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Influence of in Vitro Simulated Gastroduodenal Digestion on Methylglyoxal Concentration of Manuka (*Lectospermum scoparium*) Honey

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ABSTRACT: Manuka honey (MH) is a functional food that shows in vitro antimicrobial activity and to which wound healing properties, positive effects on oral health, and beneficial properties during the treatment of gastrointestinal infection diseases and upper gastrointestinal dyspepsia are assigned. The antibacterial activity of MH is mainly due to its high concentration of methylglyoxal (MGO), a highly bifunctional alkylating agent that can induce rapid nonenzymatic modifications of proteins. The aim of the present study was to investigate the influence of in vitro simulated gastric and gastroduodenal digestion on MGO content of MH. To this aim commercial MH samples, with different MGO concentrations, were submitted to digestion, and MGO was determined before and after digestion by a validated RP-HPLC-DAD method. Moreover, the role of MGO in causing carbonylation of the digestive proteins and influencing their enzymatic activities was investigated. The results showed that after digestion MGO concentration decreases because it reacts with digestive enzymes by carbonylating their free amino groups. Nevertheless, carbonylation of pepsin and pancreatin does not influence their physiological activity and therefore does not seem to interfere with the digestion process.

KEYWORDS: *manuka honey, methylglyoxal, gastroduodenal enzymes, protein carbonylation, enzymatic activity*

■ INTRODUCTION

Honey is a natural product that is consumed for its relevant nutritional and healthy properties. Carbohydrates are the main constituents, which represent about 95% of the honey dry weight. Beyond fructose and glucose, honey contains prebiotic oligosaccharides such as panose, which is the most active in promoting the growth of bifidobacteria and lactobacilli. Honey contains also a number of nutrients, such as proteins, mainly enzymes (diastase, invertase, and glucose oxidase); amino acids; minerals; and vitamins, like ascorbic acid.[1] Polyphenols, especially phenolic acids and flavonoids, are another important group of compounds with functional properties, the concentrations of which range from 50 to 500 mg/kg depending on the honey's botanical origin.[2,3]

Due to its complex composition, consisting of over 100 different compounds, honey shows many biological effects, such as antimicrobial, antioxidant, antimutagenic, antitumor, and anti-inflammatory properties, that are correlated to its healthy effects in otorhinolaryngology, pediatrics, cardiology, and gastroenterology.[1] Among these properties, the antibacterial activity of honey, which has been known since the 19th century, is the most studied.[4−6] The broad-spectrum antibacterial activity of honey is ascribed to its high osmolarity (due to the concentration of sugars combined with a low moisture content), low pH, and hydrogen peroxide, produced mainly during glucose oxidation catalyzed by α-glucosidase. Recent studies underlined that other bioactive substances derived from the floral source are responsible for the antibacterial activity of honey. The nonperoxide antibacterial activity was first identified in manuka honey (MH). In in vitro assays, MH has been shown to inhibit a wide range of bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus mutans*, and *Pseudomonas aeruginosa*.[7−10] Moreover, most gastrointestinal bacteria, including multiantibiotic resistant strains (such as *Salmonella typhimurium* DT104) are susceptible to the antimicrobial activity of MH with minimum inhibitory concentration and minimum bactericidal concentration values ranging from 5 to 17% of honey.[11] The outcome of MH antimicrobial activity is its commercialization for both nutritional and pharmaceutical usage due to its wound healing properties that accelerate wound recovery, its positive effects on oral health, and its beneficial properties during the treatment of gastrointestinal infection diseases and upper gastrointestinal dyspepsia. The antibacterial activity of MH is associated with the high concentration of methylglyoxal (MGO), up to 100-fold higher in comparison with conventional honeys (whose concentration ranges from 0.4 to 5.4 mg/kg): MGO derives from the conversion of dihydroxyacetone, which is present at high concentrations in the nectar of the flowers of the New Zealand and Australian indigenous tree *Leptospermum scoparium*.[12]

