Mol. Nutr. Food Res. 2006, *50*, 1105 – 1117 DOI 10.1002/mnfr.200600065

Review

Methylglyoxal in food and living organisms

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Methylglyoxal (MG) is a highly reactive α-oxoaldehyde formed endogenously in numerous enzymatic and nonenzymatic reactions. It modifies arginine and lysine residues in proteins forming advanced glycation end-products such as N_δ-(5-methyl-4-imidazolon-2-yl)-L-ornithine (MG-H1), 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2), 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3), argpyrimidine, N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (THP), N_ε-(1-carboxyethyl)lysine (CEL), MG-derived lysine dimer (MOLD), and 2-ammonio-6-({2–[4-ammonio-5-oxido-5-oxopently)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate (MODIC), which have been identified *in vivo* and are associated with complications of diabetes and some neurodegenerative diseases. In foodstuffs and beverages, MG is formed during processing, cooking, and prolonged storage. Fasting and metabolic disorders and/or defects in MG detoxification processes cause accumulation of this reactive dicarbonyl *in vivo*. In addition, the intake of low doses of MG over a prolonged period of time can cause degenerative changes in different tissues, and can also exert anticancer activity. MG in biological samples can be quantified by HPLC or GC methods with preliminary derivatization into more stable chromophores and/or fluorophores, or derivatives suitable for determination by MS by use of diamino derivatives of benzene and naphthalene, 6-hydroxy-2,4,5-triaminopyrimidine, cysteamine, and *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine. The methods include three basic steps: deproteinization, incubation with derivatization agent, and chromatographic analysis with or without preliminary extraction of the formed products.

Keywords: Advanced glycation end products / Diabetes / Food / Maillard reaction / Methylglyoxal

Received: April 28, 2006; revised: June 28, 2006; accepted: July 31, 2006

1 Introduction

Proteins are subjected to spontaneous post-translation modifications by reducing sugars in the processes collectively called the Maillard reaction, browning reactions, or nonenzymatic glycation. Although various reducing sugars or sugar derivatives are able to react with free amino acids or protein residues, glucose is most relevant in food chemistry and pathologic implications *in vivo*. The Maillard reaction begins with reversible formation of unstable Schiff base formed by condensation of the aldehyde group of open-chain glucose with the amino group of proteins. The formed base undergoes slow, yet reversible chemical transformation known as Amadori rearrangement. These initial stages are followed by a series of complex, only partially understood reactions of oxidation, reduction, condensation, fragmentation, and cyclization, which result in the formation of stable products known as advanced glycation end-products

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Abbreviations: 5-MQ, 5-methylquinoxaline; **AGEs,** advanced glycation end products; **ALDH,** aldehyde dehydrogenase; **ALR,** aldose reductase; **CBA**, *o*-chlorobenzaldehyde; **CEL**, N_ε-(1-carboxyethyl)lysine; **DA,** derivatization agent; **DAN**, 2,3-diaminonaphthalene; **DAP,** dihydroxyacetone phosphate; **DDB,** 1,2-diamino-4,5-dimethoxybenzene; **DDQ**, 2,3-dimethyl-6,7-dimethoxyquinoxaline; **DMB,** 1,2-diamino-4,5-methylenedioxybenzene; **DMQ**, 2,3-dimethylquinoxaline; **EDC**, electron-capture detector; **EtOAc,** ethyl acetate; **GA3P,** glyceraldehyde 3-phosphate; **GSH,** reduced glutathione; **HD**, 3,4-hexanedione; **HOAc,** acetic acid; **IS,** internal standard; **MG,** methylglyoxal; **MPQ**, 1-methyl-2-propylquinoxaline; **NPD,** nitrogen phosphorus detector; **2-ODH,** 2-oxoaldehyde dehydrogenase; **OPD,** 1,2-diaminobenzene; **PCA,** perchloric acid; **PD**, 2,3-pentanedione; **PFBOA,** *o*-(2,3,4,5,6-pentaflorobenzyl) hydroxylamine; **TK,** transketolase; **TRI,** 6-hydroxy-2,4,5-triaminopyrimidine

(AGEs). In distinction from the Amadori product, which is in equilibrium with free glucose, AGEs are irreversibly linked to proteins. The consequences of these reactions are damaging of proteins and changing their physicochemical and biochemical properties as well as their stability [1, 2]. Studies of the contribution of glycation to diseases have been primarily focused on its relationship to diabetes and diabetes-related complications [3, 4], but it has become clear that the glucose-induced damage also affects physiological aging and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [5]. However, it has recently been shown that AGEs can be formed from a variety of compounds besides glucose and that α-oxoaldehydes are the key intermediates in the formation of most of the AGEs identified *in vivo*; the importance of methylglyoxal (MG) in the formation of AGE structures has been increasingly recognized [6, 7].

MG is also known as 2-oxopropanal, pyruvaldehyde, pyruvic aldehyde, 2-ketopropionaldehyde, acetylformaldehyde, propanedione, or propionaldehyde. It is a yellow hygroscopic liquid which polymerizes readily and forms a variety of cyclic and acyclic structures. MG is a normal body constituent formed endogenously in numerous enzymatic and nonenzymatic reactions, although external sources have also been identified, and several enzymes are involved in its detoxification. In water, MG is present mostly in the mono- and dihydrate forms, while nonhydrated MG is only present in traces [8]. However, MG can change from less reactive noncarbonyl form to more reactive carbonyl and dicarbonyl forms and *vice versa* in the presence of some organic compounds. These changes are dependent on temperature and the amount of water, and can strongly influence MG reactivity in different parts of cells or tissues [9]. Numerous studies have demonstrated modifications of biological macromolecules, mostly proteins, with this reactive α-oxoaldehyde. It has been reported that MG primarily reacts with arginine residues to form imidazolones: N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-L-ornithine (MG-H1), 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)-pentanoic acid (MG-H2), and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3) [10–12]. With arginine residues, MG also forms N_δ-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine) [13] and N_δ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (THP) [14]. It also reacts with lysine residues to generate MG-derived lysine-lysine crosslinks N_ε-(1-carboxyethyl)lysine (CEL) [15] and MG-derived lysine dimer 1,3-di(N_ε-lysino)-4-methyl-imidazolium (MOLD) [16]; while with one lysine and one arginine residue, it forms 2-ammonio-6-({2-[4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate (MODIC) [17] (Fig. 1). These MG-derived AGE structures have been demonstrated *in vivo* and are associated with complications in diabetes and other diseases [15, 18–23].

