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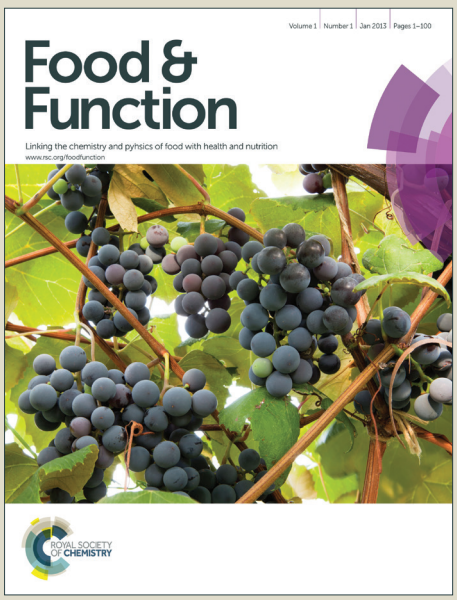

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Antioxidant properties of chemical extracts and bioaccessible fractions obtained from six Spanish monovarietal extra virgin olive oils. Assays in Caco-2 cells

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ABSTRACT

The antioxidant activity and the total phenolic content (TPC) of six Spanish commercial monovarietal extra virgin olive oils (Arbequina, Cornicabra, Hojiblanca, Manzanilla, Picual and Picudo) were evaluated in chemical extracts and in bioaccessible fractions (BF) obtained after *in vitro* digestion. Moreover, the effects of the BF on cell viability and the generation of reactive oxygen species (ROS) were investigated in Caco-2 cell cultures. The *in vitro* digestion process increased the TPC and antioxidant activity evaluated by different methods (ABTS, DPPH and FRAP) compared with chemical extracts. After digestion, the Picual variety showed better beneficial effect in preserving cell integrity that the other varieties studied. Significant reductions of ROS production were observed after incubation of Caco-2 cells with the BF of all the varieties and, moreover, a protective effect against the oxidative stress induced by *t*-BOOH was showed for Arbequina, Cornicabra, Hojiblanca, Manzanilla and Picual. These findings seem an additional reason supporting the health benefits of Spanish extra virgin olive oils varieties. Multivariate factor analysis and principal component analysis were applied to assess the contribution of antioxidant activity and TPC, before and after digestion, to the characterization of the different varieties.

KEYWORDS: Extra virgin olive oil, antioxidant activity, bioaccessibility, Caco-2 cells, reactive oxygen species.

1. Introduction

Monovarietal extra virgin olive oils (EVOO) are edible oils prepared from a single variety of olive fruit and play an important role in creating blended oils (i.e., from different varieties), which represent a high percentage of the olive oil market.[1,2] In consequence, their characterisation is of great importance for purposes of authentication.

The chemical composition of monovarietal EVOO exhibits considerable variability because it is influenced by the agronomic practices, geographical origins, harvesting periods and processing technologies.[2] Many studies of monovarietal EVOO have been conducted in recent years to differentiate the oil composition according to varieties and to know more about each variety. [1, 3- 9]

Phenolic compounds are considered in many studies as the main responsible for the antioxidant capacity of olive oils. This capacity is typically estimated by *in vitro* assays after chemical extraction, which are usually focused to assess the radical scavenging capacity (DPPH and ABTS) and the ferric-reducing antioxidant power (FRAP).[10-13]

However, the use of chemical extraction to determine antioxidant properties by *in vitro* assays is controversial, because it may be affected by factors such as the extraction procedure and the solubility of the compounds responsible for the antioxidant activity.[14] Moreover, it is generally accepted that the first requirement of a dietary compound to be considered a potential *in vivo* antioxidant is to be bioaccessible, and this depends on the compound release from the food matrix during the digestion process.[15] Consequently, while it is important to know the total quantity of a nutrient present in a food, it is also essential to know how much of that is bioaccessible. *In vitro* digestion models permit the characterisation of the compounds under physiological conditions caused by

digestive enzymes and can provide more information than that gained from the chemical analysis of food.[16] Thus, *in vitro* digestion models have been developed as a first approach to studying the bioavailability of compounds from foods, including olive oil.[14,15,17-19]

In combination with *in vitro* digestion, cell culture models facilitate the study of small-intestinal absorption and metabolism and thus elucidate the potential impact of these compounds on human health.[16] Antioxidant and chemo-protective properties of individual compounds from olive oil extracts have been reported in cultured cells such as Caco-2 and HepG2 cells, and protective effects against induced-oxidative stress have been demonstrated.[11,16,20-25] However, little is known about the antioxidant properties of monovarietal virgin olive oils after the digestion process, nor about their antioxidant effects at the cellular level.

In this study, we examined six commercial monovarietal EVOO (Arbequina, Cornicabra, Hojiblanca, Manzanilla, Picual and Picudo) with three main aims: i) to determine the total phenolic content (TPC) and the antioxidant activity by DPPH, ABTS and FRAP assays of chemical extracts and bioaccessible fractions (BF) obtained after *in vitro* digestion; ii) to investigate the effects of the BF on cell viability in Caco-2 cells; iii) to evaluate the protective effect of the BF in reactive oxygen species (ROS) generation against induced oxidative stress.

2. Materials and methods

2.1. Standards and Reagents

Alcohol, methanol, n-hexane, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid (ABTS), 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ) and iron (III) chloride were purchased from Fluka Chemicals (Madrid, Spain). Sodium

dihydrogen phosphate dehydrate, potassium hexacyanoferrate (III) and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Hydrochloric acid, anhydrous sodium carbonate and di-sodium hydrogen phosphate dehydrate were obtained from Panreac (Barcelona, Spain). The standard antioxidant 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and caffeic acid were obtained from Sigma-Aldrich (St. Louis, USA). Pepsin (P7000), pancreatin (P1750), bile salts (B8756), HEPES and tert-butyl hydroperoxide (*t*-BOOH) were purchased from the same company, as were all cell culture media and cell culture-grade chemicals. Bidistilled deionised water was used and all reagents were of suitable analytic purity.

