 doi:10.20944/preprints201805.0409.v1

Article

New Methodology for rapid formation and prevention of advanced glycation end products (AGEs) in vitro coupled with the hypoxanthine/xanthine oxidase assay system

Samuel Marques[1], Teresa Trevisan[2] Carlos Maia[3] Andrea Breuer[4] and Robert Owen [5]*

[1] Instituto Federal de Educação, Ciência e Tecnologia do Ceará. Departamento de Química, Av. José de Freitas Queiroz, 5000, Quixadá - CE, Brasil, CEP: 63902-580; e-mail: samuel.marques@ifce.edu.br
[2] Universidade Federal do Ceará, Campus do Pici – Bloco 935 superior – Laboratório de Produtos Naturais e Biotecnologia e (LPNBio), CP: 60451-970; e-mail: Maria Teresa Salles Trevisan [mariattre@hotmail.com]
[3] Instituto Federal de Educação, Ciência e Tecnologia do Ceará. Departamento de Química, Rua Juvêncio Luiz Pereira, S/N - Monte Castelo, Ubajara - CE, Brasil, CEP: 62350-000; e-mail: educadu20@hotmail.com.br
[4] National Center for Tumor Diseases (NCT)/German Cancer Research Center (DKFZ), Division of Preventive Oncology, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany; e-mail: andrea.breuer@nct-heidelberg.de
[5] National Center for Tumor Diseases (NCT)/German Cancer Research Center (DKFZ), Division of Preventive Oncology, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany; e-mail: robert.owen@nct-heidelberg.de*
Correspondence: r.owen@dkfz-heidelberg.de; Tel.: +49 6221 423317

Abstract: Advanced Glycation End Products (AGEs) represent a set of substances that contribute directly to the triggering and/or aggravation of pathologies associated with ageing. AGEs are produced by the reaction between reducing sugars (or α-dicarbonyl compounds) proteins and amino acid residues. Current methodologies require an incubation period of 1-3 weeks to generate AGEs. In this study the reaction time for the formation of AGEs (48 and 3 hours) is significantly reduced by coupling and adapting procedures already existing in the literature to the free radical generation system called the hypoxanthine/xanthine oxidase assay. The capacity of different classes and chemical compounds (aminoguanidine, chlorogenic acid, rutin, extracts of *Hancornia speciosa* Gomes) were evaluated to inhibit the protein glycation process, acting as capturing agents of α-dicarbonyl species. Aminoguanidine, rutin and the leaf extracts of *Hancornia speciosa* Gomes show a high capacity to act as α-dicarbonyl compound scavengers (RCS-trapping) and resulting in the inhibition of AGEs formation.

Keywords: advanced glycation end products; glyoxal; hypoxanthine/xanthine oxidase; methyl glyoxal; RCS-trapping

1. Introduction

The world population is undergoing a period of significant change in terms of increased life expectancy and the provision of therapeutic options for diseases that predominantly affect older people. In the field of chemistry, the evidence and elucidation of oxidative stress caused by the action of reactive oxygen species (also known as free radicals) on organisms, contribute significantly to the understanding of several pathologies associated with the ageing process. Understanding the effects produced by free radicals in human beings stimulated, for example, the increase in the consumption of foods with antioxidant capacity, such as fruits and vegetables in the diet [1].

Nevertheless, recent studies indicate that, in addition to free radicals, there is another group of substances that contributes directly to trigger and/or aggravate ageing-related pathologies called Advanced Glycation End Products (AGEs). Research has revealed a crucial relationship between the

 doi:10.20944/preprints201805.0409.v1

formation of AGEs (glycation of proteins) and various age-related diseases such as diabetes, atherosclerosis, renal and neurodegenerative diseases such as Alzheimer's disease [2, 3]. The formation of AGEs, in vivo, causes non-enzymatic modifications in protein structure, affecting their functions in numerous ways and causing the aforementioned pathologies.

The endogenous and exogenous production of AGEs is explained by the reaction mechanism that gives aroma and color to foods when processed, also known as the Maillard reaction. It consists of a complex series of parallel and subsequent chemical reactions that produce an enormous class of compounds described by the French biochemist Louis Camille Maillard [4].

Initially, a condensation reaction occurs, in which the carbonyl groups of reducing sugars (or α-dicarbonyls formed by their fragmentation) undergo nucleophilic attack on the non-ligand electron pair of amino groups present in proteins or amino acid residues (arginine, lysine, etc.) [4]. This condensation produces an intermediate carbinolamine called the Schiff base. Then, through a metameric effect and oxidation and dehydration reactions, the Schiff bases become more stable species termed Amadori compounds. Finally, the Amadori compounds produce AGEs [4 - 7].

Amadori products, through oxidative fission or retro-aldolic fragmentation, can give rise to new protein residues which react with the α-dicarbonyl compounds and thus increase the amount of AGEs formed. Depending upon the pH of the environment, the Amadori compounds may also undergo enolization and produce other α-dicarbonyl compounds. During inflammatory processes, through the action of the enzymes myeloperoxidase and NADPH oxidase, excessive formation of dicarbonyl compounds (RCS) occurs, promoting a biochemical state known as dicarbonylic stress [8]. The presence, in excess, of these dicarbonyl species further favors the formation of AGEs in the human organism [4, 8, 9, 10].

Chemically AGEs are covalently linked (fluorescent and non-fluorescent) protein adducts, or cross-linking products between different amino acid residues. Several techniques can be used for the identification and quantification of AGEs, among which the following are notable: spectrofluorimetry, liquid and gas chromatography coupled to mass spectrometry and ELISA [10, 11].

Numerous studies have been carried out in the last two decades in order to produce AGEs in vitro in order to elucidate their main pathways of formation, chemical structures and biologic effects [10]. The procedures currently described in the literature to obtain AGEs, require 1 to 3 weeks for their formation, using non-enzymatic reaction pathways [12, 13].

Several authors have proposed methodologies to inhibit the formation of AGEs, analyzing the capability of various natural and synthetic chemical compounds to act as scavengers of α-dicarbonyl species (RCS-Trapping) and thus minimize dicarbonyl stress. Guanidines (aminoguanidine), catechins (epigallocatechin), proanthocyanidins and curcumin are among some of the classes of substances capable of inhibiting the formation of AGEs by acting as α-dicarbonyl species scavengers (RCS-Trapping) [14 - 19].

It is known that the AGE generation process, in vivo, is coupled to the generation of free radicals that promote the early glycation process of proteins. Despite this evidence, only a very small number of studies have been carried out to date, relating the generation of free radicals to the formation of AGEs, even though both are related to oxidative stress and consequently to premature ageing [20 - 23].

Therefore, this work aimed to significantly reduce the reaction time for AGEs formation, by coupling and adapting procedures already reported in the literature [24-29], to the free radical generation system termed the hypoxanthine/xanthine oxidase assay [26, 28]. Different classes of chemical compounds (aminoguanidine, chlorogenic acid and rutin), as well as the methanol extracts of leaves, bark and fruits of *Hancornia speciosa* Gomes (HSG) were evaluated for their capacity to inhibit the glycation process of proteins.

 doi:10.20944/preprints201805.0409.v1

2. Experimental Section

Reagents

Hydrochloric acid, sodium chloride, bovine serum albumin, glucose, fructose, methylglyoxal, glyoxal, Trizma, salicylic acid, cinamic acid, aminoguanidine hydrochloride, chlorogenic acid and rutin from Sigma-Aldrich Chemie (Steinheim, Germany); acetonitrile from Fluka/Riedel de Haen (Seelze, Germany); acetic acid, dimethyl sulfoxide (DMSO), EDTA, ferric chloride hexahydrate, hypoxanthine, methanol, xanthine, and xanthine oxidase from Merck (Darmstadt, Germany); K_2HPO_4 and KH_2PO_4 from Serva (Heidelberg Germany). All solutions were made up in double distilled water, DMSO or methanol.

2.1 General description of the methods developed for the formation and combat of advanced glycation products (AGEs)

Maillard's description demonstrates that the reaction between the reducing sugars in their open form (or of carbonyl compounds generated in their oxidation) with proteins or amino acid residues, produces Amadori compounds which convert into AGEs. Therefore, the methodologies presented in this work consist in initially promoting the reaction of sugar solutions (glucose and fructose), with bovine serum albumin protein solution (BSA) in different reaction media and subsequently replacing the sugar solutions with dicarbonyl compounds namely glyoxal (GO) and methylglyoxal (MGO) adding to the system the enzyme xanthine oxidase (XO) to promote the formation of AGEs in reaction medium with excess hydroxyl free radicals ($HO^{\bullet}$).

