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ORIGINAL PAPER

# Enhanced crocin stability and controlled release in saffron-based emulsions via optimized ultrafiltration

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## Abstract

This study presents an integrated workflow to enhance crocin stability and controlled release in saffron-based emulsions through optimized ultrafiltration and double-emulsion encapsulation. Saffron extracts were standardized according to ISO 3632 and concentrated via ultrafiltration using a PESU membrane with a 1 kDa molecular weight cut-off. Under optimized conditions (4 °C, 2 bar, 80:20 permeate-to-retentate ratio), the crocin concentration increased 6.1-fold (7568.45 mg/L) with a total recovery of 97.8 ± 2.1% and process losses below 3%. D-optimal mixture design identified the optimal W/O formulation (67% sunflower oil, 6% PGPR, 27% ultrafiltrated extract), which showed minimal droplet growth and superior color retention ($R^2 = 0.96$, $p < 0.05$). The most stable W/O/W emulsion incorporated β-cyclodextrin (1.7%) and whey protein isolate (8%), maintaining 85.3 ± 2.7% crocin retention and stable chromatic parameters over 35 days at 5–45 °C. First-order kinetic modeling revealed a degradation rate constant ($k = 0.012$ $day^{-1}$ at 45 °C), confirming temperature-dependent stability. This integrated process bridges laboratory optimization and potential industrial application, providing a reproducible and scalable platform for formulating saffron-derived functional food systems.

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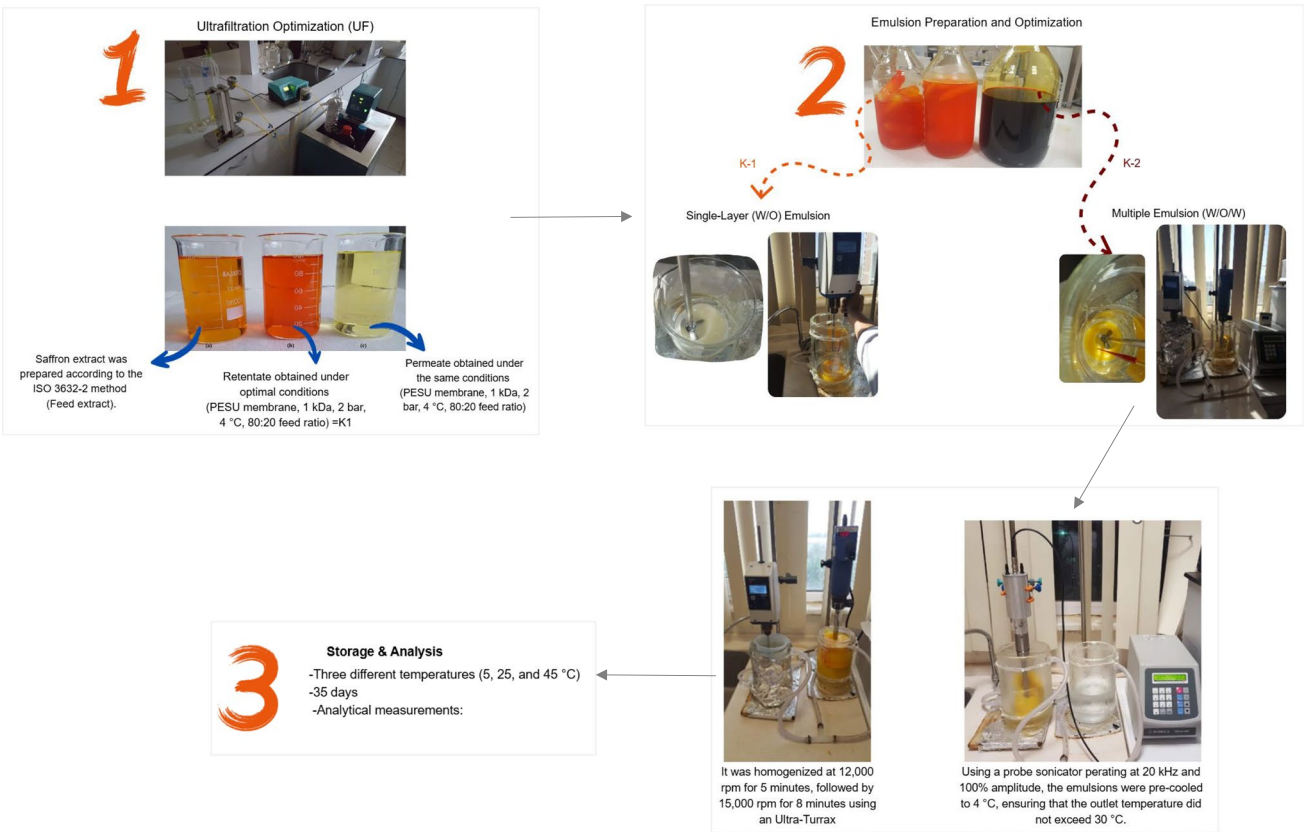
## Graphical abstract




## Highlights

- Formed an integrated workflow by integrating the ISO-standardized saffron extraction, ultrafiltration, and W/O–W/O/W.
- Attained six-fold crocin increase and proved β-cyclodextrin + whey protein isolate significantly increased emulsion stability 5–45 °C.
- Confirming stability and color preservation through the saffron rice model, bridging the encapsulation of the lab scale to practical food application.

**Keywords** Saffron extract · Crocin · Ultrafiltration · Encapsulation · Functional food · Stability

## Introduction

Saffron (*Crocus sativus* L.), belonging to the *Iridaceae* family, is the world's most valuable spice, derived from the stigmas of its flowers and cultivated since 1500 BCE [1]. Iran remains the leading producer, accounting for nearly 60% of global supply and over USD 200 million in annual export value [2]. Beyond its culinary importance, saffron is rich in bioactive molecules—mainly crocins, crocetin, picrocrocin, and safranal—known for antioxidant, anti-inflammatory, antidepressant, cardioprotective, and neuroprotective activities [3, 4]. Recent evidence highlights saffron's therapeutic potential against neurodegenerative disorders: crocetin inhibits α-synuclein aggregation, crocins improve synaptic plasticity through PI3K/Akt/mTOR pathways, and safranal offers dopaminergic neuroprotection [5]. Floral by-products such as tepals and stamens, rich in flavonoids and anthocyanins, also exhibit nutraceutical and cosmetic potential [6].

However, saffron's major bioactives are extremely sensitive to light, heat, and oxygen, making their stabilization a key technological challenge [1]. Emulsion-based encapsulation systems—especially water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsions—have shown promise for protecting pigments, extending shelf life, and enabling controlled release [7]. Previous research explored protein–polysaccharide-stabilized emulsions [8, 9], crocin-loaded nanoemulsions [10, 11], polymeric encapsulation [12, 13], and ultrasound-assisted extraction [14, 15].

Ultrafiltration (UF), on the other hand, offers a mild, solvent-free approach to concentrate and purify bioactives

with minimal thermal degradation. Unlike conventional concentration (e.g., solvent evaporation), UF allows selective retention of target molecules according to membrane molecular-weight cut-off [16, 17].

In this study, a 1 kDa polyethersulfone (PESU) membrane was selected after preliminary screening of higher cut-offs (5 kDa PESU and Hydrosart), as the dominant crocin esters (trans-crocetin di- and tri-gentiobiosyl esters, MW ≈ 976–995 Da) lie close to this threshold, ensuring selective retention of crocins while eliminating smaller impurities.

Despite advances in emulsion technology, the integration of membrane separation in saffron processing is scarce. Only a few reports have applied UF for saffron extract purification—mostly for pigment recovery or non-food uses. Sánchez et al. [18] demonstrated that centrifugal UF can selectively concentrate crocetin esters from aqueous saffron extracts. Later, Nthogiani et al. [19] and Zarkogianni et al. [20] utilized UF-purified fractions in cosmetic and nanocarrier emulsions, while Naidis et al. [21] improved colorant stability using UF. Nevertheless, no study has simultaneously optimized both ultrafiltration and double-emulsion parameters within a single experimental workflow.

Recent works on emulsion-based delivery of carotenoids [22, 23] and bioactives [22, 24] further emphasize the need for integrated, scalable approaches. To address this gap, the present study combines ISO-standardized extraction, UF optimization, and D-optimal mixture-design-based W/O/W emulsion formulation into a unified framework to enhance crocin stability, scalability, and functional delivery potential for saffron-derived food systems.

## Materials and methods

### Raw material

Commercial saffron stigmas of Crocus sativus L. (ISO 3632–2, Category I) were bought from certified dealers in Iran (Saharkhiz). Refining sunflower oil and sodium benzoate (E211) were bought through local food-grade dealers. Polyglycerol polyricinoleate (PGPR), and maltodextrin (DE 10–12) were generously donated by Palsgaard. Whey protein isolate (WPI), and β-cyclodextrin (β-CD) were bought through Sigma-Aldrich (St. Louis, MO, USA). Polyethersulfone (PESU), and Hydrosart ultrafiltration membranes of MWCO 1, 5, and 10 kDa were provided by Sartor.

