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Metabolic Transit of Dietary Methylglyoxal

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ABSTRACT: Methylglyoxal (MGO) is responsible for the pronounced antibacterial activity of manuka honey, in which it may reach concentrations up to 800 mg/kg. As MGO formed in vivo is discussed to play a role in diabetic complications, the metabolic transit of dietary MGO was studied within a 3 day dietary recall with four healthy volunteers. Determination of MGO in 24 h urine was performed with GC-MS after derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine, and D-lactate was quantified enzymatically. Following a diet virtually free from MGO and other glycation compounds, a defined amount of MGO (500 μmol in manuka honey) was administered in the morning of day 2. Renal excretion was between 0.1 and 0.4 μmol/day for MGO and between 50 and 220 μmol/day for D-lactate. No influence on excretion of both compounds was observed following administration of MGO. To investigate the stability of MGO under physiological conditions, a simulated in vitro gastrointestinal digestion was performed with MGO-containing honey. After 8 h of in vitro digestion, only 5−20% of the initial methylglyoxal was recovered. This indicates that dietary MGO is rapidly degraded during the digestion process in the intestine and, therefore, exerts no influence on the MGO level in vivo.

KEYWORDS: *methylglyoxal, D-lactate, urinary excretion, in vitro digestion, manuka honey*

INTRODUCTION

Under physiological conditions, the α-oxoaldehyde methylglyoxal (MGO) is mainly formed via the spontaneous degradation of triosephosphates, intermediates of the glycolysis pathway. Moreover, MGO is formed in the catabolism of ketone bodies and amino acids, such as threonine.[1] The formation of MGO via degradation of glycated proteins and monosaccharides in vivo[2] also occurs in food in the course of the Maillard reaction via a variety of reactions.[3] Plasma levels of MGO in healthy subjects are analyzed to range between 0.1 and 0.5 μM and are elevated under diabetic conditions.[4,5] The capability of MGO to react with amino acid side chains of proteins is about 10000-fold higher than that of glucose to form advanced glycation endproducts (AGEs).[6] The AGE derived from the reaction of MGO with the side chain of peptide-bound arginine, namely, the methylglyoxal-derived hydroimidazolone (MG-H1), was found to occur in vivo and in foods,[7,8] emphasizing the importance of MGO-related protein modifications within the course of glycation reactions (Figure 1). Minor protein-bound amino acid modifications of MGO are *N*-ε-carboxyethyllysine and argpyrimidine. Endogenously formed MGO is metabolized via the glutathione-dependent glyoxalase system to less reactive D-lactate (Figure 1). The glyoxalase is omnipresent in the cytosol of all pro- and eukaryontes.[9] Baskaran et al. reported the expression of glyoxalase 1 also in intestinal epithelial cell.[10] In addition, MGO is transformed via the enzyme aldose reductase to acetol and via 2-oxoaldehyde dehydrogenase to pyruvate.[9]

In food products, MGO was detected at first in coffee with amounts ranging between 1.6 and 7.0 mg/L and in fermented foods such as wine (0.1−2.9 mg/L), yogurt (0.6−1.3 mg/kg), and soy sauce (8.7 mg/kg).[11−13] In comparable levels MGO was recently analyzed in a variety of foodstuffs, mainly in sugar-rich and intense heat-treated products such as balsamic vinegar and soy sauce as well as cookies and jams and at very low levels in milk products (<1.6 mg/L). In this study, median MGO levels did not exceed 10 mg/kg or 10 mg/L.[14,15]

MGO was identified as the compound responsible for the pronounced antibacterial activity of manuka honey,[16] which has been linked to several medicinal properties, in particular wound healing.[17] MGO in manuka honey varies between 40 and 760 mg/kg and was shown to arise from dihydroxyacetone, which is uniquely present in the nectar of manuka flowers.[18,19] On the basis of recently published data, the daily ingested amount of MGO from the diet was calculated to range between 5 and 20 mg/day (0.1−0.3 mmol/day).[14] Wallace et al. observed no adverse (elevation of *N*-ε-carboxymethyllysine in plasma) but neither beneficial effects (gut microbial profile) when 20 g of manuka honey was consumed daily for 4 weeks.[20] When high amounts of MGO (60 mg/kg body weight) were administered intraperitoneally or subcutaneously for 4 weeks in rats, biochemical and molecular abnormalities characteristic of type 2 diabetes were observed.[21,22] Together with the observation that methylglyoxal showed cytotoxic properties in in vitro experiments,[23,24] questions may arise concerning possible health risks resulting from methylgloxyal ingested with the daily diet, in particular from MGO-containing manuka honey. The aim of our study, therefore, was to investigate whether dietary MGO present in honey can reach the systemic circulation and thus can contribute to the total methylglyoxal load of the human body. An obvious way to investigate if ingested MGO has reached the systemic circulation is to analyze the compound or a specific metabolite in urine. To obtain basic information of the metabolic fate of ingested

