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Review

Post-translational non-enzymatic modification of proteins I. Chromatography of marker adducts with special emphasis to glycation reactions

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Abstract

Analytical methods for marker compounds formed during post-translational modifications of proteins are reviewed. Only adducts arising either in vivo or under in vitro conditions simulating the in vivo situations are discussed. All of these compounds stem from either the reaction of free amino groups (i.e., lysine, arginine or N-terminal amino acid). In most cases the reactive counterpart is an aldehydic moiety containing endogenous compound; however, other functional groups containing metabolites are considered as well. The main demand put upon such marker compounds is that they are stable in acid or enzymatic hydrolysis or, alternatively, can be stabilized by simple sample pretreatment (e.g., by reduction). Practically all categories of separation procedures can be applied provided that the chemical characteristics of a particular marker are adequately respected; frequently combination of two different separation procedures based on different principles must be used. Because of the low level of such marker compounds under in vivo conditions, an appropriate sample enrichment step must be involved. Emphasis is put upon the analysis of Amadori products, pentosidine (and pentosidine related compounds), pyrraline, furosine, N-carboxymethylamino acids, amino acid hydantoins and stabilized dicarbonyl intermediates © 1997 Elsevier Science B.V.

Keywords: Reviews; Proteins; Amadori products; Pentosidine; Pyrraline; Furosine; N-Carboxymethylamino acids; Amino acids hydantoins

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1. Introduction

The analytical armamentarium used in the separation of non-enzymatically-modified proteins (free amino group of lysine, arginine or the N-terminal amino acid) by aldehydic sugars (so-called glycation products) involves practically all currently available technologies starting from the low pressure gel permeation procedures (used mainly for preparative purposes) up to modern electrokinetic separations. For these assays (typically glycated haemoglobins) a large number of commercially available kits is available; these kits exploit either some separation procedures (boronate affinity chromatography seems to be quite popular), both chromatographic or electromigration, but there are also direct immunoassays which do not involve any separation step at all (see also review by Frantzen in this Volume).

The question may arise why these post-translationally-modified proteins are so extensively studied today. There are several reasons for that. First, post-translational non-enzymatic modifications obviously affect the biological functions of a number of proteins: they may lead to increased protein insolubilization, its exclusion from normal metabolic pathways or alteration of its physiological function. This appears to be the case of, e.g., glycated crystallins, collagens, lens membrane proteins, enzymes etc. There even may be associates of different proteins creating new chemical entities (lens membrane protein associates with crystallins), they may affect the enzymatic activity or even, in combination with peroxy radicals they may lead to protein cleavage. Most of the currently investigated modifications reflect increased glycemia [1,2].

As indicated already, post-translational modifications mentioned here need not necessarily stem from the reaction of aldehydic sugars only, but they may reflect a reaction of any aldehydic moiety or even another reactive species in the body. The scattered evidence about involvement of lipid peroxidation products, typically malondialdehyde is just an illustrative example [3,4]. Perhaps there are additional, yet unknown non-enzymatic protein modifications occurring in vivo as well.

There are several approaches, all of which involve more-or-less sophisticated separation techniques and their combinations by which post-translational non-enzymatic modifications can be revealed.

First it is possible to study the altered proteins as such. In this respect collagen, eye crystallins, blood proteins of which haemoglobin is the target of many studies and is routinely assayed in diabetology represent the most frequently analyzed proteins.

The other possibility is to look for marker compounds (hydrolytically stable) which may serve as indicators of non-enzymatic protein modifications. From the analyst's point of view the problem here is how to determine those compounds which are in the tissues present nearly always in minute quantities, though their biological effects can be quite profound. Trace concentrations of these acid stable biomarker compounds are often interpreted as evidence of more extensive underlying damage to the protein. Another fact to be taken into account is that there is a large number of compounds arising during post-translational modifications of proteins (typically glycation) most of which are hydrolytically unstable, many of them representing monotopical (non-cross-linking) modifications; some of these adducts can be made resistant to acid hydrolysis by, e.g., sodium borohydride (sodium borotritide) reduction while others may be hydrolytically stable as such. Some of these adducts possess a characteristic luminescence, which can add to selective detection.

In this review we attempt to summarize the current analytical approaches for the so-called hydrolytically stable products of post-translationally-modified pro-

teins (or products that could be made hydrolytically stable by a simple treatment of the parent-modified protein). It must be mentioned that the Amadori compound is not hydrolytically stable, but it is converted to a readily analyzed stable product, furosine, during acid hydrolysis. However, the separation of modified proteins, their polymers arising by post-translational modifications on their large fragments (allowing localization of the modification in the molecule) or peptides reflecting fragmentation of the protein molecule by, e.g., the action of free radicals reaction has been deferred to another contribution.

It should be emphasized, that in this review we limit ourselves to reactions and products which are formed under physiological conditions, i.e., at temperatures about 37°C and pH 7.4, and no attempt has been made to review analogous modifications (typically Maillard reaction products) arising at elevated temperatures which are the domain of food analysis (for a survey of chromatographic analysis of Maillard products see, e.g., the review by Porretta [5]). In this review we pay attention mainly to the recently developed analytical approaches. For separation, characterization and identification of arising compounds high-performance liquid chromatography (HPLC) technologies are frequently used. The fast developing analytical technique of, capillary electrophoresis, is also applicable for the analysis of these compounds (for a survey of separation of Maillard products by capillary electrophoresis see, e.g., Tomlinson et al. [6]).

If one limits oneself to glycation reactions of body proteins only (the most intensively studied post-translational modifications), the reaction scheme, generally accepted today, can be summarized as indicated in Fig. 1; from this scheme the genesis of individual markers becomes obvious. It should be mentioned that the number of compounds arising during glycation in the human body and related Maillard products found in foods is very broad and exceeds the extent of this paper (for details see special reviews, e.g., Ledl and Schleicher [7]).

A flow chart that can be generally applied to the characterization of nearly all post-translationally-modified proteins (though worked out for glycated species only) has been published by Lapolla et al. [8] (Fig. 2).

2. Sample enrichment

Due to the paucity of glucose derived cross-linking elements in most tissue hydrolysates it is frequently necessary to undertake a prefractionation step. In the particular case of collagen this involves also naturally (physiologically) occurring cross-linkings including pyridinoline. It has to be emphasized that the trace enrichment procedures have been so far elaborated only for pentosidine present in the collagen matrix, particularly because, similarly to glycation arising adducts, it is a fluorescent compound.

The first attempt in this respect was reported by Black et al. [9] who used collagen as matrix, pyridinoline as the glycation unrelated cross-link and fibrous cellulose as the pre-concentration sorbent. Later, Sims and Bailey [10] used the same sorbent for pentosidine trace enrichment but, as reported by Avery [11] their procedure is not quite reliable as pentosidine is desorbed to a variable degree by the organic eluent. According to Avery [11] this variability can be controlled, but not eliminated, by keeping the volume of the wash and eluting solvents as small as technically possible. As reported by Takahashi et al. [12] the losses can be kept under 88% which is still quite high.

Sell and Monnier [13] used sulphopropyl Sephadex, while Dyer et al. [19] used C_{18} SepPack cartridge for the prefractionation of pentosidine (called MFP1 at the time when this paper was published).

According to Takahashi et al. [12] 70% recovery of pentosidine and pyridinoline can be obtained from the sulphopropyl prefractionation procedure.

Avery [11] has compared twelve different solid-phase systems for the trace enrichment of pentosidine in collagenous samples. Propylsulphonic acid solid-phase extraction cartridges have proven superior to all other cartridges used (twelve types investigated). There were some minor alterations introduced into the procedure compared to manufacturer's instructions:

The initial cartridge wetting with methanol was modified by incorporating 1% triethylamine in the methanol and increasing the wash volume to ten cartridge volumes. The methanol was displaced with three volumes of 10% aqueous methanol followed by three volumes of 0.1 *M* HCl and finally equilibrated

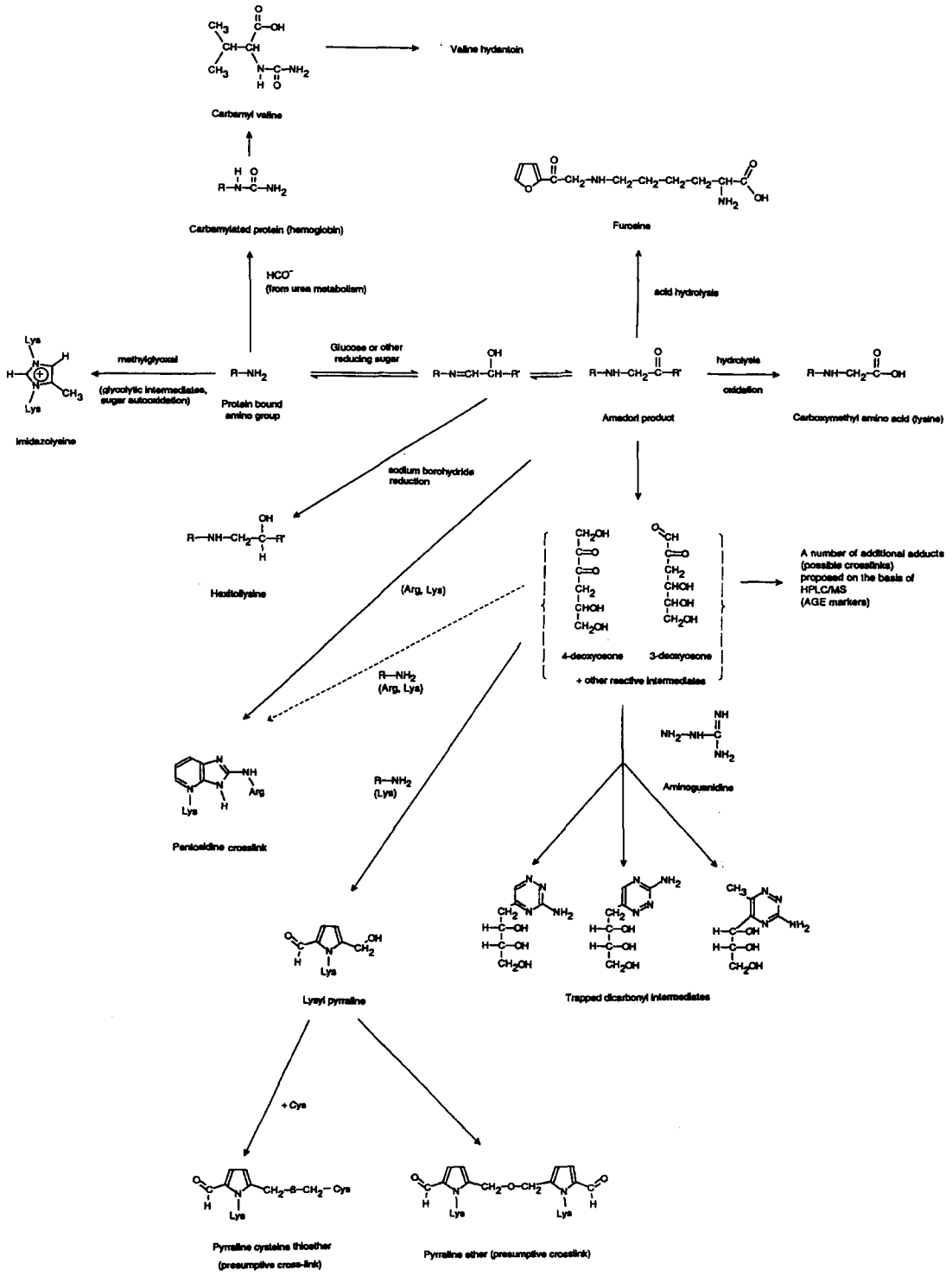


Fig. 1. Pathways leading to different marker adducts of proteins reacted with aldehydic sugar, methylglyoxal and isocyanate (uremic patients) (for additional heterocyclic compounds see, e.g., Ledl and Schleicher [7]).

