Journal of Analytical and Applied Pyrolysis, 24 (1992) 87–103
Elsevier Science Publishers B.V., Amsterdam

Pyrolysis–gas chromatography/mass spectrometry in the characterization of glycated albumin

A. Lapolla, C. Gerhardinger, L. Baldo, G. Crepaldi and D. Fedele
Università di Padova, Via Giustiniani 2, I-35100 Padova (Italy)

D. Favretto, R. Seraglia, O. Curcuruto and P. Traldi
CNR–Area di Ricerca, Corso Stati Uniti 4, I-35100 Padova (Italy)

(Received January 14, 1992; accepted in final form May 14, 1992)

ABSTRACT

Pyrolysis–GC/MS has been employed to characterize the products arising from the interaction between glucose and albumin. The pyrolysis products of genuine bovine serum albumin and glycated bovine serum albumin have been compared, after an extensive study devoted to the optimization of the pyrolysis conditions. Clear differences between the pyrograms of glycated and non-glycated samples have been found, confirming the validity of such an analytical approach. The structures of some components, characteristic of glycation, have been tentatively assigned on the basis of a mass spectra library search.

Advanced glycation products (AGE); GC/MS; Maillard reaction; non-enzymatic glycation; pyrolysis.

INTRODUCTION

Efforts have been made in the last decade to identify the complex products arising from the reaction of glucose with amino groups of proteins [1,2]. Such compounds are of wide interest, being important in the fields of both biomedical and food chemistry [3–4]. In particular, our interest is in discovering the diagnostic value of these compounds in the long-term complications of diabetes. The non-enzymatic protein glycation leads to the so-called "Advanced Glycation End Products" (AGE) which are retained and may be responsible for determining diabetes complications [5]. The main analytical approaches in AGE quantification are based on spectroscopic (UV and fluorescence) methods [6] and, more recently, on the development of reactive antibodies to be employed in radio-immuno assay (RIA) procedures [7].

Correspondence to: Dr. A. Lapolla, Istituto di Medicina Interna, Patologia Medica I, Università di Padova, Via Giustiniani, 2, I-35100 Padova, Italy.

Much of the data on AGE have been obtained by in vivo and in vitro experiments of protein glycation, followed by extensive hydrolysis procedures. The comparison between hydrolysis products from genuine and glycated proteins has led, in some cases, to the identification of products present in the glycated substrate only [8]. However, it must be stressed that some such compounds were proved to be, by MS/MS, artifacts related to the hydrolysis procedures [9–12].

The analytical procedures, which allow for more detailed structural information on the whole glycated substrate, should be employed. Among the ionization techniques in mass spectrometry, fast atom bombardment (FAB) [13] and electrospray (ES) [14] are particularly useful in the structural characterization and/or molecular weight determination of macromolecules, i.e. proteins. Unfortunately, both these approaches failed in the investigation on glycated albumin, leading to results of poor analytical value [15].

Consequently, we were forced to consider the employment of a mass spectrometric approach necessarily passing through an extensive degradation of the glycated protein, alternative to the chemical or enzymatic hydrolysis.

Py–GC/MS [16] is a promising and useful technique in the characterization of glycated poly-L-lysine, and has given interesting results [17]. The pyrograms arising from glycated poly-L-lysine and genuine poly-L-lysine resulted in differences in the low GC retention time region. For genuine poly-L-lysine a single, main component was detected, while for glycated poly-L-lysine many different components were observed. By using the mass spectra library it was possible to identify six compounds which originate from thermal degradation of the substructures present in the glycated poly-L-lysine only; e.g. propylester of acetic acid (mw = 102), 2-furaldehyde (mw = 96), 5-methyl-2-furaldehyde (mw = 110), 2-methyl-4,5-dihydrofuran (mw = 84), 5-hydroxymethyl-2-furaldehyde (mw = 126) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (mw = 144). Many of these pyrolysis products can originate from carbohydrate moieties.

This paper reports the use of Py–GC/MS in an attempt to characterize glycated albumin.

EXPERIMENTAL

Samples

Bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) (5 g) and D-glucose (Sigma, St. Louis, MO, USA) (50 g) were dissolved in 50 ml of sodium phosphate buffer (pH 7.5, 0.5 M) under sterile conditions. Following the addition of 0.5% toluene, to avoid bacterial contamination, the solution was incubated at 37°C for 40 days. The sample was dialyzed

extensively against distilled water, in order to remove free glucose and large amounts of Na^+, PO_4^{3-} salts, and then lyophylized. As control, albumin was dissolved in the same proportions in the same buffer, incubated and dialyzed in the conditions described above.

Spectroscopic measurements

Absorption at 350 nm was measured with a Varian Cary 17D spectrometer. Fluorescence was determined at 440 nm on excitation at 370 nm by a Perkin Elmer LS3B spectrophotofluorometer and the results were expressed as arbitrary units of fluorescence (AUF) per milligram of protein.

Py–GC/MS

The pyrolysis was performed by a CDS Pyroprobe 100 solid pyrolyzer using a coil/quartz tube. The best pyrolysis conditions were found to be: filament temperature 900°C, heating rate 5°C/ms and heating time 1 s. The interface between pyrolyzer and GC injector was kept at 220°C. The pyrolyzer was linked to a Carlo Erba MEGA gas chromatograph coupled with a Finnigan ITD 800 mass spectrometer, operating in EI conditions (70 eV, 750 μA). All gas chromatographic separations were performed on a DB1 capillary column (30 m × 0.32 mm I.D. fused silica, 0.25 μm film thickness). The oven temperature was programmed to increase from 100 to 300°C at a rate of 15°C/min. The temperatures of transfer line and ion source were kept at 230°C. All the mass spectra were recorded in full scan mode (mass range from 20 to 650 Da).

RESULTS AND DISCUSSION

Bovine serum albumin has received particular attention as a model protein compound employed in pyrolysis experiments [18–20]. The general approach employed consisted of direct pyrolysis (by filament heating or Curie point filament) into the mass spectrometer (Py–MS), operating under different ionization conditions (electron impact and atmospheric pressure chemical ionization) [20]. In all cases, molecular species and fragment ions led to the identification of pyrolysis products closely related to the protein structure.

