Carbonyl trapping and antiglycative activities of olive oil mill wastewater

Marta Navarro,^a Alberto Fiore,^{b,c} Vincenzo Foglianod and Francisco J. Morales^{*a}

^a Institute of Food Science, Technology and Nutrition, ICTAN-CSIC, E-28040 Madrid, Spain. E-mail: fjmorales@ictan.csic.es; Tel: +34 91 549 2300

^b School of Science, Engineering & Technology, Division of Food & Life Sciences, Abertay University, Dundee DD1 1HG, UK

^c Department of Agriculture and Food Science, University of Naples "Federico II", Naples, Italy

^d Food Quality and Design group, Wageningen University & Research Centre, PO Box 8129, 6700 EV Wageningen, The Netherlands

ABSTRACT

The use of natural compounds as antiglycative agents 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 a high content of hydroxytyrosol, hydroxytyrosol derivatives and molecules containing odihydroxyl functions such as verbascoside. Two powders were obtained after the ultrafiltration and nanofiltration of olive mill wastewater, and successive spray drying with maltodextrin and acacia fiber. The 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 ultrafiltered and nanofiltered olive mill wastewater powders had an activity comparable to quercetin and hydroxytyrosol against the inhibition of protein glycation (IC₅₀ = 0.3 mg mL⁻¹). The antiglycative activity of the powder was further investigated after separation by reverse phase solid extraction. Fractions extracted with the methanol content higher than 40% and rich in hydroxytyrosol and verbascoside exerted the highest reactivity against dicarbonyls. Data confirmed that the direct trapping of dicarbonyl compounds is the 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 ingredients in foods or in pharmacological preparations in future.

INTRODUCTION

The health benefits of a Mediterranean diet have been firmly established, including the lowering of degenerative pathologies, 1 cardiovascular diseases² and cancer.^{3,4} In this respect, olive oil's effects, as an 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 an olive plant (*Olea europaea* L.) is the oleuropein, which is hydrolyzed into hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) and tyrosol during the extraction of olive oil.⁵ Several epidemiological studies suggest that HT is an efficient scavenger of peroxyl radicals⁶ and prevents damage due to oxidative stress. In the 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, neuroprotective⁷ and cardio protective activity as well as hypoglycemic effect which could prevent the glucose auto-oxidation reaction, a process related to the formation of AGEs.⁸

Advanced glycation endproducts (AGEs) are the outcome of a non-enzymatic glycation reaction between the carbonyl group of a reducing sugar and the 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 the protein structure and their biochemical properties.⁹ AGEs are a 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 reaction to the accelerating rate of AGE formation, since radicals and α -dicarbonyls, highly reactive intermediates such as methylglyoxal (MGO), glyoxal (GO) or 3-deoxyglucosone, are generated. Previous studies have suggested that antioxidants protect against glycation since they reduce the formation of reactive carbonyl species which is a consequence of oxidative stress generated by the formation of reactive oxygen species (ROS).⁹ AGEs can also be introduced in the systems of living bodies by exogenous sources like diet. Basically, AGEs, also named Maillard reaction products, are

formed during the thermal processing of food. The AGE and dietary AGEs contributed to the total human body AGE pool.¹¹

Due to AGE 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 alternatives is very appealing. Some bioactive phytochemicals naturally occurring in plants and possessing redox properties have been related to the inhibition of the glycation process.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 involved¹³ 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 Maillard reaction products during the thermal processing of UHT milk.

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

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-pyridyl)-s-triazine (TPTZ), 2-2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and phenolic acid standards were provided by Sigma (St Louis, MO, USA). Folin–Ciocalteu reagent and iron(III) chloride were 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.

2.2. Equipment

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

2.3. Preparation of olive mill wastewater powder

Olive oil polyphenol powders were obtained from Azienda Agricola Fangiano (Calabria, Italy) starting from the water fraction resulting from virgin-olive oil production (Carolea variety) as follows. Olive water was separated from the oil and olive paste by centrifugation; it was treated with pectinases for 2 hours at 37° C then fractionated by a filtration plant made up of three membranes having different cut offs. Olive water was forced to pass through the microfiltration (cut off 25 kDa), ultrafiltration (cut off 8 kDa), and nanofiltration (cut off 0.3 kDa) membranes. At each stage a retentate containing the compounds not passing through the membrane pores was collected while the permeate moved on to the next filtration step. For this investigation, the ultrafiltration and nanofiltration retentates were concentrated by inverse osmosis (cut off 0.1 kDa) up to 20% dry weight and spray dried adding maltodextrin and acacia fiber in a ratio 2 : 1 of the

water mill dry weight (66.6% olive mill water and 33.3% maltodextrin and acacia fiber 1 : 1). Finally, a fine pale yellow ultrafiltered (UOMW) and nanofiltered (NOMW) powder with moisture content below 4% was obtained and used in this study.

