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Study of the antioxidant potential of Arbequina extra virgin olive oils from Brazil and Spain applying combined models of simulated digestion and cell culture markers

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ABSTRACT

A physiological approach to assessing the antioxidant potential of Arbequina EVOO from different zones of Brazil and Spain was performed, applying a combined model of simulated digestion and cell cultures, using the Caco-2 cell line. Our results showed an increasing of total phenolic content (TPC) and antioxidant properties promoted by the *in vitro* digestion. Preincubating Caco-2 cells with bioaccessible fractions of oils counteracted the cytotoxic effect promoted by an oxidising agent (*t*-BOOH), preserving cell viability and reducing the generation of reactive oxygen species (ROS). The protective effect on ROS production was associated with the antioxidant activity (DPPH assay), but no relation with the TPC of the digested samples was found. Differences in the parameters evaluated were observed among the samples, which were related to climatic characteristics of the production zones. It was concluded that transformations during the digestive process are important for establishing the antioxidant potential of the oils.

1. Introduction

Extra virgin olive oil (EVOO) consumption has been intensely associated with multiple healthy properties, such as prevention of coronary and degenerative disease (Chiesi, Fernandez-Blanco, Cossignani, Font, & Ruiz, 2015; Servili et al., 2014). These beneficial effects have been mainly attributed to polyphenols, which are powerful antioxidants, and also possess other potent biological activities such as anti-inflammatory, anti-cancer, antimicrobial, antiviral, anti-atherogenic or hypoglycemic (Barbaro et al., 2014). Special attention has received the study of the antioxidants of EVOO, due to their importance from health, biological and sensory points of view (Servili et al., 2014).

Several studies have been focused on the characterisation of phenolic content and antioxidant capacity of monovarietal olive oils of different areas from the Mediterranean basin and, more recently, from newly introduced varieties worldwide (Bouarroudj, Tamendjari, & Larbat, 2016; Dabbou et al., 2010; Köseoğlu, Sevim, & Kadiroğlu, 2016; Uluata, Altuntaş, & Özçelik, 2016; Xiang et al., 2016). In this regard, Brazil has beginning to cultivate olives and has shown a huge potential to produce olive oil. Currently, 70% of Brazilian olive oil production is from Arbequina cultivar (Olive Oil Times. Brazil looks for its own Olive, 2017); however, information on the antioxidant power of Brazilian virgin olive oil is still lacking.

Antioxidant properties of oils have been traditionally studied in chemical extracts by applying assays to evaluate radical scavenging capacity (DPPH and ABTS) and ferric-reducing power (FRAP) (Kalogeropoulos, Kaliora, Artemiou, & Giogios, 2014; Samaniego Sanchez et al., 2007). Nevertheless, the use of extraction procedures using organic solvents may not be a real physiological approach to measure the potential *in vivo* effects of the oils, owing

Abbreviations: ABTS, 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid; BF, bioaccessible fraction; DMEM, Dulbecco's modified minimal essential medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EVOO, extra virgin olive oil; FRAP, ferric reducing power; FBS, heat-inactivated fetal bovine serum; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; RF, residual fraction; ROS, reactive oxygen species; *t*-BOOH, tert –butylhydroperoxide; TPC, total polyphenol content.

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to that the first requirement for a dietary compound to exert a biological activity is the maintenance of its properties after the digestion process (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011). This statement implies that bioactive compounds must be bioaccessible, i.e., released from the food matrix during digestion and, thus, available to be absorbed by the intestinal cells (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). The *in vitro* static methods simulating gastrointestinal digestion allow mimicking physiological conditions *in vivo*, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time and salt concentrations, among other factors (Minekus et al., 2014). In addition, coupled to cell line models enable studies of antioxidant cell markers and intestinal absorption, elucidating the potential impact of these compounds on human health (Borges, Cabrera-Vique, & Seiquer, 2015; Soler et al., 2010). Previously, our research group has applied successfully Caco-2 cell lines as a model to study absorption and metabolism in small intestine of minerals and trace elements, products of Maillard reaction and phenolic compounds from various foodstuff, including EVOO (Mesías, Seiquer, & Navarro, 2009; Rueda, Cantarero, Seiquer, Cabrera-Vique, & Olalla, 2017; Ruiz-Roca, Navarro, & Seiquer, 2008; Seiquer, Rueda, Olalla, & Cabrera-Vique, 2015).

Antioxidant and chemoprotective properties of individual components of olive oil extracts and olive byproducts have been reported in cell cultures such as Caco-2, HepG2 and red blood cells, as well as the protective effects against induced oxidative stress (Chiesi et al., 2015; Deiana et al., 2010; Incani et al., 2016; Paiva-Martins et al., 2015; Rubió et al., 2014). However, there is scarce information concerning the potential antioxidant effect of all complex bioactive components of EVOO and their metabolites after the digestive process, or about the subsequent intestinal absorption.

The influence of different factors on the antioxidant properties of EVOO has been well-documented, such as cultivar, storage period, stages of olives ripening and technologic aspects of oil production (Köseoğlu et al., 2016; Uluata et al., 2016). However, up to the date, there are limited data regarding the antioxidant activity of monovarietal EVOO from different geographical areas, after the biotransformation underwent during gastrointestinal digestion. In a previous paper we have shown that some characteristics of Arbequina EVOO, such as oxidative stability or colour, are related with geographic and climatic factors of the production zones (Borges et al., 2017). Thus, it is possible that antioxidant properties of oils could also be influenced by geoclimatic conditions.

Therefore, the aim of the current study was to perform a physiological approach of the antioxidant potential of Arbequina EVOO from different Brazilian and Spanish producing regions. With this purpose, a combined model of *in vitro* digestion and cell cultures was applied. The antioxidant activities of oils after digestion have been studied by *in vitro* assays (DPPH, ABTS and FRAP) and cell antioxidant markers (cell proliferation and reactive oxygen species production), testing, moreover, the protective effect against an induced oxidative stress. Finally, the absorption of polyphenols and antioxidant activity across Caco-2 monolayers was also performed.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical reagent grade or high purity. Bidistilled deionized water was obtained from a Milli-Q purification system (Millipore, Bedford, MA). Folin –Ciocalteau reagent, 6 -hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pepsin, pancreatin, bile salts, HEPES and tert -butylhydroperoxide (*t*-BOOH), as were all cell culture media and cell culture-grade chemicals were provided by Sigma (Sigma–Aldrich, St. Louis, MO). 2,2-azinobis-(3-ethylben sothiazoline)-6-sulfonic acid (ABTS) was obtained from Amresco (Solon, USA). Ethanol and methanol were purchased from VWR (Barcelona, Spain). Sodium bicarbonate, acetate sodium, sodium carbonate, hydrochloric acid (37%), caffeic acid, hydrochloric acid, anhydrous sodium carbonate and potassium hexacyanoferrate(III) were obtained from Merck (Darmstad, Germany). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) and iron (III) chloride for the ferric reducing power (FRAP) method was obtained from Fluka Chemicals (Fluka Chemicals, Madrid, Spain).

