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Review Article

Kinetic Stability of Esterified and Unesterified Encapsulated Capsanthin from Red Pepper (*Capsicum annuum* L.) by Spray-Drying using Capsul® as Encapsulating Agent

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Abstract

Capsanthin is a colorant with health benefits. However, its application in the food industry is limited due to its susceptibility to degradation. In this study, pepper oleoresin was encapsulated in esterified (EO-C), unesterified (USO-C) and isolated (IC-C) forms using Capsul® as encapsulating agent by spray drying. A 22 statistical factorial design was used to evaluate the EO-C and USO-C systems; IC-C was encapsulated with the optimal conditions obtained from these systems. The objective of this study was to evaluate the effect of the esterification of capsanthin with respect to the encapsulation efficiency (EE) and the kinetic stability at three temperatures. The systems showed that the esterification of capsanthin (EO-C) increased the EE. The degradation of capsanthin followed a first order kinetics. No significant differences were found in the degradation rate, activation energy, activation enthalpy ((ΔH≠) and activation entropy (ΔS≠) between the microparticles systems. A linear relationship was obtained in the graph ΔH≠ vs ΔS≠ (r^2 = 0.99). This compensatory effect suggests that capsanthin, independent of the form in which it is presented, is degraded by a similar mechanism and the esterification process had no effect on the thermodynamic parameters.

Keywords: Capsanthin; Microencapsulation; Kinetics Parameters; Spray-Drying; Esterification

Abbreviations

EO-C: Esterified Oleoresin-Capsul®; USO-C: Unesterified Oleoresin-Capsul®; IC-C: Isolated Capsanthin Oleoresin-Capsul®.

Introduction

Currently, there is a growing interest in developing food or food supplements based on natural and functional ingredients with health benefits. Capsanthin, present in red pepper, (*Capsicum annuum* L.) is in this tendency. Red peppers contain a wide range of carotenoids, up to 30 of them identified in the esterified extract [37], capsanthin being the most abundant [25], comprising 70 - 80% of the total carotenoids in the ripe fruit [12]. Capsanthin is a natural colorant and antioxidant without cytotoxic or genotoxic effects, and it is an alternative to the use of synthetic colorants and antioxidants. Also, Aizawa and Inakuma showed that capsanthin intake significantly increases HDL cholesterol levels [1].

Paprika oleoresin concentrate, with a high level of capsanthin, is widely used as a coloring and/or antioxidant in the food, pharmaceutical and cosmetic industry [10,30]. In the food industry, the oleoresin concentrate is used as a colorant in meats, sausages, pickles and other processed foods [39], and it has the advantage of standardizing the content of capsanthin with respect to the ground product. However, the carotenoids are susceptible to degradation because they are unsaturated molecules and can be affected by factors such as temperature, oxygen and light [22,27]. Also, the stability of paprika oleoresin is further affected by the degree of esterification of xanthophylls, processing and subsequent storage [5,28]. For this reason, the encapsulation process is presented as a technology for its protection.

In recent years, encapsulation technology has increased in importance in the food industry, particularly, in the development of functional and health foods. The encapsulation of oleoresin

Capsicum annuum L. and capsanthin had been performed using different techniques. These include spray drying with different encapsulants such as gelatin, gum arabic, sodium caseinate, modified starch, a mixture of maltose-dextrin and a mixture of rice starch with gelatin [4,36,43]; coacervation using calcium alginate and an intermediate barrier composed of whey protein isolate [34], soybean protein isolate (SPI)-chitosan [44], cross-linking of soybean protein isolate-chitosan with transglutaminase [11]; self-emulsifying nanoemulsion [3]; and spray cooling using canola oil [23]. The objectives of these studies have been to evaluate the loss of color under different light, temperature and humidity conditions [4], the encapsulation efficiency and recovery of oleoresin (Xi43), morphology and particle size [36], the oxidative stability of encapsulated oleoresin [34] and the kinetics of release [23]. However, there are no studies about the effect of the esterification of capsanthin with respect to the encapsulation efficiency and stability at different temperatures.

The objective of this study was to evaluate the effect of the esterification of capsanthin with respect to the encapsulation efficiency and the stability during storage at different temperatures using Capsul® as encapsulating agent.

