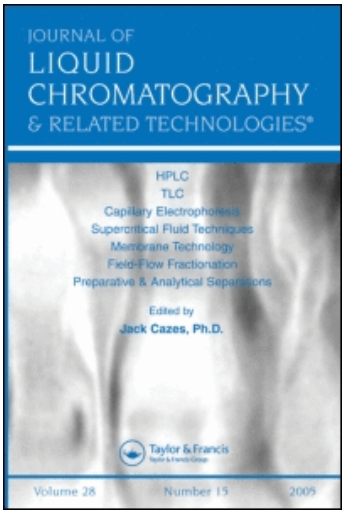

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A QUANTITATIVE ASSAY FOR THE DETERMINATION OF PROLINE AND HYDROXYPROLINE BY CAPILLARY ELECTROPHORESIS

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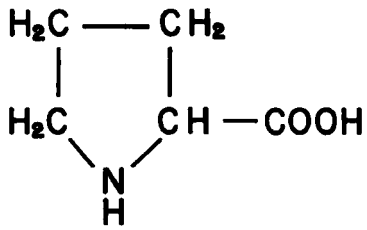
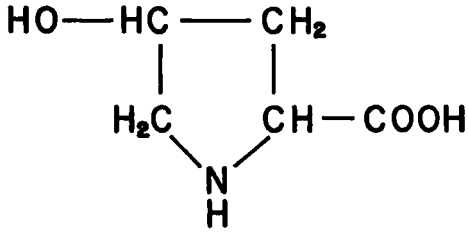
ABSTRACT

A quantitative spectrophotometric assay for the determination of proline and hydroxyproline is described. The assay is based on the derivatization of the imino acids with the fluorogenic chromophore fluorescamine and the separation of the derivatized analytes by capillary electrophoresis. Fluorescamine reacts efficiently with secondary amino acids to form nonfluorescent aminoenone type chromophores which are easily detected at the low UV region. The assay is simple, rapid, and offers a significant improvement in detection sensitivity when compared with standard colorimetric methods for determining these imino acids. Derivatization with fluorescamine also enhances the degree of resolution of the two imino acids, enabling baseline separation of the derivatized analytes.



PROLINE



4-HYDROXYPROLINE

FIGURE 1. Schematic representation of the molecular structure of L-proline and 4-hydroxy-L-proline.

INTRODUCTION

Proline and hydroxyproline are the only mammalian imino acids (1,2). As such, they differ from conventional amino acids in that they contain a secondary amine functional group linked internally in a cyclic five-membered pyrrolidine ring (Figure 1). Because of this unique structure, the incorporation of proline residues into a protein imparts a slight bend (or kink) to its secondary structure. For globular proteins, proline has indeed been found to be a key amino acid, playing an essential role in the folding-refolding stability (3-9), enzyme activity (10), and transport-translocation (11) processes.

Hydroxyproline, which is synthesized as the 3-hydroxy- and/or 4-hydroxy-derivatives of L-prolyl residues in peptide linkages, also appears to be a structurally important amino acid. It is produced almost exclusively in collagens and collagen-like peptides (for a review, see ref. 1), its presence usually being a criterion for identification of this class of protein. It is well known that in animal cells, peptidyl-4-hydroxy-L-proline residues are necessary to the formation of a stable triple-helical collagen configuration and to the secretion of procollagen molecules at a normal rate (12-15).

The importance of these secondary amino acids in biological systems has led to the development of various methods for their qualitative and quantitative analysis. A variety of procedures have been described, including colorimetric (paper chromatography, thin-layer chromatography, spectrophotometric), liquid chromatography (radioactive, spectrophotometric, fluorimetric), gas chromatography, and mass spectrometry (16-23). In this report, we have evaluated capillary electrophoresis (CE) as a complementary or alternative technique to assess proline and hydroxyproline. The method involves derivatization of the imino acids with the fluorogenic reagent fluorescamine (4-phenylspiro[furan-2(3H),1'phthalan]-3,3'-dione) to form fairly stable derivatives. These nonfluorescent type aminoenone chromophore-derivatives can then be separated by capillary electrophoresis and detected with conventional UV detector at 214 nm. The proposed scheme of fluorescamine with secondary functional amines (24,25) is shown in Figure 2. The utility of CE for the separation of fluorescamine-derivatized proteins, peptides, and amino acids has recently been reported (26).