2.2. Samples

A total of 33 samples of Arbequina EVOO from 11 different geographic zones (n = 3 from each producing region) were studied. Nine regions of olive oil production in Spain (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid and Lérida, samples 1–9) and two regions in Brazil (Rio Grande do Sul and Minas Gerais, samples 10 and 11) were selected. The olives were harvested always at the early stage of harvest; the harvest dates were: late October to mid-November of 2014 for Spanish samples and March to early April of 2015 for Brazilian samples. The oil was extracted within 24 h, under a two-phase extraction system. The oils were directly donated by the producers, adequately packaged for preserving from light and high temperatures and sent to CSIC laboratories (Granada, Spain) to perform the analysis. Information on the geographic coordinates and geo climate characteristics of the different locations of the oils is shown as supplementary material (S1). All the samples were according with the normative established by the European Union regulation n° 2568/91 for extra virgin olive oil, as was showed previously (Borges et al., 2017). The scheme of the general procedure applied on the EVOO samples in the present assay can be observed in Fig. 1.

All the determinations were done in triplicate, unless otherwise stated.

2.3. In vitro digestion

The *in vitro* digestion was performed as described by Borges et al. (2015), including sequential steps of gastric and intestinal digestion. Briefly, the oil samples were mixed with Mili-Q water (1:10, w/v) and subjected to gastric (pH 2, pepsin solution) and intestinal (pH 7, pancreatin/bile salts solution) phases (110 oscillations/min; 37 °C, 2 h). The samples were then centrifuged at 10,000 rpm for 30 min at 4 °C (Sorvall RC 6 Plus centrifuge) to separate the soluble or bioaccessible fraction (BF) and the residual fraction (RF). The samples were protected from light all over the process and submitted to sonication previous to each step. Blanks with no sample were run in parallel and analysed to discard interferences due to the reagents of the digestion process.

The BF and RF were stored at −80 °C in bottles protected from the light under a nitrogen blanket until used to determine the total polyphenol content (TPC) and antioxidant activity. Aliquots of the BF were also used for Caco-2 cell experiments.

2.4. Chemical extraction

The chemical extraction was carried out previous to analysis of TPC and antioxidant assays, in oil samples and in the RF obtained after the *in vitro* digestion. Two grams of oil, or the total RF, were mixed with 1 mL of *n*-hexane and the mixture was vigorously shaken. 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,

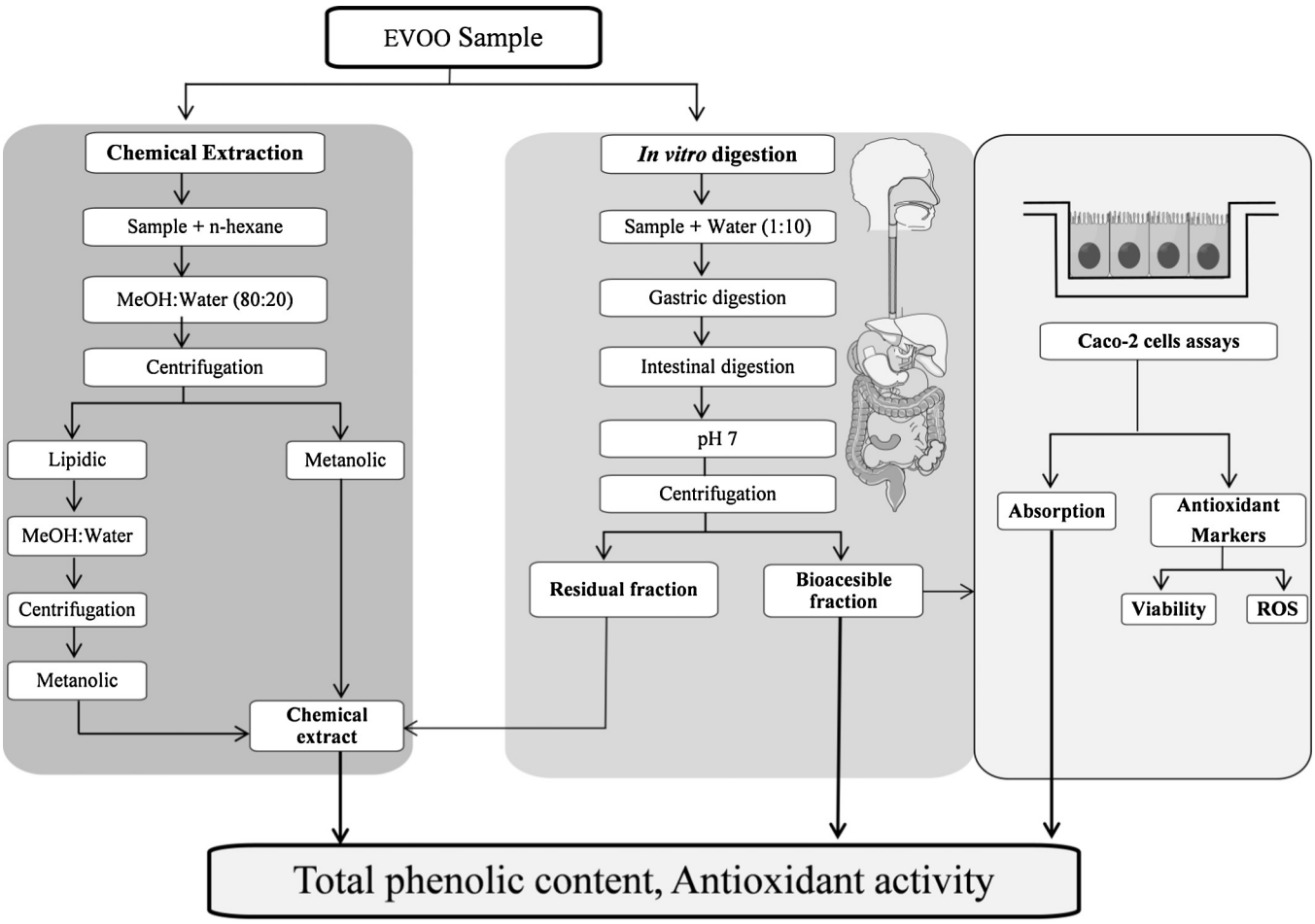

Fig. 1. Scheme of the general procedure applied to the EVOO samples.

Madrid, Spain), the extraction was repeated in the lipophilic part and the methanolic extracts were combined to obtain the chemical extract.

2.5. Total phenolic content and antioxidant activity

The methodologies previously described by Borges et al. (2015) were followed. TPC was determined using the Folin – Ciocalteu reactive and measuring the absorbance at 750 nm in a Victor X3 multilabel plate reader (Waltham, Massachusetts, USA). The results were expressed in mg of caffeic acid equivalents (CAE) per kg of sample.

To study the antioxidant capacity of the samples the ABTS and DPPH assays (for measuring the free radical scavenger activity) and the FRAP method (for assessing the reducing power) were performed. The final absorbance was measured at 750, 520 and 595 nm, for ABTS, DPPH and FRAP assays, respectively, using the Victor multilabel plate reader. A calibration curve of Trolox was performed in each method and the results were expressed in mM of Trolox equivalents per kg of sample.