### Preparation of saffron extracts

Two saffron extracts were made, one for each purpose:

- **K-1 (Ultrafiltration-purified extract) –** Made according to ISO 3632–2 with minor adjustments to avoid light bleaching. Ground saffron (0.500 ± 0.001 g, 500 µm mesh) was immersed in 900 mL distilled water in 1 L amber volumetric flask, kept under magnetic stirrer for 1 h at ~ 25 °C, and then diluted to 1 L. Filtrate (Whatman No. 1) was kept at 4 °C throughout until ultrafiltration.
- **K-2 (Concentrated preparation of W/O/W emulsions) –** Made without ultrafiltration to preserve higher concentration of the pigment for colorimetric measurements. Ten grams of saffron were dispersed in 150 mL of distilled water and kept under 1000 rpm, 24 h, ~ 25 °C, filtered, and frozen at − 80 °C until preparation.

### Experimental design and treatment selection

The overall experimental design was developed based on established protocols for saffron extraction, ultrafiltration, and double-emulsion encapsulation reported in previous literature. The extraction and grading of saffron followed ISO 3632 standards. The formulation of W/O and W/O/W emulsions was adapted from Esfanjani et al. [8, 9] and Jafari & McClements [7] with modifications to optimize crocin retention and stability. Ultrafiltration parameters (membrane material, MWCO, temperature, and pressure) were selected according to previously reported methods for concentrating carotenoids and phenolic compounds using PESU membranes [14, 18]. All experimental treatments were performed independently and in triplicate ($n = 3$) for statistical reliability. The procedures were refined through factorial and D-optimal mixture design approaches to determine optimal process parameters specific to saffron extracts. Optimization parameters and formulation steps are summarized in Table 1.

### Ultrafiltration optimization

A laboratory-scale cross-flow membrane module (Sartorius, Germany) connected to a peristaltic pump (Ismatec Reglo Digital, Germany), inlet/outlet pressure gauges, silicone tubing, stainless-steel membrane module holder (active surface area: 40 $cm^2$), and thermostatic water bath was utilized.

1. Membrane Screening: Two 5 kDa membranes, namely PESU and Hydrosart, were screened under 25 °C, 2 bar, and 70:30 permeate-to-retentate ratios. For higher crocin
2. Optimization of the Process: In 1 kDa PESU membranes, three parameters were tested by the full factorial

    - Temperature: 4 °C, 25 °C
    - Pressure: 2, 3

**Table 1** Experimental design and formulation steps used for the optimization of saffron-based emulsions

***Step a. Replacement of PESU filter with 1 kDa separation limit***

| Heat (°C) | Pressure (bar) | P:R |
|---|---|---|
| 4 | 2 | 60:40 |
| | 2 | 70:30 |
| | 2 | 80:20 |
| 4 | 3 | 60:40 |
| | 3 | 70:30 |
| | 3 | 80:20 |
| 25 | 2 | 60:40 |
| | 2 | 70:30 |
| | 2 | 80:20 |
| 25 | 3 | 60:40 |
| | 3 | 70:30 |
| | 3 | 80:20 |

***Step b. Single-layer W/O emulsions (D-optimal mixture design)***

| Run | Oil (%) | PGPR (%) | K-1 Extract (%) | Total (%) |
|---|---|---|---|---|
| **1** | 71.06 | 10.00 | 18.94 | 100 |
| **2** | 60.00 | 9.40 | 30.60 | 100 |
| **3** | 77.79 | 10.00 | 12.21 | 100 |
| **4** | 60.00 | 9.40 | 30.60 | 100 |
| **5** | 80.00 | 5.70 | 14.30 | 100 |
| **6** | 66.07 | 0.50 | 33.43 | 100 |
| **7** | 75.52 | 7.70 | 16.78 | 100 |
| **8** | 69.65 | 4.01 | 26.34 | 100 |
| **9** | 74.58 | 3.57 | 21.85 | 100 |
| **10** | 80.00 | 0.56 | 19.44 | 100 |
| **11** | 60.00 | 0.50 | 39.50 | 100 |
| **12** | 80.00 | 0.56 | 19.44 | 100 |
| **13** | 77.79 | 10.00 | 12.21 | 100 |
| **14** | 65.09 | 9.98 | 24.93 | 100 |
| **15** | 80.00 | 5.70 | 14.30 | 100 |
| **16** | 60.00 | 0.50 | 39.50 | 100 |

***Step c. External aqueous phase formulations for W/O/W emulsions (factorial design)***

| Formulation Code | Composition |
|---|---|
| **MD** | 8% maltodextrin |
| **WPI** | 8% whey protein isolate |
| **β-CD** | 1.7% β-cyclodextrin |
| **MD-WPI** | Final volume containing 8% MD and 8% WPI |
| **MD-β-CD** | Final volume containing 8% MD and 1.7% β-CD |
| **MD-WPI-β-CD** | Final volume containing 8% MD, 8% WPI, and 1.7% β-CD |
| **WPI-β-CD** | Final volume containing 8% WPI and 1.7% β-CD |

***Step d. Stored sample formulations***

| Code | Description |
|---|---|
| **A** | Prepared according to ISO 3236–2 (saffron:water ratio 0.5:1) and concentrated by ultrafiltration (PESU, 1 kDa, 2 bar, 4 °C, feed ratio 20% retentate) |
| **B** | Ten grams of saffron transferred into a 150 mL amber volumetric flask and extracted at approximately 25 °C for 24 h using a magnetic stirrer at 1000 rpm |
| **C** | 67% sunflower oil, 6% PGPR, and 27% K-1 extract |
| **D** | 67% sunflower oil, 6% PGPR, and 27% K-2 extract |
| **E** | W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied) |
| **F** | W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied) |

**Table 1** (continued)

| | |
|---|---|
| **G** | W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax + ultrasonication applied) |
| **H** | W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax + ultrasonication applied) |

Step a: Ultrafiltration (UF) optimization using 1 kDa PESU membranes under varying temperature, pressure, and permeate-to-retentate ratios

Step b: D-optimal mixture design for W/O emulsions using sunflower oil, PGPR, and K-1 extract

Step c: Factorial design of external aqueous phases for W/O/W emulsions, comprising maltodextrin (MD), whey protein isolate (WPI), and β-cyclodextrin (β-CD) combinations

Step d: Final W/O/W formulations (A–H) subjected to storage stability tests at 5, 25, and 45 °C. All formulations contained 0.05% sodium benzoate as a preservative

Analytical parameters included particle size distribution ($D_{43}$, $D_{32}$, span), stability index (ESI), crocin release (%FR), encapsulation efficiency (%EE), color parameters (L*, Hue, C*), and LC–MS/MS quantification of crocin isomers on days 0 and 35

- Permeate:Retentate ratio: 60:40, 70:30, 80:20 Optimal conditions were 4 °C, 2 bar, and 80:20. The resulting K-1 retentate was stored at − 80 °C.

Membranes were washed once every run by 1 M NaOH at 50 °C for 1 h, and then they were washed by deionized water. To validate the mass balance of crocin recovery, replicate ultrafiltration runs ($n = 3$) were performed under identical optimized conditions (1 kDa PESU, 2 bar, 4 °C). The total difference between the initial crocin load and the sum of permeate and retentate fractions remained within 2.9 ± 0.8%. The membranes were subsequently rinsed with deionized water, and the wash solution showed negligible absorbance at 440 nm, confirming minimal pigment adsorption on the membrane surface.

Based on the screening results, the 1 kDa PESU membrane was selected for process optimization, since its molecular-weight cut-off closely matches the size range of major crocin esters (≈ 976–995 Da), enabling selective concentration of glycosylated crocins.

## W/O emulsion optimization (Double Layer)

Statistical analysis of D-optimal and factorial designs was conducted using Design-Expert v7.0 software (Stat-Ease Inc., Minneapolis, USA). All models were validated through ANOVA, and adequacy statistics (F-values, p-values, $R^2$, and lack-of-fit tests) are summarized in the Table 1.

Formulations were tuned through D-optimal mixture design (Design-Expert v7.0, Stat-Ease Inc.), as per Esfanjani et al. [8] and Mehrnia et al. [10]. Components and test.

- Sunflower oil: 60–80%
- PGPR: 0.5–10%
- K-1 extract: 10–39%

Pre-mixing of sunflower oil and PGPR was carried out at 700 rpm for 30 min (IKA RW20, Germany) in a 10 °C jackets beaker maintained by Julabo F25. Dropwise addition of the saffron extract, under constant stirring, continued for 90 min.

Sixteen formulations (Table 1, Section a) were set up and kept under 30 °C for 35 days. The subsequent were evaluated every 5 days:

- Particle size ($D_{43}$, $D_{32}$, span)
- Color characteristics (L*, Hue, C*) of rice model system

Results were simulated to determine the best W/O composition, and this composition was utilized as the oil drop in W/O/W emulsions.

## W/O/W multiple emulsion optimization

First, K-1 extract (ultrafiltrated) was initially tested in the W/O/W emulsions; however, its higher viscosity and lower aqueous volume limited droplet formation and resulted in unstable emulsions with inconsistent pigment distribution. Therefore, a second extract K-2 (non-ultrafiltrated extract) – prepared with higher aqueous phase volume to facilitate W/O/W emulsion formation and allow accurate colorimetric analysis. Although K-2 appeared more intensely colored, its crocin extraction efficiency was lower, as part of the pigment remained bound within the saffron stigma residue. This adjustment ensured stable multilayer emulsions suitable for reliable analysis of droplet size, color, and crocin retention.

