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A fast and sensitive method for measuring picomole levels of total free amino acids in very small amounts of biological tissues

G. H. Fisher[1], **I. Arias**[1], **I. Quesada**[1], **S. D'Aniello**[2], **F. Errico**[2], **M. M. Di Fiore**[2,3], and **A. D'Aniello**[2]

[1] Department of Chemistry and School of Natural and Health Sciences, Barry University, Miami Shores, Florida, U.S.A.
[2] Department of Neurobiology, Zoological Station of Naples, Napoli, Italy
[3] Department of Scienze della Vita, Second University of Naples, Caserta, Italy

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Summary. In the present study we describe a simple and fast method to measure the concentration of total free amino acids in very small amounts of biological tissues. The procedure described here is based on the reaction of free amino acids with *o*-phthaldialdehyde (OPA) in the presence of a reducing agent, β-mercaptoethanol (MET), to give a complex which can be measured by fluorescence. It is a very rapid process and has the same reliability as the conventional ninhydrin method of Moore and Stein but is about 500 times more sensitive. The sensitivity of the new protocol is such to permit the determination with high reliability of very small amounts of free amino acids at picomole levels, either in a standard amino acid mixture or in biological tissues, without chromatographic separation of the amino acids. It is particularly useful when the amount of the sample is very low, e.g. on a single pituitary or pineal gland of small animals or on single cells, such as oocytes or eggs, as well as single ganglions or axons of marine invertebrates.

Keywords: Amino acids – Fluorometric method – OPA-β-Mercaptoethanol – Ninhydrin

Abbreviations: OPA, *o*-phthaldialdehyde; MET, β-mercaptoethanol; PCA, perchloric acid; GABA, gamma amino butyric acid

Introduction

Numerous chromatographic methods have been developed for separation and analysis of amino acids by gas chromatography (GC) and high performance liquid chromatography (HPLC). Although these methods are highly sensitive for measuring picomole levels of individual amino acids, they require time consuming pre- or post-column derivatization and long chromatography

times. It is often necessary to determine total free amino acids in very small amounts of biological tissues without chromatographic separation of individual amino acids. Thus, routine determination of total amino acids requires a rapid and sensitive method.

About half a century ago, Moore and Stein (1948, 1954) pioneered the development of a reliable method for the determination of total amino acids using the ninhydrin reagent. Their method was further developed and utilized for the determination of amino acids in biological samples by automated amino acid analyzers. More recently, Doi et al. (1981) modified the Moore and Stein procedure using cadmium instead of tin in the preparation of the ninhydrin reagent. As a result of this modification, many kinds of peptides were not detected; therefore, this latter method was proposed as a suitable method for the determination of peptidase activity. Although the ninhydrin methods are precise and reliable for the determination of total amino acids, they have limited sensitivity and are not suitable for the determination of the amino acid concentration for those samples in which the amount of available tissue is very small.

The importance and utility of determining small amounts of amino acids in samples is widely recognized, for example those amino acids utilized in the metabolic pathway of cells constituting tissue or of a specific amino acid which has a particular role, such as the neurotransmitters L-Asp, L-Glu, GABA. Therefore, we developed a protocol to determine total free amino acids in very small amounts of sample in a short time. This proposed method has a very high sensitivity, and at the same time is as reliable as the conventional ninhydrin methods. For this reason, the procedure presented here is suitable for routine determination of total free amino acids in biological tissues and in particular is adaptable for cases where the amount of sample available is very small (about a mg), e.g. pituitary or pineal glands etc. of small vertebrates, or single cells such as ganglions or eggs, as well as for cultured cells.

Materials and methods

Reagents

OPA, all the amino acids, amino acid derivatives and peptides were purchased from Sigma. β-mercaptoethanol was purchased from Merck. All other reagents used in this study were analytical grade and were used without further purification.

Preparation of individual amino acids, standard mixture of amino acids, and peptides

Each amino acid and peptide (as reported in Table 1) was prepared at the concentration of 10 mM in 0.1 M HCl and was used as such or diluted 10, 100, or 1,000 times in distilled water. A standard mixture of amino acids was prepared in order to have a general composition of free amino acids similar to that occurring in biological tissues. This standard mixture was obtained by mixing stock solutions of the following 22 amino acids plus ammonia: L-Ser, L-Asp, L-Met, L-Glu, L-Asn, L-Thr, L-Leu, L-Gln, L-Ile, Gly, Taurine, L-Arg, L-Phe, L-His, L-Ala, L-Trp, L-Val, L-Tyr, GABA, L-Lys, L-Cys, L-Pro,

and NH_4SO_4, such that each amino acid had the concentration of 0.4 μmol/mL (total 8.8 μmol/ml) in 0.1 M HCl.

