Italian Journal of Food Science, 2025; 37 (4): 244–254

Metallo-chlorophyll derivatives as food colorant: Intact chloroplasts from spinach leaf recovered by enzyme-assisted extraction

Caterina Mazzocchi, Claudio Lombardelli, Ilaria Benucci*, Marco Esti

Department of Agriculture and Forest Sciences (DAFNE), Tuscia University, Viterbo, Italy

***Corresponding author:** Department of Agriculture and Forest Sciences (DAFNE), Tuscia University, Via S. Camillo de Lellis snc, 01100 Viterbo, Italy. E-mail: ilaria.be@unitus.it

Academic Editor: Prof. Carla Di Mattia, University of Teramo, Italy

Received: 9 February 2025; Accepted: 16 April 2025; Published: 1 October 2025

ORIGINAL ARTICLE

Abstract

This study presents tailored enzyme-assisted extraction (EAE) to recover intact chloroplasts from spinach, using a synergistic combination of metal ions, in comparison to traditional chlorophyll (Chl) solvent extraction. The content of pigment in intact chloroplasts recovered by EAE is affected by time and metal dose; the highest concentration is achieved using 165 ppm of metals for 2 h. With regard to solvent extraction, the amount of intact chloroplasts is remarkably lower and affected only by process time. Colorimetric analysis confirms a good correlation between the concentration of metal ions and greenness. Applied to meringues, the Chl-based extract (<3% w/w) enhances color without affecting flavor, fragrance, and mouthfeel.

Keywords: Food pigment; green colorant; copper; zinc; meringues

Introduction

Chlorophyll (Chl), the vibrant green pigment found in plants and algae, plays a crucial role in photosynthesis and has garnered interest for its potential applications as a food colorant and for its health benefits (Hsiao *et al.*, 2020). The commercial Chl-based colorants are usually obtained by organic solvent extraction (Viera *et al.*, 2019), as the pigment is not soluble in water. Organic solvents (e.g., acetone, ethanol, or hexane) efficiently dissolve Chl because of their compatibility with its chemical structure, allowing for effective extraction. This issue of solubility is the main reason why Chl cannot be directly extracted with water or easily incorporated into water-based food products (Ebrahimi *et al.*, 2023).

Chl stability is often compromised by heat, oxygen, light, and acidic conditions, leading to color changes and degradation (Indrasti *et al.*, 2018), because of its structural characteristics. When Chl loses its magnesium ion (Mg^{2+}), and undergoes a pheophytinization reaction, it is transformed into pheophytin, a derivative that appears olive-green (Amin *et al.*, 2021). This transformation is heavily influenced by environmental factors such as pH and temperature (Lombardelli *et al.*, 2024).

In nature, Chl is stored inside chloroplasts, organelles found in plant, that protect and stabilize the pigment (Indrasti *et al.*, 2018; Staehelin, 2003). Indeed, Chl is bound to proteins in a protein–Chl complex, surrounded by a lipidic bilayer, which helps shield it from harmful external factors. This structure allows Chl to perform its function without undergoing rapid degradation (Miazek & Ledakowicz, 2013). When plant cell structures are damaged, the release of Chl leads to an increased susceptibility to degradation (Indrasti *et al.*, 2018).

ISSN 1120-1770 online, DOI 10.15586/ijfs.v37i4.3028

Given the challenges posed by Chl sensitivity, several strategies have been employed to enhance its stability during the extraction phase (Hu *et al.*, 2022): (i) inhibition of enzyme activity—high-temperature blanching can effectively inhibit the activity of Chl-degrading enzymes, although this process may also lead to nutrient loss and accelerate the demagnetization of Chl (Managa *et al.*, 2020); (ii) alkalization—alkaline conditions favor the preservation of Chl, although this effect may diminish over time (Kwartiningsih *et al.*, 2021); (iii) ion replacement—substitution of the Mg^{2+} ion with more stable bivalent cations, such as zinc (Zn^{2+}) or copper (Cu^{2+}), leading to metal–Chl complexes (Amin *et al.*, 2021).

These derivatives retain the green color while being more resistant to heat and acidic conditions (Amin *et al.*, 2021). Notably, Cu^{2+} not only stabilizes Chl but also exhibits protective effects against degradation. Research has shown that the addition of Cu^{2+} may enhance the retention of Chl pigments during processing, helping to maintain color and nutritional quality (Indrasti *et al.*, 2018). Research by Zheng *et al.* (2014) demonstrated that adding Cu^{2+} to grape puree significantly improved Chl retention compared to Mg^{2+}, Zn^{2+}, and K^{+}. Moreover, considering that the tolerable upper intake limit for Zn^{2+} in adults is 40 mg/day, its addition in the Chl extract may represent a safe option besides enhancing Chl stability (Chasapis *et al.*, 2020). Treatments with Zn^{2+} salts have been shown to form stable green Chl derivatives in various vegetables. Senklang & Anprung (2010) reported the formation of Zn-pheophytin in pandan leaves, enhancing their color and antioxidant properties. Mazzocchi *et al.* (2023) and Lombardelli *et al.* (2024) proved that the protective effect of $ZnCl_2$ (150 ppm) was more evident toward Chl-a rather than Chl-b, already during the enzyme-assisted extraction (EAE) process from spinach. Both in the stabilization of pigments and in their use as dietary supplements, Zn^{2+} and Cu^{2+} play a crucial role (Artar *et al.*, 2024).

These trace elements, when combined, enhance the stability and vibrancy of pigments, as well as support essential physiological functions if taken as dietary supplements, ensuring a balanced intake for overall health. Indeed, daily intakes of Cu (0.07 mg/kg of body weight; EFSA, 2014) and Zn (0.18 mg/ kg of body weight; EFSA, 2023) are required to maintain a steady state because the body has no specialized storage system for these two microelements. Several studies have demonstrated that the ideal Zn:Cu ratio is between 8:1 to 15:1 (Osredkar & Sustar, 2011) and 4:1 to 12:1 (Watts, 2010). This balance is crucial for various physiological functions, including immune response, oxidative stress management, and enzyme function. Maintaining an optimal ratio ensures that both minerals can support their respective biological roles effectively, preventing deficiencies and potential health issues related to imbalances (Escobedo-Monge *et al.*, 2023; Matuszczak *et al.*, 2024).