2.2. Samples

Two different brands of Spanish monovarietal EVOO from the different varieties (Arbequina, Cornicabra, Manzanilla, Hojiblanca, Picual and Picudo) were obtained in local stores of Granada (Spain). The reason for this approach was to assess a broad range of the EVOO available for consumers in the Spanish market. All the samples were from the same harvest (2013/2014) and were stored protected from the light at 4 °C until analysis. Figure 1 shows a simplified squeme of the general process applied to the oil samples, as described below.

2.3. Chemical extraction

For the chemical extraction, 2g of oil were mixed with 1 mL of n-hexane and the mixture was vigorously stirred until dissolution. Then, 2 mL of methanol/water (80:20 v/v) were added in order to assay the polar fraction. The solution was centrifuged at 4000 rpm for 5 min (Sorvall RC 6 Plus centrifuge, Thermo Scientific, Madrid, Spain),

the extraction was repeated twice and the methanolic extracts were combined.[26] The extracts were obtained in triplicate for each brand.

2.4. *In vitro* digestion

For *in vitro* digestion, sequential steps similar to those present in gastric and intestinal digestion were simulated, as described by Mesías et al,[27] with some modifications. The olive oil sample was mixed with Milli-Q water (1:10, w/v), sonicated (Vibracell VCX 130, Sonics & Materials INC, Danbury, Connecticut, USA) and acidified to pH 2.0 using 6N HCl. The sample was then mixed with 0.313 mL of pepsin/0.1N HCl (160 mg pepsin/mL) and stirred (110 oscillations/min) for 2 h at 37°C in a water bath (Bunsen, Madrid, Spain). After gastric digestion, the pH was increased to pH 6 with 1M $NaHCO_3$ and then 2.5 mL of pancreatin/bile salts solution (pancreatin 4 mg/mL; bile salts 2.5 mg/mL) in 0.1M $NaHCO_3$ were added. The pH was checked and the samples were stirred in the same conditions in the water bath (110 oscillations/min; 37 °C for 2h). After gastrointestinal digestion, the digestive enzymes were inactivated by heat treatment for 4 min at 100 °C in a polyethylene glycol bath. The samples were then cooled by immersion in an ice bath and centrifuged at 10000 rpm for 30 min at 4 °C (Sorvall RC 6 Plus centrifuge) to separate the soluble or bioaccessible fractions (BF). The BF were stored at -80 °C in bottles protected from the light under a nitrogen blanket. Blanks with no sample were run in parallel and analysed to discard interferences from the reagents in the digestion process.

The BF were obtained in triplicate for each brand and used to determine the TPC and antioxidant activity and for the Caco-2 cells experiments.

2.5. Determination of total phenolic content

147 TPC was determined according to the method described by Saura et al,[28] Aliquots of 10
148 µL of sample (chemical extracts or BF) were mixed with 10 µL of the Folin-Ciocalteau
149 reactive and allowed to stand for 3 min. Then, 200 µL of sodium carbonate (75 g/L) and
150 30 µL of Milli-Q water were added and allowed to stand for 60 minutes in the dark. The
151 reaction was measured at 750 nm using a Victor X3 multilabel plate reader (Waltham,
152 Massachusetts, USA). A calibration curve was determined using a 0-0.2 mg/mL
153 concentration of caffeic acid. The results were expressed in mg of caffeic acid
154 equivalents (CAE)/ kg of sample.
155
156 **2.6. DPPH assay**
157 The capacity to scavenge the DPPH free radical was determined as described by
158 Morales and Jiménez-Pérez,[29] The chemical extracts or the BF (50 µL) were mixed with
159 250 µL of methanolic solution of DPPH (74 mg/L). The mixture was shaken, left to
160 stand for 60 minutes in the dark and the absorbance at 520 nm was measured using a
161 Victor X3 multilabel plate reader (Waltham, Massachusetts, USA). The calibration
162 curve was done using a concentration range of 0.01-0.1 mg/mL of Trolox and the
163 results were expressed in mmol Trolox equivalent/kg of sample.
164
165 **2.7. ABTS assay**
166 The $ABTS^{+}$ solution was obtained by mixing 7mM of aqueous solution of ABTS with
167 2.45 mM potassium persulfate (1:1) and maintaining it for 12-16 hours in the dark at
168 room temperature. The solution was diluted with ethanol to an absorbance of 0.70 ±
169 0.02 at 750 nm. 280µL of ABTS solution was mixed with 20 µL of the extracts or BF
170 and the solution was maintained for 20 minutes in the dark.[30] Then, the absorbance was
171 measured at 750 nm. A calibration curve of Trolox (concentration range of 0.01-0.4

mg/mL) was performed and the results were expressed in mmol of Trolox equivalent/kg of sample.

2.8. Ferric reducing antioxidant power (FRAP)

The FRAP reagent was prepared from 0.3 M acetate sodium buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ in 40 mM HCl. The three solutions were mixed in the ratio 10:1:1. 20 µL of extracts or BF were mixed with 280 µL of FRAP solution, and incubated at 37 °C for 30 min. The absorbance reading was taken at 595nm.[30]. The ferric reducing ability of the samples was determined against a calibration curve of Trolox (0.01-0.2 mg/mL), and the results were expressed in mmol Trolox equivalent/kg of sample.

2.9. Cell cultures assays

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University at passage 45, and were used in the experiments at passages 49-53. Culture flasks and bicameral chambers were purchased from Corning Costar (Cambridge, MA, USA). The cells were maintained by serial passage in 75 cm^2 plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (FBS) (10%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 UI/mL), and 1% antibiotic-antimycotic solution. The cells were grown under an atmosphere of air/CO_2 (95:5) at 90% humidity and 37°C and given fresh medium every 2 days.