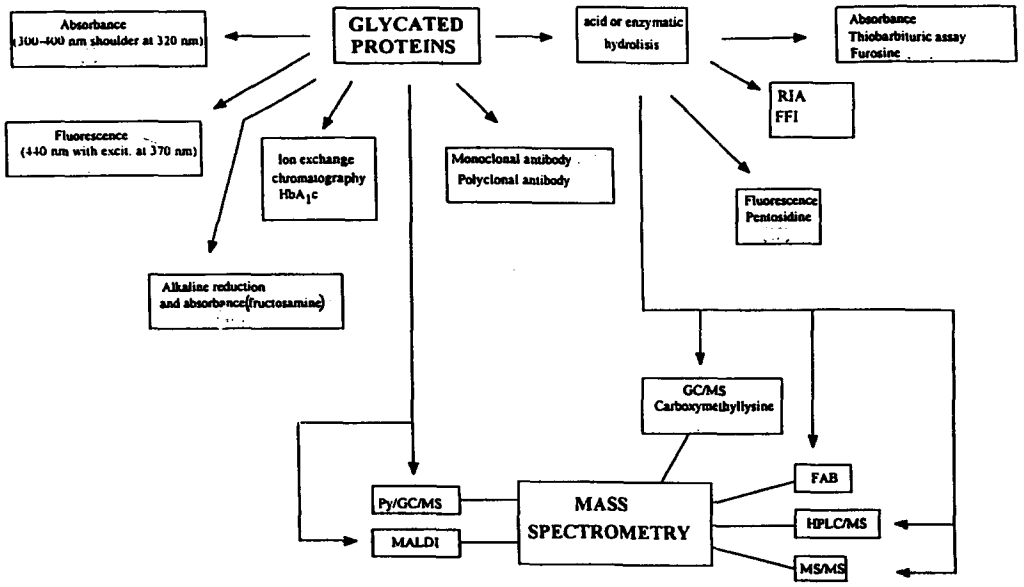


Fig. 2. Strategies for glycated protein analysis (reproduced from Lapolla et al. [8] with permission of John Wiley&Sons, Ltd.).

with three volumes of loading buffer containing also 10% methanol. The inclusion of methanol was intended to prohibit interactions with the propylsulphonic acid functional group. For pentosidine and pyridinoline pH 4.7 and 3.5 buffers were used (25 ml pyridine, 25 ml glacial acetic acid and 100 ml methanol to 1 l for the pH 4.7 buffer and 5 ml pyridine, 50 ml glacial acetic acid and 100 ml methanol to 1 l for the pH 3.5 buffer; 500 mg and 1 g cartridges were routinely used).

3. Direct assay of glycated amino acids and Amadori products

This procedure using the conversion of glycated amino acids in the corresponding phenylthiohydantoins (in the conventional way used for amino acid analysis) has been worked out by Walton and McPherson [14].

Glycated protein after borohydride reduction is hydrolysed in 4 *M* HCl at 110°C for 24 h. If glycated haemoglobin is used as test protein, about 45% of ε-GlcH-Lys and 27% of GlcH-Val are destroyed which has to be considered when quantitating the results. The separation system described involved a Bio-Rad Microguard column (ODS 5S, 4.6×40 mm) and a Whatman C_{18} analytical column (4.6×250 mm, Partisil 5 ODS-3, 5 μm particle size); gradient elution from 20 to 80% solvent A (acetate 70 m*M*–triethylamine 0.5 ml/l, pH 4.5) to solvent B (acetonitrile–water, 3:2, v/v) over a period of 18 min was applied. Typical runs are shown in Fig. 3.

Liquid column separations can be also used for the determination of Amadori products derived from tryptophan [15] (it is not quite clear to what extent these products are stable upon acid hydrolysis). Ultrasphere C_{18} column (5 μm, 2.0×150 mm) with different concentrations of H_3PO_4 in CH_3CN was used as mobile phase. Only fresh samples should be used as upon standing in the acid milieu (1% phosphoric acid) the different tautomers (keto, β-furanose, β-pyranose, α-furanose, α-pyranose) were seen on the chromatogram; however the reproducibility was rather poor. If the sample was equilibrated in 0.01 *M* H_3PO_4 in acetonitrile and let stand for 24 h, a single peak of the glycated tryptophan was observed; if the proportion of the phosphoric acid in the mobile phase is increased to 5%, well reproducible results of separating the individual tautomers were obtained.

It has to be stressed that fluorescent artifacts may

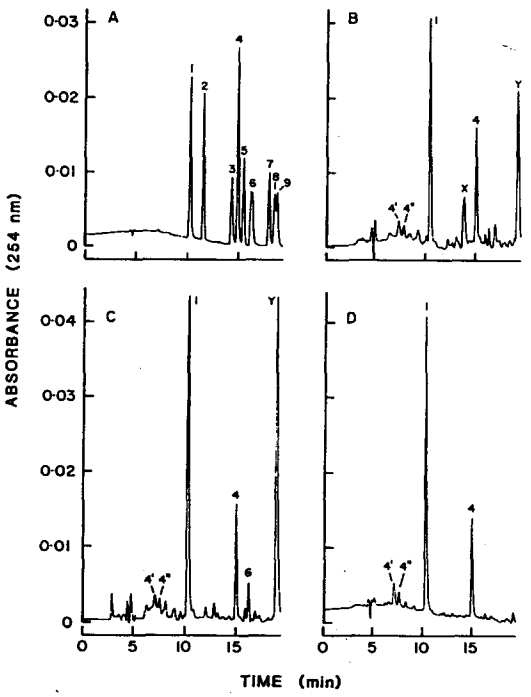


Fig. 3. Separation of phenylthiocarbamyl (PTC)-hexitolamino acids by HPLC. (A) Mixture of N-methylglucamine (1), ManH-Gly (2), GlcH-Tyr (3), ε-GlcH-Lys (4), valine (5), ManH-Val (6), ManH-Leu (7), GlcH-Phe (8) and lysine (9); (B) water insoluble fraction of lens proteins; (C) haemoglobin and (D) albumin. Panels B–D represent purified hydrolysates of borohydride-treated human proteins (1-N-methylglucamine; 4-ε-GlcH-Lys and ε-ManH-Lys; 4′ and 4″-acid degradation products of 4; 6-GlcH-Val and ManH-Val; X and Y are unidentified compounds). Reproduced from Walton and McPherson [14] with permission of Academic Press, Inc.

be formed upon acid hydrolysis of glycated proteins. The most typical example is, perhaps 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (the so-called FFI product) which originates from a non-fluorescent precursor formed during acid hydrolysis in a subsequent reaction with ammonia [16,17].

4. Pentosidine

Pentosidine represents the only so far well characterized post-translationally arising amino acid adduct leading to cross-link formation, arising by reaction between arginine, lysine and sugar (hexose or pentose) [18–21]. Its presence is characterized by a typical fluorescence at 335/385 nm and routinely is quantified by reversed-phase HPLC on a 30 nm pore size column with water–acetonitrile gradient (with 0.1% heptafluorobutyric or trifluoroacetic acid as counter ion). Typically, Dyer et al. [19] used Supelcosil LC-318 column 250×4.6 mm with gradient 0–9% B in 15 min, 10 min isocratic elution, next 45 min gradient to 60% B and last 10 min to 100% B, flow-rate 1 ml/min, where A=water and B=50% acetonitrile, both with 0.1% trifluoroacetic acid. Pentosidine was eluted at 25 min. However, this is not the only fluorescent cross-link at least in connective tissue. In mature collagen a hydroxypyridinium cross-link, pyridinoline [12,22] has been described. This cross-link is absent in skin collagen, but is present in tendons and other soft connective tissues. In bones a related cross-link, deoxypyridinoline, is present. Pyridinolines exhibit fluorescence parameters 297/390 while pentosidine, as indicated already exhibits the 335/385 nm fluorescence. Direct spectroscopic differentiation between these two luminescent entities in connective tissue is thus difficult.

As shown by Takahashi et al. [12] separation of pentosidine, pyridinoline and deoxypyridinoline in a urinary hydrolysate is possible by using SP-Sephadex C25 in the prefractionation step [23]. While in a previous communication urine prefractionation was done with CF1 cellulose columns (applicable to pyridinoline, deoxypyridinoline, desmosine and isodesmosine[1], this procedure does not allow the recovery of pentosidine from the sample as it remains tightly bound to the column. Typically the very separation of both pyridinolines and pentosidine can be done on a 8 mm×10 cm Radial Pak C_{18} column (10 μm particle size) with acetonitrile–30 m*M* heptafluorobutyric acid (27:73, v/v) with a flow-rate of 1.0 ml/min and 160 μl sample injected. Minimum detectable amounts are reported of about 0.6 pmol for any of the pyridinolines and 1.6 pmol for pentosidine. Typical separations are in Fig. 4. A

[1]Desmosine and isodesmosine represent the physiological fluorescent cross-links in elastin, a structural protein codistributed with collagen, e.g., in blood vessel walls.

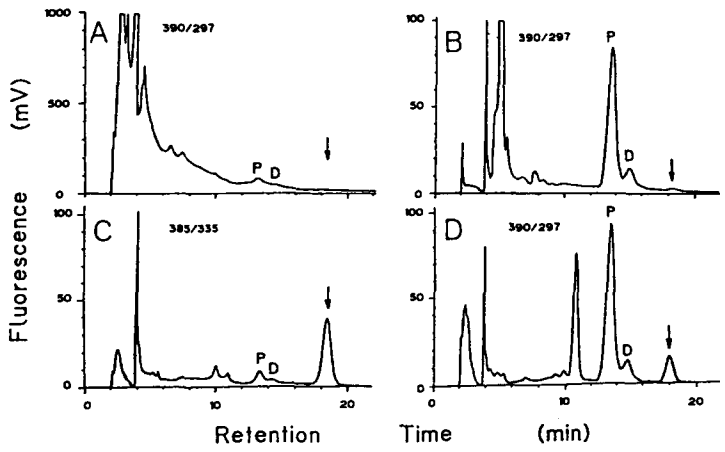


Fig. 4. HPLC chromatogram of an urinary hydrolysate. Sample pretreatment: (A) none; (B) CF1 cellulose; (C and D) SP Sephadex C25. Emission/excitation wavelengths: A, B and D 297/390 nm; C 335/385 nm. Identification: P, pyridinoline; D, deoxypyridinoline; arrow, pentosidine. Reproduced from Takahashi et al. [12] with permission of Marcel Dekker, Inc.

slight variation of this procedure was reported in Refs. [24,25].

