA (*R,S*)-2-alkyl esters on a chiral phase is demonstrated by the resolution of enantiomeric TFA-Gly (*R*)-alkyl esters and TFA-Gly (*S*)-alkyl esters on Chirasil-D-Val.

INTRODUCTION

For the separation of enantiomers of volatile amino acid (AA) derivatives by gas chromatography (GC), a number of chiral stationary phases have been described [1–9]. In order to increase the thermal stability of the stationary phases, Bayer *et al.* [10] coupled L-Val *tert.*-butylamide as a chiral selector to a copolymer of dimethylpolysiloxane together with (2-carboxypropyl)methylsiloxane and named this phase

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Chirasil-L-Val. Saeed *et al.* [11,12] synthesized chiral phases by coupling L-Val *tert.*-butylamide to modified polycyanopropylmethyl phenylmethyl silicone, and König and Benecke [13] coupled benzyloxycarbonyl(Z)-L-valine and Z-L-leucine to functionalized OV-225.

Optical isomers of AA are also separable as diastereomeric derivatives [14–17], preferably diastereomeric N-acyl AA alkyl esters, on non-chiral stationary phases such as alkylpolysiloxanes. For the determination of AA enantiomers as diastereomeric esters, derivatization with amino alcohols of the highest optical purity is essential in order to achieve satisfactory accuracy, as enantiomeric AA esters are not resolved on non-chiral columns. It was shown, however, that the enantiomeric and the diastereomeric approach can be combined by use of suitable chiral stationary phases and that mixtures of the two enantiomeric and two diastereomeric esters (*i.e.*, four stereoisomers), as obtained by esterification of (*R,S*)-AA by (*R,S*)-alkanols, are completely separable by capillary GC.

Using this approach, König [18,19] resolved the four possible stereoisomeric N-pentafluoropropionyl (PFP)-DL-Leu DL-3-methyl-2-butyl esters on the chiral phases N-trifluoroacetyl (TFA)-L-phenylalanyl-L-aspartic acid bis(cyclohexyl ester) (TFA-L-Phe-L-Asp(OcHex)-OcHex) and TFA-DL-Val (±)-3-methyl-2-butyl esters as well as PFP-DL-Leu (±)-3-methyl-2-butyl esters on the chiral phase XE-60-L-Val-(*S*)-α-phenylethylamide [XE-60-L-Val-(*S*)-PEA]. König determined and rationalized [18] the retention times of the isomers as D/*S*(+) < L/*R*(−) < D/*R*(−) < L/*S*(+) (*i.e.*, *R*–*S* < *S*–*R* < *R*–*R* < *S*–*S*; the first letter refers to the configuration of the AA). Liu [20] separated the isomers of TFA-DL-Leu (and Ala) (±)-2-butyl esters on the chiral stationary phase Chirasil-L-Val and used the method for the determination of the enantomeric purity of a sample of L-Ala. He found the retention times to increase in the order D–D < D–L < L–L < L–D (*i.e.*, R–S < R–R < S–R < S–S). This is the same elution order for the diastereomeric pairs *R*–*S* and *S*–*S* and the opposite elution order for the diastereomeric pairs *R*–*R* and *S*–*R* on an L-stationary phase.

The separation of diastereomeric N-acyl-AA esters on chiral stationary phases had been demonstrated for the few AA and chiral alcohols mentioned above; the elution orders of the *S*–*R* and *R*–*R* diastereomers of 2-butyl esters on Chirasil-L-Val were found to be the opposite of those of the 3-methyl-2-butyl esters on TFA-L-Phe-L-Asp(OcHex)-OcHex and XE-60-L-Val-(*S*)-α-PEA. No satisfactory explanation has been given for this phenomenon so far, nor has the a possible contribution of the chiral alcohol in the diastereomeric N-acyl-AA esters or a possible temperature dependence of the elution order of stereoisomers been discussed [18–20]. We have therefore determined the resolution and elution order of the stereoisomers of a series of N-PFP-(*R,S*)-AA (*R,S*)-2-alkyl esters on Chirasil-Val. Moreover, in continuation of our work on the chiral resolution of diastereomeric esters of non-protein α-alkyl-α-amino acids (AAA) [21–24] we have investigated the question of whether the stereoisomers of various TFA-(*R,S*)-AAA (*R,S*)-2-alkyl esters are resolvable on Chirasil-Val.

