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Studies on advanced glycation end products by recent mass spectrometric techniques

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Summary. The results obtained by different mass spectrometric approaches in the field of advanced glycation of proteins are reported and discussed in detail in comparison with those obtained by other analytical methodologies (fluorescence and absorbance spectroscopies, radioimmunoassay, enzyme-linked immunosorbent assay). They have been subdivided in three main groups: analysis on degraded glycated proteins, direct analysis of glycated proteins and studies on the reaction between protected lysine and glucose. The general overview so achieved indicate mass spectrometry as a particularly valid analytical method in this field of research.

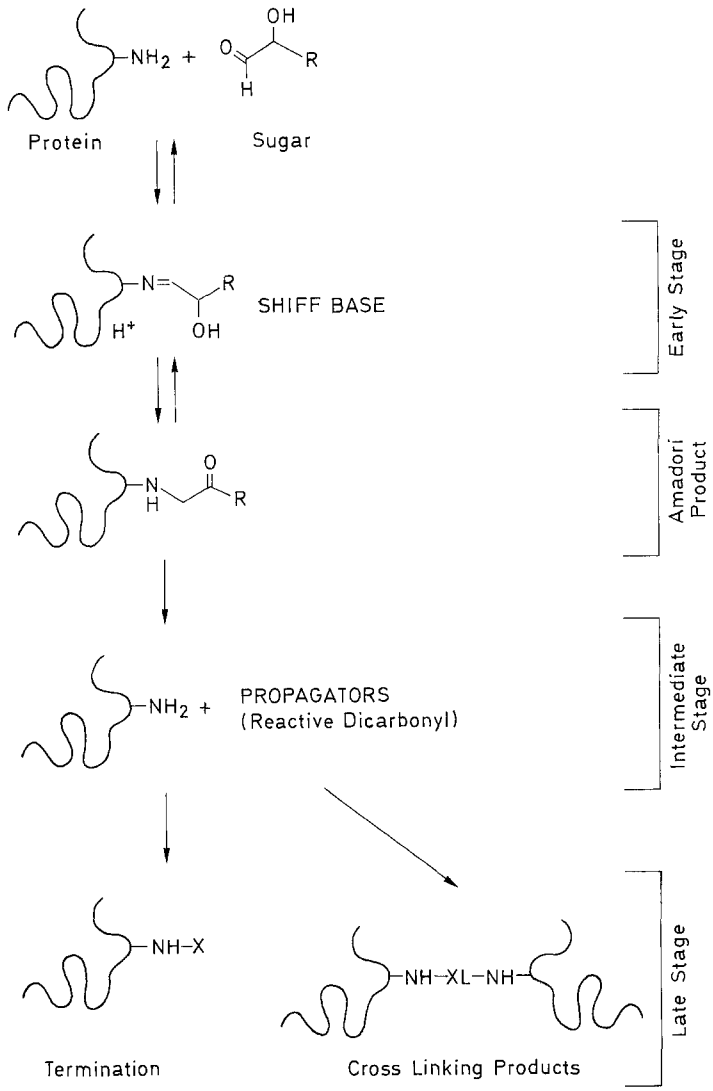
Keywords: Amino acids – Diabetes – Non enzymatic glycation – Advanced glycation end products (AGE) – Mass spectrometry – HPLC – Laser desorption

Introduction

The interaction between glucose and proteins is a topic of wide interest, ranging from food chemistry to medicine (Monnier and Cerami, 1982; Maillard, 1916).

From the early studies of Maillard (1912) it was found that the reaction of amino groups of proteins with glucose, as well as other reducing sugars, leads to highly reactive intermediates which can react with other aminic sites (or more generally, with other reactive sites) leading to intra or inter-molecular cross-linking.

The Maillard reaction proceeds classically through early, intermediate and late stages (see Scheme 1). In the early stage glucose reacts with free amino groups of proteins to form a labile Schiff base; this base then undergoes an Amadori rearrangement (Reynolds, 1963, 1965) to form a stable adduct: the 1-amino-1-deoxy-2 ketose. This Amadori product is degraded into a series of carbonyl compounds (deoxyglucosones and sugar fragmentation products) that act as propagators of the reaction (Ledl et al., 1986). In the late stage these propagators



Scheme 1. (from A Lapolla, D. Fedele, R. Seraglia and P. Traldi, "Mass Spectrometry in the Study of Advanced Glycation Processes, Responsible for Long Term Diabetes Complication", 1991. Reprinted by permission of John Wiley and Sons, Ltd)

react again with free aminogroups and through dehydration and rearrangement reactions lead to yellow-brown, insoluble and often fluorescent products, called advanced glycation end products (AGE) (Monnier and Cerami, 1983; Brownlee et al., 1984).

In medicine such reactions are of wide interest. In badly controlled diabetic conditions, the glucose concentration in blood is higher than 200 mg/dl and this significantly increases the rate, and consequently the yield, of glycation processes.

Such reactions, in particular those leading to the formation of cross-linking advanced glycation products, are retained responsible for the long-term diabetic complications (nephropathy, retinopathy, neuropathy, macroangiopathy) (Lubec and Pollak, 1980; Monnier and Cerami, 1981; Vlassara et al., 1984; Brownlee

et al., 1988; Tanaka et al., 1988; Tsilbary et al., 1988; Lyons et al., 1991; Makita et al., 1991; Patel et al., 1991; Ruderman et al., 1992). Such considerations well explain the efforts which have been done, in particular in the last decade, in order to find an AGE marker with high diagnostic value to be employed for the evaluation of tissutal modifications. In this contest 2-(2furoyl)-4-(5)-(2-furanyl)1H-imidazole, FFI, and pentosidine were recently proposed as AGE markers (Pongor et al., 1984; Sell and Monnier, 1989). FFI, identified among the hydrolysis products of glycated proteins, was proposed to originate from a condensation reaction between two glucose molecules and two amino groups of lysine, through cyclization and further autoxydation reactions (Pongor et al., 1984). The *in vitro* glycated proteins and the synthetized FFI gave the same fluorescence spectra and this led to consider FFI a possible cross-link product.

Pentosidine, a compound with fluorescence properties similar to those of Maillard compounds, whose structure was firstly identified *in vivo* on collagen samples and further confirmed *in vitro* by the non enzymatic reaction of pentoses with lysine and arginine, is considered an AGE responsible for protein cross-links. More recently it was shown that not only pentoses but also glucose, fructose and ascorbate can originate pentosidine through a glycoxydation reaction (Grandhee and Monnier, 1991; Dyer et al., 1991). The high correlations found between plasma and collagen pentosidine levels and the severity of long-term diabetic complications confirm the importance of this compound as a marker of tissue modifications by AGE (Sell et al., 1991, 1992). However pentosidine accounts for only a part of carbohydrate dependent, non enzymatic cross-links of collagen; in fact there are also other compounds capable to induce the chemico-physical alteration of proteins characteristic of diabetes; being them actually unknown, efforts must be spent for their identification.

The analytical techniques usually employed in the protein glycation field are the spectroscopic ones, mainly fluorescence and absorption spectroscopies (Monnier and Cerami, 1983). In fact AGE s synthetized *in vitro* show a characteristic increase in fluorescence at 440 nm with excitation at 370 nm and an increase in absorbance between 300 and 400 nm with a shoulder at 320 nm. Furthermore brown pigments with fluorescence and absorbance properties very similar to those of *in vitro* AGE have been evidenced *in vivo* on collagen, lens proteins and other tissues of diabetic patients (Monnier and Cerami, 1982). Recently Odetti et al. (1990) obtained interesting results by evaluating fluorescence levels in skin collagen of diabetic and normal rats and by rationalizing them *vs* age of the animals. In fact they found different trends of increase of fluorescence in healthy and diabetic rats, also if glucose and glycated haemoglobin levels were constant in the blood. These data suggest that many factors could contribute to the fluorescence levels, i.e. chemical degradation of glycated proteins and unknown accelerating factors (as the saturation of receptor-mediated removal of AGE).

However spectroscopic techniques, also if exhibit a good sensitivity with respect to the analytical problem, lack of specificity, in particular considering the complexity of the natural substrate under investigation.

A more recently employed analytical method with a surely higher specificity is the RIA one, based on the development of antibodies specific *vs* substructures significative for glycation products. A RIA method was proposed in fact for FFI

evaluation (Chang et al., 1985). The authors, by developing an antibody reactive against 4-furanyl-2-furoyl-1H imidazole-1hexanoic acid, reported different FFI levels in relation to different glucose concentrations. Unfortunately the antibody developed exhibits a not enough high specificity, showing a high binding affinity not only to FFI but also to different FFI-related compounds, all containing various furanyl and imidazole moieties (e.g. FFI-BSA, FFI-lysine, MeFFI, furoine, furoic acid) (Chang et al., 1985).

More recently an enzyme-linked immunosorbent assay (ELISA) was developed to detect glucose derived pyrroles and in particular 5-hydroxy-methyl-1-alkylpyrrole-2-carbaldehyde (pyrraline) (Miyata and Monnier, 1992). Monoclonal antibodies were developed against immobilized caproyl/pyrraline. Using this method higher levels of pyrraline in plasma were found in diabetic rats and patients in comparison with respective healthy controls. Furthermore a preferential localization of pyrraline immunoreactivity was found in the extracellular matrix of different diabetic tissues; the effective role of this compound in determining long-term diabetic complications is currently under study.

Finally Makita et al. (1992) using an ELISA method with a polyclonal antiserum to an AGE epitope, which is formed *in vitro* after the incubation of glucose with ribonuclease, demonstrated that this antiserum is able to dectect AGE *in vivo* from different tissues, as plasma and collagen. This antiserum can recognize cross-reactive epitopes which are formed from the reaction of different sugars (glucose 6-phosphate, fructose, etc.) with different carrier proteins, suggesting that tissue AGEs which form *in vivo* have a common immunological epitope which cross-reacts with *in vitro* synthesized AGE. The fact that none of the known AGE (FFI, pyrraline, pentosidine, carboxymethyl lysine) were able to compete for binding to anti-AGE antibody could mean that these structurally defined AGE may be only a part of the products formed and it points the need of further structural studies of the advanced glycation pathways that really occur *in vivo*.

Mass spectrometry represents one of the analytical methods with higher specificity and sensitivity nowadays available. In fact, if on one hand detection limits of 10^{-12} g are currently available, on the other hand the identification of molecular weight and the fingerprint obtained by the fragmentation patterns are highly diagnostic from the structural point of view (Gelpi, 1992). The development of "hyphenated methods", based on the coupling of a separative technique with mass spectrometry has furtherly increased the specificity of the analytical procedure, due to a sinergic effect of the coupling (Gelpi, 1992). With this respect the data obtained by Yost (1983) in calculating the informing power (P_{inf}, as defined by Kaiser (1978)) of different hyphenated mass spectroscopic techniques are note-worthy. Thus, while $P_{inf} = 100$ for a single quadrupole mass spectrometer with 1000 Da mass range and unitary resolution, $P_{inf} = 1000$ for a HRGC/MS and $P_{inf} = 10{,}000$ for a MS/MS system. It follows that the most powerful method currently available results to be the MS/MS one, consisting in the use of two different mass (momentum, or traslational energy) analysers for both separative and analytical purposes. From the operative point of view, the ion of interest is selected by a first analyser, decomposed by suitable methods (usually by collision with target, non-reactive, gases) and its decomposition products

separated by the second analyser and detected. Such method has been successfully applied in many analytical problems (Busch et al., 1988).

