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Stabilized chlorophyll-based food colorants from spinach: Kinetics of a tailored enzymatic extraction

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Abstract: An organic solvent-free method based on limited dosing options (biocatalyst and zinc chloride) for the quick and mild recovery of chlorophyll (Chl) from spinach has been proposed. This tailored, custom-made protocol has been designed to produce stable green natural colorants. The kinetics of pigment extraction turned out to be a very useful tool to identify the proper conditions, in terms of biocatalyst dose (0.10–50 U/g), extraction time (1–48h), and ZnCl₂ amount (50–300ppm), both for enhancing the recovery yield and preserving the green color. Considering the extraction kinetics, the recovery yield, and the colorimetric data, the suitable conditions to produce stable green and food-grade colorants are 0.10 U/g of enzyme, 3h, and 150ppm of ZnCl₂ at 25°C. The extraction yield of Chl (4863µg/U) was about 51% greater than control, with a higher extraction rate constant (5.43×10^{-4} g/(µg min)). Considering the impact of ZnCl₂ amount on Chl, its protective action resulted to be more noticeable toward Chl a: at 150ppm, an increased amount of about 2.5 and 1.5 times was found for Chl a and Chl b, respectively, in comparison to the reference (0ppm ZnCl₂).

KEYWORDS

chlorophyll a, chlorophyll b, enzyme-assisted extraction, green pigment, zinc chloride

Practical Application: This research demonstrates how a suitable kinetic approach helps to provide a tailored protocol, customized for the vegetable matrix, to produce stable green natural colorants from spinach. Lowering enzyme dosage and ZnCl₂ amount during the extraction of chlorophyll at low temperature is crucial for its potential use as a colorant in food industry, providing high economic values through saving time and environmental protection.

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1 | INTRODUCTION

Chlorophylls (Chls) consist of a hydrophilic head (porphyrin), a lipophilic hydrocarbon tail (phytol group), and a central magnesium ion bonded to the tetrapyrrole ring (Nabi et al., 2023). Due to their lipophilic hydrocarbon chains, Chls are generally considered to be insoluble in polar solvents (Ebrahimi et al., 2023). Stored mainly in chloroplasts, only two types of Chls (a and b) are present in higher plants, whereas others (c–f) are found in algae and bacteria. Chl a and Chl b differ structurally by the presence of a methyl or aldehyde group at the Carbon-7 position, respectively (Paliwal et al., 2016). These distinct structural features result in variations in their color: Chl a has a blue–green nuance, whereas Chl b has a green–yellow color (Carrillo et al., 2022). Chl a and b from plants, and recently authorized algal Chl (Chen et al., 2023), are used as colorants in food and pharmaceutical industries (Nabi et al., 2023), also for their recognized health properties (e.g., antioxidant [Li et al., 2023], anti-inflammatory, anticancer [Derrien et al., 2018], and antimutagenic properties [Ebrahimi et al., 2023]).

Several factors (e.g., heat, low pH, light, or enzymes) may degrade Chl during the extraction process. The replacement of Mg^{2+} with two H^+ ions (pheophytinization) triggers the conversion of blue–green Chl a into gray pheophytin a, whereas green–yellow Chl b turns into a brown pheophytin b (Indrasti et al., 2018). The degradation of Chl a occurs 2.5–10 times faster than that of Chl b (Eckardt, 2009). The loss of Mg^{2+} already starting from the initial stages of the extraction process, and the resulting color change affected Chl applications in food (Ghosh et al., 2022). The use of Cu^{2+} has been suggested as a valid approach to obtain commercial stable green copper Chls (E141(i) and E141(ii)) (Viera et al., 2019), which are more resistant to acids and heat and have improved antioxidant properties (Ebrahimi et al., 2023). However, the toxicity of copper has led to the investigation of other metal bivalent ions, such as zinc (Mazzocchi et al., 2023; Özkan & Bilek, 2015). The usefulness of zinc chloride during the Chl extraction has been documented in other studies (Mazzocchi et al., 2023; Özkan & Bilek, 2015; Senklang & Anprung, 2010). It is conceivable that the early addition of the Zn^{2+} ion, since the beginning of pigment extraction, may contribute to readily re-establish the correct conformation of the molecule, thus preserving its coloring properties.

Actually, all the natural Chl-based food colorants ((E140(i), E140(ii), E141(i), and E141(ii)) permitted in the EU are produced by the conventional solvent extraction (Viera et al., 2019). Despite the fact that solvent extraction is simple and does not require complex/expensive equipment, this non-environmentally friendly methodology shows numerous disadvantages, including the need for

a large amount of solvent, a long extraction time, along with the possible pigment degradation, which affects the recovery yield (Mazzocchi et al., 2023). Green processing for the recovery of Chl from plant tissues, such as enzyme-assisted extraction (EAE), is gaining more attention (Benucci et al., 2022) due to the high recovery yield, fast extraction, low energy consumption, and no solvent usage (Saad et al., 2019). The major downside of that strategy is the high cost of biocatalysts, mainly due to the use of unspecific commercial preparations usually applied in unbalanced doses (Özkan & Bilek, 2015) because they are not based on a reasoned approach.

Kinetic studies of Chl extraction from vegetable tissue using solvents (e.g., methanol and ethanol) (Miazeck & Ledakowicz, 2013), microwave-assisted extraction (Nguyen et al., 2021), or pulsed electric field (Zhang et al., 2020) turned out to be a very useful tool to identify the proper conditions for enhancing the recovery yield.

In our previous work (Mazzocchi et al., 2023), an EAE protocol for the recovery of Chl from spinach has been developed by using a tailored enzymatic mix, designed considering the polysaccharide composition of spinach cell wall, instead of commercial preparations that are not flexible in relation to the vegetable tissue composition.

