Asian Journal of Chemistry Vol. 18, No. 2 (2006),1366–1376

Identification of Ethylchloroformate Derivatives of Amino Acids and Hydrolysis of Myoglobin and Small Peptides Using Gas Chromatography-Mass Spectrometry

FARID R. ZAGGOUT*, SIMON J. GASKEL† and JABER S. HAJJAJ
Department of Chemistry, UMIST, P.O. Box 88, U.K.
E-mail: Zaggout2004@yahoo.com

Derivatization of twenty amino acids was investigated, using ethanol, methanol, propanol, ethyl chloroformate and pyridine, to provide volatile products which are easily amenable to GC-MS. This method presents the rapidity of sample preparation and speed of GC-MS analysis. The results of the analysis of the hydrolysis products of protein and peptides indicated that this technique could be used to identify most of the amino acid contents of peptides and proteins.

Key Words: Gas chromatography-mass spectrometry, Amino acids, Derivatization, Protein hydrolyzates.

INTRODUCTION

Amino acids are very important in living systems; they are among the most commonly analyzed biological compounds. Amino acids serve as the building blocks for literally thousands of different proteins and peptides which are critical for all living organisms[1].

A variety of analytical methods have been developed for the separation and identification of amino acids such as high performance liquid chromatography[2–4] and capillary electrophoresis[5, 6]. However, mass spectrometry can readily be interfaced with gas chromatography for the separation and identification of low concentration amino acids with high sensitivity. The gas chromatography-based methods for amino acid analysis require derivatization of the amino acids to produce volatile products[7–13]; one of these derivatization techniques was reported by Husek[14–16] in which a solution of ethyl chloroformate reacts instantaneously with amino acids in the presence of ethanol and pyridine as a catalyst. In this study we used alkyl chloroformate derivatization technique using ethyl chloroformate with ethanol, methanol and propanol to derivatize twenty amino acids prior

†Department of Chemistry, Al-Azhar University of Gaza, P.O. Box 1277, Gaza, PNA (Via Israel).

to analysis by GC-MS and this technique was applied for the investigation of the hydrolysis products of myoglobin and three peptides.

EXPERIMENTAL

Ethyl chloroformate, amino acids, myoglobin and peptides were obtained from Sigma (Pierce, Rockford, U.S.A). Methanol, ethanol and propanol were obtained from Rathman Chemical Ltd., Walkesburn, UK. Pyridine was obtained from Lancaster, Morecombe, UK. Chloroform was used as GC-MS grade. All chemicals were used as received.

Derivatized amino acid samples were analyzed using an HP 5971 gas chromatograph configured for capillary column and interfaced to a mass selective detector. The detector was operated in electron impact mode (EI) and the data were recorded in scan mode from 10–500 m/z. The mass spectrometer was used to identify all the components produced from derivatization of amino acids, myoglobin and peptides. The mass spectral behaviour of these derivatives is quite simple, allowing their fragmentation to be rationalized.

The separation of amino acids was accomplished using capillary columns (HP-1) 25 m × 0.32 mm ID, 0.52 μm film thickness (dimethyl polysiloxane).

Methods

The gas chromatograph was operated using a programmed temperature as follows: 110°C for 1 min, 12–270°C, hold 5 min and injection volume 1 μL. Splitless injection was used at flow rate of 0.9 mL min^{-1}. Helium was used as the carrier gas. The derivatization of amino acids was performed in a 0.3 mL reactivial. 30 μL of a solution containing 9.1×10^{-9} mole of each amino acid of the solution of twenty amino acids were treated with 50 μL (water + ethanol)-(methanol + propanol)-pyridine (30 : 16 : 4) and 2.5 μL of ethyl chloroformate was added to the solution and mixed by shaking the tube for about 3–5 s. 50 μL of chloroform (containing 1% of ethyl chloroformate) was added and the derivative is extracted into the organic phase (chloroform layer) by vortex mixing for about 5 s. The chloroform layer cleared during this process and the aqueous phase turned opaque. Anhydrous sodium sulphate was added to the chloroform layer to remove any traces of water. Aliquot of the organic phase is injected (1 μL) into the GC-MS. All derivatizations were performed in triplicate. This technique was applied on myoglobin and peptides where 30 μg of myoglobin or peptides were hydrolyzed using 150 μL of 6 M HCl for 12 h at 100°C in a closed reactivial. The acidic medium was evaporated under a stream of nitrogen and the contents were redissolved in 30 μL of water and derivatized using the procedure described above and then analyzed using GC-MS.

RESULTS AND DISCUSSION

The results were obtained from derivatization of amino acids using alkyl chloroformate as shown in Fig. 2, where the mixed anhydride formed by the reaction between the alkyl chloroformate and carboxyl group should decarboxylate to yield the ester containing the alkyl group derived from the alkyl chloroformate. The results presented in Figs. 3 and 4 show that the substituted alkyl group on the carboxylic side of amino acid is provided by the alcohol, whereas the amine side is provided by the alkyl group of the chloroformate as shown in Fig. 1.

