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Enantiomeric determination of amino compounds with high sensitivity using the chiral reagents (+)- and (−)-1-(9-anthryl)-2-propyl chloroformate

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Abstract

New chiral precolumn reagents, (+)- and (−)-1-(9-anthryl)-2-propyl chloroformate (APOC), are introduced for the chiral separation of amino acids and small peptides in capillary electrophoresis. Chiral separation of 17 amino acids and four small peptides as their diastereomeric 1-(9-anthryl)-2-propyl carbamate derivatives have been achieved by micellar electrokinetic chromatography. The detection limit for the derivatives is in the femtomole range with UV detection and in the attomole range with laser-induced fluorescence (LIF) detection. LIF detection was used to determine the enantiomeric excess of four APOC-derivatised peptides. The use of the new, anthracene-based reagents in conjunction with argon ion LIF makes enantiomeric determinations at ppm levels feasible. In this paper determinations below promille levels are performed without overloading the separation system. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Derivatization, electrophoresis; Amino acids; Peptides; 1-(9-Anthryl)-2-propyl chloroformate

1. Introduction

The stereoisomers of optically active compounds often differ in biological activity. The determination of those in biological systems is of increasing importance, as is the assessment of the optical purity of pharmaceuticals. Strict regulations on enantiomeric purity in the production of drugs will increase the need for reliable methods for the determination of the enantiomeric composition of chiral compounds. Chiral impurity quantifications at trace levels may well be routine procedures in the future [1,2].

Both direct and indirect chiral separation can be performed in capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC). In direct chiral separation a chiral additive, for instance copper(II)–aspartame [3], crown ether [4], chiral surfactant [5–7], vancomycin [8], or cyclodextrin [9–12], is included in the background electrolyte. Indirect chiral separation is performed by reacting the analyte enantiomers with the (+)- or (−)-form of a chiral reagent molecule, which will result in a diastereomeric pair that can be separated in CZE or MEKC without chiral additives. Chiral reagents that

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have been used for chiral resolution of amino acids or peptides in CE or MEKC include Marfey's reagent [13], 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [14], *o*-phthaldialdehyde / 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (OPA/TATG) [15], and 1-(9-fluorenyl)ethyl chloroformate (FLEC) [16]. If derivatisation of an analyte is necessary for detection purposes, then the incorporation of chiral selectivity by the formation of diasterioisomers is an attractive solution.

Wan et al. [16] compared direct and indirect chiral separation of amino acids by capillary electrophoresis. A number of amino acids could be separated by both methods. They succeeded in separating 10 FLEC-derivatised amino acids in one run with MEKC, but when a more complex mixture was injected many peaks comigrated. The indirect method generally provided higher separation efficiencies than the direct method. Tivesten and Folestad [15] separated 17 amino acids derivatised with OPA/TATG in less than 5 min with only a few comigrating pairs. Nishi et al. [14] separated 19 amino acids after derivatisation with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). Thirteen of these could be separated in one run. Tran et al. [13] separated some amino acids and small peptides by the use of Marfey's reagent.

The optical properties of five chiral reagents for primary amino compounds were examined by Houben et al. [17]. All five of the investigated reagents exhibit high molar absorptivity in the low UV region. Marfey's reagent and the OPA reagents possess absorbance at 340 nm, which increases the selectivity of the analysis. The OPA reagents react rapidly with primary amines at room temperature, whereas Marfey's reagent requires high temperature and prolonged reaction time for the formation of the derivatives. OPA/TATG have been used with UV-absorbance and lamp-based fluorescence detection in CE [15]. This reagent has also been used with the 325-nm emission line on the He–Cd laser in liquid chromatography [18]. The FLEC reagent has high absorbance at 260 nm and has been used as pre-column reagent in connection with micellar electrokinetic chromatography (MEKC) [14,19–21]. The derivatisation reaction of the FLEC reagent with primary and secondary amines is rapid (<5 min) at room temperature [22]. Further, it is suitable for laser-induced fluorescence (LIF) detection, as was shown by Chan et al. [19,20]. They used a pulsed KrF laser with a wavelength close to the absorption maximum at 260 nm. One disadvantage of using such a short wavelength is the high background fluorescence from buffer additives and contaminants.

