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Retention and pre-colon bioaccessibility of oleuropein in starchy food matrices, and the effect of microencapsulation by using inulin

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ABSTRACT

Oleuropein is a phenolic compound that is present in fruits and olive tree leaves, which has shown enormous health benefits. This study aimed to assess the effects of the baking and boiling cooking methods and the inclusion of extract of olive leaf and inulin microparticles on oleuropein retention and bioaccessibility in a food matrix. Retention was measured after cooking, and bioaccessibility was measured in cooked samples subjected to *in vitro* digestion. Our findings showed that oven cooking generated matrices that possess oleuropein retention 15% higher than those presented by food cooked in boiling water, while bioaccessibility at small intestine conditions was 27.5% lower when microparticles, rather than an extract in the starchy matrix, were included, which proves the benefits of using inulin microcapsules to enhance the amount of oleuropein that reaches the colon.

1. Introduction

Currently, the design of functional and/or healthy foods has attracted increasing interest. Hence, the addition of bioactive compounds to normally consumed food has in recent years become increasingly common (Boroski et al., 2011). The olive tree is well-known for its high content of biophenols, which are present both in the fruits and leaves (Al-Rimawi, 2014). Hydroalcoholic olive leaf extract (OLE) is a dark, bitter liquid that is derived from leaves of the olive tree (*Olea europaea* L., Oleaceae). This extract has high potential as a functional ingredient due its cardio-protective and chemo-protective characteristics, in addition to its proven capacity to reduce colonization by the micro-organisms *H. pylori* and *C. jejuni*, which are both related to some diseases that affect the digestive system and can even trigger the development of cancer (Sudjana et al., 2009). Additionally, it has also been used to treat malaria and malaria-associated fever (Ahmadvand, Noori, Dehnoo, Bagheri, & Cheraghi, 2014). Its effect as a modulator of digestive metabolism has been established as it stimulates pepsin enzymatic activity, diminishes lipase enzymatic activity, slows triacylglycerol metabolism and inhibits its absorption (Polzonetti, Natalini, Vincensetti, Vita, & Pucciarelli, 2010, chap. 148). All of these beneficial effects are conferred by the major bioactive compound that is present in OLE, the oleuropein (OE). This molecule is composed by a 4-(2-hydroxyethyl)-1,2 benzenediol (Hydroxytyrosol), an elenolic acid and one glucose unit (Omar, 2010).

Regarding nutrient bioaccessibility, which is defined as the amount of compound liberated from the matrix after digestion and available for absorption (Carbonell-Capella, Buniowska, Barba, Esteve, & Frigola, 2014), studies carried out in olive polyphenols established that the concentration of phenolic compounds rapidly increases after the ingestion of olive oil and reaches a maximum in the plasma and urine after approximately 1 and 2 h, respectively (Weinbrenner et al., 2004).

In contrast, starch is known to be one of the most common components of the human diet, as food based on this biopolymer is easy to prepare and obtain (Boroski et al., 2011). This makes the possibility of including OLE in starchy foods fairly attractive; nonetheless, to overcome the unwanted organoleptic characteristics presented by extracts of olive oil, such as bitterness and colour, micro-encapsulation may represent a practical improvement. In this manner, those unwanted characteristics might be mitigated. Additionally, most processes used in food elaboration, such as cooking, influence the matrix and present the possibility that those compounds into food could be degraded (Boroski et al., 2011), thus influencing the amount of compound that remains in a matrix and modifying the bioaccessible fraction of such compound.

Micro-encapsulation represents the action of generating a “cover” for a specific compound, which acts to mitigate compound deterioration and/or loss, hiding any unwanted organoleptic characteristics, providing stability and introducing the option to achieve controlled

