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THIN-LAYER CHROMATOGRAPHY USING A LAYER CONTAINING A MIXTURE OF FLUORESCENT ADDITIVES

APPLICATION TO AMINO ACID PHENYLTHIOHYDANTOINS AND OTHER DERIVATIVES

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SUMMARY

Amino acid phenylthiohydantoins (PTH) and other amino acid derivatives were analyzed by thin-layer chromatography using glass plates coated with polyamide containing three fluorescent additives. All PTH-amino acids were seen as colored spots when viewed with a UV lamp emitting light over the range 250 to 400 nm. By heating the plate after spraying with an alkaline solution, characteristic changes in the color of some PTH-amino acids were observed. About 0.1 nmole of PTH-amino acid could be detected. The method was also used for the detection of other derivatives of amino acids and peptides having UV absorption maxima above 250 nm.

INTRODUCTION

Thin-layer chromatography (TLC) of amino acid phenylthiohydantoins (PTH) produced by Edman degradation[1] is often used in the amino acid sequence analysis of polypeptides. In addition to being widely applicable and economical, the sensitivity of the TLC methods employing polyamide sheets[2–4] is comparable to gas chromatography[5] and high-performance liquid chromatography[6]. In commonly used fluorescent TLC methods[2–4], PTH-amino acids appear as dark spots on a fluorescent background. Useful characteristic fluorescent or colored spots also may be produced on silica gel plates when certain PTH-amino acids are treated with ninhydrin[7–9]. This technique has been employed to distinguish amino acids with similar R_F values[10]. Unfortunately, a 10-fold decrease in sensitivity was observed.

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The mixed fluorescent material method[11] for detecting PTH-amino acids and other UV absorbing amino acid derivatives and peptides is a non-destructive (unlike treatment with ninhydrin) technique in which UV-absorbing materials appear as colored spots on a white background. This is achieved by mixing three selected fluorescent substances with polyamide prior to spreading the plates. Alone, each fluorescent material has a characteristic color when suitably irradiated, but the three-component mixture appears white on irradiation with a light source providing continuous radiation in the range of 250–400 nm. An UV-absorbing spot on the TLC plate reduces radiation in the region of its absorption maximum, and the spot appears colored on a white background.

EXPERIMENTAL

Reagents and materials

The following were purchased from commercial sources: 2,4-dinitrophenyl (DNP)-amino acids, glycine-*p*-nitroanilide, L-leucine-*p*-nitroanilide·HCl, N-phthalylglycine, N-phthalyl-L-leucine, N-phthalyl-L-phenylalanine, L-leucyl-β-naphthylamide·HCl, L-valine-β-naphthylamide, α-N-benzoyl-DL-alanine, N-benzoyl-DL-phenylalanine, N-benzyloxycarbonyl (CBZ)-L-valine, N-CBZ-glycylglycyl-L-leucine, L-alanyl-L-histidine, glycyl-L-histidylglycine, N-methyl-DL-alanine, N-methyl-DL-leucine, N-methyl-DL-valine, N,N-dimethylglycine·HCl, L-tryptophyl-L-phenylalanine and L-valyl-L-tryptophan (Sigma, St. Louis, Mo., U.S.A.); fluorescamine (FLA), phenyl isothiocyanate, PTH-amino acids and 1-dimethylamino-naphthalene-5-sulfonyl (Dns)-amino acids (Pierce, Rockford, Ill., U.S.A.); N-CBZ-glycine and L-pyroglutamic acid (Aldrich, Milwaukee, Wisc., U.S.A.); amino acid thiohydantoins (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.); N-acetyl amino acids (Eastman-Kodak, Rochester, N.Y., U.S.A.); N-CBZ-L-phenylalanine and N-CBZ-L-arginine·HCl (Schwartz Bioresearch, Orangeburg, N.Y., U.S.A.); L-amino acids and methylthiohydantoin (MTH)-amino acids (Mann Research Labs., New York, N.Y., U.S.A.); *p*-toluenesulfonyl (tosyl)-L-arginine, tosyl-DL-arginine methyl ester·HCl and benzoyl-DL-arginine-*p*-nitroanilide·HCl (Schwartz/Mann, Orangeburg, N.Y., U.S.A.) and other amino acid derivatives used (Cyclo Chemical, Los Angeles, Calif., U.S.A.). *tert.*-Butyloxycarbonyl (BOC)-glycine-2,4,5-trichlorophenyl ester (TCP), BOC-L-valine-TCP and N-CBZ-L-proline-*p*-nitrophenyl ester were synthesized in our laboratory.