This paper offers a deep extension of the precedent work aimed at increase Chl recovered yield per unit of enzyme and, concurrently, the greenness of the extract. The novelty and significant contribution of this research consist of providing useful information to interpret, and possibly foresee, the Chl release trend from a leaf vegetable matrix by the tailored EAE protocol. The kinetic study, along with the resulting modeling parameters, may be additionally helpful for the extraction of other pigments from different vegetable sources. Moreover, this study contributes to provide the most suitable operating conditions for the production of stable green natural colorants.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Biocatalysts (Merck) applied in this research were as follows: polygalacturonase (≥ 0.3 U/mg) and cellulase (0.8 U/mg) from *Aspergillus niger*; xylanase (≥ 2500 U/mg) from *Aspergillus oryzae*. All reagents used were purchased from VWR International.

2.2 | Plant material preparation

Unsold spinach (*Spinacia oleracea*) was kindly provided by a local supermarket (Unicoop Tirreno S.C.) and kept under refrigerated conditions until extraction. Spinaches of 25 g

TABLE 1 Experimental conditions applied for the recovery of chlorophyll (Chl) by enzyme-assisted extraction.

Experimental conditions	
Temperature (°C)	25
Time (h)	1, 3, 6, 16, 24, 36, 48
Total enzyme dose (U/g)	0.10, 0.25, 0.50, 1.0, 3.0, 6.0, 10, 30, 50
ZnCl ₂ dose (ppm)	0, 50, 100, 150, 200, 300
Buffer/Substrate ratio	17.5
pH	5.0

Note: For each treatment, three experimental replicates were conducted.

were subjected to blanching (5 s at 100°C) to neutralize the chlorophyllases. After that, they were mixed using a mixer (Phillip HR 2068 mixer) for 1 min at maximum power using extraction buffer (McIlvaine 0.1 M, pH 5). According to our previous study, the ratio of biomass (spinach pulp) to buffer was set at 17.5 (Mazzocchi et al., 2023). In order to have a more representative starting vegetable material (with a more representative average concentration of target compounds), three different batches of spinach (1 kg each) were combined before the enzymatic extraction. Experimental trials were replicated three times separately.

2.3 | Effect of the biocatalyst dose

As reported in our previous study (Mazzocchi et al., 2023), using the polysaccharide cell wall composition of the vegetable, a tailor-made enzyme blend containing cellulase (40%), xylanase (41%), and polygalacturonase (19%) was formulated. The homogenized spinach, resuspended in McIlvaine buffer, was kept in a bench-scale stirred bioreactor (1 L, in the dark) by regulating the temperature (25°C) using an external water jacket and connected to a thermoregulator (MPM instrument, type M 900-TI). The treatment was carried out using 300 ppm of ZnCl₂, adding different total doses of biocatalyst: 0.10, 0.25, 0.50, 1.0, 3.0, 6.0, 10, 30, and 50 U/g at various extraction times (1, 3, 6, 16, 24, 36, and 48 h). A control sample (Ctrl) was prepared without the addition of the enzyme mixture. For the experimental plan (Table 1), three batch replicates were conducted for each sample.

2.4 | Effect of zinc chloride

In order to verify the effect of the zinc chloride during the EAE of Chl from spinach, different ZnCl₂ concentrations were tested (50, 100, 150, 200, and 300 ppm), according to the study of Özkan and Bilek (2015). The procedure was done at 25°C for 3 h by applying the lowest tested enzyme dose (0.10 U/g) and an intermediate biocatalyst

dose (1.0 U/g). A reference sample (Ref = 0 ppm ZnCl₂) was made without adding ZnCl₂. Three experimental replicates were conducted.

2.5 | Chlorophyll content and colorimetric characterization

After enzymatic extractions, the obtained pulp was centrifuged (Heraeus Megafuge 16R centrifuge, Thermo Scientific) for 5 min at 4500 rpm (10°C), and the clear supernatant was removed. Afterward, 10 mL of absolute ethanol was used to solubilize 1 g of pellet, and the obtained solution was centrifuged (under the same conditions previously used). Absorbance analysis (λ : 230–700 nm, UV-visible, Shimadzu UV 2450) was carried out on the recovered supernatant using a 1 mm quartz cuvette (Hellma). The amount of Chl a, Chl b, and total Chl was determined through the following equation with some modifications (Lichtenthaler, 1987; Özkan & Bilek, 2015):

$$\text{Chl a } (\mu\text{g/g}) = (13.36 \times A_{664} - 5.19 \times A_{649}) \times \text{DF} \quad (1)$$

$$\text{Chl b } (\mu\text{g/g}) = (27.43 \times A_{649} - 8.12 \times A_{664}) \times \text{DF} \quad (2)$$

$$\text{Tot Chl } (\mu\text{g/g}) = \text{Chl a} + \text{Chl b} \quad (3)$$

where A_{664} is absorbance at λ 664 nm; A_{649} is absorbance at λ 649 nm; DF is dilution factor.

