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Determination of hydrogen sulfide and acid-labile sulfur in animal tissues by gas chromatography and ion chromatography

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Abstract

A sensitive and reliable method was developed for the determination of hydrogen sulfide and acid-labile sulfur (ALS) in animal tissues using gas chromatography with flame photometric detector (GC-FPD) and ion chromatography (IC). Hydrogen sulfide trapped in alkaline solution was determined by GC-FPD as hydrogen sulfide or by IC as sulfate after oxidation with hydrogen peroxide. Sodium sulfide used as a source of hydrogen sulfide was standardized by IC. Fresh rat liver and heart tissues contained 112.2±23.0 and 274.1±34.6 nmol/g of ALS respectively. Free hydrogen sulfide was not detected.

Keywords: Hydrogen sulphide; Acid-labile sulphur

1. Introduction

Hydrogen sulfide (H_2S) is well known as an environmental toxic substance and numerous studies have been reported [1]. Hydrogen sulfide is also formed endogenously in the metabolism of L-cysteine in animal tissues [2,3], and its possible roles were suggested [4,5].

Animal tissues contain bound sulfur such as acid-labile sulfur (ALS) and sulfane sulfur. ALS can be liberated as hydrogen sulfide by treating tissues with acid [6] and sulfane sulfur is liberated as thiocyanate by treating tissues with cyanide [7]. A possible regulatory role of sulfane sulfur has been suggested [8], but physiological and pathological status of these bound sulfur including protein-associated sulfur [6,9] has not been fully established.

Methods of hydrogen sulfide determination include the colorimetric method utilizing methylene blue formation [2,10], the method using ion-specific electrode [11,12], high- performance liquid chromatography after derivatization [13], and gas chromatography with flame photometric detector (GC-FPD) [14]. ALS can be determined after conversion to hydrogen sulfide by acid treatment of tissues.

We have investigated L-cysteine metabolism via 3-mercaptopyruvate pathway in mitochondria, and suggested that this pathway served for ALS formation and that hydrogen sulfide was formed as an intermediate leading to sulfate formation [15,16]. In order to extend these studies, we developed a sensitive and reliable method to determine hydrogen sulfide and ALS in animal tissues. In the present

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method, hydrogen sulfide trapped in alkaline solution was determined by GC-FPD as hydrogen sulfide and by ion chromatography after oxidation to sulfate.

2. Experimental

2.1. Materials

Sodium sulfate (Na_2SO_4), sodium sulfite (Na_2SO_3), sodium sulfide (Na_2S), phthalic acid, phosphoric acid and sodium hydroxide of reagent grade were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan). Sodium thiosulfate ($Na_2S_2O_3$) was a product of Merck (Darmstadt, Germany). L-Cysteine and tris(hydroxymethyl)aminomethane (Tris) were of Sigma (St. Louis, MO, USA). Glutathione (GSH) was obtained from Boehringer (Manheim, Germany). Male rats of Wistar strain weighing 305–510 g were fed a laboratory diet of MF Oriental Yeast (Tokyo, Japan) and used in this study.

2.2. Collection of hydrogen sulfide and ALS as sodium sulfide from tissue samples (gas transfer step)

Fresh rat liver was homogenized with 5 volumes of 0.9% sodium chloride solution at 0–4°C using a Potter–Elvehjem type glass homogenizer. In case of the heart, pooled heart tissues taken freshly or stored at −30°C for 5 days–3 months were homogenized as above. The homogenate (10 ml or less) was placed in an impinger (24 mm ID×118 mm, Shibata Glass Co., Tokyo, Japan), and water was added to make up to a final volume of 20 ml. After adding 5 ml of 50% phosphoric acid or water (control), nitrogen gas, which was passed through a gas wash-bottle containing 100 ml of 0.1 *M* sodium hydroxide solution, was introduced at a flow rate of 200–250 ml/min to the above impinger for 20 min at room temperature. The nitrogen gas from the impinger was then introduced to a gas bubbler (24 mm ID×118 mm, Shibata Glass Co.) containing 10.0 ml of 0.1 *M* sodium hydroxide solution in order to trap hydrogen sulfide as sodium sulfide. This solution, which contained sodium sulfide, was further used for the sample preparation for gas chromatography.

As a standard material, a solution containing a known amount of sodium sulfide was processed as above.