Materials and Methods

Materials

Commercial pepper flakes (Capsicum annuum L.) were obtained from Invertec Foods (Rengo, Chile), Capsul® from National Starch, Sunflower oil Natura® from the supermarket, Na_2SO_4, Celite 545 and Magnesium oxide from Merck, different solvents of analytical grade and HPLC grade from Merck.

Preparation of oleoresin

The extraction of carotenoids from the pepper flakes was performed according to the method described by Mínguez-Mosquera and Hornero-Méndez [24]. The isolated capsanthin was obtained by open column according to the method described by Rodríguez-Amaya (33). Sunflower oil was then added to each extract to obtain esterified oleoresin (EO), unesterified oleoresin (USO) and isolated capsanthin oleoresin (IC) with an equal quantity of capsanthin in each oleoresin. In all systems, capsanthin was analyzed without considering the other carotenoid pigments.

Standard solution

Isolated capsanthin was obtained from the pepper flakes using acetone for the extraction and purified by open column chromatography with stationary phase MgO: Celite (1:1), according to the method described by Rodríguez-Amaya (1999). The concentration of capsanthin was determined by spectrophotometry in a Unicam UV-3 equipment at 460 nm using the extinction coefficient in acetone (E1% = 2300) [42].

Preparation of the microparticles

Emulsions were prepared containing Capsul® as encapsulating agent, water, EO or USO, with a ratio of oleoresin/encapsulating agent of 1:1, 1:2 and 1:3 (w/w), considering 100 g solution as follows: Capsul® (10-30 g) was dissolved in water at 70°C with constant stirring, then cooled to 30°C and the oleoresin (10 g) added. The resulting emulsions were homogenized at 15.000 rpm for 2 min, 19.000 rpm for 2 min and 22.000 for 1 min with a Polytron PT 2100 (Kinematica AG, Switzerland), and were fed into a B-290 mini spray-dryer (Büchi, Switzerland). The spray-dryer was operated at an inlet temperature ranging from 150 to 200ºC. The air flow, rate of feeding and atomization pressure were 600 L/h, 5 mL/min and 20 psi, respectively. The powders obtained were stored in the dark at -20°C for subsequent analysis. The IC-C microparticles were prepared from the microparticles obtained under optimal conditions from the EO-C and USO-C systems.

Determination of the capsanthin encapsulation efficiency

Total capsanthin

To determine total capsanthin, EO-C, USO-C and IC-C microparticles system (100 mg) were dispersed in 1 mL of water and stirred for 1 min., then a 10 mL mix of ethyl acetate: ethanol: hexane: water (1:1:1:1 v/v) was added and stirred again. Two extractions were made until no red color remained. The supernatants were transferred into petroleum ether, then unesterified with an equal volume of 10% potassium hydroxide in methanol overnight at room temperature. The capsanthin content was determined by HPLC, using a calibration curve for capsanthin (1.04 - 3.48 $\mu g/mL^{-1}$, $R^2 = 0.996$).

Surface capsanthin

To determine surface capsanthin, the EO-C, USO-C and IC-C microparticles systems (100 mg) were dispersed in a 3 mL hexane: ethyl ether mix (50:50 v/v). Stirring was done manually for 30 seconds and the supernatants removed. The supernatants were transferred into petroleum ether, then unesterified with an equal volume of 10% potassium hydroxide in methanol overnight at room temperature. The capsanthin content was determined by HPLC.

Capsanthin encapsulation efficiency

The capsanthin surface percentage (SC) and the microencapsulation efficiency (EE) were calculated according to Eqs. (1) and (2), respectively.

$$SC\ (\%) = \frac{\text{Surface capsanthin}}{\text{Experimental total capsanthin}} \times 100 \quad \text{.....(1)}$$

$$EE = \frac{\text{Exp. total capsanthin - Surface capsanthin}}{\text{Exp. total capsanthin}} \times 100 \quad \text{.....(2)}$$

Chromatographic procedure

Capsanthin analysis was performed by HPLC using a Merck-Hitachi L-6200 pump, a Waters 996 photodiode-array detector and C18 column (3 µm, 4.6 i.d. x 50 mm, Atlantis®, Waters, Ireland). A mobile phase of acetone/water in different proportions was used at a flow rate of 1 mL/min. Capsanthin was identified by comparing the peak retention times with standard isolated capsanthin and quantified using a calibration curve (1.04 - 3.48 ug/mL, $R^2 = 0.996$). Detection was at 450 nm.

