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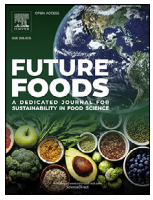

Microencapsulated olive leaf extract enhances physicochemical stability of biscuits

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ABSTRACT

Free and microencapsulated olive leaves extracts (OLE) have been proposed as natural antioxidant to prolong the stability of biscuits, under two accelerated storage conditions, with and without UV light. The equivalent of 500 μg gallic acid/ g of dough has been selected as the sensory acceptable OLE concentration. Higher total phenol content and antioxidant activity were observed in biscuits enriched with both free (B-OLE) and microencapsulated (B-MCR) OLE, in comparison to the controls (B-C), being B-MCR less affected than B-OLE during storage. Higher oxidative stability was detected for B-MCR than B-OLE and B-C, when measured by both peroxide value and Oxitest. A general hardness decrease was observed for all the formulations during storage, being however B-MCR always harder than B-OLE and B-C, probably because of the interactions of the microsphere polymers (alginate and pectin) with water. Darker colours were measured for the enriched biscuits in comparison to the control ones, being all slightly affected during storage by water migration and lipid oxidation phenomena.

1. Introduction

Olive leaves, olive pomace, olive mill wastewater, and olive stones are the main by-products of the olive oil system (Gullón et al., 2020). The disposal of these by-products represents both an environmental and economic issue (Nuneset al., 2016). Currently, the main uses of the olive oil production by-products are as animal feed, fertilizers, or source of energy in bioreactors (Berbel and Posadillo, 2018).

Olive leaves are generated during the pruning of olive trees (around 300–750 kg/ha or 25 kg per olive tree) and in olive oil industries after being separated from fruits before processing (about 10% of the weight of olives) (Clodoveo et al., 2021). Around 4.5 million tons of olive leaves are produced in the world each year (Oleaf4value 2022). After Spain, Italy is the second country both for olive production (about 2Mt) and olive tree cultivated land (about 1 Mha) (Faostat, 2022).

Olive leaves are a rich reservoir of phenolic compounds, such as oleuropein, apigenin, verbascoside, known for their antioxidant and healthy properties (Žugčić et al., 2019). In this regard, while olive leaves and their extracts are widely sold as food supplements, applications as food ingredients are still mainly discussed only at research level. Olive leaf extracts have indeed been proposed as natural additives in food formulations, both for their healthy and technological properties, with the aim to produce functional foods or to extend product's shelf-life (Difonzo et al., 2021). The use of olive leaf extracts to extend the shelf life of food is based on their well-known antimicrobial and antioxidant activities (Borjan et al., 2020; Martín-García et al., 2022).

Bakery products undergo physical, chemical, and microbiological spoilage during storage (Galanakis et al., 2018; Manzocco et al., 2020). The shelf-life of this kind of foodstuffs is influenced by both environmental and product intrinsic factors. The first ones include storage temperature, relative humidity, packaging material and packaging headspace gas composition; whereas the latter factors are represented by level of preservatives, pH and, most importantly, moisture content and water activity (a_w) (Smith et al., 2004). Biscuits are bakery products characterized by low levels of moisture content and a_w, thus the limiting factor for their shelf-life is mostly ascribable to fat oxidation, which leads to rancidity and off-flavors (Barden and Decker, 2016). The use of antioxidants in these food formulations was found to be effective in enhancing their shelf-life (Nanditha and Prabhasankar, 2008). Synthetic antioxidants have been used in the food industry for the past 50 years. Despite their high efficacy and stability, there is growing concern about their toxigenic, mutagenic, and carcinogenic potential (Nanditha and

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Prabhasankar, 2008). Hence there is an increasing demand for the use of natural antioxidants in foods, also belonging to plant by-products (Trigo et al., 2020). In this domain, fruit and vegetable peels, seeds, kernels, skins, leaves, paste, bagasse are being tested as functional ingredients, both as powder/flour or extracts, to fortify bakery products and produce functional foods with extended shelf-lives (Melini et al., 2020, Pasqualone et al., 2020). Encapsulation of natural bioactives in the form of micro/nanoparticles has been proposed as a strategy to protect these compounds from degradative reactions promoted by external factors.

Using encapsulation, natural molecules are easily handled, and their unpleasant organoleptic properties are mitigated. Moreover, encapsulation allows controlled release of the materials at a definite time and place both in the consumer body and in the food system (Nedovic et al., 2011; Rousta et al., 2021).

Several techniques have been investigated to encapsulate olive leaves extracts (González-Ortega et al., 2020; Mourtzinos et al., 2007; Moreira Oliveira et al., 2022; Soleimanifar et al., 2020). Flammini et al. (2020, 2021) used emulsification-internal gelation as microencapsulation method, by testing alginate alone or in combination with pectin, whey proteins, or sodium caseinate. Encapsulation with alginate/pectin matrix increased thermal stability of the polyphenols and allowed mitigation of the colour properties of the olive leaf extract.

Despite the different potential application of bioactive compounds recovered from olive mill waste as functional ingredients in food matrices (Flamminii et al., 2021), few studies tested olive leaf extracts as natural antioxidants to extend the shelf-life of bakery products (Difonzo et al., 2018, Conte et al., 2021). To the best of our knowledge, the effect of microencapsulated olive leaf extracts has never been tested on biscuits. In this study, olive leaf extracts, free or encapsulated in alginate/pectin microspheres (Flammini et al., 2020, 2021), have been added to biscuits formulations in order to evaluate their effect on the physicochemical properties of the final products, when kept under two different accelerated storage conditions.