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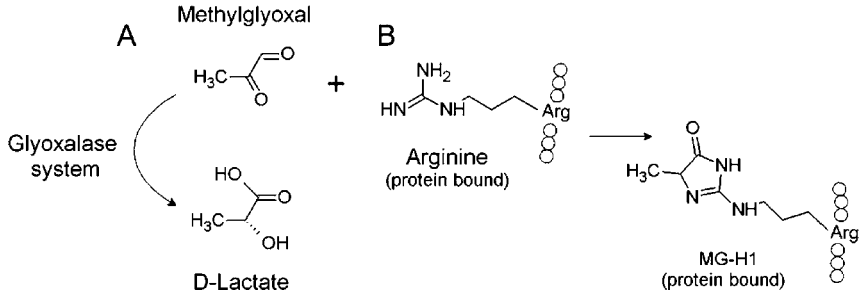

Figure 1. Schematic illustration (A) of the enzymatic transformation of methylglyoxal to D-lactate via the glyoxalase system and (B) of the reaction of protein bound arginine with methylglyoxal to form protein-bound advanced glycation endproduct MG-H1, methylglyoxal-derived hydroimidazolone.

MGO, an orienting intervention study was performed. Urinary elimination of MGO and its major metabolite, D-lactate, in subjects following a diet with no dietary restrictions was analyzed. This is the first study addressing the physiological relevance of dietary MGO in healthy subjects. Following the human studies, model experiments were performed to investigate reactions of MGO occurring during simulated gastrointestinal digestion.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from the sources given in parentheses: lyophilized porcine bile extract (SAF, Steinheim, Germany); enzyme kit "Enzytec D-/L-lactic acid", Generica Line (R-Biopharm, Darmstadt, Germany); glacial acetic acid (AppliChem, Darmstadt, Germany); lithium-D-lactate, methylglyoxal solution (40% in water), mucin from porcine stomach (SAF); *n*-hexane (Merck, Darmstadt, Germany); *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA) (SAF); *o*-chlorobenzaldehyde (Merck); pancreatin from porcine pancreas (U.S.P., SAF); pepsin from porcine gastric mucosa (10 FIP-U/mg) (Merck); trypsin from porcine pancreas (type IX-S, 15700 U/mg) and urea (SAF). Casein was extracted from raw milk via isoelectric precipitation using acetate buffer followed by an acetone/ethanol treatment. For enzymatic analysis, twice-distilled water was used, and for the determination of MGO, water was used that was distilled twice in the presence of potassium permanganate.

Urine Samples. The study comprised a 24 h dietary recall of 7 healthy, nondiabetic volunteers (ages 23−27 years, two men and five women, four nonsmokers and three smokers) who collected their 24 h urine (8:00 a.m. until 8:00 a.m. the following day) with no dietary restrictions ("common" diet). Furthermore, an intervention study with four of the seven healthy volunteers participating in the "common diet" study (ages 23−27 years, one man and three women, nondiabetic, nonsmoking) was performed over 3 days. They followed a diet consisting solely of raw fruits and vegetables, avoiding heated and fermented food such as dairy and bakery products, coffee, juices, and beer to ensure a diet virtually free of methylglyoxal and Maillard reaction products ("raw food" diet). In the morning of the second day, the participants were administered 80 g of manuka honey containing a defined amount of 500 μmol of MGO (MGO concentration of honey was 463 mg/kg). Twenty-four hour urine was collected on every day of the study as described above. Aliquots of collected 24 h urine samples were immediately stored at −18 °C until analysis. MGO and D-lactate were analyzed within 2 weeks.

Simulated in Vitro Digestion. By the means of a simulated in vitro digestion, MGO was incubated for 2 h at pH 2.0 representing the stomach and for 6 h at pH 7.5 representing the intestine at 37 °C under continuous stirring, according to the method given in ref 25. One hundred microliters of a MGO solution (c = 7.2 mM) was dissolved in 4 mL of synthetic gastric solution (290 mg of sodium chloride, 70 mg of potassium chloride, and 27 mg of potassium phosphate monobasic dissolved in 100 mL of water) followed by the addition of pepsin (2.5 mg/4 mL) and mucin (7.5 mg/4 mL) and pH adjustment to 2.0 with 3 N HCl (final concentration of MGO in the synthetic gastric solution = 0.18 mM). After 2 h, 3 mL of a synthetic intestine solution was added to the mixture (30 mg of potassium chloride, 50 mg of calcium chloride dihydrate, 30 mg of urea, 20 mg of magnesium chloride hexahydrate, 100 mg of sodium carbonate, and 900 mg of bile extract dissolved in 100 mL of water) containing 1 mg of trypsin and 32 mg of pancreatin. The pH value was adjusted to 7.5 with solid sodium carbonate. The mixture was then incubated for 6 h with hourly correction of the pH value to 7.5. Samples were withdrawn during the incubation after 10 and 120 min under simulated stomach conditions (400 μL) and after 10, 60, 180, and 360 min under simulated intestinal conditions (800 μL). After sampling, they were immediately frozen without heat or acid deactivation to preserve the samples and stored at −18 °C until analysis. The simulated in vitro digestion of MGO was also performed in the presence of casein (20 mg/4 mL simulated stomach assay). Moreover, the influence of selected components of the synthetic gastric and intestine solution was assessed. Therefore, MGO was incubated for 2 h at pH 2.0 and for 6 h at pH 7.5 (pH adjustment was performed as described above) solely in a solution containing urea (0.9 mg/3 mL synthetic intestinal solution) or solely in the presence of the proteins and the digestive enzymes pepsin, mucin and pancreatin, trypsin (concentrations were used as described above), respectively. In addition, 0.1 g of manuka honey (containing 7.2 mmol MGO/kg honey) was applied to simulated in vitro digestion without the presence of proteins and in the presence of 20 mg of casein, resulting in a concentration of 0.18 mM MGO in the simulated gastric solution. As control, MGO was incubated in aqueous solution (c = 0.18 mM) with described pH adjustment but without addition of the intestinal solutions.

