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Fortification of chocolate using *Moringa oleifera* extract encapsulated in microemulsions

Olga Kaltsa, Aggeliki Alibade, Georgia Batra, Eleni Bozinou, Dimitris P. Makris and Stavros I. Lalas*

Department of Food Science & Nutrition, University of Thessaly, Terma N. Temponera Street, GR-43100 Karditsa, Greece

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Abstract – The aim of the present study was to evaluate the physical and antioxidant properties of microemulsions containing *Moringa oleifera* leaf extract (MLE) produced by the means of a deep eutectic solvent. Selected microemulsions containing MLE were incorporated in chocolate products to enrich them. Their color properties including CIE L*, a*, b* parameters and whitening index (WI) along with DPPH radical scavenging activity were assessed during a period of 8 months. The antioxidant activity of microemulsions depended on the oil phase used, while it was unaffected by the concentration of MLE. Samples prepared with soybean oil as oil phase containing MLE presented the highest radical inhibition percentage (I% = 26.8–27.8%). Coconut microemulsions were finally incorporated at 2 and 4% w/w concentration into chocolate products, as coconut oil is a known cocoa butter substitute. Although the incorporation of MLE microemulsions did not affect the color properties of most of the chocolates, enriched products did not exhibit superior antioxidant activity compared to control samples.

Keywords: *Moringa oleifera* / chocolate / microemulsion / stability / antioxidant capacity

Résumé – Fortification du chocolat à l'aide d'un extrait de *Moringa oleifera* encapsulé dans des microémulsions. L'objectif de cette présente étude était d'évaluer les propriétés physiques et antioxydantes de microémulsions contenant de l'extrait de feuille de *Moringa oleifera* (MLE) produit avec un solvant eutectique profond. Les microémulsions sélectionnées contenant du MLE ont été incorporées dans des produits chocolatés pour les enrichir. Leurs propriétés de couleur, y compris l'espace chromatique CIE L*, a*, b* et l'indice de blanchiment (WI), ainsi que l'activité de piégeage des radicaux DPPH, ont été évaluées durant une période de 8 mois. L'activité antioxydante des microémulsions dépendait de la phase huileuse utilisée, alors qu'elle n'était pas affectée par la concentration de MLE. Les échantillons préparés avec de l'huile de soja comme la phase huileuse contenant du MLE ont présenté le pourcentage le plus élevé d'inhibition des radicaux (I% = 26,8–27,8 %). Les microémulsions de noix de coco ont finalement été incorporées à des concentrations de 2 et 4 % (en masse) aux produits chocolatés, l'huile de noix de coco étant un substitut connu du beurre de cacao. Bien que l'incorporation des microémulsions MLE n'ait pas affecté les propriétés de couleur de la plupart des chocolats, les produits enrichis n'ont pas présenté une activité antioxydante supérieure à celle des échantillons témoins.

Mots clés : *Moringa oleifera* / chocolat / microémulsion / stabilité / capacité antioxydante

*Correspondence: slalas@uth.gr

Highlights

- Deep eutectic solvent extract was incorporated in food grade micro-emulsions.
- Microemulsions were stable against centrifugation and thermal treatment.
- Microemulsion antioxidant activity was greater for soybean oil formulations.
- Microemulsion incorporation in chocolate products did not impose a significant antioxidant effect.

1 Introduction

Food bio-functionality improvement is considered as an established priority for the food industry. To help achieve the production of healthier food, numerous techniques and methodologies have been developed within the last couple decades following the nutrient fortification (Akhtar *et al.*, 2011; Shilpashree *et al.*, 2015; Zhu *et al.*, 2020) or encapsulation path when components sensitive to processing (*i.e.*, vitamins, antioxidants, enzymes, probiotics) are incorporated (Ye *et al.*, 2018; Maurya *et al.*, 2020; Castro Coelho *et al.*, 2021). For the encapsulation of bioactive ingredients, several technologies have been proposed including nano/micro-emulsification, spray drying, lyophilization, electro-spinning/spraying (Ray *et al.*, 2016; Jalali-Jivan *et al.*, 2020; Moreira *et al.*, 2020). However, micro-emulsions are considered more advantageous than nano-emulsions since they are transparent and therefore do not affect the appearance of food products nor they require expensive equipment or ingredients for their formation (Flanagan and Singh, 2006; McClements, 2018).

Moringa oleifera seed and leaf extracts (MLE) have recently drawn attention for food and medicinal applications mainly due to high levels of antioxidants that can act protectively against the oxidative stress of cells (Lalas and Tsaknis, 2002; Karageorgou *et al.*, 2017; Soliman *et al.*, 2020). These include phytochemicals such as glucosinolate, ascorbic acids, carotenoids, phenolic acids and flavonoids (Dhakad *et al.*, 2019). Although the chemical consistency and antioxidant properties of MLE have been well documented (Lin *et al.*, 2018; Suresh *et al.*, 2020; Syeda and Riazunnisa, 2020; Wang *et al.*, 2020) etc., there are limited studies on encapsulation of MLE in microemulsions (Batra *et al.*, 2017; Sermkaew and Plyduang, 2020), as well as an absence of studies with regards to possible application in food.

