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Free solution capillary electrophoresis and micellar electrokinetic resolution of amino acid enantiomers and peptide isomers with L- and D-Marfey's reagents

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ABSTRACT

Separation of amino acid enantiomers and peptide isomers has been made possible through the use of Marfey's reagent and high-performance capillary electrophoresis (HPCE). Samples of amino acids and peptides were first derivatized with Marfey's reagent and subsequently analyzed by HPCE. Different modes of separation were investigated including free solution and micellar electrokinetic chromatography.

The use of micellar electrokinetic chromatography in combination with L- and D-Marfey's reagent offered unequivocal means to confirm the presence of D-amino acid in an unknown sample. This method is also particularly useful for the analysis of peptide isomers.

INTRODUCTION

Chirality analysis of amino acids is of particular interest in both the food and pharmaceutical industries. In the food industry, the racemization of amino acids could occur during food processing such as roasting or treatment of food proteins under alkaline conditions[1]. In the pharmaceutical industry, the interest ranges from control of racemization of amino acids during peptide synthesis to post-translational protein modification caused by deamidation during protein processing. Fig. 1 illustrates the well accepted mechanism of deamidation through the formation of a cyclic imide intermediate. The latter is a reactive species, readily undergoing hydrolysis to produce the corresponding deamidated peptide or isopeptide. During hydrolysis, racemization could occur at the C^{α} chiral center of asparagine (Asn) or glutamine (Gln)[2]. A simple way to study the deamidation process is to detect the presence of D-aspartic acid (D-Asp) or D-glutamic acid (D-Glu) and the release of ammonia in the deamidated sample.

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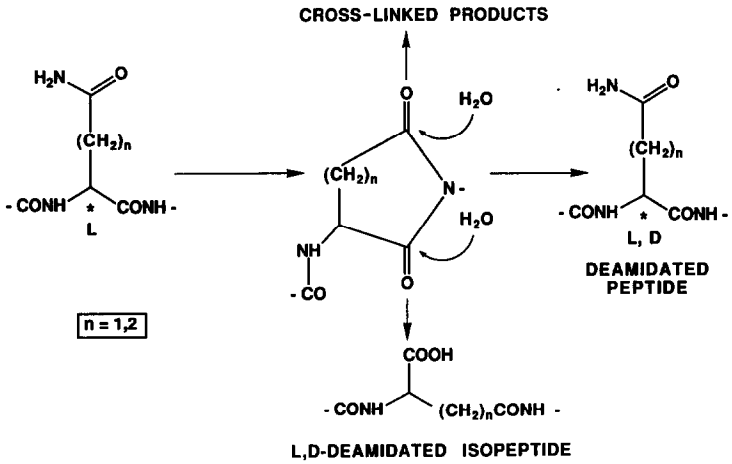
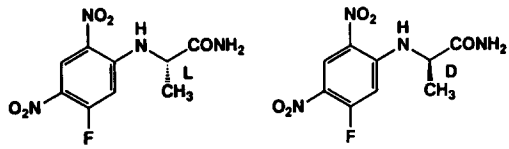


Fig. 1. Proposed mechanism for deamidation of asparaginyl and glutaminyl peptides and proteins.

Amino acid racemates are commonly resolved using high-performance liquid chromatography (HPLC) or gas chromatography (GC) and recently by capillary electrophoresis[3–5]. We report here the use of free solution capillary electrophoresis (FSCE), micellar electrokinetic capillary chromatography (MECC) in combination with L-Marfey's reagent (**1**), (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and D-Marfey's reagent (**2**), (1-fluoro-2,4-dinitrophenyl-5-D-alanine amide)[6] to provide a rapid and unambiguous technique for chiral analysis of amino acids and peptides.



1 **2**

EXPERIMENTAL

Apparatus

Separations were carried out on a capillary electropherograph P/ACE™ system 2000 (Beckman, Palo Alto, CA, U.S.A.) equipped wih a capillary cartridge 75 μm I.D. and 375 μm O.D.; the total length of the capillary was 57 cm (50 cm effective length) and was pretreated successively with 0.1 *M* hydrochloric acid and 0.1 *M* sodium hydroxide for 10 min each, then rinsed with water and electrolyte prior to use. The column temperature was maintained at 25 ± 0.1°C by means of a fluorocarbon liquid continuously circulated through the cartridge. A deuterium light source with a 214- or 340-nm bandpass filter was used and absorbance was monitored at a range of 0.02 a.u.f.s. Data collection was performed using the Beckman P/ACE system or Gold Chromatography data system version 4.0.

