

# Study of posttranslational non-enzymatic modifications of collagen using capillary electrophoresis/mass spectrometry and high performance liquid chromatography/mass spectrometry

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## Abstract

The depository effects that occur in slowly metabolized proteins (typically glycation) are very difficult to assess, owing to their extremely low concentration in the protein matrix. Collagen accumulates reactive metabolites through reactions that are not regulated by enzymes. A typical example of these non-enzymatic changes is glycation (the Maillard reaction, the formation of advanced glycation end products), resulting from the reaction of the oxo-group of sugars with the  $\epsilon$ -amino group of lysine and arginine. Collagen samples (type I) as a test protein were incubated separately with glucose, ribose and malondialdehyde. Collagen was fragmented with cyanogen bromide and then digested with trypsin. This peptide digest was separated by CE, CE–MS/MS, and HPLC–MS/MS. An ion trap MS was used and MS conditions were optimized for both methods. These on-line CE–MS/MS and HPLC–MS/MS couplings made it possible to discover specific modifications such as ( $N^{\epsilon}$ -(carboxymethyl)-lysine) in the precise location in the structure of collagen corresponding to posttranslational non-enzymatic modifications. A new CE–MS/MS technique for peptide analysis was developed, and applied in the identification of posttranslational modifications in slowly metabolized test proteins.

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**Keywords:** Collagen; Capillary electrophoresis; Posttranslational modification; CE/MS; Proteomics

## 1. Introduction

The depository effects of long-lived proteins (typically collagen) are very difficult to assess. They accumulate reactive metabolites through reactions that are not regulated by enzymes. A typical example of these non-enzymatic changes is glycation (the formation of advanced glycation end products—AGE or Maillard products) resulting from the reaction of the oxo-group of sugars with the free amino group of the protein (amino group of lysine or arginine). The initial labile Schiff base and Amadori products undergo a series of rearrangement, dehydration, and fragmentation reactions to produce more complex and irreversibly covalently cross-linked structures [1,2]. Some examples of AGEs found in glycated proteins include imidazolone A ( $\Delta m/z$ : +144.04) and

B ( $\Delta m/z$ : +142.03),  $N^{\epsilon}$ -(carboxymethyl)-lysine (CML,  $\Delta m/z$ : +58.01),  $N^{\epsilon}$ -(carboxymethylhydroxy)-lysine (CMhL,  $\Delta m/z$ : +74.00),  $N^{\epsilon}$ -(carboxyethyl)-lysine (CEL,  $\Delta m/z$ : +72.02), pyrroline ( $\Delta m/z$ : +108.02), 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP,  $\Delta m/z$ : +270.07),  $N^{\delta}$ -(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidin,  $\Delta m/z$ : +80.03),  $N^{\delta}$ -(4-oxo-5-dihydroimidazol-2-yl)-L-ornithine or 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine ( $\alpha$ NFC-1,  $\Delta m/z$ : +39.99), and  $N^{\delta}$ -(5-methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine or  $N^{\delta}$ -(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine or 2-iminoimidazolidinone ( $\beta$ NFC-1,  $\Delta m/z$ : +54.01). The most widely known cross-linked structure is pentosidine (an imidazo[4,5-b]pyridinium molecule comprised of ribose, a lysine and an arginine residue with  $\Delta m/z$ : +58.03), however, since its discovery in 1989, a few other structures were proposed and identified, including crossline ( $\Delta m/z$ : +252.11) and glucosepane ( $\Delta m/z$ : +76.03) [3–7].

We have to stress that there are two mechanisms of posttranslational modification of proteins—those that are enzymatically and non-enzymatically derived. The enzyme-derived cross-

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1	MFSFVLDRLLL	LLLAATALLT	HGQEEGQEEG	QEEDIPEVTC	VQNGLRVHDR	50
51	DVWKFPVPCQI	CVCDNGNVLC	DDVICDELKD	CENAKVPTDE	CCPVCPEGQE	100
101	SPTDQETTGV	EGPKGDTGFR	GPRGPAGPFG	RDGI PGQGL	PGFPFGPFP	150
151	GGPGLGGNFA	FQLSYGYDEK	STGISVPGPM	GPSGPRGLPG	<b>PPGAPGPQGF</b>	200
201	<b>QGGPEPEPEP</b>	<b>GASGFMGPRG</b>	<b>PPGPPGKNGD</b>	<b>DGEAGKPRP</b>	<b>GERGPPGPGG</b>	250
251	<b>ARGLPGTAGL</b>	<b>PGMKGHRGFS</b>	<b>GLDGAKGDAG</b>	<b>PAGPKGEPGS</b>	<b>PGENGAPGQM</b>	300
301	<b>GPRGLPGERG</b>	<b>RPGAPGPAGA</b>	<b>RGNDGATGAA</b>	<b>GPPGPTGPAG</b>	<b>PPGFPGAVGA</b>	350
351	<b>KGEGGPQGPR</b>	<b>GSEGPQGVVG</b>	<b>EPGPPGPAGA</b>	<b>AGPAGNPGAD</b>	<b>GQPGAKGANG</b>	400
401	<b>APGIAGAPGF</b>	<b>PGARGPSGPQ</b>	<b>GPSGPPGPKG</b>	<b>NSGEPGAPGS</b>	<b>KGDTGAKGEP</b>	450
451	<b>PTGIQGGPPG</b>	<b>PAGEEGKRGGA</b>	<b>RGEPPAGLP</b>	<b>GPPGERGGPG</b>	<b>SRGFPADGV</b>	500
501	<b>AGPKGPAGER</b>	<b>GAPGPAGPKG</b>	<b>SPGEAGRPE</b>	<b>AGLPGAKGLT</b>	<b>GSPGSPGPDG</b>	550
551	<b>KTGPPGPAGQ</b>	<b>DGRPGPPPPP</b>	<b>GARGQAGVMG</b>	<b>FPGPKGAAGE</b>	<b>PKKAGERVVP</b>	600
601	<b>GPPGAVGPAG</b>	<b>KDGEAGAQQP</b>	<b>PGPAGPAGER</b>	<b>GEQGPAGSPG</b>	<b>FQGLPGPAGP</b>	650
651	<b>PGEAGKPGEQ</b>	<b>KVPGDLGAPG</b>	<b>PSGARGERGF</b>	<b>FGERGVQGGP</b>	<b>GPAGPRGANG</b>	700
701	<b>APGNDGAKGD</b>	<b>AGAPGAPGSQ</b>	<b>GAPGLQMPG</b>	<b>ERGAAGLPGP</b>	<b>KGDRGDAGPK</b>	750
751	<b>GADGAPGKDG</b>	<b>VRGLTGP IGP</b>	<b>PGPAGAPGDK</b>	<b>GEAGPSGPAG</b>	<b>PTGARGAPGD</b>	800
801	<b>RGEPGPPGPA</b>	<b>GFAGPPGADG</b>	<b>QPGAKGEPGD</b>	<b>AGAKGDAGPP</b>	<b>GPAGPAGPPG</b>	850
851	<b>PIGNVGPAGP</b>	<b>KGARGSAGPP</b>	<b>GATGFPGAAG</b>	<b>RVGPPGPGSN</b>	<b>AGPPGPPGPA</b>	900
901	<b>GKEGSKGPRG</b>	<b>ETGPAGRPE</b>	<b>VGPPGPPGPA</b>	<b>GEKGAPGADG</b>	<b>PAGAPGTPGP</b>	950
951	<b>QGLAGQRGVV</b>	<b>GLPGQRGERG</b>	<b>FPGLPGPSGE</b>	<b>PGKQPPSGAS</b>	<b>GERGPPGPMG</b>	1000
1001	<b>PPGLAGPPPE</b>	<b>SGREGAPGAE</b>	<b>GSPGRDGSPP</b>	<b>AKGDRGETGP</b>	<b>AGPPGAPGAP</b>	1050
1051	<b>PIGNVGPAGP</b>	<b>KSGDRGETGP</b>	<b>AGPAGPIGPV</b>	<b>GARGPAGPQG</b>	<b>PRGDKGETGE</b>	1100
1101	<b>QGDRGIKGRH</b>	<b>GFSGLQGGPPG</b>	<b>PPGSPGEGQP</b>	<b>SGASGPAGPR</b>	<b>GPPGSAGSPG</b>	1150
1151	<b>KDGLNGLPGP</b>	<b>IGPPGPRGRT</b>	<b>GDAGPAGPPG</b>	<b>PPGPPGPPGP</b>	<b>PSGGYDLSEFL</b>	1200
1201	<b>FQPPQEK AHD</b>	<b>GGRYRADA</b>	<b>NVVRDRDLEV</b>	<b>DTTLKLSLQQ</b>	<b>IENIRSPFGS</b>	1250
1251	<b>RKNPARTCRD</b>	<b>LKMCHSDWKS</b>	<b>GEYWDENQGS</b>	<b>CNLDAIKVFC</b>	<b>NMETGETCVY</b>	1300
1301	<b>PTQPSVAQKN</b>	<b>WYISKNPKEK</b>	<b>RHVWYGESMT</b>	<b>GGFQFEYGGQ</b>	<b>GSDPADVAIQ</b>	1350
1351	<b>LTFRLMSTE</b>	<b>ASQNITYHCK</b>	<b>NSVAYMQQT</b>	<b>GNLKKALLLQ</b>	<b>GSNEIIRAE</b>	1400
1401	<b>GNSRFYSVT</b>	<b>YDGTSHGTA</b>	<b>WGKTVIEYKT</b>	<b>TKTSRLPIID</b>	<b>VAPLVDVAGPD</b>	1450
1451	<b>QEPFDVGP A</b>	<b>CFL</b>				1463

