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Gas chromatographic separation of diastereomeric amino acid derivatives on chiral stationary phases

Application to the determination of enantiomeric composition in (*S*)-(+)-2-butanol

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ABSTRACT

Diastereomeric derivatives of amino acid enantiomers were formed by the reaction of either their amino or carboxylic acid group with a chiral reagent. (*S*)-(+)-2-Butyltrifluoroacetyl and methyltrifluoroacetyl-(*S*)-(−)-prolyl derivatives of (*R,S*)-amino acids were resolved by gas chromatography on achiral and chiral stationary phases, respectively. The elution order of *sec.*-butyltrifluoroacetyl derivatives of (*R,S*)-leucine was established on Chirasil-Val. The combination of using (*S*)-(+)-butanol with a highly selective Chirasil-Val was used to determine the exact enantiomeric composition in the chiral alcohol. The mean value of the (*R*)-(−)-2-butanol obtained was 2.02%.

INTRODUCTION

Over the past 15 years, extensive developments have been made in the separation and analysis of amino acids by gas chromatography (GC). Undoubtedly, the most attractive application of GC in the amino acid field has been the resolution of amino acids into their optically active enantiomers. GC is the most effective and nowadays the most popular means of establishing the optical configuration or optical purity of chiral compounds, either singly or in mixtures [1–4]. Generally, GC resolution of amino acid enantiomers can be achieved by using either achiral or chiral stationary phase methods [5–9].

Resolution on an achiral phase requires the conversion of enantiomers into diastereomers with a suitable chiral reagent prior to GC analysis. (*S*)-(+)-2-Butanol and trifluoroacetyl (TFA)-(*S*)-(−)-prolyl chloride (S-TPC) have been the most popular chiral reagents in the above diastereomeric method [10–12]. One disadvantage of the method is that chiral reagents of highly optical purity are not always readily available, and optical impurity results in some determination errors in GC analyses.

With the introduction of chiral stationary phases, the direct GC resolution of amino acid enantiomers becomes possible. Since 1966, a variety of chiral phases with differing thermal stabilities have been developed [13–15]. The problem of optically impure reagents does not arise in the method which uses chiral phases. However, difficulties were encountered in separating low-volatility (*R,S*)-amino acids such as proline and aspartic acid applied in biogeochemical studies [16]. These two [*R,S*]-amino acids showed a low separation factors as TFA derivatives and were poorly resolved as heptafluorobutyryl (HFB)-alkyl derivatives [17,18]. In order to obtain high sensitivity, HFB-alkyl derivatives of amino acids are very useful when using electron-capture

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detection [19]. Hence the two methods are sometimes complementary; in other words, a certain pair of amino acids can be well resolved on an achiral stationary phase rather than on a chiral phase, and *vice versa*.

In order to overcome the difficulties of optical impurity in chiral reagents, the advantage of combining the use of a chiral reagent with a chiral phase has been demonstrated [20,21]. This procedure allows all stereomers to be completely separated and the errors due to the enantiomeric impurity of the chiral reagent can be eliminated [22].

In this work, the second chiral centre, necessary for the formation of diastereomeric derivatives, was introduced by two classes of derivatizations with chiral reagents. One was based on esterification with (*S*)-(+)-2-butanol and the other on acylation with S-TPC. These two classes of diastereomeric derivatives were then resolved on achiral and chiral phases, respectively. The (*S*)-(+)-2-butyl-TFA derivatives of (*R,S*)-leucine chromatographed on Chirasil-Val were then applied to the determination of enantiomeric composition in a chiral alcohol.

EXPERIMENTAL

Chemicals and reagents

Racemic and optically active amino acids were obtained from the Shanghai Institute of Biochemistry (Shanghai, China) and from L. Light (Colnbrook, UK). N-Trifluoroacetyl-(*S*)-(−)-prolyl chloride was synthesized as described previously [23]. Trifluoroacetic anhydride (TFAA) was purchased from BDH (Poole, UK), (*S*)-(+)-2-butanol from Fluka (Buchs, Switzerland) and *sec.*-butyl alcohol [(±)-2-butanol] from Tokyo Kasei Kogyo (Tokyo, Japan).