EXPERIMENTAL

Reagents and samples

All chemicals were obtained at the highest purity level available from the manufacturer, and were used without additional purification. Sodium hydroxide, borax ($Na_2B_4O_7 \cdot 10H_2O$), lithium chloride, L-proline, 4-hydroxy-L-proline, and fluorescamine were obtained from Sigma Chemical Company (St. Louis, MO). Acetone (HPLC grade), pyridine (Fisher Certified), and hydrochloric acid solution (12 M) were obtained from Fisher Scientific (Fair Lawn, NJ). Reagent solutions and buffers were prepared using triply distilled, deionized water, and routinely degassed and sonicated under vacuum after filtration.

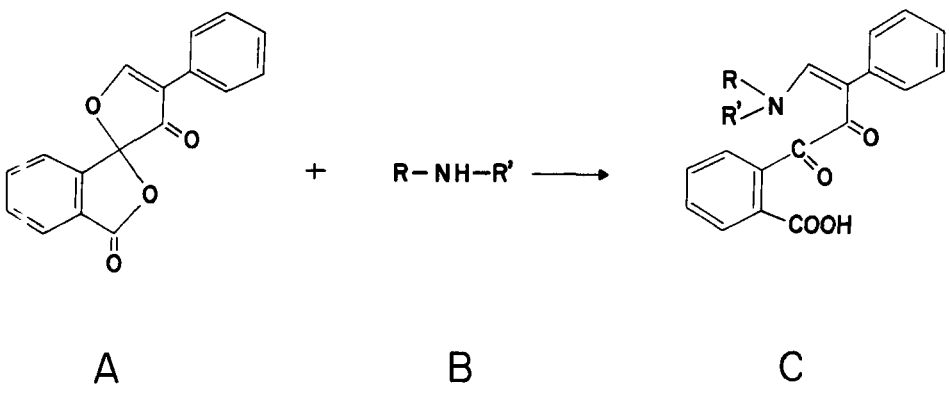


FIGURE 2. Schematic representation of the molecular structure of fluorescamine **(A)**, the reacting secondary amine functional group-containing analyte **(B)**, and the derivatized reaction product **(C)**.

Millex disposable filter units (0.22 μm) were purchased from Millipore Corporation (Bedford, MA), and fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX), and Polymicro Technologies (Phoenix, AZ).

Instrumentation

A commercially available CE instrument (P/ACE System 2000, Beckman Instruments, Palo Alto, CA), was used for this work. In this instrument, the capillary is housed in a cartridge constructed so as to allow a flow of recirculating liquid for Peltier-temperature control of the capillary column. Samples were stored in a microapplication vessel assembly, consisting of a 150 μl conical microvial inserted into a standard 4 ml-glass reservoir and held in position for injection by an adjustable spring. In order to minimize evaporation of the sample volume (100 μl), about 1-2 ml of cool water was added to the microapplication vessel housing the microvial. The external water serves as a cooling bath for the sample in the microvial, and as source of humidity to prevent sample evaporation and concentration. After insertion of the microvial, the microapplication vessel assembly was covered with a rubber injection septum and placed into the sample compartment of the CE instrument. Samples were injected into the capillary column by pressure.

Peak visualization and data acquisition were performed using the UV detection system of the CE-instrument and the System Gold Chromatography Software package (Beckman Instruments, San Ramon, CA). Data integration was also carried out with a Model D-2500 Chromato-Integrator (Hitachi Instruments, Inc., Danbury, CT).

Assay Procedure

Sample Preparation

Analyte stock solutions were individually prepared by dissolving L-proline (1 mg/ml) and hydroxy-L-proline (1 mg/ml) in 0.1 M sodium tetraborate (borax) buffer, pH 9.0.

Sample Derivatization

For CE analysis without fluorescamine conjugation, assay samples were directly transferred to the conical vial and then inserted into the microapplication vessel assembly on the CE instrument.

For CE analysis of fluorescamine conjugates, solutions of the respective analyte samples (concentration ranging from 2.5 to 40 μg, or from 19 to 305 nanomole per 100 μl reaction mixture) were transferred to a 500 μl microcentrifuge tube and their total volume adjusted to 70 μl by addition of sample dilution buffer (0.1 M sodium tetraborate buffer, pH 9.0). Derivatization was performed by the addition of 30 μl of fluorescamine solution (3 mg/ml fluorescamine in acetone, containing 20 μl pyridine) to the sample while continuously and vigorously vortexing. After approximately two minutes, the contents of the microcentrifuge tube were transferred to the conical microvial and then inserted into the microapplication vessel assembly for analysis.