Preparation of borate buffer at various pH values

In order to know the optimum pH at which the amino acids react with the OPA reagent we used borate buffers between pH 8.0 and 10.5, prepared by addition of either 0.02 M boric acid or 0.02 M NaOH to 0.02 M sodium borate until we obtained pHs 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5.

OPA-β-mercaptoethanol reagent (OPA-MET used in the present study)

This reagent was prepared by dissolving 100 mg of OPA in 10 mL of methanol, then mixing it with 200 μL of β-mercaptoethanol, and then the entire solution was mixed with 400 mL of 0.02 M borate buffer at pH 9.5.

Conventional ninhydrin reagent (Moore and Stein procedure)

This reagent was prepared according to the procedure of Moore and Stein (1948, 1954). In brief, the reagent is the following: 0.80 g of $SnCl_2 \bullet 2H_2O$ is dissolved in 500 mL of citrate buffer (0.2 M, pH 5.0) and mixed with a second solution of 20 g of ninhydrin in 500 mL of methyl cellusolve.

Modified Cd-ninhydrin reagent (Doi et al. procedure)

This reagent was prepared according to Doi et al. (1981) as follows: 0.8 g of ninhydrin was dissolved in 80 mL of absolute ethanol and 10 mL of acetic acid. After dissolving, to this mixture was added a second solution consisting of 1 g of $CdCl_2$ in 1 mL of distilled water. Before use, this reagent was diluted in distilled water 1 : 1.5.

Preparation of tissue extracts

As a general principle, the samples of biological tissue were homogenized with perchloric acid (PCA) in order to extract amino acids and precipitate proteins. The supernatant was then neutralized with KOH in order to eliminate PCA (as an insoluble precipitate of $KClO_4$). If the available tissue weight was more than 2 mg, then it was homogenized with 0.1 M PCA in ratio 1 : 20 and centrifuged at 15,000 g for 10 min. The supernatant was then neutralized with 2 M KOH (using a volume of KOH 1/19th of the supernatant). When available tissue was less than 1 mg (for example using small tissues such as young rat pineal or pituitary glands or isolated ganglion of marine mollusks) the tissue was homogenized with 0.04 M PCA, using ratio of 1 : 50 and centrifuged as above. The supernatant was neutralized with 0.8 M KOH (using a volume of KOH 1/19th of the supernatant). After the addition of KOH, the samples were cooled on ice water for about 10 min and centrifuged as above. The supernatant was used for amino acid analysis, as below.

Amino acid determination

Fluorometric method (present study): 5–40 μL of tissue supernatant (as obtained above) were mixed with 3 mL of OPA-MET reagent. After 2–10 min the fluorescence was read in cuvettes on a spectrofluorometer (Perkin-Elmer Luminescence LS50B Spectrometer)

using an excitation wavelength of 340 nm and an emission wavelength of 440 nm, against the OPA-MET reagent. In order to calculate the concentration of the amino acids in the sample, 10 μL of the standard mixture of amino acids (prepared as described above and diluted 1:10, 1:100 and 1:1,000) were mixed with 3 mL of OPA-MET reagent and the fluorescence was read as for the sample.

Conventional Sn-ninhydrin procedure: 50–100 μL of sample supernatant were mixed with 0.5 mL of ninhydrin reagent and heated for 20 min at 100°C. Then 2.5 mL of diluent reagent were added and mixed, and the color was read within 20–30 min at 570 nm against the blank reagent, using a UV/Vis spectrophotometer.

Modified Cd-nihydrin procedure: 50–100 μL of sample supernatant were mixed with 3 mL of Cd ninhydrin reagent and heated for 10 min at 84°C. After cooling the absorbance was read at 507 nm against the blank reagent.

HPLC fluorometric procedure: This analysis was based on the protocol in which the amino acids were derivatized with OPA-MET reagent and then separated by HPLC, as described by Godel et al. (1984).

