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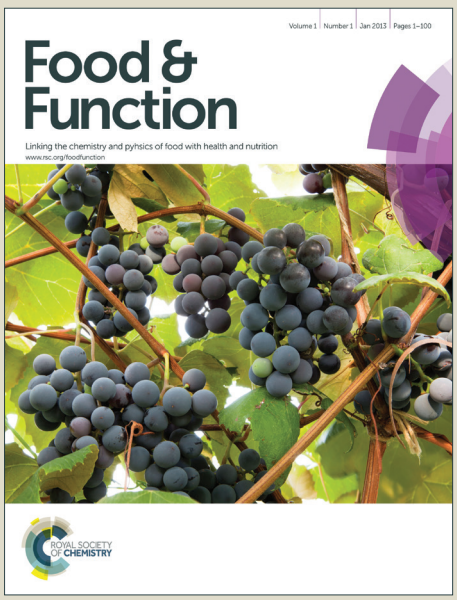

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2 **Carbonyl trapping and antiglycative activities of olive oil mill wastewater**

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Abstract

The use of natural compounds as antiglycative agent to reduce the load of advanced glycation end products from diet is very promising. Olive mill wastewater is a by-product of the olive oil extraction processes with high content of hydroxytyrosol, hydroxytyrosol derivatives and molecules containing *o*-dihydroxyl functions such as the verbascoside. Two powders were obtained after ultrafiltration and nanofiltration of olive mill wastewater, and successive spray drying with maltodextrin and acacia fiber. Samples were characterized by phenolic composition and antioxidant capacity. Antiglycative capacity was evaluated by in vitro BSA-glucose and BSA-methylglyoxal assays, formation of Amadori products and direct trapping of reactive dicarbonyls (methylglyoxal and glyoxal). Both ultrafiltrated and nanofiltrated olive mill wastewater powders had an activity comparable to quercetin and hydroxytyrosol against inhibition of protein glycation (IC_{50} = 0.3 mg mL^{-1}). The antiglycative activity of the powder was further investigated after separation by reverse phase solid extraction. Fractions extracted with methanol content higher than 40% and rich in hydroxytyrosol and verbascoside, exerted the highest reactivity against dicarbonyls. Data confirmed that direct trapping of dicarbonyl compounds is a main route explaining the antiglycative action rather than of the already known antioxidant capacity. Results support further investigations to evaluate the technological feasibility to use olive mill wastewater powders as antiglycative ingredient in foods or in pharmacological preparations in future.

Keywords Antiglycative activity; advanced glycation end-products (AGE); carbonyl trapping; olive oil mill wastewater (OMW); hydroxytyrosol.

1. Introduction

The health benefits of the Mediterranean diet have been firmly established, including the lowering of degenerative pathologies[1], cardiovascular diseases[2] and cancer[3-4]. In this respect, olive oil´s effects, as important component of the Mediterranean diet, are related not only to the peculiar fatty acid profile but also to the presence of a specific category of phenolic compounds named secoiridoids. The main phenolic compound in olive plant (*Olea europaea L.*) is the oleuropein, which is hydrolyzed into hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) and tyrosol during extraction of olive oil[5]. Several epidemiological studies suggest that HT is an efficient scavenger of peroxyl radicals[6] and prevents the damages of oxidative stress. *In vivo* and *in vitro* studies, HT has shown various biological activities with potential beneficial health effects. HT has been associated with anti-inflammatory, hypocholesterolemic, neuroprotective[7] and cardio protective activity as well as hypoglycemic effect which could prevent glucose auto-oxidation reaction, process related to the formation of AGEs[8].

Advanced glycation endproducts (AGEs) are the outcome of the non-enzymatic glycation reaction between the carbonyl group of a reducing sugar and free amino group of a protein. Several AGEs have been identified to date and many of them are fluorescent and yield protein crosslinking that alters protein structure and their biochemical properties[9]. AGEs are significant contributor to classical features of ageing and common chronic pathologies such as diabetes and its complications and Alzheimer´s disease[10]. Many researchers have linked the oxidative reactions with the accelerating the rate of AGEs formation, since radicals and α-dicarbonyls, reactive intermediates highly reactive such as methylglyoxal (MGO), glyoxal (GO) or 3-deoxyglucosone, are generated. Previous studies have suggested that antioxidants protect against glycation since they decreased the formation of reactive carbonyl species which is a consequence of oxidative stress generated by formation of the reactive oxygen species (ROS)[9]. AGEs can also be introduced in the systemic system of living bodies by exogenous sources like the diet. Basically, AGEs, also named Maillard reaction products, are formed during thermal processing of foods. The AGEs content in processed foods has increased in the past 50 years and dietary AGEs contributed to the total human body AGE pool[11].

Due to the AGEs involvement in the development of several health disorders, the search for AGE formation inhibitors in biological systems and in foods has been the target of many investigations. The use of synthetic compounds was discontinued because of the possible side effects and the use of aminoguanidine was also proposed. However the strategy of using natural compounds as antiglycative alternative is very appealing. Some bioactive phytochemicals naturally occurring in plants and possessing redox properties have been

related to the inhibition of glycation processes[12]. Studies of HT *in vivo* and *in vitro* have displayed it to possess cardio protective, anticancer and antidiabetic effects related to its ability to inhibit oxidative stress. Considering that the relationship between HT, ROS and tissue injury has been involved[13] with the pathogenesis of insulin resistance, the antiglycative activity of HT is plausible and noteworthy to be investigated. Recently, Troise et al., (2014)[14] concluded that phenol compounds from olive mill wastewater (OMW) are effective inhibitors of the extent of the Maillard reaction products during thermal processing of UHT milk.

In this framework, a food ingredient made from spray-dried OMW was selected as promising candidate for the antiglycative action. OMW is a by-product of the olive oil extraction processes with high content of HT, HT derivatives and molecules containing *o*-dihydroxyl functions such as the verbascoside[15]. The aim of the present study was assess the inhibitory capacity of different OMW powders on the formation of AGE by using different *in vitro* models of MGO and glucose as inducers of the glycation reaction. Further, the ability of the OMW ingredient for trapping of dicarbonyl compounds was also assessed. Antioxidant activity was examined in order to study their possible relationship with AGE-inhibitory activity.