MGO is a α-dicarbonyl compound that can derive from sugars, Maillard reaction products, and lipids, formed during industrial processing, cooking, and prolonged storage. MGO is widely distributed in many food products and beverages, such as fermented beverages (up to 1 mg/L in beer and 20 mg/L in sweet wine), coffee (up to 20 mg/100 g), edible oils (up to 6.5

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mg/kg), and bakery products (up to 80 mg/kg).[13−16] MH samples, aged less than 1 year, were found to contain levels of MGO ranging from 102 to 793 mg/kg, whereas after long-term storage or heat treatment MGO content significantly increases up to 1541 mg/kg.[17−19] The amount of MGO is used as a criterion for MH classification. The pronounced antibacterial activity of MH has become an important commercial factor used for marketing promotion as the so-called "Unique Manuka Factor" (UMF), leading to a classification of premium products based on in vitro microbiological assays. Nevertheless, MGO is a highly reactive metabolite, and concerns about the potential toxicity[20] of dietary MGO in honey and its effects when used for wound-healing purposes have been expressed by various researchers, and this aspect remains to be investigated.[21]

Considering that MGO is a reactive bifunctional alkylating agent that covalently binds free amino groups and is able to induce rapid nonenzymatic modification of lysine and arginine residues of proteins and peptides, the aim of the present study was to investigate the influence of gastroduodenal digestion on the MGO content of commercial MH samples. To this aim MH samples with different MGO concentrations were submitted to in vitro digestion protocols simulating either gastric digestion alone or followed by duodenal digestion. To determine the concentration of MGO before and after in vitro simulated digestion, a RP-HPLC-DAD method was developed and validated. To better investigate the interaction between MGO and the digestion enzymes, (1) aqueous solutions with standard MGO concentrations were submitted to gastric and gastroduodenal digestion and the residual MGO content was determined, (2) the role of MGO in causing carbonylation of the digestive proteins was investigated, and (3) the enzymatic activity of digestive enzymes in the presence of MGO at the concentrations occurring in MH was studied to evaluate the effect of protein carbonylation on the structure−activity relationship of the digestive enzymes.

■ MATERIALS AND METHODS

Reagents. HPLC-grade water and acetonitrile, ethyl acetate, ethanol, Tris-buffer (pH 7.20), sodium hydroxide (4 N), hydrochloric acid (4 N), sodium bicarbonate, trichloroacetic acid (TCA), o-phenylenediamine (OPD), methylglyoxal, 2-methylquinoxaline, pepsin from porcine gastric mucosa (≥400 units/mg protein), pancreatin from porcine pancreas (8×USP), bile salts, 2,4-dinitrophenylhydrazine (DNPH), guanidine HCl, pespin substrate Phe-Ala-Ala-Phe(4-NO_2)-Phe-Val-Leu (4-pyridylmethyl), and pancreatin substrate Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (DL-BApNA) were purchased from Sigma-Aldrich (Milan, Italy).

Manuka Honey Samples. Honeys from manuka (*L. scoparium*) were obtained from an Italian herbalist's shop.

In Vitro Simulated Gastric and Gastroduodenal Digestion Process. Five MH samples were submitted to in vitro simulated gastric and gastroduodenal digestion process as reported by Ames et al. with some modifications.[22] Briefly, 20 g (corresponding to a portion) of each MH sample was dissolved in 10 mL of Millipore grade water and added to 5 mL of freshly prepared pepsin (1.6 g in 10 mL of 0.1 M HCl), the pH was adjusted to 2.00 ± 0.02 using 4 M HCl, and the mixture was incubated at 37 °C for 2 h in a shaking water bath. In parallel, 10 mL of standard MGO solutions (at the same MGO concentrations occurring in a portion of each MH sample, ranging from 0.83 to 15.27 mM) were submitted to the same procedure.