Two assays were performed to observe the effects of the BF of the oils at the cellular level: modifications of cell viability and protective effect against an oxidative

197 insult, measured by ROS generation. Prior to the cell culture experiments, the
198 osmolarity of the BF was adjusted to 310 mOsm/kg (cryoscopic osmometer Osmomat
199 030-D, Berlin, Germany).
200
201 **2.9.1. Cell viability**
202 Viability of the Caco-2 cells was assessed by a neutral red (NR) cytotoxicity assay
203 procedure, based on the ability of viable uninjured cells to actively incorporate NR, a
204 supravital dye, into lysosomes. Cells were seeded in 96-well microtitre plates at a
205 density of 75000 cells/well in 100 µL of medium, and maintained for 48 hours to allow
206 adherence to the wells. Growth medium was removed and 100 µL of BF were added to
207 the cells. BF were previously diluted with FBS-free DMEM, and the following ratios
208 (BF:DMEM, v/v) were assayed: 1:1, 1:2 and 1:3. The control wells received FBS-free
209 DMEM. Caco-2 cells were harvested after 2 h exposure, and cell viability was measured
210 by staining with NR (2 h at 37°C), followed by cell fixation (0.5 % formaldehyde, 0.1
211 % $CaCl_2$ for 30 seconds at room temperature). Microtitre plates were washed by three
212 brief immersions in phosphate-buffered saline and the cells were lysed (50 % ethanol, 1
213 % acetic acid overnight at 4 ºC). The optical densities of the resulting solutions were
214 measured at 550 nm using a BioRad Model 550 microplate reader (BioRad, CA, USA).
215 Cell viability results were expressed as a percentage from the data obtained after
216 incubation with complete DMEM, from at least two independent experiments ($n \geq 5$ per
217 experiment).
218
219 **2.9.2. Reactive oxygen species generation**
220 ROS generation was determined by the dichlorofluorescein (DCF) assay described by
221 Goya et al.,[20] with modifications. Cells were seeded in 24-well multiwell plates at a

density of 2×10^5 cells/well in 1 mL of medium, and incubated at 37°C for 48 hours. The cells were pretreated with 1 mL of the BF from the oil digests and incubated for 2 hours. The control wells received FBS-free DMEM. The medium was then discarded and the cells were treated with DCFH 100 µM and incubated for 1 hour. The DCFH was removed and culture medium (for basal measurements) or the oxidising agent *tert*-butyl hydroperoxide (*t*-BOOH) 5 mM (to study the protective effect against oxidation) were added to the wells. The absorbance was immediately measured in the plate reader at a wavelength of 485 nm excitation and 535 nm emission, at a constant temperature of 37 ° C at 90 minutes. DCFH is converted into dichlorofluorescein (DCH) in the presence of ROS, and emits fluorescence.

2.10. Statistical analysis

All data are presented as the means of at least three independent experiments and in each experiment at least three replicates of each variety were obtained (n=6). Data were analysed using two-way ANOVA, with brand and variety as the main factors. As the differences between the brands were quite small and the brand effect was seldom significant, the data were re-analysed by one-way ANOVA with variety as the main factor. Statistical significance was assessed using Tukey's honest significant difference test. A probability of 5% or less was accepted as statistically significant. The relationships between the different variables were evaluated by computing the relevant correlation coefficient (Pearson linear correlation). All statistical calculations were carried out using SPSS version 21.0 (IBM Corporation, New York, USA).

To reduce the variables and explore the results, a preliminary multivariate factor analysis was performed, including all the parameters measured (antioxidant capacity, TPC from the chemical extracts and after *in vitro* digestion, cell viability and ROS

generation) using StatGraphics Centurion XV software (StatPoint Technologies, Inc. USA, 2006). We observed that the *ex vivo* values (viability and ROS) had a weak effect as regards differentiating the varieties. Therefore, we applied a multifactorial analysis using principal component analysis (PCA) for the antioxidant capacity and TPC obtained from the chemical extracts and after *in vitro* digestion. A Varimax rotation was used to facilitate the analysis.

3. Results and discussion

3.1. TPC and antioxidant capacity

The differentiation of oils based on polyphenols and antioxidant activity is a complex task, since these parameters depend on a number of variables related to olives production and extraction technology. Since this information is not available for consumers in commercial samples, only the variety has been taken into account for the oils differentiation in the present assay.

Figure 2 presents the data for TPC. In decreasing order, the TPC in the chemical extracts was as follows (mg CAE/kg): Cornicabra (317) > Picual (256) > Manzanilla (234) > Picudo (207) > Hojiblanca (169) > Arbequina (153). Significant differences ($p<0.05$) between these monovarietal oils were observed. TPC from extracts of the Cornicabra variety was higher than all the other varieties, except Picual.

The composition and concentration of phenolic compounds in EVOO vary widely, being dependent on environmental factors (soil, climate), agronomic factors (irrigation, fertilisation), cultivation (harvesting, ripeness) and technological questions (post-harvest storage and extraction system), among other aspects. For the chemical extracts, the method of extraction, concentration and polarity of the reactives used is also important. Furthermore, EVOO contain about 36 structurally different phenolic compounds, whose

total concentrations range from 0.02 to 600 mg/kg.[5,31] Thus, a very large range of TPC has been reported in olive oils.

An earlier study reported higher TPC than those found in the present study for Picual, Hojiblanca and Picudo varieties (483, 247 and 243 mg CAE/kg, respectively).[5] Similar results to ours have been found in EVOO from Extremadura at different stages of maturation, with respect to Manzanilla (200-700 mg CAE/kg) and Arbequina (160-409 mg CAE/kg), although higher values were shown for Picual (419-670).[32] Salvador et al.,[33] reported lower values for commercial Cornicabra virgin olive oil obtained by a different extraction system from that used in the present study. It has been reported that Cornicabra and Picual are the Spanish varieties of EVOO with the highest TPC[34], which is in accordance with the results observed in our study.

The *in vitro* digestion process increased the TPC in all the samples (Figure 2). The highest values of bioaccessible phenol content of the oils (mg CAE/kg) were observed in Cornicabra (891), Picudo (764), Hojiblanca (689) and Manzanilla (685) varieties, whereas Picual (630) and Arbequina (613) had the lowest values, which were significantly different from those of the Cornicabra.

