Application of a Multisystem Coating Based on Polymeric Nanocapsules Containing Essential Oil of *Thymus Vulgaris* L. to Increase the Shelf Life of Table Grapes (*Vitis Vinifera* L.)

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***Abstract*— In developing countries, the incidence of postharvest losses reduces the quantity and quality of food for human consumption and causes an economical damage along the food chain, especially, for primary producers. In this study, a multisystem coating (NC-EOt-C) based on pullulan and polymeric nanocapsules containing EO of *Thymus vulgaris* L. (EOt) was applied to increase the shelf life of table grapes (*Vitis vinifera* L.). The major components of EOt, chemically characterized by GC-MS, were *o-cymene* (32.68%), *thymol* (31.90%), and *γ -terpinene* (15.69%). The NC-EOt were prepared by nanoprecipitation and showed a particle mean size of 153.9 nm, a polydispersity index of 0.186, a zeta potential of −4.11 mV, and an encapsulation efficiency of 52.81%. The antioxidant capacity (DPPH and ABTS$^+$ methods) of EOt was maintained, or even improved, after its incorporation into NC. The shelf life study showed that grapes having the NC-EOt-C multisystem maintained their characteristics of color, firmness, TA, and SSC for longer time than those without the multisystem. NC-EOt-C multisystem acted as a barrier which reduced the metabolism of fruits. In addition, the compounds of EOt with antimicrobial activity avoided microorganism growth, while those with antioxidant activity reduced the oxidative stress induced during postharvest of grapes. Additionally, the polymeric structure of NC prevented the rapid evaporation of volatile compounds of EOt, increasing then their residence time on the fruit. Our study demonstrated that NC-EOt-C multisystem can be a viable alternative to preserve horticultural products for longer storage periods.**

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***Index Terms*— Edible coatings, essential oils, food preservation, polymeric nanoparticles, and *Thymus vulgaris* L.**

I. Introduction

FRUITS are perishable products because of their inherent tendency to deteriorate. During the postharvest period of fruit, it is necessary to guarantee a longer useful life of the vegetable. Reports from the Food and Agriculture Organization of the United Nations (FAO) mention that, in developing countries, there is a great deficiency in marketing infrastructure, therefore, postharvest losses of fresh products reach up to 50% of total production [1]. Losses of this magnitude trigger a considerable economic damage for food productive chain, especially, for primary producers. In addition, the presence of pests represents a serious health risk for the consumer. FAO, in collaboration with the Latin American Integration Association (ALADI) and the Economic Commission for Latin America and the Caribbean (ECLAC), prepared the Food Losses and Waste Plan (FLW) which promotes the development of innovative technologies that contribute to reduce food loss at all stages of the food production chain [2]. In recent years, different alternatives have been proposed in order to preserve the horticultural products, including the use of protective coatings. An edible coating is a thin layer of edible material formed as a coating on a food product. Using coatings modifies the interaction of the fruit with the environment due to their physicochemical properties, prolonging the shelf life of the treated fruits [3]. Different coating-forming compounds have been used, including chitosan, alginate, starch, and pullulan [4]–[7].

Pullulan is a polysaccharide produced by *Aureobasidium pullulans*; it can form edible coatings with several advantages over other polysaccharides. Concerning its properties, it has limited permeability to oxygen and carbon dioxide, has good adhesive properties, is colorless, and has no flavor [8]–[10]. The pullulan coating can influence on the physiology of fruit since it acts as a barrier between the environment and fruit. The protective effect of this coating can also be improved

by adding compounds that exhibit some biological activity, such as essential oils (EO). Due to their antioxidant and antimicrobial activities, EO have emerged as an important and innovative alternative for the control and reduction of postharvest losses [11]. In fact, the antimicrobial properties of EO have been used to control fungi and phytopathogenic bacteria [12]. In particular, the EO obtained from the *Thymus vulgaris* L. (EOt) plant have been widely used as additives in food, pharmaceutical and cosmetics. For example, in the alternative medicine, EOt have been useful for their antiseptic, carminative, expectorant, antimicrobial and antioxidative effects [13]–[16]. On the other hand, in the food industry, EOt and their constituents, are used as flavoring agents, aroma additives, antioxidants and preservatives. In addition, FDA has classified EOt as GRAS (Generally Recognized As Safe) [17], [18].

In general, the application of EO as additives is often troublesome because they can be deteriorated by environmental factors, such as light and oxygen, and are prone to evaporation [19]. In addition, EO have low solubility in water, which makes difficult to incorporate them into commercial products [20]. To overcome these effects, nanoencapsulation of EO in polymeric nanoparticles has become an interesting alternative. Nanoencapsulation is a process in which the active ingredient (e.g. essential oils, extract plants, drugs, vitamins) is surrounded by a coating wall of polymer to form nanocapsules or is embedded in a polymeric matrix to form nanospheres. Therefore, besides protecting the encapsulated EO against harsh environments, nanoencapsulation can offer a controlled release and improves the handling of the EO [21]–[23]. For example, nanoparticles of *Lippia sidoides* EO were prepared via spray-drying using biopolymer blends of alginate/cashew gum. Formulations of nanoparticles exhibited different release profiles of *Lippia sidoides* EO dependent on the alginate/cashew gum ratio. The most significant *in vitro* release profile showed that between 45 and 95% of oil was released within 30–50 h [24]. In another study, eugenol, a volatile constituent of EO, was nanoencapsulated by an emulsion– diffusion method. The study of oxidation stability revealed that eugenol loaded polycaprolactone nanoparticles were effective to protect eugenol from light oxidation, enhancing then its stability. The protective effect was attributed to the polymer wall that was surrounding the eugenol core [25]. Finally, nanocapsules (NC) containing lemongrass EO were prepared using polylactic acid as polymer. This formulation was more efficient in inhibiting bacteria growth that non-encapsulated EO. In addition, a biofilm formation assay demonstrated that lemongrass EO nanocapsules reduced the ability of microorganisms to develop biofilms. This could be explained by a more efficient delivery of the active compounds of EO from nanocapsules [26].