2. Materials and methods

2.1. Materials

D (+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO, 40% aqueous solution), glyoxal (GO, 40% aqueous solution), aminoguanidine (AG), quercetin (QE), verbascoside (VB), 5-methylquinoxaline (5-MQ), nitrotetrazolium blue chloride (NBT), o-phenyldiamine (OPD), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tris(2-piridil)s-tirazina (TPTZ), 2-2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and phenolic acids standards were provided by Sigma (St Louis, MO, USA). Folin-Ciocalteu reagent, iron (III) chloride was purchased from Panreac (Madrid, Spain). Hydroxytyrosol standard (HT, purity > 99 %) was acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Sodium dihydrogen phosphate monohydrate, sodium carbonate anhydrous, formic acid, glacial acetic acid and high-performance liquid chromatography (HPLC)-grade methanol were purchased from Merck (Darmstadt, Germany). C18 cartridges Sep-Pak Plus were supplied by Waters (Ireland). The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

105 *2.2. Equipments*

106 Synergy™ HT-multimode microplate reader with an automatic reagent dispense and
107 temperature control from Biotek Instruments (VT, USA). HPLC Shimadzu (Kyoto, Japan)
108 equipped with a LC-20AD pump, a SIL-20AHT autosampler, a CTO-10ASVP oven, and a DAD
109 (SPD-M20A).

110 *2.3. Preparation of olive mill wastewater powder*

111 Olive oil polyphenols powders were obtained at Azienda Agricola Fangiano (Calabria, Italy)
112 starting from the water fraction resulting from virgin-olive oil production (Carolea variety) as
113 follows. Olive water were separated from the oil and the olive paste by centrifugation; they
114 were treated with pectinases for 2 hours at 37° C then fractionated by a filtration plant made
115 up with three membranes having different cut off. Olive water was forced to pass through
116 microfiltration (cut off 25 kDa), ultrafiltration (cut off 8 kDa), and nanofiltration (cut off 0.3
117 kDa) membranes. At each stage a retentate containing the compounds not passing the
118 membrane pores was collected while the permeate move on to the next filtration step. For
119 this investigation, the ultrafiltration and nanofiltration retentates were concentrated by
120 inverse osmosis (cut off 0.1 kDa) up to 20 % dry weight and spray dried adding maltodextrin
121 and acacia fiber in a ratio 2:1 with the water mill dry weight (66,6% olive mill water 33,3%
122 maltodextrin and acacia fiber 1:1). Finally, a fine pale yellow of ultrafiltrated (UOMW) and
123 nanofiltrated (NOMW) powder with moisture content below 4% were obtained and used in
124 this study.

125 2.4. *Analysis of phenolic compounds in OMW powder*

126 The analysis of the phenolic component was carried out as described by *Kokkinidou &*
127 *Peterson*[16] with some modifications. Briefly, the OMW powder was dissolved in distilled water,
128 in order to prepare a solution at concentration of 20 mg mL^{-1}. To 1 mL of this solution, 10 µL of
129 a 5 mg L^{-1} solution of butyl -4-hydroxybenzoate as internal standard were added. The phenolic
130 fraction was extracted through the use of SPE cartridges Strata C18-E, and dried under a
131 nitrogen stream. Thereafter the precipitate was recovered in 500 µL of a solution of water-
132 methanol/95-5 (v/v) ready to HPLC analysis. The instrument used for the chromatographic
133 analysis was a LC-20AD HPLC with UV-Vis detector SPD20A, set at 279 nm, and SCL-20A
134 controller (Shimadzu, Japan). The mobile phases were H_2O 0.1% formic acid (A) and methanol
135 (B). The flow was 0.8 mL min^{-1}. A Prodigy ODS3 column was used (250 x 4.60 mm, 5 micron,
136 100 A, Phenomenex, USA). The sample (20 µL) were separated with next gradient as follow; 0
137 min (5% B); 4 min (5% B); 40 min (98% B); 43 min (98% B);46 min (5% B); 49 min (5% B).

Hydroxytyrosol, tyrosol and verbascoside were quantified by external calibration with standards. Peaks are identified by retention time, DAD spectra and spiking with sample with pure standards.

2.5. Determination of total phenolic content

Total phenolic content (TPC) was estimated by the Folin-Ciocalteu method as described by *Contini et al.*[17]. Absorbance readings were recorded using a microplate spectrophotometer and quantified using gallic acid as a standard. Results were expressed as mg gallic acid equivalent (GAE) g^{-1} sample and all measurements were carried out in triplicate. The limit of quantification was set at 0.5mg GAE g^{-1} sample.

2.6. Determination of antioxidant capacity according to ABTS assay

Antioxidant activity was estimated in terms of radicals scavenging capacity of the extracts as described by *Mesías et al.*[18]. Aqueous solutions of Trolox at different concentrations were used for calibration. The limit of quantification was set at 1.1 µmol TEAC g^{-1} sample. All measurements were performed in triplicate.

2.7. Determination of antioxidant capacity according to FRAP assay

The formation of Fe^{2+}-TPTZ complex from Fe^{3+}-TPTZ complex was determined as described by *Morales et al.*[19]. Results were expressed as µmol Trolox equivalent antioxidant capacity (TEAC) g^{-1} sample.

2.8. Fractionation of the olive mill wastewater powdery ingredient

The ultrafiltrate olive mill wastewater (UOMW) powder was further fractionated by reversed phased solid phase extraction (C18 SepPak cartridge) with sequential extraction in methanol. A solution of UOMW powder (1 mg mL^{-1}) was loaded onto a pre-activated cartridge. The first fraction was collected as eluted and termed as fraction UOMW-A. Then 1 mL of formic acid (0.1%) with 5% of methanol was passed through cartridge and the resulting fraction (UOMW-B) was collected. UOMW-C is the fraction that eluted with 1 mL of formic acid with 40% of methanol. At last, UOMW-D fraction was eluted with 1 mL of methanol was gone through cartridge. Chromatographic separations of the whole extract and fractions were performed on a C18 Kinetex column (75 mm x 4.6 mm, 2.6 µm, Phenomenex, USA). The temperature was maintained at 40ºC and the flow rate was 0.6 mL min^{-1}. The mobile phase was 0.5 % acetic acid in water (solvent A) and methanol (solvent B) and the total running time was of 40 min. The gradient changed as follows: 5 %B was held for 5 min, then increased to 100 %B in 25 min, held for 1 min, and decreased to 5 %B in 9 min.

