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Surfactant-Enhanced Extraction of Lutein from Marigold Petals using an Aqueous Two-Phase System

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Abstract: The extraction of lutein from marigold petals using a surfactant-based aqueous two-phase system is reported. In this work, the effectiveness of the hydrophilic-lipophilic balance of surfactants on extraction performance for the extraction of lutein from marigold petal powder was demonstrated using aqueous solutions of a wide range of non-ionic surfactants. The response surface methodology was applied to obtain optimised conditions for maximum extraction of lutein. At the optimised conditions (Temperature = 37.5 °C, S/L = 0.00375, and surfactant amount = 1.5% (v/v)), 12.12 ± 0.16 mg/g of lutein was obtained. Furthermore, the surface morphology of marigold petal powder (MPP) was analysed using SEM micrographs. Significant changes in surface morphology were observed which suggested better access of surfactant solution to the targeted biomolecule implanted in the matrix. Finally, the antioxidant activity of the obtained lutein extract was analysed using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Results suggest that the antioxidant activity of the lutein extract obtained by the surfactant-based system is more than that of the lutein extract obtained by organic solvents. The aforementioned results suggest that the lutein can be extracted using a surfactant-based aqueous two-phase system (ATPS).

Keywords: lutein; surfactant-based ATPS; HLB value; non-ionic surfactant

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1. Introduction

Lutein is a naturally occurring yellow compound found in a variety of foods [1–3]. Lutein is a member of the xanthophyll family of carotenoids [4]. The marigold flower is one of the commercial sources for the extraction of lutein [5,6]. The major xanthophyll constituent of the marigold flower is lutein accounting for nearly 90% of the total [7]. The structure of lutein comprises a long conjugated double-bond carbon chain with aromatic rings at either end [8]. Due to its unique structure, it has the ability to neutralise free radicals and singlet oxygen and works as an antioxidant [9]. Lutein is known as a macular pigment. It is one of the dominant carotenoids present in the human retina. Carotenoids help in preventing cataracts [10]. Lutein accounts for 66–77% of the total carotenoids in the eye, making lutein a key player in maintaining eye and brain health [11]. Lutein helps in preventing atherosclerosis and other cardiovascular illnesses and it also helps in boosting the human immune system [12]. There has been a growing interest in natural products for the treatment of cardiovascular disease. Experimental evidence suggests that lutein-rich foods or lutein supplementation have an anti-atherosclerotic effect [13,14]. Lutein also helps in the treatment of neurodegenerative diseases like Alzheimer, which afflicts more than 26 million people globally [15]. Lutein also protects photosystems from oxidative damage when exposed to high light intensities, particularly blue light [16].

Considering the aforementioned benefits of lutein, various researchers have demonstrated different methods for the extraction and isolation of lutein from marigold flow-

ers. These include soxhlet extraction [17], the supercritical carbon dioxide extraction method [18,19], enzyme-assisted aqueous two-phase extraction [20], extraction of lutein using vegetable oil as solvent [5], and the microemulsion technique [21]. The most commonly used method for the extraction of lutein is solvent extraction. Some of the examples of solvents used by previous researchers for the extraction of lutein are liquefied dimethyl ether [22], hexane [23], petroleum ether-acetone [24], solution of ethanol, and water [25]. The organic solvents described above for lutein extraction are mostly of a petrochemical origin. They can pose a health concern and can pollute the environment [25,26]. These organic solvents also have low selectivity [27]. Furthermore, these solvents may lead to the irreversible degradation of the product [28]. To address the aforementioned issues, there is a need to develop environmentally friendly methods of extraction and purification of lutein. Therefore, extraction using an aqueous two-phase system (ATPS) has gained importance amongst researchers working in the areas of food technology, biomedical research, and wastewater treatment due to better selectivity and higher separation efficiency. In addition, the use of ATPS causes relatively less damage to the target molecule [29].

The present work deals with the extraction of lutein from marigold petals using surfactant-based ATPS. The primary aim of this study is to develop a low-cost, long-term, and environmentally friendly process for the extraction of lutein. This process began with a preliminary screening of several non-ionic surfactants, followed by the application of response surface methodology to obtain the optimised parameters for maximum extraction of lutein. For this, the studied parameters were S/L ratio, surfactant concentration, extraction time, and temperature. Furthermore, cloud point extraction was performed to get a more concentrated form of lutein in the coacervate phase. Finally, the scavenging activity of lutein extract obtained by surfactant-based ATPS was studied to appraise the direct use of the obtained lutein extract in the cosmetics and pharmaceutical sectors.

2. Materials and Methods

2.1. Material

Fresh marigold flowers (*Tagetes erecta*) were purchased from a local market (Nagpur, India). The flowers were dark orange in colour. Solvents required for experimental and quantification purposes were acquired from Merck, India. Non-ionic surfactants: pluronic L101 (hydrophilic-lipophilic balance (HLB) 1), pluronic L121 (HLB 1), pluronic L81 (HLB 2), pluronic L62 (HLB 7), lutensol XL 50 (HLB 11.5), lutensol TO 7 (HLB 12), pluronic L64 (HLB 15), lutensol TO 89 (HLB 13), lutensol ON 60 (HLB 12), lutensol XL 80 (HLB 13), and plurafac LF 120 (HLB 10) were provided by BASF, India and used without further purifications. The HLB values of the above-mentioned surfactants were taken from the literature or the manufacturer's data.

2.2. Marigold Petal Powder (MPP) Preparation

The marigold flowers were washed and the petals were manually removed from the flower. The petals were washed with distilled water and then air dried (Hot Air Oven, model BT1-29, 24 h at 35 °C) away from light. The dried petals were ground into small particles ranging in size from 212 μm to 220 μm. The marigold petal powder obtained was stored at 4 °C and in a dark place for further use.

2.3. Experimental Section

The water employed in all assessments was uncontaminated, double-distilled water, passed through ion exchange resin for deionization and treated with a Merck Millipore direct Q3 water purification device. The samples were always covered with aluminum foil to avoid contact with direct light to avoid degradation and photooxidation of the samples.