**Step 1 – External aqueous phase (Solution A):** Seven formulations were synthesized through the help of factorial design (Table 1, Section b), comprising:

- MW (8%), WPI (8%), β-CD (1.7%), and their com MIXTUREs WPI-based solutions were stored overnight at 4 °C, the rest were stored at RT.

**Step 2 – Initial W/O stage (Solution B):** W/O optimized of the preceding step (67% sunflower oil, 6%

PGPR, 27% K-2) homogenized 12,000 rpm for 5 min, 15,000 rpm for 8 min (Ultra-Turrax IKA T18). All the formulations were added by 0.05% of sodium benzoate. **Step 3 –** Preparation of Multiple Emulsion: W/O/W emulsions were made according to Bathaie & Mousavi (2010) [18], Esfanjani et al. [8, 9], and Lutz et al. (2009) [19]. Kept under 30 °C for 35 days; particle size and pigmentation were evaluated every 5 days.

## Ultrasonication-assisted optimization

Both most stable W/O/W systems were subsequently subjected to probe ultrasonication (HD3200, Bandelin, Germany), 20 kHz, 100% amplitude. Emulsions were cooled to 4 °C pre-temperature; outlet temperature did not exceed 30 °C.

Tested sonication time: 15–240 s. Emulsions were assayed for stability and dye retention and optimum time was chosen accordingly.

## Storage conditions

Optimized formulations (Table 1, Section c) were prepared in triplicate and kept at three temperatures (5, 25, and 45 °C) for 35 days. All the samples were added 0.05% w/w of sodium benzoate as a preservative. At 5-day intervals, analytical assays were carried out and included the particle size distribution ($D_{43}$, $D_{32}$, and span), stability index (ESI), crocin release (%FR), encapsulation efficiency (%EE), microscopy, and color parameters (L*, Hue, C*). Furthermore, LC–MS/MS assay was assayed to measure the crocin isomers contents on days 0 and 35.

Sodium benzoate (0.05% w/w) was added to all samples as a preservative to ensure microbial stability. The concentration was low enough not to influence pH or ionic strength, and no measurable absorbance was detected at 440 nm in blank emulsions containing sodium benzoate.

## Analytical methods

### 1. Color measurement

Colour measurements of permeates and retentates recovered subsequent to ultrafiltration were conducted by means of an UltraScan-VIS HunterLab colorimeter (Virginia, USA), and by means of the application of a model food system (saffron rice) by the use of a Konica Minolta CR-400 colorimeter (Osaka, Japan). For the case of transparent liquids, the use of the Konica Minolta instrument was avoided by sensitivity towards interferences by ambient light; the UltraScan-VIS HunterLab, which has a dark chamber for measurement, was utilized. Duplicate analyses were conducted with three technical replicates. Hue angle and chroma (C*) were computed as:

$$\text{Hue} = \arctan\frac{b*}{a*} \tag{1}$$

$$C* = \sqrt{(a*^2 + b*^2)} \tag{2}$$

For negative values of a and positive values of b, the hue angle was modified to 180° + arctan(b*/a*).

### 2. Total crocin analysis

Crocin analysis was conducted on prepared extracts, the permeates and retentates of the UF treatment, and the emulsion systems. Before the analysis, the prepared samples were dilution-treated accordingly, and the absorbance of the prepared samples was also calculated using the spectrophotometer (Shimadzu UV-1800) off 440 nm. Crocin measurement was carried out by using the calibration curve prepared by crocin standard solutions (Sigma-Aldrich) and 440 nm. From the linear regression equation, the resulting equation was $y = 76.046x - 0.3346$ and the determination coefficient of the resulting equation was found to be $R^2 = 0.9988$, which showed excellent linearity of the tested concentration level of the tested concentration level of about 10–75 mg/L.

### 3. UF yield

Permeation rates of the permeate and the retentate, and the net recovery and losses calculated by using the PESU membrane of molecular weight cut-off (MWCO) 1 kDa, under various temperatures, pressure, and feed ratio conditions, were calculated by means of Eqs. 3, 4, 5, and 6. These parameters are defined by the volume of the feed extract, $V_0$, the volume of the permeate, $V_t$, and the volume of the permeate, $V_R$. Likewise, the concentration of the feed extract, $C_0$, the concentration of the permeate, $C_t$ and the concentration of the permeate, $C_R$, are all defined by their content of crocin content (mg/L) [20].

$$\text{Permeate recovery } (\%) = \left(\frac{V_F x C_F}{V_0 x C_0}\right) \times 100 \tag{3}$$

$$\text{Retentate recovery } (\%) = \left(\frac{V_R x C_R}{V_0 x C_0}\right) \times 100 \tag{4}$$

$$\text{Total recovery } (\%) = \%\ \text{Permeate recovery} + \%\ \text{Retentate recovery} \tag{5}$$

$$\%\ \text{Loss} = 100 - \%\ \text{Total recovery} \tag{6}$$

## 4. Particle size analysis

Emulsion particle size distribution was analyzed by laser diffraction by means of a Mastersizer 3000 (Malvern Instruments Ltd., UK) by the procedure of McClements (2005) [21]. Samples were 1:100 dilution (v/v) in distilled water to avoid the effects of multiple scattering and soft inversion to avoid disturbing the emulsion structure. Volume-weighted mean size, $D_{43}$, and surface-weighted mean size, $D_{32}$, were automatically calculated by the software of the instrument. Span index, distribution width, was calculated by the formula:

$$D_{43} = \left(\sum n_i d_i^4\right) / \left(\sum n_i d_i^3\right) \tag{7}$$

$$D_{32} = \left(\sum n_i d_i^3\right) / \left(\sum n_i d_i^2\right) \tag{8}$$

$$\text{Span} = \frac{\text{d}(0.90) - \text{d}(0.10)}{\text{d}(0.50)} \tag{9}$$

where d(0.10), d(0.50), and d(0.90) represent particle diameters at 10%, 50%, and 90% cumulative volume, respectively.

## 5. Encapsulation efficiency (EE%) and crocin release

Crocin release (%) and encapsulation efficiency (EE%) were determined spectrophotometrically at 440 nm (crocin λmax) using a UV–Vis spectrophotometer (Shimadzu UV-1800, Japan) following the procedure described by Gahruie et al. (2020) [22].

$$\%\text{FR} = \left(\frac{\text{C}_\text{t}}{\text{C}_0}\right) \times 100 \tag{10}$$

$$\%\text{EE} = \left(\frac{\text{C}^\text{e} - \text{C}_\text{s}}{\text{C}^\text{e}}\right) \times 100 \tag{11}$$

where $C_t$ is the quantity of crocin liberated at time t, $C_0$ represents the overall crocin content, $C_e$ represents the start-up crocin focus, and $C_s$ represents the supernatant of non-encapsulated crocin subsequent to spinal execution (5000 rpm, 10 min).

## 6. Stability index

Stability of emulsion was observed by observing the separation of phases by means of calibrated ruler and presented as the percentage of initial emulsion height stored. Turbidity was estimated at 600 nm [20] to estimate the amount of destabilization.

## 7. Microscopy

Microstructure of emulsions was examined through an optical microscope (Olympus CX31, Japan) at 100× magnification, following Dickinson's procedure (2012) [23]. Digital droplet coalescence and flocculation were recorded on days 0 and 35 to monitor the same.

Optical microscopy (100×, Carl Zeiss, Germany) was employed to visualize droplet morphology under hydrated conditions, as SEM imaging was not suitable due to the aqueous sensitivity of the emulsions.