Results and discussion

Until the 1970s–80s, when other derivatives of amino acids were studied [*o*-phthadialdehyde (OPA) (Roth and Hampai, 1973; Benson and Hare, 1975; Hill et al., 1979; Godel et al., 1984; Lindroth and Mopper, 1979), fluorescamine (Undenfriend et al., 1972; Weigele et al., 1972), dansyl (Spiess et al., 1981), dabsyl (Chang et al., 1981), phenylisothiocyanate (Bidlingmeyer et al., 1984), fluorenylmethyl (Einarsson et al., 1983), etc.], ninhydrin was the primary method for determination of amino acids. Since we had the necessity to determine total amino acids in very small amounts of tissue extracts, e.g. pituitary or pineal glands of young rat or mouse or individual ganglion or eggs of marine invertebrates, we devised the present protocol. The method developed here is based on the reaction between free amino acids and *o*-phthaldialdehyde in the presence of β-mercaptoethanol used as a reducing agent. The results obtained from this study are presented in Tables 1 through 4 and Figs. 1 and 2.

Table 1 shows a comparison of the relative reactivity of individual amino acids, derivatized amino acids, and small peptides by the present OPA-MET method and each of the conventional ninhydrin methods. Due to the different types of reagents, invidual amino acids react to different extents (percent reactivity compared to the amino acid with maximum optical density). Ser has the highest reactivity (taken as 100%) with the OPA-MET reagent, whereas Lys has the maximum reactivity (100%) with the conventional Sn-ninhydrin reagent, and finally Met reacts the most (100%) with the modified Cd-ninhydrin reagent. Most all of the other amino acids, except for taurine, GABA and cysteine, react with each of the three reagents at approximately the same efficiency. Taurine reacts maximally with the present OPA-MET reagent and the conventional Sn-ninhydrin reagent, but not with the modified Cd-ninhydrin method. Asn and Gln also react by both the OPA-MET and conventional Sn-ninhydrin reagents, but to a lesser extent with the modified Cd-ninhydrin reagent. GABA reacts mainly with the OPA-MET reagent. Finally, as expected, Pro, with a secondary amino group, is not detected by any of these reagents.

Table 1. Compative study of the reactivity of amino acids, amino acid derivatives, and peptides by OPA-β-mercaptoethanol and ninhydrin methods

	Present method (OPA-MET reagent)*	Conventional $SnCl_2$-ninhydrin method** (Moore and Stein, 1948, 1954)	Modified Cd-ninhydrin method* (Doi et al., 1981)
		% Reactivity	
Amino acids			
Ser	100	84	75
Asp	90	78	87
Met	90	89	100
Glu	89	94	98
Asn	88	84	20
Thr	88	82	72
Leu	86	89	90
Gln	86	88	40
Ile	85	89	85
Gly	85	90	91
Taurine	85	86	2–3
Arg	85	89	80
Phe	85	78	70
His	80	92	65
Ala	79	89	85
Trp	75	64	67
Val	72	91	75
Lys	76	100	85
Tyr	64	79	65
GABA	53	20	2–3
Cysteine (Cys)	5	48	25
Pro	<0.1	4	2
NH_3	5	87	4
Amino acid derivatives and peptides			
N-Methyl-D-Ala	<0.1	8	3
N-Methyl-D-Asp	<0.1	8	3
Arg-Glu	8	48	2
Leu-Phe	9	15	2
Ala-Val	10	34	3
Leu-Tyr	10	40	3
Phe-Ala	11	45	3
Leu-Gly	14	82	2
Gly-Asp	20	90	39
Gly-Leu	26	93	20
Gly-Ala	33	95	40
Ala-Gly-Gly	6	50	2
Ala-Leu-Glu	6	44	2
Leu-Gly-Gly	9	52	2
Gly-Gly-Gly	15	42	20
Tyr-Ala-Gly-Phe-Met (Met Enkephalin)	2	8	2
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Phe-Gly-NH_2 (mGnRH)	<1	3	<1

For the present OPA-MET method, 10 μL of each amino acid at concentration of 0.10 μmol/mL (1.0 nmol) were mixed with 3 mL of the OPA-MET reagent. For the other two methods, 10 μL of each amino acid at the concentration of 10 μmol/mL (100 nmol) were mixed with 3 mL of the respective reagents and analyzed as described in the "Materials and methods" section.

