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Analytical Study of Total and Free Amino acids in Liquid Food Additive Preparations

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ABSTRACT

On pharmaceutical market exist many multicomponent food additives, containing animoacids in different quantity. The enlarged application of these food supplements leads to the increasing of the problem with their quality control. Because of these reasons, the aim of current study is to apply a selective gas chromatographic method with flame-ionization detector (GC-FID) for fast separation of different aminoacids in liquid food additive and for determination of total content and quantity of free aminoacids. Acid and alkaline (for L-Triptophan) hydrolysates were purified by cation-exchange solid-phase extraction, followed by the derivatization of aminoacids with ethylchloroformate. The dissolved in isooctane derivatives were analysed by GC-FID. The identity was proved by the fact, that all data for the retention time (t_R) of aminoacids in food additive correspond to the values of t_R of the respective aminoacid in reference solution. 13 aminoacids were separated in range of time: 2.1 min.-6.55 min. In the examined food additive free L-Aspartate existed in the highest concentration (29.81 mg/dl). The proposed GC-FID method is characterized with the following advantages: 1) the high selectivity give the opportunity for separation of different aminoacids; 2) the method is appropriate for application for routine quality control of aminoacids in liquid food additives, because provides fast identification and deternination with good precision and accuracy of total, free and connected content of aminoacids; 3) the neutralized hydrolysate sample can be applied directly to an ion exchange column; 4) analytical procedure is easy for application.

Keywords: Aminoacids, gas chromatography, flame-ionization detector, liquid preparations, food additive.

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INTRODUCTION

The human body cannot synthesize from other compounds, at the level needed for normal growth, the so called essential aminoacids: L-Leucine, L-Isoleucine, L-Lysine, L-Methionine, L-Phenylalanine, L-Threonine, L-Tryptophan and L-Valine [1]. L-Triptophan is of great importance, because its following effects: induces the normal sleep, reduces the anxiety, stabilizes the mood, helps in the treatment of seasonal affective disorder [2], increases the brain levels of serotonin [3] and fructose absorption [4]. L-Arginine, L-Cysteine, L-Histidine, L-Taurine and L-Tyrosine are semiessential in children, because the metabolic pathways for their synthesis are not fully developed [5]. L-Arginine stimulates wound healing and immune function [6]. L-Tyrosine reduces stress hormone levels [7] and improves cognitive and physical performance [8]. Essential only in certain cases are L-Glutamine, L-Glycine, L-Ornithine, L-Proline and L-Serine. L-Proline aids the production of collagen and gives the skin a youthful look. L-Serine is a component of the protective myelin sheath that covers nerve fibers and is important in DNA and RNA function and cell formation. L-Serine helps formation of L-Tryptophan, phospholipids, immunoglobulins, anti-bodies, stimulates fat and fatty acid metabolism and aids in the absorption of creatine. The low levels of L-Serine may contribute to the development of depression, insomnia, confusion, anxiety, chronic fatigue syndrome (CFS) and fibromyalgia (FM) [9]. Sulfur-containing amino acids L-Cysteine, L-Cystine, L-Homocysteine, L-Methionine and L-Taurine are important for glutathione and protein synthesis, methylation and sulfation and are used as supplementation in several clinical applications, including the treatment of: depression, detoxification, diabetes, AIDS, allergy, arthritis and pain syndromes. L-Cysteine is an antioxidant and a detoxifier, promotes burning of fat and building up of muscles and helps to forming healthy skin, hair, bones and connective tissue. L-Methionine reduces the level of inflammatory histamines in the body and is needed for the production of L-Cysteine, L-Taurine, creatine and collagen, which are needed to form skin, nails and connective tissue. L-Taurine is used in treatment for epilepsy, cardiovascular diseases, diabetes, alcoholism, cystic fibrosis and Alzheimer's [10] disease.

Methods for determination of aminoacids include postcolumn derivatization with ninhydrin or o-phthalaldehyde (OPA) by commercially available aminoacid analyzers or HPLC with precolumn derivatization with different reagents, such as dansyl chloride, phenylisothiocyanate, fluorenylmethyl, chloroformate [11]. In physiologic fluid and tissue extracts is used precolumn convertion of primary and secondary amines to their colored derivatives with dabsyl chloride and separation by HPLC [12, 13]. Capillary zone electrophoresis (CZE) with pre-capillary derivatization is applied for simultaneous analysis of L-Cystine, L-Lysine, L-Cystine, L-Methionine, L-Threonine with phenylisothiocyanate and detection at λ = 190 nm of phenylthiocarbamyl-derivative [14]. The reported methods for analysis of some aminoacids are as follows: 1) L-Aspartate and L-Glutamate in rat brain microdialysate samples: gradient RP-HPLC with fluorimetric detection at λexcitation = 330 nm, λemission = 440 nm, after derivatization at 10°C with o-phthaldialdehyde-2-mercaptoethanol on Hypersil® C18 column (5 µm, 150 mm/3.2 mm) and a mobile phase: methanol and 0.05 M sodium acetate buffer, pH = 7 [15]; 3) L-Aspartate, L-Glutamate and L-Glycine in human brain: HPLC with precolumn derivatization of aminoacids with diethylaminoazobenzene sulphonylchloride [16]; 4) L-Cystine,

L-Lysine, L-Methionine: HPLC, after oxidation of L-Cystine to cysteic acid and L-Methionine to methionine sulfone, followed by 6M HCl hydrolysis at 145 ^{o}C for 4 h [17]; 5) L-Histamine: a) RP-HPLC after derivatization with 2-hydroxy-1-naphthaldehyde [18]; b) high-performance capillary electrophoresis [19]; 6) L-Lysine in human plasma and serum by L-Lysine epsilon-oxidase [20].