2.9. Assessment of in vitro glycation of BSA by methylglyoxal and glucose

The BSA-MGO assay and BSA-GLC were carried out as described by *Mesías et al.*[18]. Aminoguanidine, hydroxytyrosol and quercetin were used as standards at a concentration of 0.1 mg mL^{-1}, being AG (0.57 mg mL^{-1}) the positive control of the assay. The systems containing samples/control/blank were incubated at 37°C for 14 days (BSA-MGO assay) or 21 days (BSA-GLC assay). Reference was prepared without incubation and stored at -80 °C until analysis. In parallel, samples were dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.4) and incubated at 37 °C for 14 days (BSA-MGO assay) or 21 days (BSA-GLC assay) in order to measure their intrinsic fluorescence. The fluorescence intensity of all systems (sample/control/blank) was measured in a microplate spectrophotometer. AGE formation was characterized by measuring fluorescence with excitation and emission maxima at 340 and 420 nm for the BSA-MGO assay and 360 and 420 nm for the BSA-GLC assay. The intrinsic fluorescence of the samples incubated under the same conditions was subtracted to the overall fluorescence in each BSA-MGO or BSA-GLC system.

The percentage inhibition of AGEs formation was calculated according to following equation: inhibition (%) = {1 – [(fluorescence of solution with inhibitor – intrinsic fluorescence of sample)/fluorescence of solution without inhibitor]} x 100.

The IC_{50} (the concentration required to inhibit glycation by 50%) were calculated from the dose-response curve using Microsoft-Excel computer software.

2.10. Fructosamine adduct assay

The fructosamine formation was tested following the procedure described by *Baker et al.*[20] with slight modifications. The fructosamine has the ability to reduce NBT to tetrazinolyl radical (NBT^{+}) in alkaline solution forming a coloured compound called monoformazan (MF^{+}). Thereby, 50 µL of samples for the BSA-GLC assay were mixed with 450 µL of NBT and the mixture was incubated in darkness at 37 °C for 60 min. Then, in the microplate was added 100 µL of mixture and 100 µL of sodium carbonate buffer (0.1 mol L^{-1}, pH 10.35), in quadruplicate, except the blank that samples was replaced by sodium carbonate buffer. The presence of fructosamine was characterized by absorbance of MF^{+} at 350 nm using a microplate spectrophotometer.

The percentage of inhibition of the fructosamine formation was calculated using the following equation:

Inhibition (%) = 100 – [(fluorescence of solution with inhibitor – intrinsic fluorescence of sample – fluorescence of blank) x 100/(fluorescence control positive – intrinsic fluorescence of control positive – fluorescence of blank)].

2.11. Direct MGO and GO trapping capacity

Direct MGO or GO trapping capacity of samples was determined as described by *Mesías et al.*[18] with modifications. The mixture of MGO (0.04 mg mL^{-1}) or GO (0.03 mg mL^{-1}) with different concentration of the extracts was incubated at 37 °C for 168 h. Pyridoxamine (PM, 0.1 mg mL^{-1}) instead samples were used as positive control. OPD was added in order to derivatize of unreacted MGO or GO into 2-methylquinoxaline (2-MQ) or 1-quinoxaline (1-Q), respectively. 5-quinoxaline (5-MQ) was used as internal standard. Chromatograms were recorded at 315 nm being the retention times of 2-MQ, 1-Q and 5-MQ, 4.1, 3.4 and 5.6 min respectively. The amount of unreacted MGO and GO was calculated from the ratio of 2-MQ or 1-Q and 5-MQ to the control at time zero after preparation.

The MGO trapping capacity was calculated from the following equation:

MGO decrease (%) = [(amount of MGO in control – amount of MGO in sample with tested standard or PM solution)/amount of MGO in control] x 100%. IC_{50} value (mg mL^{-1}) was obtained from the dose-response curves of each experiment.

Similarly, the GO trapping capacity was calculated.

2.12. Statistical analysis

Data were analysed by using the software Statgraphics Centurion XV Statistical program (Herndon, VA). Homogeneity of variances was assessed via Levene´s test. Analysis of variance (ANOVA) was followed by Bonferroni´s or Tamhane´s test when the variances were homogeneous or non-homogeneous, respectively. Differences were considered to be significant at $P < 0.05$. Data from at least three independent assays were used to obtain a mean value and its standard deviation.

3. Results

A polyphenol-rich powder was obtained from olive mill wastewater through ultrafiltration/nanofiltration and spray dried, and it was investigated as a promising functional ingredient to decrease the non-enzymatic protein glycation. Two types of powders were obtained, one after ultrafiltration (UOMW) of the olive mill wastewater extract and a second from the retentate after nanofiltration (NOMW). Both extracts were spray dried adding

maltodextrins and acacia fiber, and the powders were characterized by total polyphenols content and antioxidant capacity (FRAP and ABTS) as summarised in **table 1**. The content in polyphenolic compounds and the antioxidant capacity, regardless the method used, were significantly ($P< 0.05$) higher in the UOMW powder as compared with NOMW powder. It is known that certain bioactive compounds present in plants possess antioxidant capacity as well as ability to inhibit protein glycation as flavonoids[21]. During the spray drying of the extracts, the yield of nanofiltration powder was lower than that of ultrafiltration, particularly when the concentration of added dietary fibre was reduced to 33.3%. The average content in hydroxytyrosol, tyrosol and verbascoside were 33.0, 2.1 and 2.8 μg g^{-1} for the UOMW powder and 13.8, 0.2, and 1.3 μg g^{-1} for the NOMW powder, respectively. The higher concentration of polyphenols in the UOMW powder was due to the presence of bound polyphenols link to the polysaccharide moiety (pectins and arabinans) that remains in the retentate of the extract[22].

The antiglycative properties of OMW powders were evaluated by BSA-MGO and BSA-GLC assays. **Figure 1a** showed the formation of fluorescent AGEs in the BSA-MGO assay (37°C/14 days). UOMW and NOMW powders behave in a dose-dependent manner and both reduced significantly the formation of AGEs, with an IC_{50} of 0.886 and 1.029 mg mL^{-1}, respectively for the ultrafiltrated and nanofiltrated powder (**table 2**). But ultrafiltrated powder was significantly more effective for antiglycative activity mediated by MGO. AG and QE were used as positive controls due to their well-known effectiveness against the glycation process. Suppression of dicarbonyls formation and direct reaction with dicarbonyls are the main mechanism of action of AG and QE[23-24]. The antiglycative activities of AG, QE, HT, and VB solutions (0.14 mg mL^{-1}) in the BSA-MGO assay were 68, 86, 70 %, and 84 %, respectively. The IC_{50} in the BSA-MGO assay were 0.090, 0.053, 0.086, and 0.053 mg mL^{-1} for AG, QE, HT, and VB, respectively (**table 2**). Control for maltodextrin and acacia fiber (MD+AF) shown a residual 3.5% antiglycative activity for BSA-MGO assay.