* The data of the % of reactivity of the amino acids in columns 1 and 3 represent the mean value of 4 experiments determined by us, using the respective methods. The % reactivity for individual amino acids in each column is relative to the amino acid with the maximum O.D. The data for the peptides were determined by us, using L-Lys as 100% reactivity. ** The data in column 2 are those reported by Moore and Stein.

Concerning the reactivity of amino acid derivatives and peptides with the three reagents, as is shown in Table 1, the conventional Sn-ninhydrin method reacts well with the small peptides (di- and tripeptides) but very little with the larger peptides (e.g. Met-Enkephalin or mGnRH). The small peptides react to a lesser extent by the modified Cd-ninhydrin reagent, and very little by the OPA-MET reagent. However, the two derivatized amino acids, N-methyl-D-Asp and N-methyl-D-Ala, react little with the conventional Sn-ninhydrin reagent, very little with the modified Cd-ninhydrin reagent, and almost not at all with the OPA-MET reagent.

Independent from the consideration of the above results (Table 1), the goal of developing the present method was to emphasize its sensitivity in the determination of total free amino acids. Although individual amino acids have different percent reactivity with a given reagent, there should be no overall effect on the determination of the total amino acid content by a given reagent. Table 2 gives a comparison of the sensitivity of the present OPA-MET method with that of the conventional Sn-ninhydrin method for determination of the total amino acid content. Clearly, the fluorescence method is able to detect very low concentrations of total free amino acids (at the picomole level), whereas the ninhydrin method requires much higher concentrations (nanomoles) before it is possible to detect amino acids. With the present OPA-MET method we are able to detect a minimum amount of total amino acids between 2–10 picomoles in both the standard mixture of amino acids and in tissue extracts (Table 2); e.g. 88 picomoles of amino acids (10μL of a solution 0.0088μmol/mL) give a reading of 0.270 O.D. in 3 mL of assay mixture. If we consider that the reading of 0.050 O.D. could still be considered as a reliable reading, then this means that this O.D. reading corresponds in proportion to 16 picomoles. In addition, if we reduce the volume of the assay mixtures to read in a micro cuvette of 0.5 mL, then we are able to detect a minimum of 2.5 picomoles of total amino acid. Contrary to this, in order to obtain an appreciable reading using the conventional Sn-ninhydrin method, a concentration of amino acids about 500 times more is needed (Table 2).

In order to determine the time course of the development of the fluorescence and the time at which the maximum fluorescence occurs with the OPA-MET reagent, a study was carried out on the amino acid standard and on a rat brain tissue (supernatant of the deproteinized tissue) to measure the fluorescence at given times after mixing with the reagent. Taking as an example the results of L-Ala compared with the tissue sample (Table 3), the maximum fluorescence is reached within 1 to 2 minutes after mixing the sample with the reagent, and the fluorescence is stable for approximately 10–30 minutes. In addition, a study of the dependence of the fluorescence on the pH of the reagent was carried out. Figure 1 shows that the optimum pH for reaction of amino acids with the OPA-MET reagent is at pH 9.5. Concerning the suitable buffer to use for the reaction, we have observed that borate buffer is the best because it is good for buffering solutions in the range of pH 9 to 10 and at the same time gives a minimum basal fluorescence.

The total amino acid concentrations found in some biological tissues detemined using the present OPA-MET method and the HPLC fluorometric

Table 2. Comparative sensitivity of the present OPA-MET method vs the conventional ninhydrin method

	Present OPA-MET fluorescence method	Conventional Sn-ninhydrin Moore and Stein Method
	O.D.[a]	O.D.[b]
Amino acid mixture		
0.0088 μmol/ml	0.270	Not detected
0.088 ″	Over 1.0	″
0.88 ″	″	0.050
8.8 ″	″	0.430
L-Ser		
0.01 μmol/ml	0.350	Not detected
0.1 ″	Over 1.0	″
1.0 ″	″	0.070
10 ″	″	0.620
L-Ala		
0.01 μmol/ml	0.220	Not detected
0.1 ″	Over 1.0	″
1.0 ″	″	0.050
10 ″	″	0.460