After the gastric digestion step, the samples were divided into two aliquots: one aliquot was brought to 10 mL with Millipore grade water, ultrafiltrated with Amicon Ultra centrifugal filter units (Ultracel low binding regenerated cellulose, cut off 3000 Da) for 30 min at 8000 rpm at room temperature, and then used for the chemical assays. The second aliquot was adjusted to pH 7.00 ± 0.02 with 0.1 M $NaHCO_3$ and added to 1.25 mL of freshly prepared pancreatin bile mixture (0.04 g of pancreatin and 0.25 g of bile salts in 10 mL of 0.1 M $NaHCO_3$), before incubating for 1 h at 37 °C. At the end of the gastroduodenal digestion, the samples were brought to 10 mL, submitted to ultrafiltration as described above, and then used for the chemical assays.

Sample Preparation for RP-HPLC-DAD Analysis. MGO was analyzed as the corresponding quinoxaline after derivatization with OPD, according to Silva Ferreira et al. with some modification.[15] Quantification was performed with the spiking method: before the derivatization reaction, the honey samples and the standard MGO solutions, before and after the in vitro gastric and gastroduodenal digestion process, were divided into three aliquots. Each aliquot was added to standard MGO solution (spikes of 0, 50, and 100%) and subjected to SPE according to Daglia et al.[14] Briefly, a tC_{18} Sep-Pak Vac 6 cm^3 cartridge (Waters, Milford, MA) containing 1 g of stationary phase was conditioned with methanol (10 mL) and Millipore grade water (20 mL). A 10 mL aliquot of each spiked solution was passed through a cartridge at a flow rate of ≤2 mL/min. MGO was eluted from the cartridge with 2 mL of distilled water and the whole 12 mL (SPE1 fraction) was subjected to derivatization: the SPE1 fraction was adjusted to pH 3.00 ± 0.02 with HCl solution (4 N), added to 6 mg of OPD, and kept at 37 °C for 1 h. The derivatized SPE1 fraction was filtered with nonsterile disposable syringe filters (0.22 μm) and then analyzed by RP-HPLC-DAD in triplicate.

RP-HPLC-DAD Analysis. All experiments were performed using an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump and diode array detector. The Agilent Chemstation software was used for control of the HPLC system and data processing. 2-Methylquinoxaline (methylglyoxal quinoxaline derivative) was separated by a Zorbax Eclipse XDB-C18 analytical column (150 mm × 4.6 mm i.d., 5 μm particle size) connected to a Hypersil Gold C18 guard column (10 mm × 2.1 mm i.d., 5 μm, both from Phenomenex, Torrance, CA), using a gradient of increasing acetonitrile concentrations in water acidified with 0.1% formic acid (v/v) (acetonitrile concentration: 0 min, 10%; 0−5 min, 15%; 5−30 min, 15%; 30−45 min, 30%; 45−50 min, 50%; 50−60 min, 100%; 60−65 min, 100%; 65−70 min, 10%; 70−80 min, 10%). The mobile phase flow rate was 0.3 mL/min. 2-Methylquinoxaline was identified by comparing its retention time and UV spectrum with that of the standard compound.

RP-HPLC-DAD Method Validation. Quantification of MGO as 2-methylquinoxaline was performed following ICH procedures.[23] To evaluate the concentration ranges useful for the method validation, MGO was determined in MH1 sample with the matrix spiking method. The linearity was studied through the matrix spiking method by testing MH1 sample at three standard compound concentration levels. The spiked samples at each concentration level were injected in triplicate. A calibration curve was generated to confirm the linear relationship between the analyte peak areas versus the analyte concentrations. Calibration curves (slope and intercept) and correlation coefficient (r) were calculated as regression parameters by linear regression.

The accuracy of the method was measured through a recovery assay, where spiked MH1 samples at the same concentration levels as the standard concentrations were analyzed. The study was performed in triplicate, and the accuracy is expressed as a percentage of the amount recovered compared with the standard concentrations. The precision was evaluated using the measurements of the repeatability (intraday) and intermediate precision (interday). The repeatability was investigated using three replicate injections of each spiked sample at the same concentration levels that were used in the accuracy study. The intermediate precision was determined using freshly prepared solutions at the same concentration levels used for the repeatability study after two consecutive days. The results are expressed as the relative standard deviation percentage of the measurements (RSD%).