MATERIALS AND METHODS

Materials

I) Food additive: Aminogame 1500 solution (Biogame OOD, Bulgaria, 492 ml).
II) Reference serfied standard solution with 200 nmol/l of standard substance of each of the following aminoacids: L-Alanine, L-Glycine, L-Valine, L-Leucine, L-Isoleucine, L-Threonine, L-Serine, L-Proline, L-Aspartate, L-Methionine, L-Glutamate, L-Phenylalanine, L-Cystine, L-Lysine, L-Histidine, L-Tyrosine, L-Triptophan.
III) Internal standard: 200 nmol/l Norvaline solution.
IV) Reagents with analytical grade quality: 0.1M HCl, 6M HCl, 1M KOH, 4% thioglycolic acid, sodium carbonate, ethylchloroformate, isooctane, destilled water.

Methods. Gas chromatography (GC)

I) Chromatogtaphic conditions:

GC-FID chromatograms were measured on a Thermo Scientific GC-FID device, supplied with: Faast analytical column (l = 10 m, d = 0.25 mm, 1.0 µm 5% diphenylpolysiloxane, 95% dimethylpolysiloxane film); injector temperature (split-splittless in split regime)-250 ^{o}C, temperature of flame-ionization detector-320^{o}C, the carrier gas flow rate-2.0 ml/min.

The initial oven temperature (110 ^{o}C) was held for 0.3 min., subsequently was ramped at a rate of 27 ^{o}C/min until 320 ^{o}C was reached and was held for 5 min. at 320 ^{o}C.

II) Preparation of solutions of food additive for the determination of free aminoacids.

An accurately weighed quantity of 1.0 g of Aminogame 1500 solution was extracted with 1000.0 ml 0.1 M HCl. An aliquot part of 1.0 ml of the extract was neutralyzed by adding of sodium carbonate to pH: 2.5 ÷ 5.0 to obtain 100.0 ml. To 100 µl of the neutralyzed sample were added 100 µl of solution of internal standard Norvaline. The obtained solutions were purified by cation-exchange solid-phase extraction. The aminoacids in purified samples were derivatized with ethylchloroformate. The derivatizing reagent was removed by scavenge with nitrogen. The derivatives of aminoacids were dissolved in aliquot part of isooctane and were analysed by gas chromatography with flame-ionization detector.

II) Preparation of solutions for determination of total content aminoacids in acid hydrolysate.

To an accurately weighed quantity (50 mg) of food additive Aminogame 1500 solution were added 1000 ml 6M HCl, containing 4% thioglycolic acid. Solution was incubated for 72 h at

110°C in hermetically closed glass container. An aliquot part of 1.0 ml of hydrolysates were neutralyzed by adding of sodium carbonate to pH: 2.5 ÷ 5.0 to obtain 100.0 ml. To 100 µl of the neutralyzed hydrolysed sample were added 100 µl of solution of internal standard Norvaline. The obtained solutions were purified by cation-exchange solid-phase extraction. The aminoacids in purified sample were derivatized with ethylchloroformate. The derivatizing reagent was removed by scavenge with nitrogen. The derivatives of aminoacids were dissolved in aliquot part of isooctane and were analysed by gas chromatography with flame-ionization detector.

III) Preparation of solutions for determination of L-Thyptophan in alkaline hydrolysate.

Because of its unstabillity and destruction as a result of acid hydrolysis, for the determination of L-Thyptophan the samples were prepared by the following method: to an accurately weighed quantity (50 mg) of Aminogame 1500 solution were added 1000 ml 1M KOH and the solution was incubated for 48 h at 110°C in hermetically closed glass container. An aliquot part of 1.0 ml of the alkaline hydrolysate was neutralyzed with sodium carbonate to pH: 2.5 ÷ 5.0 to obtain 100.0 ml. 100 µl of solution of internal standard Norvaline was added to 100 µl of the neutralyzed hydrolysate and cation-exchange solid-phase extraction is carried out for purification. After the derivatization procedure of aminoacids in purified sample with ethylchloroformate, the derivatizing reagent was removed by scavenge with nitrogen. The derivatives of aminoacids were dissolved in aliquot part of isooctane and were analysed by gas chromatography with flame-ionization detector.

RESULTS AND DISCUSSION

I) Selectivity of GC-FID method

A serfied standard solution, containing 200 mmol/l of each aminoacid was used for the calibration of gas chromatograph. In the same manner like the solution of serfied standard solution, was prepared placebo solution, containing all labeled in food additive supplements, without the active aminoacids. The selectivity of the applied method is confirmed by the fact that on chromatograms with placebo preparation did not exist peaks with retention time (t_R), corresponding to t_R of the respective aminoacid in serfied standard solution.

II) Derivatization reaction of aminoacids

The derivatization of aminoacids with ethylchloroformate is described by the following reaction:

