



Electrospinning Encapsulation of Chlorophylls Microwave-Extracted from Spinach Waste and Utilization as Coloring Agent in Yogurt

Beyza Sukran Isik¹ · Sinan Bayram² · Filiz Altay¹

Received: 1 October 2024 / Accepted: 26 December 2024 / Published online: 10 January 2025
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Abstract

Natural colorants have become a developing market because of consumer preferences, and more cost-effective and stable production is necessary. In the present study, a natural green colorant was obtained via microwave-assisted extraction from spinach waste to produce affordable, environmentally-friendly, and innovative colorants. The extracts were encapsulated with zein via uniaxial and coaxial electrospinning techniques to overcome the stability issues associated with their natural green color. The encapsulation efficiencies of uniaxially encapsulated chlorophyll extracted from spinach waste by microwave were 63.50 and 57.18 for chlorophyll *a* and *b*, respectively. The efficiencies of chlorophyll *a* and *b* by coaxial electrospinning were found 49.53% and 88.58%, respectively. The microwave-assisted extraction and the addition of eumelanin may contribute positively to the antioxidant activity and thermal stability of electrospun samples. The stability test against light revealed that 61% of chlorophyll *a* could be preserved by uniaxial electrospinning. In addition, when chlorophyll-loaded nanofibers were incorporated into yogurt, a more stable green color was achieved during storage.

Keywords Chlorophylls · Uniaxial electrospinning · Coaxial electrospinning · Electrospinning encapsulation · Spinach waste · Microwave extraction

Introduction

Spinach is a green leafy vegetable and a good source of chlorophyll pigments. While there are more than 100 different chlorophyll structures in nature, chlorophyll *a* and *b* are the most abundant in spinach (Murcia et al., 2020; Scheer, 2006). However, spinach is a highly perishable vegetable, and between 13 and 25% of its waste is produced from damaged leaves and solid residues during the juice production process (Derrien et al., 2017). Valuable bioactive compounds are still present in wastes, which can make spinach waste a considerable raw material for natural green colorants. The extraction of bioactive compounds from waste via novel methods has become popular especially microwave extraction which is considered a sustainable and

green technique, providing an efficient solution for industrial applications (Dai & Mumper, 2010). The application of microwaves results in the rupture of plant cell walls, thereby decreasing the resistance to extraction by enhancing internal diffusion processes (Braga et al., 2022).

Chlorophylls are lipid-soluble green pigments present in the plastids of a wide range of plants, algae, and certain bacterial species. There are numerous health advantages of chlorophylls. Chlorophyll has strong antioxidant characteristics and helps prevent cancer, diabetes, cataracts, and heart disease (Murcia et al., 2020). In recent years, a growing body of scientific literature has demonstrated the significant health benefits associated with chlorophyll consumption, including antimutagenic and antigenotoxic effects. Moreover, various chlorophyll derivatives have been extensively investigated for their potential in preventing and treating specific conditions, such as cancer, infections, and dermatological disorders (Perez-Galvez et al., 2017). Owing to their vivid green color, chlorophylls also serve as food colorants and are safely incorporated into various products either in pure form or in complex with copper, such as food, cosmetics, and oral hygiene products. However, some countries limit the use of copper complexes of chlorophylls in drugs and

✉ Filiz Altay
lokumcu@itu.edu.tr

¹ Department of Food Engineering, Faculty of Chemical-Metallurgical, Istanbul Technical University, Maslak, Sarıyer, Istanbul 34469, Turkey

² Medical Laboratory Techniques, Vocational School of Health Services, Bayburt University, Bayburt, Turkey

foods because of concerns regarding the potential toxicity of copper (Simpson et al., 2012). There has been a growing interest in natural food colorants, including chlorophylls. The global market for natural food color was valued at an estimated USD 1.33 billion in 2022. Projections indicate that this market will expand at a compound annual growth rate (CAGR) of 8.3% from 2023 to 2030 (Natural Food Color Market Size, 2023). However, the instability of natural chlorophyll pigments due to exposure to light, heat, air, and extreme pH conditions limits their use in commercial areas (Hsiao et al., 2020). This problem can be overcome with encapsulation. There are many studies on the encapsulation of chlorophylls by spray drying (Kang et al., 2019; Ledri et al., 2024; Rajabi et al., 2024; Zhang et al., 2019, 2020), freeze drying (Dewi et al., 2022; Ledri et al., 2024; Rajabi et al., 2024), fluid bed technology (Yang et al., 2022), liposomes (Chu et al., 2014), complex coacervation (Agarrry et al., 2022a, 2022b), droplet microfluidics (Hsiao et al., 2020), and ionic gelation (Tekin & Ersus, 2021). However, there is currently no documentation of the encapsulation of natural chlorophyll extracts by electrospinning techniques. Electrospinning is a simple, versatile top-down process that is promising because of its high encapsulation efficiency; improved bioavailability, light, thermal, and storage stability; and advanced protection against chemical degradation (Anu Bhushani & Anandharamakrishnan, 2014; Rostambadi et al., 2020). In this study, zein was selected as a coating material for the electrospinning process. Zein is a type of prolamin storage protein that is typically found within protein bodies located in the endosperm of maize. Zein is recognized as an exceptional material for electrospinning owing to its biodegradability, flexibility, nontoxicity, and fiber-forming characteristics (Wang et al., 2019). It was also selected specifically because of its thermal and water stability and high oxygen barrier properties (Deng et al., 2018; Wang et al., 2019). To improve chlorophyll sensitivity, this study aimed to utilize the melanin pigment in addition to zein. Melanins, heterogeneous polymeric pigments, show high levels of antioxidant activity because of their phenolic and indolic components and contribute to the removal of free radicals through reduction reactions (Altındağ et al., 2022; Kumar et al., 2024). They can absorb low-wavelength, high-energy rays such as gamma rays, X-rays, and UV rays and may provide increased photostability to light-sensitive products. In addition, research emphasizes that there is an increase in the thermal stability of polymer products provided by the addition of melanin (Di Mauro et al., 2019; Shanmuganathan et al., 2011).

In this study, the aim was to extract natural green colorants from spinach waste via different extraction techniques and improve their stability via uniaxial and coaxial electrospinning. The morphology, physicochemistry, and stability of the fabricated nanofibers were further investigated, and

these materials were subsequently added to yogurt as a coloring agent.

Materials and Methods

Materials

Spinach was purchased from a local market in Istanbul (Türkiye). Spinach leaves were washed, dried, and kept at room temperature. After 4 days, spinach was no longer suitable for consumption because undesirable color change was observed and it was too wilted for consumption. After that point, spinach was considered spinach waste.

Ethanol (purity of 99.9%) was purchased from Isolab. Liquid chromatography (LC)-grade methanol, acetone, and dimethyl sulfoxide (DMSO) were acquired from Merck. Chlorophyll *a*, chlorophyll *b*, and zein were procured from Sigma Aldrich. Alkali salts of copper chlorophyllins (E141 (ii)) were purchased from Alfasol Türkiye.

Methods

Extraction

Fresh spinach and spinach waste were extracted via two different extraction methods which were traditional and microwave extraction.

For traditional extraction, chlorophyll was extracted from fresh spinach and spinach waste via a previously described method with some modifications (Zhang et al., 2017). One hundred grams of spinach was milled with a grinder for 15 s using deionized water at a ratio of 1:2 (w/v) to remove water-soluble compounds. The resulting precipitate was then extracted with 200 mL of ethanol at 60 °C for 30 min in a water bath. The solid and liquid parts were separated. The supernatant was transferred to a beaker, and the precipitate was re-extracted. The extraction mixture was placed in a refrigerator at 4 °C overnight and centrifuged at 4000 rpm for 10 min at 4 °C to separate the proteins. The supernatant was concentrated with a rotary evaporator at 40 °C. Ethanol was added to the obtained solid extract at 0.05 g/mL, and the extract was stored at −20 °C until further analysis. The chlorophyll extracts extracted from fresh spinach and spinach waste via traditional method were coded as FTE and WTE, respectively.

Microwave extraction was performed with a microwave oven (Samsung GE83XIAND). Spinach waste and fresh spinach were homogenized with a grinder for 15 s with deionized water at a ratio of 1:2 (w/v) to remove water-soluble compounds, and the precipitate was collected as in the traditional method. One hundred grams of spinach was extracted in 1000 mL of ethanol. One hundred watts was

applied for 10 min in the microwave oven, and the beaker was sealed properly with parafilm. After application, the temperature was measured as 55 °C. The solid and liquid parts were separated. The supernatant was incubated at 4 °C overnight, centrifuged at 4000 rpm for 10 min at 4 °C, and concentrated with a rotary evaporator, similar to the traditional method. Ethanol was added to the obtained solid extract at 0.05 mg/mL, and the mixture was stored at −20 °C until further analysis. The chlorophyll extracts extracted from fresh spinach and spinach waste via the microwave method were coded FMWE and WMWE, respectively.

Production of Eumelanin (Eu) Pigment The Eu pigment was produced by the *Streptomyces parvus* BSB49 strain. The production, polymerization, and purification of the Eu pigment were performed according to the procedure outlined by Bayram et al. (2020).

Uniaxial and Coaxial Electrospinning Encapsulation The electrospinning was conducted with electrospinning equipment (Inovenso NE100, Türkiye) at room temperature. The equipment included a syringe pump (New Era Pump Systems Inc., NE-300, USA), tip of the needle, collector plate, and high-voltage power supply (Nanofen, Ankara, Türkiye). The compositions and preparation conditions of the feed solutions are given in Table 1. Zein was dissolved in 80% ethanol at 400 rpm for 2 h at room temperature. Chlorophyll extracts were mixed with zein solution, and the concentration of zein in the final solution was 30% (w/v) for uniaxial electrospinning. Zein solution was prepared at 30% (w/v) for coaxial electrospinning. The Eu solution was prepared with DMSO at 1 mg/mL. The shell and core materials were mixed before electrospinning via uniaxial electrospinning. The fabrication of electrospun samples is demonstrated in Fig. 1, and the flow rates of the zein solution and chlorophyll extracts are listed in Table 1. The nanofibers were fabricated on parchment paper, which was used for covering the collector plate.

Determination of Solution Properties To assess the electrospinnability of the feed solutions, electrical conductivity, surface tension, and viscosity were measured in triplicate.

The electrical conductivity of the feed solutions was determined via a conductometer (WTW LF95, Germany) at room temperature in triplicate for each solution.

The surface tension of the feed solutions was measured by a tensiometer (Dataphysics DCAT 11 E, Germany) at room temperature in triplicate for each solution.

The viscosity of each solution was measured with a rheometer (Haake Rheostress 1, Germany) via a plate-plate sensor ($D=35$ mm, gap = 1 mm) at room temperature in triplicate for each solution. The shear rate was between 0.1 and 100 s^{-1} . The results were modeled via software (Haake

RheoWin3 Data Manager, Germany) according to the power-law equation:

$$\tau = K\dot{\gamma}^n \quad (1)$$

where τ is the shear stress (Pa), K is the consistency index ($Pa \cdot s^n$), n is the flow behavior index, and $\dot{\gamma}$ is the shear rate (s^{-1}). The apparent viscosity (η) of the samples was calculated at 100 s^{-1} via the following equation:

$$\eta = K\dot{\gamma}^{n-1} \quad (2)$$

Characterization of Nanofibers The zeta potential of each sample was measured in triplicate via a dynamic light scattering instrument (Malvern Zetasizer Nano ZS, Worcester-shire, UK) at room temperature. Distilled water was used as a dispersant. The nanofibers were dispersed into 0.2% (w/v) distilled water.

