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Research article

Fabrication and optimization of ultra-long stable microencapsulated chlorophyll using combinations of wall material via response surface methodology

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ABSTRACT

Nowadays, the need to use natural pigments instead of artificial colorants in food has increased due to health risks. Chlorophyll is a natural colorant with antioxidant properties. Chlorophyll is a natural pigment with superior antioxidant properties. However, this pigment is unstable in the different conditions of food processing. To increase the stability of chlorophyll extracted from *Ulva intestinalis* algae, encapsulation of chlorophyll with maltodextrin (MD) and whey protein isolate (WPI) carriers by two drying methods including spray drying and freeze drying was achieved. The optimum combination of wall and core materials to achieve the highest response including encapsulation efficiency (EE) and chlorophyll content (CC) was obtained by response surface methodology and central composite design. The optimal chlorophyll microcapsule (Chl-M) obtained was chosen for subsequent tests containing solubility, moisture content, and antioxidant properties. The results showed that the highest EE and CC were 90.27 ± 0.21 %, 55.36 ± 0.36 μg/mL, and 90.46 ± 0.62 %, 85.85 ± 0.43 μg/mL, respectively in SD and FD. The microcapsules produced by the freeze dryer (FD) had higher antioxidant activity (79.1 ± 0.24) than the microcapsules produced by the spray dryer (SD) (67.5 ± 0.16). The highest solubility (95.32 %) and the lowest moisture content (3.7 ± 0.05) were related to the SD. Freeze drying method (FDM) had the highest EE (91.2 %), CC (89.67 μg/mL), and antioxidant properties (79.1 %). It is hoped that the encapsulated chlorophyll can be used as a health-promoting food color additive in the food industry.

1. Introduction

Chlorophyll is used to color dairy products, cooking oils, cakes, beverages, fruit juices, jellies, pasta, infant formula, and confectionery products, as well as maintain the color of frozen or canned vegetables. Due to the health effects of chlorophyll, many products containing it such as dietary supplements and fruit juices have become popular. Some producers claim that these products may protect the human body against a vast range of diseases [1]. Chlorophyll extracted from nature sources is not a good choice as a dye, due to its

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chemical instability, destroying and changing the appearance and quality of the products. Chlorophyll can be quickly decomposed by enzymatic reactions or other factors such as heat, light, oxygen, and acid; Consequently, chlorophyll derivatives such as *pyropho-phorbide*, *pyropheophytin*, pheophorbide, and pheophytin are created [2].

The development of affordable and applicable technologies for the preparation of natural food color and its application in foods is a great challenge and a basic need of the time. Encapsulation is one of the options to prevent the degradation of biological compounds. Encapsulation is the most common method of protecting active and sensitive fat-soluble substances such as oil essences, oleoresins, spices, fatty acids, edible oils, antioxidants, fish oil, probiotics, and color substances [3].

There are various techniques for food encapsulation, among which spray drying and freeze drying are used owing to the wide availability of the necessary equipment; the simplicity of their process; significant reduction in product volume, transportation cost, and storage space; and high stability of the product because of their reduced moisture content. The final products are readily used by reconstitution or combined with other formulated materials, as they are in powder form [4].

To guarantee the success of the microencapsulation, it is important to properly select suitable biopolymers as wall material because the type of wall materials determines the morphological and physicochemical features of the produced microcapsules. In addition, the efficiency of encapsulation affects the durability and degree of protection of sensitive core materials. Maltodextrins provide good oxidative stability for the encapsulation of oily compounds. Maltodextrins (MD) with 10-20DE due to low cost and high effectiveness, mild taste, low viscosity in high solid content ratio, and solubility in water, are considered one of the most interesting wall materials in microencapsulation. Since MD lacks emulsifying properties and interfacial activity, required for high efficiency of encapsulating, it is used as a wall material along with surface active wall constituents and usually in combination with other biopolymer materials such as whey proteins [5]. Whey protein isolate (WPI) has eutrophic character and useful functional characteristics such as the capability of emulsifying and forming films, common features for wall materials used in encapsulation [6].

Some studies have been conducted so far on the encapsulation of natural chlorophyll [7, 6, 8, 9, 10, 11], However, no study has yet been conducted on the encapsulation of chlorophyll using MD and WPI as wall materials, and comparing the two encapsulation methods by SD and FD. This study aims to optimize the encapsulation of chlorophyll extracted from *Ulva intestinalis* by different encapsulation (SD and FD) techniques and carrier agents (WPI and MD). The microcapsules with the highest EE and CC were selected for the next tests according to the optimum conditions obtained from the design software. The fabricated Chl-M was further assessed for encapsulation efficiency, chlorophyll content, and physicochemical properties.

2. Materials and methods

2.1. Reagents and chemicals

Whey protein isolate (Hilmar Co., America), maltodextrin (DE 16.5–19.5, Sigma Aldrich), Tween 80 (Merck Co., Germany), acetone (Asia Research Co., Iran), ethanol (Khorasan Distillation Co., Iran) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased. Other chemicals used for the experiment were of analytical grade.

2.2. Chlorophyll extraction from Ulva intestinalis

The seaweed *Ulva intestinalis* was collected for in September 2023 from the shores of the Caspian Sea. The fresh seaweed samples were first washed with seawater and then with fresh water to remove sand and impurities. The seaweed was then finely chopped to reduce its size. For pigment extraction, 90 % acetone, 70 % ethanol, and a blend of 50-50 % of acetone and ethanol were used as solvents. Following mixing 25 mg of the moist sample with 10 mL of the solvent, it was placed in a shaking incubator (Pars Azma. Co., Iran) at a temperature of 20 °C, away from light, for 4–6 h [12]. The extract was then filtered with a nylon syringe filter of 0.22 μm pore size and centrifuged (T-4, Arum Tajhiz Gostar, Iran) at 5000 rpm for 10 min. A rotary evaporator (IKA, RV10) under vacuum at 35 °C was used to concentrate the extracted chlorophyll and separate the solvent. Finally, chlorophyll extract was delivered to a Schott container with a parafilm-covered top and refrigerated at −18 °C.