In recent years, food production processes have prioritized the adaptation of technologies that minimize the impact to the environment. Deep eutectic solvents (DES) are a neoteric class of liquids that can be composed easily using affordable food grade compounds (polyols such as glycerol, organic acid salts and amines). Their main advantages include feasibility of tailor-made properties, preparation simplicity, absence of flammability, biodegradability and low toxicity. These characteristics make them suitable solvents for use in extraction of antioxidant compounds like polyphenols (Lakka *et al.*, 2020).

Therefore, the main objective of this work was to examine the properties of MLE microemulsions formed with a natural deep eutectic solvent (NADES) and examine the effect of its addition in chocolate products to enhance their antioxidant capacity.

2 Material and methods

2.1 Materials

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) reagent, Span80 and Tween80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cocoa butter and whey protein concentrate (80% in protein) were kind donations from Cocoowa (Thessaloniki, Greece) and Tyrokomiki Karditsas S.A. (Karditsa, Greece) respectively. Soybean oil and coconut oil, cocoa powder and sugar were purchased from a local store. Plant material was collected from *Moringa oleifera* trees grown in the Agioi Apostoloi area of Karditsa prefecture and the moringa leaf extract (MLE) was prepared according to the optimum conditions (ultrasonic pretreatment 30 min/23 °C, 100 ml of 75% w/w DES aqueous solution/g of dry plant powder, 200 RPM stirrer speed/150 min/50 °C) as established in Lakka *et al.* (2020). A detailed analysis on the polyphenolic profile of the extract by HPLC means is also given in the same study.

3 Methods

3.1 Microemulsion preparation

In this study a low energy method was used to prepare W/O microemulsions containing *Moringa* leaf extract with modifications (Yew and Misran, 2015). Initially, the surfactants Span80 and Tween80 (1:1 ratio) and selected oil (soybean or coconut oil) were mixed with a magnetic stirrer for 30 min at 600 rpm. Subsequently, the mixing speed was raised to 1 000 rpm and the MLE extract was added dropwise within 40 min. The mixture was left to equilibrate until a homogenous clear microemulsion was formed. Microemulsions containing 4 and 8% w/w MLE and respective blanks containing deep eutectic solvent aqueous solution were prepared as presented in Table 1.

3.2 Preparation of chocolate bars with microencapsulated MLE

Chocolate bars were prepared by mixing melted cocoa butter and dry ingredients (whey protein isolate, cocoa powder, sugar) in a lab-scale chocolate melanger (Chocogrind, Elgi Ultra, India). The main methodology for chocolate manufacture involves four distinctive stages: dry mixing, grinding/refining, conching and moulding (Becket, 2009). Briefly in this study, all dry ingredients (32% w/w sugar, 12% cocoa powder and 20% w/w whey protein concentrate) were initially ground in a ball mill for 1 h, melted cocoa butter (36% w/w) was added and the mixture was conched for 2 h at 70 °C. Chocolate masses were then cooled to 27 °C and allowed for tempering. Microemulsions containing MLE were incorporated at the end of the tempering phase to prevent the loss of antioxidants. Finally, the temperature was raised to 32 °C, chocolate was poured in high-density polyethylene molds and placed in a cool environment for 24 h before de-molding. The finished product was sealed in aluminum foil and kept at ambient conditions

Table 1. Percentage formulation (% w/w) of coconut and soybean oil microemulsions.

Microemulsion formulation	Surfactant mix (Tween80 + Span80, (1:1))	Oil phase (coconut or soybean oil)	Dispersed (DES) phase
4% control*	40	56	4
4% MLE	40	56	4
8% control*	40	52	8
8% MLE	40	52	8

*For control microemulsions the dispersed phase was the DES solution used for MLE preparation.

Table 2. Composition and coding of chocolate samples containing different concentrations of MLE microemulsions.

Chocolate formulation	Type of microemulsion added in chocolate	Concentration of microemulsion added in chocolate (% w/w)
Control	No added microemulsion	No added microemulsion
F4-2	with 4% w/w MLE	2
F4-4	with 4% w/w MLE	2
F8-2	with 8% w/w MLE	4
F8-4	with 8% w/w MLE	4

until further analysis. In total, 5 different formulations were produced with different concentrations of coconut microemulsions containing 4 or 8% w/w MLE. For example, chocolates prepared with 4% w/w MLE microemulsion at 2% w/w concentration are referred as samples F4-2, whereas when the same microemulsion is added at 4% w/w concentration, it is referred as F4-4. Control chocolate bars prepared without microencapsulated MLE served as a reference. Sample codes according to microemulsion formulation and concentration used are summarized in Table 2.