2.6. Cell culture assays

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University (Spain). Culture flasks 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 atmosphere of air/CO_2 (95:5) at 90% humidity and 37 °C and given fresh medium every 3 days.

2.6.1. Antioxidant cell markers

The antioxidant potential of the digested oils (BF) was also assessed at the cell level, performing two assays: modifications on cell proliferation and reactive oxygen species (ROS) generation. Determinations were carried out both at basal conditions and against an induced oxidative stress.

The viability and quantification of cell proliferation of the Caco-2 cells was assessed by the colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Roche, Mannheim, Germany). Cells were seeded in 96-well microtiter plates at a density of 25×10^4 cells/mL (100 μL/well) and incubated for 24 h at 37 °C to allow adherence. Basal effect of the samples on cell viability was studied at different concentrations BF: DMEM FBS-free (1:2 and 1:3, v/v). Spent medium was removed and the diluted samples (100 μL/well) were added to the cells and incubated during 2 h. The control wells received DMEM FBS-free. Immediately, 10 μL of MTT were incorporated followed by 4 h of incubation. Finally, 100 μL of solubilisation solution were added to each well and the plates were stood overnight in incubation, prior to measure cell viability. To study the protective effect against oxidative damage, after 2 h of initial exposure with the diluted BF, samples

solutions were removed and 100 μL of tert-butyl hydroperoxide (*t*-BOOH) at different concentrations (3 mM and 5 mM) were added as oxidising agent, and incubated for 1 h. Then, the same procedure as above was applied. After overnight incubation, the plates were mixed during 1 h in a plate stirrer until complete solubilisation of the purple formazan crystals and the absorbance was measured at 595 nm using a Victor X3 multilabel plate reader. The cell viability was expressed as a percentage of the data obtained from control wells. Data are means from at least two independent experiments (n = 6 per experiment).

For determination of ROS generation we used the dichlorofluorescin (DCFH) assay as described by Seiquer et al. (2015). In presence of free radicals (ROS), the DCFH become dichlorofluorescein (DCF) and emit fluorescence; thus, quantifying fluorescence under different situations (basal conditions and oxidising conditions with *t*-BOOH) the ROS production may be estimated. The data were expressed as fluorescence units, from at least two independent experiments (n = 3 per experiment).

2.6.2. Absorption assays

Trypsinization, seeding into permeable supports (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size, Costar) and absorption assays across Caco-2 cell monolayers were performed as described elsewhere (Seiquer et al., 2015). BF were used for the Caco-2 experiments diluted 1:3 (v:v) with DMEM FBS-free, since cell viability after 2 h of exposure to the samples in such conditions, assessed by MTT, was never <90%. After the incubation period, the buffer from the basolateral chamber was removed and used to determinate total polyphenols and antioxidant activity (DPPH and FRAP), according to the methods described. Absorption across the cell monolayers was expressed as the percentage (%) transported/well from the initial solution added to the apical chamber.

2.7. Statistical analysis

The data obtained were analysed applying analysis of variance (one-way ANOVA), with the geographic origin of oils (regions 1–11) as the main factor. Tukey's test was used to compare mean values between oils from the different regions, and differences were established at $P < 0.05$. Moreover, the data were grouped by countries, and the overall differences between Brazilian and Spanish oils were also studied by ANOVA. The relationships between the different variables and with the climate characteristics and the altitude of the producing regions were evaluated by Pearson's coefficient. All statistical calculations were carried out using SPSS version 21.0 (IBM Corporation, New York, USA).

3. Results and discussion

3.1. TPC and antioxidant capacity before and after gastrointestinal digestion

Total phenolic content and antioxidant activity of oils were measured before and after the *in vitro* digestion, i.e. in the chemical extracts and in the fractions obtained from the digestive process, both bioaccessible and residual (BF and RF).

3.1.1. Chemical extracts

Results of TPC and antioxidant activity (ABTS, DPPH and FRAP) in chemical extracts are shown in Table 1. TPC of the Arbequina EVOO analysed presented a large range of variation between 75 and 302 mg CAE/kg oil, concerning samples 11 (Minas Gerais, Brazil) and 3 (Málaga, Spain) respectively ($P < 0.05$). Likewise, a wide range of phenolic compound content in EVOO (from 50 to more than 1000 mg/kg oil) and different chemical structures have been reported in literature, linked to numerous factors such as cultivar, geo-climatic conditions, degree of fruit ripeness and processing methods (Samaniego Sanchez et al., 2007; Servili et al., 2014). Previous data on Arbequina virgin olive oil show TPC values comparable with those of the present assay (Borges et al., 2015; Uluata et al., 2016).

The antioxidant activity measured in the chemical extracts of the oils was significantly affected by growing regions ($P < 0.05$). The lowest values were observed for samples 4 (Cádiz, Spain) and 11, whereas Spanish oils 3 (Málaga) and 8 (Valladolid) presented the highest antioxidant capacity. It has been shown than geographic and climatic conditions of production areas may affect the quality and composition of olive oils (Mailer, Ayton, & Graham, 2010). Therefore, relationships of TPC and antioxidant properties with altitude, maximum and minimum temperature and rainfalls of the different region were analysed in the current assay. It was found that the maximum temperature of the growing zone was positively related ($P < 0.001$) with the TPC ($r = 0.801$) and with antioxidant properties of the oil extracts (ABTS $r = 0.624$; DPPH $r = 0.715$ and FRAP $r = 0.664$), whereas rainfalls correlated negatively with TPC ($P < 0.01$, $r = -0.520$). On accordance, Brazilian oils, produced in regions with high levels of rainfalls (Table S1), showed on average lower values of TPC than Spanish oils. No global differences of antioxidant activity were found between oils from the different countries.

The antioxidant effect of EVOO has been mainly ascribed to polyphenols (Samaniego Sanchez et al., 2007) and, in agreement, strong relationships ($P < 0.001$) were found in chemical extracts between TPC and ABTS ($r = 0.673$), DPPH ($r = 0.709$) and FRAP ($r = 0.786$) assays.

3.1.2. Bioaccessible fractions

Measuring TPC and antioxidant properties in the BF may be indicative of the impact of the digestive process and provides complementary information comparing with chemical analysis of foods (Soler et al., 2010).

The present study found a positive effect of the digestive process in TPC and antioxidant properties of all the samples (Table 2), showing increased values of these parameters in the BF compared with the chemical extracts. These results agree with previous studies that found a rise of TPC and antioxidant capacity after digestion of oils (Borges et al., 2015; Seiquer et al., 2015) and other food matrices (Arques, Pastoriza, Delgado-Andrade, Clemente, & Rufián-Henares, 2016; Pastoriza et al., 2011; Szawara-Nowak, Bączek, & Zieliński, 2016), although negative effects have been also shown (Dinnella, Minichino, D'Andrea & Monteleone, 2007). Thus, our results support that *in vitro* digestion is a crucial step that releases a high amount of phenolic and antioxidant compounds, which seem difficult to extract from the food matrix with the solvents traditionally used (Szawara-Nowak et al., 2016).