Currently, despite extensive experiments focused on the extraction and quantification of Chl from vegetable sources, no single method that combines broad applicability, ease of reproduction, and high sensitivity has been identified. Bearing in mind several factors during Chl extraction and their interaction, the identification of a single procedure capable of efficiently recovering the pigment, also preserving its greenness, is quite complex (Berhe *et al.*, 2024).

In light of these challenges, the enzymatic recovery of intact chloroplasts and their use as natural food colorants have not been thoroughly explored. Thus, we propose a novel, mild, and tailored approach for the extraction of intact chloroplasts from spinach leaves by EAE in the presence of metal ions (Zn^{2} and Cu^{2+}) in a synergistic combination. By keeping Chl within its natural cellular environment, this method may protect the pigment from oxidation and degradation. In addition, this extraction process explores the synergistic effects of Cu^{2+} and Zn^{2+} to further enhance the stability of Chl, providing a food green colorant to be applied for staining a baked good usually white, such as the meringue.

Materials and Methods

Raw material, enzymes, and chemicals

Spinach (*Spinacia oleracea*) was provided by Unicoop Tirreno S.C. (Viterbo, Lazio, Italy) and kept at 4°C until use. Enzyme preparations were as follows: cellulase (0.8 U/mg) and polygalacturonase (≥0.3 U/mg) from *Aspergillus niger* and xylanase (≥2500 units/mg) from *Aspergillus oryzae*, all obtained from Merck (Milan, Italy). In addition, all reagents, including acetone for solvent extraction and chloroplast isolation kit were purchased from Merck (Milan, Italy).

Extraction protocols

Enzyme-Assisted Extraction (EAE). The EAE was performed following the method developed by Lombardelli *et al.* (2024). Briefly, 25 g of spinach was blanched at 100°C for 5 sec, blended and added to 440 mL of McIlvaine buffer (0.1 M, pH 5) containing the tailored enzyme mix (total dose 0.1 U/g consisting of: cellulase 40% w/w, xylanase 41% w/w, and pectinase 19% w/w). The incubation time (1–3 h) (Lombardelli *et al.*, 2024; Mazzocchi *et al.*, 2023) and the total dose of metal ions (165 ppm) (Cu^{2+}: Zn^{2+}, 1:10) were varied according to the experimental plan drawn up by the design of experiment

(DOE, Table 1). The temperature was set at 25°C. At the end of the incubation, the extract was centrifuged (4500 rpm, 10 min at 4°C, Heraeus Megafuge 16R Centrifuge, Thermo Scientific, Milan, Italy) and the pellet was subjected to analysis using a chloroplast isolation kit.

Solvent Extraction (SE). The SE was performed following the method developed by Ašimović *et al.* (2016) using 80% (v/v) acetone as a solvent. The extraction was performed maintaining the same solid–liquid ratio and the same operating conditions applied for EAE (Table 1). After centrifugation, the pellet was used for chloroplast isolation kit.

Optimization of the extraction process conditions

In order to determine (i) the effect of extraction time and the total dose of metal ions on the Chl-containing chloroplast and (ii) the best conditions to maximize the recovery of intact chloroplast, a DOE based on a central composite design with the quadratic model was applied. Considering that the yield of isolated chloroplasts is expressed on a unit Chl basis (mg of Chl/mL), the measured dependent variable (y) was the concentration of Chl (mg/mL). The variance for each evaluated factor was divided into linear, quadratic, and interactive components, and represented using the second-order polynomial function as follows:

$$y = b_0+b_1x_1+b_2x_2+b_{11}x_1^2+b_{22}x_2^2+b_{12}x_1x_2 \quad (1)$$

The coefficients of the polynomial were represented by b_0 (constant term), b_1 and b_2 (linear coefficient), b_{11} and b_{22} (quadratic coefficient), and b_{12} (interactive coefficient). The significance of all the terms in the polynomial function was statistically assessed using the F-value at a probability (p) of 0.001, 0.01, or 0.05. The regression coefficients were then used to generate contour plots. The experimental design, statistical analysis, and response surface methodology (RSM) were performed using Minitab 17.1 (Minitab Inc., Pennsylvania, USA). RSM was used to verify the presence of the combined effect between the total dose of metal ions and extraction time (independent variables). These variables were coded as X1 and X2, respectively, and each had three levels: –1, 0, and +1. A total of 18 combinations, which included three replicates of the center point, were carried out in random order according to the central composite design. The level of variables for (x1 and x2) and (X1 and X2) are shown in Table 1.

Table 1. Design of experiment (DOE) for the determination of optimal process parameters (total metal ions, ppm and extraction time, h) on the amount of chlorophyll into intact chloroplast.

Treatment trial	Total metal ions dose (ppm) X1 (×1)	Extraction time (h) X2 (×2)
1	0 (–1)	1 (–1)
2	165 (+1)	1 (–1)
3	165 (+1)	3 (+1)
4	82.5 (0)	2 (0)
5	82.5 (0)	2 (0)
6	165 (+1)	1 (–1)
7	0 (–1)	1 (–1)
8	0 (–1)	3 (+1)
9	165 (+1)	3 (+1)
10	0 (–1)	3 (+1)
11	82.5 (0)	2 (0)
12	82.5 (0)	1 (–1)
13	82.5 (0)	2 (0)
14	165 (+1)	2 (0)
15	82.5 (0)	2 (0)
16	0 (–1)	2 (0)
17	82.5 (0)	3 (+1)
18	82.5 (0)	2 (0)

Isolation of chloroplast

The isolation of intact chloroplast was performed using the specific kit supplied by Merck (Milan, Italy), following the procedure reported in the technical data sheet.