3.3 Microemulsion droplet size

The particle size of the microemulsions was estimated by means of dynamic light scattering (DLS) method (Zetasizer NanoZs, Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a He-Ne laser beam with a wavelength of 632.8 nm. Measurements were carried out at 90° scattering angle at 25 °C. Results are expressed as z-average diameter (nm).

3.4 Radical scavenging activity determination

The antioxidant activity of the microemulsions and chocolate samples was determined by the scavenging of DPPH.

To determine the antioxidant activity of microemulsions a pretreatment methodology was performed. 0.5 ml of sample was diluted 1:1 with methanol in a 1.5 ml Eppendorf tube and thoroughly mixed with a vortex mixer. Following this, the tubes were centrifuged at 9164 RCF (= 10 000 RPM)/10 min/20 °C (Ortoalresa Digicen 20-R, Los Frailes, Spain) and supernatants were collected (Poomanee *et al.*, 2017).

In the case of chocolate bars, a pretreatment methodology was followed with modifications (Gültekin-Özgüven *et al.*, 2016). Chocolate samples (1 g) were ground and mixed with 40 ml of 80% w/v ethanol aqueous solution and sonicated in a water bath (40 °C/25 min). Finally, the samples were centrifuged as in the case of the microemulsions and the supernatants were collected.

The radical scavenging activity of microemulsion and chocolate supernatants was estimated according to Lakka *et al.* (2020). In brief, 25 μl of each supernatant was mixed with 975 μl of DPPH solution (100 μM in methanol) and left in the dark for 30 min. Then the absorbance was measured at 515 nm (Shimadzu UV-1700). Results were expressed as percentage of Inhibition (I%) against blank according to equation (1):

$$(I\%) = 100 \times (A_0 - A_s) \div A_0, \quad (1)$$

where, A_0 and A_s are the absorbance at 515 nm of the blank and the sample respectively (Poomanee *et al.*, 2017).

3.5 Thermal stability of microemulsions

To determine the stability of the microemulsions against high temperatures 2 ml of each sample was placed in glass tubes and placed in thermostated water baths at 50, 60 and 70 °C for 1 h. At the end of the thermal treatment the stability of the samples was checked visually for phase separation.

3.6 Accelerated stability test of microemulsions

Microemulsion samples were centrifuged at 3 000 rpm for 20 min (25 °C) (Ortoalresa Digicen 20-R, Los Frailes, Spain) and visually examined for phase separation (Rashid *et al.*, 2019).

3.7 Color measurement

Color properties of the chocolate samples were estimated by a color and appearance measurement system (Lovibond

Table 3. Average particle size (z-average diameter) of microemulsions prepared with coconut and soybean oil.

Microemulsion formulation	z-average diameter (nm)
Coconut oil microemulsions	
4% Control	$15.1^{a} \pm 2.2$
4% MLE	$28.3^{bc} \pm 5.4$
8% Control	$29.2^{bc} \pm 7.3$
8% MLE	$63.8^{e} \pm 12.0$
Soybean oil microemulsions	
4% Control	$23.3^{b} \pm 1.2$
4% MLE	$32.8^{c} \pm 1.4$
8% Control	$40.1^{d} \pm 3.3$
8% MLE	$82.6^{f} \pm 4.6$

CAM-System 500, Great Britain). The system was calibrated using the Gretamacbeth mini color checker model P/N:50111, to obtain L*, a*, b* CIE values. The Whiteness Index (WI) was also calculated according to equation (2) as follows (Briones and Aguilera, 2005):

$$WI = 100 - \left[(100 - L^*)^2 + (a^*)^2 + (b^*)^2\right]^{0.5}. \quad (2)$$

3.8 Statistical analysis

All physicochemical analyses were performed in triplicate. The statistical analysis was performed with StatGraphics Centurion XV software by means of analysis of variance (ANOVA) at 95% level of confidence ($p < 0.05$).

4 Results and discussion

4.1 Microemulsion size

In Table 3, the z-average diameter of the blank and MLE microemulsions is presented.