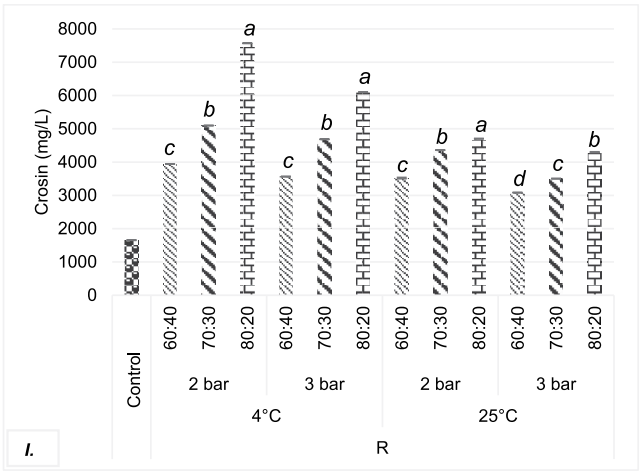
## 8. LC–MS/MS analysis of crocins

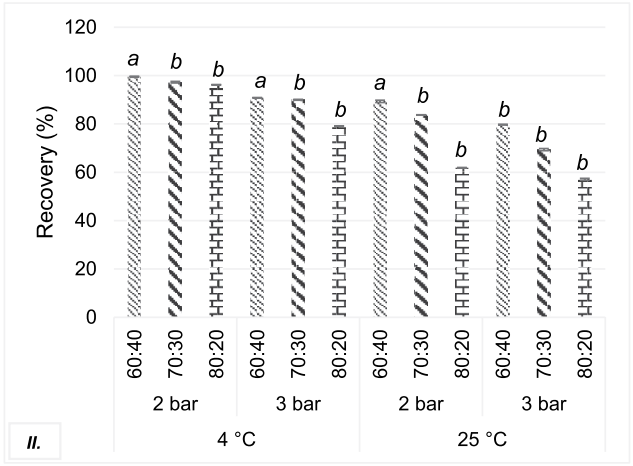
Crocin isomer profiles were analyzed using a triple quadrupole UHPLC–MS/MS system (Accela UHPLC coupled with a TSQ Quantum Access Max, Thermo Fisher Scientific Inc., Waltham, MA, USA), following the method of García-Rodríguez et al. (2020) [24] with minor modifications. Samples were extracted with methanol:water (80:20, v/v), filtered through a 0.22 µm PTFE membrane, and a 10 µL aliquot was injected into a C18 column (Phenomenex Kinetex, 100×2.1 mm, 2.6 µm). The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), delivered using a gradient elution program. Detection and quantification were performed in multiple reaction monitoring (MRM) mode, allowing selective and sensitive identification of crocin-1–1, crocin-1–2, and crocin-3. Calibration curves were prepared using authentic standards across a concentration range of 1–80 ppm for crocin-1–1, 0.5–14 ppm for crocin-1–2, and 0.5–5 ppm for crocin-3. All calibration curves exhibited for crocin-1–1, crocin-1–2, and crocin-3 exhibited excellent linearity ($R^2$=0.9871, 1.0000, and 0.9980, respectively), ensuring method reliability (Figs. 1, 2, 3, 4 and 5).

Representative chromatograms and calibration curves for crocin-1–1, crocin-1–2, and crocin-3 are presented in Fig. 6. The method was validated in terms of linearity, detection limits (LOD), quantification limits (LOQ), recovery, and repeatability. Validation results for all analytes, including intra- and inter-day RSDs, are detailed in Supplementary Table S47.

## 9. Statistical analysis

All experiments were carried out in duplicates ($n$=2), and each measurement was performed three times as technical replicates ($n$=3). Data were expressed as mean±SD. One-way and two-way analyses of variance (ANOVA) were performed using SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA) to evaluate the effects of formulation, storage time, and storage temperature on dependent variables



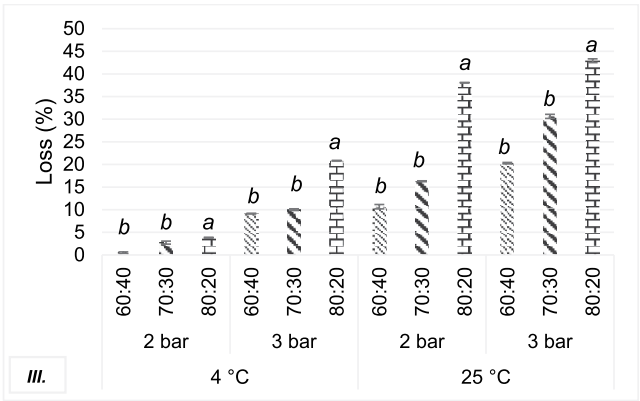




**Fig. 1** Effect of ultrafiltration parameters (temperature, transmembrane pressure, and permeate-to-retentate ratio) on (I) crocin concentration (mg/L) in retentate, (II) total recovery (%), and (III) process loss (%) for saffron extract using PESU 1 kDa membranes. Data are presented as mean ± SD ($n = 3$). *Different lowercase letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test*

(e.g., particle size, color parameters, encapsulation efficiency, crocin release, and stability index). Duncan's multiple range test was used for pairwise mean comparisons, and differences were considered statistically significant at $p < 0.05$.

For optimization of emulsion formulation, D-optimal mixture and response surface methodology were utilized by applying the Design-Expert® software (Version 7.0, Stat-Ease Inc., Minneapolis, MN, USA). Regression models were constructed for major responses ($D_{43}$, Hue, C*, etc.) to detect the best composition. Adequate model fitness was evaluated through the lack-of-fit test, the values of $R^2$, and the significance of the terms of the regression.

## Results and discussion

### Ultrafiltration optimization

Ultrafiltration conditions were optimized using a full factorial experimental design, enabling quantitative evaluation of the main and interaction effects of pressure, temperature, and permeate-to-retentate ratio on crocin recovery and pigment loss. This systematic design allowed a statistically sound assessment of performance limits rather than single-factor screening.

Crocin concentration in the retentate varied significantly ($p < 0.05$) with operating conditions (Fig. 1I). Maximum concentration (7568.45 mg/L) was achieved at 4 °C, 2 bar, and an 80:20 feed ratio, representing an approximately sixfold enrichment compared to the crude extract. Lower temperatures favored crocin stability due to the known thermal lability of carotenoid glycosides [25]. Similarly, moderate pressure (2 bar) minimized pigment losses by reducing shear-induced fouling and adsorption on the PESU membrane. The 80:20 ratio effectively concentrated hydrophilic crocin, consistent with the selective molecular-weight cut-off behavior described by Sánchez et al. [18].

Total yield (Fig. 1II) followed the same trend, with the highest recovery (87.97%) at 4 °C and 2 bar. Factorial ANOVA confirmed significant main effects of temperature

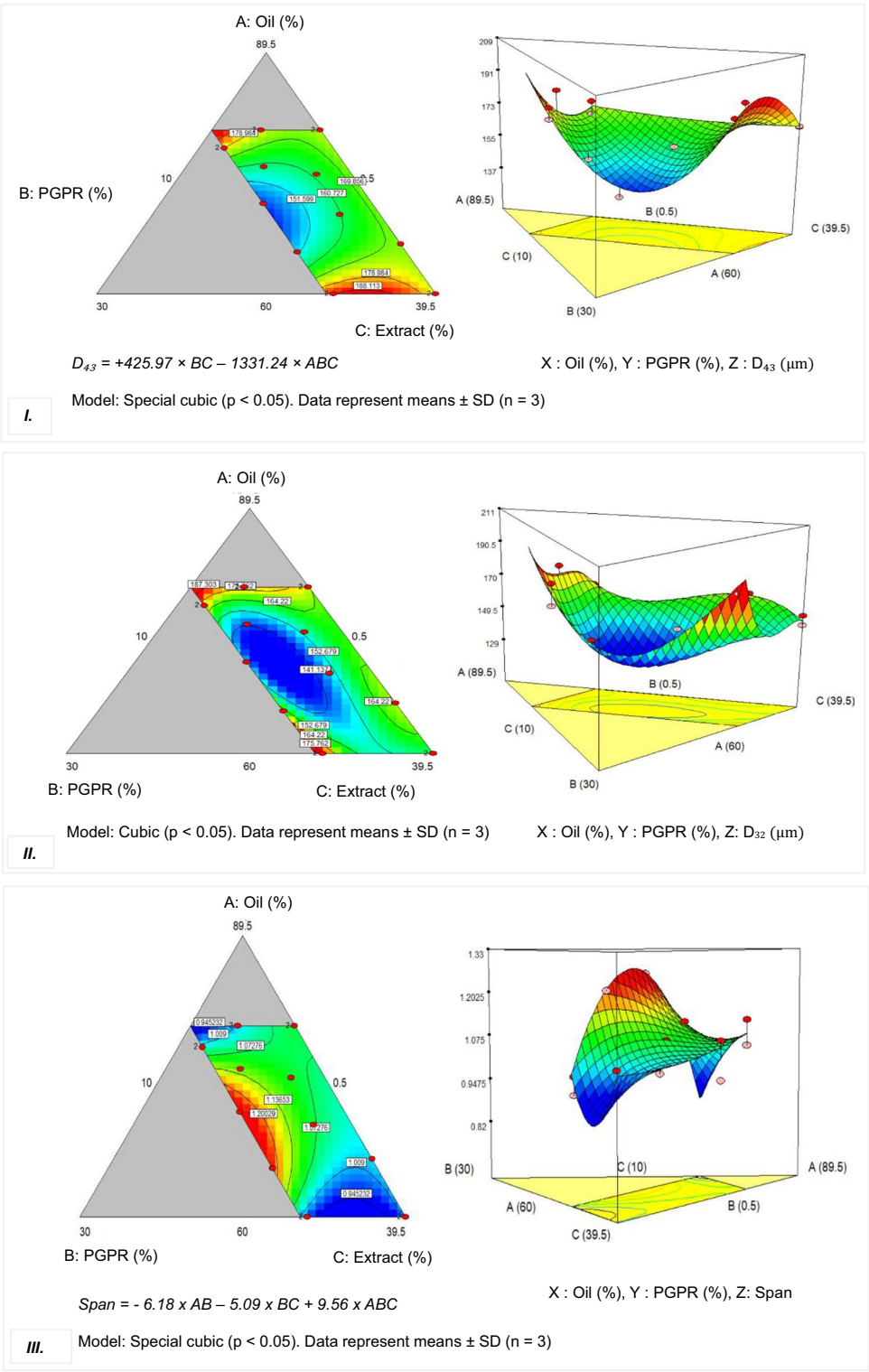
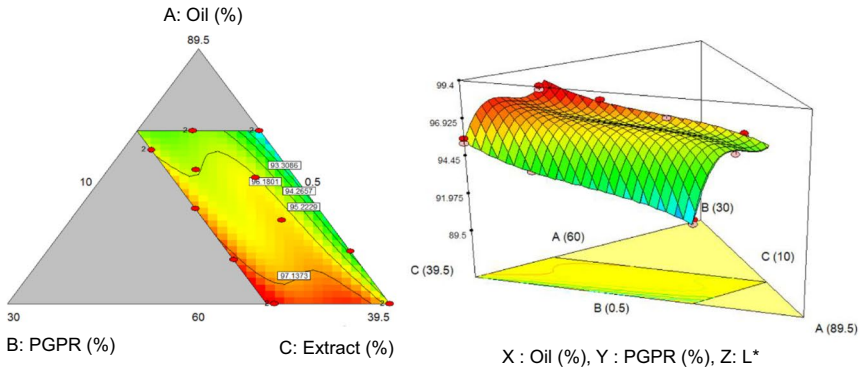