In this method, free radicals are produced by the oxidation process of hypoxanthine to xanthine (by the action of the enzyme xanthine oxidase), as described by Owen (2000) and collaborators [26]. The presence of these radicals in excess during the glycation and glycooxidation of the protein can promote the oxidation of the chain of amino acid residues thereof, as well as of the sugars and thus form dicarbonyl derivatives in a rapid way, significantly reducing the time of the glycation process of proteins. The enzyme catalyzed reactions have rate constants that are 100,000 times faster than non-enzymatic reactions and should be considered physiologically meaningful [29]. The qualitative formation of AGEs was verified by spectrofluorimetry measurements, with excitation and emission at wavelengths of 350 to 450 nm respectively [10, 11].

The final procedures developed to produce AGEs using the reaction pathway with sugars (glucose and fructose) or the dicarbonyl compounds (MGO and GO) are respectively termed the **hypoxanthine/xanthine-BSA-glucose/fructose** and **hypoxanthine/xanthine-BSA-MGO/GO** assay systems.

2.2 Formation of AGEs induced by the hypoxanthine/xanthine-BSA-glucose/fructose system

To evaluate the formation of AGEs, four different reaction media were initially tested: KH_2PO_4 (0.1 M), K_2HPO_4 (0.1 M), Tris-HCl buffer + 0.3 M NaCl (pH 8.3), and phosphate buffer (KH_2PO_4 + K_2HPO_4 (0.1 M, pH = 6.6) with the addition of EDTA (73. 0 mg), $FeCl_3.6H_2O$ (32.7 mg), hypoxanthine (20.5 mg), and salicylic acid (138.0 mg) to 500 mL of each buffer. The reaction mixtures added to Eppendorf microfuge tubes (2.0 ml) contained 93.75 µL of bovine serum albumin-BSA (16 mg/mL buffer), 25 µL glucose (1.67 M in buffer) and 881.75 µL of the various buffers.

Incubation was conducted in the absence of light at 37 °C with constant stirring (450 rpm) for one week, which is the incubation period used in most procedures currently described in the literature [24-28]. After incubation samples were transferred to 96-well plates and the measurement of total fluorescence intensity of the AGEs was performed by reading the micro plates in a Cytoflour fluorescence spectrophotometer (Perspective Biosystems, Minnesota, USA) with excitation and maximum emission at 360 and 460 nm respectively. Samples were analysed in duplicate and the fluorescence measurements were performed in triplicate.

After verification of the formation of the AGEs in all media using spectrofluorimetry, the same procedure described above was repeated, but with the addition of 10 µL of xanthine oxidase (XO; 18mU-Sigma Aldrich) to the reaction mixtures, using this time only phosphate buffer (0.1 M) plus

 doi:10.20944/preprints201805.0409.v1

salicylic acid (pH = 6.6) as the reaction medium. The incubation periods used for this procedure were 3 and 48 hours, and the reactions were terminated by the addition of 10 µL of concentrated hydrochloric acid. Fluorescence was determined as described above.

After the determination of the coupling efficiency of the medium containing xanthine oxidase (which promotes the generation of free radicals), this procedure was repeated using 24 and 48 hours of incubation, and again only with the phosphate buffer (pH = 6.6) in three distinct formulations (in the absence or presence of cinnamic acid or salicylic acid). Controls in the absence of XO, were used for comparison.

Due to the formation of fluorescent compounds resulting from hydroxylation of salicylic and cinnamic acids by hydroxyl radicals, which caused interference in the measurement of the fluorescence of the AGEs, only the phosphate buffer (pH = 6.6) was used for all further experiments. These were performed at different time intervals (24 hours, 48 hours, 1 week, 2 weeks, 3 weeks) following the methodology described above (with addition of XO). In all tests, controls were performed in the absence of XO for comparison.

To evaluate the reactivity of different sugars, glucose was replaced by the same amount of fructose (1,67 M). Initially the incubation period was 48 hours (37 °C), in the presence and absence of XO. This procedure was repeated for different incubation periods and temperatures: one week (37 °C) and 48 hours (90 °C), in the absence of XO.

2.3 Formation of AGEs induced by the system Hypoxanthine/xanthine-BSA-MGO/GO

The reaction between dicarbonyl compounds such as MGO and GO, with proteins or amino acid residues, promotes the glycation process more rapidly than the sugars in their open form. When in excess, in the human body (mainly during inflammatory processes), these dicarbonyl species generate a biochemical process known as "dicarbonyl stress" that induces harmful effects in humans due to an increase in AGEs formation.

Thus, the proposed methodology consists basically of the same procedure described for glucose and fructose (sugar + BSA + buffer + enzyme), but replacing sugar solutions with an equal amount (25 µL) of GO and MGO (5.55 $x10^{-2}$ and 6.89 $x10^{-2}$ M respectively), dissolved in 0.1 M phosphate buffer (pH = 6.6).

The intrinsic fluorescence of MGO and GO was initially determined using a calibration curve in the range 0-400 mg/mL in phosphate buffer (pH = 6.6).

To Eppendorf microfuge tubes (2.0 mL), 93.75 µL of BSA (16 mg/ml), 25 µL of MGO or GO solutions (5.55 $x10^{-2}$ and 6.89 $x10^{-2}$ M respectively) and 881.75 µl phosphate buffer (pH = 6, 6) was added. The reactions were conducted in duplicate and analyzed in the presence and absence of 10 µL XO (18 mU). These were incubated at 37 °C at 450 rpm for 3 hours. Controls were produced by replacing the BSA with an equal amount of phosphate buffer (pH = 6.6) to verify the intrinsic fluorescence of MGO and GO in the reaction mixture. The reaction completion procedure (addition of 10 µL of concentrated HCl) and determination of fluorescence were conducted in the same manner as for the glucose and fructose assays.

To evaluate the linearity of the processes in the presence and absence of XO, a similar test to the previous one was performed, in which the protein concentration (BSA 0-4 mg/mL) was varied, maintaining the concentration of MGO at 5.55 $x10^{-2}$ M. The samples were evaluated in the presence and absence of XO during one hour of incubation at 37 °C. With this experiment we also sought to verify the reproducibility of the method using XO.

2.4 Inhibition of advanced glycation products (AGEs) formation

The procedures with glucose, MGO and GO were repeated with the purpose of analyzing the capacity of different classes of compounds (guanidines, flavonoids and phenolic acids) and plant extracts to inhibit the formation of AGEs. For this, different pure compounds (aminoguanidine, rutin and chlorogenic acid) were added to the reaction mixtures, as well as methanol extracts of the leaves, bark and fruits of *Hancornia speciosa* Gomes (HSG) at varying concentrations. The aim of this was to demonstrate the possible action of these chemical species as scavengers of the 1-2-dicarbonyl

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

compounds MGO and GO, acting as trapping agents (RCS-trapping) of these species to form adducts and leading to a decrease in the amount of AGEs formed [14,30-38].

As a positive control for the methods developed to combat the formation of AGEs, aminoguanidine hydrochloride ($CH_6N_4.HCl$) at 98% purity (Sigma Aldrich) was used. Methanol solutions of the compounds were prepared at different concentrations (0.5-5.0 mM). Subsequently 1.0 mL of these solutions were added to Eppendorf microfuge tubes (2.0 ml), and the methanol removed on a Speedvac (M + S Laborgerate GMBH, 69168 Wiesloch, Germany). After removal of the solvent, the residues were dissolved in the appropriate buffer and incubated at 37 °C for 48 hours (sugar buffer) and 3 hours (MGO/GO) buffer respectively.

Methanol extracts of HSG leaves, bark and fruits were also evaluated. Methanol solutions (10 mg/mL) of the extracts were prepared and 0.5 mL (5 mg of extract) were added to Eppendorf microfuge tubes (2.0 mL) and the solvent removed on a Speedvac ((M + S Laborgerate GMBH, 69168 Wiesloch, Germany). The residues were dissolved in the sugar buffer and incubated at 37 °C for 48 hours in the presence and absence of XO.

Only the results obtained for the leaf methanol extract of HSG, was considered significant, which was subjected to more detailed analysis. The procedure was the same as for the pure compounds but using different amounts of the extract (2.5 - 20 mg).