In order to gain more definitive information on the pyrolysis products of genuine and glycated albumin, the technique of Py–GC/MS was employed.

Classical filament pyrolysis is known to lead generally to less reproducible results with respect to those achieved by the Curie point approach unless preliminary experiments are done to optimize conditions. Heating rate, final temperature and heating time, as well as morphology and quantity of the sample, were investigated as important parameters. Both albumin and

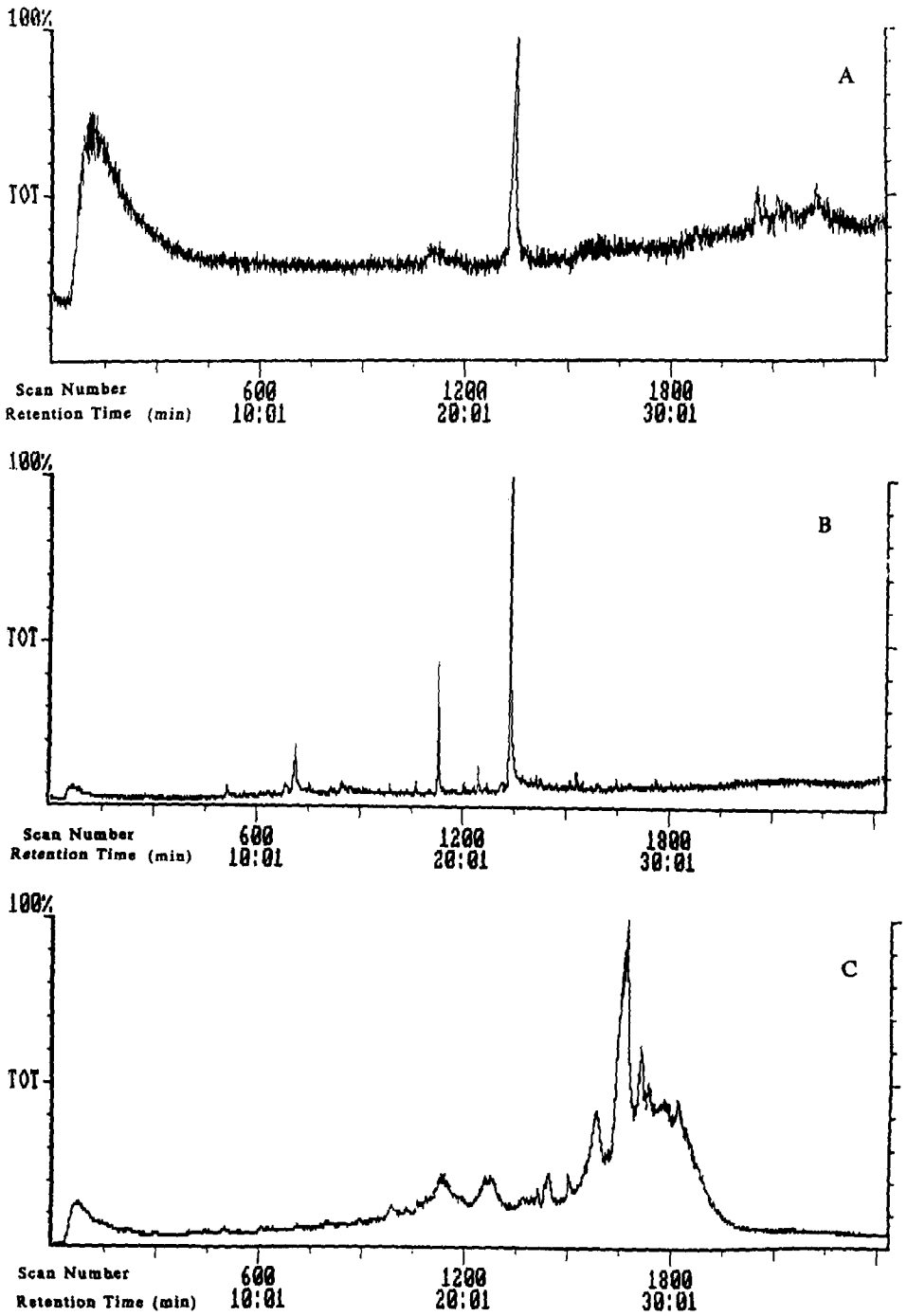


Fig. 1. Reconstructed total ion chromatogram of Py–GC/MS runs of: (A) 0.3 mg of bovine serum albumin unpowdered; (B) 0.3 mg of bovine serum albumin finely powdered; (C) 1.5 mg of bovine serum albumin finely powdered.