EXPERIMENTAL

Gas chromatography

GC was carried out on a Carlo Erba Model HRGC 5160 gas chromatograph with a flame ionization detector, a Shimadzu C-R3A integrator and (a) a Chirasil-L-Val column (25 m × 0.25 mm I.D.; Macherey–Nagel. Düren, Germany) or (b) a Chirasil-D-Val fused-silica capillary column (25 m × 0.25 mm I.D.; made by G. J. Nicholson, University of Tübingen). The carrier gas was hydrogen at 50 kPa (0.5 bar), the splitting ratio *ca.* 1:30 and the injector and detector temperatures 250°C with both columns.

α-Amino acids, α-alkyl-α-amino acids and reagents

DL-and L-α-amino acids (AA) were purchased from Fluka (Buchs, Switzerland). The (*R,S*)-α-alkyl-α-amino acids (AAA) α-amino-*n*-butyric acid (Abu), norvaline (Nva), norleucine (Nle) and α-aminooctanoic acid (Aoc) were synthesized in our laboratory according to the Strecker procedure, and optically pure and enantiomerically enriched AAA were obtained by enzymic digestion with acylase I or carboxypeptidase A [25]. AAA ar considered as being formally derived from protein α-amino acids by C^{α}-hydrogen substitution by alkyl groups [24]: α-Ethylalanine, "isovaline" (α-Et-Ala), α-butylalanine (α-But-Ala), and α-methylleucine (α-Me-Leu).

(*R,S*)- and (*S*)(+)-2-butanol, (*R,S*)- and (*S*)(+)-2-octanol and (*R,S*)-3-methyl-2-butanol were purchased from Fluka and (*S*)(+)-3-methyl-2-butanol from Chemical Dynamics (South Plainfield, NJ, U.S.A.). Esterification of AA and AAA with acetyl chloride and the respective alcohol and acylation with pentafluoropropionic anhydride were carried out as described previously [24]. The elution orders of amino acid stereoisomers were determined with the help of enantiomerically enriched AA (AAA) and 2-alkanols.

RESULTS AND DISCUSSION

Separation of N-PFP-(R,S)-2-alkylesters of α-amino acids on Chirasil-L-Val

Sections of selected gas chromatograms, taken on Chirasil-L-Val, of the separation of PFP-(*R,S*)-α-amino acid (*R,S*)-alkyl esters are shown in Fig. 1a–l. Net retention times at isothermal temperatures, resolution factors α and the number of stereoisomers resolved are shown in Table I. With the PFP-(*R,S*)-AA (*R,S*)-2-butyl esters, four stereoisomers are completely (Fig. 1a–d) or partially (Fig. 1e) resolved with the elution order *R–S* < *R–R* < *S–R* < *S–S*, or the *R–R* and *S–R* diastereoisomers are eluted together (Fig. 1f).

It is of interest that with the use of racemic AA and 2-alkanols the peak areas of the *R–S* and *S–R* derivatives are equal to each other, as also are those of the *R–R* and *S–S* derivatives. However, the peak areas of the *R–S* (*S–R*) derivatives were found to be significantly larger than those of the *R–R* (*S–S*) derivatives. This is attributed to kinetic (steric) discrimination in the course of the esterification process. The relative percentage of *R–S* with respect to *R–S* plus *S–S* was found to be 52.4% for PFP-AA 2-butyl esters on average.