2.3.1. Extraction of Lutein Using Surfactant-Based ATPS

Lutein extraction was carried out using surfactant-based ATPS. The material was extracted with different surfactants under the same conditions—e.g., temperature, extraction

time, surfactant concentration, and S/L ratio—to achieve the highest amount of lutein. In brief, 0.5 g of petal powder was added to 20 mL of a solution containing double distilled water (DDW) and non-ionic surfactant in a 19:1 ratio and mixed for 2 h using an orbital incubator shaker (Model No. CIS-18 Plus, Remi, India). The sample was maintained at 30 ± 01 °C. All solutions containing a known amount of surfactant and S/L ratio were prepared gravimetrically. In all the experiments, the stirring speed was kept constant at 110 rpm. For each process condition, experiments were conducted in triplicate. After the extraction step, the samples obtained were filtered using dustless lab filter paper to obtain the supernatant for further use. The mixtures of different surfactants with a required HLB value were also evaluated, and calculated using Equation (1):

$$HLB = HLB_{S1}W_{S1} + HLB_{S2}W_{S2} \quad (1)$$

where HLB_{S1} and HLB_{S2} are the HLB values for the pluronic L101 and pluronic L64, respectively, and W_{S1} and W_{S2} are the weight fraction of pluronic L101 and pluronic L64, respectively.

2.3.2. Cloud Point Extraction

After the extraction step, the obtained lutein extract was concentrated by raising the temperature of the lutein extract above the surfactant cloud point temperature. This resulted in preferential partitioning of the sample between the two phases (coacervate phase—surfactant-rich—and non-coacervate phase—water-rich). The two phases obtained were then separated carefully. The separation efficiency of lutein was determined by the ratio of lutein present in the coacervate phase to the content of lutein in the extract before cloud point extraction. For both phases, the volumes were recorded. Acetone was used to dilute the concentrated phase prior to the quantification of lutein.

2.4. Spectrophotometric Analysis of Lutein

Lutein assay was carried out as per the literature [21]. The total amount of lutein in the sample was quantified using lutein's molar extinction coefficient in acetone. The analysis of the extracts was performed by a spectrophotometer (Agilent Spectrophotometer, Cary60, path length = 1 cm). The maximum peak was obtained at a wavelength of 446 nm using a UV–VIS Spectrophotometer. All extracts were collected and their absorbance readings were recorded at 446 nm, the wavelength with the minimum interference from other carotenoids [30]. A blank, without lutein, was prepared for each extraction condition and used as an analytical blank for the corresponding phases.

The amount of lutein extracted from marigold petal powder was calculated using Equation (2).

$$C = \frac{A}{(14.45 \times 10000) \times (b)} \times 568.88 \times \frac{V}{M} \times \left(\frac{1\ \mathrm{L}}{103\ \mathrm{mL}}\right) \times \left(\frac{10^3\ \mathrm{mg}}{\mathrm{gm}}\right) \quad (2)$$

where C is the amount of lutein obtained (mg/g MPP), A is maximum absorbance wavelength (λ_{max}, nm), b is path length (cm), 568.88 is the molar mass of lutein (g mol^{-1}), V is the volume of extracted sample (mL), M is the weight of the consumed MPP (g), and 14.45×10^4 is the molar extinction coefficient (L mol^{-1} cm^{-1}) of lutein in acetone [30].

2.5. Optimisation of Parameters by Response Surface Methodology

RSM (Minitab version 17) was used to examine various experimental conditions and to find the effect of independent parameters on the extraction of lutein. This model provides response surface curves and also provides the extraction conditions to get the highest amount of lutein. In accordance with the results obtained from preliminary experiments (Figure 1) with various non-ionic surfactants, plurafac LF 120 was selected to perform a Box–Behnken Design (BBD) optimisation with the aim of optimising the amount of lutein extracted. The empirical ranges of the selected independent parameters, including temper-

ature (30 °C–45 °C), surfactant concentration (0.5–2.5% (v/v)) and biomass weight—solvent ratio (S/L: 0.0025–0.005). S/L ratio and temperature were selected according to the reported literature [22,31,32] and also selected on the basis of preliminary experiments. The surfactant concentration range was selected on the basis of the critical micelle concentration of the surfactant and also on the trial experiments based on the temperature-concentration relation. For this study, a three-level BBD was applied to explore the effects of various combinations of the selected process parameters. For the optimisation of operating conditions, 15 randomised experimental runs were performed (Table 1) and the lutein content was quantitatively studied by UV-VIS spectrophotometer.

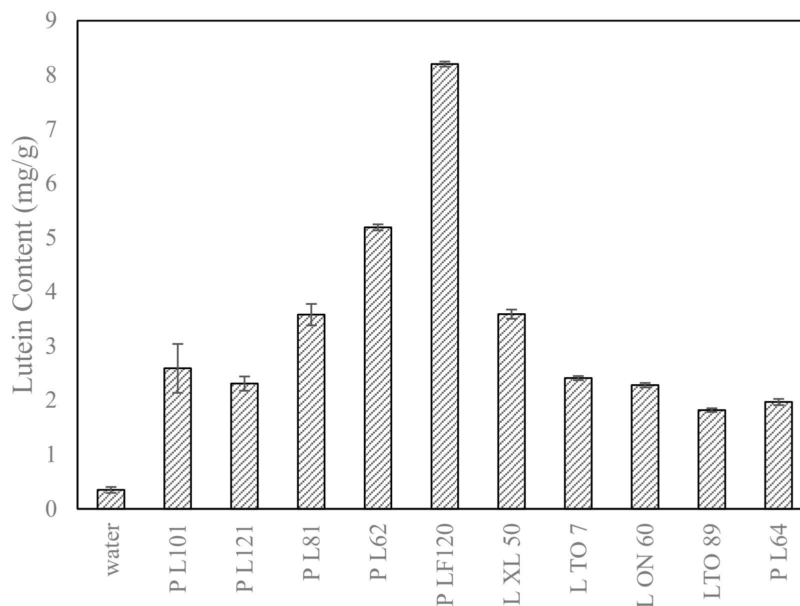

Figure 1. Extraction of lutein using different non-ionic surfactants.