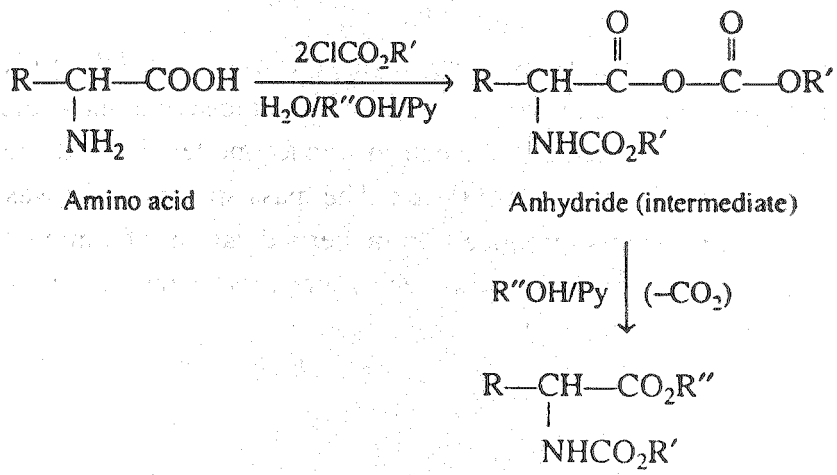

Fig. 1. Alcohol-anhydride exchange reaction

In this study different alcohols including methanol, ethanol and propanol were used for the derivatization of a mixture of equimolar 20 amino acids as shown in Figs. 2–4. From these chromatograms, it is found that differences in the retention times between the types of derivatives, as methyl derivatives, were eluted faster than ethyl derivatives and propyl derivatives, this is attributed to the volatility of these derivatives.

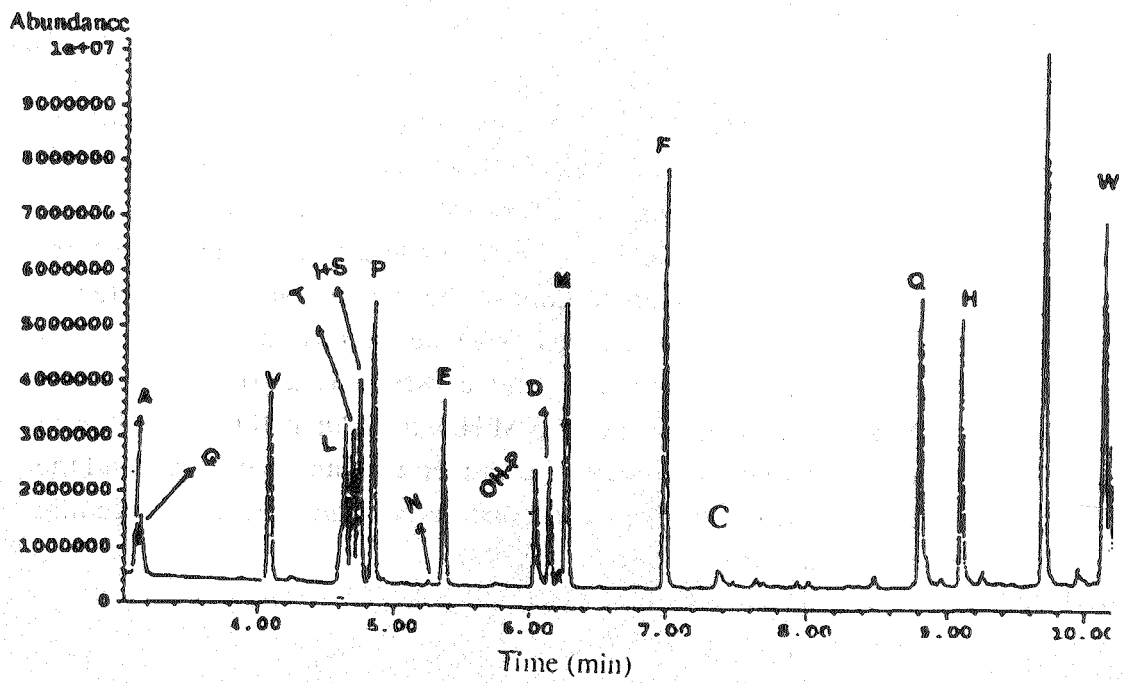

Fig. 2. Gas chromatogram of 20 amino acids as ECME derivatives (using methanol)

Identification derivatives of the component peaks of the total ion chromatogram were confirmed by the mass spectrum for each component as shown in Figs. 5–7 which presented the mass spectra of methyl derivatives (ECME)*, ethyl derivatives (ECEE)† and propyl derivatives (ECPE)‡ for threonine respectively as an example.