Materials

The phosphate buffer (pH 3.3) was prepared using 50 m*M* of ammonium phosphate monobasic from Fisher Scientific (Springfield, NJ, U.S.A.) and the pH of the solution was adjusted with a solution of orthophosphoric acid (0.05 *M*). The borate buffer (pH 8.0) was purchased from Beckman; sodium dodecyl sulfate (SDS) from Bio-Rad Labs. (Richmond, CA, U.S.A.); L-Marfey's **1** reagent from Pierce (Rockford, IL, U.S.A.); and acetonitrile from Baxter Health Care, a Burdick & Jackson Division (Muskegon, MI, U.S.A.). Dipeptides Ala–Ala–OH, Ala–Phe–OH and tripeptide Ala–Ala–Ala–OH isomers used in this study were purchased from Chemical Dynamics (Plainfield, NJ, U.S.A.). D-Marfey's reagent **2**, a new product, was synthesized in our laboratory following a similar procedure used for L-Marfey's reagent **1**[6]. The identity of this reagent was confirmed by ^{1}H-NMR, mass spectrometry, elemental analysis and optical rotation.

Derivatization methods

Method A. Marfey's reagent in acetonitrile (70 μl, 1% solution) was added to an aqueous solution of amino acid or peptide (50 μl, 0.05 *M*) in a 1-ml Reacti-Vial™ from Pierce, containing a micromagnetic stirring bar. To this mixture was added a solution of sodium bicarbonate (20 μl, 1 *M*). The vial was sealed with a screw cap and stirred at 35 ± 1°C for 90 min. After cooling to room temperature, the mixture was neutralized by addition of hydrochloric acid (20 μl, 1 *M*). The resulting solution was degassed. The mixture was then diluted with sodium borate buffer, pH 8.5 (340 μl, 0.1 *M*). Stock solutions of amino acids and peptides derivatized with Marfey's reagent are stable when stored at 25 ± 5°C in the dark. Electrophoresis was performed using solutions made by diluting the stock solutions with a running buffer (dilution 1:3). All running buffer solutions were filtered through an Acrodisc®-CR (1.0 μm) from Gelman Sciences (Ann Harbor, MI, U.S.A.) before use.

Method B[7]. The procedure for method B was similar to method A with the exception that Marfey's reagent was dissolved in acetone (1% solution) and the stirring was done at 55–60°C for 15 min. This method offered a much shorter reaction time when compared to method A.

RESULTS AND DISCUSSION

Determination of D,L-amino acids by free solution capillary electrophoresis

Amino acids are derivatized with Marfey's reagent as described under Experimental. The short reaction time at a moderate temperature (15 min, 50°C in acetone, method B) makes this method attractive. The derivatized amino acids have a strong absorbance at 340 nm ($\varepsilon_M = 3.10^4$ l mol^{-1} cm^{-1})[6]. Fig. 2 outlines the reaction sequence for the derivatization of racemic mixtures of amino acids with L-Marfey's reagent **1**. The resulting products are a pair of diastereoisomers L-1-aminylacid-2,4-dinitrophenyl-5-L-alanylamide (LL-**3**) and D-1-aminylacid-2,4-dinitrophenyl-5-L-alanylamide (DL-**4**).

If derivatization is achieved with D-Marfey's reagent **2**, the resulting products are a pair of diastereoisomers L-1-aminylacid-2,4-dinitrophenyl-5-D-alanylamide (LD-**5**) and D-1-aminylacid-2,4-dinitrophenyl-5-D-alanylamide (DD-**6**). Note that the two compounds **3** and **6** are enantiomers and the same holds true for compounds **4** and **5**.