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release in a specific site (Ray, Raychaudhuri, & Chakraborty, 2016). Inexpensive, flexible, continuous and capable of being produced in powdered particles, spray drying represents the most widely used mechanism in the food industry for encapsulation (Fang & Bhandari, 2010; Ray et al., 2016). Inulin, which belongs to the fructan group, is a polymer that is currently used in the food industry as an encapsulant material due its beneficial prebiotic properties (Nazzaro, Orlando, Fratianni, & Coppola, 2012; Ronkart et al., 2007), and is specially abundant in chicory (*Chicorium intybus* L., *var. Sativum*), which is one of the most important sources of this compound (Beirao-Da-Costa et al., 2013). Several reports have suggested the beneficial effects of both inulin and OE, supporting the idea of include these compounds into common, easily accessible foods, such as wheat flour-based food (Ahmadvand et al., 2014; Beirao-Da-Costa et al., 2013; Lee & Lee, 2010; Polzonetti et al., 2010; Ronkart et al., 2007; Sudjana et al., 2009; Vissers, Zock, Roodenburg, Leenen, & Katan, 2002). The present study aimed to assess the effect of including OLE and encapsulated OLE in a food matrix, and comparing the effects of the final cooking method (baking vs boiling) on the retention and bioaccessibility of OE.

2. Materials and methods

2.1. Materials and enzymes

Olive leaves (O. *europaea* cv Arbequina) were collected from "El Oliveto" farm (Cholqui Valley, Melipilla, Metropolitan Region, Chile). Wheat flour Collico was purchased at Kunstmann Mill (Valdivia, Chile); Coumarin (≥99% HPLC), Oleuropein (OE, ≥98% HPLC) and Digestive enzymes and bile salts (α-amylase 300–1000 U mg^{-1} protein, A1031-5KU; pepsin from porcine gastric mucosa ≥250 U mg^{-1} solid, P7000-100 G; and Pancreatin 4 × USP, P1750-100 G; Bile extract porcine, B8631-100 G) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Inulin HP (IN, DP > 23, Raftilina) was purchased from the Alfa-Chilena S.A. (Santiago, Chile).

2.2. Obtaining of olive leaves extract (OLE)

The olive leaves (7 kg) were scalded at 95 °C for 4.5 min and then was quickly cooled in cold water. Next, leaves were dried at 45 °C in an air forced oven (WTE, Germany) at 8% final moisture (18 h). Dry olive leaves (760 g) were ground and macerated in ethanol:water (50:50 v/v; 3 L) for 24 h at room temperature. Subsequently, extract was separated by filtration in Buchner funnel; this procedure was carried out twice with 2.5 L. Extracts were combined, and the volume was reduced by using a rotary evaporator (Büchi R-205, Switzerland) at 40 °C until it reached a final volume of 1500 mL. The resulting extract was frozen at −20 °C. Physical and chemical characteristics of OLE are shown in Table 1.

2.3. Microencapsulation of olive leaves extract

The encapsulation of OLE was performed by spray drying, using inulin (IN) as encapsulating agent. The OLE-IN infeed solution (400 g) was prepared as follows: Inulin (36 g) was dissolved in distilled water (344 mL) and heated to 65–70 °C with stirring, then cooled to 30 °C, and mixed with OLE (20 mL, 31.05 mg OE/mL OLE). The resulting solution was homogenized at 11,000 rpm for 5 min with a Polytron PT 2100 homogenizer (Kinematica A.G, Switzerland) and fed into a B-290 mini spray-dryer (Büchi, Switzerland). The parameters used were as follows: inlet air temperature, 136 °C; air flux, 600 L/h; feeding rate, 2 mL/min; and atomization pressure, 0.5 MPa. At the end of the process, 23.09 g of microparticles (MP) were obtained.

Table 1
Physical and chemical characteristics of olive leave extract (OLE).

Parameter	Olive leave extract (X ± SD)
Moisture (%)	90.1 ± 0.03
Soluble solids (°Brix at 20 °C)	11.3 ± 0.1
Oleuropein content (mg OE/mL extract)	31.1 ± 0.9
Total polyphenols (mg gallic acid equivalent/mL extract)	25.7 ± 0.82
Antioxidant capacity EC_{50} (mg gallic acid equivalent/mL extract)	0.15 ± 0.01

2.4. Microparticles powder analysis

2.4.1. Encapsulation efficiency and recovery

2.4.1.1. Total OE. OLE-IN microparticles (100 mg) were dispersed in water (2 mL), then put into a water bath at 65 ± 5 °C. The resultant solution was transferred to a volumetric flask, filled up 10 mL with water:acetonitrile (80:20 v/v) containing 0.1% glacial acetic acid. An aliquot was injected into the HPLC.