R' = C_2H_5

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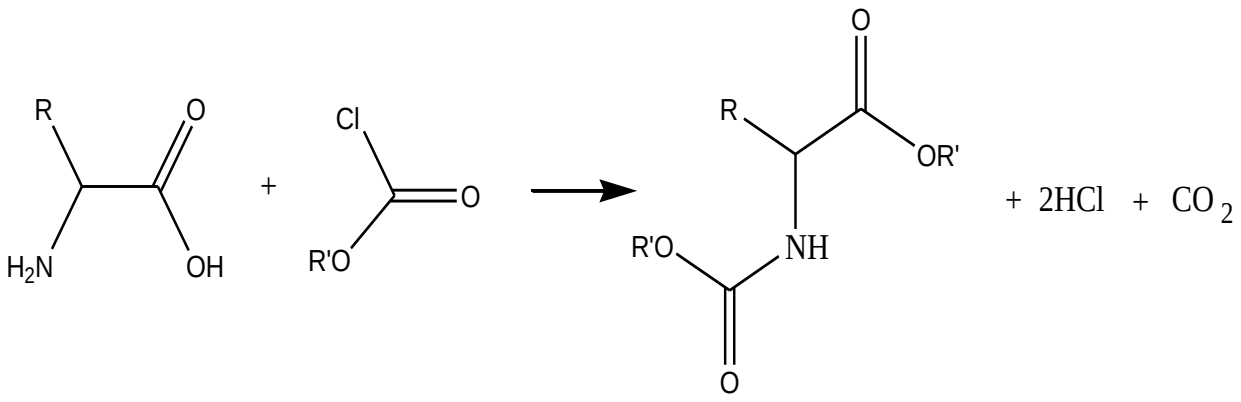

III) Identification of animoacids in food additive Aminogame 1500 solution

Table 1. Retetion time (t_R) and peak area (A) for free aminoacids in Aminogame 1500 solution.

N :	Aminoacid	Reference standard for free aminoacids		Free aminoacids in Aminogame 1500 solution	
		t_R	Peak area [uV sec.]	t_R	Peak area [uV sec.]
Internal standard	Norvaline	2.554	22011.10	2.551	9933.95
1.	L – Alanine	2.092	23179.74	2.092	183.32
2.	L – Glycine	2.214	17918.37	2.209	338.68
3.	L – Valine	2.417	18844.45	2.417	122.98
4.	L – Leucine	2.641	26847.77	2.636	178.26
5.	L – Isoleucine	2.704	16308.07	2.730	68.74
6.	L – Proline	3.077	26557.31	3.078	545.49
7.	L – Aspartate	3.753	28781.16	3.695	1452.03
8.	L – Methionine	3.804	23676.62	3.797	332.60
9.	L – Glutamate	4.141	21030.56	4.133	199.19
10.	L – Phenylalanine	4.205	40474.83	4.280	759.22
11.	L – Cystine	4.592	29937.50	4.617	98.70
12.	L – Lysine	5.576	31180.66	5.611	202.48
13.	L – Triptophan	6.508	47321.47	6.546	145.27

Table 2. Retetion time (t_R) and peak area for total content of aminoacids in Aminogame 1500 solution

N :	Aminoacid	Reference standard for total content of aminoacids		Total content of aminoacids in Aminogame 1500 solution	
		t_R	Peak area [uV sec.]	t_R	Peak area [uV sec.]
Internal standard	Norvalin	2.864	13063.52	2.888	7338.06
1.	L – Alanine	2.331	12585.93	2.361	9617.69
2.	L – Glycine	2.469	10216.19	2.498	8724.91
3.	L – Valine	2.707	11255.75	2.733	9801.43
4.	L – Leucine	2.964	14558.44	2.991	24386.62
5.	L – Isoleucine	3.036	10286.64	3.059	11609.91
6.	L – Proline	3.456	11979.44	3.474	12855.91
7.	L – Aspartate	4.225	9514.03	4.238	14450.74

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8.	L – Methionine	4.280	11798.77	4.290	1868.61
9.	L – Glutamate	4.661	5314.38	4.678	18306.14
10.	L – Phenylalanine	4.729	19208.57	4.743	19895.80
11.	L – Cystine	4.999	5649.46	5.009	2909.68
12.	L – Lysine	6.270	13946.90	6.282	30397.28
13.	L – Triptophan	7.300	18983.87	7.319	262.81

All retention times (t_R) of aminoacids in food additive sample correspond to the values of t_R of the respective aminoacid in reference solution. This fact proves the identity of the examined animoacids. The values for t_R and peak area for each aminoacid are summarized on Table 1. (free aminoacids) and Table 2. (total content of aminoacids). The most close result of correspondence of t_R of aminoacids in sample (S) and in reference standard solution (RS) are obtained as follows: 1) for free aminoacids analysis: L-Alanine ($t_RS = t_RRS = 2.092$); L-Valine ($t_RS = t_RRS = 2.417$); L-Proline ($t_RS = 3.077$, $t_RRS = 3.078$); L-Histidine ($t_RS = 5.797$, $t_RRS = 5.798$); L-Tyrosine ($t_RS = 6.112$, $t_RRS = 6.113$); 2) for total content of aminoacids: L-Histidine ($t_RS = 6.521$, $t_RRS = 6.523$); L-Methionine ($t_RS = 4.280$, $t_RRS = 4.290$); L-Cystine ($t_RS = 4.999$, $t_RRS = 5.009$).