The 1-(9-anthryl)-2-propyl chloroformate (APOC) reagent presented in this work, is analogous to the FLEC reagent. The difference is the chromophore. In APOC an anthracene moiety has been introduced instead of the fluorene chromophore of FLEC. The optical properties of the anthracene chromophore have previously been described for the achiral reagent 1-(9-anthryl)ethyl chloroformate (AEOC) [23,25]. A molar absorption coefficient of 190 000 l mol^{-1} cm^{-1} was reported [25]. Further, the absorption maxima at longer wavelengths permits the use of the 351 nm line of an argon ion laser for fluorescence excitation [24].

2. Experimental

2.1. The synthesis of the APOC reagent

The (+)- and (−)-APOC reagent were produced from (+)- and (−)-1-(9-anthryl)-2-propanol (APOH). The (+)- and (−)-APOH were synthesised from 9-bromoanthracene by the addition of *n*-butyllithium and 1,2-epoxipropane in ether. The reaction was allowed to proceed for 35 min at 0°C. The work-up comprised addition of aqueous ammonium chloride and extraction of the aqueous layer with dichloromethane. Flash chromatography was then performed on silica gel to separate (+)- and (−)-APOH from the undesired reaction products (+)- and (−)-2-(9-anthryl)propanol. The (+)- and (−)-APOH enantiomers were separated in preparative indirect chiral HPLC after (−)-camphanic acid chloride had been used to form the diastereoisomers that could be separated in this system. The (−)-camphanic acid-APOH esters were then subjected to acid hydrolysis. The chloroformates were formed by reacting the APOH enantiomers separately with phosgene in dry toluene [25].

2.2. Chemicals

Boric acid, phosphoric acid, borax, acetic acid, 2-propanol and methanol were from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was from USB (Cleveland, OH, USA). All amino acids and peptides were from Sigma (St. Louis, MO, USA), the γ-cyclodextrines used were from Sigma and Acros (Geel, Belgium) and the β-cyclodextrin was from Sigma. All buffer solutions were made with water from an Elgastat UHQII (Elga Ltd., High Wycombe, UK).

2.3. Apparatus

Two CE systems were used: a Prince auto sampler (Lauerlabs Inc., Emmen, The Netherlands) with an ISCO CV^4 UV detector (Lincoln, NE, USA) used at 256 nm for UV detection and an in house built system with argon ion LIF detection (exc, 351 nm; em, 20-nm wide bandpass filter centred at 412 nm). The laser used was an Innova argon 304 (Coherent, Palo Alto, CA, USA) with an optical arrangement similar to that described by Yeung et al. [26]. The separation capillaries were from Polymicro Technologies Inc. (Phoenix, AZ, USA). The HPLC system used to determine the yield of the amino acid reaction consisted of a Shimadzu LC-10AD pump (Kyoto, Japan), a Varian AminoTag column (Sunnyvale, CA, USA) and a Shimadzu RF-530 fluorescence detector. The mobile phase consisted of 31% 100 m*M* acetic acid, pH 4.35, and 69% methanol. Data collection was made with ELDS 900 (Chromatography Data System, Kungshög, Sweden). The molar absorption coefficient was measured on a Varian Cary-1E UV/VIS spectrometer (Mulgrave, Australia).

2.4. Derivatisation procedure

The amino acids and peptides were dissolved in 0.1 *M* HCl and these solutions were mixed with borate buffer (pH 8.6) to a final buffer concentration of 200 m*M*. From this solution 200 μl was mixed with 10 m*M* APOC reagent solution in acetone. The volume of reagent solution used was sufficient to maintain at least a 10-fold excess of reagent. After 10 min the reaction mixture was extracted with 0.5 ml *n*-hexane to terminate the reaction by removing excess reagent. The aqueous phase was diluted with water prior to injection so that the buffer concentration in the sample was lower than that of the separation media. This was done to ensure that the sample plug injected is not subjected to electrophoretic bandbroadening due to differences in conductivity from the separation buffer.