Takahashi et al. [26] also developed a method for direct measurement of pyridinoline, deoxypyridinoline and pentosidine in the hydrolysate of tissues (Fig. 5). Separations were made on ODS reversed-phase column (TSK-GEL ODS-80T, Tosoh; 4.6×150 mm) with gradient 35–50% (see Fig. 5) acetonitrile with 30 m*M* heptafluorobutyric acid, flow-rate 1 ml/min, detection fluorescence at 307/390 nm. The minimum detectable amount was 1.9 p*M* for pyridinoline, 1.3 p*M* for deoxypyridinoline and 0.06 p*M* for pentosidine with sample injection volume 5–160 μl.

Direct analysis of pentosidine in urine and serum is possible by column switching [27]. Sample is injected directly onto a gel-permeation precolumn (TSK PW; 4.6 mm×3.5 cm), eluted with 5% acetonitrile containing 30 m*M* heptafluorobutyric acid and the fraction containing pentosidine is introduced (switched) into an octadecylsilyl column (TSK-GEL ODS-80T; 4.6 mm×15 cm) and then eluted with 20% acetonitrile containing 30 m*M* heptafluorobutyric acid at flow-rate 1 ml/min and monitored at absorbance 297 nm (for the first column) or fluorescence 335 nm excitation/385 nm emission (second column). Detection limit for pentosidine is ca. 0.33 pmol/injection.

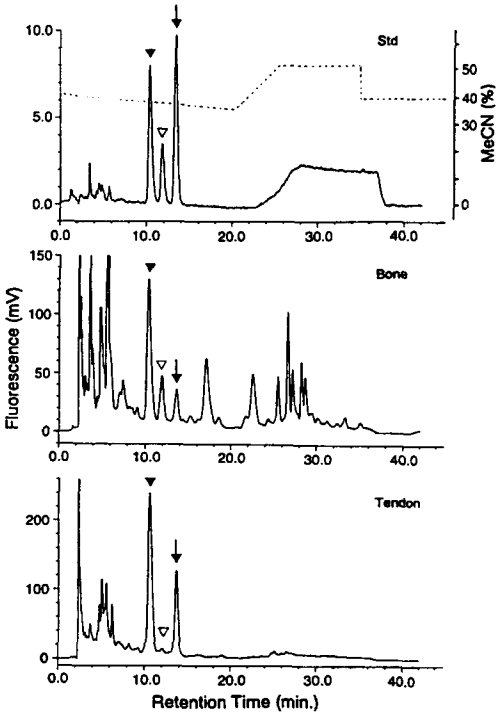


Fig. 5. HPLC chromatograms of standards of pyridinoline (▼), deoxypyridinoline (▽) and pentosidine (↓); their determination in bone and tendon hydrolyzates. Reproduced from Takahashi et al. [26] with permission of Academic Press, Inc.

As reported by Odetti et al. [25], however, there are two problems related to the pentosidine assay; if acid hydrolysis of the sample is carried out under nitrogen, the recovery of pentosidine is much lower than if the cleavage of the protein is done in the presence of oxygen. The other problem is that high protein content and presence of basic amino acids (and oxygen) during hydrolysis leads to the formation of fluorescent artifacts that can be separated by combined reversed-phase and ion-exchange chromatography. In the first phase, pentosidine is accumulated in the reversed-phase separation step (Vydac 218TP column), the fractions collected are vacuum concentrated and subjected to cation-exchange chromatography (Waters SP-5PW column using a curvilinear gradient of NaCl (0–0.06 *M*) from 0 to 40 min in 0.02 *M* sodium acetate buffer (pH 4.7). Separation from fluorescent artifacts is complete (Fig. 6), however no fluorescence parameters of the artifacts were reported.

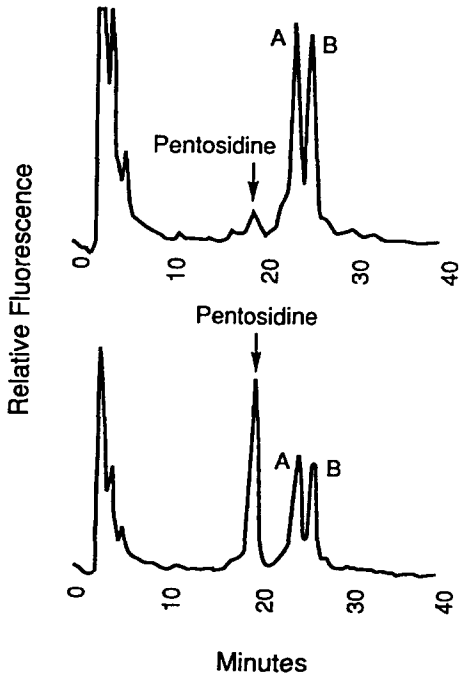


Fig. 6. Separation of true pentosidine from fluorescent artifacts collected from reversed-phase column (peaks A and B) and rechromatographed on ion-exchange column (top panel); bottom panel, standard pentosidine was added. Reproduced from Odetti et al. [25] with permission of American Diabetes Assoc.

5. Pentosidine-like fluorescent adducts (compounds K_1–K_4)

Recently Bailey et al. [28] reported formation of four different fluorescent compounds designated as compounds K_1, K_2, K_3, K_4. The former two were formed upon incubation of collagen preparations with glucose while the latter two were products arising upon ribose treatment. They were shown to have luminescence parameters close to pentosidine (340/390 nm) and were present in the incubation mixtures at a considerably higher concentrations then pentosidine. Compounds K_1 and K_2 accounted for 45% of total fluorescence (compared with 15% for pentosidine) upon glucose incubation and 25% of total fluorescence (compared with 30% for pentosidine) when the samples were treated with ribose. Compounds K_1 and K_2 (compound K) were proven not to be an artifact as they could be isolated from the dermal tissue of diabetic patients.

The incubated and control tendons were freeze dried and hydrolysed by 6 *M* HCl in the routine way. The hydrolysates can be either separated directly by HPLC to reveal the presence of the fluorescent components, or chromatographed on the TSK 40S column or eluted from the cellulose fibre columns. Samples for the cellulose column (sample pretreatment) were dissolved in *n*-butanol–acetic acid–water (4:1:1) and eluted from the column. In order to retain pentosidine on the column, the mass loaded onto the column had to be 0.5–1% of the cellulose packing dry mass and the volume of the organic phase had to be kept to three column volumes, otherwise pentosidine is eluted in the organic phase. The fluorescent compounds were then eluted with water, freeze dried and separated by reversed-phase chromatography.

Two systems can be used for the separation of the fluorescent compounds. The first employs a C_{18} column operated with a linear gradient from 5 to 35% acetonitrile in water each containing 0.05 *M* heptafluorobutyric acid at a flow-rate of 1 ml/min.

The other system employs a Hypercarb column using a shallow gradient from 0 to 11% tetrahydrofuran in water both containing 0.5% trifluoroacetic acid as counter ion (flow-rate 1 ml/min). The results obtained with the Hypercarb column are presented in Fig. 7.

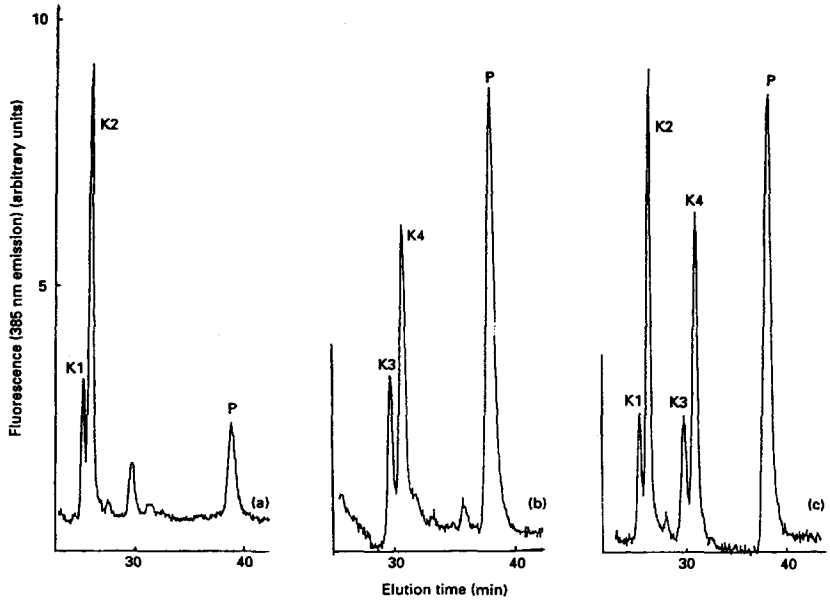


Fig. 7. HPLC chromatograms of of pentosidine (P) and compound K by Hypercarb column after preconcentration by CF1 cellulose column. (a) Glucose incubation showing pentosidine and compounds K_1 and K_2. (b) Ribose incubation showing pentosidine and compounds K_3 and K_4. (c) Profile of combined glucose and ribose incubations. Reproduced from Bailey et al. [28] with permission of the Biochemical Soc.

The pentosidine peak had identical chromatographic behavior no matter whether it was isolated from collagen incubated with glucose or ribose which is taken as a proof that in both incubations the same compound is formed (see also Refs. [29,19]).

Generally the same procedure, i.e., sample enrichment with the cellulose column followed by HPLC with the Hypercarb system can be used for diabetic skin samples documenting the formation of these compounds under in vivo conditions.

6. Imidazolysine

Recently methylglyoxal has been shown as a potential cross-linking reagent in vivo and suggested a common intermediate in the Maillard reaction involving glucose. This cross-linking agent can be derived from glycolytic intermediates by retroaldol cleavage of sugars. Velíšek and coworkers [30,31] described imidazolium salt formation during the Maillard reaction of glycine or glycylglycine with glyoxal in the presence of formaldehyde at high temperature. Nagaraj et al. (Ref. [32], see also Ref. [33]) have recently identified a model of a cross-linked peptide dimer, in which two lysines were bound through a methylimidazolium moiety. The product was obtained first by incubation of N^{α}-acetylglycyllysinemethyl ester with N^{α}-*t*-BOC (N-*tert.*-butoxycarbonyl) arginine or N^{α}-*t*-BOC lysine with methylglyoxal. A complex purification procedure involving gel permeation chromatography, C_{18} reversed-phase and even thin-layer chromatography (TLC) was used to obtain purified products. For the analysis of imidazolysine in serum proteins, human serum was precipitated with 10% trichloroacetic acid, precipitated proteins collected at 5000 *g* for 30 min and lyophilized; next, they were subjected to routine acid hydrolysis (6 *M* HCl, 110°C, 20 h), the hydrolysate was vacuum dried and the dried residue was reconstituted in 0.5 ml of water.