The **figure 1b** showed the formation of fluorescent AGEs for BSA-GLC assay (37°C for 21 days). Ultrafiltration and nanofiltration powders presented a dose-dependent inhibitory activity with not significant differences between them (IC_{50} of 0.457 and 0.429 mg mL^{-1}, respectively) as described in **table 2**. The IC_{50} for the BSA-GLC assay were 0.100, 0.101, 0.400, 0.243 mg mL^{-1} for AG, QE, HT, and VB, respectively (**table 2**). HT and VB are also effective in the BSA-GLC system where the autoxidation of glucose is a side reaction to form reactive dicarbonyl compounds that will promote the formation of AGEs. Control for maltodextrin and acacia fiber did not show antiglycative activity for BSA-GLC assay.

On the other hand, methylglyoxal (MGO) and glyoxal (GO) are key promoters of the glycation process where the formation of AGEs and irreversible modification of proteins are boosted. For this reason, the direct MGO and GO trapping capacity of UOMW and NOMW extracts were investigated. **Figures 2a** and **2b** depicted the MGO and GO trapping ability of ultrafiltration and nanofiltration powders in the range from 0.1 to 2.5 mg mL^{-1}. Both extracts showed similar dose-dependent behavior and reached the highest MGO trapping abilities at 1 mg mL^{-1}. It is noteworthy that control sample (maltodextrin + acacia fiber) showed a minor dicarbonyl trapping capacity, representing nearly the 20% of the overall response. Results shown that efficacy of OMW powder for trapping GO was lower as compared with the MGO trapping ability. The IC_{50} in the GO-trapping assay was calculated to be 0.562 mg mL^{-1} and 0.811 mg mL^{-1} for UOMW and NOMW powders, respectively. In contrast, both powders were more effective for trapping MGO with IC_{50} values of 0.294 and 0.473 mg mL^{-1}, respectively. The ultrafiltrated powder was significantly ($P<0.05$) more reactive against MGO and GO than the nanofiltrated powder for concentration higher than 0.1 mg mL^{-1}.

In the early phase of the glycation reaction, the reversible Schiff base leads a rearrangement where a more stable Amadori product is formed. In an advanced phase of glycation, irreversible AGEs are formed. Therefore, inhibition of fructosamine adducts could reduce AGEs formation in foods and hence potentially ameliorate the diabetic complications *in vivo* by a restriction of the dietary AGE intake and reduction of the circulating AGEs. The Amadori products were quantified principally on the basis of the reduction of NBT by Amadori products to monoformazan dye, which bears strong absorbance at 530 nm. Our results pointed out that antiglycative activity and dicarbonyl trapping ability of ultrafiltrated and nanofiltrated powders are relevant to the potential mitigation of diet-derived and endogenously formed AGEs. As shows in the **table 3**, both extracts performed their inhibitory effects also at an early stage of the glycation since a significant reduction in the formation of fructosamine was observed at all concentrations (0.36-3.57mg mL^{-1}). The IC_{50} for the fructosamine assay was calculated to 2.34 and 2.48 mg mL^{-1} for the ultrafiltrated and nanofiltrated powder, respectively.

Since the antiglycative activity of both ultrafiltrated and nanofiltrated powders were in the same order of magnitude, the ultrafiltrated sample was selected for a more detailed investigation to elucidate the mechanisms of the antiglycative action considering its higher yield during the production process and consequently the potential economic advantages in the scale up process.

Sequential fractionation of the UOMW powder by reverse phase extraction was applied to further investigation of the antiglycative capacity. **Figure 3** described the chromatographic profile of the whole extract and relative fractions A, B, C and D, eluted with in a gradient with methanol. In the chromatograms were identified the peaks of HT, tyrosol and verbascoside. A hump corresponding to the high molecular weight polysaccharides present in the olive pulp was clearly detectable. Since UOMW powder is particularly rich in HT (42% of the total polyphenols content), further investigations were focused in the HT content. The highest concentration of HT (representing approx. 80% of the initial HT content) was collected in the fraction C which is eluted with 40% MetOH. Fraction D (corresponding to fraction eluted with 100% MetOH) contains the lowest lower amount of HT corresponding to approximatively the 20% of the initial content. However verbascoside is eluted at fraction D together a major proportion of the polysaccharides rich in polyphenols.

The antiglycative activity of each fraction obtained by solid phase extraction was estimated by the BSA-MGO (**Figure 4a**) and BSA-GLC (**Figure 4b**) assays. In both BSA-MGO and BSA-GLC assay, fractions A and B did not exert protective effect on the formation of fluorescent AGEs, while C and D fractions shown an important antiglycative capacity. Fractions C and D were able to inhibit from 30 to 49% of the formation of fluorescent AGEs in the BSA-MGO assay, and 31.3 to 55.2% in the BSA-GLC assay. Antiglycative activity of fraction eluted with methanol was significantly higher than fraction eluted with 40% methanol. The partial contribution of the antiglycative capacity of UOMW-C and UOMW-D fractions could explain the activity of whole UOMW extract that involved the 76.3 and 88.4% of inhibition, BSA-MGO, and BSA-GLC, respectively.

Figure 5 shows the kinetic of the MGO trapping ability of the ultrafiltrated powder and fractions over time. UOMW-C and UOMW-D fractions were highly efficient for trapping MGO as compared with UOMW-A and UOMW-B fractions. In fact, UOMW-C and UOMW-D fractions reached a trapping capacity of 80.2 and 71.4% during incubation with MGO up to 168h respectively. Both C and D fractions follow the same trend and a significant MGO-trapping capacity is recorded in the first 24h of incubation. The MGO trapping capacity of whole powder (1 mg mL^{-1}) peaked at 97.6% after 168 h of incubation, but system was almost saturated (percentage of inhibition higher than 85%) at 72h of reaction. In contrast, fractions A and B just accounted for the 11 and 14.5 % of the MGO trapping ability after 168h, respectively. It was selected the reaction time at 48h to compare the relative reactivity of the fraction with the whole UOMW powder. At 48h, UOMW powder reached a MGO-trapping capacity of 78.5% and the sum of fractions accounted to the 99.3%.