2.3. Determining the content of pigments in the extract

The UV–Vis spectrophotometer (Alpha-1502-Laxco America) was used to determine the content of pigments according to the equations described below Eqs (1)–(5), and the results were expressed in μg/g [13].

$$\text{Chl a } (\mu g/mL) = -0.3319 \times (A_{630} - A_{750}) - 1.7485 \times (A_{647} - A_{750}) + 11.9442 \times (A_{664} - A_{750}) - 1.4306 \times (A_{691} - A_{750}) \ (\pm 0.0020) \quad (1)$$

$$\text{Chl b } (\mu g/mL) = -1.2825 \times (A_{630} - A_{750}) - 19.8839 \times (A_{647} - A_{750}) - 4.8860 \times (A_{664} - A_{750}) - 2.3416 \times (A_{691} - A_{750}) \ (\pm 0.0076) \quad (2)$$

$$\text{Chl c } (\mu g/mL) = -23.5902 \times (A_{630} - A_{750}) - 7.8516 \times (A_{647} - A_{750}) - 1.5214 \times (A_{664} - A_{750}) - 1.7443 \times (A_{691} - A_{750}) \ (\pm 0.0075) \quad (3)$$

$$\text{Chl d } (\mu g/mL) = -0.5881 \times (A_{630} - A_{750}) + 0.0902 \times (A_{647} - A_{750}) - 0.1564 \times (A_{664} - A_{750}) + 11.0473 \times (A_{691} - A_{750}) \ (\pm 0.0030) \quad (4)$$

$$\text{Total Chl } (\mu g/mL) = \text{Chl a} + \text{Chl b} + \text{Chl c} + \text{Chl d} \quad (5)$$

2.4. Fourier-transform infrared spectroscopy (FT-IR)

For this purpose, chlorophyll extract at a ratio of 1 mg extract to 100 mg KBr was thoroughly mixed with KBr and then compressed into a KBr pellet. Scanning was done at an ambient temperature in the spectrum range of 400–4000 cm^{-1}, resolution of 4 cm^{-1}, and scanning speed of 16. The obtained spectra were then analyzed to determine the types of possible bonds and functional groups. The spectra of each sample were measured three times.

2.5. Preparation of chlorophyll microcapsules (Chl-M)

2.5.1. Design of experiments by RSM

In this optimization study, a rotatable Central Composite Design (CCD) with three independent factors at three levels was employed. Maltodextrin (X_1), whey protein isolate (X_2), and chlorophyll (X_3) were selected as the independent variables for optimizing the chlorophyll encapsulation process. The encapsulation efficiency (EE%) and the amount of encapsulated chlorophyll (CC μg/mL) were considered as the experimental responses. For each drying method, 20 experiments were conducted separately by CCD (Table 1). Finally, to estimate the pure error sum of squares, six replicates were performed at the central point.

The evaluation of the results was done using the Design-Expert Software, V13 (Stat- Ease, Inc., MN, USA), producing response surfaces whereas holding a variable constant in the second-order polynomial model. The quality of suitable models was evaluated through correlation coefficient and Adjusted Correlation Coefficient.

The analysis of variance (ANOVA) for each answer was used to check the statistical significance of each term of the regression equations. The adequacy of the model was checked via accounting R^2 and adjusted R^2. Adequate precision, which indicates the signal-to-noise ratio and the standard deviation were also examined for each response.

The synchronized optimization of the various responses was done using the numerical and graphical optimization techniques of the Design Expert software. Finally, the experimental data of the responses were obtained according to the optimal conditions and the utility desirability function, and the confirmation of the models was obtained using the coefficient of variance and according to the by equation:

Table 1
Central composite design and results for encapsulation efficiency (EE) and chlorophyll content (CC) of Chl-M.

Independent variables			Factor level		
			−1	0	+1
Maltodextrin (g) (X_1)			6	15	24
Whey Protein Isolate (g) (X_2)			6	15	24
Chlorophyll (g) (X_3)			12	18.75	25.5

Std	Run	Independent variables			Response variables							
		X_1 (g)	X_2 (g)	X_3 (g)	EE (%) Actual (F)*	EE (%) Predicted (F)[a]	CC (μg/mL) Actual (F)[a]	CC (μg/mL) Predicted (F)[a]	EE (%) Actual (S)[b]	EE (%) Predicted (S)[b]	CC (μg/mL) Actual (S)[b]	CC (μg/mL) Predicted (S)[b]
9	1	0	15	18.75	76.01	74.81	89.49	82.45	85.42	84.86	53.25	53.46
11	2	15	0	18.75	86.81	87.19	81.27	81.38	79.91	80.22	50.01	49.55
17	3	15	15	18.75	90.53	90.61	85.26	85.31	90.67	90.32	53.87	54.22
13	4	15	15	7.5	82.75	82.26	58.23	60.71	86.93	87.12	27.90	25.86
10	5	30	15	18.75	81.58	83.09	77.7	77.27	90.03	90.77	29.15	30.94
18	6	15	15	18.75	90.68	90.61	85.67	85.31	90.09	90.32	54.16	54.22
8	7	24	24	25.5	90.05	89.70	64.19	64.48	90.48	89.93	48.11	46.85
14	8	15	15	30	89.97	90.77	76.32	75.37	85.08	85.07	44.26	43.54
5	9	6	6	25.5	82.98	83.58	79.19	79.02	80	79.81	51.80	52.43
19	10	15	15	18.75	89.94	90.61	84.93	85.31	89.84	90.32	53.96	54.22
3	11	6	24	12	74.99	76.32	80.03	77.87	85.45	85.37	34.51	32.46
16	12	15	15	18.75	91.2	90.61	84.97	85.31	90.46	90.32	54.96	54.22
12	13	15	30	18.75	88.57	88.51	67.78	69.20	82.77	82.64	46.43	48.89
15	14	15	15	18.75	90.96	90.61	85.26	85.31	89.95	90.32	54.95	54.22
6	15	24	6	25.5	87.07	85.52	89.67	90.75	86.27	86.23	31.60	32.24
2	16	24	6	12	86.65	86.66	74.17	73.57	84.89	84.08	32.25	30.76
4	17	24	24	12	85.06	84.23	60.89	59.98	85.92	85.98	53.87	54.22
7	18	6	24	25.5	87.81	87.58	78.62	78.14	80.10	80.79	56.49	56.56
20	19	15	15	18.75	90.04	90.61	86.03	85.31	90.95	90.32	53.87	54.22
1	20	6	6	12	78.81	78.94	67.45	66.08	85.77	86.19	47.99	47.84

[a] Freeze drying.
[b] Spray drying.

$$\text{CV}\ (\%) = \left(\frac{\Delta X}{X}\right) \times 100 \quad (6)$$

where CV is the coefficient of variance, ΔX is the difference between predicted and experimental values, and X is the average of experimental data.

Adequate precision, which indicates the signal-to-noise ratio and the standard deviation were also examined for each response.

After choosing the desired goal for the response and variables, all the independent variables were maintained within range, whereas maximizing the response (EE and CC).