The separative method usually employed in the biomedical field is HPLC, due to the physico-chemical properties of the molecules of interest (e.g. polarity). Hence the hyphenated HPLC/MS method is of high interest, also if from the technological point of view the coupling of a liquid phase system with the vacuum conditions present in a mass spectrometer is a severe technological problem. This has been overcomed by the use of different ionization methods, among which thermospray (and plasmaspray) and particle beam represent the more used ones (Arpino, 1992).

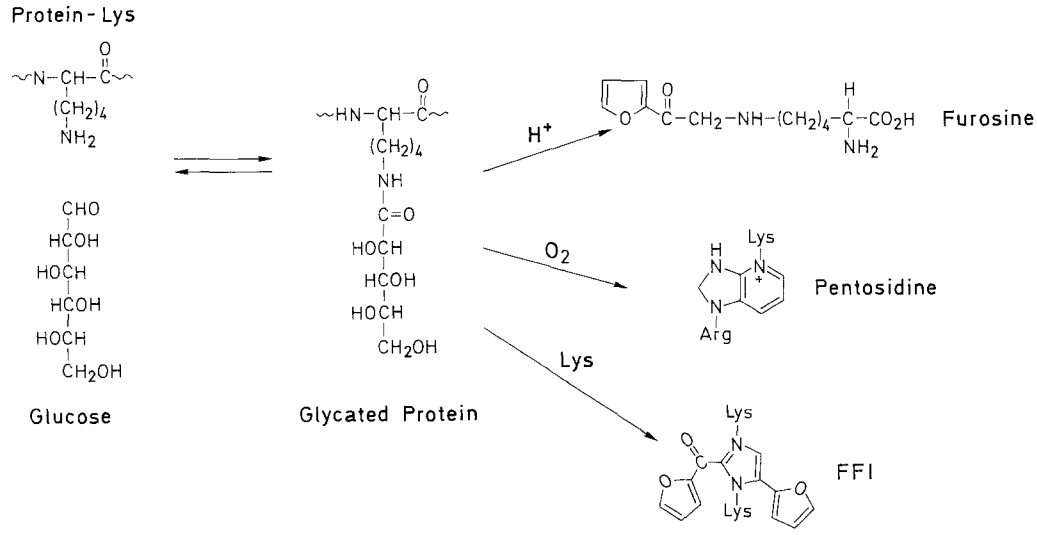
Finally for the direct analysis of high molecular weight compounds, without any preliminary decomposition procedures, two different approaches can be nowadays employed. The first consists in the controlled thermal degradation (pyrolysis) of the biomolecule in a chamber directly connected to the analytical device (usually GC/MS) (Boon, 1992), the second consists in the ionisation/desorption or desolvatation/ionisation of the intact molecule. In the former case FAB (Barber et al., 1981) and MALDI (Karas et al., 1985) can be employed, in the latter electrospray is used (Smith et al., 1990).

All the above described mass spectrometric methods have been successfully applied in the study of protein glycation. Thus while either MS/MS or HPLC/MS have been used for the structural identification of the products arising from hydrolysis of glycated proteins, Pyr/GC/MS and MALDI have been employed for the direct characterization of glycated substrates, through the comparison with the behaviour of non-glycated ones.

Analysis on degraded glycated proteins

The first success in the study of glycation processes on proteic substrates was obtained in the investigation on the products arising from hydrolysis of glycated proteins and their comparison with those derived from the non glycated ones. Among these hydrolysis products, in the early days of such investigation, the attention was focalised mainly on three different products, i.e. 2-(2 furoyl)-4-(5)-(2-furanyl)1H-imidazole (FFI) (Pongor et al., 1984), furosine (Schleicher and Wieland, 1981) and pentosidine (Sell and Monnier, 1989). For their formation the pathways reported in Scheme 2 were proposed, and their structural identification was done on the basis of spectroscopical data (fluorescence, UV absorption, ^{1}H NMR and mass spectroscopies).

In particular FFI was proposed as an interesting AGE: the two nitrogen atoms were suggested to originate from the ε-amino groups of lysines present in two different proteins, while the furane moieties were proposed to originate from a rearranged glucose molecule. Hence it gave account not only to glycation but it could also be invoked as one of the cross-linking products, responsible for the tissutal modifications. Consequently, FFI was employed as a possibly effective marker of advanced glycation: its structure was unequivocally proved by ^{1}H NMR, mass spectrometry and the comparison with an "ad hoc" syntetised molecule.



Scheme 2

Successively it was dosed on the basis of fluorescence data and a specific antibody was developed to be employed in RIA. By such approach mean FFI levels ($\pm$ ES) of 5.22 $\pm$ 0.25 pmol/mg and 8.4 $\pm$ 1.4 pmol/mg were respectively found for samples of globine and albumin of healthy controls, while they became 1744 $\pm$ 274 pmol/mg for samples of *in vitro* glycated albumin (Chang et al., 1985).

Looking at the relevance of FFI in the determination of the end-glycation levels, it was thought of interest to employ a tandem mass spectrometric technique for its detection in the hydrolysis mixture. As it has been reported in the Introduction, tandem mass spectrometry (MS/MS) is an analytical method highly effective for the identification (and possibly, quantification) of molecules of interest in complex substrates without, or only with minor, sample treatment. In the particular case of FFI determination in *in vitro* glycated proteins, this was achieved by a double focusing, reverse geometry mass spectrometer. The selection, by means of the magnetic sector, of the ionic species possibly corresponding to molecular ion (m/z 228) of FFI, their further decomposition by collision with a target gas and the analysis of the collisional products by scanning the electrostatic sector led to the MS/MS spectra. These spectra if identical to that of $M^{+\cdot}$ of a pure sample of FFI, make more than confident on the FFI presence in the complex hydrolysis substrate. In Fig. 1 the MS/MS spectrum of $M^{+\cdot}$ of FFI is reported. The collisionally generated daughter ions are well related to the structure, as shown in Scheme 3. Hence the MS/MS spectrum can be considered a valid fingerprint for FFI structure. The analysis of the ions at m/z 228 generated by EI on the hydrolysis mixture obtained by incubating polylysine and albumin (sodium phosphate buffer pH 7.5, 0.5 M Na, for 28 days at 37°C) without any glucose, led to spectra showing the presence of scarcerly abundant daughters ions without any significance, being completely unrelated to FFI structure. On the contrary the introduction of hydrolysed glycated albumin and polylysine (obtained by 5 g albumin, and 1 g polylysine incubated with 50 g glucose in sodium phosphate buffer at pH 7.5, 0.5 M Na, for 28 days at 37°C) into the EI

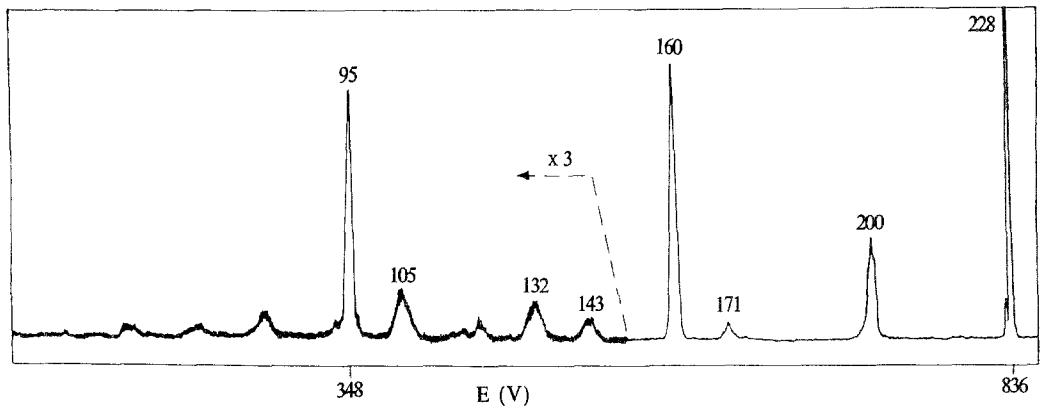


Fig. 1. CAD MIKE spectrum of $M^{+\cdot}$ ion of FFI (m/z 228) (from A. Lapolla, D. Fedele, R. Seraglia and P. Traldi, "Mass Spectrometry in the Study of Advanced Glycation Processes, Responsible for Long Term Diabetes Complications", 1991. Reprinted by permission of John Wiley and Sons, Ltd)

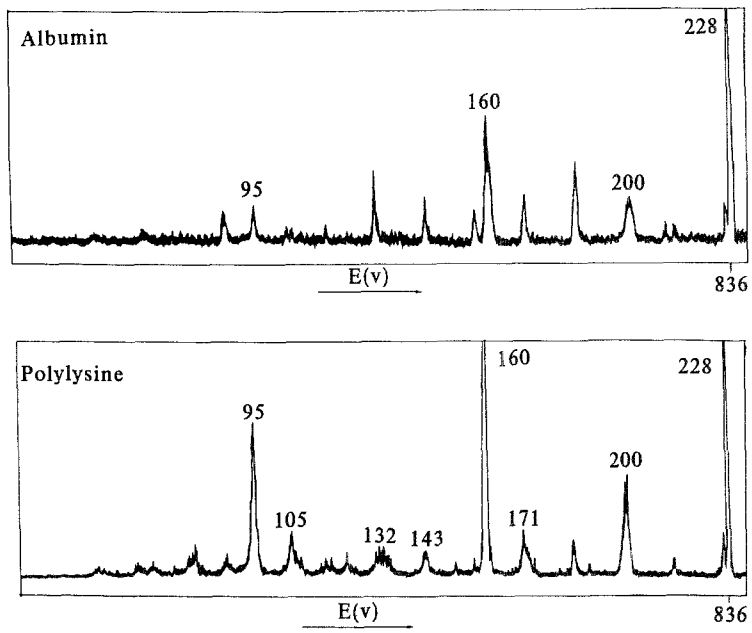
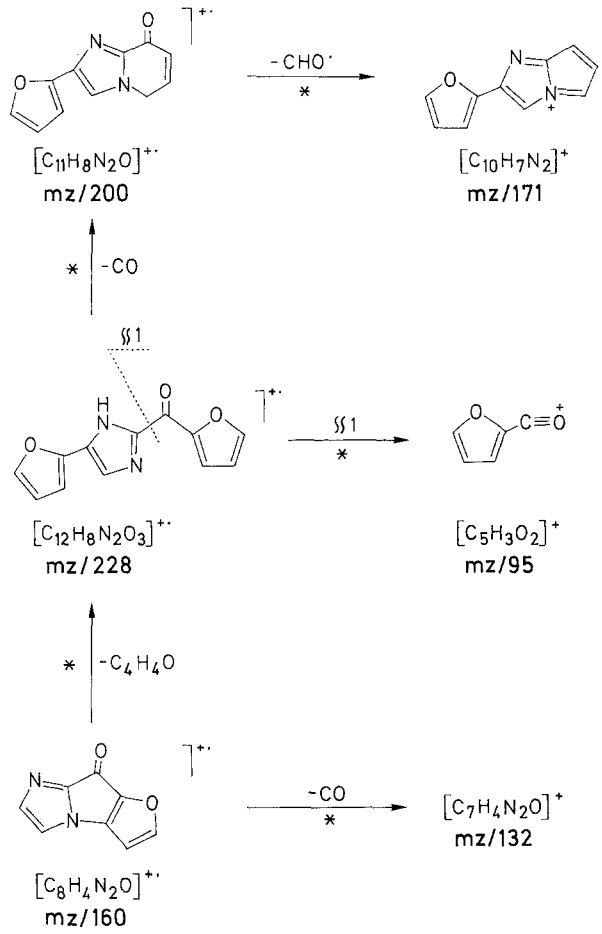


Fig. 2. CAD MIKE spectra of ionic species at m/z 228 present in glycated albumin (up) and glycated polylysine (down) (from A Lapolla, D. Fedele, R. Seraglia and P. Traldi, "Mass Spectrometry in the Study of Advanced Glycation Processes, Responsible for Long Term Diabetes Complications", 1991. Reprinted by permission of John Wiley and Sons, Ltd)

source and the further MS/MS experiments on the ions at m/z 228, led to the spectra shown in Fig. 2, practically identical to that achieved by pure FFI (see Fig. 1), proving the high specificity of the analytical procedure (Pelli et al., 1986).