Hexane, 5%, 10%, 25% and 50% MeOH in acetic acid (2%) fractions of the methanol extract of the HSG leaves obtained by fractionation on SPE C18 columns (Octadecyl-modified silica, CHROMABOND) were also evaluated. The objective was to identify which classes of substances, present in HSG leaves, showed higher inhibition of AGEs formation. This test was performed only with glucose, using 5.0 mg of the fractions, with XO only. Finally, the same procedures were repeated with a concentration range of rutin and chlorogenic acid (250-2000 μM) detected in HSG leaf extracts. In all procedures performed (pure compounds or extracts), the samples were centrifuged at 13,000 rpm for 5 minutes prior to fluorescence determination.

3. Results

3.1 Formation of AGEs induced by the system Hypoxanthine/xanthine-BSA-glucose/fructose

Four different reaction media were initially tested to establish optimum conditions for AGEs formation in vitro, using the same concentration of glucose and BSA. The results based on the increase of fluorescence minus the control values after one week incubation at 37 °C are shown in Figure 1a. The data also shows that the K_2HPO_4 (0.1 M) and phosphate buffer (0.1 M) (pH = 6.6) also containing EDTA, $FeCl_3.6H_2O$, hypoxanthine and salicylic acid were far superior to Tris buffer and KH_2PO_4. Therefore phosphate buffer (0.1 M) (pH = 6.6) containing EDTA, $FeCl_3.6H_2O$, hypoxanthine and salicylic acid was used for all further experiments.

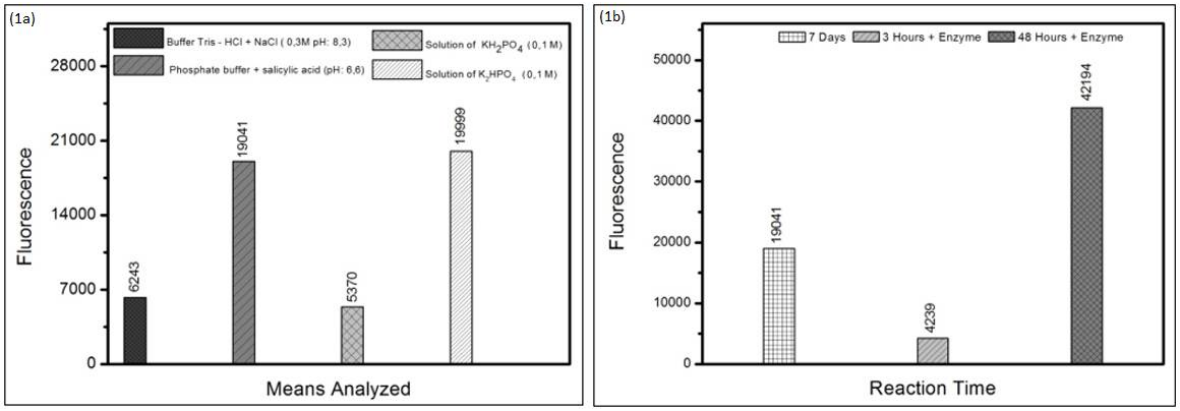


Figure 1. Formation of AGEs using glucose at 37 °C without the addition of XO - classic method. (1a) in different media evaluated after one week of incubation (1b) in phosphate buffer (pH = 6.6) with salicylic acid added for different incubation periods.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

The data for experiments performed with the addition of XO to this buffer system enzyme after 3 and 48 hours of incubation at 37 °C are shown in Figure 1b in comparison to 7 days incubation in the absence of XO. The efficiency of coupling the free radical generation system to generate AGEs is observed, considering after only 48 hours of reaction (using XO), there is already an increase of over 122% in the formation of AGEs when compared to the methods currently used, which require at least one week to produce such results. It should also be noted that in the procedure with XO, performed for only 3 hours, already 25% fluorescence is produced compared to the traditional method in seven days.

A comparison of three different compositions of phosphate buffer pH 6.6 (1. EDTA, $FeCl_3$, hypoxanthine, salicylic acid; 2. EDTA, $FeCl_3$, hypoxanthine, cinnamic acid and 3. EDTA, $FeCl_3$, hypoxanthine) in the procedure with glucose using 24 and 48 hours of incubation (Figure 2) revealed a significant increase in fluorescence in the reaction mixtures in which either salicylic or cinnamic acids were added. This elevation can be explained by the formation of fluorescent compounds from the reaction between hydroxyl radicals and salicylic and cinnamic acids (formation of 2,3 and 2,5-dihydroxybenzoic acid -DBHA- and the constitutional isomers of cinnamic acid), as demonstrated by Owen et al. [26] and confirmed by HPLC in these experiments. The major contribution to this increase in fluorescence was due to 2, 5-DBHA and *o*-coumaric acid formed by hydroxyl radical attack on salicylic acid and cinammic acid respectively as shown in Figure 3. In order to avoid interference in the measurement of AGEs, only phosphate buffer (pH = 6.6) plus EDTA, $FeCl_3$, hypoxanthine in the absence of salicylic and cinnamic acids was used for further experimentation.

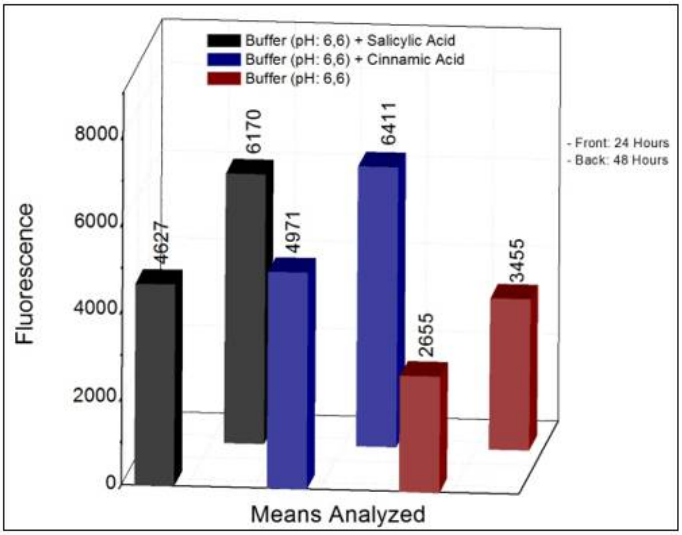
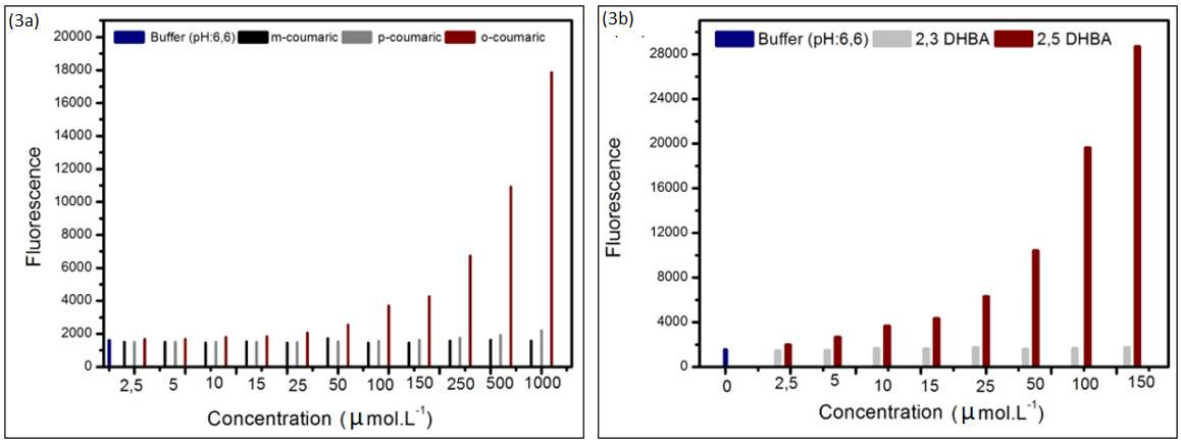


Figure 2. Formation of AGEs using glucose (37 °C) with different phosphate buffer compositions (pH = 6.6) with addition of XO and incubation at 37 °C for 24 and 48 hours.



 doi:10.20944/preprints201805.0409.v1

Figure 3. Intrinsic fluorescence at different concentrations of pure phosphate buffer (pH = 6.6): (3a) constitutional isomers of coumaric acid (3b) 2, 3 and 2, 5-dihydroxybenzoic acids (DHBA).

The analysis of the reaction occurring in the presence and absence of XO, at different time intervals (24, 48 hours, 1 week, 2 weeks and 3 weeks) using glucose, is shown in Figure 4. Here it is evident that the generation of free radicals accelerates the formation of AGEs, considering that at all intervals evaluated, at least a doubling of fluorescence was observed in the samples in which XO was added. Specific analysis of the procedure for a 1-week (168 hours) incubation period revealed an increased fluorescence of more than 500% with the addition of XO.