For the OPA-MET method, 10 μL of the samples (standard mixture of amino acids, or each single amino acid) were mixed with 3 mL of the OPA-MET reagent and the fluorescence was read using the maximum sensitivity of the spectrofluorometer. The data are the means of 4 experiments.
For the conventional Sn-ninhydrin method, 10 μL of each of the same samples were mixed with 3 mL of ninhydrin reagent, heated, and the absorbance was read on the spectrophotometer at 570 nm.
"Over 1.0" means that the amount of amino acids was too high, giving a fluorescence reading greater than the upper detection limit of the spectrofluorometer. "Not detected" means that the amount of the amino acids was so low that the absorbance reading was below the minimum dectection limit of the spectrophotometer.
[a] O.D. indicates the fluorescence read at 340 nm excitation and 440 emission with 15/18 slit widths, respectively. [b] O.D. indicates the absorbance read at 570 nm on the UV/Vis spectrophotometer.

method were compared with those found using the two ninhydrin procedures. As seen in Table 4, the total amino acid concentrations obtained by the two fluorometric protocols and by the conventional Sn-ninhydrin method are comparable. The correlation of the OPA-MET method with the Sn-ninhydrin method for these tissues is shown in Fig. 2. As seen from this figure, the correlation is very high, $r = 0.999$. However, when the available tissue is small (e.g. rat and octopus brain or marine animal ganglia or gonads), the conventional Sn-ninhydrin procedure (Moore and Stein, 1948, 1954) is not able to detect the amino acids in these tissues. In addition, the modified Cd-ninhydrin method (Doi et al., 1981) is also able to detect total amino acids only when the tissue is large, but the concentrations found using this procedure are not comparable to those found using the fluorescent methods (Table 4).

Table 3. Fluorescence developed from the reaction of amino acids with the OPA-MET reagent as a function of time

	Fluorescence O.D. at times after mixing sample with OPA-MET reagent							
	15 s	30 s	45 s	60 s	2 min	10 min	30 min	2 hrs
L-Ala	0.370	0.575	0.600	0.615	0.614	0.611	0.585	0.545
Rat brain homogenate	0.360	0.400	0.420	0.428	0.430	0.428	0.420	0.390

The L-Ala was analyzed using 10 μL of a solution at 0.1 μmol/mL plus 3 ml of the OPA-MET reagent.
Rat brain homogenate was analyzed using 10 μL of a supernatant of rat brain homogenate (1:50, see "Materials and methods") plus 3 mL of OPA-MET reagent.
The fluorescence was read at different times at 340 nm excitation wavelength and 440 nm emission wavelength, with 10/15 slit widths, respectively of the fluorometer.
The data are the means of 4 experiments.

Table 4. Total free amino acids found in some biological tissues determined by the present fluorometric method and other methods

	Present OPA-MET fluorometric method (μmol/g)	HPLC fluorometric method (Godel et al.) (μmol/g)	Conventional Sn-ninhydrin method (Moore and Stein) (μmol/g)	Modified Cd-ninhydrin method (Doi et al.) (μmol/g)
Young rat				
Brain	60.5	61.2	63.4	42.5
Pituitary gland	55.2	57.6	Not detected	Not detected
Pineal gland	45.6	46.2	" "	" "
Octopus vulgaris				
Brain optical lobe	133.5	138.1	137.3	60.2
Optical gland	121.1	122.3	Not detected	Not detected
Stellate ganglion	68.3	69.8	" "	" "
Hepatopancreas	95.4	96.7	95.3	84.5
Ciona intestinalis				
Gonad	44.2	46.4	45.3	33.4
Neural ganglion	16.3	17.3	Not detected	Not detected
Aplysia fasciata				
Single buccal ganglion	41.2	42.3	Not detected	Not detected
Single abdominal ganglion	42.4	43.4	" "	" "
Starfish				
Single egg	65.4	63.3	Not detected	Not detected

The values are the means obtained from 5 determinations carried out on each tissue.
"Not detected" means that the tissue was too small to be able to detect amino acids according to the method used.
Octopus vulgaris is a marine mollusk belonging to the Cephalopods class. *Ciona intestinalis* is a marine tunicate belonging to the Protochordate phyla. *Aplysia fasciata* is a marine mollusk belonging to the Opistobranchia class. *Starfish* is a marine invertebrate belonging to the class of Asteroids.