The average diameter of microemulsion droplet ranged from 15.1 to 82 nm, typical of microemulsion formation. Typically, droplet sizes below 100 nm ensure the clear transparent/translucent appearance of microemulsions (McClements, 2012). The increase of dispersed phase (DES aqueous solution or MLE) from 4 to 8% w/w resulted in larger droplet formation (Lou *et al.*, 2013). The type of oil phase used had also a profound effect on particle size. Coconut oil microemulsions are characterized by finer droplet compared to soybean oil samples ($p < 0.05$). Coconut oil has been used successfully to fabricate microemulsions with similar formulation of low molecular weight emulsifiers (Rukmini *et al.*, 2012; Ja'afar *et al.*, 2019). It contains high amounts of medium chain triglycerides (MCTs) including lauric, myristic and caprylic acid (Pengon *et al.*, 2018). On the other hand, soybean oil is mainly composed of long chain triaglycerols (LCTs) that are less soluble in microemulsions due to long chain fatty acids which are bulky enough and unable to reach the interfacial film (Flanagan *et al.*, 2006). Similarly, in a comparative study for microemulsions formed with vegetable oils (coconut, palm kernel and soybean oil) larger droplet sizes are reported for LCT oils (Lee *et al.*, 2017).

4.2 Stability evaluation

In Figures 1 and 2, the stability of coconut and soybean oil microemulsions after centrifugation and thermal treatment is depicted. Microemulsion stability can be affected by various environmental conditions including pH, ionic strength, centrifugation and temperature (Li *et al.*, 2017; Cortés *et al.*, 2019). Moderate temperatures can favor microemulsion formation by altering the solubility of surfactants (Li *et al.*, 2017) resulting in lower interfacial tension. However, high temperatures promote inter-droplet interactions leading to droplet aggregation, subsequent coalescence and finally phase separation (Shi *et al.*, 2015). Centrifugation tests are considered as an effective way to evaluate microemulsion stability because they show good correlation with traditional gravitational stability testing, which is more time consuming as it requires prolonged periods of evaluation, often of several months (Mouri *et al.*, 2016). In the case of W/O microemulsions, destabilization is recognized by the sedimentation of aqueous phase at the bottom (Poomanee *et al.*, 2017). As seen in Figure 1, all microemulsion formulations were stable against centrifugation regardless of the oil phase used (soybean or coconut oil) and percentage of added moringa leaf extract, as they appeared transparent even after strong agitation. Microemulsion stability remained also unaffected by thermal treatment at 50, 60 or 70 °C/1 h as demonstrated in Figure 2. Researchers who studied the stability of coconut W/O microemulsions at elevated temperatures found that formulations were unstable at 70 °C, although samples were kept at that temperature for longer time intervals (5 h) (Rukmini *et al.*, 2012). Similarly, o/w microemulsions were subjected to a high temperature treatment (105 °C) for several days for destabilization to occur (Cho *et al.*, 2008).

4.3 Antioxidant activity of microemulsions

The DPPH radical scavenging activity of microemulsions prepared with different types of oil phase is shown in Figure 3. Percentage inhibition (I%) values for soybean oil microemulsions ranged between ~23 to 28.6%, significantly higher than those of microemulsions prepared with coconut oil (11.6–15.8%) ($p < 0.05$). Comparative studies regarding the antioxidant activity of vegetable oils have shown that soybean oil demonstrates superior radical scavenging properties than coconut oil (Bhatnagar *et al.*, 2009; Goldson *et al.*, 2018). Indeed, it is reported that soybean oil is rich in tocopherols which account for 990–1670 mg/kg, whereas for coconut oil a concentration as low as 17–29 mg/kg is noticed (Hildebrand *et al.*, 1984; Jung *et al.*, 1989; Bhatnagar *et al.*, 2009; Raja *et al.*, 2010). On the contrary, higher phenolic contents up to 29 mg/100 g have been reported for coconut oil depending on processing (Marina *et al.*, 2009), whereas soybean oil contains phenolic compounds in small quantities (1.4 mg/100 g oil) (Siger *et al.*, 2008).

Consequently, the increase of MLE concentration from 4 to 8% w/w did not significantly increase the inhibition values