Preparation of Urine Samples for GC-MS Analysis. After the urine had been thawed and centrifuged at 10000 rpm for 10 min, an aliquot of 400 μL was mixed with 400 μL of acetic acid−acetate buffer (0.5 M, pH 5.0) and spiked with 50 μL of the internal standard *o*-chlorobenzaldehyde (c = 17.8 μM in ethanol/water (1:1, v/v)). For derivatization 100 μL of a freshly prepared *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine) hydrochloride (PFBOA) solution (15 mg/L in water) was added to the sample solution and vigorously mixed by vortex. After 6 h of derivatization at room temperature, subsequent extraction with 250 μL of *n*-hexane for 5 min was conducted. An aliquot of 100 μL of the organic layer was transferred to a GC vial and then analyzed.

Preparation of Samples from the Simulated in Vitro Digestion for GC-MS Analysis. The thawed samples were diluted with water prior to derivatization to be in the calibration range. Then an aliquot of 100 μL was spiked with 100 μL of the internal standard *o*-chlorobenzaldehyde (c = 0.89 mM in ethanol/water (1:1, v/v)) with subsequent addition of 800 μL of ice-cold ethanol for protein precipitation. After vigorous mixing by vortex, the sample solution was centrifuged at 10000 rpm for 10 min. A 1:10 dilution step with water was performed with 100 μL of the supernatant. One hundred microliters of the diluted sample solution was mixed with 400 μL of acetic acid−acetate buffer (0.5 M, pH 5.0), 350 μL of water, and 100 μL of a freshly prepared PFBOA solution (15 mg/L in water). The following steps of the derivatization were as described above.

GC-MS Analysis of Methylglyoxal. GC-MS analysis was performed using an HP6890 gas chromatograph, coupled with an

HP7683 automatic liquid sampler and an HP5973 mass spectrometer equipped with an HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; all from Agilent, Germany) and a Zebron Z-guard guard column (deactivated, 5 m, 0.25 mm i.d.; Phenomenex, Germany). The carrier gas was helium with a constant flow at 1.0 mL/min, the injector temperature was 250 °C, and 1 μL of sample was injected using the pulsed splitless injection mode. MS quadrupole temperature was set at 150 °C and MS source temperature at 230 °C. The oven temperature program started at 120 °C (hold time of 1 min) elevated to 230 °C at a rate of 25 °C/min (hold time of 2 min), and finally heated to 300 °C at 30 °C/min with a subsequent post run at 300 °C for 3 min, resulting in a complete run time of 13 min. The mass spectrometer was used with electron impact ionization (70 eV) in SCAN and SIM modes. For detailed SIM parameters, see Table 1. Data acquisition and evaluation were performed with MSD ChemStation software (Agilent, Germany).

Table 1. GC-MS Parameters (SIM Mode) for the Determination of MGO

	quantifier ion (m/z)	qualifier ion (m/z)	retention time (min)
MGO	265	462	7.32 (*syn*/*anti*) 7.60 (*anti*/*anti*)
o-chlorobenzaldehyde (internal standard)	335	300	6.50 (*syn*) 6.78 (*anti*)

For external calibration, stock solutions of MGO in water (0.10−1.95 μM) were prepared, and 100 μL of each stock solution was applied to the derivatization procedure, resulting in 0.010−0.195 nmol MGO per derivatization assay. For the quantification of MGO in urine and in vitro digestion samples, the diluted stock solutions were subjected to the corresponding sample preparation protocols. Due to the formation of stereoisomers with PFBOA, MGO and *o*-chlorobenzaldehyde were quantified on the basis of the sum of their signals. Quantitative evaluation was then carried out by the use of the ratio of the area of summarized analyte peaks and the summarized internal standard peaks.

The limits of detection (LOD) and quantification (LOQ) in urine were calculated as the concentrations of the analyte necessary to show a peak at a signal-to-noise ratio of 3 and 10, respectively. For the determination of the interday repeatability, one urine sample was applied to sample workup and derivatized five times on different days. The recovery of MGO was calculated from the slope of the recovery function after spiking 400 μL of urine with ascending concentrations of MGO via stock solutions (0.01−0.16 nmol per derivatization assay) with subsequent sample workup.