2. Material and methods

2.1. Materials

Soft wheat flour (type 00, according to Italian regulations), powdered sugar, leavening agent (sodium bicarbonate and tartaric acid), salt and butter were bought from a local food supermarket. Dried olive leaf extract (OLE) - standardized concentration of oleuropein (40%) - was donated by Oleafit srl (Isola del Gran Sasso, Teramo, Italy). These extracts showed a total phenol content of 347.24 ± 12.43 mg GAE/g. Dried microencapsulated OLE (MCR) was obtained by applying an emulsion/internal gelation technique, by using alginate/pectin as carrier material that, according to preliminary measurements, provided the highest encapsulation efficiency, phenolic loading and phenols/matrix interactions (Flamminii et al., 2020). The microbeads were prepared as follow: briefly, a polymeric solution of alginate (2% w/v), pectin (2% w/v) and Olive Leaf Extracts (OLE) (2% w/v) was mixed using an Ultra-Turrax model T25 Basic (Ika- Werke GmbH & Co, Germany) and stored overnight under refrigeration to allow complete hydration and deareation. Twenty grams of the polymeric solution was mixed with 1 mL of a suspension of calcium citrate eptahydrate (500 mM Ca2+ equivalents) and dispersed in 100 g of sunflower oil containing 2% (w/w) Span 80. Twenty millilitres of sunflower oil containing 500 µL of glacial acetic acid was added and stirred for 30 min. The gelled microparticles were then separated by adding 150 mL of 0.05 M CaCl2 with 0.5% (w/v) of Tween 20 and OLE (2% v/v) and gently agitated for 30 min. The gelled microbeads were recovered under vacuum filtration and washed with 30 mL of a 70% ethanol and OLE 2% (v/v) solution to remove oil traces (Flamminii et al., 2020). The OLE-loaded microcapsules had an average volume weighted mean diameter ($D_{4,3}$) of 62.6 ± 0.2 µm and a total phenol content of 92.42 ± 0.61 mg GAE/g sample. All the used reagents were of analytical grade.

Table 1
Accelerated storage conditions.

AS1	AS2
Temperature (T): 55 ± 0.1°C; Relative humidity (RH): 50 ± 0.5%; Dark conditions; Packaging: 10 × 18 cm bags of coextruded oriented polypropylene (OPP) (thickness 40 μm) and metallized OPP (thickness 20 μm), sealed with a domestic packaging machine (Severin Sundern, Germany).	Temperature (T): 55 ± 0.1°C; Relative humidity (RH): 50 ± 0.5%; UV light: 315-400 nm, light intensity 5 W/m^2. Distance light-biscuits: 30 cm. No packaging.

2.2. Biscuits preparation

Three biscuit formulations were prepared:

B-C: Control biscuits
B-OLE: Biscuits enriched with free olive leaf extract
B-MCR: Biscuits enriched with microencapsulated olive leaf extract

The recipe for producing 12 control biscuits was the following: 100 g soft wheat flour, 50 g powdered sugar, 1 g leavening agent, 1 g salt, 30 g butter, 17 g water (20 °C). B-OLE and B-MCR were prepared by substituting soft wheat flour with OLE or MCR in an amount corresponding to 500 μg GAE/g, measured by Folin Ciocalteau assay. In particular, 0.14 g of OLE and 0.55 g of MCR were used.

The amount of OLE and MCR (500 μg GAE/g) was selected based on a sensory acceptability test with 35 untrained panelists (18 women, 17 men), by using a 9 points hedonic scale. Biscuits with three increasing amounts of OLE (500, 600, 700 μg GAE/g) (Conte at al., 2021) were coded and tested randomly. Samples with an average score higher than 6 were considered acceptable.

After weighing, the ingredients were mixed with a Kitchen-Aid Professional mixer (KPM5, KitchenAid, St. Joseph, Michigan, USA) by using a dough hook (K45DH) at speed 2 for 9 min. OLE and MCR were manually sieved (10 mesh sieve) to reduce the presence of lumps. After 10 min rest at 4 °C, the dough was molded in 3 × 5 cm and 0.8 mm thickness rectangular pieces. Lastly, the biscuits were baked in a ventilated electrical oven (Whirlpool, Michigan, USA) at 180 °C for 20 min. The biscuits were then cooled in a thermostatic chamber (Memmert Schwabach, Germany) until reaching 25 ± 1 °C. Two batches were produced for each formulation.

2.3. Storage conditions

Aliquots from each of the three biscuits' formulations were subjected to 2 different accelerated storage (AS) tests (Table1) in thermostatic chambers. The AS temperature (55°C) was selected on the base of the study of Verardo et al., (2009). 50% RH has been reported to be an average value registered in a supermarket in one year (Robertson, 2006), being also the minimum value influencing the rate of fat oxidation (Lu and Xu, 2009). The UV light has been used in order to monitor the effect of photo-oxidation other than thermo-oxidation on the quality of cookies during storage, by selecting ultra-strong conditions (Lu and Xu, 2009).

The co-extruded metalized films used as packaging material in AS1 had the following technical properties: water vapor transmission rate (WVTR) <2 g/m2/24h; oxygen transmission rate (O2TR) <200 cm3/m2/24h. To avoid the light barrier effect and over-stress the photo-oxidation phenomena, no packaging was used in AS2.

For AS1 the samples were analyzed after 0 (t0), 5 (t5), 10 (t10) and 20 (t20) d from the production. For AS2 the samples were analyzed after 0 (t0), 5 (t5), 10 (t10) d from the production.

2.4. Physicochemical analysis

2.4.1. Moisture content and water activity (a_w)

Moisture content was measured according to the gravimetric method AACC 44–15.02 (AACC, 2000). a_w was detected using an electronic dew point water activity meter (4TE, AquaLab, Decagon Devices, Inc., WA, USA). Three replicates were analyzed for each studied condition.

2.4.2. Total phenol content (TPC) and antioxidant activity (AA)

For the bioactive compound's extraction, 1g of dough or ground biscuits was mixed with 10 ml of a methanol/water (70:30 v/v) solution, for 120 min at room temperature. The suspension was then centrifuged for 10 min at 3820 g, the supernatant was then filtered on a PTFE membrane (0.45 μm) and used for the TPC or AA analysis. The extraction was conducted twice for each studied condition.

The TPC analysis was conducted by means of the Folin-Ciocalteu assay: 50 μl of phenolic extract were mixed with 1160 μl of bi-distilled water, 100 μl of Folin-Ciocalteu reagent (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 300 μl of a water solution of sodium carbonate (20% w/v). After an incubation of 30 min at 40°C, the absorbance at 760 nm was measured using a UV-Vis spectrophotometer (Thermo Scientific™ Evolution™ 201/220). Total phenols were expressed as gallic acid equivalents (GAE)/g dw, based on a calibration curve prepared using a standard solution of gallic acid (ppm).