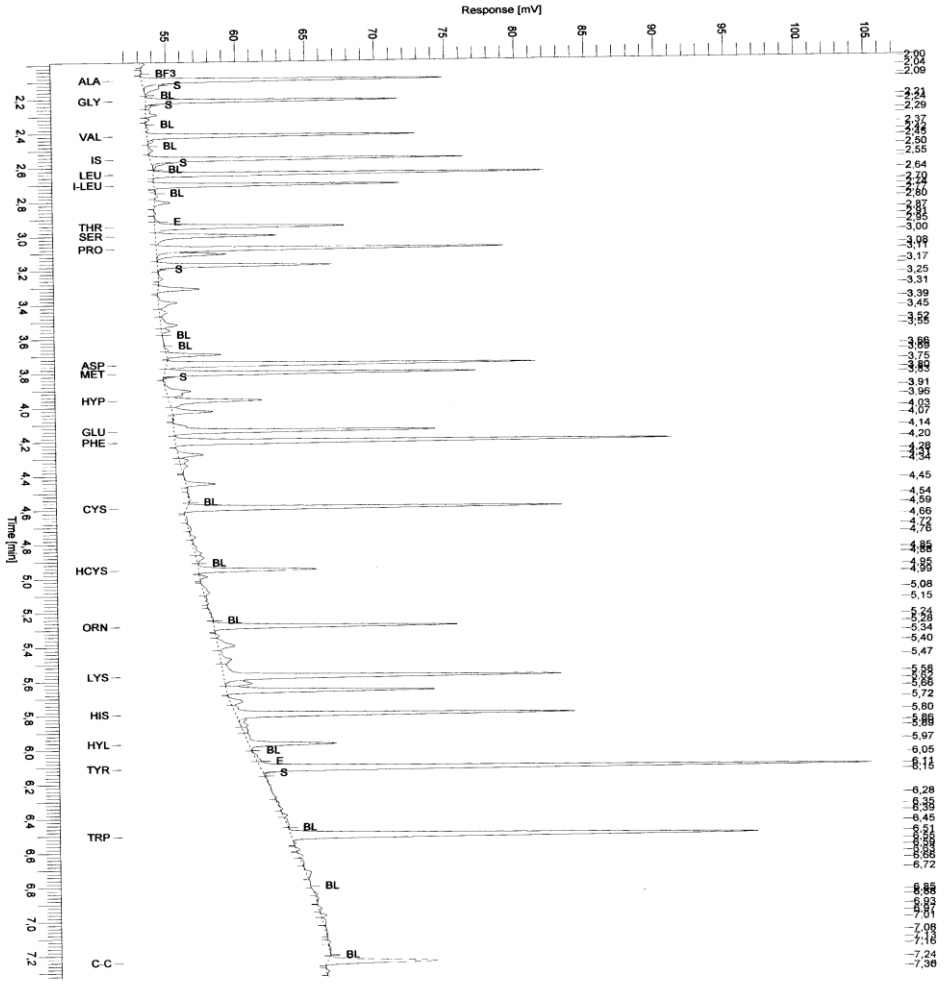

Fig. 1. Chromatogram of reference standard solution of aminoacids, used for the calibration before the analysis of free aminoacids in sample of Aminogame 1500 solution.

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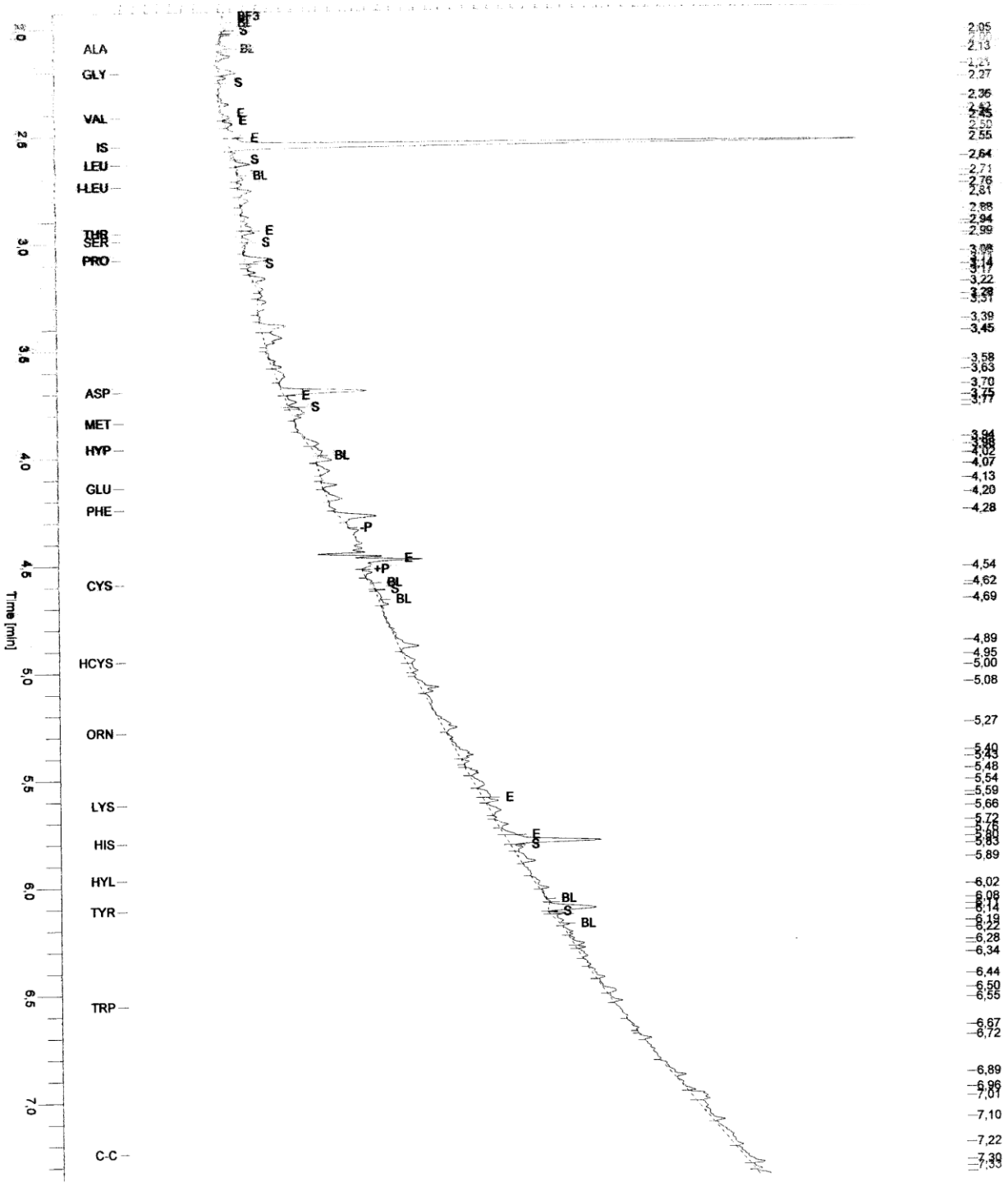

Fig. 2. Chromatogram of derivatized extract of free aminoacids in sample of Aminogame 1500 solution.