The morphologies of the electrospun samples were determined by scanning electron microscope (SEM) (Zeiss Evo LS10, Germany) at a high vacuum with an accelerating voltage. Electrospun samples measuring 1 cm^2 were coated with Au–Pd via a sputter coater (Quorum, SC7620). Images were taken at certain magnifications. The mean diameter of the fibers was determined via SEM images via ImageJ software with the DiameterJ plugin (National Institutes of Health, USA). The size distribution was evaluated via OriginLab software.

The infrared spectra of the samples were obtained via an FTIR spectrometer (Jasco-4000, UK) with an attenuated total reflection (ATR) unit attached. The spectra of each sample were measured with 64 scans and a resolution of 4 cm^{-1} . Scans were conducted over a spectral range of 400–4000 cm^{-1} .

The thermal properties of the samples were assessed via differential scanning calorimetry (DSC) (Q10, New Castle, USA) in triplicate for each sample. The spinach extracts, zein, synthetic colorant (E141 (ii)) (5 ± 0.1 mg), and nanofibers (3 ± 0.1 mg) were weighed in a DSC aluminum pan. An empty DSC-pan was used as an inert reference. The sample and the reference pans were then placed inside the calorimeter, cooled to 20 °C at a speed of 5 °C·min $^{-1}$, and heated to 300 °C at a heating speed of 10 °C·min $^{-1}$.

HPLC Analysis

The chlorophyll amount was determined via reversed-phase high-performance liquid chromatography (HPLC). Analysis was performed via an Agilent 1260 Infinity HPLC system (USA) coupled to a DAD detector (Agilent, G131D, USA). The compounds were separated via an ACE 5 C18 column (150 × 4.6 mm), and the column temperature was maintained at room temperature. The injection volume was

Table 1 Composition of the feed solution, electrospinning conditions, and characteristics of the feed solutions

Sample number	Feed solutions		Electrospinning conditions			Feed solution characteristics*			
	Core composition (A)	Shell composition (B)	Electrospinning type	Flow rate (mL/h)	Applied voltage (kV)	Distance to the collector plate (cm)	Electrical conductivity (mS/cm)	Surface tension (mN/m)	Apparent viscosity (mPa.s) at 10 s ⁻¹
1	-	30% zein (6 g) 80% ethanol (20 mL)	-	0.8	17	15	0.90 ± 0.07 ^c	25.37 ± 0.05 ^a	0.12 ± 0.01 ^c
2	-	40% zein (6 g) 80% ethanol (15 mL)	FTE (5 mL)	0.8	17	15	0.81 ± 0.02 ^b	25.20 ± 0.56 ^a	0.14 ± 0.01 ^d
3	-	40% zein (6 g) 80% ethanol (15 mL)	WTE (5 mL)	0.8	17	15	0.82 ± 0.04 ^b	25.45 ± 0.53 ^a	0.14 ± 0.01 ^d
4	-	40% zein (6 g) 80% ethanol (15 mL)	FMWE (5 mL)	0.8	17	15	0.83 ± 0.01 ^b	25.36 ± 0.61 ^a	0.15 ± 0.01 ^d
5	-	40% zein (6 g) 80% ethanol (15 mL)	WMWE (5 mL)	0.8	17	15	0.79 ± 0.03 ^b	24.71 ± 0.13 ^a	0.15 ± 0.01 ^d
6	-	20% Zein (6 g) 80% ethanol (30 mL) + Eu (5 mg) DMSO (5 mL)	WMWE (5 mL)	1.5	25	10	0.73 ± 0.01 ^a	29.26 ± 0.07 ^b	0.09 ± 0.01 ^b
7	-	25% zein (6.25 g) 80% ethanol (25 mL) + Eu (17.5 mg) DMSO (17.5 mL)	WMWE (5 mL)	8.5:1	25	10	0.72 ± 0.01 ^a	29.13 ± 0.01 ^b	0.05 ± 0.01 ^a
8	FTE	30% zein (6 g) 80% ethanol (20 mL)	-	A: 0.3 B: 0.6	17	13	-	-	-
9	WTE	30% zein (6 g) 80% ethanol (20 mL)	-	A: 0.3 B: 0.6	17	13	-	-	-

Table 1 (continued)

Sample number	Feed solutions		Electrospinning conditions			Feed solution characteristics*			
	Core composition (A)	Shell composition (B)	Electrospinning type	Flow rate (mL/h)	Applied voltage (kV)	Distance to the collector plate (cm)	Electrical conductivity (mS/cm)	Surface tension (mN/m)	Apparent viscosity (mPa.s) at 10 s ⁻¹
10	FMWE	30% zein (6 g) 80% ethanol (20 mL)	-	A: 0.3 B: 0.6	17	13	-	-	-
11	WMWE	30% zein (6 g) 80% ethanol (20 mL)	-	A: 0.3 B: 0.6	17	13	-	-	-

Eu, eumelanin; *FTE*, chlorophyll extracted from fresh spinach via traditional extraction; *WTE*, chlorophyll extracted from spinach waste via traditional extraction; *FMWE*, chlorophyll extracted from fresh spinach via microwave extraction; *WMWE*, chlorophyll extracted from spinach waste via microwave extraction

*Data are presented as the mean ± standard deviation of three replicates. The values in each column with different letters indicate statistically significant differences ($p < 0.05$)

10 µL, and the samples were injected with an autosampler (Agilent 1329B, USA). An isocratic method was used, and the mobile phase was water/methanol/acetone (4:36:60, v/v/v). The flow rate was 0.6 mL/min. The eluted peaks were detected at 661 nm (Li et al., 2014). Chlorophyll standards and extracts were diluted with methanol/dichloromethane (65/35; v/v) (Gleize et al., 2012). The electrospun samples were first dissolved in methanol/dichloromethane (65/35; v/v), and then, the zein was removed with water and acetone before HPLC analysis.

Antioxidant Activity

The antioxidant activity of the extracts and nanofibers was determined via 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH) analysis (Braca et al., 2001). A total of 25 mg of nanofibers was dissolved in methanol. One thousand-microliter extracts or nanofiber solutions were mixed with 3 mL of a 0.004% methanol solution of DPPH. After 30 min in the dark, the absorbance was read at 517 nm against methanol ($R^2 = 0.96$).

Light Stability of Chlorophylls

The extracts and nanofibers were exposed to direct sunlight to determine the stability of the chlorophyll. First, 0.1 g of nanofibers was dissolved in 2 mL of ethanol (80%, v/v). Samples were collected at days 0, 1, 4, 7, and 10. The chlorophyll content was determined according to the aforementioned method.

The Addition of Chlorophylls to Yogurt

Yogurt was chosen as a food sample. Due to the lower stability of chlorophyll at low pH, yogurt, a low-pH product, was chosen to examine how the processes affect chlorophyll stability in a positive manner. Furthermore, as yogurt is a white food product, it provides a clearer contrast for observing any color changes in the green pigment over time, facilitating a more effective assessment of visual appearance.

The chlorophyll-loaded electrospun samples (0.1 g) were mixed with yogurt. Five milliliters of chlorophyll extracts and synthetic colorant (E141 (ii)) were added into yogurt and homogeneous end products were obtained. A colorimeter (Konica Minolta Chromameter CR-400, Japan) was used to determine the CIE L^* , a^* , and b^* values of the samples, where L^* is the lightness of color (100 = white, 0 = black), a^* value ($+a^*$ = red, $-a^*$ = green), and b^* value ($+b^*$ = yellow, $-b^*$ = blue). The measurements were conducted at days 0, 1, 3, 5, and 7. The total color difference (ΔE^*) is determined via Eq. 3, as outlined in a previous study (Mercali et al., 2014). The formula is as follows:

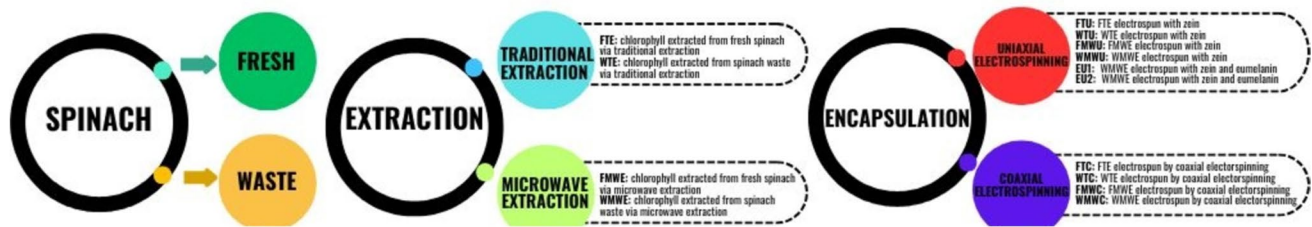


Fig. 1 An illustrative scheme for fabrication of electrospun samples

$$\Delta E^* = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2} \quad (3)$$

where ΔE^* represents the total color difference, Δa^* represents the difference between the a^* values of the control and the sample, Δb^* represents the difference in b^* values between the control and the sample, and ΔL^* represents the difference in L^* values between the control and sample. The color at day 0 was selected as the control. The synthetic colorant (E141 (ii)) was also examined for comparison.

Statistical Analysis

IBM SPSS Statistics 28 (Chicago, IL, USA) software was used for the statistical analysis. The data are presented as the mean $\pm$ standard deviation ($n=3$). One-way analysis of variance (ANOVA) was conducted via Duncan's multiple range test. Differences between means were considered significant at $p < 0.05$.

Results and Discussion

Effect of the Extraction Method on the Chlorophyll Content

The chlorophyll a and b contents of fresh and waste spinach extracted via traditional and microwave extraction are shown in Table 2. According to the results, fresh samples contained higher chlorophyll a and b contents than waste samples. While the highest chlorophyll a content (13.62 ± 0.10 mg/g dry weight (DW)) was measured for fresh spinach extracted via the traditional extraction method (FTE), fresh spinach extracted via microwave extraction (FMWE) presented the highest chlorophyll b content (6.98 ± 0.22 mg/g DW).