Methanol and dichloromethane (DCM) were dried and fractionated before use. Solutions of 1.25 *M* methanol, 3 *M sec.*-butyl alcohol and 3 *M* (*S*)-(+)-2-butanol in hydrochloric acid were prepared by bubbling dry hydrogen chloride gas into the respective alcohols until the required weights were obtained.

Apparatus and GC conditions

GC was carried out using the following instruments and achiral and chiral columns: (A) HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) and an HP-5 fused-silica column (25 m × 0.32 mm I.D.) (Hewlett-Packard); (B) the same instrument as in (A) but with a Chirasil-L-Val fused-silica column (25 m × 0.25 mm I.D.) (Chrompack, Middelburg, Netherlands); (C) GC-5A gas chromatograph (Shimadzu, Kyoto, Japan) and an OV-17 glass capillary column (30 m × 0.37 mm I.D.) (Shanghai Reagent Plant, Shanghai, China); (D) same instrument as in (C) but with a Chirasil-Val glass capillary column [24] (23 m × 0.25 mm I.D.) (Dalian Institute of Chemical Physics, Dalian, China). The instruments were equipped with a flame ionization detector and fitted with an HP 3396A and Shimadzu E-1A integrator, respectively. The injector and detector temperatures were 250°C and the splitting ratio was *ca.* 1:100. The temperature programmes were as follows: (A) 5 min at 80°C, then increased at 4°C/min to 185°C; (B) 95°C isothermal; (C) 3 min at 180°C, then increased at 2°C/min to 230°C; and (D) 105°C isothermal for (*S*)-(+)-2-butyl TFA derivatives and 150°C isothermal for methyl TFA-(*S*)-(−)-prolyl derivatives. The carrier gas was nitrogen, except (A) hydrogen.

Formation of derivatives

(S)-(+)- and (±)-2-butyl-TFA derivatives. A solution containing 50–500 nmol of each (*R,S*)-amino acid was added to a vial and evaporated to dryness at 40°C with a stream of dry nitrogen. Remaining traces of water were removed by re-evaporation with DCM and the amino acids were esterified by the addition of 200 μl of acidic 2-butanol, with vortex mixing and heating at 105°C for 1 h. After cooling, the solution was removed with nitrogen at 45°C and the last traces of 2-butanol were removed by re-evaporation with DCM. N-Trifluoroacetyl derivatives were prepared by dissolving the residue in 200 μl of DCM and 100 μl of TFAA with heating at 120°C for 20 min in an oil-bath. After cooling to room temperature, the solution was removed using a stream of nitrogen and the residue was dissolved in 100 μl of DCM.

Methyl-TFA-(S)-(−)-prolyl derivatives. A solution containing 35–70 μmol of each (*R,S*)-amino acid was dried at 50°C with dry nitrogen. The residue of the amino acid mixture was dissolved in 1 ml of acidic methanol and heated at 90°C, without capping, to synthesize the amino acid methyl esters. The methyl esters were then dissolved in 1 ml of

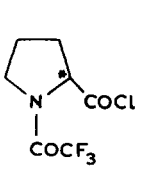
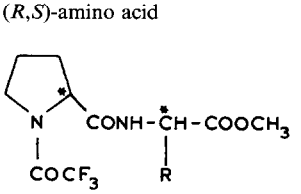
DCM and made to react with 1 ml of S-TPC together with 3–6 drops of triethylamine to synthesize the methyl-TFA-(*S*)-(−)-prolyl esters. With the addition of triethylamine, the pH of the mixture was checked to verify that it was basic (pH 10–12). This derivatization was carried out at room temperature for 1 h, and 1 ml of 6 *M* hydrochloric acid was added to terminate the reaction. The solution was then stirred and centrifuged to separate the aqueous and organic layers. The organic layer containing the diastereomeric amino acid esters was removed and dried by shaking with anhydrous magnesium sulphate. The magnesium sulphate was washed with 1.5 ml of DCM, filtered and then combined with the initial solution. The DCM was evaporated with a stream of dry nitrogen at room temperature and the residue was dissolved in 200 µl of DCM.