Although MG has been implicated in a number of diseases, its real metabolic role and its potential in the pathogenesis of different diseases have not yet been fully understood. Proper identification of MG sources, which is unavoidably associated with accurate analytical methods for its determination, and recognition of metabolic disturbances that lead to MG accumulation would help us understand this α-oxoaldehyde.

2 Determination of methylglyoxal in biological samples

Several methods for quantification of MG in biological samples have been developed over time. Some of the most extensively published HPLC and GC methods for MG determination are described here.

MG has been analyzed in different samples including food, beverages and biological matrices such as urine, blood plasma, and organs. Not only the fact that the amount of

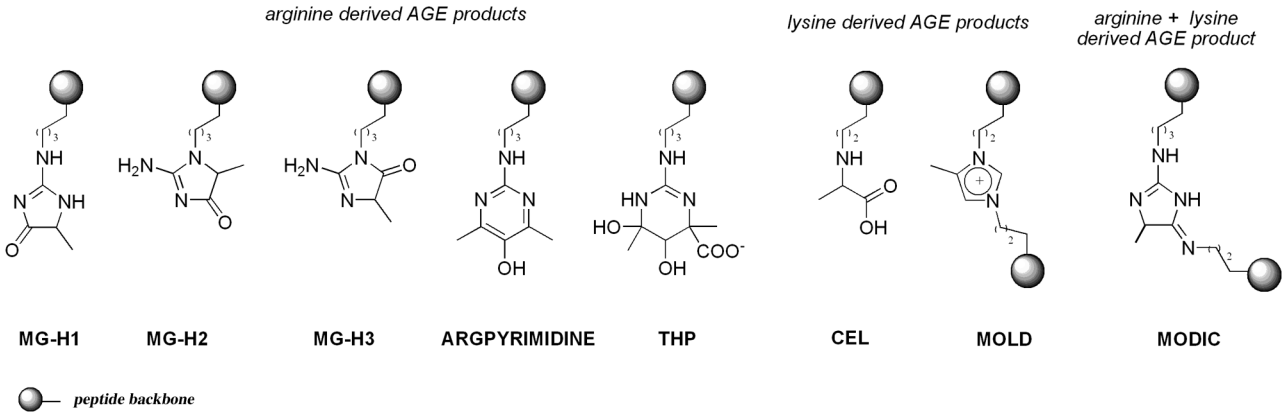

Figure 1. AGEs formed in the reaction between MG and arginine and lysine protein residues.

MG in biological matrices is low (measured values are usually in the range of picomole *per* gram of biological sample), but also the complexity of food or body fluids which requires a multistep technique on its quantification, and sometimes may make the determination of MG difficult to perform. There are several major problems connected with MG quantification. First of all, the commercially available MG contains many impurities and as such it is unacceptable as a standard without preliminary purification. This step is difficult to perform by reason of its high reactivity and tendency to polymerization [9]; therefore, the synthesis of pure MG is recommended before setting a method for its quantification. Pure water solution of MG can be prepared following the method of Kellum *et al*. [24] with additional redistillation [25], or by hydrolyzing 1,1-dimethoxypropanone in the presence of ion exchange resin (Dowex 50×8 H^+) as a catalyst [26]. In addition, the concentration of the prepared solution can be measured by Friedemann's titration after MG oxidation into formic and acetic acid (HOAc) performed by H_2O_2 [27] and/or enzymatically with glyoxalases [28, 29]. Other problems encountered in MG quantification are related to its low concentration in biological samples, its high reactivity with other components of biological matrix, and the possibility of its formation from other components of a biological sample. At the end, the impossibility of its direct measurement requires derivatization into more stable chromophores and/or fluorophores or derivatives suitable for determination by MS.

2.1 Sample preparation

Although sometimes complex, all methods of MG determination in biological samples can be separated into several basic steps: (i) sample deproteinization (if necessary) in the presence of internal standard (IS), (ii) incubation with derivatization agent (DA), and (iii) chromatographic analysis with or without preliminary extraction of the formed products.

2.1.1 Deproteinization

Protein insolubility during chromatographic analysis and the necessity of the reversibly bound MG liberation from proteins makes deproteinization an important step during MG quantification, especially in body fluids and tissues. Namely, it was shown that in the presence of protein (*e.g.*, albumin), only 1% of MG was in the free form, whereas more than 90% was reversibly bound to proteins; it was demonstrated that the MG reversibly bound to protein could be liberated by use of a deproteinization agent (perchloric acid, PCA) [30].

As low pH prevents degradation of dihydroxyacetone phosphate (DAP) and glyceraldehyde 3-phosphate (GA3P) to MG through phosphate elimination, acidic deproteinization agents are the only suitable agents to be used in the methods of MG quantification. For this reason, PCA is widely used as an agent for deproteinization; moreover, it was demonstrated that glucose, pyruvate, D-lactate, D,L-glyceraldehyde, DAP, and GA3P, all serving as potential sources of undesirably formed MG in assay conditions, did not cause an increase of MG in the presence of PCA [29]. However, a significant increase in the MG level was caused by PCA due to oxidative degradation of nucleic acids to MG [31], and a prederivatization SPE step was necessary to reduce the interferences to acceptable levels [32]. In addition, TFA can be used for deproteinization of human plasma samples [26].

Deproteinization of samples should be done in the presence of IS. Most IS used in the published methods are structurally related to MG such as 2,3-butanedione [26, 29, 33], 2,3-pentanedione [34], 3,4-hexanedione [35], or 2,3-hexanedione [36, 37]. These IS can be added to the sample as a nonderivatized compound or as a compound previously derivatized with corresponding DA. *o*-Chlorobenzaldehyde (CBA), an IS structurally related to DA, was used as IS in the GC/MS method [38].