Running Conditions

Sample solutions for analysis in microapplication vessels were placed into the sample holder of the analyzer. The analysis program was initiated and the first sample automatically injected into the capillary by a positive nitrogen pressure of 0.5 psi for 4 seconds. At the completion of each run, the capillary column was sequentially washed by injection of 2.0 N sodium hydroxide solution, 0.1 N sodium hydroxide solution, distilled-deionized water, and then regenerated with running buffer.

The CE separations reported were performed using 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl. The CE instrument was equipped with a 57 cm (50 cm to the detector) x 75 μm I.D. capillary column for all experiments, except one experiment

in which a 107 cm (100 cm to the detector) x 75 μm I.D. capillary column was used. The CE separation was performed at 18 kV and 12 kV respectively. Capillary temperature was maintained at 25°C during the run. Under these conditions, approximately 24 nl/4 sec (6 nl/sec) was injected into the capillary column (27). Monitoring of the analytes was performed at a wavelength of 214 nm.

RESULTS

The electropherograms for a 4-hydroxy-L-proline solution, analyzed with and without fluorescamine derivatization, are shown in Figures 3 and 4. For conventional CE analysis, performed without fluorescamine derivatization, approximately 24 nanoliters of the 4-hydroxyproline stock solution (1 mg/ml) was injected into the capillary column. As shown in Figure 3A, the underivatized amino acid migrates as a single peak, appearing at about 9.3 minutes. The observed peak, which represents an injection of approximately 183 picomoles of 4-hydroxyproline, is quite small and approaches the limits of detection for this system (UV at 214 nm).

The effects of derivatization with fluorescamine on the determination of 4-hydroxyproline are shown in Figure 3B. The analysis mixture, consisting of 4-hydroxyproline (40 μl, 305 nanomole), 30 μl of reaction buffer, and 30 μl of fluorescamine reagent (30 μl, 324 nanomole) in a total reaction mixture of 100 μl, was injected (24 nl, 73 picomole of 4-hydroxyproline) into the capillary column and CE analysis performed as usual. As shown in the figure, coupling of 4-hydroxyproline to fluorescamine results in a decreased mobility for hydroxyproline and its derivative (peak 3), appearing at about 23 minutes. The derivatization of 4-hydroxyproline also results in an approximately 800-fold increase in its detected peak area (Table I).

As shown in Figure 4, the derivative is well separated from the additional peaks observed, which correspond to excess fluorescamine reagent (peak 4), reaction solvents acetone and pyridine (comigrating at peak 1), and an unknown reagent impurity (peak 2), which were identified by CE analysis of an appropriate blank solution (Figure 4A). The observed decreased peak area for the fluorescamine reagent (Figure 4B, peak 4) in comparison with the control value (Figure 4A, peak 4), confirms that a significant amount of the reagent is consumed in reaction with the imino acid analytes. For all experimental conditions, the same amount of fluorescamine reagent was used (324 nmole/100 μl reaction mixture), as previ-