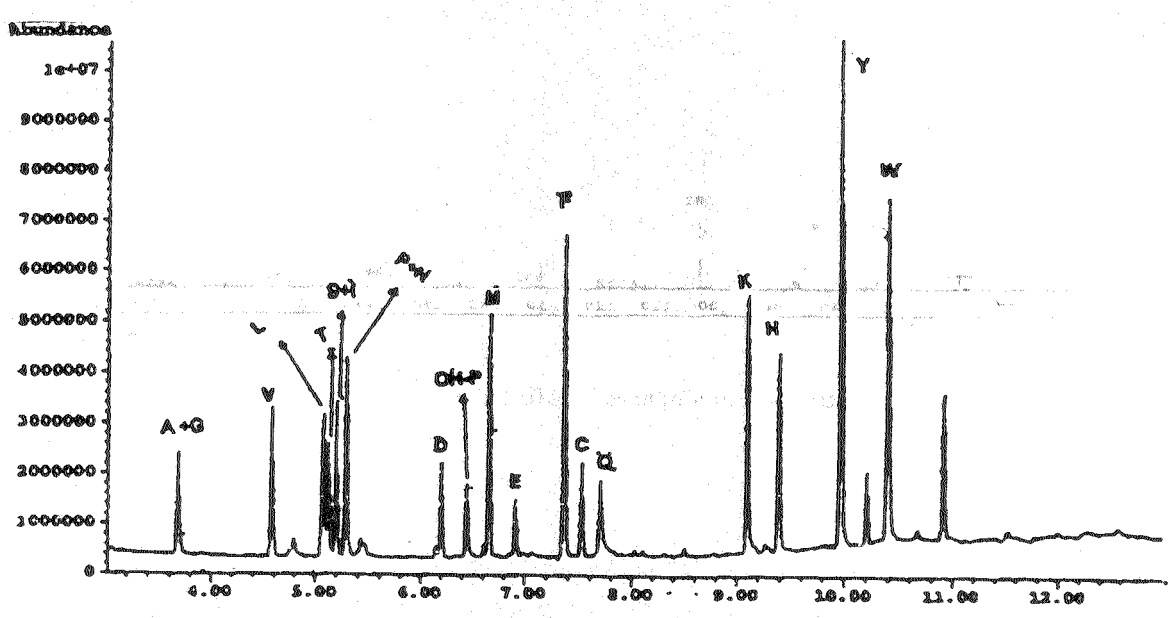

Fig. 3. Gas chromatogram of 20 amino acids as ECEE derivatives (using ethanol)

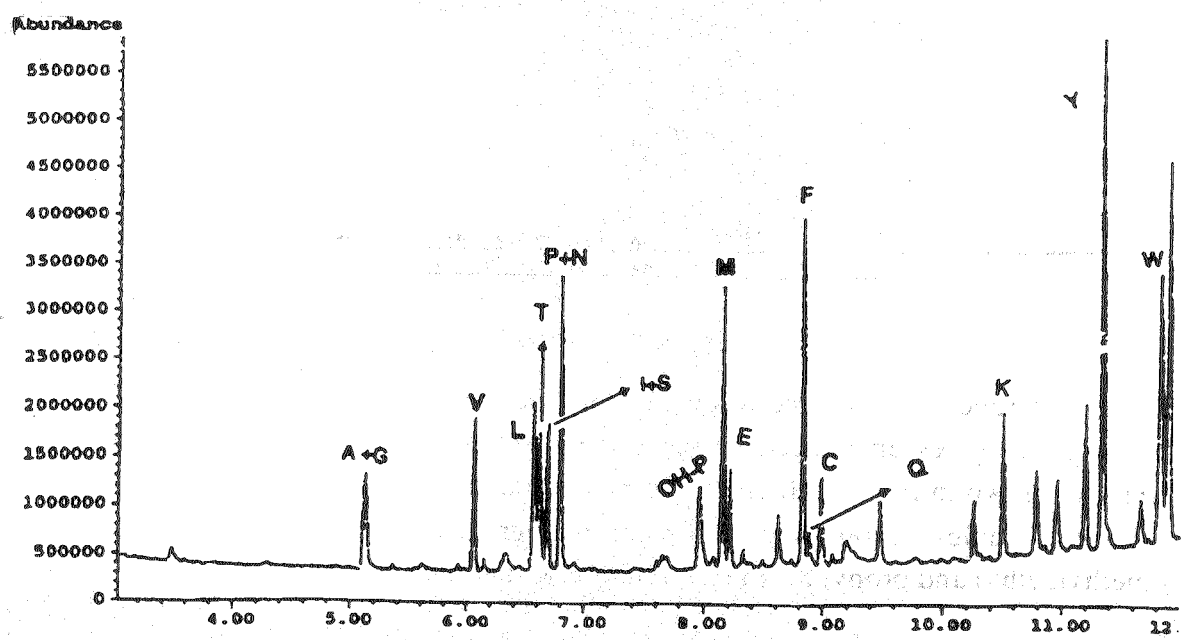

Fig. 4. Gas chromatogram of 20 amino acids as ECPE derivatives (using propanol)

*ECME = N-ethoxycarbonyl methyl ester, †ECEE = N-ethoxycarbonyl ethyl ester and ‡ECPE = N-ethoxycarbonyl propyl ester.