Imidazolysine was detected and quantified by post column *o*-phthalaldehyde derivatization. A C_{18} column eluted with a 0–25% acetonitrile gradient in water with 0.01 *M* heptafluorobutyric acid was used at a flow-rate of 1 ml/min. The *o*-phthalaldehyde-derivatized products were detected by fluorescence at

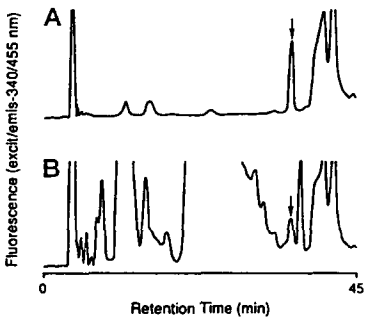


Fig. 8. HPLC chromatogram of imidazolysine standard (A) and serum protein hydrolysate (B), arrows are imidazolysine. Reproduced from Nagaraj et al. [32] with permission of the Am. Soc. Biochem. Mol. Biol.

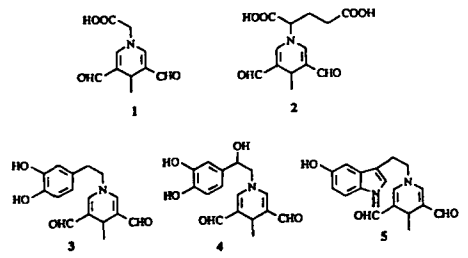


Fig. 9. Structures of fluorescent dihydropyridine adducts formed by reaction of malondialdehyde with glycine (1), glutamic acid (2), dopamine (3), norepinephrine (4) and serotonin (5). Reproduced from d'Ischia et al. [34] with permission.

340/455 nm. Under these conditions imidazolysine eluted between 29 and 30 min.

For the purpose of liquid chromatography (LC)–MS spectra [fast atom bombardment (FAB)-LC–MS] the HPLC conditions were as follows:

A reversed-phase (C_{18} 5 μm, 15 cm×2.1 mm, column) was used with a mobile phase consisting of linear gradient of acetonitrile in water (0–8%, 32 min) with 1% glycerol and 0.1% trifluoroacetic acid at a flow-rate of 250 μl/min.

A typical chromatogram of the imidazolysine standard and serum protein hydrolysate is shown in Fig. 8.

Due to the post column *o*-phthalaldehyde derivatization approach used in the analysis of this cross-link no information is available at present whether or not this cross-linking amino acid possesses a typical fluorescence as, e.g., pentosidine or pK_2 (see Section 4 Section 5).

7. Malondialdehyde adducts

As mentioned in Section 1, post-translational non-enzymatic modifications discussed in this review can arise from the reaction of nearly any aldehydic moiety in the body. From this point of view, lipid peroxidation products, typically malondialdehyde are highly interesting. Studies on this matter subject (from the analytical point of view) are restricted mainly to model mixtures with amino acids.

d'Ischia et al. [34] showed that iron-promoted autooxidation of arachidonic acid in acetate buffer (pH 6.0) at 37°C in the presence of amino acids (particularly glycine) leads to the formation of fluorescent products. Though no direct evidence about in vivo presence of these products exists so far, the broad gamut of potential targets available for interaction with malondialdehyde in vivo would be expected to lead to infinitely complex mixtures of fluorescent pigments (lipofuscin-like).

Reaction of malondialdehyde with glycine (leading to 1-carboxymethyl-4-methyl-1,4-dihydropyridine-3,5-dicarboxaldehyde) can be followed by HPLC chromatography using a C_{18} column with 0.1 *M* formic acid–acetonitrile 85:15 (v/v) as mobile phase or a gradient of 0.1 *M* formic acid to 0.1 *M* formic acid–acetonitrile (1:1, v/v) over 30 min. Though the compound (and related dihydropyridine adducts derived from glycine, glutamic acid, dopamine, norepinephrine and serotonin— Fig. 9) exhibit typical fluorescence (e.g., 385/457 for glycine adducts). Detection was done at 254 or 366 nm.

HPLC elution profiles obtained with iron-induced autooxidation of arachidonic acid in the presence of glycine (pH 6.0, in vitro incubation) using the C_{18} column eluted with an acetonitrile–0.1 *M* formic acid gradient ranging from 7.5:92.5 (v/v) to 80:20 (v/v) are shown in Fig. 10.

8. Pyrraline

Pyrraline has been identified as one of the major advanced glycation products formed in the reaction of 3-deoxyglucosone with proteins; its presence has been proven in both Descement's membrane and

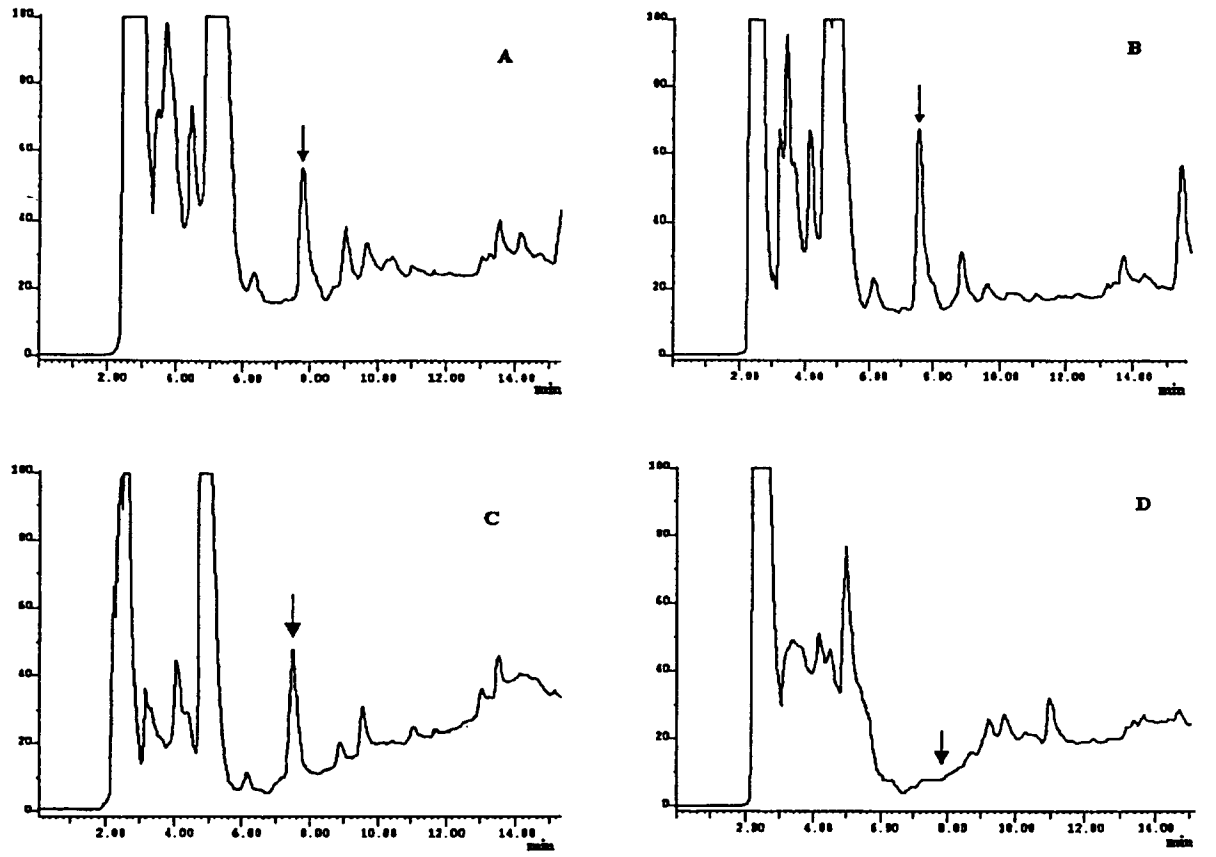


Fig. 10. HPLC elution profiles (spectrofluorimetric detection) of reaction mixtures obtained by iron-induced autoxidation of arachidonic acid in the presence of glycine: (A) 1 m*M* glycine; (B) 1 m*M* glycine+dihydropyridine 1 (see Fig. 8); (C) 1 m*M* glycine added after 24 h autoxidation and (D) 10 m*M* glycine. Arrows indicate dihydropyridine 1. Reproduced from d'Ischia et al. [34] with permission.

plasma proteins [35–39]. Synthetic lysyl pyrraline can be most easily separated on a C_{18} reversed-phase column eluted at a flow-rate of 1.0 ml/min with an acetonitrile–water gradient [40]. The following programme is used: mobile phase A: water containing 0.1% trifluoroacetic acid; mobile phase B 50% acetonitrile in water containing 0.1% trifluoroacetic acid; 0–100% B over 30 min was applied (column eluate monitoring at 298 nm and 500 nm) (Fig. 11).

Upon standing pyrraline yields coloured products through the reaction with cysteine or glutathione. Hydroxyl groups possessing amino acid are generally non-reactive, except hydroxylysine and hydroxyproline which show some affinity for this reaction. Incubation of caproyl pyrraline with N-α-acetyl cysteine showed two products upon TLC (silica plate developed in *n*-butanol–acetic acid–water 75:15:15, R_F 0.77 and 0.57). The conclusion was that 50% of pyrraline is readily converted in vitro into a major thioether product the structure of which was confirmed by ^{1}H NMR spectra.

The general concept of protein cross-linking through pyrraline was proposed by Nagaraj et al. [40]; the reaction of a protein with glucose yields the Amadori product; this is converted to 3-glucosone and with another protein molecule a possessing free NH_2 group, protein–pyrraline is formed, which in a subsequent step can yield a pyrraline–pyrraline cross-link or, if an SH-moiety is present a pyrraline–cystein cross-link. The resulting structures may look as follows as shown Fig. 12.

Though dipyrraline is the presumptive cross-link, it has so far not been identified (in contrast to pyrraline) in naturally occurring proteins. No ana-

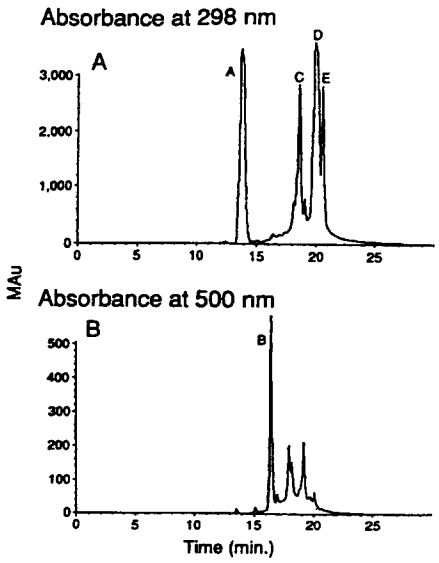


Fig. 11. HPLC chromatograms of a pyrraline incubated samples (D-lysyl pyrraline). Reproduced from Nagaraj et al. [40] with permission of Academic Press, Ltd.

lytical (separation) methods are available for this compound.