Extraction yield was estimated as the ratio between total Chl ($\mu\text{g/g}$) and biocatalyst dose (U/g):

$$\text{Yield } (\mu\text{g/U}) = \text{Tot Chl } (\mu\text{g/g}) / \text{biocatalyst dose (U/g)} \quad (4)$$

The green extract was calorimetrically characterized using a CR-5 colorimeter (Konica Minolta) with the CIELa*b* system (illuminant D65 and visual angle of 10°). Three analytical replicates were conducted for each sample (five measurements for each sample unit). Moreover, two derived color parameters were determined: hue angle (h°) and chroma (C^*) (Lombardelli et al., 2021; Mazzocchi et al., 2023):

$$h^\circ = 180^\circ + (\arctan b^* / a^*) \quad (5)$$

$$C^* = (a^{*2} + b^{*2})^{0.5} \quad (6)$$

2.6 | Mathematical model for chlorophyll extraction process

As reported by Nguyen et al. (2021), the second-order model has been proven to be appropriate for describing the extraction process of active compounds from plants. It has also been successfully applied for pigment recovery by microwaves (Nguyen et al., 2021) and pulsed electric fields (Zhang et al., 2020). The second-order equation applied for Chl recovery by EAE is

$$C = \frac{C_{\infty}^2 kt}{1 + C_{\infty} kt} \quad (7)$$

where C_{∞} is the saturated concentration ($\mu\text{g/g}$), and k is the second-order extraction rate constant ($\text{g}/(\mu\text{g min})$) calculated by linear regression.

2.7 | Statistical analysis

Data were analyzed using (i) two-way analysis of variance (ANOVA) to verify the effect of the enzyme dose and treatment time and their interaction on the colorimetric parameters ($p < 0.01$); and (ii) one-way ANOVA to test for significant differences between enzyme dose and process time. When significance was reached, Tukey's honestly significant difference (HSD) test ($p < 0.05$) was applied (EXCEL® Add-in macro DSASTAT). A Pearson correlation analysis ($p < 0.01$) was performed between the k values (second-order kinetic constant) and the different enzyme doses (0.1–50 U/g) computed with GraphPad Prism 5.0 (GraphPad Software, Inc.).

3 | RESULTS AND DISCUSSION

3.1 | Chlorophyll kinetics during enzyme-assisted extraction: effect of the biocatalyst dose

The changes in Chl a, Chl b, and total Chl concentration ($\mu\text{g/g}$) during the EAE from spinach leaves under different conditions (e.g., enzymatic dose and extraction time) are shown in Figure 1. In all trials, the content of recovered Chl was dose-dependent and increased remarkably as the amount of biocatalyst increased (Figure 1, Table S1). Irrespective of the biocatalyst dose, the concentration of Chl recovered by EAE was always greater as compared to that without enzyme (Ctrl). Özkan and Bilek (2015) observed a 39% increase in Zn–Chl yield by applying EAE with respect to solvent extraction alone. It is interesting to highlight that the Chl a/b ratio was approximately 2.7, in line with

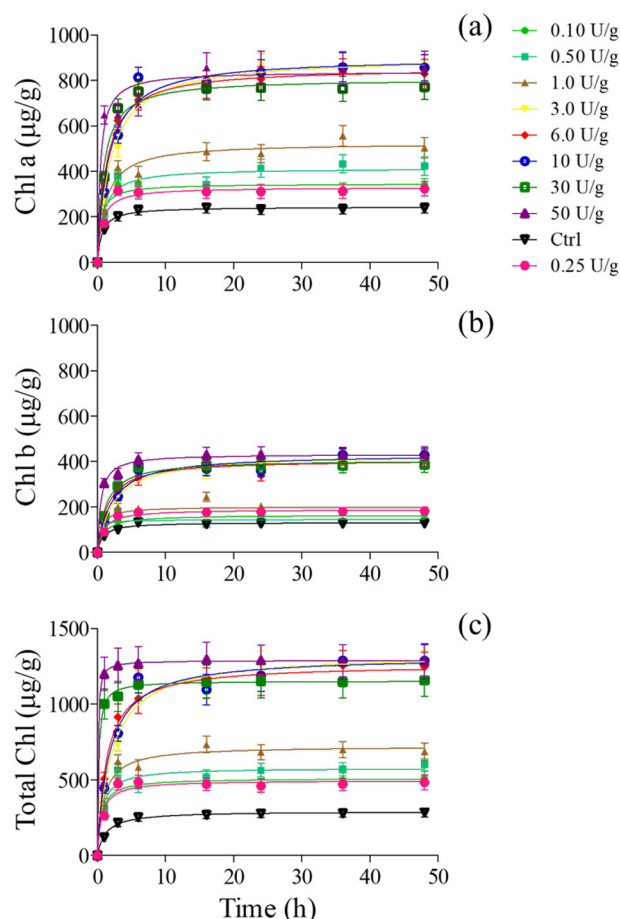


FIGURE 1 Extraction kinetics of (a) chlorophyll (Chl) a, (b) Chl b, and (c) total Chl (Chl a + Chl b), during enzyme-assisted extraction (EAE) from spinach, at different enzyme doses (0.1–50 U/g) and extraction times (1–48 h). Recovery of Chl ($\mu\text{g/g}$) was performed at 25°C and 300 ppm of ZnCl_2 . Ctrl is the control sample (treatment without enzyme). Reported values are mean $\pm$ 95% confidence interval of triplicate measurements.

those reported in the literature (Indrasti et al., 2018), and remained constant as the enzyme dose changed.

The results in Figure 1a, b (Table S1) suggest the Chl (a and b) extraction curves followed a hyperbolic behavior and were consistent with the ideal two-phase extraction kinetic of plant pigment. Indeed, it is widely recognized that the extraction process involves two phases (Nguyen et al., 2021). The first phase (faster) is characterized by the hydrolytic degradation of cellular structures thanks to the action of enzymes, subsequently followed by the dissolution of the soluble constituents in the solvent. The second stage consists in the external diffusion of the soluble components through the porous structures and, therefore, in their solubilization (Goula et al., 2017). As depicted in Figure 1a,b, the greater part of Chl a and Chl b was quickly recovered during the first hour of extraction only at the highest enzyme dose (30 and 50 U/g). A longer time (3 h)

TABLE 2 Second-order kinetic constant (k , g/(μ g min)) and R^2 values obtained for chlorophyll (Chl) extracted at different enzyme doses (0.1–50 U/g) and times (1–48 h).