For PFP-(*R,S*)-AA (*R,S*)-3-methyl-2-butyl esters the elution orders of the

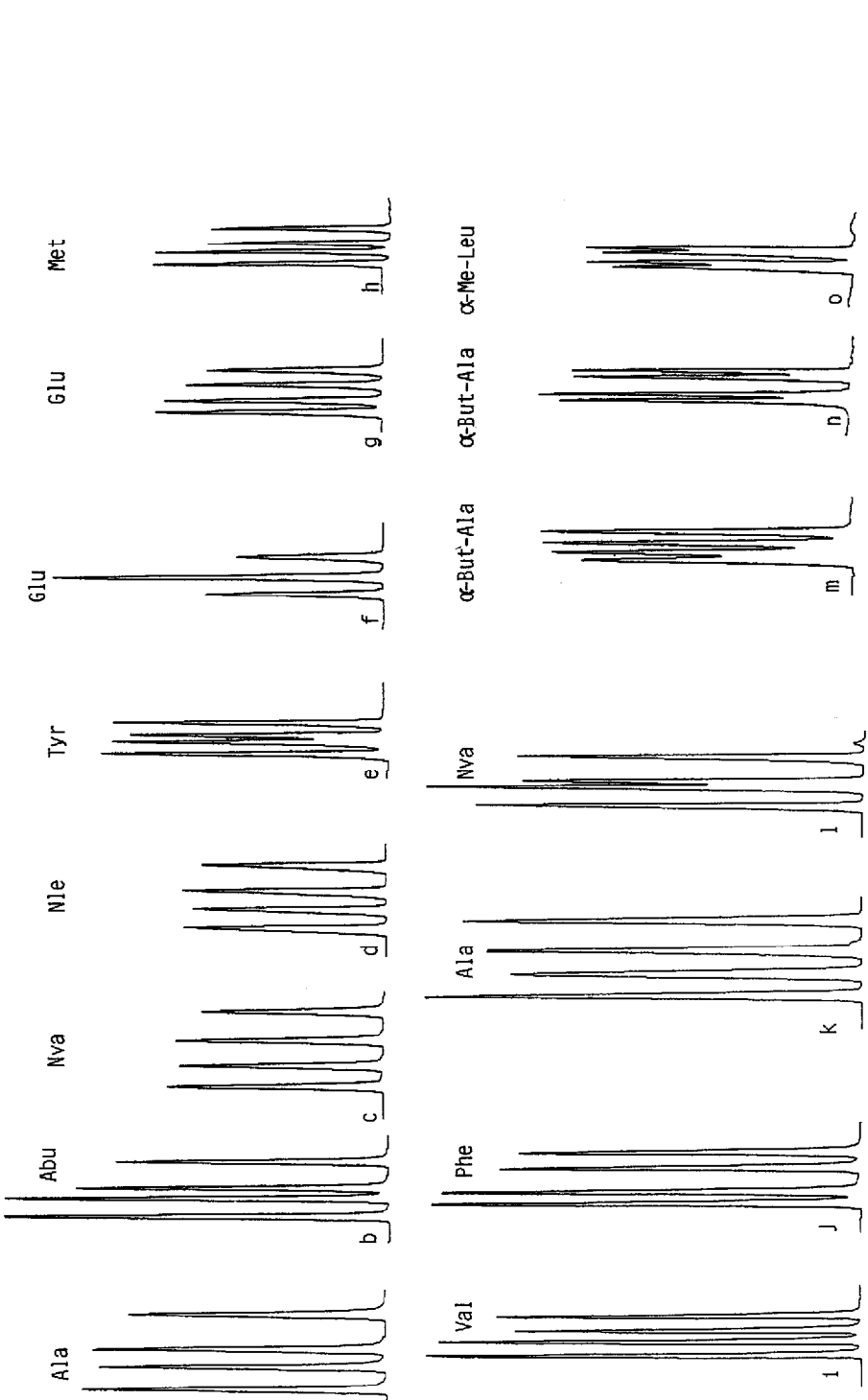

Fig. 1. Sections of gas chromatograms of the separation of stereoisomers of (a–l) PFP-(*R,S*)-AA (*R,S*)-2-alkyl esters and (m–o) TFA-(*R,S*)-AAA (*R,S*)-2-alkyl esters. (a–f) 2-Butyl esters; (g–j) 3-methyl2-butyl esters; (k and l), 2-octyl esters; (m) 2-butyl esters; (n and o) 2-octyl esters. Column, (a–l) Chirasil-L-Val, (m–o) Chirasil-D-Val. For GC conditions, retention times and elution orders see Tables I and II.

TABLE I

ELUTION ORDER OF STEREOISOMERS OF PFP-(*R,S*)-AA (*R,S*)-ALKYL ESTERS ON CHIRASIL-L-VAL

Net retention times, *t* (min), isothermal temperatures, *T* (°C), resolution factors, α, and number, *n*, of separated stereoisomers are given. For GC conditions, see Experimental.