**Fig. 2** D-optimal mixture design: Response surface plots showing the effects of oil, PGPR, and saffron extract concentrations on droplet size parameters ($D_{43}$, $D_{32}$, and Span) and color attributes (L, a*, b*, Hue, and Chroma) of W/O emulsions. Data represent mean ± SD ($n = 3$). ANOVA results are provided in Tables S8–S15

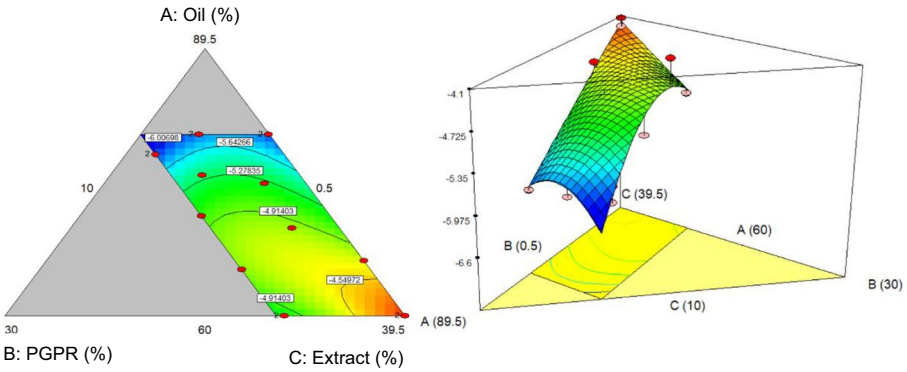
and pressure, as well as a temperature × ratio interaction ($p < 0.05$). Higher temperatures (> 25 °C) led to pronounced pigment losses, in line with the thermal and oxidative degradation reported for similar carotenoid systems [26].

Loss proportions (Fig. 1III) were minimal (< 5%) under optimal UF conditions but exceeded 30% at elevated temperatures or prolonged filtration, likely due to oxidation and hydrolysis of crocins to crocetin and volatile safranal [27].



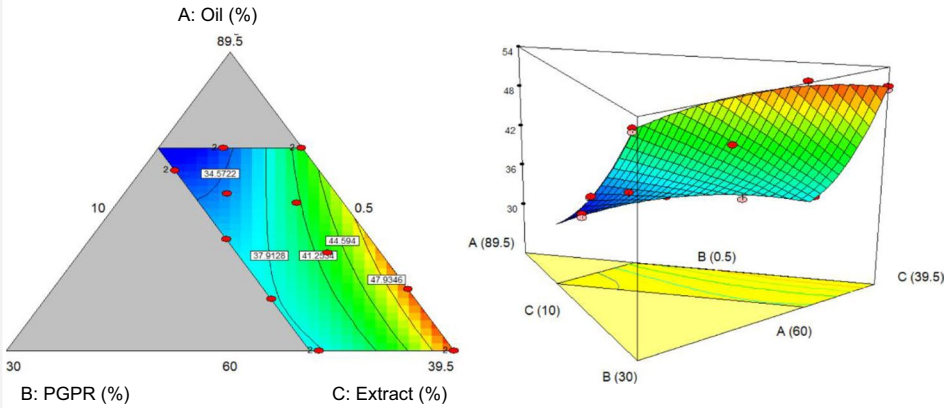

$L^* = -460.39xAB - 459.92xBC + 504.44xABC + 289.09xAB(A-B) - 242xBC(B-C)$

***IV.*** Model: Cubic ($p < 0.05$). Data represent means ± SD (n = 3)




$a^* = -6.91 \times A - 6.82 \times B - 4.19 \times C$

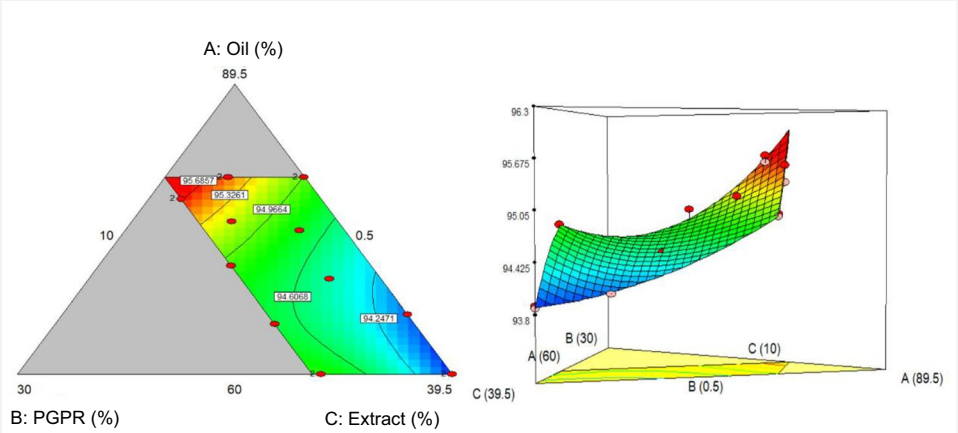
***V.*** Model: Special cubic ($p < 0.05$). Data represent means ± SD (n = 3)




$b^* = +31.91 \times A + 75.82 \times B + 51.28 \times C - 68.43 \times AB + 21.23 \times AC - 99.42 \times BC$

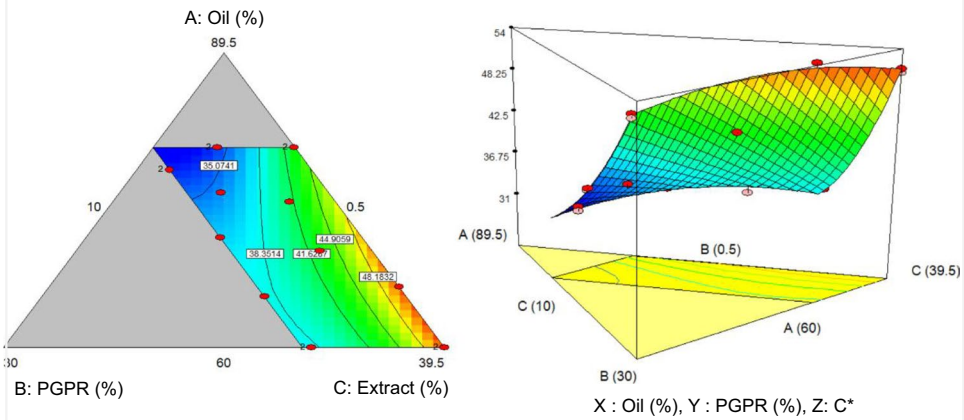
***VI.*** Model: Quadratic ($p < 0.05$). Data represent means ± SD (n = 3)

**Fig. 2** (continued)




*Hue* = +96.00 x A + 93.17 x B + 93.89 x C – 11.78 x ABC*

***VII.*** Model: Special cubic ($p < 0.05$). Data represent means ± SD (n = 3)




*C* = +32.70xA+76.62xB+51.46xC–69.43xAB+20.47xAC–99.66xBC*

***VIII.*** Model: Quadratic ($p < 0.05$). Data represent means ± SD (n = 3)

**Fig. 2** (continued)

Overall, the optimized parameters (4 °C, 2 bar, 80:20 ratio) offered the best compromise between yield, pigment retention, and operational efficiency. These statistically verified conditions were subsequently used for the preparation of ultrafiltrated extracts (K-1) applied in emulsion formulations.

## Optimization of single-layer (W/O) saffron emulsions

Formulation of W/O emulsions was optimized via a D-optimal mixture design considering oil, PGPR, and saffron extract as mixture components. This approach effectively captured compositional interdependencies inherent to emulsion systems ($\Sigma = 100\%$).

Regression models exhibited excellent predictive quality ($R^2 = 0.86–0.99$, CV = 0.24–4.75%). Adequate precision values (>4) confirmed the reliability of model predictions. For droplet size parameters ($D_{43}$, $D_{32}$, Span), significant interaction terms (BC and ABC, $p < 0.05$) indicated that both surfactant and extract concentration influenced droplet distribution. Contrary to typical emulsification trends, excessive PGPR levels did not always yield smaller droplets, suggesting formation of high-viscosity, liquid-crystalline structures that hindered effective dispersion [10].