2.5.2. Preparation of reagents

WPI was dissolved in distilled water and stirred overnight (12 h) with a magnetic stirrer to ensure complete swelling. MD (DE 16.5–19.5) was dissolved in distilled water, heated to 65 °C, and stirred for 30 min. Afterward, WPI and MD were mixed in the ratios defined by the Design Expert software as a mixture of encapsulating agents and stirred for 40 min to obtain the aqueous phase. Following cooling the solutions to room temperature (23 ± 1 °C), they were stored in a refrigerator overnight at 4 ± 0.5 °C to ensure full hydration of the polymer molecules. Subsequently, 1.5 % (w/v) of the emulsifier Tween 80 was added to the hydrated wall material solutions, followed by vigorous stirring at room temperature to achieve complete dissolution.

2.5.3. Microencapsulation

The chlorophyll extract solution as the core material was added to the wall material solution in specified ratios and then homogenized at 1000 rpm for 5 min.

2.5.4. Drying process

A spray dryer and freeze dryer were used for drying the microcapsules. The FD process began with freezing. The emulsions were frozen at −18 °C for 12 h. Glass containers connected to the freeze dryer unit were used to store the frozen samples. The FD process was carried out with a laboratory-scale freeze dryer (ZiRBUS, VaCo 5) with a condenser temperature of −50 °C and a vacuum of 0.1 mbar. The spray dryer (Dorsa Behsaz Company, model D26) was operated with an inlet air temperature of 145 °C, an outlet air temperature of 110 °C, a rotary atomizer at 10 × 10 kPa, a pump speed of 1.5 mL/min, and a blower speed of 0.6 m^3/min.

2.6. Encapsulation efficiency of Chl-M

EE, considered as the ratio of the core material in microcapsules to the main emulsion, was evaluated using fresh chlorophyll-loaded microparticles according to the method Agarry et al., 2022 [8] (Eq. (7)):

$$\text{Encapsulation efficiency}\ (\%) = \frac{\text{Total chlorophyll amount} - \text{Surface chlorophyll amount}}{\text{Total chlorophyll amount}} \times 100 \quad (7)$$

The sum of the surface chlorophyll (SC), core chlorophyll (CC), and residual chlorophyll (RC) in the supernatant of fresh microcapsules provides the total chlorophyll (TC). The amount of CC in the microcapsules was obtained by centrifuging the fresh microparticle suspension at 10,000 rpm for 15 min to get the pellet. UV–Vis spectrophotometer was used to immediately determine chlorophyll in the supernatant and calculated as by Eq. (8) [6]:

$$\text{chlorophyll}\ \left(\frac{\mu g}{ml}\right) = 6.10 \times A_{665} + 20.04 \times A_{649} \quad (8)$$

where A_{665} and A_{649} represent the absorbance at 665 and 649 nm, respectively, and 6.10 and 20.04 are the transfer coefficients.

SC assessment was done via pipetting 10 mL of hexane into the microparticles and then gently shaking for 5 min. Following centrifugation at the same conditions, the supernatant was collected to determine the SC content. Then pipetting 20 mL of 80 % acetone into the pellets, they were ultrasonicated (Ultrasonic bath of Elma Co. Germany) (100 % power) for 30 min at room temperature aimed at breaking the Chl-M membrane and discharging the CC. Finally, the suspension was centrifuged and the supernatant's CC was determined. To ensure the complete extraction of chlorophyll from Chl-M, the process was repeated three times to achieve colorless microcapsules.

2.7. Determination of chlorophyll content of Chl-M

The method developed by Agarry et al. (2022) [8] was used to determine CC in each Chl-M. UV–Vis spectrophotometer was used to determine CC. A given amount of Chl-M was dissolved in 20 mL of 80 % acetone by vortexing at 3000 rpm for 2 min, followed by its transfer to an ultrasonic bath at 100 % power for 30 min. Then, the suspension was centrifuged at 10,000 rpm for 15 min. The extracted core materials containing chlorophyll were measured to determine CC in absorbances at 646 and 662 nm by the equations (2) and (2):

$$\text{Chlorophyll } a\ (\mu g/mL) = 11.24A_{662} - 2.04A_{646} \quad (9)$$

$$\text{Chlorophyll } b\ (\mu g/mL) = 20.13A_{646} - 4.19A_{662} \quad (10)$$

where, A_{646} and A_{662} represent absorbance at 646 and 662 nm, respectively. TC (μg/mL) is defined as the sum of chlorophyll *a* and

chlorophyll *b*.

2.8. Moisture content and solubility of Chl-M

The evaluation of the moisture content (MC) of Chl-M was done by the (AOAC) method [34]. Following placing 1 g of each Chl-M on an aluminum plate, it was dried in an oven (UF110, Co. Memmert Germany) at 105 °C for until a constant weight. MC of each Chl-M was calculated according to Eq (6) [14]:

MC (%) = (Wet powder weight – Dried powder weight)/ (Wet powder weight) × 100 (6)

To measure the solubility of Chl-M, 1 g of Chl-M was dissolved in 10 mL distilled water with stirring, then it centrifuged at room temperature at 500 rpm for 30 min, followed by a 5-min centrifuge at 5000 rpm. Finally, following transferring the supernatant to the plate and drying overnight at 105 °C, solubility was calculated as a percentage according to Eq (7) [15]:

Solubility (%) = Weight of dried supernatant (g) / Weight of the original powder × 100 (7)

2.9. Antioxidant properties of Chl-M

The antioxidant properties of Chl-M were measured by the DPPH free radical scavenging activity [6]. The sample mixture included 0.4 mL of sample solution (50 mg of sample in 5 mL of distilled water), 0.5 mL of distilled water as well as 2.5 mL of 0.1 mmol/L DPPH solution. It was maintained in the dark at ambient temperature for 20 min. After centrifuging the sample at 5000 rpm for 10 min, the supernatant absorbance was determined at 517 nm by UV–Vis spectrophotometer. Eq (8) was used to calculate DPPH scavenging activity:

$$SA(\%) = \left(1 - \frac{A1 - A2}{A0}\right) \times 100 \quad (8)$$

where, SA denotes the scavenging activity of DPPH free radical (%) of, A_0 represents the absorbance of the control sample replacing the sample solution with distilled water, A_1 is the sample's absorbance, A_2 denotes the absorbance of the blank sample in which methanol and DPPH are replaced for the sample solution.

2.10. Statistical analysis

The optimal microcapsule obtained by FD and SD methods was evaluated for further analysis. All experiments were carried out in triplicates, and results were reported as the mean ± standard deviation. Significant differences ($p < 0.05$) among samples were evaluated using a one-way ANOVA of the SPSS 27.0 software version. Duncan's multiple comparison test was employed to identify differences between means.