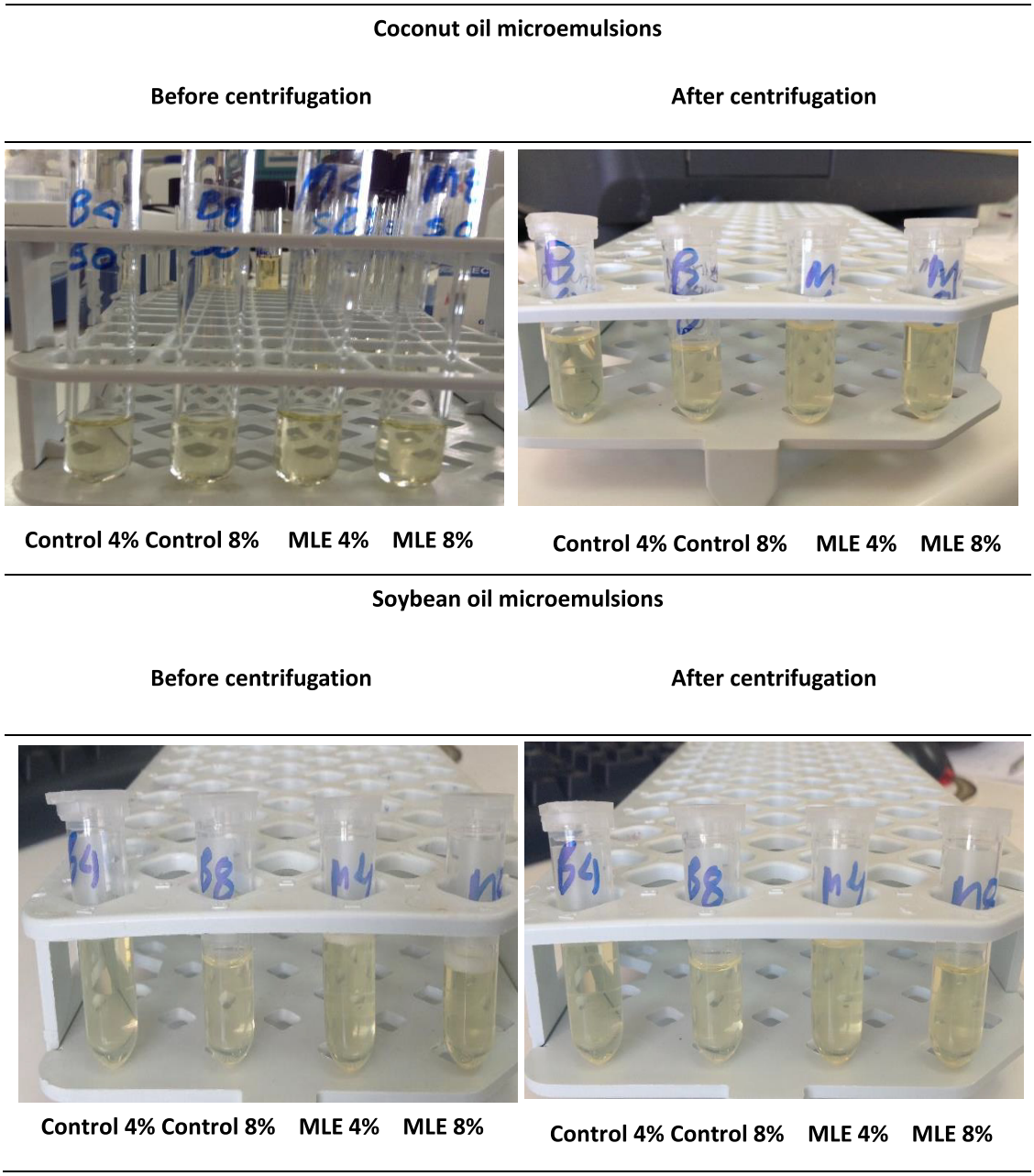

Fig. 1. Microemulsion stability assessed by accelerated centrifugal testing (3000 RPM/20 min) (from left to right: Blank microemulsion 4%, blank 8%, with 4% MLE and with 8% MLE).

($p > 0.05$). In general, the addition of antioxidants is expected to increase the radical scavenging capacity of such systems. For instance, the addition of purple sweet potato extract in W/O microemulsion formed with olive oil presented 30% higher DPPH inhibition values compared to the control sample (Desnita et al., 2016). However, the concentration of the purple potato extract used in this case was considerably higher (~38% w/w). Our finding is in accordance with Vorarat *et al.* (2010) who reported almost equal I% values for microemulsions containing 20% rice bran oil and 10% rice bran oil + oryzanol. This could be due to the subsequent decrease of the oil phase and respective antioxidants contained. Indeed, a positive "quasi-linear" effect of oil concentration on radical inhibition values has also been reported in emulsions formed with plant derived oil (sea buckthorn oil) which has considerable amounts of antioxidants (Zheng *et al.*, 2020).

4.4 Antioxidant activity and color properties of chocolate products during storage

In this section, coconut oil microemulsions were incorporated in chocolates since coconut oil is a known substitute for cocoa butter, although restrictions may apply depending on national legislations and this type of products are referred as compound chocolate (Halim *et al.*, 2019).