Colorimetric responses (L*, a*, b*, Hue, C*) were primarily governed by extract concentration, with increased extract levels deepening yellowness (higher b* and C*) and lowering hue angle (movement toward saturated golden

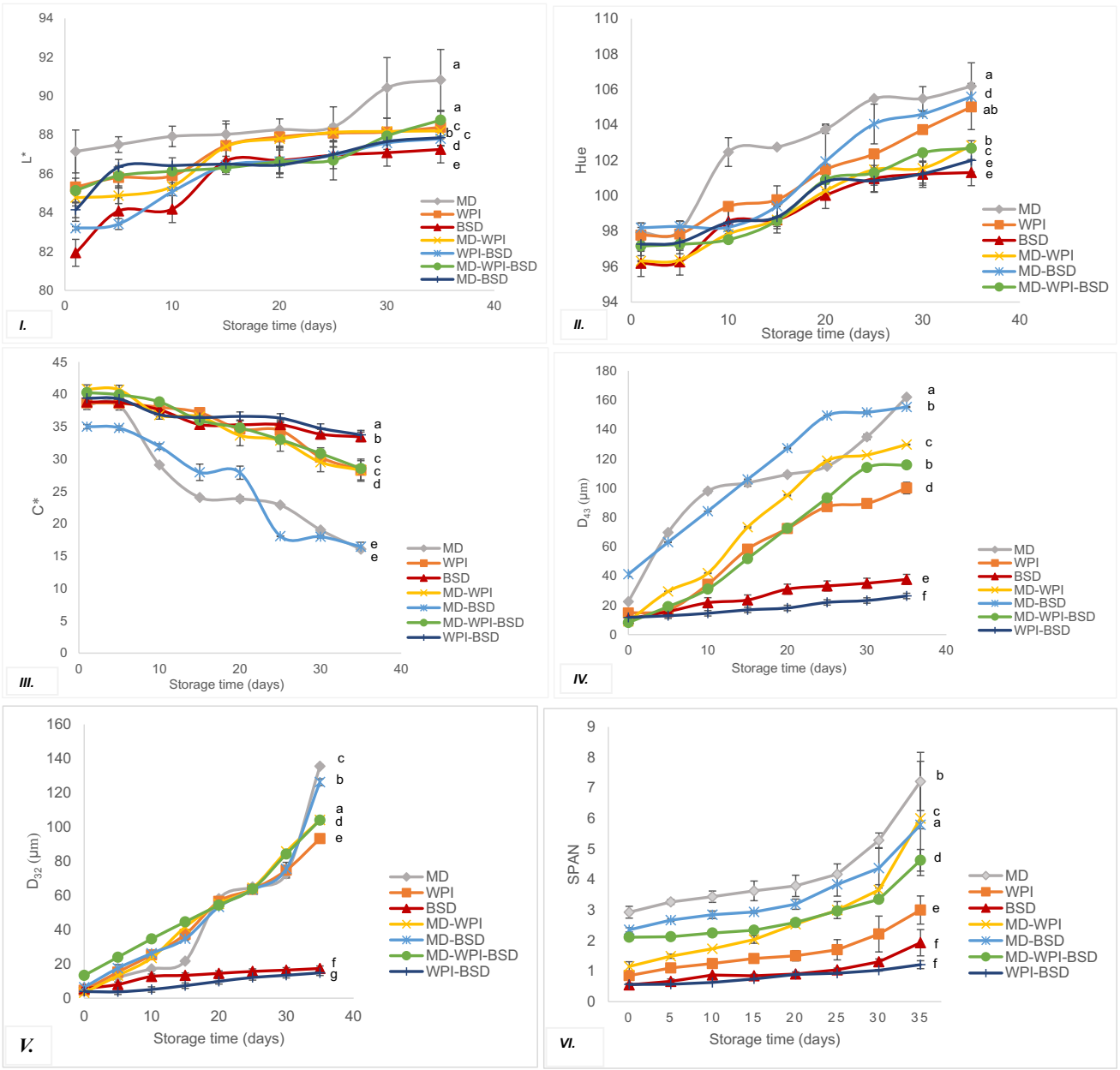



**Fig. 3** Changes in Color and Particle Size Parameters of W/O/W Emulsions During Storage: **(I)** Lightness (L*), **(II)** Hue, **(III)** Chroma (C*), **(IV)** Volume-Weighted Mean Diameter ($D_{43}$), **(V)** Surface-Weighted Mean Diameter ($D_{32}$), and **(VI)** SPAN. Different lowercase letters indicate significant differences ($p<0.05$) according to Duncan's multiple range test

tones). These results align with known sensory and spectroscopic behaviors of saffron-pigmented systems [9].

The optimized formulation (67% sunflower oil, 6% PGPR, 27% extract) achieved 94–99% agreement between predicted and experimental responses, demonstrating high model validity. While $D_{32}$ and a* exhibited slightly lower predictive $R^2$ values (0.57 and 0.05), this variation reflects inherent measurement sensitivity rather than model inaccuracy. Collectively, these data validate the robustness of the D-optimal mixture design for predicting and optimizing saffron-based emulsion systems.

## Optimization of W/O/W emulsion formulations for enhanced stability

The dual-layer (W/O/W) emulsions were further designed to enhance crocin retention and color stability. Storage stability was evaluated for 35 days at 5, 25, and 45 °C (Figs. 4