Dose (U/g)	k (g/(μ g min))	R^2
0.10	5.43×10^{-4a}	0.97
0.25	5.51×10^{-4a}	0.98
0.50	4.70×10^{-4b}	0.97
1.0	3.88×10^{-4c}	0.99
3.0	2.08×10^{-4c}	0.98
6.0	2.04×10^{-4c}	0.98
10	1.62×10^{-4d}	0.97
30	1.68×10^{-4d}	0.98
50	1.63×10^{-4d}	0.98

Note: Statistical analysis of data was performed by one-way analysis of variance (ANOVA). Values with different small letters (a–d) differ significantly (Tukey's test, $p = 0.05$) according to enzyme dose.

was required to achieve the maximum Chl amount when a lower dose of biocatalyst was used (0.10–1.0 U/g) (Figure 1). In all trials, no remarkable increase in pigment content was revealed over extended times (>3 h), suggesting the solid–liquid system reached the saturation state (Table S1).

Similarly, the concentration of total Chl followed a comparable behavior (Figure 1c), and the greatest value (about 1200 μ g/g) was recovered after 1 h of extraction at the dose of 50 U/g. At the lowest dose (0.10 U/g), 490 μ g/g of Chl were extracted after 3 h of treatment (Figure 1c, Table S1). In any case, the suggested biocatalytic approach, based on a tailored enzymatic mix, turned out to be more effective in the recovery of pigment than commercial standard preparation (50.747 mg_{TCC}/100 g_{spinach pulp}, at 45°C, Özkan & Bilek, 2015), also considering the low process temperature applied (25°C).

Similar results have been reported for Chl solvent extraction (methanol: 51 mg/100g_{fresh spinach}, acetone: 90 mg/100g_{fresh spinach}) (Khalyfa et al., 1992).

To develop a kinetic model for predicting the pigment extraction behavior, the rate of Chl recovery was expressed by a second-order extraction rate constant (k). The determination coefficients ($R^2 = 0.97$ –0.99) in Table 2 suggest the model was adequate for fitting the experimental data. Indeed, the second-order model has been proven to be appropriate for describing the extraction process of active compounds from various plants (Nguyen et al., 2021; Zhang et al., 2020). Significantly, greater k values (5.43×10^{-4} and 5.51×10^{-4} g/(μ g min)) were obtained for Chl recovered by EAE from spinach leaves at 0.10 and 0.25 U/g (Table 2). At higher enzyme doses (10–50 U/g), k decreased by approximately three times (1.68×10^{-4} and 1.62×10^{-4} g/(mg min)), proving the loss of process efficiency. As a confirmation of this, a correlational analysis between the k values and the different enzyme doses

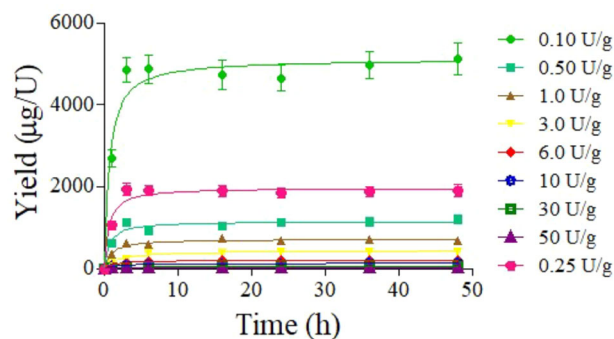


FIGURE 2 Extraction yield (μ g/U) of total chlorophyll (Chl). Treatments were carried out using different enzymatic doses (0.1–50 U/g) for various process times (1–48 h) at 25°C and 300 ppm of ZnCl₂. Reported values are mean $\pm$ 95% confidence interval of triplicate measurements.

(0.1–50 U/g) was conducted. The value of the Pearson correlation coefficient ($r = -0.618$, $p < 0.01$) suggests a moderate negative correlation.

Frequently, in order to guarantee an efficient process, a high extraction yield is essential, even if this does not necessarily guarantee a high concentration of molecules with high added value (Aspé & Fernández, 2011). Therefore, in this study, attention has been given to the enzymatic extraction yield, calculated as the ratio between the pigment concentration (μ g/g) and the enzymatic dose (U/g). Even though this trend followed a hyperbolic behavior (Figure 2) resembling that previously described, the highest extraction yield (about 4860 μ g/U) was revealed using the lowest enzyme dose (0.10 U/g) after 3 h of extraction. At 50 U/g, the extraction yield significantly diminished (about 30 μ g/U) after 1 h, reaching a plateau (Table S2). The amount of Chl recovered by the tailored enzymatic mix at low temperature (25°C) was in line with the results found by Ahmadi et al. (2022) and Mehdiipoor Damiri et al. (2021), who applied a commercial pectolytic preparation (PectineX Ultra SP L) for the recovery of Chl from alfalfa and clover under different extraction conditions (40°C, in the absence of bivalent ions able to preserve the pigment structure). In addition to determine the concentration and yield of pigment, it is essential to take into account the quality of the green color obtained through the CIELa*b* parameters (L^* , a^* , b^*) and the color indices (C^* , h°) (Mazzocchi et al., 2023). As is well documented, the evaluation of greenness is mainly expressed by the parameter a^* (Özkan & Bilek, 2015; Senklang & Anprung, 2010). The values of L^* , a^* , b^* , h° , and C^* and the corresponding resulting color of Chl-based extracts are summarized in Table 3. All samples obtained by EAE were characterized by a significantly more intense greenness than Ctrl.

