Antioxidant Stability Study of Oregano Essential Oil Microcapsules Prepared by Spray-Drying

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Abstract: Release kinetics of the volatile compounds of oregano EO microcapsules and the relation with the antioxidant activity were studied. Different wall material (WM) to core (C) ratios (1:1 and 2:1; WM:C), addition of colloidal silicon dioxide (CSD); and different storage conditions: 23 °C (room temperature; R) and 4 °C (fridge temperature; F) were evaluated for 90 d. Volatile compounds, total phenolic content (TPC), free radical scavenging activity (FRSA), and Trolox equivalent antioxidant capacity (TEAC) were measured. The formulas 2:1 (WM:C) (R and F) without CSD behaved differently from the rest, exhibited a higher antioxidant activity, and released less amount of volatile compounds after 90 d. These treatments grouped together in the cluster analysis, showing the highest TPC (81.54 mg gallic ac/g), FRSA (8.66%), and TEAC (12.35 μg Trolox/g). The addition of CSD facilitated the released of volatile compounds through storage time and promoted losses in the antioxidant activity. The temperature had a significant effect in most of the evaluated variables. However, this effect was more noticeable in F2 (1:1, CSD).

Keywords: antioxidant capacity, microencapsulation, *Origanum vulgare*, spray-drying, volatiles

Practical Application: Oregano essential oil has antioxidant, antimicrobial, and sensory preserving properties. However, it is susceptible to volatilization and is degraded by external factors. Its addition into food matrices is restricted due to low solubility and hydrophobicity. The antioxidant activity of oregano EO is preserved after the process of microencapsulation by spray-drying that extends its stability during storage. Oregano EO microcapsules are an alternative of delivery which protects and extends the shelf life of this essential oil, overcomes stability related limitations and preserves its desirable characteristics allowing these kind of microcapsules to be later incorporated into food products. These microcapsules could be used as a natural additive/flavouring with antioxidant properties.

Introduction

Substantial efforts have been made to develop novel natural preserving ingredients with application in the food industry. Essential oils (EOs) obtained from aromatic plants are generally recognized as safe (GRAS) (Asensio and others 2015a). Oregano is an aromatic plant used as a food ingredient due to its pleasant flavor (Asensio and others 2012; Olmedo and others 2015) and antioxidant (Kulisic and others 2004; Fasseas and others 2008) and antimicrobial properties (Gallucci and others 2009; Olmedo and others 2013; Asensio and others 2014; Asensio and others 2015b) properties.

The addition of lipophilic compounds, such as EOs, into a food matrices, particularly in water-based carriers, is restricted due to a low solubility and hydrophobicity (López and ohters 2014; González and others 2016). Moreover, EOs are susceptible to degradation by external factors like UV radiation, oxygen, and high temperatures. Controlling the release of volatile EOs under various conditions is another important issue to increase their effectiveness. Consequently, new methods of preserving the properties of EOs should be investigated.

The microencapsulation technique is being widely used in the pharmaceutical industry for controlled delivery of drugs, but also it is currently used in the food industry for many purposes: flavor, antimicrobial and/or antioxidants stabilization. Microencapsulation is an effective method to overcome stability-related limitations for the utilization of volatile ingredients (Calvo and others 2012). Microencapsulation of food ingredients is highly used to deliver bioactive compounds to the consumers without any destruction, preserving their activity. This technique provides a stable environment for the encapsulated compounds protecting them from the external adverse environment, assisting in preserving their flavor, and limiting aroma degradation or loss during processing and storage. Spray-drying is the most widely used technique for microencapsulation in the food industry because it is a low-cost, simple, inexpensive, reproducible and scalable operation. Moreover, it converts the liquid oil into a free-flowing powder that can be readily incorporated into food formulations. It is also considered one of the most effective methods to achieve a constant release of bioactive compounds (Xiao and others 2014).

The cost of spray-drying is 6 times lower, per kg of water removed, than the cost of freeze-drying (Bhargava and others 2015). In the food industry, the microencapsulated core ingredient is protected by wall materials (WM) typically formulated with sugars, gums, proteins, natural modified polysaccharides or lipids, or their combinations. The properties of the wall and core materials, and the prepared emulsion of lipid core and aqueous phase along with the drying process conditions, influence the efficiency

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and the retention of the core. For instance, the WM must have a high stability and water solubility, and a low viscosity. In addition, the WM should have a tendency to form a fine and dense network during drying, and should not permit a lipid separation from the emulsion during dehydration (Gharsallaoui and others 2007; Calvo and others 2012). The spray-drying process has been used to encapsulate extracts of *Ilex paraguariensis* (Nunes and others 2015), *Capsicum annuum* L. (Guadarrama-Lezama and others 2012), *Opuntia ficus-indica* (Saenz and others 2009), black currant polyphenols (Bakowska-Barczak and Kolodziejczyk 2011), *Averrhoa carambola* (Saikia Mahnot and Mahanta 2015), clove (Chatterjee and Bhattacharjee 2013), pomegranate peel (Çam Içyer and Erdoğan 2014), fish oil, olive, chia, and flax oils (Polavarapu and others 2011; Quispe-Condori and others 2011; González and others 2016), rosemary EO (De Barros Fernandes and others 2014), *Schinus molle* L. (López and others 2014), oregano, citronella and marjoram EOs (Baranauskienė and others 2006; Beirão Da Costa and others 2012), among others.

The progressive release of the volatile compounds and the maintenance of the antioxidant capacity of microcapsules with oregano EOs have not been deeply studied. This information is vital if they are intended to be used as an additive to control oxidation of different food products. The objectives of this study were to evaluate how the WM ratio, presence of CSD, and storage temperature influence the antioxidant activity, total phenolic content, and release kinetics of volatile compounds from oregano EO spray dried microcapsules during storage.

