PAPER

Fortification of dark chocolate with microencapsulated phytosterols: chemical and sensory evaluation

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Chocolate is one of the most consumed delicacies in the world. Nowadays high-cocoa polyphenol-rich chocolates, probiotic chocolates, and prebiotic chocolates are getting more attention. In light of this, dark chocolate containing microencapsulated phytosterols (MPs) has been developed to reduce cholesterol in individuals. In particular, different dark chocolates containing 64, 72 and 85% of cocoa, fortified with 0, 5, 10 and 15% MP have been produced. The obtained chocolates were characterized by a particle size distribution lower than 30 μm and were stable from a chemical point of view. Specifically, peroxide values were always lower than 2 meq O_2 per kg of fat, also after three months of storage. The bioaccessibility of phytosterols was comparable with literature values and the antioxidant activity reached a value of 92 μg trolox per g chocolate for samples obtained from 85% of cocoa. Moreover, sensory evaluation demonstrated a positive effect on the acceptability of the functional chocolate produced and a significant effect of the information on the final sample acceptability.

Introduction

Chocolate represents a functional product due to its high level of flavonoid content and its beneficial impact on human health.[1] In the last few years, consumers are becoming more demanding in the food market and they would like to have more options to choose from than ever before. In view of the above, organic chocolates, high-cocoa polyphenol-rich chocolates, probiotic chocolates, and prebiotic chocolates can be found in the market.[2] In fact, at present, functional foods are gaining a prominent position in the food market because of their ability to provide positive health effects beyond their traditional nutritional value.[3] Two types of functional foods can be found in the market bearing the health claim label "able to lower cholesterol levels in serum". One contains phytosterols and the other the water-soluble fiber β-glucan from oats. Both food components decrease the cholesterol absorption from the diet, through different mechanisms of action.[4] The major application of phytosterols is their addition into spreads, yogurt and vegetable oils. However, their incorporation into other food products, characterized by a low humidity level or subjected to high temperatures of the process, is still limited due to the loss in functionality resulting from the deterioration caused by processing conditions.[5] Formulation of food products with phytosterols is difficult from a technological and food quality point of view, since phytosterols are insoluble in water and poorly soluble in fats and oils. At the same time, because of their waxiness and chalky taste, phytosterols may have an adverse effect on the sensorial properties of foods. When formulating phytosterol-containing foods, it is important to use a particle size preferably lower than 20 μm, so that foods can be perceived by consumers as smooth in texture and acceptable in 'mouthfeel'. However, it is possible to purchase phytosterols in the crystalline form in various particle sizes, typically 30–300 μm and obviously, this particle size has a significant impact on sensorial attributes of the phytosterol enriched food.[6] Insoluble particles could contribute to grittiness, and coarseness, which define in part the overall 'mouthfeel' of foods and could affect a central chocolate sensory character: melting properties. Melting rate has been defined as "the time required to melt half of a chocolate square when chewed in mouth". A consumer's liking study showed that quickly melting chocolates in the mouth are preferred to slow melting chocolates.[7] Furthermore, it has been demonstrated that an average particle size of about 25 μm could enhance the incorporation of phytosterols into the micellar phase in the

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intestine, improving their bioavailability.[8] Regarding the use of phytosterols for food fortification it is necessary to consider that, like all unsaturated lipids, phytosterols are liable to oxidation giving rise to a family of compounds termed phytosterol oxidation products (POPs) which include keto-, hydroxyl-, and epoxy-derivatives. Inhibition of phytosterol oxidation has become a subject of research. The usual approach used to minimize the POP generation involves the addition of antioxidants.[9] However, a recent study reported that the oxidative stability of dark chocolate bars enriched with phytosterols, with or without the addition of ascorbic acid and α-tocopherol, at a concentration of 0.90 mg per 100 g of the product, did not allow the reduction of produced phytosterol hydroperoxides over a 5 month period of storage.[10] In this context, microencapsulation could be a viable alternative. Microencapsulation is defined as the technology that permits the inclusion of a sensitive ingredient within several matrices, since that it is entrapped or embedded by the protective matrix/matrices and protected against the external environment. The majority of the studies about the microencapsulation of sensitive compounds do not include their incorporation into food matrices. However, this step is crucial for the food formulation, because it allows us to evaluate the stability and the acceptability of the final product.[11] For this reason, we explored the possibility of using spray dried phytosterol microcapsules during the production of several functional dark chocolates containing 64, 72 and 85% of cocoa. The functional chocolates, fortified with 5–10 and 15% of microencapsulated phytosterols (MPs), were subjected to chemical and sensory analyses. Moreover, the shelf-life evaluation during 3 months of storage has been carried out. Peroxide value, water activity, humidity content, antioxidant activity and colour were determined immediately after the production and during 3 months of storage at ambient temperature. Sensory evaluation was carried out in order to evaluate the profile of the different samples. Furthermore, to assess the sensory acceptability of samples, a consumer test was conducted. The study involved 116 consumers of dark chocolate who evaluated the overall liking score under blind (tasting only) and under informed (tasting and product information) experimental conditions.