Table 1. Box-Behnken Matrix with experimental response.

Run No.	Temperature (X1)	S/L (X2)	Surfactant Concentration (X3)	Lutein Content (Y)
1	30	0.0025	1.5	6.01 ± 0.13
2	37.5	0.0025	0.5	7.89 ± 0.14
3	37.5	0.0025	2.5	6.20 ± 0.14
4 *	37.5	0.00375	1.5	12.12 ± 0.16
5	37.5	0.00375	1.5	11.98 ± 0.13
6	45	0.0025	1.5	4.90 ± 0.07
7	30	0.00375	0.5	7.52 ± 0.04
8	45	0.005	1.5	5.01 ± 0.03
9	37.5	0.005	0.5	7.89 ± 0.07
10	37.5	0.005	2.5	9.56 ± 0.07
11	30	0.005	1.5	8.23 ± 0.13
12	45	0.00375	2.5	6.72 ± 0.08
13	30	0.00375	2.5	6.98 ± 0.10
14	37.5	0.00375	1.5	10.96 ± 0.08
15	45	0.00375	0.5	6.01 ± 0.13

* Optimized operating conditions for the maximum extraction of lutein

The relation of the independent and dependent variables is given by a quadratic regression model as follows:

$$Y = \beta_0 + \sum_{i=0}^{4} \beta_i X_i + \sum_{i=0}^{4} \beta_{ii} X_i^2 + \sum_{i=0}^{4} \sum_{i=0}^{4} \beta_{ij} X_i X_j \quad (3)$$

where Y is the predicted response (separation efficiency (%) of lutein); β_0 is the constant coefficient; β_i is the linear coefficient; β_{ii} is the quadratic coefficients; β_{ij} is the two-factor interaction coefficient, and X_i and X_j the independent variables [33].

The goodness of the model obtained was evaluated by estimating the value of regression coefficient (R^2), adjusted regression coefficient, *p*-value, and F-value obtained by ANOVA. Minitab version 17.0 was used for all data interpretation and contour plots.

2.6. Antioxidant Activity Test

The scavenging tendency of different extracts containing lutein was assessed by the 2,2-diphenyl-1-picrylhydrazyl free radical. In this study, 3 mL of DPPH solution (0.2 mM) was dissolved in methanol and mixed with 1 mL of lutein extract and the sample was kept for 30 min at 30 °C in the dark. After 30 min, the absorbance value was measured at 517 nm [34]. A blank used was prepared by the use of methanol and DPPH. The DPPH free radical reaction with a lutein extract was determined as described previously [35]. The positive control was ascorbic acid purchased from Sisco Research Laboratories, Nagpur, India. The antioxidant activity was expressed in terms of IC_{50} values. The inhibition (%) was determined using Equation (4).

$$\text{Inhibition (\%)} = [(A_c - A_s)/(A_c)] \times 100 \quad (4)$$

where A_c represents the absorbance value of the control and A_s represents the absorbance value of the tested sample.

2.7. Data Analysis

All experimental studies were performed in triplicate on newly prepared samples. The results were reported as mean ± standard deviations of the experimental data. One-way ANOVA was carried out using Tukey's test to calculate the significance of variation between the mean values. The variations were considered significant only at $p < 0.05$.

3. Results and Discussions

3.1. Effect of the Surfactant Type Used for Extraction of Lutein

Preliminary screening of several surfactants for the extraction of Lutein was performed at concentrations above their critical micelle concentration. This was done to obtain the most effective surfactant for lutein extraction. The surfactants utilised for this study are given in the experimental section. The experimental conditions were the same for all experiments (DDW: Surfactant = 19:1 (*v*/*v*), mixing time = 2 h, temperature = 30 °C). The effect of different surfactants on the extracted amount of lutein is shown in Figure 1. The amount of lutein extracted by the surfactant solutions was also compared with the amount of lutein obtained by double-distilled water under the same conditions. The outcome of the experiments shows that the amount of lutein obtained using aqueous solutions of surfactants (at a lower amount) is notably higher than that achieved with DDW. The above results reveal the effectiveness of surfactants in interacting with hydrophobic compounds from marigold petals. However, the amount of lutein obtained was highly dependent on the type of surfactant used. Among the used surfactants, plurafac LF 120 (HLB = 10) gave the highest extraction amount (8.2 ± 0.05 mg/g) of lutein, while the use of surfactants like pluronic and lutensol surfactants resulted in a lower extraction amount of lutein in comparison to plurafac LF 120, but extracted a significantly higher amount of lutein than DDW.

The extraction of lutein from marigold petals was also performed using different organic solvents (acetone, methanol, ethanol, tetrahydrofuran (THF), chloroform, xylene,

hexane) for comparison purposes under the same experimental conditions (DDW: Surfactant = 19:1, mixing time = 2 h, temperature = 30 °C). From the results, it was observed that THF extracted the highest amount (12.92 ± 0.0813 mg/g) of lutein followed by chloroform (10.18 ± 0.201 mg/g) and acetone (11.01 ± 0.511 mg/g). This may be due to the fact that the solubility of lutein is very high in THF (8000 mg/L) followed by chloroform (6000 mg/L) and acetone (800 mg/L) [30]. Amongst the solvents used for this study, methanol extracted the lowest amount of lutein (2.97 ± 0.15 mg/g) followed by ethanol (3.39 ± 0.30 mg/g) and hexane (4.45 ± 0.22 mg/g). This may be due to the lower solubility of lutein in these solvents [6,30]. The aforementioned results suggest that an aqueous solution of alkoxylate unbranched fatty alcohols surfactant at low concentration extracted the highest amount of lutein (8.2 ± 0.05 mg/g) while synthetic block copolymer surfactants extracted the lowest amount of lutein. These results suggest that alkoxylate unbranched fatty alcohol surfactants can be used for the efficient extraction of lutein.