Briefly, the extract is gradually passed through a filter mesh into 50 mL tubes. The filtrate is then divided into four 50 mL tubes, ensuring that the volume in each tube does not exceed two-thirds of its total capacity. To remove unwanted whole cells and cell wall debris, each tube is centrifuged for 3 min at 200 rpm, resulting in the precipitation of a white pellet at the bottom of the tube. For the isolation of chloroplast, the supernatant is carefully transferred into a fresh, pre-chilled 50 mL tube and centrifuged for 7 min at 1,000 rpm. This step sediments the chloroplasts, which form a distinct green pellet. The supernatant is discarded, and the pellet is resuspended in 2 mL of 1× chloroplast isolation buffer solution containing bovine serum albumin by gently pipetting up and down, ensuring that foaming is avoided during the process. The resuspended pellet is pooled into a single tube for subsequent steps. To purify intact chloroplasts, the separation of intact from broken chloroplasts is performed by centrifugation, applying a 40% Percoll® layer to achieve effective segregation.

At the end of the procedure, a suspension containing only intact chloroplasts was obtained. This suspension was used to determine the yield of chloroplast, expressed on a unit Chl basis (mg of Chl/mL). This requires the extraction of the Chl from the chloroplast suspension with an organic solvent: 10 µL of the chloroplast

suspension was added to 1 mL of an 80% acetone solution and mixed. Then, the sample was centrifuged for 2 min at 3000 rpm, retaining the supernatant and reading the absorbance at 652 nm (A_{652}) (UV-visible, Shimadzu UV 2450, Milan, Italy), using 80% acetone solution as reference blank. To obtain the mg of Chl per milliliter of chloroplast suspension, the following equation was applied:

$$\frac{mg_{chlorphyll}}{mL} = \frac{A_{652} \times 100}{36} \quad (2)$$

As reported in literature (Gedi *et al.*, 2017; Henriques, 2001), the Chl concentration serves as an indirect indicator of the amount of intact chloroplasts.

Meringue preparation

The optimal freeze-dried extract, characterized by the highest Chl content and the most suitable colorimetric parameters, was used in the preparation of a real food. Meringue, a sweet baked good typically white, has been selected in order to better appreciate the coloring impact of the recovered green pigment.

Meringues were prepared by mixing three egg whites (100 mL) with 220 g of powdered sugar (added in two steps) using electric whisks (Phillip, Milan, Italy) at speed 3 (600 rpm) for 10 min to obtain soft/stiff peak batter structure. The prepared meringue batter was divided into six equivalent portions, and the appropriate dosage of freeze-dried colorant was added (0, 1, 2, 3, 5, and 7% w/w sugar basis) and squeezed into waxed paper trays before baking. The baking temperature was adjusted to 75°C for 3 h using an oven (Venticell, MMM Medcenter Einrichtungen GmbH, Planegg/München, Germany). The cooked meringues (n = 150) were subjected to colorimetric and sensory analyses.

Colorimetric determinations

Color measurement of the extract and meringue was carried out using a CR-5 colorimeter (Konica Minolta, Tokyo, Japan) by a D65 illuminant on the basis of L*, a*, and b* values, where the L* value indicates lightness, and its value ranges from 0 to 100; the a* value gives the degree of the red-green color, with a lower negative a* value suggesting more green (–a); and the b* value indicates the degree of the yellow-blue color, with a higher positive b value suggesting more yellow (+b). The colorimeter was calibrated using a standard white and black plate. The ΔE (color difference) between samples [estimated as the difference with trial 3 (reference sample with the lowest a*)] was calculated by equation reported in Lombardelli *et al.* (2021).

Sensory analysis

Meringue samples were analyzed for their organoleptic characteristics by a panel of 25 trained members (26–47 years of age, included 14 female and 11 male panelists) using a five-point hedonic scale. The panelists scored different attributes, described each one to facilitate scoring (Table 2). The meringues were evaluated for their brightness, color homogeneity, green color intensity, smell (herbaceous), crumb color, internal color, texture, hardness, taste, herbaceous flavor, and overall acceptability. The samples were placed on white plates and identified with random three-digit numbers. The panelists evaluated all samples in a testing area with good light condition and were instructed to rinse their mouth with water between samples to minimize any residual effects.

Statistical analysis

All trials were performed in triplicate, and results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) to find the effect of individual factors and their interaction on colorimetric parameters ($p < 0.01$). Tukey's post-hoc test (HSD) was also performed ($p < 0.05$) using EXCEL® extension DSAAS-TAT for multiple comparisons between samples.

Results and Discussion

Chloroplast recovery by enzyme-assisted- and solvent extraction

The recovery yield of intact chloroplasts by EAE and SE, varying the total metal dose (0–165 ppm) and the

Table 2. List of attributes and their descriptor scale for sensory analysis.

Attributes	Descriptor scale
Brightness	1 (opaque) → 5 (bright)
Color homogeneity	1 (inhomogeneous) → 5 (homogeneous)
Green color intensity	1 (pale) → 5 (intense)
Smell (herbaceous)	1 (very herbaceous) → 5 (herbaceous absent)
Exterior color	1 (uninviting) → 5 (very inviting)
Internal color	1 (not very uniform) → 5 (uniform)
Texture	1 (granulous) → 5 (homogeneous)
Hardness	1 (soft/too hard) → 5 (correct)
Taste	1 (unpleasant) → 5 (pleasant)
Herbaceous flavor	1 (excessive) → 5 (absent)
Overall acceptability	1 (negative) → 5 (positive)