2.1.2 Derivatization

The second step in MG measurement, which cannot be avoided because of the impossibility of direct MG measurement, is derivatization. Most of HPLC methods for MG quantification in biological samples are based on MG derivatization into quinoxaline adducts with diamino derivatives of benzene or naphthalene [26, 29, 32–37, 39–42] (Fig 2). These quinoxalines can easily be monitored either by UV detector at 300–360 nm, by fluorescent detector with excitation wavelengths at 300–360 nm and emission wavelengths at 380–450 nm (depending on the diamine used), or by MS detector. HPLC methods where MG was derivatized into the corresponding pteridin derivative with 6-hydroxy-2,4,5-triaminopyrimidine (TRI) [43] or 2-acetylthiazolidine by cysteamine [44] have also been described. GC methods were based on MG derivatization with 1,2-diaminobenzene (OPD) with detection either on MS/SIM or on specific nitrogen phosphorus detector (NPD) [37, 45], by *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA) with detection either on MS/SIM detector [38], or electron-capture detector (EDC) [46], NPD, or flame photometric detector [42] (Fig. 2).

Incubations of samples were performed with the excess of DA, the incubation period depending mostly on both the derivative and the temperature applied, which varied between 45 min at 60°C to 18–20 h at 4°C. Degradation of compounds for derivatization provoked by incubation conditions occasionally posed a great problem. Therefore, the incubations had to be performed in the dark and even in the atmosphere of nitrogen. It has been shown that some com-

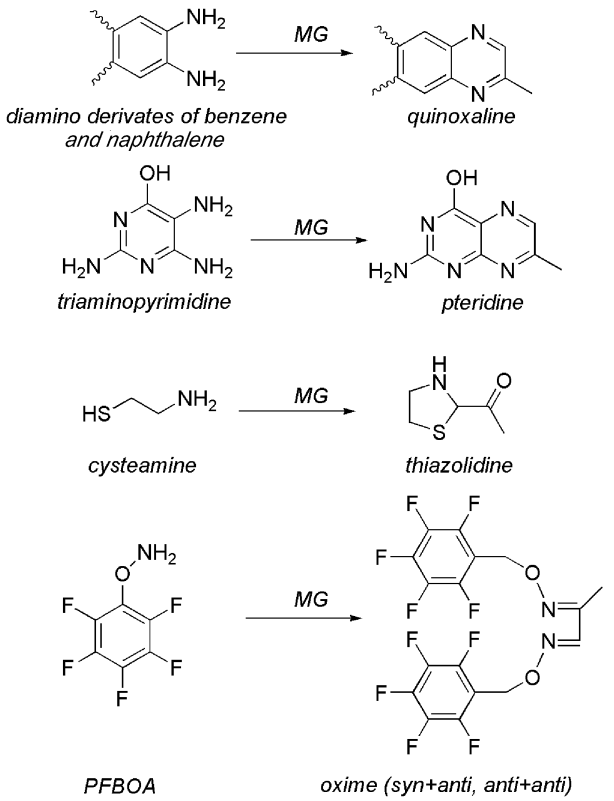

Figure 2. Derivatization of MG with diamino derivatives of benzene and naphthalene into quinoxaline derivatives, 6-hydroxy-2,4,5-triaminopyrimidine (TRI) into 6-methylpteridine, cysteamine into 2–acetylthiazolidine, and *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBOA) into oxime derivatives.

pounds that stabilize diamino derivatives (such as β-mercaptoethanol [40]) significantly decrease MG level [26], and that even the osmolality of incubation mixtures has the same effect on the derivatization process [34].

Unfortunately, some DA suitable for MG quantification are not commercially available and have to be synthesized and characterized before analysis as well as the products of reaction of MG and IS with DA. Diamino derivatives of benzene or its derivatives or naphthalene can be obtained by nitration of aromates by nitric acid, followed by additional reduction of dinitro derivatives in conc. HCl in the presence of methanol and Fe [47], Pb [48], or $SnCl_2$ [49], then by hydrogenation of dinitro compounds in absolute ethanol in the presence of Pd/C [25, 26] or PtO_2 [50] as catalysts, or by refluxing dinitro derivative with hydrazine hydrate in the presence of Pd/C [51].

2.2 Chromatographic analysis

Chromatographic analysis is the final step in the determination of MG. When the amount of MG is very low, preliminary concentration of the sample is necessary. This can be performed by liquid–liquid [34, 35, 38, 39] or SPE [29, 32, 33, 36] with additional evaporation of organic solvent or by freeze drying of samples deproteinized by TFA [26]. HPLC analysis was usually performed on the RP HPLC columns which were eluted with water solutions of acids or buffers in combinations with methanol or ACN under the gradient or isocratic conditions, while GC analysis was performed on fused-silica capillary columns with He as carrier gas.

2.3 Quantification of methylglyoxal in food samples and cigarette smoke

Methods for the determination of MG in different food samples and beverages were mostly based on HPLC or GC analysis of derivatized MG with commercially available OPD, which makes these methods widely used. Detectors used in chromatographic analysis were UV, fluorescence, MS, EDC, or NPD, with different sensitivity and LODs. OPD instead of HPLC and GC analysis can be used as a DA in CZE with DAD [52]. In addition to these methods, a method using 1,2-diamino-4,5-methylenedioxybenzene (DMB) [41] as well as cysteamine [44] and PFBOA [46] as DAs is also described.

The methods, validated for specific samples, were used for determination of MG in different wines [37, 53, 54], beer [54], lipids [42, 55], dairy products [56, 57], honey [58], and many others.

Since most of foodstuffs in which MG content was determined are protein free or have a low protein content, the deproteinization step was avoided making these methods easier to perform. However, in some cases, such as beer [54], the level of MG was difficult to measure due to the complex composition of the matrix, while on MG measurement in cigarette smoke, some additional efforts were invested in sample preparation, *i.e.*, a special chamber for accumulation of smoke had to be constructed [45, 59].