Alkyl group in alkyl ester	(*R,S*)-AA	*t* (min)				*T* (°C)	α			*n*
		R–S	*R–R*	*S–R*	*S–S*		*S–S*/*R–S*	*S–R*/*R–S*	*S–S*/*R–R*	
2-Butyl	Ala	8.87	9.39	9.78	10.55	80	1.19	1.10	1.12	4
	Abu	7.69	8.11	8.34	8.92	90	1.16	1.08	1.10	4
	Nva	10.27	10.72	11.29	11.96	95	1.16	1.10	1.12	4
	Nle	9.69	10.12	10.53	11.10	105	1.14	1.09	1.10	4
	Aoa	9.98	10.33	10.58	11.02	125	1.10	1.06	1.07	4
	Val	8.18	8.58	8.58	9.02	90	1.10	1.05	1.05	3
	Leu	7.79	8.18	8.65	9.23	105	1.19	1.11	1.13	4
	Met	10.25	10.61	10.78	11.22	135	1.10	1.05	1.06	4
	Ser	9.80	10.08	10.08	10.47	100	1.07	1.03	1.04	3
	Glu	11.18	11.58	11.58	12.02	150	1.08	1.04	1.04	3
	Phe	9.64	9.96	9.96	10.37	145	1.08	1.03	1.04	3
	Tyr	8.81	9.08	9.23	9.51	160	1.08	1.05	1.05	4
		R–S	*S–R*	*R–R*	*S–S*					
3-Methyl-2-butyl	Ala	8.04	8.70	8.88	9.84	90	1.22	1.08	1.11	4
	Abu	8.84	9.58	9.71	10.68	95	1.21	1.08	1.10	4
	Nva	8.68	9.36	9.36	10.29	105	1.19	1.08	1.10	3
	Nle	10.88	11.72	11.72	12.83	110	1.18	1.08	1.09	3
	Aoa	10.67	11.23	11.41	12.12	130	1.14	1.05	1.06	4
	Val	7.29	7.60	7.85	8.16	100	1.12	1.04	1.04	4
	Leu	8.72	9.54	9.54	10.67	110	1.22	1.09	1.12	3
	Met	7.18	7.45	7.65	7.98	150	1.11	1.04	1.04	4
	Ser	8.39	8.51	8.90	9.19	110	1.10	1.01	1.03	4
	Glu	9.69	9.95	10.29	10.61	165	1.10	1.03	1.03	4
	Phe	10.27	10.53	11.06	11.41	150	1.11	1.03	1.03	4
	Tyr	8.89	9.09	9.71	9.99	165	1.12	1.02	1.03	4
		R–S	*R–R*	*S–R*	*S–S*					
2-Octyl	Ala	9.91	10.38	10.91	11.59	120	1.17	1.10	1.12	4
	Abu	7.91	8.18	8.48	8.99	130	1.14	1.07	1.10	4
	Nva	9.50	9.91	10.05	10.62	135	1.12	1.06	1.07	4
	Nle	10.90	11.25	11.25	11.97	140	1.10	1.03	1.06	3
	Aoa	9.85	10.05	10.05	10.40	160	1.06	1.02	1.03	3
	Leu	11.75	12.41	12.41	13.42	135	1.14	1.06	1.08	3
	Met	9.97	10.19	10.19	10.52	170	1.06	1.02	1.03	3
	Phe	11.78	11.95	12.31	12.56	175	1.07	1.04	1.05	4
	Tyr	9.23	9.55	9.55	9.81	190	1.05	1.03	1.04	3

respective stereoisomers are *R–S* < *S–R* < *R–R* < *S–S*, or *R–S* < *S–R* together with *R–R* < *S–S*; (*cf.*, Table I). The elution order *S–R* before *R–R* for the 3-methyl-2-butyl esters is the opposite of that found for 2-butyl esters (*cf.*, Table I).