D-Lactate Assay of Urine Samples. The quantitative determination of D-lactate in urine is based on the commercially available enzyme kit Enzytec D-/L-Lactic Acid (R-Biopharm, Darmstadt, Germany). The procedure was transferred to a 96-well microtiter plate method. All employed chemicals were included in the test kit, except for Carrez solutions I and II, NaOH solution, and stock solution of the standard lithium-D-lactate. A dilution step of the glutamate pyruvate transaminase (GTP) suspension (1:6) and the D-

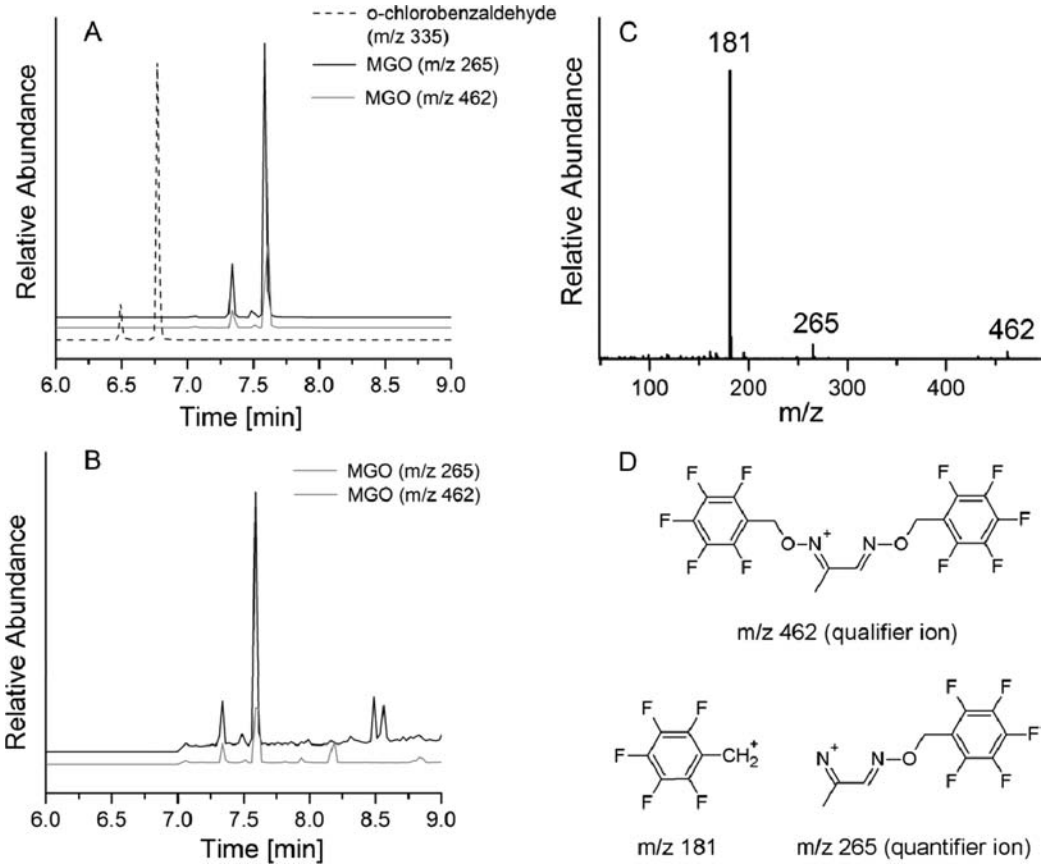

Figure 2. (A) Selected ion monitoring chromatogram of an MGO standard solution (m/z 265 and 462) and the internal standard *o*-chlorobenzaldehyde (m/z 330, quantifier ion) as their bis(pentafluorobenzoyl) (PFB) oximes acquired by GC-MS. (B) Selected ion monitoring chromatogram of a urine sample (m/z 265 and 462) after derivatization with PFBOA. (C) EI-mass spectrum of the peak eluting at 7.70 min of an MGO standard solution acquired by GC-MS after derivatization to the PFB oxime (SCAN mode). (D) Structures of the major peaks detected in the EI-mass spectrum of the peak eluting at 7.70 min of an MGO standard solution: the bis(pentafluorobenzoyl) oxime of MGO $[M]^+$ (m/z 462) and the fragments $[C_6F_5CH_2]^+$ (m/z 181) and $[M - 197]^+$ (m/z 265).

Table 2. Performance Parameters for the GC-MS Method for the Determination of MGO in Urine

	R^2	linear range (μmol/L)	LOD[a] (nmol/L)	LOQ[a] (nmol/L)	relative interday repeatability (%)	recovery[b] (%)
MGO	0.999	0.10−1.56	9.8	29.3	6.9	92.8 ± 3.3

[a]LOD and LOQ were calculated on the basis of signal-to-noise-ratio. [b]Recovery was determined by addition of various concentrations of MGO (0.10−1.56 μM, $n = 5$) to 400 μL of urine under inclusion of all steps of analysis. The value was calculated from the slope of the recovery function and is given in percent ± SE.

