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Ceratonia siliqua L. kibbles, seeds and leaves as a source of volatile bioactive compounds for antioxidant food biopackaging applications

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ABSTRACT

Macerates of kibbles, seeds and leaves of *Ceratonia siliqua* L. were prepared and analysed to apply them for the development of a new multilayer active packaging. The profile of forty-three different bioactive volatile compounds was obtained employing headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS). The antioxidant capacity was investigated using the following methods: 2,2-diphenyl-1-picrylhydrazyl and phosphomolybdenum method, which demonstrated stronger antioxidant capacity in the case of seeds macerates. However, the analysis of the antimicrobial properties of the different macerates versus *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Escherichia coli* revealed a weak antibacterial activity. Additionally, the different macerates were incorporated into an adhesive used to build a new biopackaging multilayer film based on cellulose polymer, whose antioxidant capacity was evaluated by two different methods (2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical assay). The films with the best antioxidant activity, containing carob seeds macerates, were selected for the food safety assessment through migration assay. The obtained results showed no migrants, neither in the case of the volatile compounds nor non-volatile ones. Besides, the CIE $L^*a^*b^*$ colour of the active films was evaluated. The obtained results are therefore promising for future production of active biopackaging at an industrial scale.

1. Introduction

The demand for healthy, nutritious, safe, and more natural products has recently increased the search for new food production, processing, and preservation technologies. Consumer concerns about the quality of foods are reflected in an awareness of nutritional composition, bioactive components, and safety issues (Castro, Andrade, Silva, Vaz, & Vilarinho, 2019).

Food oxidation is a significant cause of food quality drop, affecting both the nutritional and sensory properties and its safety. Thus, it is a challenge for the food preservation industry to maintain refrigerated foods fresh (Carrizo, Taborda, Nerín, & Bosetti, 2016). Consequently, considerable research has been directed toward various preservation technologies (Sallam, 2007), such as the addition of synthetic antioxidants directly into food (Mohan, Ravishankar, Lalitha, & Srinivasa Gopal, 2012). As a result, health concerns about food ingredients have led to an increase in the request for more natural foods free of additives (Djenane, 2015). In this sense, the use of natural antioxidants is emerging as an effective alternative for product preservation (Sabeena Farvin, Grejsen, & Jacobsen, 2012). To this end, many sources of natural antioxidants have been investigated, such as herbs, plants, fruits, essential oils, natural pigments, and apiculture products (Oudjedi, Manso, Nerin, Hassissen, & Zaidi, 2019). Nevertheless, the direct addition of natural antioxidants into food formulations is challenging because they tend to be less potent than synthetic additives and therefore must be added in more significant amounts (Belasli et al., 2020; Carrizo, Gullo, Bosetti, & Nerín, 2014; Echegoyen & Nerín, 2015; Nerin, Astudillo, Covían, & Mujika, 2006; Nerín, Tovar, & Salafranca, 2008). This fact presents some disadvantages from the technological point of view, due to the intense flavour and smell, causing the alteration of the sensory characteristics of the product (El-Sayed, 2014). To overcome this challenge, the alternative approach is to incorporate them into the

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polymeric packaging instead of food (Akrami et al., 2015; Camo, Lorés, Djenane, Beltrán, & Roncalés, 2011; Djenane, Beltrán, Camo, & Roncalés, 2016; López, Sánchez, Batlle, & Nerín, 2007). Active antioxidant packaging is a novel concept and emerging packaging technology that acts by releasing, in a controlled way, the active agents to the surface or the inner atmosphere of the product, or just by scavenging radicals responsible for deleterious effects on food quality (Nerin et al., 2006). Several antioxidant packaging materials have been proposed (Carrizo et al., 2014, 2016; Tovar, Salafranca, Sánchez, & Nerín, 2005). They have proven that the addition of natural antioxidants into packaging present significant advantages, due to the improvement of the safety and the sensory properties, delay of the food spoilage, slowdown of the oxidation, and extension the shelf life of packaged foods without compromising their quality (Borzi, Torrieri, Wrona, & Nerín, 2019; Dong, Xu, Ahmed, Li, & Lin, 2018; Sanches-Silva et al., 2014). Moreover, there is an increasing interest in the use of bio-based polymer films (Al-Tayyar, Youssef, & Al-Hindi, 2020a, 2020b), which are produced from the waste of the food industry or from underutilized means of polysaccharides, lipids, and proteins. This innovative approach has been originated from environmental concerns, the increasing burden of disposing of plastic waste and the industrial use of food waste. In a recent review (Al-Tayyar et al., 2020a, 2020b), the use of carboxymethyl cellulose/polyvinyl alcohol/CuO bionanocomposites in the coating of processed cheese demonstrated a reduction in the growth of microbial contaminants, and noticeably extended and enhanced its shelf-life. Also, a covered cheese with a probiotic, edible coating based on chitosan, alginate and carboxymethyl cellulose gained sensory properties during storage (El-Sayed, El-Sayed, Mabrouk, Nawwar, & Youssef, 2021). Another study on coating fruits with a combined chitosan-beeswax pollen grain emulsion demonstrated to be the best treatment for maintaining the quality of Le Conte pears during storage life and marketing periods (Sultan, Hafez, Saleh, & Youssef, 2021).

Several approaches have been proposed to manufacture active packaging without modifying the production line or the characteristics of the packaged product, but all of them fail by one or another reason. Extrusion of polymers involves high temperature, causing the decomposition of active agents. Coating systems could affect the sensory properties of the packaged food. However, in all these approaches, the antioxidant agent is incorporated in the layer in contact with food (Carrizo et al., 2016). A new system has been explored by eliminating the free radicals responsible for the initiation of the oxidation phenomenon. Since the free radicals can efficiently diffuse through the polymer, they can be trapped by the antioxidant agents incorporated in the adhesive between polymer films of a multilayer packaging, thus avoiding the direct contact of active agents with the packaged food (Djebari et al., 2021; Moudache, Colon, Nerín, & Zaidi, 2016; Oudjedi et al., 2019).

Carob (*Ceratonia siliqua* L.) is a flowering evergreen tree grown in the Mediterranean area (Rtibi et al., 2015). The scientific name of this tree came from the Greek word “kera” connected with the shape of the carob fruit, and the Latin word “siliqua”, associated with the hardness of the pods (Papaefstathiou, Agapiou, Giannopoulos, & Kokkinofta, 2018). Carob fruits consist of pods and seeds. They are considered powerful antioxidants due to the high content of bioactive phytochemicals (Santonocito et al., 2020). Moreover, their antimicrobial activity has been recently demonstrated against different bacteria (Ben Othmen, Garcia-Beltrán, Elfalleh, Haddad, & Esteban, 2021; Fidan et al., 2019; Goulas et al., 2019). Deseeded broken carob fruits, commonly known as kibble, are also a source of natural bioactive compounds. The effective utilization of this industrial by-product is becoming the focus of researchers (Nasar-Abbas et al., 2016; Owen et al., 2003). Furthermore, carob leaves are rich in polyphenols and flavonoids (Rtibi et al., 2015).

Various in vitro studies have shown that extracts of the carob tree have a potent antioxidant effect (Roseiro, Tavares, Roseiro, & Rauter, 2013; Sebai et al., 2013). They also have strong scavenging activity on reactive oxygen and free radicals, thus reducing oxidative damage (Roseiro, Duarte, et al., 2013; Roseiro, Tavares, et al., 2013).

To the best of our knowledge, macerates of *Ceratonia siliqua* L. kibbles, seeds and leaves have not been tested as potentially active agents in antioxidant multilayer biopackaging for food applications. Moreover, a complex profile of volatile bioactive compounds from the Algerian carob variety has been determined.