When sodium sulfite was examined instead of sodium sulfide, formation of sulfur dioxide (SO_2) and its transfer to 0.1 *M* sodium hydroxide solution were performed as above.

2.3. Sample preparation for gas chromatography (gas equilibration step)

An aliquot (usually 4.0 ml) of the 0.1 *M* sodium hydroxide solution obtained above in the gas transfer step was placed in a vial (19 mm×70 mm) for automatic sampler with an open top screw cap which was sealed with a Teflon/silicone septum (type SY-5, Nichiden-Rika Glass Co., Ltd., Kobe, Japan). After addition of 0.5 ml of 70% phosphoric acid through the septum using a syringe, the vial was incubated at 40°C for 60 min.

During this incubation, the content in the vial was stirred with a magnetic stirring bar (4.5 mm×12 mm) in order to accelerate rapid evolution of hydrogen sulfide from the aqueous phase and equilibration of gas phase in the head-space. Fifty μl of the head-space gas was subjected to gas chromatography.

In some experiments, tissue homogenates or sample solutions were directly placed in the vial, and the gas equilibration step was performed without the above gas transfer step. This procedure was mentioned as the direct method, and the standard procedure consisted of gas transfer and gas equilibration steps was as the indirect method.

2.4. Gas chromatography

A gas chromatograph G-5000 A equipped with a flame photometric detector (GC-FPD) and a data processor D-2500 (Hitachi Seisakusho, Ltd., Tokyo, Japan) were used. Column used was 5% polyphenyl ether 5 rings on Uniport HP 80/100 packed in Teflon tube (3 mm ID×6 m) (GL Sciences, Tokyo, Japan). Temperature was regulated as follows: column oven, 70°C; injector, 100°C; FPD, 180°C. Nitrogen gas was used as a carrier gas and the pressure was regulated at 180 kPa. Gas pressure for FPD was regulated as follows: hydrogen, 80 kPa; oxygen, 60 kPa; nitrogen, 60 kPa.

2.5. Sample preparation for ion chromatography

In preliminary experiments, it was found that sodium sulfide in 0.1 *M* sodium hydroxide solution was easily oxidized to sulfate by hydrogen peroxide. This was applied to the determination of hydrogen sulfide, which was oxidized to sulfate and determined by ion chromatography. An aliquot (usually 1.0 ml) of 0.1 *M* sodium hydroxide solution containing sodium sulfide obtained by the gas transfer step was placed in a test tube (15×124 mm) with a Teflon-lined screw cap. After addition of 30 μl of 30% hydrogen peroxide, the mixture was incubated at 50°C for 60 min. After cooling, 0.5 ml of the mixture was placed on the top of a Dowex 50 W column (0.7×2.5 cm, 200–400 mesh, H^+ form, washed thoroughly with pure water) in order to eliminate sodium ions which interfered with ion chromatography. The column was washed with several portions of water and 3.0 ml of the effluent was collected, 100 μl of which was subjected to ion chromatography.

Sodium sulfite and sodium thiosulfate solutions were also analyzed by a direct and an indirect method similar to the determination of hydrogen sulfide using gas chromatography. In the case of the indirect method, the 0.1 *M* sodium hydroxide trap solution was treated with hydrogen peroxide as above. In the case of the direct method, 1.0 ml of a sample solution was treated with hydrogen peroxide as above without the gas transfer step, and analyzed by ion chromatography.

2.6. Ion chromatography

The ion chromatography system (LaChrom; Hitachi Co., Ltd., Tokyo, Japan) consisted of the following parts: an isocratic pump L-7110, a column oven L-7300, a conductivity detector L-7470 and a data processor D-7500. The ion chromatography column system used was a Shodex IC I-524A column (4.6 mm Diameter×100 mm) connected to a guard column of Shodex IC IA-G (4.6 mm Diameter×10 mm). Both columns are products of Showa Denko Co., Ltd, Tokyo, Japan. The chromatography was performed at a flow-rate of 1.5 ml per min and at a temperature of 40°C using buffer solution which contained 2.5 m*M* phthalic acid in water–methanol (85:15, v/v) adjusted to pH 3.80 with Tris.