To better acknowledge the role of different surfactant solutions, the relationship between the amount of lutein extracted and the HLB value of an individual surfactant was evaluated. Surfactants with HLB values between 7 and 10 were found to be most effective for the extraction of lutein. It was also observed that the amount of lutein extracted by surfactants having an HLB range outside of 7 to 10 was significantly low.

To further understand whether the extracted amount of lutein is dependent on the surfactant structure or on the micelle formation property of the surfactant (where HLB plays an important role), the extraction of lutein was performed using aqueous solutions of pluronic L121 (HLB value = 1) and pluronic L64 (HLB value = 15). Surfactant solutions with different HLB values (1,2,7,10,11.5,12,13,15) were prepared using pluronic L121 (HLB = 1) and pluronic L64 (HLB = 15) according to Equation (1). Extractions were performed under the same experimental conditions (DDW: Surfactant = 19:1, mixing time = 2 h, temperature = 30 °C) as mentioned in the experimental section. The results obtained (Figure 2) show that the maximum extraction of lutein was in the HLB value of 7 to 10. Outside this range, a remarkable decrease in the amount of extracted lutein was observed. It was also observed that aqueous solutions of a mixture of surfactants and aqueous solutions of individual surfactants having the same HLB values extracted almost the same amount of lutein. Hence, it can be concluded from these results that no surfactant—lutein interaction is responsible for the extraction of lutein.

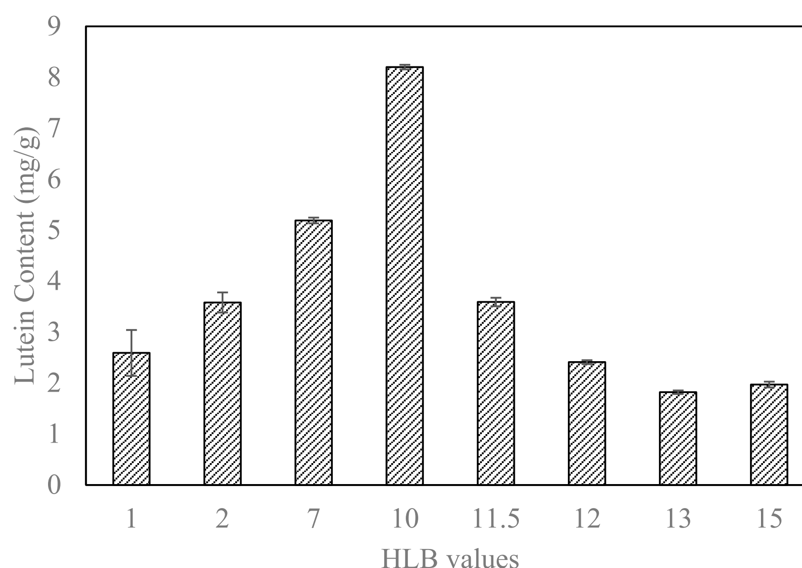

Figure 2. Effect of HLB value (hydrophile—lipophile balance) on the extraction of lutein by a combination of two surfactants.

Preliminary screenings for extraction of lutein from marigold petals powder. The results attained manifest that the extraction of lutein by non-ionic surfactant solutions,

specially plurafac LF 120, gave the most lutein (8.197 ± 0.046 mg/g) among all surfactants studied. Based on these results, plurafac LF 120 was selected for further studies on the optimisation of process parameters for the extraction of lutein.

3.2. Optimization of Process Parameters for Extraction of Lutein

Previously, the optimisation of operational parameters was carried out using univariate methods. However, their main drawback is that they do not consider the effect of the interaction of different parameters. Hence, they may not produce the desired optimum results. For this study RSM using BBD was chosen for the optimisation of process conditions for the extraction of lutein. BBD was performed using an aqueous solution of plurafac LF 120 by taking a constant extraction time of 60 min. The extraction time was kept constant based on preliminary experiments in which the extraction time was varied from 30 min to 120 min. After 60 min of extraction, there was not much difference in the amount of extracted lutein. The outcome of BBD was studied in terms of lutein content (mg/g) (Y). Fifteen randomized runs were obtained by BBD as shown in Table 1.

The results obtained by the BBD model gave us the combined effect of S/L ratio—surfactant concentration, S/L ratio, temperature, and temperature-surfactant concentration on the extraction amount of lutein. After applying the multiple regression equations, a quadratic equation was selected to attain maximum lutein content. ANOVA was used to estimate the importance of the coefficients of Equation (2). The importance of the coefficients was determined on the basis of *p*-value and F-value. When the *p*-value is smaller and F-value is higher, the coefficient will be more valuable. Analysis of the variance in the regression model showed that the model is significant, as it has a high F-value (23.60) with a very low *p*-value (0.001), as can be seen in Table 2. Parameters can also be considered as having significance if the *p*-value is less than 0.05 ($p < 0.05$) [36]. An R^2 near 100% indicates a better response, as it shows that the model fits well. The R^2 for the extraction of lutein was 97.70%. This signifies that only a 3% variation in the results could not be explained by the model. In addition, the value of the adjusted R^2 (93.56%) was high. This indicates a high significance of the model [37]. Statistically, the significant conditions ($p< 0.05$) are temperature (X1), S/L ratio (X2) and the interaction between surfactant concentration (X3) and S/L ratio (X2). The contour plots for the independent variables are shown in Figure 3. They express the dependency of lutein content on the interaction of two independent factors at the same time. Each contour plot has a middle parameter kept constant to monitor the effect of other parameters.

The contour plot for lutein content versus surfactant concentration and S/L ratio is shown in Figure 3A. It was observed from Figure 3A that, on increasing the S/L ratio from 0.003 to 0.0045, the extraction amount of lutein increased from 9 mg/g to 11 mg/g. This may be due to the sufficient mass transfer between the biomass and the solvent. Further increases in the S/L ratio caused a decrease in lutein content. This may be due to the hindrance caused by biomass in the driving force for mass transfer [38].