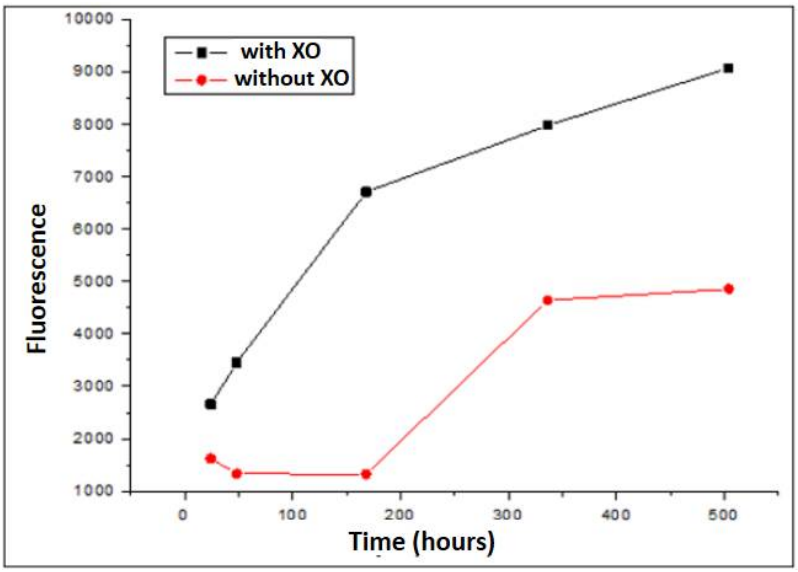
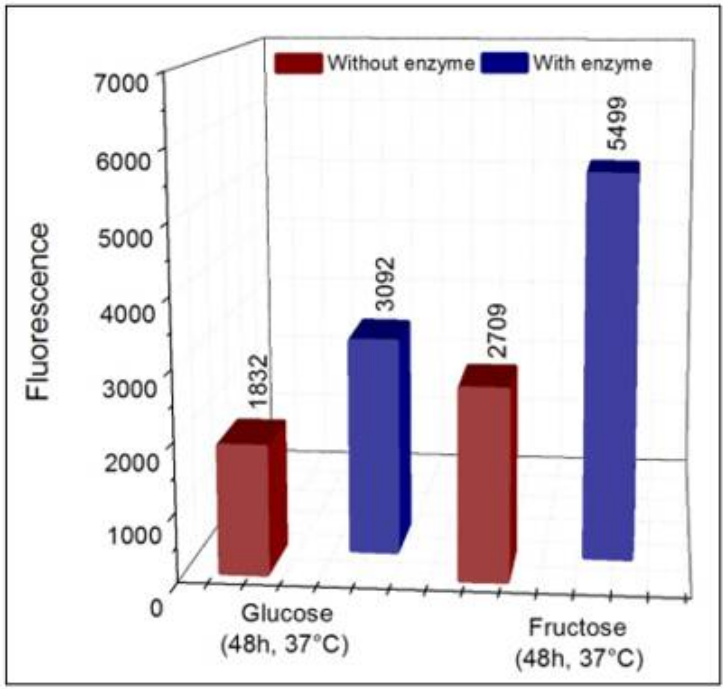


Figure 4. Formation of AGEs using glucose (37 ° C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6) using different incubation times (24 hours, 48 hours, 1 week, 2 weeks, 3 weeks).

In experiments where glucose was replaced by fructose (Figure 5), there was a considerable increase in the formation of AGEs compared to the glucose method. This result was repeated under different reaction conditions (Figure 6), indicating that fructose (ketose) is more reactive for the formation of AGEs than glucose (aldose), as already reported [25, 39,40].



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

Figure 5. Formation of AGEs using glucose and fructose in the presence and absence of XO in pure phosphate buffer (pH = 6.6) and incubation for 48 hours at 37° C.

It is known, based on the theoretical studies of organic chemistry, that aldehyde carbonyl groups are more reactive than those of ketones if we consider a condensation reaction that produces Schiff bases, with subsequent formation of AGEs (as described for the Maillard reaction). The more pronounced reactivity of the aldoses can be justified by the more electrophilic character of the aldehyde carbonyl carbon, since it has a number of inductive electron-receiving effects smaller than a ketone. In addition, the carbonyl of an aldehyde has, around itself, a smaller space impediment, facilitating its reaction with a nucleophile [41]. However, what happens in experimental procedures and in human metabolism (in vivo and in vitro), is exactly the opposite, that is: the formation of AGEs increases significantly when glucose (aldose) is substituted by fructose (ketose), and is confirmed in the results obtained in this work.

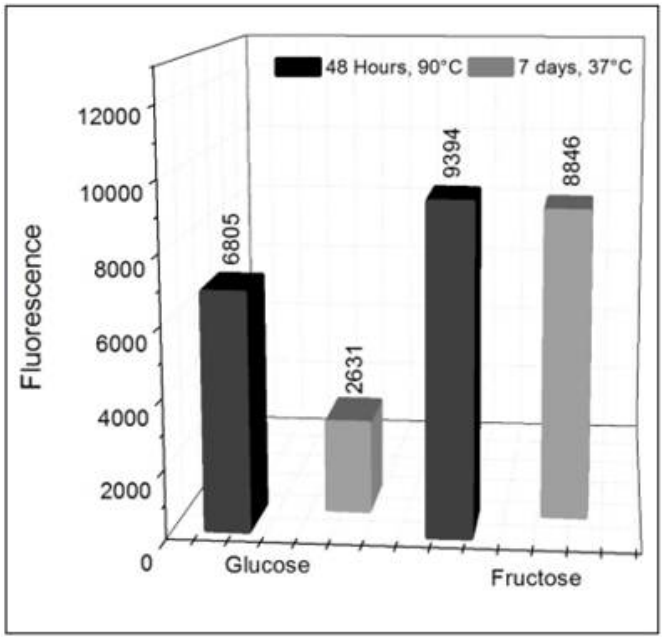


Figure 6. Formation of AGEs using glucose and fructose in absence of XO in pure phosphate buffer (pH = 6.6) with incubation for 48 hours (90° C) and 1 week (37° C).

The data obtained indicate the critical and harmful role that this sugar may cause to human health. Fructose is a main carbohydrate in the human diet, mainly due to the addition of fructose syrup to industrialized beverages [10]. This situation becomes more critical in people with hyperglycemia, such as diabetes mellitus, in whom blood sugar levels are naturally elevated, leading to increased formation of AGEs.

Figure 6 also shows that the food cooking process may also contribute significantly to the formation of AGEs since all the experiments performed at 90 °C (with glucose and fructose) showed almost twice the fluorescence when compared to the experiments performed at 37 °C. This indicates that the cooking process may significantly increase the amount of AGEs in food, which corroborates with some theories linked to vegetarianism that advocate the consumption of raw foods as a way of preserving health. The defense of raw food consumption is based in part by the presence of antioxidant species in these foods, as well as avoiding the formation of AGEs from the cooking process.

5.2 Formation of AGEs induced by the hypoxanthine/xanthine BSA-MGO/GO system

The calibration curves of MGO and GO (0-400 mg) demonstrating the intrinsic fluorescence are shown in Figure 7. Here high fluorescence is observed for MGO, which could indicate an

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

interference factor in the quantification of AGEs. As a way of avoiding this possible interference, controls were used in the absence of BSA, to discount the intrinsic fluorescence of the MGO and GO contained in the reaction mixture.

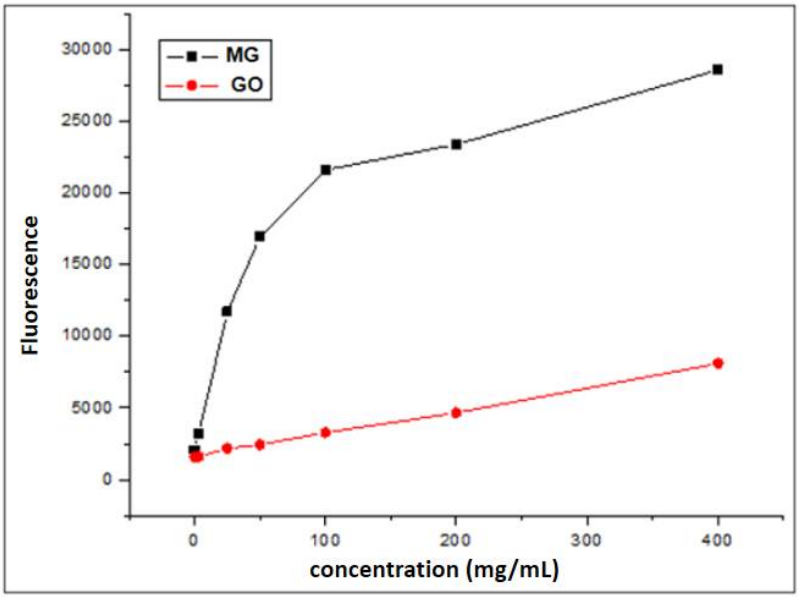


Figure 7. Intrinsic fluorescence at different concentrations of methylglyoxal (MGO) and glyoxal (GO) in pure phosphate buffer (pH = 6.6).