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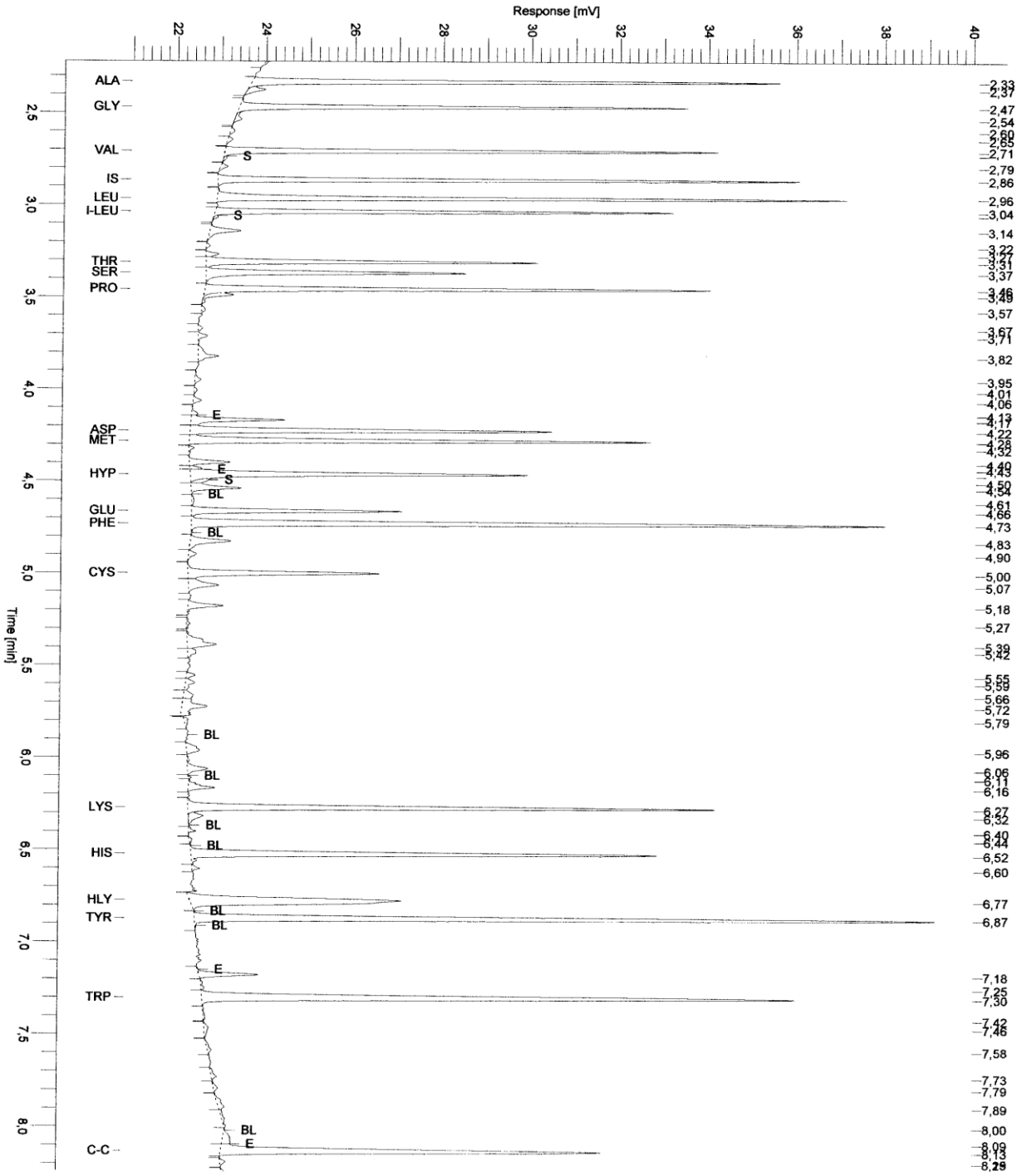

Fig. 3. Chromatogram of reference standard solution of aminoacids, used for the calibration before the analysis of acid hydrolysed sample of Aminogame 1500 solution.