The main aim of this investigation was to design, optimize and prepare new multilayer active biopackaging films based on cellulose and the macerates of leaves, seeds, and kibbles of *Ceratonia siliqua* L. from Algeria incorporated in the adhesive between polymer layers. First of all, the profile of volatile bioactive compounds was studied, and the compounds were identified and quantified. Then, the antioxidant and antimicrobial activities of the macerates were measured. Afterwards, the antioxidant capacity of active packaging materials was tested using the in-situ hydroxyl radical generation method developed by Pezo, Salafranca, and Nerín, (2006), Pezo, Salafranca, and Nerín (2008) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Finally, the safety of the new antioxidant films for biopackaging applications has been evaluated by migration tests. The material selected as the substrate is from natural resources, which is interesting and emerges as a response to growing attention to environmental pollution and environmental footprint.

2. Material and methods

2.1. Chemicals

Acetic acid ($\geq$ 99.7%, CAS 64-19-7), ascorbic acid ($\geq$ 99.0%, CAS 50-81-7), ammonium heptamolybdate tetrahydrate ($\geq$ 99.0%, CAS 12054-85-2), 2,2-diphenyl-1-picrylhydrazyl (DPPH, CAS 1898-66-4), hydrogen peroxide (30%, CAS 7722-84-1) and sodium salicylate ($>$ 99.5%, CAS 54-21-7) were supplied by Sigma-Aldrich (Madrid, Spain). Sodium dihydrogen phosphate monohydrate (99%, CAS 7558-80-7) was acquired from Merck (Madrid, Spain). Sulphuric acid (H_2SO_4, 96%, CAS 7664-93-9) was from Panreac Química SLU (Barcelona, Spain). Phosphoric acid (85%, CAS 7664-38-2) was purchased from Scharlab (Barcelona, Spain). The plants were macerated using ethanol (99.9%, CAS 64-17-5) and acetone (99.9%, CAS 67-64-1) from Panreac. Methanol ($>$ 99.9%, CAS 67-56-1) was from Honeywell (Madrid, Spain). Butylated hydroxyanisole (BHA, $\geq$ 98.5%, CAS 25013-16-5) was purchased from Sigma Aldrich, represented by the Algerian Chemical Society. Ultrapure water was produced in a Wasserlab Ultramatic GR system (Barbatáin, Spain).

2.2. Samples

The fruits and leaves of carob (*Ceratonia siliqua* L.) were collected after fruit harvesting during December 2018 from carob trees located in the region of Tizi-Ouzou (Algeria, coordinates: 36°43′ N, 4°3′ E), where they are naturally grown.

2.3. Sample preparation

Carob pods (CP) and leaves (CL) were cleaned with tap water, the carob seeds (CS) were separated manually from the fruits, and the carob kibble (CK) were recuperated. The photograph of the samples is shown in Fig. 1.

The samples were air-dried at room temperature for ten days. Then, dried samples were ground by an electric grinder to obtain a mean particle size below 0.5 mm. The grinded samples were stored in airtight glass containers in the dark until being macerated as described below.

2.4. Maceration of samples

An amount of 30 g of powdered samples was macerated with 500 mL of 80% aqueous solution of ethanol and 80% aqueous solution of acetone at room temperature. The process was sequentially repeated

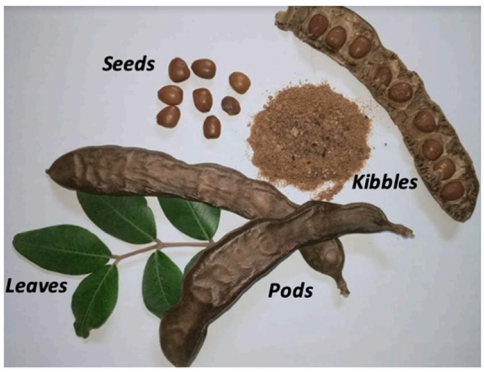

Fig. 1. Photograph of *Ceratonia siliqua* L. samples such as pods, leaves, seeds and kibbles.

three times with the renewal of the solvent each 24 h. The macerates were filtered through Whatman filter paper of 0.22 μm pore size and stored in the dark at 4 °C until further use. The following abbreviations of macerates were applied: CLE, CSE and CKE, for leaves, seeds and kibbles respectively, macerated in 80% ethanol, and CLA, CSA and CKA for leaves, seeds and kibbles macerated in 80% acetone.

2.5. Active packaging material

The material selected as substrate was cellulose film NK from the Natureflex product range supplied by Futamura UK Ltd (Burgos, Spain). A multilayer active material was prepared using two cellulose polymer sheets glued together using a water-based biodegradable adhesive for food packaging from Samtack (Barcelona, Spain). Details about the adhesive formula cannot be disclosed because of confidential reasons. Different concentrations of 5%, 8%, and 10% (w/w) of each macerate were prepared in the adhesive and tested. The mixture was vortexed for 1 min and allowed to stand at room temperature until total homogenization. The maximum concentration chosen for further preparation of active biopackaging was 8%. The selected concentration was optimum as it did not affect the adhesion properties of adhesive.

The active adhesive was spread on the cellulose sheet using a K Control Coater from RK Print Coat Instruments (Litlington, UK). The coating was performed by wire wound bar number 4 (colour code: black; wire diameter: 0.51 mm; wet film deposit: 40 μm). After air-drying of the solvent, the cellulose sheet with adhesive was covered by another cellulose layer. Finally, multilayer biofilm was pressed at 40 °C and speed set to 5, using BiO 330 A3 Heavy Duty Laminator (South Korea). In addition, a blank biomaterial was prepared under identical conditions but without active macerate.

2.6. Analysis of macerates

2.6.1. Identification of volatile compounds

The volatile compounds of macerates of leaves, seeds and kibbles of *Ceratonia siliqua* L. were analysed by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS). Each macerate was diluted 5 times with ultrapure water. Chromatographic analysis was performed using a CTC Analytics autosampler from Agilent technologies (Madrid, Spain). A GC Agilent 6890N coupled to a mass-selective detector Agilent MS 5975B was used to analyse the samples. HS-SPME-GC-MS analyses were carried out with a 100 μm polydimethylsiloxane (PDMS) fibre from Supelco (Bellefonte, PA, USA) previously conditioned before the first extraction according to the manufacturer specifications. The extraction conditions were as follows: 80 °C extraction temperature, 15 min extraction time, and 2 min desorption time. The injection was performed in splitless mode. Helium was used as carrier gas at a flow rate of 1.0 mL/min. The oven temperature was as follows: 40 °C for 2 min, with a rate of 10 °C min up to 300 °C held for 2 min. Volatile compounds were separated on HP-5MS GC column (30 m × 0.25 mm × 0.25 μm film thickness, Agilent). The mass detector was used in scan mode in the *m/z* range from 45 to 350. The inlet, ion source, and quadrupole temperatures were 250, 230 and 150 °C, respectively. Analysis of blanks was also performed. All samples were analysed in triplicate.

Bioactive volatile compounds identification was based on the GC-MS spectrum interpretation compared with the spectrum database of the National Institute Standard and Technology (NIST) library. It allowed the confirmation of the name, molecular weight and structure of the chemical compounds from *Ceratonia siliqua* L. macerates. Compounds with experimental retention index close to those reported in the literature and match values higher than 80% were considered as correctly identified.

Bioactive volatile compounds semi-quantitative analysis was based on the percentage analysis. The percentage abundance of compounds in the analysed samples was expressed as the integrated area of each compound divided by the total area of all compounds. The sum of areas of all determined compounds was considered as 100% (Ruiz-Hernández, Roca, Egea-Cortines, & Weiss, 2018).