Materials and methods

Microencapsulated phytosterol production

Microencapsulated phytosterols (MPs) have been prepared by using Whey Protein Isolate (WPI) as the coating polymer. In detail, the dissolution of WPI at 8% was carried out in distilled water followed by the dispersion, in the obtained aqueous suspension, of 7% of free phytosterols provided by Phytopin (DRT, France). In order to aid the phytosterol dispersion, Tween80 at 1.25% was used as the surfactant. Fine suspensions were produced by homogenizing with an Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) at 20 000 rpm for 10 minutes in the solution. The obtained solution has been sprayed using a co-current spray dryer (FT 80 Tall Form Spray

Dryer, Armfiled) by using the following operation conditions: inlet air drying temperature 155 °C, flow rate 0.5 L h^{-1}, spray flow of 600 L h^{-1} and nozzle cup diameter 0.7 mm. The used formulation was selected according to preliminary tests.

Functional chocolate production

Different types of dark chocolates have been fortified at 0, 5, 10 and 15% with the microencapsulated phytosterols (MPs) in order to obtain the formulation reported in Table 1. In detail, commercial chips containing 64, 72 or 85% of cocoa (Valrhona chocolate, France) have been melted at 45 °C using a Selmi One Continuous Chocolate temper machine (Selmi Srl, Santa Vittoria d'Alba, Italy). The molten chocolate has been tempered at 30.5 °C and the weighted microcapsules were added to the molten chocolate. Finally, a thin layer of chocolate was placed in the mould (4 cm length and 0.5 cm height) and left to cool. The moulded finished chocolates were packed into polypropylene bags and were analysed immediately after the production and during 3 months of storage at ambient temperature (20 ± 2 °C).

Determination of particle size distribution (PSD)

A method for determining the particle size distribution (PSD) was adapted from the method used by Afoakwa et al.[12] A Mastersizer laser diffractor equipped with a Hydro 2000s wet dispersion unit (Malvern Instruments) was used. About 0.05 g of ground chocolate sample was dispersed in the dispersion unit filled with approximately 140 ml of sunflower oil at ambient temperature until an obscuration of 20% was reached, following the user guide of the instrument. Ultrasonic dispersion was maintained by stirring for 2 min to ensure that the particles were freely dispersed. The speed of stirring was maintained at 3000 rpm for all the measurements. Diffraction data were analyzed based on Mie theory using the refractive indices of 1.590 and 1.450 for chocolates and sunflower oil. The corresponding size distribution was quantified as the volumetric fraction of particles in different size intervals and presented as cumulative size distribution curves and probability density size distribution curves The obtained PSD parameters included the volume moment mean, $D[4:3]$, the size

Table 1 Formulation of the different fortified chocolates produced

Sample	Cocoa content (%)	Microencapsulated phytosterols (%)
64%-MP 0%	64	0
64%-MP 5%	64	5
64%-MP 10%	64	10
64%-MP 15%	64	15
72%-MP 0%	72	0
72%-MP 5%	72	5
72%-MP 10%	72	10
72%-MP 15%	72	15
85%-MP 0%	85	0
85%-MP 5%	85	5
85%-MP 10%	85	10
85%-MP 15%	85	15

for which 10% of particles by volume are smaller (10th percentile size), D_{10}, the size for which 50% of particles by volume are smaller and 50% are larger (median size), D_{50}, the size for which 90% of particles by volume are smaller (90th percentile size), D_{90}, the reported values result from the average of 5 measurements.

Fat extraction

The fat from chocolate bars was extracted according to Mexis.[13] In detail, 5 g of crushed dark chocolate was transferred into a separatory funnel with 100 mL of diethyl ether and 10 mL of distilled water. The separatory funnel was agitated for 2 minutes and left to equilibrate for 24 h. The supernatant was transferred into a flask and diethyl ether was evaporated in a water bath at 40 °C. The extracted fat was dried in an oven at 105 °C for 3 min, and the residue was used to determine the peroxide value.

Peroxide value

The peroxide value (PV) of the chocolate fat was measured according to the method of the American Oil Chemist's Society,[14] as follows: 2 g of the fat extracted was weighed into a 250 mL Erlenmeyer flask, 25 mL acetic acid/chloroform mixture (3 : 2 v/v) was added and the mixture was swirled for the dissolution of chocolate fat. 1 mL of fresh saturated aqueous potassium iodide solution was added, the flask was gently mixed for 1 min and left to stand in darkness for 5 min at room temperature. Then 75 mL distilled water were added and the content was titrated against 0.01 N sodium thiosulphate ($Na_2S_2O_3$) (using a starch indicator). PV has been expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2 per kg). The evaluation was repeated immediately after the production, and after 45 days up to 3 months of storage.

