OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF CHLOROPHYLL USING DESIGN OF EXPERIMENTS AND STABILITY IMPROVEMENT VIA ENCAPSULATION

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The study shows the possibility of improving the extraction efficiencies of chlorophylls a and b from fresh spinach leaves by using the Design of Experiments (DOE) approach. The chlorophyll extracts were obtained by ultrasound assisted extraction (UAE). Optimal parameters for maximizing the concentration of extracted chlorophyll were established. The storage stability of the natural compound was improved by encapsulation in silica matrix. The stability of the chlorophyll loaded silica particles were characterized by UV-VIS spectroscopy analyses over a 24-day period. The encapsulation method led to an improved color stability in time for all samples.

Keywords: Design of Experiments, Box-Behnken, ultrasound-assisted extraction, chlorophyll, encapsulation, silica matrix, screen-printing

1. Introduction

The industry of textile manufacturing, dyeing included, negatively impacts the environment by employing high quantities of water in the process and considerable amounts of energy. These are some of the reasons why the scientific community has begun studying natural dyes and pigments as substitutes for the synthetic options. The interest points are both the extraction and the stabilization of the natural choices.

The discussions regarding the replacement of the currently used dyes are focused on their toxicity – especially during production. The current tendency to use natural products also has an important part here. However, there are noteworthy differences regarding the dyeing process involving natural colorants.

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These variances occur at nearly every lot and the adjustment of formulation or hue is more difficult than for synthetic dyes.

In later years, important research was conducted regarding the standardization of both extraction and application of natural dyes.

Chlorophyll is wide-spread in nature and is being successfully utilized as food dye. Using it in the leather (dyeing process) and beauty (cosmetic products and hair dyes) industries is the natural succeeding step [1]. Chlorophylls are tetrapyrrole derivatives with a coplanar system of conjugated double bonds which form an aromatic structure with delocalized electron density at the orbitals level. Typically found in higher plants are chlorophyll-a and chlorophyll-b. Their proportions depend on the maturity and species of the plant and also on the exposure to light or stress [2, 3]. One drawback is its easily degradable nature which prevents the use of chlorophyll with high efficiency.

Numerous extraction methods have been reported regarding conventional and unconventional procedures to separate chlorophyll. These can be intensified when using heating and/or stirring during extraction. The conventional methods [4-7] utilize a high amount of solvent and require extensive time periods. To overcome these disadvantages, the unconventional extraction methods (super-critical fluids [8], enzyme-assisted [9] , ultrasound-assisted [10, 11] , microwave-assisted [12] , ultrasound and microwave-assisted [13] , solvent free [14] extractions) were introduced. However, to achieve the optimal parameters that maximize the extraction yield of the interest compound numerous trials have to be executed. A valuable tool which optimizes the process can be Design of Experiments. It is a statistical method that maximizes the existing resources and increases the overall efficiency [15].

The aim of this study was to develop an empirical model which focuses on the optimization of chlorophyll extraction from fresh spinach leaves using UAE and ethanol 96% as extractive solvent. The DOE approach was carried out by employing the Box-Behnken response surface methodology (RSM) and its application with selected input variables to the determination of the optimal conditions to maximize the concentration of chlorophyll extraction. Furthermore, the study aims to determine if the encapsulation in silica can minimize the

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degradation effects during storage and whether it allows the preservation of specific characteristics of the analyzed dye.

2. Materials and methods

2.1. Materials

Fresh spinach was purchased from a local store. Technical grade ethanol (96 % vol.) was acquired from the Chemical Company SA (Iasi, Romania) and used as extractive solvent. Tetraethyl orthosilicate (TEOS) (Aldrich), 3-aminopropyltriethoxysilane (APTES) (Aldrich), cetyltrimethylammonium bromide (CTAB) (Aldrich), and sodium hydroxide (NaOH) (Fluka) were of high purity grade. Furthermore, Hydra Clear 77 (a transparent, flexible textile ink base designed for pigmentation) and a white fabric (20 % polyester and 80 % cotton) with a specific weight of 195 g/m^2 were used.

2.2. Extraction of chlorophyll

The extraction was performed using a UP200H Hielscher ultrasonic processor (200 W, 24 kHz). The sonotrode, mounted on a stand, was lowered into a cylindrical jacketed reactor of 100 mL, at a distance of 3 cm from the bottom of the vessel. A heating plate equipped with a magnetic stirring unit was used. The temperature of the extraction mixture was maintained constant by circulating cold water through the reactor jacket.