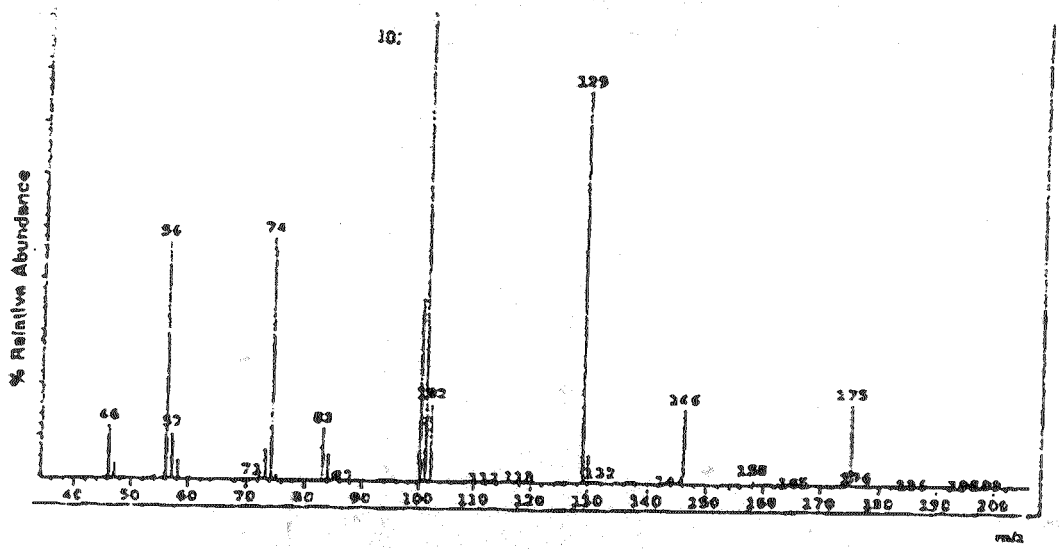

Fig. 5. Mass spectrum of threonine as ECME derivative (methanol derivatives)

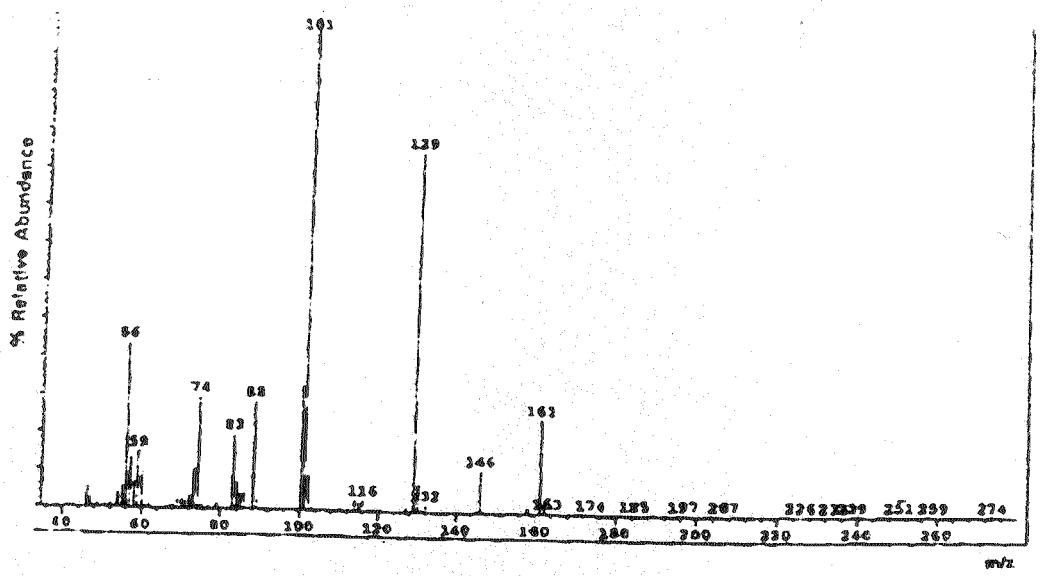

Fig. 6. Mass spectrum of threonine as ECEE derivative (ethanol derivatives)

The difference of 14 u between the ions peaks 161, 175 and 189 due to $—CH_2$ group gives an indication about the substitution in the carboxylic side chains as shown in Fig. 1. All amino acids were identified from the mass spectra as shown in Table-1. From the total ion chromatogram of amino acids, derivatives of methyl, ethyl and propyl for the 20 amino acid mixtures are shown in Figs. 2–4.

TABLE-1
CHARACTERISTIC ION PEAKS IN EI SPECTRA OF ECME, ECEE AND ECPE DERIVATIVES OF AMINO ACIDS

Amino acid	M^+			Base peak	$M-CO_2R_1$	$M-CO_2R_1-(CO_2Et-H)$
	ECME	ECEE	ECPE			
(Gly) G	161	175	189	102	102	
(Ala) A	175	189	203	116	116	
(Val) V	*203	217	*231	144	144	72
(Leu) L	217	*231	245	158	158	86
(Ile) I	217	231	*245	158	158	86
(Pro) P	201	215	229	142	142	70
(Hpr) OH—P	*231	*245	*259	158	158	86
(Ser) S	*191	*205	*219	132	132	60
(Thr) T	*205	*219	*233	101	146	74
(Cys) C	279	293	307	102	220	148
(Met) M	235	249	263	61	176	
(Lys) L	*304	318	332	158		
(Asp) D	*247	261	*275	174[a]	188	116
				188[b]	188	
				202[c]	188	
(Glu) E	*261	275	*289	84	202	
(Asn) N	200	214	228	141	141	69
(Gln) Q	232	246	260	84	173	
(Phe) F	251	265	279	162[a]	192	120
				176[b]	192	
				74[c]	192	
(Tyr) Y	*239	*253	*267	107	280	208
(Trp) W	290	304	318	130	231	
(His) H	313	327	341	81	254	182

a, b and c are base peaks of ECME , ECEE and ECPE, respectively,
* = absence of the molecular ion peak, R_1 = methyl, ethyl and propyl.