Antioxidant determination by DPPH radical scavenging assay

The polyphenols were extracted according to Batista et al.[15] In detail, 5 mL of the solvent extraction solution (acetone/distilled water/acetic acid; 70 : 29.5 : 0.5 v/v/v) were added to 1 g of ground sample and stirred by vortexing for 1 min followed by sonication for 10 min in an ultrasonic bath. The mixture was centrifuged at 2086g for 10 min, filtered with cellulose paper and used for DPPH radical scavenging assay. The DPPH radical scavenging assay was performed as described by Brand-Williams et al.[16] Briefly, 100 µL of sample was added to 3.9 mL of the DPPH radical solution (0.06 mM) and stored in the dark for 2 h at room temperature. The free radical scavenging capacity was then evaluated by measuring the absorbance at 515 nm (Cary 1E UV-VIS spectrophotometer, Varian-Agilent). The results were expressed as mmol Trolox Equivalents (TE). The analyses were performed in triplicate.

Color measurement

Colour was measured by using a Minolta Chroma meter CR-300 with a D 65 illuminant, and expressed as color L^* (lightness), a^* (redness) and b^* (yellowness) values using CIELab parameters. The measurements were carried out in triplicate and in three different places on each sample. The reported results (L^*, a^*, b^*) are the mean of twelve determinations. The evaluation was repeated every month during 3 months of storage.

Water activity and moisture content

Water activity (a_w) was measured on ground chocolate using a HygroPalm (series 21/22/23/23-AW/TP22) with a HC2-AW sensor (Rotronic Italia Srl, Milano, Italy) at 25 °C. Regarding the moisture content, 5 g of sample was dried to a constant weight at 100 °C in a moisture dish for one hour. The weight loss was used to calculate the percent moisture. The evaluation was repeated every month during 3 months of storage.

Simulated in vitro gastrointestinal digestion

The bioaccessibility (percentage of soluble compound available for absorption) of phytosterols in these chocolates, after the simulated in vitro digestion involving three stages has been evaluated. The in vitro gastrointestinal digestion model applied in this study consisted of a three step procedure, which sequentially simulated the digestion in the mouth, stomach and intestine as described by Minekus et al.[17] with some modification. Briefly, 2.5 g of ground samples were mixed with 1.75 mL of Simulated Salivary Fluid (SSF), 0.25 mL of salivary a-amylase solution of 1500 U mL^{-1} made up in SSF electrolyte stock solution (a-amylase from human saliva, Sigma), 12.5 µL of $CaCl_2$ and 487.5 µL of Milli-Q water. After 3 minutes, 3.75 ml of Simulated Gastric Fluid (SGF) was added to the salivary phase with 0.8 mL of porcine pepsin (EC 3.4.23.1), 2.5 µL of $CaCl_2$ and 0.375 ml of Milli-Q water. 1 M HCl was added to reduce the pH to 3.0 After 1 h of incubation at 37 °C, 3.85 ml of Simulated intestinal fluid (SIF) was added to 1.75 ml of porcine pancreatic lipase (EC3.1.1.3) (2000 U ml^{-1}), 0.875 ml of biliary salts, 14 µL of $CaCl_2$ and 0.458 ml of Milli-Q water. The pH in the intestinal phase has been adjusted to 7 by using NaOH 1 M and the samples were digested for 2 h at 37 °C with shaking. The SSF, SGF and SIF have been prepared following in detail the indications from Minekus et al.[17] Three collections of samples were performed in the gastric phase, at time 0, after 30 and 60 min, and three collections in the intestinal phase, at time 0, after 1 and 2 hours were performed. Aliquots (1 g) were collected at 30 min intervals in the gastric phase and at 1 h in the intestinal phase, added to 9 ml of chloroform and used for phytosterol quantification. Phytosterol determination has been carried out by the calibration curve method using the Liebermann-Burchard reagent as reported by Tolve et al.[18] Bioaccessibility, the fraction of a compound released from the food matrix in the gastrointestinal lumen with respect to its total content in the food, has been reported as the mean of three repetitions.

Sensory evaluation

The sensory evaluation was conducted on the chocolate samples. The descriptive terminology and the sensory profile

of chocolates were developed by using Quantitative Descriptive Analysis (QDA). A twelve-member trained sensory panel (6 females and 6 males), aged between 21 and 32 years, recruited from the University of Basilicata was involved. Quantitative Descriptive Analyses were performed to select key-attributes (*i.e.* the most effective attributes in discriminating the products) to be used for the following sensory analysis. The judges have been trained to recognize different intensities of 20 sensory attributes (Appearance: colour uniformity, brightness, and presence of crystals; Aroma: butter aroma, cocoa, coffee, milk, and off-odour; Taste and Flavour: bitter, sweet, and pungent; Texture: astringent, coffee, salty, sour, off-flavour, firm, melting, adhesive, and gritty) obtained from the bibliography.[19] Moreover, to help judges to recognize the attributes of interest appropriate reference standards were created. The experiment consisted of 3 repetitions. Judges evaluated a total of 12 samples. All the samples were completely randomized and then divided into 3 balanced groups. Each repetition was divided into three sub sessions of 4 samples. Judges evaluated 4 samples at the same time. The evaluation was performed in individual booths using white light during the evaluation of visual attributes and using red light for other attributes. Before sensory evaluation, all participants signed an informed consent form.