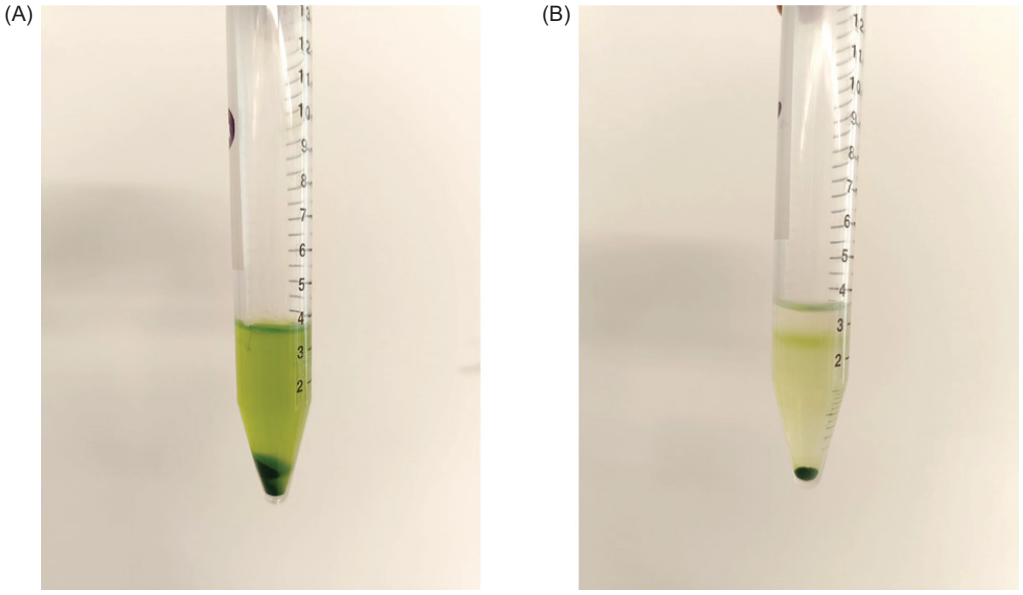

Figure 1. Percoll gradient allowing the separation of broken (top) and intact (bottom) chloroplast extracted by (A) enzyme-assisted extraction and (B) solvent extraction.

extraction time (1–3 h), was investigated. From a first visual analysis, the difference between the two extraction methods was already evident. In fact, in Figure 1, it is possible to distinguish broken (top) and intact (bottom) chloroplasts; the latter seems to be more numerous in Figure 1A, suggesting EAE allowed the recovery of a greater amount of intact chloroplasts compared to SE (Figure 1B).

The enzymatic approach for extracting pigments retained within their structural environment has already proven successful. Cuccolini *et al.*, (2013) utilized a commercial enzymatic preparation, whereas Lombardelli *et al.*, (2020) applied a tailored enzymatic mixture specifically designed according to the characteristics of the vegetable matrix. Akin to the approach adopted in this study for the recovery of chloroplast, carotenoids still contained within chromoplasts were obtained, thereby preventing their degradation (Cuccolini *et al.*, 2013; Lombardelli *et al.*, 2020).

Extraction process optimization

The effect of process variables (time and total metal dose) on the amount of Chl in intact chloroplasts was evaluated by RSM. Table 1 shows the results of the full factorial design, and Table 3 displays the coefficients of the mathematical model and statistical parameters, proving that the RSM developed was adequate (Models p-value = 0.000). For the response variable considered (Chl concentration), the R^2 values were equal to 0.96 for both extraction methods (EAE and SE), indicating that the regression models were able to effectively explain how the variables and their interactions affected the response.

Table 3. Regression coefficients, model p-value, R^2, and adjusted R^2 for the different polynomial models (Note: Subscripts: 0 = constant term; 1 = metals dose; 2 = time).

Regression coefficient	EAE Chl	Solvent Chl
b_0	+0.2243	+0.07911
b_1	–0.000342***	–0.000009
b_2	+0.0656***	+0.001964***
b_{11}	+0.000007**	+0.0000001
b_{22}	+0.0076	–0.00831***
b_{12}	+0.000125	–0.000017
Model p-value	0.000	0.000
R^2	0.96	0.96
R^2 adj	0.93	0.94

*significant at 0.05 level, **significant at 0.01 level, ***significant at 0.001 level.

Furthermore, the significance of each equation coefficient was determined using the p-value (Table 3) (Mazzocchi *et al.*, 2023). The analysis of variance was performed to determine the significance of the linear, quadratic, and interaction effects of the independent variables (extraction time and total metals dose) on the dependent variable (Chl concentration). Considering

the EAE process, both the extraction time and metal dose had a statistically significant linear effect (p-value < 0.001, Table 3), contributing to the recovery of intact chloroplasts. The quadratic effect was significant (p-value <0.05) only for metal dose (Table 3). Otherwise, no interactions among the variables were revealed (p-value > 0.05). The results allowed us to develop a useful equation to predict the amount of intact chloroplasts recovered with varying metal dose (X1) and extraction time (X2). Considering SE, only the linear and the quadratic terms of time were significant (p-value < 0.001).

The interactions between the variables are depicted through the contour plots (Figure 2). It is interesting to note that for EAE, the greatest amount of recovered pigment and, therefore, the highest yield of intact chloroplasts was obtained as the extraction time and metal dose increased (Figure 2A). For the SE, however, the yield of intact chloroplasts was greater as the extraction time decreased, regardless of the metal dose (Figure 2B), proving the detrimental effect of solvents on the integrity of organelles (Harwood, 1998).

Considering data reported in Figure 3A, it was confirmed that the amount of Chl in intact chloroplasts depends both on the extraction time and on the presence of metals. Indeed, the greatest concentration was obtained in the samples recovered using the maximum dose of metals (165 ppm) for 2 h (trial 14) with no remarkable difference extending the extraction time up to 3 h (trials 3 and 9). Irrespective of the process time, a notable difference in the amount of intact chloroplasts was observed between the sample with the maximum dose of metals (trials 2, 6, 14, 3, and 9) and the sample without metals (trials 1, 7, 16, 8, and 10). Trials with halved metal dose were characterized by an intermediate Chl content (Figure 3A). For the SE, data reported in Figure 3B confirmed that the amount of intact chloroplasts depended on the extraction time, whereas the presence of metals was not relevant. Indeed, with the same extraction time and different metal dose, the concentration of chloroplasts was similar (trial 1 and 7 vs trials 2 and 6 vs trial 12). Unlike the enzymatically extracted samples, the greater content of intact chloroplasts was identified in the tests with a shorter extraction time (1 h), probably because a prolonged contact with organic solvent destroyed the organelles (Lombardelli *et al.*, 2020).

Colorimetric properties of green extract

In addition to the quantification of pigment, CIELa*b* parameters have been considered for characterizing the extracts (Tables 4 and 5). In particular, the greenness is expressed by the a* value; the more negative it is, the greener the sample is. For EAE trials, the total color difference (ΔE) was also evaluated in order to better highlight the color changes. In detail, a total color difference ranging from 0 to 2 is considered unrecognizable, a $\Delta E \geq 5$ is rated significant and recognizable, whereas a remarkable variation is found when $5 \leq \Delta E < 12$, and a different color if $\Delta E \geq 12$.