It is found that the results of the reaction of ethyl chloroformate with different alcohols (methanol, ethanol and propanol) indicated that the type of ester formed during the derivatization depends on the type of alcohol present in the reaction medium. For example, when phenyl alanine reacts with ethyl chloroformate in aqueous medium containing methanol, the derivative produced is that in which the carboxylic group is esterified with methyl group not with ethyl group. These

observations of the involvement of alcohol constituents in the aqueous reaction medium containing the ethyl chloroformate reagent are confirmed as shown in Fig. 8, where these data are obtained during the analysis of isoleucine after treatment with ethyl chloroformate in an aqueous solution containing five alcohols (methanol, ethanol, propanol, butanol, hexanol) where the total ion chromatogram results from analysis of the reaction mixture by GC-MS are represented. Five peaks are obtained corresponding to the esters formed by the reactions with the alcohols in the reaction medium, the different intensities of the peaks result from the difference in reactivity of these alcohol with the anhydride formed during the reaction. So the use of different alcohols was to confirm that the esterification of carboxyl group of amino acids occurred by alcohol, not by ethyl chloroformate as shown in Fig. 1.

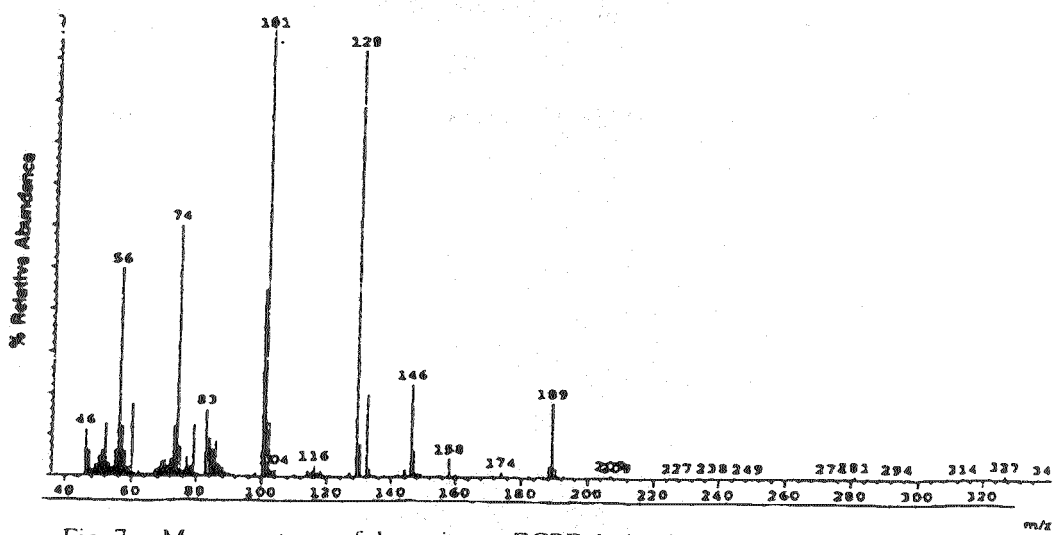

Fig. 7. Mass spectrum of threonine as ECPE derivative (propanol derivatives)

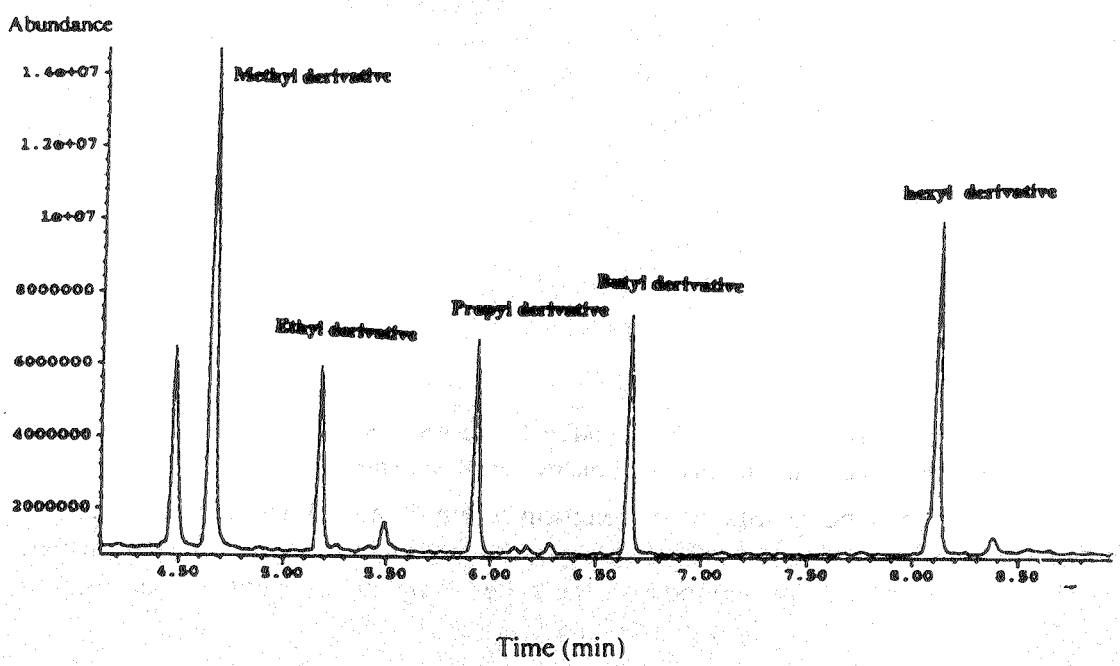

Fig. 8. Gas chromatogram of isoleucine as ECME (methanol), ECEE (ethanol), ECPE (propanol), ECBE (butanol) and ECHE (hexanol) derivative