The radical scavenging AA was measured by using the DPPH assay, following the method of Pasqualone at al. (2014), with slight modifications. 500 μl of extract were mixed with 1500 μl of 60 μM DPPH methanolic solution; after incubation in the dark for 30 min at room temperature the absorbance at 517 nm was read using a UV–visible spectrophotometer (Thermo Scientific™ Evolution™ 201/220). Following the same procedure, a blank sample was also read.

The DPPH radicals scavenging activity was determined using equation (1):

$$I\% = \left[\left(A_0 - A_1/A_0\right) * 100\right] \quad (1)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the samples.

Three measurements were performed for each sample and reported as %.

2.4.3. Peroxide value (PV) and oxidative stability (OSI)

The lipids of grinded biscuits were extracted using diethyl ether in a ratio 1:2.5 (w/v) on a laboratory shaker in ambient conditions for 60 min. After filtration, the lipid fraction was separated by evaporating the solvent under vacuum condition onto the rotary evaporator (Steroglass Perugia, Italy). The extracted fat was immediately used to calculate the peroxide value (PV) according to the European Regulation n. 2568/91, with slight changes: 10 mL of dichloromethane were added to the obtained fat fraction, then 15 mL of glacial acetic acid and 1 mL of saturated potassium iodide solution were added. The solution was stirred and kept in the dark for 5 min. At the end, 75 mL of distilled water and few mL of starch solution were added as an indicator. Finally, the free iodine was titrated with a sodium thiosulfate solution (0.002 N or 0.01 N solution, for expected values below 12 or above 12, respectively). PV was calculated according to Eq. (2)

$$PV = VxT/m * 1000 \quad (2)$$

V is the volume (ml) of sodium thiosulfate, T is the concentration of sodium thiosulfate, m is the weight (g) of the fat fraction. The values are expressed as milli-equivalents of active oxygen per kilogram of oil (meqO2/kg). Three measurements were performed for each sample.

OXITEST (VELP, Usmate, MB, Italy) was used for measuring OSI, according to Paciulli et al., (2018). The accelerated oxidation test was performed on 30 g of grinded biscuits exposed to constant high temperature (90 °C) and over-pressure of pure oxygen (0.6 MPa, degree 5.0). This test delivers results in terms of induction period (IP): the time (min) requested for fat oxidation, corresponding to a drop of O_2 pressure when it is consumed by the sample; the longer the IP, the higher the sample's oxidative stability. The IP value (min) is calculated from the oxidation curve by using the software OXISoft™ and the graphical two tangent method. The results are reported as the average of three replications.

2.4.4. Dimensions and texture

Length, width, and thickness of the biscuits were measured using a Vernier caliper, the same day of production (t0).

Cutting test, performed with a TA. XT2i Texture Analyser, equipped with a 25 kg load cell (Stable Micro Systems, Godalming, UK), was used to measure maximum cutting force (N), taken as reference for hardness, and distance at the maximum force (mm), according to the method of Paciulli et al., (2018). Eight replicates were analyzed for each studied condition.

2.4.5. Colour

The CIE-Lab colour parameters L^*, a^* and b^* of doughs and biscuits were measured by means of the Minolta Colourimeter (CM 2600d, Minolta Co., Osaka, Japan) equipped with a standard illuminant D65 (Commission Internationale de l'Eclairage CIE, 1978). The Spectramagic Software (version 3.6) was used for data analysis, using 10° position of the standard observer. The colour difference (ΔE) between doughs and biscuits at t0, between fresh and stored biscuits, as well as between B-C and the other biscuits formulations at the same storage time, was calculated as $\Delta E = \sqrt{[(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]}$. Eight measurements were analyzed for each studied condition.

2.4.6. Statistical analysis

The statistical software SPSS (Version 27.0, SPSS Inc., Chicago, USA) was used to measure means and standard deviations. One way ANOVA, coupled with a Least Significance Difference (LSD) post-hoc test, was performed by means of the same software to evaluate the differences among formulations and during storage, at a significance level of $p < 0.05$. The *t*-test for independent samples was also carried out to determine significant differences ($p < 0.05$) between the same formulations of doughs and biscuits. Pearson correlation coefficients ($p < 0.01$; 0.05) between variables were also calculated.

3. Results and discussions

3.1. Preliminary acceptability test

Olive leaves extract (OLE) is characterized by a persistent bitter taste, due to its chemical composition rich in several classes of polyphenols (Ataei and Hojjatoleslamy, 2017; Kranz et al., 2010). This sensory feature of the olive leaves extracts limits their addition in food formulations, when used as natural ingredients. In order to select the maximum acceptable concentration to be used in biscuits formulations, three OLE levels have been tested: 500, 600 and 700 μg gallic acid equivalents (GAE)/g of dough. The scores expressed by the tasters on the 9-point hedonistic scale showed an inverse trend with respect to the OLE level: 6.6±1.4, 5.8±2.0, 5.2±1.9 for 500, 600 and 700 μg GAE, respectively. 500 μg GAE was the formulation that received the highest score, exceeding also the average of 6, considered as the minimum score for sensory acceptability; for this reason, it was selected to enrich the biscuits. Concentrations higher than 500 μg GAE/g were considered too much bitter from the consumers.