2.4. Analysis of phenolic compounds in OMW powder

The analysis of the phenolic component was carried out as described by Kokkinidou & Peterson¹⁶ with some modifications. Briefly, the OMW powder was dissolved in distilled water, in order to prepare a solution at a concentration of 20 mg mL⁻¹. To 1 mL of this solution, 10 µL of a 5 mg L⁻¹ solution of butyl-4-hydroxybenzoate as internal standard were added. The phenolic fraction was extracted through the use of SPE cartridges Strata C18-E, and dried under a nitrogen stream. Thereafter the precipitate was recovered in 500 µL of a solution of water-methanol/95 : 5 (v/v) ready for HPLC analysis. The instrument used for chromatographic analysis was an LC-20AD HPLC with a UV-Vis detector SPD20A, set at 279 nm, and an SCL-20A controller (Shimadzu, Japan). The mobile phases were 0.1% formic acid in H₂O (A) and methanol (B). The flow was 0.8 mL min⁻¹. A Prodigy ODS3 column was used (250 × 4.60 mm, 5 micron, 100 Å, Phenomenex, USA). The sample (20 µL) was separated with the next gradient as follows; 0 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 the standards. Peaks were identified by retention time, DAD spectra and spiking the 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) per g sample and all measurements were carried out in triplicate. The limit of quantification was set at 0.5 mg GAE per g sample.

2.6. Determination of antioxidant capacity according to ABTS assay

The antioxidant activity was estimated in terms of the radical 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 the Fe²⁺-TPTZ complex from the Fe³⁺-TPTZ complex was determined as described by Morales et al.¹⁹ Results are expressed as µmol Trolox equivalent antioxidant capacity (TEAC) per g sample.

2.8. Fractionation of the olive mill wastewater powder ingredient

The ultrafiltrate olive mill wastewater (UOMW) powder was further fractionated by reversed phase solid phase extraction (C18 SepPak cartridge) with sequential extraction in methanol. A solution of the UOMW powder (1 mg mL⁻¹) was loaded onto a pre-activated cartridge. The first fraction was eluted and collected and termed as fraction UOMW-A. Then 1 mL of formic acid (0.1%) with 5% of methanol was passed through the cartridge and the resulting fraction (UOMW-B) was collected. UOMW-C is the fraction that was eluted with 1 mL of formic acid with 40% of methanol. Finally, the UOMW-D fraction was eluted with 1 mL of methanol passed through the cartridge. Chromatographic separations of the whole extract and fractions were performed on a C18 Kinetex column (75 mm × 4.6 mm, 2.6 µm, Phenomenex, USA). The temperature was maintained at 40 °C and the flow rate was 0.6 mL min⁻¹. The mobile phases were 0.5% acetic acid in water (solvent A) and methanol (solvent B) and the total running time was 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 and BSA-GLC assays 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, AG (0.57 mg mL⁻¹) being the positive control of the assay. The systems containing the samples/control/ blank were incubated at 37 °C for 14 days (BSA-MGO assay) or 21 days (BSA-GLC assay). The 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 using a microplate spectrophotometer. AGE formation was characterized by measuring fluorescence with the excitation and emission maxima at 340 and 420 nm, respectively, for the BSA-MGO assay and at 360 and 420 nm, respectively, for the BSA-GLC assay. The intrinsic fluorescence of the samples incubated under the same conditions was subtracted from the overall fluorescence in each BSA-MGO or BSA-GLC system. The percentage inhibition of AGE formation was calculated according to the following equation:

$$\text{inhibition (\%)} = \{1 - [(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence of sample}) / \text{fluorescence of solution without inhibitor}] \} \times 100$$

IC₅₀ (the concentration required to inhibit glycation by 50%) were calculated from the dose–response curve using the Microsoft-Excel computer software.