Significant variations were found in bioaccessible TPC between oils, but no relationships with altitude or climatic factors of the regions were detected, contrary to those observed in the chemical extracts. Thus, the negative effect of high rainfall in EVOO TPC (Inglese et al., 2010) was only found in the chemical extracts, but no in the BF of the oils.

Polyphenols are strongly affected during the digestive process, mainly due to changes of pH and interactions with other compounds, which differ depending on their chemical structure (Dinnella, Minichino, D'Andrea, & Monteleone, 2007). Some of the major olive oil phenols (tyrosol, hydroxytyrosol and lignans) are relatively stable after gastrointestinal digestion and are able to generate derivate compounds owing to digestion, whereas other (flavonoids and secoiridoids) are unstable (Soler et al., 2010).

Table 1
Total phenolic content (TPC, mg of CAE per kilogram of oil) and antioxidant activity (ABTS, DPPH and FRAP, mmol of Trolox per kilogram of oil) determined in the chemical extract of Arbequina EVOO from Spain (samples 1–9) and Brazil (samples 10 and 11).

Oil sample	TPC	ABTS	DPPH	FRAP
1	168 ± 1.14^{b,c}	0.41 ± 0.04^{b,c}	0.93 ± 0.05^{b,c,d}	0.93 ± 0.02^{a,b}
2	163 ± 13.519^{b,c}	0.59 ± 0.14^{c,d,e}	1.45 ± 0.18^{e}	1.35 ± 0.40^{b,c}
3	302 ± 29.45^{e}	0.73 ± 0.01^{e}	1.58 ± 0.01^{e}	2.22 ± 0.23^{e}
4	196 ± 13.5^{c,d}	0.20 ± 0.01^{a}	0.52 ± 0.02^{a}	0.54 ± 0.03^{a}
5	227 ± 9.27^{d}	0.60 ± 0.13^{d,e}	1.22 ± 0.30^{d,e}	1.35 ± 0.38^{b,c}
6	197 ± 13.5^{c,d}	0.46 ± 0.05^{b,c,d}	0.74 ± 0.05^{a,b,c}	0.83 ± 0.05^{a,b}
7	174 ± 2.85^{b,c}	0.46 ± 0.03^{b,c,d}	0.81 ± 0.03^{a,b,c}	0.90 ± 0.09^{a,b}
8	290 ± 15.61^{e}	0.73 ± 0.01^{e}	1.52 ± 0.04^{e}	1.64 ± 0.14^{c,d}
9	104 ± 21.3^{a}	0.34 ± 0.01^{a,b}	0.65 ± 0.20^{a,b,c}	0.70 ± 0.24^{a}
10	151 ± 4.44^{b}	0.56 ± 0.02^{c,d,e}	0.97 ± 0.01^{c,d}	1.06 ± 0.02^{a,b,c}
11	75.0 ± 2.18^{a}	0.33 ± 0.01^{a,b}	0.56 ± 0.06^{a,b}	0.57 ± 0.01^{a}
P-value Spain × Brazil	**	n.s	n.s	n.s

Values are expressed as mean ± SE. Means values in each column with different letters are significantly different ($p < 0.05$). Comparing countries: ns, not significant $P > 0.05$;
* $P < 0.05$.
** $P < 0.01$.

Table 2
Total phenolic content (TPC, mg of CAE per kilogram of oil) and antioxidant activity (ABTS, DPPH and FRAP, mmol of Trolox per kilogram of oil) determined in bioaccessible fraction and residual fraction after *in vitro* digestion of Arbequina EVOO from Spain (samples 1–9) and Brazil (samples 10 and 11).

Oil sample	TPC		ABTS		DPPH		FRAP	
	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction
1	451 ± 29.8^{a,b,c,d}	110 ± 12.7^{a,b}	4.70 ± 0.31	1.35 ± 0.10^{d,e}	2.58 ± 0.31^{b,c}	0.38 ± 0.07^{c}	4.04 ± 0.42^{d,e}	1.53 ± 0.13^{a,b}
2	538 ± 53.1^{c,d}	101 ± 25.1^{a,b}	4.53 ± 0.12	0.92 ± 0.17^{a,b,c}	2.27 ± 0.45^{b}	0.42 ± 0.02^{c}	3.58 ± 0.79^{c,d,e}	1.51 ± 0.22^{a,b}
3	506 ± 3.06^{a,b,c,d}	140 ± 16.2^{b,c}	4.42 ± 0.13	1.01 ± 0.14^{a,b,c,d}	0.99 ± 0.08^{a}	–	2.54 ± 0.40^{b,c}	2.14 ± 0.17^{b,c,d}
4	392 ± 66.1^{a}	67.7 ± 24.8^{a}	4.59 ± 0.09	1.03 ± 0.02^{a,b,c,d,e}	2.54 ± 0.13^{b,c}	0.41 ± 0.04^{c}	3.08 ± 0.44^{c,d}	1.39 ± 0.21^{a}
5	411 ± 48.0^{a,b}	194 ± 33.6^{c}	4.28 ± 0.10	0.71 ± 0.20^{a}	2.08 ± 0.27^{b}	–	3.25 ± 0.10^{c,d}	2.26 ± 0.24^{c,d}
6	566 ± 38.9^{d}	127 ± 41.2^{a,b}	4.45 ± 0.10	0.99 ± 0.18^{a,b,c}	2.56 ± 0.19^{b,c}	0.18 ± 0.18^{a,b}	3.85 ± 0.39^{d,e}	2.47 ± 0.25^{d}
7	436 ± 4.02^{a,b,c}	109 ± 12.2^{a,b}	4.53 ± 0.19	1.37 ± 0.06^{e}	1.91 ± 0.24^{b}	0.29 ± 0.04^{b,c}	3.68 ± 0.52^{c,d,e}	2.14 ± 0.41^{b,c,d}
8	432 ± 18.9^{a,b,c}	136 ± 15.7^{b,c}	4.26 ± 0.12	1.27 ± 0.03^{c,d,e}	2.33 ± 0.12^{b,c}	–	4.62 ± 0.35^{e}	2.19 ± 0.24^{c,d}
9	391 ± 81.1^{a}	96.8 ± 16.1^{a,b}	4.56 ± 0.05	1.19 ± 0.11^{b,c,d,e}	2.51 ± 0.07^{b,c}	0.35 ± 0.04^{b,c}	3.51 ± 0.15^{c,d,e}	1.73 ± 0.05^{a,b,c}
10	531 ± 12.1^{b,c,d}	121 ± 9.21^{a,b}	4.29 ± 0.16	0.80 ± 0.08^{a}	2.97 ± 0.26^{c,d}	–	1.40 ± 0.18^{a,b}	1.63 ± 0.18^{a,b,c}
11	473 ± 41.6^{a,b,c,d}	92.6 ± 4.83^{a,b}	4.39 ± 0.15	0.85 ± 0.04^{a,b}	3.30 ± 0.12^{d}	–	1.24 ± 0.18^{a}	1.52 ± 0.16^{a,b}
P-value Spain × Brazil	n.s	n.s	n.s	*	**	n.s	**	n.s

Values are expressed as mean ± SE. Means values in each column with different letters are significantly different ($p < 0.05$). Comparing countries: ns, not significant $P > 0.05$.
* $P < 0.05$.
** $P < 0.01$.