In order to introduce a novel strategy to prevent quality and quantity losses in postharvest storage of horticultural products, in this work, a multisystem coating based on pullulan and polymeric nanocapsules containing essential oil of *Thymus vulgaris* L. (NC-EOt-C) was evaluated to increase the shelf life of the table grapes (*Vitis vinifera* L.).

II. Methodology

A. Plant Collection and Identification

Fresh stems and leaves of the *Thymus vulgaris* L. plants were purchased from the local market in Monterrey, México in June 2016. The plants were identified with the number 010970 and deposited at the herbarium of the School of Biological Sciences of the Autonomous University of Nuevo León, Monterrey, México.

B. Essential Oil Extraction

The EOt was obtained by hydrodistillation [27]. Briefly, fresh-cut plant was hydrodistilled using a Clevenger-type apparatus (100 g· L^{-1} water) for 4 h. The oil was collected and kept at 4 °C until use. The yield of EOt was calculated by using (1):

$$Yield = (EOt/F.plant) \times 100 \quad (1)$$

where *EOt* are the grams of obtained oil and *F. plant* is the total weight, in grams, of fresh *Thymus vulgaris* L.

C. Gas Chromatography-Mass Spectrometry (GC-MS) and GC With Flame Ionization Detection (GC-FID) Analysis

The composition of volatile constituents of EOt was analyzed using a gas chromatograph 6890N (Agilent Technologies, USA) equipped with a 5973 INERT mass selective spectrometer (ionization energy 70 eV) and a HP-5MS capillary column (5% phenylmethylpolysiloxane, 30 m × 0.25 mm, 0.25 μm, Agilent J and W). The ionization-source temperature was 230 °C, the quadrupole temperature was 150 °C and the injector temperature was 220 °C. Data acquisition was performed in the scan mode. The oven temperature was programmed as follows: 35 °C for 9 min, increased to 150 °C at 3 °C· min^{-1} and held for 10 min, increased to 250 °C at 10 °C· min^{-1}, and increased to 270 °C at 3 °C· min^{-1} and held for 10 min. The flow rate of the helium carrier gas (99.999% purity) was 0.5 mL· min^{-1}. EOt components were identified by comparing retention indices relative to C8-C20 n-alkanes (Sigma-Aldrich), and MS were compared with the mass spectra from the US National Institute of Standards and Technology (NIST) library and reference data [28]. To determine the proportion of each component, a quantitative analysis was performed with a GC-FID (Autosystem XL, Perkin Elmer, USA) using the same HP-5MS column. The injector temperature was 270 °C, the oven temperature program was same as the GC-MS analysis. The percentage composition of EOt was calculated using the peak normalization method.

D. Formulation of the Multisystem Coating Based on Polymeric Nanocapsules Containing Thymus Vulgaris L. Essential Oil (NC-EOt-C)

The NC were prepared by the nanoprecipitation method as described by Lugo-Estrada *et al.* [29]. Briefly, an organic phase (5 mL) composed of a mixture of acetone:isopropanol (1:1),

Eudragit L 100-55 (60 mg), and EOt (90 mg) was prepared. This was injected into an aqueous phase (10 mL) containing 0.5% (w/w) of poly(vinyl alcohol) (Mowiol 4-88 with a MW 26,000 and a hydrolysis degree of 88%) under magnetic stirring. Diffusion of the organic phase into the aqueous phase induced the aggregation of polymer and encapsulation of EOt into nanocapsules (NC-EOt). The solvent was then evaporated under reduced pressure (Laborota 4003 control, Heidolph Instruments, GER). Unloaded NC (NC-BCO), without EOt, were obtained following the same procedure described above.

The physicochemical characterization of the formulations was determined in an aqueous suspension of NC-EOt. The mean particle size, and polydispersity index (PI) were measured at 90 degree scattering angle using Dynamic Light Scattering, while the zeta potential measurement was by Laser Doppler Microelectrophoresis (Zetasizer Nano-ZS90, Malvern Instruments, UK).

For the formation of the multisystem coating, pullulan was used as film-coating. Pullulan powder was incorporated into an aqueous suspension of NC-EOt under magnetic stirring. The final concentration of pullulan was of 1.5% (w/v). On the other hand, an emulsion of EOt (700 μg· mL^{-1}) in 3% (w/v) Tween 80® was prepared at 11000 rpm (Homogeniser VDI 12, VWR, USA) to be used as a control.