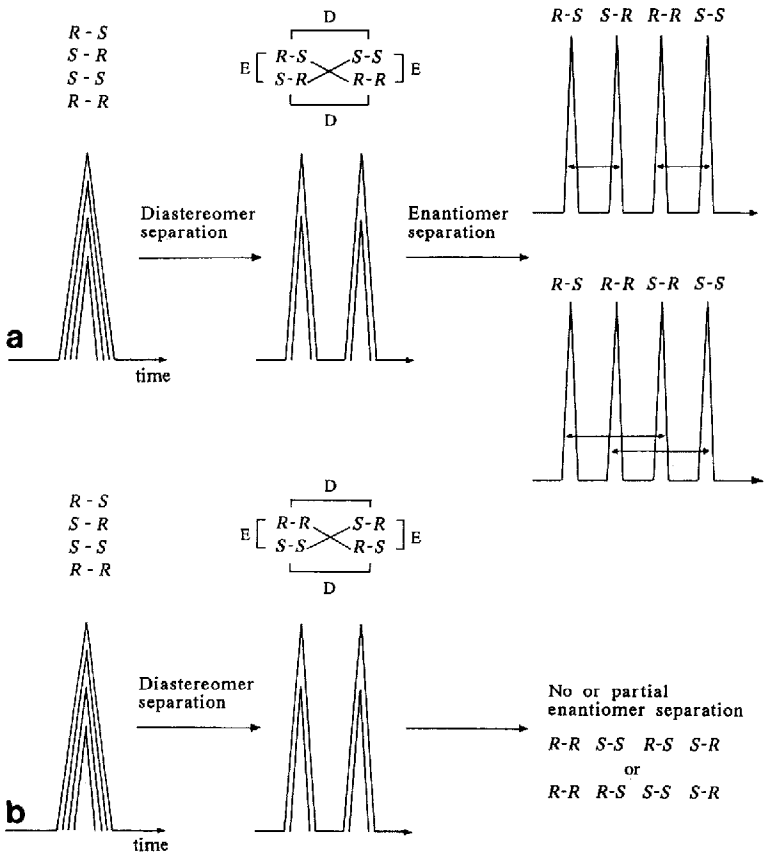

Fig. 2. Scheme of the GC separation on chiral L-α-amino acid phase (Chirasil-L-Val) of stereoisomers of (a) PFP-(*R,S*)-AA (*R,S*)-2-alkyl esters and (b) TFA-(*R,S*)-AAA (*R,S*)-2-alkyl esters. The first letter in the abbreviations of the stereoisomers (*e.g.*, *R–S*) refers to the configuration of the amino acid and the second to that of the alcohol. (*R*)- and (*S*)-α-amino amino acids correspond to D- and L-α-amino acids, and (*S*)(+)- and (*R*)(−)-2-alkanols to D- and L-2-alkanols [18–20]; diastereomers (D) and enantiomers (E) are assigned in the scheme.

3-Methyl-2-butanol also exerts an increased kinetic (steric) discrimination effect as compared with 2-butanol. The relative percentage of *R–S*, calculated with respect to *R–S* plus *S–S*, was found to be 55.7% for PFP-AA 3-methyl-2-butyl esters on average.

For PFP-(*R,S*)-AA (*R,S*)-2-octyl esters, the diastereomeric pairs *S–S* and *R–S* show smaller resolution factors than to 2-butyl and 3-methyl-2-butyl esters (*cf.*, Table I and Fig. 1k and l), and the diastereomeric pairs *S–R* and *R–R* of the 2-octyl esters of Nle, Aoa, Leu, Met and Tyr are eluted together (*cf.*, Table I). In summary, however, the selection of an appropriate ester makes the resolution of all four stereoisomers of the PFP-AA 2-alkyl esters possible (*cf.*, Table I).

The elution order of stereoisomers on Chirasil-L-Val is, in agreement with an explanation given by König [18] for other L-phases, rationalized as follows (Fig. 2a). On esterification with (*R,S*)-2-alkanols (*R,S*)-AA form two diastereomeric and two enantiomeric derivatives. On an appropriate non-chiral stationary phase the diaster-

eomeric esters, but not the enantiomeric esters, are separable, whereas on a chiral stationary phase, in principle, both the diastereomeric esters and the enantiomeric esters are separable. Therefore, if optimally separated, four baseline-resolved peaks of the respective stereoisomers should appear in the chromatograms. The elution order of the four possible stereoisomers on Chirasil-L-Val is explained below (*cf.*, Fig. 2a).

The stationary phase Chirasil-L-Val can be considered to consist of a non-chiral backbone of dimethylpolysiloxane to which the chiral selector L-Val *tert.*-butylamide has been covalently bonded via a spacer group [10,26].

On a non-chiral stationary phase diastereomeric, but not enantiomeric, PFP-AA esters are separated. This leads to the resolution of *R–S* and *S–S* diastereomers and of *S–R* and *R–R* diastereomers, respectively. The enantiomeric pair *R–S* and *S–R* will elute together and first; *S–S* and *R–R* also elute together but second (cf. Fig. 2a). This resolution effect is called the "diastereomeric resolution effect" in the following discussion. Diastereomers of AAA show the opposite elution order to AA, *i.e.*, *R–R* and *S–S* are eluted first and together, and *S–R* and *R–S* are eluted second and together (*cf.* Fig. 2b; for discussion, see below).

On a chiral stationary phase, such as Chirasil-L-Val, the diastereomeric resolution effect will be superimposed by an "enantiomeric resolution effect", and therefore simultaneously the separation of the enantiomeric pairs *R–S* and *S–R* or *S–S* and *R–R* will occur. As a result of chiral interactions on Chirasil-L (= *S*)-Val [and also other chiral phases composed of L (= *S*)-AA], (*R*)-AA derivatives are less retarded than (*S*)-AA derivatives. AAA enantiomers show the same elution order as AA enantiomers. The diastereomeric and enantiomeric effects are additive for the diastereomeric pairs *S–S* and *R–S*. For the diastereomeric pair *S–R* and *R–R* both effects are opposite. Depending on the dominance of the diastereomeric or the enantiomeric resolution effect (which is dependent on the alcohol used; *cf.* Table I), S–R is eluted before *R–R* or *vice versa*. It might also be possible that *S–R* and *R–R* are eluted together from the column or are incompletely resolved.