The limits of detection (LOD) and limits of quantification (LOQ) were estimated using the six calibration curves calculated during the validation procedure, from which the average of the slope (S) and the

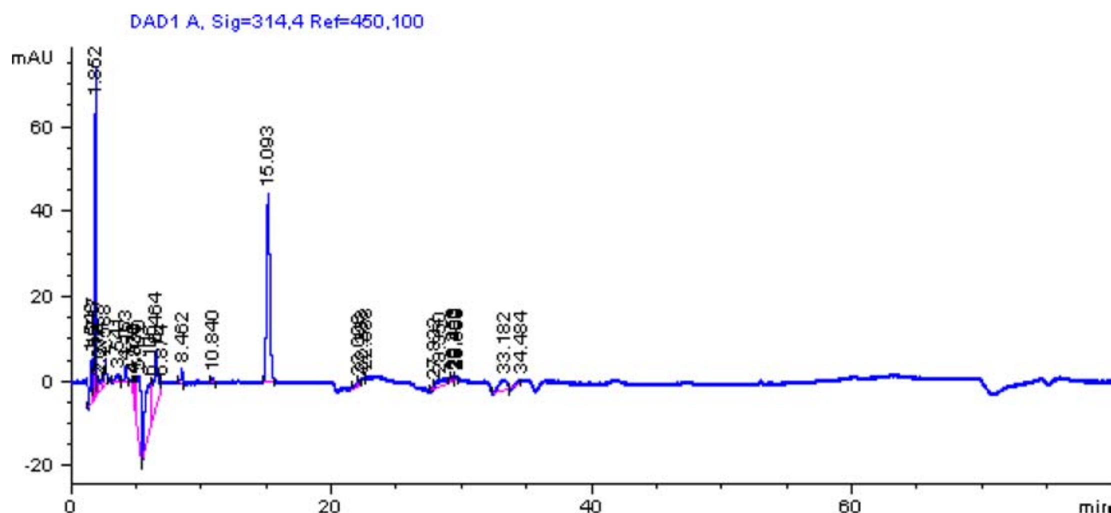

Figure 1. RP-HPLC chromatogram with UV detection at 314 nm of derivatized SPE1 fraction obtained from undigested MH1. Solvent gradient as reported in Materials and Methods.

standard deviation of intercept (δ) were calculated. LOD and LOQ were obtained as follows: LOD = $3.3\delta/S$, LOQ = $10\delta/S$.

Carbonylation by MGO of Digestive Enzymes. Pepsin and pancreatin at the same concentrations as used in the in vitro simulated gastroduodenal digestion were incubated with protease inhibitors and MGO at increasing concentrations. The total protein-bound carbonyl content (protein carbonylation) was determined by derivatizing the protein carbonyl adducts with DNPH, as reported by Dong et al. with some modifications.[24] Briefly, five standard MGO solutions (1 mL) at the same MGO concentrations occurring in each different MH portion, ranging from 0.83 to 15.27 mM, were added to 0.5 mL of pepsin (1.6 g in 5 mL of 0.1 M HCl), 10 μL of phenylmethanesulfonyl fluoride (2 mg/mL), 1 μL of leupeptin hemisulfate salt (10 mg/mL), and 1 μL of aprotinin from bovine lung (10 mg/mL). The solution was brought to pH 2.00 ± 0.02 with HCl (1 M) and incubated at 37 °C for 2 h in an oscillating water bath. The solutions were subdivided into two aliquots. The first aliquot was submitted to protein carbonyl content determination, while the second aliquot was brought to pH 7.00 ± 0.02 with $NaHCO_3$ (1 M), added to 62.5 μL of pancreatin (0.04 g of pancreatin in 5 mL of 0.1 M $NaHCO_3$), incubated at 37 °C for 1 h, and then submitted to the determination of protein carbonyl content. The 100 μL portions of both aliquots were brought to 2 mL and incubated for 1 h in the dark at room temperature with 2 mL of DNPH (0.1%, w/v in 2 M HCl). Then, an aliquot of 4 mL of TCA (20%, w/v) was added to the suspension to stop the reaction. The sample was centrifuged at 8000 rpm at room temperature to obtain the protein pellet, and the supernatant was discarded. DNPH was removed by extracting the pellet three times using 1 mL of ethyl acetate:ethanol (1:1, v/v) solution. After the extraction, the pellet was dried under a gentle stream of nitrogen and dissolved in 4 mL of Tris-buffered 8 M guanidine HCl (pH 7.20 ± 0.01). Pepsin and pepsin plus pancreatin without MGO were submitted to the same assay (control). The solubilized hydrazones were measured spectrophotometrically using an extinction coefficient of 22 000 M^{-1} cm^{-1} at 374 nm.