3. Results and discussion

Fig. 1 shows chromatograms obtained by gas chromatography. Fig. 1A shows peaks of hydrogen sulfide and sulfur dioxide when sodium sulfide and sodium sulfite were processed by the standard (indirect) method and the head-space gas samples were mixed. Similar chromatograms were obtained when sodium sulfide and sodium sulfite were analyzed by the direct method. Retention time of hydrogen sulfide was 3.06 min and that of sulfur dioxide was 3.57 min. Detection limit of hydrogen sulfide was 6.0 pmol at a signal-to-noise ratio >3. Fig. 1B is a chromatogram obtained when rat heart was analyzed by the standard method. Fig. 1C is a chromatogram showing that the peak obtained from rat heart coincides with that obtained from sodium sulfide.

Fig. 2 shows the effect of incubation time of the vial containing various amounts of sodium sulfide at 40°C on the equilibration of head-space gas phase.

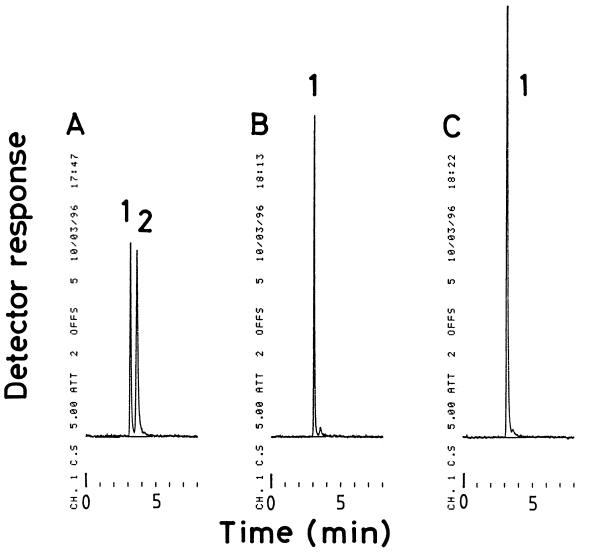

Fig. 1. Gas chromatograms of hydrogen sulfide (peak 1) and sulfur dioxide (peak 2). A: Head-space gas samples of sulfur dioxide and hydrogen sulfide were mixed in an injection syringe and applied. B: Head-space gas sample prepared from heart tissue. C: Head-space gas samples prepared from heart tissue and from sodium sulfide were mixed in an injection syringe and applied.

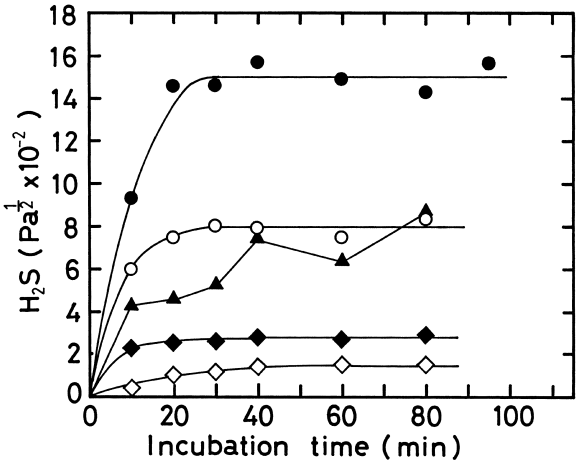

Fig. 2. Effect of incubation time on the equilibration of hydrogen sulfide into the head-space gas. Incubation vials contained 100 (●), 60 (○), 20 (♦) and 10 nmol of sodium sulfide (◇), and solutions were stirred with magnetic stirrer as described under Experimental. One of the vials containing 60 nmol of sodium sulfide (▲) was incubated without stirring.

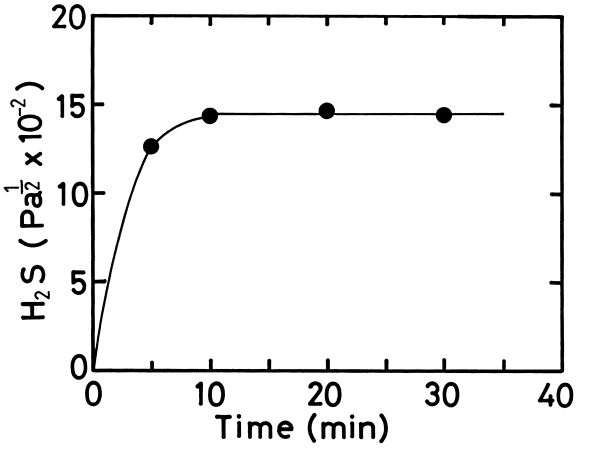

Fig. 3. Effect of time on the transfer of hydrogen sulfide from the impinger to the sodium hydroxide solution. One hundred nmol of sodium sulfide was added to 10 ml of liver homogenate and hydrogen gas transfer was performed as described under Experimental.

