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# Immunochemical and Mass Spectrometric Analysis of $N^{\varepsilon}$-(Carboxymethyl)lysine Content of AGE−BSA Systems Prepared with and without Selected Antiglycation Agents

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The present study was designed to compare surface plasmon resonance (SPR) biosensor, enzyme-linked immunosorbent assay (ELISA), and ultraperformance liquid chromatography−tandem mass spectrometry (UPLC-MS/MS) methods for the analysis of $N^{\varepsilon}$-(carboxymethyl)lysine (CML) in glucose−bovine serum albumin (BSA) model systems and to investigate the possible inhibitory effect of selected compounds (α-tocopherol, ferulic acid, rutin, thiamin, thiamin monophosphate, and thiamin pyrophosphate) on CML formation. The reported levels of CML detected were dependent upon the method of analysis employed. The highest reported concentrations were obtained with the SPR biosensor, whereas the lowest were found by ELISA. However, a high correlation was observed between these two immunochemical procedures. CML concentrations were dependent upon the type and concentration of the candidate CML inhibitor. All inhibitory compounds investigated, with the exception of α-tocopherol, decreased the level of CML formation in the glucose−BSA system.

**KEYWORDS: $N^{\varepsilon}$-(Carboxymethyl)lysine (CML); advanced glycation endproducts (AGEs); CML inhibitor; enzyme-linked immunosorbent assay (ELISA); surface plasmon resonance (SPR) biosensor; ultraperformance liquid chromatography−tandem mass spectrometry (UPLC-MS/MS)**

## INTRODUCTION

Advanced glycation endproducts (AGEs) are a class of Maillard reaction (MR) products (MRPs). Chemically, the MR involves a reaction between a free amino group, for example, the ε-amino groups of lysine residues within protein, with the carbonyl group of a reducing sugar, such as glucose (*1*). $N^{\varepsilon}$-(Carboxymethyl)lysine (CML), one of the best known AGEs, can be formed through a number of different pathways. The condensation reaction between glucose and the ε-amino group of lysine forms fructoselysine (the Amadori rearrangement product, ARP, of the reaction), which is subsequently oxidized to form CML. Glyoxal can be formed from the oxidation of glucose, and it can also react directly with the ε-amino group of lysine to form CML (*2*). CML has been associated with aging and diseases such as renal failure and diabetes (*3*, *4*). Furthermore, the accumulation of CML in the hearts of patients with diabetes may contribute to the increased risk of heart failure associated with hyperglycemia (*5*). Therefore, the search for inhibitors of CML formation is of significant medical interest. The effectiveness of various potential glycation inhibitors has been tested in model systems (*6*,*7*), in foods (*8*), and in vivo (*9*). Mechanisms include reactive carbonyl trapping (*6*, *10*), antioxidant activity (*7*, *8*), sugar autoxidation inhibition (*6*), and amino group binding inhibition/competition (*9*, *11*).

Several analytical methods have been reported for the detection and quantification of CML, including LC-MS/MS (*12*), GC-MS (*13*), and ELISA (*14*). Recently, new analytical approaches such as UPLC-MS/MS (*15*) and surface plasmon resonance imaging (SPRI) biosensor assays (*16*) have been employed for the quantitative analysis of CML. Only a small number of reports on inter-/intralaboratory comparisons of CML data obtained via different analytical approaches have been published to date in the scientific literature (*13*, *16*). Biosensor assays have proved to be versatile, robust, and capable of producing rapid and reliable data for the analysis of a wide range of components in complex food matrices with minimal sample preparation (*17*−*20*). The main difference between ELISA and SPR biosensor assays is that the SPR biosensor approach is a label-free technique which relies for detection upon an increase in molecular mass due to antibody−analyte interactions on a chip surface.

Research has been conducted on AGE inhibitors (*8*, *21*), but a small number of reports have been focused on CML. No comparison of levels of CML determined by SPR, ELISA, and UPLC-MS/MS has been reported. Therefore, the present study aimed to employ three analytical methods in the detection of CML and to investigate the effect of three antioxidants

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(α-tocopherol, ferulic acid, and rutin hydrate) and competitors (thiamin, thiamin monophosphate, and thiamin pyrophosphate) on the formation of CML, in AGE−bovine serum albumin (BSA) model systems.

## MATERIALS AND METHODS

**Reagents and Apparatus.** All of the reagents used in this study were of analytical grade. α-Tocopherol, bovine serum albumin (BSA, fraction V), ferulic acid, glucose, glyoxylic acid, keyhole limpet hemocyanin (KLH), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), polyethylene glycol sorbitan monolaurate (Tween 20), rutin hydrate, sodium cyanoborohydride, sodium phosphate monobasic, sodium phosphate dibasic, sodium pyruvate, thiamin hydrochloride, thiamin monophosphate, thiamin pyrophosphate, and other chemicals were purchased from Sigma-Aldrich (Gillingham, U.K.). Dimethyl sulfoxide (DMSO), Hybridoma Feeder Supplement (Doma-Drive), Dulbecco's modified eagle's medium (DMEM), heat-inactivated fetal calf serum (HI-FCS), hypoxanthine aminopterin thymidine (HAT) medium, penicillin streptomycin (pen strep), and polyethylene glycol (PEG) were from Invitrogen (Paisley, U.K.). Gelatin and horseradish peroxidase-linked anti-mouse immunoglobulin were obtained from DAKO (Cambridge, U.K.). 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was from Chemicon International (Temecula, CA). The optical surface plasmon resonance (SPR) biosensor system (Biacore Q), Biosensor chip (CM5), ethanolamine hydrochloride (1 M), and HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) were supplied by GE Healthcare (Uppsala, Sweden). Nunc-Immuno 96 microwell plates (NUNC Brand Products) were from Thermo-Scientific, Denmark. The Tecan Safire plate reader was from Vector Scientific, Ireland. The Genevac evaporator (EZ-2) was from Ipswich, U.K. A Waters Acquity UPLC triple-quadrupole MS/MS (Manchester, U.K.) was used for mass spectrometric analyses.