Fig. 1. Sequence of the bovine collagen (P02453 [49], alpha 1, type I) before glycation. The matched peptides are in bold. Propeptides are highlighted by small font.

linking in which the *N*- and *C*-terminal lysines are oxidatively deaminated by lysyl oxidase to form lysyl aldehyde is now well established [8]. The non-enzymatic modification by sugars is known as glycation and plays a central role in the pathogenesis of ageing [9]. The glycation of proteins, including collagen, is initiated by the reversible Schiff base condensation of the oxo-group of the sugar (e.g. glucose) with amino groups, such as lysine side-chains, followed by a largely irreversible rearrangement that yields ketoamine Amadori products [10]. There are a number of situations in which the proteins present in tissues undergo minor non-enzymatic modifications, which are extremely difficult to assess.

Glycation products lead to tissue damage through a series of mechanisms, of which the above mentioned crosslink formation, intracellular accumulation, and interactions with specific receptors are the most important. AGEs can affect the interaction of proteins with cells, e.g. by altering the charge profile of the protein molecule and, more importantly, can modify the physicochemical properties of the protein by the formation of intermolecular cross-links. With fibrous collagen, this additional cross-linking has the effect of rendering the protein less soluble, more resistant to enzyme digestion and less flexible.

The term collagen applies to a broad group of proteins. They are a family of extracellular matrix proteins that play a dominant role in maintaining the structure of various tissues and also have many other important functions (for example, adhesion, tissue remodelling) [11,12].

Collagens possess some typical features: they consist of three polypeptide chains (called  $\alpha$  chains) and contain at least one domain composed of a repeating tripeptide sequence:

-Gly-X-Y-. The protein chains are coiled together into a left-handed helix and are then wound around a common axis to form a triple helix. The typical presence of glycine at every third position is essential for this packing to a coiled-coil structure and is one of the ways to determine the presence of collagen in a tissue sample. Triple helical molecule of collagen is matured after the cleavage of propeptides ( $\alpha$  chain consists of only 1054 aminoacids (Fig. 1)) [13]. At present, there are at least 28 known collagen types in vertebrates, containing a total of 42 distinct  $\alpha$  chains [14].

Collagen, which is a typical long-life protein, loses flexibility and enzymatic digestivity in parallel with aging [15]. Similar changes in collagen are known to occur in diabetic patients [16].

Collagen is the main structural protein in the body and therefore posttranslational modifications have marked effects on vital tissue and organ function. These peptide changes are associated with inter- and intra-molecular cross-linking and side-chain modifications. Cross-linking involves two different mechanisms, a precise enzymatic process during development and maturation, and a subsequent non-enzymatic adventitious reaction with reactive compounds (e.g. sugars) during ageing. The chemical modifications and damage to proteins and other biomolecules are thought to be produced during the later stages of life of the parent organism. Acceleration of these reactions as a result of hyperglycemia in diabetes and the consequent increase in the structural and functional modification of long-lived proteins are implicated in the pathogenesis of the chronic complications of diabetes [17].

In order to identify such alterations, a deep enzymatic fragmentation resulting in a set of small peptides is often used.

The changes in these peptides caused by modifications can be observed in their chromatographic and electrophoretic profile (peptide mapping). In protein chemistry, peptide mapping, using both chromatographic and electromigratory methods, is a widely-used approach [18].

For the bioanalysis of peptides and proteins in biological samples, one-dimensional separation methods often do not have sufficient selectivity. In these cases, multidimensional methods may offer a higher selectivity [19–25]. LC/CE coupling instead of an LC/LC combination is generally more orthogonal. Also, the high separation speed of CE is advantageous, especially in the development of compatible multidimensional systems. However, especially when trace level concentrations have to be determined, the analyte concentration is often insufficient to be detected. The on-line coupling of LC and CE is complicated by two additional problems: the large difference between the peak volume of LC and the injection volume of CE and the way the electrode is used at the coupling end of the capillary when the voltage is applied [26–33].

Mass spectrometry (MS), especially when combined with HPLC or CE, is by far the most powerful technique when the determination of both identity and site of modification is required and it has been successfully employed in investigating non-enzymatic protein glycation, a process relevant to diabetic disease. Several ionization techniques such as FAB-MS [34], ESI-MS [4,5,34,35] and MALDI-MS [3,6] have been used to identify or quantify the AGEs in protein digests. In addition to that, MS and MS<sup>n</sup> experiments in combination with proteomics software or PERL script search algorithms allow the determination of the modification sites [6,35,36], while high resolution MS can provide information about the structure and can therefore be helpful in identifying new AGEs [37].