Glass-backed, pre-coated polyamide plates (Polyamide FM Plate, 5 × 10 cm) and silica gel plates (Wakogel FM Plate, 5 × 10 cm) were kindly provided by Wako, Osaka, Japan. Both plates contained three fluorescent mineral additives, $Sr_2P_2O_7$/Sn, Zn_2SiO_4/Mn, and YVO_4/Eu. They were irradiated with a Pan UV lamp (Type PUV-1A, Tokyo Kogaku Kikai, Tokyo, Japan), which gives continuous radiation in the range of 250–400 nm.

Analysis of PTH-amino acids

PTH-Amino acids were dissolved in methanol separately or in a mixture to make stock solutions of 1 mg/ml. An aliquot of 1 μl of the stock solution or a solution diluted with methanol was applied with a micro-pipette ("Microcaps", Drummond, Broomall, Pa., U.S.A.) under a stream of cold air from a hairdryer (to

minimize the size of the spot) at a point 5 mm above the lower edge (5 cm side) and 5 mm from the left edge (10 cm side) of Polyamide FM plates.

Plates were developed in toluene–*n*-pentane–acetic acid (6:3:2) at room temperature[2]; the solvent front migrated the full 10 cm in *ca.* 18 min. After drying in air, they were developed to the top in the perpendicular direction in acetic acid–water (1:3)[4] in *ca.* 11 min. The plate was briefly air-dried and placed face down on the Pan UV lamp as described previously[12–14]. The spots were traced on the glass, sprayed with 0.05 *M* sodium hydroxide solution in 50% methanol, heated for 30 min at 115° in an oven and re-examined with the Pan UV lamp and with a long-wavelength (365 nm) UV lamp.

Determination of limits of detection

Compounds were dissolved in methanol, water, acetone, or mixtures of the solvents to make 2–10 m*M* stock solutions. Tryptophan, phenylalanine, tyrosine and cystine were made up in methanol–0.2 *M* sodium hydroxide (1:1). Dilutions were made with the same solvents.

FLA derivatives were formed with the aid of a vortex mixer. A 50-μl aliquot of an acetonitrile solution of FLA (20 mg/100 ml) was rapidly added to a mixture of 10 μl of 10 m*M* aqueous solution of compound and 10 μl of 0.2 *M* sodium borate buffer, pH 9.0. Phenylthiocarbamyl (PTC) derivatives were prepared by mixing 10 μl of 10 m*M* aqueous solution of compound, 10 μl of 0.2 *M* sodium borate buffer, pH 9.0, 20 μl of ethanol and 10 μl of phenyl isothiocyanate and then heating the mixture (with a hairdryer) to 60° for 30 min while on a vortex mixer. The FLA and PTC reaction mixtures were diluted with methanol and used as standard solutions.

A 1-μl aliquot of solution was applied 0.5 cm from the lower edge of a Wakogel FM plate as described above. After complete evaporation of the solvent, the plate was placed in a 400 ml glass beaker containing freshly prepared *n*-butanol–acetic acid–water (5:2:3). The beaker was tightly capped with aluminum foil. When the solvent front reached the end of the plate (*ca.* 2 h), the plate was removed and the solvent completely evaporated with a hairdryer. The plate was observed in the dark with the Pan UV lamp.

Unlike Polyamide FM plates, Wakogel FM plates contain starch as a binder and often turn yellow during storage. The yellow material migrated with the solvent front and appeared as a strong greenish band under the Pan UV lamp. Also, the lower 1.0 to 1.5 cm of the plate appeared pink under the Pan UV lamp owing to partial degradation of the fluorescent additives by the acid. This is of no consequence since the compounds studied migrated beyond the discolored portion of the plate. To obtain maximal sensitivity, it is advisable to develop the plate with methanol, acetone or another neutral, volatile, organic solvent prior to applying the sample.

RESULTS

Characteristic colors of PTH-amino acids

All PTH-amino acids appeared as red spots on a white background when irradiated with the Pan UV lamp just after spotting on the polyamide plate.