Hydroxytyrosol seems to be decisive for phenolic bioaccessibility, as during digestion it is released from its precursors, the secoiridois, and its bioaccessibility may be over 100% (Rubió et al., 2014). A limitation of this study is that individual phenols were not characterised, but, according to previous bibliography, phenol profile within genotype may be affected by environmental conditions of oil producing regions (Inglese et al., 2010). Therefore, probably due to the different composition, the phenolic fraction of the oils of the current assay underwent different bio-transformation rates during digestion. As a result, TPC and antioxidant activity after digestion of the oils were different comparing with values found in the chemical extracts, and statistical differences between samples also varied.

Average values of antioxidant activity after digestion of Spanish oils differed significantly from Brazilian samples, being DPPH values higher and FRAP values lower in Brazilian BF than in Spanish samples. This suggests that in biological systems compounds than scavenge free radicals are not necessarily capable of reducing oxidants (Frankel & Meyer, 2000) and that transformations during the digestive process are decisive for establishing the antioxidant potential of the oils.

Relationships of antioxidant activity in the BF with climatic factors were also different than those observed in the chemical extracts, since bioaccessible DPPH was negatively correlated with maximum temperature ($P < 0.01$, $r = -0.697$) and positively with rainfalls ($P < 0.01$, $r = 0.583$). Moreover, whereas antioxidant properties were strongly related with TPC in oil extracts, no relationships was found in the BF after the digestive process, suggesting that after digestion other compounds than polyphenols could also be responsible for antioxidant properties. In agreement, other authors have found that correlations between TPC and antioxidant activity (measured by ABTS, DPPH and FRAP assays) are reduced after the digestion of fruits, compared with the initial values in chemical extracts (Chen et al., 2014).

3.1.3. Residual fractions

After *in vitro* digestion of Arbequina EVOO significant amounts of TPC and antioxidant activity were detected in the RF (Table 2). This fraction accounted a 20% of TPC, 19% ABTS, 7% DPPH and 39% FRAP from the total recovered after digestion (Fig. 2).

Traditionally, the residual fraction after digestion is not considered when studying phenolic bioavailability, but certain absorption cannot be discarded as it has been described that polyphenols that remain in the large intestine after digestion may be transformed by the intestinal microbiota into a series of low molecular-structures potentially absorbable (Cardona, Andres-Lacuerva, Tulipania, Tinahonesb, & Queipo-Ortuno, 2013). In fact, certain polyphenols are very poorly absorbed in the small intestine in their intact

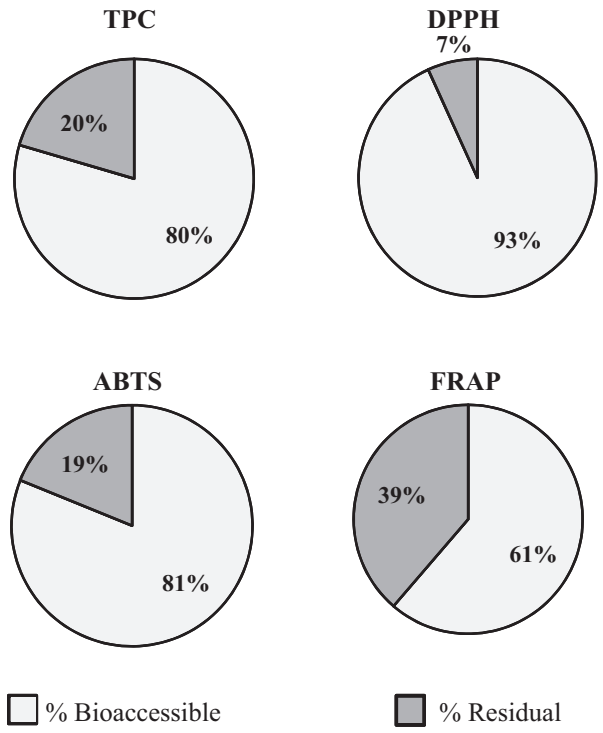

Fig. 2. Average distribution of TPC and antioxidant activity recovered after digestion corresponding to bioaccessible (%) and residual (%) fractions.

forms, but are extensively catabolized buy the gut microbiota and the products are efficiently absorbed through the colonic epithelium (Williamson & Clifford, 2017). Moreover, these residues deserve to be considered in the global antioxidant power of the digested oils, especially as a source of reducing capacity agents (Seiquer et al., 2015), as it has been suggested by others after digestion of different food matrices (Pastoriza et al., 2011; Szawara-Nowak et al., 2016). It has also been shown that unabsorbed polyphenols may exert beneficial local effects at the intestinal tract, interacting with the microbiota, mucosal cells and dendritic projections in the lumen (Martin & Bolling, 2015). Therefore, both fractions obtained after the *in vitro* digestion process may be responsible for health effects derived from polyphenol-rich foods.

3.2. Antioxidant cell markers

In order to assess the effects of the BF of the oils at the cellular level, two antioxidant markers on Caco-2 cells were studied: cell viability and ROS generation, both at basal conditions and against induced oxidative stress.

Firstly, BF was mixed with increasing proportions of DMEM FBS-free to evaluate the effect of the sample concentration in the viability of Caco-2 cells. Fig. 3A shows the viability obtained, expressed as percentage in relation to the control value (DMEM FBS-free), after 2 h of incubation with dilutions 1:2 and 1:3 (v/v) BF: DMEM FBS-free (differences are not indicated to avoid overlapping). When samples were diluted 1:2 (v/v), statistical differences of cell proliferation were observed after exposure to the different samples ($P < 0.01$), ranging from 70% (corresponding to Brazilian sample 11) to 130% (in Spanish sample 3). Treatment with BF from samples 2 (Jaén), 6 (Albacete), 7 (Toledo), 9 (Lérida), 10 (Rio Grande do Sul) and 11 (Minas Gerais) led to cell viability values lower than 85%, showing that monovarietal oils from different producing areas had different effects on cell integrity. When dilution of BF was increased to 1:3, high values of viable cells were observed after exposure to all samples (≥90%) and, therefore, dilution 1:3 was selected for further assays, as it was shown not damage cellular integrity in any case.