2.5. Separation conditions

The capillary was rinsed with five column volumes of each 0.1 *M* NaOH and water, then equilibrated with 10 column volumes of buffer before each run. For other separation conditions see the figure legends. Buffer concentration, SDS concentration, cyclodextrin concentration and pH are given as they were before the addition of organic modifiers. The pH was adjusted with NaOH or HCl. Samples were injected by the application of pressure on the injection side when the Prince instrument was used. When using the home-made apparatus with LIF detection, samples were injected gravimetrically by raising the inlet vial 11 cm for a specific time interval between 20 and 80 s. The pluglength injected of the peptide mixture was 0.3% of the length of the capillary. This corresponds to a volume of 2 nl. When the purity of the (+)-APOC reagent was determined the injected plug was 0.7% of the capillary or a volume of 10 nl.

3. Results and discussion

The conditions for the derivatisation were based on those earlier found to be suitable for AEOC [27]. A pH of 8.2 was used to prevent the phenolic side chain of tyrosine from reacting with APOC. No difference was found in the reaction rate between the enantiomers. This was investigated by adding APOC to racemic mixtures of alanine and proline and allowing the reaction to proceed for given time intervals. The diastereomers were then monitored on an isocratic HPLC system with fluorescence detection. The derivatisation reaction for the investigated amino acids was found to have gone to completion in less than 1 min. The reaction was found to be linear in the interval 10 n*M*–1 μ*M*. The

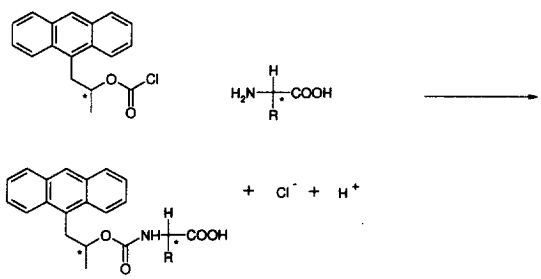

Fig. 1. Reaction of 1-(9-anthryl)-2-propyl chloroformate with a chiral amino acid.

reaction of APOC with a chiral amino acid is shown in Fig. 1.

When using chiral derivatising agents the enantiomeric purity of the reagent has to be exactly known [21,28–30]. The enantiomeric purity of (+)- and (−)-APOC was determined by reacting each enantiomer with glycine, thereby producing (+)- and (−)-APOC-glycine (APOC-Gly). A CD modified MEKC separation was used to separate the (+)- and (−)-APOC-Gly enantiomers (Fig. 2).

The enantiomeric purity of (+)-APOC was found to be 99.91±0.03% with a 95% confidence interval by standard addition procedure with 0.050, 0.10, and 0.15% additions of (−)-APOC-Gly. It is likely that the impurity of the reagent originates from an incomplete chiral separation of the APOH-camphanic ester enantiomers. This statement is supported by the fact that the impurity content of the (+)-APOC enantiomer in the (−)-APOC reagent was found to be 1.5%. LIF detection was employed in the purity determination of (+)-APOC whereas UV-absorbance detection was sufficient to detect the impurity in (−)-APOC without the need to overload the separation system.