There is, however, one important point to be stressed; in order to see pyrraline in glucose incubated samples (the more in natural samples) the hydrolysis must not be done in acid media. Either $Ba(OH)_2$ or enzymatic hydrolysis (peptidase from *Streptomyces griseus*) should be applied. $Ba(OH)_2$ hydrolysis yields better chromatograms, peptidase digests are frequently obscured by high baseline noise [41]. On the other hand it is worth mentioning that the presence of pyrraline has been reported as being observed in human skin collagen preparations, though this result has not been confirmed so far.

9. Furosine

Furosine is formed by hydrolysis of 1-deoxy-fructosyl lysine arising in glycated proteins. The product is stable and presently it can be relatively routinely assayed by reversed-phase HPLC [42–44]. One of the advantages of this analysis is the commercial availability of furosine standard (Neosystem, Strasbourg, France). Separations can be performed with a system consisting of Guard Pak in-line filter and a C_8 furosine dedicated column (250×4.6 mm, Altech, Milan, Italy) with 0.4% acetic acid as solvent A and 0.27% potassium chloride in solvent A (solvent B). The elution gradient was as follows

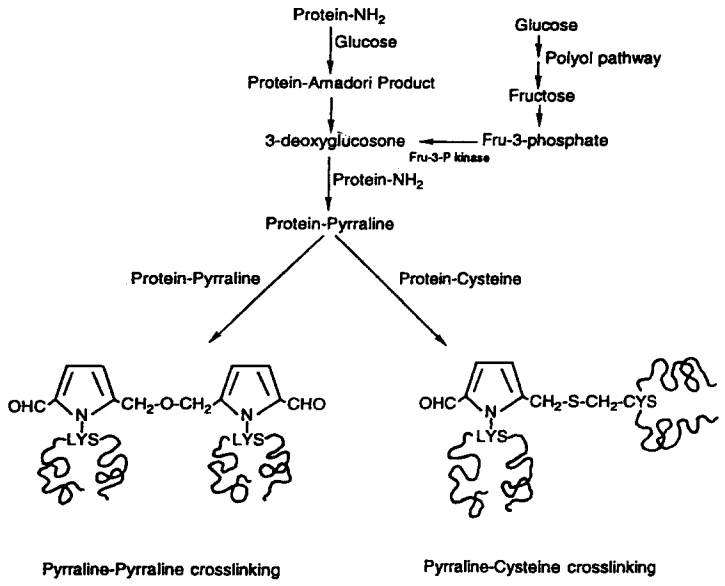


Fig. 12. Conceptual mechanisms of pyrraline ether formation on proteins. Reproduced from Nagaraj et al. [40] with permission of Academic Press, Ltd.

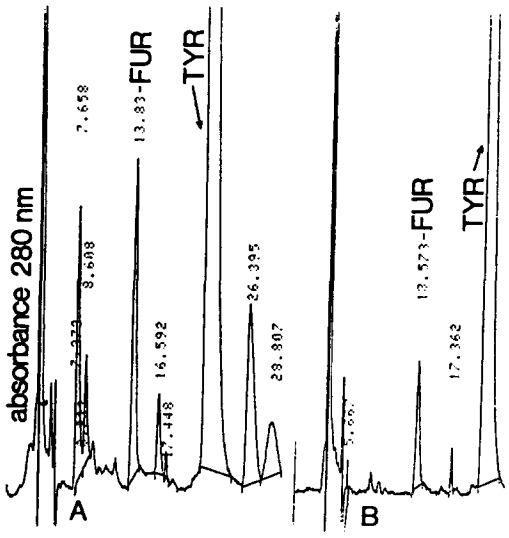


Fig. 13. Chromatographs of a hydrolysate of the bulk serum proteins (A) and of human serum albumin (B). Abbreviations: FUR, furosine; TYR, tyrosine. Reproduced from Drexel et al. [43] with permission of Am. Assoc. Clin. Chem.

(proportion of solvent B) 0–12.5 min, 0%; 12.5–22 min, 50%; 22–32 min, 0% (flow-rate 1.2 ml/min). Furosine elutes around 29 min and can be detected by UV at 280 nm [42].

It is also possible to use an acid resistant C_{18} column for the above purpose [43]. In this case the column (25×0.46 cm I.D., Altex Ultrasphere TM, C_{18} with 5 μm particle size), guarded by a 4.00× 0.46 cm I.D. C_{18} precolumn is used with 3.5 mmol H_3PO_4 containing 30 ml acetonitrile per liter. Typical examples of analysis of both serum proteins and human serum albumin are shown in Fig. 13.

Yet another paper [44] describes the possibility of using an ODS 2 column (5 μm, 0.46×25 cm, Phenomenex, Macclesfield, UK) with 5 m*M* sodiumheptane sulphonate with 20% acetonitrile and 0.2% formic acid (1.2 ml/min flow-rate) for the same purpose.

10. N-Carboxymethylamino acids

Oxidation (e.g., by periodate) of free and protein bound Amadori compounds formed by the condensation of reducing sugars with primary amino groups generates on acid hydrolysis N-carboxymethyl derivatives of amino acids. Their separation and quantitation can be used to estimate both the extent and the site of protein glycation. Methods applicable to this task were published by Badoud and Fay [45] and Badoud et al. [46]. Separation is done by gas chromatography (GC) using capillary columns (DB-5 or DB-1701, 30 m×0.32 mm, J&W Scientific, Folsom, CA, USA) operated in a splitless injection mode at 250°C with temperature programming from 50–200°C at 30°C/min, from 200–300°C at 5°C/min with He as carrier gas. Electron impact spectra can be recorded at 70 eV and positive chemical ionization mass spectra were obtained with ammonia as reagent gas. The GC–MS–MS spectra were obtained with positive chemical ionization using ammonia as the reagent gas to generate $[M+NH_4]^+$ ions analyzed by collision induced dissociation (6 eV, argon as collision gas) of N,O-pentafluoropropionyl derivatives prepared by reacting acetic anhydride and isobutyl alcohol with the acid hydrolysate. This was prepared from the sample protein by adding stepwise 100 m*M* periodic acid and 2 *M* sodium thiosulphate followed by 37% HCl to make the final concentration 6 *M* with respect to HCl. The hydrolysate was purified by passing through a small Dowex 50W-X4 column, the amino acids were eluted with 3 *M* ammonia, brought to dryness and derivatized.

A typical chromatogram of nine isobutyl ester N,O-pentafluoropropionyl derivatives of carboxymethylamino acids is shown in Fig. 14. Practical applicability of this method was shown with glycated haemoglobin in which both carboxymethylvaline and carboxymethyllysine could be assayed.

In the preceding version of the analytical approach from the same laboratory GC only was used. The procedure was essentially the same as just described except that the Dowex eluate was derivatized with ethylchloroformate in ethanol–pyridine–water to modify the eluting N-carboxymethylamino acids to the respective ethyl ester N-ethoxycarbonyl derivatives. A typical GC run with flame ionization detection (FID) is demonstrated in Fig. 15.

Dunn et al. [47] determined N^{ε}-carboxymethyllysine and N^{ε}-carboxymethylhydroxylysine including fructose–lysine (as furosine) in the skin collagen as trifluoroacetylmethyl ester derivatives. These derivatives were analysed by GC–MS using 30 m DB-5 capillary column (J&W Scientific). The

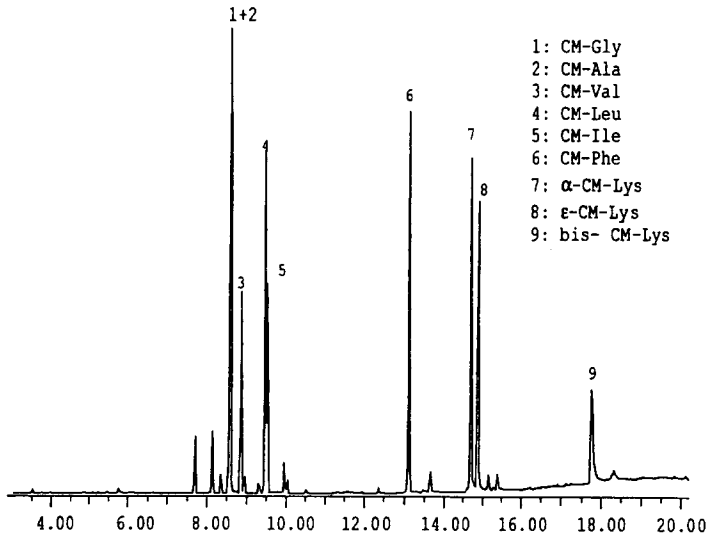


Fig. 14. GC–MS separation of the isobutyl ester N,O-pentafluoropropionyl derivatives carboxymethylamino acids (CM-AAs). Reproduced from Badoud and Fay [45] with permission of Springer Verlag.

temperature program used ran as follows: 2 min at 70°C, ramp to 260°C at 5°C/min and then to 290°C at 15°C/min, hold for 4 min at 290°C. The products were determined by selected-ion monitoring (SIM-GC–MS).

On the other hand N^{ε}-carboxymethyllysine can be also determined after reduction of the glycated protein and hydrolysis through reversed-phase-HPLC separation of its *o*-phthalaldehyde derivative. This method has been worked out by Hartkopf et al. [48] for estimation of this lysine derivative in foods, but can be easily applied to other biological matrices as well. The samples are reduced by prior hydrolysis which is done under the standard conditions (110°C, 7.8 *M* HCl, 20 h). The hydrolysates can be concentrated by rotary evaporation if necessary, or adjusted

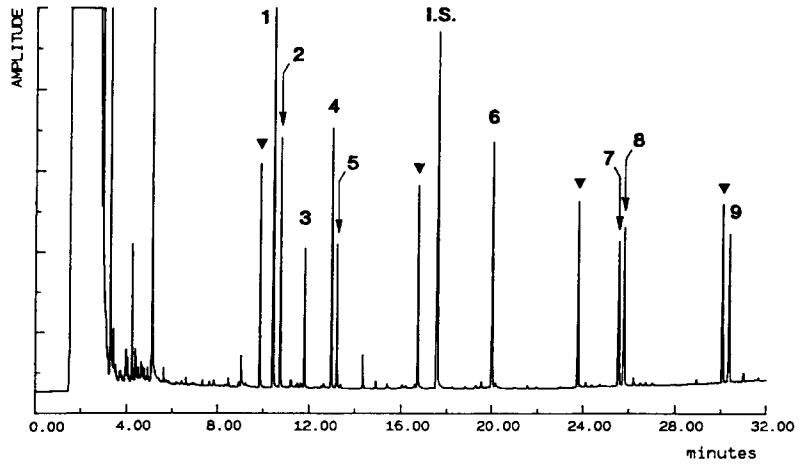


Fig. 15. GS separation (with FID detection) of a standard mixture of carboxymethylamino acids after derivatization with ethylchloroformate. For identification see Fig. 14. Reproduced from Badoud et al. [46] with permission.