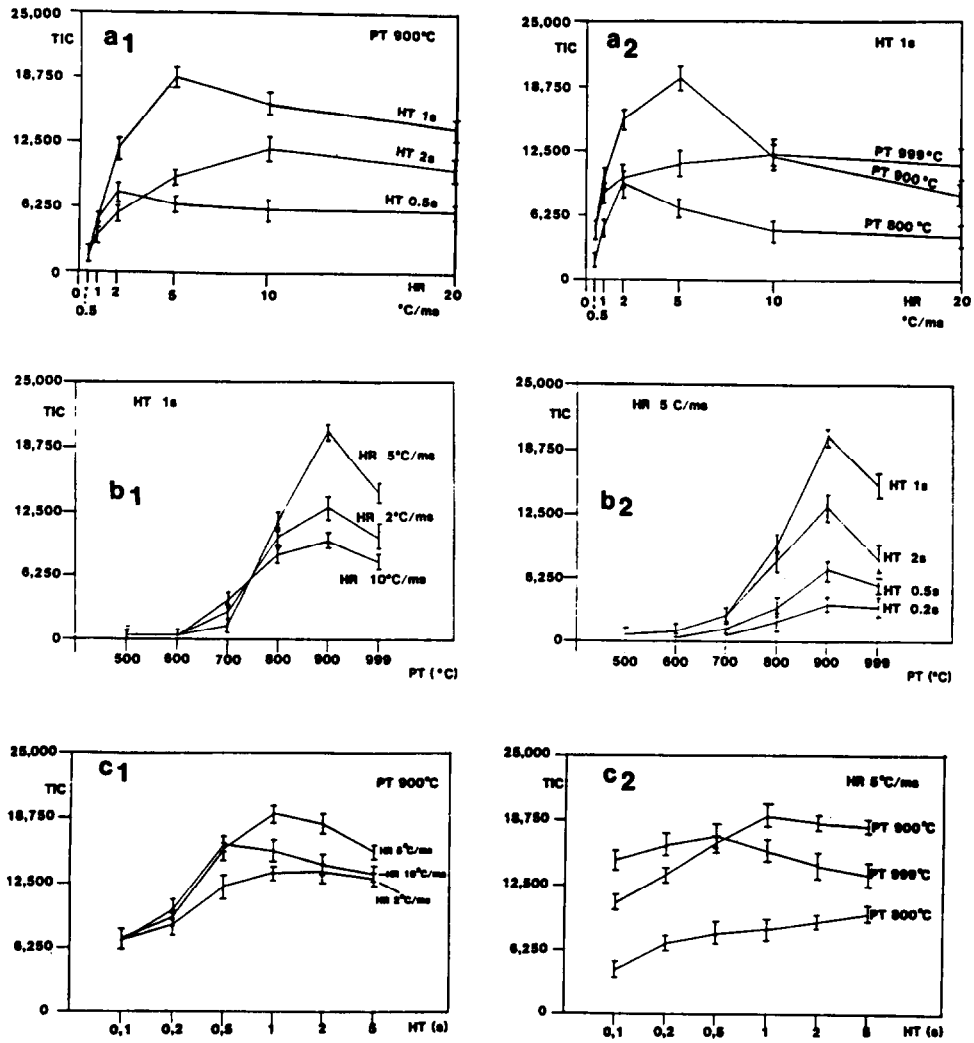


Fig. 2. (a_1) Plots of total ion current (TIC, arbitrary units) vs heating rate (HR, °C/ms) at different values of heating time (HT, s) for a constant pyrolysis temperature (PT = 900°C). (a_2) Plots of total ion current (TIC, arbitrary units) vs heating rate (HR) at different values of pyrolysis temperature (PT, °C) for a constant heating time (HT, 1 s). (b_1) Plots of total ion current (TIC) vs pyrolysis temperature (PT, °C) at different values of heating rate (HR, °C/ms) for a constant heating time (HT, 1 s). (b_2) Plots of total ion current (TIC) vs pyrolysis temperature (PT, °C) at different values of heating time (HT, s) for a constant heating rate (HR, 5°C/ms). (c_1) Plots of total ion current (TIC) vs heating time (HT, s) at different heating rate (HR, °C/ms) for a constant pyrolysis temperature (PT, 900°C). (c_2) Plots of total ion current vs heating time (HT) at different values of pyrolysis temperature (PT, °C) for a constant heating rate (HR, 5°C/ms).

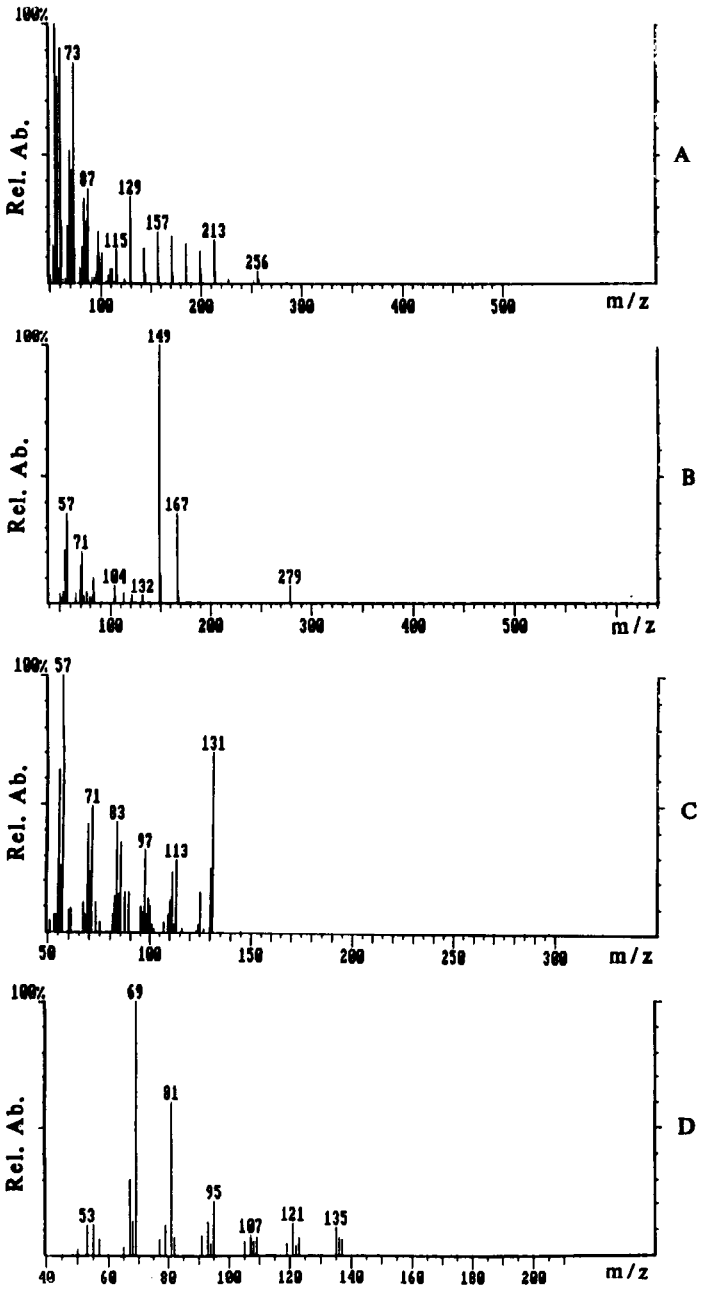


Fig. 3. Mass spectra obtained at different elution times by Py–GC/MS analysis of untreated bovine serum albumin: (A) mass spectrum of scan 701 (see peak a of Fig. 1B); (B) mass spectrum of scan 1116 (see peak b of Fig. 1B); (C) mass spectrum of scan 1126 (see peak c of Fig. 1B); (D) mass spectrum of scan 1321 (see peak d of Fig. 1B).

glycated albumin were finely powdered by ball mill and sample amounts were normally in the range 0.2–0.5 mg.