PTH-Dehydroserine and PTH-dehydrothreonine were green. After developing in the first direction, the colors remained unchanged. However, after the second development a change in color was observed with PTH-S-carboxymethylcysteine, which turned from red to green, and with PTH-threonine and PTH-serine, which were partially degraded to their dehydro derivatives.

Certain PTH-amino acids after heating the developed plate for 20 min at 90° fluoresced:

TABLE I

CHARACTERISTIC COLORS OF PTH-AMINO ACIDS ON POLYAMIDE FM PLATES CONTAINING MIXED FLUORESCENT ADDITIVES

The plates were viewed under a Pan UV lamp after two-dimensional chromatography in toluene–*n*-pentane–acetic acid (6:3:2) (first direction) and acetic acid–water (1:3). Unless otherwise specified, the colors were the same before and after chromatography. Alkaline treatment involved spraying with 0.05 *M* sodium hydroxide in methanol–water (1:1) and heating at 115° for 30 min. Colors seen under the long-wavelength UV lamp are shown in parentheses.

PTH-amino acid	*Color after*	
	Second development	*Alkaline treatment*
Valine	Red	Red
Proline	Red	Red
Alanine	Red	Red
Glycine*	Red	Brownish red
Serine	Red	Brownish red (blue)
Dehydroserine	Green (blue)	Bluish green (blue)
Asparagine*	Red	Greenish brown (bluish green)§
Asparagine by-product	Colorless	White blue§ (greenish blue)§
Aspartic acid	Red	Brownish red (dark brown)
S-Carboxymethylcysteine	Green** (blue)	Brownish red§§ (blue)
Methionine*	Red	Brownish red
Methionine sulfone*	Red	Brownish red (blue)
Leucine	Red	Brownish red
Isoleucine	Red	Red
Lysine	Red	Red
Tyrosine*	Red	Red (bluish green)§
Tyrosine by-product*	Greenish yellow	Green (green)§
Threonine	Red	Bluish green (blue)
Dehydrothreonine	Green (blue)	Bluish green (blue)
Glutamine*	Red	Greenish brown (white yellow)§
Glutamine by-product*	Colorless	Green (white yellow)§
Glutamic acid	Red	Red
Phenylalanine*	Red	Greyish red (white blue)§
Tryptophan*	Red	Greyish red (white blue)§
Histidine*	Red	Blue§ (light blue)§
Histidine by-product	Red (blue)***,§	Purple (bluish purple)§
Arginine*	Red (blue)***,§	Purple (blue)§
Cysteic acid	Red	Brownish red (dark brown)

* Spots appear yellow to the naked eye. The glycine derivative is an exception. It is uniquely pink, especially after spraying with 1 *M* NaOH.

** Red after the first development. The red color sometimes persisted after the second development.

*** No fluorescence after the first development.

§ Fluorescent.

§§ Sometimes mixed with green color.

PTH-histidine, blue; PTH-arginine, purple; PTH-phenylalanine, white-blue; PTH-asparagine, blue-white. In addition, PTH-tyrosine gave a second yellow spot near the origin, and PTH-histidine and PTH-tryptophan appeared yellow when viewed with the naked eye. Additional discrimination was achieved with the alkaline spray, 0.05 *M* sodium hydroxide in 50% methanol. On heating for 30 min at 115°, the PTH derivatives of histidine, arginine, phenylalanine, asparagine, glutamine, tyrosine and tryptophan changed color. Fluorescent color changes were also observed with certain derivatives viewed under a long-wavelength UV lamp (Table I). Spraying with 1 *M* sodium hydroxide allowed the specific detection of PTH-glycine, which turned reddish pink. The other derivatives all turned brown, and the plates became discolored.

The two-dimensional TLC separation of 24 PTH-amino acids is shown in Fig. 1. Twenty-two derivatives were completely separated, including PTH-methionine from PTH-methionine sulfone and PTH-arginine from PTH-histidine. These derivatives were not always separated by previous TLC methods[3,4]. The separation of PTH-leucine from PTH-isoleucine has not been achieved in this or previous investigations[2–4]. However, they exhibited slightly different colors after sodium hydroxide treatment (Table I).