As neither a contribution of the chiral alcohol in N-acyl-AA 2-alkyl esters nor a possible temperature dependence on the resolution and elution order of stereoisomers on chiral stationary phases had been discussed previously [18–20], we investigated the separation of N-TFA-Gly (*R,S*)-2-alkyl esters and N-PFP-(*R,S*)-Ala (*R,S*)-2-octyl ester on the Chirasil-D-Val column. Achiral Gly was used in order to eliminate the influence of the chiral AA on resolution, and a laboratory-made, fused-silica Chirasil-D-Val column was selected as it showed exceptionally high resolution coefficients or DL-AA. [In agreement with the literature [3] and from our experience with the determination of D-AA in food [27,28], commercially available Chirasil-L-Val columns from various manufacturers (Macherey-Nagel; Chrompack, Middelburg, The Netherlands; C.G.C. Analytic, Mössingen, Germany; laboratory-made fused-silica and glass capillar columns) showed different selectivities for amino acid derivatives.] It was found that TFA-Gly (*R,S*)-2-alkyl esters (alkyl = 2-butyl, 3-methyl-2-butyl and 2-octyl) were significantly resolved on Chirasil-D-Val, the (*S*)(+)-derivative eluting before the (*R*)(−)-derivative (Fig. 3a); with a Chirasil-L-Val column the opposite elution order is to be expected. Therefore a (minor) contribution of the chiral alcohol to the resolution of AA enantiomers on chiral column also has to be taken into account.

On an L-stationary phase for the enantiomeric pair *R–S* and *S–R*, the

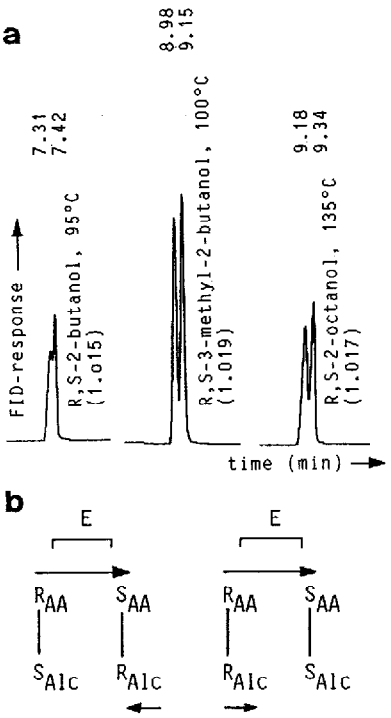

Fig. 3. (a) Resolution of TFA-Gly (*R,S*)-2-alkyl esters on Chirasil-D-Val. Alcohols used for esterification, net retention times (min), isothermal temperatures and resolution factors α are given. Derivatives with (*S*)(+)-alcohol elute before those with (*R*)(−)-alcohol on Chirasil-D-Val and the opposite on Chirasil-L-Val. Carrier gas, hydrogen at 0.5 bar. (b) Scheme of the influence (indicated by direction and size of the arrows) of the chiral alcohol in the N-acyl-(*R,S*)-AA (*R,S*)-2-alkyl esters on the separation of the enantiomeric pairs on Chirasil-L-Val. E, Enantiomers; R_{AA} and S_{AA}, S_{Alc} and R_{Alc}, configurations of the amino acid (AA) and alcohol (Alc), respectively, in diastereomers (*e.g.*, R_{AA}–S_{Alc}, arranged vertically in scheme).

contributions of the chiral AA and chiral alcohol are opposite with respect to the elution order, and for the enantiomeric pair *S–S* and *R–R* they are additive. This is shown schematically in Fig. 3b.

A comparison of resolution factors α for PFP-(*R,S*)-AA 2-alkyl esters (*cf.*, Table I) shows that in most instances the α-values of the enantiomeric pairs *S–S* and *R–R* are higher than those of the pairs *S–R* and *R–S*. In a few instances equal α-values were found; it is assumed that in these instances the chiral alcohol does not affect he resolution of enantiomers. In no case does the enantiomeric pair *S–R* and *R–S* show a higher α-value than the pair *S–S* and *R–R* (*cf.*, Table I).