Based on the results obtained from this method, this technique was applied on myoglobin and peptides for the investigation of the hydrolysis products of their composition. As shown in Fig. 9, the results obtained from the hydrolysis and derivatization of myoglobin with ethyl chloroformate using ethanol were compared with the total ion chromatogram of the known amino acids (Fig. 3). Fourteen amino acids produced from the hydrolysis and derivatization of myoglobin were investigated except tyrosine, asparagine and glutamine; this could be due to the destruction of these amino acids during the acid hydrolysis or unbreak some peptidic bonds. The mass spectra of N-ethoxycarbonyl ethyl ester of amino acids of myoglobin are summarized in Table-2.

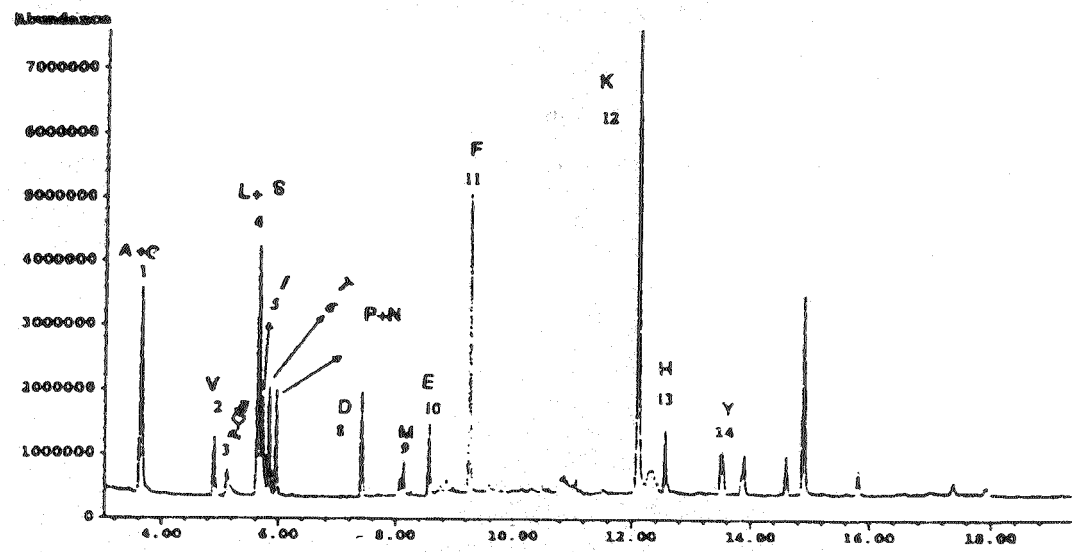

Fig. 9. Gas chromatogram of 14 amino acids as ECEE (ethanol derivatives) derived from the hydrolysis of myoglobin

The technique was also applied on three peptides: peptide 1 which consists of sequences (Phe-Ser-Trp-Gly-Ala-Glu-Gln-Arg), peptide 2 (Trp-Ala-Val-Gly-Leu-Met) and the third peptide has the sequence (Val-Thr-Cys-Gly). By comparison of the total ion chromatogram of the hydrolysis products of peptides 1, 2 and 3 as shown in Figs. 10–12, respectively with the total ion chromatogram for 20 amino acids as standard (Fig. 3.).

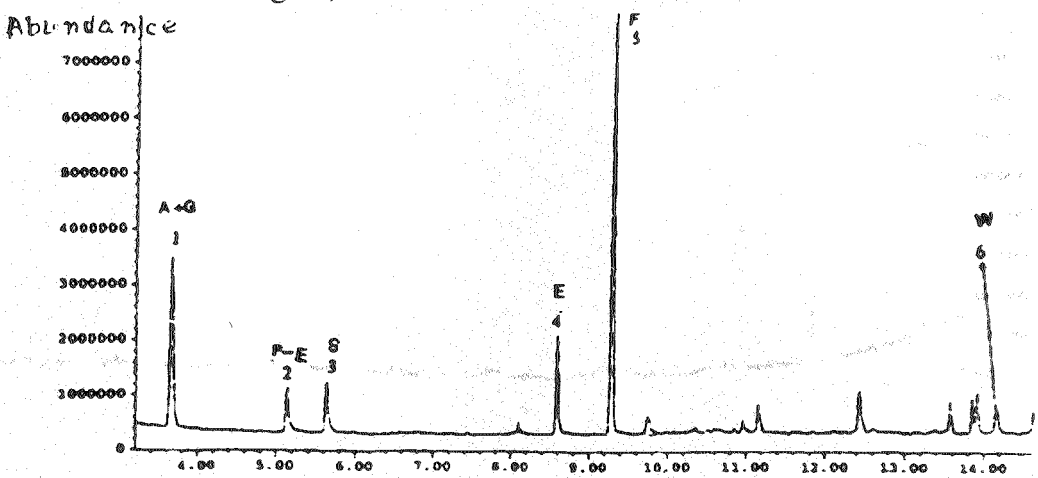

Fig. 10. Gas chromatogram of amino acids as ECEE (ethanol derivatives) derived from the hydrolysis of peptide-1