Irrespective of the enzyme dose, the lowest a^* values (< -35 , nuance toward green) were obtained at the

TABLE 3 Visual color attributes [a^* , b^* , L^* , hue (h°), chroma value (C^*)] of the chlorophyll (Chl)-based extracts.

Dose (U/g)	Time (h)	L^*	a^*	b^*	C^*	h°	Simulated color
0.10 U/g	1	71.83 ^{b,A}	-39.94 ^{ab,B}	96.56 ^{ab,A}	104.5 ^{a,A}	112.5 ^{ef,AB}	
	3	49.13 ^{e,D}	-44.77 ^{a,A}	84.55 ^{ab,AB}	93.0 ^{ab,BC}	114.6 ^{bc,A}	
	6	63.72 ^{d,B}	-33.19 ^{cd,C}	89.88 ^{abc,AB}	95.8 ^{abc,B}	110.3 ^{bc,B}	
	16	58.15 ^{b,C}	-16.51 ^{de,F}	86.67 ^{ab,AB}	88.2 ^{ab,C}	100.8 ^{f,D}	
	24	50.76 ^{d,D}	-13.56 ^{d,F}	79.65 ^{ab,B}	80.8 ^{b,D}	99.7 ^{bc,D}	
	36	63.15 ^{c,B}	-20.91 ^{b,E}	84.01 ^{a,AB}	86.6 ^{ab,C}	104.0 ^{b,C}	
	48	33.91 ^{c,E}	-25.05 ^{a,D}	54.66 ^{de,C}	60.1 ^{d,E}	114.6 ^{a,A}	
0.25 U/g	1	80.32 ^{a,A}	-34.64 ^{cd,B}	98.67 ^{a,A}	104.6 ^{a,A}	109.3 ^{f,BC}	
	3	69.41 ^{b,B}	-41.68 ^{b,A}	93.58 ^{a,AB}	102.4 ^{a,AB}	114.0 ^{cd,A}	
	6	75.53 ^{a,AB}	-35.64 ^{c,B}	95.51 ^{ab,A}	101.9 ^{a,AB}	110.5 ^{bc,B}	
	16	77.45 ^{a,A}	-28.45 ^{ab,C}	87.16 ^{ab,ABC}	91.7 ^{a,BC}	108 ^{abc,BC}	
	24	79.88 ^{a,A}	-23.89 ^{b,D}	80.37 ^{ab,BC}	83.8 ^{b,CD}	106.6 ^{a,CD}	
	36	70.11 ^{b,B}	-20.48 ^{b,E}	77.89 ^{ab,C}	80.5 ^{bc,CD}	104.7 ^{ab,D}	
	48	70.85 ^{a,B}	-20.01 ^{b,E}	75.95 ^{ab,C}	78.5 ^{ab,D}	104.8 ^{c,D}	
0.50 U/g	1	65.51 ^{cd,A}	-41.27 ^{a,A}	89.65 ^{ab,A}	98.7 ^{b,A}	114.7 ^{de,A}	
	3	67.92 ^{b,A}	-35.09 ^{cd,B}	92.43 ^{a,A}	98.9 ^{ab,A}	110.8 ^{de,B}	
	6	69.83 ^{bc,A}	-39.47 ^{b,A}	91.42 ^{abc,A}	99.6 ^{ab,A}	113.4 ^{ab,AB}	
	16	67.86 ^{b,A}	-21.21 ^{c,C}	86.81 ^{ab,AB}	89.4 ^{ab,B}	103.7 ^{ef,C}	
	24	52.9 ^{d,B}	-16.29 ^{c,D}	80.01 ^{ab,B}	81.7 ^{b,C}	101.5 ^{b,C}	
	36	43.93 ^{e,C}	-5.87 ^{f,E}	70.49 ^{bc,C}	70.7 ^{cd,D}	94.8 ^{ef,D}	
	48	44.09 ^{b,C}	-23.69 ^{a,C}	59.85 ^{cd,D}	64.4 ^{cd,E}	111.6 ^{b,B}	
1.0 U/g	1	61.38 ^{def,A}	-37.43 ^{abc,A}	86.32 ^{ab,A}	94.1 ^{bc,A}	113.4 ^{de,A}	
	3	55.75 ^{cd,B}	-31.7 ^{de,B}	83.31 ^{ab,B}	89.1 ^{b,B}	110.8 ^{de,B}	
	6	52.98 ^{e,BC}	-25.5 ^{fg,C}	80.45 ^{cd,C}	84.4 ^{cd,C}	107.6 ^{c,C}	
	16	49.20 ^{f,CD}	-11.46 ^{f,D}	77.03 ^{b,CD}	77.9 ^{b,D}	98.5 ^{f,D}	
	24	49.86 ^{de,CD}	-2.66 ^{h,F}	74.25 ^{abc,D}	74.3 ^{c,D}	92.1 ^{d,F}	
	36	43.65 ^{e,E}	-1.96 ^{g,F}	60.94 ^{cd,F}	61.0 ^{de,F}	91.8 ^{f,F}	
	48	46.07 ^{b,DE}	-6.82 ^{ef,E}	69.81 ^{bc,E}	70.1 ^{bc,E}	95.6 ^{de,E}	
3.0 U/g	1	60.6 ^{def,B}	-40.62 ^{ab,B}	85.93 ^{ab}	97.8 ^b	118.5 ^{bc,A}	
	3	60.4 ^{c,B}	-44.74 ^{a,A}	85.59 ^{ab}	96.6 ^{ab}	117.6 ^{ab,A}	
	6	63.78 ^{d,AB}	-44.13 ^{a,AB}	88.6 ^{abc}	99.0 ^{ab}	116.5 ^{a,A}	
	16	69.02 ^{b,A}	-30.26 ^{a,C}	87.5 ^{ab}	92.6 ^a	109.1 ^{a,B}	
	24	68.45 ^{b,A}	-25.98 ^{a,D}	86.4 ^a	90.2 ^a	106.7 ^{a,B}	
	36	66.55 ^{bc,A}	-26.25 ^{a,D}	85.9 ^a	89.8 ^{ab}	107.0 ^{a,B}	
	48	67.98 ^{a,A}	-20.15 ^{b,E}	85.1 ^a	87.5 ^a	103.3 ^{c,C}	
6.0 U/g	1	42.18 ^{g,B}	-40.32 ^{ab,A}	65.4 ^{c,B}	80.1 ^{c,B}	125.3 ^{a,A}	
	3	50.85 ^{de,A}	-41.53 ^{ab,A}	76.06 ^{c,A}	86.7 ^{b,A}	118.6 ^{a,B}	
	6	41.21 ^{fg,B}	-24.33 ^{fg,B}	65.95 ^{de,B}	70.3 ^{e,C}	110.2 ^{bc,C}	
	16	34.96 ^{g,C}	-18.78 ^{cd,C}	56.61 ^{d,BC}	59.6 ^{c,D}	108.4 ^{a,C}	
	24	31.7 ^{f,D}	-11.27 ^{e,D}	53.23 ^{d,CD}	54.4 ^{de,E}	102.0 ^{ab,D}	
	36	28.81 ^{g,DE}	-3.47 ^{g,E}	48.02 ^{e,CD}	48.1 ^{f,F}	94.1 ^{f,E}	
	48	28.08 ^{d,E}	-3.55 ^{g,E}	46.22 ^{e,D}	46.4 ^{e,F}	94.4 ^{e,E}	
10 U/g	1	56.42 ^{f,A}	-40.93 ^{ab,A}	82.2 ^{b,A}	94.2 ^{bc,A}	119.2 ^{b,A}	
	3	56.95 ^{c,A}	-38.99 ^{bc,A}	82.78 ^{ab,A}	91.5 ^{ab,A}	115.2 ^{bc,B}	
	6	36.94 ^{g,B}	-22.19 ^{g,B}	60.14 ^{e,B}	64.1 ^{f,B}	110.3 ^{bc,C}	
	16	34.21 ^{g,B}	-15.09 ^{e,C}	56.29 ^{d,BC}	58.3 ^{c,C}	105.0 ^{b,D}	
	24	30.69 ^{f,C}	-9.02 ^{f,D}	52.34 ^{d,BCD}	53.1 ^{de,D}	99.8 ^{b,E}	