Digestive factors are among the most important ones affecting phenol bioavailability and conflicting results have been reported for TPC after digestion of the oils. Some authors suggest that only a minor fraction of the phenols in olive oil can be considered bioaccessible[15] while others believe that a large fraction is bioavailable.[31] In consequence, different studies have reported either increased or decreased phenol contents after the digestion process.[14,18,19] The different phenolic profile of the EVOO varieties[5] is probably responsible for the variations observed between oils after the digestion process of the oils, since, depending on their chemical structure, polyphenols are hydrolysed by the intestinal enzymes in a different way and also undergo different

297 structural modifications due to the conjugation process.[31] Thus, the effect of the
298 digestive process on TPC varied from a 2.5 fold increase for the Picual variety to a 4.1
299 fold for Hojiblanca. A significant positive correlation ($r= 0.3714$; $p<0.05$) was observed
300 between TPC before and after *in vitro* digestion, suggesting that TPC in chemical
301 extracts could be indicative of TPC post-digestion.

302 Table 1 shows the antioxidant activity of monovarietal olive oils obtained by
303 chemical extraction and *in vitro* digestion assessed by ABTS, DPPH and FRAP assays.
304 In the chemical extracts, the Picual variety showed significantly higher values of ABTS
305 activity compared with all the other varieties studied. Cornicabra, Manzanilla and
306 Picudo presented the highest DPPH values, whereas Picual had the highest reducing
307 power, although differences with the other varieties were not always significant.

308 The behaviour of the chemical extracts obtained from Arbequina and Hojiblanca
309 was similar, with a lower phenol content level and lower antioxidant activity. Moreover,
310 significant correlations were found in the chemical extracts between phenol content and
311 antioxidant activity, measured by different methods ($r=0.574$ ABTS; $r= 0.416$ DPPH; $r=$
312 0.631 FRAP; $p<0.01$), which supports the view that phenolic compounds may be the
313 main responsible for the antioxidant activity of the samples, as has been suggested
314 previously.[13,15]

315 In the bioaccessible fraction, ABTS values differed between Arbequina (the
316 highest) and Cornicabra (the lowest), which in turn were similar to the other varieties.
317 Significant differences were also found for the capacity to scavenge the DPPH radical,
318 but in the opposite sense, i.e., Arbequina showed the lowest value and Cornicabra the
319 highest one. No differences were found for FRAP values after *in vitro* digestion of the
320 samples. The antioxidant activity of the samples increased after the digestion process,
321 with greater values of ABTS, DPPH and FRAP from 2 to 6 fold being observed in the

322 BF compared with the chemical extracts. Therefore, enzymatic modifications during the
323 digestion of oils produce derivatives which are still bioactive, thus maintaining or even
324 enhancing the antioxidant activity of the resulting compounds. It is widely accepted that
325 polyphenols are affected by digestive modifications; the mechanisms by which
326 glycosides may be hydrolysed in the small intestine, as well as other changes caused in
327 the conjugation process, could strongly affect the biological and antioxidant activity of
328 polyphenols.[31] In the present study bioaccessible polyphenols were positively correlated
329 with DPPH ($r=0.530$, $p<0.001$) and FRAP ($r=0.713$, $p<0.001$) assays, but not with
330 ABTS values after digestion. Thus, minor components of oils other than polyphenols
331 should also be considered as contributors to antioxidant properties after digestion.

332 Few data have been reported in the literature about the stability of antioxidant
333 properties of oils during the digestion process. In this respect, Dinella et al, [15] in a study
334 of Italian EVOO, found a negative effect of the *in vitro* digestion followed by dialysis
335 on the antioxidant activity determined by the ABTS procedure. On the other hand, Soler
336 et al,[16] measured the individual phenolic compounds in oil digesta and aqueous micellar
337 phases and observed good stability of the major compounds, especially hydroxityrosol
338 and tyrosol, under gastric and intestinal conditions. Taking into account that these
339 polyphenols have been associated with a high level of antioxidant activity in oils, we
340 hypothesise that their stability may contribute to increasing antioxidant activity during
341 digestion.

342 The antioxidant response of foods during the digestion process has been studied in
343 fruits[18] and in different food matrices.[30] In general, increased activity, measured by
344 ABTS, DPPH and FRAP, has been recorded after the *in vitro* digestion of foods,
345 compared with solvent extraction procedures, and this method has been proposed as a
346 more physiological approach to determining the real antioxidant capacity of foods.[30] We

consider that the in vitro digestion procedure may contribute to a better knowledge of the real bioactive power of olive oils from a nutritional standpoint. To the best of our knowledge, no previous data have been obtained about the effect of *in vitro* digestion on the content of phenols or on antioxidant activity in Spanish monovarietal olive oils.

3.2. Cell culture assays.

In addition to measuring the antioxidant properties of oil digests by *in vitro* methods, we examined the antioxidant effects of the BF at the cellular level, as a main requirement to be a potential *in vivo* antioxidant.

3.2.1. Viability

The bioaccessible fractions were mixed with increasing proportions of FSB-free DMEM in order to evaluate the effect of the sample concentration on the viability of Caco-2 cells. Figure 3 shows the viability, expressed as a percentage with respect to the control value, after 2 hours of incubation with dilutions 1:1; 1:2 and 1:3 (v/v) of the samples.

Severe cell damage was observed after the incubation at the minimum dilution (1:1) of all samples (only 23-40% of viable cells), with the sole exception of the Picual variety, which had cell viability values (87%) that were significantly higher ($p<0.05$) than those of all the other varieties. When the dilution of the BF was increased to 1:2, a parallel increase in the proportion of viable cells was observed, reaching values ranging from 56% (Picudo) to 105% (Picual). Finally, cell viability after exposure to samples diluted 1:3 presented values always ≥80%. Thus, this dilution was selected to study effects on ROS generation. The results showed that the Picual variety had a more beneficial effect on cell viability than the other varieties studied.