3. Results and discussion

3.1. Chlorophyll extraction

The amount of chlorophyll obtained from extraction with three different solvents is shown in Table 2. Among all the chlorophyll contents determined, acetone had the highest chlorophyll extraction value. Ethanol is one of the preferred solvents for chlorophyll extraction due to its suitable polarity, non-toxicity, and biodegradability. However, because of its low selectivity (also extracting other compounds such as carotenoids and lipids), additional purification steps may be required to obtain pure chlorophyll. On the other hand, acetone is usually an acceptable solvent for chlorophyll extraction acetone, due to its suitable polarity, high extraction efficiency, and the ability to yield a purer product (extracting chlorophyll with higher purity) and is one of the appropriate solvents for

Table 2
The Chlorophyll content extracted from *Ulva intestinalis* algae using different solvents.

	Ethanol-Acetone	Ethanol 70 %	Acetone 90 %
Chlorophyll *a*	29.65 ± 0.51^{ab}	26.43 ± 3.36^{a}	31.88 ± 0.77^{b}
Chlorophyll *b*	18.93 ± 0.67^{b}	10.76 ± 0.51^{a}	$42.02 \pm 0.0.28^{c}$
Chlorophyll *c*	10.25 ± 0.23^{b}	11.32 ± 0.32^{c}	8.72 ± 0.32^{a}
Chlorophyll *d*	21.57 ± 0.34^{b}	28.79 ± 0.91^{c}	8.09 ± 0.28^{a}
Total Chlorophyll	$80.4 \pm 0.1.37^{b}$	77.3 ± 0.51^{a}	90.71 ± 0.82^{c}

Data are expressed as mean ± standard deviation in μg/g of algae. Within each row, different letters represent significant differences between results obtained with different extraction solvents, at $p < 0.05$.To ensure the accuracy of responses, the experiments were conducted in three repetition.

extracting compounds such as chlorophylls.

Chlorophyll is located in a thin layer of internal organelles of green plants known as chloroplasts, attached to weak covalent bonds that can easily be broken. The extraction of chlorophyll is carried out by soaking plant tissue in organic solvents. Chlorophyll is a compound soluble in fat, as a result, oily solutions and polar solvents such as acetone, ethyl acetone, ethanol, and methanol are more effective than non-polar ones such as petroleum ether and hexane. Acetone is an organic solvent that can break the dissolved impurities in the leaves of plants and algae. Chlorophyll has the most, easiest and fastest dissolution in this solvent. In previous studies [12,13,16], it was stated that the best solvents for optimal chlorophyll extraction are acetone and ethanol. Accordingly, in preliminary experiments using 70 % ethanol and 90 % acetone, 90 % acetone was chosen as the solvent for chlorophyll extraction.

3.2. FT-IR of algae extract

Fig. 1 shows the FT-IR spectrum of chlorophyll extracted from the algae. The observed signals for chlorophylls were assigned according to previous studies [14,17–20]. The observed peak in the range of 2920 and 2850 cm^{-1} was ascribed to anti-symmetric and symmetric C-H stretching vibrations of methyl, methylene, or methine group in phytol. The peak in the range of 1730 cm^{-1} was associated with the stretching vibrations of C-17^3 = O and C-13^3 = O of the ester group, whereas the peak at 1690 cm^{-1} was related to the stretching vibrations of the free keto group at C-13^1. The peak observed in the range of 1610 cm^{-1}, corresponded to C=C and C=N skeletal stretching vibrations of the aromatic system in chlorophyll. Finally, C-17^3-O and C-13^3-O stretching vibrations of ester groups were detected in 1280 cm^{-1}.

Chlorophyll was present in the extract from the algae, as proven by the analysis of the peaks obtained from FT-IR.

3.3. Optimization of EE and CC of Chl-M

3.3.1. Effect of independent variables on EE and CC of Chl-M

The encapsulation efficiency of chlorophyll microcapsules using different percentages of MD, WPI, and Chl is shown in Table 1. EE for the microcapsules obtained by SD was 79.91–90.95 and for FD was 74.99–91.2, significantly influenced by the composition of the wall material and the type of drying method. The highest EE in SD was 90.95 %, belonging to treatment No. 19, consisting of MD, WPI, and Chl with ratios of 15, 15, and 18.75 (central point). In FD, the highest EE was 91.2 % belonging to treatment No. 12 (central point).

To examine the effect of independent variables on the responses at different points, a single-factor plot was used (Fig. 2). In these graphs, the mentioned effect was studied when the other two factors were fixed in the central points.

In the treatment in which MD was used alone as a wall (treatment No. 2), a good emulsion was not formed and a sticky powder was produced, showing that MD alone did not have a good ability to form a film. This result was not in agreement with those of Kang et al. (2019) [14] which stated that MD alone can be used as a wall for chlorophyll encapsulation with high CC and EE. Fig. 2 (a and d) demonstrates the effect of MD (g) on the EE of Chl-M when the two factors of WPI and Chl were fixed at the central point. Fig. 2a shows the EE of SD and Fig. 2d depicts the EE in FD. By increasing the concentration of MD in SD, an increase in EE was witnessed. However, in FD, by increasing the percentage of MD up to the central point, an increase in EE was evident; however, an increase in concentration led to a decrease in EE.

The effect of WPI on the EE in two drying methods is shown in Fig. 2b and e, according to which the increase in WPI up to the central point (15 g) resulted in an increase in EE, and then with the increasing concentration, the EE decreased. According to Fig. 2f, in FD with the increase in Chl, EE increased; however, in SD (Fig. 2c), with the increase in Chl up to the central point, EE increased and then, with raising Chl concentration, EE decreased. The decrease in EE through the increase in concentration can be justified

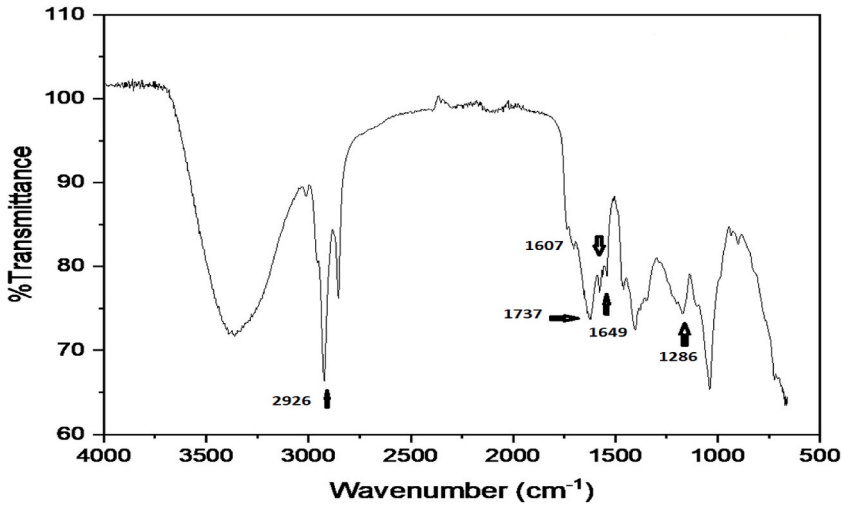

Fig. 1. FT-IR spectrum of chlorophyll extracted from *Ulva intestinalis*.