The optimization of the method was carried out using the DOE approach in order to decrease the volume of used solvent (96 % ethanol) and, consequently, the number of experiments. The Box-Behnken RSM was employed. The selected input variables are of two types: continuous on interval (solid/solvent ratio – from 0.1 to 0.5 – and stirring rate – from 500 to 1500 rpm) and discrete (ultrasound (US) power – 40, 60, 80, 100, 120 W). These variables were used for the optimal conditions determination in order to maximize the concentration of the extracted chlorophyll. The JMP software (Cary, N.C., USA) was used to design the experiment. The parameters used for the 15 initial experimental trials are presented in Table 1.

Table 1

Selected input variables

No. Crt.	Solid-liquid ratio, x_1	US power, W, x_2	Stirring speed, rpm, x_3
1	0.1	40	500
2	0.1	60	1000
3	0.1	100	1500
4	0.1	120	500
5	0.236	40	1500
6	0.3	60	1000
7	0.3	80	500

8	0.3	100	1000
9	0.3	100	1000
10	0.378	120	1500
11	0.402	60	500
12	0.5	40	790
13	0.5	60	1500
14	0.5	100	1000
15	0.5	120	500

The extracts were spectrophotometrically analyzed in triplicate and the concentrations of chlorophyll-a and chlorophyll-b were calculated using molar extinction coefficients, according to eq. 1 and 2.

$$Chl_a = 13.36 * A_{664} - 5.19 * A_{649} \quad (1)$$

$$Chl_b = 27.43 * A_{649} - 8.12 * A_{664} \quad (2)$$

where A_{649} is the absorbance value at 649 nm and A_{664} is the absorbance value at 664 nm.

The answers evaluated in this experimental set were the concentrations of chlorophyll-a and chlorophyll-b. The levels of the evaluated factors were determined.

2.3. Stabilization of chlorophyll

The extract stabilization is an important step (see Fig. 1), due to the light and heat sensitive nature of chlorophyll. The encapsulation strategy involved two steps. The used equipment for both phases comprises a round-bottomed flask with two necks and joint sleeves, thermocouple, and a heating plate with a magnetic stirrer unit (model ARE, producer VELP SCIENTIFICA).

The first step is the silanization of the chlorophyll extract. The latter was concentrated using a rotary evaporator until a volume of 7 mL (approximatively 25% of the initial amount) remained. Next, 0.15 mL of APTES were added to the concentrated chlorophyll extract. The blend was heated, and the temperature was maintained at 70 °C for 1-2 h, ensuring a mixing speed of 800 rot/min. During this time, the carbonyl groups of chlorophyll reacted with the amino group of APTES, leading to the silanization of the pigment. A drop of HCl was also added to control the pH. This was followed by a hydrolysis step, performed in the presence of TEOS and CTAB – as stabilizing agent – for the generation of silica. Three experiments were performed, in duplicate. In each installation 90 mL water, 0.25 g CTAB, and 0.7 mL NaOH solution 2M were added. After 15 min at 80 °C, different volumes of silanized chlorophyll were added (0.5, 1, and 2 mL) and after 5 more minutes, 0.9 mL of TEOS were poured in each flask. The mixing speed was constant and maintained at 800 rot/min.