Consumer test

To assess the sensory acceptability of samples, a consumer test was conducted. 116 regular consumers of chocolate (65 females and 51 males) aged between 18 and 64 years participated in the study. Samples were served in completely randomized and balanced order among subjects and evaluated at room temperature (20 ± 2 °C). Subjects were instructed to observe, smell and taste the samples and to report their overall liking score on a nine-point hedonic scale ranging from 'dislike extremely' (1) to 'like extremely' (9). A score of 5 was taken as the lower limit of acceptability. Panelists were instructed to consume the sample and rinse their mouth with water after each sample evaluation. In the information test participants tasted each sample with the product information related to the fortification with MPs and again, they rated their overall liking score on the hedonic scale. The samples were served in a randomized order. At the beginning of the information test session, participants were asked to fill the neophobia questionnaire developed by Pliner and Hobden[20] in order to evaluate the reluctance to try and eat unfamiliar foods. Before the consumer test, all participants signed an informed consent form. Moreover, all experiments were performed in compliance with the relevant laws and institutional guidelines.

Statistical analysis

Chemical data were processed by ANOVA. Moreover, the least significant difference (LSD) test was performed to compare the means ($p \leq 0.05$). Statistical procedures were computed using the statistical package SYSTAT for Windows (ver. 10, 2003) (Systat Software, Chicago, IL). Sensory data were collected and elaborated by using the software FIZZ. Statistical procedures were computed using the statistical package SYSTAT for Windows (ver. 2.47, Biosystèmes, Couternon, France).

Results and discussion

Microencapsulated phytosterol production

Microencapsulated phytosterols used for chocolate fortification were obtained by feeding a co-current spray dryer with an aqueous suspension prepared by using phytosterols, Tween 80 and WPI. The obtained microcapsules were characterized by a very low water activity and moisture content, 0.32 and 4.53%, respectively, peroxide value of 0.46 meq O_2 per kg of fat and a loading capacity, that represent the amount of the bioactive compound loaded into the capsules, of about 24.7%. The size of the obtained microcapsules ranged from 20 to 35 μm.

Determination of particle size distribution (PSD)

The particle size distribution of samples was measured to characterize chocolates with and without MPs. The results showed relatively narrow unimodal particle size distribution for all the samples. Table 2 reports the influence of the fortification with MPs on the particle size parameters $D[4:3]$ (volume moment mean), D_{10} (10th percentile size), D_{50} (median size), and D_{90} (90th percentile size). A two-way ANOVA was used to investigate the effect of the two factors (cocoa content and microcapsule addition) and their interaction on the dependent variables. Based on the results of the statistical analysis, it can be observed that the microcapsule addition significantly affected the PSD parameters. In particular, for all the chocolate samples the two factors considered, and their interaction, showed a significant effect on the volumetric fraction of particles belonging to larger size intervals, that is the relative amount of coarse particles. Therefore, the most significant increase of the characteristic size values with the addition of microcapsules was observed for D_{90}. This produced a slight increase of the volume moment mean $D[4:3]$ as well. However, the D_{90}, that is the size for which 90% of particles are smaller, was lower than 25 μm for all the samples. This was a good

Table 2 Particle size distribution of the different chocolates produced

Particle size distribution

Sample	$D[4,3]$ (μm)	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)
64%-MP 0%	9.7 ± 0.1	2.4 ± 0.0	7.2 ± 0.0	19.6 ± 0.1
64%-MP 5%	9.9 ± 0.0	2.6 ± 0.0	7.7 ± 0.1	20.6 ± 0.1
64%-MP 10%	11.1 ± 0.4	2.8 ± 0.0	8.6 ± 0.1	23.1 ± 0.8
64%-MP 15%	9.8 ± 0.2	2.6 ± 0.2	7.4 ± 0.0	19.7 ± 1.0
72%-MP 0%	10.7 ± 0.1	2.6 ± 0.0	7.5 ± 0.0	19.9 ± 0.1
72%-MP 5%	11.0 ± 0.0	2.4 ± 0.0	8.0 ± 0.0	23.5 ± 0.1
72%-MP 10%	11.4 ± 0.3	2.8 ± 0.0	8.9 ± 0.1	23.6 ± 0.6
72%-MP 15%	13.2 ± 0.0	3.0 ± 0.0	9.8 ± 0.0	24.4 ± 0.1
85%-MP 0%	10.2 ± 0.1	2.6 ± 0.0	7.5 ± 0.0	19.5 ± 0.2
85%-MP 5%	10.61 ± 0.1	2.7 ± 0.0	7.8 ± 0.0	20.7 ± 0.2
85%-MP 10%	10.4 ± 0.1	2.8 ± 0.0	7.9 ± 0.0	21.1 ± 0.1
85%-MP 15%	10.8 ± 0.2	2.6 ± 0.2	7.8 ± 0.0	22.2 ± 1.1

Results are expressed as mean ± SD (standard deviation).

result, considering that a good dark chocolate requires a maximum particle size of 35 μm.[21] Furthermore, a smaller particle size in chocolate is known to improve the sensory properties.