**Sample Preparation.** *Glycated Bovine Serum Albumin* (*Glycated BSA*). BSA (10 mg, equivalent to 8.85 mM lysine), glucose (90 mg, 0.5 M), and inhibitors (8.85 and 88.5 mM α-tocopherol, ferulic acid, rutin hydrate, thiamin hydrochloride, thiamin monophosphate, thiamin pyrophosphate) were mixed in sodium phosphate buffer (0.2 M, pH 7.2, 1 mL) to bring the molecular ratio of lysine/inhibitor to 1:1 and 1:10. The mixed samples were incubated, in a 5 mL glass bottle with a screw-tight lid, at 50 °C for 10 days and vortexed once per day. Glycated BSA, without addition of an inhibitor, was prepared by mixing BSA (10 mg) with glucose (90 mg) in phosphate buffer (0.2 M, pH 7.2, 1 mL) and used as a positive control to calculate the percentage inhibition of CML formation caused by the trialed compounds. All of the samples were prepared in triplicate and were stored at −20 °C prior to analysis.

*$N^{\varepsilon}$-(Carboxymethyl)lysine-Bovine Serum Albumin/Keyhole Limpet Hemocyanin Protein* (*CML-BSA/KLH*). CML-BSA was prepared by incubating BSA (10 mg, equivalent to 8.85 mM lysine) with glyoxylic acid (8.85 mM) in phosphate buffer (pH 7.5, 0.5 M) for 1 h at 37 °C, and then sodium cyanoborohydride (17.70 mM) was added and the incubation continued for a further 23 h. The pH was adjusted to 7.4 with NaOH (0.1 M), if required. CML-modified KLH was prepared by incubating KLH (10 mg) with glyoxylic acid (260 mM) in phosphate buffer (pH 7.5, 0.5 M) for 1 h at 37 °C, and then sodium cyanoborohydride (520 mM) was added and the incubation continued for a further 23 h. Blank samples were also prepared as previously stated, but with the omission of glyoxylic acid. The incubated solutions were dialyzed against phosphate buffer (0.05 M, pH 7.2) containing 0.15 M NaCl and stored at −20 °C. The conjugates were used to prepare immunogens to raise antibodies and for use in the ELISA and SPR assays.

*$N^{\varepsilon}$-(Carboxyethyl)lysine-Modified Bovine Serum Albumin* (*CEL-BSA*). CEL-BSA was prepared by incubating BSA (20 mg/mL), sodium pyruvate (17.14 mM, 17.14 μL), and sodium cyanoborohydride (25.71 mM, 25.7 μL) in phosphate buffer (0.2 M, pH 7.4, 1 mL). The solution was incubated at 37 °C for 24 h. A control was also prepared using the same conditions but with the omission of sodium pyruvate.

**Preparation of a Monoclonal Antibody to CML.** The immunization and fusion procedure described previously by Stewart et al. (*17*) was used to prepare anti-CML monoclonal antibody. Briefly, three BALB/c mice were immunized at 3 week intervals with CML-KLH immunogen (20 μg of protein). Primary and secondary booster immunizations were administered using Quil A adjuvant by subcutaneous injection. Third (20 μg protein) and fourth (80 μg protein) boosters were administered by intraperitoneal injection with Freund's complete adjuvant. Tail bleeds taken from mice 10 days after each booster were tested using ELISA and SPR assays. The most responsive mouse, as determined by antibody titer, was selected and, 4 days prior to the fusion being performed, received a final booster intraperitoneally of the immunogen (100 μg of protein) in phosphate-buffered saline (pH 7.2). The fusion was performed according to a modification of the method of Kohler and Milstein (*22*). A single cell suspension was collected from the spleen of the immunized mouse and fused with SP2/O-Ag14 myeloma cells using polyethylene glycol. After 10−14 days of fusion, the resulting hybrid cells (hybridomas) were screened using ELISA and SPR assays. Serum from the final heart bleed of the fusion mouse was used as a positive control, and cell culture medium buffer was used as a negative control, in the screening assays. The hybridomas that produced antibodies specific for CML but did not bind to CEL were selected for further investigation. All cell lines that gave a strong binding to CML-coated biosensor chips (as detected by SPR) were cloned twice and selected for scale-up antibody production, and the products were stored in liquid nitrogen.