In order to understand the influences of morphology and quantity of sample on pyrolysis, the pyrograms of 0.3 mg unpowdered albumin, and 0.3 mg and 1.5 mg powdered albumin are reported in Fig. 1. The most suitable pyrogram is obtained when the sample is small in size and pow-

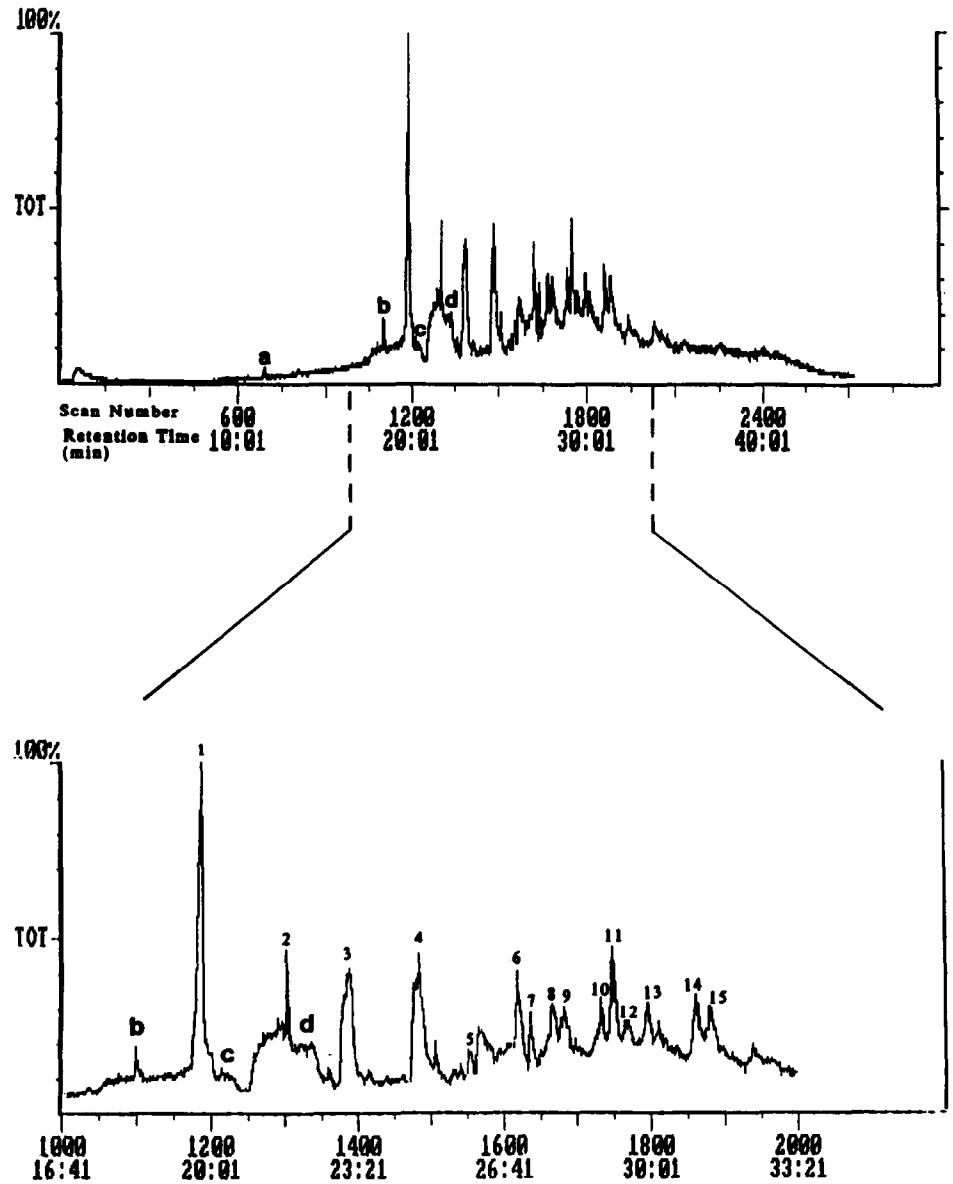


Fig. 4. Reconstructed total ion chromatogram of Py–GC/MS run of 0.3 mg of finely powdered glycated bovine serum albumin.

dered in nature. Statistical data, on repetitive Py–GC/MS analysis on such samples, unequivocally prove that the best reproducibility (% sd = 4.4–7.4) is obtained in this case (Fig. 1B). The higher complexity of the chromatogram reported in Fig. 1C can be explained by the occurrence of secondary reactions. Such reactions become operative at higher reactant pressure in the pyrolysis chamber [21].

Further investigations were carried out to determine the best pyrolysis conditions. Plots of total ion current, directly related to the total amounts of the pyrolysis products, vs. heating rate (HR), (at constant pyrolysis temperature (PT) or heating time (HT)), vs PT (at constant HR or HT) and vs HT (at constant HR or PT) for the pyrolysis of 0.3 mg finely powdered albumin are shown in Fig. 2. The data show that the best values of such parameters in terms of pyrolysis yield and reproducibility, are: HT = 1 s; PT = 900°C; HR = 5°C/ms.

The pyrogram of albumin, as obtained under the above described conditions, is shown in Fig. 1B. It mainly consists of four distinguishable peaks. The mass spectra of these compounds are reported in Fig. 3.

The peak corresponding to spectrum 1116 (b in Fig. 1B) is due to dibutyl phthalate, a widespread contaminant commonly present.