Several assays have established a relationship between antioxidant effects of certain plant extracts with the inhibition of protein modifications in the glycation process[25]. *Jemai et al.*[26] suggested that the antidiabetic effect of olive leaves might be due to antioxidant activity of HT and oleuropein. In this regard, total antioxidant capacity by FRAP and ABTS were evaluated in the samples. Total phenolic content of the powder and its fraction was assessed since phenolic compounds have been proposed as major contributors to antiglycative activity[27]. **Table 1** summarized the results of TPC, FRAP and ABTS for the UOMW powders and its respective fractions A, B, C, and D. The reducing ability of the UOMW powder (780.4 µmol TEAC g^{-1}) was higher than the sum of the reducing activity of its UOMW-C and UOMW-D fractions (199.2 and 241.7 µmol TEAC g^{-1} respectively). ABTS results showed that UOMW powder had a TEAC value of 605.6 µmol g^{-1} and the free radical scavenging activity of UOMW was due to its UOMW-C and UOMW-D fractions. The total phenolic content of UOMW powder was 61.8 mg GAE g^{-1} and UOMW-C and UOMW-D fractions accounted practically the total phenol content of the whole powder. Data suggested that the antiglycative action was related to the phenolic moiety of the ingredients, separating the polysaccharides that were added in the formulation steps, the biological activity is linearly correlated with the antioxidant ability.

4. Discussion

Olive mill wastewater is a by-product of the olive oil extraction industry posing a serious environmental problem due to its highly organic load. However, OMW is also an important source of polyphenol, such as hydroxytyrosol, tyrosol verbascoside and oleuropein, with potential health benefits. Consequently, during recent years, new technologies for the recovery of phenols compounds from OMW have been studied[28].

It is known that under hyperglycaemic conditions, the glycation process leads to the formation and accumulation of AGEs, compounds able to modify function and structure proteins of tissues and organs inducing some metabolic diseases. *Ramkissoon et al.*[21] reported that many constituents in plants possess a high TPC and antioxidant potential as well as high ability to inhibit protein glycation. The high incidence of chronic diseases such as diabetes, Alzheimer or cardiovascular diseases and the key role of glycation process in the production of these had been a high relevance in several reports that have appeared in the literature following such benefit[9-29]. In the organism the total pool of circulating AGEs is the sum of those with dietary origin, including AGEs absorbed as such products as well as those formed in vivo from the absorbed dicarbonyl compounds, and those endogenously formed[30]. There is a direct

correlation between circulating AGE level, of which two-thirds remain in the body for 72 hours, and those consumed[11]. Consequently, the dietary AGE restriction is an efficient tool to ameliorate the pathological consequences of the glycation process *in vivo* until effective and safe drugs become available[11]. Our investigation point out that the by-products from olive oil must be considered as an efficient inhibitor of the formation of AGEs, and in extension very likely will act as effective inhibitors of the glycation process. OMW extracts spray dried with maltodextrin and acacia fiber interfered with the formation of Amadori product and also reduced the formation of fluorescent AGE by trapping reactive dicarbonyls, such as MGO and GO. After partial fractionation of the OMW powder, hydroxytyrosol and verbascoside revealed as the most active compound exerting the antiglycative and dicarbonyl trapping activity. Despite the promising data obtained *in vitro*, *in vivo* studies are needed in a further step as well as metabolic studies to clarify its mechanism of action.

5. Conclusion

Results suggest that OMW powder would also act at the early stage of the glycation process being effective inhibitors of the fructosamine formation. The inhibition of dicarbonyl compounds formation by OMW powder was in the same magnitude than that of pyridoxamine, hydroxytyrosol, and verbascoside. On the other hand, it is plausible that the antiglycative capacity and antioxidant properties of extract are due to its phenolic content, especially hydroxytyrosol and verbascoside, but also by the presence of other high molecular compounds. The high molecular weight compounds present in the olive as cellulose, arabinans and pectins are present as well in the OMW powder and a significant part of the phenolic compounds are bound to the polysaccharide moiety[31] as shown by the fact that they are present in the ultrafiltration fraction. Olive mill wastewater powder could be considered as a potent antiglycative ingredient which was found to inhibit AGEs formation at different stages of the pathway. In addition, direct trapping of dicarbonyl compounds have been confirmed as a novel mechanism of action apart of the already known antioxidant capacity. Nevertheless, further research is ongoing in food models to confirm the mitigation of AGE formation and the mechanism of action before to conclude firmly that OMW powder is a diet AGE inhibitor.

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494 **Table 1.** Antioxidant activity and total phenol content of nanofiltrated olive mill wastewater
495 (NOMW), ultrafiltrated olive mill wastewater (UOMW) powders and its fractions obtained by
496 reverse-phase extraction. Results are expressed as mean ± SD for n = 4. Different letters in the
497 same column denote significant differences $P < 0.05$. LoQ < 0.5 GAE mg g^{-1} or < 1.1 TEAC µmol
498 g^{-1}.

499

	TFC GAE mg g^{-1}	FRAP TEAC µmol g^{-1}	ABTS TEAC µmol g^{-1}
Sample			
NOMW	49.9 ± 5.5 [b]	439.4 ± 15.5 [b]	333.7 ± 34.9 [b]
UOMW	61.8 ± 3.4 [a]	780.4 ± 23.0 [a]	605.6 ± 28.8 [a]
Fractions			
UOMW-A	< LoQ	< LoQ	2.0 ± 0.4 [c]
UOMW-B	< LoQ	5.0 ± 0.1 [c]	5.0 ± 0.3 [c]
UOMW-C	24.6 ± 0.2 [c]	199.2 ± 2.6 [d]	249.3 ± 29.6 [d]
UOMW-D	30.0 ± 0.2 [c]	241.7 ± 17.1 [d]	379.8 ± 34.0 [b]

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Table 2. Concentration (mg mL^{-1}) of aminoguanidine, pyridoxamine, quercetin, hydroxytyrosol, tyrosol, verbascoside, and ultrafiltrated (UOMW) and nanofiltrated (NOMW) olive mill wastewater powders exerting the 50% inhibition of BSA glycation. Different letters in the same column denote significant differences $P < 0.05$ in the same column. IC_{50} higher than 1 mg/mL (n.a.)