In order to evaluate the sensitivity of the method, a hydrolysed non glycated albumin sample was spiked with known amounts of pure FFI, allowing the evaluation of a detection limit in the range 50–100 pg. Considering that the detectable levels reported in literature correspond to the range from 912 to



Scheme 3. (from A Lapolla, D. Fedele, R. Seraglia and P. Traldi, "Mass Spectrometry in the Study of Advanced Glycation Processes, Responsible for Long Term Diabetes Complications", 1991. Reprinted by permission of John Wiley and Sons, Ltd)

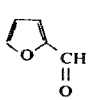
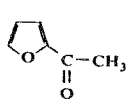
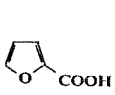
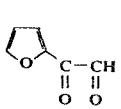
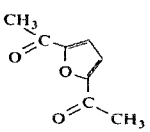
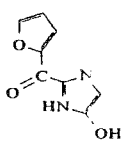
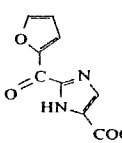
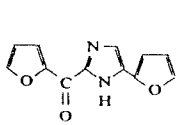
6.1×10^4 pg, MS/MS resulted a more than suitable analytical method for FFI determination.

Such a judgment seemed to be not valid when, instead of *in vitro* samples, *in vivo* glycated substrates were analyzed by MS/MS.

RIA data were in agreement with the fluorescence ones in determining high FFI levels in globine and in glycated proteins. Hence it was surprising how, by MS/MS, no FFI was evidenced in *in vivo* glycated samples. In an investigation on collagen of diabetic rats, while fluorescence and absorbance data were typical for the AGE presence, by MS/MS was impossible to detect any presence of FFI, also if, by spiking, the sensitivity was proved to be analogous to that obtained in *in vitro* samples. Looking at the high specificity of MS/MS, the obtained results could be explained not on the basis of the unvalidity of the analytical procedure, but for the real absence of FFI among the hydrolysis products of glycated collagen (Lapolla et al., 1990a).

In the same period FFI was proved to be an artifact, originating from the NH_4OH neutralization after HCl hydrolysis (Njoroge et al., 1988). In fact, by

Table 1. Precursor ion spectra of furoyl cations (m/z 95) as obtained by B^2/E linked scans[a]

Compounds	Ionic species							
	96	110	112	124	152	178	206	228
HCl hydrolyzed NaOH neutralized glycated albumin		++	++	++	++	++	++	++
Protease hydrolyzed glycated albumin	+	+						
HCl hydrolyzed NaOH neutralized glycated polylysine		++	++	++	++	++	++	++
Protease hydrolyzed glycated polylysine	+	+		+				
Proposed structures								

[a] The ion abundances were calculated comparing the absolute signal obtained introducing comparable amounts of samples and measuring the peak intensities with respect to the most abundant one, kept equal to 100. Relative abundances: $^{+}1-50\%$, $^{++}50-100\%$ (from Gerhardinger C. et al., 1990. Reprinted by permission of Elsevier Science Publishers BV)

using labelled $^{15}NH_4OH$, an increase of FFI molecular weight of 2 Da was found, proving that the imidazole nitrogen atoms do not originate from ε-aminogroups of lysine, but from the neutralization media. The absence of FFI in hydrolysed collagen samples, as determined by MS/MS, is in agreement with such findings and clearly in contrast with the RIA data.

Such a discrepancy could be reasonably explained by a RIA positive response on molecules structurally related to FFI but with different structures and molecular weights. In order to investigate on such hypothesis, further mass spectrometric experiments were carried out. Looking at the fact that the RIA antibody was developed against 4-furanyl-2-furoyl-1H-imidazole-1-hexanoic acid, it was considered of interest to put in evidence, in the hydrolysis mixture, all the molecular species containing the furoyl moiety, which in principle could lead to a positive RIA response.

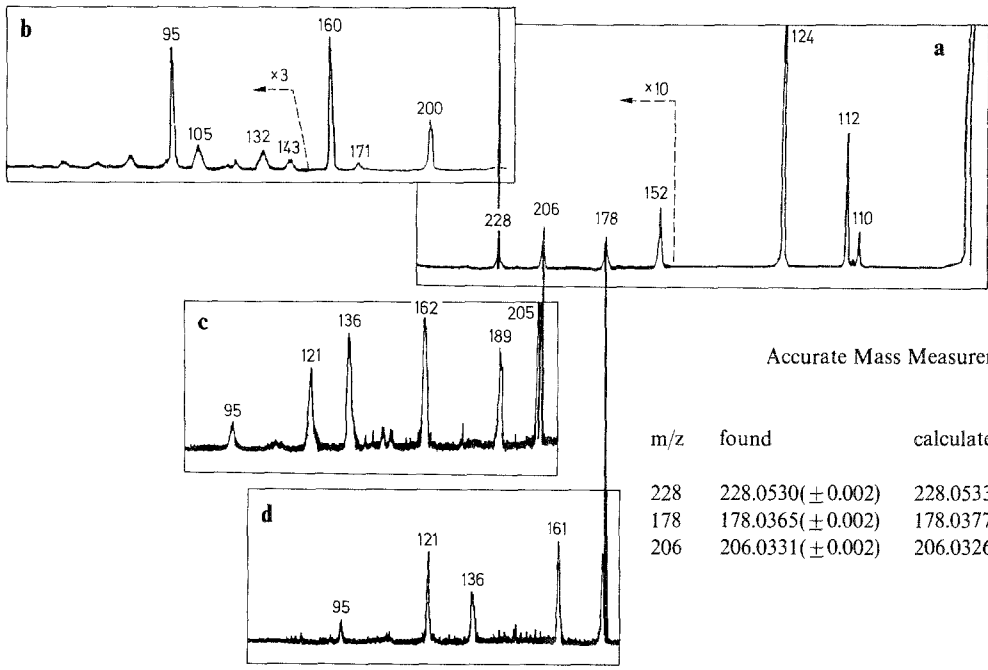
This could be easily obtained by "linked scan" of both magnetic (B) and electrostatic (E) sectors, performed keeping constant the ratio B^2/E (Busch et al., 1988). Operatively, once selected the daughter ion of interest (in this case the furoyl cation at m/z 95), the B^2/E = const linked scan evidences only its precursor ions. For example, by such approach in a hydrolyzed mixture from glycated albumin it was proved that, together with FFI, many other molecules containing the furoyl moiety, namely 2-(2-furoyl)-4-hydroxy-1H-imidazole (m/z 178), 2-(2-furoyl)-4-carboxy-1H-imidazole (m/z 206), furan-2,5-diacetyl (m/z 152), 2-furanglyoxal (m/z 124), 2-acetylfuran (m/z 110), and 2-furanaldehyde (m/z 96), were present (see Table 1 and Fig. 3) (Lapolla et al., 1989; Gerhardinger et al., 1990).

The same method was applied for the identification of furoyl-containing advanced glycation products in collagen samples from diabetic and healthy rats, by both acid and enzymatic hydrolysis of the proteinic substrate (Lapolla et al., 1990b). The results so obtained are summarized in Table 2. No great differences were found among the different samples, showing the presence of the same molecular species with differences only in relative abundances. 2-Furanaldehyde (responsible for the peak at m/z 96), 2 acetylfuran (m/z 110) and furanglyoxal (m/z 124) were already found in *in vitro* glycation of albumin and polylysine (Lapolla et al., 1989; Gerhardinger et al., 1990). In particular the presence of furanglyoxal is significant, being proposed by Njoroge et al. (1988) as intermediate in the FFI synthesis, before the reaction with ammonia. These molecular species were found in larger amounts in the samples originating from HCl hydrolysis, and this led to the conclusion that acidification and further neutralization are important for their production.

The molecular species at m/z 150 and 154 were not previously found among the hydrolysis products of *in vitro* glycated albumin (Lapolla et al., 1989; Gerhardinger et al., 1990). Further mass spectrometric measurement devoted to their structural identification (accurate mass measurements and daughter ion spectroscopy) were successful only for the latter species, indicating for it the structure of 2-carboxy-2-acetylfurane.

In conclusion, the data obtained by the above described investigations led to the following considerations:

i) glycated and not glycated hydrolyzed collagen samples contain the same furoyl-containing compounds;



Accurate Mass Measurements

m/z	found	calculated	elemental formula
228	228.0530(±0.002)	228.0533	$C_{12}H_8O_3N_2$
178	178.0365(±0.002)	178.0377	$C_8H_6O_3N_2$
206	206.0331(±0.002)	206.0326	$C_9H_6O_4N_2$

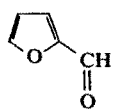
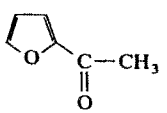
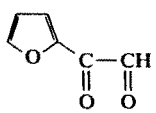
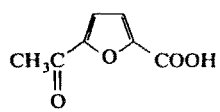
Fig. 3. a Parent ion spectrum (linked scans at constant B^2/E) of ionic species at m/z 95 generated by EI of glycated and HCl-hydrolyzed albumin; **b** CAD MIKE spectrum of the ion at m/z 228; **c** CAD MIKE spectrum of the ion at m/z 206; **d** CAD MIKE spectrum of the ion at m/z 178; and accurate mass values of the same ionic species (from A Lapolla, D. Fedele, R. Seraglia and P. Traldi, "Mass Spectrometry in the Study of Advanced Glycation Processes, Responsible for Long Term Diabetes Complications", 1991. Reprinted by permission of John Wiley and Sons, Ltd)

ii) from a qualitative point of view, hydrolysis does not affect the production of such compounds, and it only slightly affects their relative abundance.

The mixtures arising from hydrolysis of proteins and glycated proteins are usually particularly complex and in order to obtain a their valid description, further investigations by HPLC and HPLC/MS techniques were carried out. The first study on this topic was devoted to the identification of the products arising from enzymatic digestion of advanced glycated albumin (Lapolla et al., 1991a). In Fig. 4 the HPLC chromatograms with UV detection (320 nm) of hydrolysed albumin (up) and hydrolysed glycated albumin (down) are reported. Their comparison shows that the glycated protein leads to a complex mixture, with a clear increase of the abundance of compounds with retention times between 11 and 18 min. Such molecules must necessarily contain a chromophoric moiety responsible for the absorption at 320 nm. However these chromatograms cannot exclude the presence of other compounds which do not contain such chromphores in their structure. This limitation of UV detection can be effectively overcome by using a HPLC/MS system.