3.2. Moisture content (MC) and water activity (a_w)

In Table 2, the values of MC and a_w measured on biscuits the same day of production (t0) and during storage under the two selected conditions are reported. At t0, no differences were observed among the formulations, for both MC and a_w, indicating that the addition of the olive leaves extract, both in free and incapsulated form, did not affect these

Table 2
Moisture content (%) and water activity of biscuits.[a]

	MC (%)			a_w		
	B-C	B-OLE	B-MCR	B-C	B-OLE	B-MCR
AS1						
t0	4.02±0.36^{aA}	4.10±0.21^{aA}	4.24±0.41^{aAB}	0.28±0.02^{aA}	0.28±0.02^{aA}	0.29±0.02^{aA}
t5	3.73±0.23^{bA}	3.88±0.08^{bAB}	4.37±0.06^{aA}	0.25±0.01^{bB}	0.26±0.00^{bAB}	0.28±0.00^{aA}
t10	3.82±0.15^{bA}	3.77±0.09^{bB}	4.11±0.06^{aAB}	0.26±0.01^{bAB}	0.24±0.01^{bB}	0.29±0.01^{aA}
t20	3.75±0.19^{bA}	3.71±0.20^{bB}	4.03±0.06^{aB}	0.24±0.01^{aB}	0.23±0.03^{aB}	0.25±0.01^{aB}
AS2						
t0	4.02±0.36^{aC}	4.10±0.21^{aC}	4.24±0.41^{aC}	0.28±0.02^{aC}	0.28±0.02^{aC}	0.29±0.02^{aC}
t5	4.97±0.05^{aB}	5.04±0.04^{aB}	5.01±0.04^{aB}	0.30±0.00^{bB}	0.31±0.01^{bB}	0.32±0.1^{aB}
t10	5.39±0.01^{aA}	5.39±0.11^{aA}	5.34±0.09^{aA}	0.34±0.00^{cA}	0.34±0.00^{bA}	0.35±0.0^{aA}

[a] Data are reported as mean ± standard deviations (n=6). Different small letters in the same row indicate significant differences between formulations at the same storage time ($p<0.05$). Different capital letters in the same column indicate significant differences between storage times for the same formulation($p<0.05$). Abbreviations: MC: moisture content; a_w: water activity; B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract; AS1: accelerated storage 1; AS2: accelerated storage 2.

parameters, as expected. The observed results were in line with those reported for this product category, having a MC lower than 6 g/100 g and a_w around 0.3 (Curti et al., 2018). During the accelerated storage 1 (AS1) - 55°C, 50%RH, OPP/OPP metallized packaging, darkness - both MC and a_w decreased for all the formulations, in comparison to t0, probably due to water evaporation in the bag's headspace. It should be noted that B-MCR maintained a higher MC and a_w than B-C and B-OLE throughout the entire AS1. This trend was observed even at t0, despite the difference among the samples was not significant. Such result may be related to the presence of alginate and pectin (~ 0.8% on flour weight), the hydrocolloids forming the microspheres; indeed, thanks to the presence of different hydroxyl groups in their molecular structure, even in small amounts, hydrocolloids tend to interact with water through hydrogen bonds (Guarda et al., 2004; Lazaridou et al., 2007); this would justify the greater ability of B-MCR to retain and / or absorb water compared to the other two formulations.

During the Accelerated Storage 2 (AS2) - 55°C, 50% RH, UV light – a progressive increase of both MC and a_w has been observed during the 10 storage d (Tab.2), for all the formulations. No differences in MC were observed at the same storage time for the three formulations, while a_w resulted always significantly higher for B-MCR, followed by B-OLE and B-C. These data show the importance of a moisture barrier packaging for biscuits like products; indeed, the absorption of water would lead to organoleptic modifications, as well as increasing in chemical and microbiological vulnerability.

3.3. Total phenol content (TPC) and antioxidant activity (AA)

The TPC has been measured both on doughs and biscuits, in order to detect the amount of polyphenols lost during baking. Although no polyphenols have been added to the control dough, the sample generated a signal probably due either to the presence of phenolic acids, including ferulic acid, deriving from the pericarp and endosperm of the wheat kernel (Wang et al., 2013) or to the ability of the Folin-Ciocalteu reagent to react also with molecules different from phenolic compounds capable of reducing it (Ikawa et al., 2003; Sánchez-Rangel et al., 2013). After baking, a loss of around 41.5, 30 and 34 % of total phenols has been detected for B-C, B-OLE and B-MCR, respectively, in comparison to the doughs. A lower retention has been observed for the control formulation, which probably lost the phenolic acids of the flour, reported to be sensitive to the baking process (Yu et al., 2013). Also, other authors have observed loss of phenolic compounds, both free and encapsulated, after the biscuits baking (Kaderides et al., 2020; Saponjac et al., 2016). They attributed this phenomenon to the reaction of polyphenols with other cookies ingredients, such as sugar fragments generated from caramelization and Maillard reactions. After baking (t0), the TPC of B-OLE and B-MCR resulted significantly higher than that of B-C (Fig. 1), as expected, being enriched with olive leaves phenolic extracts (Urzùa et al., 2017). Moreover, B-OLE showed significantly higher TPC than B-MCR. As suggested by Gómez-Mascaraque et al. (2017), it may be due to the difficulty in extracting some polyphenols from the microspheres, which are held by the matrix both physically (Fang and Bhandari, 2010) and by hydrogen bonds (Flamminii et al., 2020). It must be also considered that, during kneading, a low energy input was used for dough formation; therefore, microparticles were subjected to a limited stress that was not able to cause the disruption of the polymer matrix and favor the phenolic release during the extraction procedure.

During AS1 (Fig. 1a) the TPC of B-C resulted stable and lower than that of B-OLE and B-MCR. On the other hand, the enriched formulations showed a slight TPC decrease during storage, being however B-MCR more stable than B-OLE. At t20 no TPC differences were found between B-OLE and B-MCR (Fig. 1a). The decrease in TPC observed for both B-OLE and B-MCR could have occurred due to the reaction between the polyphenols and the radical products developed during storage (Spigno et al., 2013). Moreover, Taghvaei et al. (2014) observed that olive leaves phenolic extracts added to soy oil, in order to improve the oxidative stability, were partially degraded during a 20 d storage at 55°C; among the polyphenols, flavonoids were found to be the most stable.