The technique most frequently used in proteomics is the off-line combination of two-dimension gel electrophoresis and mass spectrometry. As for on-line coupling, the most common technique is an HPLC–MS approach [38]. The separation of peptides by capillary electrophoresis (CE) is more effective than HPLC [39]. There are currently many methods of peptide/protein analysis using this technique, but the most promising approach in proteomic research is CE–MS coupling [40]. The first reported CE–ESI–MS interface did not incorporate a supplemental fluid [41,42] but at present the most widely used interfaces designed for CE–ESI–MS are based on the sheath-flow interface [43]. The main disadvantage of this approach is the high dilution of the peptide/protein mixture by the sheath liquid, and for this reason the sensitivity of this technique is lower.

This work is the continuation of our previous research of collagen in which we used offline combination of HPLC and CE [44]. In this study, peptides were generated using combined CNBr and trypsin digestion of collagen. The peptides were subsequently analyzed using CE–MS/MS and HPLC–MS/MS in an approach to identify posttranslational modifications of collagen by sugars. While previously published methods for collagen analyses by CE used unvolatile buffers (typically phosphate buffer), in this paper we present method involving volatile background electrolyte suitable for CE/MS analyses.

## 2. Material and methods

### 2.1. Chemicals

All chemicals used were either of analytical grade or the highest available purity. Phosphate (NaH<sub>2</sub>PO<sub>4</sub>) was obtained from Lachema (Brno, Czech Republic), acetic acid from Lach-Ner (Neratovice, Czech Republic), 2-mercaptoethanol from Merck (Darmstadt, Germany), collagen type I (bovine Achilles tendon), cyanogen bromide, glucose, ribose, malondialdehyde and sodium 1-heptane-sulfonate were obtained from Sigma (St. Louis, MO, USA), trifluoroacetic acid and acetonitrile (HPLC gradient grade) were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO, USA). All solutions were prepared in MilliQ Water (Millipore, Bedford, MA, USA).

### 2.2. Instruments

The HPLC apparatus used was an HP 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment, and a diode array detector. The instrument was controlled, and the data collected and manipulated by the program ChemStation B.01.03. It was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra); for details on the instrument conditions, see conditions for HPLC–MS.

Capillary zone electrophoresis (CZE) was performed on a P/ACE 5000 instrument (Beckman Instruments, Fullerton, CA, USA) with UV detection set to 214 nm. The instrument was controlled, and the data collected and manipulated by the Beckman P/ACE Station program version 1.21. A fused-silica capillary of 100 cm total length, 75 μm I.D. was used for all experiments. The instrument was coupled to the ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra) using a grounded needle carrying a flow of sheath liquid; for details about the instrument conditions, see conditions for CE–MS.

Analysis of MS/MS data (peptide/protein identification) was carried out using the software SpectrumMill (v.3.02, Agilent). The searches were performed in the full protein databases SwissProt and NCBIInr and then on the data extracted from these databases.

### 2.3. Sample preparation

#### 2.3.1. Preparation of posttranslationally modified collagen

Collagen was suspended in a pH 7.4 0.2 M phosphate buffer at a concentration of 10 mg/5 ml and incubated at 37 °C for 7 days with one of the following oxo-compounds: 180 mg/10 ml glucose, 150 mg/10 ml ribose or 164 μl/10 ml malondialdehyde. A control group of collagen samples was incubated under the same conditions in only the buffer.

#### 2.3.2. Protein used

Commercially available collagen type I was used. Each group contained at least five samples.

1. Control collagen (group Cc) samples of collagen incubated for 7 days at 37 °C.
2. Glucose collagen (Gc)—samples of collagen incubated with glucose for 7 days at 37 °C.
3. Ribose collagen (Rc)—samples of collagen incubated with ribose for 7 days at 37 °C.
4. Malondialdehyde collagen (Mc)—samples of collagen incubated with malondialdehyde for 7 days at 37 °C.

After incubation, all collagen samples were centrifuged (10,000 × *g*) and subsequently washed-off with water to remove the remaining modifiers and buffer.

### 2.3.3. Collagen digest

To obtain peptide maps, the incubated collagen was reduced with mercaptoethanol then collagen was digested with CNBr and subsequently with trypsin (collagen/trypsin ratio, 50:1, w/w). The samples were incubated at 37 °C for 48 h in a trypsin buffer (200 mmol/L NH<sub>4</sub>HCO<sub>3</sub>, pH 7.8). Blank samples were prepared by incubating the enzyme solution alone under identical conditions. After the incubation was complete, the vials were centrifuged for 5 min at 2000 × *g* and the supernatants removed to other vials and stored at −18 °C.

## 2.4. Separation conditions

### 2.4.1. Conditions for HPLC–MS

Chromatographic separation was carried out on a Jupiter Proteo 90 A, 250 mm × 2 mm (Phenomenex, Torrance, CA, USA).

Separation was achieved by a linear gradient between mobile phase A (water–trifluoroacetic acid, 100:0.03, v/v) and B (acetonitrile–trifluoroacetic acid, 100:0.025, v/v). Separation was initiated by running the system isocratically for 2 min with 2% mobile phase B, followed by a gradient elution to 35% B at 40 min. Finally the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with 2% of mobile phase B for 10 min. The flow-rate was 0.25 ml/min, the column temperature was held at 25 °C and UV absorbance detection was done at 214 nm.

Atmospheric pressure ionization–electrospray ionization (API–ESI) positive mode ion-trap mass spectrometry was used. Operating conditions: drying gas (N<sub>2</sub>), 10 l/min; drying gas temperature, 350 °C; nebulizer pressure, 25 psi; ions were observed over the mass range *m/z* 100–2200 (MS—standard mode, MS/MS—enhanced mode). Analysis was done in auto MS/MS mode (10 precursor ions, excluded after 2 spectra for 0.5 min). The Spectrum-Mill autovalidation of spectra was performed using default settings, but all spectra were then evaluated manually.

### 2.4.2. Capillary electrophoresis

Separations were run at 10 kV in a bare fused silica capillary (57 cm, 50 cm to the detector, 75 μm I.D.) at 20 °C. UV absorbance at 214 nm was used for detection. A pH 2.5, 100 mmol/l phosphate buffer was used as the background electrolyte with sodium 1-heptane-sulfonate as the ion-pairing agent (100 mmol/l).

Injection was done hydrodynamically by overpressure (3.45 kPa, 10 s). Before analysis, the capillary was conditioned with the run buffer (4 min). The capillary was flushed stepwise with the run buffer (1 min), water (1 min), 1 mol/l NaOH (3 min), water (1 min), 3 mol/l HCl (1 min) and water (1 min) every day.