372

373 **3.2.2. ROS generation**

374 ROS production can induce oxidative stress, leading to cell damage that can culminate
375 in cell death. This damage is linked to the onset of many degenerative diseases,
376 including cancer, cardiovascular disease, cataracts and aging. Antioxidants can attenuate
377 the damaging effects of ROS and delay many events that contribute to cellular aging.[35]

378 In basal conditions, the incubation for 2h of cells with the BF of all the varieties led
379 to a significant reduction in ROS generation compared with the control cells,
380 numerically greater after incubation with the Picual variety, but with no significant
381 differences between the samples (Figure 4A). In this respect, it has been shown that
382 when Caco-2 cells are incubated with hydroxytyrosol, one of the main phenolic
383 compounds from virgin olive oil, ROS generation is reduced.[36]. This supports the idea
384 that the antioxidant properties of initial phenolic compounds may be retained during the
385 digestive process.

386 In order to induce oxidative stress, differentiated Caco-2 cells were treated with
387 5 mM *t*-BOOH for 2 h. The damage produced by *t*-BOOH provoked an increase in ROS
388 generation in the cells and thus enabled us to estimate the protective effect of
389 monovarietal EVOO (Figure 4B). When the cells were pretreated with the BF of the
390 oils, a significant protective effect was observed, since the high ROS production
391 observed in cells stressed with *t*-BOOH was neutralised to a notable extent. Reductions
392 in ROS levels were in the range 17-55 %, and differences with oxidised cells were
393 always significant except with the Picudo variety (17% reduction in ROS). Incubation
394 with the Cornicabra variety produced the strongest protective effect against induced
395 oxidative stress, although the differences with Picual, Manzanilla, Hojiblanca and
396 Arbequina did not reach significance. These findings confirm the positive effect of the

digested olive oil at the cellular level, which may be an additional reason in assessing the health benefits of the varieties assayed. Previous research has demonstrated the beneficial effects of specific phenol compounds from olive oils on oxidation in cell models[20-24], which has been related to the reduced production of free radicals or to an enhancement of enzyme antioxidant defences.[11,20,21,24,25,36]

There is scarce information about the antioxidant effects of digested foods in cell oxidative markers[37], and no data have been reported concerning the effects of the different Spanish monovarietal olive oils. The findings of the present study show that all the EVOO varieties analyzed in the present study had a similar positive effect on reducing basal ROS generation and, moreover, the Arbequina, Cornicabra, Hojiblanca, Manzanilla and Picual varieties were able of preventing against an induced oxidative stress.

3.3. Multivariate factor analysis

A multivariate factor analysis using PCA was performed using data obtained from the chemical extracts and the BF, concerning TPC and antioxidant activity. The aim of this approach was to reduce the number of variables into a small number of factors and thus explore the global differences between the monovarietal olive oils tested, according with the variables analysed. Figure 5 shows the vector arrows of the variables used to perform the PCA (A) and the graphic distribution of the different EVOO varieties (B). The two main factors obtained explained 66.16% of the total variance (factor 1, 21.32%; factor 2, 44.84%). Factor 1 was mainly contributed by the determinations carried out after *in vitro* digestion (Figure 5, A), and the variables with the greatest influence were PFT, FRAP and DPPH (loadings of 0.9084, 0.8029 and 0.7361, respectively). The variables with the greatest loadings for factor 2 were those performed in the chemical

extracts (FRAP 0.8950, ABTS 0.8726, PFT 0.7618). Taking into account factor 1, we observed a separation of the Cornicabra variety from the other samples (Figure 5, B), which may be related to its particular values of TPC and antioxidant activity after digestion. On the other hand, factor 2 showed a different behaviour in the Picual, Cornicabra and Manzanilla varieties compared to Arbequina, Hojiblanca and Picudo, probably due to the different antioxidant activity in the chemical extracts among the two groups of oils. Therefore, both chemical extraction and *in vitro* digestion should be considered in the characterisation of different monovarietal olive oils.

4. Conclusions

In our study, the antioxidant activity after in vitro digestion of six monovarietal extra virgin olive oils was reported, as well as their protective effect against induced oxidative stress at the cell level. The findings show that the digestion process should be taken into account to evaluate the release of the antioxidant compounds from oil matrix, as the results in the bioaccessible fractions may largely differ from those of chemical extracts. After digestion, the tested monovarietal olive oils present different capacities for preserving cellular integrity, but all of them show promising protecting activities against free radical generation.

Conflict of interest

The authors declare that they have no conflict of interest.

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ABBREVIATIONS USED

ABTS, 2,2-azinobis-(3 ethylbensothiazoline)-6-sulfonic acid; BF, Bioaccessible fraction; CAE, caffeic acid equivalents; DMEM, Dulbecco's modified minimal essential medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EVOO, Extra virgin olive oils; FBS, fetal bovine serum; FRAP, Ferric reducing antioxidant power; PCA, principal component analysis; *t*-BOOH, tert-butyl hydroperoxide; TPC, Total phenolic contain; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

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526

527 **Figure captions**

528

529 **Figure 1.** Scheme of the general procedure applied on the samples.

530 **Figure 2.** Total phenolic contain (TPC) from chemical extracts and bioaccessible
531 fractions of monovarietal EVOO. Values are expressed as mean ± SE of mg of caffeic
532 acid per kilogram of sample (n = 6). Equal bars with different letters differ ($P < 0.05$).

533 **Figure 3.** Cell viability (%) after 2h of incubation of Caco-2 cells with the BF of
534 monovarietal EVOO. BF were diluted with FBS-free DMEM at 1:1, 1:2 and 1:2 (v/v).
535 Values are expressed as mean ± SE (n = 6). Bars with different letters within each
536 dilution differ ($P < 0.05$).

537 **Figure 4.** ROS generation in Caco-2 cells expressed as fluorescence intensity ($\times 10^3$).
538 Data are means ± SE (n=6). A: basal effect after 2 h of incubation with the BF of
539 monovarietal EVOO. B: protective effect against oxidation with 5mM *t*-BOOH. Bars
540 with different letters differ ($P < 0.05$).

541 **Figure 5.** Representation of the two main factors of the PCA, considering TPC and
542 antioxidant parameters from chemical extracts and BF of monovarietal olive oils (n =
543 36). A: vector arrows of the variables used to perform the PCA. B: distribution of the
544 different oils.