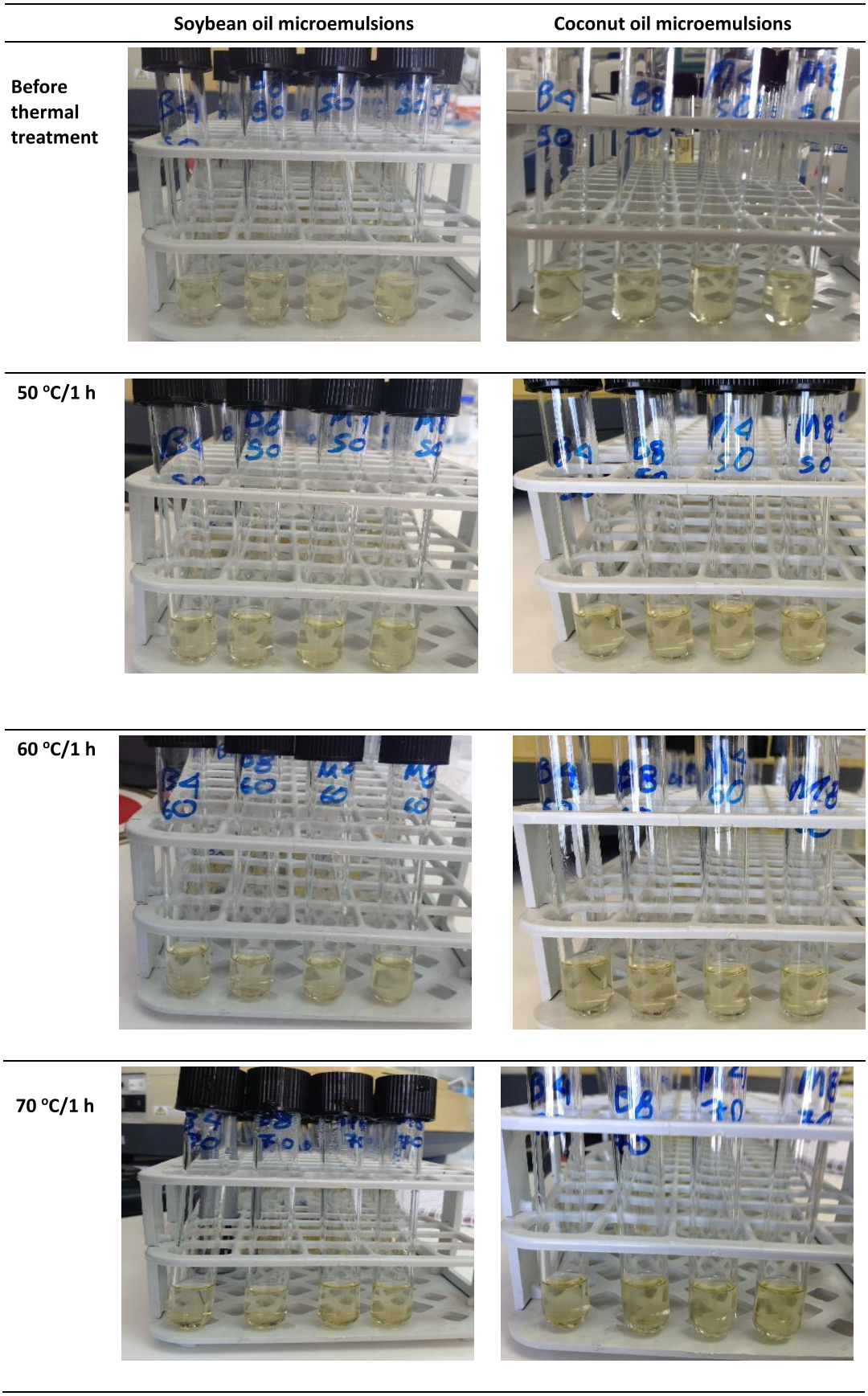

Fig. 2. Stability of microemulsions against thermal treatment (50, 60 and 70 °C/1 h) (from left to right: control microemulsion 4%, control 8%, with 4% MLE and with 8% MLE).

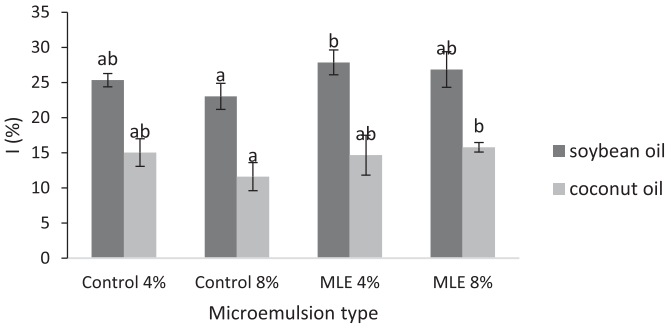

Fig. 3. Antioxidant capacity of microemulsions as affected by oil phase type and MLE concentration.
Different small letters among samples prepared with the same oil indicate statistically significant differences ($p < 0.05$).

Color is considered as a very important property as it can affect consumer behavior and modify other organoleptic characteristics such as taste and/or flavor of food (Williams, 1992; Spence and Piqueras-Fiszman, 2016). The main phenomenon governing the appearance of chocolate during storage is blooming. Blooming is the migration of fat to the surface of chocolate caused by exposure to high ambient temperatures leading to loss of gloss, irregular whitish flaky pattern formation and color degradation (Briones and Aguilera, 2005).