### 2.4.3. Conditions for capillary electrophoresis-MS

Separations were run at 15 kV in a bare fused silica capillary (100 cm, 75 μm I.D.) at 20 °C. The samples were injected hydrodynamically (50 s, 3.45 kPa). UV absorbance at 214 nm was used for detection. 3.3 M acetic acid was used as the background electrolyte for all separations. The instrument was coupled to the ion-trap mass spectrometer using a grounded needle carrying a flow of sheath liquid (5 mM ammonium acetate/isopropanol 1:1 at a flow-rate of 3 μl/min).

Before running the sample, the capillary was washed with 1 mol/l NaOH, followed by a 20 min wash with water and 20 min wash with 1 mol/l HCl. Then it was washed with water again for 20 min and finally with the running buffer (20 min). Between runs, the capillary was merely rinsed with the running buffer (5 min).

The conditions used with the MS instrument were the same as with HPLC–MS, except for those at the interface: drying gas (N<sub>2</sub>): 8 l/min; drying gas temperature: 150 °C; nebulizer pressure: 5 psi; capillary voltage: 3500 V; ions were observed over the mass range *m/z* 100–1500; analysis was done in auto MS/MS mode (10 precursor ions, excluded after 2 spectra for 0.5 min).

### 2.4.4. *t*-Test evaluation of the electropherograms

The collagen incubated with ribose, malondialdehyde (MDA), and glucose was compared with the control collagen. Capillary electrophoresis peptide profiles of collagen obtained after CNBr/trypsin cleavage were compared: 46 peptide peaks of the profile were quantified using the valley-to-valley integration method, and the total peak area was calculated (UV detection at λ = 214 nm). The total peak area was used as 100%. The area percentage was calculated for each peak, and the peak area difference (if any) was calculated using a conventional *t*-test (significant change (*P* < 0.05) compared to the controls) [45].

## 3. Results

### 3.1. CE-UV

Fig. 2 demonstrates the total UV (214 nm) electrophoretic profile of the collagenous peptides obtained by CNBr cleavage and subsequent trypsin digest of all collagen groups. Significant differences in the profiles revealed by valley-to-valley integration relative to the controls are indicated with arrows. Because it is assumed that not many (if any at all) of the separated peaks represent a pure peptide, the results can only be considered as qualitative differences (the differences were evaluated by a simple *t*-test of the compared peak areas). Both increased and decreased peaks (indicated with arrows) were observed. All

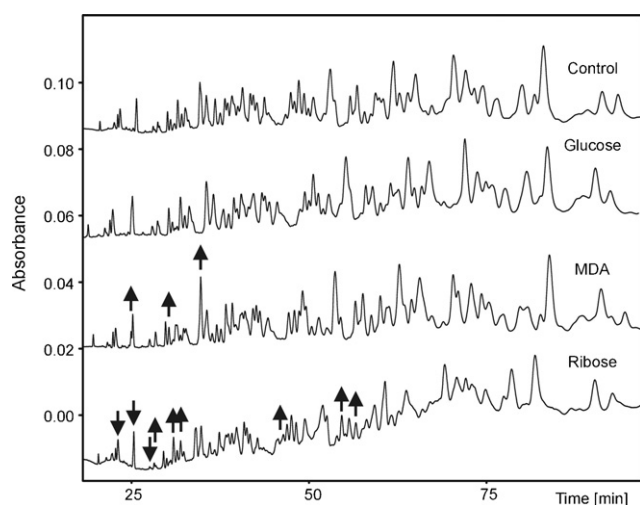


Fig. 2. Capillary electrophoresis peptide profiles arising after CNBr/trypsin cleavage. Collagen incubated with ribose (Ribose), malondialdehyde (MDA), and glucose (Glucose) was compared with control collagen (Controls). Increased and decreased peaks are indicated with arrows (UV detection at  $\lambda = 214$  nm).

profiles were normalized with the respect to the dominant peaks, which were used as internal standards to negate migration time differences between individual runs. The peptide map of the preparation obtained from collagen treated with ribose differed most from the control preparation (9 changes), MDA samples showing the second most differences (3 changes) and treatment with glucose showing the least, with no significant difference. Ribose was confirmed as the most reactive agent in comparison with other two compounds.

### 3.2. MS/MS analyses

LC–MS/MS and CE–MS/MS analyses were performed in order to identify the changes caused by glycation. The identification of AGEs that were thought to be present in glycated collagen was carried out using Spectrum Mill software.

Not only we were able to determine the type of modification, but we also were able to determine the precise site of modification, i.e. a particular amino acid in the sequence where glycation takes place.

As the purpose of this work was primarily to identify the type and position of posttranslational modifications, no quantitative analyses have been performed.

#### 3.2.1. HPLC–MS/MS

We can divide the HPLC–MS analyses of artificially glycated collagens into two groups: (i) glycation by sugars and (ii) glycation by lipid-peroxidation product (malondialdehyde).

**3.2.1.1. Ribose and glucose-modified samples.** Samples glycated by glucose showed similar results to those glycated by ribose. The main type of modification was CML. Although a few other possible modifications were found, such as imidazolone A, they were either not present in all the glycated samples or, in the case of lysine at position 1095 (K1095) modification by CEL, they were found in a non-glycated collagen.

We were able to find two marker ions in both samples glycated by ribose or glucose—with  $m/z$  of 857.4 and 517.3, belonging to two peptides with CML-modified lysines at positions 504 and 1032, respectively (Table 1).

The original non-glycated peptide sequences from which these two marker ions originate were GFPGADGVAGPK where **P** is a hydroxylated proline P495 and DGSPGAK with hydroxylated proline P1029 (Table 1). In non-glycated collagen, the sample is digested by trypsin at K504 and K1032. However, when modified by CML, these peptides could not be digested at their modified lysines and therefore the digestion occurred at the next arginine in the sequence. The additional sites of CML modification in the samples glycated by ribose were K750, K861 and K519.

The MS/MS spectrum of a non-glycated peptide and that of glycated peptide are compared for K504 modification in Figs. 3 and 4a, respectively, their assignment can be found in Tables 2 and 3.

The peptide maps for glycated and non-glycated collagen are in good accordance with respect to the cleavage sites in non-modified peptides and high sequence coverage. For example, for a ribose-modified sample, the matched peptides covered 77% of the matured protein (after the propeptides' split-off) with 29 lysines out of 38 identified (Fig. 5). The same sequence coverage was obtained for control sample (Fig. 1).