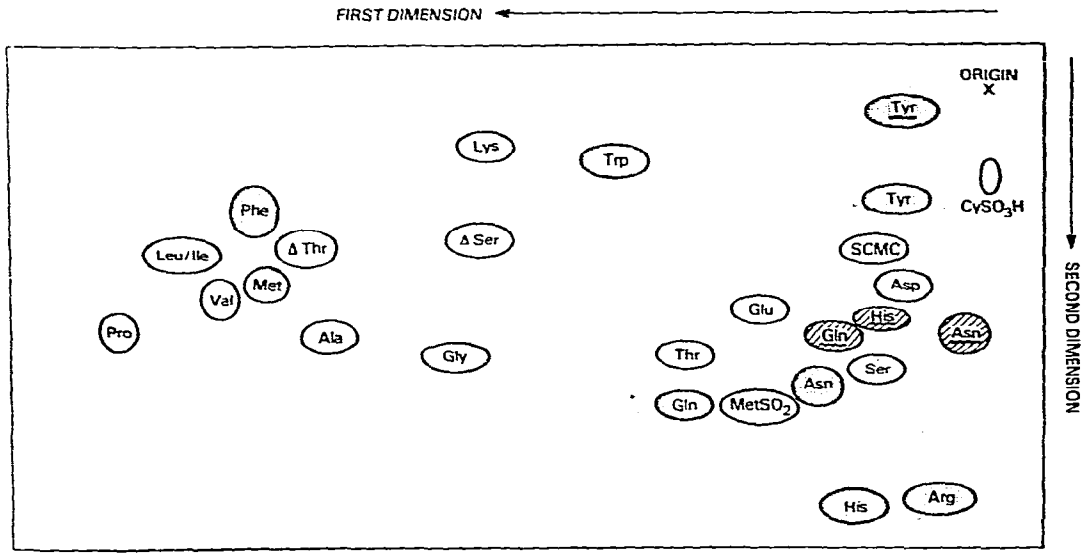

Fig. 1. Two-dimensional chromatographic separation of 24 PTH-amino acids on a Polyamide FM plate. Sample size: *ca.* 0.5 μg each of PTH-amino acid. Solvent: first dimension, toluene–*n*-pentane–acetic acid (6:3:2), second dimension, acetic acid–water (1:3). An underlined amino acid name designates the by-product formed from the PTH derivative during storage in methanol. Open circle, detected only by color; hatched circle, detected only by fluorescence after the alkaline treatment; stippled circle, detected by both color and fluorescence after the alkaline treatment.

Other derivatives

Table II contains R_F values, colors, and limits of detection of various amino acid derivatives on Wakogel FM plates. Derivatives with aromatic protecting groups usually were detected below the 1-nmole level. Highest sensitivity was obtained with DNP-PTH and PTC derivatives: 0.08 nmole was detected after two-dimentional chro-

matography. MTH-Amino acids behave similarly. DNP, *o*-nitrophenylsulfenyl (ONPS), DNS, FLA, *p*-nitroanilide, *p*-nitrophenyl ester and β-naphthylamide derivatives were detected at the 0.2–0.3-nmole level and gave characteristic green, reddish brown, yellowish green, bluish green, bluish green, green and red colors, respectively. N-Benzoyl (red), N-phthalyl (grayish green), tosyl (pink) and thiohydantoin (red) derivatives were detected with slightly lower sensitivity (0.6–0.8 nmole). CBZ and BOC derivatives were not detectable even at the 10-nmole level. Amino acids and peptides without an aromatic ring also gave no color at the 10-nmole level. Cystine was an exception; 2 nmoles were detected. Tryptophan and its derivatives gave red spots visible at the 0.5-nmole level; tyrosine and phenylalanine were visible at the 1–2-nmole level.

TABLE II

COLORS AND LIMITS OF DETECTION OF AMINO ACID AND PEPTIDE DERIVATIVES ON WAKOGEL FM PLATES

Plates were developed in *n*-butanol–acetic acid–water (5:2:3, v/v). Colors in parentheses were observed only before development.