The measurements performed by this approach, using a plasmaspray interface, led to the results reported in Fig. 5. The chromatograms strongly differ from those obtained by UV detection, proving the high effectiveness of MS as chromatographic detector. The mass spectra of the major component of the hydrolysed mixture arising from glycated albumin led to the identification of the

Table 2. Ionic species detected by parent ion scans (B^2/E = const) on furoyl cation (m/z 95) performed on the compounds under study

	Ionic species				
Compounds	96	110	124	150	154
HCl hydrolyzed normal collagen	+ +	+ +	+	+	
Proteinase hydrolyzed normal collagen	+	+	+	+	
HCl hydrolyzed diabetic collagen	+ +	+ +	+ +	+	
Proteinase hydrolyzed diabetic collagen	+	+	+ +	+	
Collagen incubated with 0.5 g glucose and HCl hydrolyzed	+	+ +	+	+	
Collagen incubated with 10 g glucose and HCl hydrolyzed	+	+ +	+	+	
Collagen incubated with 0.5 g glucose and proteinase hydrolyzed	+	+ +	+	+	
Collagen incubated with 10 g glucose and proteinase hydrolyzed	+	+ +	+ +	+	+
Structures assigned on the basis of accurate mass measurements and daughter ion spectroscopy				??	

The ion abundance was calculated by comparing the absolute signal obtained introducing a comparable amount of samples and measuring the peak intensities with respect to the most abundant one, kept equal to 100. Relative abundance, + = $(1-5)\cdot 10^{-8}$ ampères; + + = $(5-10)\cdot 10^{-8}$, ampères (from Lapolla A. et al., 1990b. Reprinted by permission of Elsevier Science Publishers BV)

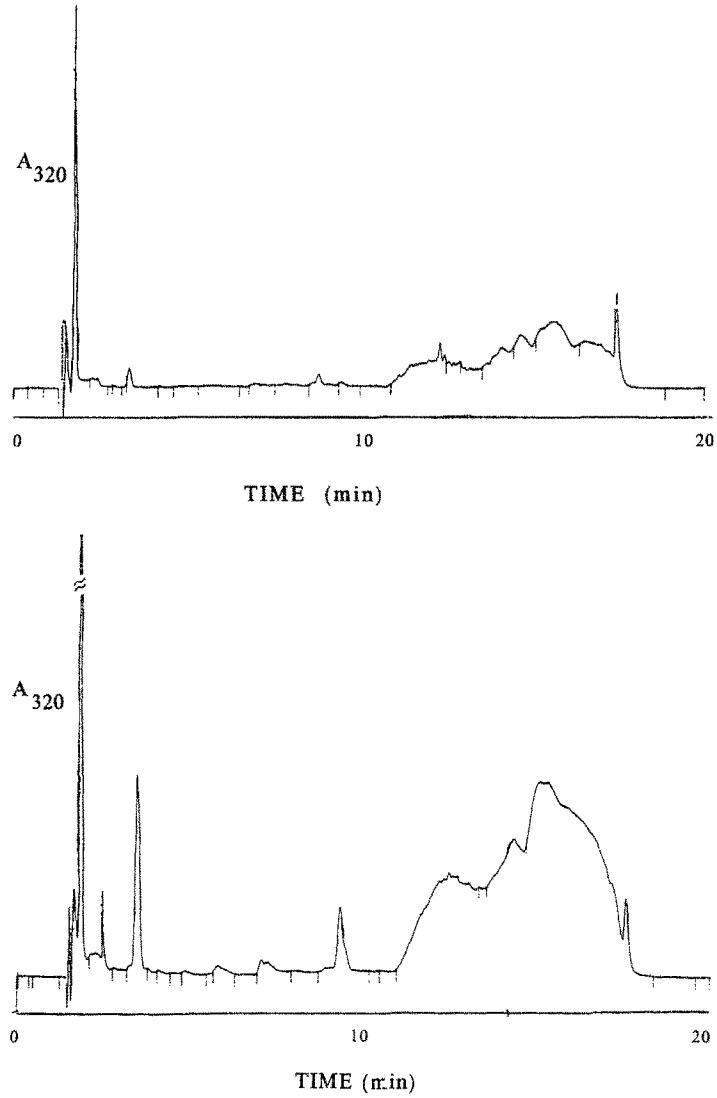


Fig. 4. HPLC chromatogram obtained by detection at 320 nm of reaction mixtures: – protease hydrolyzed albumin (up); – protease hydrolyzed glycated albumin (down) (from Lapolla A. et al, 1991a. Reprinted by permission of John Wiley and Sons, Ltd)

structures reported in Table 3. In particular structure 1 can be proposed for ionic species at m/z 194: it can arise from the condensation reaction between ε-aminogroup of lysine and glucose, followed by dehydration and oxidation processes. Ionic species at m/z 206 could correspond to structure 2: an analogous compound was found by Olsson et al. (1977) in the reaction mixture of glucose and methylamine. For the ion at m/z 132 a lactone structure, that could arise from an α-diketo cleavage from a 3-deoxyosone, could be proposed (Sengl, 1988). Two different structures (4 and 5), both originating from dehydration and further decomposition of 3-deoxyosone, could be assigned for ionic species at m/z 144 (Ledl et al., 1986; Hodge, 1955). Also for ionic species at m/z 233 two different structures (6 and 7) both originating from a dimerization of 2-hydroxymethyl-

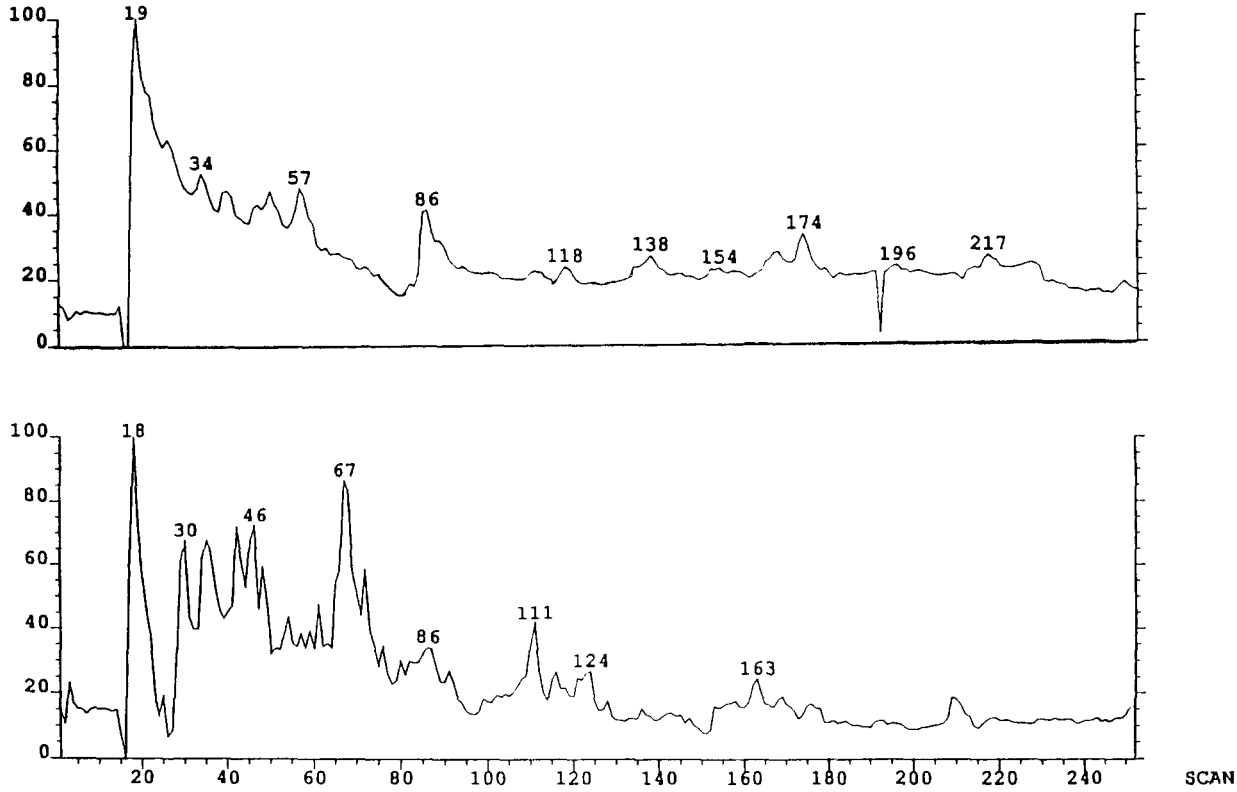


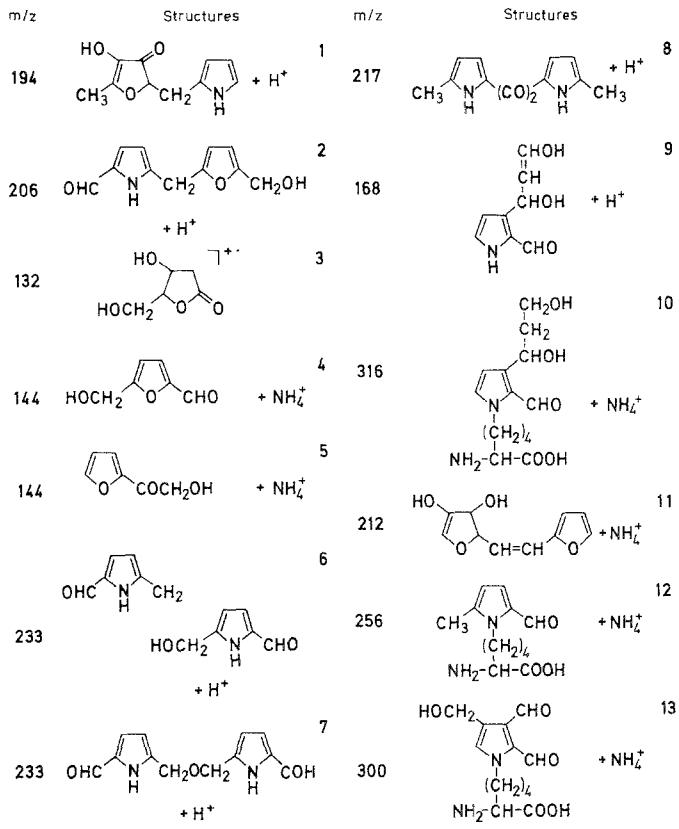
Fig. 5. Reconstructed ion chromatogram of HPLC/MS run of reaction mixtures: – protease hydrolyzed albumin (up); – protease hydrolyzed glycated albumin (down) (from Lapolla A. et al, 1991a. Reprinted by permission of John Wiley and Sons, Ltd)

pyrrolaldheyde, as suggested by Olsson et al. (1977), could be assigned. A dimerization of methyl-pyrrolaldehyde, a product of Strecker degradation, could determine the formation of structure 8 (m/z 217). The structures 9 and 10 could be attributed to ions at m/z 168 and 316 respectively, as originating from the reaction between an aminoketose with 3-deoxyosone, as previously described (Njoroge et al., 1987). A yellow compound, just described by Ledl et al. (1986) as derived from the condensation of 1-deoxyosone with carbonyl compounds, could correspond to ionic species at m/z 212. Finally for ionic species at m/z 256 and 300 the structures 12 and 13 could be assigned: these pyrroles could arise from 3-deoxyosone via a Strecker degradation (Njoroge et al., 1987). Such structures must be considered propositive, being obtained just on the basis of molecular weight and literature data.