Table 1

Peptides found by HPLC–MS/MS in non-glycated (top) and glycated (bottom) collagen (**P**: hydroxylated proline, **K**: CML-lysine)

Site of modification	Peptide	RT (min)	$m/z$ Measured
Glycation with ribose and glucose common to both sugars			
P: 495	GFPGADGVAGPK	24.7	544.8
P: 495; K: 504	GFPGADGVAGPKGPAGER	24.9	857.4
P: 1029	DGSPGAK	3.2	647.3
P: 1029; K: 1032	DGSPGAKGDR	6.1	517.3
Glycation with ribose unique to ribose			
P: 756	GADGAPGK	5.5	688.3
P: 756; K: 750	GDAGPKGADGAPGK	14.3	636.8
P: 840, 851, 858, 860	GDAGPPGPAGPAGPPGPIGNVGA <b>PGPK</b>	25.9	775.4
P: 858, 860; K: 861	VGAP <b>PGPK</b> GAR	15.5	500.2
P: 513	GAP <b>PG</b> PAGPK	15.1	384.2
P: 513, 522; K: 519	GAP <b>PG</b> PAGPK <b>KGSP</b> GEAGR	16.8	777.3

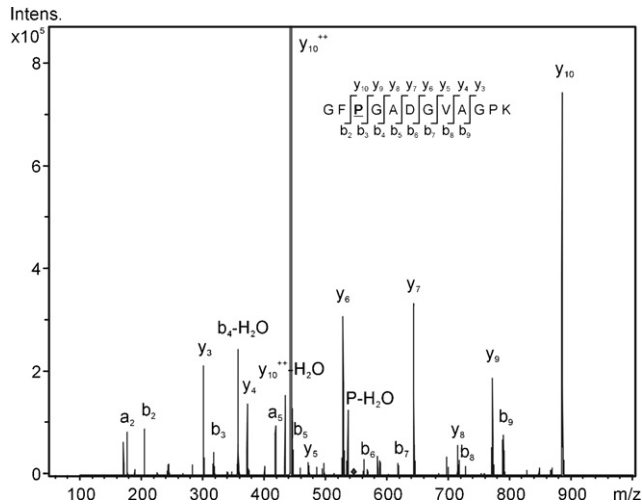


Fig. 3. Fragmentation mass spectra measured by HPLC-MS/MS of non-glycated peptide ( $MH^+ = 1088.5$ ,  $m/z = 544.8$ ,  $z = 2$ ) with hydroxylated proline P495.

Table 2

Assignment of MS/MS peaks for original peptide (before glycation)

Fragment ion ( $m/z$ )	Ion	Theoretical mass (Da)	Delta mass (Da)
<b>y-Ions</b>			
301.12	$y_3$	301.20	-0.08
372.20	$y_4$	372.24	-0.04
471.29	$y_5$	471.30	-0.01
528.35	$y_6$	528.33	0.02
643.30	$y_7$	643.35	-0.05
714.39	$y_8$	714.39	0.00
771.37	$y_9$	771.41	-0.04
884.43	$y_{10}$	884.46	-0.03
442.83	$y_{10}^{2+}$	442.74	0.09
433.97	$y_{10}^{2+}-H_2O$	433.73	0.24
<b>b-Ions</b>			
788.05	$b_9$	788.36	-0.31
717.24	$b_8$	717.33	-0.09
618.23	$b_7$	618.26	-0.03
561.08	$b_6$	561.24	-0.16
446.12	$b_5$	446.21	-0.09
357.13	$b_4-H_2O$	357.15	-0.17
318.05	$b_3$	318.15	-0.10
204.87	$b_2$	205.10	-0.23
<b>a-Ions</b>			
177.02	$a_2$	177.10	-0.08
418.11	$a_5$	418.21	-0.10

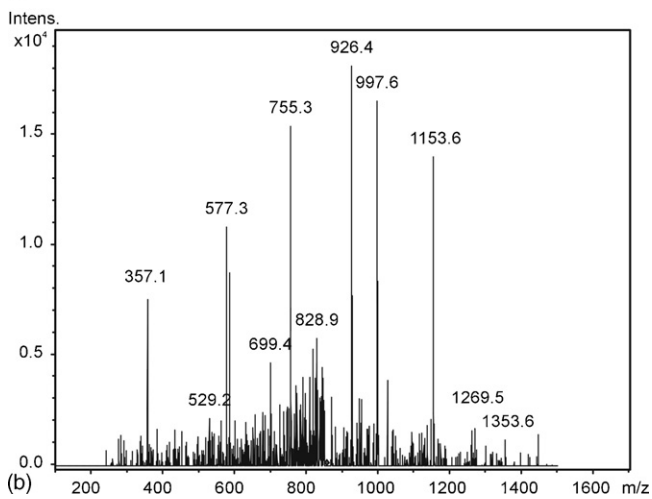
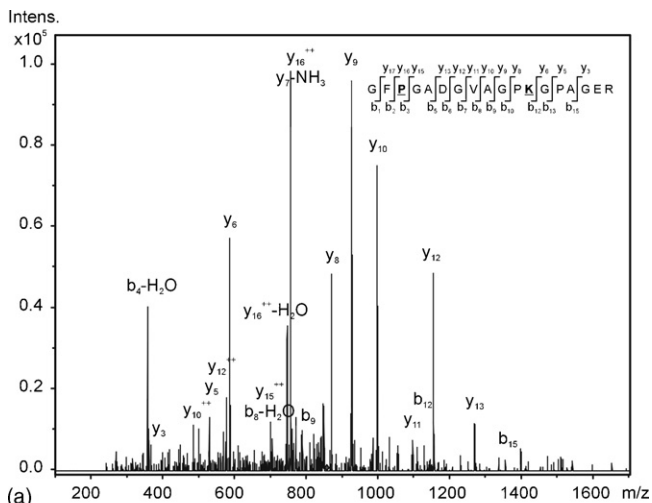


Fig. 4. Fragmentation spectra of CML-modified peptide GFPGADGVAGPK-GPAGER ( $MH^+ = 1713.8$ ,  $m/z = 857.4$ ,  $z = 2$ ,  $\Delta m/z$  associated with CML modification: +58.0) measured by (a) HPLC-MS/MS and (b) CE-MS/MS.

Table 3

Assignment of MS/MS peaks for K504-CML-modified peptide (after glycation)

Fragment ion ( $m/z$ )	Ion	Theoretical mass (Da)	Delta mass (Da)
<b>y-Ions</b>			
361.14	$y_3$	361.19	-0.05
529.23	$y_5$	529.28	-0.05
586.33	$y_6$	586.31	0.02
755.61	$y_7-NH_3$	755.39	0.22
869.45	$y_8$	869.47	-0.02
926.49	$y_9$	926.49	0.00
997.56	$y_{10}$	997.53	0.03
1096.56	$y_{11}$	1096.60	-0.04
1153.75	$y_{12}$	1153.62	0.13
1268.79	$y_{13}$	1268.65	0.14
699.41	$y_{15}^{++}$	698.86	0.55
755.61	$y_{16}^{++}$	755.38	0.23
828.37	$y_{17}^{++}$	828.92	-0.55
<b>b-Ions</b>			
1353.74	$b_{15}$	1353.66	0.08
-	$b_{13}$	1185.58	-
1128.32	$b_{12}$	1128.55	-0.23
-	$b_{11}$	942.44	-
-	$b_{10}$	845.39	-
788.31	$b_9$	788.36	-0.05
770.31	$b_9-H_2O$	770.35	-0.04
699.41	$b_8-H_2O$	699.31	0.10
-	$b_7$	618.26	-
-	$b_6$	561.24	-
-	$b_5$	446.21	-