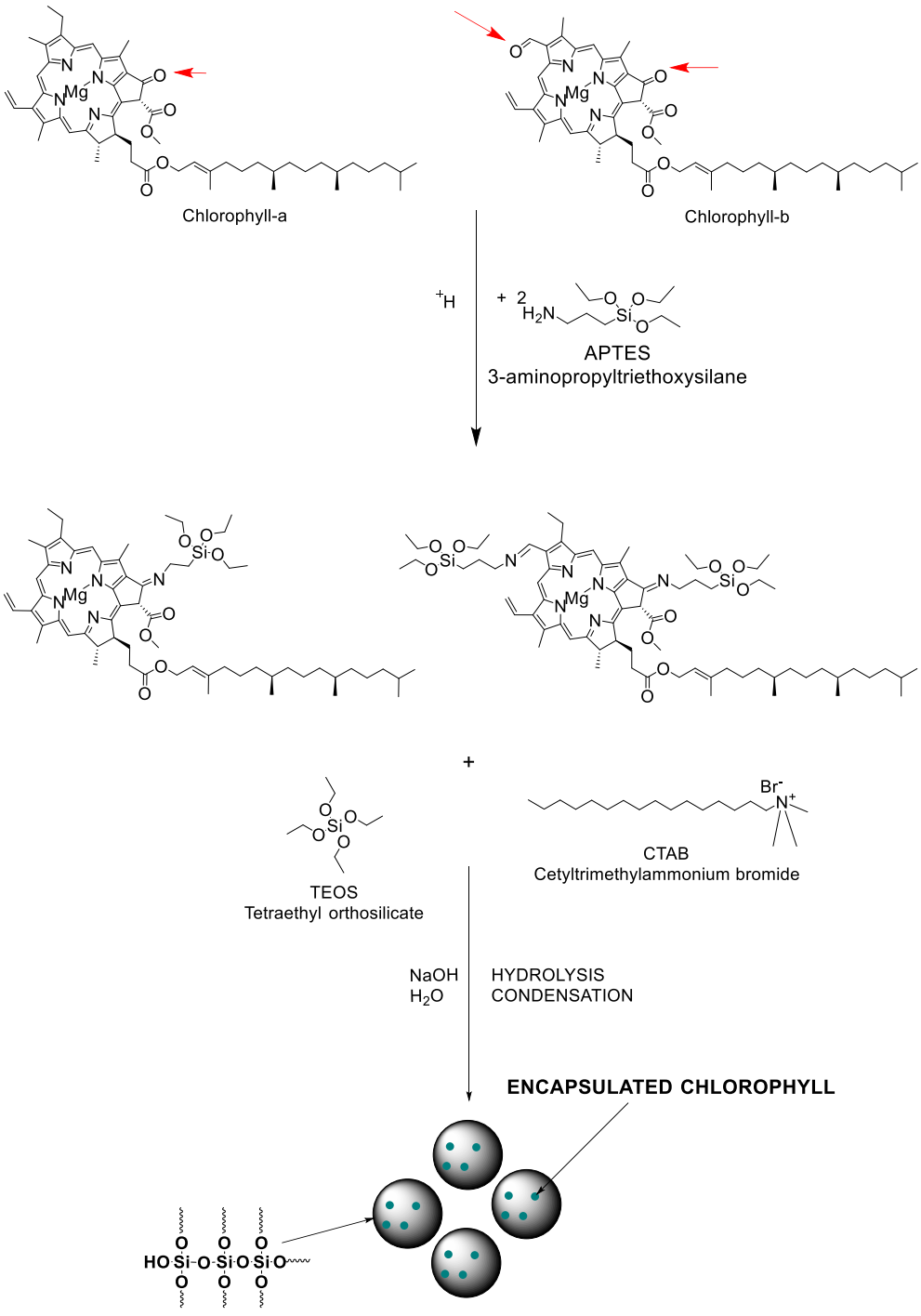

Fig. 1. The silanization of chlorophyll

The operating parameters were maintained for 8 h, before separating the silica capsules by centrifugation (10 min at 4000 rot/min) and washing them with water. They were further dried. The stability in time of the encapsulated chlorophyll was studied over the next 24 days.

2.4. Color stability in time

The color resistance was observed over a period of 24 days. The samples were kept in the dark and only removed when performing the absorbance spectra, aided by the Jasco V-550 spectrophotometer. The analyses were carried out directly on the capsules, in powder form. The results were compared with unstabilized chlorophyll extracts added as pigment in a dye formulation. From this point forward, these will be named standards. The concentration of the standards was equal to the one found in the capsules.

The method of preparing the standards involves mixing the extract with the polyurethanic base, Hydra Clear 77, and water – to reduce viscosity. To imprint the textile material, a mesh with a wooden frame, attached to a special screen-printing flatbed table, was utilized. The stencil containing 61-64 holes/cm^2 allows the ink to pass through when pressed with a silicone squeegee. To properly clean the mesh, an industrial solvent is used after each use to prevent clogging the stencil holes. The imprinted fabric was placed, together with the encapsuled chlorophyll, in the dark at room temperature. The absorbance spectra were measured after 12 and 24 days.

The stability in time of chlorophyll-a and chlorophyll-b was calculated as percentage from the initial concentration values according to eq. 3.

$$St_{Chl-a/b} = \frac{Chl-a/b_0 - Chl-a/b_n}{Chl-a/b_n} * 100 \quad (3)$$

where $St_{Chl-a/b}$ is the stability to storage of chlorophyll after encapsulation, Chl-a/b_0 is the concentration of chlorophyll-a/chlorophyll-b corresponding to day 0 and Chl-a/b_n is the concentration of chlorophyll-a/chlorophyll-b corresponding to each 12-day increment, up until 24 days.