Peroxide value

As the oxidation of sterol reactions can start with the hydroperoxide formation, the primary oxidation of unsaturated lipids was measured by the hydroperoxide concentration. Changes in the peroxide value of different kinds of dark chocolates with or without MPs, during three months of storage in the dark at 18 °C are shown in Fig. 1. The peroxide value was always very low and ranged from 0.679 to 1.429 immediately after the production. This low hydroperoxide content observed in chocolates was a consequence of the high proportion of saturated (50 g per 100 g) and monounsaturated (40 g per 100 g) fatty acids present in the cocoa butter.[10] Despite this, during the storage period it was possible to observe a slight increase in

the peroxide value that ranged from 1.200 to 1.895 after 45 days of storage and from 1.966 to 2.545 meq O_2 per kg of fat after 90 days of storage. This increase was more pronounced in samples obtained by using chocolate with 72% of cocoa. With respect to this, it should be noted that different kinds of chocolate which probably do not differ only by the cocoa percentage have been used. It is well known, for example, that an increase in the roasting time of cocoa leads to a consistent increase in the peroxide value.[22] However, it is not possible to ascribe this slight increase in the peroxide value to the fortification with MPs, as this is also observed in control samples obtained without fortification.

### Antioxidant determination by DPPH radical scavenging assay

The antioxidant capacity of chocolates with 64, 72 and 85% of cocoa, with MPs at 0–5–10 and 15%, was assessed after production and during three months of storage by DPPH assay. The results showed a significant effect of the cocoa content and of the microcapsule addition and of their interaction ($p < 0.05$) on the antioxidant activity. Considering the types of chocolates, the one characterized by the presence of cocoa at 85% showed a higher antioxidant activity. This might be due to the properties of this chocolate characterized by higher levels of cocoa due to a higher concentration in phenolic compounds (Fig. 2).

Color measurement

Colour is one of the key attributes for consumer acceptance. Colour changes in chocolates are often due to the difference in their composition and processing parameters during production. In order to evaluate the effect of the storage on the colour, the CIELab parameters (L^*, a^* and b^*) have been evaluated (Table 3). The brightness values (L^*) for chocolate increased with the time of storage and the microcapsule concentration. Instead, as expected, L^* decreased with the increase of cocoa concentration because lower values for L^* indicate a darker appearance. Regarding the redness (a^*) and yellowness (b^*) their values showed an increase according to the microcapsule concentration and a reduction correlated with the cocoa percentage and the time of storage. The microencapsulated phytosterol addition caused an increase in L values for all the chocolates. Similar results, regarding the effect of microencapsulated phytosterols on the L^* value, were also reported by Botelho et al.,[10] who evaluated the effect of the phytosterol addition on the chemical properties of dark chocolate.