	IC_{50} - Antiglycative activity	
	BSA-GLC assay	**BSA-MGO assay**
Aminoguanidine	0.100[a]	0.090[a]
Pyridoxamine	0.283[b]	0.046[b]
Quercetin	0.101[a]	0.053[b]
Hydroxytyrosol	0.400[c]	0.086[a]
Tyrosol	n.a.	n.a.
Verbascoside	0.243[b]	0.053[b]
UOMW powder	0.457[c]	0.886[c]
NOMW powder	0.429[c]	1.029[d]

510 **Table 3.** Percentage of inhibition of the formation of fructosamine by ultrafiltrated olive mill
511 wastewater (UOMW) and nanofiltrated olive mill wastewater (NOMW) powders in the range
512 from 0.36 - 3.57 mg mL^{-1}. Results are expressed as mean ± SD for n = 4. Different letters in the
513 same column denote significant differences $P < 0.05$.

514

mg mL^{-1}	UOMW	NOMW
0.36	10.02 ± 5.45 [a]	10.54 ± 2.65 [a]
0.71	25.48 ± 1.90 [b]	33.52 ± 1.61 [b]
1.43	44.67 ± 2.92 [c]	43.67 ± 0.94 [c]
3.57	57.31 ± 1.90 [d]	56.75 ± 6.25 [d]

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516

Figure 1. Antiglycative activity of ultrafiltrated (solid bar) and nanofiltrated (empty bar) olive mill wastewater OMW extracts in the range from 0.14 to 1.43 mg mL^{-1} on the formation of fluorescent AGEs in (A) BSA-MGO and (B) BSA-GLC assays. Aminoguanidine (AG), quercetin (QE), hydroxytyrosol (HT), and verbascoside (VB) at 0.14 mg mL^{-1} were used as reference and showed an antiglycative activity of 67.7%, 86.3%, 69.8%, and 84.4% respectively in BSA-MGO and 64.3%, 63.1%, 26.5% and 54.4% respectively in BSA-GLC. AG was used as control of the assay. Maltodextrin and acacia fiber (MD+AF, 1.43 mg/mL) was used as control of the powder. Results are expressed as mean ± SD for n = 4. Different letters mean significant differences ($P < 0.05$) between samples for a same concentration.

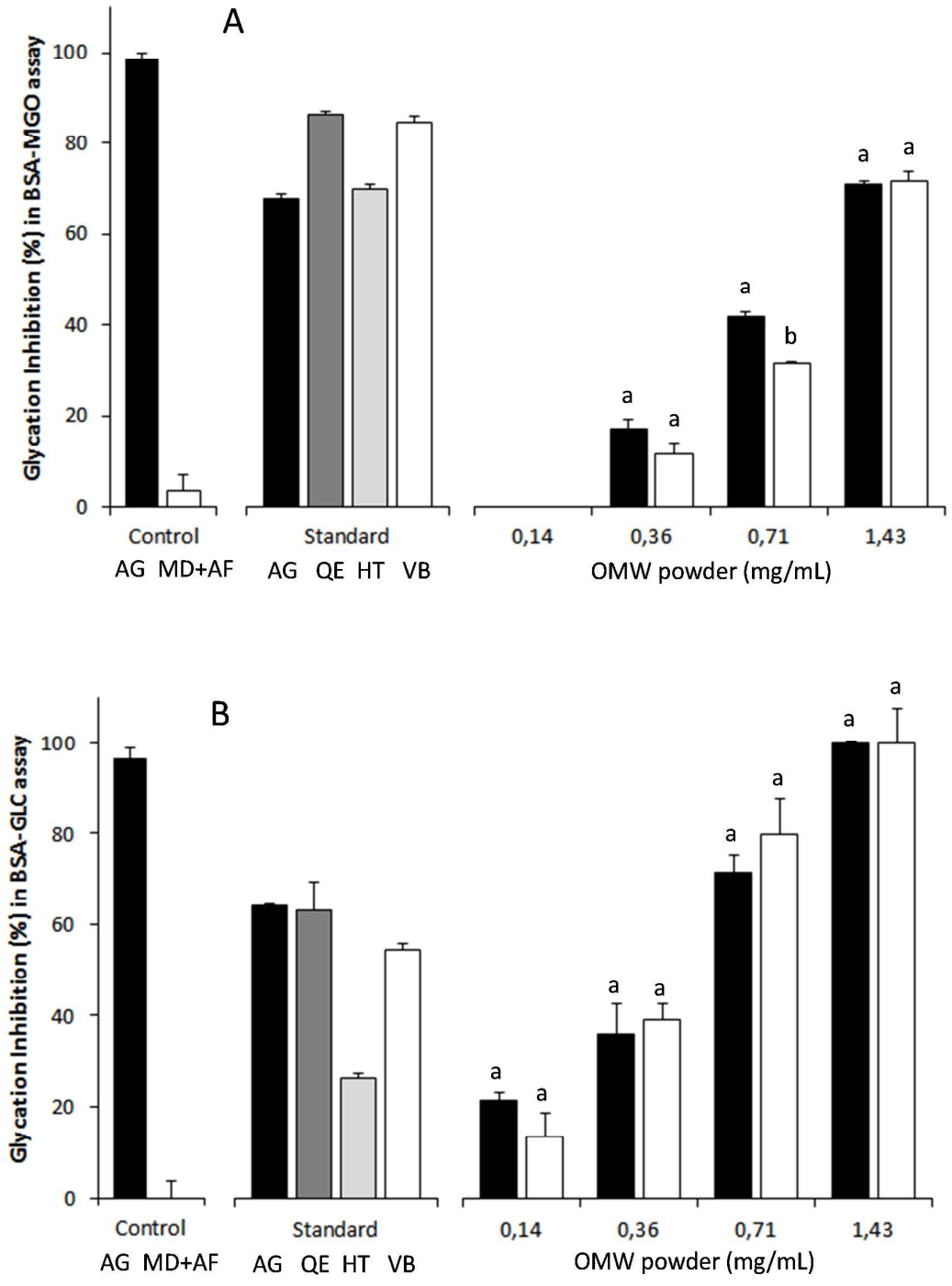
Figure 2. MGO-trapping capacity (A) and GO-trapping capacity (B) of ultrafiltrated olive mill wastewater (UOMW,●) and nanofiltrated olive mill wastewater (NOMW, ○) powders (0.1 - 2.5 mg mL^{-1}) after incubation (168h). Results are expressed as mean ± SD for n = 4. PM (0.1 mg mL^{-1}) was used as control positive and presented and MGO and GO trapping capacity of 99.6% and 14.5% respectively. Control for maltodextrin + acacia fiber depicted as dotted line.