The mass spectrum related to peak a (Fig. 3A) shows a possible molecular ion at m/z 256, with abundant fragments at m/z 129, 87, 73, 71 and 55. Analogously the spectrum related to peak c (Fig. 3C) shows abundant ionic species at m/z 131, 113, 97, 83, 71 and 57, while in the mass spectrum of peak d (Fig. 3D) abundant ions at m/z 135, 121, 107, 95, 81 and 69 are detected. Structural assignment of the compounds related to peaks a, c and d could not be done on the basis of simple mass spectra.

It is worth noting, however, that some of the same ionic fragments have been detected and described among the pyrolysis products of albumin, as obtained by direct probe pyrolysis (Py–MS) [20]. In fact, Snyder et al. [20] considered, in their work on Curie point pyrolysis of albumin, that ions at m/z 92 and 104 arise from the thermal decomposition of phenylalanine, ions at m/z 94 and 108 from tyrosine, and that ions at m/z 131 are characteristic decomposition products of tryptophan. In the same paper the authors suggested that ions at m/z 69 can be considered to be due to $M^{+\cdot}$ of pyrroline, arising from the pyrolysis of proline, while ions at m/z 124, 138, 152 and 166 are due to diketopiperazines, pyrolysis products formed by the condensation of two amino acid moieties.

In our previous work on the Py–GC/MS of poly-L-lysine [17], low molecular weight species were detected at low retention times in the case of glycated poly-L-lysine only. For such reasons the related molecular ions at m/z 84, 96, 102, 110, 126 and 144, were proposed as being due to diagnostic species, arising from the interaction between poly-L-lysine and glucose. Some of the same components were also detected by Boon et al. [22] among the Curie point pyrolysis products of some model melanoidinis.

One of the aims of the present work was to test their diagnostic validity in the comparison between albumin and glycated albumin. Single ion monitoring Py–CG/MS experiments on genuine albumin show the presence of products having molecular weights of 84, 96, 102, 110, 126 and 144 with relative abundances that, as will be discussed later, are very similar to those found in the case of glycated albumin. Consequently, in the present case such compounds cannot be considered diagnostic for glycation processes.

After incubation of albumin with glucose, as described in the experimental section, the occurrence of glycation reactions was verified by classical spectroscopic measurements. The higher absorbance and fluorescence values found in the sample of glycated albumin with respect to those of control sample, confirm the presence in the former of a large amount of AGE. In particular, the absorbance at 350 nm passes from 0.041 ($\pm$0.01) to 0.105 ($\pm$0.1), while the AUF passes from 10 ($\pm$0.1) to 55 ($\pm$0.05). The significant difference is well evidenced by statistical calculation (Student's t test) [23], from which p values lower than 0.001 were found.

The typical pyrogram of glycated albumin is shown in Fig. 4. Numerous components are present with retention times in the range of 16–33 min. While some components are common to that of albumin (Fig. 1B, peaks a, b, c and d), the new chromatographic peaks may account for glycation products.

The mass spectra of peaks 1–15, present in the pyrolysis of glycated albumin only, are reported in Figs. 5–8, while the relevant data on the chromatographic peaks, i.e. retention times, possible molecular weights and structures, are reported in Table 1.

Among the pyrolysis products identified by the mass spectra library search, those containing furan and pyrrole moieties, often described in the literature as arising from glycation processes [1,2], are worth noting. Thus, for example, the mass spectrum of peak 2 is in agreement with the structure of 1-(2-furanyl methyl)-1H pyrrole with an FIT value[a] of 745. Analogously, compounds corresponding to peaks 4, 5, 6, 9, 11, 13 and 14 have been identified as derivatives of oxygen- and/or nitrogen-containing heterocyclic compounds.

Four of the chromatographic peaks are tentatively assigned as esters of fatty acids. Of course, they cannot originate from the pyrolysis of both protein chain and AGE. Their presence could be justified by a chemical contamination of the sample.

Finally, peak 3 is due to column bleeding. Such a decomposition of the column phase is not due to the chromatographic conditions (i.e. high

[a] FIT values are a measure of the degree to which the library spectrum is included in the unknown spectrum. An FIT value of 1000 indicates that all library peaks occur as peaks in the unknown; for those common peaks all intensities are exactly proportional.