3. Results and discussion

3.1. Extraction of chlorophyll

The experimental runs from Table 1 were performed and analyzed in triplicate. The obtained chlorophyll-a and chlorophyll-b concentrations are presented in Table 2. The maximum concentration for chlorophyll-a (0.149 mg/mL) was obtained for a solid-liquid ratio (x_1) of 0.3, a US power (x_2) of 120 W, and a stirring rate (x_3) of 1000 rpm.

Table 2

Initial chlorophyll concentration

No. Crt.	Chlorophyll-a concentration, mg/mL	Chlorophyll-b concentration, mg/mL
1	0.062	0.025
2	0.085	0.038
3	0.073	0.025
4	0.099	0.038
5	0.086	0.035
6	0.094	0.047
7	0.079	0.042
8	0.125	0.053
9	0.149	0.066
10	0.098	0.088
11	0.032	0.025
12	0.04	0.047
13	0.041	0.061
14	0.043	0.060
15	0.027	0.028

The linear model was generated (see eq. 4). It describes the empiric relation between the three parameters and the desired responses (in this case, the maximum chlorophyll concentration).

$$Conc_{chlorophyll-a} = 0.114 + (-0.024) * \frac{x_1 - 0.3}{0.2} + 0.016 * \frac{x_2 - 80}{40} + \frac{x_2 - 80}{40} * \frac{x_2 - 80}{40} * 0.013 + 0.007 * \frac{x_3 - 1000}{500} + \frac{x_1 - 0{,}3}{0.2} * \frac{x_1 - 0.3}{0.2} * (-0.043) + \frac{x_1 - 0.3}{0.2} * \frac{x_2 - 80}{40} * (-0.006) + \frac{x_1 - 0.3}{0.2} * \frac{x_3 - 1000}{500} * 0.012 + \frac{x_2 - 80}{40} * \frac{x_3 - 1000}{500} * (-0.003) + \frac{x_3 - 1000}{500} * \frac{x_3 - 1000}{500} * (-0.031) \quad (4)$$

For chlorophyll-b, the maximum concentration (0.088 mg/mL) resulted for x_1 0.378, x_2 120 W, and x_3 1500 rpm. Similarly, the linear model was computed and represented in eq. 5.

The screening of the model was performed by analysis of the variance (ANOVA). The quality of the fitted model was expressed via the coefficient of determination, R^2. According to Fig. 2, the coefficients (0.93 for chlorophyll-a and 0.94 for chlorophyll-b) demonstrate a good compatibility between the predicted and real data.

$$Conc_{chlorophyll-b} = 0.055 + 0.010 * \frac{x_1 - 0.3}{0.2} + 0.01 * \frac{x_2 - 80}{40} + \frac{x_2 - 80}{40} * \frac{x_2 - 80}{40} * 0.007 + 0.099 * \frac{x_3 - 1000}{500} + \frac{x_1 - 0.3}{0.2} * \frac{x_1 - 0.3}{0.2} * (-0.011) + \frac{x_1 - 0.3}{0.2} * \frac{x_2 - 80}{40} * (-0.001) + \frac{x_1 - 0.3}{0.2} * \frac{x_3 - 1000}{500} * 0.014 + \frac{x_2 - 80}{40} * \frac{x_3 - 1000}{500} * 0.006 + \frac{x_3 - 1000}{500} * \frac{x_3 - 1000}{500} * (-0.012) \quad (5)$$

As a general rule, for a good fit, the experimental results (represented by black dots) should be close to the regression line (characterized by the red line). Also, the experimental results must be placed inside the trust interval – region with reddish background.