2.6.2. Antioxidant activity – DPPH method

The scavenging activity of carob leaves, seeds and kibble macerates against DPPH radical was examined (Brand-Williams, Cuvelier, & Berset, 1995). Before the test, a new 60 μM methanolic solution of DPPH was prepared. Briefly, 50 μL of each macerate were added to 1950 μL of DPPH solution. The absorbance of the mixture was determined at 515 nm with a Shimadzu UV-1700 PharmaSpec spectrophotometer (Duisburg, Germany) after 30-min incubation. Five concentrations of ascorbic acid and BHA (40, 80, 120, 160, 200 μg/mL) were prepared and used as a positive control. All the measurements were performed in triplicate. Radical scavenging activity was expressed as the inhibition percentage of free radicals by the sample and calculated according to Eq. (1) (Taylor & Todd, 1995).

$$\text{Scavenging effect}(\%\ \text{inhibition}) = (A_{\text{control}} - A_{\text{sample}})/(A_{\text{control}}) \cdot 100\% \quad (1)$$

Where $A_{control}$ corresponds to the absorbance of the blank (1950 μL of DPPH with 50 μL of methanol), and A_{sample} corresponds to the absorbance of the sample (1950 μL of DPPH with 50 μL of the sample). The IC_{50} (concentration providing 50% inhibition) values were calculated from the plotted graph of scavenging activity against the concentration of the samples.

2.6.3. Total antioxidant capacity (TAC)

The total antioxidant capacity of the macerates was evaluated by adapting the method used by Mekhoukhe et al. (2019). A volume of 400 μL (25–85 μg/mL) of each macerate were mixed with 1 mL of reagent solution consisting of a mixture of 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. Then, it was incubated at 90 °C for 90 min and was cooled down at room temperature. The absorbance of the solution was read at 695 nm. The total antioxidant activity was expressed as ascorbic acid equivalent from a calibration curve ($y = 0.0146\ x + 0.0097$).

2.6.4. Antimicrobial activity

Antimicrobial activity of macerates was tested against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 by the disk diffusion method. Briefly, 100 μL of a suspension of the tested microorganism (10^8 CFU/mL) was spread on Mueller-Hinton agar plates. Sterile paper disks (9 mm) were impregnated with 75 μL of the macerated solution and placed on inoculated plates. After 24 h at 37 °C the diameters of the inhibition zones were measured. Each test was performed in triplicate.

2.7. Analysis of active biopackaging

2.7.1. Migration tests

One-side migration tests have been performed using two different food simulants (10% and 95% solutions of ethanol) for 3 days at 5 °C. The simulants and parameters were based on recommendations of legislation for food contact materials, Commission Regulation (EU) No 10/2011, and its amendments (Union Europea, 2011). The conditions of migration tests were selected according to the intended use of the developed active biofilm such as the conservation of fresh, processed or marinated meat, fish or seafood. Also, it could be applied for preserved vegetables, cheese and meat in the oil medium; fried or roasted foods of animal origin and fried potatoes, cakes and the like; sandwiches, toasted bread, pizza and similar containing any kind of foodstuff with fat matter on the surface. Contact time between food and active biopackaging in worst foreseeable use should be between 1 and 3 days, and the worst foreseeable contact temperature of stored food should be > 5 °C.

Two sets of multilayer materials containing CSE and CSA macerate (8%) were studied. Three replicates of each sample were prepared. Blank samples consisting of only pure simulants were prepared. In addition, blank material without any active agent was considered. Qualitative analysis of volatile and non-volatile compounds was performed using the method described below.

2.7.2. Volatile compounds

To determine the volatile compounds migrated from the active biofilms into 10% ethanol food simulant, HS-SPME-GC-MS already described in Section 2.6.1. has been used. In contrast, samples of 95% ethanol simulant were analysed in splitless mode (2 min) by direct injection-gas chromatography-mass spectroscopy (DI-GC-MS). In this case, the solvent delay was 5 min, and injection volume was 1 μL. The chromatographic conditions were the same as those used for the HS-SPME-GC-MS method.

2.7.3. Non-volatile compounds

Both simulants, 10% and 95% ethanol, were analysed by the ultra-high performance liquid chromatography coupled to quadruple time-of-flight with MSE technology (UPLC-ESI-Q-TOF-MS^E) to determine the non-volatile migrants. An Acquity UPLC system (Waters, Milford, MA) equipped with a quadrupole time-of-flight mass spectrometer (Xevo G2-XS QTOF, Waters) was used. Chromatography was carried out using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm × 1.7 μm particle size). The autosampler temperature was 10 °C, the column flow was 0.3 mL/min, and the column temperature was 35 °C. Water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) were used as mobile phase, and the volume of sample injected was 10 μL. The optimized UPLC conditions were as follows: 0–6 min 5% A/95% B; 6–8 min 5% A/95% B; 8–8.1 min, 95% A/5% B; 8.1–10 min, 95% A – 5% B. The electrospray ionization interface was used in both positive (ESI+) and negative (ESI-) modes. Sensitivity mode was used to operate the mass spectrometer with a capillary voltage of 3 kV and a sampling cone voltage of 30 V. Source temperature was set at 150 °C, and the flow rate of desolvation gas (nitrogen) was 450 L/h at 400 °C. MS^E mode selected for the data acquisition involves the fast alternation between two energy conditions: low energy without collision ramp, and high energy with collision ramp from 5 to 30 eV. The mass range considered was 10–1200 Da. Centroid mode was used for data collection. The software Masslynx (version 4.1, Waters) was used for data acquisition and processing.

2.7.4. DPPH radical scavenging capacity

The DPPH radical scavenging capacity of multilayer films was determined according to the method described by Wrona, Cran, Nerín, and Bigger (2017). Sonication was performed for 30 min to extract active substances from 1 dm^2 of film, both active and blank, previously placed in an 18 mL glass vial with 6 g of methanol. Then, 50 μL of different concentrations of macerates (40, 80, 120, 160, 200 μg/mL) were mixed with 1950 μL of DPPH methanolic solution (60 μM) and kept in the dark at room temperature for 30 min. The absorbance of the solution was measured against methanol as blank at 515 nm using a spectrophotometer. The results were expressed as IC_{50}, meaning the concentration providing 50% inhibition.

2.7.5. Hydroxyl radical assay

The multilayer biofilms containing 8% of each macerate of *Ceratonia siliqua* L. were exposed to an atmosphere enriched in OH· free radicals passed through bag-shaped samples prepared according to the procedure previously developed by Pezo et al. (2008). The method consists of salicylic acid hydroxylation by the OH· radicals generated from aqueous hydrogen peroxide (0.29 mol/L) in vapour phase under UV-light irradiation. The generated atmosphere enriched in OH· radicals is carried by air through the bag made of active material, and finally it bubbles into a salicylic acid solution. Salicylic acid reacts with the not scavenged OH· radicals and forms 2,5-dihydroxybenzoic acid (2,5-DHB) as a primary compound. Quantitative analysis of 2,5-DHB and the residual salicylic acid was performed by high-performance liquid chromatography (Waters Alliance 2695 Separations Module with a 474 Scanning Fluorescence Detector). A Waters reversed-phase column (100 mm × 4.6 mm × 3 μm particle size) Atlantis dC18 was used. The mobile phase was a mixture of acetate buffer (40 mM, pH = 5.9) and methanol (90:10, v/v), with an injection volume of 20 μL. Excitation and emission wavelengths were set at 324 and 448 nm, respectively. Radical scavenging activity was calculated using Eq. (2).

$$\text{Radical scavenging activity } (\%) = \text{Area}_{\text{2,5-DHB AOX}}/\text{Area}_{\text{2,5-DHB BK}} \cdot 100\% \quad (2)$$

Where: $\text{Area}_{\text{2,5-DHB AOX}}$ is the area of peak of 2,5-DHB in case of active biofilm, and $\text{Area}_{\text{2,5-DHB BK}}$ is the area of peak of 2,5-DHB in case of a blank film.

2.7.6. Characterization of the packaging colour

The colour of the active biofilms containing CL, CS and CK macerates, and the control material, was determined by using a Chroma Meter CR-400 from Konica Minolta (Osaka, Japan) as described by Marcos et al. (2014). C illuminant and 2° standard observer conditions were chosen. L* represents lightness (L* = 100) and darkness (L* = 0), a* represents redness and greenness for positive/negative values, and b* represent yellowness and blueness colour for positive/negative values. The values were determined in the 1976 CIELAB colour space system. The chroma meter was calibrated with a standard whiteboard before each series of measurements (Y = 93.70, x = 0.3130, y = 0.3191). The mean of 18 measurements was recorded for each film. Three replicates were tested.