E. Analysis of Nanocapsules by GC-FID

The NC-EOt were centrifuged at 25,000 rpm for 2 h (Allegra 64R centrifuge, Beckman Coulter, USA), and then the pellet was dissolved in methanol (Tedia). The quantitative analysis of *o*-cymene, thymol, and γ-terpinene in NC-EOt was performed with a GC-FID (Clarus 480, PerkinElmer, USA) using a capillary column (30 m x 0.25 mm x 0.25 μm) (Elite-5, PerkinElmer, USA). The injector and detector temperatures were 280 °C and 260 °C, respectively. The oven temperature was programmed as follows: 70 °C for 1 min, increased to 116 °C at 4 °C· min^{-1} and held for 1 min, increased to 200 °C at 20 °C· min^{-1}, and increased to 230 °C at 14 °C · min^{-1}. The flow rate of the helium carrier gas (99.999% purity) was 1.0 mL· min^{-1}. This temperature program was used during the validation of the method. *o*-cymene, thymol, and γ-terpinene were selected as test compounds because they were the main components of the EOt. Subsequently, encapsulation efficiency percentage (EE%) was calculated by using (2):

$$EE\% = (Ce/Ct) \times 100 \tag{2}$$

where *Ce* is the amount of a component in the NC-EOt (mg), and *Ct* is the amount of the component in the total EOt (mg) used in organic phase.

F. Antioxidant Activity

The radical scavenging activities of NC-EOt and non-encapsulated EOt were first determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) method [13]. Briefly, solutions of NC-EOt, EOt and carvacrol (positive control) were prepared in methanol (Tedia) at a concentration of 1000 μg · mL^{-1}. A volume of 1 mL of each solution was mixed with 1 mL of freshly prepared DPPH methanol solution (80 μg· mL^{-1}). These systems were left at room temperature in the dark for 30 min. Absorbances of these solutions were measured at 517 nm (Epoch Microplate Spectrophotometer, Biotek, USA). On the other hand, the ABTS$^+$ method [27] is based on the reduction of the green ABTS$^+$ radical cation. A volume of 25 μL of each solution (NC-EOt, EOt or carvacrol, at a concentration of 1000 μg· mL^{-1}) was added to 1 mL of ABTS$^+$ methanol solution (7 mM). After 7 min, absorbances were measured at 734 nm (Epoch Microplate Spectrophotometer, Biotek, USA). In both cases, the percentage inhibition of free radical (I%) was calculated by using (3):

$$I\% = [(ABSo - ABSsample)/ABSo] \times 100 \tag{3}$$

where *ABSo* is the absorbance of the radical (DPPH or ABTS$^+$) in methanol and *ABSsample* is the absorbance of the sample with the radical.

G. Effect of NC-EOt-C Multisystem on Postharvest Quality and Shelf Life of Grapes Fruits (Vitis Vinifera L.)

Grapes with homogeneous characteristics of color, size, and without mechanical damage were selected. They were washed with distilled water and dried. Fruits were distributed in four groups. The first group was used as a control (without treatment), while the second one was immersed for 1 min in the EOt emulsion (700 μg· mL^{-1}). Then, the third group was immersed in an aqueous dispersion of pullulan (1.5%, w/v), which contained NC-BCO (NC-BCO-C). Finally, the fourth group was immersed for 1 min in an aqueous dispersion containing the components of the multisystem, the pullulan and NC-EOt (NC-EOt-C); EOt was at the same concentration of EOt group. All groups were maintained at 25 °C for 13 days.

Titratable acidity (TA) was determined by titration of grape juice using 0.1 N NaOH solution until the end of titration (pH = 8.2). It was expressed as grams of tartaric acid per 100 mL.

The total soluble solid content (SSC) of grape juice was obtained by refractometry (Abbemat 200, AntonPaar, AUT).

The color values (CIE L∗a∗ and b∗) of grapes were determined by using a colorimeter (ColorFlex EZ, HunterLab, USA). The firmness of grapes was measured by using a texture analyser (CT3 Texture Analyzer, Brookfield-Ametek, USA), which was equipped with a cylindrical probe having 4 mm diameter.

The firmness was expressed in Newton (N). All parameters were determined at the beginning and at the end of the shelf life study.

In another evaluation, the presence of microbiological damage on the fruits stored at 4 °C was monitored for 6 months.

III. Results and Discussion

A. Chemical Characterization of Thymus Vulgaris L. Essential Oil

EOt had a yield of 0.378 ± 0.159% fresh weight. Chemical analysis of the EOt by GC-MS led to identification of 20 compounds (Table I). As is shown in Fig. 1, the major components

TABLE I
CHEMICAL COMPOSITION OF *Thymus Vulgaris* L. ESSENTIAL OIL BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

No.[a]	t_r[b] (min)	Area (%)	Compound
1	13.79	0.78	*α-thujene*
2	13.93	0.95	*α-pinene*
3	14.75	1.55	*Camphene*
4	16.76	0.40	*β-pinene*
5	17.81	0.40	*1-octen-3-ol*
6	18.03	1.52	*Myrcene*
7	19.35	1.03	*α-terpinene*
8	20.26	32.68	*o-cymene*
9	20.36	0.75	*Sylvestrene*
10	20.59	0.58	*1,8-sineole*
11	22.35	15.69	*γ-terpinene*
12	22.68	0.47	*cis-sabinene hydrate*
13	24.78	3.54	*Linalool*
14	26.78	1.25	*Camphor*
15	28.06	2.75	*Borneol*
16	28.70	0.75	*Terpinen-4-ol*
17	32.16	0.98	*Carvacrol methyl ether*
18	35.44	31.90	*Thymol*
19	35.58	1.16	*Carvacrol*
20	40.14	0.88	*E- caryophyllene*

[a] Elution order.
[b] Retention time.