The contour plot for lutein content versus surfactant concentration and temperature is shown in Figure 3B. It was observed that the extraction of lutein markedly increased from 6 mg/g to 11 mg/g (with an increase in the concentration of surfactant). This increase may be attributed to the formation of micelles, which could have helped to capture most of the lutein present in biomass [39]. However, a further increase in surfactant concentration didn't affect the extraction of lutein [40]. Similar studies were reported by Goswami et al. for cloud point extraction of nitrobenzene using Triton X-100 [41]. This may be due to a reduction in micelle size on increasing surfactant concentration. Also, an excess quantity of surfactant can also cause wastage of surfactant. Similarly, increasing the temperature, increased the extraction of lutein. The highest lutein extracted amount was at approximately 36 °C. A further increase in temperature caused lower extraction. A similar finding was reported by Boonnoun et al. [22] using liquefied dimethyl ether. This may be due to the fact that higher temperatures could have increased the diffusivity of the solvent into MPP, which could have resulted in a higher rate of dissolution of lutein into the solvent [6,42]. At

very high temperatures, the decline in the amount of lutein extracted could be explained by the degradation of lutein at high temperatures [22]. Moreover, the stability of lutein decreases with an increase in temperature. Similarly, from the contour plot in Figure 3C, it was observed that with an increase in temperature from 34 °C to 40 °C, the lutein content increased from 9 mg/g to 11 mg/g. A further increase in the temperature caused a decrease in the amount of lutein extracted. The decline in lutein content could be explained by the degradation of lutein at temperatures above 40° C [22].

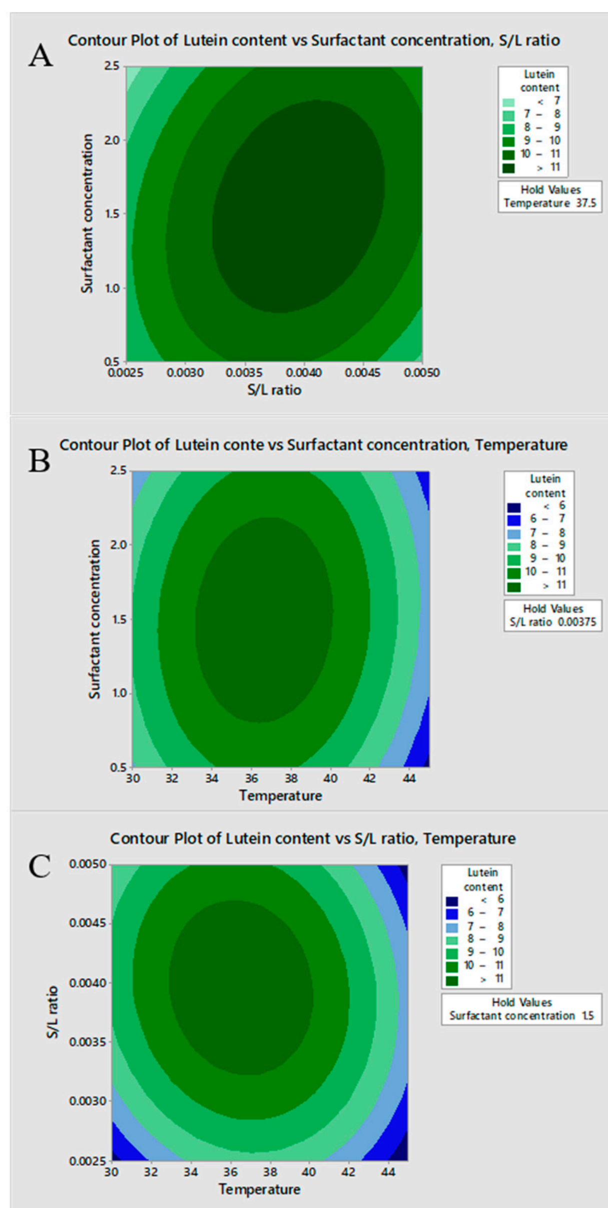

Figure 3. Contour plots representing the effect of the interaction between two independent parameters on lutein extraction. (**A**): represents the effect of interaction of surfactant concentration and S/L ratio on lutein content, (**B**): represents the effect of interaction of surfactant concentration and temperature on lutein content, (**C**): represents the effect of interaction of S/L ratio and temperature on lutein content).

Table 2. Estimated coefficients for Lutein content.

Source	F-Value	*p*-Value
Model	23.60	0.001
Linear	17.74	0.022
X_1	31.76	0.015
X_2	20.20	0.019
X_3	11.24	0.932
Square	58.44	0.000
${X_1}^2$	118.78	0.000
${X_2}^2$	54.86	0.000
${X_3}^2$	24.13	0.058
2—way Interaction	4.10	0.081
$X_1 \times X_2$	3.17	0.135
$X_1 \times X_3$	1.11	0.340
X2 × X3	8.03	0.035
R^2	97.70%	
${R^2}_a$	93.56%	

3.3. Validation of Process Parameters

At the predicted optimum conditions (surfactant concentration 1.53% (v/v), S/L ratio 0.004, and temperature 36.5 °C), the predicted outcome by the selected model for the optimum production of lutein extract was 11.79 mg/g. Experiments were performed at these predicted conditions. This resulted in 12.01 mg/g of lutein extracted. The obtained results showed that the experimental outcome was in good agreement with the predicted outcome. It also confirms that the BBD design approach was successfully used for the optimization of independent parameters for the extraction of lutein by surfactant-based ATPS.

$$\begin{aligned} Y = -96.52 + 4.531\ X_1 + 12643\ X_2 + 0.48\ X_3 - 0.05979\ {X_1}^2 - 14662933{X_2}^2 \\ -1.516\ {X_3}^2 - 56.3\ X_1X_2 + 0.0417\ X_1X_3 + 672\ X_2X_3 \end{aligned} \quad (5)$$

where Y is the lutein content (mg/g), X_1 is the temperature, X_2 is the S/L ratio, and X_3 is the surfactant concentration