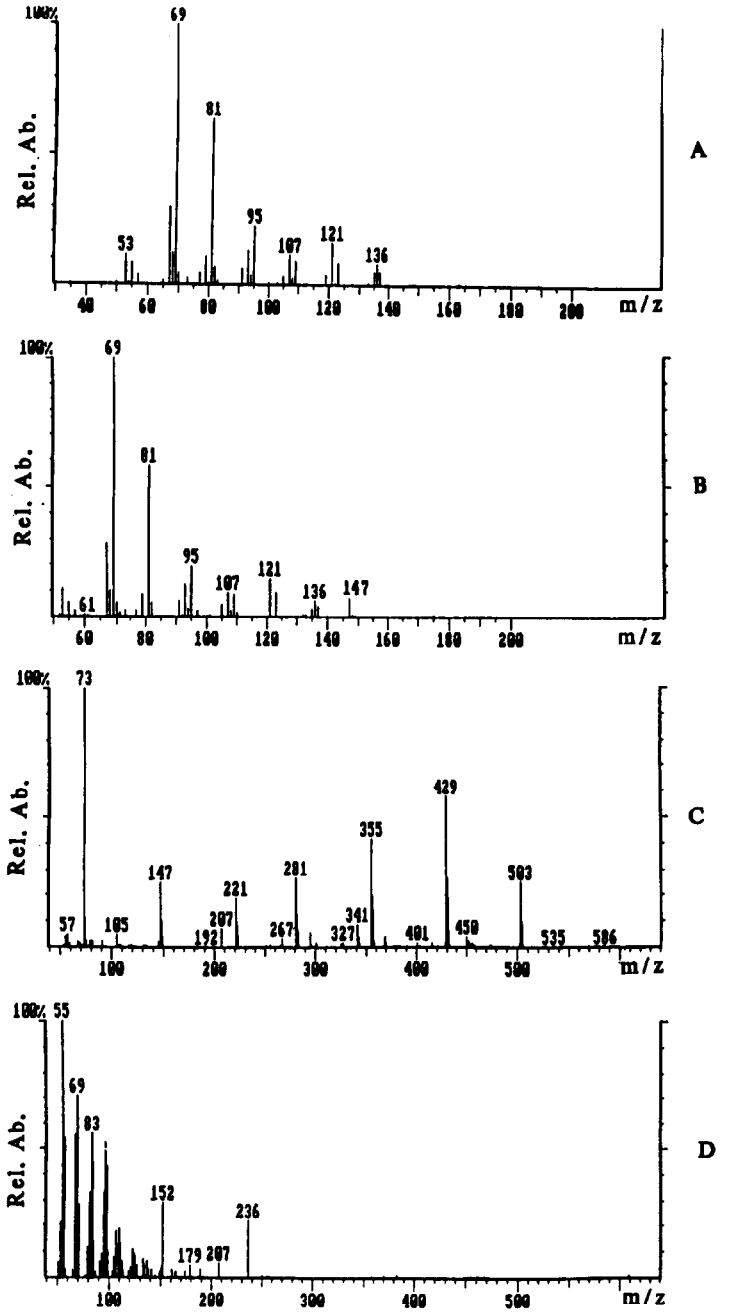


Fig. 5. Mass spectra obtained at different elution times by Py–GC/MS analysis of glycated bovine serum albumin: (A) mass spectrum of scan 1188 (see peak 1 of Fig. 4); (B) mass spectrum of scan 1302 (see peak 2 of Fig. 4); (C) mass spectrum of scan 1388 (see peak 3 of Fig. 4); (D) mass spectrum of scan 1484 (see peak 4 of Fig. 4).

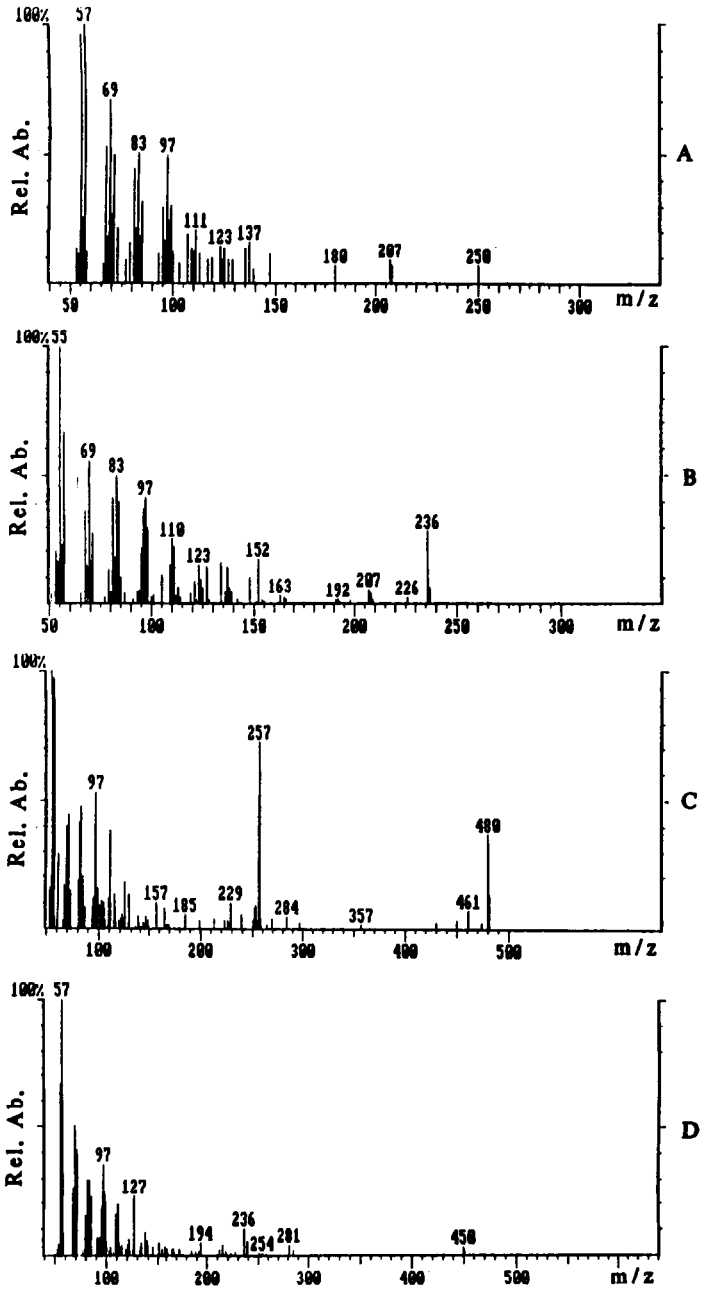


Fig. 6. Mass spectra obtained at different elution times by Py–GC/MS analysis of glycated bovine serum albumin: (A) mass spectrum of scan 1554 (see peak 5 of Fig. 4); (B) mass spectrum of scan 1620 (see peak 6 of Fig. 4); (C) mass spectrum of scan 1637 (see peak 7 of Fig. 4); (D) mass spectrum of scan 1666 (see peak 8 of Fig. 4).