Enzymatic Activity of Pancreatin and Pepsin in the Presence of MGO. The activity of pepsin in the presence and absence of MGO was assayed with the chromogenic substrate (Phe-Ala-Ala-Phe(4-NO_2)-Phe-Val-Leu-O4MP). In these assays, 50 nM of pepsin were incubated with or without increasing concentrations of MGO (ranging from 0.83 to 15.27 mM) at 37 °C and pH 2.00 for 2 h. Samples were added with the substrate (200 μM final concentration), to make 1 mL final volume. The extent of the reaction was followed by monitoring the changes in absorbance at 300 nm using a JASCO V-550 spectrophotometer.

The activity of pancreatin in the presence and absence of MGO was assayed using the chromogenic substrate DL-BApNA. In these assays, an aliquot of 0.25 mg of pancreatin was incubated with or without MGO at 37 °C and pH 7.00 for 2 h. Samples were added with DL-BApNA (0.312 μM final concentration, from a 5 mM stock solution in 100% DMSO) to make 1 mL final volume. The *p*-nitroaniline released was measured at 405 nm, using a JASCO V-550 spectrophotometer.

Statistical Analysis. The data regarding MGO concentrations in digested and undigested manuka honey represent the mean values obtained from a triplicate analysis of the honey samples. The data regarding the carbonylation and enzymatic activity of the digestive enzymes in the presence of MGO represent the mean values obtained from at least five analyses.

RESULTS AND DISCUSSION

RP-HPLC-DAD Method Development and Validation. In the initial phase of the investigation a RP-HPLC-DAD method was developed and validated to determine the concentration of MGO in MH before and after gastric and gastroduodenal digestion. To this aim, the amount corresponding to a portion (20 g) of a commercial MH honey sample (MH1) was treated with pepsin at pH 2.00 at 37 °C for 2 h, to mimic the gastric digestion, and then with pancreatin and bile salts at pH 7.00 at the same temperature for 1 h, to mimic duodenal conditions. At the end of the digestion process, both gastric and gastroduodenal digested MH1 samples were submitted to ultrafiltration with Amicon Ultra centrifugal filter units for 30 min at 8000 rpm. The undigested (UN-D), the gastric digested (GA-D), and the gastroduodenal digested (GD-D) MH1 samples were then purified by SPE as reported by Daglia et al.[14] The obtained SPE1 fractions were derivatized with the widely used method commonly applied to food matrixes, involving the reaction with OPD and the formation of quinoxaline derivatives as reported in Materials and Methods (37 °C for 1 h at pH 3.00). The choice of the acidic conditions for the derivatization avoids the fragmentation of sugars, particularly abundant in honey. Each derivatized SPE1 fraction (UN-D MH1, GA-D MH1, and GD-D MH1) was properly diluted and then analyzed by RP-HPLC-DAD. The chromatograms showed a peak with the same retention time (15.093 min) and UV spectrum as the standard 2-methylquinoxaline, confirming the presence of MGO both in undigested (Figure 1) and digested MH1 samples.