The chlorophyll content of fresh spinach reported in the literature significantly varies. While one of the studies reported chlorophyll a and b contents in spinach at 37.4 ± 3.4 and 13.9 ± 2.0 mg/100 g, respectively (Dermeşonluoglu et al., 2015), another study reported that 100 g of spinach contained 103.37 ± 3.94 mg of chlorophyll a and 32.89 ± 0.76 mg of chlorophyll b (Lee et al., 2005). Another

Table 2 Chlorophyll content of the extracts

Sample	Chlorophyll a (mg/g DW)	Chlorophyll b (mg/g DW)	Yield (%)
FTE	13.62 ± 0.10^d	5.76 ± 0.11^c	41.90 ± 1.69^b
WTE	9.87 ± 0.19^b	3.89 ± 0.01^a	30.42 ± 2.34^a
FMWE	11.52 ± 0.14^c	6.98 ± 0.22^d	80.57 ± 2.99^d
WMWE	5.74 ± 0.01^a	4.63 ± 0.11^b	65.11 ± 1.19^c

The data are displayed as the means $\pm$ standard deviations of three replicates. The values in each column with different letters are statistically significant ($p < 0.05$)

FTE, fresh spinach extracted via traditional extraction; WTE, spinach waste extracted via traditional extraction; FMWE, fresh spinach extracted via microwave extraction; WMWE, spinach waste extracted via microwave extraction

study revealed 71 different spinach genotypes and reported that the chlorophyll a and b contents ranged from 475.76 to 1196.45 and from 200.91 to 522.60 nmol/g FW, respectively, in fall-grown spinach. The chlorophyll content of spinach was reported to range between 13.61 and 15.82 mg/g for chlorophyll a and between 9.16 and 10.18 mg/g for chlorophyll b in a recent study (Nipa et al., 2023). In spring-grown spinach, the chlorophyll a content varies from 646.66 to 1400.00 nmol/g, and the chlorophyll b content fluctuates from 219.26 to 474.46 nmol/g (Hayes et al., 2020). The phytochemical composition of spinach is affected by climatic conditions during the growing season (de Azevedo-Meleiro & Rodriguez-Amaya, 2005), nitrogen status (Rorie et al., 2011), leaf maturity (Farnham et al., 2012), cultivation methods (Koh et al., 2012), postharvest processing conditions (Leong & Oey, 2012; Martínez et al., 2013; Mendelová et al., 2014), and plant genotypes (Hayes et al., 2020; Kidmose et al., 2001). In addition, different extraction conditions and determination methods can lead to different results. Therefore, the chlorophyll content of the spinach extracts determined in this study may differ from the results reported in the literature.

The chlorophyll contents of the waste samples were lower than those of the fresh samples. Chlorophyll degrades when exposed to light, air, and heat, resulting in the loss of the phytol side chain or the removal of central Mg^{2+}

(Simpson et al., 2012). The lowest chlorophyll *a* content (5.74 ± 0.01 mg/g DW) was detected in spinach waste extracted via microwave extraction (WMWE) and spinach waste extracted via traditional extraction (WTE) presented the lowest chlorophyll *b* content (3.89 ± 0.01 mg/g DW). In this study, spinach was kept at room temperature for 4 days, after which it was considered spinach waste. There are several studies concerning the chlorophyll content of spinach during storage. One of the studies measured the chlorophyll *a* and *b* contents of spinach stored at 5 °C and 20 °C. The chlorophyll *a* content decreased from 103.37 ± 3.94 mg/100 g to 24.53 ± 4.84 mg/100 g at 20 °C. Even though the chlorophyll *b* content increased from 32.89 ± 0.76 mg/100 g to 33.47 ± 2.02 mg/100 g, the content was 42.02 ± 2.26 mg/100 g on the first day of storage at 20 °C. The chlorophyll *b* content first increased during storage but then started to decrease on day 2 (Lee et al., 2005). Another study reported that the chlorophyll *a* content decreased continually to $\approx 65\%$ of the initial level by day 4 at 25 °C. Moreover, the chlorophyll *b* content decreased similarly to that of chlorophyll *a* but at a lower rate (Yamauchi & Watada, 1991). Similar results were also observed in this study. Although the rates of decrease in chlorophyll *a* and *b* changed, a decrease was recorded for both extraction methods.

According to the results, the traditional extraction method resulted in a higher chlorophyll *a* content than microwave extraction. These results can be explained in several ways. The most likely explanation could be the conversion of chlorophyll *a* to chlorophyll *b* because the chlorophyll *b* contents of the microwave samples were higher than those of the samples extracted via traditional method. The transformation of chlorophylls *a* and *b*, which is also called the chlorophyll cycle, is believed to yield either chlorophyll *a* or *b*, according to particular physiological needs (Rüdiger, 2002). The metabolism of chlorophyll *b* plays a crucial role in the mechanisms of light acclimation in plants (Hu et al., 2021). Chlorophyll *a* is crucial for photochemical processes, whereas chlorophyll *b* enhances a plant's ability to capture a broader spectrum of light, particularly because it has strong absorption at approximately 450 nm, a wavelength that chlorophyll *a* does not absorb efficiently. This characteristic makes chlorophyll *b* particularly important for increasing a plant's light-harvesting ability (Larkum et al., 2018). Furthermore, the biosynthesis and degradation of chlorophyll *b* are closely associated with the assembly and disassembly of light-harvesting complexes, which can be regulated according to varying light conditions (Tanaka & Tanaka, 2011). Vegetables continue to undergo biochemical processes due to their modular structure and ability to maintain physiological autonomy once harvested and placed on supermarket shelves. One of the studies investigated whether endogenous rhythms persist in commercial vegetables and, if so, how

these cycles might influence their nutritional quality. It was observed that Chl *a/b* ratios oscillated under both constant light and darkness in packaged rocket leaves, whereas oscillations in total Chl *a + b* were detected exclusively under darkness conditions (De Larrinaga et al., 2019). The conversion could also be triggered during microwave extraction.

The chlorophyll structure consists of four pyrrole groups linked by a central magnesium ion to form a tetrapyrrole (or porphyrin) ring system, with a 20-carbon hydrocarbon side chain known as a phytol group. The central magnesium atom of chlorophyll may be sensitive to microwaves as a result of the ionic conduction mechanism (Yan et al., 2023). Interactions between the central magnesium atom and microwaves may cause localized heating or changes in the electronic structure around the magnesium atom, potentially affecting the stability of the chlorophyll molecule.

The chlorophyll binding site is situated in a predominantly hydrophobic environment, with an estimated dielectric constant (ϵ) of approximately 2, which is representative of the typical optical dielectric constant observed in organic molecules (Krishtalik et al., 1997). The dielectric constant is a measure of a material's ability to reduce the electric field within it, essentially indicating how easily the material can be polarized by an electric field. The estimated value of $\epsilon = 2$ is quite low, implying that the binding site does not easily polarize in response to an electric field. Materials with a low dielectric constant absorb microwaves less efficiently because of their reduced ability to interact with the oscillating electric field of microwaves. This reduced interaction means that they convert microwave energy into heat less effectively. Another explanation could be the duration of interaction with the solvent, which was nine times lower in microwave extraction than in traditional extraction, so extraction could not be completed completely.

In the extraction of chlorophyll from fresh and waste spinach, the extraction yield using microwave extraction has been found to be higher compared to the traditional method. The extraction yields from fresh and waste spinach using microwave-assisted extraction were found 80.57 and 65.11%, respectively, while the yields obtained through traditional extraction methods were 41.90 and 30.42%, respectively. The yields of chlorophyll extracted from spinach byproducts via conventional green extraction and supercritical extraction methods were determined in consecutive studies, and the highest yields were 96% and 50% for conventional and supercritical extraction methods, respectively (Derrien et al., 2017, 2018). The chlorophyll contents of green gooseberry plants extracted via conventional and ultrasonic-assisted extraction were analyzed, and the highest extraction yield was obtained via traditional extraction (Hussain et al., 2023). One of the studies on chlorophyll extraction from microalgae via traditional, microwave, and supercritical fluid extraction methods reported that a relatively high chlorophyll content

was obtained via microwave extraction (Georgiopoulou et al., 2023). On the other hand, another study compared traditional, microwave, and pressurized liquid extraction methods, and the extraction yield and extraction amount were lower for microwave extraction (Gilbert-López et al., 2017). The recovery of pigments from a macroalgae, *Ulva rigida*, by traditional, microwave-assisted, and ultrasound-assisted extraction methods was found to be statistically similar in another study (Martins et al., 2021). Even though the results seem contradictory, the nature of the samples and solvents, appliance used in the studies, and process conditions might have significantly affected the results. Another important result from this study is that although microwave-assisted extraction provided higher yields, the chlorophyll content was found to be lower compared to that obtained through traditional extraction methods.

Electrospinnability and Properties of the Feed Solutions

The feed solution properties help explain electrospinnability. The electrical conductivity, surface tension, and viscosity of the solutions are presented in Table 1.

The electrical conductivity of the solution should be greater than zero because only conductive polymers have a high repulsion force to overcome the surface tension of a droplet to form a nanofiber (Wang et al., 2013a, 2013b). The highest conductivity was measured for the zein solution (0.90 ± 0.07 mS/cm). The addition of chlorophyll to the zein solution decreased the conductivity. Furthermore, there was no significant difference between solutions prepared with different chlorophyll extracts. However, the solutions prepared with Eu (solutions 6 and 7) were less conductive than the other solutions ($p < 0.05$). The conductivity of both synthetic and natural Eu has been documented to range from 10^{-13} to 10^{-5} S/cm (Meredith & Sarna, 2006; Osak et al., 1989), and this variation can be explained by the measurement conditions, especially the humidity in the environment (Jastrzebska et al., 1995). The fact that the solution prepared with Eu is less conductive can be explained by the quinones and hydroquinones found in the structure of eumelanins. These reduced and oxidized components give melanins semiconductor properties. Owing to their biocompatible and biodegradable semiconductor properties, the Eu pigment, which is an organic semiconductor, has the potential to be used in the field of organoelectronics (Matos-Peralta et al., 2023). The mildly conductive property of Eu might affect the conductivity and cause less conductive last solutions. While zein and zein with chlorophyll extract solutions produced by uniaxial electrospinning were successfully electrospun and resulted in unbeaded nanofiber formations, nanofiber formations were not obtained with solutions containing Eu

(Solutions 6 and 7), which presented the lowest conductivity results. The lower electrical conductivity of the solutions might have caused the beaded structure in this study.

To achieve nanofiber morphology by electrospinning, the repulsion force should surpass the surface tension. When the surface tension exceeds a certain threshold, droplet formation occurs instead of the desired nanofiber structure (Ki et al., 2005). The surface tension of the solutions was measured the same statistically for solution prepared with different chlorophyll extracts and zein ($p < 0.05$). Higher surface tension results were measured for solutions with Eu (solutions 6 and 7). The surface tension of water is greater than the surface tension of dimethyl sulfoxide (DMSO). The surface tension of DMSO at 20 °C is 43.5 mN/m, and the surface tension of water is 72.8 mN/m at 20 °C (Kalová & Mareš, 2022; Zhao et al., 2021). In this study, an 80% ethanol solution was used, and its surface tension was measured as 24.8 mN/m at 20 °C (Organisation Internationale de Métrologie Légale, 1975). According to these measurements, the surface tension of the 80% ethanol solution is lower than that of DMSO, and the higher surface tension of EU1 and EU2 could be explained in this manner. Additionally, the negatively charged eumelanin pigment may have increased the surface tension of the DMSO solution. The second assumption explaining the high surface tension of the Eu/DMSO solution may be that this solution was prepared at a low concentration. On the basis of these results, when we evaluate the eumelanin/DMSO solution, it can be said that eumelanin prepared in a DMSO solution is a disadvantageous product in electrospinning applications. Nanofiber formation was not achieved with solutions 6 and 7 (Fig. 1i and j). High surface tension could affect the morphology of the electrospun samples.