1	MFSFVLRLL	LLAATALLT	HQEEGQEEG	QEEDIPPVTC	VQNGLRYHDR	50
51	DVWKVPCQI	CVCINGVLC	DDVICDELKD	CPNAKVPTDE	CCFVCEGQE	100
101	SPTDQETGV	EGPKGDTGPR	GPRGPAGPPG	RDGIPGQPL	PGFPGPPPP	150
151	GPPGLGNFA	PQLSYGYDEK	STGISVPGPM	GPSGPRGLPG	<b>PPGAPGQGF</b>	200
201	<b>QGPPGEGEP</b>	<b>GASGPMGPRG</b>	<b>PPGPPGKNGD</b>	<b>DGEAGKGRP</b>	<b>GERGPPGQG</b>	250
251	<b>ARGLPGTAGL</b>	<b>PGMKHGRFS</b>	<b>GLDGAKGDAG</b>	<b>PAGPKGEPGS</b>	<b>PGENGAPGQM</b>	300
301	<b>GPRGLPGERG</b>	<b>PGAPGPAGA</b>	<b>RGNDGATGAA</b>	<b>GPPGPTGPAG</b>	<b>PPGFPGAVGA</b>	350
351	<b>KGEGGPGPR</b>	<b>GSEGPQVVRG</b>	<b>EPGPPGAGA</b>	<b>AGPAGNPGAD</b>	<b>GQPGAKGANG</b>	400
401	<b>APGIAGAPGF</b>	<b>PGARGPSGQ</b>	<b>GPSGPPGPKG</b>	<b>NSGEPGAPGS</b>	<b>KGDTGAKGEP</b>	450
451	<b>GPTGIQPPG</b>	<b>PAGEEGKRG</b>	<b>RGEPGAGLP</b>	<b>GPPGERGGPG</b>	<b>SRGFPGADGV</b>	500
501	<b>AGPKGAPGER</b>	<b>GAGPAGPKG</b>	<b>SPGEAGRPE</b>	<b>AGLPAGAKLT</b>	<b>GSPGSPGPDG</b>	550
551	<b>KTGPPGPAQ</b>	<b>DGRFPGPPGP</b>	<b>GARGQAGVMG</b>	<b>FPGPKGAAGE</b>	<b>PGKAGERGVP</b>	600
601	<b>GPPGAVGPAG</b>	<b>KDGEAGAQQP</b>	<b>PGPAGPAGER</b>	<b>GEQGPAGSPG</b>	<b>FQGLPGPAGP</b>	650
651	<b>PGEAGKPEQ</b>	<b>GVPDLGAPG</b>	<b>PSGARGERGF</b>	<b>PGERGVQGP</b>	<b>GPAGPRGANG</b>	700
701	<b>APGNDGAKGD</b>	<b>AGAPGAPGSQ</b>	<b>GAPGLQGMG</b>	<b>ERGAAGLPGP</b>	<b>KGDFGDAGPK</b>	750
751	<b>GADGAPKDG</b>	<b>VRGLTGPIGP</b>	<b>PGPAGAPGDK</b>	<b>GEAGSPGAP</b>	<b>PTGARGAPGD</b>	800
801	<b>RGEPGPPGPA</b>	<b>GFAGPPGADG</b>	<b>QPGAKGEPGD</b>	<b>AGAKGDAGPP</b>	<b>GPAGPAGPPG</b>	850
851	<b>PIGNVAPGP</b>	<b>KGARGSAGPP</b>	<b>GATGFPGAAG</b>	<b>RVGPPGPSGN</b>	<b>AGPPGPPGPA</b>	900
901	<b>GKEGSKGPRG</b>	<b>ETGPAGRPE</b>	<b>VGPPGPPGPA</b>	<b>GEKAPGADG</b>	<b>PAGAPGTPGP</b>	950
951	<b>QGIAGQGVV</b>	<b>GLPGQRGERG</b>	<b>FFGLPSPSE</b>	<b>PGKQGPSGAS</b>	<b>GERGPPGPMG</b>	1000
1001	<b>PPGLAGPPGE</b>	<b>SGREGAPGAE</b>	<b>GSPGRDGSFG</b>	<b>AKGDRGETGP</b>	<b>AGPPGAPGAP</b>	1050
1051	<b>GAPGVVPAG</b>	<b>KSGDRGETGP</b>	<b>AGPAGPIGPV</b>	<b>GARGPAGPQG</b>	<b>PRGDKGETGE</b>	1100
1101	<b>QDGRGKGRH</b>	<b>GFSGLQGGP</b>	<b>PPGSPGEGP</b>	<b>SGASGAPGR</b>	<b>GPPGSGAGSPG</b>	1150
1151	<b>KDGLNGLPG</b>	<b>IGPPGPRGRT</b>	<b>GDAGPAGPPG</b>	<b>PPGPPGPPGP</b>	<b>PSGGYDLSFL</b>	1200
1201	<b>PQPPEKAHD</b>	<b>GGRYYRADA</b>	<b>NVVRDRDLEV</b>	<b>DTTLKSLSP</b>	<b>IENIRSEGS</b>	1250
1251	<b>RKNPARTCRD</b>	<b>LKMCHSDWKS</b>	<b>GEYWDPNQG</b>	<b>CNLDAIKVFC</b>	<b>NMETGETCVY</b>	1300
1301	<b>PTQPSVAQKN</b>	<b>WYISKPKKEK</b>	<b>RHVWYGESMT</b>	<b>GGFQFEYGGQ</b>	<b>GSDPADVAIQ</b>	1350
1351	<b>LTFLRLMSTE</b>	<b>ASQNIYHCK</b>	<b>NSVAYMDQQT</b>	<b>GNLKKALLQ</b>	<b>GSNEIEIRAE</b>	1400
1401	<b>GNSRFTYSVT</b>	<b>YDQTSHTGA</b>	<b>WGKTVIEYKT</b>	<b>TKTSRLPIID</b>	<b>VAELEVGAPD</b>	1450
1451	<b>QEGFDVGA</b>	<b>CFL</b>				1463

Fig. 5. Sequence of the bovine collagen (P02453 [49], alpha 1, type I) after glycation by ribose. The matched peptides are in bold, modified lysines are underlined. Propeptides are highlighted by small font.

It is worth mentioning that the matured collagen molecule does not contain any cysteines and therefore no disulfide bridge exists (it could be reformed by prolonged enzymatic incubation).

**3.2.1.2. Malondialdehyde-modified samples.** While CML is the most pronounced type of modification for both ribose and glucose modified samples, the reaction of collagen with MDA did not show the same sites and types of modification. Taking the quality of MS/MS spectra into account, the only modification found in two samples was argpyrimidine-modified R321 in the peptide sequence **PGPAGARGN** which was also found in one control sample. This is in agreement with the differing chemistry of MDA (a lipid-peroxidation product) with protein.

### 3.2.2. CE-MS/MS

Similarly to HPLC-MS results, CE-MS analyses can be divided to two sections based on the chemistry of the reactants.