Material and Methods

Hydroxypropyl methyl cellulose (HPMC) (Methocel® K100, donated by Colorcon, Buenos Aires, Argentina.), maltodextrin (MD) (Todo Droga, Córdoba, Argentina), and colloidal silicon dioxide (CSD) (Aerosil 200®, Evonik Degussa, Essen, Germany) were used as WM. CSD provided non-hygroscopic medium, good flow-ability, compressibility, and led to dry samples with low inlet temperatures (Moreira and others 2009; Gallo and others, 2011). Neutralized peanut oil (NPO) was obtained from NutrIn SA (Ticino, Córdoba, Argentina). All other reagents were pro-analysis grade. All the experiments were performed with MiliQ water.

Plant material and essential oil extraction

Aerial parts of *Origanum vulgare subsp. hirtum* clone 'Cordobes' were collected from the Experimental Station of the Agronomy Collage (National University of Córdoba) (April, 2015). Essential oils were extracted by steam distillation using a Clevenger-type apparatus (Asensio and others 2015a).

Preparation of emulsions and microcapsules

The aqueous phase was prepared previously dissolving HPMC and MD, both under magnetic stirring for 12 h at 40 °C then CSD was dispersed into the solution. Oregano EO was dissolved in neutralized peanut oil (10% w/w) and employed as the lipid core. For the emulsion preparation, the lipid core (C) was incorporated into the aqueous phase using an auto mixer homogenizer (SMT Company, Japan) at 10000 rpm for 5 min and immediately after, the solution was spray-dried. Two different WM:C ratios were prepared (1:1 and 2:1) and 2 different ratios of WM components: 1:1:0 and 1:0.5:0.5 (HPMC:MD:CSD) were tested. Therefore, 4 different formulas were spray-dried: F1 (1:1, without CSD), F2 (1:1 with CSD), F3 (2:1, without CSD), and F4 (2:1 with CSD).

Spray-drying conditions

The spray-drying process was performed using a laboratory scale Mini Spray Dryer (Büchi B-290, Büchi Labortechnik AG, Flawil, Switzerland) according to López and others (2014) with slight modifications. Briefly, the samples were atomized with a hot air stream in the drying chamber. A 2-fluid nozzle of 0.5 mm cap orifice diameter was used. The following parameters were fixed: pump (10%); aspirator (100%); Q-flow (600 L/h); inlet temperature (160 °C); and outlet temperature (100 °C).

Recovered solid yield

The recovered solid yield (SY) was calculated as ratio of powder weight collected after each drying experiment (Wf, on dry basis) and initial weight of components in the prepared emulsions (except water) (Wi, on dry basis), as indicated in Eq. (1).

$$SY = (Wf/Wi) \times 100 \quad (1)$$

Physical characterization of microcapsules

Optical microscopy. A droplet of the emulsion was examined with an optical light microscopy (Olympus BX41, Tokyo, Japan) applying 100X magnification to check whether the emulsion was formed.

Moisture content. Moisture content was measured with a moisture analyzer with halogen heating (model MB45 OHAUS, Bradford, United States). The moisture content analysis was carried out immediately after the drying process.

Essential oil analysis. The essentials oils were analyzed with a Perkin Elmer Clarus 600 GC–MS (Shelton, Conn., U.S.A.) coupled with an ion trap mass detector (MS) and non-polar capillary column Elite-5 MS (methylpolysiloxane, 5% phenyl, 30 m, 0.25 mm id, and 0.25 mm coating thickness. The compounds were identified by comparing their mass spectra with those from literature (Adams 1995) and libraries (NIST). The main components were further identified by co-injection of authentic standards (Sigma®, St. Louis, Mo., U.S.A.). The quantitative composition was obtained by peak area normalization, and the response factor for each component was considered equal one (Asensio and others 2015a).

Antioxidant capacity of the microcapsules during storage

The 4 oregano EO spray-dried microcapsule formulas (F1, F2, F3, and F4) were stored in 2 different temperatures, at 23 ± 1 °C considered room temperature (R) and 4 ± 0.5 °C considered fridge temperature (F), during 90 d. Every 30 d, samples were analyzed for antioxidant activity, total phenolic content, and volatile compounds.

Antioxidant activity tests and total phenolic compounds. Before antioxidant activity assays, the microcapsules were dissolved in DMSO which assured destruction of microparticles and release of the essential oil to the medium (Beirão Da Costa and others 2012). The dispersions were agitated using a vortex (2 min), put in an orbital shaker for 30 min at 1500 rpm, and then, centrifuged at 10000 rpm for 5 min in a Mini-spin centrifuge (Eppendorf, Hamburg, Germany). The supernatant was analyzed for antioxidant activity and total phenolic content.

Trolox equivalent antioxidant capacity (TEAC- ABTS assay). ABTS (Sigma®) stock solution (7 mM) was mixed with 2.45 mM potassium persulfate solution and incubated at room temperature in the dark for 16 h. Afterwards, the solution was diluted with water to an absorbance value of 0.7 ± 0.1 at 734 nm. ABTS reagent (990 μL)

Table 1–Means and standard deviations ($n = 3$) of physical and chemical variables determined on EO microcapsule formulas after spray-drying process in fresh samples (storage time = 0 d): solid yield, moisture content, total phenolic content (TPC), free radical scavenging activity-DPPH test (FRSA), and Trolox equivalent antioxidant activity (TEAC).

	F1[a]			F2[a]			F3[a]			F4[a]		
	Mean	SD	[b]	Mean	SD	[b]	Mean	SD	[b]	Mean	SD	[b]
Physical characterization												
Solid Yield (%)	39.9	0.89	A	31.1	1.20	B	51.8	0.38	C	52.6	0.55	C
Moisture content (%)	5.1	0.55	B	5.1	0.89	B	3.2	0.33	A	3.1	0.21	A
Chemical characterization												
TPC (mg gallic acid/g)	46.9	2.37	B	25.0	1.68	A	84.6	1.95	D	56.3	0.36	C
FRSA (% inhibition)	6.5	0.71	A	5.6	0.92	A	11.8	1.19	C	9.4	0.61	B
TEAC (μg Trolox/mg)	5.7	0.17	B	2.0	0.11	A	12.7	1.25	D	7.8	0.28	C

[a]EO microcapsule formulas: F1 = 1:1 WM:C without CSD, F2 = 1:1 WM:C with CSD, F3 = 2:1 WM:C without CSD, and F4 = 2:1 WM:C with CSD.
[b]Different letters in each raw indicate significant differences between samples (ANOVA, LSD test, $\alpha = 0.05$).

was added to 10 μL of microcapsules extract. The concentration of sample giving the same percentage of absorbance inhibition than 1 mM Trolox was calculated in terms Trolox equivalent antioxidant capacity (TEAC). The TEAC of microcapsules were expressed as μg Trolox/mg sample (Asensio and others 2015a).