The total colour difference (ΔE) was determined as an estimate of colour changes according to Eq. (3).

$$\Delta E = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (3)$$

The colour values of control films (cellulose/adhesive/cellulose) were used as reference values for ΔE calculation (L_0^*, a_0^*, b_0^*).

2.8. Statistics

Three replicates of all samples were performed and mean ± standard deviation was obtained. Therefore, error bars on the figures are standard deviations. Significant differences ($p \leq 0.05$) between samples in the case of all performed analytical tests were determined using *t*-test. At the beginning, the null hypothesis assumption was considered: analysed samples are equal. To confirm it, the experimental *t*-test statistic value was calculated compared to the theoretical value *t*-test statistic from tables. Null hypothesis was true if compared values were equal or theoretical *t*-test statistic value was higher than the experimental one.

Otherwise, analysed samples were significantly different ($p \leq 0.05$).

3. Results and discussion

3.1. Identification of volatile compounds

The volatile compounds of *Ceratonia siliqua* L. were extracted by SPME and analysed by GC-MS. This technique is solvent-free, cheap, easy, fast, and sensitive. In addition, it needs lower volume of sample than hydrodistillation, the most common method for plant extraction, which is time-consuming and requires a large amount of plant material (Zouaoui, Chenchouni, Bouguerra, Massouras, & Barkat, 2020).

Table 1 shows the identified volatile bioactive compounds, numbered according to their elution order. Also, their relative retention time (RT), retention index (RI), relative area (%), chemical formula and class are provided. A total of forty-three compounds were identified, but only a few of them were similar to those described in previous works (Ben Ayache et al., 2020; Farag & El-Kersh, 2017). This dissimilarity can be certainly explained by the differences in cultivars and the ecological factors affecting the growth process. Moreover, different extraction methods and sampling techniques may influence qualitative analysis (Ben Ayache et al., 2020). CLE was the sample that contained more volatile compounds (21), followed by CKE (18), CKA (15), CLA (13), CSE (9) and CSA (6). The same compounds were identified at trace level or with lesser relative abundance in the other macerates. Besides, in some cases, no compounds were detected. All determined substances were classified according to their chemical structure, and the distribution of volatile compounds represented by a pie chart is depicted in Fig. 2.

According to the results from Table 1, thirteen different chemical classes were determined, which indicates that the obtained extracts have a different composition of volatile compounds. Semi-quantitative analysis of the volatile bioactive compounds was based on the percentage analysis, where the abundance of compounds in each macerate was expressed as the integrated area of each compound divided by the total area of all compounds and results were represented in Fig. 2, which helped us to determine the predominant volatile compounds contained in the different macerates. Esters had a higher abundance in CSE, CKE and CLE samples. They reached 96.56%, 48.07% and 18.09% of the total area, respectively, where the ethyl hexadecanoate was recorded as the main component in such samples (53.45%, 27.19% and 14.32%, respectively). In contrast, the relative peak areas for terpenoid alcohols and sesquiterpenes were much higher in CLE (33.18% and 21.15%, respectively). A significant level of sesquiterpenes was determined in CLA (7.55%). At the same time, the CKE had the opposite tendency (0.15%). Carboxylic acids were found mainly in CKE and CKA, particularly n-hexadecanoic acid (5.70% and 24.79%, respectively). Nevertheless, the main compounds of CLA, CKA and CSA were diterpenoids (68.09%), ketones (47.14%) and sesquiterpenoids (39.48%), respectively. Several other less abundant volatile compounds were detected including monoterpenes (0.28–0.52%), alkanes (0.59–9.24%), alkenes (0.65–1.29%), alkylbenzenes (0.48–10.29%) alcohols (4.71%) and aldehydes (1.43–2.86%).

Natural antioxidants can work as scavengers, either by preventing the formation of reactive species or by removing them before they start damaging food processes. According to the literature (Djebari et al., 2021; Pinto et al., 2021), the antioxidant properties can be related to various bioactive volatile compounds.

Monoterpenes and sesquiterpenes are widely present in plants because they act as allelopathic agents. Their antioxidant capacity has been studied and proven. They can act as free radical quenchers and function through either the hydrogen donor or electron donor mechanism (Djebari et al., 2021). In fact, it has been previously shown that species rich in these compounds possessed appreciable antioxidant activity. Kelen and Tepe (2008), Mimica-Dukic, Bozin, Sokovic, and Simin (2004), Wei and Shibamoto (2007) demonstrated the antioxidant activity of the monoterpenes, especially the limonene. Singh et al. (2010) reported that the antioxidant activity of essential oils in terms of free radical scavenger was related to the antioxidant activity of DL-limonene. Previous studies have demonstrated that the species rich in caryophyllene possessed appreciable antioxidant activity (Nafis et al., 2019; Salleh, Kammil, Ahmad, & Sirat, 2015; Sarikurkcu, Ozer, Calli, & Popović-Djordjević, 2018). In addition, it was reported that the sesquiterpene (caryophyllene) showed an antioxidant activity concerning the neutralization of the DPPH radical (Mimica-Dukic et al., 2004). The sesquiterpene germacrene D was demonstrated as a strong antioxidant due to its extra cyclic methylene chemical structure (Victoria et al., 2012). Badr, Badawy, and Taktak (2021) reported that camphene, as the most significant component of *Lavandula spica* essential oil, exhibited a moderate scavenging capacity of the radical DPPH. In contrast, a study of the antiradical activities of six camphene-based thiosemicarbazones was investigated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and peroxyl radical scavenging capacity (PSC) assays, respectively, and the results revealed that this compound exhibited a good ability for scavenging free radicals in a dose-dependent way (Yang, Liu, Xia, & Wang, 2020). Bakkali, Averbeck, Averbeck, and Idaomar (2008) found that camphene showed a significant antioxidant property against oxidative stress in murine alveolar macrophage induced by tert-butyl hydroperoxide, causing a significant decrement in the lipid peroxidation.

It is known that the antioxidant properties of active extracts cannot be related to only their major constituents (Bakkali et al., 2008). Other minor components could also contribute by synergistic effects to the obtained antioxidative activity, as well as by the interaction among the compounds (Kasrati et al., 2015; Pereira, Severino, Santos, Silva, & Souto, 2018). The analyses revealed that carob leaves, seeds, and kibbles were rich in volatile bioactive compounds characterized by antioxidant properties. Therefore, *Ceratonia siliqua* L. macerates can be considered as a good source of active agents for being used in active biopackaging films.

3.2. Antioxidant capacity of macerates

3.2.1. DPPH radical assay

The antioxidant activity of *Ceratonia siliqua* L. macerates was performed using the DPPH method. This in vitro test was chosen due to its simplicity, stability, and reproducibility (Benchikh, Louaileche, George, & Merlin, 2014). It is mainly associated with the ability of bioactive compounds to adsorb and neutralize free radicals (Popovici & Saykova, 2009). As given in Fig. 3a, the obtained results showed that CL, CS and CK macerates have powerful antioxidant activity. The antioxidant activity of the different macerates was evidenced from the obtained results compared to the performances of the standards used.