To evaluate the protective effect on cell viability, an induced oxidative stress was promoted by *t*-BOOH at different concentrations, 3 mM and 5 mM (Fig. 3B). When a slight damage was caused (*t*-BOOH 3 mM), control cells showed a drastic reduction in cell viability (67%), while cells preincubated with the samples were able to preserve the viability (>80%), thereby preventing cell damage due to the oxidising agent, without significant differences among oils. At stronger oxidising conditions (*t*-BOOH 5 mM) the proportion of viable cells was reduced to approximately 62%, and pretreatment for 2 h with the digested oils also neutralised the oxidative damage and improved the cell proliferation. However, in such conditions, certain differences were observed between samples, and some of them (samples 6 and 7) did not reach significant protective effects compared to control oxidized cells. Similar protective effect of cell viability has been shown with digested samples of Picual EVOO and also, although to a lesser extent, of extra virgin argan oil (Seiquer et al., 2015). Other studies have confirmed a protective effect in Caco-2 cells viability of EVOO extracts and phenolic compounds such as oleuropein and tyrosol, against the cytotoxic damage promoted by the mycotoxin alternariol or a pro-oxidant agent (Chiesi et al., 2015; Incani et al., 2016). It has been suggested that ingested polyphenols have a direct protective effect in the gastrointestinal tract by scavenging reactive species and/or preventing their formation (Halliwell, Rafter, & Jenner, 2005). However, in the present assay no relationships were found among bioaccessible TPC of the samples and cell proliferation under stressed conditions.

The ROS generation by Caco-2 cells after exposure to the different Arbequina samples is shown in Fig. 4. Under basal conditions, a slight ROS cell production was detected, as result of the normal cell functionality. In such conditions, a reduction of the ROS levels was obtained after exposure to BF of oils compared to control wells, ranging from 16 to 47%. Statistically significant reductions were observed for samples 4 (Cádiz), 5 (Sevilla), 8 (Valladolid), 9 (Lérida), 10 (Rio Grande do Sul) and 11 (Minas Gerais) in relation to control ($P < 0.05$), showing that digested oils are able to modify the redox status of intestinal cells, even under non-stressed conditions. The intestinal tract is continually attacked by luminal microbes and by oxidized compounds from the diet, exposing it to frequent oxidative changes and, thus, there is a need of maintenance of the redox intestinal homeostasis (Biasi et al., 2014). Although homeostatic mechanisms is one of the uncontrolled aspects in cell cultures, decreasing intracellular levels of ROS can act as biological signal molecules to regulate the redox homeostasis *in vivo* and should be considered positive in the antioxidant cell response.

In order to induce an oxidative injury, Caco-2 cells were treated with *t*-BOOH (5 mM for 2 h). The damage produced by *t*-BOOH caused a drastic increasing of ROS generation in the cells (Fig. 4) and, thus, the possible protective effect of oils was evaluated. Preincubating the cells with BF of oils prevented the free radical production due to the pro-oxidant agent from 16% (sample 6, Seville) to 53% (sample 10, Rio Grande do Sul), although in some cases (oils 5 and 6) differences comparing with oxidized cells were not significant. Our results are in line with previous data that relate a protective effect on induced ROS production of digested samples from different monovarietal EVOO (Borges et al., 2015). The beneficial effect of EVOO at intestinal level has been directly related to the presence of phenolic compounds, able to exert antioxidant actions (Biasi, Astegiano, Maina, Leonarduzzi, & Poli, 2011) or preserving the cellular antioxidant defences (Di Benedetto et al., 2007). Thus, several studies have been mainly focused on exploring the role of individual polyphenols (Chiesi et al., 2015; Di Benedetto

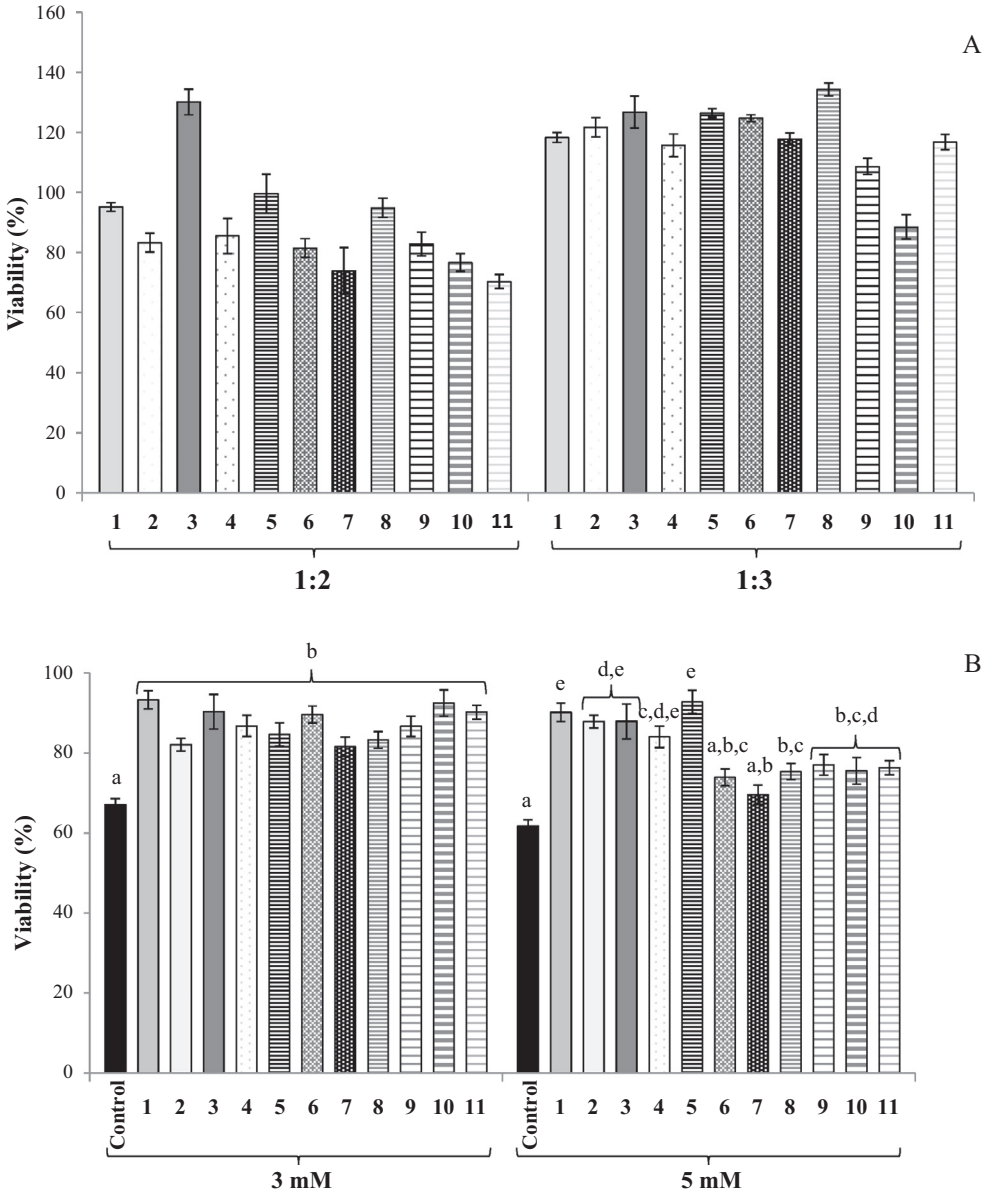

Fig. 3. (A) Cell viability (%) in Caco-2 cells after 2 h of incubation with bioaccessible fractions of Arbequina EVOO from Spain (samples 1–9) and Brazil (samples 10 and 11), diluted 1:2 and 1:3 (v/v) with DMEM FBS-free. (B) Cell viability in Caco-2 cells preincubated with the different samples and oxidized with *t*-BOOH 3 mM and 5 mM. (mean ± SE, n = 6). Bars with different minor letters differ significantly within 3 mM or 5 mM *t*-BOOH effect ($P < 0.05$).

