Chiral Amino Acid Microanalysis by Direct Optical Resolution of Fluorescent Derivatives on BSA-Based (Resolvosil) Columns

S. Allenmark[1]* / S. Andersson[2]

[1]Laboratory of Microbiological Chemistry, University of Gothenburg, Guldhedsgatan 10A, S-41346 Gothenburg, Sweden
[2]IFM, Department of Chemistry, University of Linköping, S-58183 Linköping, Sweden

Key Words

Column liquid chromatography
Chiral separations
Amino acids
Fluorogenic derivatization
N-(Chloroformyl)-carbazole

Summary

A new reagent for fluorescence labelling of amino acids, N-(chloroformyl)-carbazole, has been used in order to achieve high sensitivity of detection as well as good optical resolution. The derivatization reaction is fast and conveniently carried out at room temperature by shaking a buffered aqueous solution of the amino acids with an acetone solution of the reagent. The individual derivatized amino acids are first separated and collected via chromatography on a standard octadecyl-silica column. Each amino acid derivative is then analyzed for enantiomer composition by re-injection on a BSA-silica (Resolvosil®) column using fluorescence detection. For some amino acids (alanine, threonine, phenylalanine, tryptophan) enantiomeric separation factors exceeding $\alpha = 3$ have been obtained. The phosphonic acid analogue of alanine (1-aminoethylphosphonic acid) was also found to be well resolved into its enantiomers.

Introduction

Determination of amino acid configuration and optical purity on a small scale is of increasing importance in many areas of peptide and polypeptide chemistry. Characterization of synthetic peptides and structure elucidation of new peptide antibiotics of microbial origin are well-known examples of procedures which require such techniques. Furthermore, with the evolution of protein engineering, methods for microscale chiral amino acid analysis will be even more required in the future.

Although chiral, fluorogenic derivatization reagents for indirect determination of enantiomer composition, such as Marfey's reagent [1] and FLEC [2], have been developed and are often found to be useful, a direct technique is inherently more reliable.

Chiral liquid chromatography columns based on bovine serum albumin (BSA) have previously been shown [3–6] to be suitable for direct optical resolution of a number of different N-derivatives of amino acids. By the introduction of a fluorescent group, it is possible to bring into being a chiral amino acid analysis technique requiring only very small amounts of analyte. Dansyl- (5-dimethylamino-1-naphthalene-sulfonyl) [6] and FMOC- (9-fluorenyl-methoxycarbonyl) [7] amino acids have earlier been studied by us and shown to be optically resolvable. However, certain disadvantages were found to be associated with both of these derivatives and a reagent combining ease of derivatization with favourable chromatographic properties and giving high sensitivity in fluorescence detection was therefore sought. In this paper, we describe the use of a new derivatization reagent for amino acids, N-(chloroformyl)-carbazole [8], in combination with the separation and fluorimetric detection of enantiomers of amino acids, as well as an amino phosphonic acid, on BSA-based columns. A comparison with some corresponding FMOC-derivatives is also made.

Structures of the compounds discussed are given in Figure 1.

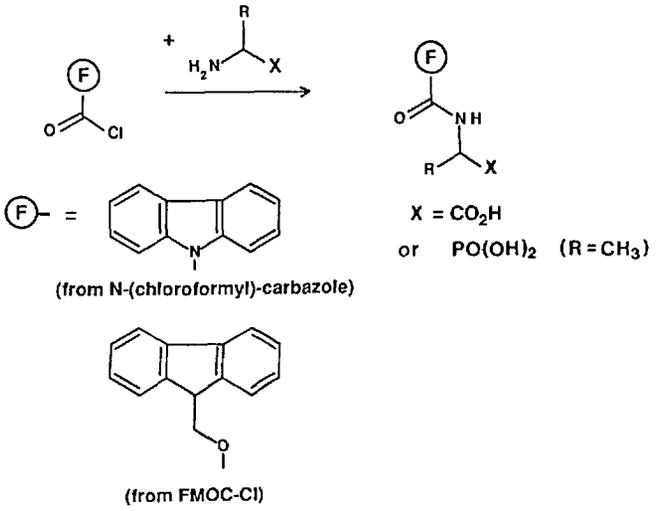

Figure 1
Chemical structures of reagents and analytes used in this study.