2.10. Fructosamine adduct assay

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

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

$$\begin{aligned} \text{inhibition (\%)} &= 100 \\ &- [(\text{fluorescence of solution with inhibitor} \\ &- \text{intrinsic fluorescence of sample} \\ &- \text{fluorescence of blank}) \\ &\times 100 / (\text{fluorescence control positive} \\ &- \text{intrinsic fluorescence of control positive} \\ &- \text{fluorescence of blank})] \end{aligned}$$

2.11. Direct MGO and GO trapping capacity

Direct MGO or GO trapping capacity of the 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 concentrations of the extracts was incubated at 37°C for 168 h. Pyridoxamine (PM, 0.1 mg mL^{-1}) were used as the positive control. OPD was added in order to derivatize the unreacted MGO or GO into 2-methylquinoxaline (2-MQ) or 1-quinoxaline (1-Q), respectively. 5-quinoxaline (5-MQ) was used as an internal standard. Chromatograms were recorded at 315 nm with the retention times of 2-MQ, 1-Q and 5-MQ being 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:

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) followed 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.

RESULTS AND DISCUSSION

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 non-enzymatic protein glycation. Two types of powders were obtained, one after the ultrafiltration (UOMW) of the olive mill wastewater extract and the second from the retentate after nanofiltration (NOMW). Both extracts were spray dried adding maltodextrins and acacia fiber, and the powders were characterized by the total polyphenol content and antioxidant capacity (FRAP and ABTS) as summarised in Table 1. The content of polyphenolic compounds and the antioxidant capacity, regardless of the method used, was significantly ($P < 0.05$) higher in the UOMW powder as compared with the NOMW powder. It is known that certain bioactive compounds present in plants possess antioxidant capacity as well as the ability to inhibit protein glycation as flavonoids.²¹ During the spray drying of extracts, the yield of the nanofiltration powder was lower than that of ultrafiltration, particularly when the concentration of the added dietary fibre was reduced to 33.3%. The average content in hydroxytyrosol, tyrosol and verbascoside was 33.0, 2.1 and 2.8 $\mu\text{g g}^{-1}$ for the UOMW powder and 13.8, 0.2, and 1.3 $\mu\text{g g}^{-1}$ for the NOMW powder, respectively. The higher concentration of polyphenols in the UOMW powder was due to the presence of bound polyphenols linked to the polysaccharide moiety (pectins and arabinans) that remain in the retentate of the extract.²²

The antiglycative properties of OMW powders were evaluated by the BSA-MGO and BSA-GLC assays. Fig. 1a showed the formation of fluorescent AGEs in the BSA-MGO assay (37 °C/14 days). The UOMW and NOMW powders behave in a dose dependent manner and both reduced the formation of AGEs significantly, with an IC₅₀ of 0.886 and 1.029 mg mL⁻¹, respectively for the ultrafiltered and nanofiltrated powders (Table 2). But the ultrafiltered 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. The suppression of dicarbonyl formation and direct reaction with dicarbonyls are the main mechanisms 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. IC₅₀ 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). The control for maltodextrin and acacia fiber (MD + AF) showed a residual 3.5% antiglycative activity for the BSA-MGO assay.

Fig. 1b showed the formation of fluorescent AGEs for the BSA-GLC assay (37 °C for 21 days). Ultrafiltration and nanofiltration powders presented a dose-dependent inhibitory activity with no significant differences between them (IC₅₀ of 0.457 and 0.429 mg mL⁻¹, respectively) as described in Table 2. IC₅₀ for the BSA-GLC assay were 0.100, 0.101, 0.400, and 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 autooxidation of glucose is a side reaction to form reactive dicarbonyl compounds that promote the formation of AGEs. The control for maltodextrin and acacia fiber did not show any antiglycative activity for the BSA-GLC assay.

On the other hand, methylglyoxal (MGO) and glyoxal (GO) are the key promoters of the glycation process where the formation of AGEs and the irreversible modification of proteins is boosted. For this reason, the direct MGO and GO trapping capacities of the UOMW and NOMW extracts were investigated. Fig. 2a and b depicted the MGO and GO trapping abilities of the 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 the control sample (maltodextrin + acacia fiber) showed a minor dicarbonyl trapping capacity, representing nearly 20% of the overall response. Results show that the efficacy of the OMW powder for trapping GO was lower as compared with the MGO trapping ability. IC₅₀ for the GO-trapping assay was calculated to be 0.562 mg mL⁻¹ and 0.811 mg mL⁻¹ for the UOMW and NOMW powders, respectively. In contrast, both powders were more effective for trapping MGO with IC₅₀ values of 0.294 and 0.473 mg mL⁻¹, respectively. The ultrafiltered powder was significantly ($P < 0.05$) more reactive against MGO and GO than the nanofiltered powder for a concentration higher than 0.1 mg mL⁻¹.