The lowest a* value for EAE samples (Table 4) was observed by applying the highest metal dose at 2h (trial 14) and 3h (trials 3 and 9), while preserving the greenness. By decreasing the metal dose, the color shifted towards duller nuances until obtaining the worst green color (higher a* value) for the tests without metal ions (trials 1,7 and 16), as also highlighted by the ΔE value.

Considering the colorimetric data of the SE samples (Table 5), the presence of metal ions did not preserve the color which tended to turn on duller and less brilliant

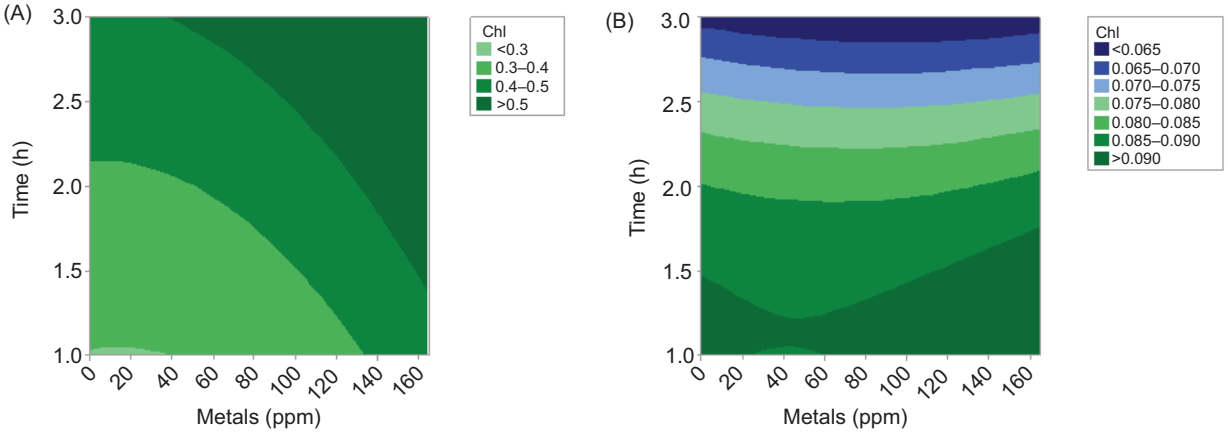

Figure 2. Contour plots showing the interactions between the variables on the amount of chlorophyll into intact chloroplast (mg/mL): (A) time (h) × metals dose (ppm) for enzyme-assisted extraction and (B) time (h) × metals dose (ppm) for solvent extraction.

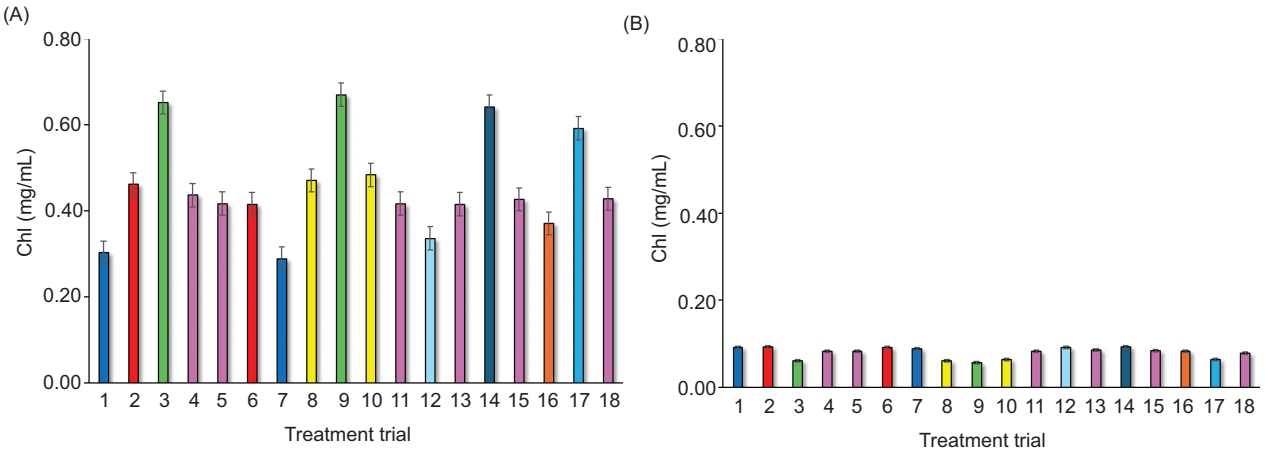

Figure 3. Chlorophyll concentration (mg/mL) into intact chloroplast obtained by (A) enzyme-assisted extraction and (B) solvent extraction.

Table 4. Visual color attributes (L*, a*, and b*) and total color difference (ΔE) of chlorophyll into intact chloroplast recovered by enzyme-assisted extraction at different conditions.

Treatment trial	L*	a*	b*	ΔE and simulated color
1	76.14	−27.79	99.8	17.1
2	75.89	−30.63	97.07	13.8
3	74.45	−44.35	95.96	0.0
4	77.38	−34.5	97.82	10.4
5	79.71	−32.51	98.64	13.2
6	75.78	−29.27	98.77	15.4
7	76.78	−28.01	99.5	16.9
8	75.96	−29.57	98.1	15.0
9	75.05	−43.55	96	1.0
10	76.1	−30.02	98.2	14.6
11	78.25	−33.99	97.87	11.2
12	76.56	−28.99	99.01	15.8
13	78.15	−34.65	97.33	10.5
14	75.23	-42.99	98.24	1.8
15	76.01	−33.99	99.21	11.0
16	76.85	−30.12	97.25	14.5
17	75.03	−34.52	98.75	10.2
18	77.25	−34.02	98.41	11.0

nuances, tending to grayish hue when the extraction time extends. These results fit with what has been reported in the literature. It is well known that the use of organic solvents may easily lead to a partial degradation of the extracted molecules and, consequently, to a qualitative color deterioration (Taghavi *et al.*, 2023).

Considering the obtained results, it is possible to hypothesize that the synergistic use of a tailored enzymatic mix and a suitable combination of metal ions (Zn^{2+} and Cu^{2+}) may be an efficient method for the recovery of Chl within intact chloroplasts.