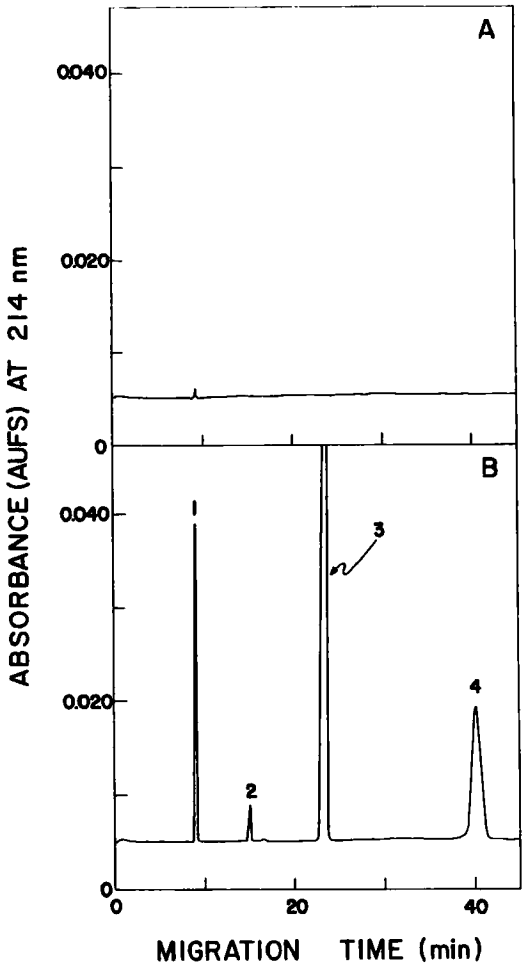


FIGURE 3. Comparative profile of underivatized and fluorescamine-derivatized 4-hydroxyproline. **(A).** Electropherogram of underivatized 4-hydroxyproline (1 mg/ml). **(B).** Electropherogram of fluorescamine-derivatized 4-hydroxyproline. Peak 1, represents acetone; peak 2, represents an apparent reagent contaminant; peak 3, represents fluorescamine-derivatized 4-hydroxyproline; and peak 4, fluorescamine reagent (324 nanomole/100 µl reaction mixture).

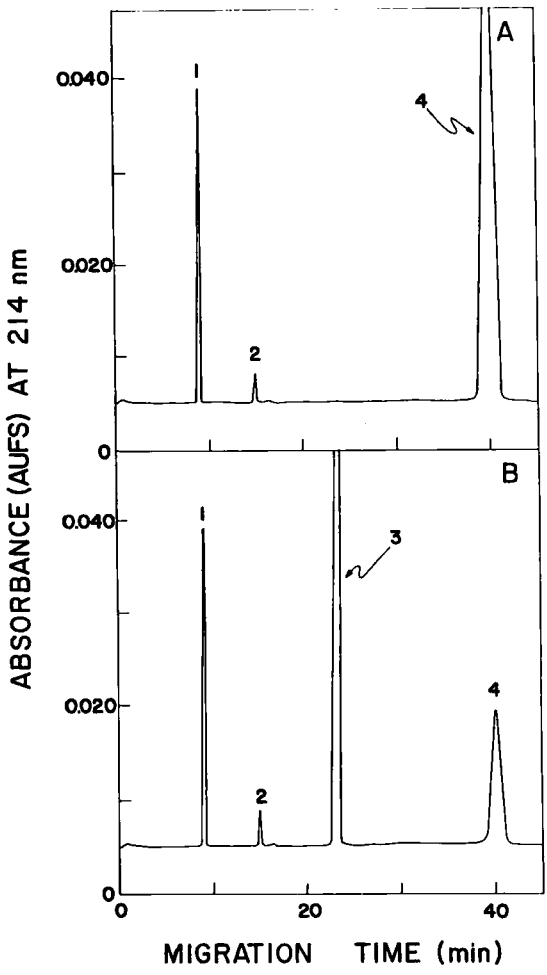


FIGURE 4. Capillary electrophoresis profile of fluorescamine-control reagent and fluorescamine-derivated 4-hydroxyproline reaction products. **(A).** Electropherogram of the fluorescamine solution control. **(B).** Electropherogram of fluorescamine-derivatized 4-hydroxyproline. All peaks as described in Figure 3.

TABLE I. COMPARISON IN PEAK AREA BETWEEN UNDERIVATIZED 4-HYDROXY-L-PROLINE AND FLUORESCAMINE-DERIVATIZED 4-HYDROXY-L-PROLINE.

Sample	Peak Area (arbitrary units)	Fold Increase
Underivatized-Imino Acid	75,497	1.0
Derivatized-Imino Acid	58,335,732	773

TABLE II. MOLAR RELATIONSHIP BETWEEN FLUORESCAMINE AND 4-HYDROXYPROLINE

Amount of 4-Hydroxyproline (nanomole)	Amount of Fluorescamine (nanomole)	Molar Ratio (Fluorescamine/4-Hydroxyproline)
19	324	17.1
38	324	8.5
76	324	4.3
153	324	2.1
229	324	1.4
305	324	1.1