2.4 Quantification of methylglyoxal in body fluids and tissues

The methods of MG quantification in human and/or mouse whole blood [29, 33, 36, 40], human plasma [26, 35, 38], human urine [34, 43], yeast cell culture [33], Chinese hamster ovary (CHO) cells [32], rat heart, liver, kidney, and aorta [36, 39] are presented in Table 1. As body fluids and tissues (except for urine) have a high content of protein, deproteinization is an unavoidable step in sample processing and all the above-mentioned facts connected with deproteinization should be considered. However, although all the methods described have a similar groundwork, the

Table 1. Methods of MG determination in body fluids and tissues

Matrix/sample preparation	• IS • DA • Technique	• Detection • LOD • Recovery	Ref.
Rat liver/The sample was homogenized with PCA (0.5 M, 2 mL/g of liver) and centrifuged. To the supernatant (1 mL), OPD (1% aq., 0.1 mL) was added. The mixture was allowed to rest at 25°C for 1 h. Then NaOH (5 M, 0.2 mL) was added, and the reaction mixture was extracted with CH_2Cl_2 (3 mL). To the organic layer (2 mL), HCl (6 M, 0.05 mL) was added, the vial was tightly capped and shaken well. The mixture was evaporated; the residue was dissolved in the mobile phase (1 mL) and subjected to analysis.	• No • OPD • HPLC[a)]	• UV (315 nm) • 10 nmol • 74.2 ± 1.8%	[39]
Mouse whole blood/To the sample (5 μL), water was added (100 μL). The resulting lysate was poured onto ultrafilter and centrifuged. The elute (50 μL) in a screw-capped vial was diluted with the DMB solution (7.0 mM in water containing 1.0 M β-mercaptoethanol and 28 mM sodium dithionite). The vial was tightly closed and warmed at 60°C for 40 min in the dark and the resulting mixture (10 μL) was subjected to analysis.	• No • DMB • HPLC[b)]	• Fluor. (ex 355 nm; em 393 nm) • 400 pmol • 88.0–93.1%	[40]
Human whole blood/PCA (0.6 M, 2 mL) was added to the sample (1 mL) and vortex mixed. The IS was added (50 μM in 0.5 M HCl, 20 μL). The sample was held on ice for 10 min and then centrifuged. The supernatant (2 mL) was removed, DBB (1.1 mM in 0.5 M HCl, 200 μL) was added and the sample was incubated at r.t. for 4 h in the dark. The pH of the sample was then adjusted to 2.3 with Na_2HPO_4 (0.5 M, 850 μL) and the sample applied to SPE cartridge, equilibrated with $NH_4H_2PO_4$ buffer (20 mM, pH 2.3). The SPE cartridge was washed with the same buffer (6 mL); the retentate was eluted with MeOH (3 mL). The sample was evaporated to dryness at r.t., dissolved in the mobile phase (200 μL), filtered, and injected for analysis.	• DDQ • DDB • HPLC[c)]	• UV (352 nm); Fluor. (ex 352 nm; em 385 nm) • 45 pmol (UV) 10 pmol (fluor.)	[29]
Yeast cells and whole rat blood/To a sample (1 mL) PCA (5 M, 0.2 mL), IS (10 μM, 0.1 mL), OPD (9.2 mM, 0.2 mL), and water to the final volume (2 mL) were added and incubated at r.t. for 30 min. The sample was added to SPE, the column was washed twice with $HCOONH_4$ buffer (20 mM, pH 2.3, 2.5 mL) and the retentate was eluted with MeOH (2.5 mL). The sample was evaporated at 40°C under a stream of N_2. The residue was dissolved in the mobile phase (0.2 mL) and injected for analysis.	• DMQ • OPD • HPLC[d)]	• UV (320 nm) • 0.12 μM • No	[33]
CHO Cells/A sample (0.45 g of cells suspended in 4.5 mL of the buffer) was lysed over ice by sonication, followed by the addition of PCA (5 M, 0.45 mL). The samples were incubated on ice for 10 min and centrifuged. The supernatant was applied to SPE equilibrated with ACN (6–8 mL) and eluted with KH_2PO_4 (10 mM, pH 2.5, 6–8 mL). To the sample IS (1.25 nmol) and OPD (125 nmol) were added. The samples were incubated at 20°C for 3.5–4 h. The samples were applied to SPE at a rate of 1–2 mL/min, rinsed with KH_2PO_4 (10 mM, pH 2.5, 1–2 mL), the retentate was eluted with ACN (2 mL) and evaporated to a volume of approximately 400 μL and injected for analysis.	• 5-MQ • OPD • HPLC[e)]	• UV (315 nm) • 44 pmol • 72%	[32]
Human urine/To aliquots of fresh samples (1 mL) TRI solution (7.02 mM, 1.5 mL), 1 mL of a 0.02 M NaOAc/HOAc (pH 4.05) buffer solution, and deionized water were added to complete 10 mL. The samples were heated at 60°C for 45 min. The resulting solutions were filtered and injected for analysis.	• No • TRI • HPLC[f)]	• Fluor. (ex 352 nm; em 447 nm) • 11 pmol • 111–115%	[43]
Human plasma/The sample (1 mL) was incubated overnight at 4°C with DAN (0.1%, 100 μL) in the presence of IS (0.01%, 50 μL). The reaction mixture was extracted by ethyl acetate (EtOAc) (4 mL), and the solvent was dried under N_2. The dried extract was reconstituted with MeOH (200 μL) and filtered for analysis.	• HD • DAN • HPLC[g)]	• ESI/MS and ESI/MS/MS • No • No	[35]