In the early phase of the glycation reaction, the reversible Schiff base leads to a rearrangement where a more stable Amadori product is formed. In the advanced phase of glycation, irreversible AGEs are formed. Therefore, the inhibition of fructosamine adducts could reduce AGE formation in foods and hence

potentially ameliorate the diabetic complications in vivo by a restriction of the dietary AGE intake and a 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 the antiglycative activity and dicarbonyl trapping ability of the ultrafiltered and nanofiltered powders are relevant to the potential mitigation of diet-derived and endogenously formed AGEs. As shown in Table 3, both extracts performed their inhibitory effects at an early stage of glycation since a significant reduction in the formation of fructosamine was observed at all concentrations (0.36–3.57 mg mL⁻¹). The IC₅₀ for the fructosamine assay was calculated to be 2.34 and 2.48 mg mL⁻¹ for the ultrafiltered and nanofiltered powders, respectively.

Since the antiglycative activity of both ultrafiltered and nanofiltered powders was in the same order of magnitude, the ultrafiltered sample was selected for a more detailed investigation to elucidate the mechanisms of 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 for a further investigation of the antiglycative capacity. Fig. 3 described the chromatographic profile of the whole extract and the relative fractions A, B, C and D, eluted 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 the UOMW powder is particularly rich in HT (42% of the total polyphenols content), further investigations focused on the HT content. The highest concentration of HT (representing approx. 80% of the initial HT content) was collected in the fraction C which was eluted with 40% MetOH. Fraction D (corresponding to fraction eluted with 100% MetOH) contains the lowest amount of HT corresponding to approximately 20% of the initial content. However verbascoside is eluted at fraction D together with a major proportion of the polysaccharides rich in polyphenols.

The antiglycative activity of each fraction obtained by the solid phase extraction was estimated by the BSA-MGO (Fig. 4a) and BSA-GLC (Fig. 4b) assays. In both BSA-MGO and BSA-GLC assays, fractions A and B did not exert a protective effect on the formation of fluorescent AGEs, while C and D fractions showed 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. The antiglycative activity of the fraction eluted with methanol was significantly higher than the fraction eluted with 40% methanol. The partial contribution of the antiglycative capacity of the UOMW-C and UOMW-D fractions could explain the activity of the whole UOMW extract that involved 76.3 and 88.4% of inhibition in BSA-MGO, and BSA-GLC, respectively.

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

Several assays have established a relationship between the 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 the antioxidant activity of HT and oleuropein. In this regard, the total antioxidant capacity was evaluated in the samples by FRAP and ABTS. The 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 $\mu\text{mol TEAC g}^{-1}$) was higher than the sum of the reducing activity of its UOMW-C and UOMW-D fractions (199.2 and 241.7 $\mu\text{mol TEAC g}^{-1}$ respectively). ABTS results showed that UOMW powder had a TEAC value of 605.6 $\mu\text{mol g}^{-1}$ 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 the 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.

DISCUSSION

Olive mill wastewater is a by-product of the olive oil extraction industry posing a serious environmental problem due to its high organic load. However, OMW is also an important source of polyphenols, such as hydroxytyrosol, tyrosol, verbascoside and oleuropein, with potential health benefits. Consequently, during recent years, new technologies for the recovery of phenol 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 the function and structure of proteins in 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 a high ability to inhibit protein glycation. The high incidence of chronic diseases such as diabetes, Alzheimer's or cardiovascular diseases and the key role of the glycation process in causing these diseases has been intensively studied on the literature and several reports with a high relevance could be found thanks the beneficial effect of the polyphenols.^{9–29} In an 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 the circulating AGE level, of which two-thirds remain in the body for 72 hours of those consumed.¹¹ Consequently, 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 points out that the byproducts from olive oil must be considered as efficient inhibitors 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 the 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 were revealed as the most active compounds exerting antiglycative and dicarbonyl trapping activity. Despite the promising data obtained in vitro, in vivo studies are needed as a further step and so are metabolic studies to clarify its mechanism of action.