2.4.1.2. Surface OE. OLE-IN microparticles (100 mg) were dispersed in ethanol (2 mL) with soft stirring. The dispersion was centrifuged at 2906*g* for 10 min. The supernatant was transferred to a volumetric flask filled up 10 mL with water:acetonitrile (80:20 v/v) containing 0.1% glacial acetic acid. An aliquot was injected into the HPLC.

Encapsulation efficiency (EE) and recovery (R), were calculated according to Eqs. (1) and (2), respectively:

$$EE(\%) = \frac{OE_{Total} - OE_{Surface}}{OE_{Total}} \times 100 \quad (1)$$

$$R(\%) = \frac{OE_{Total}}{OE_{Total\ theoretical}} \times 100 \quad (2)$$

where OE_{Total} corresponds to total OE content in the MP powder (mg/g), $OE_{Surface}$ corresponds to the OE content in the surface of MP powder (mg/g), and $OE_{Total\ theoretical}$ corresponds to the theoretical content of OE in the feed solution (mg/g).

2.4.2. Scanning electron microscopy

Morphological descriptions of the structure of MP were carried out according to the methodology used by Robert, García, Reyes, Chávez, and Santos (2012) and Palma, García, Márquez-Ruiz, Vergara, and Robert (2014), which consists of using a scanning electron microscope (SEM). This approach was used to analyse the superficial structure of the OLE-IN microparticles. The samples were covered with gold/palladium using a vacuum evaporator and analysed with an electronic microscope (LEO Electron Microscopy Ltd., Cambridge, UK). Digital images (1024 × 768 pixels) were saved as 8 bits TIFF image file, without compression (EDS INCA × sight, Oxford Instruments, Oxford, UK).

2.5. Preparation of starchy matrix with OLE inclusion

Dough matrices were prepared by mixing sifted wheat flour (200 g) and distilled water (120 mL) for 12 min using a semi-industrial mixer (Kitchenaid 4.3 Liters 5 QT). The dough was sheeting (ATLAS, 150 Chrome line Marcato design) to obtain sheets 2 mm thickness. Finally, the sheets were cut with an iron circular mould 50 mm diameter. Cooking was performed in the following two ways: in a water bath (distilled boiling water for 20 min) or in an oven (air forced oven at 190 °C for 12 min). After cooking, samples were cooled at room temperature for 20 min. For water cooking, samples were placed over an absorbent paper to eliminate water excess (Aravena, Garcia, Muñoz, Pérez-Correa, & Parada, 2016). OLE (2 mL OLE, 31.05 mg OE/mL) or OLE-IN (5 g MP, 11.45 mg OE/g MP) were incorporated to dough during mixing.

2.6. Retention and bioaccessibility

2.6.1. Retention

Retention of a compound represents the amount of such compound retained in the food matrix after processing; it is calculated using the following equation (Aravena et al., 2016):

$$\text{Retention}(\%) = \frac{M_P}{M_R} \times 100 \quad (3)$$

where M_P are OE mg in the processed matrix (cooked) and M_R corresponds to OE mg in the raw matrix (dry basis).

Moisture content in the samples was measured with an electronic moisture analyser (Startorius moisture analyser model MA35).

2.6.2. Oleuropein bioaccessibility after in vitro digestion

Bioaccessibility is the fraction of a bioactive compound that is released from the matrix by the action of digestive enzymes. After the cooking process, samples were submitted to an *in vitro* digestion process, which simulates the three main stages of human digestion (oral, stomach and intestinal). This method was implemented according to Aravena et al. (2016), Rubió et al. (2014) and Giao et al. (2012). Samples were cut into small pieces (3–5 mm) to simulate the chewing process and, subsequently, allow for the enzymatic digestion process.

2.6.2.1. Oral digestion. First, 5 g sample was weighed and 9 mL artificial saliva was added, which was composed of 14.4 mM sodium bicarbonate ($NaHCO_3$), 21.1 mM potassium chloride (KCl), 1.59 mM calcium chloride ($CaCl_2$) and 0.2 mM magnesium chloride ($MgCl_2$); the pH values of samples was adjusted at 7 with 1 N HCl and the enzyme α-amylase was added to the solution. Samples were incubated in a shaking water bath (Zhicheng ZHWY – 110 × 30) for 5 min at 37 °C and agitated at 185 rpm.