0009-5893/91/5 0429-05 $ 03.00/0

Experimental

Chemicals

The reagent was synthesized by phosgenation of carbazole as described previously [8, 9] and was used as a 15 mM solution in acetone. Both FMOC-Cl and di-(N-succinimidyl)-carbonate (DSC) were obtained from Fluka AG (Buchs, Switzerland). *Rac.* 1-amino-ethylphosphonic acid was from Sigma (St. Louis, MO) as were most of the amino acids used. All solvents and buffer chemicals were of analytical grade quality.

Column Preparation

The columns were prepared by immobilization with crosslinking of BSA to 100 Å Nucleosil 7 µ (Macherey-Nagel GmbH, Düren, FRG) or 100 Å Kromasil 7 µ (EKA-Nobel AB, Surte, Sweden), essentially according to the method described by Miwa et al. [10]. The silica was first 3-aminopropyl-functionalized using a standard procedure [11]. The BSA was allowed to adsorb to the support from a concentrated aqueous solution and immobilized with crosslinking by addition of the DSC reagent. This material was then packed into 150 × 4.6 mm steel columns.

Derivatization

The procedure used was a minor modification of the method described earlier for derivatization with FMOC-Cl [9, 10]. To 200 µl of the amino acid or amino acid mixture (1–100 µM) in 200 mM borate buffer (pH 9) 200 µl of the reagent solution was added. The mixture was then shaken for 1 min. The reagent excess and possible hydrolysis products were removed by extraction with 3 × 200 µl of hexane. An aliquot of the remaining aqueous phase was diluted with the mobile phase prior to injection. Usually, a 50-fold diluted sample was used when a direct injection of a single, derivatized amino acid was made onto the chiral column. In those cases where a mixture of derivatized amino acids was first separated on the achiral column and fractions collected, the sample was diluted five-fold or less. The same method was also used for the derivatization of the phosphonic acid.

A slightly modified procedure was used for the determination of enantiomeric purity. Here 500 µl of a 4 mM solution of the amino acid in 100 mM borate buffer (pH 9) was vigorously mixed with 900 µl of a 5 mM acetone solution of the reagent for 1 minute. Then about 0.5 ml of diethyl ether was added to yield a clear solution and shaking was continued for another 30 seconds. After mixing with ca 0.5 ml of water, giving phase separation, the organic upper phase was removed and the aqueous phase extracted twice with the same volume of ether. A 20 µl sample of the remaining aqueous phase was then injected into the achiral LC system and the eluate corresponding to the peak obtained for the derivatized amino acid was collected. This eluate was then analyzed for enantiomer composition by injection of 20 µl into the chiral system.

Liquid Chromatography

The chromatographic system was composed of an ERC mod. 64 high-pressure pump, a Rheodyne mod. 7125 injector valve equipped with a 20 µl loop, the analytical, chiral column, a Shimadzu mod. 510 fluorimetric detector and a Waters mod. 740 electronic integrator. In some of the chromatographic runs a Shimadzu mod. 535 fluorimetric detector coupled to a Shimadzu mod. C-R5A Chromatopac integrator was used. With the mod. 510 detector, the excitation and emission bandwidths used were 10 and 40 nm, respectively, whereas the bandwidth settings in the mod. 535 detector were both 13 nm. The carbazole derivatives were monitored at $\lambda_{exc.}$ = 287 nm and $\lambda_{emiss.}$ = 340 nm and the FMOC derivatives at $\lambda_{exc.}$ = 260 nm and $\lambda_{emiss.}$ = 313 nm. Mobile phase buffer concentrations were 50 mM unless otherwise indicated. Achiral, reversed-phase CC-amino acid separation was carried out with a 150 × 4.6 mm Nucleosil C-18 7 µ column, usually coupled to a variable wavelength UV detector (ISCO mod. V^4, Lincoln, NE, USA) for detection at 290 nm.

The elution order of the enantiomers was determined by separate chromatographic runs of derivatized L-amino acids, under the same conditions as used for the racemates.