Alternatively to hydrolysis procedures, experiments based on the controlled thermal degradation (pyrolysis) of glycated and not glycated polypeptidic molecules were carried out. As analytical method devoted to the identification of the pyrolysis products, GC/MS was choosen.

Firstly, in order to study a simple substrate, the pyrolysis of polylysine and glycated polylysine were studied (Lapolla et al., 1992a). In Fig. 6 the gas chromatograms of the related pyrolysis products are reported. Clear differences are

Table 3. Possible structures identified by HPLC/MS



(from Lapolla A. et al., 1991a. Reprinted by permission of John Wiley and Sons, Ltd)

evidenced, with the presence of more numerous products in the case of glycated polylysine. Such compounds must necessarily arise from the interaction between polylysine and glucose. In Table 4 the structural assignment, achieved on the basis of EI mass spectra of the various components, is resumed. The structure of propylacetate (m/z 102) could be attributed to compound 1, even if with a low fit value (719). Compound 2 (m/z 96), by library search was identified as furan-2-aldehyde. Compound 3 (m/z 110), the most abundant among the pyrolysis product arising from interaction between glucose and polylysine, was identified as 5-methylfuran-2-aldehyde. Then the structures of 2-methyl-4, 5-dihydrofuran and 5-hydroxymethylfuran-2-aldehyde could be assigned to the components 4 (m/z 84) and 5 (m/z 126) respectively. These furane derivatives have been already reported among the acid and enzymatic hydrolysis products of glycated proteins (Lapolla et al., 1991a, Ledl et al., 1986) and among the pyrolysis products of protein polysaccaride mixtures (Snyder et al., 1988). Finally the structure of 2,3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one (m/z 144), could be assigned to compound 6 even if with a relative low fit value (823): this compound was already described among the reaction products between protected lysine and glucose (Lapolla et al., 1991b).

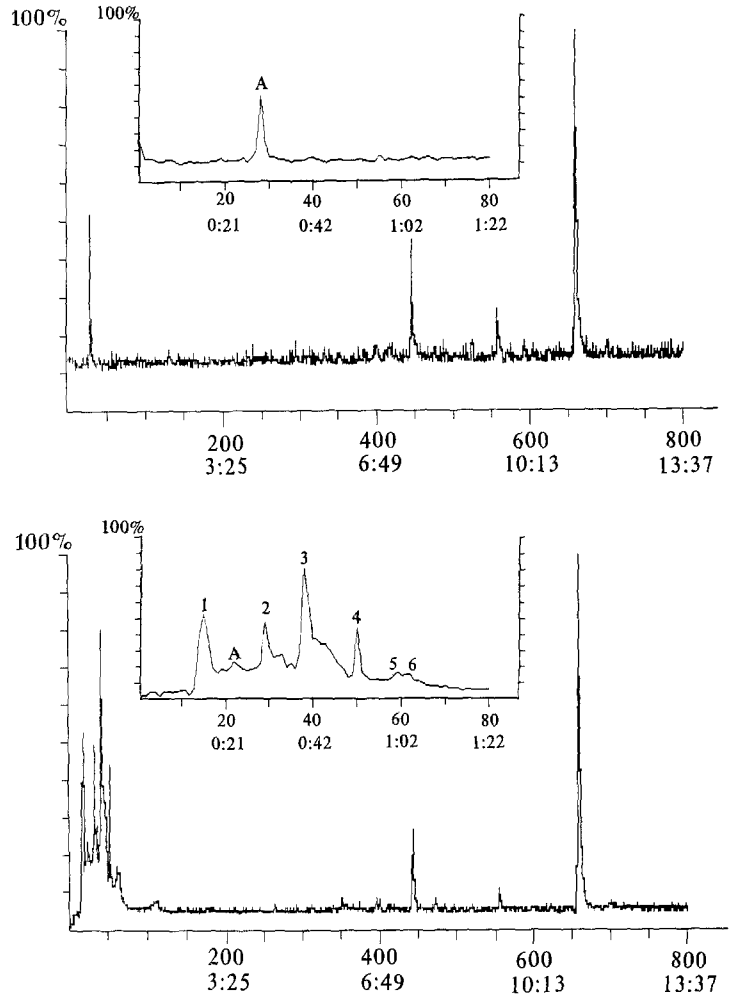
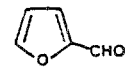
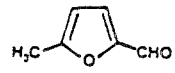
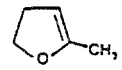
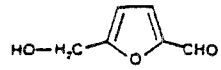
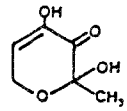


Fig. 6. Reconstructed total ion chromatogram from pyrolysis/GC/MS of – control poly-L-lysine (up); – glycated poly-L-lysine (down) (from Lapolla A. et al, 1992a. Reprinted by permission of John Wiley and Sons, Ltd)

The same approach, when employed in the comparison of glycated and not glycated albumin (Lapolla et al., 1992b), led to the chromatograms reported in Fig. 7. Again clear differences are present between the glycated and not glycated substrates, but, quite surprisingly, the molecular species characteristic for glycated polylysine are in the present case of very poor abundance, proving that the complexity of the proteinic substrate leads to a more complicated thermal decomposition pattern.

However, diagnostic components for the glycation process are still present (see Table 5), proving that the Pyr/GC/MS approach can be validly employed for the evaluation of glycation levels. In fact, among the pyrolysis products identified by the mass spectra library search, those containing furane and pyrrole moieties, i.e. compounds 2, 4, 5, 6, 9, 11, 13, 14, are of interest, because often described as arising from glycation processes (Ledl et al., 1986; Paulsen and Pflughaupt, 1980).

Table 4. Retention times, relative molecular masses (RMM), molecular structures and fit values for compounds 1–6

Compounds	Retention time (s)	RMM	Structure	Fit value
1	15	102	$CH_3\overset{\overset{O}{\|}}{C}CH_2CH_2CH_3$	719
2	33	96		800
3	36	110		790
4	49	84		837
5	59	126		908
6	62	144		823

(from Lapolla A. et al., 1992a. Reprinted by permission of John Wiley and Sons, Ltd)

Direct analysis of glycated proteins

From the early days of the investigation on glycated proteins, only spectroscopical methods (i.e. fluorescence and absorbance spectroscopies) were employed on the untreated proteins.

Such methods result effective for an evaluation of glycation levels, but they lack of the specificity necessary to give any structural information on the glycation products. As discussed in the introduction section, this was the main reason for the employement of hydrolysis procedures, thus to investigate on smaller glycated moieties by different and more specific spectroscopical methods.

As shown above, mass spectrometry, and in particular MS/MS, demonstrated to be highly valid in this contest, but neither EI nor FAB MS were able to give any direct information on the whole glycated protein.

Recently new ionisation techniques have become available for the analysis of intact macromolecules, i.e. electrospray (ES) (Smith et al., 1990) and matrix assisted laser desorption/ionisation (MALDI) methods (Karas et al., 1985). The first is based on the deposition, on the macromolecule under study, of a large number of positive charges. Usually a mass analyser (employing magnetic sectors or quadrupolar fields) separates the ions with respect to their mass-to-charge ratio. Hence by electrospray, also if the mass analyser has an its own mass range of one or few KDa, the deposition of *n* charges on the macromolecule

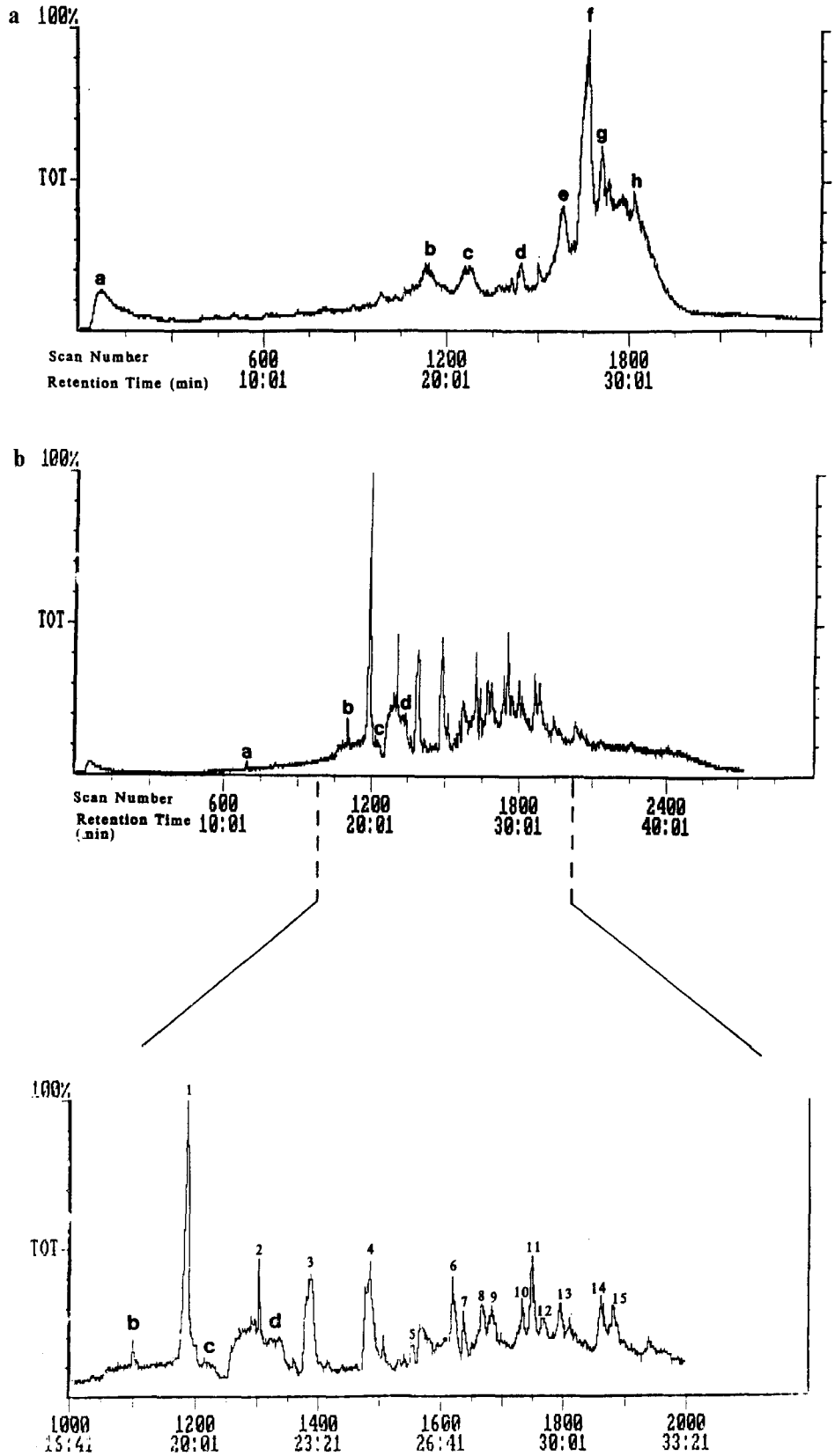
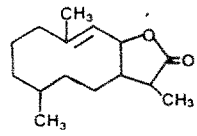
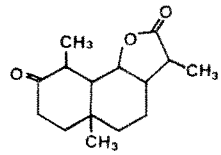
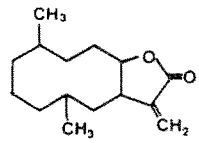
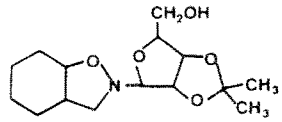
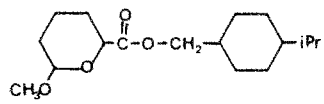


Fig. 7. Reconstructed total ion chromatogram of pyrolysis/GC/MS runs of: **a** 0.3 mg of bovine serum albumin; **b** 0.3 mg of glycated bovine serum albumin (from Lapolla A. et al, 1992b. Reprinted by permission of Elsevier Science Publishers BV)

Table 5. 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 (from Lapolla A. et al., 1992b. Reprinted by permission of Elsevier Science Publishers BV)

allows its employment in a virtual mass range increased *n* times. MALDI is based on the laser induced desorption/ionisation of the compound of interest when it is mixed with a suitable matrix. Such matrix works as intermediate in the ionisation of the macromolecule. Being the ionisation by laser pulse a particular fast phenomenon ($\sim 10^{-3}$–10^{-4} s) the employment of a fast mass analyser is needed, and in this respect time-of-flight devices result the most effective.