RESULTS AND DISCUSSION

Different kinds of derivatizing reagents used in derivatizations and the diastereomeric derivatives formed are listed in Table I. Four stereomers can be formed when an (*R,S*)-amino acid, having one chiral centre, reacts with an optically impure reagent. The stereomers are designated *RS*, *SR*, *RR* and *SS*, where the first letter indicates the configuration of the amino acid and the second the configuration of the chiral reagent. For example, when (*R,S*)-valine is reacted with optically impure S-TPC, four reaction products are obtained. Their interrelationships are shown in Fig. 1. The separation parameters of (*S*)-(+)-2-butyl-TFA esters and methyl-TFA-(*S*)-(−)-prolyl esters of (*R,S*)-amino acids, which were obtained on HP-5 and OV-17 stationary phases, re-

TABLE I

DIASTEREOMERIC AMINO ACID DERIVATIVES FOR GAS CHROMATOGRAPHY

Classification	No.	Esterifying reagent[a]	Acylating reagent	Derivative[b]
I	1	$CH_3CH_2\overset{*}{C}HCH_3 \cdot HCl$ (OH)	$(CF_3CO)_2O$	$R\text{-}\overset{*}{C}H\text{-}COO\overset{*}{C}HCH_2CH_3$ (CH_3; $NHCOCF_3$)
		(*S*)-(+)-2-Butanol	TFAA	(*S*)-(+)-2-Butyl-TFA esters of (*R,S*)-amino acid
	2	$CH_3CH_2\overset{*}{C}H(OH)CH_3 \cdot HCl$	TFAA	$R\text{-}\overset{*}{C}H\text{-}COO\overset{*}{C}H(CH_3)CH_2CH_3$ ($NHCOCF_3$)
		(±)-2-Butanol		(±)-2-Butyl-TFA esters of (*R,S*)-amino acid
II	1	$CH_3OH \cdot HCl$		
		Methanol	S-TPC	Methyl-TFA-(*S*)-(−)-prolyl esters of (*R,S*)-amino acid

[a] Asterisks denote chiral centres.
[b] R = Radicals of different complexities.

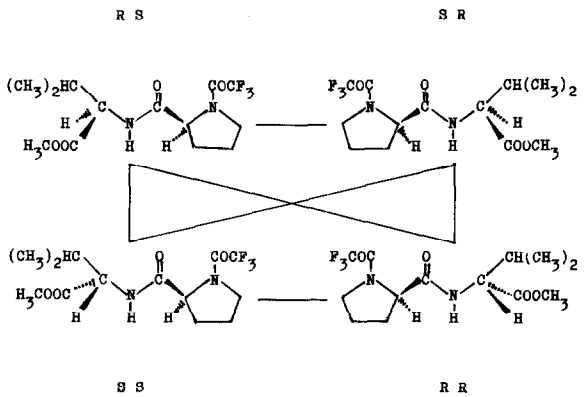


Fig. 1. Structures of methyl-TFA-(*R,S*)-prolyl derivatives of (*R,S*)-valine. Horizontal lines connect enantiomers; vertical and diagonal lines connect diastereomers.

spectively, are listed Table II. As *RS* and *SR*, and *RR* and *SS*, are enantiomers of each other, they cannot be resolved on achiral stationary phases. Therefore, separation parameters were based on the value of *RR* + *SS*/*RS* + *SR*. Figs. 2 and 3 show the GC resolution of these two classes of diastereomeric amino acid derivatives on the above stationary phases. It can be seen from the chromatograms that (*S*)-(+)-2-butyl-TFA esters and methyl-TFA-(*S*)-(−)-prolyl esters of (*RS*)-Pro and -Asp show a good resolution obtained by the diastereomeric method. The first peak of each enantiomeric pair of amino acids represents the *RS*/*SR* enantiomers on the chromatograms and the second the *RR*/*SS* enantiomers. They can only appear as diastereomeric pairs. This means that they co-elute with their enantiomers and add to the peak area. Hence an inherent error is present in GC analysis.