Total phenolic content (TPC). Phenolic content was determined by Folin–Cicolteau reagent according to Olmedo and others (2015). The concentration was calculated using gallic acid as standard (Sigma®). Phenolic content was measured at 760 nm and was expressed as mg gallic acid/g sample.

Free-radical scavenging activity (FRSA-DPPH test). Microcapsule extracts were added to 0.05 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) methanolic solution. The absorbance of the samples was measured at 517 nm on a spectrophotometer. The radical-scavenging activity was expressed as percentage of DPPH inhibition (PI) (Olmedo and others 2014).

Volatile release analysis. The volatile compounds of essential oils were studied to analyze the realise profile of EO from the MC samples (Olmedo and others 2014; Quiroga and others 2014). Microcapsules (2 g) were carefully weighted and added into 20 mL capacity vials, covered and sealed. Volatile compounds were captured using a solid phase micro-extractor fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Sigma®). The vials were heated at 70 °C for 20 min without stirring. The fiber was exposed into the vial headspace for 10 min and then, injected into the CG-MS. The samples were separated in a non-polar column Elite-5MS (Perkin Elmer). The identification of volatile compounds was performed in full scan mode (m/z range 40 to 550) via a combination of the NIST mass spectral library and gas chromatographic retention indices of standard compounds (Sigma®). Acetaldehyde (Sigma®) was running as an internal standard in all samples. Individual volatiles were quantified from the FID peak areas relative to that of the internal standard (Yin and others 2012).

Statistical analysis

The experiments were carried out 3 times and results were expressed as mean ± standard deviations. Analysis of variance (ANOVA, $\alpha = 0.05$), factorial analysis of variance (F-ANOVA, $\alpha = 0.05$) and LSD Fisher's multiple range test were performed to determine significant differences between means. Pearson´s correlation coefficients were determined for the analyzed dependent variables. Principal component analysis (PCA) was performed on the correlation matrix of normalized data from storage study (Asensio and others 2012). Associations between microcapsules, antioxidant activity, and volatile compounds were assessed by PCA. A biplot from PCA was obtained to show those associations. Vectors and points represented dependent variables and treatments, respectively. The angle formed between vectors indicates correlation between variables. Cluster analysis (CA) was carried out to obtain groups of microcapsules with similar characteristics. Sample similarities were calculated based on Euclidean distance, and the groups with similar characteristics were obtained using the unweighted pair-group method (UPGMA). Data were analyzed using the InfoStat software, version 2015 (Di Rienzo and others 2015).

Results and Discussion

Characterization of spray-dried microcapsules

The solid yields (SY) and moisture contents ranged from 31.1% to 51.8% and 3.1% to 5.1%, respectively (Table 1). Solid yield represents the weighted material recovered in the dryer after the encapsulation process (Roccia and others 2014), this is particularly interesting because essential oils are volatile compounds highly susceptible to volatilization during the drying process. Microcapsules with 2:1 WM:C ratios had higher SY than those with 1:1 ratio, meaning that a higher percentage of EO may be entrapped in these microcapsules. Reineccius (2004) found that the amount of wall materials exercises a fundamental role in the retention of volatile particles, they shorten the length of the diffusion path to the air/particle interface. De Barros Fernandes and others (2014) observed that increasing the WM concentration a higher proportion of rosemary essential oil was entrapped. In contrast, an excessive increase in the concentration of solids in the feed led to a decrease in the encapsulation yield (Huynh and others 2008). Significant differences were also found between F1 and F2 formulas, the presence of CSD seemed to lessen the SY. The F2 (1:1 with CSD) displayed the lowest SY value (31.1%).

The samples with the same WM:C ratios exhibited similar moisture contents (Table 1). A lower moisture content was found in formulas with 2:1 WM:C ratios (F3 and F4) (3.2% and 3.1%) compared to formulas with 1:1 WM:C (F1 and F2) (5.1% and 5.1%, respectively). Gallo and others (2011) found a negative correlation between MC and solids concentration that indicates that a higher solid concentration produced powders with a lower moisture contents, in concurrence with the current observations presented in this study.

Antioxidant activity values and TPC varied significantly in the oregano EO microcapsules, immediately after the drying process (Table 1). The samples with 2:1 WM:C ratios exhibited higher TPC, FRSA and TEAC values than those with 1:1 ratio. F3 (2:1 without CSD) had the highest TPC (84.6 mg gallic acid/g), FRSA

Table 2–Means and standard deviations ($n = 3$) of volatile compounds (areas relative composition, %) of oregano essential oil and EO microcapsule formulas analyzed on fresh samples before storage.