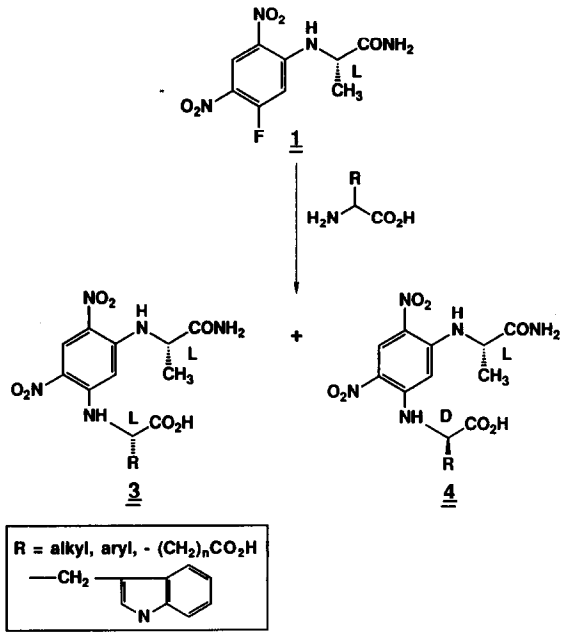


Fig. 2. Derivatization of racemic mixtures of amino acids with L-Marfey's reagent.

Fig. 3 represents a typical electropherogram of racemic mixtures of alanine (Ala), valine (Val), leucine (Leu), phenylalanine (Phe) and tryptophan (Trp) derivatized with L-Marfey's reagent **1**. The electropherogram was obtained by free solution capillary electrophoresis at 10 kV with sodium borate as the running buffer (pH 8.5). No separations were observed and the mixture of derivatized products coeluted as a single peak. When the same mixture was run at 30 kV, good separations were obtained

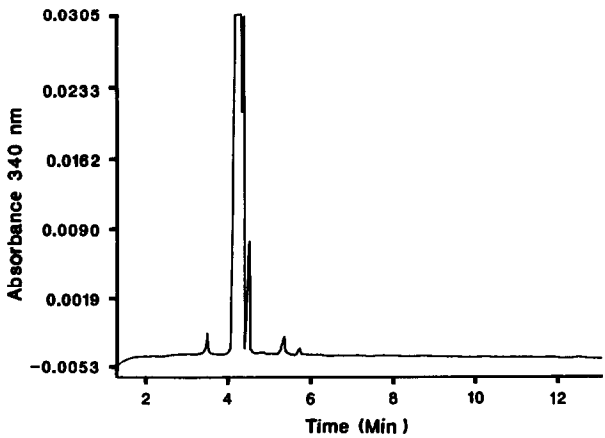


Fig. 3. Electropherogram of racemic mixtures of Ala, Val, Leu, Phe and Trp derivatized with L-Marfey's reagent. The electropherogram was obtained by free solution capillary electrophoresis using a 75 μm I.D. capillary; 100 m*M* sodium borate buffer, pH 8.5; 10 kV, 25 $\pm$ 0.1°C; detection at 340 nm.

between different derivatized amino acids; however, the lack of separation between the pair of amino acid enantiomers could result from the remote location between the two chiral centers of the derivatized products. This hypothesis is corroborated by additional experiments showing that dipeptides L-Ala–L-Ala–OH and L-Ala–D-Ala–OH are well separated under similar conditions, using sodium borate buffer (pH 8.5), at 30 kV.

The separation of derivatized amino acids is not detected when a buffer with a low pH such as ammonium phosphate (pH 3.3) is used, and no bands elute through the detector. Since the electroosmotic flow is significantly reduced at low pH due to the protonation of the silanolate group of the capillary wall (p*I* of the silanol group is around 1.0, ref. 8), the electrophoretic migration of the negatively charged species overcomes the electroosmotic flow and migrates in an opposite direction towards the anode (+) where the inlet buffer reservoir is located. If the polarity of the electrodes is reversed after the injection is made, the derivatized amino acid will migrate towards the detector as illustrated in Fig. 4.

A typical electropherogram of Ala, Leu, Asp and Glu derivatized with L-Marfey's reagent **1** is represented in Fig. 5. All L-amino acids were eluted first when compared to the corresponding D-amino acids. The sequence of migration increased from Asp < Glu < Ala < Val < Leu < Trp in direct correlation with the increase in hydrophobicity of the side chain of the amino acid. It is of special interest to observe that the order of migration is reversed with D-amino acids eluting first when D-Marfey's reagent **2** is used for the derivatization step. Such reversal offers an unequivocal means to confirm the presence of D-amino acids in an unknown sample.

As expected, no separation was observed when enantiomers **3** and **6** or **4** and **5** are coinjected. The wide and asymmetrical peak shapes observed in the FSCE mode operated at low pH could result from differences in electrophoretic mobilities between the solute and the buffer constituents.