A racemic mixture of the 20 protein amino acids and norvaline was derivatised and separated by MEKC. Selectivity was found for 17 of these. Two of the amino acids, cystine and histidine, are doubly labelled and migrates with the micelles under the investigated conditions. Separation and determination of all protein amino acids in one run could not be achieved when using the procedure we apply here. When 10 m*M* SDS was used D- and L-arginine were well separated (Fig. 3), but the low SDS

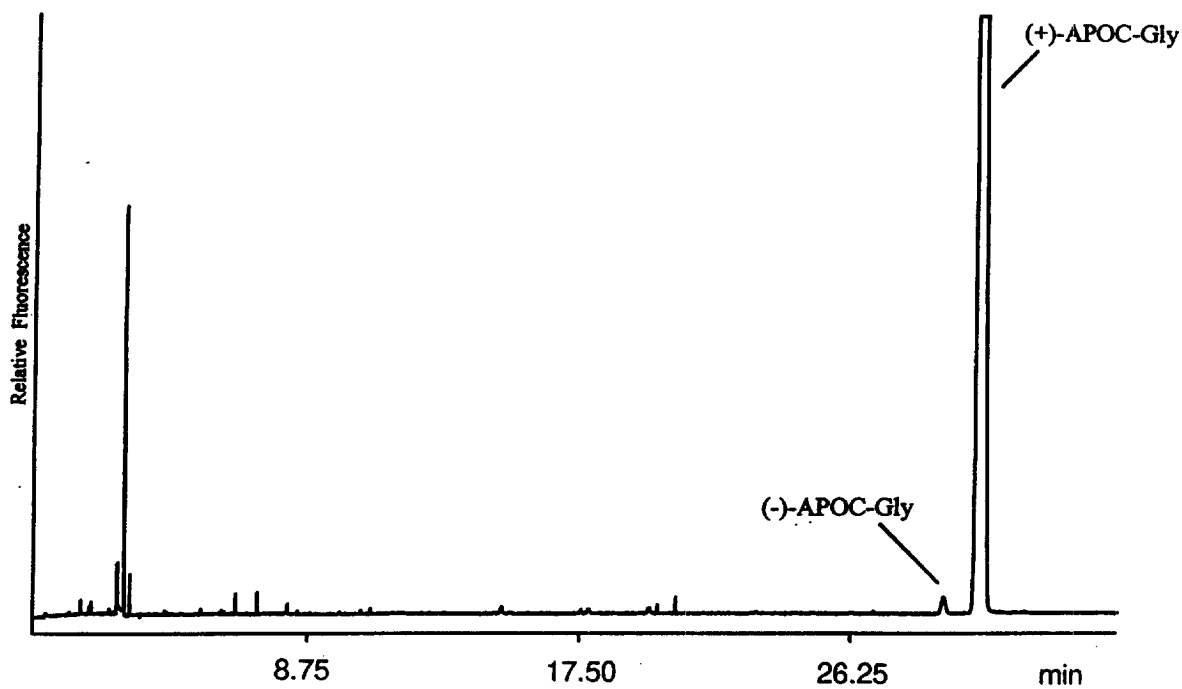

Fig. 2. Enantiomeric purity testing of (+)-APOC. Separation buffer: 10 m*M* MES buffer at pH 6.7, 30 m*M* SDS, and 5 m*M* γ-CD.