	Oregano EO			F1[a]			F2[a]			F3[a]			F4[a]		
Volatile compounds	**Mean**	**SD**	**d**	**Mean**	**SD**	**d**	**Mean**	**SD**	**d**	**Mean**	**SD**	**d**	**Mean**	**SD**	**d**
α-Thujene	1.20	0.03	A	1.17	0.03	A	2.40	0.04	C	1.21	0.08	A	1.38	0.00	B
α-Pinene	0.81	0.004	A	1.18	0.04	C	1.28	0.01	C	2.16	0.15	D	1.01	0.00	B
β-Pinene	1.41	0.08	A	1.44	0.13	A	6.97	0.07	B	3.34	0.26	C	1.74	0.01	D
o -Cymene	**7.44**	**0.042**	**C**	**6.30**	**0.20**	**B**	**10.51**	**0.16**	**D**	**10.70**	**0.43**	**D**	**4.81**	**0.04**	**A**
β -*trans*-Ocimene	3.87	0.12	B	2.26	0.86	A	5.17	0.06	C	3.10	0.11	A	2.69	0.06	A
γ-Terpinene	**7.64**	**0.11**	**B**	**5.36**	**0.21**	**A**	**28.29**	**0.32**	**D**	**8.55**	**0.32**	**C**	**5.24**	**0.11**	**A**
Isoterpinolene	3.39	0.03	D	0.83	0.05	A	3.03	0.04	C	1.64	0.06	B	0.85	0.02	A
Terpinolene	**25.77**	**0.01**	**D**	**11.67**	**0.46**	**B**	**1.37**	**0.02**	**A**	**13.89**	**1.35**	**C**	**12.97**	**0.22**	**C**
Borneol	0.88	0.02	C	0.80	0.08	C	nd		A	0.70	0.01	B	1.04	0.06	D
Terpinen-4-ol	**9.38**	**0.05**	**B**	**8.24**	**0.20**	**A**	**11.24**	**0.08**	**D**	**10.57**	**0.04**	**C**	**9.34**	**0.47**	**B**
α-Terpineol	2.73	0.04	B	2.79	0.13	B	1.42	0.03	A	2.93	0.19	B	3.17	0.20	C
Thymol methyl ether	0.72	0.03	B	0.41	0.03	C	1.51	0.04	A	0.39	0.03	A	0.44	0.06	A
Carvacrol methyl ether	0.84	0.03	A	1.98	0.05	B	2.45	0.04	B	2.11	0.003	C	2.30	0.16	D
Thymol	**23.58**	**0.22**	**B**	**37.34**	**1.77**	**E**	**10.04**	**0.43**	**A**	**23.33**	**1.06**	**C**	**32.84**	**0.96**	**D**
iso-Caryophyllene	1.91	0.001	A	3.52	0.03	D	1.87	0.03	B	2.58	0.12	C	3.91	0.53	E
α-Caryophyllene	0.51	0.02	A	1.20	0.03	C	0.95	0.01	B	0.72	0.07	A	1.26	0.15	C
Germacrene D	0.87	0.003	A	2.88	0.15	D	1.20	0.09	B	1.02	0.11	B	2.34	0.64	C
β-Cadinene	0.06	0.002	A	0.37	0.01	D	0.26	0.02	C	0.16	0.02	B	0.37	0.002	D
Hexadecane	Nd		A	Nd		A	0.37	0.00	B	0.18	0.00	B	0.19	0.15	B
Spathulenol	0.09	0.004	A	0.77	0.06	C	0.09	0.09	A	0.15	0.07	B	0.53	0.01	C
Caryophyllene oxide	Nd		A	0.34	0.03	E	0.01	0.00	B	0.12	0.00	C	0.30	0.01	D
Decane, 5,6-bis dimethylpropylidene	Nd		A	1.97	0.50	B	3.30	0.56	B	0.68	0.09	B	3.49	3.58	B
Main compounds[b]	**73.8**			**78.9**			**76.1**			**79.0**			**76.7**		
Other compounds[c]	**19.3**			**13.9**			**18.2**			**11.1**			**15.5**		
Total identified compounds	**93.1**			**92.8**			**94.3**			**90.1**			**92.2**		

[a]EO microcapsule formulas: F1 = 1:1 WM:C without CSD, F2 = 1:1 WM:C with CSD, F3 = 2:1 WM:C without CSD, and F4 = 2:1 WM:C with CSD.
[b]Main compounds: sum of compounds in high percentages marked in bold letters.
[c]Other compounds: sum of compounds in low percentages.
[d]Different letters in each raw indicate significant differences between samples (ANOVA, LSD test, $\alpha = 0.05$).
nd: not detected.

(11.8%), and TEAC (12.7 μg/mg sample). The wall membrane can act as a barrier to most flavor compounds but remains permeable for water molecules, avoiding the loss of volatile flavors (Huynh and others 2008). Comparing samples formulated with the same WM:C ratio those with CSD (F2 and F4) had lower antioxidant activity values for both FRSA and TEAC. Colloidal silicon dioxide has been used as a carrier that let to dry samples with different moisture contents even at low inlet temperatures, and can hold substantial quantities of liquid within the interstitial volume of its agglomerates. However, previous results showed that, in samples with low solids concentration of *Rhamnus purshian* a plant extract, the amount of CSD was not enough to totally confine the plant extract within the agglomerates' interstitial volume (Gallo and others 2011). Similar results were observed in the present study, where the amount of CSD was not high enough to retain the EO, then, the antioxidant activity of the microcapsules was lost. This effect was more evident in F2, which had lower WM:C ratio and presence of CSD.

Pearson´s coefficients were calculated to evaluate the correlation among the dependent variables after the encapsulation process. Solid yield was highly ($P \leq 0.01$) and positively associated with TPC (0.87), FRSA (0.88), and TEAC (0.87), suggesting that higher SY resulted in higher antioxidant activity and, as a consequence, higher carrying capacity of these formulas. On the contrary, moisture content was negatively associated with SY (–0.82), TPC, FRSA, and TEAC.

The results of chemical composition (Table 2) showed that significant differences were found between oregano EO and its microcapsules ($P \leq 0.05$). Cordobes EO contained 5 major compounds (concentrations higher than 5%), which included *o*-cymene (7.44%), γ-terpinene (7.64%), terpinen-4-ol (9.38%), terpinolene (25.78%), and thymol (23.58%). The same principal compounds (concentrations higher than 5%) were found within all microcapsule formulas, except for *o*-cymene in F4 (4.81%). However, significant differences were found in the profile after the encapsulation process. These differences could be attributed to the spray drying process. De Barros Fernandez and others (2014) found in rosemary EO microcapsules that the compounds present in the pure and in the encapsulated EO were similar in the relative percentages, but not the same. In this study, F2 exhibited the highest concentrations of *o*-cymene (10.51%), γ-terpinene (28.29%), and terpinen-4-ol (11.24%) but the lowest concentrations of terpinolene and thymol (1.37% and 10.40%, respectively). This sample also presented a similar percentage of other compounds released than the pure EO (18.2 compared with 19.3). However, this percentage was the highest with respect to the other microcapsules, meaning that F2 can release more compounds after the spray drying process. Previous works demonstrated that differences in released EO were possibly due to differences in wall structure or way that the oil was retained (Beirão da Costa and others 2013). Baranauskienė and others (2006) suggested that hydrophobic volatile compounds of essential oils, mostly present on the surface of particles, would be less protected and, consequently, more susceptible to evaporation, which may explain a higher release of *o*-cymene and γ-terpinene in F2.