Table 1 Antioxidant activity from chemical extracts and bioaccessible fractions (BF) of the different EVOO varieties measured by ABTS, DPPH and FRAP assays (mmol Trolox equivalents/kg oil)[a]

	ABTS		DPPH		FRAP	
Variety	Chemical extracts	BF	Chemical extracts	BF	Chemical extracts	BF
Arbequina	2.09 ± 0.13 b	4.08 ± 0.73 b	0.44 ± 0.12 a,b,c	0.84 ± 0.39 a	0.68 ± 0.09 a	3.76 ± 0.62 a
Cornicabra	2.62 ± 0.32 c	3.52 ± 0.11 a	0.60 ± 0.13 b,c	2.51 ± 0.90 c	0.94 ± 0.95 a,b,c	4.47 ± 0.73 a
Hojiblanca	1.34 ± 0.32 a	3.75 ± 0.77 a,b	0.36 ± 0.13 a	1.08 ± 0.56 a,b	0.75 ± 0.10 a,b	3.99 ± 0.26 a
Manzanilla	2.83 ± 0.30 c	3.74 ± 0.08 a,b	0.60 ± 0.09 b,c	1.95 ± 0.24 b,c	1.00 ± 0.10 b,c	4.17 ± 0.17 a
Picual	3.46 ± 0.05 d	3.73 ± 0.08 a,b	0.40 ± 0.13 a,b,c	0.95 ± 0.24 a	1.15 ± 0.08 c	3.76 ± 0.49 a
Picudo	2.5 ± 0.44 b,c	3.68 ± 0.06 a,b	0.62 ± 0.13 c	1.55 ± 0.30 a,b	1.00 ± 0.36 b,c	4.67 ± 0.81 a

[a] Values are expressed as mean ± SE (n = 6). Means values in each column with different letters are significantly different ($P < 0.05$).

Fig.1.

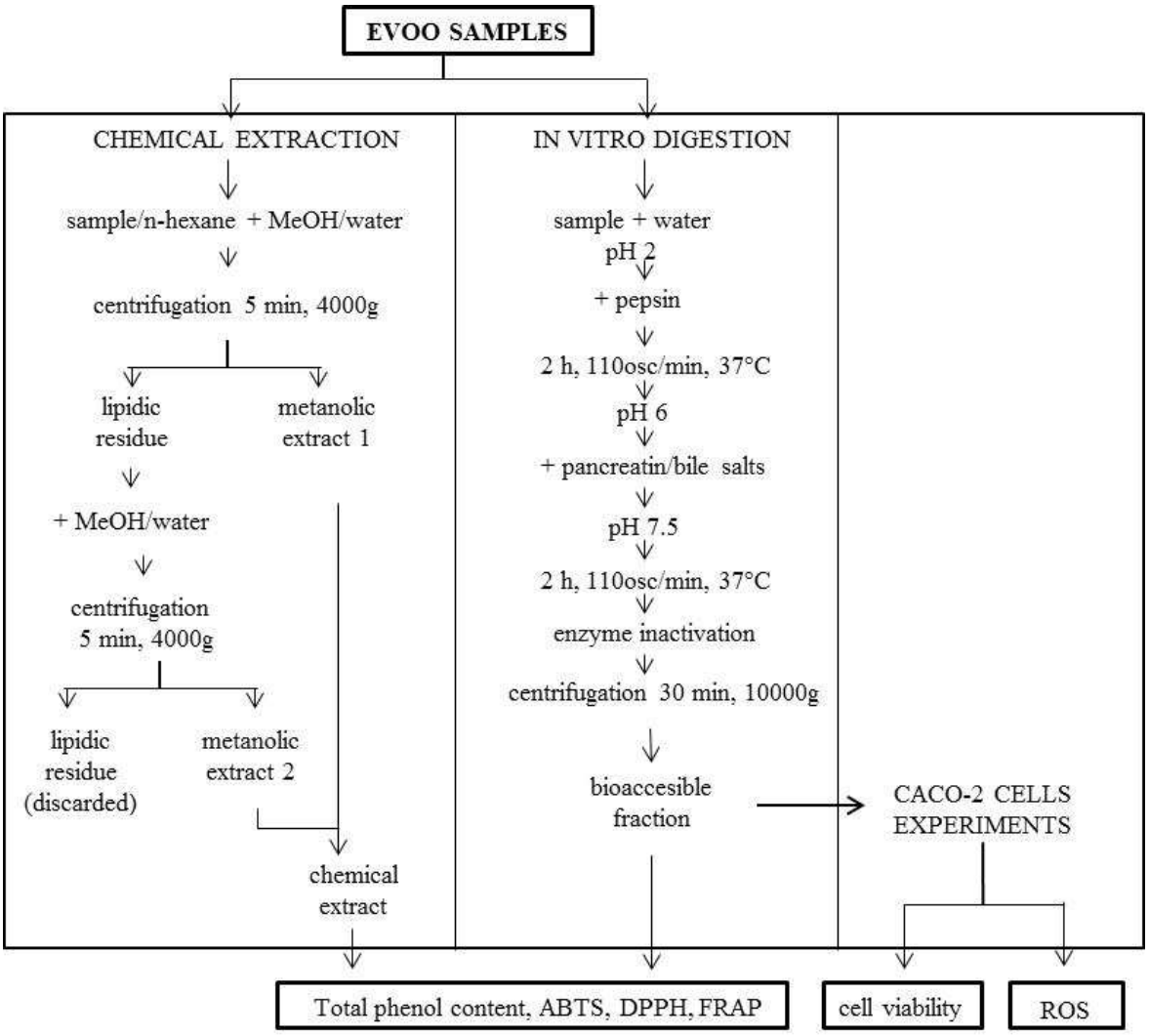
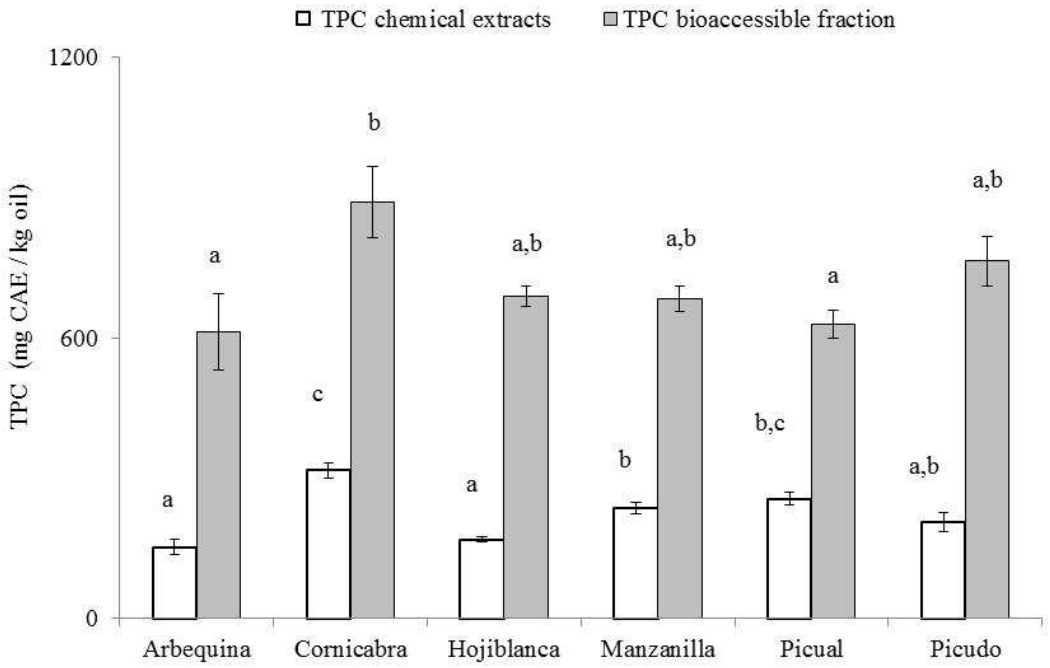