Application of the green extract in a food matrix: the case study of meringues

Meringues are a baked mixture of egg white and sugar, and the quality is mainly linked to the processing methods, the ingredients of the product recipes (Yüceer & Caner, 2021), as well as to the typical pure white. The freeze-dried Chl-based sample, containing intact chloroplast (trial 14), was used in the formulation of meringues at different doses (0, 1, 2, 3, 5, and 7 % w/w sugar based). The pictures and the coordinates of the tristimulus values (CIELa*b*) in Table 6 suggested a significant variation of the colorimetric parameters L*, a*, and b* between the meringue without colorant (0% w/w) and the product at lowest concentration (1% w/w). Concerning a*, it significantly varied toward negative values (more intense green nuance) up to a Chl-based extract concentration equal to 3% w/w. Over this dose, the addition of increased amount of colorant did not affect the colorimetric parameters a* and b* (Table 6).

The effect of the incorporation of powder colorant on the organoleptic characteristics of meringues is presented in Figure 4. The sensory evaluation suggested that, irrespective of the dosage, the addition of colorant had a significant effect on the quality of meringues ($p < 0.05$) except for internal color. Excluding the attributes related to the intensity of the green color and the overall acceptability, it may be noted that, considering the dose of the colorant added, no significant differences were observed among the control (0%) and the 1 and 2% samples for taste, smell (herbaceous), texture, hardness, and herbaceous flavor.

Table 5. Visual color attributes (L*, a*, and b*) of chlorophyll into intact chloroplast recovered by solvent extraction at different conditions.

Treatment trial	L*	a*	b*	Simulated color
1	80.99	–20.97	32.85	
2	82.25	–21.32	34.22	
3	95.24	–10.23	24.44	
4	88.79	–19.88	20.54	
5	90	–18.96	20.54	
6	79.47	–20.13	31.98	
7	79.12	–19.98	30.54	
8	96.47	–9.37	24.47	
9	96.24	–11.43	24.87	
10	95.22	–11.04	24.95	
11	87.25	–18.35	19.55	
12	97.01	–10.1	23.89	
13	88.52	–18.25	21.01	
14	79.35	–22.01	32.54	
15	89.57	–19.58	20.96	
16	91.24	–17.87	21.02	
17	97.01	–10.11	23.89	
18	88.78	–17.99	20.56	

The sample containing 3% pigment slightly differed from the 0–2% samples, with the only exception of brightness (Figure 4).

The meringues with the highest dose of colorant (5 and 7%) were similar, but completely different from the other samples with the exception of crumb color and color homogeneity. Regarding the intensity of the green color, as expected, the 3%, 5%, and 7% samples obtained the highest score (Figure 4). The overall acceptability on a five-point hedonic scale was higher than 4.6 for meringues at 1–3% and did not differ from the control. However, for 5 and 7%, the overall acceptability was rated as poor (3.2 and 2.6, respectively) because of the strong taste and aroma of these samples. The sensory analysis data suggested that the most suitable dose of colorant that can be used in an original and innovative green meringue preparation is 3% (w/w), without significantly affecting the sensory characteristics.

Conclusions

This study demonstrates that Chl may be effectively recovered inside their natural envelope (chloroplast) by a tailored approach, which combines the simultaneous use of EAE in the presence of metal ions (zinc and copper), applied in an ideal ratio for health benefits. This method exploits the mild and selective hydrolytic action

Table 6. Visual color attributes (a*, b*, and L*) of meringues prepared by adding different amounts of green chlorophyll-based colorant recovered by enzyme-assisted extraction (0–7 % w/w sugar based).

Colorant (%)	L*	a*	b*	Sample
0%	95.29^{e}	-0.30^{d}	9.48^{a}	
1.0%	86.69^{d}	-6.51^{c}	23.2^{b}	
2.0%	81.91^{c}	-8.29^{b}	29.15^{c}	
3.0%	75.72^{b}	-9.35^{a}	33.16^{d}	
5.0%	68.89^{a}	-9.38^{a}	34.11^{d}	
7.0%	64.45^{a}	-9.52^{a}	32.86^{d}	

Values with different letters (a–e) differ significantly (Tukey's test, p = 0.05) according to color.

of enzymes, preserving the structural integrity of chloroplast, and concurrently stabilizing the pigment, thanks to the protective action of the two bivalent ions.

The combined use of enzymes and metal ions allowed for a more efficient extraction and better preservation of Chl, maintaining its vibrant green color and minimizing the degradation of pigment, compared to traditional organic solvent extraction.

Colorimetric analysis revealed that the stability of the color is strongly correlated with the concentration of metal ions, and the dose of 165 ppm helped to better preserve an intense green color even at longer extraction times.

The application of the extracted Chl, as a natural colorant in a typical pure white food matrix, such as meringues, confirmed that at lower concentrations (1–3% w/w), the pigment not only gives the desired color but also did not