Further, in order to determine a possible temperature dependence of the elution order of stereoisomers on the chiral stationary phase, the resolution of PFP-(*R,S*)-Ala (*R,S*)-2-octyl esters [enriched with PFP-(*S*)-Ala (*S*)-2-octyl ester] was investigated on Chirasil-D-Val at the isothermal temperatures 125, 150 and 175°C (Fig. 4). It was found that at all temperatures *S–R* eluted first and *R–R* last, but at 125°C *S–S* eluted

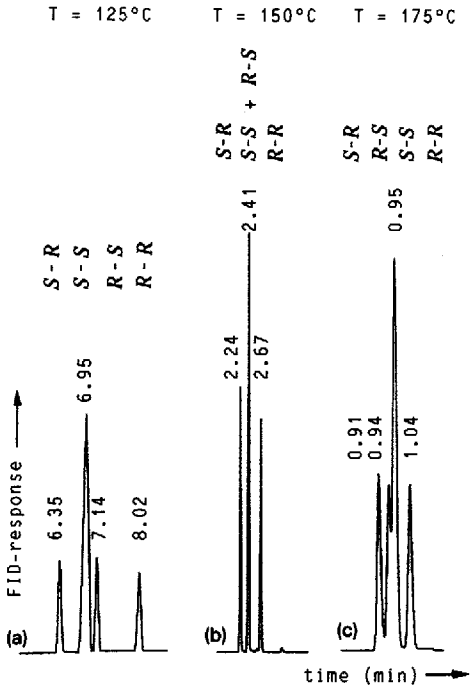

Fig. 4. Temperature dependence of the elution order of the diastereomeric pair *S–S* and *R–S* of PFP-(*R,S*)-Ala (*R,S*)-2-octyl esters on Chirasil-D-Val [sample enriched with PFP-(*S*)-Ala (*S*)-2-octyl ester]. (a) *S–S* is eluted before *R–S*; (b) *S–S* and *R–S* are eluted together; (c) *R–S* is eluted before *S–S*. Isothermal temperatures *T* and net retention times (min) are given; carrier gas, hydrogen at 0.5 bar.

before *R–S* and at 150°C *S–S* and *R–S* eluted together whereas at 175°C *R–S* eluted before *S–S*.

In summary, the resolution of (*R,S*)-AA as diastereomeric 2-alkyl esters on a chiral L-phase (Chirasil-L-Val) ideally will lead to the separation of four stereoisomers, *R–S* being eluted first and *S–S* last (fourth) in all instances. The elution order on an L-phase of the second and third stereoisomers (*i.e.*, *S–R* before *R–R* or *vice versa*) depends mainly on the structure of the chiral alcohol. The *S–R* and *R–R* diastereomers might also elute together or might be incompletely resolved (*cf.*, Fig. 1), depending on the selectivity of a certain stationary phase. A temperature dependence of the elution order has been demonstrated so far for the example PFP-(*R,S*)-Ala 2-octyl ester (*cf.*, Fig. 4).

Separation of TFA-α-alkyl-α-amino acid 2-alkyl esters on Chirasil-D-Val

In previous investigations it was found [22] that enantiomeric N-acyl-AAA *n*-propyl esters were satisfactorily resolved on commercial fused-silica capillary columns coated with Chirasil-L-Val, although the resolution coefficients in general were lower than those of AA. In spite of these findings, it was of interest to determine whether or not stereoisomers of representative AAA, in analogy with AA, were separable as 2-alkyl esters on Chirasil-Val, as in particular diastereomeric PFP-AAA

TABLE II

ELUTION ORDER STEREOISOMERS OF TFA-(*R,S*)-AAA (*R,S*)-ALKYL ESTERS ON A 25-m CHIRASIL-D-VAL CAPILLARY COLUMN

Net retention times, t (min), isothermal temperature, T (°C), number, n, of separated stereoisomers and resolution factors, α, are given. For GC conditions, see Experimental.

Alkyl group in alkyl ester	(*R,S*)-AAA[a]	t (min)				T (°C)	α (*R–S*/*S–S*)	n
		1st peak (*S–S*)	2nd peak	3rd peak	4th peak (*R–S*)			
2-Butyl	α-Et-Ala	6.46	6.46	6.57	6.65	75	1.029	3
	α-Bu-Ala	10.73	10.92[b]	11.13[c]	11.38	85	1.061	4
	α-Me-Leu	8.06	8.06	8.19	8.30	85	1.030	3
	α-Me-Met	9.60	9.60	9.77	9.87	115	1.028	3
	α-Me-Ser	8.12	8.12	8.22	8.22	85	1.012	2
	α-Me-Phe	9.11	9.11	9.11	9.11	130	1.000	1
3-Methyl-2-butyl	α-Et-Ala	6.72	6.72	6.88	6.88	85	1.024	2
	α-Bu-Ala	8.10	8.30	8.30	8.55	100	1.056	3
	α-Me-Leu	6.58	6.58	6.68	6.75	90	1.026	3
	α-Me-Met	8.92	8.92	9.24	9.24	130	1.036	2
	α-Me-Ser	7.68	7.68	7.97	7.97	95	1.038	2
	α-Me-Phe	8.76	8.76	8.76	8.76	140	1.000	1
2-Octyl	α-Et-Ala	7.63	7.73	7.73	7.86	120	1.030	3
	α-Bu-Ala	7.75	7.89[c]	8.27[b]	8.41	135	1.085	4
	α-Me-Leu	8.77	8.88[c]	9.10[b]	9.20	125	1.049	4
	α-Me-Met	7.86	7.86	8.57	8.57	160	1.090	2
	α-Me-Ser	7.11	7.11	7.56	7.56	130	1.063	2
	α-Me-Phe	8.78	8.78	8.91	8.91	170	1.015	2