**Development of an Enzyme-Linked Immunosorbent Assay (ELISA).** Ninety-six-well Nunc Maxisorp plates were coated with CML-BSA (1 μg/mL, 100 μL) and blocked with PBS/gelatin (1%) blocking buffer (0.1 M, pH 7.2, 200 μL) overnight at room temperature. After the blocking buffer was discarded, 50 μL of glycated BSA (with or without inhibitors, 300 μg/mL) and 50 μL of anti-CML antibody, 1:15000-fold dilution, were added to the wells and incubated by shaking at 37 °C for 90 min. The supernatant was discarded and the plate washed three times with wash buffer (1% Tween 20 and 0.9% NaCl). The secondary goat anti-mouse HRP antibody was added (1:2000 dilution) and incubated at 37 °C for 60 min. The supernatant was again discarded, and the plate was washed three times with wash buffer. TMB (100 μL) was added to each well and developed in darkness for 5 min. The substrate reaction was stopped using sulfuric acid (2.5 M, 25 μL/well). Absorbance was read at 450 nm using a microplate reader. A range of CML-BSA standards (50%, 0−100 μg of BSA/mL, equivalent to 0−3500 μg of CML/g BSA) were also added to a number of wells and used to generate a calibration curve. Prism 5 software was used to calculate CML concentration in the samples. Buffer (no antibody) was used as a negative control. Unheated glycated BSA (prepared with and without an inhibitor) was tested for background effects.

**Development of the SPR Biosensor Assay.** *CML-BSA Immobilization onto a Biosensor Chip* (*CM5*). The CM5 sensor chip is composed of a glass slide coated with a thin layer of gold to which a carboxymethylated dextran matrix is covalently attached. The conditions for the immobilization of the CML-BSA were optimized (e.g., concentration of CML-BSA, speed of injection, and contact time) to ensure a high surface coverage of the protein. The immobilization procedure was performed within the biosensor unit using Biacore control software. Briefly, the concentration, injection speed, and contact time of each solution were carried out as follows: EDC (0.4 M, 50%) and NHS (0.1 M, 50%) were mixed and injected for 7 min (10 μL/min) onto the CM5 chip (flow cell 1), to activate carboxyl groups on the chip. Jeffamine diluted (1:5) in sodium borate buffer (pH 8.5, 63 mM) was injected (5 μL/min, 7 min) to cover the surface of the flow cell with amino groups. The flow cell was then deactivated with ethanolamine−HCl (10 μL/min, 3 min). After deactivation, CML-BSA solutions were coated for 10 min (1 mg/mL). The coated chip was ready for use immediately or could be stored at 4 °C for several months under dry conditions. After use, the chip was washed with distilled water and dried under a gentle stream of nitrogen.

*Samples Analysis Using the SPR Biosensor Assay*. Glycated BSA samples (10 mg of BSA/mL, 2 μL) and HBS-EP buffer (pH 7.4, 38 μL) were pipetted and mixed in a 96-well plate to bring the final concentration to 0.5 mg of BSA/mL. The CML-specific monoclonal antibodies were diluted in HBS−EP buffer (60-fold dilution). The antibody was mixed automatically with glycated BSA (50:50) in wells of a 96-microtiter plate and then injected over the CML-BSA coated chip at a flow rate of 10 μL/min and a contact time 2 min. The chip surface was regenerated

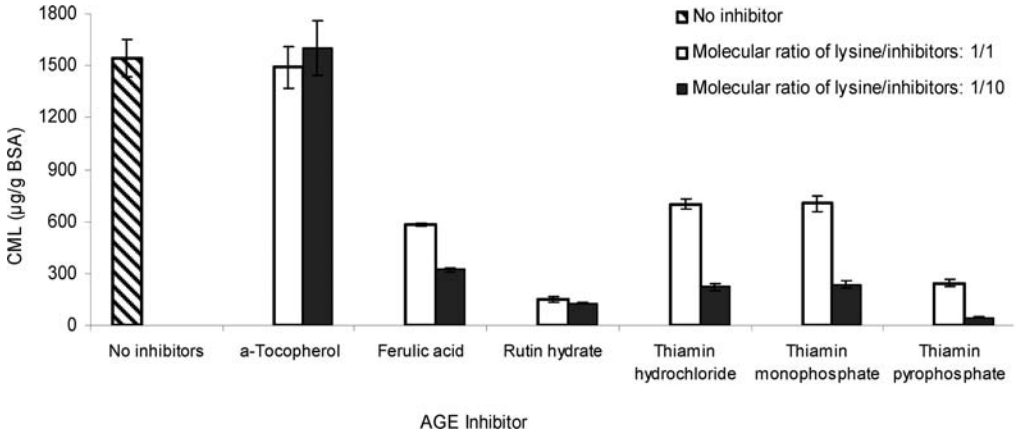


**Figure 1.** CML concentration in glycated BSA as determined by SPR biosensor analysis. No inhibitor, AGE−BSA without addition of any inhibitor; α-tocopherol, AGE−BSA with addition of α-tocopherol; ferulic acid, AGE−BSA with addition of ferulic acid; rutin, AGE−BSA with addition of rutin; thiamin hydrochloride, AGE−BSA with addition of thiamin hydrochloride; thiamin monophosphate, AGE−BSA with addition of thiamin monophosphate; thiamin pyrophosphate, AGE−BSA with addition of thiamin pyrophosphate. Data are expressed as the mean ± SD ($n$ = 3).

with 50 mM NaOH (flow rate = 25 μL/min, 1 min contact time). The concentration of CML in glycated samples was calculated against a CML-BSA calibration curve (50%, 0−100 μg of BSA/mL, equivalent to 0−3500 μg of CML/g BSA, $R^2$ = 0.997) and using Prism 5 software.