Therefore, as shown in Figure 8, it can be seen that in comparison to the controls with intrinsic fluorescence from both MGO and GO, the increase of fluorescence in the presence of BSA due to AGEs formation is comparable to our standard sugar procedure. However this is only after 3 hours incubation as compared to 48 hours.

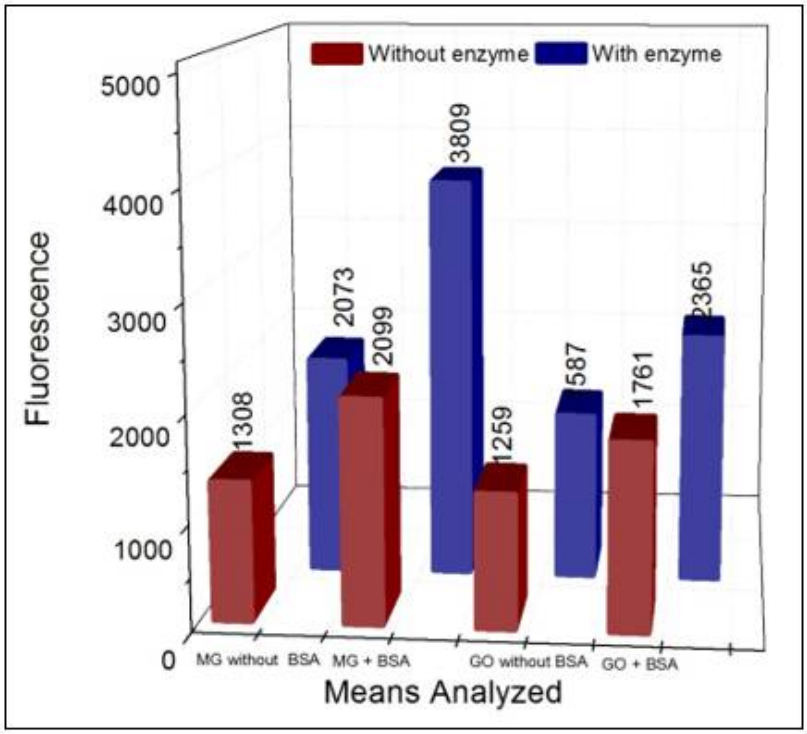


Figure 8. Formation of AGEs using MGO and GO (37 °C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6) and incubation for 5 hours.

 doi:10.20944/preprints201805.0409.v1

When MGO was incubated in the presence of a concentration range of BSA (0-4 mg/mL) at 37 °C for one hour plus or minus XO a superior linearity is evident in the presence of XO showing the importance of controlled generation of free radicals (Figure 9).

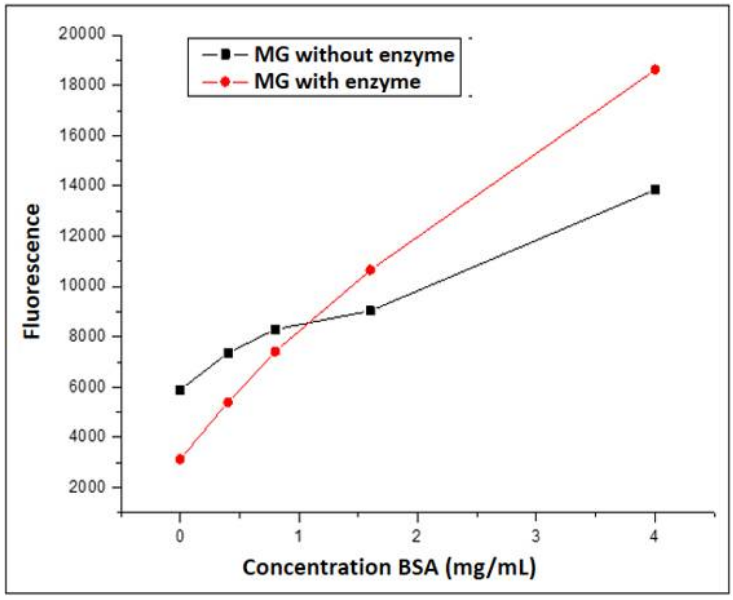


Figure 9. Formation of AGEs using MGO (37° C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6) with incubation for 1 hour and varying concentrations of BSA 0-4 mg/mL).

5.3 Inhibition of AGEs using extracts and fractions of HSG and pure compounds.

5.3.1 Inhibition using aminoguanidine (positive control)

As demonstrated by Nilsson [30] and Thornalley [31], aminoguanidine (AG) may act as a positive control in AGEs assays. Aminoguanidine reacts rapidly with dicarbonyl compounds (such as MGO and GO) acting as a scavenging agent.

Aminoguanidine (AG) worked moderately well (Figure 10) in the presence of XO but was ineffective in its absence and at a concentration of 5.0 mM was more effective in the GO assay (38 % inhibition) compared to the glucose (29 % inhibition) and MGO (9 % inhibition) assays. This compares well with the data of Thornalley (2003) [31], where 25.0 mM aminoguanidine was required to demonstrate inhibition.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

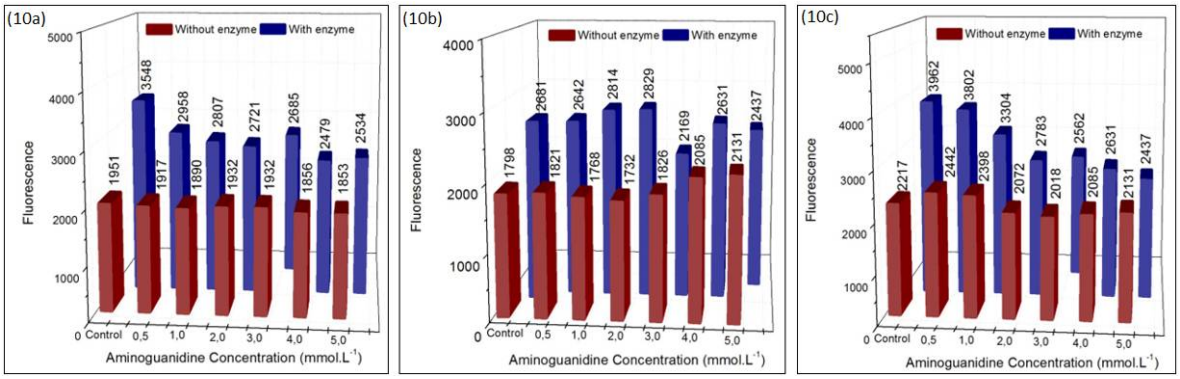


Figure 10. Inhibition of AGEs formation using different concentrations of aminoguanidine (37°C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6). (10a) procedure with glucose - 48 hours (10b) procedure with MGO - 3 hours (10c) procedure with GO - 3 hours.

5.3.2 Inhibition using HSG extracts and fractions

Literature surveys reveal that leaf extracts of HSG are used in traditional medicine to treat diabetes and contains rutin as a major phytochemical in organic extracts. Therefore, the capacity of methanol extracts of the leaves, bark and fruit of HSG to inhibit AGEs formation was evaluated in the glucose assay (Figure 11). Whereas the bark and fruit extracts showed intrinsic fluorescence the leaf extract was effective in the presence of XO. Therefore this extract was studied in more depth using the three newly developed methods in the presence of XO only.