[a] Et = Ethyl; Bu = butyl; Me = methyl.
[b] *S–R*.
[c] *R–R*.

(*S*)(+)-3-methyl-2-butyl esters and PFP-AAA (*S*)(+)-2-octyl esters showed good resolution properties on achiral methylpolysiloxane (CP-Sil-5) [24].

The laboratory-made Chirasil-D-Val column, specially designed for the highest resolution of TFA-DL-AA 1-propyl esters, achieved the resolution of the four possible stereoisomers of selected TFA-(*R,S*)-AAA (*R,S*)-2-alkyl esters, namely for TFA-(*R,S*)-α-But-Ala (*R,S*)-2-butyl ester, TFA-(*R,S*)-α-But-Ala (*R,S*)-2-octyl ester and TFA-(*R,S*)-α-Me-Leu (*R,S*)-2-octyl ester (Table II; Fig. 1m–o). If separated, the highest values were found for the diastereomeric pair *R–S* and *S–S* (*cf.*, Table II).

The elution order of stereoisomers of TFA-(*R,S*)-AAA (*R,S*)-2-alkyl esters is explained as follows. On non-chiral stationary phases *S–S* diastereomers are eluted before *R–S* diastereomers and *R–R* diastereomers before *S–R* diastereomers. This is the opposite elution order to that found for AA [21,24]. In contrast, on chiral stationary phases AAA and AA derivatives show the same elution order [21]. With Chirasil-D-Val *S–S* diastereomers are eluted before *R–R* diastereomers and *S–R* diastereomers before *R–S* diastereomers. (The possible elution order of stereoisomers of AAA on Chirasil-L-Val and Chirasil-D-Val as compared with those of AA is shown in Table III; see also Fig. 2b.)

TABLE III

POSSIBLE ELUTION ORDERS (WITH INCREASING RETENTION TIME FROM LEFT TO RIGHT) BY SUPERIMPOSITION OF THE DIASTEREOMERIC AND ENANTIOMERIC RESOLUTION EFFECTS FOR AA AND AAA ON CHIRASIL-L-VAL AND CHIRASIL-D-VAL

Acids	Chirasil-L-Val	Chirasil-D-Val
AA	*R–S* < *S–R* < *R–R* < *S–S* or	*S–R* < *R–S* < *S–S* < *R–R* or
	R–S < *R–R* < *S–R* < *S–S*	*S–R* < *S–S* < *R–S* < *R–R*
AAA	*R–R* < *S–S* < *R–S* < *S–R* or	*S–S* < *R–R* < *S–R* < *R–S* or
	R–R < *R–S* < *S–S* < *S–R*	*S–S* < *S–R* < *R–R* < *R–S*

As with AA, diastereomeric and enantiomeric resolution effects for AAA are additive for *S–S* and *R–S* diastereomers; of the four possible stereoisomers *S–S* is eluted first and *R–S* is eluted last (fourth) in all instances. The elution order of the second- and third-eluted stereoisomers is *R–R* before *S–R* or *vice versa*. With TFA-(*R,S*)-α-But-Ala (*R*)-2-butyl ester *S–R* is eluted before *R–R*, and with TFA (*R,S*)-α-But-Ala (*R*)-2-octyl ester and TFA-(*R,S*)-α-Me-Leu (*R*)-2-octyl ester *R–R* is eluted before *S–R* at the temperatures used (*cf.*, Table II).

The results presented are of interest for (a) the separation and determination of (*R,S*)-AA and (*R,S*)-AAA as diastereomeric esters on chiral stationary phases, in particular when the esterification reagent is not optically pure, (b) for the chromatographic determination of the absolute configuration of non-protein AAA and (c) for obtaining an insight into chromatographic resolution mechanisms of stereoisomers.

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