Table 1. Continued

Matrix/sample preparation	• IS • DA • Technique	• Detection • LOD • Recovery	Ref.
Human plasma/The physiological solution (1200 μL) was added to the sample (600 μL), which was then centrifuged. To the filtered aliquot (200 μL) HCl solution (pH 3, 4.6 mL) and IS (0.023 mM, 200 μL) were added. The pH was adjusted to 3 with HCl (0.1 M). The resulting solution was treated with PFBOA (10 mg) and stirred at r.t. for 1 h. After addition of NaCl (0.5 g), the obtained oxime was extracted with diethylether-hexane (1:1 v/v, 3 × 2 mL) with stirring for 5 min, and then the organic phase was dried over Na_2SO_4 and filtered. The volume was reduced to 200 μL under N_2 flow before analysis.	• CBA • PFBOA • GC/MS[h)]	• GC/MS • No • No	[38]
Human urine/The sample was immediately diluted to reach 100-120 mOsm/kg H_2O. Diluted sample (0.5 mL) was mixed with NaOAc buffer (0.2 M, pH 4.5, 0.5 mL), spiked with IS (46 μM, 100 μL) and DDB (50 mM, 100 μL) and heated at 60°C for 15 h in the dark. The pH value of the solution before heating was in the range of 4.5–4. 9. The reaction mixture was vortexed with EtOAc (2 × 1 mL), and the organic layers were evaporated to dryness by mild stream of dry N_2. The residue was dissolved in ACN (5 mL), filtered, and injected for analysis.	• PD • DDB • HPLC[i)]	• Fluor. (ex 352 nm; em 385 nm) • No • 57.3–64.7%	[34]
Human Plasma/TFA (99%, 100 μL) was added to the sample (1 mL), vortexed, and then IS (50 μM in 1.18 M TFA, 20 μL) was added. The mixture was allowed to rest at 0°C for 15 min and then centrifuged. To the supernatant (800 μL) DDB (3.6 M in 1.18 M TFA, 100 μL) was added and the mixture kept (under N_2 and protected from light) at r.t. for 2 h. The mixture was freeze dried. The residue was dissolved in the mobile phase (100 μL) and subjected to HPLC.	• DDQ • DDB • HPLC[j)]	• UV (352 nm; 215 nm) • 30.6 pmol (215 nm) 45.9 pmol (352 nm) • 69%	[26]
Rat heart, liver, kidney, aorta, and blood/Tissue samples were homogenized over ice for 1–2 min in 5 volumes of ice-cold phosphate buffer (10 mM, pH 7.4). IS1 (2.5 nmol) was added to homogenates and each was further sonicated using 3 × 5-s 10 W bursts. Then PCA (5 M, 0.1 volume) was added and the sample was incubated on ice for 10 min and then centrifuged. The supernatants were passed through SPE columns that were previously primed using ACN (6 mL) and phosphate buffer (10 mM, pH 2.5, 6 mL). To the eluate, IS2 (2.5 nmol) and OPD (125 nmol) were added and the mixture was kept at 4°C for 18–20 h. The sample was then applied to SPE, rinsed twice with phosphate buffer, and the retentate was then eluted with ACN (2 mL). The eluate was evaporated to dryness under a stream of N_2 and the residue was dissolved in MeOH (300 μL), filtered, and subjected to HPLC.	• 5-MQ (IS1); MPQ (IS2) • OPD • HPLC[k)]	• ESI/LC/MS • No • 63.9%	[36]

Chromatographic conditions: column/mobile phase/flow rate.

a) Unisil Q ODS T (150 × 4.6 mm, 5 μm)/$KHPO_4$-H_3PO_4 (10 mM; pH 2.1)-ACN (80:20, v/v).
b) Radial-Pak C18 (100 × 8 mm, 5 μm)/MeOH-ACN-phosphate buffer (0.04 M, pH 7.0) (44:7:49, v/v/v)/1 mL/min.
c) Nova-Pak ODS (100 × 8 mm, 4 μm)/MeOH-$HCOONH_4$ (20 mM, pH 3.4) (42:58, v/v)/2 mL/min.
d) Merck RP-18 (250 × 4 mm, 5 μm)/MeOH-$HCOONH_4$ (25 mM, pH 3.4) (60:40, v/v)/1 mL/min.
e) Adsorbosphere C-18 (250 × 4.6 mm)/KH_2PO_4 (10 mM, pH 2.5)-ACN (68:32, v/v)/1 mL/min.
f) Nova-Pack C-18 (150 × 3.9 mm)/chloroacetic acid-sodium chloroacetate (0.04 M, pH 2.9)/1.3 mL/min.
g) Excelpak SIL-C18 (150 × 4.6 mm, 5 μ m)/MeOH-water-HOAc (40:60:0.2%)/0.4 mL/min.
h) Innowax fused-silica capillary column (30 m × 0.25 mm; 0.25 μm), injection temperature 250°C, injected volume (0.5 μL), carrier gas He, 5 min 60°C, 3°C/min to 210°C, 5 min at 210°C.
i) Inertsil ODS-2 (250 × 4 mm; 5 μm)/ACN-$HCOONH_4$ (20 mM, pH 3.4) (solvent A, 1:9 v/v; solvent B, 1:1 v/v). Solvent A: 0–20 min, 80%; 20–30 min, 80% → 7%; 30–35 min, 7%; 35–35.1 min, 7% → 80%; 35.1–45 min, 80%/1 mL/min.
j) Eurospher 100 RP C-18/MeOH (52% in 0.1% aq. TFA)/0.8 mL/min.
k) Waters Wat058961RP C8 (100 × 2.1 mm; 25 μm)/MeOH (40% in 0.2% TFA (solvent A) and MeOH (solvent B). Solvent A: 0–0.5 min, 100%; 0.5–4 min, 100% → 0%; 4–6 min, 0%/0.25 mL/min.

measured values varied in some cases and more efforts are required to determine absolute MG concentrations in some biological samples.

3 Sources of methylglyoxal and its effects on living organisms

There are several sources of this very reactive α-oxoaldehyde in human and other living organisms. According to origin, the sources can be classified as exogenous and endogenous. Exogenous sources include MG, mostly from food and beverages, while endogenous sources include enzyme-catalyzed MG and spontaneous reactions that lead to MG formation.

3.1 Exogenous sources of methylglyoxal

3.1.1 General

Many food products, beverages, water, rain, clouds, fog water, and urban atmosphere as well as cigarette smoke represent exogenous sources of MG. The origins of MG in food and beverages are sugars, the products of the Maillard reaction, lipids and microorganisms formed during industrial processing, cooking, and prolonged storage.

From carbohydrates, MG can be formed by fragmentation of the sugar moiety during retro-aldol condensation and auto-oxidation. Although this process is highly favored by alkaline conditions, the formation of fragments with an α-dicarbonyl moiety also takes place under conditions that are more relevant for food processing. Moreover, the formation of MG was observed during the heating of glucose, fructose, maltose, and maltulose, where the amount of MG obtained from monosaccharides was markedly higher than that from disaccharides, and so was its amount obtained from glucose in comparison to fructose [60]. MG formation was performed by carbohydrate decomposition during caramelization of mono-, oligo- and polysaccharides (glucose, dextrin 15, and starch) [61].