**UPLC-MS/MS Analysis.** The CML content of glycated BSA was determined by UPLC-MS/MS (*15*). Samples were prepared for analysis by sodium borohydride reduction, protein isolation using TCA precipitation, protein hydrolysis with 6 M HCl at 110 °C for 24 h, and solid phase extraction using a $C_{18}$ cartridge. Protein hydrolysates (equivalent to 7.5 μg of protein, 7.5 μL) were injected into a BEH $C_{18}$ UPLC column (Waters, 2.1 × 50 mm, 1.7 μm) housed in a column oven at 50 °C in gradient elution mode. Solvent A was nonafluoropentanoic acid (NFPA, 5 mM), and solvent B was acetonitrile. The injection time was 7.5 min. The analysis was performed using a Waters Acquity UPLC coupled to a Waters Premier triple-quadruple MS operating in multiple reaction monitoring (MRM) mode. The flow rate was 0.2 mL/min. The MS was operated in electrospray ionization (ESI) positive mode using MRM mode. The CML data were analyzed using MassLynx software. CML concentrations in the samples were quantified by means of reference to the internal standard. Data were reported as the mean ± SD.

**Statistical Analysis.** Statistical analysis (ANOVA) was performed to determine differences between three groups of means ($P$ < 0.01). Limits of detection (LOD) and limits of quantification (LOQ) of CML concentration analysis by immunochemical methods (SPR and ELISA) were determined from three independent runs of 20 unheated AGE−BSA samples. The LOD and LOQ of CML concentration, analysis by UPLC-MS/MS method, were 1.62 and 5.41 μg of CML/g of BSA, respectively (*15*).

## RESULTS

**Anti-CML Monoclonal Antibody.** Fourteen days after fusion, 447 hybridoma supernatants were screened by two methods (ELISA and SPR biosensor). ELISA screening of the 447 hybridoma supernatants gave 23 positive reactors to CML-BSA, whereas screening by SPR biosensor revealed 5 positive reactors. These 5 positive reactors (shown by SPR) were also found to be positive binders by ELISA and were considered to be true positive binders. After further testing using CML-BSA and CEL-BSA in assay inhibition studies, 1 of the 5 positive reactors was selected for full assay development and designated 2C1. This monoclonal antibody was chosen because it exhibited the highest specificity of the five positive reactors for CML-BSA but not CEL-BSA as observed in inhibition binding ELISA. 2C1 was applied to ELISA and SPR assays for the determination of CML in glycated BSA.

**SPR Analysis of CML in Glycated BSA.** The SPR-measured level of CML in glycated BSA model samples is shown in **Figure 1**. CML levels in the samples were (45 ± 4.76)−(1603 ± 156) μg of CML/g of BSA. The highest level of CML was in BSA glycated either in the absence of any inhibitor (1543 ± 119 μg of CML/g of BSA) or in the presence of the antioxidant α-tocopherol (1603 ± 156 μg of CML/g of BSA). The lowest concentrations of CML were found in BSA glycated in the presence of rutin or thiamin pyrophosphate (127 ± 5.20 and 45 ± 4.76 μg of CML/g of BSA, respectively). BSA glycated in the presence of thiamin or thiamin monophosphate gave similar concentrations of CML (223 ± 20.1 and 233 ± 20.2 μg of CML/g of BSA), about 5-fold higher than in the presence of thiamin pyrophosphate.

**ELISA Analysis of CML in Glycated BSA.** CML levels in glycated BSA measured by ELISA are shown in **Figure 2**. Concentrations were (6 ± 0.80)−(900 ± 99.0) μg of CML/g of BSA. Again, the highest levels were in BSA glycated without an inhibitor and in BSA glycated in the presence of α-tocopherol (852 ± 27.82 and 900 ± 99.0 μg of CML/g of BSA, respectively). Once more, BSA glycated in the presence of rutin or thiamin pyrophosphate provided the lowest concentration of CML (8 ± 0.55 and 6 ± 0.37 μg of CML/g of BSA, respectively), whereas samples containing thiamin and thiamin monophosphate gave very similar levels of CML formation (41 ± 2.20 and 66 ± 3.43 μg of CML/g of BSA, respectively), which were about 7-fold higher than those obtained in the presence of thiamin pyrophosphate.

**UPLC-MS/MS Analysis of CML in Glycated BSA.** CML levels of glycated BSA measured by UPLC-MS/MS are shown in **Figure 3**. The concentrations of CML in samples varied between 194 ± 19.7 and 1075 ± 68.6 μg/g of BSA. The highest concentrations of CML were determined in glycated BSA in the absence of an inhibitor and samples containing α-tocopherol (960 ± 14.4 and 1075 ± 68.6 μg of CML/g of BSA, respectively), whereas the lowest concentrations of CML were found in glycated samples containing rutin and thiamin pyrophosphate (389 ± 17.7 and 194 ± 19.7 μg of CML/g of BSA, respectively). The level of CML in glycated BSA in the presence of thiamin or thiamin monophosphate gave similar data (467 ± 25.4 and 433 ± 39.7 μg of CML/g of BSA, respectively), which were 2.4-fold higher than those of thiamin pyrophosphate.