Water activity and moisture content

Microencapsulated phytosterols were obtained by using WPI as the coating polymer. WPIs are hydrophilic substances and could influence the a_w of the foodstuff to which they are added. However, no significant ($p > 0.05$) effect of the microencapsulated phytosterols on the water activity has been found. In the same way, no significant effect of the cocoa percentage has been obtained ($p > 0.05$). a_w values slightly increased during three months of storage in all the samples (Fig. 3a).
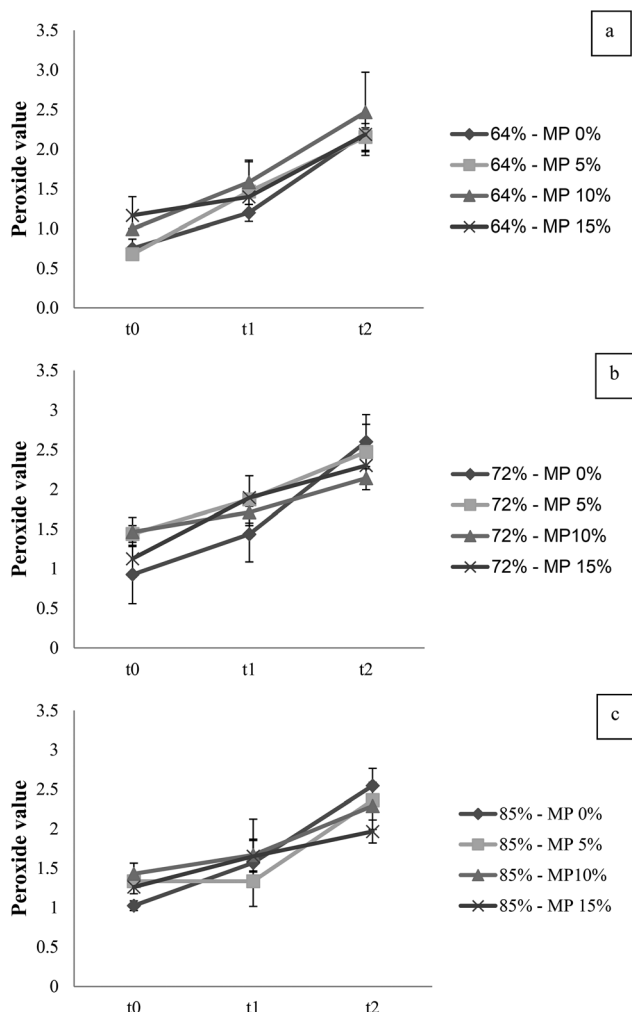
Fig. 1 Effect of microcapsule concentration on the peroxide value for different kinds of chocolates 64% (a), 72% (b) and 85% (c) of cocoa during 3 months of storage. t_0: immediately after the production; t_1: after 45 days; t_2: up to 3 months of storage.
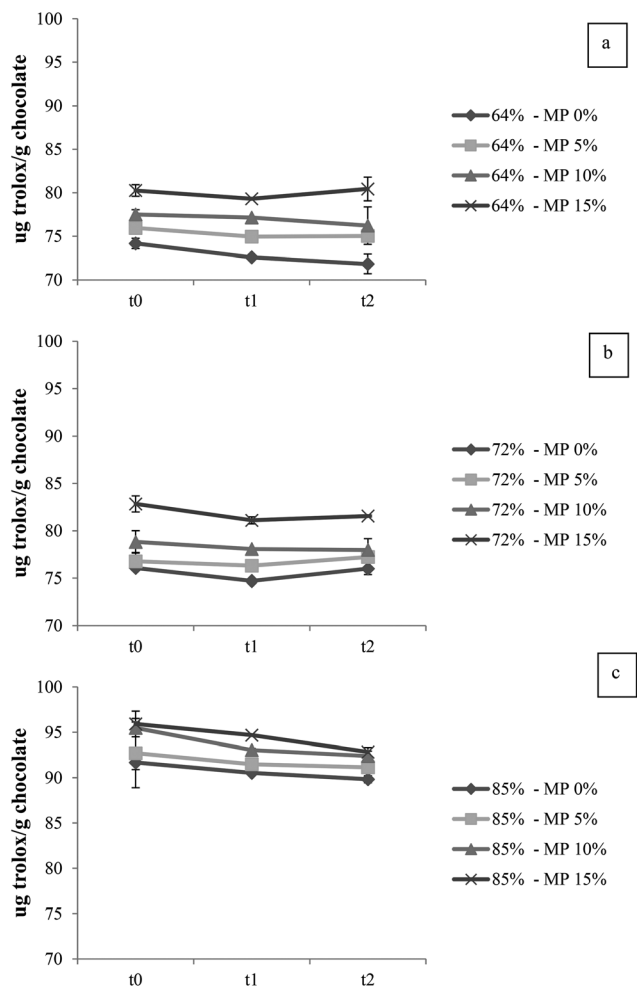
Fig. 2   Effect of microcapsule concentration on the antioxidant activity determined by DPPH for different kinds of chocolates 64% (a), 72% (b) and 85% (c) of cocoa during 3 months of storage. t_0: immediately after the production; t_1: after 45 days; t_2: up to 3 months of storage.
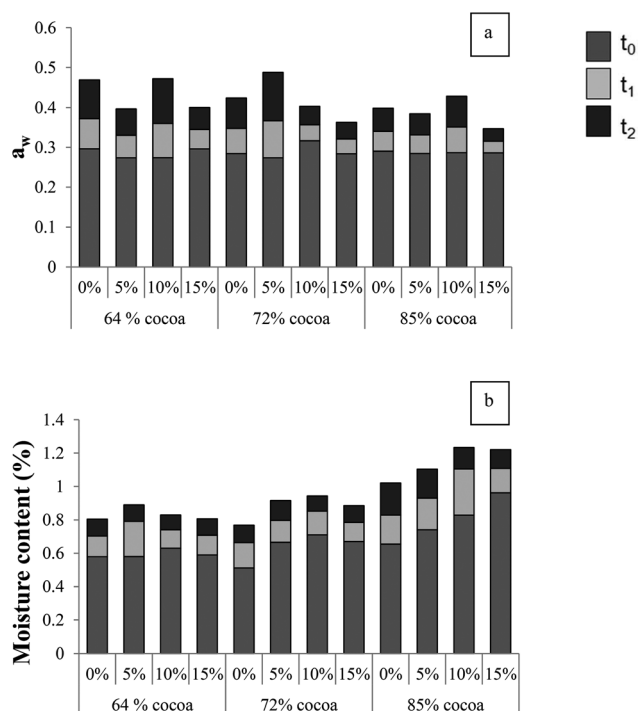

Fig. 3   Effect of microcapsule concentration on the water activity (a) and on the moisture content (b) for different kinds of chocolates 64%, 72% and 85% of cocoa during 3 months of storage. t_0: immediately after the production; t_1: after 45 days; t_2: up to 3 months of storage.

The results related to the moisture content of the different samples are reported in Fig. 3b. The obtained results showed a significant effect of the cocoa content on the moisture ($p < 0.05$). This effect could be due to the different process conditions used for chocolate chip production.