The formation of MG was also demonstrated in the Maillard reaction. Moreover, a higher concentration of MG was obtained in the presence of OPD and glycine in comparison with the process of caramelization, although, the concentration of MG was higher in the presence of water in both caramelization and the Maillard reaction [61]. Thorough analysis of the reaction mechanism in the degradation pathway of model Amadori compound (*N*-(1-deoxy-D-fructose-1-yl)-glycine (DFG)) has shown that pH increase had almost no effect on MG formation, in contrast to raised temperature that induced its markedly higher release [62, 63]. A very low increase in the MG amount was observed in a glycine/β-alanine reaction mixture heated in phosphate buffer for 12 h, when pH was changed from 5 to 7 [64]. No influence of pH on MG formation justified the previously proposed mechanism of MG formation from both 1- and 3-deoxyosones through retro-aldolization [65]. However, as opposed to pH, the effect of temperature was quite significant: temperature increased from 100 to 120°C more than doubled the formation of MG with no decrease in its amount with time [62]. Another possibility of MG formation as the result of degradation pathway of Amadori compounds could be direct cleavage of DFG into MG [62]. The supposed conversion of glyceraldehydes into MG by the catalytic action of amino acid was also observed in the glucose/glycine model systems [66]. Honey, as a sample with a high content of simple carbohydrates, is a system which is prone to sugar degradation during the heating processes applied in manufacturing and storage. The measured concentrations of MG in honey were in the range of 0.4–5.4 mg/kg (median 2.4) and these amounts were not affected by storage condition either at 35 or 45°C [58].

Decomposition of different lipids, caused by storage and processing, could also affect the accumulation of MG in food samples. Depending on oil origin (tuna, salmon, cod liver, soybean, olive, and corn oils) under accelerated storage conditions (60°C for 3 and 7 days) or cooking (200°C for 1 h), a broad range in MG levels were obtained. In such a manner, the amount of MG formed in fish oils heated at 60°C for 7 days ranged from 2.03 ± 0.13 (cod liver oil) to 2.89 ± 0.11 mg/kg (tuna oil), whereas among vegetable oils under these conditions, only olive oil yielded MG (0.61 ± 0.03 mg/kg) [55]. Photodegradation of lipids and related compounds also yielded MG as a degradation product [42]. In these experiments, squalene, cod liver oil, ethyl esters of fatty acids, and volatile aldehydes were exposed upon UV irradiation and the maximum amount of MG (1008 mg/kg) was formed from squalene after 10 h of UV irradiation. Cod liver oils also produced MG (410.4 mg/kg) as well as ethyl esters of fatty acids, acetaldehyde, acrolein, and propanal, although in minor yields [42].

The accumulation of MG in the growth medium of microorganisms may be an important danger for many fermentation processes. During fermentation, different microorganisms release MG causing an increase of this α-oxoaldehyde in alcohol drinks and dairy products. In wine, MG is synthesized by *Saccharomyces cerevisiae* during alcoholic fermentation as well as by *Oenococcus oeni* (*Leuconostoc oenos*) during malolactic fermentation [37]. The releasing of MG by microorganisms is a common process during milk fermentation by *Lactobacillus* sp. (*L. casei* and *L. delbrueckii*, subsp. *bulgaricus*) [56, 57]. In bacteria, most of the evidence favor the production of MG from DAP *via* the enzyme MG synthase, encoded in *Escherichia coli* by the mgsA gene [67]. In addition to the formation of MG in the metabolic pathways of microorganisms it should not be for-

gotten that most foodstuffs were or are living organisms and MG can be formed in their physiological processes. For example, in rice (*Oryza sativa*), millet (*Pennisetum glaucum*), tobacco (*Nicotina tabacum*), and mustard (*Brassica juncea*), MG concentration varies in the range of 30–75 μM indicating very high levels of MG in plants. Moreover, stress conditions (drought, salinity, and cold) may increase MG concentration up to six-fold [68].

Besides the MG sources in food and beverages, drinking water can also be an exogenous source of MG. Ozonation and chlorination of natural water, usually applied processes in the treatment of drinking water, result in the formation of MG from humic substances [46, 52, 69, 70]. MG has also been identified in urban atmosphere, probably as the result of the reaction of ozone with aromatic hydrocarbons and toluene, compounds generally found in motor exhaust due to incomplete combustion [71]. Rainwater droplets take up MG from the polluted air and convey it to water resources on the earth.

At the end, MG was quantified in cigarette smoke where the measured levels of MG from different cigarette samples were 13.4–59.6 μg/cigarette, which is of great importance because tobacco smoke is one of the major sources of indoor air contamination with toxic aldehyde [45].

3.1.2 Effects of exogenous methylglyoxal

Once formed in food, MG can react with other food components causing the loss of nutritional quality as well as the production of toxic compounds [72–76]. Although the MG concentration in food is relatively low, it has been shown that MG leads to vascular/connective tissue changes when given to healthy animals. The accumulation of collagen in mouse kidneys caused by the intake of low levels of MG over a prolonged period of time (50 mg/kg of body mass/day for 5 months) was observed. Fluorescence in the proteins from extracted kidneys of MG treated animals was about twice that of untreated animals and the glomerular basement membrane thickness was significantly higher in MG treated animals [77]. The adverse effects of exogenous MG on healthy animals were also noticed when a daily intake of 1.7 μmol/mouse/day of MG for 5 days in drinking water resulted in a significant decrease (30.6%) of the blood reduced glutathione (GSH) levels [78]. This indicates that the capacity of red blood cells to refract oxidative stress could decrease by chronic intake of low levels of MG. It is known that red blood cell GSH sufficiency is of utmost importance in protection from endogenous oxidative agents or induced free radical reactions that are involved in red cell hemolytic and hemoglobin precipitin reactions *in vivo* [78]. The same study also demonstrated a highly significant decrease in the activity of blood GSH-*S*-T, the enzyme important in the metabolism and detoxification of various toxic and carcinogenic chemicals [79], in MG exposed mice. In addition to these findings, it has been reported that the systolic blood pressure, platelet [Ca^{2+}], and kidney aldehyde conjugates were significantly higher and serum nitric oxide levels were lower in MG treated rats [80]. Moreover, MG treated rats also showed smooth muscle cell hyperplasia in the small artery and arterioles of the kidney.