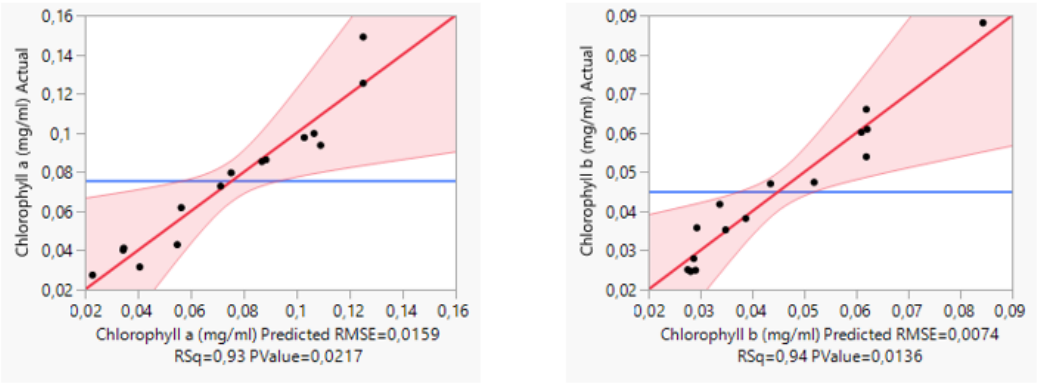

Fig. 2. Chlorophyll-a and chlorophyll-b concentrations predicted by the empirical model as compared with the experimentally obtained results

The prediction profiler provides the optimal parameters, with a desirability of 0.96 (see Fig. 3). The optimal parameters are x_1 0.23, x_2 120 W, and x_3 1000 rpm. By respecting these parameters, the maximum concentration of chlorophyll-a (0.148 mg/mL) and chlorophyll-b (0.067mg/mL) can be reached.

The extractions performed employing the optimal parameters expressed in the prediction profiler led to the expected concentration only for chlorophyll-a. The obtained value fits the reference interval, with a concentration of 0.119 mg/mL. The influence of the three parameters on the concentration of chlorophyll-a was represented as surface plots (see Fig. 4).