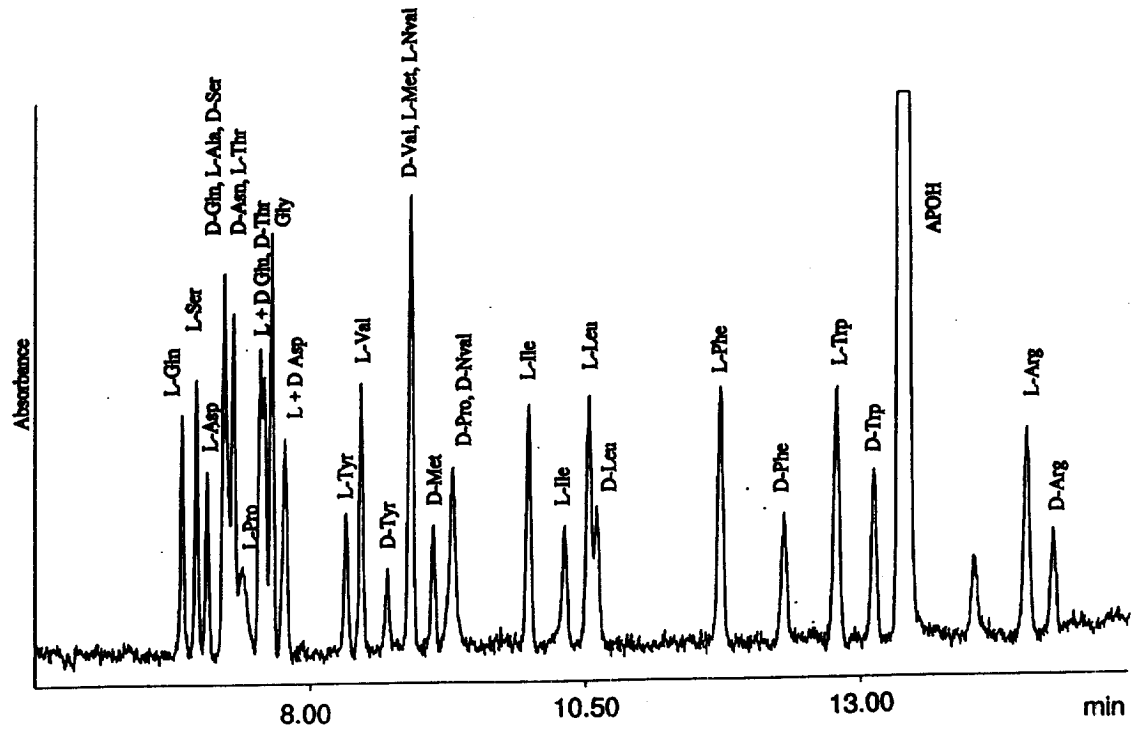

Fig. 3. Separation of a racemic amino acid mixture. Separation buffer: 28.75 m*M* borax, 10 m*M* SDS, pH 9.95. Capillary: 70 cm (57 cm to detection window), 50 μm I.D.; 30 kV. UV detection.

concentration resulted in the comigration of many of the early eluting amino acids. With 15 m*M* SDS a much better resolution of these compounds was achieved (Fig. 4). This, however, resulted in almost total loss of selectivity in the later part of the chromatogram. The proline peak was much broader than the other peaks which makes simultaneous baseline resolution of all amino acids difficult to achieve. This broad peak has also been observed for AEOC- [24], FMOC- [20], and FLEC- [16] derivatised proline in MEKC mode. It is likely that this broad peak depends upon the slow transitions between the *cis*- and *trans*-forms of the carbamate bond between the secondary amine function of proline and the carbonyl carbon of the reagent [31].

Four small peptides were derivatised with APOC and separated by MEKC (Fig. 5). The addition of 2-propanol to the buffer increased selectivity for the two last migrating peptides Leu–Gly and Leu–Gly–Gly. The selectivity for Ala–Gly and Ala–Gly–Gly was, however decreased by this addition. The same effects were found when using lower concentrations of SDS. Those observations are in line with the results of Wan and Blomberg [22], who separated these peptides after derivatisation with FLEC. The APOC derivatives are more hydrophobic than the FLEC derivatives, thus a lower concentration of SDS is optimal for the diastereomeric separation of the APOC-tagged species.

The molar absorptivity of APOC-derivatised glycine was investigated by HPLC-fractionation of the reaction products, APOC–glycine and 1-(9-anthryl)-2-propanol, with subsequent UV-absorption analysis. The molar absorptivity at 255 nm was found to be 224 000 l mol^{-1} cm^{-1} in an 55% acetate buffer (pH 4.35); 45% acetonitrile mixture. The same mixture of buffer and acetonitrile was used as a blank. The detection limits found for APOC-tagged phenylalanine with UV-absorbance and laser-induced fluorescence were 2 fmol and 0.5 amol, respectively. This is similar to those reported for AEOC derivatives [25]. Furthermore, the sensitivity with UV-