However, although the previously cited studies report on the toxic effects of MG given to healthy animals, the intake of MG has also exerted an anticancer effect, which was significantly augmented by ascorbic acid and by a combination of ascorbic acid and creatine. Nearly 80% of animals treated with MG plus ascorbic acid plus creatine were completely cured and devoid of any malignant cells within peritoneal cavity. In addition to these results, the same study showed no toxic effects of MG on the four different animal species (mice, rats, rabbits, and dogs) [81]. The results obtained in this study are in disagreement with many studies which showed toxic effects of MG, where the increase in its concentration was associated with complications of different diseases, especially diabetes. Although the efficacy of a particular drug and the mode of treatment should be assessed by balancing the benefits and adverse effects, some additional efforts are needed to clarify these findings.

3.2 Endogenous sources of methylglyoxal

In vivo MG can be formed in many enzymatic and nonenzymatic pathways. Enzymatic pathways include reactions catalyzed by triosephosphate isomerase, cytochrome P450 2E1, myeloperoxidase, and aminooxidase, whereas nonenzymatic pathways include decomposition of DAP, the Maillard reaction, oxidation of acetol, and lipid peroxidation. Fasting and metabolic disorders such as diabetes can induce an increase in the formation of this highly reactive α-oxoaldehyde.

One of the most important ways of MG production occurs from the triose phosphate intermediates in the glycolytic pathway, which include DAP and GA3P by two processes: spontaneous nonenzymatic elimination of the phosphate group, or decomposition of ene-diol triose intermediate that leaks from the active site of triose phosphate isomerase [82, 83]. Elevated levels of MG measured in body fluids of diabetic patients [29, 84, 85] have been connected with the formation of MG in this pathway as the result of an increased glycolytic flux in erythrocytes and accumulation of triose phosphates with the subsequent production of MG, wherefrom it enters the circulation, most probably by passive diffusion [86]. Although Ahmed *et al*. [87] showed an increased formation of MG in triosephosphate isomerase deficiency, in our study, where MG was determined in whole blood and plasma samples, collected simultaneously under identical conditions from the same subjects, we recorded a higher MG

level in plasma than in whole blood, with a significantly higher MG concentration only in plasma samples as well as ΔMG (plasma MG – whole blood MG) in patients with diabetes [88]. These results are identical to those published earlier, where MG levels were approximately 10–15 times higher in plasma than in erythrocytes of diabetic animals. Moreover, MG levels increased in both erythrocytes and plasma, but the increase was only significant in plasma [89]. All these results indicate that a reduced ability of the glycolytic enzymes downstream of triose phosphates, particularly glyceraldehyde-3-phosphate dehydrogenase [90, 91] and/or defects in MG detoxification processes cause an increase inits levels in diabetes mellitus. In addition to the previously described observations, a significant postprandial increase in MG levels in diabetic patients [92] and a significant decrease in MG levels in patients with reduced diurnal glucose variability in both whole blood and plasma samples [88] were also demonstrated, suggesting the possible utilization of MG level as an additional indicator of diabetes regulation. Namely, many studies have shown a strong correlation between long-term average glycemia and the development of diabetic complications such as retinopathy, nephropathy, neuropathy, and cardiovascular diseases in both type 1 and type 2 diabetes. In these studies, the primary method used to assess long-term glycemic control was the measurement of HbA_{1c}. However, many studies suggest that HbA_{1c} is relatively insensitive to glycemic fluctuation, because diabetic populations with either high or low glycemic excursions but with steady mean glucose levels have similar levels of HbA_{1c}. For this reason, it has been suggested that glucose variability, considered in combination with HbA_{1c}, is a more reliable indicator of blood glucose control for the risk of long-term complications than mean HbA_{1c} alone [93], and the presence of MG could be an indicator of this variability.

The formation of MG in the Maillard reaction takes place not only at high temperatures characteristic of food cooking, but also under physiological conditions. Moreover, MG formation was observed by slow degradation of glucose and Amadori product under physiological-like conditions (phosphate buffer, pH 7.4, 37°C), indicating the possibility of MG formation *in vivo* by these reaction pathways [94]. The other possible sources of MG include catabolism of threonine *via* aminoacetone mediated by semicarbazide-sensitive amine oxidase [95]. The catabolism of threonine normally produces glycine and acetyl-CoA; however, in a low CoA state, such as the one that would exist in diabetic ketoacidosis, where much of the CoA is in the form of acetyl-CoA, threonine is catabolized to aminoacetone [96]. Enzymatic oxidation of ketone bodies (acetoacetate and acetone) can also generate MG. Its production from acetoacetate is mediated by myeloperoxidase [97], whereas P450 2E1 cytochrome catalyzes conversion of acetone into MG in two consecutive steps *via* acetol as an intermediate and consumption of NADPH [98]. In addition, acetol can be transformed back into MG in the presence of copper ions [99], whose disregulation is also observed in diabetes patients [100]. The latter pathways of MG formation are also significant in special body states such as ketosis and diabetic ketoacidosis. Moreover, it has been shown that the popular and widely used Atkins diet, which is characterized by low carbohydrate intake (less than 20 g/day for the first 2 wk and less than 40 g thereafter), leads to a significant increase in MG levels [101]. Otherwise, the increase in MG was greater in healthy people than in those with poorly controlled diabetes. The highly significant relationship between MG and acetol levels, demonstrated by the same authors, suggests that MG is produced directly from acetol by oxidative mechanisms. Although ketone bodies are likely to be an important source of MG, it is also possible that some MG is derived from increased triose phosphate resulting from the increased production of glycerol caused by accelerated triglyceride breakdown, or from lipoxidation products [102]. We have shown that diabetic ketoacidosis, an acquired metabolic disorder, could produce a considerable amount of MG [103]. In that study, it was demonstrated that MG was elevated before the ketoacidosis treatment relative to the values recorded in the control group of diabetic patients, and showed further increase over 12–24 h of therapy initiation. However, in five of seven patients, its fasting level was still increased at 168 h of treatment, indicating that the treatment for ketoacidosis and glycemia control failed to produce a significant reduction of MG toward control values of diabetic population. These findings suggest the necessity of discovering and analyzing other possible sources of MG.