Fig. 4 illustrates the resolution of the conversion from three (*R,S*)-amino acids to the (*S*)-(+)-2-butyl-TFA derivatives by reaction with (*S*)-(+)-2-butanol. The two small peaks between two dominant peaks are due to the reaction of a small amount of (*R*)-(−)-butanol reagent. (*S*)-(+)-2-Butyl-TFA esters of glycine cannot be resolved on Chirasil-L-Val under the same isothermal conditions. Therefore, no small peak of the (*R*)-(−)-derivative [25] was observed on the chromatogram. When *sec.*-butyl alcohol, instead of (*S*)-(+)-2-butanol, reacted with a mixture of (*RS*)- and (*S*)-leucine (1:1, w/w), the resulting derivatives yielded four peaks [14] with relative areas of 1:1:3:3 on a Chirasil-Val column, as shown in Fig. 5. Each (*R*)- or (*S*)-leucine derivative can give a pair of peaks, which are approximately equal in area and appear close to each other. Similarly, four separate peaks were observed, as shown in Fig. 6, when methyl-TFA-(*S*)-(−)-prolyl esters

TABLE II

COMPARISON OF RETENTION TIMES (t_R) AND SEPARATION FACTORS (α) OF (*S*)-(+)-2-BUTYL-TFA (BTFA) DERIVATIVES AND METHYL-TFA-(*S*)-(−)-PROLYL (MTFP) DERIVATIVES OF (*R,S*)-AMINO ACIDS ON HP-5 AND OV-17 COLUMNS, RESPECTIVELY

Amino acid	Abbreviation	BTFA[a]		MTFP[b]	
		t_R (min)	α	t_R (min)	α
(*RS*)-Alanine	Ala	8.26 8.30	1.005	10.80 12.16	1.126
Glycine	Gly	8.87		13.14	
(*RS*)-Valine	Val	11.76 12.05	1.025	13.78 15.36	1.115
(*RS*)-Leucine	Leu	14.15 14.44	1.021	15.96 17.18	1.076
(*RS*)-Proline	Pro	19.49 19.87	1.020	22.72 24.96	1.099
(*RS*)-Aspartic acid	Asp	25.55 25.79	1.009	25.90 26.64	1.029

[a] Resolved on HP-5 column.
[b] Resolved on OV-17 column.

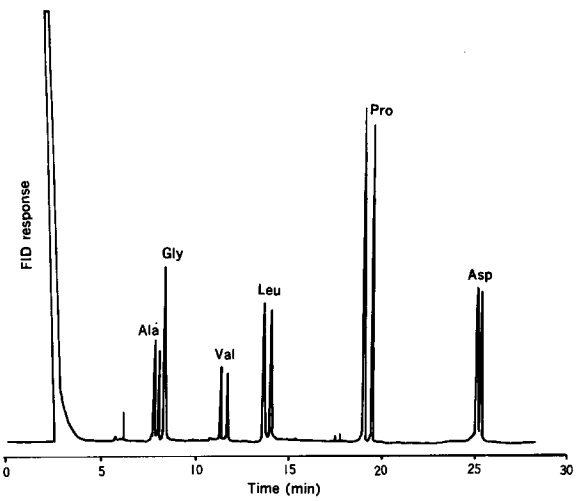


Fig. 2. GC resolution of (*S*)-(+)-2-butyl-TFA derivatives of (*R,S*)-amino acids on an HP-5 cross-linked column. Peaks: *RS*/*SR*, (*S*)-(+)-2-butyl-TFA esters of (*R*)-Ala, -Val, -Leu, -Pro and -Asp, and (*R*)-(−)-2-butyl-TFA esters of (*S*)-Ala, -Val, -Leu, -Pro and -Asp; *RR*/*SS*, (*R*)-(−)-2-butyl-TFA esters of (*R*)-Ala, -Val, -Leu, -Pro and -Asp, and (*S*)-(+)-2-butyl-TFA esters of (*S*)-Ala, -Val, -Leu, -Pro and -Asp; *R*/*S*, (*R*)-(−)-2-butyl-TFA ester and (*S*)-(+)-2-butyl-TFA ester of Gly.