*Compound**	R_F	*Color*	*Limit of detection (nmole)*
1 N-DNP-L-Alanine	0.82	Green	0.2
2 N,N-di-DNP-L-Lysine	0.84	Green	0.1
3 N-DNP-L-Phenylalanine	0.85	Green	0.2
4 ONPS-L-Alanine·DCA salt	0.86	Reddish brown	0.2
5 ONPS-Glycine·DCA salt	0.83	Reddish brown	0.2
6 ONPS-L-Proline·DCA salt	0.89	Reddish brown	0.2
7 N-Pht-Glycine	0.76	Grayish green	0.7
8 N-Pht-L-Leucine	0.85	Grayish green	0.7
9 N-Pht-L-Phenylalanine	0.84	Grayish green	0.7
10 Tosyl-L-arginine	0.81	Pink	0.8
11 Tosyl-DL-arginine methyl ester·HCl	0.74	Pink	0.8
12 PTC-L-Alanine	0.92	Red	0.08
13 PTC-L-Ala-L-His	0.67	Red	0.08
14 PTC-Gly-L-His-Gly	0.64	Red	0.08
15 α-N-Benzoyl-DL-alanine	0.83	Red	0.6
16 N-Benzoyl-DL-phenylalanine	0.89	Red	0.6
17 Dns-Glycine	0.79	Yellowish green	0.2
18 Dns-L-β-Phenylalanine	0.86	Yellowish green	0.2
19 Dns-L-Valine	0.88	Yellowish green	0.2
20 FLA-L-Alanine	0.81	Bluish green	0.2
21 FLA-L-Ala-L-His	0.67	Bluish green	0.2
22 FLA-Gly-L-His-Gly	0.66	Bluish green	0.2
23 BOC-L-Valine	—	(Grayish red)	>10
24 BOC-L-Glutamine	—	(Grayish red)	>10
25 BOC-L-Glutamic-γ-benzyl ester·DCA salt	—	(Grayish red)	>10
26 CBZ-L-Valine	—	(Faint red)	>10
27 CBZ-Glycine	—	(Faint red)	>10
28 CBZ-L-Phenylalanine	—	(Faint red)	>10
29 CBZ-L-Arginine·HCl	—	(Faint red)	>10
30 CBZ-Gly-Gly-L-Leu	—	(Faint red)	>10
31 CBZ-Nitro-L-arginine	0.77	Reddish brown	0.3
32 Glycine-*p*-nitroanilide	0.78	Bluish green	0.2

TABLE II *(continued)*

*Compound**	R_F	*Color*	*Limit of detection (nmole)*
33 L-Leucine-*p*-nitroanilide·HCl	0.85	Bluish green	0.2
34 L-Leucine-β-naphthylamide·HCl	0.87	Red	0.3
35 L-Valine-β-naphthylamide	0.85	Red	0.3
36 L-Glutamic acid dibenzyl ester·HCl	0.82	Red	8
37 CBZ-L-Glutamic-α-benzyl ester	0.95	Red	8
38 CBZ-L-Alanine-*p*-nitrophenyl ester	0.90	Green	0.2
39 CBZ-L-Proline-*p*-nitrophenyl ester	0.91	Green	0.2
40 BOC-L-Glutamine-*p*-nitrophenyl ester	0.90	Green	0.2
41 BOC-L-Valine TCP ester	0.95	Light purple	8
42 BOC-Glycine TCP ester	0.95	Light purple	8
43 L-Pyroglutamic acid	—	(Faint red)	>10
44 Histidine thiohydantoin	0.68	Red	0.7
45 Tryptophan thiohydantoin	0.91	Red	0.7
46 Benzoyl-DL-arginine-*p*-nitroanilide·HCl	0.83	Bluish green	0.05
47 α-CBZ-L-lysine benzyl ester-*p*-tosylate	0.69	Red	7
48 PTH-DL-Alanine	0.88	Red	0.08
49 PTH-DL-Phenylalanine	0.90	Red	0.08
50 PTH-L-Arginine	0.77	Red	0.08
51 MTH-Glycine	0.83	Red	0.1
52 MTH-DL-Asparagine	0.77	Red	0.1
53 MTH-DL-Histidine	0.68	Red	0.1
54 N-Acetyl-L-(+)-tryptophan	0.79	Red	0.5
55 N-Acetyl-L-(+)-phenylalanine	—	None	>10
56 N-Acetyl-L-(−)-leucine	—	None	>10
57 L-Glutamic-γ-hydrazide	—	None	>10
58 N-Methyl-DL-alanine	—	None	>10
59 N-Methyl-DL-leucine	—	None	>10
60 N-Methyl-DL-valine	—	None	>10
61 N,N-Dimethylglycine·HCl	—	None	>10
62 L-Tryptophan	0.72	Red	0.3
63 L-Tryptophyl-L-phenylalanine	0.79	Red	0.3
64 L-Valyl-L-tryptophan	0.74	Red	0.3
65 L-Tyrosine	0.63	Red	1
66 L-Phenylalanine	0.69	Red	2
67 L-Cystine	0.43	Red	2