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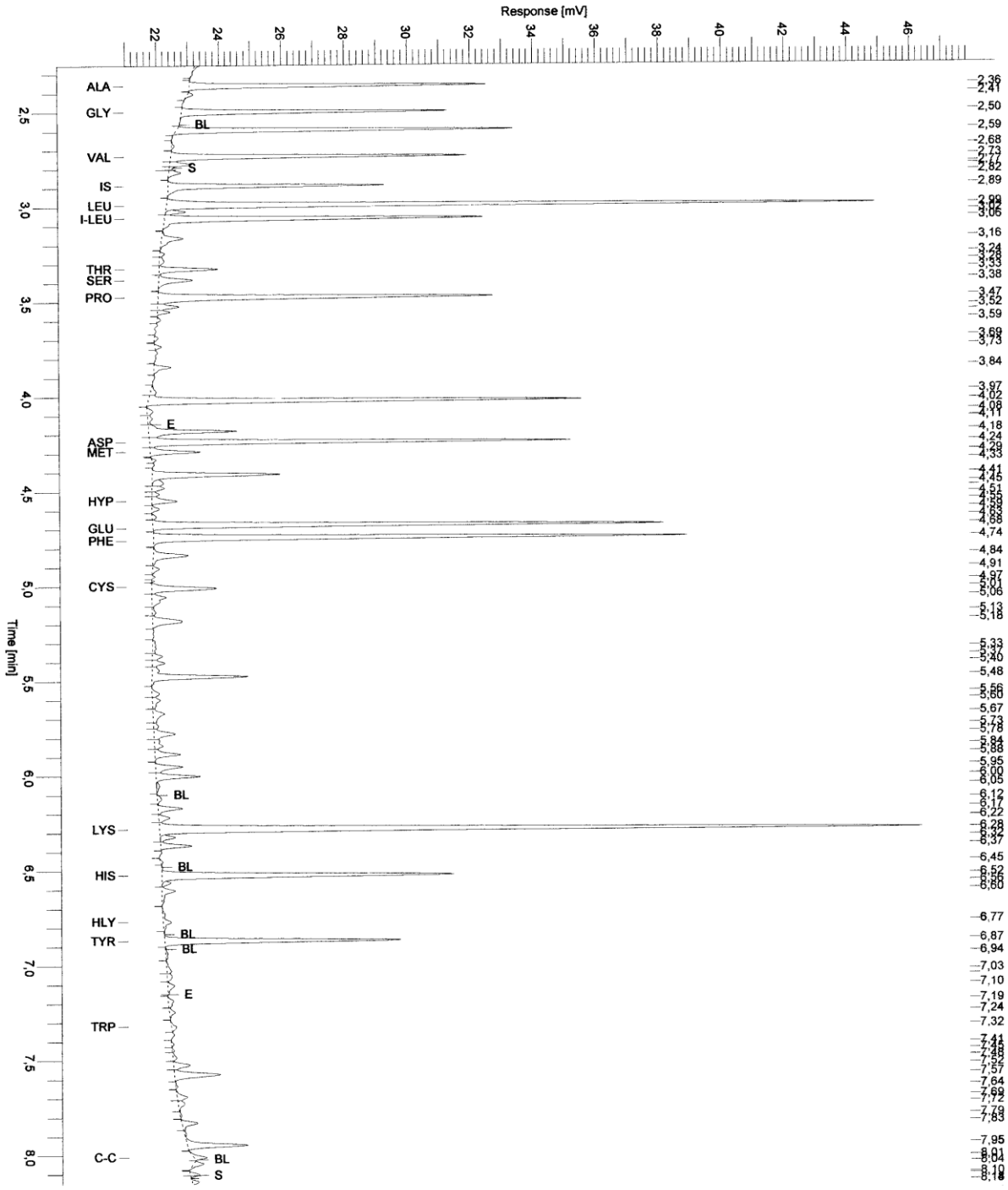

Fig. 4. Chromatogram sample of Aminogame 1500 solution, hydrolysed with 6M HCl for 72 h at 110°C.

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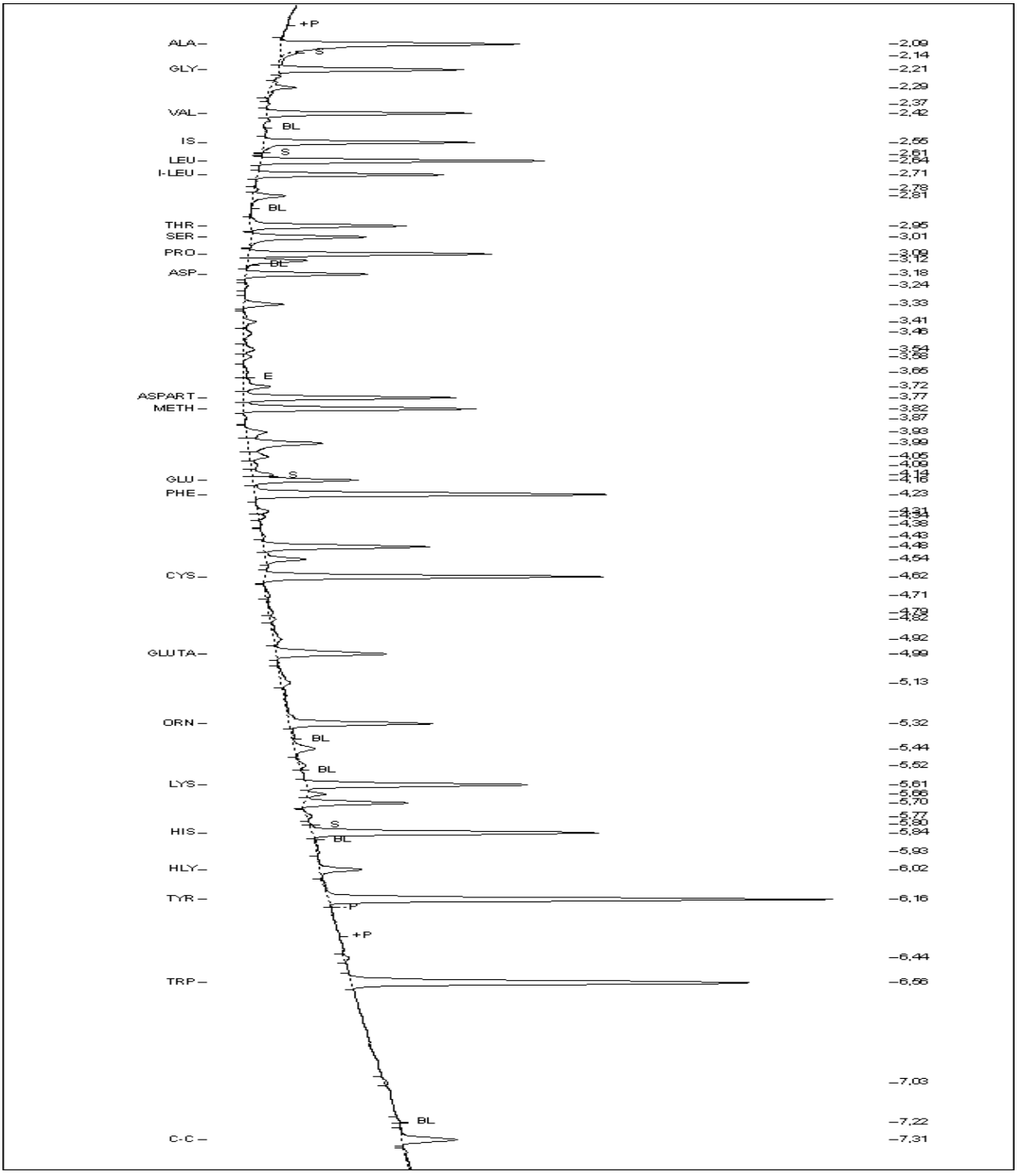