lactate dehydrogenase solution (D-LDH) (1:10) of the enzyme kit with bidistilled water was conducted immediately before analysis. For protein elimination, an aliquot of 325 μL tempered urine (20 °C) was mixed with 100 μL of bidistilled water, 25 μL of NaOH solution (30 mM), and 25 μL of Carrez solution I (3.6 g of potassium hexocyanoferrate dissolved in 100 mL of bidistilled water) and shaken for 30 s. Then, 25 μL of Carrez solution II (7.2 g of zinc heptasulfate dissolved in 100 mL of bidistilled water) was added, and after 5 min, the sample solution was centrifuged at 10000 rpm for 10 min. Absorbance at 340 nm was measured by a Multiskan AscentV 1.2 photometric microplate reader (Thermo Labsystems) at room temperature. In each well the following reagents and samples were added: 100 μL of clarified sample, 60 μL of glycylglycine buffer, 20 μL of NAD solution (15 mg/mL), and 20 μL of diluted GPT suspension. After mixing and 5 min of incubation at room temperature, the absorption was read at 340 nm (A_1). Then 20 μL of a diluted D-LDH solution was added to each well and mixed, and after 30 min, the absorption A_2 was read at 340 nm in 1 min intervals until the absorbance revealed constant values. The final volume of the reaction mixture in each well was 220 μL. The difference between the two absorptions (A_1 and A_2) was employed in the calculation, which is proposed from the manufacturer of the enzyme kit. Within each run calibration standards and a blank sample were carried along. Quantitative evaluation was carried out via external calibration, which was performed with a stock solution of lithium-D-lactate (1 mM) within a range of 10−60 μM.

The limit of detection (LOD) in urine was calculated as the concentration of D-lactate necessary to show a difference in the absorption of A_1 to $3A_2$ of 0.01. The limit of quantification (LOQ) was calculated as the triple concentration of the LOD. For the determination of the interday repeatability, one urine sample was separately worked up and analyzed five times on different days. The recovery of D-lactate was calculated from the slope of the recovery function after spiking 325 μL of urine with ascending concentrations of D-lactate (10−60 μM) and subsequent sample workup.

Data Analysis. Quantitative data of MGO and D-lactate in urine are expressed as micromoles per day corresponding to the content in the total volume of the 24 h urine. In vitro digestion was performed in duplicate, and results are given at the mean value ± SE. For evaluation of the in vitro digestion, quantitative data of MGO are given in percent referring to the MGO concentration present at 10 min (first sampling).

Statistical Treatment. Comparisons of means between the daily urinary excretions of the different days were examined using Student's t test. P values of ≤0.05 were considered to be significant (two-tailed) using OriginPro 8.6.

RESULTS AND DISCUSSION

Evaluation of the GC-MS Method for MGO Determination. Quantitative data for MGO in urine reported in the literature differ strongly from 0.28 to 100 μM.[26−29] All data were obtained by using different analytical methods. Therefore, it was important to establish a reliable method with appropriate validation parameters for the quantification of MGO in urine. The method set up in this work is based on methods already described in the literature comprising the essential step of derivatization of the carbonyl groups with PFBOA (Figure 2D) to give the corresponding bis(pentafluorobenzoyl) (PFB) oxime with subsequent GC-MS analysis.[4,5] Furthermore, it was necessary to establish a suitable sample cleanup for the urine matrix and adapt the GC-MS parameters from the literature. This protocol was then transferred to samples of the in vitro digestion experiments. MGO in urine was unequivocally identified via comparison of the chromatographic characteristics of the PFB derivative of MGO in standard solution and in urine together with the corresponding mass spectrum (Figure 2). The derivatization of MGO with PFBOA gives rise to three possible stereoisomers (*anti*/*anti*, *anti*/*syn*, *syn*/*syn*). In the chromatogram two major peaks can be detected at 7.32 and 7.60 min, which can be assigned to the *syn*/*anti* and *anti*/*anti* forms of the PFB oxime of MGO (Table 1), and one minor peak at 7.50 min, which can be presumably assigned to the *syn*/*syn* form, the formation of which is reported to be sterically hindered.[4] For quantification, the summarized peak areas of the two major peaks are employed, which account for >95% of the total peak area. The structures and mass-to-charge ratios of the qualifier and quantifier ions of the PFB oxime of MGO are displayed in Figure 2D, as well as the structure of the base peak of the spectrum (m/z 181), which is generated as a nonspecific fragment of the derivatization agent. Lapolla et al. and Wu et al. examined the use of *o*-chlorobenzaldehyde as the internal standard for the determination of MGO[4,5] and observed good performance, which was proven in this work. A derivatization time of 6 h was found to be adequate on the basis of time course studies. The MGO−PFB oxime was found to be stable for a minimum of 16 h. Extraction with hexane was highly reproducible. No formation of the MGO−PFB oxime was detected when the derivatization was carried out in the presence of only glucose.

The accuracy of the sample preparation and GC-MS analysis is proven with the good performance in the tested validation parameters (Table 2). The main problem of the developed method is the contamination of the derivatization agent with MGO itself and the contamination of distilled water, and therefore these contaminations were revealed to be inevitable. Hence, it is necessary for the MGO determination to use water, which was twice distilled in the presence of the oxidizing agent potassium permanganate to ensure MGO-free water. Furthermore, a blank sample was carried along in every test series, consisting of buffer, internal standard, and PFBOA solution. The peak area of the PFB oxime of MGO obtained in the blank sample was then always subtracted from the peak area of the MGO−PFB derivative detected in standards and samples, where it accounted for only up to 10% of total quantified MGO−PFB oxime. In summary, a reliable method for the analysis of MGO at low concentration has been established which features simple sample preparation and short analysis time. The method can easily be transferred to the analysis of other 1,2-dicarbonyl compounds as well as to the analysis of other short-chained carbonyl compounds when a sensitive detection is required.