Results and Discussion

We have previously shown that derivatization of amino acids with N-(chloroformyl)-carbazole at pH 9.0 is a rapid reaction even at room temperature [8]. Essentially, the derivatization reaction is carried out in much the same way as with FMOC [12, 13]. The derivatives formed (here called CC-derivatives) are, however, completely stable as a result of the urea-linkage and show shorter retention times and a higher fluorescence intensity. They are very well optically resolved on BSA-silica (Resolvosil®) columns and each enantiomer can be determined down to one picomol or even lower.

In Figure 2 the separation of five common protein amino acids as FMOC- and CC-derivatives, on an octadecyl-silica column, are compared. There is no change in elution order and when detected fluorimetrically under optimal conditions the CC-derivatives were found to give ca. three times higher sensitivity. The CC-derivatives were also less retained than the FMOC-derivatives by a factor of ca. 0.6 under identical mobile phase conditions [8].

With excess reagent, double derivatives are formed with amino acids having a nucleophilic functional group in the side chain. It has been reported [12, 14] that the double FMOC-derivative of histidine is unstable and slowly forms the mono-derivative when kept in solution. Such a behaviour could not be found with the corresponding CC-derivative. It is worth recalling that all FMOC-derivatives are alkali-labile and easily undergo β-elimination, particularly by secondary amines [14], with the formation of dibenzofulvene or a dibenzofulvene-amine adduct and regeneration of the amino acid after loss of carbon dioxide.

A comparatively rapid screening of the optical resolution of racemic amino acids as CC-derivatives on a BSA-silica column could be performed by using a mobile phase

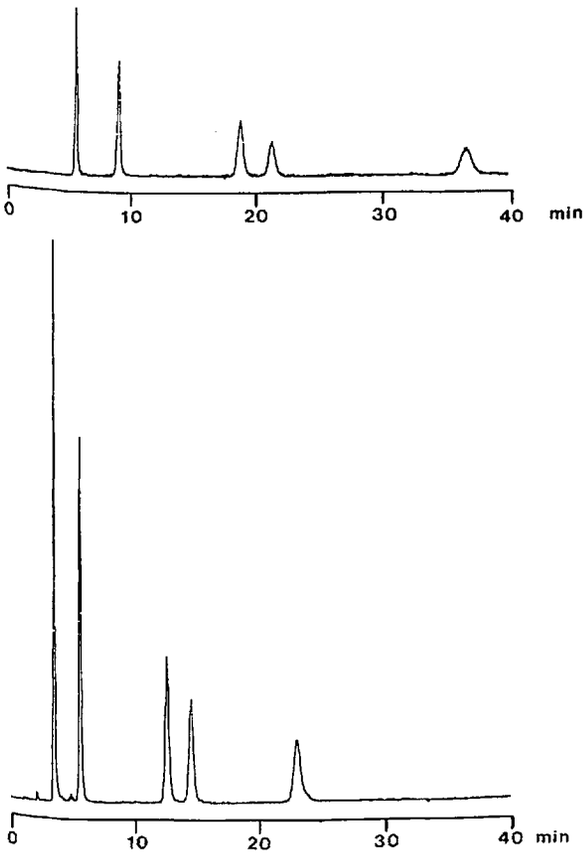

Figure 2

Chromatograms showing separation of a mixture of five protein amino acids (Ser, Ala, Val, Met, Leu (in order of elution)) as FMOC-derivatives (top) and CC-derivatives (bottom) on an octadecyl-silica column using identical chromatographic conditions. Mobile phase: Phosphate buffer (5 mM), pH 6.0, 25 % acetonitrile; 1.0 ml min^{-1}. Fluorescence detection at optimal wavelengths (260/313 and 287/340 nm, respectively). Each peak represents 20 pmol.

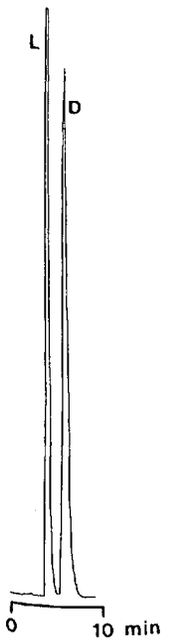

Figure 3

Optical resolution of DL-proline as CC-derivative. Mobile phase: 50 mM phosphate buffer containing 50 % of methanol, pH 8.5; 1.5 ml min^{-1}. Fluorescence detection.

containing 50 % of methanol. The least hydrophobic compounds (derivatives of e.g. ser, thr, his) were then run with mobile phases containing less organic modifier. In Figure 3 a separation of the enantiomers of proline is shown.