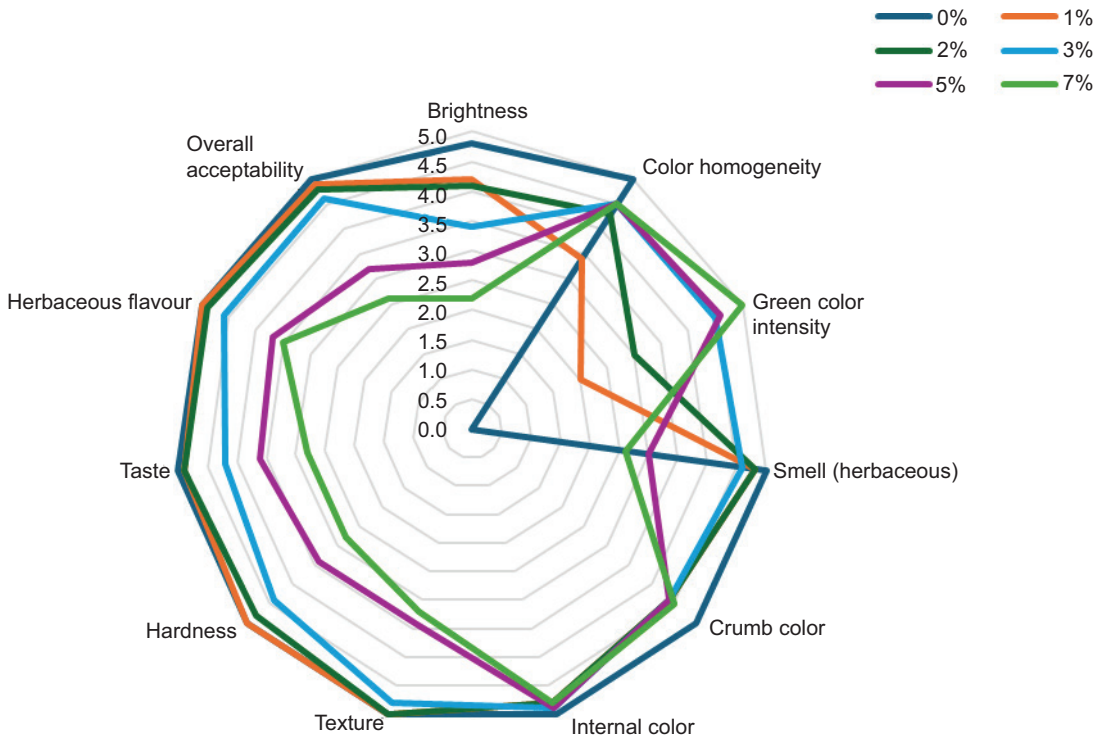

Figure 4. Spider plot representing the mean sensory analysis scores for meringues at different concentrations of green chlorophyll-based colorant (0–7 % w/w sugar based).

compromise on the organoleptic characteristics of the product, including taste, aroma, and texture. In contrast, higher concentrations (5–7% w/w) led to an overpowering herbal flavor, reducing the overall acceptability of the product.

Overall, the proposed methodology not only offers a more efficient process for extracting Chl, avoiding the use of organic solvents, but also enhances the quality and stability of natural colorants for industrial applications.

Data Availability

Data will be made available on request.

Acknowledgments

The research was carried out within the framework of the Ministry of University and Research (MUR) initiative "Departments of Excellence" (Law 232/2016) DAFNE Project 2023-27 "Digital, Intelligent, Green and Sustainable (acronym: D.I.Ver.So)."

Author Contributions

Caterina Mazzocchi was responsible for writing—original draft, methodology, investigation, and formal analysis; Claudio Lombardelli looked into writing—review & editing, methodology, conceptualization, and formal analysis; Ilaria Benucci was concerned with writing—review & editing, conceptualization, visualization, validation, data curation, resources, and supervision; Marco Esti did writing—review & editing, conceptualization, funding acquisition, and project administration.

Conflicts of Interest

The authors declare no conflicts of interest.

Funding

Project ECS 0000024 Rome Technopole—CUP B83C22002820006; PNRR Mission 4 Component 2 Investment 1.5; European Union—NextGenerationEU; Unicoop Tirreno S.C. (Viterbo, Lazio region, Italy).

References

Amin, S.F.M., Muhammad, K., Karim, R., Yusof, Y. A., Mamat, H., Sintang, M.D. 2021. A short review on the stability of chlorophylls and metallo-chlorophyll complexes in fruits and vegetables. Trans. Sci. Technol. 8(3–3): 419–424.

Artar, E., Olgunoglu, M.P., Olgunoglu, I.A. 2024. Evaluation of heavy metal accumulation and associated human health risks in three commercial marine fish species from the Aegean Sea, Türkiye. IJFS. 36(2): 136–149. https://doi.org/10.15586/ijfs.v36i2.2474

Ašimović, Z., Čengić, L., Hodžić, J., Murtić, S. 2016. Spectrophotometric determination of total chlorophyll content in fresh vegetables. God. LXI Broj. 66: 104–108.

Berhe, M., You, J., Dossa, K., Li, D., Zhou, R., Zhang, Y., Wang, L. 2024. Examining chlorophyll extraction methods in sesame genotypes: Uncovering leaf coloration effects and anatomy variations. Plants. 13(12): 1589. https://www.mdpi.com/2223-7747/13/12/1589#

Chasapis, C.T., Ntoupa, P.S.A., Spiliopoulou, C.A., Stefanidou, M.E. 2020. Recent aspects of the effects of zinc on human health. Arch. Toxicol. 94: 1443–1460. https://doi.org/10.1007/s00204-020-02702-9

Cuccolini, S., Aldini, A., Visai, L., Daglia, M., Ferrari, D. 2013. Environmentally friendly lycopene purification from tomato peel waste: Enzymatic assisted aqueous extraction. JAFC. 61(8): 1646–1651. https://doi.org/10.1021/jf3027815

Ebrahimi, P., Shokramraji, Z., Tavakkoli, S., Mihaylova, D., Lante, A. 2023. Chlorophylls as natural bioactive compounds existing in food by-products: A critical review. Plants. 12(7): 1533. https://doi.org/10.3390/plants12071533

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). 2014. Scientific opinion on dietary reference values for zinc. EFSA J. 12(10): 3844. https://doi.org/10.2903/j.efsa.2014.3844

EFSA Scientific Committee, More, S.J., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T. I. ..., Leblanc, J.C. 2023. Re-evaluation of the existing health-based guidance values for copper and exposure assessment from all sources. EFSA J. 21(1): e07728. https://doi.org/10.2903/j.efsa.2023.7728

Escobedo-Monge, M.F., Barrado, E., Parodi-Román, J., Escobedo-Monge, M.A., Torres-Hinojal, M.C., Marugán-Miguelsanz, J.M. 2023. Copper/zinc ratio in childhood and adolescence: A review. Metabolites. 13(1): 82. https://www.mdpi.com/2218-1989/13/1/82#

Gedi, M.A., Briars, R., Yuseli, F., Zainol, N., Darwish, R., Salter, A.M., Gray, D.A. 2017. Component analysis of nutritionally rich chloroplasts: Recovery from conventional and unconventional green plant species. J. Food Sci. 54:2746–2757. https://doi:10.1007/s13197-017-2711-8