This activity was higher in CSE and CSA. All obtained values were significantly different ($p \leq 0.05$). Additionally, very high reproducibility of analysed replicated was obtained. Indeed, powerful antioxidant activity depends on the part of the plant and the solubility of bioactive compounds in the chosen solvent. In this sense, our results agree with previous findings. Mekhoukhe et al. (2019) demonstrated that the antioxidant activity of CS depended on the solvent polarity (aqueous acetone 70% > aqueous ethanol 80% > aqueous methanol 80%). In another study, the ethanolic extract of CL showed a promising antiradical effect (Hajaji et al., 2011). However, the acetonic extract of CK provided the strongest activity, according to Makris and Kefalas (2004). Benchikh et al. (2014) demonstrated that all assayed extracts of carob pulp varieties from Algeria can scavenge hydrogen- or electron-donating mechanisms and were highly correlated ($p < 0.001$) with their bioactive phytochemical contents. The efficacy of solvents on the antioxidant activity of the extracts was also studied by Goulas et al. (2019) with DPPH assay, and the results demonstrated that the ethyl acetate and acetone extracts had the lowest antioxidant activity, while it was revealed that the most promising solvents for the recovery of carob antioxidants were acetone–water (80:20, v/v), acidic acetone, acidic methanol, and water. As a result of the literature search, the antioxidant

Table 1
Results of identification and semi-quantification (expressed as percentage) of bioactive volatile compounds of CL, CS and CK of *Ceratonia siliqua* L. macerates. Compounds not detected in the different samples are marked with the symbol "–".

No.	Retention time (min)	Retention index	Compound	Formula	CAS	Chemical class	Relative percentage (%)					
							CLE	CLA	CKE	CKA	CSE	CSA
1	8.531	920	Camphene	$C_{10}H_{16}$	79-92-5	Monoterpene	–	0.61 ± 0.07	–	–	–	–
2	8.778	952	4-Ethyl-octane	$C_{10}H_{22}$	15869-86-0	Alkane	–	–	0.28 ± 0.03	–	–	–
3	9.755	1000	Decane	$C_{10}H_{22}$	1Z24-18-5	Alkane	–	0.52 ± 0.07	–	0.26 ± 0.03	–	–
4	10.197	1022	o-Cymene	$C_{10}H_{14}$	527-84-4	Alkybenzene	–	–	–	–	0.48 ± 0.22	10.29 ± 1.07
5	10.285	1030	DL-Limonene	$C_{10}H_{16}$	138-86-3	Monoterpene	–	–	0.28 ± 0.07	–	0.52 ± 0.22	–
6	13.237	1200	Dodecane	$C_{12}H_{26}$	112-40-3	Alkane	–	0.65 ± 0.08	0.78 ± 0.28	2.06 ± 0.09	–	16.17 ± 4.12
7	14.707	1300	Tridecane	$C_{13}H_{28}$	629-50-5	Alkane	–	0.40 ± 0.04	–	–	–	–
8	15.049	1322	Isocetane	$C_{16}H_{34}$	224-506-8	Alkane	–	0.87 ± 0.08	–	–	–	–
9	15.199	1325	4,6-dimethyl-dodecane	$C_{14}H_{30}$	61141-72-8	Alkane	–	–	–	0.83 ± 0.07	–	–
10	15.442	1351	α-Cubebene	$C_{15}H_{24}$	17699-14-8	Sesquiterpene	0.34 ± 0.12	–	–	–	–	–
11	15.943	1392	1-Tetradecene	$C_{14}H_{30}O$	1120-36-1	Alkene	0.65 ± 0.29	1.29 ± 0.29	–		–	–
12	16.047	1400	Tetradecane	$C_{14}H_{30}$	629-59-4	Alkane	0.67 ± 0.19	2.76 ± 0.13	1.97 ± 0.69	3.87 ± 2.54	0.59 ± 0.23	20.19 ± 3.63
13	16.406	1419	Caryophyllene	$C_{15}H_{24}$	87-44-5	Sesquiterpene	11.04 ± 3.45	6.88 ± 0.09	–	–	–	–
14	16.602	1427	Nonyl 2-methylpropanoate	$C_{13}H_{26}O_2$	10522-34-6	Ester	–	–	–	0.35 ± 0.04	–	–
15	16.849	1454	Humulene	$C_{15}H_{24}$	6753-98-6	Sesquiterpene	0.83 ± 0.26	–	–	–	–	–
16	17.103	1477	γ-Muurolene	$C_{15}H_{24}$	30021-74-0	Sesquiterpene	1.74 ± 0.61	–	–	–	–	–
17	17.187	1481	Germacrene D	$C_{15}H_{24}$	23986-74-5	Sesquiterpene	3.17 ± 0.87	–	–	–	–	–
18	17.575	1485	α-Amorphene	$C_{15}H_{24}$	483-75-0	Sesquiterpene	1.09 ± 0.39	0.67 ± 0.02	–	–	–	–
19	17.622	1497	2-Tridecanone	$C_{13}H_{26}O$	593-08-8	Ketone	–	–	0.05 ± 0.02	–	–	–
20	17.675	1524	δ-Cadinene	$C_{15}H_{24}$	483-76-1	Sesquiterpene	2.94 ± 1.02	–	0.15 ± 0.02	–	–	–
21	17.680	1531	cis-Calamenene	$C_{15}H_{22}$	72937-55-4	Sesquiterpenoid	–	–	–	–	–	17.92 ± 7.33
22	18.427	1595	Ethyl dodecanoate	$C_{14}H_{28}O_2$	106-33-2	Ester	–	–	–	–	1.66 ± 0.83	–
23	18.498	1600	Hexadecane	$C_{16}H_{34}$	544-76-3	Alkane	0.46 ± 0.14	–	1.32 ± 0.11	2.22 ± 0.29	–	–
24	18.844	1623	α-Corocalene	$C_{15}H_{20}$	20129-39-9	Sesquiterpenoid	0.27 ± 0.08	–	–	–	–	–
25	19.450	1674	Cadalene	$C_{15}H_{18}$	483-78-3	Sesquiterpenoid	0.66 ± 0.23	–	1.53 ± 0.19	1.72 ± 0.03	0.77 ± 0.50	21.56 ± 2.21
26	19.613	1698	2-Pentadecanone	$C_{15}H_{30}O$	2345-28-0	Ketone	–	–	20.87 ± 2.28	31.80 ± 1.39	–	–
27	19.671	1710	2-Pentadecanol	$C_{15}H_{32}O$	1653-34-5	Alcohol	–	–	–	4.71 ± 0.23	–	–
28	19.792	1715	Pentadecanal	$C_{15}H_{30}O$	2765-11-9	Aldehyde	1.43 ± 0.50	2.45 ± 0.19	2.86 ± 0.30	2.49 ± 0.09	–	–
29	20.185	1780	Dodecyl butyrate	$C_{16}H_{32}O_2$	3724-61-6	Ester	–	–	4.27 ± 0.36	3.49 ± 0.25	–	–
30	20.636	1794	Ethyl tetradecanoate	$C_{16}H_{32}O_2$	124-06-1	Ester	0.51 ± 0.16	–	–	–	–	–
31	21.128	1837	Neophytadiene	$C_{20}H_{38}$	504-96-1	Diterpenoid	8.75 ± 1.34	68.09 ± 2.79	–	–	–	–
32	21.174	1844	6,10,14-Trimethyl-2-pentadecanone	$C_{18}H_{36}O$	502-69-2	Ketone	–	–	10.28 ± 0.47	11.63 ± 0.53	1.20 ± 0.24	14.67 ± 0.11
33	21.746	1902	2-Heptadecanone	$C_{17}H_{34}O$	2922-51-2	Ketone	–	–	4.93 ± 0.28	3.71 ± 0.19	–	–
34	21.984	1926	Methyl hexadecanoate	$C_{17}H_{34}O_2$	112-39-0	Ester	–	–	4.33 ± 0.38	3.96 ± 0.31	–	–
35	22.214	1948	Isophytol	$C_{20}H_{40}O$	505-32-8	Terpenoid alcohol	0.78 ± 0.10	0.79 ± 0.09	–	–	–	–
36	22.331	1968	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	57-10-3	Carboxylic acid	–	–	5.70 ± 6.23	24.79 ± 3.69	–	–
37	22.640	1993	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	628-97-7	Ester	14.32 ± 4.20	–	27.19 ± 1.83	–	53.45 ± 13.55	–
38	23.650	2110	(*E*)-Methyl-9-octadecenoate	$C_{19}H_{36}O_2$	1937-62-8	Ester	–	–	0.88 ± 0.11	–	–	–
39	23.771	2114	Phytol	$C_{20}H_{40}O$	150-86-7	Terpenoid alcohol	32.40 ± 4.60	32.40 ± 4.60	–	–	–	–
40	24.047	2139	9,12,15-Octadecatrienoic acid, (*Z*,*Z*,*Z*)-	$C_{18}H_{30}O_2$	463-40-1	Carboxylic acid	8.96 ± 10.95	–	–	–	–	–
41	24.205	2162	Ethyl linoleate	$C_{20}H_{36}O_2$	544-35-4	Ester	1.79 ± 1.42	–	–	–	36.55 ± 20.43	–
42	24.251	2174	(*E*)-Ethyl-9-octadecenoate	$C_{20}H_{38}O_2$	6114-18-7	Ester	–	–	11.40 ± 1.18	–	24.90 ± 5.14	–
43	24.477	2175	Methyl-17-methyl octadecanoate	$C_{20}H_{40}O_2$	55124-97-5	Ester	1.47 ± 1.42	–	–	–	–	–