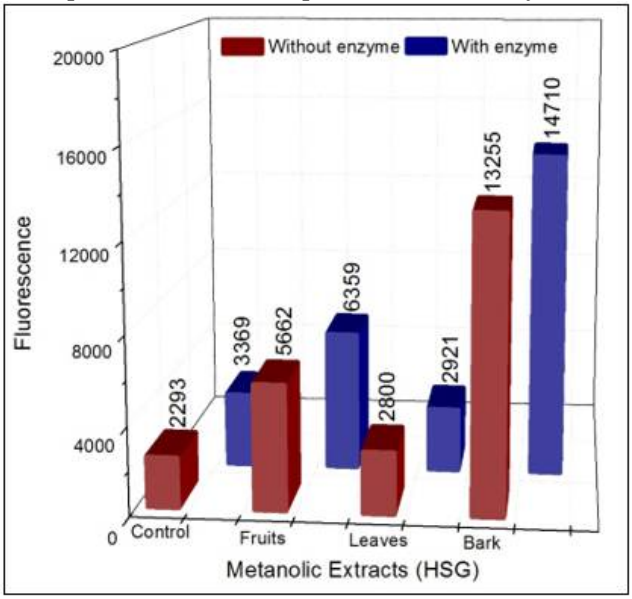


Figure 11. Inhibition of AGEs formation using 5 mg/mL of different HSG methanol extracts with glucose (37 °C), in the presence and absence of XO in pure phosphate buffer (pH = 6.6) and incubation for 48 hours.

A dose (2.5-20.0 mg/mL of HSG leaf extract) dependent decrease in AGEs formation was observed in both the MGO and sugar assays (Figure 12). At 20 mg/mL compared to the controls, inhibition was similar at 43 and 46 % in the sugar and MGO assays respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

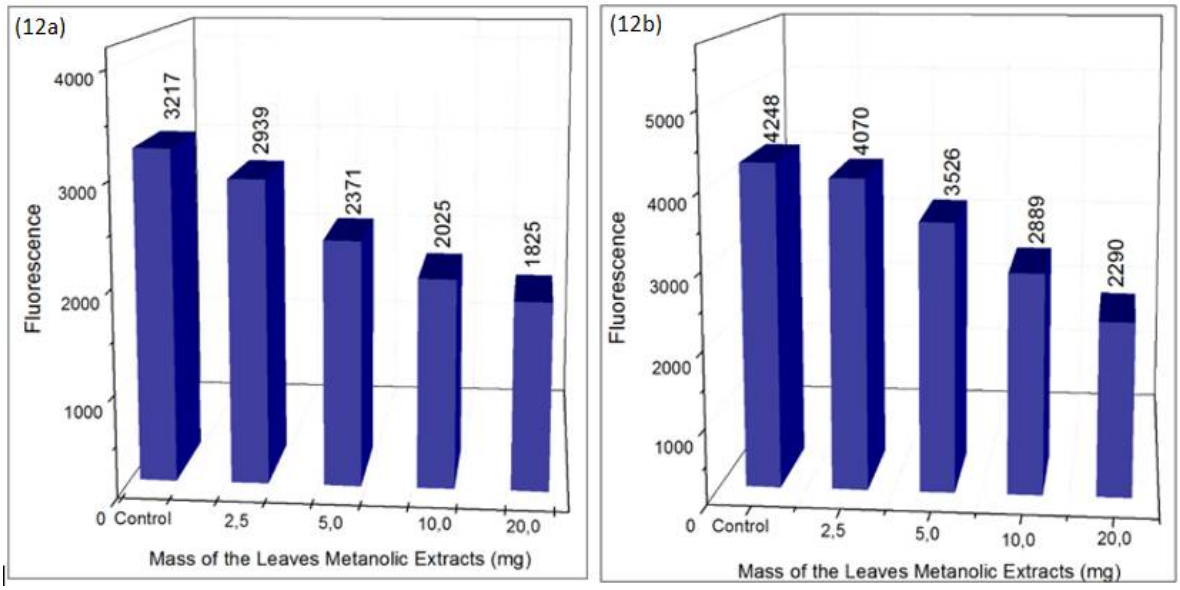


Figure 12. Inhibition of AGEs formation using different concentrations of methanol leaf extract of HSG (37 ° C) in the presence of XO in pure phosphate buffer (pH = 6.6). (12a) procedure with glucose - 48 hours (12b) procedure with MGO - 3 hours.

Therefore fractions obtained by fractionation on SPE C18 columns of HSG leaf extracts were also evaluated. The only fraction which exhibited inhibition (24 %) capacity was that eluted with 50 % methanol in 2% aqueous acetic acid (Figure 13). The only polyphenols present in this fraction were flavonoids dominated by rutin and isorutin whereas the 5 % methanol fraction (with very high intrinsic fluorescence) contained appreciable amounts of chlorogenic acid, also evaluated in the new assays and are presented below.

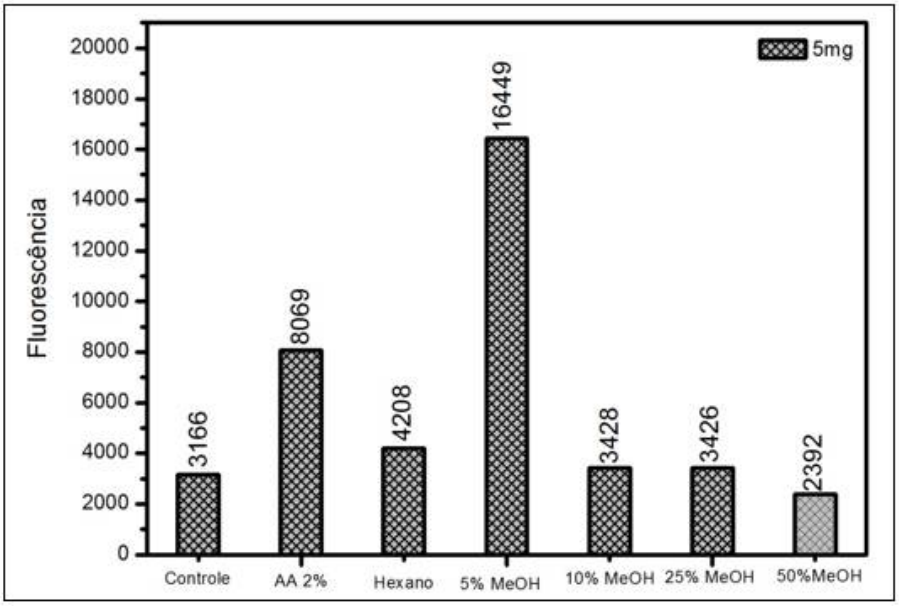


Figure 13. Inhibition of AGEs formation using 5 mg of various fractions (obtained by SPE C18) of the methanol extract of the leaves of HSG with glucose (37 ° C), only in the presence of XO enzyme in pure phosphate buffer (pH = 6.6) and incubation for 48 hours.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 May 2018 doi:10.20944/preprints201805.0409.v1

5.3.3 Inhibition using the flavonoid Rutin

The ability of some flavonoids to act as scavenging agents of 1, 2-dicarbonyl compounds has previously been demonstrated using classical procedures with an incubation period of 1-3 weeks. (+)-Catechin, (-)-epicatechin and procyanidins are reported to be active inhibitors [14, 32, 33]. It has also been demonstrated that epigallocatechin-3-gallate (EGCG) is a good scavenger of MGO and GO under neutral or slightly alkaline pH conditions, producing mono- and di-substituted adducts (3: 1 molar ratio) from condensation reactions [36]. These reactions occur preferentially at the C6 and/or C8 positions of the A ring. The higher reactivity of these positions is attributed to the increase in nucleophilicity of these carbons due to resonance effects, activated by the phenolic groups through a condensation reaction [36].

Rutin, is a flavonoid with a structure very similar to EGCG, in which gallate is replaced by a rhamnoglucoside group. In all the assays performed (glucose, MGO and GO), rutin presented a dose-response capacity to scavenge 1-2-dicarbonyl compounds and consequently inhibit the formation of AGEs (Figure 14). As opposed to the positive control aminoguanidine, the decrease in fluorescence occurred in both the presence and absence of XO.