Retention data, obtained from various mobile phase compositions, are assembled in Table I. In general, a satisfactory separation of those enantiomers with reasonable retention times could be achieved at pH 7–8 with a methanol concentration between 30 and 50 %. From an analytical point of view, α-values much larger than necessary for baseline resolution are clearly undesirable, which means that an $\alpha = 1.5$ will usually be optimal. It is evident, from the data collected, that this condition is readily reached by a variety of mobile phase conditions for most of the amino acids investigated. To achieve a high detection sensitivity, it is also necessary to reduce the k'-values while maintaining a near optimal α-value. A reduction of k' can always be achieved by an increase in the organic modifier content. However, in many cases this is also associated with a reduction of α. Usually, a more profitable way to reduce k' of protolytes is to change the pH of the mobile phase. However, since the response of the α-value of the analyte to a pH change is rather unpredictable, this kind of optimization is time-consuming and has to be carried out for each particular analyte. For example, if the carbazole derivatives of serine and threonine are compared, it is found that in 50 mM phosphate buffer with 30 % of methanol, a pH increase from 7.3 to 8.0 leads to an almost 50 % reduction of the k'-values of serine with a small increase in α, whilst in the case of threonine the same change leads to a larger reduction of k_2', resulting in a considerable decrease in α. Similarly, in 50 mM phosphate buffer with 12 % of 1-propanol, a pH increase from 7.3 to 8.5 leads to a decrease in the α-value of alanine, whilst for leucine in the same buffer with 20 % of 1-propanol, a pH increase from 6.0 to 8.0 leads to a significant increase in α.

Another illustration of how small structural changes in the analyte will influence the retention behaviour of the enantiomers is found by a comparison of serine and alanine in 30 % of methanol when the pH is changed from 8.0 to 7.3. As expected, k_1' and k_2' of both analytes are increased. Although this change yields a small decrease in α for serine, it gives rise to a large increase in α for alanine, since in this case k_2' is influenced to a much larger extent than k_1'. A further interesting result from a comparison of these two analytes is that 1-propanol is a more favourable organic modifier than methanol for serine, whereas the reverse holds for alanine.

A common behaviour of the acidic amino acids aspartic and glutamic acid is found in their relatively small variation in α with the mobile phase composition. This seems to be most pronounced in the case of glutamic acid.

The basic amino acids have to be investigated in some further detail since these are capable of forming double derivatives. It has been shown previously in FMOC-derivatizations that an excess of reagent will yield doubly labelled derivatives with lysine, ornithine and histidine [12]. It is therefore reasonable to assume that the same holds for derivatizations with the carbazole reagent. These

Table I. Selected retention data (on CC-derivatives) from different mobile phase compositions