**Comparison of CML Data Determined by SPR, ELISA, and UPLC-MS/MS.** SPR, ELISA, and UPLC-MS/MS methods for CML analysis were validated and the results of a range of samples compared by linear regression. The three methods showed similar trends with regard to analysis of samples of BSA glycated in the presence of different inhibitors. CML concentrations obtained by

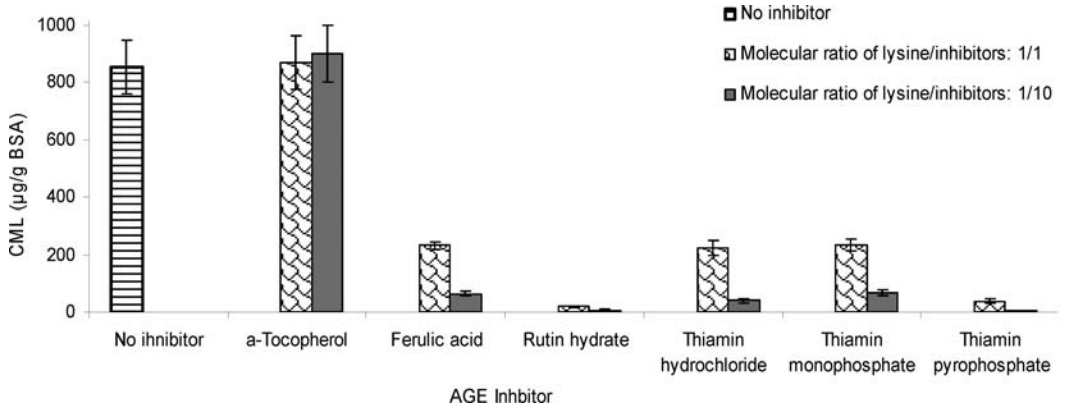


**Figure 2.** CML concentration in glycated BSA determined by ELISA assay. No inhibitor, AGE−BSA without addition of any inhibitor; α-tocopherol, AGE−BSA with addition of α-tocopherol; ferulic acid, AGE−BSA with addition of ferulic acid; rutin, AGE−BSA with addition of rutin; thiamin hydrochloride, AGE−BSA with addition of thiamin hydrochloride; thiamin monophosphate, AGE−BSA with addition of thiamin monophosphate; thiamin pyrophosphate, AGE−BSA with addition of thiamin pyrophosphate. Data are expressed as the mean ± SD ($n$ = 3).

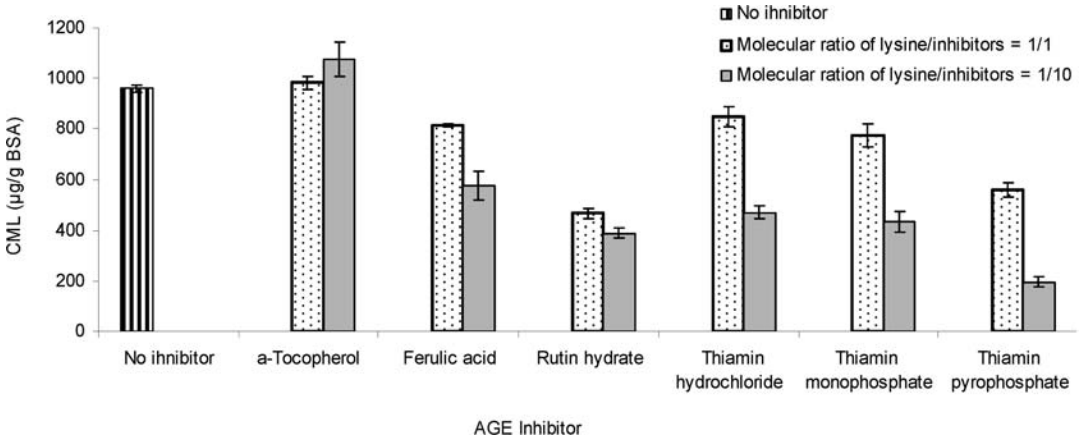


**Figure 3.** CML concentration in glycated BSA determined by UPLC-MS/MS analysis. No inhibitor, AGE−BSA without addition of any inhibitor; α-tocopherol, AGE−BSA with addition of α-tocopherol; ferulic acid, AGE−BSA with addition of ferulic acid; rutin, AGE−BSA with addition of rutin; thiamin hydrochloride, AGE−BSA with addition of thiamin hydrochloride; thiamin monophosphate, AGE−BSA with addition of thiamin monophosphate; thiamin pyrophosphate, AGE−BSA with addition of thiamin pyrophosphate. Data are expressed as the mean ± SD ($n$ = 3).

SPR, ELISA, and UPLC-MS/MS methods are shown in **Figure 4**. Correlations between CML levels determined by SPR versus ELISA, UPLC-MS/MS versus SPR, and UPLC-MS/MS versus ELISA methods of analysis are shown in panels **a**, **b**, and **c**, respectively, of **Figure 5**.