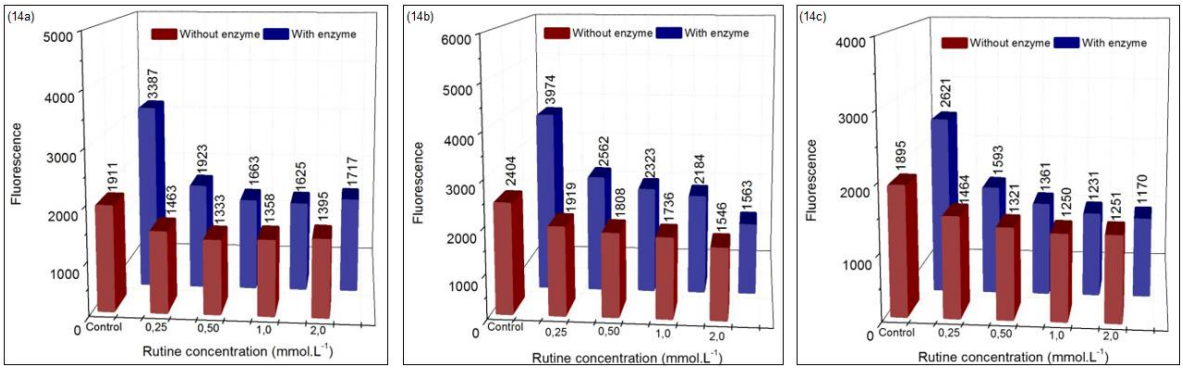


Figure 14. Inhibition of AGEs formation using different concentrations of rutin (37°C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6). (14a) procedure with glucose - 48 hours (14b) procedure with MGO - 3 hours (14c) procedure with GO - 3 hours.

Of interest is that in the presence of XO, rutin was very effective at only 0.25 mM, exerting an inhition capacity of 43, 36 and 39 % in the glucose, MGO and GO assays respectively and increasing the concentration to 2.0 mM only increased this by 6, 25 and 16 %. Therefore it can be concluded that rutin is a far more effective inhibitor of AGEs formation than the positive control aminoguanidine.

5.3.4 Inhibition using chlorogenic acid

The inhibition capacity of chlorogenic acid a major polyphenol detected in the 5 % methanol SPE fraction with high intrinsic fluorescence was also evaluated. The data shows (Figure 15) conclusively that in the glucose assay, chlorogenic acid, due to its high intrinsic fluoresecent behavior cannot be evaluated by these methods.

 doi:10.20944/preprints201805.0409.v1

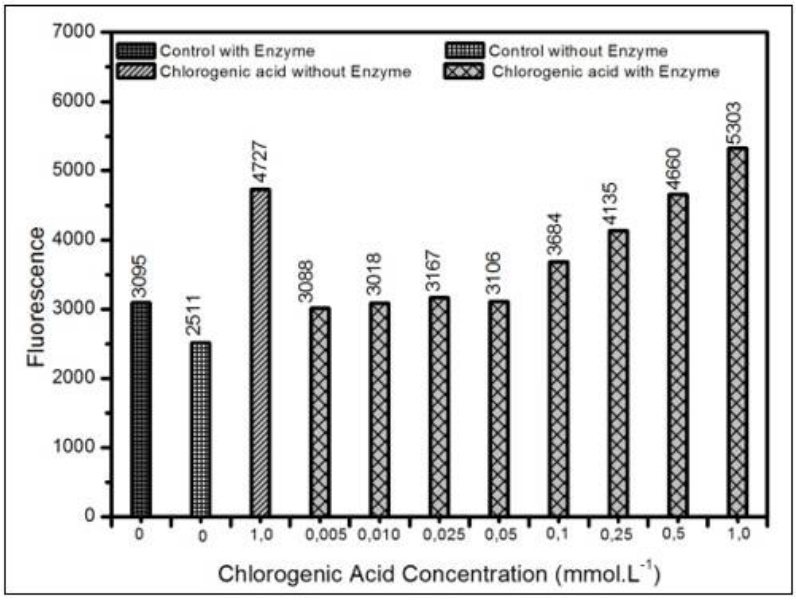


Figure 15. Inhibition of AGEs formation using different concentrations of chlorogenic acid with glucose (37 ° C) in the presence and absence of XO in pure phosphate buffer (pH = 6.6) and incubation for 48 hours.

.

4. Conclusions

It is concluded that coupling of a free radical generating system such as the hypoxanthine/xanthine oxidase assay, to existing published methods, promotes and accelerates the formation of AGEs in vitro. The assay time by the classical traditional method is reduced from 1-3 weeks to 48 hours and when glucose is replaced by MGO and GO to only 3 hours. Because this acceleration occurs in the presence of XO, indicates very strongly that free radicals are important in the generation of AGEs. It is noteworthy that rutin in these newly developed assays is demonstrated to be a far more efficient inhibitor of AGEs formation than the positive control aminoguanidine.

Our data is supported by a recent clinical study showing that serum XO levels are significantly increased in both type 2 diabetes mellitus patients with and without diabetic peripheral neuropathy in comparison to healthy controls. Xanthine oxidase activity was also directly correlated to the formation of AGEs in these patients [43].

Acknowledgements: This work was supported by CNPq

Conflicts of interest: All authors confirm that no conflicts of interest exist regarding this research.

References

1. Halvorsen, B.L.; Carlsen, M.H.; Phillips, K.M.; Bøhn, S. K.; Holte, K.; Jacobs, D.R.J.; Blomhoff, R. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. *Am. J. Clin. Nutr.* **2006,** *84*, 93-135, DOI: 10.1.1.562.9976.
2. Uribarri, J.; Cai, W.; Peppa, M.; Goodman, S.; Ferruci, L.; Striker, G.; Vlassara, H. Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress and aging. *J. Gerontol. A Biol. Sci. Med. Sci.* **2007**, *62*, 427-433.
3. Vlassara, H.; Uribarri, J. Advanced glycation end products (AGE) and diabetes: cause, effect, or both? *Curr. Diab. Rep.* **2014**, *14*, 1-17, DOI: 10.1007/s11892-013-0453-1.
4. Yu, L.L.; Wang, S.; Sun, B.G. *Food safety chemistry: toxicant occurrence, analisys and mitigation*, 1 rd ed.; CRC: New York, USA, 2014, pp. (60-65), ISBN-10: 1466597941.
5. Bruice, P. Y. Química Orgânica, 4rd ed.; Pearson: São Paulo, Brasil, 2006; pp. ISBN: 3226132261.
6. Hidalgo, F.J.; Zamora, R. Interplay between the maillard reaction and lipid peroxidation in biochemical systems. *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 319-326, DOI: 10.1196/annals.1333.039.
7. Fan, X.; Reneker, L.W.; Obrenovich, M.E.; Strauch, C.; Cheng, R.; Jarvis, S.M.; Ortwerth, B.J.; Monnier, V.M. Vitamin C mediates chemical aging of lens crystallins by the Maillard reaction in a humanized mouse model. *Proc. Natl. Acad. Sci.* **2006**, *103*, 16912-16917, DOI: 10.1073/pnas.0605101103.
8. Ledl, F.; Schleicher, E. New aspects of the Maillard reactions in foods and in the human body. *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 565-706, DOI: 10.1002/anie.199005653.
9. Poulsen, M.W.; Hedegaard, R.V.; Andersen, J.M.; Courten, B.; Bugel, S.; Nielsen, J.; Skibsted, L.H.; Dragsted, L.O. Advanced glycation endproducts in food and their effects on health. *Food. Chem. Toxicol.* **2013**, *60*, 10-37, DOI: 10.1016/j.fct.2013.06.052.
10. Vistoli, G.; Maddis, D.; Cipak, A.; Zarkovic, N.; Carini, M.; Aldini, G. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. *Free Radic Res.* **2013**, *47*, 3-27, DOI: 10.3109/10715762.2013.815348.
11. Barbosa, J.H.P.; Souza, I.T.; Santana, A.E.G.; Goulart, M.O.F. A determinação dos produtos avançados de glicação (AGES) e de lipoxidação (ALES) em alimentos e em sistemas biológicos: avanços, desafios e perspectivas. *Quim. Nova.* **2016**, *XY*, 1-13, DOI: 10.5935/0100-4042.20160048.
12. Vinson, J.A.; Howard, T.B. Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *J. Nutr. Biochem.* **1996**, *7*, 659-663, DOI: 10.1016/S0955-2863(96)00128-3.
13. Bousova, I.; Pruchova, Z.; Trnkova, L.; Drsata, J. Comparison of glycation of glutathione S-transferase by methylglyoxal, glucose or fructose. *Mol. Cell. Biochem.* **2011**, *357*, 323–330, DOI: 10.1007/s11010-011-0903-5.
14. Jiang, Y.; Hengel, M.; Pan, C.; Seiber, J.N.; Shibamoto, T. Determination of toxic alpha-dicarbonyl compounds, glyoxal, methylglyoxal, and diacetyl, released to the headspace of lipid commodities upon heat treatment. *J. Agric. Food Chem.* **2013**, *61*, 1067-1071, DOI: 10.1021/jf3047303.
15. Shao, X.; Bai, N.; He, K.; Ho, C.T.; Yang, C.S.; Sang, S. Apple polyphenols, phloretin and phloridzin: new trapping agents of reactive dicarbonyl species. *Chem. Res. Toxicol.* **2008**, *21*, 2042-2050, DOI: 10.1021/tx800227v.
16. Tan, D.; Wang, Y.; Lo, C.Y.; Ho, C.T. Methylglyoxal: its presence in beverages and potential scavengers. *Ann N Y Acad Sci.* **2008**, *1126*, 72-75, DOI: 10.1196/annals.1433.027.
17. Lo, C.Y.; Hsiao, W.T.; Chen, X.Y. Efficiency of trapping methylglyoxal by phenols and phenolic acid. *J. Food Sci.* **2011**, *76*, H90-H96, DOI: 10.1111/j.1750-3841.2011.02067.
18. Peng, X.; Ma, J.; Chen, F.; Wang, M. Naturally occurring inhibitors against the formation of advanced glycation end-products. *Food. Funct.* **2011**, *2*, 289–301, DOI: 10.1039/c1fo10034c.
19. Hu, T.Y.; Liu, C.L.; Chyau, C.C.; Hu, M.L. Trapping of methylglyoxal by curcumin in cell-free system and in human umbilical vein endotelial cells. *J. Agric. Food Chem.* **2012**, *60*, 8190-8196, DOI: 10.1021/jf302188a.
20. Wang, Y.; Ho, C.T. Flavour chemistry of methylglyoxal and glyoxal. *Chem. Soc. Rev.* **2012**, 41, 4140-4149, DOI: 10.1039/c2cs35025d.
21. Hiramoto, K.; Sekiguchi, K.; Aso-o, R.; Ayuha, K.; Ni-iyama, H.; Kato, T.; Kikugawa, K. DNA strand breaks induced through active oxygen radicals by fragrant component 4-hydroxy-2-hydroxymethyl-5-methyl-3(2h)-furanone in Maillard reaction of hexose/amino acid. *Food Chem. Toxicol.* **1995**, *33*, 803-814, DOI: 10.1016/0278-6915(95)00053-5.