Table 6. Fluorescence and absorbance data of samples 1–5. Fluorescence is expressed in emission unit (EU)/mg protein; absorbance is expressed in absorbance (ABS)/mg protein

Samples	1		2		3		4		5	
Time (days)	EU	ABS	EU	ABS	EU	ABS	EU	ABS	EU	ABS
0	13.6	0.10	25.2	0.09	22.4	0.10	20.4	0.11	27.5	0.09
7	12.8	0.08	40	0.08	108	0.12	168	0.20	307	0.30
14	28	0.08	69.2	0.10	312	0.12	440	0.26	514	0.47
21	40	0.10	80	0.12	468	0.17	828	0.34	895	0.58
28	50	0.12	100	0.14	820	0.25	1104	0.54	1180	0.80

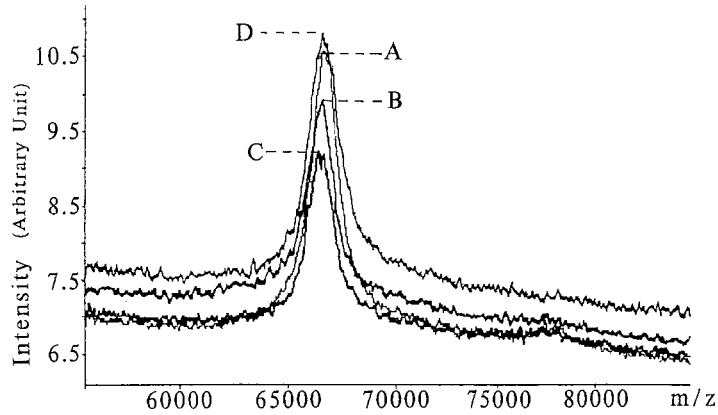


Fig. 8. MALDI spectra of BSA incubated with phosphate buffer without glucose (pH 7.5; 37°C) (samples 1) recorded at different incubation times: **A** Incubation time = 0 days (Molecular weight = 66429 Da); **B** Incubation time = 7 days (Molecular weight = 66449 Da); **C** Incubation time = 14 days (Molecular weight = 66434 Da); **D** Incubation time = 28 days (Molecular weight = 66464 Da)

Both these techniques have been successfully applied in the field of protein chemistry and for such reason the application of MALDI in the investigation of glycation processes was thought of interest. Until now the only data published on this argument pertain the study of *in vitro* glycation of albumin. In this work a parallel investigation on the basis of fluorescence and MALDI data was carried out (Lapolla sub. Biochem Biophys Acta). Incubating a sample of albumin without any glucose (samples 1 of Table 6) (1.5 g bovine serum albumin in sodium phosphate buffer 0.05 M at pH 7.5 at 37°C from 0 to 28 days), an increase in fluorescence is observed, while no increase in molecular weight is detected (see Table 6 and Fig. 8). Such a discrepancy can be explained by considering that the fluorescence data originate by intramolecular reactions of the protein without any detectable release of chemical moieties. In other words the practical constancy of molecular weight with respect to incubation time demonstrates that the elemental formula of BSA remains necessarily the same and that some structural modifications due to rearrangement processes are the responsible for the fluorescence data. Another possibility could be related to the production of particularly small amounts of different products not detectable by MALDI but, possibly due to their optical properties, easy detectable by fluorescence methods.

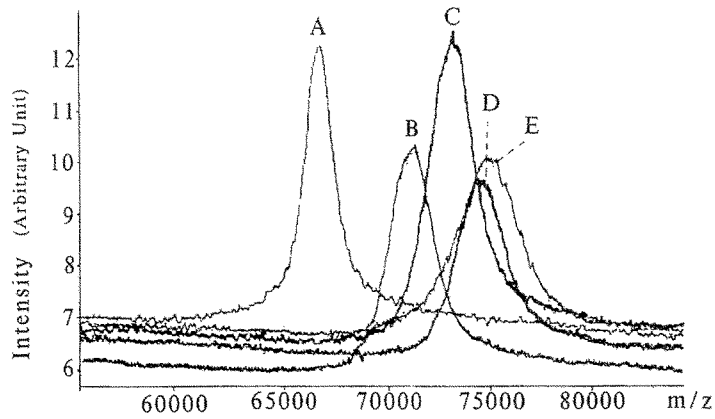


Fig. 9. MALDI spectra of BSA incubated with glucose at 2 M (pH 7.5; 37°C) (samples 4) recorded at different incubation times: **A** Incubation time = 0 days (Molecular weight = 66429 Da); **B** Incubation time = 7 days (Molecular weight = 71103 Da); **C** Incubation time = 14 days (Molecular weight = 73099 Da); **D** Incubation time = 21 days (Molecular weight = 74279 Da); **E** Incubation time = 28 days (Molecular weight = 74682 Da)

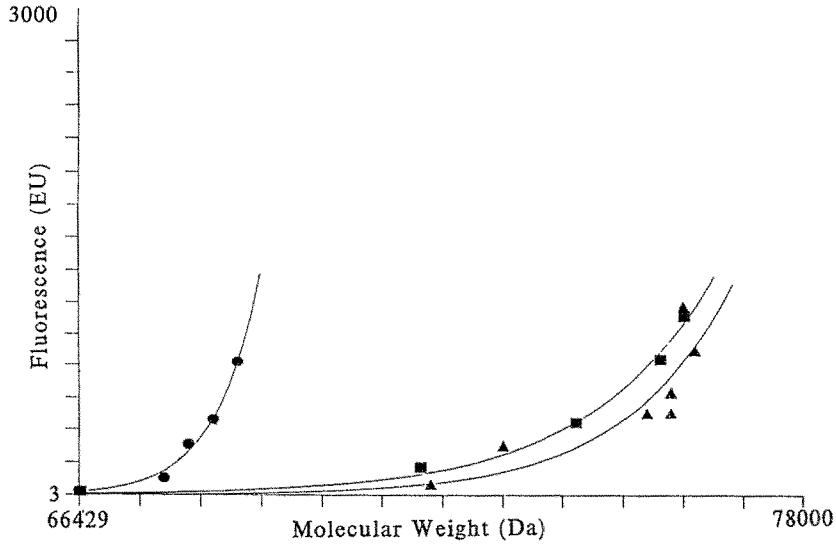


Fig. 10. Plots of fluorescence (Emission unit/mg protein) *vs* molecular weight (Da) related to BSA incubated with glucose at different concentration (pH 7.5; 37°C): – ● glucose concentration 0.2 M (samples 3); – ■ glucose concentration 2 M (samples 4); – ▲ glucose concentration 5 M (samples 5)

In presence of glucose (1.5 g BSA, glucose 2 M, in sodium phosphate buffer 0.05 M at pH 7.5 at 37°C from 0 to 28 days) (samples 4 of Table 6) a clear increase in molecular weight with respect to incubation time is observed (see Fig. 9) proving that in such conditions condensation reactions of glucose on BSA take place. The yields in glycated products are influenced, as to be expected, by both glucose concentration and incubation time. An increment of molecular weight corresponding to a condensation of about 52 glucose units on BSA was detected.

The plot of fluorescence data *vs* molecular weights obtained by MALDI gives rise to a trend (see Fig. 10) analogous to that obtained by Odetti et al. (1990)

relating the fluorescence data *vs* age of diabetic rats. Such exponential trends were rationalized by considering how many factors, among which chemical degradation of glycated proteins and unknown accelerating factors, can contribute to the fluorescence response.

The data obtained by MALDI investigation suggest that, after the initial glucose addition to the ε-aminogroups, some further reactions must take place leading to the formation of more fluorescent moieties. Such reactions could be, in principle, either intramolecular or due to the reactivity of modified condensed glucose moieties *vs* free glucose.

Studies on the reaction between protected lysine and glucose

Alternatively to the studies above described on the glycation products of proteins and mainly devoted to the identification of AGEs, in order to gain general information on the intimate mechanisms of the reaction between ε-amino group of lysine and glucose, it was thought of interest to undertake a study on the reaction between protected lysine and glucose. Such approach would in principle give rise to an easier description of the final products as well as of possible

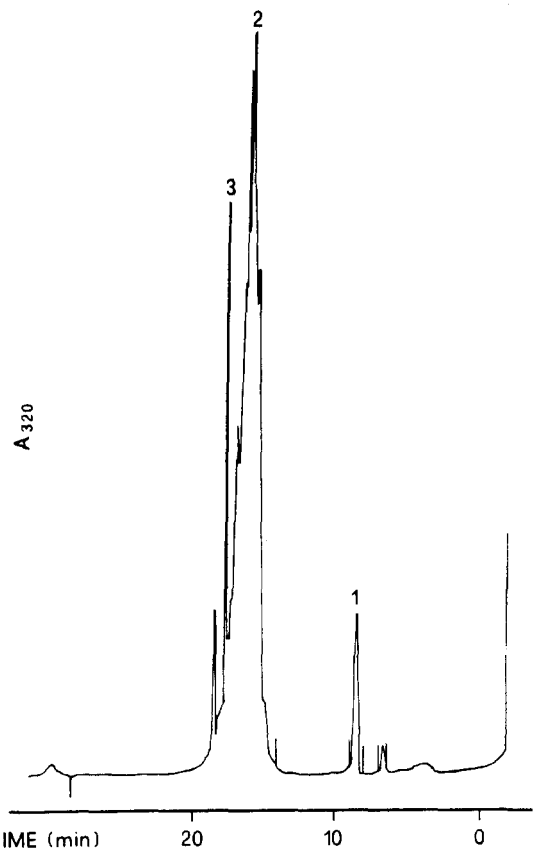


Fig. 11. HPLC chromatogram of the reaction mixture of protected lysine and glucose, obtained by detection at 320 nm (from Lapolla A. et al, 1991b, with kind permission from Pergamon Press Ltd, Headington Hill Hall, Oxford OX3 OBW, UK)

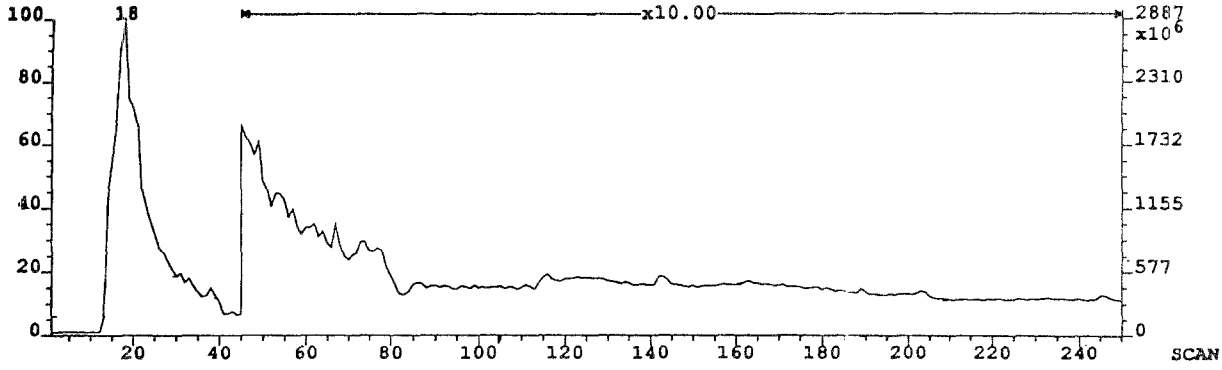


Fig. 12. Reconstructed ion chromatogram for HPLC/MS analysis of the reaction mixture of protected lysine and glucose under plasma-spray conditions (from Lapolla A. et al, 1991b, with kind permission of Pergamon Press Ltd, Headington Hill Hall, Oxford OX3 OBW, UK)

intermediates of the glycation reactions; protected lysine should in principle mimick the lysine contained in proteinic chain, without all the problems related to the protein degradation.