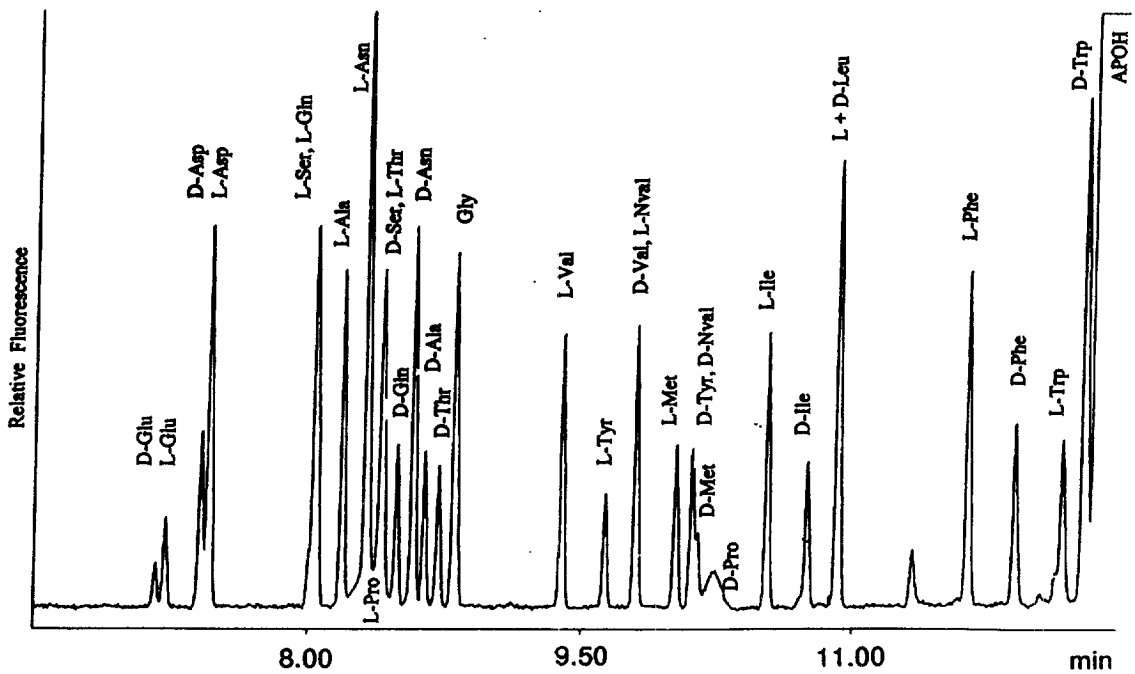

Fig. 4. Separation of a racemic amino acid mixture. Separation buffer: 20 m*M* borax, 15 m*M* SDS, pH 9.95. Capillary: 70 cm (62 cm to detection point), 21 μm I.D.; 30 kV. LIF detection.

absorbance detection for the APOC derivatives was 10-times higher than for FLEC derivatives using the same instrumentation. This corresponds to the differences in molar absorptivity for the chromophores.

The linear range of the detector may limit the determination of impurities at extremely low levels. This is due to the large concentration interval between the main compound and the impurity. In on-column UV-absorbance detection the lower limit of the linear range is determined by the short pathlength of light passing through the capillary while the high absorbance is limited mainly by stray light. Often, overloading of the separation system is necessary for the detection of the smaller impurity peak. The high concentration of the main compound can then cause serious peak distortion through electromigration dispersion, which in turn makes quantification difficult. With LIF detection this can be avoided because the detection limit is several orders of magnitude lower than with UV detection. In addition, the linear range of LIF is larger than that of UV-absorbance detection. In LIF the detection limit is mainly determined by stray light and background fluorescence. Fig. 5 shows the determination of the L-enantiomers in the D-form standards of the four small peptides performed with LIF detection. The enantiomeric purity of these peptides is presented in Table 1. The other impurities present in the chromatogram were also present in the water blank.