2.6.2.2. Gastric digestion. The pH of samples was adjusted to 2.0 using 1 N HCl and 36 mL pepsin solution was added (25 mg/mL in 0.02 N HCl). Once the enzyme was added, the mixture was incubated for 60 min at 37 °C and shaken at 130 rpm.

2.6.2.3. Duodenal digestion. The pH of samples was adjusted to 6.0 with 1 M $NaHCO_3$; subsequently, 0.25 mL pancreatin solution (2 g/L) and biliary salts (12 g/L) dissolved in aqueous 0.1 M $NaHCO_3$ were added to each sample. Incubation was performed for 120 min at 37 °C and shaken at 45 rpm.

Next, the digestion product was transferred to 50 mL Falcon tubes and centrifuged for 10 min at 5000 rpm to obtain a liquid fraction; this allowed the OE compounds released by the digestion stages to be quantified. From the obtained liquid fraction, 1 mL was extracted and transferred to an Eppendorf tube. Next, internal standard (cumarin) was added and tube was ultra-centrifuged for 10 min at 12.000 rpm.

The liquid product of digestion was analysed to quantify the OE content. Once data were obtained, Eq. (4) was used to obtain a percentage of bioaccessibility (Aravena et al., 2016) as follows:

$$\text{Bioaccessibility}(\%) = \frac{M_D}{M_P} \times 100 \quad (4)$$

where M_D corresponds to OE (mg) present in the digestion product, and M_P the OE (mg) present in the processed matrix (cooked); both values are expressed on dry basis.

2.6.3. High-Performance Liquid Chromatography (HPLC)

OE quantification was performed by HPLC using a Merck Hitachi L-6200 A pump, Merck Hitachi L-4250 UV–VIS detector, and a C18 column (5 µm × 4.6 mm i.d. × 250 mm, Symetry®, Waters, Ireland). For the determination of OE an isocratic mobile phase of water:-acetonitrile (80:20 v/v) containing 0.1% glacial acetic acid was used at a flow rate of 1 mL/min. The injection volume was 20 µL, and the wavelength was 280 nm (Al-Rimawi, 2014; Savournin et al., 2001). OE was quantified using an OE standard calibration curve (20–200 µg/mL; $y = 5760.9x + 5628$; $R^2 = 0.9997$).

2.7. Statistical analyses

Data were analysed by Multifactorial Analysis of Variance (ANOVA) along with the Tukey test with 95% confidence using the Statgraphics Centurion XVI software package.

3. Results and discussion

3.1. Oleuropein quantification methodology by HPLC

To validate the methodology used in this study, four parameters were determined; decision limit (Ccα), detection capacity (Ccβ), precision and exactness. For Ccα and Ccβ, values were close and low (5.11 µg/g and 6.01 µg/g, respectively). These values represent a minimal concentration of analyte that can express a positive result, with error probabilities of 1% and 5%. On the other hand, precision is related to the range of values for a mean and indicates the degree of concordance among measurements, while exactness corresponds to the obtained average value compared with the true value. Precision and exactness yielded consistent results (0.54% and 102.55%, respectively), which agree with results reported by Al-Rimawi (2014), who obtained a precision between 0.71% and 0.92%, and exactness between 98.4% and 100.5% for OE.

3.2. Characterisation of OLE microparticles with inulin

The total OE content of MP was 11.45 ± 0.23 mg of OE/g MP and the encapsulation efficiency was 78.7 ± 0.42%. These results agree with outcomes for other polyphenols microencapsulated by spray-drying (Robert et al., 2012).

The OE recovery reached a value of 70.5 ± 1.43%, lower than that described by Robert et al. (2012) for microparticles of gallic acid with inulin (83.9–94.2%) using spray drying as an encapsulation method.