TABLE III. TYPICAL AMOUNTS OF FLUORESCAMINE-DERIVATIZED 4-HYDROXYPROLINE ANALYZED BY CAPILLARY ELECTROPHORESIS AT 214 nm

Amount of 4-Hydroxyproline Present in 100 µl Reaction Mixture			Amount of 4-Hydroxyproline Injected into the Capillary Column			
µL	µg	nmole	nL	ng	pmole	Peak Area*
2.5	2.5	19	24	0.6	4.6	0.5
5.0	5.0	38	24	1.2	9	0.9
10	10	76	24	2.4	18	2.2
20	20	153	24	4.8	37	4.2
30	30	229	24	7.2	55	6.6
40	40	305	24	9.6	73	8.5

For this experiment, increasing amounts of 4-hydroxyproline were reacted with a fixed concentration of fluorescamine reagent (324 nmole/100 µl reaction mixture). The separation buffer consisted of 0.05 M sodium tetraborate buffer, pH 8.3, containing 0.025 M LiCl.
*Peak area, expressed as arbitrary units x 10^{-6}.

ously described (26). This amount of fluorescamine reagent (ranging from 1.1 to 17 molar excess) should be sufficient to saturate the reaction mixture and form an optimal fluorescamine-imino acid derivative (Table II).

Under the running conditions used, the linearity of derivatized-4-hydroxyproline peak area, as a function of 4-hydroxyproline concentration, was investigated. As shown in Table III and Figure 5, a linear response (at 214 nm) was observed for 4-hydroxyproline concentrations ranging from 19 nanomole/100 µl to 305 nanomole/100

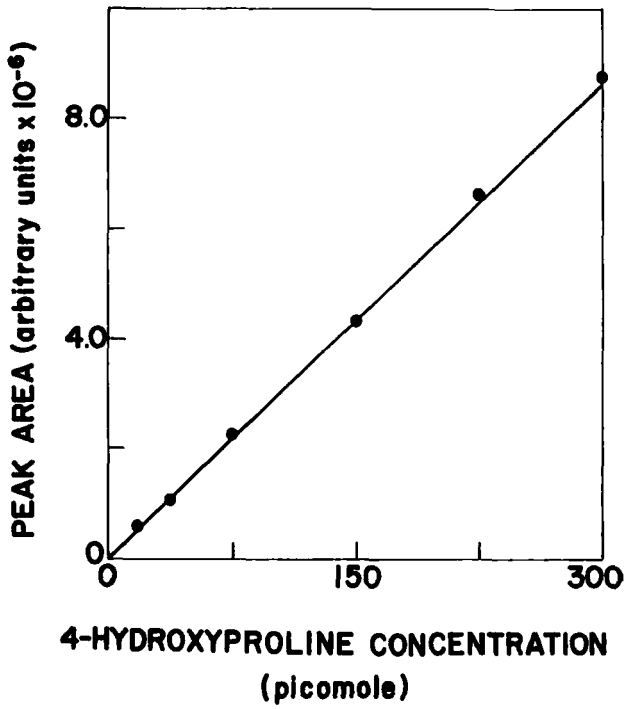


FIGURE 5. Relationship between peak area of derivatized analyte formed and the concentration of reacting 4-hydroxyproline. The linearity of formation of fluorescamine-derivatized imino acid was calculated by reacting increasing concentrations of 4-hydroxyproline with an excess amount of fluorescamine reagent (324 nanomole/100 µl reaction mixture). The procedure was carried out as described in Experimental Section. The values represent the concentrations of the fluorescamine-derivatized 4-hydroxyproline in the 24 nl volume injected into the capillary column.