Table 1
Composition of the solvent during the gradient time of the HPLC analysis of *o*-phthalaldehyde derivative of N^{ε}-carboxymethyllysine [48]

Time (min)	A (%)	B (%)
0	85	15
5	85	15
10	84	16
15	80	20
20	75	25
25	65	35
30	50	50
35	30	70

otherwise and finally taken to dryness. Then they are reconstituted in 0.4 *M* borate buffer, pH 9.5, ultrafiltrated and derivatized with *o*-phthalaldehyde (54 mg of *o*-phthalaldehyde reagent dissolved in 1 ml methanol, 50 μl of 2-mercaptoethanol and borate buffer pH 9.5, 0.4 *M* to make 10 ml). For the precolumn derivatization 100 μl of the sample were reacted with 500 μl of the *o*-phthalaldehyde reagent. Separation was done with Spherisorb 5 C_{18} (Promochem, Wesel, Germany) using a rather complex gradient composed of buffer A (sodium acetate, 0.05 *M*, pH 6.7–methanol 96:4, v/v) and solvent B (pure methanol). The time course of the gradient is seen from Table 1 with a result shown in Fig. 16.

Another approach for the separation of *o*-phthalaldehyde derivatives was reported by Weninger et al. [49]. After derivatization of N^{ε}-carboxymethyllysine with *o*-phthalaldehyde-3-mercaptopropionic acid, the separation was done on Hypersil ODS 3 μm (125×4 mm). The following gradient was used: 0–30% B/0–9 min, 30–50%/9–11 min, 50–100%/13–14 min and next 4 min 100%B [A: 25 m*M* sodium acetate, pH 7.2 with 0.7% tetrahydrofuran; B: 100 m*M* sodium acetate, pH 7.2 in acetonitrile (1:4, v/v)]. Retention time of carboxymethyllysine was at 9 min.

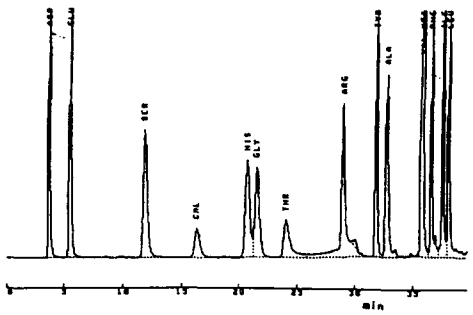


Fig. 16. HPLC chromatogram of a *o*-phthalaldehyde derivative of N^{ε}-carboxymethyllysine and amino acid standard. Reproduced from Hartkopf et al. [48] with permission.

Glomb and Monnier [50] separated modified amino acids (carboxymethyllysine and glucitolyllysine) on reversed-phase column by a solvent containing sodium dodecyl sulphate (SDS) with postcolumn derivatization with *o*-phthalaldehyde. They used C_{18} column Vydac 218TP54 (0.4×25 cm, 5 μm) with 5% propanol (A) and 60% propanol in water (B) both with 3 g of SDS/l, 1 g of monobasic sodium phosphate monohydrate/l and adjusted to pH 2.8 with phosphoric acid; gradient from 15% B to 22% B in 30 min, to 40% B in 20 min and to 100% B in 5 min at flow-rate 1 ml/min. Retention time of carboxymethyllysine was 26.3 min and glucitolyllysine 39.1 min.

11. Amino acid hydantoins

A rather similar approach was developed for assaying carbamylated haemoglobin through quantification of valine hydantoin arising after acid hydrolysis from N-terminal carbamyl valine (Fig. 17) [51]. This modification occurs in patients with sickle cell anaemia during cyanate therapy.

The sample was obtained from heparinized blood by hydrolysis with 11 *M* HCl and 17 *M* acetic acid (added 1 ml each to 1 ml sample) at 110°C for 2 h. The hydrolysate was cooled, brought with 2 ml 10 *M* NaOH to pH about 4; in this stage the internal standard was added; the sample was extracted with 5 ml of ethylacetate, centrifuged at 5000 *g*; 4.5 ml of this extract was shaken with 2 ml of 1 *M* $NaHCO_3$, the sample was centrifuged and dried under nitrogen at 70°C.

The dried sample was reconstituted in 0.5 ml mobile phase (water containing 60 ml of acetonitrile and 1 ml 17 *M* acetic acid per liter, pH about 4.0). Separation was isocratic using a 250×4.6 mm (5 μm particle size) octadecylsilica-bonded reversed-phase column maintained at 45°C. Quantitation was done by using internal standard addition (100 and 2 μl of 32 mg/l carbamyl valine) (Fig. 17).

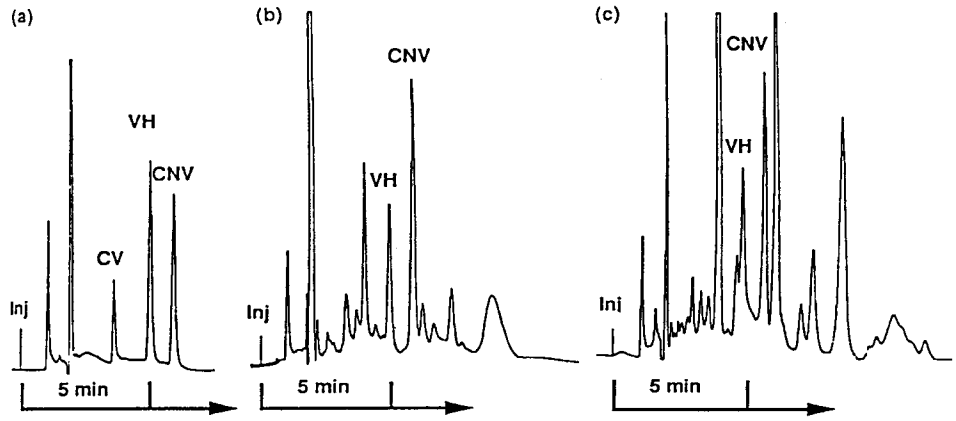


Fig. 17. HPLC chromatograms of (a) standards with carbamyl valine added to the final extract, (b) washed blood sample and (c) unwashed whole-blood sample. Abbreviations: CV, carbamyl valine; VH, valine hydantoin and CNV, carbamyl norvaline. Reproduced from Kwan et al. [51] with permission of Am. Assoc. Clin. Chem.

12. Analysis of dicarbonyl intermediates (3-deoxyglucosone)

In the initial phase of the glycation reaction 1-amino-1-deoxy-2-ketose derivatives (Amadori compounds) arise. Their formation is followed in vivo by a complex set of reactions involving deoxydicarbonyl intermediates (e.g., 3-deoxy-D-erythro-hexos-2-ulose=3-deoxyglucosone). These intermediates are under examined physiological conditions very reactive and so they are not usable as such (underivatized) as glycation markers. This reactivity, however, may be used in their determination, e.g., in a reaction with *o*-phenylenediamine, quinoxalines are formed which may be easily quantified by reversed-phase HPLC [7]. Dicarbonyl intermediates also react readily with aminoguanidine to give stable 3-aminotriazine derivatives and this can be exploited for their assay and quantitation as well (for three arising compounds see Fig. 1) [52] (as a matter of fact aminoguanidine can be used to prevent glycation reactions both in vivo and in vitro).

For gas–liquid chromatography (GLC) separation trimethylsilyl derivatives [52] (prepared by the standard silylation with N,O-bis(trimethylsilyl)trifluoroacetimide in pyridine) was recommended: 0.2 mm×25 m Quadrex 007OV-17 GLC column, starting temperature 120°C, followed by 2 min hold, then a ramp of 8°C/min to a final temperature of 250°C. Retention times reported for the three arising compounds were 16.6, 16.75 and 15.4 min.

Alternatively it is also possible to use TLC separation using silica gel plates (Whatman K5F) with $CHCl_3$–MeOH–H_2O (7:3:0.3, v/v) as mobile phase. The R_F values obtained were 0.5, 0.45 and 0.48 [52].

Yamada et al. [53] converted 3-deoxyglucosone (for determination in rat plasma) to a stable compound, 2-(2,3,4-trihydroxybutyl)-benzo[*g*]quinoxaline by reacting it with 2,3-diaminonaphthalene. This derivative was separated on TSK ODS-80TM (4.6×250 mm) with linear gradient 100% A to 100% B from 30 to 35 min at flow-rate 1 ml/min. Mobile phase A was composed from 70% 50 m*M* phosphate, 15% acetonitrile and 15% methanol; mobile phase B was composed from 20% 50 m*M* phosphate, 40% acetonitrile and 40% methanol. Detection was by absorbance at 268 nm or fluorescence at 271/503 nm.

Another approach for the analysis of glucosone intermediates has been reported by Fuji et al. [54]. This method is specific for 3-deoxyglucosone assay and exploits the oxidation of this analyte with crude oxoaldehyde dehydrogenase yielding 2-keto-3-deoxygluconic acid. This product is derivatized with 1,2-diamino-4,5-methylenedioxybenzene and the fluorescent products can be detected and quantified by HPLC using an aqueous or borate containing mobile

phase. The borate phase is used to separate 2-keto-3-deoxygluconic acid from N-acetylneuraminic acid which may interfere (if applicable). The very separation was done on a reversed-phase column (Intersil ODS-2, Gasukoro, Kogyo, Tokyo, Japan, 250×4.6 mm, particle size 5 μm) with acetonitrile–methanol–water or 10 m*M* sodium borate buffer pH 7.0 (9:7:84, v/v). Detection was done by fluorescence at 373/448 nm. Derivatization is simple as it involves just adding the derivatizing reagent to the pre-purified sample and heating at 50°C for 2.5 h. Baseline separations are obtained with runs not exceeding 20 min (limit of detection 2.5 pmol/injection).

Knecht et al. [55] detected 3-deoxyfructose and 3-deoxyglucosone in human urine by GC–MS. In the first stage dicarbonyls were reduced by $NaBH_4$, cleaned up by mixed-bed resin AG 501-X8 (Bio-Rad) and anion- (Dowex 1-8X) and cation- (Dowex 50W-8X) exchange resin. Then alditols were converted to per-O-acetyl derivatives. GC–MS of urinary 3-deoxyhexitols was performed on 30 m DB-5 capillary column (J&W Scientific) with the following program: 2 min at 150°C, ramp to 240°C at 5°C/min, then to 290°C at 15°C/min, and hold for 4 min at 290°C. Plasma 3-deoxyhexitols were analysed using a more gradual temperature program: 2 min at 100°C, ramp to 240°C at 4°C/min, then to 290°C at 15°C/min and hold for 4 min at 290°C.