TABLE-2
CHARACTERISTIC ION PEAKS IN EI SPECTRA AS ECEE DERIVATIVES FROM THE HYDROLYSIS OF MYOGLOBIN

Peak No.	R_t (min)	M^+	Base peak	M-73*	M-145†	Other minor ions	Identification
1	3.640	189	116	116			Ala
2	4.897	217	144	144	72	116	Val
3	5.109	157	84	84			P-Glu
4	5.641	231	158	158	86	102	Leu
4	5.641	205	132	132	60	175, 86	Ser
5	5.660	231	158	158	68	102	Ile
6	5.708	219	101	146	74	129, 175	Thr
7	5.950	215	142	142	70	98	Pro
8	7.409	261	188	188	116	142, 74, 70	Asp
9	9.257	249	61	176		101, 188, 142, 129	Met
10	8.551	275	84	202		156, 128 Glu	
11	9.257	265	176	192	120	131, 102, 91, 74	Phe
12	12.091	318	156			84, 226, 272	Lys
13	12.556	327	81	254	182	238, 154	His
14	13.464	304	130	231		246, 192	Trp

*73 = CO_2Et,
†145 = CO_2Et + (CO_2Et—H).

It is found that all amino acids of peptides were investigated except glutamine and arginine of peptide-1 and troptophane for peptide-2. The mass spectra of ECEE (ethanol derivatives) for the three peptides 1, 2 and 3 are summarized in Tables 3–5, respectively.

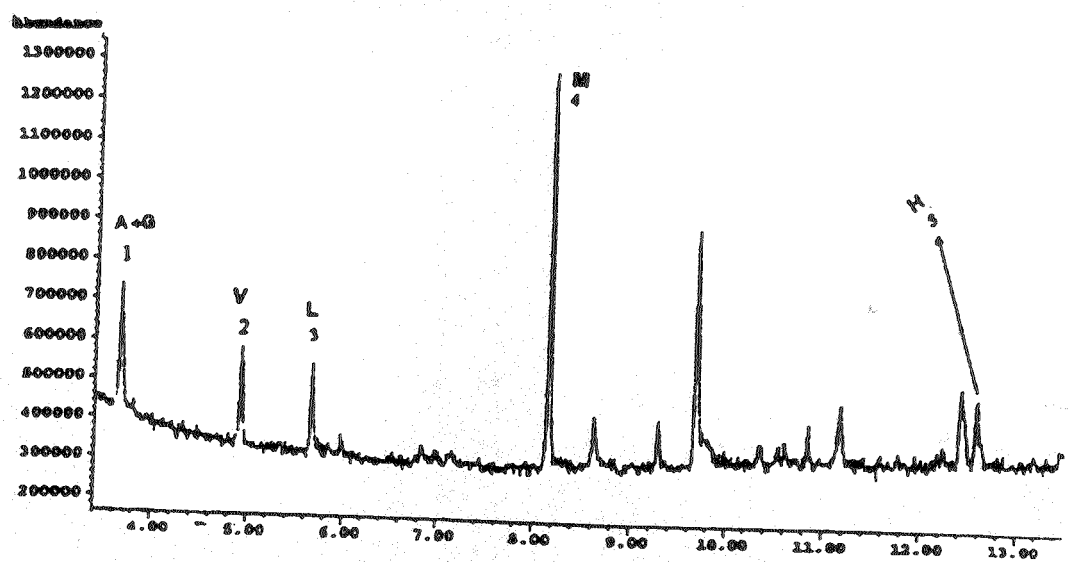

Fig. 11. Gas chromatogram of amino acids as ECEE (ethanol derivatives) derived from the hydrolysis of peptide-2