Evaluation of the Enzymatic D-Lactate Assay. Talasniemi et al. have already described a 96-microplate enzymatic assay for D-lactate in urine with good performance parameters.[30] In the present work, the method was adopted and the volumes of

Table 3. Performance Parameters of the Enzymatic D-Lactate Assay

	R^2	linear range (μmol/L)	LOD[a] (μmol/L)	LOD[a](μmol/L)	relative interday repeatability (%)	recovery[B] (%)
D-lactate	0.999	10 − 60	4.7	14.0	7.7	94.9 ± 2.0

[a]LOD and LOQ were calculated on the basis of signal-to-noise-ratio. [B]Recovery was determined by addition of various concentrations of D-lactate (10−60 μM, $n = 6$) to 325 μL OF urine under inclusion of all steps of analysis. The value was calculated from the slope of the recovery function and is given in percent ± SE.

added reagents and solutions, especially of D-lactate dehydrogenase (D-LDH) and glutamate pyruvate transaminase (GPT), were adjusted. The adopted method combines the reliability of enzyme kits with the high throughput of microplate assay and allows a selective and rapid quantification of D-lactate in urine with only a minimal sample volume. Although D-lactate is present in urine at only low concentrations, sometimes even below the LOQ, the in-matrix validation parameters, recovery, and interday repeatability show good performance (Table 3). These data are in line with recently published data.[30]

Urinary Excretion of MGO and D-Lactate. We then monitored the urinary excretion of MGO and D-lactate in the urine of healthy, normoglycemic subjects. When following a diet with no restrictions ("common" diet, day 0, Figure 3), an

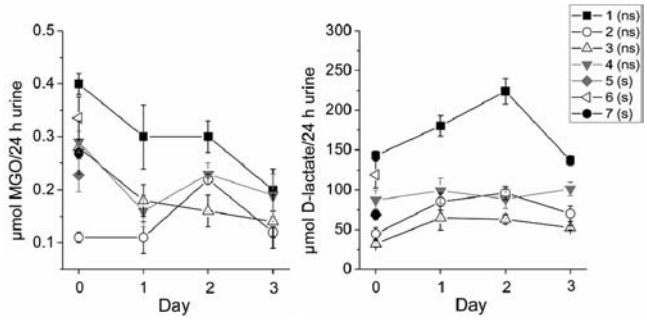

Figure 3. Urinary excretion of (left) MGO and (right) the metabolite D-lactate in 24 h urine of healthy subjects. Day 0, seven test persons followed a "common" diet, among them four nonsmokers (ns) and three smokers (s); days 1−3, four persons followed a "raw food" diet with administration of 500 μmol of MGO on the morning of the second day. *Y*-error bars indicate SE.