et al., 2007; Rodríguez-Ramiro, Martín, Ramos, Bravo, & Goya, 2011) or methanolic extracts from EVOO (Incani et al., 2016) and olive leaves (Difonzo et al., 2017) in preventing oxidative damage in cell cultures. A clear difference between the referred studies and the present assay is that the complete bioaccessible fraction obtained after digestion of EVOO, instead of chemical extracts o isolated compounds, was used, as a more physiological approach to probe the protective role of oils at intestinal level. In the current study, no relationship was found between the positive role of the samples on preventing ROS generation and the phenolic content in the BF. However, a correlation was observed with the DPPH assay ($P < 0.01$; $r = -0.450$), i.e., the higher the DPPH value, the lower the ROS production in stressed conditions (see samples 10 and 11- Table 2 and Fig. 4). Thus, compounds originated from the EVOO digestion may prevent or delay the progression of intestinal diseases characterized by oxidative stress, as it has been shown that diet components, together with antioxidant enzymes, are involved in the intestinal mucosa response aimed at preventing oxidative damage (Biasi et al., 2014).

When data were grouped by countries, it was observed that cells treated with Brazilian oils were on average more protected against *t*-BOOH- induced ROS generation than those exposed to Spanish oils ($P < 0.001$), according to the highest DPPH values found in Brazilian BF.

Our data suggest that, after biotransformations undergone during the digestive process, the resulting phenolic fraction of oils could not be the only responsible for protecting cells from oxidation, which was also supported by the lack of correlation between TPC and antioxidant activity found in the BF of oils (commented in Section 3.1.2). Further studies on the phenolic profile of the oils of the present study could help to elucidate the possible role of individual compounds in the protective effect against oxidation.

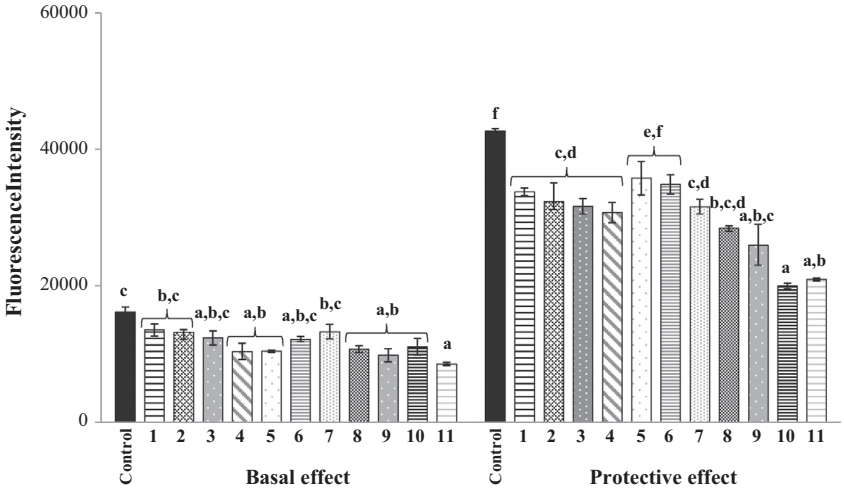

Fig. 4. ROS generation in Caco-2 cells expressed as fluorescence intensity at 90 min after incubation (basal effect) with bioaccessible fractions of Arbequina EVOO from Spain (samples 1–9) and Brazil (samples 10 and 11) or preincubated with samples and oxidized with *t*-BOOH 5 mM (protective effect). Control cells received culture medium. (Mean ± SE, n = 3). Bars with different minor letters differ significantly within basal or protective effect ($P < 0.05$).

3.3. Absorption across Caco-2 cells

The Caco2 cell lines derived from human intestinal epithelial adenocarcinoma is a suitable model system for the evaluation of intestinal functions and nutrient absorption (Mesías et al., 2009; Seiquer et al., 2015). In this study, the absorption of TPC and antioxidant properties of digested oils across Caco2 cell monolayers was studied. For the scavenging ability the DPPH assay was selected, due to DPPH activity was mainly located in the bioaccessible fraction (93%) and also due to its positive relationships with the protecting effect from oxidative damage. Non-significant amounts of FRAP activity (less than 0.1% in all the cases) were recovered in basolateral chambers after incubation (data not shown), probably due to that significant proportions of reducing activity (nearly 40%) remains in the residual fractions after digestion or to a short time of exposure at experimental conditions (2 h).

Results are presented in Table 3. Total TPC and DPPH activity recovered in basal chambers after incubation was measured and absorption was calculated as the percentage of the total initial content exposed to cell monolayers.

Absorption of TPC ranged from 32.5 to 110%, corresponding to samples 6 and 8, respectively with statistically significant differences between oils ($P < 0.05$). Average absorption of phenolic compounds was higher ($P < 0.05$) from Brazilian oils than from Spanish oils (97% *vs* 67%, respectively). Low proportions of TPC absorption (32–55%) were observed for samples 1, 2, 3, 4 and 6, despite the high bioaccessibility previously detected (Table 1). However, it must be taken into account that phenolic compounds could continue the absorption process in the lower digestive tract, as it has been recently suggested (Pereira-Caro et al., 2015). On the other hand, incubation with samples 5 and 8 surpassed 100% of TPC absorption. This finding could be explained by two hypotheses. Firstly, Folin - Ciocalteu method is a not a selective assay, determining all kinds of phenolic molecules and, thus, a variety of compounds may interfere with the reactive to give apparently elevated phenolic concentrations (Cerretini & Bendini, 2010). Secondly, the metabolites present in the basal medium could have been produced intracellularly and excreted to the exterior or produced directly by secreted enzymes (Soler et al., 2010). In this sense, different mechanism has been described in the Caco-2 cell model to evaluated the permeability of phenolic compounds (passive paracellular transport or active efflux process) and potential interference by either efflux or metabolic process have been described for some compounds, causing deviation from the predicted perme-

Table 3
Total initial content, total recovered and absorption of TPC and DPPH (% from the initial solution) across Caco-2 monolayers after 2 h of incubation with the BF of Arbequina EVOO from Spain (samples 1–9) and Brazil (samples 10 and 11).