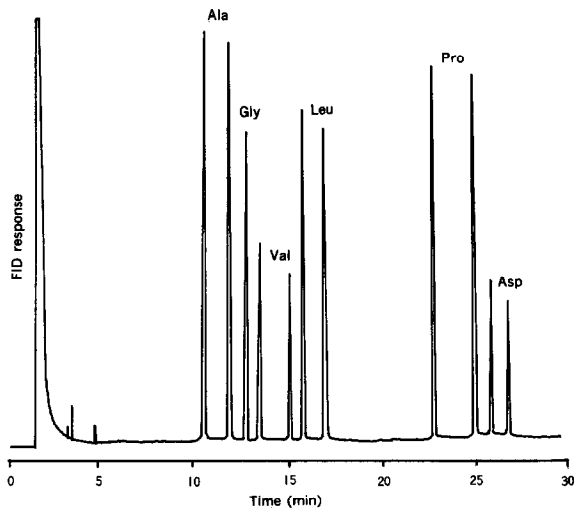


Fig. 3. GC resolution of methyl-TFA-(*S*)(−)-prolyl derivatives of (*R,S*)-amino acids on OV-17 support-coated open-tubular column. Peaks: *RS*/*SR*, methyl-TFA-(*S*)(−)-prolyl esters of (*R*)-Ala, -Val, -Leu, -Pro and -Asp, and methyl-TFA-(*R*)-(+)-prolyl esters of (*S*)-Ala, -Val, -Leu, -Pro and -Asp; *RR*/*SS*, methyl-TFA-(*R*)-(+)-prolyl esters of (*R*)-Ala, -Val, -Leu, -Pro and -Asp, and methyl-TFA-(*S*)-(−)-prolyl esters of (*S*)-Ala, -Val, -Leu, -Pro and -Asp; *R*/*S*, methyl-TFA-(*R*)-(+)-prolyl ester and methyl-TFA-(*S*)-(−)-prolyl ester of Gly.

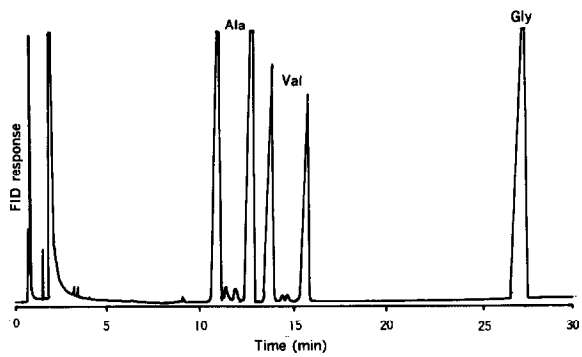


Fig. 4. GC resolution of (*S*)-(+)-2-butyl-TFA derivatives of (*R,S*)-amino acids on Chirasil-L-Val wall-coated open-tubular (WCOT) column. Peaks: *RS*, (*S*)-(+)-2-butyl-TFA esters of (*R*)-Ala and -Val; *RR*, (*R*)-(−)-2-butyl-TFA esters of (*R*)-Ala and -Val; *SR*, (*R*)-(−)-2-butyl-TFA esters of (*S*)-Ala and -Val; *SS*, (*S*)-(+)-2-butyl-TFA esters of (*S*)-Ala and -Val; *R* + *S*, (*R*)-(−)-2-butyl-TFA and (*S*)-(+)-2-butyl-TFA esters of Gly.

of (*R*)- and (*S*)-valine (1:2, w/w) were chromatographed on Chiralsil-Val. The behaviour of two classes of diastereomeric amino acid derivatives on chiral stationary phases is shown in Table III. (*R,S*)-Leucine shows the best resolution as (*S*)-(+)-2-butyl-TFA derivatives.