The antioxidant activity of oregano EO is associated with its chemical composition, and particularly with the content of monooxygenated terpenes (Dambolena and others 2010; Quiroga and others 2013; Asensio and others 2015a; Olmedo and others 2015). A similar relation can be established within the antioxidant activity of each formula and the amount of components released after the encapsulation process.

Table 3–Means (n = 3) of mayor volatile compounds (areas relative composition, %), total phenolic compounds (TPC, mg gallic acid/g), free radical scavenging activity (FRSA, % inhibition), and Trolox equivalent antioxidant capacity (TEAC, μg Trolox/mg) determined on EO microcapsule formulas stored during 90 d under different storage conditions (SC).

Formula	SC[b]	Time (d)	o-Cymene[c]	γ-Terpinene[c]	Terpinolene[c]	Terpinen-4-ol[c]	Thymol[c]	Other compounds[c]	TPC[c]	FRSA[c]	TEAC[c]
F1[a]	R	0	6.30B	5.36D	11.67A	8.24A	37.34A	23.93D	46.94B	6.25B	5.69A
		30	5.02B	3.71C	11.76A	8.73A	45.85B	19.79C	41.54B	6.06B	5.47A
		60	4.07A	2.65B	11.69A	8.92A	48.90B	18.47B	52.02B	5.53B	7.69C
		90	2.58A	1.42A	11.12A	8.59A	59.71C	13.51A	32.70A	4.31A	6.36B
	F	0	6.30B	5.36D	11.67A	8.24A	37.34A	23.93D	46.94B	6.81B	5.69A
		30	5.54B	4.18C	12.61A	8.64A	43.98B	20.35C	39.18A	6.29B	5.37A
		60	3.47A	2.72B	11.53A	8.16A	47.04B	21.63C	45.39B	5.73B	7.43C
		90	2.76A	2.23B	11.06A	8.64A	54.36C	17.04B	36.40A	4.70A	6.49B
F2[a]	R	0	10.51B	28.29C	1.37B	11.24A	10.04A	32.86B	25.03C	5.63C	2.04B
		30	8.64B	23.68C	1.55B	12.90A	24.05B	24.91A	22.63C	4.81B	1.98B
		60	8.04B	16.15B	0.28A	17.05B	28.65B	22.79A	19.26B	4.10B	1.78A
		90	3.83A	6.46A	0.00A	17.17B	43.0C	19.61A	13.49A	3.01A	1.72A
	F	0	10.51B	28.29C	1.37B	11.24A	10.04A	32.86B	25.03C	5.63C	2.04B
		30	10.41B	25.26C	1.62B	13.26A	23.28B	22.80A	23.43C	4.75B	2.03B
		60	9.90B	24.44C	1.57B	11.68A	17.38B	28.94B	22.87C	3.77B	2.10B
		90	8.93B	24.98C	1.63B	13.12A	21.52B	24.78A	16.20A	3.23C	2.15B
F3[a]	R	0	10.70A	8.55D	13.89A	10.57B	23.33A	23.03B	84.57B	11.81D	12.69B
		30	9.81A	5.86B	14.49A	11.67C	29.87B	21.09A	82.18B	9.49C	12.40B
		60	8.32A	5.68B	14.50A	10.63B	34.52B	19.18A	81.42B	6.54A	9.46A
		90	9.46A	4.27A	12.70A	11.94C	33.84B	20.42A	76.65A	5.71A	12.69B
	F	0	10.70A	8.55D	13.89A	10.57B	23.33A	23.03B	84.57B	11.81D	12.69B
		30	8.59A	7.18C	14.25A	10.17A	26.32A	25.13C	82.50B	10.21C	12.40B
		60	8.86A	6.20B	13.98A	10.66B	32.03B	20.35A	83.12B	8.40B	11.47B
		90	7.96A	5.51B	15.03A	10.78B	34.19B	19.47A	73.31A	7.38B	14.05B
F4[a]	R	0	4.81D	5.24E	12.97A	9.34A	32.84A	27.01D	56.32C	9.45C	7.80B
		30	4.18C	4.08B	12.98A	9.67A	43.96C	19.97B	51.29B	7.88B	7.66B
		60	3.44B	3.17A	12.40A	9.05A	46.92D	19.97B	45.19A	6.02A	6.32A
		90	3.40B	2.80A	13.93B	10.01A	42.84C	21.22B	43.78A	5.02A	6.53A
	F	0	4.81C	5.24E	12.97A	9.34A	32.84A	27.01D	56.32C	9.44C	7.80B
		30	4.33C	4.64D	12.91A	9.46A	39.79B	23.01C	52.40B	8.20B	7.20B
		60	3.04A	3.28B	12.33A	8.82A	44.98C	22.98C	50.18B	7.23B	7.48B
		90	3.06A	3.32B	14.58B	8.86A	48.12D	17.55A	42.26A	5.17A	7.31B
Factorial ANOVA[d]											
Formula (p-value)			<0.001	<0,001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
F1[d]			B	A	B	A	D	A	B	B	B
F2 [d]			C	C	A	B	A	C	A	A	A
F3 [d]			D	B	D	C	B	B	D	D	D
F4 [d]			A	A	C	B	C	B	C	C	C
SC (p-value)			0.016	<0,001	0.041	<0.001	<0.001	0.001	ns	0.001	0.004
R [d]			A	A	A	B	B	A		A	A
F [d]			B	B	B	A	A	B		B	B
Time (p-value)			<0.001	<0.001	ns	<0.001	<0.001	<0.001	<0.001	<0.001	0.026
0 [d]			D	D		A	A	C	C	D	BC
30 [d]			C	C		B	B	B	B	C	AB
60 [d]			B	B		B	C	B	B	B	A
90 [d]			A	A		C	D	A	A	A	C
Interactions:											
Formula x Condition (p-value)			<0.001	<0.001	ns	<0.001	0.002	Ns	ns	0.032	0.040
Formula x Time (p-value)			<0.001	<0.001	0.02	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Condition x Time (p-value)			ns	<0.001	ns	<0.001	ns	0.044	ns	ns	0.008
Formula x Condition x Time (p-value)			<0.001	<0.001	ns	<0.001	0.001	0.006	0.02	ns	ns