Determination of L,D-amino acids by micellar electrokinetic capillary chromatography using L- and D-Marfey's reagents

MECC was introduced by Terabe *et al.*[9] for separations of neutral compounds. The technique involves the addition of an anion surfactant, particularly SDS, to the operating buffer at a concentration exceeding the critical micelle concentration (CMC). When a mixture of five pairs of diastereoisomers of Ala, Val, Leu, Phe and Trp

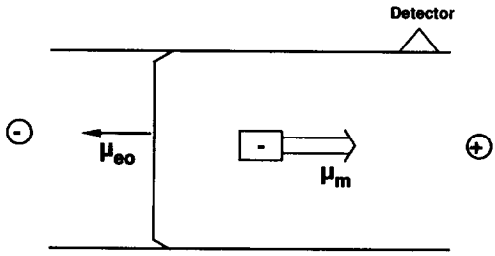


Fig. 4. Schematic representation of free solution capillary electrophoresis operated at low pH; polarity of the electrodes is reversed after the injection is made. Anionic species migrates towards the detector or the anode (+) in the opposite direction of the osmotic flow; μ_{eo} = electroosmotic flow factor, μ_m = electrophoretic mobility of the anionic species.

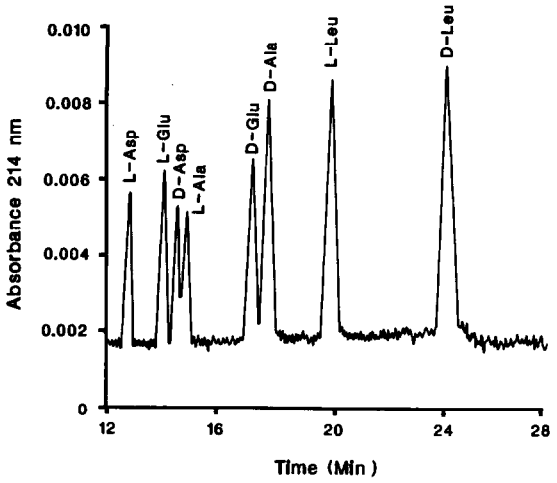


Fig. 5. Electropherogram of racemic mixtures of Ala, Asp, Glu and Leu derivatized with L-Marfey's reagent. The electropherogram was obtained by free solution capillary electrophoresis using a 75 μm I.D. capillary; 50 mM of ammonium phosphate, pH 3.3; 20 kV, 120 mA, 25 $\pm$ 0.1°C; detection at 214 nm.

derivatized with L-Marfey's reagent **1** are separated under the MECC mode, good separations and peak shapes are observed as represented in Fig. 6. The presence of 5% acetonitrile in the running buffer is critical for good separation between L-Trp and D-Val; the level of SDS was selected at 200 mM because of the acceptable separation times and current values.

All L-amino acids of the five pairs of diastereoisomers migrated earlier from the capillary when compared to the corresponding D-amino acids. It is tempting to suggest that derivatized products of the DL-**3** configuration are more hydrophobic and interact

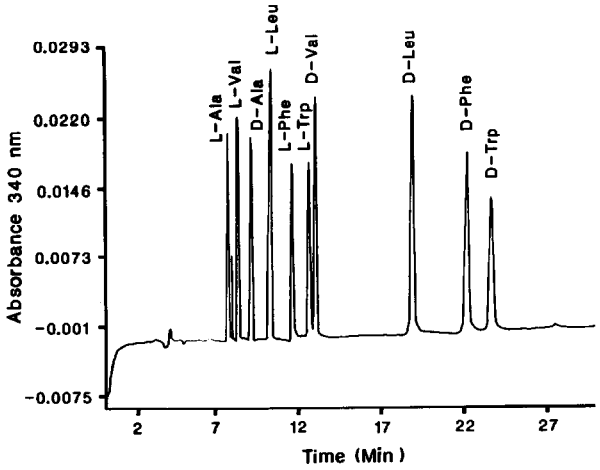


Fig. 6. Electropherogram of racemic mixtures of Ala, Val, Leu, Phe and Trp derivatized with L-Marfey's reagent. The electropherogram was obtained by micellar electrokinetic capillary chromatography using a 75 μm I.D. capillary; 100 mM sodium borate buffer, pH 8.5; 20 kV, 20 mA, 25 $\pm$ 0.1°C; detection at 340 nm.