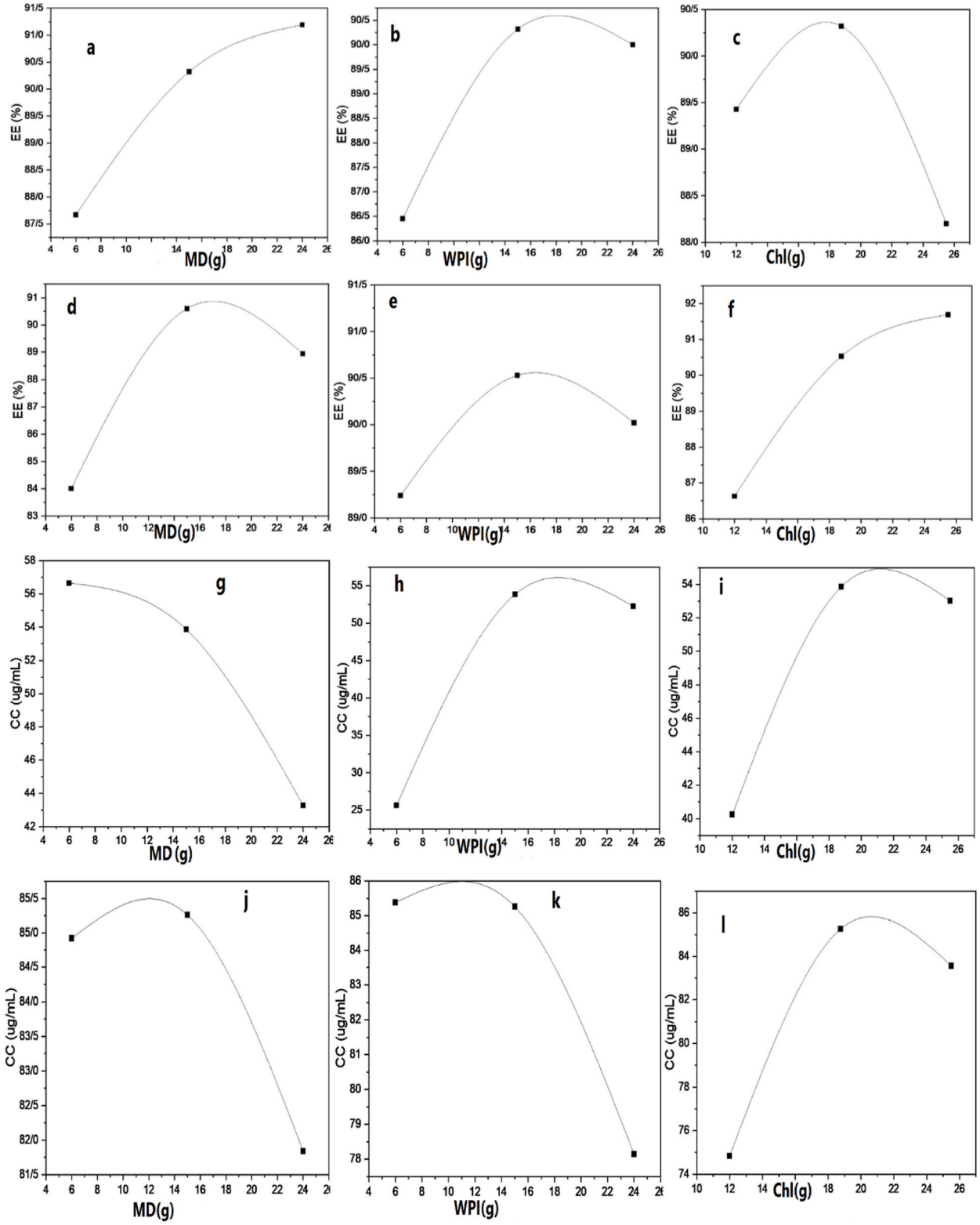

Fig. 2. The effect of maltodextrin, whey protein isolate, and chlorophyll according to SD (a-b-c) and FD (d-e-f) on encapsulation efficiency (EE); chlorophyll content (CC) according to SD (g-h-i) and FD (j-k-l) of Chl-M.

considering the fact with an increase in the concentration of one wall material, the concentration of another wall decreases. According to the data in Tables 1 and it was also observed that the highest EE was obtained when an equal ratio of two wall compositions was used, and this was the same in the two drying methods. This factor was probably due to the good interaction of carbohydrates and proteins as a good preservative compound in the protection of the core and as a result of increasing EE. The extent to which the loaded compounds are protected by carrier materials and drying aids against adverse environmental conditions illustrates the importance of EE [21].

The effect of variables on CC is depicted in Fig. 2. In both SD and FD, with an increase in MD concentration, CC decreased (Fig. 2 g and j). Increasing WPI concentration up to the central point (SD) led to an increase in CC (Fig. 2h); however, in FD, the relationship between increased WPI concentration and CC was inverse (Fig. 2k). Regarding the increase in the percentage of Chl and its effect on the CC of Chl-M in both SD and FD, the increase in concentration up to the central point leads to an increase in CC, and after passing the central point, with an increase in concentration, the CC decreases (Fig. 2 i and l).

Concerning the CC, according to Fig. 2, and the study of the 3D plot, it can be concluded that the simultaneous use of MD and WPI as a wall had a greater effect than the use of only MD and WPI on the CC of Chl-M.

According to the findings, the interaction forces between the wall material and core material could contribute to the retention of encapsulated compounds. The high encapsulation efficiency confirmed the success of various biopolymers in the encapsulation process [8]. Basically, due to their amphiphilic nature and surface activity, proteins can migrate to the interface between the drop and air, and as a result, they are placed around the core drops before drying, causing the trapping of loaded compounds effectively and protecting them against adverse drying conditions. On the other hand, MD lacks surface activity and emulsifying properties [22], probably increasing the amount of MD to reduce the EE and CC, and when it is combined with protein, its emulsifying properties are improved.