During AS2 (Fig. 1b), results similar to AS1 have been observed, despite the much stronger storage conditions due to the presence of UV light. Fig. 1c and d show the data related to the radical scavenging activity (DPPH) of the studied biscuits. The addition of the olive leaves extract, both free and microencapsulated, produced a significant increase in antioxidant activity in comparison to the control; indeed, the antioxidant activity of B-OLE and B-MCR resulted significantly higher ($p \leq 0.05$) than that of B-C during the entire storage period, for both AS1 and AS2. During AS1 (Fig. 1c), the antioxidant activity of B-C remained constant; on the other hand, a slight decrease in DPPH was observed for B-OLE and B-MCR, being however this latter more stable and significantly higher ($p \leq 0.05$) than B-OLE. A positive correlation between TPC (Fig. 1a) and DPPH (Fig. 1c) has been found ($R= 0.943$; $p \leq 0.01$), confirming the antioxidant activity of the olive leaves polyphenols. During AS2 (Fig. 1d), the antioxidant activity of B-C decreased, being however stable for B-OLE and B-MCR. Some studies justify the increase or preservation of plant phenolic antioxidant activity, even during accelerated storage, through the fact that polyphenols oxidation products may also act as antioxidant (Spigno et al., 2013; Sui, 2017). This aspect could impact both on the results of the Folin-Ciocalteu assay (Spigno et al., 2013) and on the ones of DPPH assay (Sui, 2017).

3.4. Peroxide value (POV) and oxidative stability

POV has been monitored as primary oxidation index during both the accelerated storage tests. From Table 3 it is visible how POV was

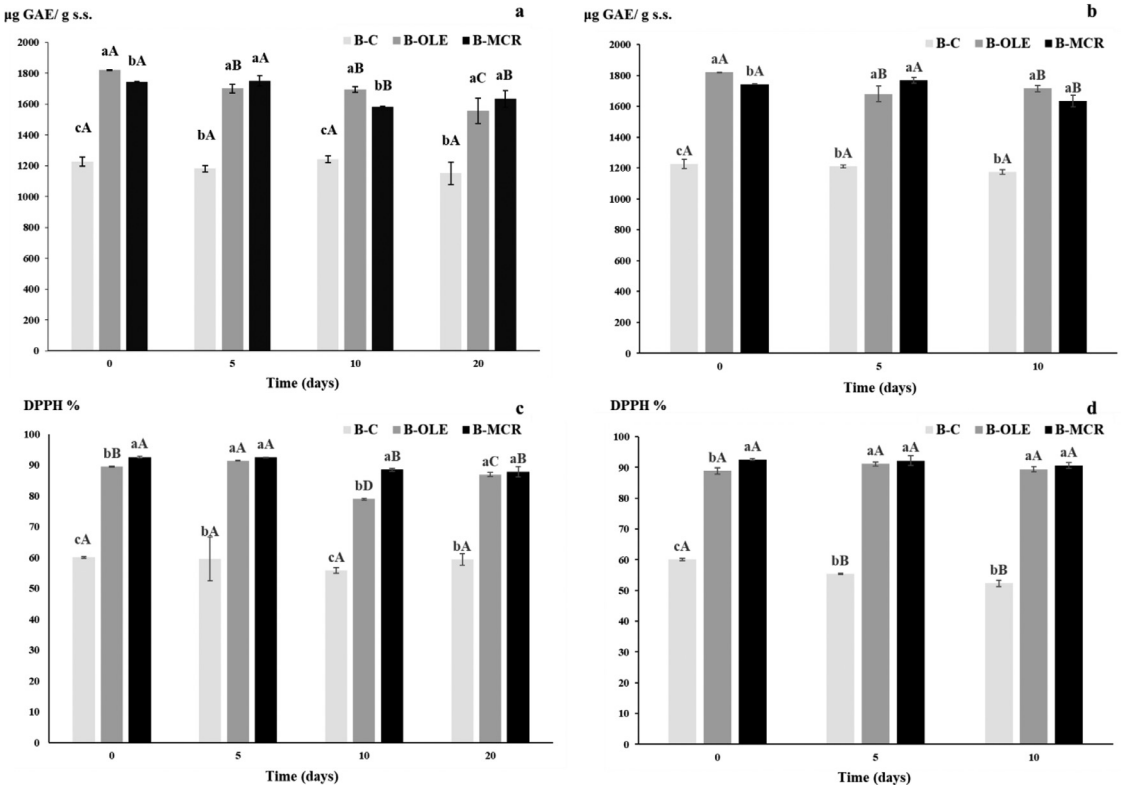

Fig. 1. Total phenol content and radical scavenging activity (DPPH) of biscuits during AS1 (a, c) and AS2 (b, d)[a].

[a] Data are reported as mean ± standard deviations (n=3). Different small letters indicate significant differences between formulations at the same storage time ($p<0.05$). Different capital letters indicate significant differences between storage times for the same formulation ($p<0.05$). Abbreviations: B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract.

Table 3
Peroxide value (POV) (meqO_2/Kg fat) of biscuits during AS1 and AS2.[a]

	POV		
	B-C	B-OLE	B-MCR
AS1			
t0	2.21±0.42^{aA}	2.35±0.50^{aA}	1.89±0.37^{aA}
t5	1.99±0.23^{aA}	1.87±0.43^{aA}	1.93±0.21^{aA}
t10	1.77±0.20^{aA}	1.94±0.52^{aA}	1.96±0.15^{aA}
t20	1.63±0.21^{aA}	1.56±0.34^{aA}	1.48±0.28^{aA}
AS2			
t0	2.21±0.42^{aC}	2.35±0.50^{aC}	1.89±0.37^{aC}
t5	87.76±10.12^{aB}	52.19±2.31^{bB}	64.03±5.30^{bB}
t10	151.57±0.12^{aA}	143.44±6.26^{aA}	121.04±8.86^{bA}

[a] Data are reported as mean ± standard deviations (n=3). Different small letters in the same row indicate significant differences between formulations at the same storage time ($p<0.05$). Different capital letters in the same column indicate significant differences between storage times for the same formulation($p<0.05$). Abbreviations: B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract; AS1: accelerated storage 1; AS2: accelerated storage 2.