Simulated *in vitro* gastrointestinal digestion

The bioaccessibility of phytosterols in the chocolates fortified with MPs has been evaluated. Significant differences in the phytosterol release in gastric and intestinal phases have been

Table 3   Colour changes in chocolate during 3 months of storage

Sample	Brightness (L^*)			Redness (a^*)			Yellowness (b^*)		
	t_0	t_1	t_2	t_0	t_1	t_2	t_0	t_1	t_2
64%-MP 0%	29.03 ± 0.77	29.01 ± 0.28	28.84 ± 0.19	8.01 ± 0.18	8.04 ± 0.09	7.75 ± 0.10	6.48 ± 0.35	6.42 ± 0.19	6.21 ± 0.13
64%-MP 5%	30.17 ± 0.79	30.55 ± 0.17	33.61 ± 0.03	9.39 ± 0.11	8.62 ± 0.14	7.23 ± 0.03	8.32 ± 0.27	7.20 ± 0.21	6.02 ± 0.03
64%-MP 10%	30.53 ± 0.56	32.04 ± 0.25	32.47 ± 0.07	9.21 ± 0.27	9.83 ± 0.15	8.88 ± 0.01	7.95 ± 0.39	8.94 ± 0.19	8.17 ± 0.02
64%-MP 15%	31.78 ± 0.65	31.96 ± 0.19	32.55 ± 0.01	9.64 ± 0.36	9.69 ± 0.10	9.33 ± 0.02	8.79 ± 0.42	8.77 ± 0.14	8.89 ± 0.02
72%-MP 0%	28.22 ± 0.38	28.96 ± 0.31	28.71 ± 0.02	7.02 ± 0.18	7.07 ± 0.10	6.82 ± 0.02	4.99 ± 0.19	5.21 ± 0.21	5.13 ± 0.02
72%-MP 5%	30.06 ± 1.00	30.60 ± 0.29	30.42 ± 0.02	7.15 ± 0.53	7.66 ± 0.13	7.51 ± 0.02	5.95 ± 0.71	5.79 ± 0.07	5.89 ± 0.02
72%-MP 10%	32.62 ± 0.51	30.55 ± 0.40	30.82 ± 0.01	8.70 ± 0.24	8.50 ± 0.13	8.27 ± 0.01	7.05 ± 0.34	6.93 ± 0.22	6.85 ± 0.02
72%-MP15%	33.00 ± 0.45	31.26 ± 0.52	32.46 ± 0.07	9.02 ± 0.17	8.99 ± 0.24	8.56 ± 0.05	9.69 ± 0.35	7.60 ± 0.21	6.97 ± 0.03
85%-MP 0%	28.04 ± 0.53	27.95 ± 0.19	27.37 ± 0.02	6.34 ± 0.19	6.36 ± 0.07	6.34 ± 0.01	4.77 ± 0.23	4.69 ± 0.15	4.94 ± 0.02
85%-MP 5%	28.16 ± 0.42	28.14 ± 0.28	28.48 ± 0.01	6.84 ± 0.21	6.81 ± 0.09	6.71 ± 0.04	5.12 ± 0.25	5.16 ± 0.08	5.87 ± 0.02
85%-MP 10%	28.22 ± 0.66	28.80 ± 0.38	29.25 ± 0.00	7.17 ± 0.26	6.90 ± 0.24	6.81 ± 0.03	5.33 ± 0.32	5.23 ± 0.35	5.82 ± 0.01
85%-MP 15%	29.04 ± 0.35	29.23 ± 0.25	29.45 ± 0.02	7.35 ± 0.08	7.59 ± 0.37	6.91 ± 0.04	5.94 ± 0.30	6.14 ± 0.46	5.70 ± 0.04

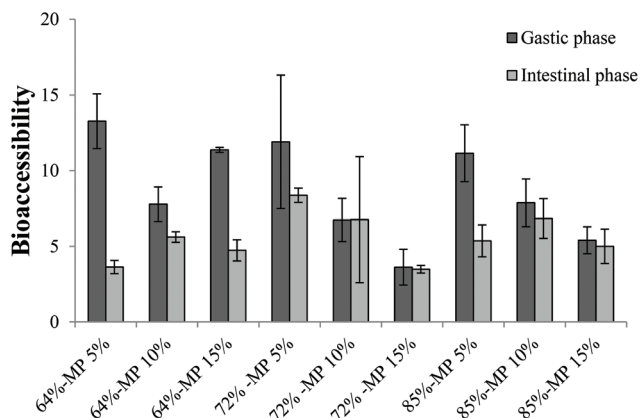Results are expressed as mean ± SD.

Fig. 4 Bioaccessibility of phytosterols in samples with MP addition.

melting and adhesiveness. The interaction between the chocolate cocoa content and microcapsule concentration did not show a significant effect ($p > 0.05$). Microcapsule's concentrations had a significant effect only on the chocolate attributes reported in Fig. 5. However, the interaction between the cocoa content and MPs did not show a significant effect ($p > 0.05$). For this reason, taking into account the results of the chemical evaluation (*e.g.* the antioxidant activity), it was decided to submit only the four samples obtained by using cocoa at 85% to the consumer test.