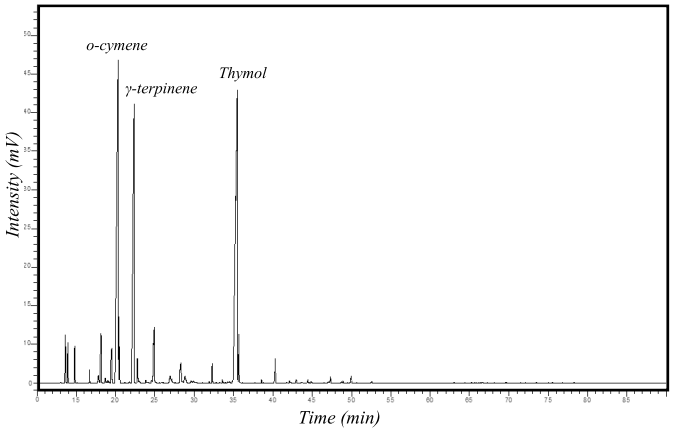

Fig. 1. Chromatogram by gas chromatography with a flame ionization detector of *Thymus vulgaris* L. essential oil.

were *o*-cymene (32.68%), thymol (31.90%), and γ-terpinene (15.69%). In general, the EOt was composed of 56.69% of aliphatic terpenes and 43.31% of oxygenated terpenes. Guerra-Boone *et al*. [28] found the same components in EOt, but with different percentages, 62.6% of aliphatic terpenes and 35.6% oxygenated terpenes.

Results of chemical characterization of EOt by GC-MS were also similar to those reported by Satyal *et al.* [30], who found that the main components were thymol (23-60%), γ-terpinene (18-50%), *p*-cymene (8-44%), carvacrol (2-8%), and linalool (3-4%); percentages of these components may vary depending on harvest season and habitat of plant, as well as the extraction method of EO [13].

Several studies are focused on showing the antimicrobial and antioxidant activities of EOt [15], [31]. Some authors have suggested that the antimicrobial effects of EO could mainly be attributed to their major constituants. Regarding our study, it has been reported thymol to be active against a broad spectrum of microorganisms, including phytopatogen [14], [17], [32], [33]. Furthermore, *o*-cymene, and γ-terpinene have also shown antimicrobial actvity [15], [34], [35]. However, there is not much information about the application of these two compounds for fruit conservation.

In general, fruits are products that easily deteriorate due to different types of microorganisms. Particulary, grapes of *Vitis vinefera* L. are susceptible to fungal decomposition by the genus *Aspergillus*, and more importantly, by the *Botrytis cinerea* species [36], [37]. In our study, it was important to found that the major compounds of EOt (i.e. thymol and carvacrol) had previously demonstrated relevant antifungal activities.

TABLE II
PHYSICOCHEMICAL CHARACTERIZATION OF THE FORMULATION OF NANOPARTICLES LOADED WITH *Thymus Vulgaris* L. ESSENTIAL OIL

Mean size (nm)	PI[1]	Zeta potential (mV)	EE%[2]
153.9 ± 4.7	0.186 ± 0.011	-4.11 ± 0.170	52.81 ± 2.69

[1] *Polydispersity index which ranges from 0 to 1, a higher value corresponds to less homogeneous NC size distribution.*
[2] *Encapsulation efficiency percentage.*
(n=3; $\bar{X}$±DS).

B. Physicochemical Characterization of NC-EOt

EOt has a great potential as a natural preservative of fruits and vegetables, mainly due to the biological activities reported for many of its components. In fact, EOt is included in the GRAS list of the FDA [38]. However, its use and application have some limitations as a food additive because it has low solubility in water, and it is susceptible to degradation by environmental factors (e.g. oxygen and UV radiations) [22].

Using the nanoprecipitation technique, EOt was nanoencapsulated into a polymeric structure to protect it from such natural agents [39]. The formation of NC by the nanoprecipitation technique is explained in terms of an interfacial turbulence. This occurs when an organic phase containing polymer, EOt, and an organic solvent miscible in water diffuses into an aqueous phase under slight magnetic stirring [40]. This technique has been successfully used for the nanoencapsulation of natural products, such as EO [41], [42]. In the present study, polymeric nanoparticles containing EOt (NC-EOt) were obtained with the physicochemical characteristics showed in Table II.

Formulation of NC-EOt showed a mean size of 153.9 ± 4.7 nm. When compared to non-encapsulated EOt, its incorporation into NC can offer several advantages for its application as preservative of fruit. First, due to their size and multiparticulate character, EOt-loaded nanoparticles can be more uniformly distributed on the fruits and then, they can gradually release EOt for covering all fruit surface [43], [44].