Viscosity is a crucial parameter in the electrospinning process. Notably, all the solutions displayed Newtonian characteristics, and all the solutions with extracts were found to be significantly the same except for EU1 and EU2 ($p < 0.05$). The viscosity of the zein solution was lower than that of the zein and chlorophyll solutions. The interaction between the extracts and zein might cause an increase in viscosity. A previous study reported that there is a direct proportional relationship between the viscosity of a solution and the average diameter of ultrathin fibers (İnanç Horuz & Belibağlı, 2018). According to the results of this study, as the viscosity of the feed solution increased, the average diameter decreased. The thinnest nanofibers (557.90 ± 129.41 and 652.80 ± 151.10 nm) were obtained from the solutions with the highest viscosity (0.15 ± 0.01 mPa.s). The lowest viscosity value was obtained for solution 7 (0.05 ± 0.01 Pa.s). The lower viscosity values were probably due to the lower zein concentrations used in solutions 6 and 7. An increase in viscosity promotes the formation of smooth, bead-free fibers (Lu et al., 2016). The entanglement of molecules and

viscosities of EU1 and EU2 were not enough for nanofiber formation.

Characterization of the Electrospun Samples

Zeta potential Measurements

The zeta potentials of the electrospun samples were measured, and the results are shown in Table 3. All of the electrospun samples had negative zeta potentials except zein. The zeta potential of samples prepared via chlorophyll extracts derived from microwave-assisted extraction (FMWU and WMWU) was greater than that of their counterparts prepared from extracts obtained via traditional extraction methods (FTU and WTU). A similar pattern was also observed with the coaxial electrospinning method. To maintain the suspension in a stable and dispersed state, the zeta potential values should exceed +25 mV or fall below −25 mV (Anonymous, 1999). According to the results, the electrospun samples produced with fresh and waste spinach extracts by microwave extraction were more stable than the other samples prepared with extracts obtained via traditional extraction. The EU 1 and EU 2 samples were prepared with WMWE, and their zeta potential values were lower than those of WMWU ($p < 0.05$). The addition of Eu and a lower amount of zein might lead to a decrease in the zeta potential.

After the addition of chlorophyll extracts into the nanofiber system, the zeta potential of the nanofibers

transitioned from a positive charge to a negative charge. Considering that the zeta potential is a measurement of the electrical repulsive forces between particles and is an expression of surface charge, reconfiguration of the surface coverage and greater unfolding of anionic groups could explain the measurement results (Agarry et al., 2022a, 2022b). The change in zeta potential was greater in the microwave samples than in the traditional samples for both electrospinning methods. Therefore, the extraction method has a significant effect on the morphology of the nanofibers.

SEM The surface morphologies of the samples are shown in Fig. 2. SEM analysis revealed that all the samples had fibrous structures except EU1 and EU 2 (Fig. 2i and j). In addition, samples electrospun by coaxial electrospinning presented lower average diameter results but beady fiber formation. While EU 1 represented relatively thin but beady fiber formation, no fiber formation was observed for EU2.

The average diameters of zein (k) as a control and samples containing chlorophyll extracts (a, b, c, d, and i) electrospun by uniaxial electrospinning were calculated as 905.72 ± 233.30 , 899.18 ± 212.34 , 865.60 ± 191.65 , 557.90 ± 129.41 , 652.80 ± 151.10 , and 566.46 ± 207 nm, respectively. Nanofibers produced with microwave extracts (FMWE and WMWE) resulted in the formation of thinner fibrous nanofibers (Fig. 2c and d). Even though these samples had higher viscosity values than the samples prepared with extracts obtained via traditional extraction, FMWE had

Table 3 Zeta potential, diameter, and encapsulation efficiency of the electrospun samples

Sample no/code	Zeta potential (mV)*	Nanofiber diameter (nm)	Encapsulation efficiency (%)**		DPPH (mg trolox/g dry weight)***
			Chlorophyll a	Chlorophyll b	
1	0.16 ± 0.03^h	905.72 ± 233.30^{bc}	-	-	-
2	-0.47 ± 0.10^{bc}	899.18 ± 212.34^{bc}	79.31 ± 0.15^h	77.99 ± 2.10^D	21.72 ± 0.47^e
3	-0.37 ± 0.11^{cd}	865.60 ± 191.65^{bc}	63.43 ± 0.07^e	82.95 ± 0.04^E	16.02 ± 0.44^d
4	-0.68 ± 0.05^a	557.90 ± 129.41^{ab}	70.75 ± 0.25^f	72.16 ± 0.10^C	21.95 ± 0.22^e
5	-0.68 ± 0.05^a	652.80 ± 151.10^{ab}	63.50 ± 0.21^e	57.18 ± 0.74^A	21.07 ± 0.29^e
6	-0.52 ± 0.11^b	566.46 ± 207.00^{ab}	61.37 ± 0.24^d	61.45 ± 0.97^B	25.49 ± 0.53^f
7	-0.35 ± 0.01^{de}	-	57.13 ± 1.27^c	70.48 ± 1.37^C	30.46 ± 0.51^h
8	-0.17 ± 0.01^{fg}	341.68 ± 106.55^a	53.50 ± 0.77^b	126.63 ± 0.48^H	8.54 ± 0.13^{ab}
9	-0.13 ± 0.03^g	303.52 ± 86.75^a	61.59 ± 0.19^d	121.38 ± 1.96^G	8.08 ± 0.20^a
10	-0.25 ± 0.03^{ef}	1061.16 ± 319.85^c	75.47 ± 0.27^g	124.39 ± 4.03^{GH}	10.81 ± 0.36^c
11	-0.32 ± 0.05^{de}	309.32 ± 100.37^a	49.53 ± 0.36^a	88.58 ± 2.01^F	9.83 ± 1.17^{bc}
FTE	-	-	-	-	37.73 ± 1.39^i
WTE	-	-	-	-	28.84 ± 0.52^g
FMWE	-	-	-	-	49.86 ± 2.78^j
WMWE	-	-	-	-	25.31 ± 0.33^f

*Data are displayed as the mean $\pm$ standard deviation of three replicates. Different letters indicate statistically significant differences ($p < 0.05$)

**Data are displayed as the mean $\pm$ standard deviation of three replicates. The lowercase and uppercase letters indicate significant differences in chlorophyll a and b, respectively ($p < 0.05$)

***Data are displayed as the mean $\pm$ standard deviation of three replicates. Different letters indicate significant differences ($p < 0.05$)

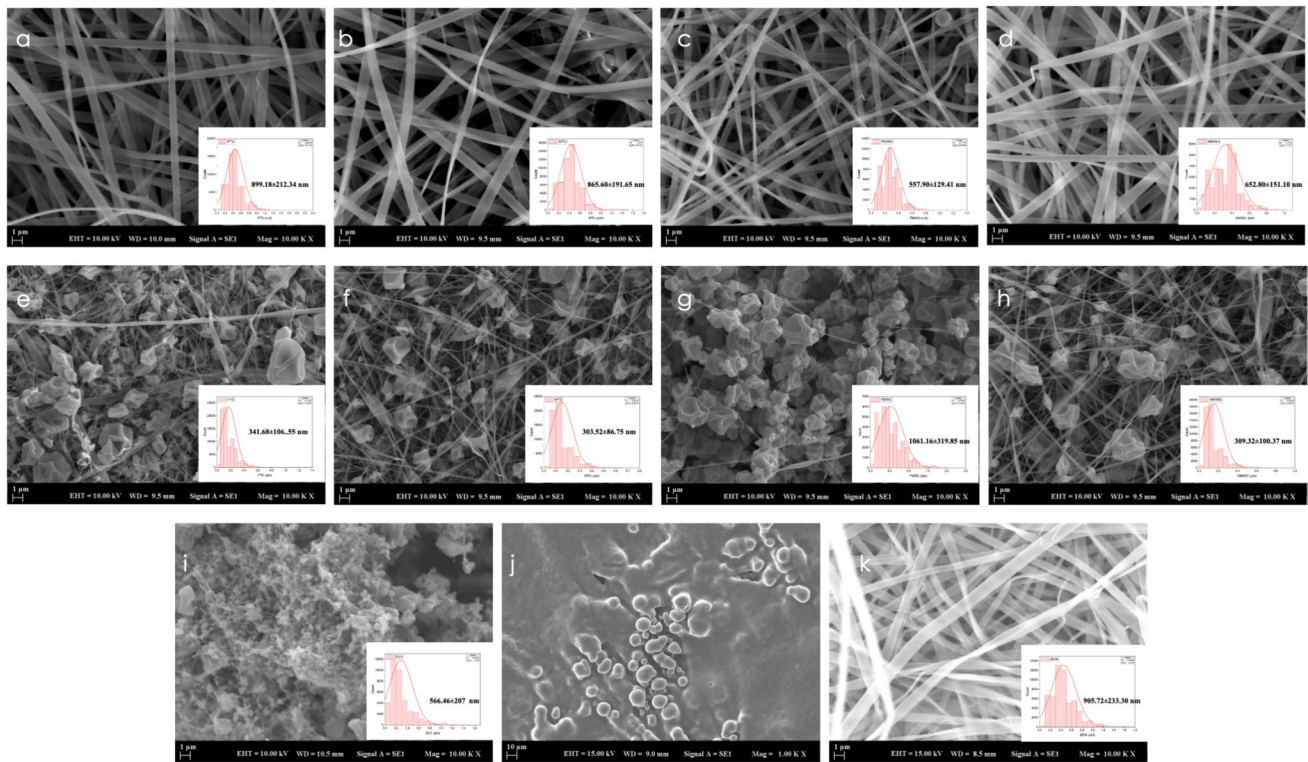


Fig. 2 SEM images of electrospun samples. FTU (a), WTU (b), FMWU (c), WMWU (d), FTC (e), WTC (f), FMWC (g), WMWC (h), EU1 (i), EU2 (j), and zein (k) samples at 10,000 $\times$ magnification. FTU, WTU, FMWU, and WMWU: electrospun samples prepared with FTE, WTE, FMWE, and WMWE, respectively, via uni-

axial electrospinning; EU 1 and 2: electrospun samples prepared with eumelanin and WMWE via uniaxial electrospinning; FTC, WTC, FMWC, and WMWC: electrospun samples prepared with FTE, WTE, FMWE, and WMWE, respectively, via coaxial electrospinning

the highest conductivity and the lowest surface tension values measured for WMWE (Table 3). These properties could lead to the formation of thinner nanofibers.

Compared with the other samples, the EU 1 and EU 2 samples presented the lowest conductivity and viscosity and the highest surface tension values and were prepared with lower zein concentrations. It is known that insufficient concentrations of zein result in bead formation due to the unavoidable occurrence of Rayleigh instability (Chang et al., 1999). In addition, high surface tension could cause bead formation in the EU 1 and EU 2 samples (Fong et al., 1999).

In coaxial electrospinning, all the samples were in beady formation. Insufficient entanglement of polymers can result in the formation of beads or droplets during the electrospinning process (Kriegel et al., 2009). Zein solution was sent to the system separately in coaxial electrospinning and the viscosity of the zein solution without chlorophyll might result in bead and beaded nanofiber formation.

FTIR Measurements The infrared spectra of the chlorophyll extracts and nanofibers are depicted in Fig. 3.