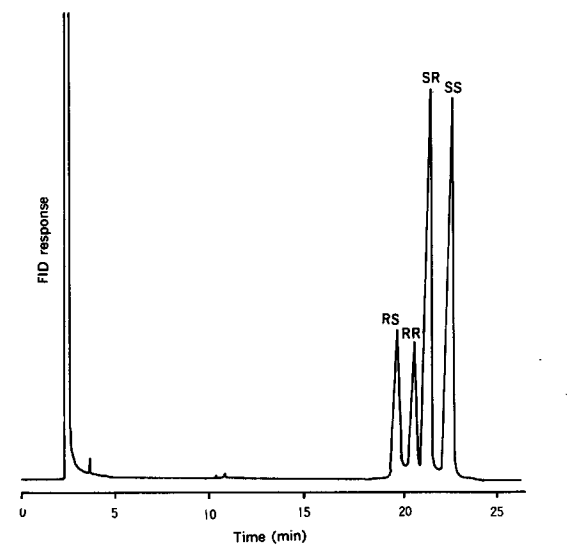


Fig. 5. GC resolution of (±)-2-butyl-TFA derivatives of (*RS*)- and (*S*)-leucine mixture on Chirasil-Val WCOT column. Peaks: *RS*, (*S*)-(+)-2-butyl-TFA ester of (*R*)-Leu; *RR*, (*R*)-(−)-2-butyl-TFA ester of (*R*)-Leu; *SR*, (*R*)-(−)-2-butyl-TFA ester of (*S*)-Leu; *SS*, (*S*)-(+)-2-butyl-TFA ester of (*S*)-Leu.

TABLE III

COMPARISON OF SEPARATION FACTORS (α) AND RESOLUTIONS (R) OF (*S*)-(+)-2-BUTYL-TFA DERIVATIVES AND METHYL-TFA-(*S*)-(−)-PROLYL DERIVATIVES OF (*R,S*)-AMINO ACIDS ON CHIRASIL-VAL COLUMNS

Amino acid	α		R		Resolving agent
	SR/RS	*SS/RR*	*SR/RS*	*SS/RR*	
(*RS*)-Alanine	1.068	1.131	1.727	1.596	(*S*)-(+)-2-butanol[a]
	1.074	1.078	1.810	2.430	S-TPC[b]
(*RS*)-Valine	1.059	1.025	3.556	2.542	(*S*)-(+)-2-butanol[a]
	1.115	1.045	1.020	2.180	S-TPC[b]
(*RS*)-Leucine	1.084	1.097	2.950	3.170	(±)-2-Butanol[b]

[a] Resolved on Chirasil-L-Val.
[b] Resolved on Chirasil-Val.

The use of two different types of diastereomeric derivatives, (*S*)-(+)- and (±)-2-butyl-TFA esters of (*R,S*)-amino acids, can readily identify the exact elution order of each stereomer, *i.e.*, the derivatives obtained by using (*S*)-(+)-2-butanol produce two dominant peaks during GC for a given racemic amino acid. It can be expected that the first and the last peaks are *RS* and *SS*, respectively, or *vice versa*. When (±)-2-butanol is used, the derivatives of (*R*)- and (*S*)- amino acids in the ratio 1:3 produce two pair of peaks with different heights. This result clearly demonstrates that the first of the four peaks is *RS* and the last is *SS*, the second *RR* and the third *SR*.