Quantification of MGO in MH1 sample before and after gastric and gastroduodenal digestion was performed by the standard addition method. The concentration ranges, the

calibration curves, and the correlation coefficients (r) for each MH1 sample are reported in Table 1. Calibration curves were linear with correlation coefficients higher than 0.998. To evaluate the accuracy and precision of the method, UN-D, GA-D, and GD-D MH1 samples at three concentration levels were analyzed in three standard compound replicates on three different days. Accuracy and intra/interday precision values are listed in Table 2. The results indicate that the developed method shows good accuracy with recoveries that ranged from 97.4 to 103.1%. The method is precise with intraday variation that ranged from 0.42 to 0.92%; the interday variation ranged from 0.43 to 1.38%. LOD and LOQ values were 0.043 and 0.130 mg/mL for UN-D MH1, 0.099 and 0.264 mg/mL for GA-D MH, and 0.082 and 0.248 mg/mL for GD-D MH1, respectively.

Table 1. Concentration Ranges, Calibration Curves, and Correlation Coefficients of the RP-HPLC-DAD Analysis of MH1 Samples before Digestion (UN-D MH1), after Gastric Digestion (GA-D MH1), and after Gastroduodenal Digestion (GD-D MH1)

sample	concn range (μM)	calibration curve	corr coeff (r)
UN-D MH1	30.0	$y = 28.1x + 1003.3$	0.998
	45.0		
	60.0		
GA-D MH1	15.0	$y = 38.7x + 783.5$	0.999
	22.5		
	30.0		
GD-D MH1	5.0	$y = 35.9x + 232.1$	0.999
	7.5		
	10.0		

Quantification of MGO in MH Samples before and after Gastric and Gastroduodenal Digestion. The developed and validated RP-HPLC-DAD method was applied to the analysis of four other MH commercial samples, with different MGO contents (MH2, 400 mg/kg, 8.0 mg/portion; MH3, 250 mg/kg, 5.0 mg/portion; MH4, 100 mg/kg, 2.0 mg/portion; MH5, 30 mg/kg, 0.6 mg/portion; a portion is assumed to be 20 g), before and after gastric and gastroduodenal digestions. Regarding undigested MH samples, the results showed that MGO concentrations correspond to those stated on the labels, with a variability lower than 3% for all the MH samples, with the exception of the MH1 samples, which showed a higher MGO concentration, by about 16% than that declared on the label. The explanation of this latter result could be that during long-term storage of manuka honey, MGO content significantly increases.[18] Anyway, the concentration determined in MH1 sample (637.4 mg/kg) remains well within the literature-based concentration limits.[12] In GA-D and GD-D MH samples, the concentrations of free and therefore detectable MGO decreased, with the gastro-duodenal digestion showing the higher decreases, ranging from 51.2 to 87.8%. These data suggest that MGO reacts with the digestive enzymes involved in both gastric and duodenal digestion (Table 3).

Table 3. Quantification of MGO (mg/kg)[a] in MH Samples before Digestion (UN-D MH) and after Gastric Digestion (GA-D MH) and Gastroduodenal Digestion (GD-D MH)

MH sample	UN-D MH	GA-D MH	concn loss (%)	GD-D MH	concn loss (%)
MH1	637.4 ± 11.8	515.0 ± 12.8	19.2	305.3 ± 6.4	52.1
MH2	388.2 ± 9.1	355.2 ± 8.2	18.5	189.4 ± 3.5	51.2
MH3	261.6 ± 3.2	189.6 ± 3.1	23.7	109.3 ± 2.2	58.2
MH4	117.7 ± 2.7	46.8 ± 0.8	60.2	16.5 ± 0.2	86.1
MH5	33.9 ± 0.5	13.6 ± 0.2	59.8	4.1 ± 0.1	87.8

[a]MGO concentration expressed as mg/kg of edible food.