**3.2.2.1. Ribose and glucose-modified samples.** Three CML-modified peptides have been identified in the electropherogram of a ribose-glycated collagen sample (Fig. 6). The peptide sequences were **GFPGADGVAGPKGPAGER** (RT = 45.1 min) with K504 modified lysine (Fig. 4b), **VGAPGPKGAR** (migration time 39.8 min) with K861 modified lysine (Fig. 7a), and **DGSPGAKGDR** (migration time 40.9 min) with K1032 modified lysine (Fig. 7b).

In a glucose-modified collagen sample, two CML-modified peptides with migration times of 45.1 and 39.6 min

were detected by CE-MS/MS. While the former peptide (**GFPGADGVAGPKGPAGER**) was also found using HPLC-MS/MS, the latter one (**VGAPGPKGAR**) could not be detected in glucose-modified samples using HPLC-MS/MS.

**3.2.2.2. Malondialdehyde-modified samples.** No modified peptides were found using CE-MS/MS. This is not surprising as CE has a lower sensitivity than HPLC.

## 4. Discussion

Although some data have been published regarding the determination and/or localization of AGEs mostly in lens crystallins [4], beta-2-microglobuline [6] or human serum albumin [46], to

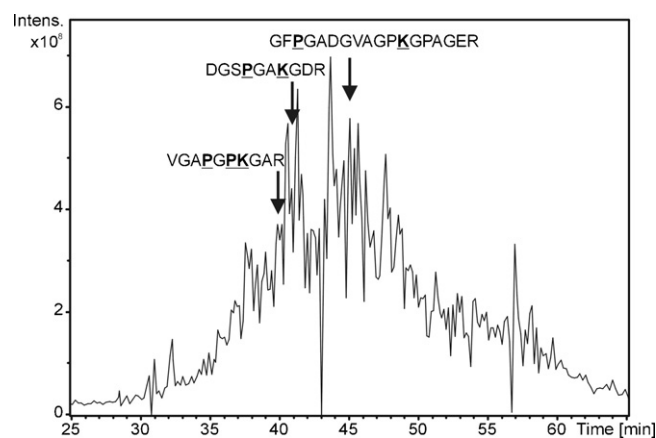


Fig. 6. CE-MS profile of collagen sample glycated by ribose.

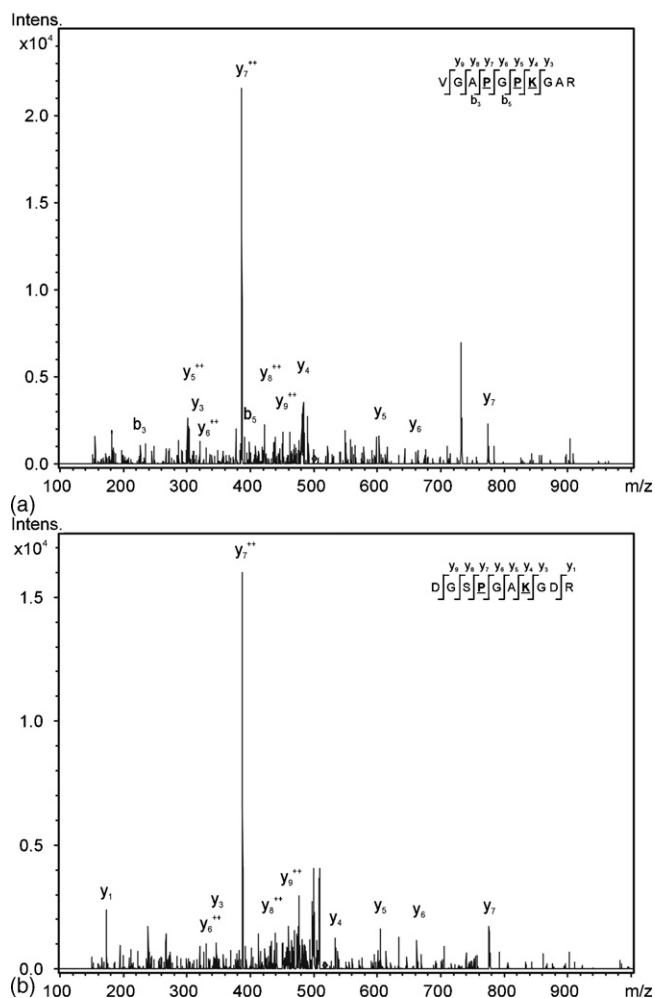


Fig. 7. Fragmentation spectra of CML-modified peptides VGAPGPKGAR ( $MH^+ = 909.5$ ,  $m/z = 500.2$ ,  $z = 2$ ,  $\Delta m/z$  associated with CML modification: +58.0) and DGSPGAKGDR ( $MH^+ = 517.2$ ,  $m/z = 959.5$ ,  $z = 2$ ,  $\Delta m/z$  associated with CML modification: +58.0) measured by CE-MS/MS.

date no attempts have been made to determine the particular peptides that are affected by glycation in water insoluble collagens. While some authors have focused their attention primarily on the isolation, characterization and formation pathways of new AGEs, such as the derivatives of ornithine found in glycated collagens [4,34], stress has been overwhelmingly laid on the quantification of known AGEs such as pentosidine and CML, which have been found to accumulate in the skin and lens collagen matrix at accelerated rates in diabetic patients [47]. Collagen cross-linking reactions are intensively studied because they contribute to diabetic circulatory complications such as vascular stiffening and myocardial dysfunction [48].

The HPLC-MS/MS and CE-MS/MS techniques used in our study enabled us to find the precise sites which were more likely to be modified by CML than the others. Our findings showed that the preferred glycation sites for collagen type I glycated by ribose or glucose were lysines K504 and K1032. All modifications took place in either PKG or AKG motifs. However, in the whole matured protein, there are 17 places comprising one of these motifs, out of which only five have

been found to be glycated (K504, K519, K750, and K861 with PKG motifs, and K1032 with AKG motif). In addition, there are four PKG-comprising and six AKG-comprising sites which provided only spectra of non-glycated peptides. One peptide with lysine K276 (AKG motif) was missing. The last one, K447 (AKG motif) was also missing, but as we can see from other peptides (GFPGADGVAGPK or DGSPGAK), in the case of glycation, the digestion did not take place at a glycated lysine but rather at the next lysine or arginine in the sequence. As we can see from the Fig. 5, the peptide which follows in the sequence (GEPGPTGIQGPPGPAGEEGK) was found, therefore confirming that the digestion occurred at this lysine. This indirect approach allows us to assume that lysine at K447 was most probably not modified. The MS/MS spectra confirmed the presence of CML in collagen after glycation. These results also agree well with other authors' findings that showed CML to be the dominant AGE in tissue proteins, particularly collagens [47].

On the other hand, the LC-MS/MS analyses of collagen glycated by lipid peroxidation product (MDA) produced different results when compared to ribose or glucose, due to the different chemistry of the reaction and higher reactivity of MDA, which leads to cross-linking reactions.

The higher number of CML-modified sites for ribose (five) in comparison to glucose (two) agrees well with the higher reactivity of ribose. This reactivity is caused by the higher content of the aldehydic form of ribose under the reaction conditions. From this differing reactivity we can conclude that the lysines at positions 504 and 1032 are more susceptible to glycation than at positions 519, 750 and 861.