ingestion of about 100−300 μmol of MGO can be estimated (compare ref 14). The MGO level in the 24 h urine was between 0.11 and 0.40 μmol and the D-lactate level between 32 and 143 μmol (Figure 3). The detected amounts of urinary MGO were in the same range as data published by Zhang et al.,[26] who have measured 0.28 μmol/L urine. Quantified urinary D-lactate levels were also comparable with literature data.[30−32] In healthy subjects, plasma MGO levels between 0.1 and 0.5 μM were determined,[4,33] the same range as present in urine. Among the seven test persons of the "common" diet group (compare Figure 3, day 0), there were three smokers who smoked 10−15 cigarettes per day. Contents of 0.2−0.8 μmol MGO were detected in the smoke of one cigarette,[34] resulting in a possible exposure to MGO through cigarette smoke of between 2 and 12 μmol/day in this study. No influence of the cigarette smoke on the excreted amount of MGO and D-lactate in urine was detectable ($p > 0.05$). During the intervention study (days 1−3, Figure 3) a "raw food" diet was followed with administration of a defined amount of 500 μmol of MGO in manuka honey on the morning of the second day. Under the assumption of a 50% absorption and elimination of unmodified MGO, an ingestion of 80 g of manuka honey would have led to an increase in the urinary excretion of MGO up to 250 μmol/24 h. Likewise, the amount of D-lactate would have risen by about 100−200% if dietary MGO is absorbed and metabolized. In this study, however, the administration of 500 μmol of MGO did not lead to a significant increase in either MGO or D-lactate excretion. Only a slight but not significant increase of the urinary MGO concentration was detected ($p > 0.05$). On average, the increase from day 1 to day 2 was <0.1% referred to the ingested amount of MGO. MGO elimination in urine was between 0.11 and 0.30 μmol/day (Figure 3, left) and that for D-lactate between 52 and 224 μmol. Mean levels did not differ significantly between the raw food diet and manuka honey ingestion (Figure 3, right). These experiments clearly indicate that even rather high amounts of MGO applied with foods such as manuka honey have no influence on the urinary elimination of MGO and D-lactate. This does not necessarily mean that MGO is unable to enter the systemic circulation. D-Lactate is no final endproduct of MGO metabolism, because it can be further degraded to pyruvate by dehydrogenases[35] and its physiological half-life is only 21 min.[36] Therefore, it accumulates only to a certain extent in urine. The detection of an influence of dietary MGO to the excreted amounts of MGO and D-lactate could also have been hampered by the endogenous formation of MGO, which is estimated between 400 and 3000 μmol/day.[37,38] If an absorption rate of dietary MGO from the intestine of 20% is assumed, the proportion of the absorbed dietary MGO to endogenously formed MGO would be between 4 and 20%. In healthy subjects, it would be readily metabolized to D-lactate by the potent glyoxalase system.[9] It must be pointed out that our results are not in line with the findings of Nakayama et al., who observed an influence on plasma MGO levels after consumption of 300 mL of carbonated soft drinks containing 2.2 μmol of MGO besides 34 g of carbohydrates. This finding could be due to absorption of dietary MGO, but may more likely result from an increased glycolytic flux due to ingestion of carbohydrates.[39] The latter conclusion is supported by studies of Beisswenger et al., who detected a postprandial increase in plasma MGO levels following a carbohydrate-rich rest meal.[40] In an animal study with rats, Golej et al. observed that daily amounts of 0.7 mmol MGO/kg body weight orally administered over 5 months induced pathological changes in the kidneys of the animals (glomerular basement membrane thickening), indicating a possible absorption of ingested MGO and transfer to the kidneys.[41] However, this "worst case scenario" is far outside realistic dietary conditions. Scaled up to a human study, this would mean a daily intake of 42 mmol of MGO, corresponding to 6.5 kg of the manuka honey used in this study, for a person weighing 60 kg. The major source of endogenously formed MGO is the glycolytic pathway, through which it is formed as a byproduct from triosephosphate intermediates.[38]

Taken together, the orally administered amount of MGO employed in this intervention study seemed to be too low to show any influence on the renal elimination of MGO and D-lactate. To obtain more information about a possible

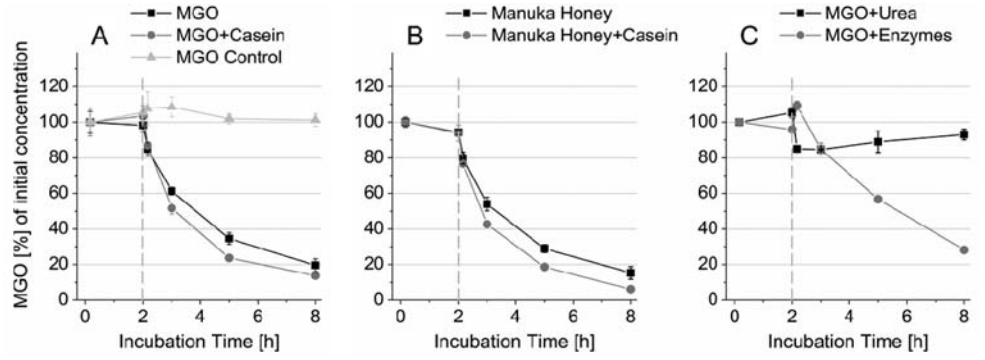

Figure 4. (A) Simulated in vitro digestion of aqueous MGO solution (c = 0.18 nm) with or without casein and a control sample of MGO (only pH adjustment, no added synthetic gastrointestinal solutions). (B) Simulated in vitro digestion of a manuka honey (c_{MGO} = 0.18 mM) with or without casein. (C) Simulated in vitro digestion of aqueous MGO solution (c = 0.18 nm) solely in the presence of urea or gastrointestinal proteins and enzymes.

absorption of reactive MGO from the diet, it is necessary to extend these preliminary experiments. On the one hand, MGO-derived AGEs should be monitored in urine and, on the other hand, analysis of MGO and D-lactate in plasma after oral administration of MGO should be conducted. The best alternative would be to administer ^{13}C-labeled MGO. This would also indicate if D-lactate is a suitable metabolite monitoring possible absorption of MGO.

Stability of MGO under Simulated Digestion Conditions. Another approach to explain why dietary MGO did not influence the urinary excretion of MGO or its metabolite D-lactate is the high reactivity of the dicarbonyl compound toward nucleophilic amino acid side chains of proteins, peptides, or amino acids, resulting in the formation of AGEs. Before absorption, formation of AGEs with food or body proteins is more presumable, because after absorption, effective detoxifying systems will most probably transform reactive MGO to less reactive compounds such as D-lactate.[9,42] Therefore, we tested the stability of MGO under simulated gastrointestinal (GI) conditions, either alone or in the presence of a food protein. Standard solutions of MGO were incubated for 2 h at pH 2 and for 6 h at pH 7.5 in the presence of peptidases to simulate the conditions of the stomach and the small intestine. The concentration of MGO in the incubation experiments was chosen according to the MGO content of manuka honey. To simulate possible interactions between dietary MGO and food proteins in the intestine, casein was added in amounts based on the protein content of milk, which resembled 200 mL of milk and 20 g of honey in an assumed stomach volume of 700 mL.