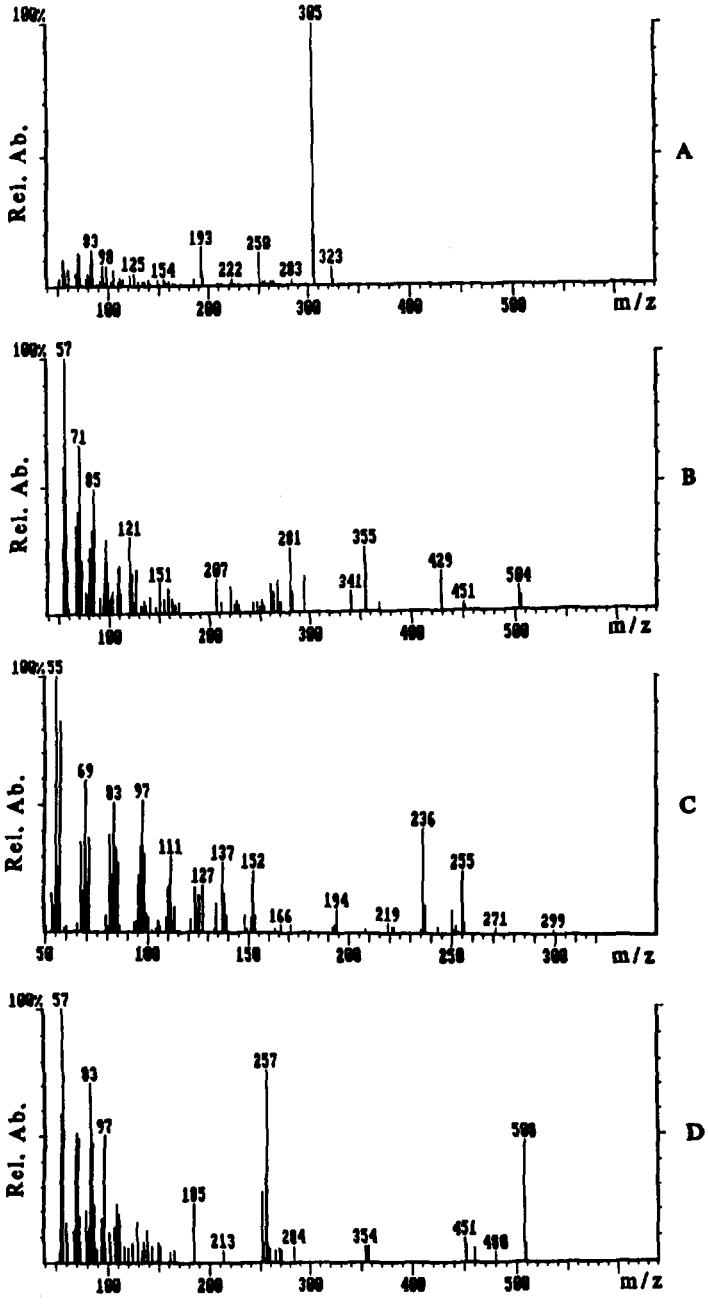


Fig. 7. Mass spectra obtained at different elution times by Py–GC/MS analysis of glycated bovine serum albumin: (A) mass spectrum of scan 1682 (see peak 9 of Fig. 4); (B) mass spectrum of scan 1732 (see peak 10 of Fig. 4); (C) mass spectrum of scan 1747 (see peak 11 of Fig. 4); (D) mass spectrum of scan 1764 (see peak 12 of Fig. 4).

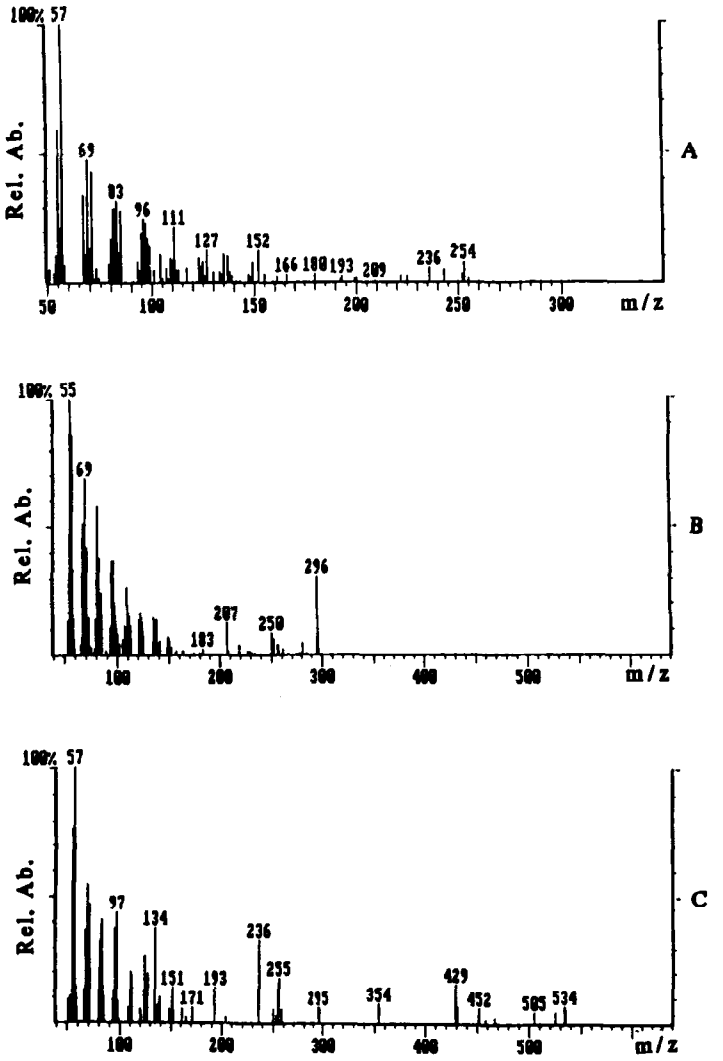
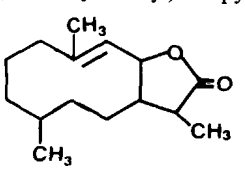
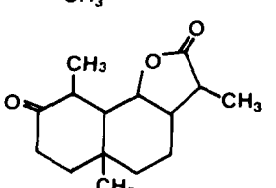
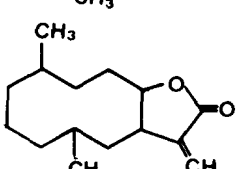


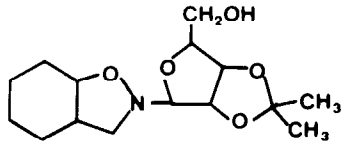
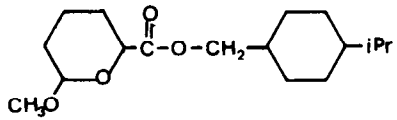
Fig. 8. Mass spectra obtained at different elution times by Py–GC/MS analysis of glycated bovine serum albumin: (A) mass spectrum of scan 1794 (see peak 13 of Fig. 4); (B) mass spectrum of scan 1810 (see peak 14 of Fig. 4); (C) mass spectrum of scan 1880 (see peak 15 of Fig. 4).

temperature), but must be ascribed to highly reactive pyrolysis products which destabilize the column stationary phase. This aspect is well evidenced by the ion chromatogram relative to an m/z value of 503, typical of column bleeding. The plot does not show any increase with respect to temperature, but a broad peak from 20 to 22 min retention time.