The figure shows that equilibration completed at 30 min, and that stirring during the incubation is necessary for the equilibration (see ▲–▲). Thus in routine analyses 60 min of incubation with stirring is employed.

A standard curve of hydrogen sulfide by gas chromatography was prepared by adding various amounts between 10 and 100 nmol of sodium sulfide to the vial of sample preparation for gas chromatography before the addition of phosphoric acid and incubating at 40°C for 60 min. The regression line was $y=15.7x-73.1$ when the square root of the peak area y (a relative number of integrator output) was plotted against the sodium sulfide x (nmol). The intercept was close to zero and the correlation coefficient was 1.000.

Fig. 3 shows the effect of time of hydrogen sulfide transfer from the rat liver homogenate to the 0.1 *M* sodium hydroxide trap solution. The figure shows that the transfer was completed at 10 min when 100 nmol of sodium sulfide was added to the homogenate placed in the impinger. In the routine experiment, 20 min of transfer time was employed.

It was found that hydrogen sulfide could be determined by ion chromatography after oxidation of hydrogen sulfide by hydrogen peroxide. Fig. 4 shows chromatograms obtained by ion chromatography. For sulfate (Fig. 4A, peak 1) and thiosulfate (Fig. 4A, peak 2) retention times of 13.0 and 19.7 min were determined respectively. Detection limit of sulfate and thiosulfate was 20.0 pmol at a signal-to-noise

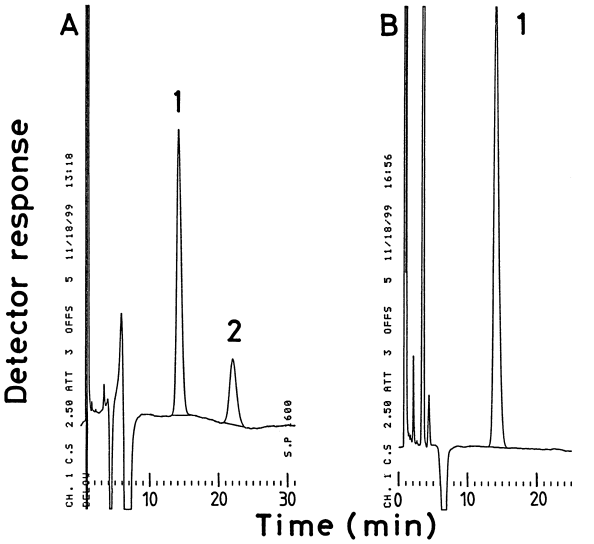

Fig. 4. Ion chromatograms of sulfate (peak 1) and thiosulfate (peak 2). A: A mixture of sodium sulfate and sodium thiosulfate was applied. B: A sample prepared by treating sodium sulfide with hydrogen peroxide was analyzed.

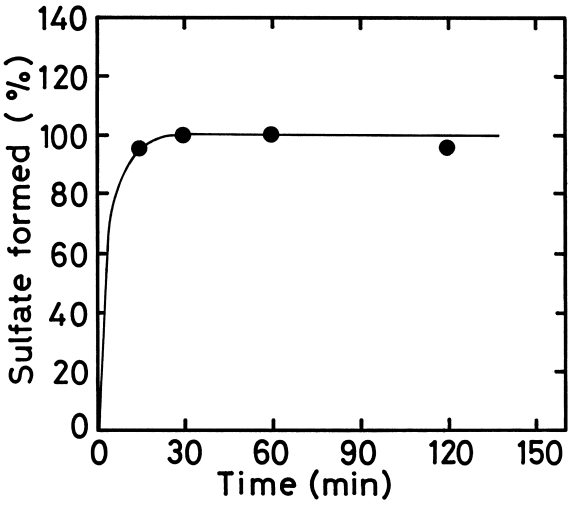

Fig. 5. Effect of time on the oxidation of hydrogen sulfide to sulfate by hydrogen peroxide treatment. Sodium hydroxide (0.1 *M*) solution (1.13 ml) containing 1.08 μmol of sodium sulfide was incubated with 0.9% hydrogen peroxide at 50°C.

ratio >3. Fig. 4B shows a chromatogram of sulfate formed by oxidation of sodium sulfide with hydrogen peroxide as described above. Fig. 5 shows effect of time on the oxidation of hydrogen sulfide to sulfate by hydrogen peroxide. The oxidation to sulfate was shown to be completed in 30 min under the present conditions. In routine experiments, 60 min of incubation time was employed.