The observed peaks in the chlorophyll extracts are consistent with previous studies (Kang et al., 2019; Sengupta

et al., 2015; Yang et al., 2022; Zhang et al., 2019). The broad absorption observed at 3305 cm^{-1} is attributed to the O–H stretching vibration of ethanol. The sharp peak at 2971 cm^{-1} could be indicative of the C–H stretching vibration of chlorophyll. The peaks at 2878 and 2880 cm^{-1} are caused by asymmetric and symmetric CH_2 and CH_3 stretching. The pronounced peak at 1640 cm^{-1} is consistent with C=O bonding, and the peaks observed in the ranges of 1045–1100 cm^{-1} and 1377 cm^{-1} could be attributed primarily to the C–C and C–N vibrations of the tetrapyrrole ring structure of chlorophyll.

The characteristic zein spectrum has an –OH group at 3340 cm^{-1} , aliphatic C–H stretching bands at 2960 and 2868 cm^{-1} , an amide peak I at ~1650 cm^{-1} , an amide II at 1530 and 1515 cm^{-1} , an amide III at 1240 cm^{-1} , and C–N stretching at 1445 cm^{-1} (Bumedi et al., 2023; Dehcheshmeh & Fathi, 2019; Sessa & Woods, 2011). The absorbance bands of zein nanofiber were observed for –OH group at 3284 cm^{-1} , carboxylic acids at 2958 and 2873 cm^{-1} , amide I (C=O stretching) at 1644 cm^{-1} , amide II (N–H bending) at 1531 and 1519 cm^{-1} , amide III (C–N stretching) at 1241 cm^{-1} , and C–N stretching at 1446 cm^{-1} .

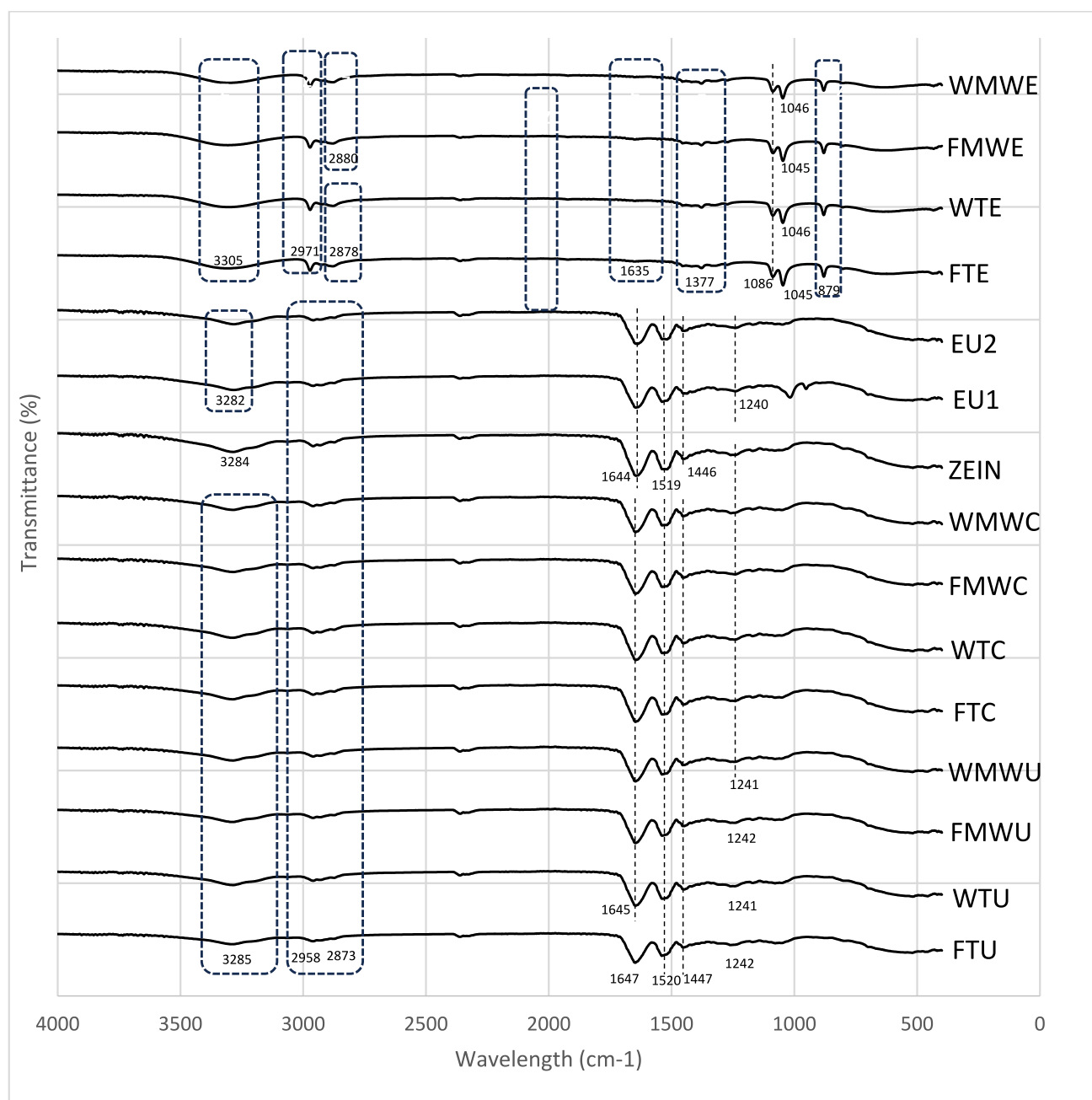


Fig. 3 FTIR spectra of the chlorophyll extracts and nanofibers

The spectrum of the chlorophyll-loaded nanoparticles underwent some changes. Compared with that of the extracts, the spectrum of the chlorophyll-loaded nanoparticles exhibited a downward shift from 3305 to 3285 cm^{-1} . Furthermore, compared with the peak intensities of the extracts in the wavenumber range of 2800–3000 cm^{-1} , a reduction in the peak intensities was observed for the nanoparticles. The observed decrease in the vibrations of the methylene and methyl groups might be attributed to the interaction between the chlorophyll phytol group, which

serves as the core, and the hydrophobic regions of zein, which act as wall (Ledri et al., 2024; Zhang et al., 2020).

The impact of incorporating chlorophyll extracts into zein is most evident, particularly in the –OH group (3284 cm^{-1}), the amide I (1644 cm^{-1}), and amide II (1531 and 1519 cm^{-1}) regions. The observed changes in these regions may be attributed to the hydrophobic interaction between chlorophyll and zein (Agarrry et al., 2023). While EU 1 and EU 2 exhibited similar spectra, alterations were detected in specified regions compared with WMWU.

The Eu samples showed a downward shift from 3285 to 3282 cm^{-1} , 1645 to 1644 cm^{-1} , 1520 to 1519 cm^{-1} , 1447 to 1446 cm^{-1} , and 1241 to 1240 cm^{-1} . The addition of Eu to the electrospun samples might interact with zein and chlorophyll and cause alterations in the spectra. Moreover, the intensities of these peaks were greater in samples electrospun by uniaxial electrospinning than in those electrospun by coaxial electrospinning.

The formation of an interaction between the core and the wall could be inferred by observing changes in the intensity of the bands or the appearance of new bands in the electrospun samples in comparison to the samples of extracts and zein nanofiber. Consequently, successful encapsulation via uniaxial and coaxial electrospinning techniques was confirmed.

DSC Measurements The thermal properties of the extracts, zein, and nanofibers were examined via DSC, and the results are shown in Fig. 4. As shown in Fig. 4a, endothermic peaks were observed for all the chlorophyll extracts. The chlorophyll extracts obtained via traditional extraction (FTE and WTE) presented similar profiles, whereas FTE had endothermic peaks at 130.25 and 134.8 °C, and peaks at 138.49 and 143.33 °C were observed in the WTE sample. According to several studies (Agarry et al., 2022a, 2022b; Kang et al., 2019; Ledri et al., 2024), these peaks could be the melting points of chlorophylls, which are known to melt at 115–133 °C. The samples extracted via microwave extraction (FMWE and WMWE) presented peaks at 101.53–107.5 °C, which can be related to the loss of bound water. Similar results were reported in a previous study (Agarry et al., 2022a, 2022b). Similar peaks at 122.86–136.22 °C and 128.83–142.47 °C, as observed in the FTE and WTE, could be related to chlorophyll. As reported in previous studies, carotenoids melt within the range of 175–200 °C (Sy et al., 2012), and since no peak within this range was observed in any of the extracts, it can be inferred that a selective extraction process for chlorophyll was applied during both traditional and microwave-assisted extraction procedures.

Zein showed an endothermic peak at 144.18 °C, and after electrospinning, the peak shifted to 158.39 °C in the zein nanofiber (Fig. 4b). The highest degradation temperatures were detected for EU 1 (170.62 °C) and EU 2 (169.93 °C). While peaks were observed between 159.82 and 162.37 °C in the electrospun samples obtained via uniaxial electrospinning, the degradation temperatures were measured between 149.58 and 157.83 °C for the samples obtained via coaxial electrospinning (Fig. 4b). Nanostructures containing chlorophyll extracts did not show any additional peak; therefore, it can be stated that chlorophyll is compatible with zein. Comparable results have also been reported in a previous study (Agarry et al., 2022a, 2022b).