TABLE 1

Possible structures identified at different retention times[a]

Peak	Retention time (min)	Spectrum	Possible M.W.	Possible structure subunits	Remarks	FIT
1	19:49	1188	136	Furane, dihydrofurane, terminal methyl group		
2	21:43	1302	147	1-(2-furanylmethyl)1*H*-pyrrole		745
3	23:09	1388	503		Column bleeding	
4	24:45	1484	236			803
5	25:55	1554	250			753
6	26:58	1620	236			720

7	27:18	1637	480	Hexadecyl hexadecanoate	Unknown origin	790
8	27:47	1666	450	Tetradecyl 9-hexadecenoate	Unknown origin	687
9	28:03	1682	323	1-Hydroxy-2,5-anisyl-3,4-dimethyl-pyrrole		
10	28:53	1732	504	9-Octadecenyl(Z,Z)-9-hexadecenoate	Unknown origin	785
11	29:08	1747	299			478
12	29:25	1764	508	Octadecyl hexadecanoate	Unknown origin	711
13	29:55	1797	254	5-Dodecyl-dihydro-2(3*H*)-furanone		720
14	31:01	1810	296			
15	31:21	1880	534	Eicosil Hexadecenoate	Unknown origin	730

[a] FIT values are a measure of the degree to which the library spectrum is included in the unknown spectrum. An FIT value of 1000 indicates that all library peaks occur as peaks in the unknown; for those common peaks all intensities are exactly proportional.

CONCLUSIONS

Py–GC/MS has proved to be a valid analytical tool for the characterization of the products arising from the interaction between glucose and albumin. After preliminary experiments devoted to the *mise au point* of the best pyrolysis conditions, clear differences have been found between the pyrolysis products of genuine bovine serum albumin and glycated bovine serum albumin. Some of the components of the pyrolysis mixture have been structurally identified on the basis of a mass spectra library search.

ACKNOWLEDGEMENT

Financial support by CNR-Roma, Progetto Finalizzato Invecchiamento Co. No. 923225, is acknowledged.

REFERENCES

1 H. Paulsen and K.W. Pflughaupt, in W. Pigman and D. Horton (Eds.), Carbohydrates, Vol. 1B, Academic Press, New York, 1980, p. 881.
2 F. Ledl, The Maillard Reaction Advances in Life, Sciences Birkhauser Verlag, Basel, 1990, p. 19.
3 T.M. Reynolds, Adv. Food. Res., 12 (1963) 1.
4 V.M. Monnier and A. Cerami, Clin Endocrinol. Metab., 11 (1982) 431.
5 M. Brownlee, A. Cerami and H. Vlassara, N. Engl. J. Med., 318 (1988) 1315.
6 V.M. Monnier, R.R. Kohn and A. Cerami, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 583.
7 J.C.F. Chang, P.C. Ulrich, R. Bucala and A. Cerami, J. Biol. Chem., 13 (1985) 7970.
8 S. Pongor, P. Ulrich, A. Bencsath and A. Cerami, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 2684.
9 A. Lapolla, C. Gerhardinger, B. Pelli, A. Sturaro, E. Del Favero, P. Traldi, G. Crepaldi and D. Fedele, Diabetes, 39 (1990) 57.
10 A. Lapolla, C. Gerhardinger, E. Ghezzo, R. Seraglia, A. Sturaro, G. Crepaldi, D. Fedele and P. Traldi, Biochim. Biophys. Acta, 1033 (1990) 13.
11 C. Gerhardinger, A. Lapolla, G. Crepaldi, D. Fedele, E. Ghezzo, R. Seraglia and P. Traldi, Clin. Chim. Acta., 189 (1990) 335.
12 F.G. Njoroge, A.A. Fernandes and V.M. Monnier, J. Biol. Chem., 263 (1988) 10646.
13 W. Heerma and E.R. Bathelt, Biomed Environ. Mass. Spectrom., 13 (1986) 205.
14 R.T. Gallagher, J.R. Chapman and M. Mann, Rapid Commun Mass. Spectrom., 4 (1990) 369.
15 A. Lapolla, C. Gerhardinger, D. Fedele, C.J. Porter, R. Seraglia, D. Favretto and P. Traldi, unpublished results, 1990.
16 T.O. Munson and D.D. Fetterolf, J. Anal. Appl. Pyrolysis, 11 (1987) 15.
17 A. Lapolla, C. Gerhardinger, L. Baldo, D. Fedele, D. Favretto, R. Seraglia and P. Traldi, Org. Mass. Spectrom., 27 (1992) 183.
18 B. Windig, P.G. Kistemaker and J. Haverkamp, J. Anal. Appl. Pyrolysis, 3 (1981/82) 199.
19 P.A. Snyder, J.H. Kremer, H.L.C. Meuzelaar and W.J. Windig, J. Anal. Appl. Pyrolysis, 13 (1988) 77.

20 P.A. Snyder, J.H. Kremer, H.L.C. Meuzelaar, W. Windig and K. Taghizadeh, Anal. Chem., 59 (1987) 1945.
21 A.J.H. Boerboon, Mass Spectrometry of Large Molecules, Elsevier, Amsterdam, 1985, p. 265.
22 J.J. Boon, J.W. De Leenw, Y. Rubinsztain, Z. Aizenshtat, P. Ioselis and R. Ikan, Org. Geochem., 6 (1984) 805.
23 K.A. Brownlee, Statistical Theory and Methodology, Wiley, New York, 1965.