more strongly with the SDS micelle compared to their counterparts of LL-**4** configuration. When D-Marfey's reagent **2** is used, the order of migration is reversed, with D-amino acids migrating first, suggesting that the product LD-**5** binds more strongly to the micelle compared to the DD-**6**. As expected, no separation was observed when the pairs of enantiomers LD-**5** and DL-**4** are coinjected; the same holds true for compounds LL-**3** and DD-**6**. The sequence and differences in migration times of the five diastereoisomers derivatized with L- or D-Marfey's reagents are in the following order: Ala (1.39 min) < Val (4.62 min) < Leu (8.62 min) < Phe (10.31 min) < Trp (10.90 min). It is clear that the migration times and resolutions increase with the increased hydrophobicity of the side chain.

Several consecutive runs with different concentrations of standard solutions of amino acids were made in order to study the reproducibility of elution times and the variation of the peak areas *versus* concentrations. The reproducibility of the elution times (1%) and the good correlation coefficients ($r = 0.99$) of the straight-line fits indicate that this method could be used as a quantitative analysis.

Under similar conditions no separation was observed for the pair of diastereoisomers of Asp or Glu. The extra negative charge of the side chain of these amino acids could be the origin for charge repulsion between micelle and amino acid, and so prohibit potential interactions between the two species.

When MECC is operated at low pH with ammonium phosphate (pH 3.3), no product was eluted through the detector, similar to the situation mentioned earlier for FSCE. Because of the significant reduction of the osmotic flow at low pH, the negative SDS micelle could now migrate in the opposite direction to the anode (+) or the inlet buffer. Good separations for neutral, basic and acidic amino acids were observed when the polarity was reversed after the injection was made, as illustrated in Fig. 7.

Fig. 8 illustrates a schematic representation of MECC mode operated at low pH. Similar to MECC operating at neutral and basic pH, the migration of the solute is based on its hydrophobicity. However, the order of migration is reversed with more

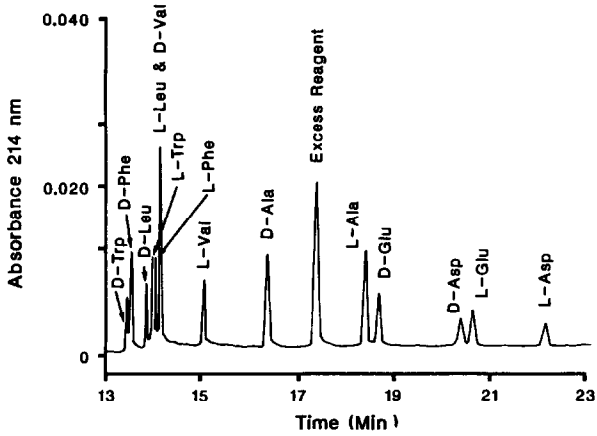


Fig. 7. Electropherogram of racemic mixtures of Ala, Asp, Glu, Val, Leu, Phe and Trp derivatized with L-Marfey's reagent. The electropherogram was obtained by micellar electrokinetic capillary chromatography using a 75 μm I.D. capillary; 50 m*M* ammonium phosphate buffer, pH 3.3; 10 kV, 60 mA, 25 $\pm$ 0.1°C; detection at 214 nm.

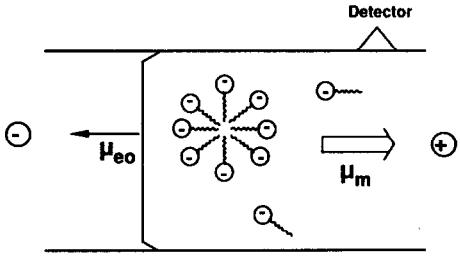


Fig. 8. Schematic representation of micellar electrokinetic capillary chromatography operated at low pH; polarity of the electrodes was reversed after the injection was made. The SDS micelle migrated towards the detector in the opposite direction of the osmotic flow; μ_{eo} = electroosmotic flow factor, μ_m = electrophoretic mobility of the micelle.

hydrophobic compounds eluted first, since the micelle migrates towards the detector. When D-Marfey's reagent **2** is used, the order of elution is reversed with L-amino acids eluted first compared to the corresponding D-amino acids. MECC mode operated at low pH is a complementary approach to MECC at basic pH. This method is especially useful for negatively charged products which may not separate under MECC at basic pH because of charge repulsion between product and micelle.