Fig. 1 shows the SEM microphotographs of OE microparticles. In

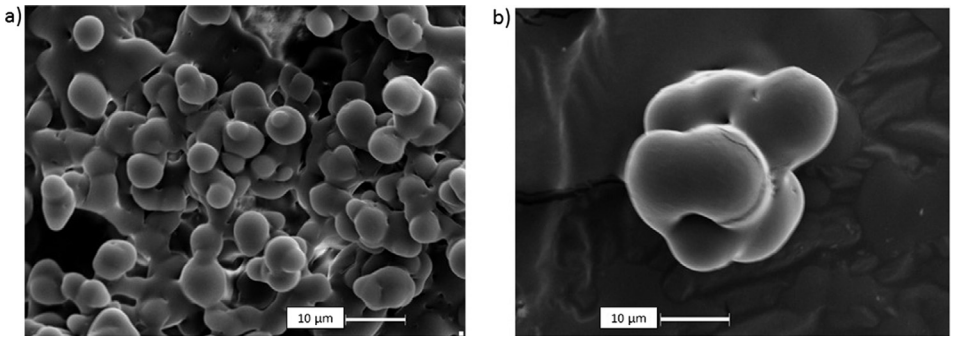

Fig. 1. Scanning electron microscopy (SEM) images of inulin microparticles with oleuropein. (a) Image of inulin microparticles with oleuropein. (b) Approximation of an isolated particle showing microparticle agglomeration.

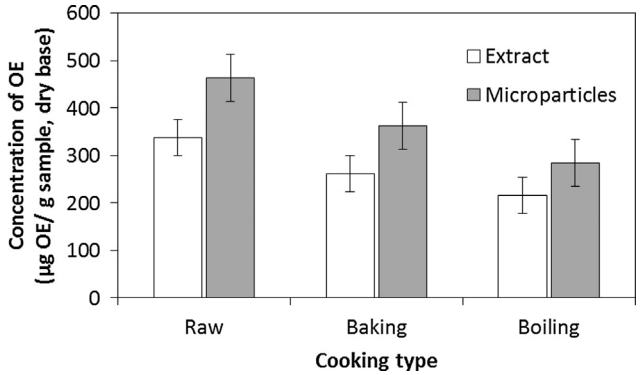

Fig. 2. Oleuropein concentrations in raw and processed starchy matrices. The values indicate the mean of measurements performed in triplicate with respective error bars.

this image, three main characteristics can be observed: size, shape and agglomeration. Most MP exhibits an approximate average size of 10 µm. Fig. 1a shows that the MPs have a spherical regular shape, which is consistent with that reported by Ronkart et al. (2007) and Robert et al. (2012). These characteristics can be influenced by the relationship between the inulin fraction and drying temperature (Beirao-Da-Costa et al., 2013). In the present study, only a few clefts could be observed in the structure of the particle, which indicates that inulin replacement and drying temperature were adequate for particles structuring. Fig. 1b shows some MP agglomeration, which are produced by collisions among particles inside the drying chamber (primary agglomeration) and collision of particles that emerge from the drying chamber as re-circulating powder (secondary agglomeration) (Ronkart et al., 2007). Frequently, a certain level of agglomeration is required by the food industry with the purpose of generating greater product solubility and avoiding the segregation of powder individual components (Ronkart et al., 2007).

3.3. OE retention in the starchy matrix after cooking

Fig. 2 shows OE content in the starchy matrices, both raw and cooked (baking and boiling), for inclusion as OLE and inulin MP, respectively.

In a comparison of raw and cooked matrices, it became clear that both cooking methods could generate a reduction in OE content; nevertheless, the magnitude of this reduction varied depending upon the cooking method. For baking, the OE was reduced by ~20%, whereas for boiling the reduction was ~35% for both OLE and MP.