Particularly, the size next to 150 nm offers advantages in comparison with large particles (e.g. microparticles). As particle size smaller, their surface area to volume ratio gets larger. Then, it is possible to have more surface of the NC directly in contact with the fruit surface, allowing a local delivery of the EOt, specially where the microorganism are preferably found [23]. Secondly, polymer wall of NC allows retaining the EOt inside the structure, thus reducing its evaporation rate.

The mean size of NC-EOt was similar to that reported by El Asbahani *et al.* [45] who obtained NC (110-150 nm) with EO from two species of *Thymus* genus. Additionally, they found similar antimicrobial activities of nanoencapsulated and non-nanoencapsulated EO against bacteria (i.e. *E. Coli, Kliebsella Pneumoniae, Staphylococcus aureus, Pseudomons aeruginosa, and Salmonella typhimurium*), yeast (i.e. *Candida albicans and Candida glabrata*) and fungi (i.e. *Aspergillus niger and Penicillium funiculosum*). In another study, Granata *et al.* [23] prepared nanoparticles of *Thymus capitatus* EO and *Origanum vulgare* EO with mean sizes of 175 ± 1.0 and 171 ± 2.0 nm, respectively. In addition, an improvement in the antimicrobial activity of EO-loaded nanoparticles was reported, showing a greater activity against foodborne pathogens compared with non-nanoencapsulated EO.

On the other hand, the PI is a parameter associated to homogeneity of the NC dispersion. For Zetasizer Nano-ZS90 (Malvern Instruments) PI, values range from 0 to 1; a value lower than 0.200 indicates a homogeneous nanoparticle size distribution [40]. For this study, the PI value of NC-EOt formulation corresponded to 0.186 ± 0.011, indicating high nanoparticle homogeneity which would allow that the individual interactions of NC (e.g. bioadhesion, EOt release) to be also homogeneous on fruit surface.

Electrokinetic properties of NC-EOt were also considering in this study. The zeta potential is the electrostatic potential at the boundary dividing the compact layer and the diffuse layer of the colloidal particles. Zeta potential value for NC-EOt corresponded to −4.11 mV. The negative sign indicates that negative charges are dominant at the surface of NC and it can be attributed to Eudragit L 100-55, which is an anionic copolymer based on methacrylic acid and ethyl acrylate. Thus, during NC-EOt formation, the polymer chains surrounded the oily core to form the polymer wall and their anionic functional groups were exposed to the external aqueous media. This negative charge is important because it could facilitate the interaction of NC-EOt with the membrane of phytopathogenic microorganisms (e.g. *Botrytis cinerea* species), which would ensure that the delivery of the EOt compounds could be directly from the NC to the bacteria cells, increasing thus their antimicrobial effectiveness [46].

In order to complete the physicochemical characterization of NC-EOt, the EOt entrapped in NC was quantified by GC-FID and the EE% was calculated by using (2). The EE% was determined considering only the three major components of EOt and it corresponded to 52.81 ± 2.69% (Table II) for *o*-cymene, γ-terpinene, and thymol. These results are consistent with those of Marcet *et al.* [47], who obtained thymol-loaded PLA NC with a mean size next to 260 nm and an EE of 60.3 ± 8%. In addition, they demonstrated that thymol-loaded PLA NC had improved antimicrobial properties in comparison with the non-encapsulated thymol. In another study, Sotelo-Boyás *et al.* [42] prepared two types of chitosan based nanoparticles loaded with EOt. Particularly, the EE% of thymol was of 68% for EOt-loaded chitosan nanospheres, whereas it was of 72% for EOt-loaded chitosan NC. In our study, GC-FID analysis revealed the presence of thymol into EOt-NC, which was relevant because several studies have reported that the antimicrobial and antioxidant activities of EOt were due partially to this compound. Thus, considering that more than 50% of the EOt added during the preparation of the NC was encapsulated, it is possible that its major compounds (i.e. thymol) could be gradually released from NC to the fruit surface.

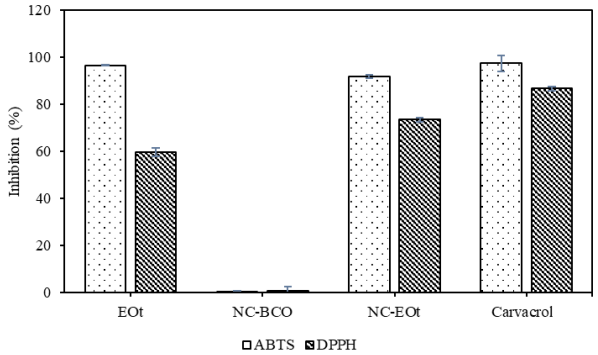

Fig. 2. Radical scavenging activity of *Thymus vulgaris* L essential oil (EOt), nanocapsules without EOt (NC-BCO), nanocapsules with EOt (NC-EOt), and standard (carvacrol) at 1000 μg· mL^{-1} by the DPPH and ABTS^{+} methods. ($n = 3$; $\bar{X} \pm DS$).