around 2 meqO_2/kg fat, without significant differences both among the formulations and during AS1. These results suggest that despite the temperature of 55°C, applied as an acceleration factor, at t20 the lag phase (or induction period) of the lipid oxidation was still in progress. A lag phase was also observed by Calligaris et al. (2007), who monitored the evolution of POV on breadsticks packaged in OPP bags and subjected to different storage temperatures (20, 30, 37, 45°C); at all temperatures the authors observed an initial phase in which the number of peroxides remained constant. During this phase, the accumulation of radical lipid oxidation products is slow, and it results in a slow formation of hydroperoxides (Barden et al., 2016). A food product can be considered stable when the peroxide values do not exceed the limit of 10 meqO_2/kg fat. Calligaris et al. (2016) report that in some food matrices, even when subjected to high temperatures, lipid oxidation proceeds slowly, however, if exposed to UV light the reaction proceeds faster and with a lower dependence from temperature. Following this goal, for AS2, the absence of packaging barrier against oxygen, combined with the ultraviolet light (315-400 nm) and high temperature (55°C) have been applied as oxidative accelerating factors, as suggested by other authors (Golmakani et al., 2019). During AS2 test, considerable POV increases have been observed for all the three biscuits formulations, as expected (Table 3). UV light induces photo-oxidation phenomena, mediated by photosensitizers, which lead to the development of hydroperoxides (Veberg et al., 2007). Radical scavengers' antioxidants are not active in inhibiting photo-oxidation reactions mediated by photosensitizers (Frankel, 2005). However, ultraviolet radiation is also involved in direct photo-oxidation (not mediated by photosensitizers) that lead to the production of free radicals through the decomposition of peroxides and hydroperoxides, with the same mechanism of auto-oxidation. On this kind of reaction, radical scavenger antioxidants are active (Frankel, E., 2005). Focusing with this last aspect, the goal of this work was also to explore the inhibitory activity of olive leaves polyphenols against the photo-oxidation mediated by the application of UV light as an accelerating factor.

From Table 3, it is possible to observe that, during AS2, at t5 the POV of both B-OLE and B-MCR was significantly lower ($p \leq 0.05$) than B-C, indicating that the olive leaves extract both in free and microencapsulated form, slowed down the lipid oxidation.

POV increased significantly ($p \leq 0.05$) between t5 and t10 for all the samples and particularly, for B-OLE, that reached the peroxide values of B-C. The lower POV observed for B-MCR in comparison to B-C and B-OLE at t10 could be due to the protection and stabilization of the microsphere

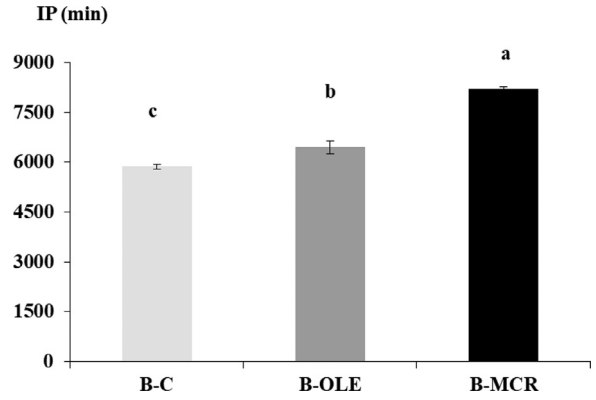

Fig. 2. Oxidative stability of biscuits measured by Oxitest[a].
[a] Data are reported as mean ± standard deviations (n=3). Different small letters indicate significant differences between formulations (p<0.05). Abbreviations: B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract; IP: induction period.

matrix over the phenolic compounds followed by a gradual release of the antioxidant molecules from the microspheres over time. Indeed, under AS2 storage conditions, the partial adsorption of water may have caused the swelling of the alginate/pectin matrix which in turn promoted the diffusion of the phenolic compounds towards the outer layers of the particles and, then, towards the food matrix; in such conditions, phenolic compounds were thus able to exert their antiradical activity.

OXITEST was used as fast method to measure the oxidative stability of the biscuits. In order to accelerate the reaction, high temperature and oxygen pressure are used as stressors. Moreover, the samples were also ground for the analysis (without affecting the integrity of the encapsulation system in B-MCR), resulting in a greater area exposed to oxygen. In Fig. 2 the induction periods (IP), measured for each sample are reported. The induction period is considered the time during which the sample remains stable under the applied conditions (Caruso et al., 2017); the longer the induction period the higher the oxidative stability (Sharma et al., 2019). The end of the induction period results in a sudden increase in oxygen consumption, and therefore in a decrease in the measured oxygen pressure. The induction period of B-C resulted significantly lower ($p \leq 0.05$) than that of B-OLE and B-MCR, indicating that the addition of the olive leaf extract, both in free and microencapsulated form, conferred greater oxidative stability to the samples under the applied analytical conditions. The induction period of B-OLE was approximately 8% higher than that of B-C, similarly to what observed by Difonzo et al. (2018) using RapidOxy (140°C, O_2 7 bar) to measure the oxidative stability of snacks enriched with olive leaf extract. B-MCR showed an increase in the induction period of about 38% in relation to B-C. As hypothesized in the previous paragraph, the higher oxidative stability of B-MCR may be due to a gradual release of the phenolic compounds from the microspheres, leading to a more extensive protection over time (Jolayemi et al., 2021), and/or the microspheres may have played a role in protecting the polyphenols from thermal and oxygen exposure (Kaderides et al., 2020).