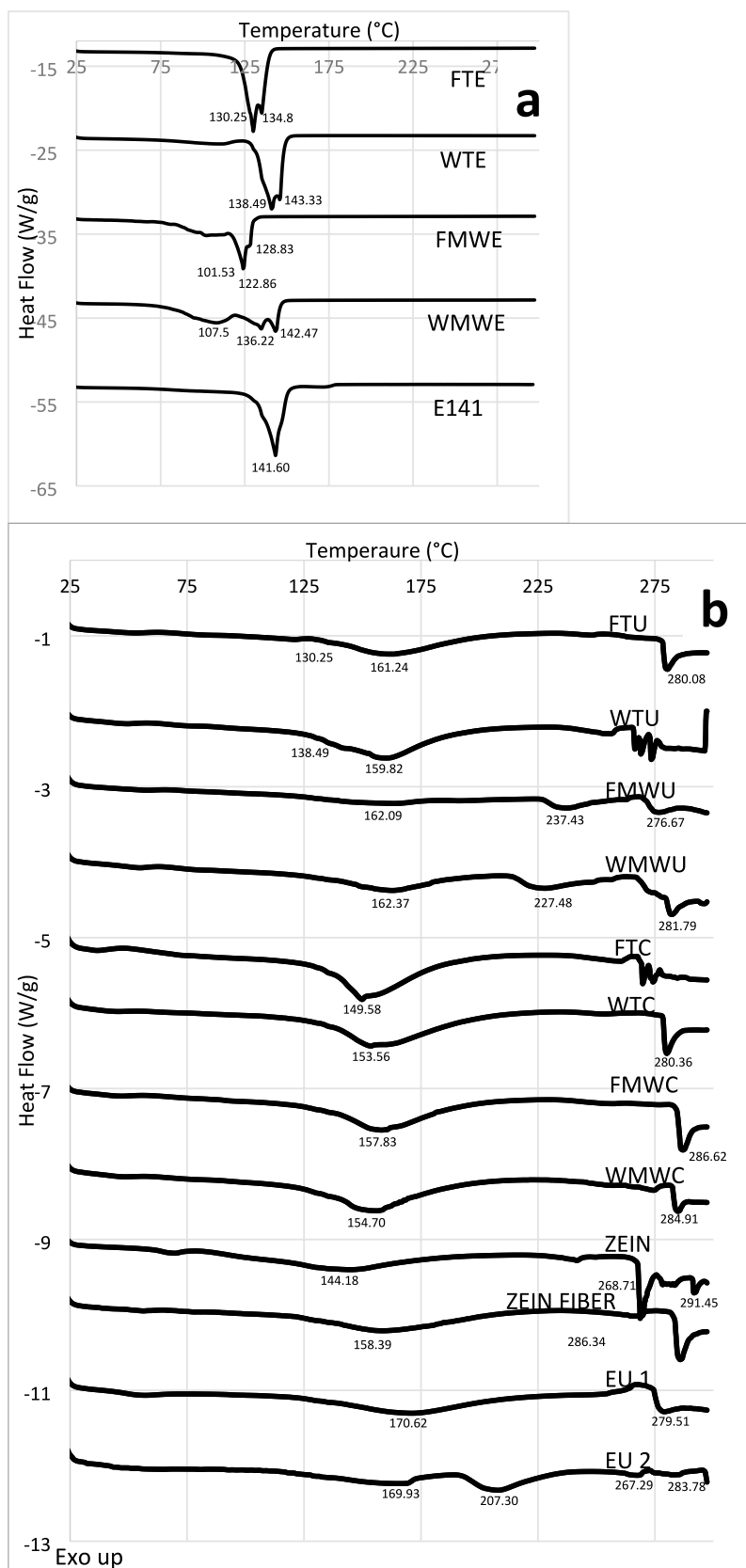
The DSC assay results demonstrated that chlorophyll was successfully encapsulated with zein by electrospinning. The chlorophyll extracts encapsulated with zein presented higher degradation temperatures than did the chlorophyll extracts and E141 (ii), indicating the thermal protection provided by the electrospinning method. In addition, because the temperatures of degradation were lower for the coaxial electrospinning samples than for the uniaxial electrospinning samples, it can be claimed that uniaxial electrospinning enables better thermal protection than coaxial electrospinning. This result could be the result of bead formation in the coaxial electrospinning samples. All of our samples electrospun by coaxial electrospinning showed beaded fiber formations (Fig. 1e, f, g, and h). In a previous study, the presence of beads within the fiber mats significantly compromised their structural integrity and resulted in increased fragility. Consequently, this weak lamination caused the beaded fibers to be more prone to breakage. This increased susceptibility to fracture may have also decreased the thermal stability of the samples. Additionally, based on the results, the incorporation of Eu into the system improved the thermal stability of chlorophyll.

Encapsulation Efficiency of Electrospun Samples

The encapsulation efficiencies of the electrospun samples produced via uniaxial electrospinning ranged from 57.13 to 82.95%. In uniaxial electrospinning, wall and core materials are mixed and delivered through the same tip into the electrical field. Therefore, the encapsulation efficiency can be affected by the interaction between the wall and core materials. The hydrophobic interactions between the nonpolar regions of zein and the hydrophobic regions of chlorophyll molecules could impact the stability of the chlorophyll molecules within the nanofibers. According to previous studies, increasing the number of hydrophobic regions and increasing the number of nonpolar interactions and polar bonds of the carbonyl group of the chlorophyll molecule improve the encapsulation efficiency (Agarry et al., 2023; Zhang et al., 2020). In this study, the highest encapsulation efficiency of chlorophyll *a* was determined for FTU and the lowest efficiency was measured for EU 2. The lowest values were determined for electrospun samples prepared with waste extracts (EU 2, EU1, WMWU, and FMWU). It can be deduced from the data that nanofibers prepared from fresh spinach extracts result in higher encapsulation efficiencies for chlorophyll *a*.

The encapsulation efficiency of chlorophyll *b* did not follow the same pattern as that of chlorophyll *a*. While one of the lowest efficiencies was for the WTU for chlorophyll *a*, the highest efficiency of chlorophyll *b* was achieved for the WTU. Additionally, higher efficiencies were calculated for the WTU and FMWU samples for chlorophyll *b* than for

Fig. 4 DSC profiles of extracts (a), zein (b), and nanofibers (b)



chlorophyll *a*. In a previous study of the microencapsulation of chlorophyll, the encapsulation efficiency of chlorophyll *b* was also greater than that of chlorophyll *a* (Dewi et al., 2022).

Importantly, the extraction technique significantly influences the encapsulation efficiency. FTU and WTU had higher encapsulation efficiencies than FMWU and WMWU for both chlorophyll *a* and *b*. Extraction by microwave energy could influence the chemical structure of chlorophyll molecules and the ability to interact between wall material and chlorophyll, resulting in lower encapsulation efficiencies in uniaxial electrospinning.

The encapsulation efficiency of the nanofibers prepared via coaxial electrospinning ranged from 49.53 to 126.64%. The encapsulation efficiency of chlorophyll *a* was lower than that of uniaxially aligned nanofibers, except for the FMWC sample. Additionally, the efficiencies of chlorophyll *b* in addition to WMWC were calculated to be higher than 100%, unusually. In coaxial electrospinning, the core and wall materials are fed into the system separately and spun simultaneously. The lower chlorophyll *a* efficiency and exceptional encapsulation efficiency of chlorophyll *b* could be related to the conversion of chlorophyll *a* to chlorophyll *b* because of the high voltage applied directly to the chlorophyll extracts during processing.

Antioxidant Activity

The antioxidant activities of the extracts and electrospun samples were determined via DPPH analysis via a spectrophotometer, and the results are presented in Table 3.

The highest antioxidant activity (49.86 ± 2.78 mg Trolox/g DW) was observed in FMWE for all the samples. Although the highest total chlorophyll content was measured for FTE (19.38 mg/g DW), the chlorophyll *b* content of FMWE (18.5 mg/g DW) was greater than that of FTE. A relatively high chlorophyll *b* content may cause greater inhibition of free radicals than chlorophyll *a*. Hsu et al., 2013 also reported that chlorophyll *b* had slightly greater scavenging activity than chlorophyll *a*. Furthermore, microwave extraction could lead extraction of other compounds. Microwave energy initiates cellular disruption within plant materials, facilitating the penetration of solvent into the solid matrix, thereby promoting the dissolution and subsequent release of compounds into the surrounding solvent (Upadhyay et al., 2012). When the DSC results were considered, the peaks observed in FMWE and WMWE were also absent in the FTE and WTE samples (Fig. 4). Therefore, the extraction method has a substantial effect on the antioxidant activity.

The first and second highest DPPH values in the electrospun samples were measured for EU 2 (30.46 ± 0.51 mg Trolox/g DW) and EU 1 (25.49 ± 0.53 mg Trolox/g DW), respectively. Considering the higher antioxidant activities

obtained from EU 1 and EU 2 than from WMWU and the higher proportion of Eu in EU 2 than in EU 1, it can be inferred that the addition of Eu into the encapsulation media positively affects the antioxidant activity. Previous studies have confirmed that Eu exhibits antioxidant activity (Bayram et al., 2020; Cecchi et al., 2020; Song et al., 2023).

When comparing electrospinning techniques in terms of antioxidant activity, nanofibers fabricated via uniaxial electrospinning showed greater antioxidant activity than those produced by coaxial electrospinning. The core-shell structure formed during coaxial electrospinning might hinder the chemical reaction between chlorophyll and the DPPH reagent and lead to decreased antioxidant activity. Furthermore, hydrophobic interactions between zein and chlorophyll molecules in uniaxial electrospinning may enhance the antioxidant activity of nanofibers.

Light Stability

The stability of the extracts and nanofibers against light was evaluated, and the retention of chlorophyll after 10 days of exposure to sunlight at room temperature is shown in Fig. 5.

The chlorophyll *a* content of the extracts decreased sharply on the first day. On day 4, the chlorophyll level decreased to very low levels and no chlorophyll was detected in the extracts thereafter. The retention of chlorophyll *a* content of nanofibers was ranged from 4 to 61%. The highest chlorophyll *a* contents were detected in the microwave samples prepared via uniaxial electrospinning at the end of the test. Only a negligible amount of chlorophyll *a* was detected in the nanofibers produced by coaxial electrospinning on day 10.

Chlorophyll *b*, like chlorophyll *a*, could not be detected in the extracts after day 4. Similarly, no chlorophyll *b* remained in the nanofibers produced by uniaxial electrospinning on day 7. Chlorophyll *b* was still detected on even day 10 for the nanofibers produced by coaxial electrospinning, although their retention percentages were quite low (18–22%) compared with that of chlorophyll *a*.

In a previous study, better stability against light was expected from Eu-added electrospun samples, because Eu was proposed to possess photoprotective properties (Bayram et al., 2020). However, in this study, the addition of Eu did not effectively protect chlorophyll from light. This result could be attributed to the low concentration of the Eu pigment in the solution. According to the results, uniaxial electrospinning improved the stability of chlorophyll *a* and provided better protection from degradation under light.

The Addition of Encapsulated Chlorophylls to Yogurt

The stability of color during storage was evaluated by measuring the L^* , a^* , and b^* values of yogurt samples prepared