As with MECC, this method could be used for both qualitative and quantitative analysis since there appears to be good reproducibility of the elution times (1%) and good linearity curves between peak areas and concentrations (r = 0.99) of the straight-line fits of different standard amino acid solutions.

Determination of dipeptide and tripeptide isomers by micellar electrokinetic capillary chromatography using L- and D-Marfey's reagents

To further extend the use of Marfey's reagent to peptides, Ala–Ala–OH was

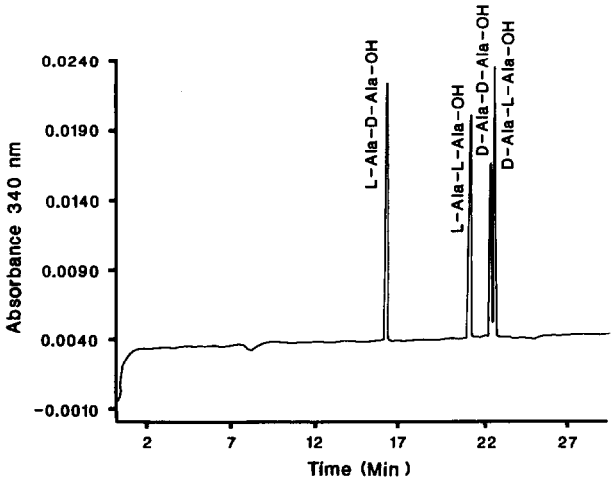


Fig. 9. Electropherogram of isomers of dipeptides Ala–Ala–OH derivatized with L-Marfey's reagent **1**. The electropherogram was obtained by micellar electrokinetic capillary chromatography using a 75 μm I.D. capillary; 100 m*M* sodium borate, pH 8.5; 12 kV, 10 mA, 25 ± 0.1°C; detection at 340 nm.

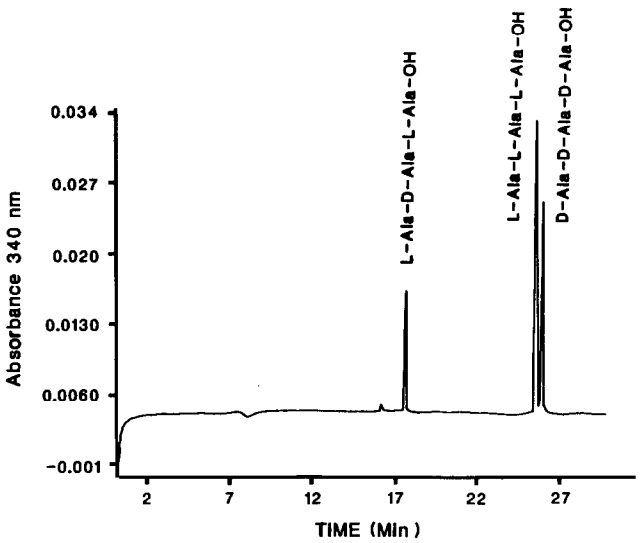


Fig. 10. Electropherogram of isomers of tripeptide Ala–Ala–Ala–OH derivatized with L-Marfey's reagent **1**. The electropherogram was obtained by micellar electrokinetic capillary chromatography using a 75 μm I.D. capillary; 100 m*M* sodium borate buffer, pH 8.5; 12 kV, 20 mA, 25 $\pm$ 0.1°C; detection at 340 nm.

selected as a model substrate since all four dipeptide isomers are commercially available. As expected under MECC mode, the mixture of underivatized isomers separated as two bands, with enantiomeric pairs L–D and D–L or L–L and D–D coeluted as a single peak. However, when derivatized with L-Marfey's reagent **1**, all four isomers are diastereoisomers and are well separated as illustrated in Fig. 9. The sequence of migration increases from L–D < L–L < D–D < D–L and varied to DL < DD < LL < LD when D-Marfey's reagent is used for the derivatization step. Unlike amino acids, the sequence of migration of dipeptides is more difficult to predict. A different order of migration is obtained with Ala–Phe–OH increasing from L–D < D–D < D–L < L–L.

The isomers of tripeptide Ala–Ala–Ala–OH were also used to test the effect of chirality in remote parts of a peptide chain. Fig. 10 illustrates the separation of L–D–L, L–L–L and D–D–D isomers derivatized with L-Marfey's reagent.

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