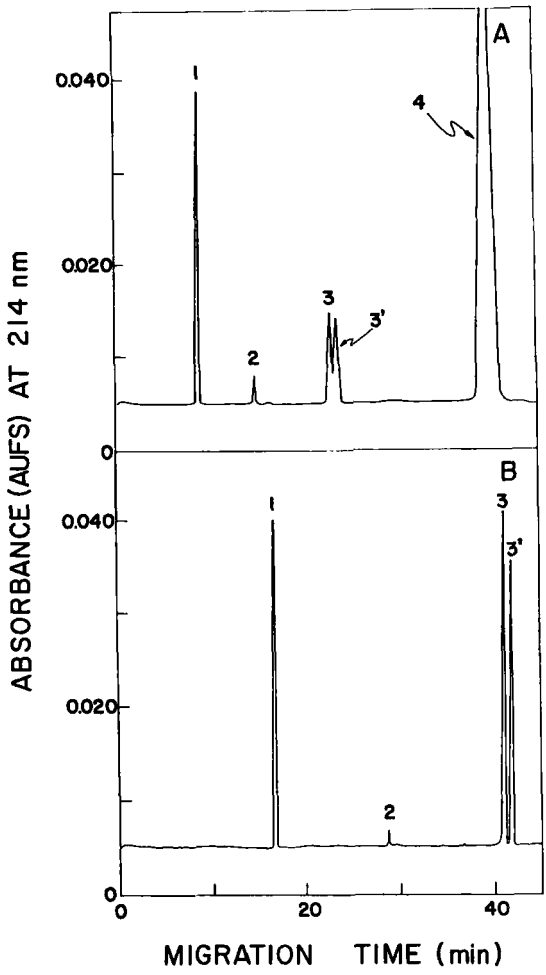


FIGURE 6. Capillary electrophoresis separation of a mixture of L-proline and 4-hydroxy-L-proline. **(A).** Electropherogram of fluorescamine-derivatized proline and 4-hydroxyproline using a 75 µm x 57 cm (50 cm to detector) capillary column. **(B).** Electropherogram of fluorescamine-derivatized proline and 4-hydroxyproline using a 75 µm x 107 cm (100 cm to detector). Peak 3, correspond to 4-hydroxyproline; peak 3', correspond to proline; all other peaks as described in legend to Figure 3.

μl reaction mixture (4.6 picomole/24 nl to 73 picomole/24 nl injected into the capillary column).

The procedures developed for the derivatization and CE analysis of 4-hydroxy-L-proline were also used to investigate solutions of L-proline, which only differs structurally in the absence of the 4-hydroxyl group (Figure 1). As shown in Figure 6A, only a partial resolution of the fluorescamine-derivatized 4-hydroxyproline (peak 3) and proline (peak 3') was achieved. This problem of partial resolution was solved by replacing the 57-cm long capillary column with an 80-cm long one. With the new column, a baseline separation of the two imino acids was achieved (Figure 6B).

DISCUSSION

During the last two decades, the determination of proline and hydroxyproline has played an essential role in elucidating the folding and stability mechanism(s) of both globular and collagenous proteins (3-15). Measurements of hydroxyproline content in a particular tissue have not only been useful in assessing the quantity and quality of collagen in that tissue, but also in determining the rate at which that collagen is synthesized or degraded. The intense interest in imino acids is evident by the great number of methods and modifications of methods developed over the years (16-23). It is also evident from these reports that a simple specific method for measuring proline and hydroxyproline is lacking.

Most earlier methods developed, i.e., colorimetric, radiochemical, conventional ion-exchange, and high-voltage paper electrophoresis assays were very time-consuming and were limited in their detection sensitivity. Furthermore, many of the earlier methods contain tedious steps involving oxidation, extraction, and desalting of the samples, which were difficult to perform and frequently resulted in sample loss. Later, with the use of modern amino acid analyzers, high-performance liquid chromatography technology, and specific derivatizing agents, it became possible to simplify the methods and achieve better sensitivities (16-23). Sophisticated applications procedures based upon stable isotope enrichment and gas chromatography/mass spectrometry have recently extended the sensitivity to the nanogram level, enabling the measurement of 4-hydroxyproline concentrations in small biopsy-sized tissue specimens and in tissue culture products (20).

The experiments described in this report demonstrate that capillary electrophoresis with fluorescamine derivatization is a simple and highly reliable method for the separation and analysis of

proline and 4-hydroxyproline. The reaction with fluorescamine is rapid, going to completion within one minute, and yields a covalently linked derivative that is stable through the period of analysis. With detection in the UV region (214 nm), fluorescamine derivatization results in approximately an 800-fold increase in the detection sensitivity for hydroxyproline, as compared to the underivatized imino acid.

In conclusion, the incorporation of fluorescamine derivatization in the analysis of proline and 4-hydroxyproline produces highly absorbent compounds in the UV region. The derivatized imino acids are separated very efficiently by capillary electrophoresis and the resulting analyte species migrating within the capillary may be detected and quantitated at the picomole range of sensitivity. The CE assay significantly improves detection sensitivity and separation. The method is simple, quite fast, and very reproducible.

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