Characterization of microparticles obtained under optimal conditions

Total and surface capsanthin, and therefore encapsulation efficiency, were determined as described above. The morphology of the microparticles obtained under optimal conditions was evaluated by Scanning Electron Microscopy (SEM). The samples were coated with gold/palladium using Varian Vacuum Evaporator PS 10E, and were analysed using a JEOL JSM-25SII (Jeol, Tokyo, Japan) scanning electron microscope operated at 30 kV. The images were obtained with a Mamiya Roll Film Holder camera (Model 2) coupled to the microscope using Kodak 120 T-Max ISO 100 film.

Storage stability assay

The EO-C and USO-C microparticles obtained under optimal conditions and the IC-C microparticles were stored at 40, 50 and 70 ± 1°C in a forced-air oven with controlled temperature (Memmert BE 500, Germany) in absence of light. 50 mg samples were transferred to glass tubes and left uncovered. For determination of capsanthin levels by HPLC, duplicate vials were removed at different times depending on the temperature. 7 points were obtained in each system to obtain the degradation curve of capsanthin in each system.

Kinetic analysis

The data were fitted to first-order kinetics. Degradation rate constants (k) were obtained from the slope of a plot of the natural log of the retention percentage of carotenoids vs. time. The activation energy (E_a) and frequency factor (A) were determined from the Arrhenius model $k = Ae^{-(Ea/R)/T}$, where E_a/R is the slope and lnA is the intercept of the relationship between the natural log k and (1/T) in kelvin degrees. For a first-order reaction, the half-life was determined at a specific temperature by the equation $t_{1/2} = \ln 2/k$.

The activation enthalpy ($\Delta H\neq$) was obtained by plotting ln(k/T) vs. (1/T), and the activation entropy ($\Delta S\neq$) was obtained from Equation 3 based on the transition state theory,

$$\ln(k/T) = \ln(k_B/h) + \Delta S\neq/R - \Delta H\neq/RT \text{.....(3)}$$

where k_B is the Boltzmann constant and h is Planck's constant.

Statistical design

The experiments were performed using a factorial 22 central composite experimental design. Ten experiments were performed for the EO-C and USO-C systems. The oleoresin/encapsulating agent ratio (1:1-1:3) and inlet air temperatures (150-200ºC) were evaluated as independent variables. The dependent variable was the capsanthin EE. A response surface methodology was applied to optimize the EE of capsanthin, using Statgraphics software version 7.0 (Manugistics Inc., Statistical Graphics Corporation, 1993, Rockville, MA, USA).

The linear regression (95% confidence limit) was used to determine the reaction order, rate constants, and activation energy. To determine the statistical differences among rate constants degradation, activation energy, activation enthalpy and activation entropy, a multivariate ANOVA and multiple range test of Tukey were performed. All the statistical analyses were calculated by using Statgraphics, version 7.0 (Manugistics Inc., Statistical Graphics Corporation, 1993, Rockville, MA, USA).

Results and Discussion

Optimization of capsanthin microencapsulation by response surface methodology (RSM)

It is known that optimal spray-drying conditions must be used to obtain high EE. Feed temperature, air inlet temperature and air outlet temperature have been reported as the important

variables in the spray-drying process [8]. In this study, the inlet air temperature and oleoresin/encapsulating agent ratio were evaluated as independent variables and capsanthin EE as the dependent variable.

The EE of capsanthin ranged from 38-92% and 35-86%, and capsanthin recovery ranged from 28-67% and 32-74% in the EO-C and USO-C systems, respectively. When spray drying is used, the results of the EE of carotenoid pigments will be different and depend on the encapsulating agent and the conditions in the process [7,18,19,40]. In the EO-C and USO-C systems, when the quantity of encapsulating agent was increased, the percentage of the superficial capsanthin decreased, and both the EE and recovery of capsanthin improved. This behavior has been reported in other studies by Rosenberg., *et al.* and Soottitantawat., *et al.* [35,41].