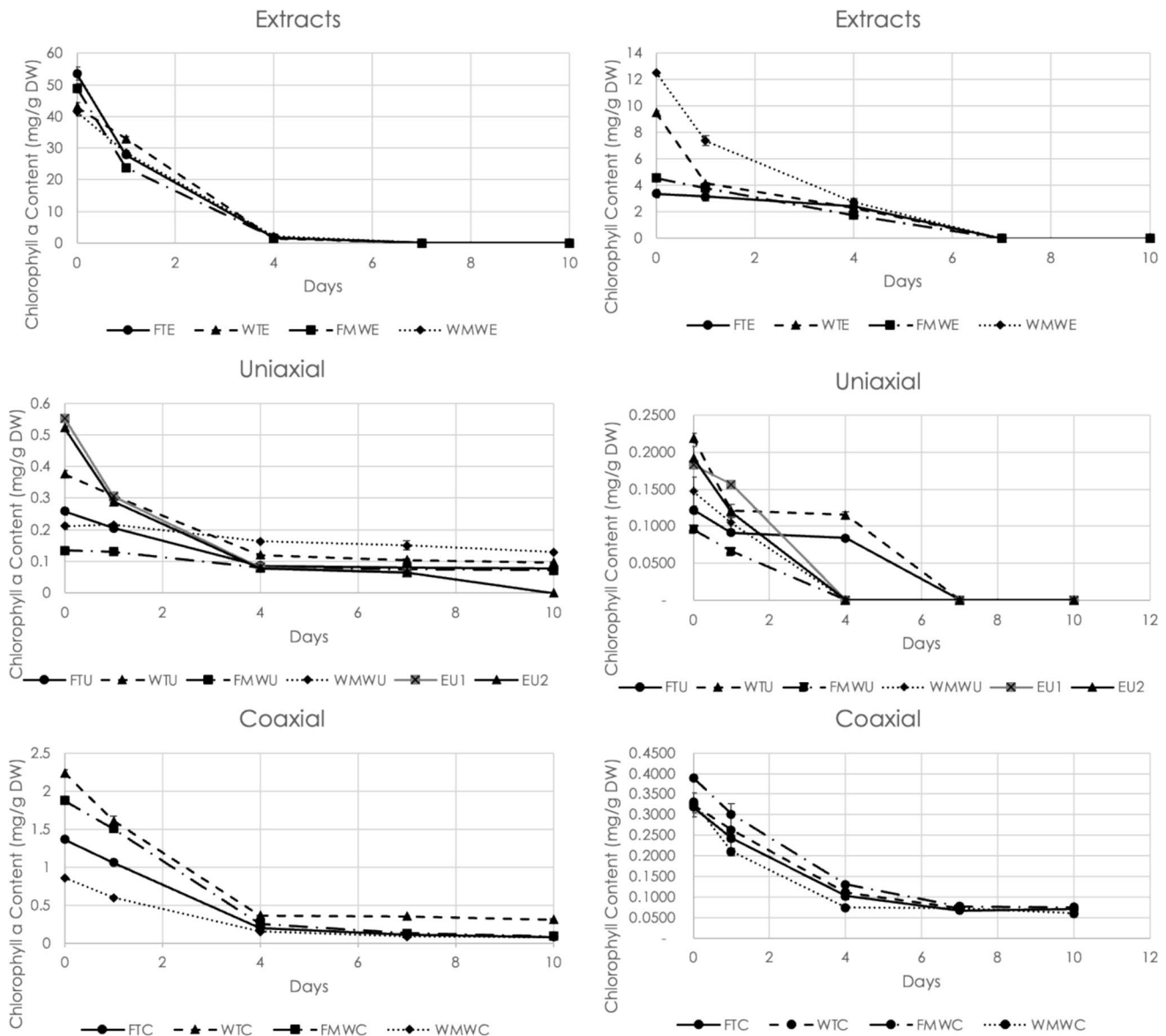


Fig. 5 Chlorophyll retention after 10 days of exposure to sunlight

with extracts, electrospun samples, and E141 (ii), the results of which are represented in Fig. 6. The changes in color of the samples during storage are shown in Fig. 7.

Compared with the control yogurt, yogurt supplemented with chlorophyll extracts, electrospun samples, and E141 (ii) presented reduced lightness (L^*). The L^* values of the chlorophyll extracts incorporated into yogurt (FTE, WTE, FMWE, and WMWE) and the yogurt sample prepared with E141 (ii) increased over the storage period (Fig. 5). A similar decrease was observed in previous studies (Chen et al., 2012; Pires et al., 2018; Wijesekara et al., 2022), and it has been associated with possible degradation or oxidation of color pigments (Wijesekara et al., 2022). In contrast, the L^* values of the electrospun samples (FTU, WTU, FMWU, WMWU,

FTC, WTC, FMWC, and WMWC) increased during storage. As the L^* index is strongly influenced by the water content in yogurt, the addition of zein might result in a decrease in the L^* value (García-Pérez et al., 2005; Jrad et al., 2019). The a^* values of samples changed significantly ($p < 0.5$). The $-a^*$ value can be used as an indicator of the greenness of a product (R. Wang et al., 2013a, 2013b). The $-a^*$ values of the samples tended to decrease across all samples at different rates during storage. Major decreases were observed in the extracts (FTE, WTE, FMWE, and WMWE) and the changes ranged from ≈ 40 to 46% at the end of storage. There was no statistically significant difference between samples with different extracts ($p < 0.5$). The bright green color faded by the end

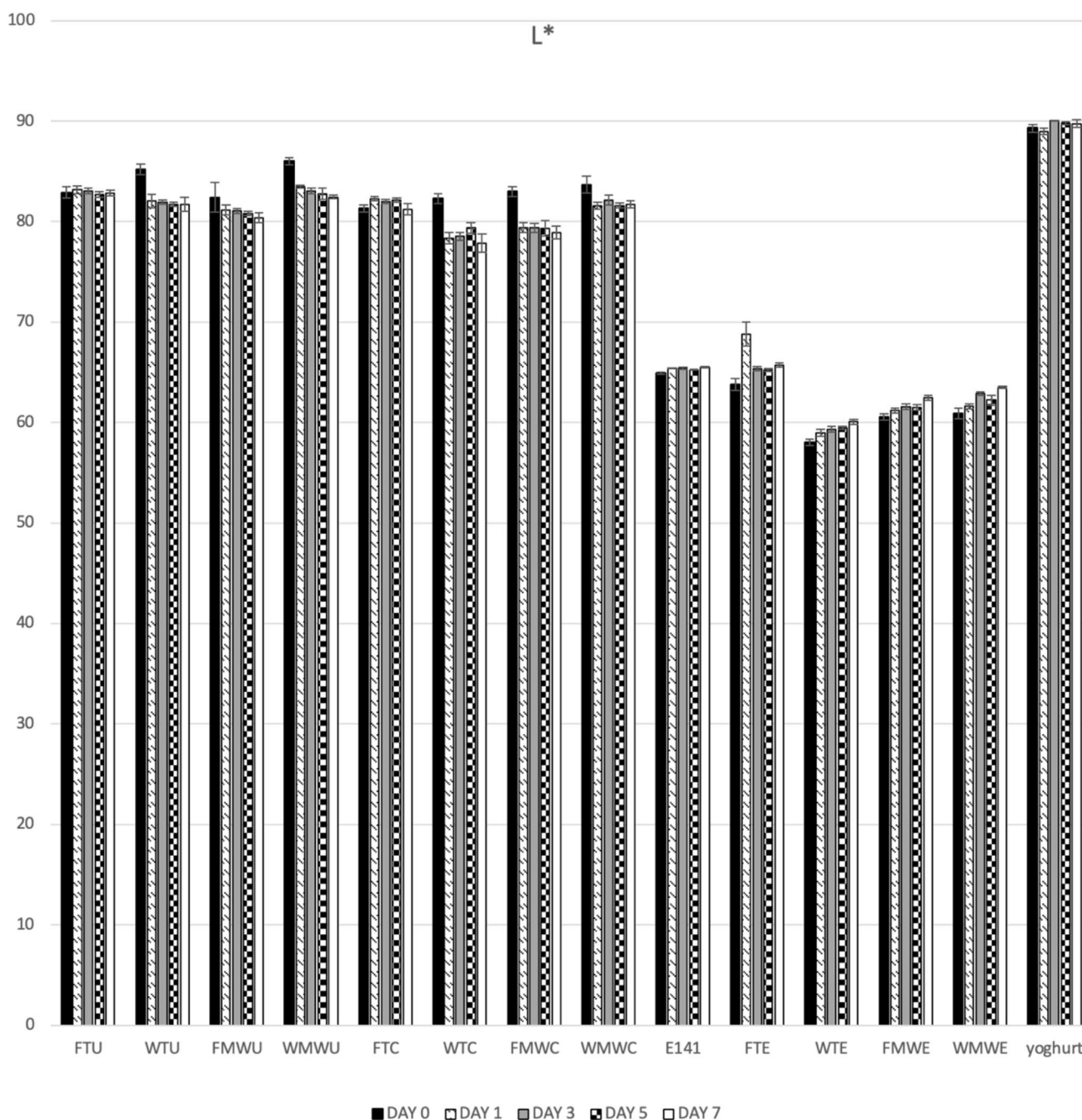


Fig. 6 L^* , a^* , and b^* values of the samples

of the storage period (Fig. 6). The reduction in yogurts with electrospun samples ranged from ≈ 17 to $\approx 24\%$. Significant differences were detected in the changes in the $-a^*$ values between yogurt samples with extracts and those with electrospun fibers ($p < 0.5$). Compared with their counterparts prepared via coaxial electrospinning (FTC, WTC, FMWC, and WMWC), electrospun samples obtained by uniaxial electrospinning (FTU, WTU, FMWU, and WMWU) presented fewer changes in a^* values; however, the differences

were not significant ($p < 0.5$). High stability and decreased susceptibility to fading or degradation of synthetic colorants were also observed in this study, and the sample prepared with E141 (ii) exhibited the smallest decrease ($\approx 3\%$). E141 (ii) is produced by adding copper to the product obtained from the saponification of a solvent extract derived from strains of edible plant materials. The incorporation of copper into the chlorophyllin structure stabilizes the vibrant green color, enabling various manufacturing processes and

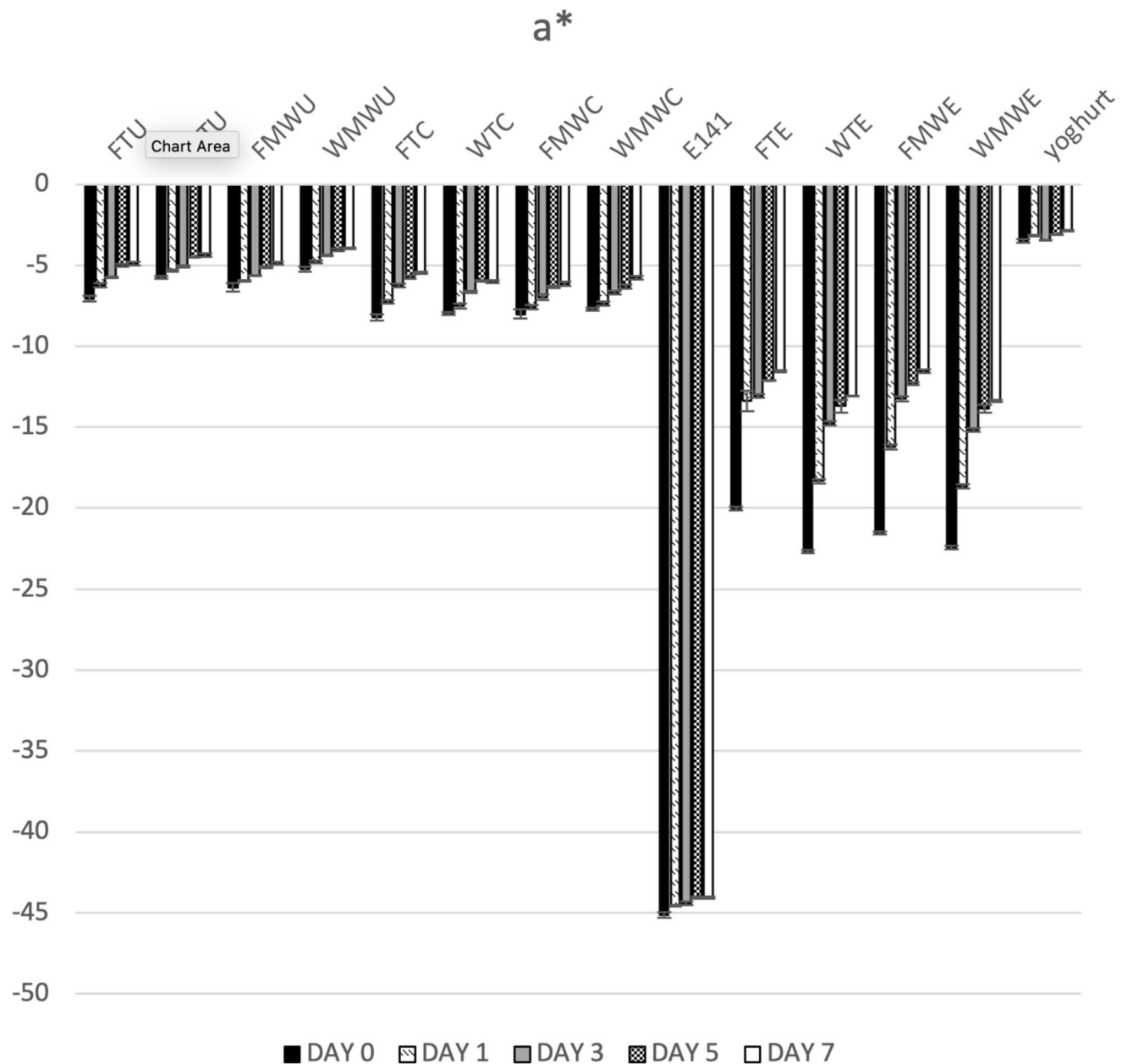


Fig. 6 (continued)

any color alteration during extended storage time (Roca & Pérez-Gálvez, 2024). The b^* values did not change considerably during the 7 days of storage.