Amino acid	Mobile phase*	k_1'	k_2'	α	First eluted enantiomer
SER	50, 8.5, 6 (P)	19.0	37.6	1.97	L
	50, 8.5, 8 (P)	11.2	20.2	1.80	
	50, 7.2, 12 (P)	6.0	10.0	1.67	
	50, 6.0, 20 (P)	2.82	4.0	1.40	
	50, 7.2, 12 (A)	9.83	13.4	1.37	
	50, 8.15, 20 (M)	25.2	44.1	1.75	
	50, 8.0, 30 (M)	5.84	8.92	1.53	
	50, 7.3, 30 (M)	11.1	16.5	1.48	
ALA	50, 8.5, 12 (P)	5.2	12.3	2.36	L
	50, 7.2, 12 (P)	7.07	19.2	2.72	
	50, 6.0, 20 (P)	3.34	5.82	1.75	
	50, 8.5, 40 (M)	1.80	3.86	2.15	
	50, 8.0, 30 (M)	5.12	15.0	2.93	
	50, 7.3, 30 (M)	10.7	38.4	3.58	
	10, 7.2, 50 (M)	1.17	2.40	2.06	
ASP	50, 8.0, 30 (M)	7.94	10.00	1.26	L
	10, 7.2, 50 (M)	3.59	5.24	1.46	
GLU	50, 8.5, 8 (P)	8.40	11.3	1.34	
	50, 8.5, 12 (P)	3.70	4.90	1.33	
	50, 7.2, 12 (P)	6.40	8.60	1.35	
	50, 6.0, 20 (P)	5.98	7.47	1.25	
	50, 8.0, 30 (M)	3.07	4.20	1.37	
MET	50, 8.5, 12 (P)	18.7	40.9	2.18	L
	50, 7.2, 12 (P)	29.6	72.0	2.44	
	50, 6.0, 20 (P)	8.02	13.1	1.63	
	50, 8.0, 30 (M)	21.8	25.3	1.16	
	10, 7.2, 50 (M)	2.98	4.07	1.37	
THR	50, 7.2, 12 (P)	5.0	15.1	3.0	L
	50, 6.0, 20 (P)	2.56	4.80	1.87	
	50, 8.0, 30 (M)	3.59	11.6	3.23	
	50, 7.3, 30 (M)	7.18	28.7	3.99	
VAL	50, 8.0, 30 (M)	8.93	13.23	1.48	L
	50, 6.0, 20 (P)	7.00	10.05	1.44	
LEU	50, 7.2, 12 (A)	56.0	119	2.13	
	50, 8.0, 20 (P)	4.08	6.04	1.48	
	50, 6.0, 20 (P)	12.9	17.1	1.32	
PHE	50, 8.0, 30 (M)	28.8	107.4	3.73	L
	10, 7.2, 50 (M)	3.73	19.7	5.28	
	50, 8.5, 50 (M)	1.20	6.93	5.78	
TYR	50, 8.0, 20 (P)	2.49	3.19	1.28	
	10, 8.0, 30 (M)	4.84	6.38	1.32	
	10, 7.4, 40 (M)	3.33	3.81	1.14	
ASN	50, 7.3, 30 (M)	4.57	9.14	2.0	L
	10, 7.2, 50 (M)	0.895	1.27	1.42	
	50, 6.0, 20 (P)	2.61	3.22	1.23	
	50, 8.5, 6 (P)	9.99	27.3	2.73	
GLN	50, 7.3, 30 (M)	4.29	4.29	1.00	
	50, 8.5, 6 (P)	9.77	11.16	1.14	
PRO	50, 8.5, 50 (M)	3.04	4.87	1.60	L
	50, 5.45, 50 (M)	2.23	2.71	1.22	
	10, 7.3, 30 (M)	5.50	10.15	1.84	
NVAL	50, 7.3, 30 (M)	32.9	71.0	2.16	
	50, 8.25, 40 (M)	4.63	7.87	1.70	
	10, 7.2, 50 (M)	2.55	4.31	1.69	
NLEU	50, 8.25, 40 (M)	10.75	13.9	1.30	
	10, 7.2, 50 (M)	4.78	6.83	1.43	
HIS	50, 7.3, 30 (M)	5.8	7.44	1.28	L
	50, 5.45, 50 (M)	1.5	1.5	1.00	
	50, 6.7, 4 (P)	20.27	33.35	1.65	
	10, 7.5, 30 (M)	5.91	8.17	1.38	

Table I (continued)

Amino acid	Mobile phase*	k_1'	k_2'	α	First eluted enantiomer
TRP	50, 8.0, 30 (M)	0.80	2.58	3.22	
ARG	50, 7.3, 30 (M)	5.02	5.02	1.00	
	50, 8.5, 6 (P)	10.75	11.84	1.10	
	10, 7.5, 4 (P)	15.8	18.2	1.16	
	50, 6.7, 4 (P)	15.0	17.0	1.135	
	25, 5.4, 10 (M)	20.9	22.8	1.09	
	10, 8.0, 30 (M)	5.51	6.97	1.26	
LYS	10, 7.2, 50 (M)	5.70	7.04	1.24	
	50, 8.0, 20 (P)	7.60	9.00	1.18	
	10, 8.0, 30 (M)	10.3	22.4	2.17	

* Values refer to: buffer concentration, pH and % organic modifier. P = 1-propanol; A = acetonitrile; M = methanol.