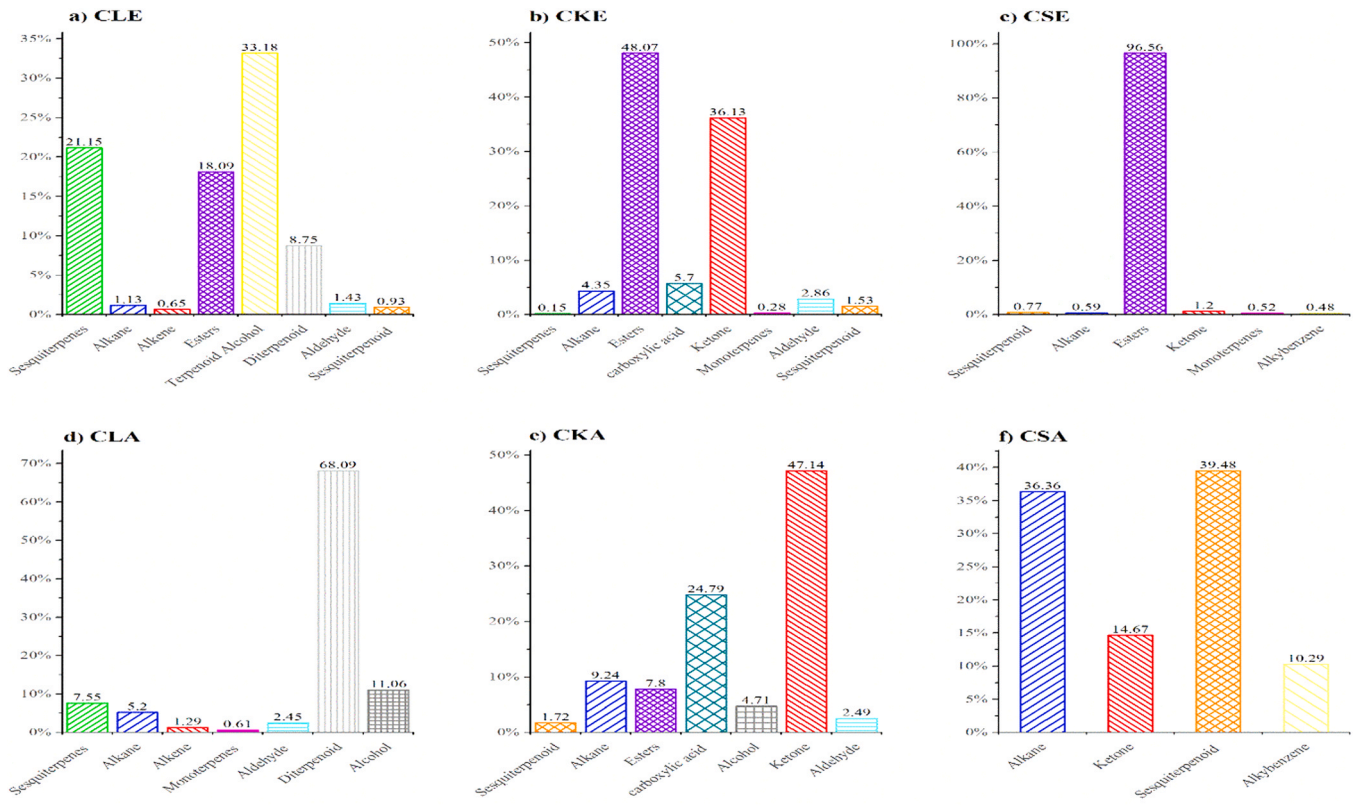

Fig. 2. Column graph representing percentage of determined compounds grouped into different chemical classes in various samples such as a) CKE; b) CLA; c) CSE; d) CKA; e) CLE; f) CSA.

activity of the different extracts is loosely related to their chemical composition, the determined major compounds and the possible synergistic effects established between them (De Saint Laumer, Frérot, & Herrmann, 2003). Bozin, Mimica-Dukic, Samojlik, and Jovin (2007) noted that the mixture of mono and sesquiterpene showed notable scavenging activity by a synergistic effect, which may explain the strong antioxidant activity that was observed through our results. Mimica-Dukic et al. (2004) reported that the monoterpenes (limonene) and sesquiterpenes (caryophyllene) are responsible for the neutralization of the DPPH radical. Wei and Shibamoto (2007) noted significant antioxidant activity of essential oils rich in monoterpenes (limonene). However, Kelen and Tepe (2008) reported that the monoterpene (limonene) individually tested did not have a significant antioxidant activity compared to the same constituent when tested together with α-pinene and β-pinene. Another in vitro study on the evaluation of the antioxidant activity of the D-Limonene confirmed a strong antioxidant activity by reducing the free radical formation in the different antioxidant assays (Shah & Mehta, 2018). DPPH scavenging capacity increased when the level of the OH- groupings present in the aromatic rings raises. However, other phenolic compounds that are present but were not determined may also have an antioxidant effect (Abidar et al., 2020). From our results, we can deduce that *Ceratonia siliqua* L. seeds, leaves and kibbles could be used as a potential source of antioxidants what can be seen in Fig. 3a.

3.2.2. *Phosphomolybdenum assay*

The phosphomolybdenum reduction is a quantitative method expressed as equivalents of ascorbic acid. It gives a direct estimation of the reducing capacity of the sample of interest. It is based on the reduction of phosphate-molybdenum (VI) to phosphate-molybdenum (V) and measurement of the absorbance at 695 nm due to the reduced green molybdenum complex (Sharadanand Phatak, Subhash Hendre, & Rohan Sharadanand Phatak, 2014). The total antioxidant capacity of CS, CL and CK macerates is shown in Fig. 3b. It decreases in the following order: CSE > CSA > CLE > CLA > CKE > CKA. Their respective total antioxidant capacities were 72.81 ± 0.09; 67.22 ± 3.91; 53.52 ± 0.09; 51.76 ± 0.20; 26.96 ± 2.14 and 12.35 ± 0.46 μg/mL (expressed as ascorbic acid equivalents).

Several in vitro studies showed that the carob tree extracts have potent antioxidant effects (Rtibi et al., 2015; Sebai et al., 2013). A study reported by Mekhoukhe et al. (2019) on the antioxidant activity of CS extracts demonstrated that TAC decreased in the following order: aqueous acetonic extract > aqueous ethanolic extract > aqueous methanolic extract. Their respective TAC were 112.59, 62.73, and 41.83 μg/mL. Another study reported that TAC decreased in this order: acetonic extract > methanolic extract > ethanolic extract (Lakkab et al., 2019). Hajaji et al. (2011) reported that the extracts from three varieties of *Ceratonia siliqua* L. leaves from Morocco showed significant antioxidant capacity compared to the reference antioxidants, butylated hydroxytoluene (BHT) and ascorbic acid (AA), in a dose dependent manner.