The type of drying method also had a significant effect on EE and CC. In general, freeze-dried microparticles showed higher EE than the spray-dried samples. In FD, the emulsion containing the wall and core material was frozen. At this stage, the water present turned into ice crystals, and the core material (chlorophyll) was locked inside the walls of MD and WPI, causing Chl to be placed inside the walls in a stable manner and with high EE. Proteins changed their structure through unfolding, and by forming a resistant multilayer around the core material by repulsive forces, they formed remarkably stable emulsions which are essential for encapsulation purposes. WPI showed a very good ability to form films [23]. Therefore, the effect of overlapping protein and carbohydrate in increasing the EE and maintaining the core content (chlorophyll) during drying was evident. The type of encapsulation method also had a significant effect on the amount of CC as the microcapsules produced by FD had more CC than SD. Guo et al. (2020) [24] stated that the milder processing conditions of FD (low temperature and absence of oxygen) compared with SD (high temperature and presence of oxygen) could be the reason for this.

3.3.2. Statistical analysis and modeling of EE and CC

The fitted model for EE and CC obtained from two drying methods to predict the relationship between the dependent and the independent variables can be expressed by:

$$Y_{EEF^*} = 54.040 + 2.276X_1 + 1.538X_3 - 0.0242 \times 1 \times {}_3 + 0.0276 \times 2 \times {}_3 - 0.0518X_1^2 - 0.0122 \times {}_2^2 \quad (9)$$

$$Y_{EES^{**}} = 90.32 + 1.76X_1 + 0.719X_2 - 0.607X_3 + 0.682 \times 1 \times {}_2 + 2.13 \times 1 \times {}_3 - 0.886 \times {}_1^2 - 3.14 \times {}_2^2 - 1.49 \times {}_3^2 \quad (10)$$

$$Y_{CCF^{***}} = -9.566 + 1.417\ X_1 + 3.122X_2 + 6.299X_3 - 0.0797 \times 1 \times {}_2 - 0.0531X_2X_3 - 0.0242 \times {}_1^2 - 0.044 \times {}_2^2 - 0.136 \times {}_3^2 \quad (11)$$

$$Y_{CCS^{****}} = +54.22 - 6.70X_1 + 6.39X_3 + 2.62 \times 1 \times {}_2 + 4.88 \times 2 \times {}_3 - 4.25 \times {}_1^2 + 1.77\ X_2^2 - 7.58 \times {}_3^2 \quad (12)$$

*: Encapsulation efficiency freeze-dried of Chl-M.

**: Encapsulation efficiency spray dried of Chl-M.

***: Chlorophyll content freeze-dried of Chl-M.

****: Chlorophyll content spray dried of Chl-M.

Table S (Supplementary data) presents the experimental data and process variables for EE and CC under different treatment conditions. The adequacy of the suggested model (9–12) was investigated by analysis of variance (ANOVA) and the significant factors were identified. The statistical significance was studied at a 95 % confidence level ($p < 0.05$). The ANOVA did not show a significant lack of fit, meaning that the model represented the data satisfactorily. The proposed regression model for EE and CC was efficient thanks to satisfactory R^2 (determination coefficient) and adj-R^2 values. A high R^2 demonstrates that the obtained equation has a good

Table 3
The signal-to-noise ratio values for the model and standard deviation for microcapsules produced by freeze-drying and spray-drying methods (FDM & SDM).

Signal-to-noise ratio (S/N)	EE_F[a]	EE_S[b]	CC_F[c]	CC_S[d]
model	21.379	25.723	28.533	27.303
Standard deviation	112.529	198.058	69.954	34.646

[a] Encapsulation efficiency freeze-dried of Chl-M.
[b] Encapsulation efficiency spray dried of Chl-M.
[c] Chlorophyll content freeze-dried of Chl-M.
[d] Chlorophyll content spray dried of Chl-M.

ability to link the experimental outcomes. The R^2 and adj-R^2 values for EE of Chl-M were 0.9771 and 0.9564 for the FD and 0.9863 and 0.9740 for SD, respectively, confirming a good accordance between the experimental data and the theoretical values anticipated by the polynomial model. This value for CC was 0.9853 and 0.9721 for FD and 0.9870 and 0.9754 for SD, respectively (Table S).

The coefficient of variation (CV) values were found to be 0.696 and 1.23 for EE of Chl-M in the SD and FD, respectively. The CV values were reported to be 3.98 and 1.97 for the CC of Chl-M in the SD and FD, respectively. Since the coefficient of variation expresses the amount of dispersion per unit of the mean, smaller values of CV result in better reproducibility. Generally, a CV of more than 10 is translated to a variation in the mean value, failing to adequately develop a proper response model [25]. The signal-to-noise ratio (S/N) is measured by adequate precision, where a ratio higher than 4 is desirable [26]. Following Table 3 for the models proposed, this value was 25.723 and 21.379 (EE), 27.303 and 28.533 (CC) for SD and FD respectively which is a very good signal-to-noise ratio. The statistical parameters determined here confirm the models' reliability. The signal-to-noise ratio values for the standard deviation are also in Table 3 provided.

The significance of each coefficient can be determined by examining the p-value. The lower p-value of each coefficient means that the more significant the corresponding coefficient is in the response. The ANOVA analysis showed that the linear effect of MD (X_1), Chl (X_3), the interaction terms of X_1X_3, X_2X_3, and all quadratic terms of X_1^2, X_2^2, and X_3^2 in the model of EE (9), in FD was significant ($p < 0.05$). In the SD, the model of EE (10), all models except the interaction terms of X_2X_3 were significant. In the model of CC (11-(12), all