Table II. FMOC-derivatives of amino acids: Capacity factors at maximum α-values obtained

Amino acid	Mobile phase*	k_L'	k_D'	α
SER	50, 7.5, 30 (M)	15.8	25.1	1.59
ALA	50, 7.5, 15 (P)	16.8	25.0	1.49
ASP	50, 7.5, 10 (P)	20.8	20.8	1.00
GLU	50, 7.5, 10 (P)	13.4	16.4	1.22
LEU	50, 7.5, 15 (P)	40.6	48.9	1.20
VAL	50, 7.5, 15 (P)	28.2	53.2	1.89
PHE	50, 8.3, 20 (P)	42.1	62.1	1.46

* Values refer to: buffer concentration, pH and % organic modifier. P = 1-propanol; A = acetonitrile; M = methanol.

studies have not yet been completed, however, and will be presented in a later paper.

In Tables II and III are given the maximum α-values obtained for FMOC- and CC-derivatives, respectively, under the relatively limited selection of mobile phase conditions used. Many of these α-values are too large to be practically useful, but they reflect the high enantioselectivity exerted by BSA towards this type of compound. The FMOC-amino acids were generally more strongly retained than the corresponding CC-derivatives, while the latter yielded higher α-values, except for valine. The derivative of the phosphonic acid was found to be more strongly retained than that of alanine and it was also found to give a lower α-value. A comparison of data is given in Table III. A baseline separation could only be achieved under conditions giving relatively large k'-values.

Due to the high sensitivity achieved, very small amounts of the minor enantiomer can be determined in the presence of a large excess of the other without exceeding the capacity of the column. This is highly important in enantiomeric purity determination. Figure 4 illustrates how precisely an enantiomeric excess of more than 99 % can be determined

Table III. CC-derivatives of amino acids: Capacity factors at maximum α-values obtained

Amino acid	Mobile phase*	k_1'	k_2'	α
SER	50, 8.5, 6 (P)	19.0	37.6	1.97
ALA	50, 7.3, 30 (M)	10.7	38.4	3.58
ASP	10, 7.2, 50 (M)	3.59	5.24	1.46
GLU	50, 8.0, 30 (M)	3.07	4.20	1.37
MET	50, 7.2, 12 (P)	29.6	72.0	2.44
THR	50, 7.3, 30 (M)	7.18	28.7	3.99
VAL	50, 8.0, 30 (M)	8.93	13.2	1.48
LEU	50, 7.2, 12 (A)	56.0	119.0	2.13
PHE	50, 8.5, 50 (M)	1.20	6.93	5.78
TYR	10, 8.0, 30 (M)	4.84	6.38	1.32
ASN	50, 8.5, 6 (P)	9.99	27.28	2.73
GLN	50, 8.5, 6 (P)	9.77	11.16	1.14
PRO	10, 7.3, 30 (M)	5.50	10.15	1.84
NVAL	50, 7.3, 30 (M)	32.9	71.0	2.16
NLEU	10, 7.2, 50 (M)	4.78	6.83	1.43
HIS	50, 6.7, 4 (P)	20.27	33.55	1.65
TRP	50, 8.0, 30 (M)	0.80	2.58	3.22
ARG	10, 8.0, 30 (M)	5.51	6.97	1.26
LYS	10, 8.0, 30 (M)	10.3	22.4	2.17

* Values refer to: buffer concentration, pH and % organic modifier. P = 1-propanol; A = acetonitrile; M = methanol.