* Abbreviations are: DNP = 2,4-dinitrophenyl; ONPS = *o*-nitrophenylsulfenyl; Pht = phthalyl; tosyl = *p*-toluenesulfonyl; PTC = phenylthiocarbamyl; PTH = phenylthiohydantoin; Dns = 1-dimethylamino-naphthalene-5-sulfonyl; FLA = fluorescamine; BOC = *tert.*-butyloxycarbonyl; CBZ = N-benzyloxycarbonyl; DCA = dicyclohexylamine; TCP = 2,4,5-trichlorophenyl; MTH = methylthiohydantoin.

DISCUSSION

Using mixed fluorescent materials in a TLC bed permits the discrimination of UV-absorbing materials, which cannot be distinguished from one another in a bed which contains only one fluorescent substance. In addition, the color and the sensitivity can be roughly estimated from a compound's absorption maximum and molar absorption coefficient, respectively[13,14]. PTH-Amino acids absorb[1] in the UV at *ca.* 268 nm and appear as red spots. Exceptions are PTH-dehydrothreonine and

PTH-dehydroserine, which have a maximum absorption[1] at *ca.* 323 nm and give green spots. The detection limit of the method is *ca.* 0.1 nmole of PTH-amino acid and is comparable to methods of Summers *et al.*[3] and Kulbe[2,4] in this respect. The detection limit of PTH-amino acids with ninhydrin is 2.5–5 nmoles in the procedure of Walz and Reuterby[10], and 5–15 nmoles in the procedure of Inagami and Murakami[8]. Furthermore, some PTH-amino acids, such as PTH-valine, PTH-leucine, PTH-isoleucine[8,10], PTH-dehydrothreonine[10], PTH-phenylalanine and PTH-tryptophan[8], do not yield a colored product in the ninhydrin reaction. In the present method, all PTH-amino acids are detected as colored spots without destroying the compounds. In addition, the characteristic fluorescence and color changes produced by the sodium hydroxide spray and heating assists in the identification of certain PTH-amino acids. However, distinguishing between PTH-leucine and PTH-isoleucine remains an unsolved problem in all TLC methods, and an alternative method such as gas chromatography[5] or high-performance liquid chromatography[6] must be used.

The characteristic green color given by PTH-dehydrothreonine, PTH-dehydroserine and PTH-S-carboxymethylcysteine after the second development is especially useful for their identification. Although PTH-dehydrothreonine and PTH-dehydroserine were stable in the chromatographic procedure employed, PTH-dehydroserine and insoluble products of its decomposition formed easily from PTH-serine during storage in methanol. PTH-Dehydrothreonine also formed in methanol, but it was stable. After alkaline treatment, the color of PTH-threonine changed to the same bluish green as that of PTH-dehydrothreonine, which readily forms in alkali[15]. The green color observed after the second development of PTH-S-carboxymethylcysteine probably results from its transformation into PTH-dehydroserine by the acetic acid solvent employed. Identification of PTH-arginine and PTH-histidine, which have in the past been detected with specific spot color reactions[16,17], is easily performed in the present method.

The present method is also useful for the non-destructive detection of other UV-absorbing derivatives of amino acids and peptides at the nanomole level. Colors observed were those expected from their UV maxima. For example, DNP, FLA, PTC, and Dns derivatives absorb strongly in the UV with maxima at *ca.* 360, 385, 240 and 340 nm respectively, and they gave yellowish green, bluish green, red and green colors, respectively (Table II). Tryptophan, tyrosine, phenylalanine and cystine absorb above 250 nm, and they were detected below the 10-nmole level. The detection of as little as 0.3 nmoles of tryptophan is noteworthy.

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