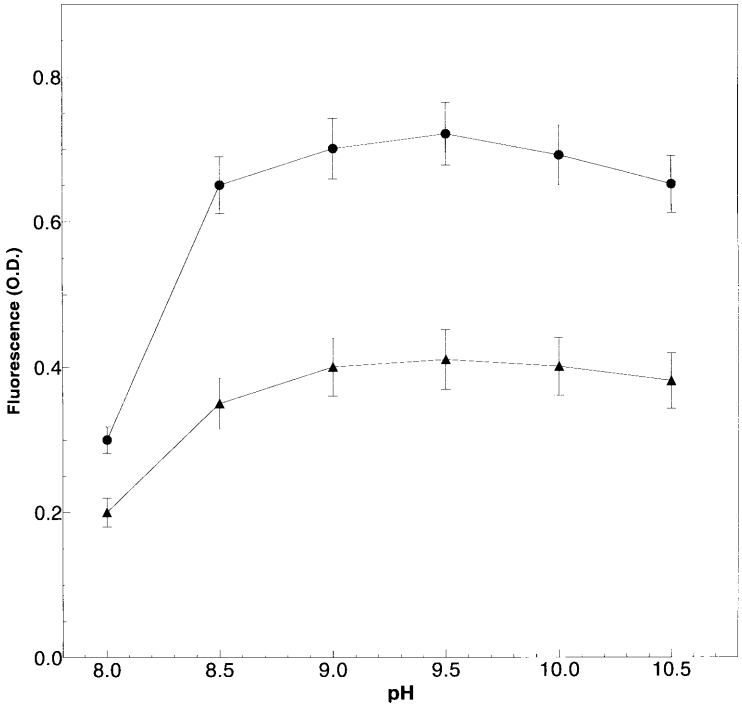

Fig. 1. Dependence of fluorescence on the pH of the OPA-MET reagent. ● Rat brain homogenate. ▲ L-Ala

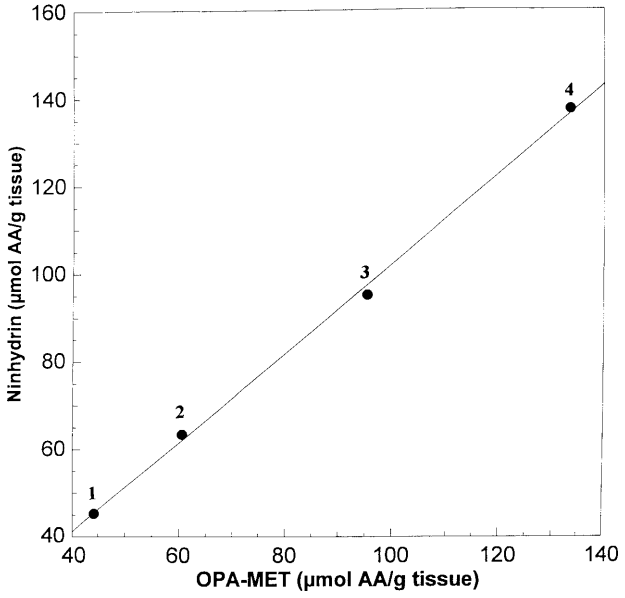

Fig. 2. Correlation of the total amino acid concentration obtained in some tissues (as reported in Table 4) using the OPA-MET fluorescence method versus the conventional Sn-ninhydrin method. Correlation r = 0.999. *1* is Ciona gonad, *2* is rat brain, *3* is Octopus hepatopancreas, *4* is Octopus brain

In conclusion, using the present OPA-MET fluorescence method, amino acids having a free α- or β-amino group react with the OPA-β-mercaptoethanol reagent to an extent similar to that of the conventional ninhydrin reagents for most amino acids and dipeptides. However, the OPA-MET procedure has certain advantages, particularly over GC and HPLC chromatographic methods: it is less time consuming, does not require heating the sample mixture, and above all it is about 500 times more sensitive than the colorimetric ninhydrin methods, thus being able to detect picomole levels of total free amino acids in small amounts of biological tissue that were not possible to detect by the conventional ninhydrin methods.

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Authors' address: Dr. George H. Fisher, Department of Chemistry, Barry University, 11300 NE 2nd Ave., Miami Shores, FL 33161 U.S.A.,
Fax +305 899-3479, E-mail: gfisher@mail.barry.edu

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