[a]EO microcapsule formulas: F1 = 1:1 WM:C without CSD, F2 = 1:1 WM:C with CSD, F3 = 2:1 WM:C without CSD, and F4 = 2:1 WM:C with CSD.
[b]SC: Storage conditions: R (room temperature), F (fridge).
[c]Different letters in the columns indicate significant differences between treatments (ANOVA, LSD test, $\alpha = 0.05$).
[d]Different letters in the columns indicate significant differences between factor levels. Factorial ANOVA and LSD test ($\alpha = 0.05$) considering factors: formula (F1, F2, F3, and F4), storage conditions (R and F), and time (0, 30, 60, and 90 d), and their possible interactions.
ns: not significant ($P > 0.05$).

Microcapsules' antioxidant stability study

Significant differences in the main volatile compounds identified, antioxidant activities, and TPC were observed between samples stored for 90 d (Table 3). In general, the storage condition (temperature) showed significant effect on the relative composition of all compounds, FRSA, and TEAC (Table 3: Storage condition, $P < 0.05$). This effect was different for each compound. o-Cymene, γ-terpinene, terpinolene, other compounds, FRSA, and TEAC decreased, and terpinen-4-ol and thymol increased while storage temperature increased. The effect of temperature on

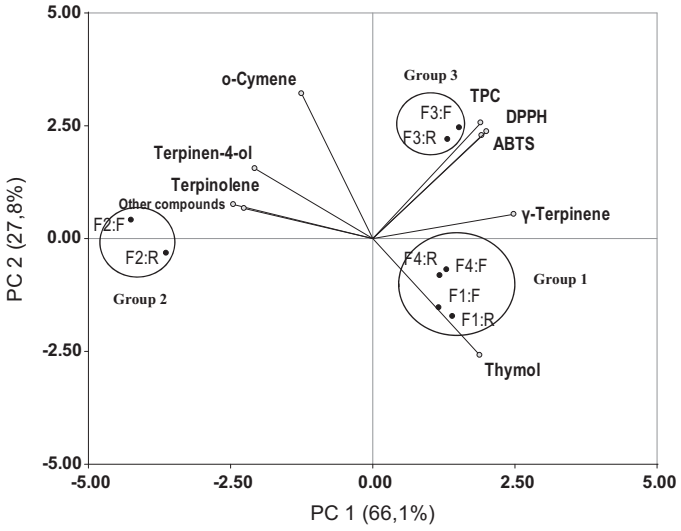

Figure 1–Biplot from the principal component analysis. Vectors are dependent variables: main volatile compounds (o-cymene, terpinen-4-ol, terpinolene, γ-terpineneand thymol), FRSA (DPPH test), TEAC (ABTS test), and TPC (Total phenolic content). Points are treatments: formulas: F1 = 1:1 WM:C without CSD, F2 = 1:1 WM:C with CSD, F3 = 2:1 WM:C without CSD, and F4 = 2:1 WM:C with CSD. Samples surrounded by circles are groups obtained by Cluster Analysis (Average: average linkage, distance: Euclidean).

Table 4–Means (n = 3) and standard deviations of the variables determined on each group of samples from cluster analysis: total phenolic compounds (TPC), free radical scavenging activity (FRSA), Trolox equivalent antioxidant capacity (TEAC), and volatile compounds (relative composition, %).

	Group 1[a]			Group 2[a]			Group 3[a]		
Variable	Mean	SD	[b]	Mean	SD	[b]	Mean	SD	[b]
TPC (mg galic acid/g)	45.7	6.94	B	21.0	4.3	A	81.5	4.00	C
FRSA (%)	6.4	1.59	B	4.4	1.1	A	8.7	2.19	C
TEAC (μg Trolox/g)	6.7	0.90	B	2.0	0.2	A	12.4	1.40	C
o-Cymene	4.2	1.19	A	8.8	2.2	B	9.4	1.19	B
γ -Terpinene	3.7	1.22	A	22.2	7.4	C	6.4	1.59	B
Terpinolene	12.4	1.04	B	1.2	0.7	A	14.1	1.06	C
Terpinen-4-ol	8.9	0.57	A	13.5	2.4	C	10.9	0.60	B
Thymol	44.2	7.20	C	22.2	11	A	29.7	5.16	B
Other volatile compounds	21.1	3.57	A	26.2	5.1	B	21.3	1.90	A

[a]Groups from cluster analysis: Group 1 (F4:R, F4:F, F1:R, and F1:F), Group 2 (F2:R and F2:F), and Group 3 (F3:R and F3:F).
[b]Different letters in the rows indicate significant differences between group of samples (ANOVA, LSD test, $\alpha = 0.05$).