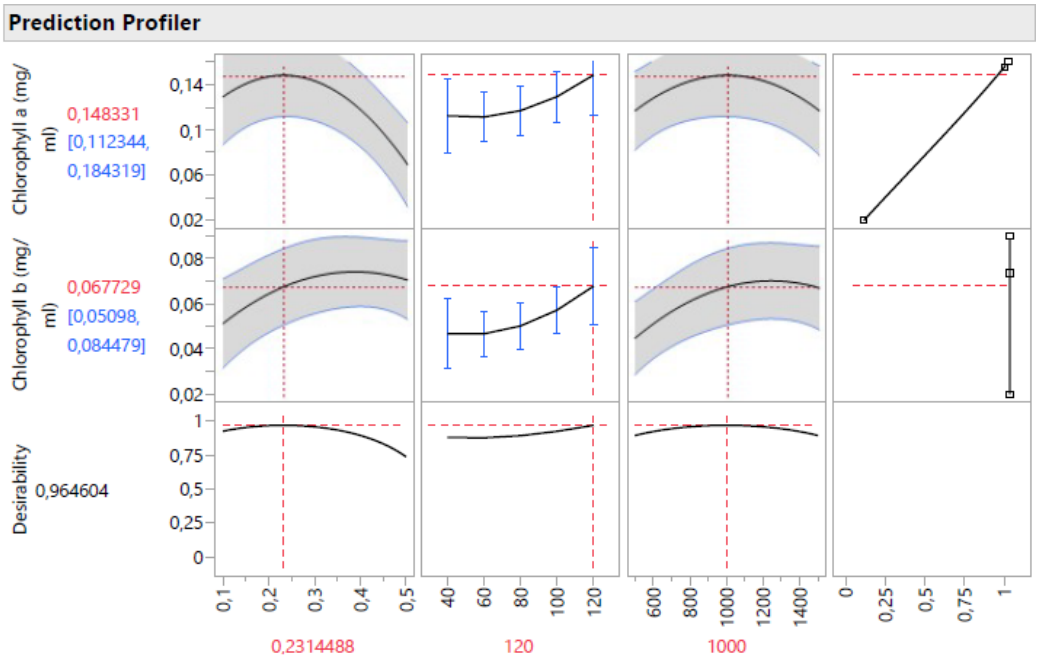

Fig. 3. Optimal parameters of chlorophyll extraction by means of UAE

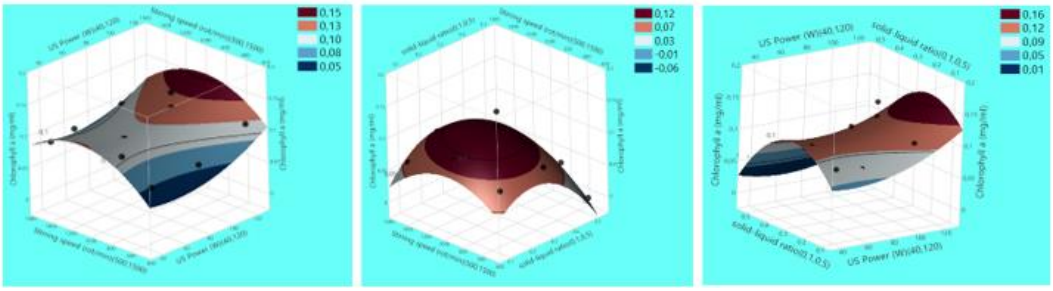

Fig. 4. Parameter influence on the chlorophyll-a concentration

The interaction profiles are shown in Fig. 5. These interactions represent the combined effects of two of the three parameters on the chlorophyll-a concentration. For instance, the resulted content of the interest compound at 500 rpm reaches its maximum value for x_1 equal to 0.2. From this point on, up until x_1=0.5, the concentration value decreases; the minimum content results whenthe maximum amount of raw material is used. For 1500 rpm, there is an insignificant variation of the chlorophyll-a concentration as compared with 500 rpm. The behavior is similar: after a maximum – obtained for x_1 equal to 0.3 – its concentration decreases.

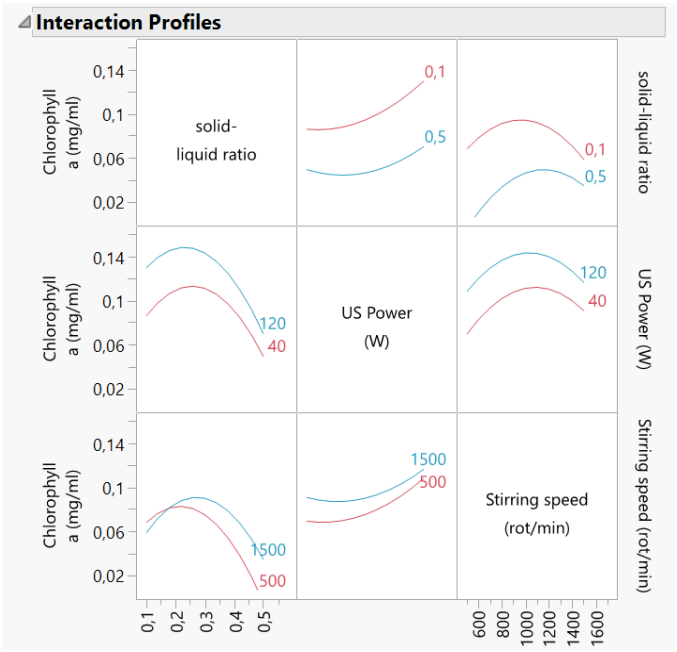

Fig. 5. Interaction profiles for chlorophyll-a

For chlorophyll-b, the extractions performed using the optimal parameters expressed in the prediction profiler from Fig. 4 led to a smaller concentration than the lower end of the interval, of only 0.49613 mg/mL. This result is with 2.68 % below the minimum predicted value of 0.051 mg/mL chlorophyll-b. The influence of the parameters was also studied and expressed as surface plots (Fig. 6).

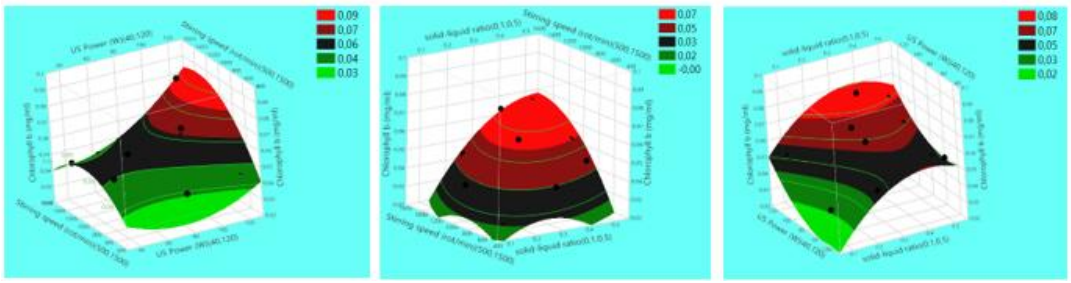

Fig. 6. Parameter influence on the chlorophyll-b concentration

The interaction profiles were computed and are shown in Fig. 7. Considering only x_1 and x_3, it can be noticed that for 500 rpm, the concentration of chlorophyll-b is approximatively 0.03 mg/mL. This value also corresponds to x_1 between 0.1 and 0.5. By increasing x_3 to 1500 rpm, the concentration of the interest compound also increases. In this manner, it reaches 0.08 mg/mL for x_1 equal to 0.5.