A standard curve of sulfate by ion chromatography was prepared using sodium sulfate between 0.3 and 10.0 nmol. The regression line was $y=3.80x-0.11$ when relative peak area y (a relative number of integrator output) was plotted against sodium sulfate x (nmol). The intercept was close to 0 and the correlation coefficient was 1.000.

Table 1 shows recovery of sodium sulfide, from which hydrogen sulfide was formed, transferred to 0.1 *M* sodium hydroxide solution, oxidized to sulfate, and finally the sulfate was determined by ion chromatography. As sodium sulfate was easily handled as a standard material, ion chromatographic determination of sulfate was used for the standardization of hydrogen sulfide determination. As shown in Fig. 1B, sulfur dioxide was formed when sodium sulfite was treated with 10% phosphoric acid and it was transferred to 0.1 *M* sodium hydroxide solution. Table 1 also shows the recovery of sodium sulfite, from which sulfur dioxide was formed, transferred to 0.1 *M* sodium hydroxide solution, and finally determined as sulfate by ion chromatography after oxidation by hydrogen peroxide. As shown in the table, the recovery was 76%, indicating its recovery of the transfer step was incomplete compared to that of hydrogen sulfide.

Table 1
Recovery of sodium sulfide, sodium sulfite and sodium thiosulfate determined as sulfate after oxidation by hydrogen peroxide

Material (μmol)	Sulfate recovered, μmol (%)	
	Indirect method	Direct method
Sodium sulfide (9.97[a])	9.894±0.329[b]	–
	(99.24±3.30)	–
Sodium sulfide (1.076)	–	1.075±0.018[b]
	–	(99.91±1.68)
Sodium sulfite (10.00[a])	7.588	–
	(75.99)	–
Sodium sulfite (1.000)	–	1.048±0.018[b]
	–	(104.8±1.8)
Sodium thiosulfate (10.00[a])	0.275	–
	(2.75)	–
Sodium thiosulfate (1.000)	–	1.993±0.017[b]
	–	(199.3±1.7)

[a] This amount of sodium sulfite or sodium thiosulfate was placed in an impinger, and hydrogen sulfide or sulfur dioxide were trapped in 10 ml of 0.1 *M* sodium hydroxide solution, 1.0 ml of which was used for oxidation step using hydrogen peroxide.

[b] Mean±SD of duplicate or triplicate determinations of two to four experiments.

Possible formation of hydrogen sulfide from other sulfur compounds commonly found in animal tissues in the hydrogen sulfide transfer step, in which samples were acidified with 10% phosphoric acid, was examined. No hydrogen sulfide formation was detected from L-cysteine, L-cystine, D,L-homocystine and GSH under the present analytical conditions.

Table 2 summarizes recovery of hydrogen sulfide from sodium sulfide which was added to rat liver homogenate and determined by gas chromatography. As shown in the table, the recoveries were good, when 10 to 65 nmol of sodium sulfide was added to 10 ml of rat liver homogenate placed in the impinger.

The present method was applied to freshly prepared homogenates of rat tissues, but free hydrogen sulfide and sulfur dioxide were not detected in the liver and heart by gas chromatographic analyses. On the other hand, when these tissues were treated with

Table 2
Recovery of hydrogen sulfide (H_2S) by gas chromatography when sodium sulfide (Na_2S) was added to rat liver homogenate[a]

Na_2S added (nmol/10 ml of homogenate)	H_2S determined (nmol)	Recovery (%)
11.14	11.47	100.3
37.10	39.47	106.4
38.03	36.43	95.8
65.20	71.98	110.4
		103.2±6.5[b]

[a] A known amount of sodium sulfide solution was added to 10 ml of rat liver homogenate placed in an impinger.
[b] Mean±SD of recoveries. Each value is mean of duplicate determinations.