Figure 4 shows the evolution of color properties (L*, a*, b*) and whiteness (WI) of chocolate samples containing MLE coconut oil microemulsions during 8 months of storage. The WI is a useful colorimetric parameter that can be used to monitor the migration of fat on the surface of chocolate accumulated in white spots (Mexis *et al.*, 2010). It can be seen that chocolate formulation had a minor effect on color properties of fresh samples. With the exception of sample 84 that contained the highest amount of MLE microemulsion, all other samples presented similar values of L*, a*, b* and WI. Storage on the other hand affected majorly all color parameters. Sample lightness (L*) of freshly prepared chocolates ranged between 37.9 and 41.8 and gradually increased to levels of 50–60. The same trend was also observed for WI values, which were increased by a factor of 1.36 on average. It should be mentioned though that reference chocolates that did not contain microencapsulated MLE presented the lowest WI values, hence they were the least ones affected by blooming during storage, whereas slightly higher values were observed for enriched chocolates. Several studies have shown that coconut oil – that is the main ingredient of MLE microemulsions incorporated in chocolates – exhibits anti-blooming properties when added in chocolate even at levels as low as ~4.5% (Putri Limbardo *et al.*, 2017; Halim *et al.*, 2019). However, in this study the amount of coconut oil incorporated in chocolate in the form of microemulsion is much lower, which could possibly justify the absence of the anti-blooming effect. Finally, the amount of microemulsion and MLE added did not affect the blooming of the chocolate samples. All samples containing microencapsulated MLE presented similar WI values ($p > 0.05$) after 8 months of storage.

The antioxidant activity of chocolates is demonstrated in DPPH inhibition (I%) (Fig. 5). Freshly prepared chocolate samples presented similar I% values ranging between 12.8–14.7% ($p > 0.05$). The fact that MLE enriched chocolates did not exhibit superior antioxidant activity compared to control chocolate could be due to the high content of chocolates in phenolic compounds derived from cocoa nib derivatives. For example, researchers have reported total phenolic contents between 47.17–57.16 and 39.1–39.9 mgGAE/g of chocolate accordingly (Batista *et al.*, 2016; Di Mattia *et al.*, 2017). However, MLE used in this study contains ~94 mgGAE/g (Lakka *et al.*, 2020) and is only added at a maximum 8% w/w in the microemulsions.

A small loss of antioxidant activity by 2.5% on average was also observed in all samples after 8 months of storage, as seen by DPPH inhibition values that decreased to 11.1–11.6% levels. This finding is in accordance with other similar studies regarding the effect of storage on the antioxidant capacity of chocolate products (Pavlovic *et al.*, 2017) and is related to the degradation of major polyphenol compounds (flaval-3-ols, anthocyanins, catechins, procyanidins and proanthocyanidins) (Laličić-Petronijević *et al.*, 2016; Pavlovic *et al.*, 2017; Roda and Lambri, 2019).

5 Conclusions

Moringa oleifera leaf extract (MLE) was successfully encapsulated in microemulsions prepared with different plant oils. The antioxidant activity of microemulsions depends on oil type used, with soybean oil demonstrating superior radical scavenging properties. Even though enrichment of chocolate products with MLE in the form of microemulsions did not affect the color properties and overall acceptance of products, nevertheless its application in chocolate products is limited due to poor beneficial antioxidant effect observed following incorporation.

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Author contributions

S.I.L., D.P.M., experimental design, funding acquisition, supervision, monitoring of research work, manuscript, reviewing; O.K. experimental design, laboratory work, analysis, writing, statistical analysis; A.A., G.B., E.B., laboratory work, analysis; All authors have read and agreed to the published version of the manuscript.

Conflicts of interest. The authors declare no conflicts of interest.