Fig. 3 shows the effect of cooking type (boiling-baking, Fig. 3A) and inclusion method (OLE and MP, Fig. 3B) on OE retention in cooked matrices. Results showed that the cooking type significant affects the retention of OE ($p < .05$), while inclusion type did not affect it. When the matrices were baked, the retention percentage was ~77 mg/100 mg, significantly higher than when boiling was used (~63 mg/100 mg; Fig. 3a). Phenolic compounds, such as oleuropein, have been found to exhibit high solubility, which could be increased by raising the temperature (Karunaratne & Zhu, 2016); therefore, by increasing solubility, the mobility of these compounds and compound diffusion through the matrix can also increase. Consequently, different interactions with starch may occur, which might favour compound retention in the matrix. These interactions may occur because of hydrogen bonds, whereby hydroxyl or carbonyl groups of the phenolic compounds may interact with both water and the hydroxyl groups of starch (Zhu, 2015). According to the studies of Zhu (2015) and Karunaratne and Zhu (2016), it is possible that by not having high water availability to migrate into baked samples, OE can interact with the matrix via these hydrogen bonds, and therefore it stays in the sample and increases the retention percentage. For boiling, various studies have established the presence of bioactive compounds that leach from the food matrix, as occurs in phenolic compounds, such as anthocyanins that are present in grape marc and can be incorporated into a paste (Sant'Anna, Porta, Ferrerira, Tessaro, & Silveira, 2014). According to the results of the present study, it is possible that a process of leaching of unretained oleuropein could be occurring in boiled samples due water excess.

Regarding the "inclusion" factor, no significant differences were observed when inclusion was considered as OLE or MP, and was ~70 mg/100 mg (i.e., the average contrast considering both cooking methods) (Fig. 3b). Since the protection provided by inulin microparticles, it was expected a higher OE retention for MP than OLE, however, such difference was not observed, suggesting that matrix biopolymers (starch and gluten) could also protect OE during cooking (as when inulin PM was used). Consistently with our finding and the study of Zhu (2015), it is possible that OE interacted with the starch matrix via hydrogen-bond interactions; hence, the compound remained trapped in the matrix during the cooking process at levels similar to those obtained for inclusion with MP.

3.4. In vitro bioaccessibility of OE in starchy matrices

Fig. 4 shows the effects of cooking (baked-boiled) and inclusion (OLE-MP) on bioaccessibility of OE in cooked matrices.

We found that only the type of inclusion (average contrast of both cooking methods by inclusion) had a significant effect ($p < .05$) on OE bioaccessibility (~90 vs ~73 mg OE/100 mg OE, for the use of OLE and MP, respectively; Fig. 4b), whereas both cooking methods that we used did not yield statistically significant differences (Fig. 4a). For both cooking types, we observed bioaccessibility greater than 60%, which agree with previous studies that arrived at values greater than 90% of OE recovery in plasm after intake, without any type of matrix or processing (Vissers et al., 2002).

For the inclusion method, the lower bioaccessibility of OE when MP

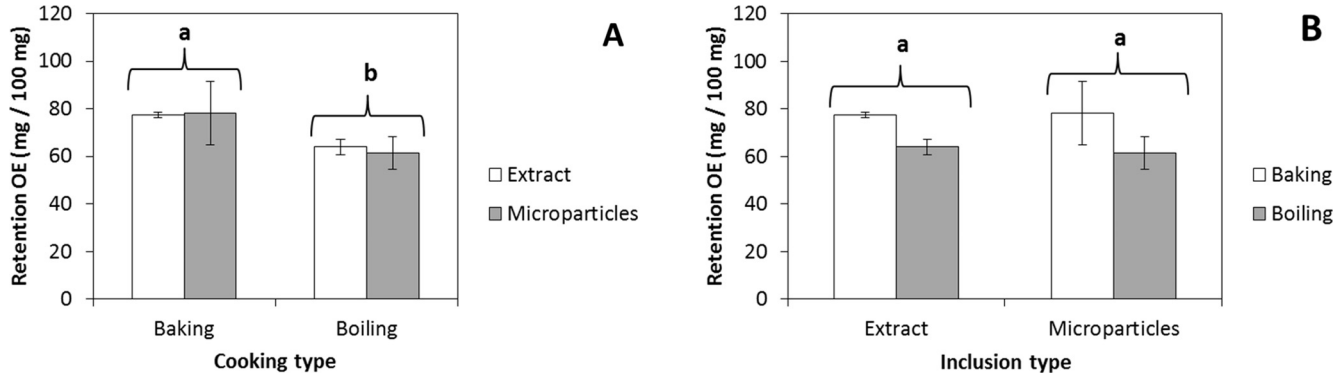

Fig. 3. Oleuropein retention in cooked matrices. (a) Cooking effect (baking-boiling); (b) inclusion effect (extract-microparticles). Different letters indicate statistically significant differences for the TUKEY multiple range test with 95% confidence. OE, Oleuropein.