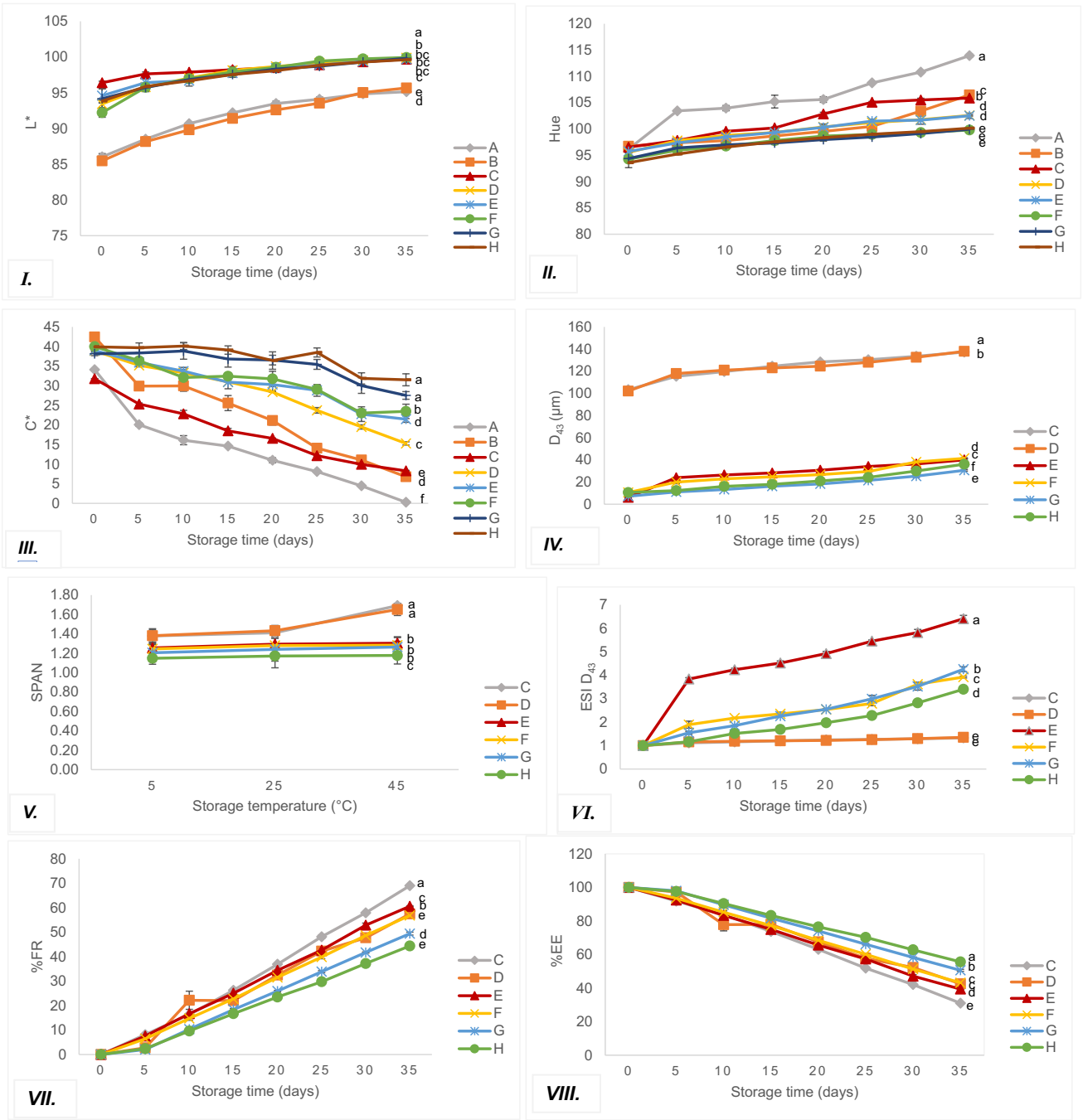



**Fig. 4** Changes in physicochemical stability parameters of selected W/O/W emulsions during 35 days of storage at different temperatures: **(I)** Lightness (L*) values, **(II)** Hue, **(III)** Chroma (C*) values, **(IV)** Volume-weighted mean diameter ($D_{43}$, μm), **(V)** Span values at different storage temperatures (°C), **(VI)** Emulsion stability index based on droplet size (ESI_$D_{43}$), **(VII)** Flocculation rate (%FR), and **(VIII)** Encapsulation efficiency (%EE). *Note: Codes; A: Prepared according to ISO 3236–2 (saffron:water ratio 0.5:1) and concentrated by ultrafiltration (PESU, 1 kDa, 2 bar, 4 °C, feed ratio 20% retentate) (K-1), B:* Ten grams of saffron transferred into a 150 mL amber volumetric flask and extracted at approximately 25 °C for 24 h using a magnetic stirrer at 1000 rpm. (K-2), C: 67% sunflower oil, 6% PGPR, and 27% K-1 extract. D: 67% sunflower oil, 6% PGPR, and 27% K-2 extract. E: W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied). F: W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied)., G: W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax + ultrasonication applied)., H:W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax + ultrasonication applied)

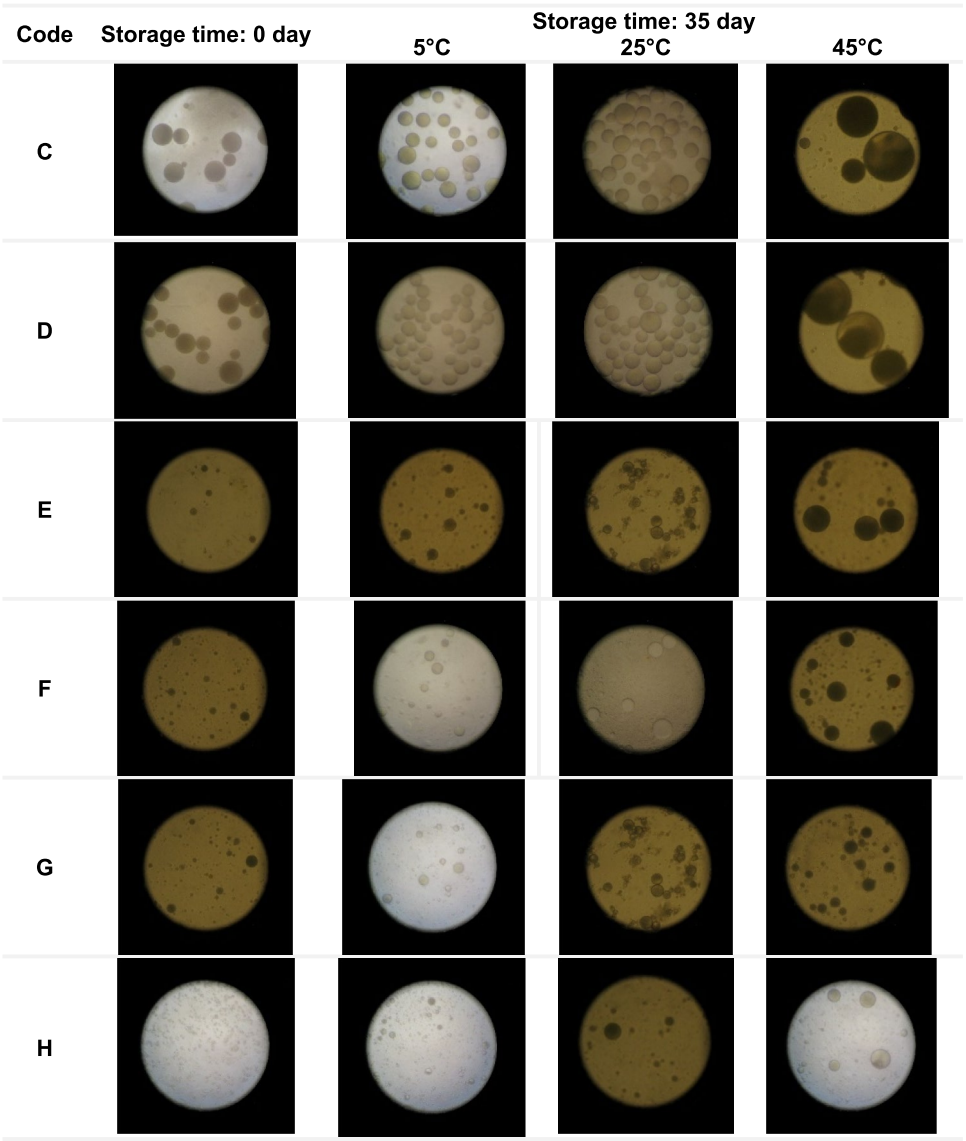



**Fig. 5** Microscopic images of selected W/O/W emulsions (codes C–H) at day 0 and after 35 days of storage at 5 °C, 25 °C, and 45 °C, showing changes in droplet morphology and distribution over time and temperature. *Note: Codes; C: 67% sunflower oil, 6% PGPR, and 27% K-1 extract. D: 67% sunflower oil, 6% PGPR, and 27% K-2 extract. E: W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied). F: W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax homogenization applied)., G: W/O-2 emulsion with 1.7% β-CD in the external aqueous phase (Ultra-Turrax+ultrasonication applied)., H:W/O-2 emulsion with 8% WPI and 1.7% β-CD in the external aqueous phase (Ultra-Turrax+ultrasonication applied).* As this figure provides qualitative microstructural visualization, statistical data are not applicable; however, quantitative comparisons based on droplet size, stability index (ESI), and encapsulation efficiency (%EE) are already presented in Figs. 3 and 4 and Supplementary Tables S42–S46

and 5), with analysis of color attributes (L*, Hue, C*) and particle size distribution ($D_{43}$, $D_{32}$, Span).

Both formulation type and storage time exerted significant effects on these parameters ($p < 0.01$). L* values (lightness) increased gradually, indicating pigment fading, while C* values decreased consistently across formulations, most sharply at 45 °C. Formulations containing β-cyclodextrin (βCD) or βCD + WPI (Figs. 4III, 5) retained color intensity most effectively, supporting prior findings that cyclodextrins form inclusion complexes with crocetin esters, reducing oxidation and thermal degradation [12, 22, 24].

Hue angle shifts indicated color movement from yellow toward duller greenish tones, particularly in maltodextrin (MD)-based systems, confirming their inferior color stability. Particle size parameters ($D_{43}$, $D_{32}$) increased over time, reflecting droplet coalescence, while βCD-based emulsions exhibited the least growth, corroborated by microscopy images showing minimal phase separation (Fig. 5).

Encapsulation efficiency (%EE) and flocculation rate (%FR) trends (Fig. 4VI–VIII) supported these observations: βCD and WPI-βCD systems preserved structural integrity and delayed droplet aggregation throughout storage, even at elevated temperatures.

These results collectively establish βCD and WPI-βCD formulations as the most stable W/O/W systems for crocin encapsulation and color preservation. The performance of these dual-stabilized emulsions substantially surpasses earlier studies limited to short-term (≤15 days) or single-temperature tests [8, 9].