Now, concerning the antioxidant properties of NC-EOt, we observed that the antioxidant capacity of EOt was maintained, or even improved, after its incorporation into NC. First, as is shown in Fig. 2, the radical scavenging activity were similar for NC-EOt (91.86 ± 0.79%) and for EOt (96.66 ± 0.26%) by using the ABTS^{+} assay. Nevertheless, the radical scavenging activity was slightly higher for NC-EOt (73.50 ± 0.76%) than for non-encapsulated EOt (59.62 ± 1.77%) by the DPPH method. The NC-BCO, it means nanoparticles without EOt, showed no antioxidant activity by any radical scavenging assay. Some authors have proposed that a good interaction between radical DPPH and the polymeric wall of NC can promote the radical scavenging activity for the encapsulated compounds [48], [49].

For instance, Zhang *et al* [50] reported that the antioxidant activity of quercetin was maintained when it was incorporated into chitosan nanoparticles. This was attributed to the complexes formed between the polymeric network of nanoparticles and quercetin, which allowed to retain and protect the quercetin molecules. Likewise, Kumar *et al.* [51] determined the antioxidant capacity of naringenin-loaded chitosan nanoparticles, a flavonoid with an anti-inflamatory activity. They found that the ability to scavenge DPPH free radicals was significantly higher (84%) for nanoencapsulated naringenin than for free naringenin (∼65%). It was relevant to verify that the antioxidant activity of EOt was maintained after

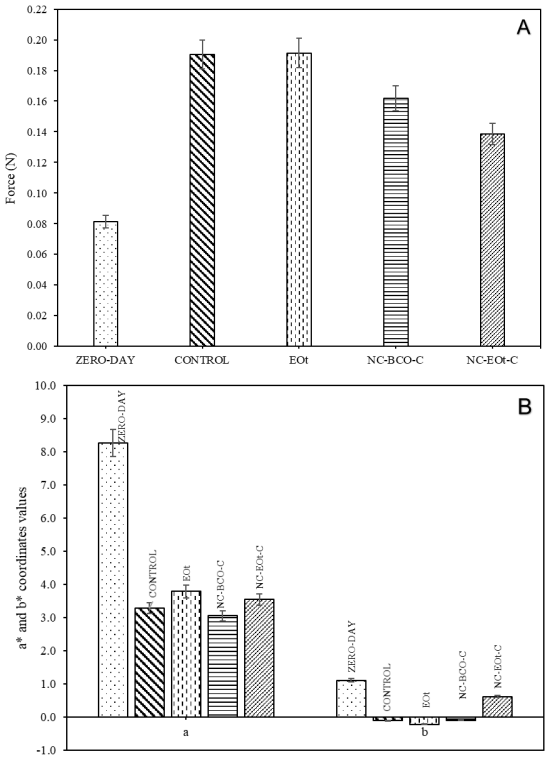

Fig. 3. Firmness (A) and color (B) of grapes after storage at 25 °C for 13 days. Bars correspond to the initial measurement (zero day) as well as groups of grapes without treatment (control), with EOt emulsion (EOt), with nanocapsules without EOt (NC-BCO-C), and with the multisystem coating (NC-EOt-C). (n = 10; $\bar{X} \pm DS$).

its nanoencapsulation because it had been established, as a hypothesis, that the oxidative stress induced during postharvest of grapes would be delayed or avoided for the NC-EOt.

So, considering all these aspects, the antimicrobial activity previously reported for EOt, the antioxidant activity tested for EOt as well as the advantages of its incorporation into NC, we established that the NCS-EOt formulation had adequate characteristics to be a part of the multisystem coating intended for fruit conservation.

C. Effect of NC-EOt-C Multisystem on Postharvest Quality and Shelf Life of Grape Fruits

Fruit firmness is an important quality attribute. The firmness (Fig. 3A) of the grapes was 0.082 ± 0.030 N at the beginning of the experiment. This parameter increased for all groups of grapes after 13 days of storage. The water loss is related to an increase of firmness of grapes because it induces a turgor loss of the mesocarp cells, making the exocarp (skin) a more flexible structure [52]. Considering our results, the control (0.19 ± 0.051 N) and non-encapsulated EOt (0.19 ± 0.037 N) groups showed the highest values of firmness while the NC-BCO-C (0.16 ± 0.043 N) and NC-EOt-C multisystem (0.14 ± 0.022 N) had the lowest ones. In this case, the pullulan coating acted as a barrier, avoiding loss of water from grapes. Similar results were obtained by Kraśniewska *et al* [10], who reported that a pullulan coating protected the blueberry fruits from loss of firmness, when they were stored at 4 ± 1 °C for 28 days and at 16 ± 2 °C for 14 days. Authors mentioned that the pullulan coating avoided the excessive transpiration of fruits. One the other hand, the NC-EOt-C multisystem group had a lower firmness value than that of NC-BCO-C group. Considering that breaking down of complex carbohydrate polymers (e.g. pectin) by enzymes led to loosening of cell walls and softening of fruit [53], the constituents of EOt released from NC could have interfered with enzymatic activity, delaying then the softening effect.