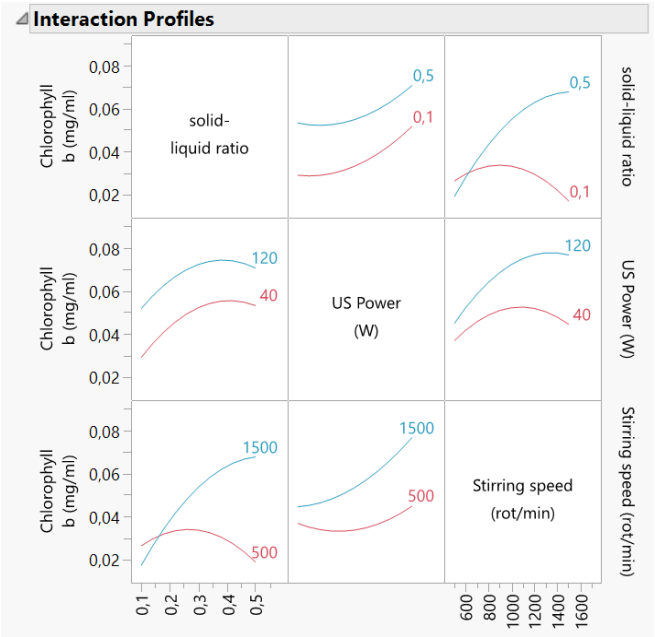

Fig. 7. Interaction profiles for chlorophyll-b

The manner in which chlorophyll-a and chlorophyll-b concentrations behave is different. Meaning that, according to the interaction profiles (Fig. 6 and Fig. 8), it is difficult to maximize the extraction of both natural dyes. For example, from the highest values of x_1 and x_3 resulted the maximum chlorophyll-b content; the same parameters led to an almost minimum amount of chlorophyll-a. Furthermore, when discussing x_1 and x_2, when the highest concentration of chlorophyll-b is reached, the lowest is obtained for chlorophyll-a.

This might be attributed to the fact that the empirical model was designed in such way that it maximizes both natural dyes concentration. In future studies, it might be of interest to focus only on one of the two. In this way, an efficient process with almost complete extraction could ensue. The choice can be made taking into account the further utilization of the extract. For example, the color might be an important aspect: chlorophyll-a for blue-green and chlorophyll-b for yellow-green. Another vital characteristic can be the chemical structure: chlorophyll-b contains a methyl group in the third position of the chlorin ring, while chlorophyll-a has an aldehyde group in the same location.

3.2. Color stability of the encapsulated chlorophyll

The samples (three for the encapsulated chlorophyll and three for screen-printed unstabilized pigment) were kept in the dark and only removed for absorbance measurements. The interest wavelengths are 649 and 664 nm. Samples A1 and A2 ($8.19 \cdot 10^{-5}$ g of chlorophyll/sample), B1 and B2 ($1.64 \cdot 10^{-4}$ g of

chlorophyll/sample), and C1 and C2 ($3.28 \cdot 10^{-4}$ g of chlorophyll/sample) are of equal chlorophyll concentrations.