The results obtained (for the extraction of lutein from MPP using surfactant-based ATPS) in this study have been tabulated in Table 3 along with results reported by other researchers. It was noted that the extraction of lutein using surfactant-based ATPS was higher than the extraction of lutein using enzyme-assisted ATPE [20] and microwave enzyme-assisted ATPE [43] techniques. This may be attributed to the good solubility of lutein in the plurafac LF 120 surfactant used in this study [44]. Boonnoun et al. reported the extraction of 16.65 mg (lutein)/g (dried marigold flower) using DME as solvent. This was attributed to the fact that the DME can dissolve most of the carotenoids below their critical temperature and pressure (126.85 °C, 53.7 bar) [45]. However, the major disadvantage of using DME lies in the solvent recovery step. Solvent recovery may degrade the extracted compound and can also have a negative impact on process economics [46]. In addition, the surfactants used for the current study are biocompatible, and hence, further purification of the extracted compound is not required.

Table 3. Different extraction methods used by different authors for the extraction of lutein.

S. No	Source	Methods of Extraction	Solvents	Conditions	Extracted Amount of Lutein Content	Year	References
1.	Marigold flowers	Supercritical CO_2 Extraction	Soyabean oil as co-solvent	58.7 °C, 35.5 MPa, CO_2 flow rate of 19.9 L/h with 6.9% of soybean oil	10.397 mg/g	2007	[42]
2.	Marigold flowers	Solvent extraction	Hexane	40 °C, solvent/material 5 L/kg	2.13 mg/g	2007	[6]
3.	Marigold flowers	(SC-CO_2) Extraction & ultrasound	Supercritical Carbon dioxide	55 °C, extraction pressure of 32.5 MPa, CO_2 flow rate of 10 kg/h	6.90 mg/g	2009	[43]
4.	Marigold flowers	Solvent extraction	(DME)–KOH–EtOH mixture	35 °C, Solvent: marigold flowers 33:0.5 (w/w), extraction time 1 h	16.65 mg/g	2017	[22]
5.	Marigold flowers	Enzyme assisted ATPE	Ethanol/ammonium sulphate system	37 °C enzymolysis temperature 30% (w/w) ethanol/19% (w/w) ammonium sulphate, and Extraction time 117 min	5.59 mg/g	2018	[20]
6.	Marigold flowers	microwave and enzyme co-assisted ATPE	ethanol/ammonium sulphate	45 °C enzymolysis temperature 28% ethanol/20% ammonium sulphate, Extraction time of 150 min	7.32 mg/g	2018	[43]
7.	Marigold flowers	Solvent free extraction	Canola oil	0.2 g dried flower/mL oil	6.05 mg/g	2019	[5]
8.	Marigold flowers	Microemulsion technique	Lecithin, sunflower oil	25 °C, S/L: 20 mg of MPP/10 mL of acetone, mixed for 30 min at 300 rpm	14.51 mg/g	2020	[21]
9.	Marigold flowers	Surfactant-based ATPS	Various non-ionic surfactant solutions	37.5 °C, S/L = 0.00375, Surfactant Concentration = 1.5% (v/v)	12.12 mg/g		This study

3.4. Phase Behaviour Study of Plurafac LF 120 Surfactant

This part of the study was carried out since the cloud point extraction process is affected by many factors like cloud point temperature, surfactant concentration, and two-phase temperature [47]. The cloud point temperature is the temperature at which clear surfactant solution becomes turbid on heating [47]. The two-phase temperature is the temperature at which two phases can clearly be observed on heating the surfactant solution above the cloud point temperature. The cloud point and two-phase temperatures were determined on the basis of visual observation. From the results of our experiments, it was observed (Figure 4) that on increasing the concentration of surfactant, the cloud point temperature and two-phase temperatures decreased. The decrease in cloud point temperature and two-phase temperature on increasing surfactant concentration may be due to the increase in micelle concentration [39]. Although, a further increase in surfactant concentration beyond 1.2% (v/v) didn't affect the cloud point temperature (Figure 4). The effect of biomass on the cloud point and two-phase temperatures at different surfactant concentrations was also studied. It was observed (Figure 4) that, on adding biomass, both the cloud point temperature and the two-phase temperature increased compared to the solution without biomass.

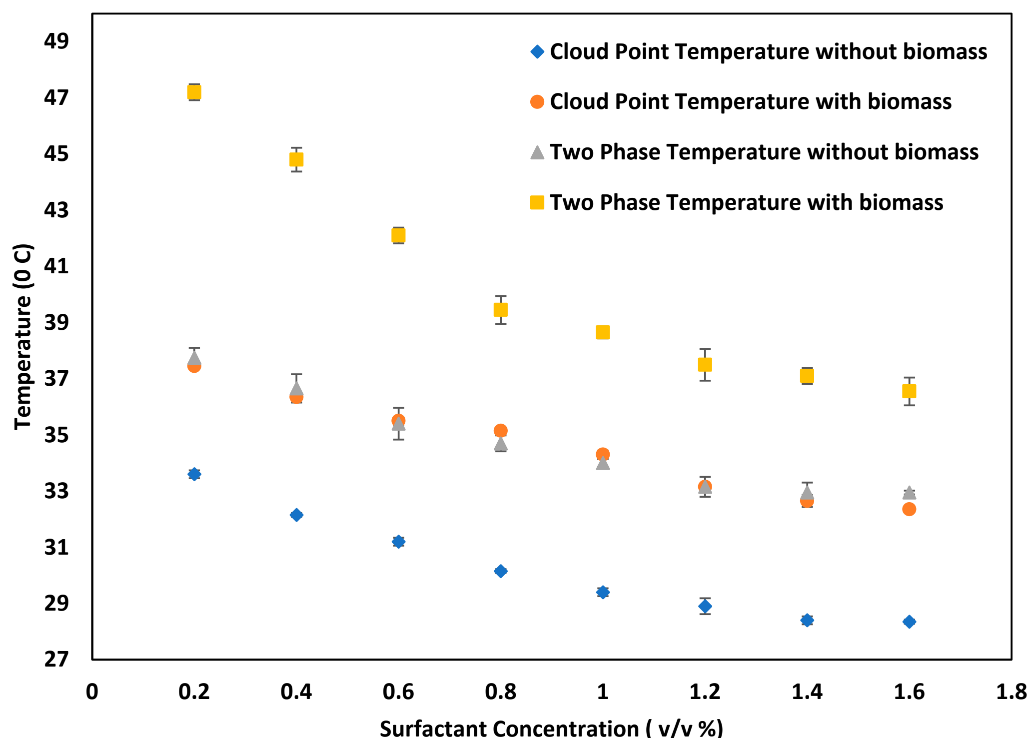

Figure 4. Phase diagram of plurafac LF 120.