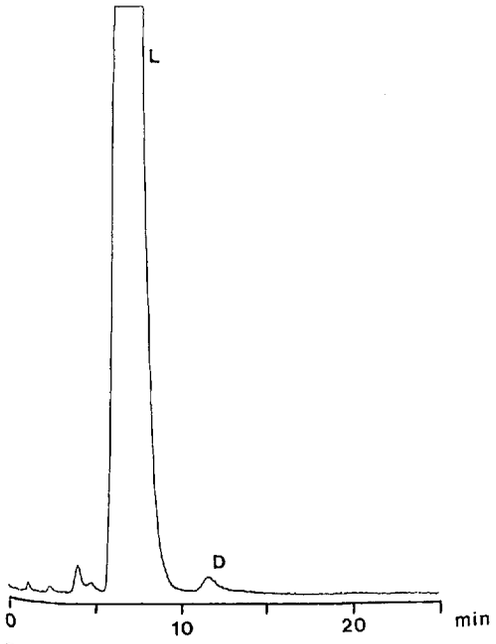

Figure 4

Chromatogram showing ca. 0.6 nmol of a commercial sample of L-alanine (as CC-derivative) showing 99.3 % enantiomeric excess (0.35 % of D-enantiomer present). Mobile phase: 50 mM phosphate buffer containing 30 % of methanol, pH 8.3; 1.5 ml min^{-1}. Fluorescence detection.

by integration over the separated peaks of the enantiomers. Since the derivatization reaction occurs without any detectable racemization, the data obtained by integration over the peaks from the separated enantiomers are highly reliable. As judged from Figure 4, even less than 0.1 % of the second enantiomer can be determined by the technique described. This means less than 600 fmol using the mod. 510 detector. Since the signal-to-noise ratio is significantly improved in modern fluorimeters such as the mod. 535, it should be possible to determine 0.01 % of an enantiomeric impurity, provided a clean, chromatographically purified, sample is used and the chiral separation shows no peak overlap.

In all the cases investigated thus far, the elution order is L- prior to D- (Table I). The method would therefore be even more favourable for D-amino acid purity determination, since then any tailing of the major peak would not interfere with the minor one.

Table IV. Differences in retention shown between CC-derivatives of alanine (1) and its phosphonic acid analogue (2)

Mobile phase*	k_1'	k_2'	α	Compound
50, 7.2, 12 % (A)	9.6	25.7	2.68	1
	27.6	42.6	1.54	2
20, 8.6, 10 % (A)	6.0	14.2	2.37	1
	41.9	65.9	1.57	2
50, 5.45, 50 % (M)	2.29	2.93	1.28	1
	2.74	2.74	1.00	2

* Values refer to: buffer concentration, pH and % organic modifier. P = 1-propanol; A = acetonitrile; M = methanol.

Acknowledgement

This work was supported by a grant (K-KU-2508-301) from the Swedish Natural Science Research Council. Financial support from the Swedish National Board for Technical Development (travel grants to S. Andersson) and from AB Hässle, Mölndal, Sweden, is also gratefully acknowledged.

References

[1] *P. Marfey,* Carlsberg Res. Commun. **49**, 591 (1984).
[2] *S. Einarsson, B. Josefsson, R. Möller, D. Sanchez,* Anal. Chem. **59**, 1191 (1987).
[3] *S. Allenmark, B. Bomgren, H. Borén,* J. Chromatogr. **264**, 63 (1983).
[4] *S. Allenmark, B. Bomgren, H. Borén,* J. Chromatogr. **316**, 617 (1984).
[5] *B. Bomgren, S. Allenmark,* J. Liquid Chromatogr. **9**, 667 (1986).
[6] *S. Allenmark, S. Andersson,* J. Chromatogr. **351**, 231 (1986).
[7] *S. Andersson, S. Allenmark,* Abstr. Th/F-P-016, The Thirteenth Symposium on Column Liquid Chromatography, Stockholm 1989.
[8] *S. Allenmark,* Tetrahedron Lett. **31**, 1455 (1990).
[9] *H. Hagemann,* In: Houben-Weyl, Methoden der Organischen Chemie, Band E4, Thieme, Stuttgart & New York, 1983, p. 50.
[10] *T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano, Y. Miyake,* Chem. Pharm. Bull. **35**, 682 (1987).
[11] *W.H. Pirkle, D.W. House, J.W. Finn,* J. Chromatogr. **192**, 143 (1980).
[12] *S. Einarsson, B. Josefsson, S. Lagerkvist,* J. Chromatogr. **282**, 609 (1983).
[13] *S. Einarsson, B. Josefsson, S. Lagerkvist,* Anal. Chem. **58**, 1638 (1986).
[14] *S. Einarsson,* Thesis, Chalmers University of Technology and University of Göteborg, Sweden, 1986 (ISBN 91-7032-270-8), p. 14.

Received: Jan. 28, 1991
Accepted: March 13, 1991
B