Harwood, J.L. 1998. Involvement of chloroplast lipids in the reaction of plants submitted to stress. Lipids in photosynthesis. 6: 287–302. https://doi.org/10.1007/0-306-48087-5

Henriques, F.S. 2001. Loss of blade photosynthetic area and of chloroplasts' photochemical capacity account for reduced CO_2 assimilation rates in zinc-deficient sugar beet leaves. J. Plant Physiol. 158(7): 915–919. https://doi.org/10.1078/0176-1617-00274

Hsiao, C.J., Lin, J.F., Wen, H.Y., Lin, Y.M., Yang, C.H., Huang, K.S., Shaw, J.F. 2020. Enhancement of the stability of chlorophyll using chlorophyll-encapsulated polycaprolactone microparticles based on droplet microfluidics. Food Chem. 306: 125300. https://doi.org/10.1016/j.foodchem.2019.125300

Hu, Y., Sun, H., Mu, T. 2022. Effects of different Zn^{2+} concentrations and high hydrostatic pressures (HHP) on chlorophyll stability. Foods. 11(14): 2129. https://www.mdpi.com/2304-8158/11/14/2129#

Indrasti, D., Andarwulan, N., Purnomo, E.H., Wulandari, N.U.R. 2018. Stability of chlorophyll as natural colorant: A review for suji (Dracaena Angustifolia Roxb.) leaves' case. Curr. Res. Nutr. Food Sci. 6(3): 609–625. https://doi.org/10.12944/CRNFSJ.6.3.04

Kwartiningsih, E., Ramadhani, A.N., Putri, N.G.A., Damara, V.C.J. 2021. Chlorophyll extraction methods review and chlorophyll stability of katuk leaves (Sauropus androgynous). Journal of Physics (Conference Series). 1858(1): 012015.. https://doi.org/10.1088/1742-6596/1858/1/012015

Lombardelli, C., Benucci, I., Marco, E. 2021. Novel food colorants from tomatoes: Stability of carotenoid-containing chromoplasts under different storage conditions. LWT. 140: 110725. https://doi.org/10.1016/j.lwt.2020.110725

Lombardelli, C., Liburdi, K., Benucci, I., Esti, M. 2020. Tailored and synergistic enzyme-assisted extraction of carotenoid-containing chromoplasts from tomatoes. Food Bioprod. 121: 43–53. https://doi.org/10.1016/j.fbp.2020.01.014

Lombardelli, C., Mazzocchi, C., Benucci, I., Esti, M. 2024. Stabilized chlorophyll-based food colorants from spinach: Kinetics of a tailored enzymatic extraction. J. Food Sci. 89(9): 5270–5279. https://doi.org/10.1111/1750-3841.17269

Managa, M.G., Mpai, S., Remize, F., Garcia, C. and Sivakumar, D. 2020. Impact of moist cooking methods on colour, anti-nutritive compounds and phenolic metabolites in African nightshade (Solanum retroflexum Dun.). Food Chem. 325: 126805. https://doi.org/10.1016/j.foodchem.2020.126805

Matuszczak, M., Kiljańczyk, A., Marciniak, W., Derkacz, R., Stempa, K., Baszuk, P., ... Lubiński, J. 2024. Antioxidant properties of zinc and copper—blood zinc-to copper-ratio as a marker of cancer risk BRCA1 mutation carriers. Antioxidants. 13(7): 841. https://www.mdpi.com/2076-3921/13/7/841#

Mazzocchi, C., Benucci, I., Lombardelli, C., Esti, M. 2023. Enzyme-assisted extraction for the recovery of food-grade chlorophyll-based green colorant. Foods. 12(18): 3440. https://www.mdpi.com/2304-8158/12/18/3440#

Miazek, K. and Ledakowicz, S. 2013. Chlorophyll extraction from leaves, needles and microalgae: A kinetic approach. IJABE. 6(2): 107–115.

Osredkar, J. and Sustar, N. 2011. Copper and zinc, biological role and significance of copper/zinc imbalance. J. Clin. Toxicol. 3(2161): 0495.

Schwartz, S.J. and Lorenzo, T.V. 1990. Chlorophylls in foods. Crit. Rev. Food Sci. Nutr. 29(1): 1–17. https://doi.org/10.1080/10408399009527511

Seigneurin-Berny, D., Salvi, D., Dorne, A.J., Joyard, J., Rolland, N. 2008. Percoll-purified and photosynthetically active chloroplasts from Arabidopsis thaliana leaves. PPB. 46(11): 951–955. https://doi.org/10.1016/j.plaphy.2008.06.009

Senklang, P. and Anprung, P. 2010. Optimizing enzymatic extraction of Zn–chlorophyll derivatives from pandan leaf using response surface methodology. J. Food Process. Preserv. 34(5): 759–776. https://doi.org/10.1111/j.1745-4549.2009.00393.x

Staehelin, L.A. 2003. Chloroplast structure: From chlorophyll granules to supra-molecular architecture of thylakoid membranes. Photosynth. Res. 76: 185–196. https://doi.org/10.1023/A:1024994525586

Taghavi, T., Patel, H., Rafie, R. 2023. Extraction solvents affect anthocyanin yield, color, and profile of strawberries. Plants. 12(9): 1833. 10.3390/plants12091833

Viera, I., Pérez-Gálvez, A., Roca, M. 2019. Green natural colorants. Molecules. 24(1): 154. https://www.mdpi.com/1420-3049/24/1/154#

Watts, D.L. 2010. HTMA mineral ratios. A brief discussion of their clinical importance. Trace Elem. Med. Biol. 21: 1–3.

Yüceer, M. and Caner, C. 2021. Effects of protease-hydrolyzed egg white on the meringue batter properties and meringue textural and sensory properties during storage. Int. J. Gastron. Food Sci. 25: 100409. https://doi.org/10.1016/j.ijgfs.2021.100409

Zheng, Y., Shi, J., Pan, Z., Cheng, Y., Zhang, Y., Li, N. 2014. Effect of heat treatment, pH, sugar concentration, and metal ion addition on green color retention in homogenized puree of Thompson seedless grape. LWT. 55(2): 595–603. https://doi.org/10.1016/j.lwt.2013.10.011