3.5. Texture and dimensions

Table 4 shows the consistency values of the three biscuit formulations, measured by means of cutting tests. The maximum force was used as an index of samples' hardness. The data show that at t0, the addition of the olive leaves extract in free form did not significantly change ($p \leq 0.05$) the hardness of the biscuits, however they became significantly harder ($p \leq 0.05$) with the addition of the microencapsulated extract. The higher hardness of B-MCR could be due to the interactions of the microsphere polymers (alginate and pectin) with water during the kneading phase, competing for hydration with the components of the flour (Ferrero, 2017). Rosell et al. (2001) and Lazaridou et al. (2007) studied the development of wheat and gluten-free bread doughs by adding various hydrocolloids, including sodium alginate (0.5% on wheat flour) and pectin (1% and 2% on rice flour). The authors observed that the interaction of hydrocolloids with water resulted in the request of a greater amount of water in comparison to the control to reach 500 BU (Brabender unit). The texture of the biscuits is strongly linked to the degree of hydration of the dough (Villemejane et al., 2013). Furthermore, the lower flour hydration could have caused lower starch gelatinization in the subsequent cooking phase; the starch gelatinization is indeed strictly related to the formation of the typical porous structure of biscuits (Manley, 2011). Less starch gelatinization may have contributed to obtain a more compact and hard structure, characterized by a lower specific volume (Demirkesen, 2016; Mandala et al., 2006). Length and width of B-MCR (5.2±0.1 and 3.6±0.2 cm) resulted indeed significantly lower ($p \leq 0.05$) compared to that of B-C and B-OLE (5.5±0.2 and 3.9±0.2 cm). This could indicate, in the case of B-MCR, a more compact structure characterized by higher hardness, confirming the above assumptions. Also Maache-Rezzoug et al. (1998) observed that the different fluidity of the dough, related to the degree of hydration, would cause a change in the size of the obtained biscuits: larger presence of water in the dough develop longer lengths on the biscuits.

During AS1, all types of biscuits showed a decrease in hardness over time (Table 4). In particular, between t0 and t5 a hardness decreases of 45-50% was observed for all samples, remaining pretty stable till t20. Similarly to hardness, also the distance underwent a sharp decrease between t0 and t5, indicating that all the samples at t0 supported a greater deformation, while at t5 they were characterized by higher fragility (Table 4). Some authors observed higher fragility for biscuits stored at 40-45°C compared to the same samples stored at 20°C (Yang et al., 2013). The physical changes that can occur in biscuits at 55°C are not easy to interpret; however, interactions between the components of the matrix, the migration and redistribution of water within the sample and with the environment, the fat melting, may have contributed to the hardness decrease (Manley, 2011, Paciulli et al., 2020; Duta et al., 2029).

During AS2, a general decrease of hardness was observed for all the samples over time. B-MRC resulted always significantly harder than B-C and B-OLE (Table 4). Similar trends were observed for the values of distance. Generally, the exchange of water with the storage environment is one of the main factors influencing the consistency of biscuits (Paciulli et al., 2018; Paciulli et al., 2020). In this study, at t0 no differences in MC or a_w were observed between the sample (Table 2), thus the texture variation was ascribable only to components' interactions. During AS2, the phenomena of water absorption (~ 1%) and a_w increase (~ 0.05) become predominant and affect the decrease in texture of the biscuits, keeping however the differences due to formulations.

3.6. Colour

Comparing the colour of the biscuits at t0 (Table 5), it is visible how the lightness L^* was significantly higher ($p \leq 0.05$) for B-C than for B-OLE and B-MCR. On the other hand, the parameters a^* and b^* resulted significantly lower for B-C than for B-OLE and B-MCR. It must be said that the extract in the two forms, free and microencapsulated, had a visually different colour, which was also confirmed experimentally (Flamminii et al., 2021); moreover, the microencapsulated extract showed a colour much more similar to the used flour. Furthermore, Ou et al. (2019) report that the oxidation of polyphenols to the form of quinone can cause the latter to react with amino acids and proteins by means of the Maillard, Michael and Strecker reactions with the formation of brown pigments; this would therefore lead to an increase of the surface darkness. B-OLE contained the extract in free form, thus it could have been involved in the above reactions more easily than B-MCR. Regarding the parameter ΔE_f, no significant differences ($p \leq 0.05$) have been found between B-OLE and B-MCR, furthermore it was lower than

Table 4
Texture of biscuits.[a]

	Hardness (N)			Distance (mm)		
	B-C	B-OLE	B-MCR	B-C	B-OLE	B-MCR
AS1						
t0	147.87±21.94^{bA}	157.57±15.45^{bA}	202.58±19.28^{aA}	1.86±0.48^{aA}	2.02±0.26^{abA}	2.17±0.20^{aA}
t5	74.70±11.36^{bB}	85.04±13.05^{bB}	110.31±15.87^{aB}	0.89±0.24^{aC}	0.94±0.27^{aB}	0.85±0.11^{aC}
t10	79.21±12.63^{aB}	60.40±9.60^{bC}	84.00±13.71^{aC}	0.85±0.24^{aC}	0.80±0.21^{aB}	0.69±0.15^{aCD}
t20	76.73±12.04^{bB}	80.05±11.71^{bB}	97.48±16.56^{aBC}	0.79±0.25^{bC}	0.89±0.23^{abB}	1.09±0.28^{aB}
AS2						
t0	147.87±21.94^{bA}	157.57±15.45^{bA}	202.58±19.28^{aA}	1.86±0.48^{bA}	2.02±0.26^{abA}	2.17±0.20^{aA}
t5	93.08±18.19^{bB}	88.67±15.96^{bB}	193.67±16.42^{aA}	0.96±0.34^{bB}	0.94±0.30^{bB}	1.45±0.17^{aB}
t10	70.96±11.91^{bC}	86.12±10.81^{bB}	128.19±33.44^{aB}	0.77±0.25^{aB}	0.80±0.13^{aB}	0.92±0.16^{aC}

[a] Data are reported as mean ± standard deviations (n=6). Different small letters in the same row indicate significant differences between formulations at the same storage time ($p<0.05$). Different capital letters in the same column indicate significant differences between storage times for the same formulation($p<0.05$). Abbreviations: B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract; AS1: accelerated storage 1; AS2: accelerated storage 2.