Furthermore, color changes were determined by the CIE L a∗ b∗ color scale, where a∗ is the red/green coordinate (+a∗ indicates redder and –a∗ indicates greener), while b∗ is the yellow/blue coordinate (+b∗ indicates yellower and –b∗ indicates bluer). As is shown in Fig. 3B, all treatments (i.e. EOt, NC-BCO-C, and NC-EOt-C) and control grapes showed a∗ values less positives (∼3) than that measured at the beginning of the shelf life study (8.27 ± 2.6). When values of a∗ are positive and close to zero, this indicates an anthocyanin accumulation in grapes, which is related to ripening of fruits [54]. Values of b∗ coordinate also decreased for all treatments compared to the b∗ initial value, although they had minor differences. Changes of color were due to enzymatic and chemical changes during ripening [55]. In particular, the contents of anthocyanins and acylated anthocyanins change during ripening and are responsible of the color of grape peel [56], which can range from light red to dark blue and almost black. Concerning fruits treated with the NC-EOt-C multisystem, values of a∗ and b∗ were slightly higher than those for fruits without treatment (control grapes), showing that red color of fruits was not altered by the NC-EOt-C multisystem during ripening.

Similar results were obtained by Lemes *et al.* [7] who reported that a coating based on gelatin hydrogel added with curcumin-loaded nanoparticles maintained the color of *Benitaka* grapes after storage of 7 days at 25 °C. Particularly for b∗ coordinate, there was a significant difference between the NC-EOt-C multisystem (0.614 ± 0.7) and EOt (−0.221 ± 0.9). This could be due to a rapid evaporation of molecules of non-encapsulated EOt, which avoided that their components could delayed the fruit ripening. The incorporation of EOt into the NC could have prolonged the interaction of EOt components on fruit surface.

The SSC of the grapes tends to increase during ripening. The SSC (Fig. 4A) of the grapes was 18.60 ± 0.042 °Brix at the beginning of the experiment. This parameter increased for control grapes (21.49 ± 0.036 °Brix) as well as grapes tested with EOt (21.76 ± 0.116 °Brix), NC-BCO-C (20.66 ± 0.181 °Brix) and the NC-EOt-C multisystem (19.05 ± 0.274 °Brix) after 13 days of storage. The increase of SSC during storage are due to respiration, the inversion of insoluble compounds to soluble forms and the moisture loss by evaporation [57]. In addition, metabolic activity continues as a result of fruit ripening, which leads to converting carbohydrates and organic acids into sugars to be used in several metabolic processes [58]. In our study, the increase of SSC in grapes with treatments and control grapes was due to conversion of sucrose into glucose and fructose [59].

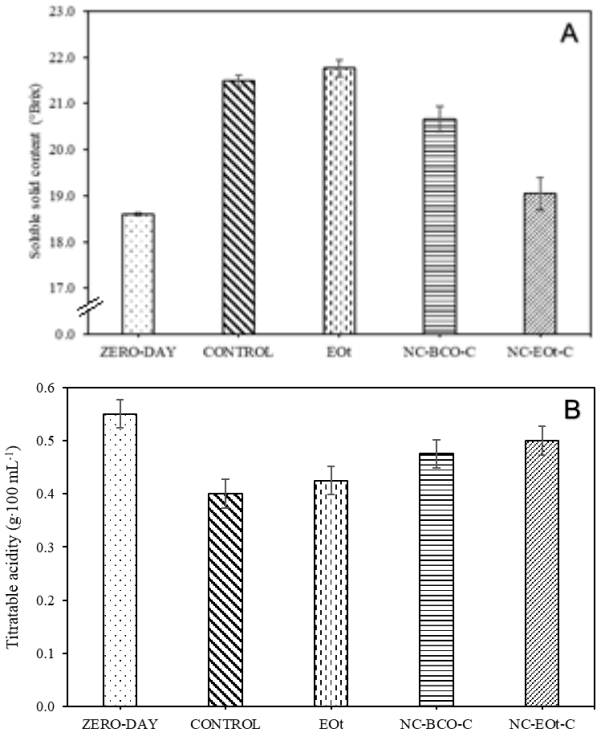

Fig. 4. Total soluble solid content (A) and titratable acidity (B) of grapes after storage at 25 °C for 13 days. Bars correspond to the initial measurement (zero day) as well as groups of grapes without treatment (control), with EOt emulsion (EOt), with nanocapsules without EOt (NC-BCO-C), and with the multisystem coating (NC-EOt-C). ($n = 3$; $\bar{X} \pm DS$).

Fig. 5. Evaluation of antimicrobial protection of grapes after storage at 4 °C for 6 months. Image correspond to the grapes without treatment (control, A), with EOt emulsion (EOt, B), with nanocapsules without EOt (NC-BCO-C, C), and with the multisystem coating (NC-EOt-C, D).

Now, comparing with control grape, the NC-BCO-C and the NC-EOt-C multisystem showed lower values of SSC. This behavior could be attributed to the pullulan coating present in both multisystem, which helped to reduce the respiration of the grapes, delaying their ripening [60]. Similar results were reported by Diab *et al.* [8], who reported that Strawberry treated with a pullulan coating maintained the SSC values lower than the uncovered fruits after 12 days of storage at 20 °C. They attribute this result to the beneficial effects of the pullulan coatings to decrease the respiration rate of the fruits.

A significant difference of SSC was observed between the NC-EOt-C multisystem (19.05 ± 0.274 °Brix) and the EOt (21.49 ± 0.036 °Brix). In fact, the EOt had 2.71 more °Brix than the NC-EOt-C multisystem. This could be due to the short residence of the EOt on the fruit surface because of its rapid evaporation when it was not nanoencapsulated. This limited its protective effect to delay the ripening of grapes. In contrast, besides to barrier properties of the NC-EOt-C multisystem, the sustained release of EOt from NC-EOt could have extended the antioxidant activity of EOt on fruit surface, which contributed to have a better conservation of grapes.