Therefore, it can be concluded that the solvent had a significant effect on the estimated antioxidant activity as it determined the rates and mechanism of the reaction of the phenolics with reagents (Nenadis & Tsimidou, 2002). It also confirms the highest antiradical effect of CS macerate determined by the DPPH assay. The slight variation detected among carob parts can be explained by the fact that the phosphomolybdenum reduction method is based on an estimation of antioxidant activity of polyphenols and other non-volatile antioxidant compounds, which were not the object of this study.

3.3. *Antioxidant capacity of active multilayer biopackaging*

As shown in Fig. 3c, the highest antioxidant capacity was obtained in active biofilm with CSE followed by CSA, CLE, CLA, CKE and CKA with a value of 180.67, 182.34, 185.06, 227.83, 343.57, and 548.26 μg/mL,

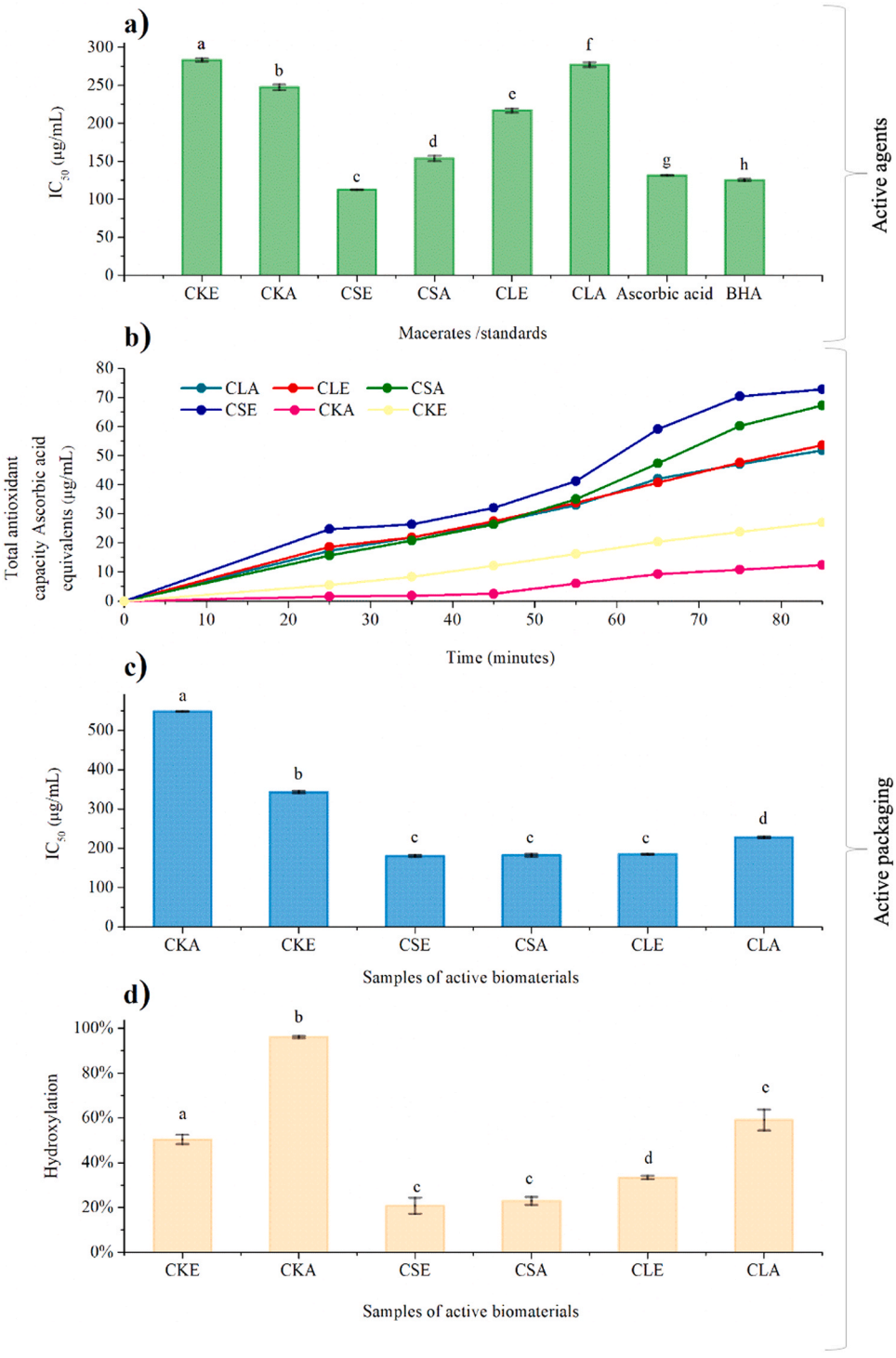

Fig. 3. Comparison of antioxidant activity of macerates and active biofilms, where: a) DPPH radical scavenging activity of *Ceratonia siliqua* L. macerates and standards; b) Total antioxidant capacity of macerates; c) IC_{50} values of active biomaterial packaging containing CL, CS and CK macerates; d) free OH· radical assay of different active biopackaging subjected to hydroxylation during 24 h (blank = 100%). Different letters (a–h) indicate statistically significant differences between samples ($p \leq 0.05$). All samples were performed in triplicate.

respectively. There was no significant difference among samples such as CSE, CSA and CLE. Blank biofilm without macerates did not change the absorbance of DPPH, which shows its absence of antioxidant properties.

The antioxidant capacities of multilayer biofilms were based on the scavenging of gas-phase OH·radicals, generated from an aqueous peroxide solution, by the carob extracts incorporated into biofilms. The obtained results after 24 h of hydroxylation are shown in Fig. 3d. As can be seen, significant differences were observed among almost all antioxidant biofilms. Samples of carob seeds extracted with ethanol and acetone and incorporated into polymer matrix provided similar results. The active biofilm containing CS macerates showed the best results with values of 20.91 ± 3.59% and 23.04 ± 1.79% for CSE and CSA, respectively, in good agreement with the DPPH test, followed by CLE (33.49 ± 0.78%), CKE (50.43 ± 2.08%), CLA (59.12 ± 4.72%), and CKA (96.12 ± 0.52%). This effect is associated with the presence of scavengers of free radicals and can be explained by the different chemical reactions taking part in each method for the determination of the antioxidant activity. Finally, based on these results, the active biopackaging with CS macerates incorporated was selected for the migration assay.

It should be highlighted that Fig. 3a–d compare the antioxidant capacity of pure macerates, as well as antioxidant packaging with

incorporated extracts to assess the behaviour of the active agents. The obtained results confirm that active films eliminate the free radicals responsible for the initiation of the oxidation phenomenon. Since free radicals can efficiently diffuse through the polymer, they can be trapped by the antioxidant agents fixed in the multilayer packaging as components of the adhesive among polymer films, thus avoiding the direct contact with the packaged food. Consequently, the results of the antioxidant activity obtained for active films are weaker than those obtained in the macerates.

3.4. Antibacterial activity of macerates

The inhibition zones of macerates, expressed in mm, are given in Table 2. The macerates generated larger inhibition against the *Staphylococcus aureus*, compared to the *Escherichia coli* and *Pseudomonas aeruginosa*, suggesting that macerates have better antimicrobial activity against gram-positive bacteria than against gram-negative bacteria. This behaviour can be due to the outer lipopolysaccharide membrane of gram-negative strains, which restricts the diffusion of hydrophobic compounds (Burt, 2004).

Macerates such as CKE, CLE and CLA demonstrated the highest antimicrobial activity against the three microorganisms tested. However, from a practical point of view this antimicrobial activity is not enough for the use of extracts in antimicrobial active packaging applications. Antimicrobials typically used in active packaging such as cinnamon essential oil or clove essential oil showed similar inhibition zones by applying 25 times less antimicrobial in the disk (López, Sánchez, Batlle, & Nerín, 2005).