The SPR biosensor analysis of CML gave the highest LOD and LOQ (1.7 and 17 $\mu$g of CML/g of BSA, respectively), whereas ELISA analysis gave the lowest (1.51 and 5.20 $\mu$g of CML/g of BSA, respectively). The LOD and LOQ for UPLC-MS/MS were 1.62 and 5.41 $\mu$g of CML/g of BSA, respectively, very similar to the values for the ELISA. The intra-assay % CV for samples analyzed by each method was found to be $<10\%$. The inter-assay % CV was also $<10\%$ for the SPR and UPLC-MS/MS methods, but the variation for the ELISA was up to 24% for some samples. CML concentrations detected by ELISA [(6 ± 0.80)−(900 ± 99.0) $\mu$g/g of BSA) were significantly lower ($P < 0.01$) compared to SPR biosensor [(45 ± 4.76)−(1603 ± 156) $\mu$g/g of BSA) and UPLC-MS/MS [(194 ± 19.7)−(1075 ± 68.6) $\mu$g/g of BSA) methods (**Figure 4**). CML levels detected in glycated BSA using the SPR biosensor were 1.6-fold higher than those obtained by ELISA, but there was good linearity between the methods ($R^2 = 0.977$) (**Figure 5a**). The concentration of CML obtained by UPLC-MS/MS was 0.79-fold lower than by SPR analysis (**Figure 5b**), but 1.35-fold higher than by ELISA assay (**Figure 5c**). The coefficients of determination of a linear correlation CML values obtained between UPLC-MS/MS versus SPR and UPLC-MS/MS versus ELISA were fairly good ($R^2 = 0.85$ and 0.75, respectively).

## DISCUSSION

An earlier study compared a SPRI biosensor method and an ELISA for the analysis of AGEs in serum from Zucker diabetic fatty (ZDF) rats and Zucker lean (ZL) rats (*16*). The authors reported that the two methods gave similar results, although the data from the two assays were expressed in different units. It is not possible to compare those data sets with CML concentrations determined by the three methods used in the current study. The differences in values obtained by the two methods may be due to the different equipment used. In the present study a SPR biosensor assay was employed, whereas Kim et al. (*16*) used a surface plasmon resonance imaging (SPRI) biosensor. A published study (*23*) reported the detection levels of progesterone in bovine milk to be higher when analyzed by a biosensor compared to an ELISA, but, in agreement with our study, a reasonable correlation ($R^2 = 0.75$) was observed between both methods of analysis. In contrast, Yman et al. (*19*), measuring tropomyosin in crabmeat (surimi), illustrated that biosensor analysis reported lower levels than an ELISA. The discrepancies observed between