Table 5
Colour of biscuits during accelerated storage AS1 and AS2.[a]

AS1	L^*	a^*	b^*	ΔE_{st}	ΔE_f
B-C					
t0	79.42±0.74^{aB}	2.65±0.17^{bC}	21.82±0.40^{cB}	-	-
t5	80.22±0.50^{aA}	3.03±0.27^{bB}	24.83±0.67^{aA}	3.06±0.70^{aB}	
t10	79.11±1.12^{aB}	3.50±0.40^{aA}	25.26±0.69^{aA}	3.64±0.64^{aA}	
t20	80.17±0.69^{aA}	3.42±0.37^{aA}	25.10±1.00^{aA}	3.44±0.91^{aAB}	
B-OLE					
t0	77.88±0.83^{cAB}	2.95±0.33^{aD}	22.71±0.61^{bC}	-	1.97±0.73^{a}
t5	77.97±0.73^{bAB}	3.96±0.31^{aA}	24.74±0.56^{aB}	2.41±0.50^{bB}	
t10	77.35±0.97^{cB}	3.55±0.36^{aB}	25.46±0.48^{aA}	3.03±0.48^{bA}	
t20	78.30±1.18^{bA}	3.22±0.23^{bC}	24.49±0.54^{bB}	2.18±0.59^{bB}	
B-MCR					
t0	78.86±0.40^{bA}	3.01±0.26^{aAB}	23.70±0.59^{aA}	-	2.04±0.61^{a}
t5	77.48±0.56^{cC}	3.18±0.28^{bA}	24.06±0.58^{bA}	1.59±0.51^{cA}	
t10	78.24±0.68^{bB}	2.60±0.23^{bC}	23.93±0.66^{bA}	1.13±0.50^{cB}	
t20	78.30±0.84^{bB}	2.96±0.26^{cB}	23.84±0.55^{cA}	1.03±0.57^{cB}	
AS2	L^*	a^*	b^*	ΔE	
B-C					
t0	79.42±0.74^{aB}	2.65±0.17^{bB}	21.82±0.40^{cA}		
				-	
t5	80.85±0.79^{aA}	2.59±0.30^{aB}	21.89±0.69^{bA}	1.67±0.58^{aB}	
t10	80.93±0.53^{aA}	2.95±0.20^{bA}	20.59±0.41^{bB}	2.10±0.44^{aA}	
B-OLE					
t0	77.88±0.83^{cB}	2.95±0.33^{aB}	22.71±0.61^{bB}	-	
t5	78.63±0.79^{bA}	3.01±0.20^{aB}	23.57±0.54^{aA}	1.40±0.53^{aA}	
t10	78.95±0.38^{bA}	3.39±0.16^{aA}	21.79±0.42^{aC}	1.54±0.38^{bA}	
B-MCR					
t0	78.86±0.40^{bA}	3.01±0.26^{aB}	23.70±0.59^{aA}	-	
t5	77.51±0.48^{cB}	2.86±0.16^{aC}	23.22±0.54^{abB}	1.55±0.41^{aB}	
t10	77.42±0.66^{cB}	3.49±0.16^{aA}	22.30±0.47^{aC}	2.32±0.62^{aA}	

[a] Data are reported as mean ± standard deviations (n=6). Different small letters in the same column, at the same storage time, indicate significant differences between formulations ($p<0.05$). Different capital letters in the same column, for the same formulation, indicate significant differences between storage times ($p<0.05$). Abbreviations: B-C: control biscuits; B-OLE: biscuits enriched with free olive leaf extract; B-MCR: Biscuits enriched with microencapsulated olive leaf extract; AS1: accelerated storage 1; AS2: accelerated storage 2; ΔE_{st}: ΔE calculated between t0 and the other storage times, for the same formulation.

3 for both formulations; such a value is reported as a threshold under which the colour difference between samples is not perceived by the human eye (Duta, Culetu, and Mohan, 2019).

During AS1 (Table 5) no clear trends were observed for all the samples regarding the colorimetric parameters. These changes may be associated to the water redistribution in the samples. The increase of b^*, observed for all the samples, especially between t0 and t5, may be associated to the butter, that being melted at 55°C, may have been partially migrated towards the surface of the biscuits (Duta et al., 2019; Onacik-Gür and Żbikowska, 2020). Looking at ΔE_{st}, it is visible how, during AS1, B-MCR and B-C were the formulations who changed less and more in comparison to t0, respectively.

During AS2 (Table 5) L^* increased significantly ($p \leq 0.05$) between t0 and t5 for B-C and B-OLE. Lu et al. (2009) report that the exposure of biscuits to UV light causes a discolouration effect on the surface. For B-MCR the opposite trend was observed, indeed for this sample L^* decreased significantly between t0 and t5; such result can be probably associated to the protective effect of the matrix over the phenolic compounds or

phenomena of water absorption and lipid oxidation. Regarding a^*, an overall increase was observed for all formulations. This trend, similar to what observed during AS1, may be associated to the absorption of water from the environment; a positive correlation has been indeed found between a^* and the product a_w ($R = 0.681$; $p \leq 0.05$). The parameter b^* decreased between t0 and t10 for all formulations, probably due to the light effect; Duta et al. (2019) claim to have observed an attenuation of the yellow component of biscuits stored in the presence of light. For all formulations the ΔE_{st} values were lower than 3, with B-OLE showing the smallest difference in comparison to t0.

4. Conclusions

This study highlights, for the first time, the potential use of olive leaf extracts (OLE) as natural additives to prolong the shelf life of biscuits. The results of the work attest that OLE, in concentration of 500 μg GAE/ g of dough, may be effectively used for this purpose, both in free and encapsulated form. Higher radical scavenging activity and oxidative stability have been detected for the enriched biscuits in comparison to the controls, being the ones enriched with encapsulated polyphenols even more stable. On the other hand, the presence of alginate and pectin as microsphere wall materials influenced the water absorption phenomena, resulting in hardening of the biscuits texture. The results have been confirmed under both the tested accelerated storage conditions (55°C; RH 50%), in presence of UV light or by storing the biscuits in an OPP bag in the dark. Further studies under real storage conditions will be conducted in order to confirm these promising results.

Ethical Statement - Studies in humans and animals

No studies on animals were conducted in this research work.

For the preliminary sensory analysis, an appropriate protocol for protecting the rights and privacy of all participants was utilized during the execution of the research (no coercion to participate, full disclosure of study requirements and risks, written or verbal consent of participants, no release of participant data without their knowledge, ability to withdraw from the study at any time).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data Availability

Data will be made available on request.

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