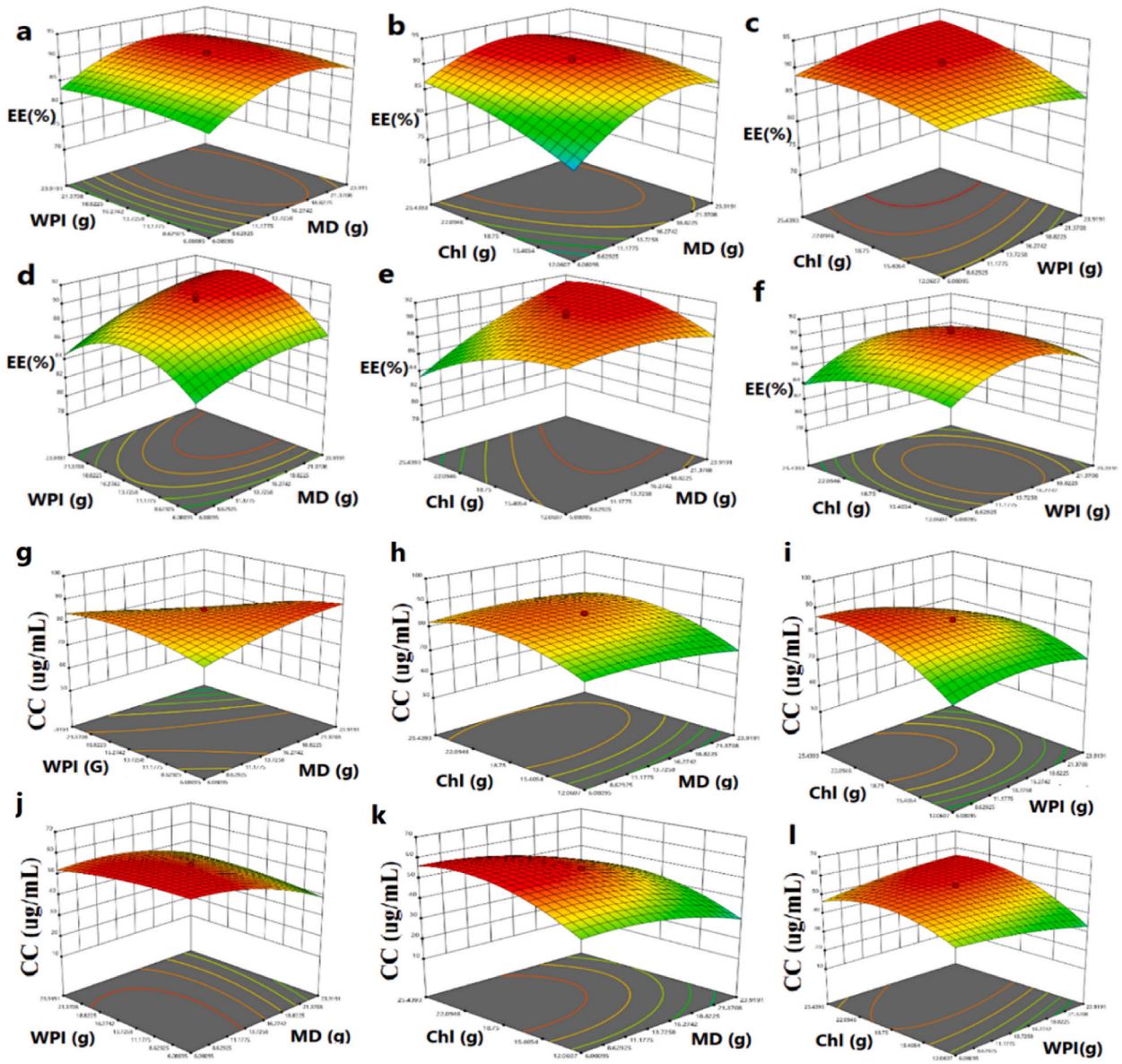

Fig. 3. 3D plot of encapsulation efficiency (EE %) using FD (a-b-c), SD (d-e-f), and chlorophyll content (CC μg/mL) by FD (g-h-i) and SD (j-k-l).

linear terms, interaction terms of X_1X_2 and X_2X_3 and all quadratic terms in FD and, all models except X_2 and X_1X_3 in SD were significant.

3.3.3. Optimization of EE and CC

Design Expert software was used to plot response surfaces, aimed at studying the effects of variables and their interactions on EE and CC of Chl-M. The results of EE and CC affected by the amount of MD, WPI, and Chl are presented in Fig. 3. The application of 3D plots of the model is vastly suggested for a graphical explanation of the interactions, facilitating the visualization of the relationship between each factor's experimental levels and the responses. These types of plots show the effects of two factors on the response at a time; however the other factor was kept at the central level [11].

The 3D response surface plot in Fig. 3 (a and d), giving the EE according to the value of MD and WPI and chlorophyll (g), is fixed at the central point. According to the findings, EE increased by increasing the amount of MD and WPI up to the center point (range 15 g). This was true in both types of drying methods. Fig. 3(b and e) depicts a 3D response surface plot for EE when WPI was fixed at the central point and the value of MD, Chl(g) was changed. According to Fig. 3b, in the freeze-drying method, when MD was 14 g, Chl was 16 g, and WPI was 15 g, the highest EE was obtained. In SD (Fig. 3e), an increase in EE by increasing the amount of MD to 19 g and Chl to 15.5 g was observed.

Fig. 3(c and f) shows the 3D response surface plot for EE when Chl and WPI were variable and MD was fixed at its central point. In the FD, when WPI was 17 g and Chl 20 g, the highest EE was witnessed, and in SD, this value was 16 g for WPI and 18 g for Chl. In the analysis of CC, Fig. 3(g and j), the variable proportions of WPI and MD on EE, considering Chl as the central point, were examined. According to Fig. 3, the highest CC was obtained when MD was 13 and WPI was 19 g.

In Fig. 3 (h and k),3D response surface plots examined CC when MD and Chl were variable and WPI was at the center point. Examining the figure showed that CC had the highest value in FD when MD and Chl were 21 g; however, this value in SD was equal to 16 and 20 g for MD and Chl, respectively. The 3D response surface plot in Fig. 3 (i and l) showed the value of CC in WPI and variable Chl and MD in the central point. The results showed that in FD and SD when WPI was equal to 16 and Chl 19 g, CC was highest.

To optimize the ratio of wall and core in microcapsules, all the variables in the investigated domain and the EE and CC were considered as the maximum. To ensure the optimal combination with the predicted ratios, a confirmation test was performed. The results obtained from the experimental data (Table 4) were contrasted with those anticipated by the model, confirming no significant difference between them. The desirability function of Design Expert was also used for the automatic identification of the best parameters in the preparation of chlorophyll microencapsulation (Fig S).

3.4. Solubility and moisture content of Chl-M

Solubility is the final stage of particle dissolution and an important attribute in the determination of the quality of food. The powders with poor solubility may bring about processing problems and economic disadvantages. The instant ability of powders is usually evaluated by their solubility in water, and usually, this index decreases with decreasing solubility. The ability of powders to reconstitute in water is significant for food development. The data obtained confirmed the good solubility of all the powders. Chlorophyll is insoluble in water at room temperature; however, the wall material improved its solubility by encapsulating the chlorophyll.