In a first study (Lapolla et al., 1991b) *N*-α acetyl-L-lysine methylester, prepared and purified according to Irwine and Gutman (1959), was employed; 100 mg of it were incubated with D glucose (5 g) in 5 ml of sodium phosphate buffer (pH 7.5, 0.05 M Na) for 28 days at 37°C and then lyophilized. The HPLC chromatogram (UV detection) of the reaction mixture after 28 days is reported in Fig. 11: three main peaks are present with retention times of 6, 11 and 12 min respectively. The same sample, when analyzed by HPLC/MS, gave rise to the chromatogram shown in Fig. 12. As it can be seen, instead of the well separated components obtained by UV detection at 320 nm, a practical continuous signal of total ion current with a maximum at 10 min, slowly decreasing, is present. The direct analysis by FAB of the whole mixture gave a spectrum clearly different from that of the pure protected lysine (see Fig. 13); the $[M + H]^+$ ion of protected lysine at m/z 203 is still present but with a low abundance. In the reaction mixture ions at m/z 285 and 263 are well detectable and, on the basis of their collisional spectra, structures 1 and 2 were respectively assigned (Scheme 4). The spectra obtained by HPLC/MS allowed to identify further possible molecular species at m/z 342, 326, 324, 270, 222 and 162, whose structure assignement is reported in Table 7. Furthermore, the presence of ions at m/z 189 demonstrates that a partial hydrolysis of the protected lysine takes place.

For such reasons other investigation were performed using *N*-α-*p*-tosyl-lysine methylester hydrochloride; looking at the complexity of the reaction mixture, different separative and spectroscopical techniques were employed (Lapolla sub. Amino Acids). 5 g of *N*-α-*p*-tosyl-lysine methylester hydrochloride were incubated with 2.57 g of anhydrous D-glucose in 6 ml of distilled water. The solution, kept at pH 7.2 was mantained at 37°C for 10 days under darkness. The free glucose was eliminated by an Extrelut cartridge and the aqueous solution was eluted with CH_2Cl_2. Different fractions were obtained by silica gel column

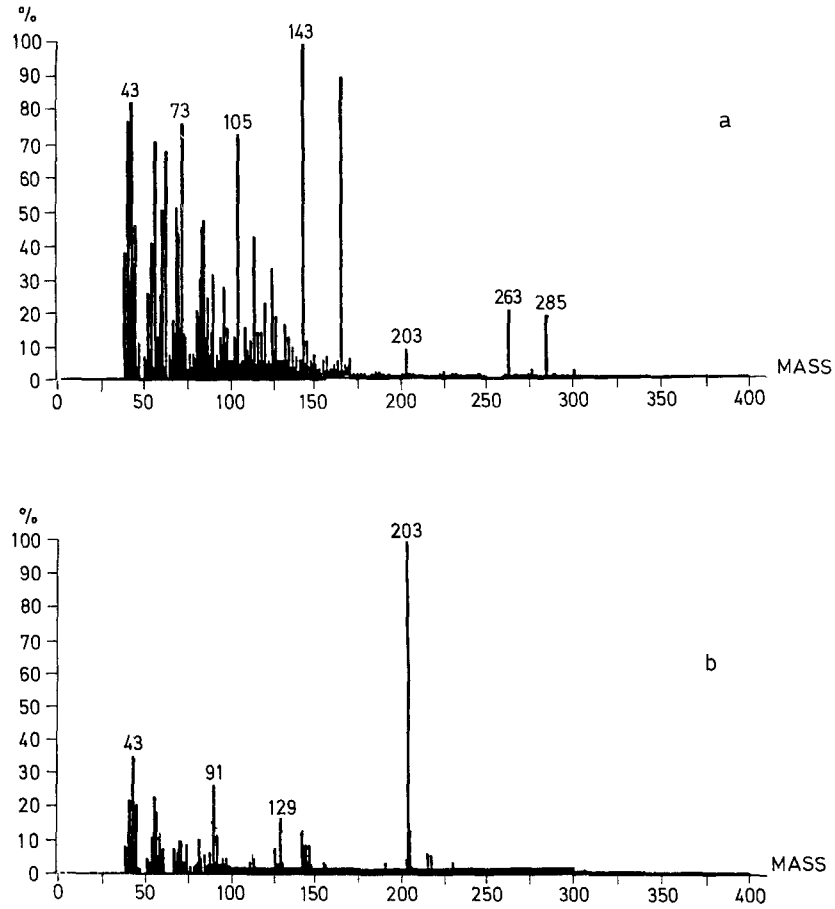
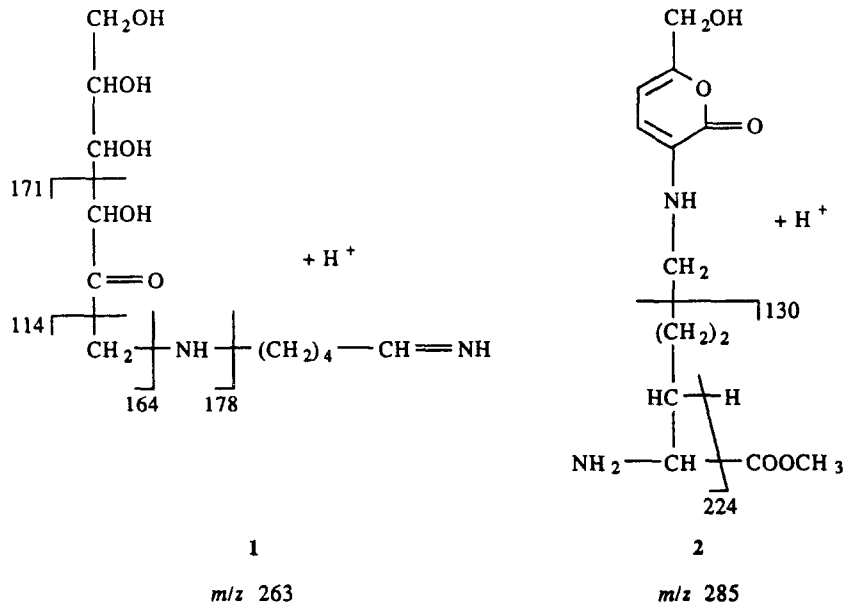


Fig. 13. FAB mass spectra of: **a** reaction mixture of protected lysine and glucose; **b** α-protected lysine (from Lapolla A. et al, 1991b, with kind permission of Pergamon Press Ltd, Headington Hill Hall, Oxford OX3 OBW, UK)



1

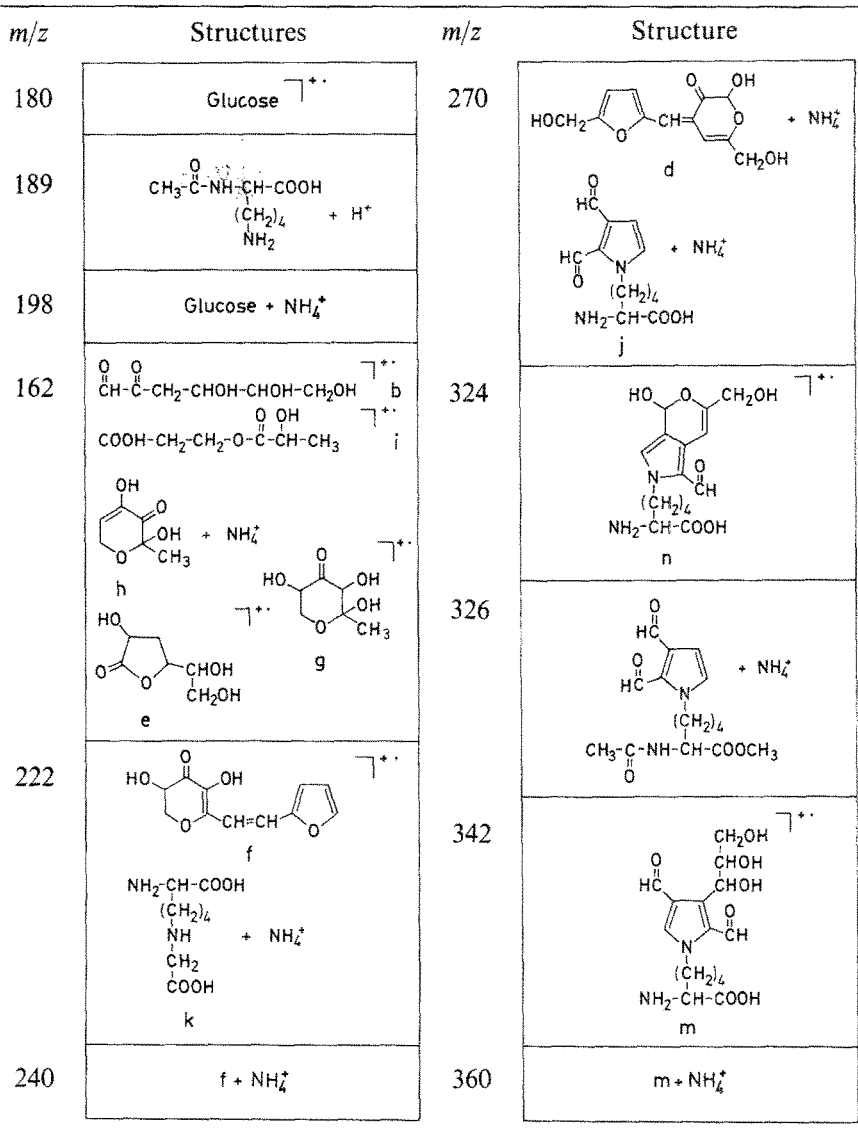
m/z 263

2

m/z 285

Scheme 4

Table 7. Possible structure detected by plasma-spray HPLC/MS



(from Lapolla A. et al., 1991b with kind permission of Pergamon Press Ltd, Headington Hill Hall. Oxford OX3 OBW, UK)

chromatography. Thus, by eluting with H_2O, fraction A was obtained, while eluting firstly with CH_2Cl_2 the colorless fraction 1 was separated. Successively two different fractions were obtained with methanol: the brownish fraction 2 and the yellow fraction 3. The Extrelut cartridge was furtherly eluted with CH_2Cl_2 obtaining the lightly yellow fraction 4.