The RSM was applied to optimize the EE of capsanthin for each system studied, considering linear, quadratic, and cross-product forms for the independent variables studied, with a significance levels $p \leq 0.05$. figure 1 shows the graphs obtained with the RSM for the USO-C and EO-C systems. The inlet air temperature did not show significant differences ($p > 0.05$) on the EE of capsanthin for any of the systems studied, due to the short drying times (5-100 s) and the rapid formation of a crust [8], allowing the diffusion of water from the interior of the microparticles and the retention of capsanthin [35,41]. The oleoresin/encapsulating agent ratio was significant ($p \leq 0.05$) in both systems studied, and when the oleoresin/encapsulating agent ratio was increased, the percentage of surface capsanthin decreased, and the EE and recovery of capsanthin improved. When Capsul® is used as encapsulating agent, it is possible to add a higher quantity of solid because it has a low viscosity, which explains the better recovery of the active compound.

Figure 1: Graph of surface response for EO-C (A) and USO-C (B) microparticles systems.

Microparticles obtained under optimal conditions

Table 1 shows EE, recovery and the size of the capsanthin particles for each microparticles system obtained under optimal conditions.

Systems	Relation O/E	Inlet Temperature (ºC)	Surface Capsanthin (X% ± DS)	Capsanthin EE(X% ± DS)	Capsanthin recovery (X% ± DS)	Particle size D (3,2)(µm)
EO-C	1:3	200	8,4 ± 0,3^{c}	91,6 ± 0,3^{a}	69,9 ± 2,5^{a}	3,9
USO-C	1:3	200	16,9 ± 1,5^{a}	83,1 ± 1,5^{c}	74,5 ± 1,8^{a}	4,4
IC-C	1:3	200	11,2 ± 0,4^{b}	88,8 ± 0,4^{b}	68,2 ± 0,2^{a}	3,9

Table 1: Conditions and characterization for EO-C and USO-C microparticles systems obtained under optimal conditions and IC-C microparticle systems.

O/E: Oleoresin/Encapsulant Agent Ratio; X: Mean; DS: Standar Deviation; EO-C: Esterified Oleoresin-Capsul®; USO-C: Unesterified Oleoresin-Capsul®; IC-C: Isolated Capsanthin Oleoresin-Capsul®.

*Different letters indicate significant differences

The recovery did not show significant difference ($p > 0.05$) between the systems studied, because the microparticles were prepared using the same optimal conditions oleoresin/Capsul® and inlet air temperature, being the effect of the oleoresin the main factor to evaluate. In relation to EE, it is possible to see a significant difference ($p \leq 0.05$) between systems. The results show that the system, where the capsanthin was free of fatty acid, (USO-C and IC-C) had a significantly lower EE ($p \leq 0.05$) with respect to the EO-C system, because the fatty acid which esterified the capsanthin in the EO-C system allows a better interaction with the hydrophobic sites of Capsul®, improving the EE.

The size of the microparticles produced under optimal conditions, is presented according to the value D (3.2), which fluctuated between 3.9 and 4.4 µm and is within the ranges reported in the literature for the spray-drying process [8]. The

size distribution for the three systems studied was unimodal, with particles in one size range. The size of the microparticles obtained by spray-drying depends on the active compound and the encapsulanting agent [20,36,38,40].

Figure 2 (A-C) shows the SEM photographs of the capsanthin microparticle powders obtained under optimal conditions. In all systems, the microparticles are spherical in shape with smooth walls, and microparticles with dents on the surface. Both types of microparticles present continuous walls without apparent cracks. The formation of microparticles with dents is a usual characteristic when spray-drying is used. The shrinkage of the particles during the drying process can occur at low or high inlet air temperatures [2]. At low temperatures, there is less diffusion of water and the particles have more time to shrink, while at higher temperatures, the rapid evaporation of water and high pressure inside the particles produces shrinkage. A similar morphology was observed with modified tapioca starch, native tapioca starch, maltodextrin, Capsul®, Hi Cap-100 and gum Arabic [15,20,32,41]. However, Krishnan., *et al.* [17] found that microparticles prepared from a mixture of rice starch and gelatin have interstices and pores, because the gelatin does not form a continuous film on the surface, compromising its ability to act as a barrier. Finally, the morphology of the microparticles depends on the different parameters involved in the drying process such as feed temperature, inlet air temperature and solvents used, among others [16].