these measured variables was weak with the exception of treatment F2 where this effect was more noticeable than in the others. The behaviors of volatile compounds captured by the SPME fiber depended on the properties of the individual compounds. In general, the release of *o*-cymene and γ-terpinene decreased during storage in all microcapsule samples. At 90 d of storage, F1 released the least amount of *o*-cymene, while no significant differences were found between F1:R (2.58%) and F1:F (2.76%), whereas, the highest value for this compound was observed in F3:R. Similar results were noted for γ-terpinene. Terpinolene did not present significant changes in quantity captured by the fiber during storage of F1 and F3 (different WM:C ratio, without CSD), regardless of the storage condition. However, the amount of terpinolene decreased significantly in F2:R (none detected). The behavior of terpinen-4-ol was similar to terpinolene, whereby the volatile quantity of this compound did not change in F1 and F4. Thymol, the oxygenated monoterpene known for being characteristic of oregano species and for its antioxidant activity (Dambolena and others 2010; Quiroga and others 2011, 2014) experienced significant changes during storage for all formulas and conditions. In general, the amount of thymol released increased during storage in all samples. This result might be directly associated with the decrease in the antioxidant activity (FRSA and TEAC) and phenolic content of the microcapsules during storage. Conversely, according to Asensio and others (2015a), no direct correlation between the antioxidant activity of EOs and their main components was demonstrated. The lowest TPC, FRSA and TEAC values at 90 d of storage were observed in F2:R (13.49 mg gallic acid/g sample, 3.01 PI and 1.72 μg Trolox/mg sample). The volatile compound release affected the antioxidant capacity of all formulas. Lower molecular weight volatile compounds (*o*-cymene and γ-terpinene) showed a decrease in their release, probably because they were largely volatilized during the spray-drying process. It was reported in oregano gelatin/sucrose microcapsules that once the temperature process was applied in the spray-drying method, some unavoidable loss of the volatile compounds occurred and, consequently, less EO was released from the microcapsule (Beirão Da Costa and others 2012).

In order to assess each factor's (formula, storage condition and time) contribution to the obtained data, the factors were first analyzed separately and then, the interactions between them were studied. A factorial analysis of variance was carried out (Table 3). An interaction between 2 or more factors signifies that their performances could not be evaluated separately. The release of *o*-cymene was affected by the formula ($P < 0.001$), storage temperature ($P = 0.016$), and the storage period ($P < 0.001$). In addition, a significant interaction between these 3 factors was evident ($P < 0.001$), so the behavior of this compound varied because of this interaction. The same results were observed for γ-terpinene, terpinen-4-ol and thymol. The TPC value was influenced by the formula and storage time but it was not affected by the storage temperature. FRSA and TEAC values depended on the formula, storage temperature, and storage time. Nevertheless, no interaction was established between these 3 factors. Therefore, the behavior of the antioxidant activity of these microcapsules needs to be predicted by analyzing the conditions under which a formula will be stored.

PCA

The biplot of the PCA results is presented in Figure 1. Principal component 1 (PC1) and principal component 2 (PC2) explained 93.9% data variability. This percentage was considered acceptable

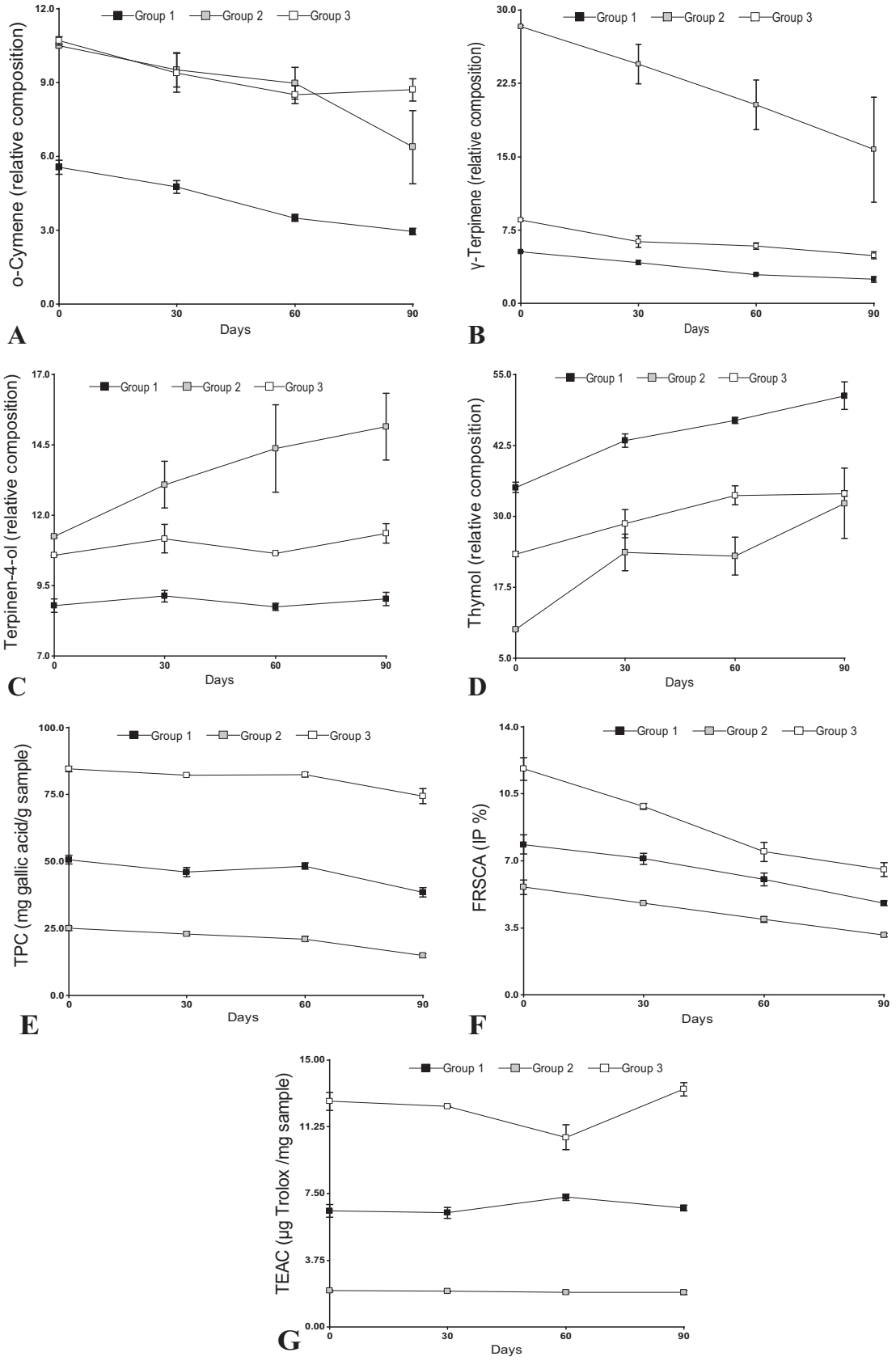

Figure 2–(A) o-Cymene, (B) γ-Terpinene,), (C)Terpinen-4-ol, (D) Thymol, (E) TPC, (F) FRSA, and (G) TEAC of microcapsules groups obtained by cluster analysis during 90 d of storage under 2 different conditions. Groups from cluster analysis: Group 1 (■) formed by F4:R, F4:F, F1:R, and F1:F; Group 2 (▣) formed by F2:R and F2:F; and Group 3 (□) formed by F3:R and F3:F.