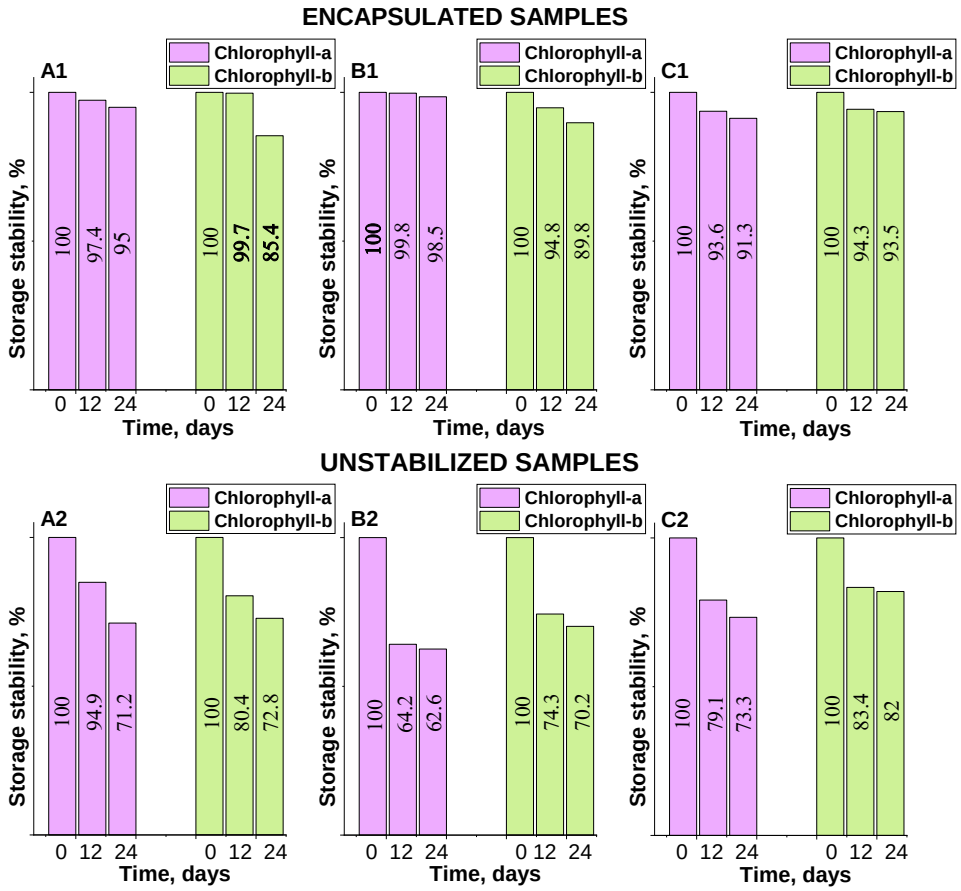

Fig. 8. Chlorophyll stability in time

According to Fig. 8, over the 24 days, both interest compounds degrade. Although at different rates, the degradation takes place for the encapsulated and unstabilized samples. Considering figures 8-A1 and 8-A2, the color resistance in time (day 24) of chlorophyll-a improves with 33.39 % when encapsulated. For chlorophyll-b, an increased stability with 17.28 % is noticed. In figures 8-B1 and 8-B2, better results are obtained: a 57.44 % and 27.97 % improvement for chlorophyll-a and chlorophyll-b time stability, respectively. In figures 8-C1 and 8-C2, the interest compounds exhibit enhanced color resistance over time when encapsulated. For chlorophyll-a, an increased stability of 24.57 % is noticed, while for chlorophyll-b a percentage of 14.06 % is achieved.

According to some studies [16], chlorophyll-a degrades more easily during storage, due to the presence of free radicals and humidity in the environment. Östbring et al. [17] explain that it degrades up to 3 times faster than chlorophyll-b.

This clarifies the behavior of the unstabilized samples. However, the validity of the studies cannot be confirmed when discussing the capsules. During the encapsulation process, the interest compounds are submitted to a series of changes which ultimately affect the degradation pathway. In this way, chlorophyll-a is better bonded inside the silica matrix due to having only one possible reaction site with APTES. The chemical structure of chlorophyll-b contains two such possible sites. Because of this, the silanzation may occur at only one carbonyl group, leaving the interest compound prone to degradation. Moreover, at higher chlorophyll concentration, the encapsulation yield might be lower, allowing the unstabilized pigment to degrade according to literature [16, 17], up until only capsules remain in the system.

4. Conclusions

Chlorophyll was extracted from fresh spinach leaves using an ultrasonic processor. The method was performed by means of the DOE approach. The Box-Behnken RSM was used and the selected input variables were either continuous on interval or discrete. The optimal parameters for the maximization of both chlorophyll-a and chlorophyll-b concentrations were solid-solvent ratio 0.23, ultrasound power 120 W, and stirring rate 1000 rpm; the desirability was 0.96. Next, silica particles containing chlorophyll were synthetized. The strategy involved the silanization of a chlorophyll extract by reaction with APTES, followed by a condensation step with TEOS. The color stability in time was studied, comparing the behavior of the capsules with standards. Although at different rates, the degradation took place for the encapsulated and unstabilized samples. An improvement of at least 24.57 % and 14.06 % was recorded for chlorophyll-a and chlorophyll-b, respectively.

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