(Continues)

TABLE 3 (Continued)

Dose (U/g)	Time (h)	<i>L</i> *	<i>a</i> *	<i>b</i> *	<i>C</i> *	<i>h</i> °	Simulated color
30 U/g	36	28.56 ^{g,D}	−6.38 ^{ef,E}	47.9 ^{e,CD}	48.3 ^{f,E}	97.6 ^{de,E}	
	48	28.21 ^{d,D}	−6.02 ^{f,E}	45.52 ^{e,D}	45.9 ^{e,E}	97.5 ^{de,E}	
	1	62.61 ^{de,BC}	−41.74 ^{a,A}	86.39 ^{ab,A}	95.9 ^{b,A}	115.8 ^{cd,A}	
	3	58.63 ^{c,C}	−38.93 ^{bc,A}	85.47 ^{ab,A}	93.9 ^{ab,A}	114.5 ^{bc,A}	
	6	67.71 ^{cd,A}	−32.06 ^{cd,B}	81.71 ^{bcd,AB}	87.8 ^{bc,B}	111.4 ^{b,B}	
	16	65.84 ^{bc,AB}	−20.25 ^{c,C}	76.87 ^{bc,BC}	79.5 ^{b,C}	104.8 ^{b,C}	
	24	52.03 ^{d,D}	−14.67 ^{cd,D}	72.32 ^{bc,BCD}	73.8 ^{c,D}	101.5 ^{b,DE}	
	36	49.25 ^{d,DE}	−12.19 ^{c,D}	67.88 ^{bcd,CD}	69.0 ^{d,E}	100.2 ^{cd,E}	
50 U/g	48	45.60 ^{b,E}	−15.04 ^{c,D}	62.44 ^{cd,D}	64.2 ^{cd,F}	103.5 ^{c,CD}	
	1	59.36 ^{ef,A}	−36.78 ^{bc,A}	85.01 ^{ab,A}	92.6 ^{bc,A}	113.4 ^{de,A}	
	3	56.9 ^{c,B}	−34.81 ^{cd,A}	84.27 ^{ab,A}	91.2 ^{ab,A}	112.4 ^{cd,A}	
	6	44.9 ^{f,C}	−27.54 ^{ef,B}	68.01 ^{de,B}	73.4 ^{de,B}	112.0 ^{b,A}	
	16	52.47 ^{ef,B}	−17.17 ^{de,C}	63.37 ^{cd,C}	65.7 ^{c,C}	105.2 ^{b,B}	
	24	45.77 ^{e,C}	−11.32 ^{e,DE}	66.2 ^{c,B}	67.2 ^{d,C}	99.7 ^{bc,C}	
	36	34.52 ^{f,D}	−9.37 ^{d,E}	57.61 ^{de,D}	58.4 ^{ef,D}	99.2 ^{de,C}	
	48	33.75 ^{c,D}	−12.89 ^{d,D}	54.93 ^{de,E}	56.4 ^{d,D}	103.2 ^{c,B}	
Ctrl	1	70.16 ^{bc,C}	−30.61 ^{d,A}	94.78 ^{ab,A}	98.6 ^{b,A}	101.1 ^{g,C}	
	3	80.01 ^{a,A}	−30.21 ^{e,A}	90.28 ^{a,B}	95.2 ^{ab,A}	108.5 ^{e,A}	
	6	73.04 ^{ab,BC}	−30.1 ^{de,A}	96.71 ^{a,A}	99.3 ^{ab,A}	107.3 ^{c,A}	
	16	61.23 ^{cd,D}	−26.61 ^{b,B}	94.78 ^{a,A}	98.4 ^{a,A}	105.7 ^{b,AB}	
	24	62.58 ^{c,D}	−6.72 ^{g,C}	64.78 ^{cd}	65.1 ^{e,D}	95.9 ^{d,D}	
	36	78.25 ^{a,A}	−7.68 ^{e,C}	89.85 ^{a,B}	92.2 ^{a,B}	103.0 ^{bc,BC}	
	48	70.23 ^{a,C}	−8.52 ^{e,C}	69.85 ^{bc,C}	70.4 ^{bc,C}	97.0 ^{de,D}	