to establish correlations between variables. The results showed a high dispersion point that indicates a great variability among samples. Considering that angles between vectors lower than 90° indicate positive associations and angles near 180° indicate negative associations between variables, the results of this study showed that thymol, TEAC, FRSA, TPC, and γ-terpinene exhibited positive association. All of them were located on the right-hand side of PC1. Particularly, a strong association between TEAC, FRSA, and TPC was established. Pearson´s correlation coefficients show that TPC was highly correlated ($P < 0.001$) with TEAC ($r = 0.956$) and with FRSA ($r = 0.845$). Also, TEAC and FRSA were correlated between them ($r = 0.733$). A positive association between the ability of antioxidants to scavenge free radicals (ABTS assay) and to reduce iron (FRAP assay) was previously reported (Juliani and others 2009; Malatova and others 2011; Asensio and others 2015a). However, inconsistent findings were reported regarding a correlation between ABTS and DPPH tests and TPC.

Surprisingly, no association was established with antioxidant activity tests and the release of thymol, explained by vectors located at 90°. Asensio and others (2015a) found a poor correlation between *trans*-sabinene hydrate and TEAC ($r = 0.26$), and with thymol and terpinen-4-ol ($r = 0.42$). Conversely, a negative association was observed between the release of thymol and *o*-cymene. When thymol levels increased, *o*-cymene levels decreased. Terpinen-4-ol, terpinolene, *o*-cymene, and other compounds were located on the left-hand side of the plot, suggesting a strong association between the performances of these compounds. In general, the same formulas, regardless of the storage condition, were associated with each other. In the PCA biplot, both F2 (1:1), stored at R and F, were placed close to the release of terpinen-4-ol and terpinolene. These 2 treatments were located opposite to F3 (R and F), the antioxidant activity tests, TPC, and γ-terpinene. In addition, lower values for these variables should be expected in the F2 formula than in the other formulas. These associations were consistent with the results of the storage study, showing poor preservation of the F2 antioxidant capacity at the end of storage.

F1 and F4 were associated with the release of thymol during storage at R and F. The presence of CSD in F4 with double WM exhibited the same effect as the 1:1 WM:C ratio (F1). Moreover, it seemed that the release of thymol could have caused a decrease in the antioxidant capacity of these formulas, with respect to F3. Data obtained from this study suggests that some formulas (F3 ≥ F4 ≥ F1) are a promising alternative to the delivery of oregano EO as a natural antioxidant agent to be applied in foods with the purpose of improving their preservation.

CA

The groups obtained from CA were surrounded by a circle in the PCA biplot (Figure 1). Three groups were identified, including Group 1 (formed by the samples F1:R, F1:F, F4:R, and F4:F), Group 2 (F2:R and F2:H), and Group 3 (F3:R and F3:F). Means and standard deviations of the variables determined on each group of samples from cluster analysis are shown in Table 4. The clustering of F1 and F4 (irrespective of the storage condition) in Group 1, suggested that the presence of CSD in F4, with double WM, produced the same effect in the release profile of volatile compounds and in the antioxidant capacity as the sample with the same ratio of wall components and lipid core (F1). Previously, Gallo and others (2011) found that in formulations with a low solids concentration, Aerosil (CSD) was not able to hold substantial quantities of liquid to totally confine the plant extract within the agglomerates' interstitial volume. In this study, the presence of CSD in F4 lessens the protective effect of the double WM:C ratio of this formula.

The release of the major volatile compounds, the antioxidant capacity, and the TPC of the CA groups during storage is shown in Figure 2. In general, o-cymene, γ-terpinene, FRSCA, and TPC decreased, and thymol increased in the 3 groups during storage. Terpinen-4-ol only increased during storage in Group 2. TEAC remained almost constant during storage in all groups. All variables differed among groups of formulas, as can be seen in Figure 2 and in Table 4. The lowest o-cymene, γ-terpinene, terpinen-4-ol, and the highest thymol amounts were found in Group 1 (F1 and F4). This group had intermediate values of TPC, TEAC, and FRSCA. Group 3 had the highest, and Group 2 exhibited the lowest TPC, TEAC, and FRSCA values. Those differences could indicate different stability properties provided by each of the matrices regarding volatile compound release and antioxidant capacity. These results are in accordance with Figure 1, were F3 (Group 3) is associated with higher phenolic compounds and antioxidant properties than the other formulas. De Barros Fernandez and others (2014) found that the concentration of starch and maltodextrin (WM) in the feed was directly associated with the viscosity of the medium, which, in turn, interfered with the retention of volatiles of rosemary EO, and that oil retention was significantly influenced ($P < 0.05$) only by the WM concentration, in agreement with the current results.

Conclusions

The release of volatile compounds from oregano EO microcapsules and their antioxidant activity is determined by each formula. The presence of CSD in microcapsules increases the release of volatile compounds and, as a consequence, the antioxidant activity is lost in a higher proportion through storage, particularly, in formulas with lower WM:C ratio. A higher WM on microcapsules keeps longer the antioxidant activity of microcapsules during storage. The studied formulas show a positive interaction between antioxidant variables and temperature. The EOs microencapsulation constitutes an alternative delivery way which makes them easy to handle and to distribute in a food matrix, and helps to preserve the antioxidant activity of these compounds. However, further studies are required to evaluate the preserving effects of these kind of microcapsules as an additive to control oxidation in various food matrices.

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Author Contributions

C. Asensio designed the experiment, collected the data and drafted the manuscript. A. Paredes and P. Martin operated the spray drying. V. Nepote collaborated with the statistical analysis. D. Alemandi and NR. Grosso were the advisors of the project.

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