3.5. Cloud Point Extraction

After demonstrating that aqueous solutions of non-ionic surfactants can be efficiently used for the extraction of lutein from MPP, we looked into concentrating lutein using cloud point extraction (CPE). Cloud point extraction allows us to concentrate lutein in the coacervate phase. This helps in reducing the water content of the lutein extract. This is one of the advantages of cloud point extraction in comparison to the extraction of biomolecules using volatile organic solvents.

CPE was carried out using plurafac LF 120 as a solvent under the optimised conditions (Temperature = 37.5 °C, S/L ratio = 0.0037, Surfactant Concentration = 1.5% (v/v)) obtained by BBD. The lutein extract obtained was placed at 40 °C for 40 min to achieve two-phase separation. The lower phase consisted of a small volume of surfactant-rich phase enriched with lutein. The upper phase consisted of a large volume of water with a much lower amount of surfactant and lutein. Using the above approach, a concentrated form of lutein could be obtained in the surfactant-rich phase with a separation efficiency of 95.6%.

3.6. SEM Analysis

The morphology of the marigold petal powder before and after extraction was studied using scanning electron microscopy (JEOL 6380 A). This analysis was carried out to check the effects of surfactants and solvents on MPP. From Figure 5A it was observed that the surface before treatment had a well-organised smooth surface, which was found to be distorted after the extraction process. After treatment with solvent and surfactant, many pores on the surface of biomass were observed as can be seen in Figure 5B,C. This may be due to the diffusion of solvent inside the MPP matrix, which might have led to the leaching of biomolecules present inside it [48].

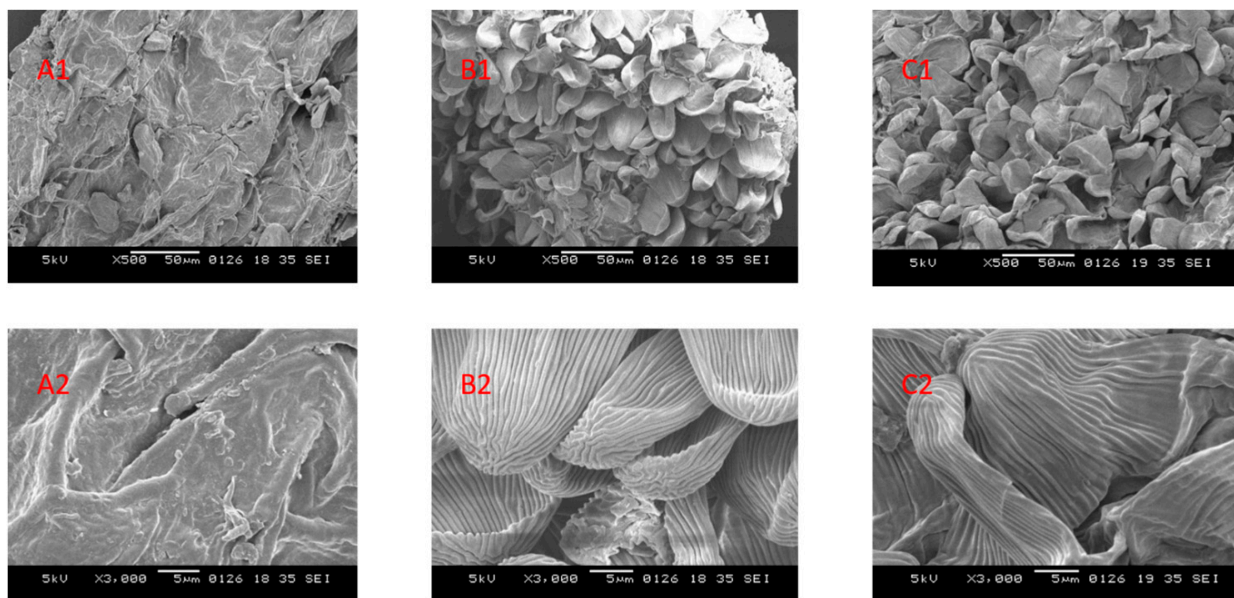

Figure 5. SEM analysis of MPP: (**A**–**C**) represent MPP before treatment, after treatment with solvent, and after treatment with surfactant. (**1**) and (**2**) represent images at two different magnifications.

3.7. DPPH Scavenging Activity

Non-ionic surfactants have been used in this study for the extraction of lutein. These surfactants are biodegradable. Hence, the antioxidant activity of surfactant–lutein extracts was evaluated to assess the feasibility of direct use of these extracts without any further recovery step. The antioxidant activity of lutein extracts was studied using DPPH free-radical scavenging assay by taking ascorbic acid as a positive control. The antioxidant activity of an aqueous solution of plurafac LF 120 surfactant containing lutein extract before and after cloud point extraction was evaluated. Also, the antioxidant activity of lutein extracts obtained by organic solvents had been evaluated for comparison. The antioxidant activity of an aqueous solution of plurafac LF 120 was also evaluated as a control. The lutein extracts obtained with THF and acetone showed comparable IC_{50} values (38.05 ± 0.95 µg/mL, 39.95 ± 0.855 µg/mL). The lutein extract obtained by plurafac LF 120 showed a lower IC_{50} value (33.7 ± 0.52 µg/mL) than the lutein extracts obtained with the solvents. The IC_{50} value of lutein (35 µg/mL) by DPPH scavenging assay was reported by Sindhu et al. [49]. It was also observed that the IC_{50} value of surfactant–lutein extract was not affected by the presence of surfactant. This was evidenced by the null IC_{50} value of the aqueous solution of surfactants.