These results are fully consistent with the recent findings showing that MGO modifies the proteinous components occurring in MH (such as defensing 1), and together they demonstrated clearly the modifying effect of MGO on proteins.[25]

Gastric and Gastroduodenal Digestion of Standard MGO Aqueous Solution. To study in more detail the interactions among MGO and digestive enzymes, five standard MGO solutions at concentrations ranging from 0.83 to 15.27 mM (corresponding to the concentrations occurring in a portion of the previously analyzed MH samples) were submitted to gastric and gastroduodenal digestion as reported above. The concentrations of free MGO after gastric and gastroduodenal digestion are reported in Table 4, and the data confirmed that MGO reacted with the digestive enzymes involved both in gastric and gastroduodenal digestion. The comparison between the data obtained from the analysis of MH samples and standard MGO solutions showed that the concentration of free MGO after digestion was higher when MH is tested. This could be attributed to the influence of the food matrix: when MGO is digested alone, it reacts with the

Table 2. Accuracy (Recovery %), Precision (Repeatability and Intermediate Precision), and Limits of Detection (LOD) and Quantification (LOQ) of the Analytical Procedure for the Analysis of MGO before Digestion (UN-D MH1) and after Gastric (GA-D MH1) and Gastroduodenal (GD-D MH1) Digestion

	concn (μM)	UN-D MH1	concn (μM)	GA-D MH1	concn (μM)	GD-D MH1
recovery %	30.0	97.4	15.0	100.2	5.0	101.9
	45.0	102.0	22.5	98.9	7.5	101.8
	60.0	103.1	30.0	102.8	10.0	100.5
repeatability (RSD%)	30.0	0.42	15.0	0.63	5.0	0.62
	45.0	0.46	22.5	0.74	7.5	0.55
	60.0	0.51	30.0	0.71	10.0	0.92
intermediate precision (RSD%)	30.0	0.52	15.0	0.43	5.0	0.54
	45.0	1.29	22.5	0.69	7.5	0.93
	60.0	0.99	30.0	0.71	10.0	1.38
LOD (μg/mL)		0.043		0.099		0.082
LOQ (μg/mL)		0.130		0.264		0.248

Table 4. Quantification of MGO (mM) Standard Solutions after Gastric Digestion (GA-D) and Gastroduodenal Digestion (GD-D)

initial concn	GA-D	concn loss (%)	GD-D	concn loss (%)
15.27	3.53 ± 0.07	76.8	1.66 ± 0.02	89.1
11.87	3.41 ± 0.06	71.3	1.60 ± 0.03	86.5
7.43	2.59 ± 0.04	65.1	0.95 ± 0.02	87.2
2.97	1.23 ± 0.02	58.4	0.64 ± 0.01	78.4
0.83	0.39 ± 0.01	52.4	0.17 ± 0.00	79.3

digestive enzymes to a greater extent, while when MGO is within the honey matrix, its reaction with digestive enzymes is partially inhibited by other components occurring in manuka honey.

Carbonylation of Pepsin and Pepsin plus Pancreatin by MGO. The ability of MGO to induce the carbonylation of pepsin and pancreatin was also investigated. The degree of carbonylation of the digestive enzymes in the presence of MGO was evaluated using five standard MGO solutions at increasing concentrations (ranging from 0.83 to 15.27 mM) incubated with pepsin and then with pancreatin in the presence of protease inhibitors to avoid the digestion of carbonylated proteins by the same digestive enzymes. As shown in Figure 2,

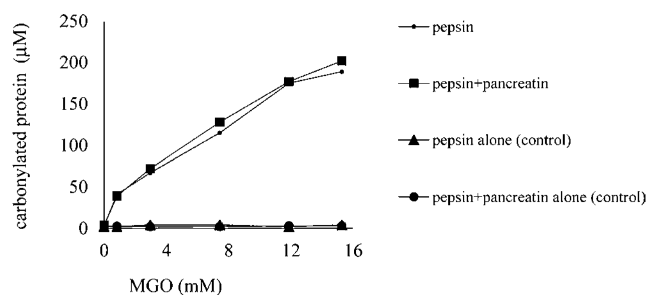

Figure 2. Carbonylation of pepsin and pepsin plus pancreatin by MGO at increasing concentration (ranging from 0.83 to 15.27 mM). Standard deviation <3.5%.

the extent of pepsin and pepsin plus pancreatin carbonylation was dose-dependent with a carbonylated protein concentration that reached 202 μM when both pepsin and pancreatin are incubated with 15.27 mM standard MGO solution.