Fig. 2



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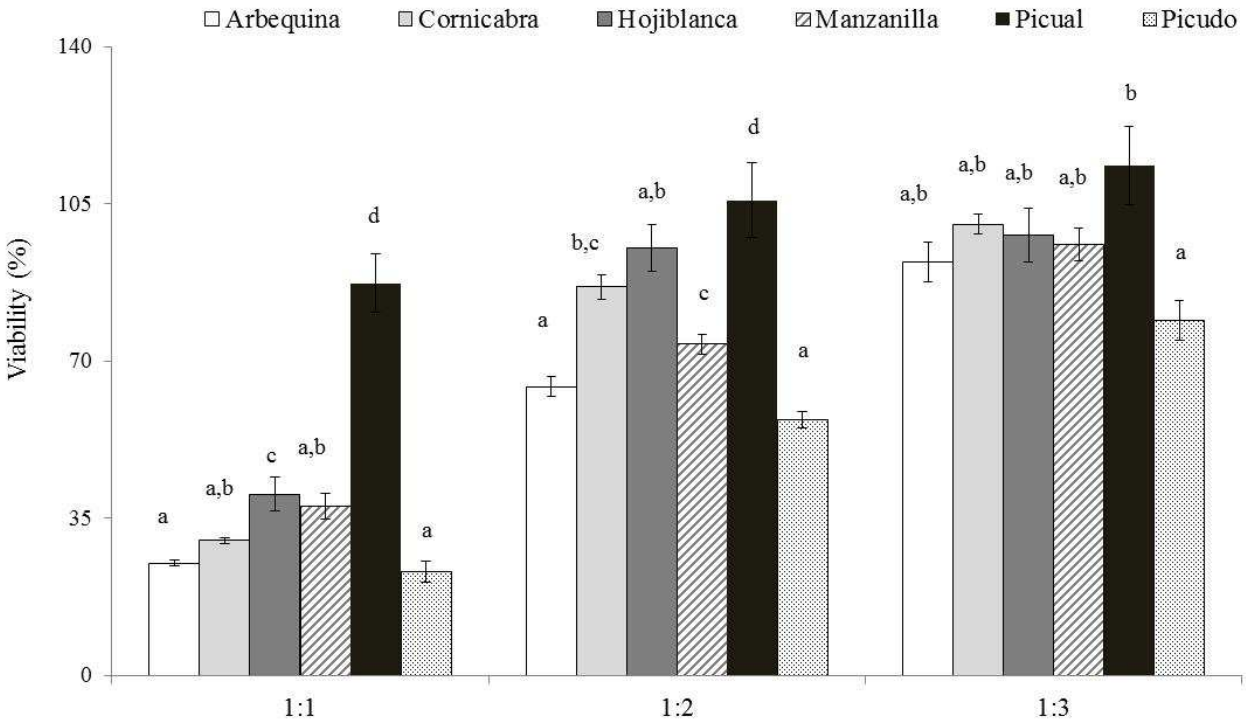