	TPC			DPPH		
Oil sample	Total initial (mg/per well)	Total recovered (mg/per well)	Absorption (%)	Total initial (mg/per well)	Total recovered (mg/per well)	Absorption (%)
1	0.227	$0.111 \pm 0.01^{a,b,c,d}$	$49.1 \pm 4.74^{a,b}$	0.109	$0.055 \pm 0.01^{a,b,c}$	50.1 ± 2.21^{b}
2	0.212	$0.118 \pm 0.01^{b,c,d,e}$	$55.9 \pm 5.88^{a,b,c}$	0.115	$0.050 \pm 0.01^{a,b}$	$43.0 \pm 0.70^{a,b}$
3	0.189	$0.070 \pm 0.01^{a,b}$	37.9 ± 7.00^{a}	0.126	$0.066 \pm 0.02^{a,b,c}$	52.4 ± 12.8^{b}
4	0.198	$0.080 \pm 0.01^{a,b,c}$	44.5 ± 3.04^{a}	0.160	$0.049 \pm 0.01^{a,b}$	30.7 ± 0.10^{a}
5	0.149	$0.169 \pm 0.01g^{,h}$	102 ± 19.4^{d}	0.154	$0.061 \pm 0.01^{a,b,c}$	$40.0 \pm 2.22^{a,b}$
6	0.209	0.068 ± 0.01^{a}	32.5 ± 3.57^{a}	0.119	0.047 ± 0.02^{a}	$39.2 \pm 5.98^{a,b}$
7	0.214	0.188 ± 0.02h	$87.7 \pm 7.86^{c,d}$	0.133	$0.069 \pm 0.02^{b,c}$	52.2 ± 4.54^{b}
8	0.146	$0.161 \pm 0.02^{e,f,g}$	110 ± 17.4^{d}	0.178	$0.069 \pm 0.01^{b,c}$	$38.9 \pm 4.76^{a,b}$
9	0.140	$0.116 \pm 0.01^{b,c,d,e}$	$83.4 \pm 9.73^{b,c,d}$	0.134	$0.060 \pm 0.01^{a,b,c}$	$44.4 \pm 6.74^{a,b}$
10	0.134	$0.130 \pm 0.02^{d,e,f}$	97.0 ± 15.4^{d}	0.140	0.072 ± 0.01^{c}	51.5 ± 5.56^{b}
11	0.127	$0.124 \pm 0.03^{c,d,e,f}$	97.6 ± 21.0^{d}	0.132	$0.066 \pm 0.01^{a,b,c}$	50.0 ± 3.64^{b}
P-value Spain × Brazil	–	n.s	*	–	*	*

Absorption was calculated as the percentage absorbed in the basal chamber from the total initial quantity in the apical chamber. Values are means ± SD. Values with different superscript letters within each file are significant different at $P < 0.05$. Comparing countries: ns, not significant $P > 0.05$, ** $P < 0.01$.
* $P < 0.05$.

ability (Farrell, Poquet, Dew, Barber, & Williamson, 2012). Previous studies on the transport of polyphenols from olive oil extracts through Caco-2 cells have shown that compounds present a high conjugation rate (including methylation, sulphation and glucuronidation) that, in some cases, such as hydroxytyrosol, led to almost complete metabolic conversion (Rubió et al., 2014).

The present findings support that olive oil phenolics could be well absorbed at the intestinal level (Tuck, Freeman, Hayball, Stretch, & Stupans, 2001). Moreover, depending on the phenolic profile, conjugation may affect the efflux of phenols from inside the enterocyte (Soler et al., 2010). The majority of previous studies have been performed with individual phenolic compounds or chemical extracts, although absorption through intestinal cells after digestion of oils has been scarcely investigated. In an earlier study, we have found a percentage of phenolic absorption of 25% from digested samples of Picual EVOO (Seiquer et al., 2015), lower than reported in the current study with oils from Arbequina cultivar.

Antioxidant compounds were well absorbed across cell monolayers, leading to 30–52% of DPPH activity recovered in the basal chambers. Statistical differences ($P > 0.05$) were found between individual samples and between countries (51% *vs* 43% for Brazilian and Spanish oils, respectively). The results suggest a good bioavailability of the antioxidant properties of oils, which were maintained and absorbed after digestion. Although this assay does not identify the compounds responsible for the activity, other than polyphenols should be implicated, as no correlation was observed between absorption of DPPH and TPC.

In general, the data concerning absorption of antioxidant compounds from digested foods are very limited. Additionally, the knowledge about which compounds could be responsible for the potential biological effects *in vivo* is controversial. Several authors related that one of major compounds linked to beneficial effects *in vitro*, hydroxytyrosol, in spite to be well absorbed in the intestinal tract, has really a poor bioavailability in its free form due to an important presystemic metabolism in gut, leading to the formation of sulfate and glucuronide conjugates (Miro-Casas et al., 2003; Pastor et al., 2016; Pérez-Mañá et al., 2015). According to Pastor et al. (2016), the concentration of hydroxytyrosol in body fluids is too low to explain the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations. Thus, without discarding the activity of secondary phenolic metabolites, it may be postulated that antioxidant properties recovered at the intestinal level are also due to compounds other than polyphenols, with could be able to cross the intestinal barrier in an effective manner. In this regard, all the bioactive compounds (tocopherols, polyphenols, carotenoids, coenzyme Q_{10}) could be linked to the antioxidant activity found in the present study.

Results of the current assay support the use of *in vitro* gastrointestinal digestion coupled to Caco-2 cells as a reliable tool for investigating antioxidant properties of the oils at the digestive level. However, aspects others than those controlled in these experiments, such as homeostatic modulation of redox status, presence of oxidative stress conditions or the impact of the intestinal microflora in the digestive process, could affect the *in vivo* produced metabolites as well as the resulting antioxidant effect of the EVOO studied.

4. Conclusions

For the first time, antioxidant activity performed by different methods and combined with cellular models of Brazilian EVOO was evaluated. At the same time, the current study contributes to the database of antioxidant proprieties of Spanish Arbequina EVOO. The *in vitro* digestion promoted the release of bioactive compounds leading to an increase of TPC and antioxidant potential in all samples, after the biotransformations undergone by the digestive process of EVOO. Thus, the bioaccessible fractions obtained after the *in vitro* digestion of oils protected cell integrity from oxidative damage, and were able to reduce the ROS generation by the cells. On average, Arbequina Brazilian oils showed higher values of antioxidant activity (measured by the DPPH assay) after digestion, and better protective effects against induced ROS production, than Spanish oils.

Findings of the present study support that modifications during digestion are decisive to establish the antioxidant potential of the oils, which is linked, among other biological activities, to the health properties of the EVOO. The results also show that climatic conditions, especially rainfalls, affected the antioxidant behavior of oils produced in different producing zones. The influence of other factors than those considered in the present study, such as the ripeness index and further environmental variables (light intensity, humidity, evapo-transpiration, soil) cannot be discarded.

The lack of information of phenolic profile of the oils is a limitation of this study. Current studies of our group are aimed at determining the composition of the minor fraction of the oils, including, phenolic compounds, tocopherols and coenzyme Q_{10} Thus, further papers could give additional information on the compounds responsible for the antioxidant properties examined in the present assay.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.07.059.

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