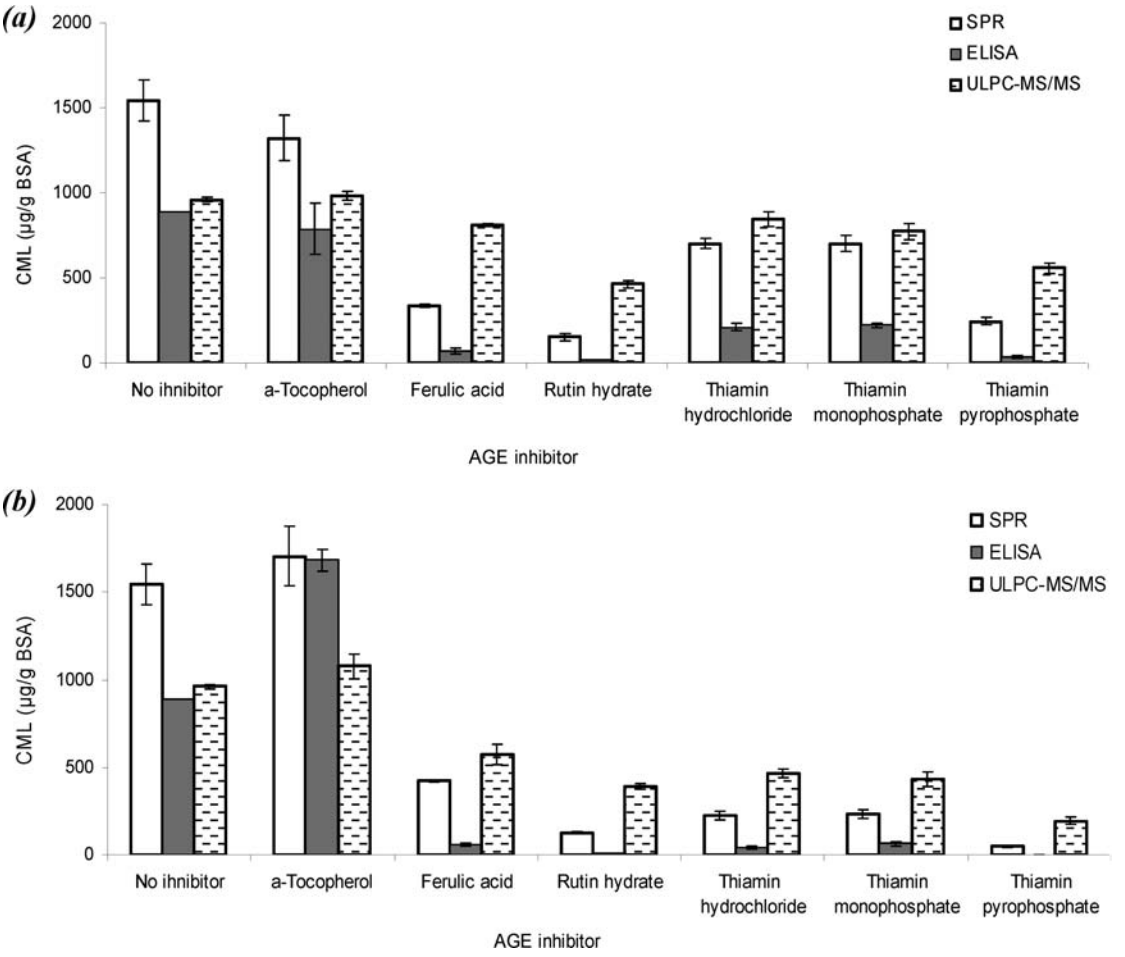


**Figure 4.** Comparison of CML concentration detected by three methods of analysis (SPR, ELISA, and UPLC-MS/MS): (**a**) amount of AGE inhibitor used was equimolar with respect to the lysine content of BSA; (**b**) amount of AGE inhibitor used was 10-fold greater than the lysine content of BSA. No inhibitor, AGE−BSA without addition of any inhibitor; α-tocopherol, AGE−BSA with addition of α-tocopherol; ferulic acid, AGE−BSA with addition of ferulic acid; rutin, AGE−BSA with addition of rutin; thiamin hydrochloride, AGE−BSA with addition of thiamin hydrochloride; thiamin monophosphate, AGE−BSA with addition of thiamin monophosphate; thiamin pyrophosphate, AGE−BSA with addition of thiamin pyrophosphate. Data are expressed as the mean ± SD ($n = 3$).

the methods applied in these different papers may be due to different instrumentations in the case of biosensor-based assay or interfering factors that are present in the samples (*24*).

Compared to UPLC-MS/MS, immunochemical (ELISA and SPR) analysis has advantages such as simpler sample preparation, speed, and cost. However, the advantages of UPLC-MS/MS include the smaller volumes of sample (7.5 μL, 7.5 μg of protein) required compared to ELISA and SPR methods. The correlation of the CML levels found in glycated samples, with or without inhibitors, analyzed by immunochemical methods and UPLC-MS/MS were also well correlated. Charissou et al. (*13*) reported a good correlation between ELISA and GC-MS data for CML levels in model milk (slope = 1.18) and powdered formulas, but satisfactory linear or nonlinear fitting in liquid formula was not observed. These authors also reported that data for CML in liquid milk were almost 10-fold higher when analyzed by ELISA compared to GC-MS (*13*). This is in contrast to the present study in which measured concentrations of CML in glycated BSA obtained by the ELISA were lower compared to those obtained by UPLC-MS/MS.

In the current study, the concentration of CML in glycated BSA was dependent on the concentration and the nature of the inhibitor used. α-Tocopherol did not affect CML formation, whatever the concentration of α-tocopherol applied. In contrast, ferulic acid, rutin, thiamin hydrochloride, and thiamin derivatives (thiamin monophosphate and thiamin pyrophosphate) inhibited CML formation, and the inhibitory effect increased with concentration. In good agreement with the current study, Yin and Chan (*25*) published that α-tocopherol did not inhibit CML and pentosidine formation in the glycated BSA model system. This is possibly due to α-tocopherol insolubility in the aqueous media (phosphate buffer, 0.2 M, pH 7.2) used in both investigations.

Ferulic acid is a free radical scavenger (*26*, *27*), and in the current study its inhibitory effect on CML formation was concentration dependent. The strong free radical scavenging (hydroxyl or superoxide radical) activity of ferulic acid is due to its phenolic nucleus and extended side-chain conjugation, which allow it to form a resonance-stabilized phenoxy radical (*8*, *26*, *27*). The anti-CML effect of ferulic acid is attributed to the second phase of the glycation reaction, that is, glyoxal production from sugar or Amadori product oxidation. The data presented here agree with an earlier study (*8*) suggesting that ferulic acid (0.25 mg/mL) inhibits AGE formation as a result of its free radical scavenging capacities. However, much lower concentrations of ferulic acid appear not to inhibit CML formation (*7*).

Rutin, a powerful antioxidant and antiglycation agent, inhibited CML formation in the current study due to its free radical scavenging capacity (*21*), which mainly inhibits glyoxal (*10*)