Figure 3. Chromatographic profile of the ultrafiltrated olive mill wastewater (UOMW) powder and fractions (UOMW-A, OUMW-B, UOMW-C and UOMW-D) fractions. See materials and methods for details. Butyl -4-hydroxybenzoate (IS).

Figure 4. Antiglycative activity of ultrafiltrated olive mill wastewater (UOMW) powder and fractions in the BSA-MGO (A) and in BSA-GLC (B) assay. UOMW was used as a reference presenting an antiglycative activity of 76.3% and 88.4% for BSA-MGO and BSA-GLC, respectively. Control for maltodextrin and acacia fiber has not activity as described in figure 1. Results are expressed as mean ± SD for n = 4. Different letters denote significant differences $P < 0.05$.

Figure 5. Methylglyoxal trapping activity of ultrafiltrate olive mill wastewater powder and fractions over time. Ultrafiltrated olive mill wastewater (UOMW) powder (◆) and UOMW-A (■), UOMW-B (□), UOMW-C (●), UOMW-D (○) fraction

544 **FIGURE 1**



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548 **FIGURE 2**

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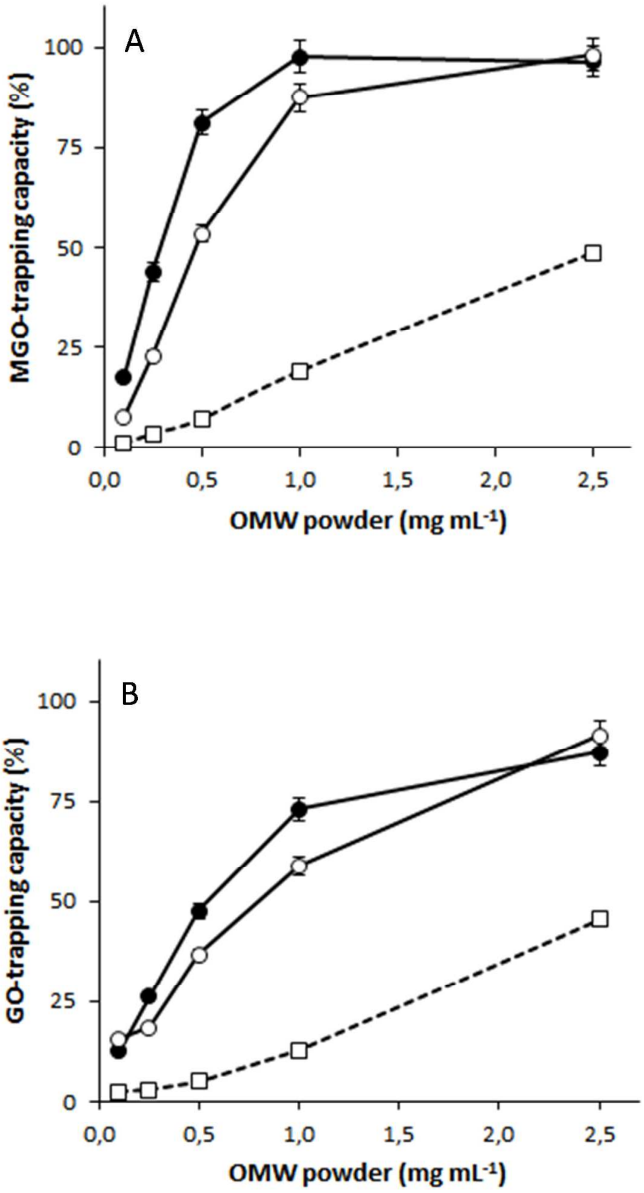

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554 **FIGURE 3**

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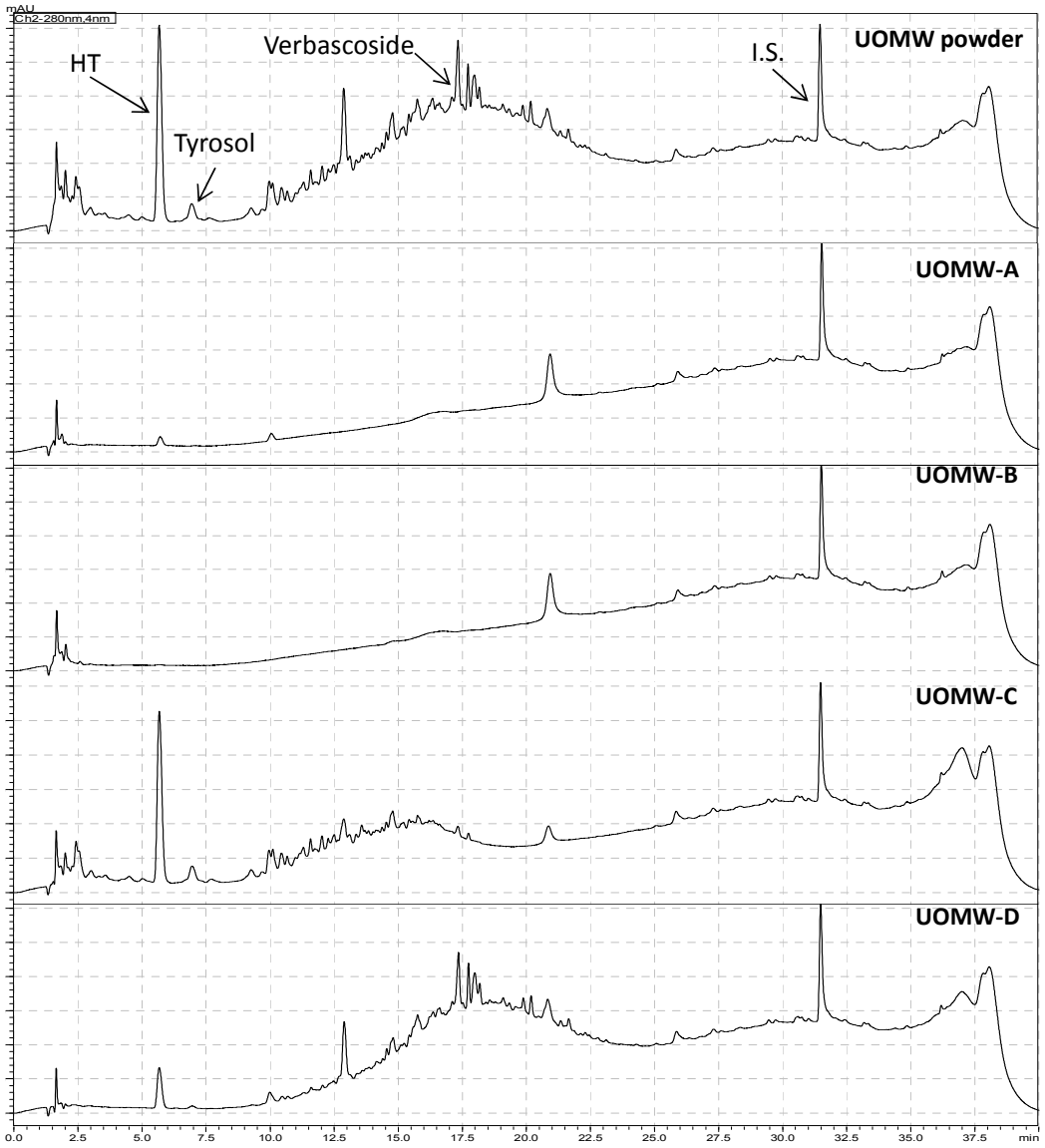