Enzymatic Activity of Pancreatin and Pepsin in the Presence of Increasing Amounts of MGO. To verify whether the enzymatic activities of the enzymes involved in the digestion were influenced by the presence of MGO, pancreatin and pepsin were treated with a chromophoric substrate after an incubation of 30 min at 37 °C in the presence of increasing concentrations of MGO up to 15.27 mM. The pancreatin reaction in the absence and presence of MGO did not show any significant changes (Figure 3), demonstrating that the carbonylation of the enzyme does not influence its hydrolytic/digestive activity. The same was true for the hydrolytic enzyme pepsin (data not shown), which also did not show any significant difference in its activity in the presence of increasing concentrations of MGO when compared to no MGO added sample.

Considering that MGO is present in many foods commonly consumed, we can hypothetically assume that the enzymatic activities are not affected even when the digestive enzymes have been incubated with a large amount of MGO, and therefore

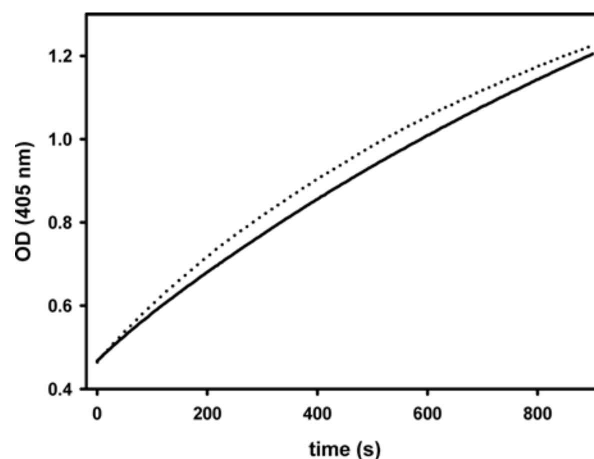

Figure 3. Enzymatic activity of pancreatin in the presence (dotted line) and absence (solid line) of MGO assayed using the chromogenic substrate DL-BApNA. See Materials and Methods for details.

large protein carbonylation has occurred, perhaps a consequence of protein evolution. We can speculate that life evolution have selected, for the digestive enzymes, polypeptide chains whose carbonylation does not affect its three-dimensional structure in a manner that prevent enzyme activity modification.

In conclusion, the study shows that MGO assumed by MH consumption reacts with digestive enzymes by carbonylating their amino groups. Nevertheless, carbonylation of the digestive enzymes does not influence their physiological activity and therefore does not seem to interfere with the digestion process. Therefore, reaction of MGO with the digestion enzymes and the other MH proteins[18] is likely to reduce the level of free MGO in the gastrointestinal tract. In the present investigation we did not examine whether MGO remains bound to proteins during its transit in the intestinal tract and the level is below the limit of toxicity for human cells and above the limit to exert antibacterial activity, because these aspects were beyond the scope of the present study. In spite of the fact that these aspects remain open, on the basis of obtained results we can assume that MGO, besides its proven positive antibacterial properties, is able to induce digestive enzyme carbonylation. This finding could be added to concerns regarding the potential negative effect of MGO on the wound-healing process of diabetic ulcers and on the structure and function of MH proteins.[21,25] Further research is needed to clarify the open questions, and an investigation is currently underway to understand how gastric digestion and gastroduodenal digestion influence the antibacterial and bactericidal activities of manuka honey.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

MH, manuka honey; MGO, methylglyoxal; UMF, unique manuka factor; RP-HPLC-DAD, reverse-phase high-performance liquid chromatography-diode array detection; OPD, *o*-phenylenediamine; TCA, trichloroacetic acid; DNPH, 2,4-dinitrophenylhydrazine; DL-BApNA, Nα-benzoyl-DL-arginine

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4-nitroanilide hydrochloride; LOD, limit of detection; LOQ, limit of quantification; SPE, solid phase extraction; UN-D MH, undigested manuka honey sample; GA-D MH, gastric digested manuka honey sample; GD-D MH, gastroduodenal digested manuka honey sample

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