A GC–MS technique was used by Niwa et al. [56] for determination of 3-deoxyglucosone in human serum. They converted samples to their methoxime derivatives by O-methoxylamine hydrochloride in pyridine and hydroxyl group converted to their trimethylsilyl derivatives with N,O-bis-(trimethylsilyl)trifluoroacetimide containing 1% trimethylchlorosilane. Analyses were made using GC equipped with a DB-17 bonded fused-silica capillary column (30 m×0.32 mm I.D., 0.15 μm film thickness; J&W Scientific). Column temperature was programmed at 5°C/min from 140°C to 200°C. Electron ionization-MS or chemical ionization were used.

It is also possible to separate dicarbonyl sugars by capillary electrophoresis. Dicarbonyl sugars were derivatized by *o*-phenylenediamine and arising quinoxalines were separated by capillary electrophoresis (70 cm×50 μm I.D. fused capillary) at 15 kV in 75 m*M* borate buffer (pH 11.0) and 8 m*M* tetrabutylammonium bromide as modifier. By this method, glucosone in the incubation mixture of lysine and glucose was successfully identified [57]. Another approach used to reveal dicarbonyl compounds (sugars) by capillary electrophoresis is based on their derivatization with N,N-diphenylhydrazine. The arising hydrazones were separated by microemulsion electrokinetic chromatography (fused-silica 70 cm×50 μm I.D.) at 20 kV in 5 m*M* borate buffer pH 8.0 (89.27%, w/w), *n*-hexanol (0.81%), SDS (3.31%) and *n*-butanol (6.61%). This method enabled separation of, e.g., galactosone, glucosone, 6-deoxyglucosone and furylglyoxal [58] (see Fig. 18).

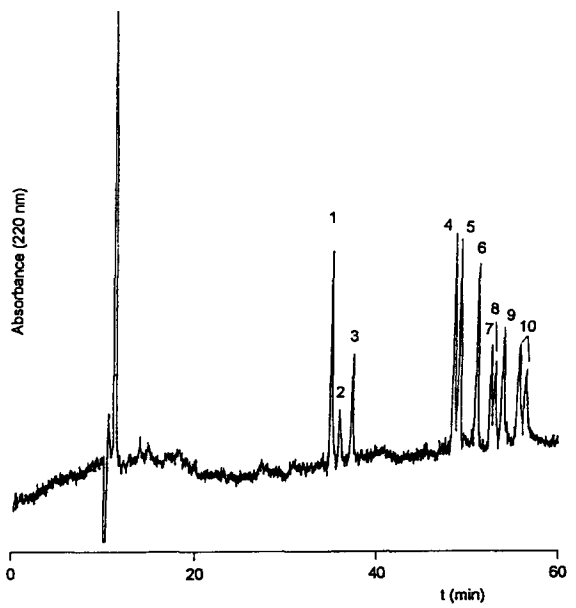


Fig. 18. Microemulsion electrokinetic chromatographic separation of mono-(1,3–7), bis-(2,8,10) and tris-diphenylhydrazones of galactosone (1,4), 5-ketofructose (2), glucosone (3,8), xylosone (5), 6-deoxyglucosone (6), 2-furylglyoxal (7,10) and 5-hydroxy-2,3-dioxopentanal (9). Reproduced from Mikšík et al. [58].

13. Multicomponent analysis of acid stable cross-links

In addition to pentosidine a new cross-link, so far characterized by its physico-chemical parameters

was reported by Graham [59]. The crude ribose amino acid mixture was incubated for 8 h at 85°C in 0.2 *M* sodium phosphate buffer pH 7.4. Then the sample pH was adjusted to 1 and the sample was loaded (300 ml) on a 150 ml Dowex 50 column (AG50W-X8, 100–200 mesh) and eluted with 300 ml 0.05 *M* HCl. Amino acids were eluted at 5 ml/min with a linear gradient beginning with 0.5 *M* acetic acid and ending with 0.5 *M* NH_4OH (total gradient volume 500 ml, 5 ml fractions were collected). The gradient was followed by 400 ml of 0.5 *M* NH_4OH and 300 ml 2 *M* NH_4OH. Fractions 16–75 (acid) 76–115 (neutral), 116–155 (basic 1), 156–195 (basic 2) and 196–255 (basic 3) were pooled and lyophilized. When necessary the pooled fractions were analysed by using the diphenylboronic acid (DPBA) method as described by Graham and Gallop [60]. In principle concentrated DPBA (~0.44 *M*) was prepared by heating 100 mg DPBA–ethanolamine complex at 90°C for 1 min; an equimolar amount of fumaric acid (25 mg/100 mg DPBA) was added, the solution was cooled and the precipitate (ethanolamine fumarate) removed by centrifugation. A 100 μl volume of the DPBA solution was mixed with an equal amount of the amino acid solution and 10 μl of acetic acid. The reaction mixture was heated to 75°C and another portion of DPBA was added and heating continued for another 10 min. Three further 200 μl aliquots of DPBA were added at 10 min intervals and the reaction mixture was taken to dryness upon a stream of nitrogen. For HPLC analysis the reaction products were redissolved in 1200 μl of 75% buffer A–25% acetonitrile and 500 μl were injected on a Zorbax PTH column (Dupont) at 40°C. Buffer A was 70% 6 m*M* phosphoric acid (pH 3.3) and 30% acetonitrile, buffer B had the same components, but with acetonitrile at 85%; the gradient started at 10% B (isocratic for 10 min), increased to 35% B from 35 to 50 min run time and then to 95% B between 50 to 90 min.

The separation strategy was based on the fact that DPBA-modified amino acids can be separated according to the number of the molecules of the derivatizing agent present. Cross-linking amino acids which have more than one amino group per molecule elute later than single amino acids as shown in Fig. 19. Consequently, the whole chromatogram can be divided into three sections, namely simple amino

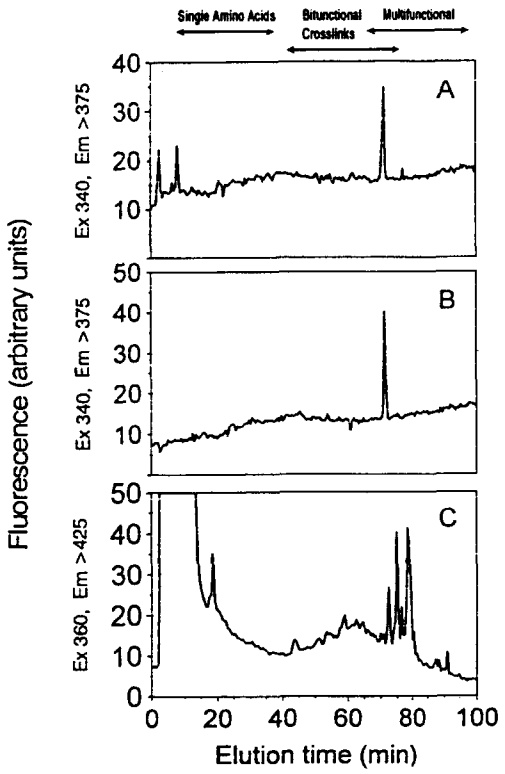


Fig. 19. HPLC chromatograms by DPBA analysis of fluorescent products formed by reaction of ribose with lysine and arginine analysed at excitation wavelength 340 nm, emission above 375 nm (A), 360/>425 nm (C) and pentosidine standard (B). Reproduced from Graham [59] with permission.

acids region, region containing bifunctional cross-links (typically pentosidine) and the region of multifunctional cross-links. Clearly (see Fig. 19) at least two peaks were seen in the cross-linking region of the chromatogram in addition to pentosidine. These peaks were also revealed (by using the DPBA procedure) in acid hydrolysates of ribose treated bovine serum albumin and lysozyme in vitro. Reaction products were detected at 360/>425 nm fluorescence. Spectral characteristics are shown in Fig. 20. The cross-link has not been fully characterized yet and is referred to as $penK_2$ (indicating pentose involvement) and is suspected to be identical with the so-called Maillard product LM1 [61]. The compound has two excitation maxima (310 and 360 nm) and emission maximum at 440 mm (in acid

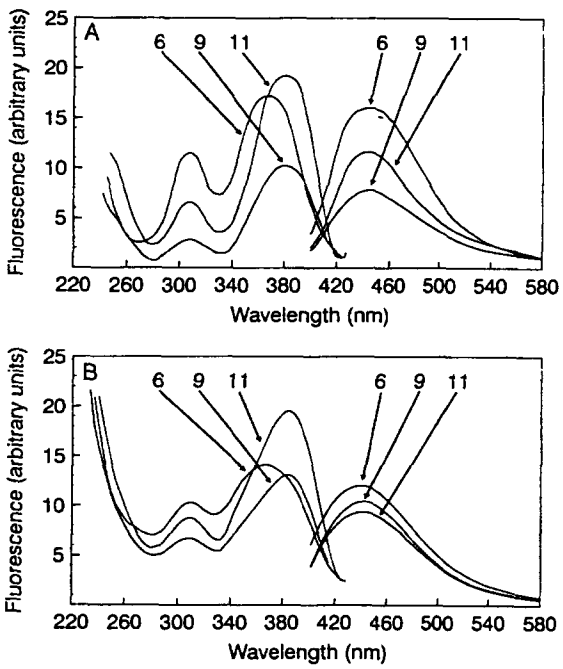


Fig. 20. Fluorescence spectra of the purified cross-link from the reaction of ribose with lysine (A) and from the reaction of ribose with bovine serum albumin (B) at pHs 6, 9 and 11. Reproduced from Graham [59] with permission.

medium). In alkaline medium a shift from 366 to 385 of the main excitation maximum peak was observed.

In conclusion it can be stated that $penK_2$ and pentosidine are the main acid stable cross-links formed by ribose treatment; pentosidine elutes in the system described before $penK_2$. It has been also stated that $penK_2$ originates from the interaction with ribose only [59]. Finally, unlike pentosidine which bridges one lysine and one arginine residue, $penK_2$ is believed to cross-link two lysine residues. It is also worth noting that the first peak eluted in the multifunctional region was identified as pyridinoline, the naturally occurring cross-link in collagen the formation of which does not involve an interaction with aldehydic sugars.