4. Conclusions

The short pathlength available for UV-absorbance detection in capillary electrophoresis necessitates the use of reagents with suitable optical properties. The high molar absorptivity found for APOC (ϵ= 224 000 l mol^{-1} cm^{-1}) makes this reagent attractive for use in chiral trace analysis of amino compounds. Good selectivity and high resolution obtained through indirect chiral separation enabled simultaneous enantiomeric purity determination of four small peptides. Selectivity could also be found for several D- and L-amino acids in complex amino acid mixtures. The compatibility of the reagent to argon

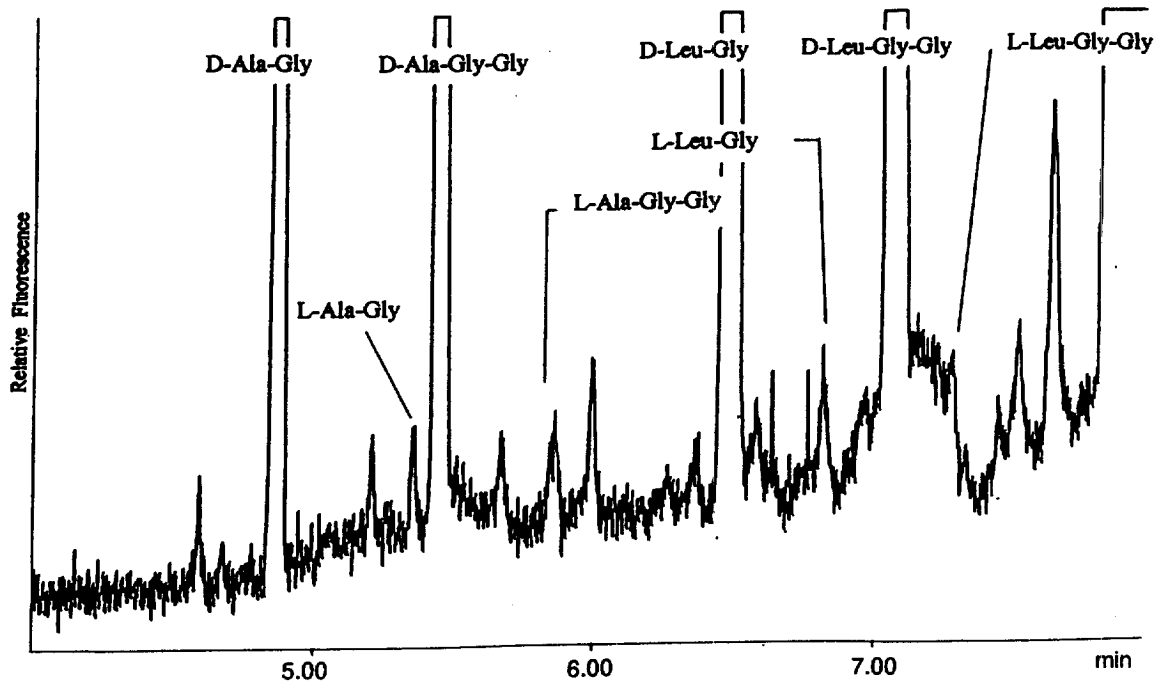

Fig. 5. Determination of chiral impurities in the D-form of the four peptides. Separation buffer: 10 m*M* borate, 7.5 m*M* phosphate, 15 m*M* SDS, pH 9.2. Capillary: 70 cm (62 cm to detection point), 21 μm I.D.; 30 kV. LIF detection. The concentration of the main components before derivatization was 500 μ*M*.

Table 1
Determination of the L-enantiomer impurities in the D-enantiomers of four small peptides using the (+)-APOC reagent

Peptide	% L-Enantiomer	Impurity of (+)-APOC (%)	Impurity of peptide (%)
Ala–Gly	0.13	0.09±0.03	≤0.07
Leu–Gly	0.28	0.09±0.03	≤0.22
Ala–Gly–Gly	0.15	0.09±0.03	≤0.09
Leu–Gly–Gly	0.12	0.09±0.03	≤0.06

ion LIF expands the possibilities for use in chiral trace analysis.

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