During the in vitro digestion of a MGO standard solution (c = 0.18 mM), a decrease of the MGO concentration to 19.6 ± 3.6% was measurable at the end of the digestion procedure. When casein was present, the decline was more distinct (to 13.8 ± 0.9%, Figure 4A). To rule out that MGO decomposed nonenzymatically to L- and D-lactate, the stability of MGO was tested under the applied pH conditions in the absence of the simulated GI solutions. MGO in aqueous solution was revealed to be stable over 8 h (Figure 4A) and no de novo formation of L- and D-lactate was observable (data not shown). No marked influence of urea, which is present in the synthetic intestinal fluid, was detected on the MGO concentration; the MGO decrease at the end of the digestion was 6.9 ± 2.9% (Figure 4C). This indicates that MGO must react with other components of the synthetic gastrointestinal fluid, probably with the proteins and enzymes present. This is supported by the observation that MGO degradation slightly increased with added casein. To display the situation when manuka honey is orally consumed, as in the above-described intervention study, the simulated digestion was performed with MGO containing manuka honey with or without the presence of casein. The decrease in the MGO concentration was comparable to the decrease during digestion of the MGO standard solution, but slightly more pronounced: When manuka honey was incubated without casein, the MGO decrease was down to 15.3 ± 3.5%. In the presence of casein, only 6.1 ± 0.3% of the initially present MGO was analyzed after the digestion procedure (Figure 4A,B). When MGO was incubated solely in the presence of gastrointestinal proteins and enzymes, a significant decline to 28.2 ± 0.1% of the initial concentration was detectable (Figure 4C). The decrease in MGO solely in the presence of proteins quantitatively explains the MGO decrease during simulated in vitro digestion (Figure 4A,C), proving the high reactivity of MGO toward proteins. The rate of decrease is in line with the results of Lo et al., who reported that about 70% of free MGO reacts within 15 h with bovine serum albumin under physiological conditions.[43] A rough estimation of the lysine and arginine residues present in the simulated gastrointestinal fluid due to the digestive enzymes/proteins yields lysine and arginine concentrations of around 1.7−2.0 mM each in the simulated GI solutions. Hence, these peptide-bound amino acids are present in an about 10-fold molar excess compared to MGO in the digestion assay. As Figure 4 displays, MGO was revealed to be stable under simulated gastric conditions (pH 2.0) compared to simulated intestinal conditions (pH 7.5), indicating that reactions with proteins at pH 2.0 either do not occur or are reverted at higher pH. The reaction of MGO with proteins at the physiological pH value is well described.[1,44] Further investigations have to be done to elucidate which of the digestive enzymes/proteins are prone to MGO modification during dwell time in the gastrointestinal tract, which protein-bound products are formed, and if a possible modification by MGO has an influence on enzyme functionality.

In conclusion, the intervention study clearly shows that even relatively high amounts of dietary MGO do not lead to an increase in urinary excretion of MGO or its metabolite D-lactate. As MGO was shown to be unstable under simulated gastrointestinal conditions, we conclude that free MGO resulting from the diet most probably does not reach the systemic circulation. The instability of MGO can be attributed

to its reactivity toward lysine and arginine side chains of proteolytic enzymes and food proteins ingested together with MGO. The gastrointestinal tract of healthy human subjects acts as a barrier for reactive dicarbonyl compounds such as methylglyoxal, which are restrained from entering the circulation by chemical reaction with amino acid side chains in a Maillard reaction. Furthermore, an enzymatic degradation of MGO via the glyoxalase system, which is putatively present in epithelial cells in the small intestine, may also contribute to the degradation of dietary MGO. According to Richards et al. and Thornalley et al., the total flux of MGO resulting from the glyoxalase system in vivo ranges between 0.4 and 3 mmol/day.[37,38] Compared to this and assuming that dietary MGO is not taken up in the intestine, exogenously applied MGO (ranging from 0.1 to 0.3 mmol/day) does not contribute to the total "MGO load" of the human body, even if consumed in high amounts with food items such as coffee or manuka honey. The observation that MGO is stable under the acid conditions simulating the stomach may be of relevance for possible health effects of manuka honey, for which antibacterial properties against *Helicobacter pylori* in the stomach were reported.[45,46] The fact that MGO reacts with enzymes within the gastrointestinal tract warrants further studies, although an adverse influence of MGO on the proteolytic activity seems unlikely due to the excess of enzymes compared to ingested MGO. Taken together, we conclude that possible "health risks" due to consumption of MGO-containing foods seem to be negligible for healthy persons.

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ABBREVIATIONS USED

AGE, advanced glycation endproduct; D-LDH, D-lactate dehydrogenase; GC-MS, gas chromatography−mass spectrometry; GTP, glutamate pyruvate transaminase; LOD, limit of detection; LOQ, limit of quantification; MGO, methylglyoxal; MG-H1, methylglyoxal-derived hydroimidazolone; PFB, bis-(pentafluorobenzoyl); PFBOA, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride

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