Note: Statistical analysis of data was performed by two-way analysis of variance (ANOVA). Treatments were carried out using different enzymatic doses (0.10–50 U/g) for various process times (1–48 h) at 25°C and 300 ppm of ZnCl₂. Values with different capital letters (A–F) differ significantly (Tukey's test, *p* = 0.05) according to process time. Values with different small letters (a–f) differ significantly (Tukey's test, *p* = 0.05) according to enzyme dose.

shortest extraction times (1 or 3 h). As regards the colorimetric parameters *C* (color saturation) and *h*° (hue), they showed the same trend as the parameter *a**, given that they are mainly determined by its value (Table 3).

3.2 | Chlorophyll kinetics during enzyme-assisted extraction: effect of zinc chloride

The data available in the literature report that using a Zn²⁺ concentration of 300 ppm, it is possible to obtain zinc–Chl complexes from spinach pulp for the production of stable food coloring (Özkan & Bilek, 2015). In this study, we investigated the possibility of preserving the color stability of Chl-based extracts by employing a lower amount of ZnCl₂ during EAE.

Figure 3a,b illustrates the effect of ZnCl₂ addition on the formation of Zn–Chl derivatives at the lowest tested enzyme dose (0.10 U/g) and at an intermediate dose (1.0 U/g). The total amount of recovered Chl increased as Zn²⁺ concentrations increased, reaching the maximum

level at 150 ppm of ZnCl₂, both at 0.10 and 1.0 U/g. No significant variations were observed at higher levels (150–300 ppm, Figure 3a). A similar trend was observed in terms of extraction yield (Figure 3b) (Table S3).

Considering the effect of ZnCl₂ concentration on Chl a and Chl b, its protective action was more evident toward Chl a (Figure 4). In detail, at 150 ppm, an increased amount in comparison to the reference sample (Ref = 0 ppm ZnCl₂) of about 2.5 and 1.5 times was revealed for Chl a and Chl b, respectively. These results appeared relevant given that Chl a is the least stable form of pigment, as described in literature. Indrasti et al. (2018) reported that Chl a degrades 2.5 times faster than Chl b due to heat, pH, and oxidation phenomena.

Colorimetric data (Table 4) show that the use of a high level of Zn²⁺ significantly improved the greenness (−*a** value) of the Chl-based extracts. The −*a** value reached the maximum level at 150 ppm, and no remarkably color value differences were observed between 150 and 300 ppm when compared to the reference sample (Ref = 0 ppm ZnCl₂). The protective effect of Zn²⁺ was more evident at the lowest enzyme dose (0.10 U/g). Accordingly, *C** and

TABLE 4 Visual color attributes [a^* , b^* , L^* , hue (h°), chroma value (C^*)] of the chlorophyll (Chl)-based extracts.

Dose (U/g)	ZnCl ₂ (ppm)	L^*	a^*	b^*	C^*	h°	Simulated color
0.10	0	53.92 ^{bc}	−33.58 ^d	80.14 ^{ab}	80.1 ^{cd}	114.5 ^{ab}	
	50	59.36 ^{ab}	−30.31 ^e	86.49 ^a	81.6 ^c	109.3 ^b	
	100	49.59 ^c	−39.66 ^b	74.76 ^b	86.6 ^b	120.3 ^a	
	150	66.08 ^a	−41.21 ^a	71.28 ^{bc}	91.8 ^a	119.4 ^a	
	200	40.99 ^d	−39.97 ^b	64.34 ^c	75.7 ^d	121.8 ^a	
	300	47.32 ^{cd}	−34.86 ^c	73.31 ^{bc}	81.2 ^c	115.4 ^{ab}	
1.0	0	54.98 ^{ab}	−34.88 ^b	80.35 ^{ab}	80.6 ^b	116.3	
	50	54.88 ^{ab}	−34.97 ^b	81.92 ^{ab}	89.1 ^{ab}	113.1	
	100	58.94 ^{ab}	−40.52 ^a	84.89 ^a	94.1 ^a	115.5	
	150	46.48 ^b	−35.58 ^{ab}	71.42 ^b	79.8 ^b	116.5	
	200	61.54 ^a	−37.23 ^{ab}	87.57 ^a	95.2 ^a	113.0	
	300	65.36 ^a	−37.79 ^{ab}	90.16 ^a	97.8 ^a	112.7	

Note: Statistical analysis of data was performed by one-way analysis of variance (ANOVA). Treatments were carried out using different enzyme doses (0.10 or 1.0 U/g) for 3 h at 25°C. Values with different small letters (a–e) differ significantly (Tukey's test, $p = 0.05$) according to ZnCl₂ amount.