Consumer test

For an industrial application and production line of a new food, it is very important to assess its acceptability. Since the interaction between the chocolate cocoa content and microcapsule concentration did not show a significant effect, the consumer test has been carried out only on the dark chocolate with 85% of cocoa (due to the higher nutritional value). When
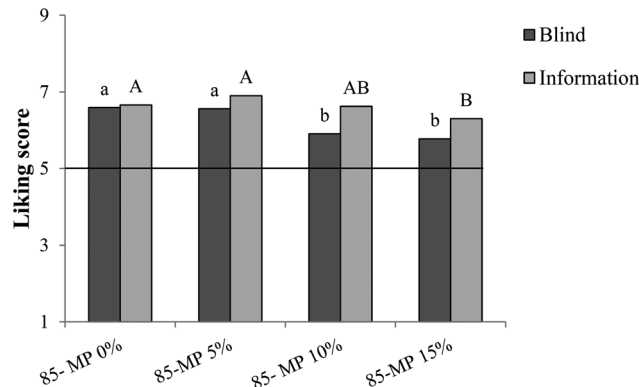
observed ($p < 0.05$). This result was expected because the protein digestion begins in the stomach and then proceeds in the small intestine. As reported in Fig. 4, the bioaccessibility of phytosterols in the gastric phase ranged from 13.3 to 3.3%, while in the intestinal phase the bioaccessibility ranged from to 6.5 to 8.4%. Although the obtained values were quite low, they are perfectly comparable to other values in the literature. In fact, the phytosterol absorption reported is generally less than 6%.[23,24]

Sensory evaluation

Regarding the sensory analysis, a three-way ANOVA has been carried out by considering factors such as the microcapsule concentration, the dark chocolate cocoa content and the replication. The replication factor had no significant effect on the chocolate attribute intensity ($p > 0.05$). As expected, the cocoa content showed a significant effect regarding the characteristic chocolate attributes, such as colour uniformity, brightness, colour intensity, bitterness, sweetness, pungency, astringency,

Fig. 6 Liking score reported for 85-MP 0%, 85-MP 5%, 85-MP 10% and 85-MP 15%, in blind and in information tests. For each sample in the different sessions, columns with different superscripts are significantly different (*T*-test at $p < 0.05$).
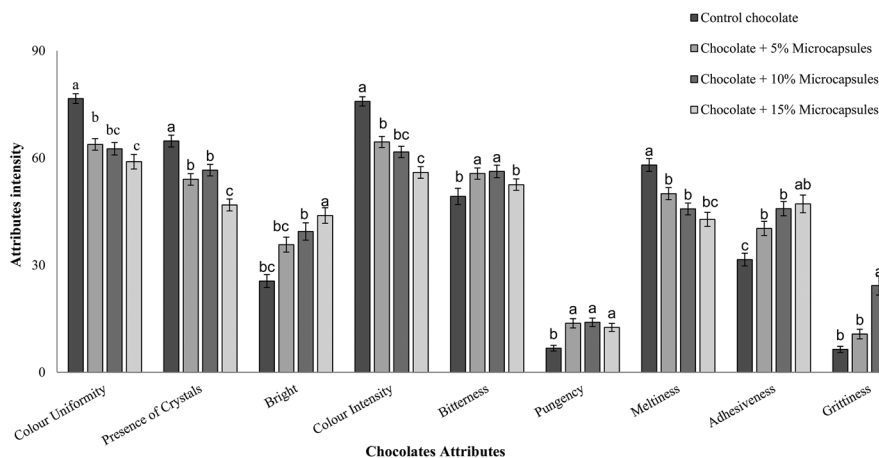

Fig. 5 Chocolate sensory attributes significantly affected by microcapsule concentrations. For each attribute, columns with different superscripts are significantly different (LSD test at $p < 0.05$).

the consumers tested the chocolate under the blind conditions, significant differences in the liking score of the samples have been observed. However, all the samples reached the threshold of acceptability (Fig. 6). In the information test, all the participants tasted each sample with the product information related to the MP fortification. No neophobia was detected according to the results of the neophobia questionnaire. The information related to the products caused a significant increase in the liking score ($p < 0.05$), except for the 85-MP 0% (control). In view of the above, it was concluded that a significant effect of the information on the liking score has been observed. In particular, after the information, the liking score of the sample 85-MP increased 10% and did not result significantly different from that of control.

Conclusion

Different functional dark chocolates enriched with optimized microencapsulated phytosterols have been produced. These chocolates attained all relevant aspects for a satisfactory functional food development. Preliminary results highlighted that microcapsule's concentration affects the chocolate sensory attributes, independently on the cocoa contents (64, 72 and 85%). For this reason, in order to obtain a functional product with an ability to modulate significantly human health, chocolates containing 85% of cocoa could be used for the fortification. If the data can be corroborated by further analyses, the daily intake of 10 g of chocolates fortified with 15% of microencapsulated phytosterols could provide about 25–30% of 0.8 g day^{-1}, the lowest amount required to observe a reduction of 10% of the LDL cholesterol. However, in order to obtain the new functional product, it will be necessary to link the sensory descriptive data with the consumer liking data, taking also into account the shelf life of the product. Moreover, to ensure that these functional chocolates could represent an interesting option for individuals with dyslipidemia, it would be necessary to carry out *in vivo* studies with subjects who suffer from these kinds of disease.

Conflicts of interest

The authors declare no conflict of interest.

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