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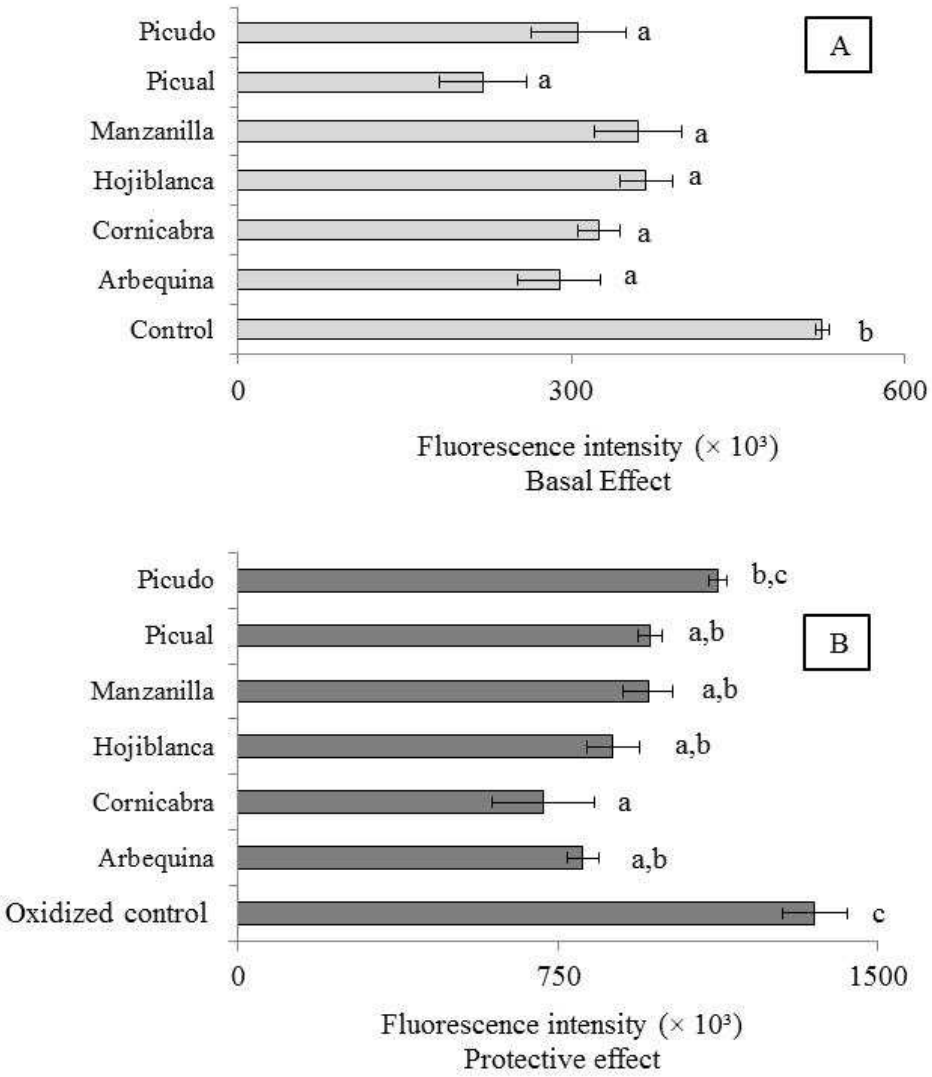
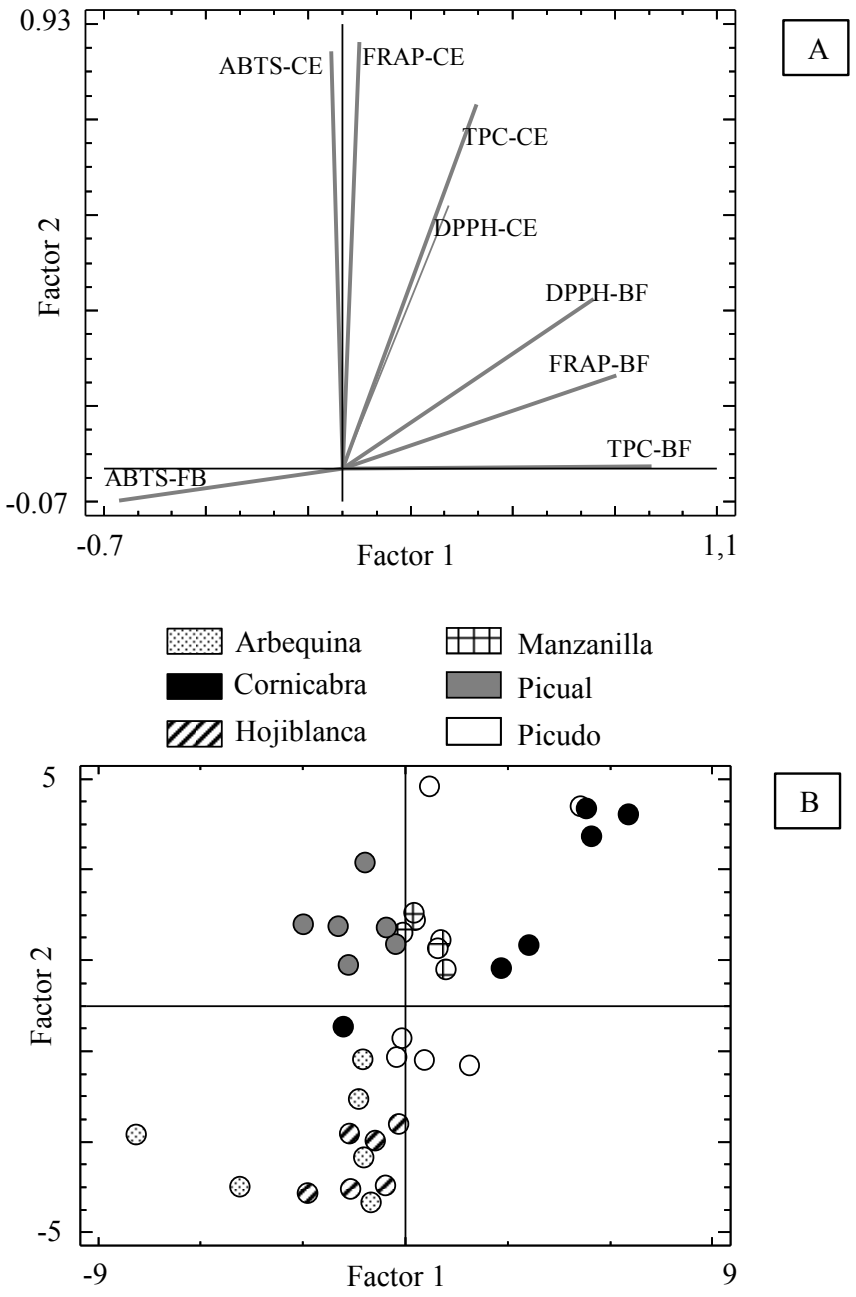
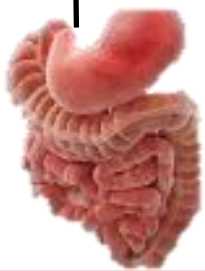


Fig. 5



Monovarietal Olive oils

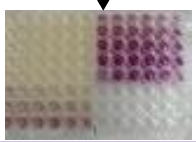
Chemical extraction



In vitro digestion

Caco-2 cells assays

Free radical generation



Antioxidant capacity