22. Yim, H.; Kang, S.; Hah, Y.; Chock, P.B.; Yim, M.B. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. *J. Biol. Chem.* **1995**, *270*, 28228–28233, DOI: 10.1074/jbc.270.47.28228.
23. Masaki, H.; Okano, Y.; Sakurai, H. Generation of active oxygen species from advanced glycation end-products (AGEs) during ultraviolet light A (UVA) irradiation and a possible mechanism for cell damaging. *Biochim. Biophys*. Acta. **1999**, *1428*, 45-56.
24. Spiteller, G. Peroxyl radicals are essential reagents in the oxidation steps of the Maillard reaction leading to generation of advanced glycation end products. *Ann. N. Y. Acad. Sci.* **2008**, *1126*, 128–133 DOI: 10.1196/annals.1433.031.
25. Lunceford, N.; GugliuccI, A. *Ilex paraguariensis* extracts inhibit AGE formation more efficiently than green tea. *Fitoterapia* **2005**, *76*, 419-427, DOI: 10.1016/j.fitote.2005.03.021.
26. Monnier, V.M.; Vishwanath, V.; Frank, K.E.; Elmets, C.A.; Dauchot, P.; Kohn, R.R. Relation between complications of type I diabetes mellitus and collagen linked fluorecence. *N. Engl. J. Med.* **1986**, *314*, 403-408 DOI: 10.1056/NEJM198602133140702.
27. Owen, R.W.; Giacosa, A.; Hull, W.E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer.* **2000**, *36*, 1235-1247, DOI: 0.1016/S0959-8049(00)00103-9.
28. Lapolla, A.; Fedele, D.; Reitano, R.; Arico, N.C.; Seraglia, R.; Traldi, P.; Marotta, E.; Tonani, R. Enzymatic digestion and mass spectrometry in the studies of advanced glycation end products/peptides. *J. Am. Soc. Mass. Spectrom.* **2004**, *15*, 496-509, DOI: 10.1016/j.jasms.2003.11.014.
29. Trevisan, M.T.S.; Pfundstein, B.; Haubner, R.; Wurtele, G.; Spiegelhalder, B.; Bartsch, H.; Owen, R.W. Characterization of alkyl phenols in cashew (*Anacardium occidentale*) products and assay of their antioxidant capacity. *Food Chem. Toxicol.* **2006**, *44*, 188-197, DOI: 10.1016/j.fct.2005.06.012.
30. Forman, H.J.; Augusto, O.; Brigelius-Flohe, R.; Dennery, P.A.; Kalyanaraman, B.; Ischiropoulos, H.; Mann, G.E.; Radi, R.; Roberts, L.J.; Vina, J.; Davies, K.J. Even free radicals should follow some rules: a guide to free radical research terminology and methodology. *Free Radic. Biol. Med.* **2015**, *78*, 233-235, DOI: 10.1016/j.freeradbiomed.2014.10.504.
31. Chen, G.; Smith, J.S. Determination of advanced glycation end products in cooked meat products. *Food Chem.* **2015**, *168*, 190-195, DOI: 10.1016/j.foodchem.2014.06.081.
32. Navarro, F.; Morales, J. Mechanism of reactive carbonyl species trapping by hydroxytyrosol under simulated physiological conditions. *Food Chem*. **2015**, *175*, 92-99, DOI: 10.1016/j.foodchem.2014.11.117.
33. Nilsson, B.O. Biological effects of aminoguanidine: an update. *Inflamm. Res.* **1999**, *48*, 509-515, DOI: 10.1007/s000110050495.
34. Thornalley, P.J. Use of aminoguanidine (pimagedine) to prevent the formation of advanced glycation end products. *Arch. Biochem. Bioph.* **2003**, *419*, 31-40, DOI: DOI: 10.1016/j.abb.2003.08.013.
35. Joglekar, M.M.; Panaskar, S.N.; Arvindekar, A.U. Inhibition of advanced glycation end product formation by cymene – a common food constituent. *J. Funct. Foods.* **2013**, *6*, 107-115, DOI: 10.1016/j.jff.2013.09.024.
36. Lo, C.Y.; Li, S.; Tan, D.; Pan, M.H.; Sang, S.; Ho, C.T. Trapping reactions of reactive carbonyl species with tea polyphenols in simulated physiological conditions. *Mol. Nutr. Food Res.* **2006**, *50*, 1118-1128, DOI: 10.1002/mnfr.200600094.
37. Tang, D.; Zhu, J.X.; Wu, A.G.; Xu, Y.H.; Duan, T.T.; Zheng, Z.G.; Wang, R.S.; Li, D.; Zhu, Q. Pre-column incubation followed by fast liquid chromatography analysis for rapid screening of natural methylglyoxal scavengers directly from herbal medicines: case study of *Polygonum cuspidatum*. *J. Chromatogr. A.* **2013**, *1286*, 102-110, DOI: 10.1016/j.chroma.2013.02.058.
38. Peng, X.; Cheng, K.W.; Ma, J.; Chen, B.; Ho, C.T.; Lo, C.; Chen, F.; Wang, M. Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **2008**, *56*, 1907-1911, DOI: 10.1021/jf073065v.
39. Endringer, D.C.; Pezzuto, J.M.; Braga, F.C. NF-kB inhibitory activity of cyclitols isolated from *Hancornia speciose*. *Phytomedicine.* **2009**, *16*, 1064–1069, DOI: 10.1016/j.phymed.2009.03.022.
40. Mcpherson, J.D.; Shilton, B.H.; Walton, D.J. Role of fructose in glycation and cross-linking of proteins. *Biochem.* **1988**, *27*, 1901-1907, DOI: 10.1021/bi00406a016.
41. Bruice, P. Y. *Química orgânica*, 4rd ed.; PEARSON: São Paulo, Brasil, 2006; pp. (747-751), ISBN: 3226132261.
42. Marques, S.P.D. Nova proposta para rápida formação/combate dos produtos de glicação avançada (AGEs) e propriedades farmacológicas de *Hancornia speciosa* Gomes. Doctoral Thesis, Universidade Federal do Ceará, 05/05/2017.

552 43. Miric, D.J.; Kisic, B.J.; Filipovic-Danic, S.; Grbic, R.; Dragojevic, I.; Miric, M.B.; Puhalo-Sladoje, D. Xanthine
553 oxidase activity in type 2 diabetes mellitus patients with and without diabetic peripheral neuropathy. *J.*
554 *Diabetes R.* **2016,** 1-7, DOI: 10.1155/2016/4370490.