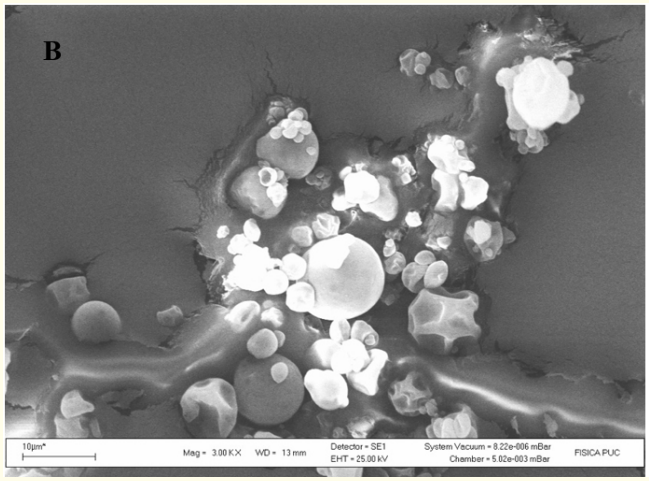

Figure 2: Scanning electron microscopic photographs for EO-C (A), USO-C (B), and IC-C (C) microparticle systems. Mag. 300 Kx.

Capsanthin stability assays

The capsanthin kinetics degradation was monitored during the storage of the EO-C, USO-C and IC-C microparticles systems at 40, 50 and 70°C, respectively. The order of the reaction, rate constants, half-life and thermodynamic parameters were determined.

Figure 3 (A-C) shows the natural logarithm of capsanthin percentage retention versus time (h) for the EO-C, USO-C and IC-C microparticles systems, stored at 40, 50 and 70°C. From the slope of the graphs, the rate constants of degradation of capsanthin were obtained for each system and temperatures studied.

Figure 3: Capsanthin degradation kinetics for microparticles from the systems EO-C (A), USO-C (B), IC-C (C) obtained under optimal conditions ● (40ºC), □ (50ºC) y ▲ (70ºC).

The degradation of capsanthin in the three systems followed first order kinetics at three temperatures studied. Similar results were reported for paprika oleoresin [14,29], paprika extract dissolved in water [27,39], and standard solution of esterified and unesterified capsanthin [28]. Conversely, a second order kinetics degradation [4] was found in microencapsulated paprika oleoresin.

Table 2 shows degradation rate constants (k) of capsanthin for the EO-C, USO-C and IC-C microparticles systems stored at 40, 50 and 70°C. An increase in the storage temperature increased the degradation rate constant (k) of capsanthin in all systems studied. The degradation rate of capsanthin did not show significant differences differences ($p > 0.05$) between systems at the different temperatures studied, showing that the esterification of capsanthin does not influence the stability of capsanthin. In studies with other carotenoids esterified with polyunsaturated fatty acids, an increase in the degradation rate constant was observed due to a more oxidative environment that accelerates the degradation, in relation to unesterified forms [28]. These differences could be attributed to the type of fatty acids used in the esterification of carotenoids; capsanthin is preferably esterified with saturated fatty acids. The studies about the effect of esterified and unesterified carotenoids on lipid oxidation are controversial. Matsufuji., *et al.* [21] reported that esterified and unesterified capsanthin inhibit oxidation by the same mechanism, and it could be due to the presence of the keto group in the structure of capsanthin. Was reported that esterified capsanthin is more stable than unesterified capsanthin to lipoxygenases in seeds [5]. While Jarén-Galan and Minguez-

Systems			
Temperature (ºC)	**EO-C k (h^{-1}) ± SD**	**USO-C k (h^{-1}) ± SD**	**IC-C k (h^{-1}) ± SD**
40	$2,4 \times 10^{-3} \pm 0,9 \times 10^{-4a}$	$2,4 \times 10^{-3} \pm 2,8 \times 10^{-4a}$	$2,0 \times 10^{-3} \pm 2,8 \times 10^{-4a}$
50	$4,0 \times 10^{-3} \pm 3,5 \times 10^{-4b}$	$4,0 \times 10^{-3} \pm 2,8 \times 10^{-4b}$	$4,2 \times 10^{-3} \pm 1,4 \times 10^{-4b}$
70	$2,3 \times 10^{-2} \pm 1,6 \times 10^{-3c}$	$2,3 \times 10^{-2} \pm 1,6 \times 10^{-3c}$	$2,2 \times 10^{-2} \pm 2,1 \times 10^{-4c}$

Table 2: Degradation rate constants (k) from EO-C and USO-C micropaticles systems obtained under optimal conditions and IC-C microparticles system, stored at 40, 50 and 70°C.