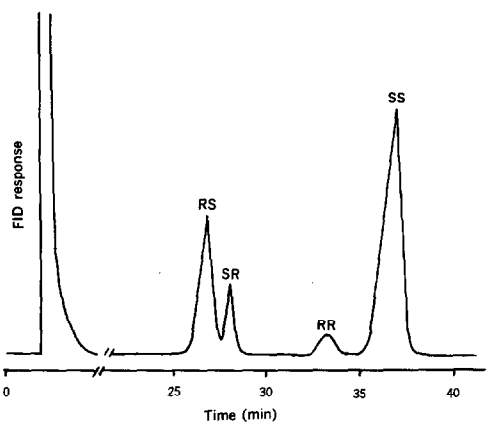


Fig. 6. GC resolution of methyl-TFA-(*S*)-(−)-prolyl derivatives of (*R,S*)-valine (1:2, w/w) on Chirasil-Val WCOT column. Peaks: *RS*, methyl-TFA-(*S*)-(−)-prolyl ester of (*R*)-Val; *SR*, methyl-TFA-(*R*)-(+)-prolyl ester of (*S*)-Val; *RR*, methyl-TFA-(*R*)-(+)-prolyl ester of (*R*)-Val; *SS*, methyl-TFA-(*S*)-(−)-prolyl ester of (*S*)-Val.

The chromatographic characteristics of the chiral stationary phase to resolve all stereomers can be utilized to determine the concentration of enantiomeric composition of (*S*)-(+)-2-butanol. Two 10 μmol/ml solutions were prepared, one containing (*RS*)-leucine and the other pure (*S*)-leucine. The two groups of leucine solutions were used to prepare a standard solution with a known *R/S* ratio. These solutions were derivatized and chromatographed as described above. The amount of each of the four isomers was determined by measuring the peak area. The percentage of (*R*)-(−)-butanol impurity was calculated from

$$\% = \frac{SR}{SR + SS} \cdot 100 \qquad (1)$$

and/or

$$\% = \frac{RR}{RR + RS} \cdot 100 \qquad (2)$$

where *SR*, *SS*, *RR* and *RS* are peak areas of the resulting isomers on the chromatogram. If a series of different enantiomeric ratios of amino acids are used, a suitable equation for calculation should be selected for the accuracy of measurement. When a low concentration of (*R*)-amino acid ($S \gg R$) is

TABLE IV

DETERMINATION OF THE PERCENTAGE OF (*R*)-(−)-2-BUTANOL IN COMMERCIAL (*S*)-(+)-2-BUTANOL USING THE CHIRASIL-VAL COLUMN

Leucine ratio[a] (*R*:*S*)	Peak area				(*R*)-(−)-2-Butanol (%)			
	RS	*RR*	*SR*	*SS*	Eqn. 1	Eqn. 2	Mean	SD
0:100	192	n.d.[b]	1508	71358	2.07	–		
15: 85	12364	152	1404	67447	2.04	n.c.[c]		
50: 50	9630	196	253	9919	2.49	2.00	2.02	0.38
	10021	170	274	10862	2.46	1.67		
	28933	423	684	28417	2.35	1.44		
	63263	957	1344	61562	2.14	1.49		

[a] Numbers in this column represent only the relative weights of (*RS*)-and (*S*)-leucine used; they do not represent true concentration ratios owing to the presence of enantiomeric impurities.
[b] Not detectable.
[c] Not calculable.

used, the peak area of *RR* is very small; likewise, when a low concentration of (*S*)-amino acid (*R* ≫ *S*) is used, the peak area of *SR* is also small. To avoid inaccuracy caused by the determination of small peak areas, the calculations involving these small peak areas should be omitted, *i.e.*, the former uses eqn. 1 and the latter eqn. 2. The results obtained from separate derivatizations are presented in Table IV. The mean value of the percentage of (*R*)-(−)-2-butanol and the standard deviation are 2.02% and 0.38% respectively.

CONCLUSION

The achiral and chiral phase methods can be combined as a new approach for separating all stereomers with a high enantioselectivity. This technique is suitable for the precise measurement of enantiomeric composition in chiral reagents and will be useful in eliminating errors when very different proportions of an enantiomeric mixture are analysed.

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