Retention time (min)

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562 **FIGURE 4**

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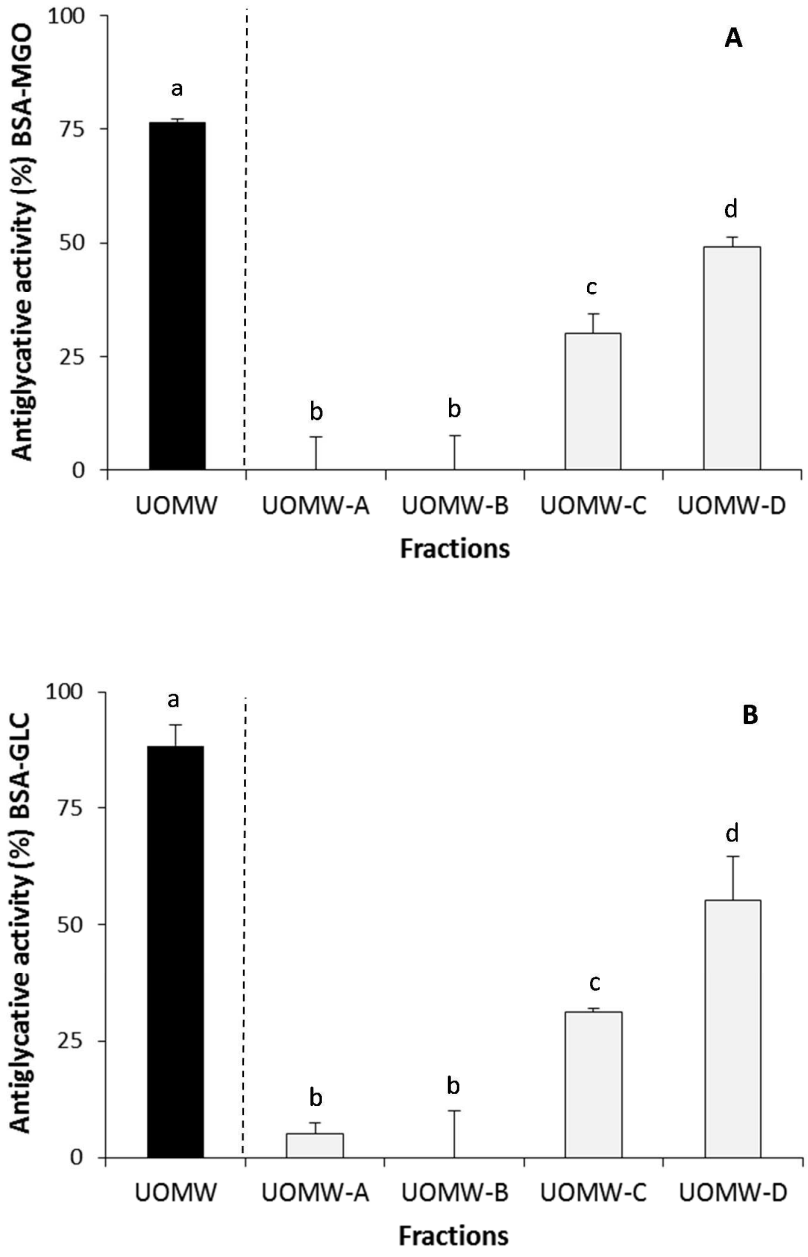

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567 **FIGURE 5**

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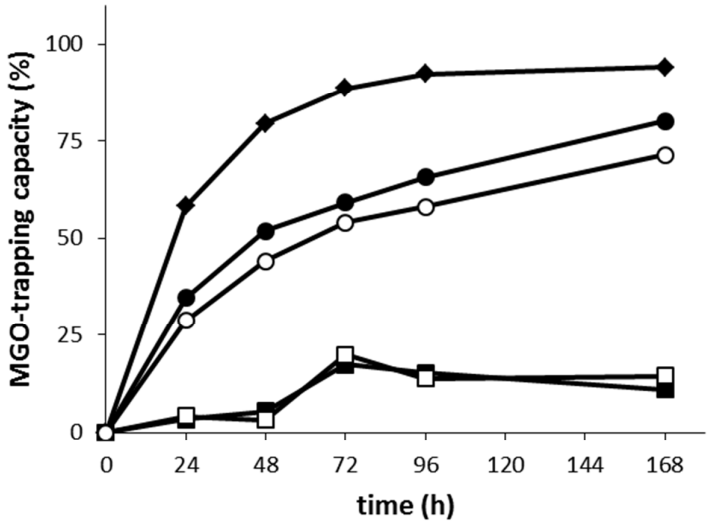

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