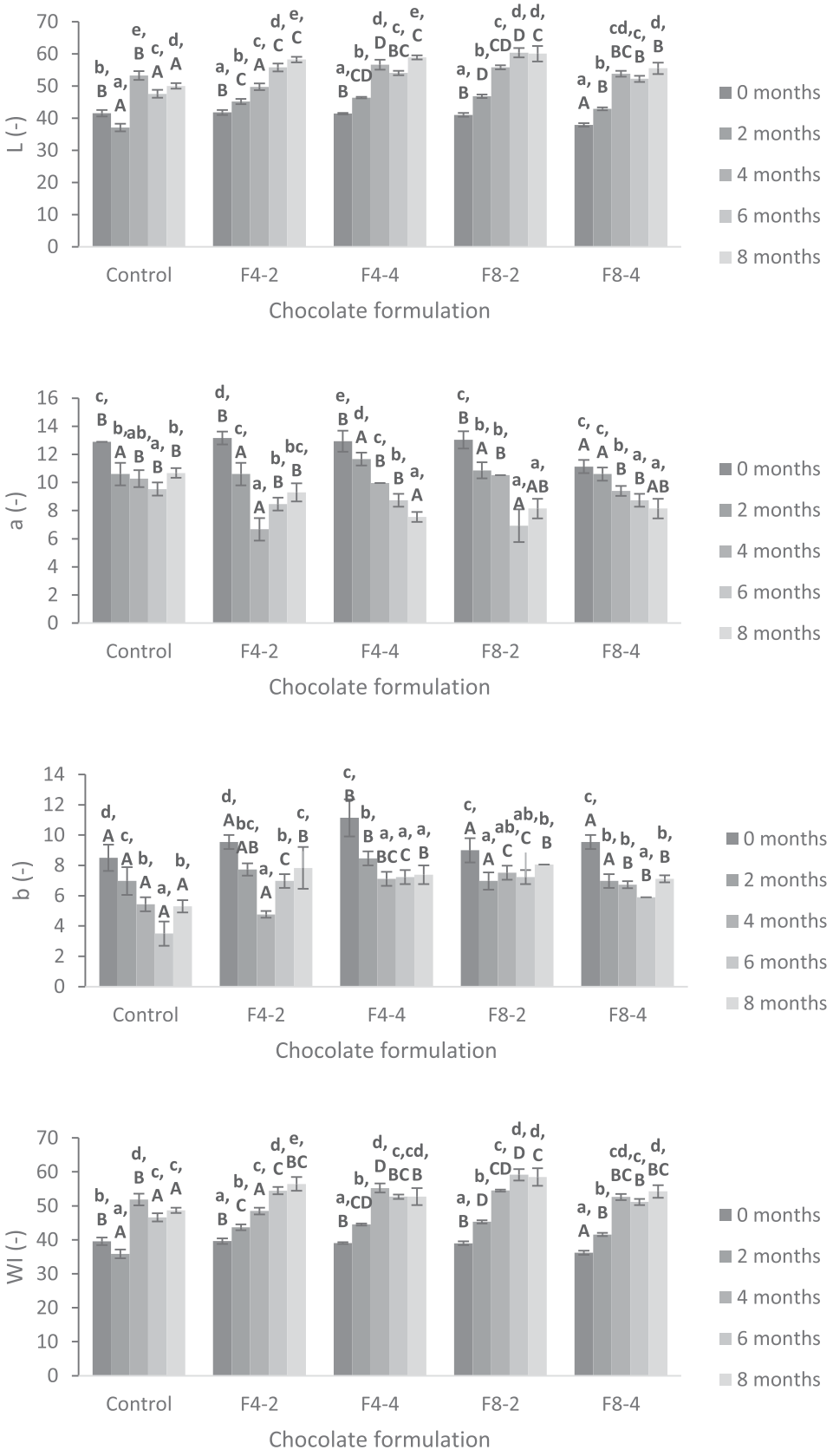

Fig. 4. Color properties of chocolate products as affected by composition and storage.
Different small letters for the same sample indicate statistically significant differences during storage ($p < 0.05$).
Different capital letters among samples of the same storage indicate statistically significant differences ($p < 0.05$).

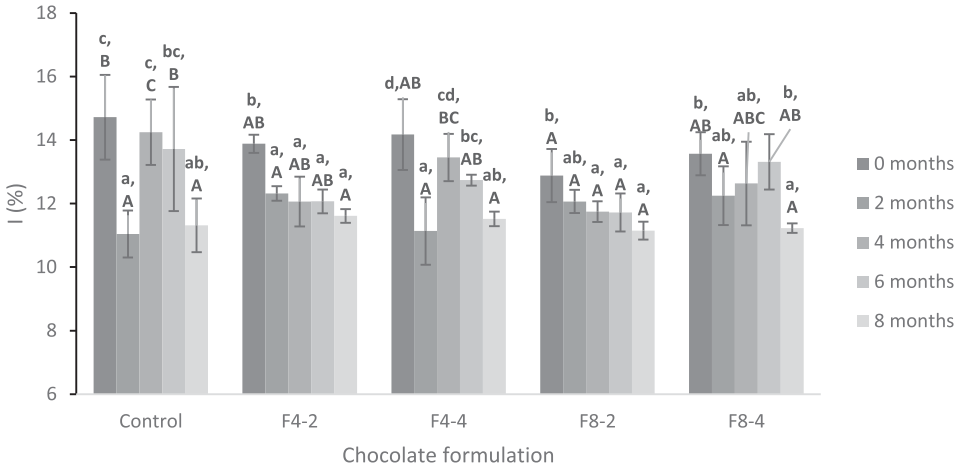

Fig. 5. Antioxidant capacity of chocolate products as affected by composition and storage.
Different small letters for the same sample indicate statistically significant differences during storage ($p < 0.05$).
Different capital letters among samples of the same storage indicate statistically significant differences ($p < 0.05$).

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