Fig. 5. Chromatogram of reference standard solution of aminoacids, used for the calibration before the analysis of alkaline hydrolysed sample of Aminogame 1500 solution

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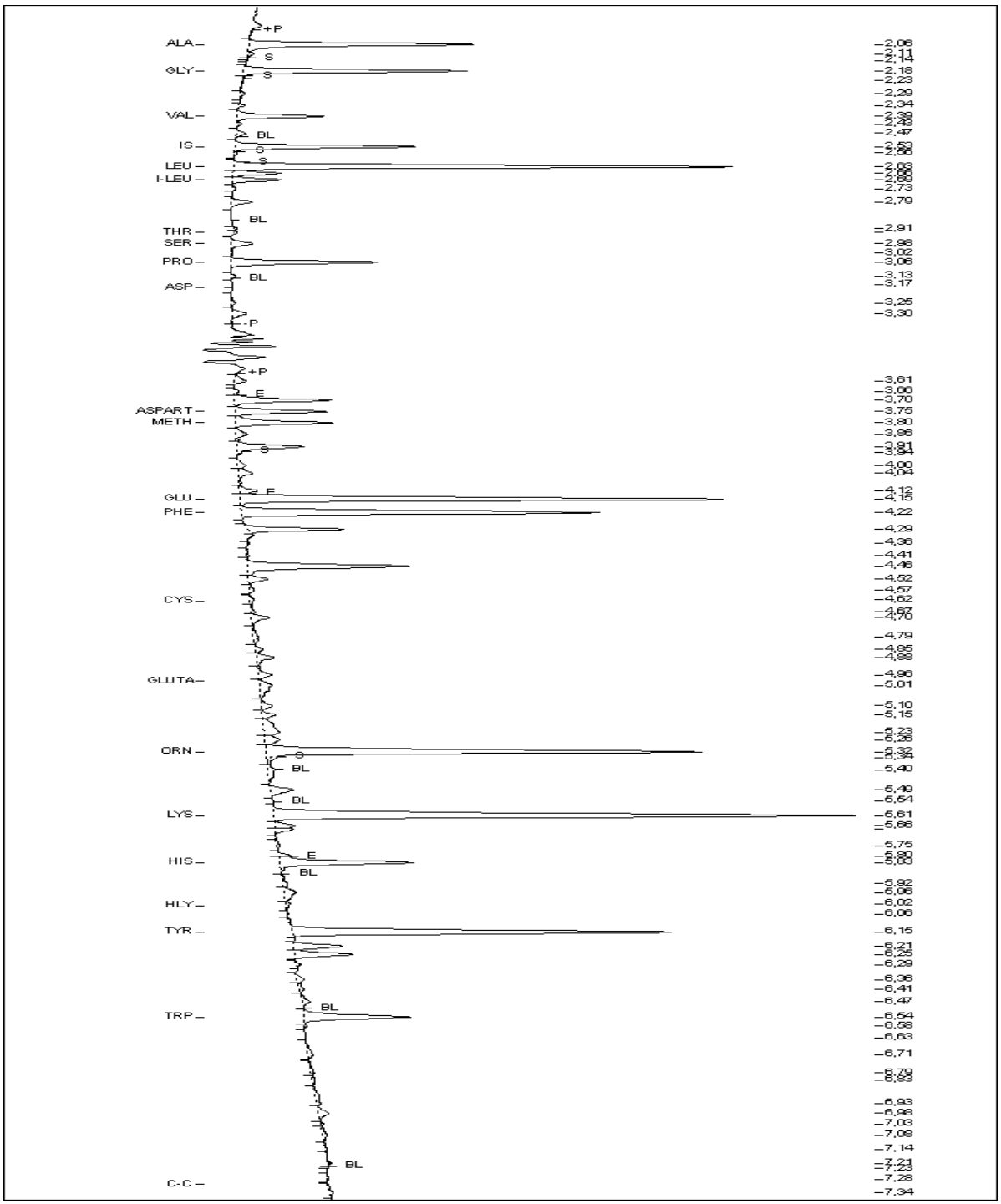

Fig. 6. Chromatogram of sample of Aminogame 1500 solution, hydrolysed with 1M KOH for 48 h at 110°C

On Fig. 1-Fig. 6. are illustrated respectively chromatograms of: reference standard solution of aminoacids, used for the calibration before the analysis of free aminoacids acids in sample of Aminogame 1500 solution (Fig. 1.); derivatized extract of free aminoacids in sample of Aminogame 1500 solution (Fig. 2.); reference standard solution of aminoacids, used for the

calibration before the analysis of acid hydrolysed sample of Aminogame 1500 solution (Fig. 3.); sample of Aminogame 1500 solution, hydrolysed with 6M HCl for 72 h at 110^{o}C (Fig. 4.); reference standard solution of aminoacids, used for the calibration before the analysis of alkaline hydrolysed sample of Aminogame 1500 solution (Fig. 5.); sample of Aminogame 1500 solution, hydrolysed with 1M KOH for 48 h at 110^{o}C (Fig. 6.).