The solubility of SD powders (95.32 %) was higher than that of the FD powders (92.77 %), attributable to the difference in the morphology of the powders and the corresponding contact surface with water (Table 4). The main functional properties of MD consist of helping dispersion and solubility, controlling freezing, preventing crystallization, and creating spreadable properties in the product. Texture and viscosity improvement, emulsification ability, gelling properties, water binding capacity, and high dissolution rate in a wide range of pH are among the favorable features of WPI as coating materials in microencapsulation [27]. Thus, MD and WPI as microencapsulating agents are capable of interacting with chlorophyll, resulting in the proper dissolution of powders achieved from Chl-M in water.

Drying at high temperatures in a spray dryer boosted the solubility of microencapsulated samples in contrast with freeze drying at low temperatures. The lower the moisture content of the powder, the more soluble it is [28]. In addition, the high temperature of the drying air generally causes the formation of larger particles, reducing the time required for the powder to dissolve, because coarser particles are easier to dissolve in water than finer ones [29]. Large particles may settle, whereas small particles are more dusty and generally float on water, causing variable and incomplete wetting and reconstitution [30].

As an important characteristic of microcapsules, moisture content (MC) is associated with adhesion, microbial growth, drying efficiency, water activity, flowability, and oxidation of bioactive agents, determining product shelf life. In addition, microcapsule moisture affects storage stability given the change of wall material from a glassy state to an amorphous rubbery state at higher moisture levels, resulting in the degradation and release of the core material during storage [14]. Low humidity in microcapsules can prevent degradation by molds and moisture absorption reduce agglomeration of active dispersants, prevent microbial growth, and improve physical and chemical stability [31].

MC of Chl-M achieved by spray drying was lower than that of freeze-drying ($p < 0.05$), as shown in Table 4, The difference in water removal methods in both methods affects the water holding capacity and consequently the final MC of the powder [27]. The type of wall composition could also affect the MC of Chl-M. The optimal microcapsule in the spray-drying method had a lower ratio of chlorophyll and MD than that in the freezing method, and the amount of WPI was higher (Table 4). MD can form a gel, leading to improved moisture retention, justifying its use as a texture in the food industry. Increasing the concentration of MD decreased the water available for evaporation owing to the chemical structure of MD and probably the difference in the number of linking groups with water, resulting in a decrease in the MC of Chl-M. According to Goula & Adamopoulos (2008) [28], the amount of moisture

Table 4
Predicted and experimental values of the response at optimal conditions and investigation of solubility (s%), moisture content (MC%), and antioxidant activity (%) of optimal microcapsules produced by spray and freeze-drying methods (SDM & FDM).

	MD (g)	WPI (g)	Chl (g)	EE (%) Predicted	EE (%) Experimental	CC (μg/mL) Predicted	CC (μg/mL) Experimental	Desirability	MC (%)	S (%)	antioxidant activity (%)
FD microcapsules	17.27	11.21	22.05	90.98	90.46 ± 0.62	87.83	85.85 ± 0.43	0.96	3.7 ± 0.05[b]	92.77 ± 0.42[a]	79.1 ± 0.24[c]
SD microcapsules	16.02	16.57	20.40	90.39	90.27 ± 0.21	54.66	55.36 ± 0.36	0.95	2/27 ± 0.18[a]	95.32 ± 1.19[b]	67.5 ± 0.16[b]
Control[a]	–	–	–	–	–	–	–	–	–	–	20 ± 0.12[a]

Each value represents the mean of triplicate experiments ± standard deviation. Mean values in a row with different letters are significantly different ($p < 0.05$).
[a] Control: WPI & MD.

increases with the increase in the concentration of MD, because water molecules are difficult to diffuse from the larger molecules of MD. Premi & Sharma (2017) [32] and Kang et al. (2019) [14] reported the relationship between higher MD values in wall materials and reduced particle moisture content. Zhang et al. (2020) [6] stated that high WPI concentration led to a significant decrease in moisture content, likely due to the increased interaction between chlorophyll and wall materials (WPI and MD). Consequently, the easy evaporation of water molecules during the drying process occurred.

3.5. Antioxidant activity of Chl-M

DPPH radical scavenging activity was used to determine the antioxidant activity of WPI MD SDM and FDM. The type of wall material can be effective on the oxidative stability of the product. The simultaneous presence of carbohydrates and proteins in the wall structure improves the emulsifying features and oxidative stability of the product [27]. Kerasioti et al. (2014) [17] stated that partial denaturation of WPI during encapsulation produce more electron-donating groups such as methionine, cysteine, and sulfur-containing amino acids exposed. The control sample (WPI & MD) had insufficient antioxidant activity (about 20 %). Due to the presence of chlorophyll, Chl-M showed higher DPPH radical scavenging activity compared with the control sample. Various studies show that chlorophyll can scavenge oxidizing compounds such as DPPH (2,2-diphenyl-1-picrylhydrazyl) [33]. Therefore, it can be used as a pigment and natural antioxidant in various food products.

The drying method significantly affected the DPPH free radical scavenging activity of the powders obtained ($p < 0.05$). Drying medium in freeze dryers protected chlorophylls better than spray dryers during the drying process, increasing DPPH free radical scavenging activity in freeze-dried powders. The reason was probably the higher chlorophyll content in the microcapsules produced by the freeze-drying method (Table 1).

4. Conclusion

Response surface methodology was applied to determine the optimum processing conditions giving the maximum EE and CC from Chl-M. The optimization of EE and CC by RSM indicated that a combination of MD (17.27 g), WPI (11.21 g), and Chl (22.05 g) in freeze drying and MD (16.02 g), WPI (16.57 g), and Chl (20.40 g) in spray drying resulted in the maximum EE and CC. Under these conditions, the EE and CC were 90.46 and 85.85 in freeze and 90.27 and 55.36 in spray drying, respectively. The effect of overlapping protein and carbohydrate in increasing the EE and CC during drying to prepare capsules was evident. Encapsulation improved the solubility of chlorophyll. The samples produced by SD had higher solubility and lower moisture content. Radical scavenging tests of Chl-M confirmed that the FD had stronger radical scavenging activities in contrast with the control sample. Therefore, WPI and MD are favorable wall materials to protect chlorophyll molecules during the drying process, and Chl-M resulting from freeze drying can be used as a natural food additive thanks to its high CC, EE, and antioxidant activity.

CRediT authorship contribution statement

Shahrbanoo Ahmadi Ledari: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Jafar M. Milani:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Seyed-Ahmad Shahidi:** Resources, Project administration, Conceptualization. **Abdolkhalegh Golkar:** Resources, Project administration, Conceptualization.

Data and code availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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