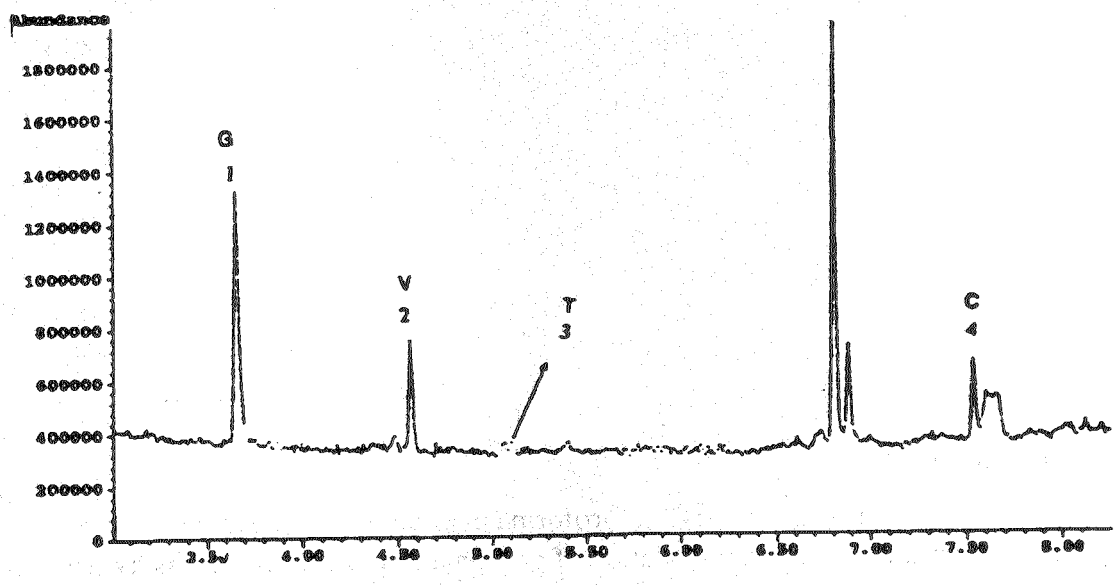

Fig. 12. Gas chromatogram of amino acids as ECEE (ethanol derivatives) derived from the hydrolysis of peptide-3

TABLE-3
CHARACTERISTIC ION PEAKS IN EL SPECTRA AS ECEE DERIVATIVES FROM THE HYDROLYSIS OF PEPTIDE-1

Peak No.	R_t (min)	M^+	Base peak	M-73	M-145	Other minor ions	Identification
1	3.659	175	102	102			Gly
1	3.695	189	116	116			Ala
2	5.136	157	84	84			P-Glu
3	5.693	205	132	132	60	175, 101, 86	Ser
4	8.585	275	84	202		156, 128	Glu
5	9.290	265	176	192	120	131, 102, 91, 74	Phe
6	14.151	304	130	231			Trp

TABLE-4
CHARACTERISTIC ION PEAKS IN EI SPECTRA AS ECEE DERIVATIVES FROM THE HYDROLYSIS OF PEPTIDE-2

Peak No.	R_t (min)	M^+	Base Peak	M-73	M-145	Other minor ions	Identification
1	3.659	175	102	102			Gly
1	3.695	189	116	116			Ala
2	4.924	217	144	144	72		Val
3	5.667	231	158	158	86	102	Leu
4	8.159	249	61	176		129, 101, 142	Met
5	12.582	327	81	245	182	238, 154	His

TABLE-5
CHARACTERISTIC ION PEAKS IN EL SPECTRA AS ECEE DERIVATIVES FROM THE HYDROLYSIS OF PEPTIDE-3

Peak No.	R_t (min)	M^+	Base peak	M-73	M-145	Other minor ions	Identification
1	3.659	175	102	102			Gly
2	4.567	189	144	144	72	116	Val
3	5.108	219	101	146	74	175, 129	Thr
4	7.543	293	102	220	148	174, 114, 204	Cys

Conclusion

Derivatization with an ethyl chloroformate, ethanol and pyridine solution is a useful technique for the identification of protein amino acids as well as hydrolysis products of myoglobin and peptides. It was indicated that the ester of amino acid derivatives is dependent on the type of alcohol used in the aqueous reaction medium. The effect of alcohol on chloroformate reaction in an aqueous medium leads to preparation of many ester derivatives that can be solving many analytical problems.

REFERENCES

1. R. McGilvery, Biochemistry: A Functional Approach, W.B. Saunders Company, Philadelphia PA (1970).
2. R. Schuster, *Anal. Chem.*, **52**, 617 (1980).
3. W.-Y. Lee and T.A. Nieman, *J. Chromatogr. A*, **659**, 111 (1994).
4. Z. Deyl, K. Macek and J. Janak, *J. Chromatogr. A*, **372**, B124 (1986).
5. M. Yu and N. Dovichi, *Anal. Chem.*, **61**, 37 (1989).
6. Y. Cheng and N. Dovichi, *Science*, **242**, 562 (1988).
7. M. Makita, S. Yamamoto and S. Kiyama, *J. Chromatogr. A*, **237**, 279 (1982).
8. T.P. Mawhinney, R.S.R. Robinett, A. Atalay and M.A. Madson, *J. Chromatogr. A*, **358**, 231 (1986).
9. S.L. MacKenzie, D. Tenashuk and G. Fortier, *J. Chromatogr. A*, **387**, 241 (1987).
10. C.-H. Oh, J.-H. Kim, K.-R. Kim, D.M. Brownson and T.J. Mabry, *J. Chromatogr. A*, **669**, 125 (1994).
11. K. Woo and D. Lee, *J. Chromatogr. B*, **665**, 15 (1995).
12. P. Simek, A. Heydova and A. Jegorov, *J. High Resol. Chromatogr.*, **17**, 147 (1994).
13. R. Zumwalt, K. Kuo and C. Gehrke, Amino Acid Analysis by Gas Chromatography, CRC Press, Boca Raton, FL, Vols. 1–3 (1987).
14. P.J. Husek, *Chromatography*, **552**, 289 (1991).
15. P. Husek and C. Sweeley, *J. High Resol. Chromatogr.*, **14**, 751 (1991).
16. P. Husek, *FEPS Letters*, **280**, 345 (1991).
17. Z.-H. Huang, J. Wang, D.A. Gage, J.T. Watson and C.C. Sweeley, *J. Chromatogr. A*, **635**, 271 (1993).

(*Received*: 27 July 2005; *Accepted*: 31 December 2005) AJC-4578