Table 3
Detection of free hydrogen sulfide (H_2S) and acid-labile sulfur (ALS) in rat tissues

Tissue (*n*)	nmol/g of wet tissue[a]	
	H_2S	ALS
Liver (7)[b]	0.0	112.2±23.0
Heart (7)[c]	0.0	274.1±34.6

[a] Duplicate vials were employed for the preparation of headspace gas sample and gas chromatography.
[b] Seven experiments using seven fresh liver tissues from seven animals.
[c] Seven experiments using 17 heart tissues from 17 animals which were stored for 0–90 days at −30°C. Two to three heart tissues were used per experiments.

Table 4
Effect of sample storage time and analysis method on the determination of acid-labile sulfur (ALS)

Storage time (days)[a]	Number of hearts	Number of experiments	Method[b]	ALS (nmol/g of tissue)
0[c]	1	1	I	267.0
5	3	2	I	245.5±9.3
7–12	5	5	D	290.9±25.4
21	6	1	I	347.4
		1	D	350.5
90	7	3	I	271.0±3.4

[a] Tissues were weighed and stored at −30°C.
[b] Indirect (I) or direct (D) methods were used. For details see Materials and methods.
[c] Fresh tissue was used immediately after taking out.

phosphoric acid, 112.2±23.0 nmol/g in liver (seven experiments using seven liver tissues from seven animals) and 274.1±34.6 nmol/g in heart (seven experiments using 17 heart tissues from 17 animals) of hydrogen sulfide were detected (Table 3). These values are four and two times higher, respectively, compared to the values obtained by determination of hydrogen sulfide using HPLC after gas dialysis as reported by Ogasawara et al. [6]. The discrepancy suggests that the transfer of hydrogen sulfide in the present method is efficient as shown above. In one experiment using two kidney tissues from one animal, 174.3 nmol/g of ALS was detected. This value is also four times higher than the value reported [6].

Table 4 summarizes effect of storage time and analysis method on the determination of ALS in the rat heart. The ALS contents did not differ significantly by storage at −30°C for up to 90 days. The result also shows that there were no significant differences between results obtained by the direct and indirect methods. However, it seems preferable to confirm the results of the direct method by the indirect method.

Determination of hydrogen sulfide by ion chromatography after its oxidation to sulfate is convenient, but it is also preferable to check the results by gas chromatography for confirmation when biological samples are examined.

Thus, the present method seems to be useful for the determination of free hydrogen sulfide and ALS in biological materials and also applicable to the metabolic studies of sulfur compounds.

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References

[1] R.J. Reiffenstein, W.C. Hulbert, S.H. Roth, Annu. Rev. Pharmacol. Toxicol. 32 (1992) 109.
[2] M.H. Stipanuk, P.W. Beck, Biochem. J. 206 (1982) 267.
[3] Y. Matsuo, D.M. Greenberg, J. Biol. Chem. 234 (1959) 516.

[4] K. Abe, H. Kimura, J. Neurosci. 16 (1996) 1066.
[5] R. Hosoki, N. Matsuki, H. Kimura, Biochem. Biophys. Res. Commun. 237 (1997) 527.
[6] Y. Ogasawara, S. Isoda, S. Tanabe, Biol. Pharma. Bull. 17 (1994) 1535.
[7] A.M. Westley, J. Westley, Anal. Biochem. 195 (1991) 63.
[8] J.I. Toohey, Biochem. J. 264 (1989) 625.
[9] U. Hannestad, S. Margheri, B. Sörbo, Anal. Biochem. 178 (1989) 394.
[10] L.M. Siegel, Anal. Biochem. 11 (1965) 126.
[11] P.F. Cook, R. T Wedding, J. Biol. Chem. 251 (1976) 2023.
[12] B.H. McAnnaley, W.T. Lowry, R. D Oliver, J.C. Garriot, J. Anal. Toxicol. 3 (1979) 111.
[13] J.C. Savage, D.H. Gould, J. Chromatogr. 526 (1990) 540.
[14] F. Bruner, A. Liberti, M. Possanzini, I. Allegrini, Anal. Chem. 44 (1972) 2070.
[15] T. Ubuka, S. Yuasa, J. Ohta, N. Masuoka, K. Yao, M. Kinuta, Acta Med. Okayama 44 (1990) 55.
[16] T. Ubuka, J. Ohta, W.-B. Yao, T. Abe, T. Teraoka, Y. Kurozumi, Amino Acids 2 (1992) 143.