All the fractions were investigated by mass spectrometry and ^1H and ^{13}C nuclear magnetic resonance spectroscopy. A portion of the reaction mixture just after glucose purification was analysed by gel permeation chromatography (GPC) in order to obtain reliable information on the molecular weight distribu-

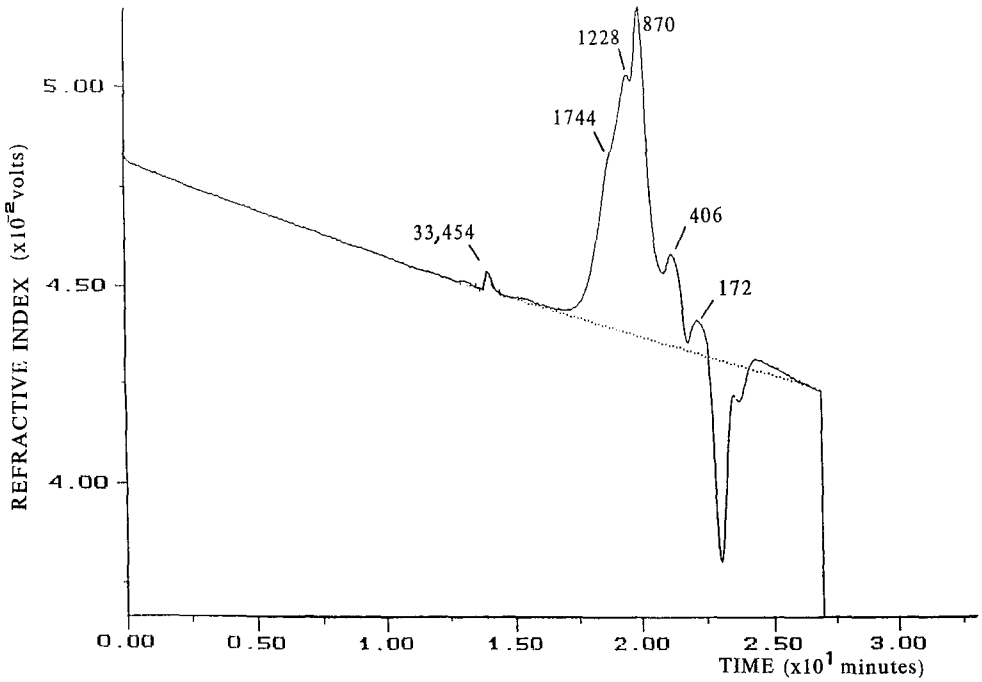


Fig. 14. Gel permeation chromatogram of the whole reaction mixture obtained by 10 days incubation of protected lysine and glucose (pH 7.2; 37°C)

tion of the reaction products. The results so obtained are shown in Fig. 14. A major peak with components corresponding to mean molecular weight of 870, 1228 and 1744 Da is evidenced. Other quite abundant components of the reaction mixture have mean molecular weights of 172 and 406, while a minor (but significant) component corresponds to a mean molecular weight of 33454 Da, indicating the occurrence of extensive polimerization processes.

This first screening allowed an estimation of the real complexity of the reaction mixture and consequently the use of column chromatography was retained essential to obtain fractions with a more limitate number of components.

The ethereal fraction A and the four fractions 1–4 were analysed by mass spectrometry and ^{1}H- and ^{13}C-NMR spectroscopy. While electron impact mass spectrometry did not lead to any significative result, possibly due to the low volatility of the reaction products, FAB mass spectrometry gave interesting analytical information. The FAB mass spectrum of the fraction A obtained by eluting with diethyl-ether (to separate lower molecular weight organic products) evidenced the presence of an highly abundant ion at m/z 223 (see Fig. 15). The ions at m/z 337, 315, 283 and 238 present in the same spectrum do not have any analytical value, being originated from protected lysine. Metastable ion studies performed on the ion at m/z 223 led to the assignement of structure **b** of Fig. 16, already proposed by Ledl et al. (1990).

The FAB mass spectrum of fraction 1 showed the presence of highly abundant ions at m/z 191, 219, 315 and 531 with relative abundances of 13, 40, 25, and 8% respectively.

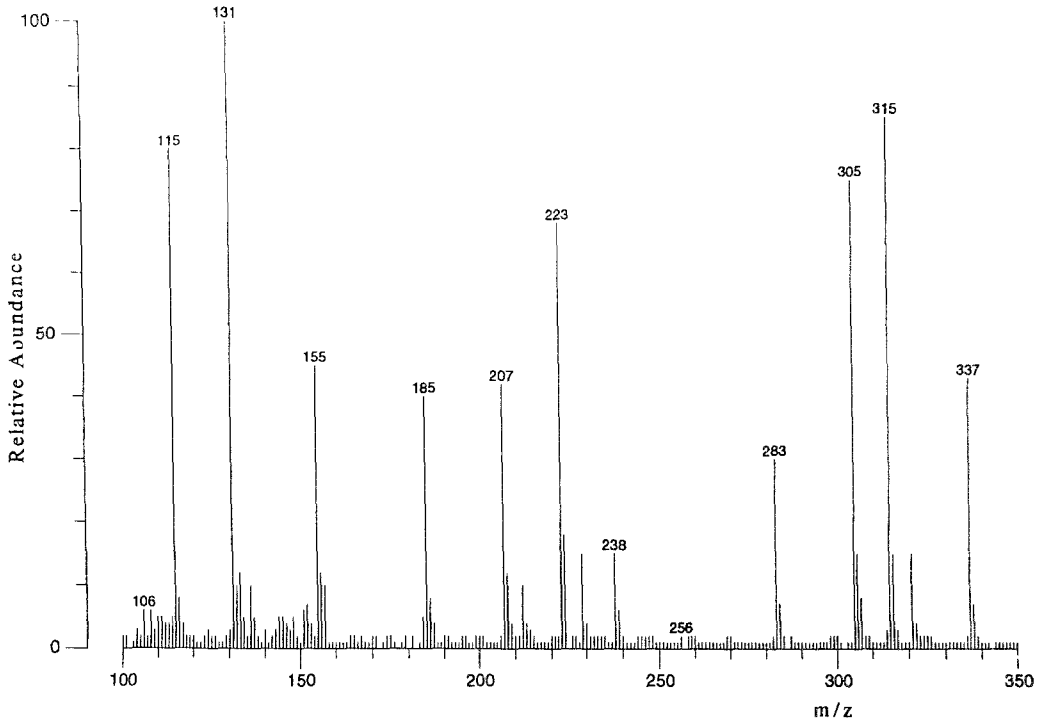
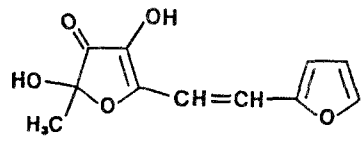


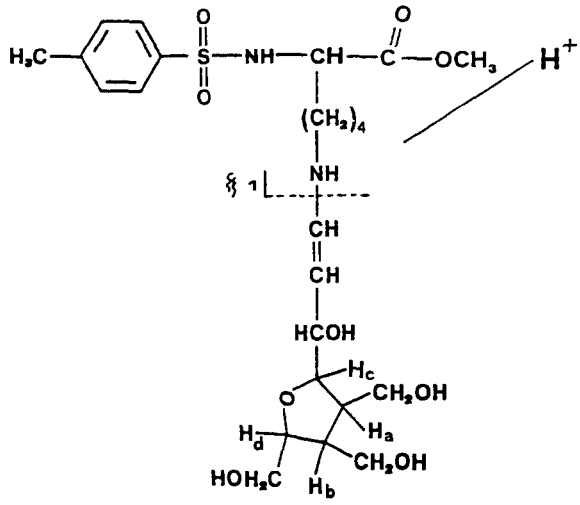
Fig. 15. FAB mass spectrum of fraction A



b

Fig. 16. Structure of the species with molecular weight of 222 Da present in the FAB spectrum of fraction A

The molecular weight of 530 Da cannot be justified by the simple condensation of a glucose molecule on the protected lysine. In such case a molecular weight of 476 Da should be obtained. Hence the detected ionic species must necessarily originate by the reaction of two glucose units with the protected lysine and further skeletal rearrangement, leading to oxigen containing heterocycles, always invoked in glycation processes. Either the fragment ions detected in the FAB spectrum or the MIKE data (showing the loss of CH_3· and the formation of the ions at m/z 315 and 222) are in agreement with structure **c** of Fig. 17. The ^{1}H-NMR spectrum of the same fraction shows the signals related to the protected lysine (1.4–1.7 ppm, m, CH_2; 2.45 ppm, s, CH_3; 2.96 ppm, t, CH_2NH_2; 3.44 ppm, s, OCH_3; 3.98 ppm, dd, CH; 7.45–7.79 ppm, m, Ph) together with an AA' BB' system centered at 4.11 ppm and a multiplet centered at 4.91 ppm, fully in agreement with the proposed structure **c**.



C

Fig. 17. Structure of the species with molecular weight of 530 Da present in the FAB spectrum of fraction 1

In the FAB mass spectrum of fraction 2 abundant ions at m/z 459 and 477 are present; on the basis of MIKE data and ^{1}H-NMR spectrum (which does not show the presence of signals attributable to heterocyclic systems) the structure of a condensation product of glucose on the ε-amino group of lysine was assigned. Fractions 3 and 4 led to molecular species at m/z 783 and 1070 respectively; in both cases highly abundant ions at m/z 477 were detected; the two ions at higher mass can be considered originating from the addition to a protected lysine of three glucose units with losses of 4 H_2O (m/z 783) and five glucose units with losses of 8 H_2O (m/z 1070). The related ^{1}H-NMR spectra show a very complex pattern. However integration data are in agreement with the proposed mechanism of multiple glucose addition on a lysine molecule and not with the possible polymerization of the Amadori products. Thus, even if fraction 3 and 4 are yellow (indicating the presence of browning products) the most abundant component in the fraction under study is the Amadori product itself. These features indicate that, under the experimental conditions employed, the late stage of the Maillard reaction is just initiated. Further confirmation of such data were also gained by ^{13}C-NMR.

Forthcoming trends

The studies until now developed in the field of advanced glycation end products and the results obtained by mass spectrometric metodologies have shown, in our opinion, the great potentialities of such technique in this field.

Its possible future use will give an important contribution to the rationalization of Maillard reaction mechanism.

In particular while the extensive use of hyphenated techniques, as HPLC/MS, MS/MS and HPLC/MS/MS, would lead to important structural information on the products arising from hydrolysis (either chemical or enzymatic) of glycated proteins, the employment of the newest desorption/ionization methods, as MALDI and Electrospray, would allow to obtain definitive results on the extention of glycation in the single proteinic system.

The rationalisation of glycation processes by the data coming from the two different approaches would lead to a general overview of the mechanisms responsible for AGE production as well as to precious information on AGE structure: it will be surely a puzzling work, but this makes it more than interesting.

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