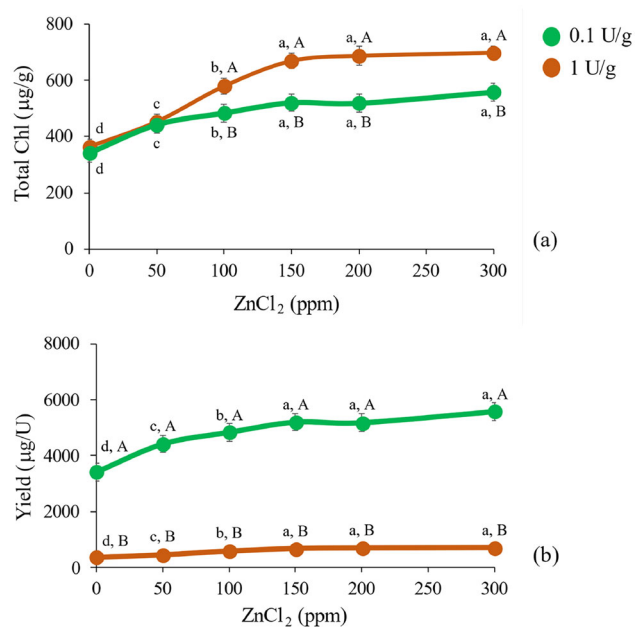


FIGURE 3 Effect of the ZnCl₂ concentration (ppm) on (a) total chlorophyll (Chl) (μg/g) and (b) extraction yield (μg/U) during enzyme-assisted extraction (EAE). Treatments were carried out for 3 h using different enzymatic doses (0.10 and 1.0 U/g) at 25°C. Reported values are mean ± 95% confidence interval of triplicate measurements. Statistical analysis of data was performed by two-way analysis of variance (ANOVA). Values with different letters (a–d) differ significantly (Tukey's test, $p = 0.05$) according to ZnCl₂ amount (ppm). Values with different capital letters (A–B) differ significantly (Tukey's test, $p = 0.05$) according to enzyme dose.

h° parameters followed the same trend of the a^* value (Table 4). Overall, our data suggested that 150 ppm ZnCl₂ was enough to ensure the formation of Zn–Chl complexes during the EAE, employing low enzyme dose at mild

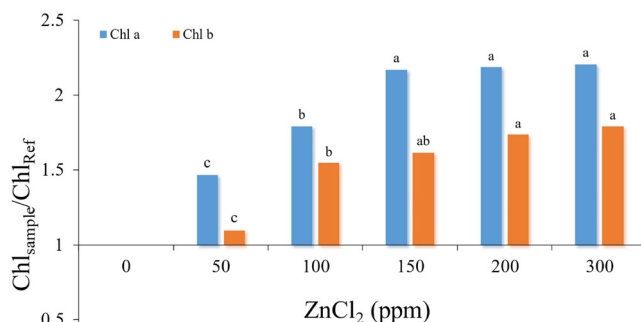


FIGURE 4 Ratio between chlorophyll (Chl) amount (μg/g) in enzyme-assisted extraction (EAE) sample (0.10 U/g) and reference sample (Ref = 0 ppm ZnCl₂) in the presence of different ZnCl₂ concentrations (ppm). Values with different letters (a–c) differ significantly (Tukey's test, $p = 0.05$) according to ZnCl₂ amount (ppm).

condition (25°C). Considering the extraction kinetics, the recovery yield and the colorimetric data, the most suitable conditions for a tailored enzymatic extraction to produce green Chl-based extracts are as follows: 0.10 U/g of enzyme dose, 3 h, and 150 ppm of ZnCl₂ at 25°C.

4 | CONCLUSION

The idea behind the present study was to apply a tailored enzyme mix *ad hoc* formulated considering the vegetable composition of the matrix cell wall, first affected by the vegetative organs (fruits, tubers, or leaves) and, second, by the vegetable species. Our results proved that the

biocatalyst dose significantly affected the recovered yield of Chl as well as the quality of greenness. At mild conditions (25°C), the highest Chl extraction yield and the best quality of green were reached using the lowest enzyme dose (0.10 U/g) for short processing time (1–3 h). The protective effect of ZnCl₂ was more evident toward Chl a, already during the extraction process. An amount of 150 ppm was enough to ensure the formation of green stable Zn–Chl complexes employing low enzyme dose and short extraction time. Our tailored and low-temperature (25°C) approach clearly appeared more effective than those based on the use of unspecific commercial enzymes (45°C), both in terms of recovered pigment and yield per enzyme unit.

The method developed for spinach is clearly applicable to other green leafy vegetables, as long as the polysaccharide composition of the pigment-holding cell wall is determined in order to optimize the *ad hoc* enzymatic formulation. Furthermore, the kinetic study applied in the present study may be helpful for the extraction of other pigments from different vegetal sources.

AUTHOR CONTRIBUTIONS

Claudio Lombardelli and **Caterina Mazzocchi**: Conceptualization; methodology; formal analysis; investigation; resources; data curation; writing—original draft; visualization. **Ilaria Benucci**: Conceptualization; methodology; validation; data curation; writing—review and editing; visualization; supervision; funding acquisition. **Marco Esti**: Conceptualization; validation; data curation; writing—review and editing; supervision; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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