In addition to all above-described sources of MG *in vivo*, identification of some MG-derived AGEs on proteins as a result of lipoproteins oxidation (EAGLEs: either advanced glycation or lipoxidation end-products) suggests lipid peroxidation processes as additional endogenous sources of MG [102].

3.3 Detoxification of methylglyoxal

Several enzymes are involved in the detoxification of MG and make a network of four recognized catabolism pathways, as follows: the glyoxalase system, aldose reductase (ALR), betaine aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase (2-ODH). MG is also excreted from organisms through retina and several pharmaceuticals successfully reduced free MG by transforming it into nonreactive compounds or by preventing its accumulation by activating metabolism.

The glyoxalase system is a metabolic pathway that catalyzes the detoxification of MG to D-lactate. It consists of two enzymes, glyoxalase I and glyoxalase II, and a catalytic

amount of GSH. Glyoxalase I (EC 4. 4. 1. 5) catalyzes the formation of *S*-D-lactoylglutathione hemithioacetal formed nonenzymatically from GSH and MG. Glyoxalase II (EC 3.2.1.6) catalyzes the hydrolysis of *S*-hydroxyacetylglutathione to D-lactate with regeneration of GSH consumed in the reaction catalyzed by glyoxalase I. D-Lactate hydrogenase transforms the formed D-lactate into pyruvate [104]. Because this system depends on the availability of GSH, its activity is severely limited by the conditions of oxidative stress that impact the levels of GSH. In diabetes, the polyol pathway flux is increased with a concomitant decrease of cytosolic NADPH. As NADPH is required for regeneration of GSH, this processes could decrease GSH level and consequently decrease glyoxalase I activity and increase MG content [91].

ALR (EC 1.1.1.21, ALR2, AKR1B1) catalyzes the NADPH-dependent reduction of MG into lactaldehyde in the presence of GSH and into acetol in the absence of GSH. The same enzyme converts both acetol and lactaldehyde into 1,2-propandiol. However, acetol is a much poorer substrate for the second reduction ($k_{cat}/K_M = 3.4 \times 10^4$ mol/L min^{-1}) than lactaldehyde ($k_{cat}/K_M = 5.1 \times 10^6$ mol/L min^{-1}), and thus the GSH decrease could cause acetol increase. As acetol can be spontaneously transformed into MG in the conditions characteristic of diabetes, the reduction of MG by ALR in the absence of GSH could actually be an undesirable reaction [99, 105].

Betaine aldehyde dehydrogenase catalyzes the NAD-dependent oxidation of MG into pyruvate. Some human aldehyde dehydrogenases (ALDH1, ALDH2, and ALDH3) exhibit broad specificity and carry out detoxification of numerous aldehydes through binding their unhydrated forms. Most of the physiological aldehydes are only partially hydrated in physiological conditions, however, α-oxoaldehydes such as MG are completely hydrated. This may explain the fact that α-oxoaldehydes are not good substrates of ALDH1 and ALDH2. However, MG is a fairly good substrate for betaine aldehyde dehydrogenase (ALDH9 or E3), yet considerably poorer than betaine aldehyde [106].

2-ODH catalyzes MG oxidation into pyruvate. The enzyme was for the first time purified from sheep liver [107]. The authors observed that the enzyme was specific for α-oxoaldehydes and that it needed NAD or NADPH for its activity. Subsequent studies of the enzyme revealed the requirement for an activator for any activity. Vicinal aminoalcohols (for example, L-2-amino-1-propanol) have been shown to be the best activators, but the activity was noticed in the presence of aminothioles as well as glycine. Nevertheless, the physiological activator of 2-ODH is still unknown. It may represent an important liver detoxification enzyme for protection against MG [108].

A significant negative correlation between MG and creatinine clearance and its elevated level discriminated patients with increased urinary albumin excretion from those with normoalbuminuria [88]. Elevation of plasma MG was also observed in nondiabetic patients with mild to moderate renal failure and in patients on hemodialysis [109]. All these observations suggest the effect of renal clearance on MG concentration in the blood stream. In addition to these findings, the influence of diet on serum MG level was also demonstrated in renal failure patients (patients with diabetes were excluded from the study). It was shown that a high dietary AGE intake increased serum MG (26%) in contrast to the diet with low AGE intake which caused a decrease of serum MG (35%) [110].

Pharmaceutical inhibition of MG accumulation *in vivo* can also participate in the detoxification processes. Trapping of MG by guanidino compounds such as aminoguanidine and metformin has shown the ability to scavenge this dicarbonyl *in vivo* [111]. It was also demonstrated that pyridoxamine significantly reduced MG levels in red blood cells and plasma, and blocked the formation of MG lysine dimer in plasma protein from diabetic rats [89].

Some new investigations have shown that MG accumulation as well as the formation of MG-derived AGE products could be suppressed with high-dose thiamine (vitamin B_1) and benfothiamine (lipophilic derivative of thiamine), important coenzymes for transketolase (TK). Activation of the reductive pentose phosphate pathway (PPP) by increasing TK activity with high-dose thiamine can stimulate the conversion of GA3P and fructose-6-phosphate (F6P) to ribose-5-phosphate (R5P) and prevent the accumulation of these glycolytic intermediates and consequently the accumulation of MG. It was also demonstrated that in human red blood cells which were incubated under hyperglycemic conditions, the accumulation of MG and triose phosphates could be prevented by thiamine [112]. Similarly, diet therapy with high doses of thiamine and benfothiamine administered to streptozotocin-induced diabetic rats prevented the diabetes-associated accumulation of MG [113], whereas the reduced dietary thiamine therapy in rats decreased TK activity and simultaneously increased the levels of MG protein adducts [114].

4 Conclusion

The knowledge of the Maillard reactions has considerably expanded over years, from classic nonenzymatic interaction between reducing sugars and free amino acids or protein residues to a much broader group of chemically heterogeneous compounds collectively known as AGEs. The recently recognized relationship among α-oxoaldehydes and biologically important macromolecules in food and liv-

ing organisms highlights the intermediate steps of the Maillard cascade. MG is considered as one of the key α-oxoaldehydes leading to AGEs formation. This compound plays a role in oxidative or carbonyl stress under physiological conditions, and its role in the changes of food quality is well documented.

We thank the Croatian Ministry of Science, Education, and Sports for support to our research program.

5 References

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