IV) Determination of total content of aminoacids in food supplement Aminogame 1500 solution.

For the simultaneous determination of total and free aminoacids in the examined food supplement Aminogame 1500 solution the method of internal standard is applied. As an analytical parameter for determination are used the respective experimentally obtained data for the area inder the curve for aminoacids. The results for the obtained content of free aminoacids (Table 3.) and total and connected content of aminoacids (Table 4.) are summarized.

The following relations for the obtained content of aminoacids in food additive are observed: 1) for free aminoacids in 100 ml product L-Aspartate is with the highest concentration (29.81 mg/dl), followed by L-Phenylalanine (13.71 mg/dl) and L-Proline (10.48 mg/dl); 2) for total content of aminoacids in 100 ml product: a) L-Cystine is with the highest concentration (4.41 g/dl), followed by L-Lysine (1.13 g/dl); b) L-Triptophan is with the lowest concentration (10.01 mg/dl).

It is reported a similar method for quantitatively analysis in blood samples of L-Leucine, L-Isoleucine, L-Phenylanaline, L-Tyrosine and L-Valine, by applying of a solid-phase microextraction technique, derivatization of aminoacids with isobutyl chloroformate, methanol and pyridine and measurement of the corresponding N(O,S)-alkoxycarbonyl alkyl esters by gas chromatography/mass spectrometry using an external standard and obtaining separation of 5 aminoacids for 5.85 min. (between 6.96 min. and 12.81 min.). In comparison the advantage of our method is the obtaining the faster separation of 13 aminoacids for 4.45 min. (between 2.1 min. and 6.55 min.) [21]. L-Alanine in human serum is determined with ammonia gas-sensitive electrode, after L-Alanine deamination to ammonia, by bacterial alanine dehydrogenase in the presence of β-NAD+ [22].

Table 3. Content of free aminoacids in Aminogame 1500 solution

N :	Aminoacid	Content of free aminoacids in Aminogame 1500 solution		
		[nmol/ml]	[mmol/l]	[mg/dl]
1.	L – Alanine	350.47	0.35	3.12
2.	L – Glycine	837.61	0.84	6.31
3.	L – Valine	289.20	0.29	3.40
4.	L – Leucine	294.24	0.29	3.80
5.	L – Isoleucine	186.79	0.19	2.49
6.	L – Proline	910.23	0.91	10.48
7.	L – Aspartate	2235.72	2.24	29.81
8.	L – Methionine	622.52	0.62	9.25
9.	L – Glutamate	419.73	0.42	6.18

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10.	L – Phenylalanine	831.25	0.83	13.71
11.	L – Cystine	146.10	0.15	3.60
12.	L – Lysine	287.77	0.29	4.24
13.	L – Triptophan	136.04	0.14	2.86

Table 4. Total and connected content of aminoacids in Aminogame 1500 solution.

N :	Aminoacid	Total content of aminoacids in Aminogame 1500 solution			Content of aminoacids in connected form in Aminogame 1500 solution	
		[nmol/ml]	[mmol/l]	[mg/dl]	[mmol/l]	[mg/dl]
1.	L – Alanine	27207.86	27.21	242.41	26.86	239.29
2.	L – Glycine	30407.52	30.41	228.29	29.57	221.98
3.	L – Valine	31004.44	31.00	363.17	30.71	359.77
4.	L – Leucine	59641.11	59.64	782.30	59.35	778.50
5.	L – Isoleucine	40185.03	40.19	527.17	40.00	524.68
6.	L – Proline	38209.84	38.21	439.91	37.30	429.43
7.	L – Aspartate	54079.73	54.08	719.80	51.84	689.99
8.	L – Methionine	5638.85	5.64	84.15	5.02	74.90
9.	L – Glutamate	122645.93	122.65	1804.55	122.23	1798.37
10.	L – Phenylalanine	36878.68	36.88	609.22	36.05	595.51
11.	L – Cystine	18337.80	183.39	4406.86	183.24	4403.26
12.	L – Lysine	77600.76	77.60	1134.43	77.31	1130.19
13.	L – Triptophan	492.91	0.49	10.01	0.35	7.15

CONCLUSION

The identity is proved by the fact, that all data for the retention time (t_R) of aminoacids in food additive correspond to the values of t_R of the respective aminoacid in reference solution. 13 aminoacids are separated in range of time: 2.1 min.-6.55 min. In the examined food additive form free aminoacids L-Aspartate is with the highest concentration (29.81 mg/dl). The total content of L-Cystine is with the highest concentration (4.41 g/dl) and L-Triptophan is with the lowest concentration (10.01 mg/dl).

The proposed GC-FID method has the following advantages: 1) high selectivity giving the opportunity for separation of different aminoacids; 2) the method is appropriate for application for routine quality control of aminoacids in liquid food additives, because provides fast identification and deternination with good precision and accuracy of total, free and connected content of aminoacids; 3) directly application of the neutralized hydrolysate to an ion exchange column; 4) easy analytical procedure.

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