In the same paper where Bailey et al. [28] reported on the presence of the pentosidine-like compounds K_1–K_4 they also isolated, from the in vitro incubates of rat tail tendons, high-molecular-mass amino acids; the incubated tendons were washed with phosphate buffered saline, hydrolysed routinely in 6 *M* HCl and the hydrolysate was chromatographed on TSK-40S gel (Fractogel) column 90×1.6 cm using 0.05 *M* acetic acid as eluent at a rate of 1 ml/min. The high-molecular-mass material (with molecular mass higher than 551 marked by adenosine triphosphate) was submitted to a technique developed for identification of lysinealdehyde derived cross-links [10]. The high-molecular-mass amino acids were pre-concentrated on the cellulose column as described for the fluorescent non-pentosidine components and separated on an amino acid analyser (Pharmacia Alpha Plus) using a modified gradient and ninhydrin detection. The gradient was extended using an additional 38 min step of 0.5 min citrate–borate buffer pH 8.6 and the column was maintained at 90°C. Known cross-linking amino acids of collagen elute between phenylalanine and hydroxylysine. A typical chromatogram showing the position of "physiological" cross-linking amino acids and the non-fluorescent cross-linking glucose derived product (as proven by using ^{14}C glucose) is shown in Fig. 21 (it emerges just before ammonia with a retention

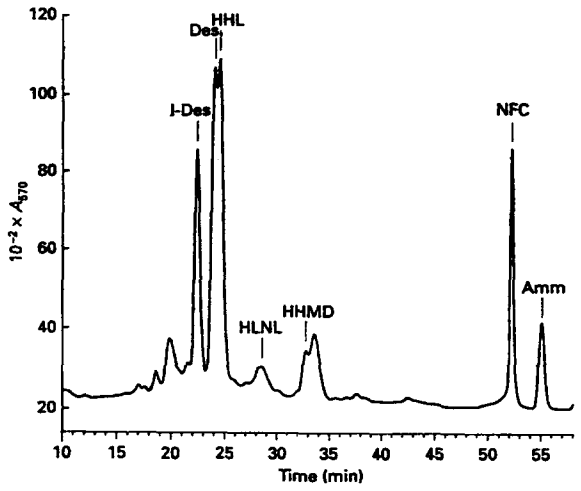


Fig. 21. HPLC chromatogram of non-fluorescent cross-linking amino acids (ninhydrine detection) after prefractionation using CF1 cellulose column. Abbreviations: I-Des, isodesmosine; Des, desmosine; HHL, histidinohydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; NFC, non-fluorescent component; Amm, ammonia. Reproduced from Bailey et al. [28] with permission of the Biochem. Soc.

~52 min). Also this compound can be found in diabetic tissue.

14. LC–MS and matrix assisted laser desorption–ionization-MS of heterocyclic glycation adducts

Matrix assisted laser desorption–ionization-MS (MALDI-MS) has been introduced into the field of protein post-translational modifications by the Italian group in Padova [62–65]. In these papers both model glycation reactions regarding bovine serum albumin and ribonuclease as the proteins involved and glucose or fructose as the sugar component were investigated under in vitro conditions. However, some in vivo results are also available from these papers.

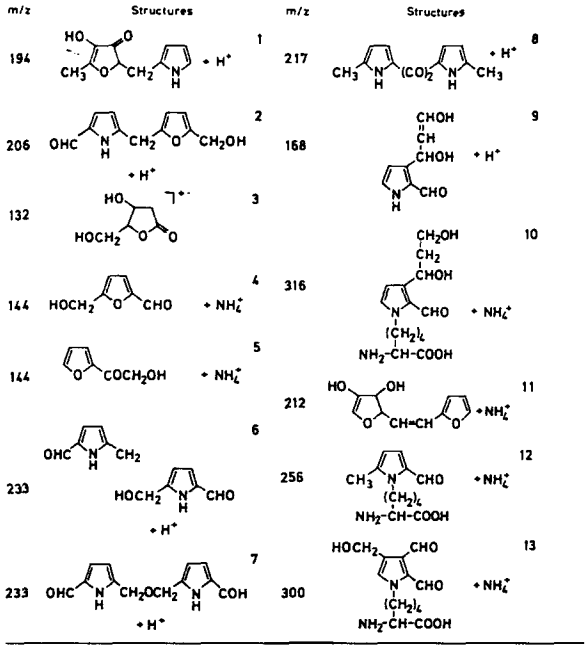
In the original version a HPLC–MS approach was used [62]. In vitro glycated bovine serum albumin was separated on a μ-Bondapak C_{18} reversed-phase column using gradient elution with an acetonitrile–water ratio progressively changed from 1:99 to 20:80 over 30 min (flow-rate 2 ml/min). The sample was prepared by incubating bovine serum albumin with a ten-fold mass excess of glucose at 37°C under sterile conditions at pH 7.5. Before chromatography the sample was digested by proteinase K (Sigma) using a protein–enzyme ratio 10:1. Detection of the eluate was done by UV at 320 nm. All MS measurements were done under plasmaspray conditions with a probe temperature of 250°C and source temperature 240°C. The chromatographic conditions used for HPLC–MS employed the addition of 0.1 *M* ammonium acetate buffer. This approach allowed the analysis of glycation-modified peptides.

A number of different compounds were detected and for most of them probable structures were reported on the basis of literature data and molecular mass assignments. Tentative formulae of the arising products are summarized in Table 2.

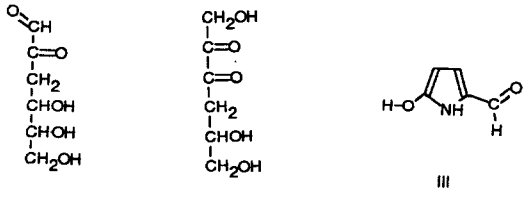
The disadvantage of this approach is that plasmaspray ionization leads to abundant molecular ions but is characteristic in paucity of fragment ions, which, consequently, causes problems in the identification of the ionized species observed.

However, at least one conclusion can be clearly drawn from this investigation, namely, the role of deoxyosones (for direct assay of these compounds see Section 12 of this review) in formation of the structures observed. Typically lactone 3 in Table 2 can be produced via 3-deoxyosone cleavage; the $[M+NH_4]^+$ ion of which can be also traced in the spectrum (compound I.).

Table 2
Possible structures identified by HPLC–MS



Reproduced from Lapolla et al. [62] with permission of John Wiley&Sons, Ltd.

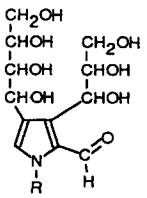


I II III

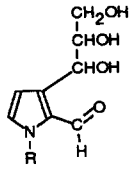
Cyclization of compound I and its dehydration leads to hydroxymethylfurfural (item 4 in Table 2). Hydroxyacetylfuran (item 5 in Table 2) can be

considered a decomposition product of 4-deoxyosone (compound II). Structures 6 and 7 in Table 2 can be both derived from 5-hydroxy-pyrrole-2-carboxyaldehyde through secondary dimerization (compound III).

The structures at positions 1 and 10 of Table 2 can be explained by the reaction an of aminoketose and 3-deoxyosone leading to substituted pyrroles-like compounds IV and V).

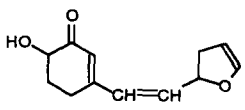


IV

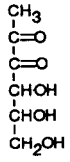


V

A yellow brown product of the structure (VI) has been described by Ledl and Severin [66] and can be derived in a condensation reaction from 1-deoxyosone (VII).



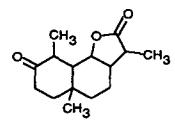
VI



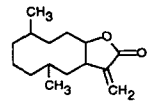
VII

In experiments using electrospray ionization in the evaluation of glycation reactions with ribonuclease as model protein under physiological conditions in vitro [67], it was demonstrated that subsequent to protein glycation a series of cross-linking products are generated, however severe degradation processes of the protein substrate take place after glycation as cross-linking proceeds.

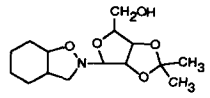
When using pyrolysis GC–MS with glycated albumin as sample it was possible to assign tentatively the structures of some compounds; however, the origin of a number of the compounds identified on the basis of mass spectra library search was difficult to trace except furan, dihydrofuran-hydroxy-2,5-anisyl-3,4-dimethylpyrrole, 5-dodecyl-dihydro-2(3H)-furanone and the following ones (VIII–XI) [68].



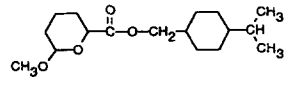
VIII



IX



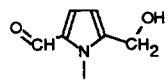
X



XI

Obviously the pyrolysis GC–MS approach adds considerably to the already complex protein (amino acid) derivatives arising after glycation reactions and seems to represent a dead-end in the investigation of these products.

On the other hand a milder approach, i.e., using MALDI-MS with bovine pancreatic ribonuclease not only revealed differences in reactivity between fructose and glucose (glucose being faster in the early stage of glycation while formation of advanced glycation products was faster with fructose-modified protein) [69]. The abundant structure revealed by this technique is monotopical binding of compound XII



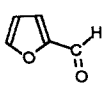
XII

in both fructose- and glucose-modified proteins. It is noticeable that this compound possesses an aldehydic group which is likely to cause further polymerization of the protein at a more advanced stage of the glycation reaction.

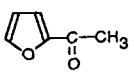
So far the MS approach has revealed the presence of pyrrole derivatives in glycated proteins. These appear to be formed after rather short term in vitro reaction. Clearly it would be interesting to have an adequate marker of the so-called advanced glycation end products. According to the results of Lapolla et al. [65] the originally proposed 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) [70] appears not to be the appropriate marker at least as far as advanced glycation products in collagen is concerned as much

as its artificial nature was proven beyond any doubt. On the other hand it seems to be well documented that both in vitro glycated and non-glycated (subjected to the action of glucose in vivo) collagen samples contain some furoyl derivatives which are not (or only slightly) affected by acid hydrolysis and it appears evident that the in vitro modifications (influenced by various model compounds) and in vivo formation of advanced glycation products runs along different pathways.

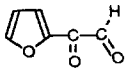
The furoyl structures proposed to occur in collagenous structures can be summarized as 2-furaldehyde (XIII), 2-acetylfuran (XIV) and furanglyoxal (XV) [71].



XIII



XIV



XV

It is worth noting that 2-furaldehyde, 2-acetylfuran and furanglyoxal (*m/z* 96, 110 and 124 respectively) were previously detected in in vitro glycation of serum albumin and polylysine. It was also observed that the above ions are more abundant in samples subjected to acid hydrolysis and neutralization which indicates the importance of these two steps with regard to any analysis directed towards direct estimation of advanced end glycation markers.

15. Conclusions

Though under the mild in vivo conditions the number of arising compounds in post-translational non-enzymatic reactions of proteins (e.g., glycation) is limited in comparison to the Maillard products relevant to food chemistry where the adducts arise at elevated temperatures, still the result is a multicomponent mixture which is very difficult to separate. Analysis (and separation) of compounds which are hydrolytically stable represents a way of simplifying the arising mixture and eliminating the role of, e.g., simultaneous forming of the same adducts in several regions of the protein molecule. In our opinion analysis of hydrolytically stable adducts offers valuable (though a bit one-sided) information about the processes affecting a particular protein during its lifetime. End hydrolytically stable products represent important markers of the modification processes.

Because the information about the analysis of such marker adducts is scattered throughout predominantly medically oriented literature and escaped the attention of professional analysts so far, we have attempted in this review to summarize the current separation procedures applicable to the main hydrolytically stable compounds, namely, Amadori adducts, pentosidine (and pentosidine related compounds), pyrraline, N-carboxymethylamino acids, amino acid hydantoins and stabilized dicarbonyl intermediates.

Acknowledgements

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