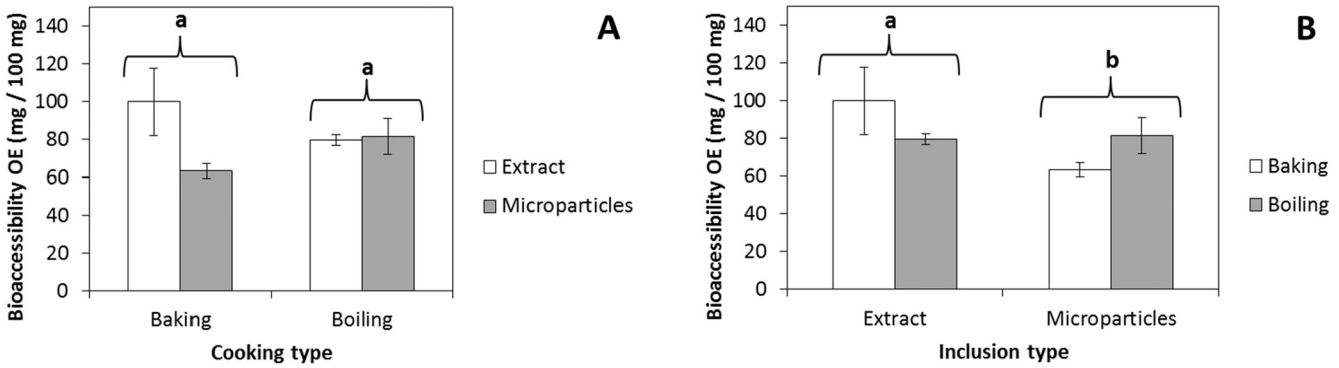

Fig. 4. *In vitro* bioaccessibility of oleuropein in cooked matrices. (a) Cooking effect (baking-boiling); (b) inclusion effect (extract-microparticles). Different letters indicate statistically significant differences for the TUKEY multiple range test with 95% confidence. OE, Oleuropein.

was used support the idea that the covering material of such MP (inulin) is not digested either during gastric digestion or in the small intestine. It is assumed that this prebiotic characteristic protected part of the internal OE contents inside throughout digestion; therefore, it is assumed that for *in vivo* models, MP would enter into the colon, where bacteria could carry out fermentation of inulin to produce short-chain fatty acids, such as propionic and butyric acids. Notably, fermentation is a fundamental process for microbiota development and to maintenance the integrity of those epithelial cells that are responsible for nutrient absorption (Dawood & Koshio, 2016; Robert et al., 2012). Additionally, microbiota stimulation yields multiple benefits, such as anti-carcinogenic and immunomodulatory properties (Beirao-Da-Costa et al., 2013).

Additionally, since OE occurs naturally as glucoside form, it is poorly absorbed in the proximal section of the small intestine. Therefore, OE will continue the digestion process until to reach the colon, where bacteria breakdown OE structure, releasing one glucose molecule and thereby facilitating OE absorption, although it should be noted that from all microbial species of our gastrointestinal tract, only a few species can modify the structure of polyphenols, limiting their actual biological action (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Chiou et al., 2014), including antimicrobial activity against pathogenic micro-organisms such as *H. pylori* and *C. jejuni* (Sudjana et al., 2009).

4. Conclusions

Our study established that MPs protect OE during *in vitro* digestion. Encapsulation was found to be more effective for diminishing bioaccessibility in the small intestine rather than increasing retention during the processing. Both retention and bioaccessibility were greater than 60% in all cases. OE retention yielded values that were 15% higher for baking than for boiling, and there were no significant differences when olive oil extract or inulin MP were included. When olive oil extract was included, even 100% bioaccessibility was observed, in contrast to inulin MP, which yielded 72.52% bioaccessibility. All these results suggest that baking is a better cooking method than boiling in order to minimize the loss of OE during processing, while the use of MPs would diminish OE bioaccessibility in the intestine, which would enhance the feeding rate and, therefore, bioaccessibility at the colonic level. Further studies will be needed to confirm these preliminary observations.

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