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## References

- [1] T. Niwa, *Mass Spectrom. Rev.* 16 (1997) 307.
- [2] M.B. Yim, H.S. Yim, C. Lee, S.O. Kang, P.B. Chock, *Ann. NY Acad. Sci.* 928 (2001) 48.
- [3] A. Lapolla, D. Fedele, P. Traldi, *Diabetes Metab. Res. Rev.* 17 (2001) 99.
- [4] D.R. Sell, V.M. Monnier, *J. Biol. Chem.* 279 (2004) 54173.
- [5] N. Ahmed, B. Mirshekar-Syahkal, L. Kennish, N. Karachalias, R. Babaei-Jadidi, P.J. Thornalley, *Mol. Nutr. Food Res.* 49 (2005) 691.
- [6] Y. Zhang, R.R. Cocklin, K.R. Bidasee, M. Wang, *J. Biomol. Tech.* 14 (2003) 224.
- [7] K.M. Biemel, D.A. Friedl, M.O. Lederer, *J. Biol. Chem.* 277 (2002) 24907.
- [8] H. Oxlund, M. Barckman, G. Ortoft, T.T. Andreassen, *Bone* 17 (1995) 365S.
- [9] A. Cerami, H. Vlassara, M. Brownlee, *Sci. Am.* 256 (1987) 82.
- [10] J.J. Harding, *Adv. Prot. Chem.* 37 (1985) 247.
- [11] D.J. Prockop, K.I. Kivirikko, *Ann. Rev. Biochem.* 64 (1995) 403.
- [12] J. Myllyharju, K.I. Kivirikko, *Trends Genet.* 20 (2004) 33.
- [13] D. Voet, J.G. Voet, in: D. Voet, J.G. Voet (Eds.), *Biochemie*, Victoria Publishing, Prague, 1992, p. 1052 (in Czech).
- [14] I. Mikšík, P. Sedláková, K. Mikulíková, A. Eckhardt, *J. Chromatogr. B* 841 (2006) 3.
- [15] S.L. Schneider, R.R. Kohn, *J. Clin. Invest.* 67 (1981) 1630.

- [16] S.L. Schneider, R.R. Kohn, *Exp. Geront.* 17 (1982) 185.
- [17] M.P. Cohen, in: M.P. Cohen (Ed.), *Diabetes and Protein Glycosylation: Measurement and Biologic Relevance*, Springer-Verlag, New York, 1986, p. 140.
- [18] A.J. Bailey, R.G. Paul, L. Knott, *Mech. Age. Dev.* 106 (1998) 1.
- [19] Z. Deyl, I. Mikšík, in: Z. Deyl, I. Mikšík, F. Tagliaro, E. Tesařová (Eds.), *Advanced Chromatographic and Electromigration Methods in Bio-Sciences*, Elsevier, Amsterdam, 1998, pp. 465–523 (Chapter 12).
- [20] J.M. Davis, J.C. Giddings, *Anal. Chem.* 55 (1983) 418.
- [21] J.M. Davis, J.C. Giddings, *Anal. Chem.* 57 (1985) 2168.
- [22] J.M. Davis, J.C. Giddings, *Anal. Chem.* 57 (1985) 2178.
- [23] J.C. Giddings, *Anal. Chem.* 56 (1984) 1258A.
- [24] J.C. Giddings, *J. High Resol. Chromatogr.* 10 (1987) 319.
- [25] H.J. Cortes, *J. Chromatogr.* 626 (1992) 3.
- [26] A.W. Moore, J.W. Jorgenson, *Anal. Chem.* 67 (1995) 3448.
- [27] M.M. Bushey, J.W. Jorgenson, *Anal. Chem.* 62 (1990) 161.
- [28] T. Stroink, P. Schravendijk, G. Wiese, J. Teeuwsen, J.C.M. Waterval, A. Bult, G.J. de Jong, W.J.M. Underberg, *Electrophoresis* 24 (2003) 1126.
- [29] T. Stroink, P. Schravendijk, G. Wiese, J. Teeuwsen, J.C.M. Waterval, A. Bult, G.J. de Jong, W.J.M. Underberg, *Electrophoresis* 24 (2003) 897.
- [30] K. Wagner, T. Miliotis, G. Marco-Varga, R. Bischoff, K.K. Unger, *Anal. Chem.* 74 (2002) 809.
- [31] A.V. Lemmo, J.W. Jorgenson, *Anal. Chem.* 65 (1993) 1576.
- [32] A.V. Lemmo, J.W. Jorgenson, *J. Chromatogr.* 633 (1993) 213.
- [33] T.F. Hooker, J.W. Jorgenson, *Anal. Chem.* 69 (1997) 4134.
- [34] R.G. Paul, N.C. Avery, D.A. Slatter, T.J. Sims, A.J. Bailey, *Biochem. J.* 330 (1998) 1241.
- [35] A. Lappolla, D. Fedele, R. Reitano, N.C. Arico, *J. Am. Soc. Mass Spectrom.* 15 (2004) 198.
- [36] P.A. Haynes, S. Miller, T. Radabaugh, M. Galligan, L. Breci, J. Rohrbough, F. Hickman, N. Merchant, *J. Biomol. Tech.* 17 (2006) 97.
- [37] S.K. Grandhee, V.M. Monnier, *J. Biol. Chem.* 266 (1991) 11649.
- [38] R. Aebersold, M. Mann, *Nature* 422 (2003) 198.
- [39] H. Nishi, K. Nakamura, H. Nakai, T. Sato, S. Terabe, *Chromatographia* 40 (1995) 638.
- [40] D.C. Simpson, R.D. Smith, *Electrophoresis* 26 (2005) 1291.
- [41] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, *Anal. Chem.* 59 (1987) 1230.
- [42] R.D. Smith, J.A. Olivares, N.T. Nguyen, H.R. Udseth, *Anal. Chem.* 60 (1988) 436.
- [43] R.D. Smith, C.J. Barinaga, H.R. Udseth, *Anal. Chem.* 60 (1988) 1948.
- [44] K. Mikulíková, A. Eckhardt, I. Mikšík, *J. Sep. Sci.* 29 (2006) 1126.
- [45] FDA, Guidance on statistical procedures for bioequivalence studies using a standard two-treatment crossover design, Division of Bioequivalence, Office Of Generic Drugs, Center For Drug Evaluation Research, Food And Drug Administration, Rockville, Maryland, 1992.
- [46] A. Lappolla, D. Fedele, R. Reitano, N.C. Arico, *J. Am. Soc. Mass Spectrom.* 15 (2004) 496.
- [47] N. Verzijl, J. DeGroot, E. Oldehinkel, R.A. Bank, S.R. Thorpe, J.W. Baynes, M.T. Bayliss, J.W.J. Bijlsma, F.P.J.G. Lafeber, J.M. TeKoppele, *Biochem. J.* 350 (2000) 381.
- [48] J.L. Wautier, P.J. Guillausseau, *Diabetes Metabol.* 27 (2001) 535.
- [49] <http://www.expasy.org/uniprot/P02453>, December 13, 2006.