Table 4 shows that the lowest total color changes (ΔE^*) were observed in yogurt and E141 (ii). The ΔE^* and $-a^*$ results revealed similar patterns in electrosun samples. Compared with their counterparts prepared via coaxial electrospinning, samples prepared by uniaxial electrospinning presented smaller color change ($p < 0.5$). Additionally, the greatest changes were observed for the spinach extracts.

These results indicate that better green color retention in yogurt can be achieved by the addition of nanofibers.

Chlorophyll is a pH-sensitive compound that is unstable at acidic pH values (3.5–5) (Rodríguez-Amaya, 2016). Yogurt is an acidic food (pH 4.0–4.4) (De Souza Oliveira et al., 2011); therefore, the chlorophyll in the extracts might degrade when yogurt is added directly into yogurt. When the chlorophyll extracts were encapsulated with zein, the stability of the chlorophyll improved. A previous study on different carrier agents for chlorophyll encapsulation reported that zein-chlorophyll nanoparticles presented significantly greater retention than whey protein isolate and casein against low pH and are recommended for use in acidic foods such as yogurt and pickles (Agarry et al., 2022a, 2022b).

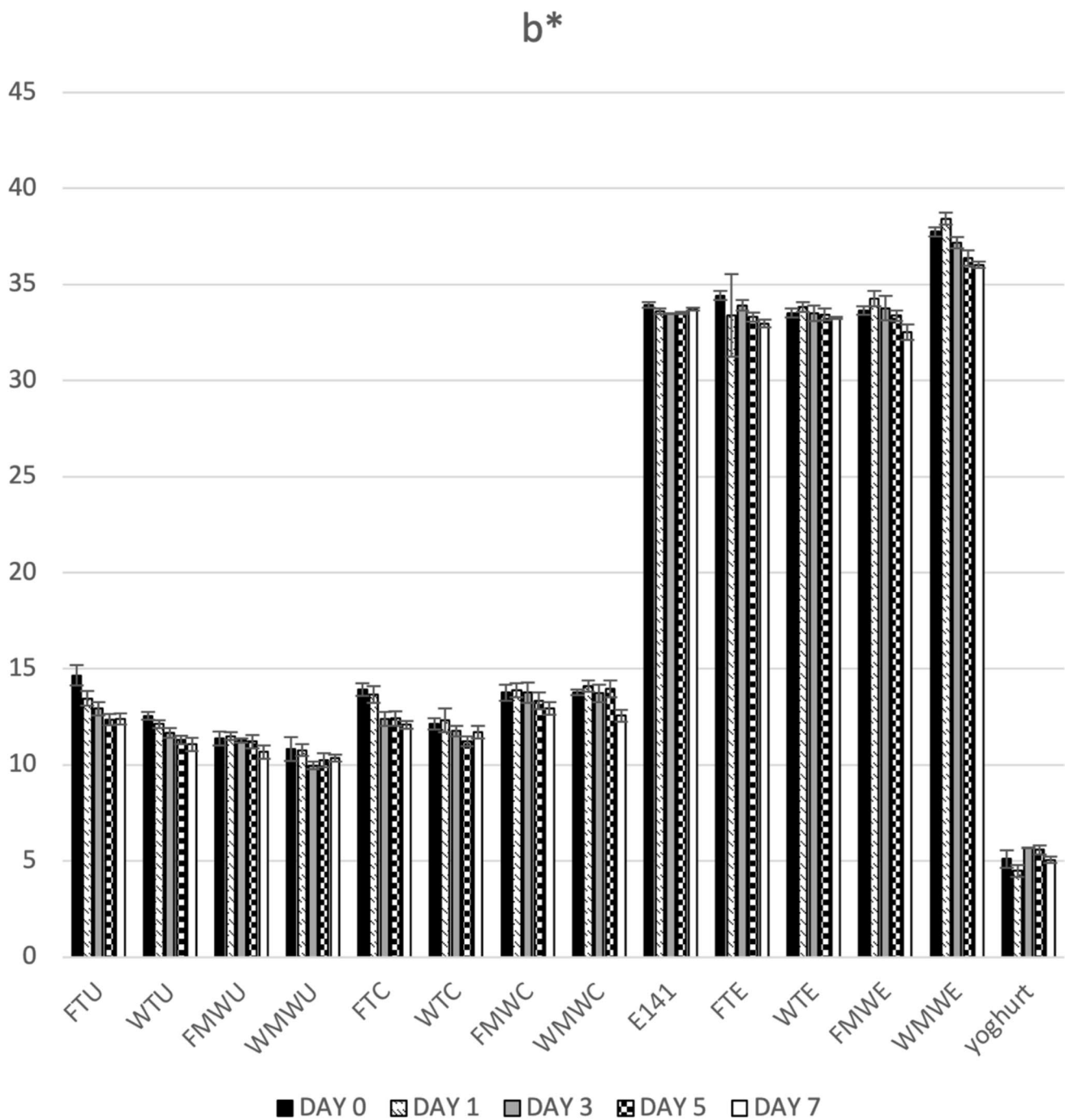


Fig. 6 (continued)

Conclusion

Chlorophyll extraction from fresh and waste spinach was performed via traditional and microwave-assisted extraction, and comparative analyses were discussed. While higher chlorophyll *b* contents were obtained through microwave-assisted extraction, traditional extraction resulted in higher chlorophyll *a* contents.

Microwave-assisted extraction can be a promising method for chlorophyll extraction because of its rapidity, cost-effectiveness, environmental friendliness, elevated thermal stability, and enhanced antioxidant activity. According to the results of this study, spinach waste, despite its relatively low chlorophyll content, could serve as a low-cost raw material source for the production of natural green colorants. Chlorophyll extracts were successfully

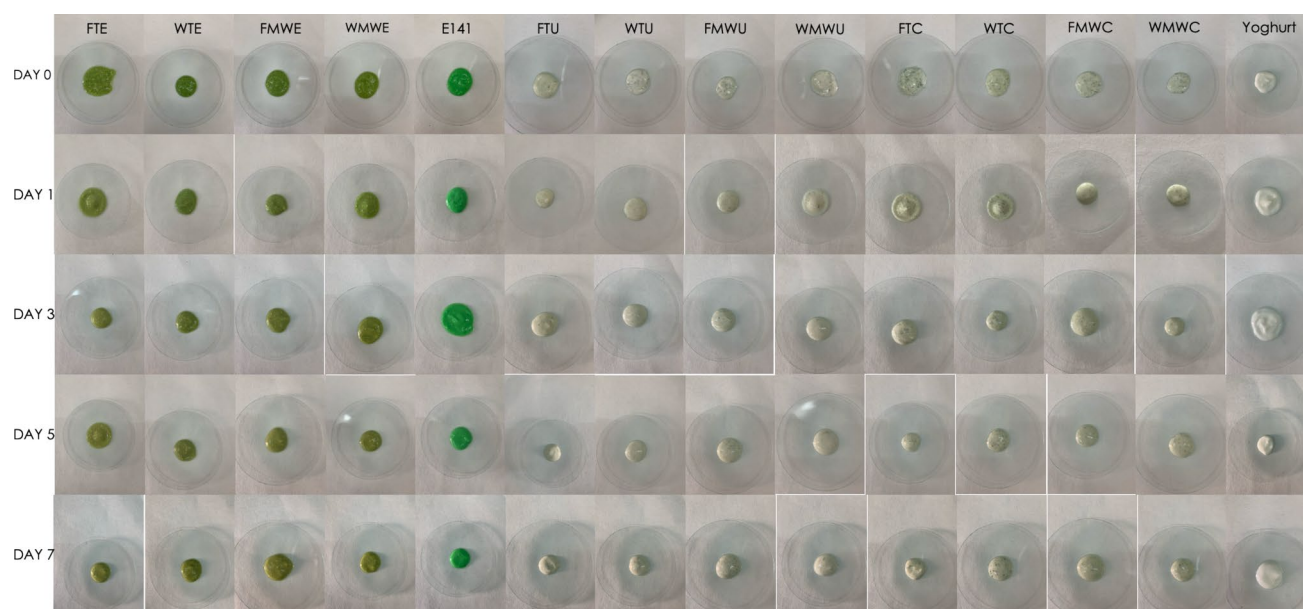


Fig. 7 Yogurt samples with extracts, electrospun samples, and E141 (ii)

Table 4 The total color difference (ΔE^*) of yogurt samples

Sample no/code	ΔE^*
2	2.71 ± 0.35^b
3	2.85 ± 0.07^{bc}
4	2.49 ± 0.74^b
5	3.46 ± 0.20^c
8	2.60 ± 0.24^b
9	4.16 ± 0.96^d
10	3.98 ± 0.97^d
11	5.10 ± 0.90^d
FTE	8.86 ± 0.12^e
WTE	9.85 ± 0.11^f
FMWE	10.26 ± 0.18^f
WMWE	9.60 ± 0.24^f
E141	1.24 ± 0.24^a
Yogurt	1.04 ± 0.36^a

Data are displayed as the mean $\pm$ standard deviation of three replicates. Different letters indicate statistically significant differences ($p < 0.05$)

encapsulated via uniaxial and coaxial electrospinning. The microscopy images of the electrospun samples revealed that smooth and unbeaded nanofibers were obtained from all the extracts via uniaxial electrospinning. The electrospinning of chlorophyll extracts with Eu did not result in nanofiber formation. The low conductivity and viscosity and high surface tension of the solutions with Eu could have contributed to this outcome. The successful

encapsulation and improved thermal stability of the chlorophyll extracts via the electrospinning method were confirmed through DSC analysis. The results of the DPPH assay revealed that higher antioxidant activity is achieved by microwave-assisted extraction. Additionally, the use of zein and Eu as core materials with electrospinning provides advantages in terms of antioxidant activity. The degradation of chlorophyll extracts when they were exposed to sunlight was determined, and enhanced stability was observed in the electrospun samples. Additionally, chlorophyll extracts and electrospun samples were incorporated into yogurt, and it was concluded that the color stability of pH-sensitive chlorophyll molecules was improved by the electrospinning method. Further investigations are warranted to explore the broader implications and potential applications of these findings across related fields.

Author Contribution Beyza Sukran Isik: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing- Original Draft, Visualization, Funding acquisition, Sinan Bayram: Methodology, Investigation, Writing—Original Draft, Writing- Review&Editing, Filiz Altay: Conceptualization, Methodology, Writing-Review&Editing, Supervision, Funding acquisition.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). This work was supported by the Research Fund of the Istanbul Technical University (Project ID: 42890).

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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