CONCLUSIONS

Results suggest that the OMW powder would also act at the early stage of the glycation process being an effective inhibitor of fructosamine formation. The inhibition of dicarbonyl compound formation by OMW powder was in the same magnitude as that of pyridoxamine, hydroxytyrosol, and verbascoside. On the other hand, it is plausible that the antiglycative capacity and antioxidant properties of the extracts are due to their phenolic content, especially hydroxytyrosol and verbascoside, but also due to the presence of other high molecular compounds. High molecular weight compounds present in olives as cellulose, arabinans and pectins are present as well in the OMW powder and a significant part of the phenolic compounds is bound to the polysaccharide moiety³¹ 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 AGE formation at different stages of the pathway. In addition, the direct trapping of dicarbonyl compounds has been confirmed to be a novel mechanism of the action apart from 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 concluding firmly that the OMW powder is a diet AGE inhibitor.

Acknowledgments

This work was funded by project AVANSECAL (S2013/ABI-3028, Madrid Community, ES), CSIC-201370E027, program PON01_02863 (Italian Ministry of University and Scientific Research, MIUR), and APQ project (Regione Calabria, Italy). M. Navarro thanks additional funding by the JAE program (Spanish National Research Council).

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FIGURES AND TABLES

Table 1. Antioxidant activity and total phenol content of nanofiltered olive mill wastewater (NOMW), ultrafiltered olive mill wastewater (UOMW) powders and its fractions obtained by reverse-phase extraction. Results are expressed as mean $\pm$ SD for $n = 4$. Different letters in the same column denote significant differences $P < 0.05$. LoQ < 0.5 GAE (mg g^{-1}) or < 1.1 TEAC ($\mu\text{mol g}^{-1}$).

	TFC GAE (mg g^{-1})	FRAP TEAC ($\mu\text{mol g}^{-1}$)	ABTS TEAC ($\mu\text{mol g}^{-1}$)
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

Table 2. Table 2 Concentration (mg mL^{-1}) of aminoguanidine, pyridoxamine, quercetin, hydroxytyrosol, tyrosol, verbascoside, and ultrafiltered (UOMW) and nanofiltered (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. IC₅₀ higher than 1 mg mL^{-1} (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

Table 3. Percentage of inhibition of the formation of fructosamine by ultrafiltered olive mill wastewater (UOMW) and nanofiltrated olive mill wastewater (NOMW) powders in the range from 0.36–3.57 mg mL⁻¹. Results are expressed as mean $\pm$ SD for n = 4. Different letters in the same column denote significant differences P < 0.05.

mg mL ⁻¹	UOMW	NOMW
0.36	10.02 $\pm$ 5.45 ^a	10.54 $\pm$ 2.65 ^a
0.71	25.48 $\pm$ 1.90 ^b	33.52 $\pm$ 1.61 ^b
1.43	44.67 $\pm$ 2.92 ^c	43.67 $\pm$ 0.94 ^c
3.57	57.31 $\pm$ 1.90 ^d	56.75 $\pm$ 6.25 ^d

Figure 1. Antiglycative activity of ultrafiltered (solid bar) and nanofiltered (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⁻¹) were used as control of the powder. Results are expressed as mean $\pm$ SD for n = 4. Different letters mean significant differences (P < 0.05) between samples for a same concentration.

Figure 2. Fig. 2 MGO-trapping capacity (A) and GO-trapping capacity (B) of ultrafiltered olive mill wastewater (UOMW, ●) and nanofiltered olive mill wastewater (NOMW, ○) powders (0.1–2.5 mg mL⁻¹) after incubation (168 h). Results are expressed as mean ± SD for n = 4. PM (0.1 mg mL⁻¹) was used as a control positive and presented 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 ultrafiltered olive mill wastewater (UOMW) powder and UOMW-A, UOMW-B, UOMW-C and UOMW-D fractions. See Materials and methods for details. Butyl-4-hydroxybenzoate (IS).

Figure 4. Antiglycative activity of ultrafiltered 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 no activity as described in Fig. 1. Results are expressed as mean $\pm$ 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. Ultrafiltered olive mill wastewater (UOMW) powder (◆) and UOMW-A (■), UOMW-B (□), UOMW-C (●), and UOMW-D (○) fraction.