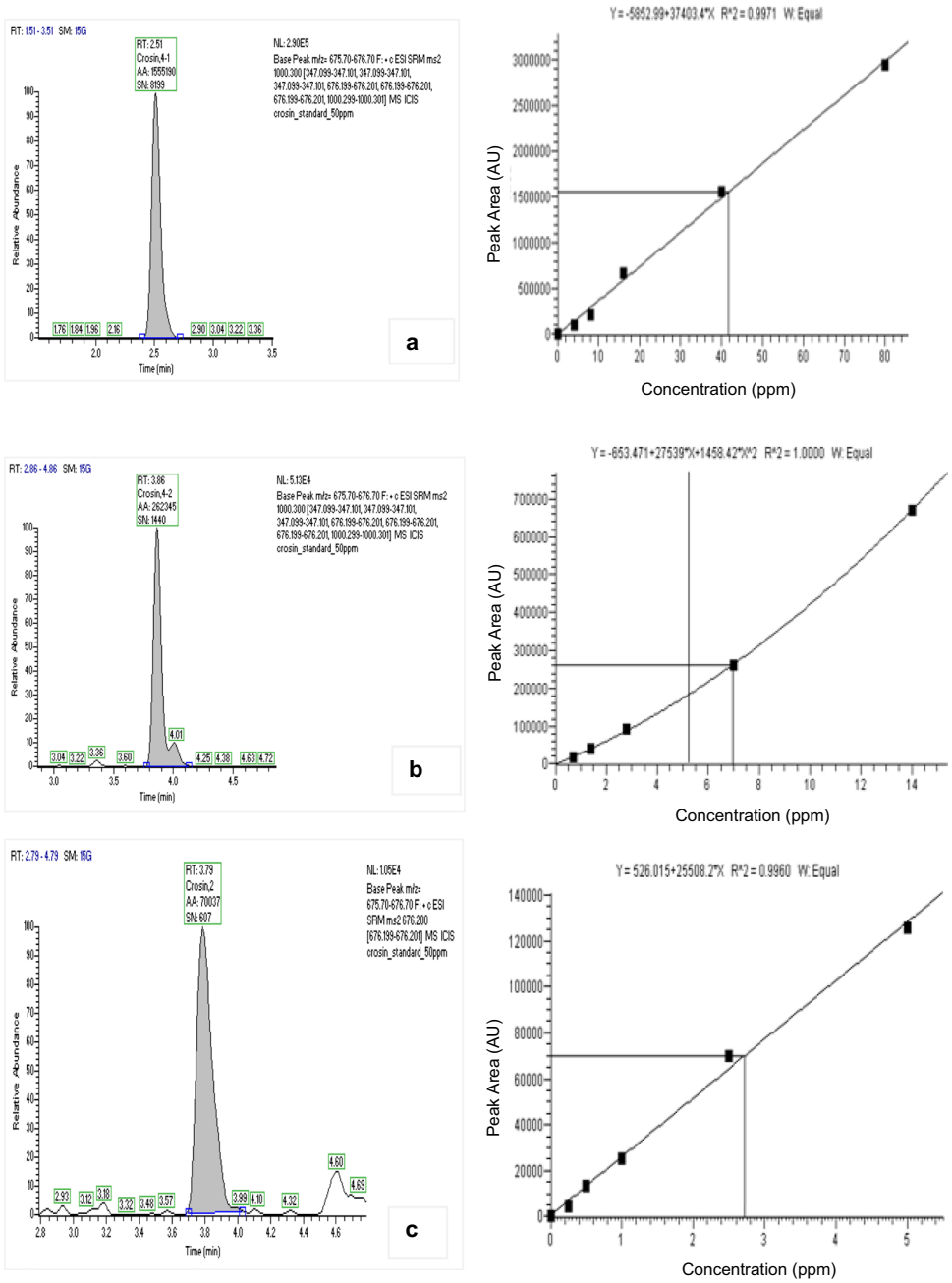



**Fig. 6** LC–MS/MS chromatograms and calibration curves of crocin isomers: (**a**) Crocin-1–1, (**b**) Crocin-1–2, and (**c**) Crocin-3. Calibration curves were obtained across 5–7 concentration levels with $R^2 \geq 0.99$ for all analytes. Detailed validation parameters (LOD, LOQ, recovery, and RSD%) are provided in Supplementary Table S47

## Storage stability and crocin degradation

Long-term storage tests revealed that temperature had a more pronounced influence on crocin degradation than time ($p < 0.01$). Crocin-1–1, the most abundant and stable isomer, exhibited the slowest degradation, followed by Crocin-1–2 and Crocin-3 (Table 2). WPI–βCD emulsions maintained significantly higher crocin retention under all conditions. At 45 °C, total crocin retention remained above 80% in these formulations compared to < 50% in MD-based systems, confirming superior protection mechanisms.

Crocin degradation kinetics followed apparent first-order trends (data not modeled), with higher rate constants at elevated temperatures—consistent with literature on thermally driven crocetin ester cleavage. The observed stabilization effect of βCD likely arises from host–guest complexation, while WPI enhances interfacial film strength, jointly limiting oxygen diffusion and pigment oxidation [13, 21, 23, 25, 26].

These observations were supported by LC–MS/MS quantification of individual crocin isomers (crocin-1–1, −1–2, and −3), which confirmed higher retention in βCD–WPI-stabilized

**Table 2** Effects of storage time and temperature on Crocin-1–1, Crocin-1–2, and Crocin-3 concentrations in W/O/W emulsions containing different formulations

| | Crocin type | Code | Concentration (mg/L) | Storage time (day) | Concentration (mg/L) | Storage temp (°C) | Concentration (mg/L) |
|---|---|---|---|---|---|---|---|
| W/O | **Crocin-1–1** | C | $127.74^{b} \pm 82.10$ | 35 | $96.64^{b} \pm 67.60$ | 45 | $144.07^{b} \pm 110.89$ |
| | | D | $208.53^{a} \pm 89.55$ | 0 | $239.64^{a} \pm 50.95$ | 25 | $154.65^{b} \pm 105.56$ |
| | | | | | | 5 | $205.69^{a} \pm 53.54$ |
| | **Crocin-1–2** | C | $16.68^{b} \pm 12.52$ | 35 | $12.45^{b} \pm 10.49$ | 45 | $19.11^{b} \pm 16.09$ |
| | | D | $28.63^{a} \pm 12.36$ | 0 | $32.86^{a} \pm 7.08$ | 25 | $20.61^{b} \pm 15.45$ |
| | | | | | | 5 | $28.23^{a} \pm 7.53$ |
| | **Crocin-3** | C | $16.93^{b} \pm 12.68$ | 35 | $12.60^{b} \pm 10.51$ | 45 | $19.40^{b} \pm 16.32$ |
| | | D | $29.04^{a} \pm 12.47$ | 0 | $33.37^{a} \pm 7.10$ | 25 | $20.91^{b} \pm 15.64$ |
| | | | | | | 5 | $28.64^{a} \pm 7.64$ |
| W/O/W | **Crocin-1–1** | F | $197.02^{c} \pm 98.02$ | 35 | $157.30^{b} \pm 82.08$ | 45 | $208.15^{c} \pm 137.54$ |
| | | E | $209.38^{c} \pm 113.76$ | 0 | $332.78^{a} \pm 58.44$ | 25 | $239.05^{b} \pm 112.96$ |
| | | G | $259.92^{b} \pm 89.30$ | | | 5 | $287.92^{a} \pm 71.23$ |
| | | H | $313.84^{a} \pm 122.42$ | | | | |
| | **Crocin-1–2** | F | $22.50^{d} \pm 9.49$ | 35 | $21.41^{b} \pm 11.48$ | 45 | $27.46^{c} \pm 18.76$ |
| | | E | $28.78^{c} \pm 15.75$ | 0 | $43.71^{a} \pm 11.01$ | 25 | $31.66^{b} \pm 15.91$ |
| | | G | $35.79^{b} \pm 12.97$ | | | 5 | $38.46^{a} \pm 10.78$ |
| | | H | $43.17^{a} \pm 17.46$ | | | | |
| | **Crocin-3** | F | $26.50^{d} \pm 10.24$ | 35 | $22.45^{b} \pm 11.20$ | 45 | $28.65^{c} \pm 18.62$ |
| | | E | $29.15^{c} \pm 15.84$ | 0 | $45.32^{a} \pm 8.82$ | 25 | $33.03^{b} \pm 15.15$ |
| | | G | $36.19^{b} \pm 12.43$ | | | 5 | $39.98^{a} \pm 9.18$ |
| | | H | $43.70^{a} \pm 17.05$ | | | | |

Values are means ± standard deviation ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$) according to Tukey's HSD test

Comparisons were made between formulations (codes) within each crocin type, as well as between storage times (0 and 35 days) and storage temperatures (5 °C, 25 °C, and 45 °C) for the same formulation. Different superscripts within the same comparison set denote significant differences, while identical letters indicate no significant difference. Statistical analysis was performed using two-way ANOVA considering formulation type and storage condition as fixed factors

emulsions (Fig. 6 and Table 2). Detailed statistical comparisons are provided in Supplementary Tables S48–S53.

### Implications and industrial relevance

These findings bridge the gap between laboratory-scale encapsulation studies and realistic food applications. The demonstrated formulations retained bioactive crocins and color integrity for over one month, fulfilling industrial shelf-life expectations for functional ingredients. The synergistic βCD–WPI system offers a scalable and food-grade solution for stabilizing carotenoid glycosides, paving the way for next-generation saffron-based functional foods.

## Conclusion

Integrating ISO-standardized extraction, statistically optimized emulsions, and multi-temperature storage testing provides a comprehensive framework for stabilizing saffron bioactives. Ultrafiltration (4 °C, 2 bar, 80:20 ratio) maximized crocin concentration, while βCD- and WPI-enriched W/O/W emulsions minimized degradation and color loss during 35-day storage. Validation in a saffron-rice model confirmed superior real-food stability. These outcomes establish a reproducible, scalable platform with strong potential for commercial development of high-value saffron formulations.

These trends were further confirmed by LC–MS/MS-based quantification of individual crocin isomers (crocin-1–1, −1–2, and −3), as illustrated in Fig. 6 and Table 2. Supplementary Tables S48–S53 provide full validation and statistical details supporting these findings.

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**Authors' contributions** • Negin Azarbadi: Conceptualization, Methodology, Data collection, Formal analysis, Writing - original draft.
• Feramuz Özdemir: Supervision, Validation, Writing - review & editing.

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**Data availability** Data supporting the findings of this study are included in the manuscript and supplementary files.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Not applicable (this study does not involve human participants or animal experiments).

**Consent to participate** Not applicable.

**Consent for publication** All authors consent to the publication of this manuscript.

**Conflicts of interest** The authors declare no conflicts of interest.



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