Moreover, as is shown in Fig. 4B, the TA of the grapes was 0.555 ± 0.086 g· 100 mL^{-1} at the beginning of the experiment and decreased slightly during storage for all groups. After 13 days of storage, the TA values were 0.400 ± 0.042, 0.425 ± 0.045, 0.475 ± 0.043, and 0.500 ± 0.044 g· 100 mL^{-1} for control grapes, EOt, the NC-BCO-C, and the NC-EOt-C multisystem, respectively. This reduction of TA values is associated to the metabolism of organic acids in grapes. Next to sugars, organic acids are the most abundant solids present in grapes. They are responsible of fruit acidity which is expressed as TA. The main organic acids found in grapes are tartaric, malic and to a small extent, citric. During the ripening of grapes, the amount of malate/citrate decreases, and this means that organic acids are being metabolized. The possible fates for these compounds are the Krebs cycle (respiration), gluconeogenesis, amino acid synthesis, production of ethanol by fermentation and synthesis of secondary metabolites (e.g. pigments) [61], [62].

Concerning the groups of grapes with treatments, results of TA had the same trend that those obtained for SSC. After 13 days of storage, control grapes had the most significant decrease of TA, while the NC-EOt-C multisystem the lowest decrease of TA.

It means that the NC-EOt-C multisystem was the most effective treatment to delay the grape ripening, which can be attributed to a double effect. First, the pullulan coating acted as a barrier and decreased the respiration rate (metabolism) of grape. Second, the NC-EOt prolonged the residence of EOt on the surface of the fruit, which led to improve its antioxidant activity. These results are consistent with those obtained by Melo *et al*. [54] who reported that, after 12 days of storage, grape fruits coated with gel and chitosan nanoparticles had lower SSC values as well as few changes in the TA content than uncoated fruits. They also attributed that trend to a slowdown in the metabolic activity of the fruit caused by the coating gel and the chitosan nanoparticles.

Finally, control grapes and groups of grapes with treatments (i.e. EOt, NC-BCO-C, and NC-EOt-C multisystem) were stored for 6 months in order to observe the presence or absence of macroscopic damage caused by microorganisms. With exception of grapes treated with the NC-EOt-C multisystem, all groups of grapes showed damage caused by microorganisms, probably, fungi (Fig. 5A, B, and C). Various kinds of growth patterns were observed on the infected grapes. Mycelia displayed powdery, cottony, and radial characteristics. Colonies were varied, from white and dirty white in color to grayish and orange; some of these characteristics could

correspond to the disease called "gray mold" caused by *B. cinerea.*

The grapes fruits having the NC-EOt-C multisystem showed no macroscopic damage (Fig. 5D). This protection can be due to EOt components with antimicrobial activity [23], [63]. Particularly, thymol and carvacrol were identified in the EOt by the gas chromatography analysis (Table I). It has been suggested that EO alter the surface and structure of fungal cell wall reducing the cell wall synthesis [5].

In addition, EOt compounds are able to interact with outer cell membrane, increasing fluidity and permeability, and to cause structural and functional damages to cytoplasmic membrane of the microorganism [23]. On the other hand, the incorporation of EOt into NC provided advantages, including its sustained release and an increase of its residence time on the surface of the grape, which favored the interaction of EOt compounds with microorganisms that cause damage to the fruit. These advantages of the NC have been also observed in other areas such as medicine, pharmacology, and agriculture [64], [65]. These results demonstrated the functionality of the NC-EOt-C multisystem as an effective preservative for the fruit of *Vitis vinifera*L. grapes.

IV. Conclusion

In this study, a multisystem coating based on pullulan and polymeric NC containing essential oil of *Thymus vulgaris* L. (NC-EOt-C) was applied to increase the shelf life of the table grape (*Vitis vinifera* L.). The results demonstrated the potential of the NC-EOt-C multisystem as an alternative to traditional methods of fruit preservation. The shelf life study showed that grapes having the NC-EOt-C multisystem maintained their characteristics such as color, firmness, TA, and SSC for longer time than those without the multisystem. Additionally, grapes with the NC-EOt-C multisystem did not show signs of microbiological damage. The physicochemical properties of the multisystem components contributed positively to delay the ripening of grapes and protect them from the damage of microorganisms. First, the pullulan coating acted as a barrier which reduced the transpiration and the respiration rate (metabolism) of fruits. Second, the compounds of EOt with antimicrobial activity avoided microorganism growth, while those with antioxidant activity reduced the oxidative stress induced during postharvest of grapes. In particular, due to their size and multiparticulate character, EOt-loaded nanoparticles were more uniformly distributed on the fruits and then, they gradually released EOt for covering all fruit surface; in addition, polymeric structure of NC prevented the rapid evaporation of volatile compounds of EOt, increasing then their residence time on fruit. Based on these results, we can conclude that the presence of the NC-EOt-C multisystem on the surface of the grape was effective to maintain the postharvest quality and to extend the shelf life of grapes. This kind of multisystem can be a viable alternative to preserve horticultural products for longer periods.

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