EO-C: Esterified Oleoresin-Capsul®; USO-C: Unesterified Oleoresin-Capsul®; IC-C: Isolated Capsanthin Oleoresin-Capsul®.

*Different letters indicate significant differences

Table 3 shows the Arrhenius parameters, half-life and thermodynamic parameters of encapsulated capsanthin. The activation enthalpy (ΔH≠) and activation entropy (ΔS≠) were obtained from the slope and intercept, respectively, from the graph of ln (k/T) vs. 1/T. Statistical analysis showed no significant differences for the activation energy, activation enthalpy and activation entropy of capsanthin, between the microparticles systems studied. Pérez-Galvez., *et al.* [26] reported the same activation energy for the red fraction of paprika oleoresin, however, the activation enthalpy was higher and the activation entropy was lower than reported in this study, because the capsorubin was included in the red fraction as it is more stable than capsanthin.

Systems	Activation energy (Kcal/mol) (X ± SD)	Intercept (ln A) (X ± SD)	R^2	Half-life to 25°C (Days ± SD	Activation enthalpy (Kcal/mol) (X ± SD)	Activation entropy (cal/mol K) (X ± SD)	R^2
EO-C	15,9 ± 0,41ª	19,5 ± 0,69a	0,98	48	15,3 ± 0,42a	-24,8 ± 1,37ª	0,98
USO-C	16,5 ± 0,28ª	20,5 ± 0,34ª	0,98	53	15,9 ± 0,28ª	-22,9 ± 0,68ª	0,98
IC-C	17,3 ± 0,86ª	21,5 ± 1,26ª	0,99	62	16,6 ± 0,86ª	-20,9 ± 2,51ª	0,99

Table 3: Arrhenius parameters, half-life and thermodynamic parameters from EO-C and USO-C microparticles systems obtained under optimal conditions and IC-C microparticles systems.

X: Mean; SD: Estándar Deviation; EO-C: Esterified Oleoresin-Capsul®; USO-C: Unesterified Oleoresin-Capsul®; IC-C: Isolated Capsanthin Oleoresin-Capsul®.

*Different letters indicate significant differences

The estimated half-life of capsanthin at 25°C ranges between 48-62 days and was higher than rosehip oleoresin microparticles [31], and β-carotene (7-27 days at 25°C) [7], all using spray drying as encapsulation method.

Figure 4 shows the graph activation enthalpy versus activation entropy (r^2 = 0,999). The graph shows that capsanthin is degraded by a similar mechanism, and it does not depend on the esterification of capsanthin. The same effect was reported in the oxidative degradation kinetics of lycopene, lutein, 9-cis and trans-β-carotene in saffron oil [9].

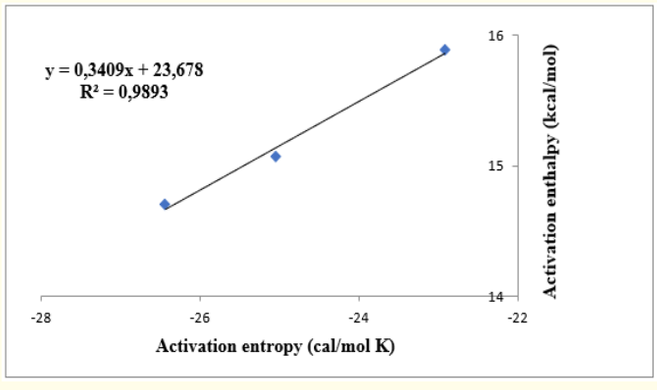

Figure 4: Isokinetic line for capsanthin degradation.

Conclusion

In the encapsulation of capsanthin, when capsanthin is esterified with fatty acids, the EE increased, suggesting that the fatty acids allow a better interaction with the hydrophobic sites of Capsul®. The degradation rate constants of capsanthin were similar for the three microparticles systems (EO-C, USO-C and IC-C) studied, showing that the esterification of capsanthin does not affect its stability. The results obtained in this study should allow the development of paprika oleoresin microparticles with potential application as functional ingredients.

Conflict of Interest

I do not declare conflicts of interest

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