3.5. Migration tests and risk assessment

Biopackaging and active antioxidant packaging are new areas of technology with protective effect against oxidative damage with the advantage of its sustainability. Nevertheless, to accomplish the legislation of food contact materials, packaging must not transfer their chemical components into the foods through the migration process. A migration test and risk assessment of new multilayer active biopackaging films based on cellulose were performed to assess their safety in the present investigation. Two sets of food packaging biofilms containing CSE and CSA (8%) were studied in this work, and 10% and 95% aqueous ethanol solutions were used as food simulants with a contact time of 3 days at temperature < 5 °C.

The obtained chromatograms of both simulants after the exposure to active biofilms were compared to those of the blank biofilm and those of pure simulants. No peaks were detected, neither in the case of the volatile compounds nor non-volatile ones. Similar results were obtained by Oudjedi et al. (2019) on a new antioxidant active packaging which incorporated Algerian extracts of sage and bay leaves, where no migration of compounds was noticed from both kinds of packaging. These results could be expected, as probably the compounds are grafted in the adhesive behind the cellulose layer in contact with the simulant, as demonstrated by previous studies (Carrizo et al., 2016). Another research on the migration of compounds from cellulose-based trays, coated with an active filler, by using the acetic acid at 3% (v/v), ethanol at 50% (v/v) and vegetable oil as food simulants, resulted in compliance with the migration limits imposed from EU regulation, thus demonstrating the suitability of the prepared material for food contact (Bugatti, Viscusi, & Gorrasi, 2020).

Therefore, the experimental results here obtained confirmed that the prepared active biofilm is safe and can be used as antioxidant food biopackaging for future preservation, according to experiment described in Section 2.7.1.

Table 2
Inhibition zones of macerates (results expressed in mm).

Sample	*E. coli*	*P. aeruginosa*	*S. aureus*
CSA	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	22.1 ± 1.0^{a}
CSE	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	22.4 ± 0.5^{a}
CKA	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	11.6 ± 1.4^{b}
CKE	16.4 ± 2.0^{b}	20.3 ± 1.0^{b}	26.3 ± 1.1^{c}
CLA	20.8 ± 1.0^{c}	22.1 ± 0.2^{c}	27.9 ± 1.9^{c}
CLE	$19.4 \pm 1.4^{b,c}$	22.9 ± 0.4^{d}	27.8 ± 1.6^{c}

Different letters (a–d) indicate statistically significant differences between samples ($p \leq 0.05$).

3.6. Colour measurement

In food packaging applications, the appearance of films is an essential issue (Urbina, Eceiza, Gabilondo, Corcuera, & Retegi, 2019). The colour of the film is often the factor determining its production on an industrial scale, introducing the product to the market and purchasing the product by the consumer.

To evaluate the influence of active agents on the polymer matrix, colour parameters of the active biofilms containing CL, CS, and CK macerates were measured. Moreover, total colour changes (ΔE) were calculated, and the obtained results are shown in Table 3. The cellulose sheet material used in the present work was colourless and translucent. The visual examination of all the biofilms containing the different macerates of leaves, seeds, and kibbles from *Ceratonia siliqua* L. showed a light painted colour compared to the blank biofilm without macerates. The instrumental colour analysis results presented in Table 3 show that L^*, a^*, b^* increased after the incorporation of the macerates. Concerning lightness, a higher value (L^*) was recorded in the CKE, followed by CKA > CLE > CSA > CLA > CSE. Nevertheless, statistical analysis showed that there were no significant differences ($p \leq 0.05$) among the L* values of all samples. Little changes were noticed (Student *t*-test, $p \leq 0.05$), in the positive and negative values of a^* and b^* parameters in all bioactive materials in comparison to the blank biofilm. Almost all values were recorded with only a slight increase. The fundamental differences in colour (ΔE) were calculated, and results showed that the highest value corresponded to CKE ($\Delta E = 2.87$), and the lowest one was found in CSE ($\Delta E = 0.76$) as the film less affected by the addition of the macerate in the adhesive formulation. Our results can be explained by the application of the minimum possible concentration of macerate (8%). The obtained results of total colour change (values from 0 to 100) should be interpreted according to the following key: $\Delta E < 1$ means that the colour change is not perceptible by human eyes; if $\Delta E = 1$–2, the change is perceptible after very close observation; when $\Delta E = 2$–10, the change is perceptible at first sight; in the range $\Delta E = 11$–49, colours are more likely similar than dissimilar, whereas $\Delta E = 50$–100 are perceived as opposite colours.

4. Conclusion

In this work, forty-three volatile compounds were identified in *Ceratonia siliqua* L. macerates and, among them, natural antioxidants have been noticed. The antioxidant activity was carried out with two different methods, and the best results corresponded to macerates of *Ceratonia siliqua* L. seeds with IC_{50} values of 112.75 ± 0.32, and 153.61 ± 3.65 μg/mL for the CSE and CSA extracts, respectively using DPPH test, while values of 72.81 ± 0.09, and 67.22 ± 3.91 μg/mL were recorded in the case of CSE and CSA extracts, respectively by the phosphomolybdenum assay.

Besides, a new antioxidant biofilm based on cellulose as the matrix was successfully developed and was evaluated for its antioxidant capacity by a method based on in situ hydroxyl radical generator and DPPH test. The one containing *Ceratonia siliqua* L. seeds macerated with 80% ethanol and 80% acetone demonstrated the strongest antioxidant activity with percentages of hydroxylation of $20.91 \pm 3.59\%$ and $23.04 \pm 1.79\%$, respectively, followed by CLE, CKE, CLA, and CKA with percentages of hydroxylation of $33.49 \pm 0.78\%$; $50.43 \pm 2.08\%$; $59.12 \pm 4.72\%$ and $96.12 \pm 0.52\%$, respectively (100% hydroxylation

Table 3
CIELAB colour parameters of the films and total colour change (ΔE).

	Biomaterials with ethanolic macerates				Biomaterials with acetonic macerates			
	Control	CL	CS	CK	Control	CL	CS	CK
L^*	88.09 ± 0.29^a	87.29 ± 0.80^a	87.81 ± 0.69^a	88.44 ± 0.18^a	88.03 ± 0.34^a	87.72 ± 0.50^a	87.86 ± 0.37^a	88.01 ± 0.58^a
a^*	2.76 ± 0.03^a	3.23 ± 0.06^b	3.31 ± 0.04^b	3.46 ± 0.03^c	2.80 ± 0.02^a	3.03 ± 0.06^d	3.28 ± 0.04^b	3.55 ± 0.21^b
b^*	-3.33 ± 0.08^a	-4.70 ± 0.08^b	-3.78 ± 3.21^c	-6.09 ± 0.04^c	-3.42 ± 0.05^a	-4.11 ± 0.14^e	-4.79 ± 0.09^b	-5.80 ± 0.58^d
ΔE	0.00	1.71	0.76	2.87	0.00	0.79	1.46	2.50

Different letters (a–e) indicate statistically significant differences between samples ($p \leq 0.05$).

means no antioxidant properties). In contrast, the antioxidant capacity was also shown in the case of seeds macerates biofilms with DPPH test (180.67 ± 2.5 and 182.34 ± 3.21 μg/mL for CSE and CSA extracts, respectively). The following macerates CKE, CLE and CLA demonstrated the highest antimicrobial activity against all microorganisms that were tested. Nevertheless, the evaluation of the antimicrobial activity of the different macerates against bacterial strains showed a weak antibacterial activity, limiting our study to evaluate the antioxidant performance of the new biofilm.

Finally, a migration test of both volatile and non-volatile compounds was carried out, and the obtained results showed the full absence of migrants in all the cases. Therefore, it can be concluded that the developed material complies with Commission Regulation (EU) No 10/2011 and all its amendments for food contact materials. These results are promising for future production of developed active biofilms at industrial scale. Nevertheless, before that, subsequent trials on real food samples are necessary.

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.

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