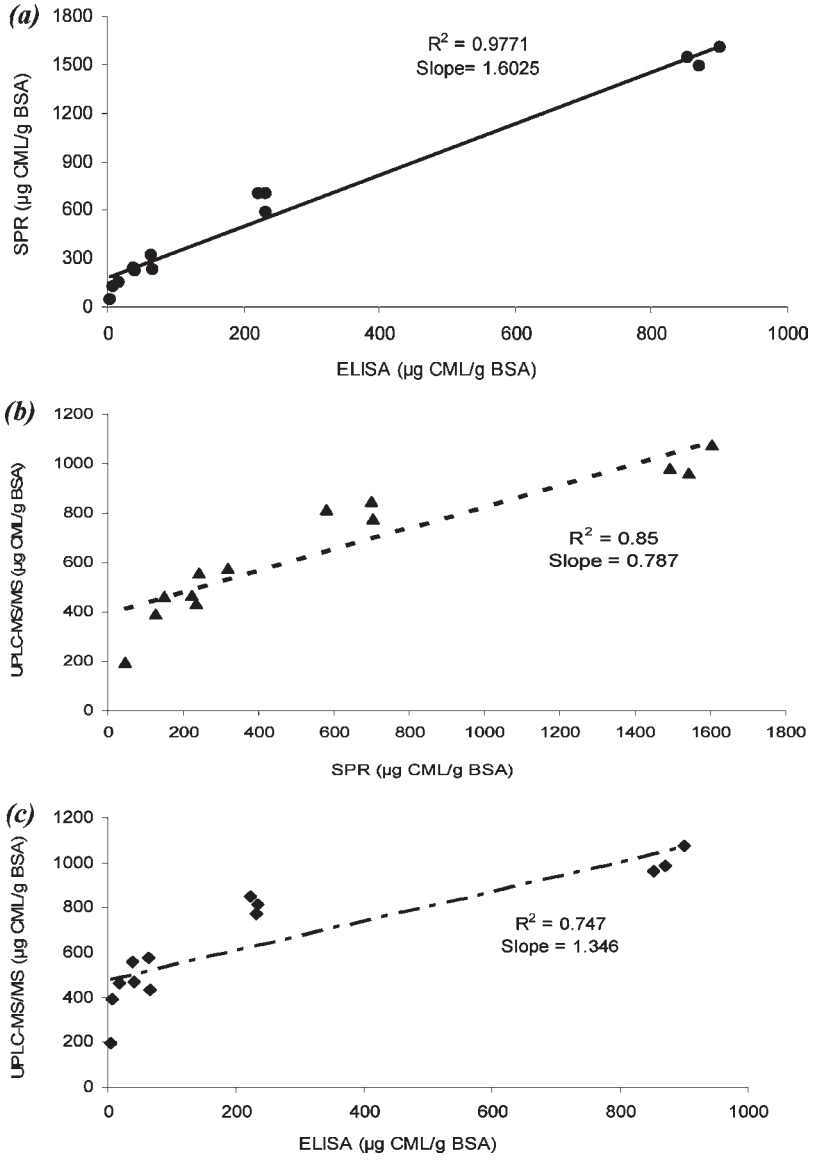


**Figure 5.** Correlation between CML concentrations obtained by SPR, ELISA, and UPLC-MS/MS analysis: (**a**) correlation of CML levels obtained by SPR and ELISA analysis; (**b**) correlation of CML levels obtained by UPLC-MS/MS and SPR analysis; (**c**) correlation of CML levels obtained by UPLC-MS/MS and ELISA analysis.

formation. The data agree with earlier studies (*6*, *10*, *21*) suggesting that rutin inhibits all stages of protein glycation formation, that is, autoxidation of glucose, glyoxal formation, retroaldo condensation of Schiff base, and oxidative degradation of Amadori products to CML. Furthermore, dietary rutin has been proven to reduce glycation in tissue protein of streptozotocin-induced diabetic rats (*28*). Data from previous studies (*6*, *10*, *21*, *28*) and our current investigation would suggest that rutin is a powerful antioxidant which inhibits CML formation both in vitro and in vivo.

Thiamin and its derivatives, thiamin monophosphate and thiamin pyrophosphate, are not antioxidants, but do inhibit CML formation, dependent on their concentration investigated, in the current study. The inhibitory effect may be through a competitive mechanism between the amino group of thiamin/thiamin derivatives with the amino group of lysine residues within protein during glycation, as well as α-oxoaldehyde formation (*29*). The data agree with CML plasma levels of diabetic rats, which were reduced by thiamin administered orally (*9*). Besides this evidence, Booth et al. (*11*) also report a similar effect of thiamin pyrophosphate on AGE formation (98%), even at low concentrations. However, the authors (*11*) did not observe any antiglycation capacity for thiamin and thiamin monophosphate. In this investigation we found thiamin pyrophosphate to exhibit a greater inhibitory effect on CML formation than thiamin and thiamin monophosphate. Each has a similar chemical structure, and all contain a functional amino group. The proposed mechanism may be due to the diphosphate group on thiamin pyrophosphate interfering in the reaction rate between the amino group of thiamin pyrophosphate and the carbonyl group of a reducing sugar during the glycation process, thus inhibiting the formation of CML through a competitive reaction with the amino group on the protein.

In conclusion, the concentration of CML formed was dependent on the types and concentrations of AGE inhibitor, and the reported level of CML was found to be dependent on the methods of analysis (SPR biosensor, ELISA, and UPLC-MS/MS). However, good correlations were observed between those three analysis methods of analysis. The concentrations of CML detected by SPR and UPLC-MS/MS were closer compared to

an ELISA analysis. The SPR biosensor has a number of advantages over ELISA such as the need for smaller volumes of reagents, no need for a labeled compound, higher repeatability, high automation, and higher precision between runs. However, the biosensor assay required a higher concentration of the antibody. Immunochemical analysis of CML, in the AGE−BSA model system, has advantages over the UPLC-MS/MS method such as reduced sample preparation, reduced analysis time, increased speed, and lower costs. α-Tocopherol had no measurable effect on CML formation in AGE−BSA model systems. In contrast, ferulic acid, rutin, thiamin, and thiamin metabolites, thiamin monophosphate and thiamin pyrophosphate, showed various degrees of antiglycation capacity on CML formation. These compounds may be used for health therapy.

## ABBREVIATIONS USED

CML, $N^{\epsilon}$-(carboxymethyl)lysine; CEL, $N^{\epsilon}$-(carboxyethyl)lysine; AGEs, advanced glycation endproducts; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; CML-BSA, $N^{\epsilon}$-(carboxymethyl)lysine-modified bovine serum albumin; CEL-BSA, $N^{\epsilon}$-(carboxyethyl)lysine-modified bovine serum albumin; CML-KLH, $N^{\epsilon}$-(carboxymethyl)lysine-modified keyhole limpet hemocyanin; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; UPLC-MS/MS, ultraperformance liquid chromatography−tandem mass spectrometry.

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