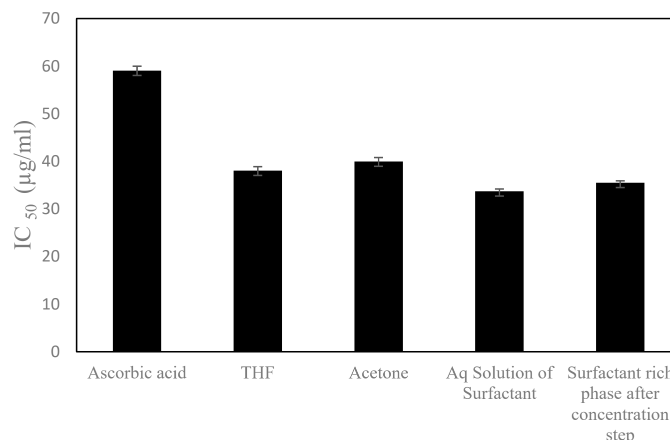

Figure 6. IC_{50} Values of different lutein extracts.

It was observed from Figure 6 that all extracts containing lutein showed lower IC_{50} than ascorbic acid. Wang et al. suggested that the high antioxidant activity in marigold petal extract is due to the presence of lutein in comparison to other biomolecules present [50]. In conclusion, these findings suggest the direct use of surfactant phase containing lutein in nutraceutical and cosmetics applications may be possible without carrying out an additional purification step.

4. Conclusions

A non-toxic, efficient, and robust extraction method for lutein from marigold flower petals has been developed. Plurafac LF 120 was selected for the extraction of lutein based on preliminary screening of multiple surfactants. Under optimized conditions (Temperature = 37.5 °C, Extraction time = 1 h, S/L = 0.00375, & Surfactant amount = 1.5% (v/v)), 12.12 ± 0.16 mg/g of lutein was obtained. Results obtained from BBD suggested that temperature, S/L ratio, and the interaction between surfactant concentration and S/L ratio played a major role in the extraction of lutein. The concentration of lutein was achieved by cloud point extraction, which led to a separation efficiency of 95.6%. It was also observed that lutein-rich extracts in an aqueous surfactant solution showed higher antioxidant activity than lutein extracts obtained by organic solvents. The results exhibit the potential use of non-ionic surfactants as solvents for the successful extraction of lutein.

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Abbreviations

MPP	Marigold petal powder
P	Pluronic surfactants
PF 120	Plurafac LF 120
L	Lutensol Surfactants
ATPS	Aqueous Two-Phase System
CMC	Critical Micelle Concentration
HLB	Hydrophilic-lipophilic balance

References

1. Ochoa Becerra, M.; Mojica Contreras, L.; Hsieh Lo, M.; Mateos Díaz, J.; Castillo Herrera, G. Lutein as a Functional Food Ingredient: Stability and Bioavailability. *J. Funct. Foods* **2020**, *66*, 103771. [CrossRef]
2. Abdel-Aal, E.S.M.; Akhtar, H.; Zaheer, K.; Ali, R. Dietary Sources of Lutein and Zeaxanthin Carotenoids and Their Role in Eye Health. *Nutrients* **2013**, *5*, 1169–1185. [CrossRef]
3. Murillo, E.; Meléndez-Martínez, A.J.; Portugal, F. Screening of Vegetables and Fruits from Panama for Rich Sources of Lutein and Zeaxanthin. *Food Chem.* **2010**, *122*, 167–172. [CrossRef]
4. Wang, L.; Li, Y.; Xiang, D.; Zhang, W.; Bai, X. Stability of Lutein in O/W Emulsion Prepared Using Xanthan and Propylene Glycol Alginate. *Int. J. Biol. Macromol.* **2020**, *152*, 371–379. [CrossRef] [PubMed]
5. Indrawati, R.; Kurniawan, J.M.; Wibowo, A.A.; Juliana; Gunawan, I.A.; Heriyanto; Brotosudarmo, T.H.P. Integrated Solvent-Free Extraction and Encapsulation of Lutein from Marigold Petals and Its Application. *CYTA—J. Food* **2019**, *17*, 121–127. [CrossRef]
6. Hojnik, M.; Škerget, M.; Knez, Ž. Extraction of Lutein from Marigold Flower Petals—Experimental Kinetics and Modelling. *LWT—Food Sci. Technol.* **2008**, *41*, 2008–2016. [CrossRef]

7. Tanaka, Y.; Sasaki, N.; Ohmiya, A. Biosynthesis of Plant Pigments: Anthocyanins, Betalains and Carotenoids. *Plant J.* **2008**, *54*, 733–749. [CrossRef]
8. Hirdyani, H. Lutein—The Less Explored Carotenoid. *World J. Pharm. Res.* **2017**, *6*, 528–553. [CrossRef]
9. Alves-Rodrigues, A.; Shao, A. The Science behind Lutein. *Toxicol. Lett.* **2004**, *150*, 57–83. [CrossRef]
10. Landrum, J.T.; Bone, R.A. Lutein, Zeaxanthin, and the Macular Pigment. *Arch. Biochem. Biophys.* **2001**, *385*, 28–40. [CrossRef]
11. Zafar, J.; Aqeel, A.; Shah, F.I.; Ehsan, N.; Gohar, U.F.; Moga, M.A.; Festila, D.; Ciurea, C.; Irimie, M.; Chicea, R. Biochemical and Immunological Implications of Lutein and Zeaxanthin. *Int. J. Mol. Sci.* **2021**, *22*, 10910. [CrossRef] [PubMed]
12. Dwyer, J.H.; Navab, M.; Dwyer, K.M.; Hassan, K.; Sun, P.; Shircore, A.; Hama-Levy, S.; Hough, G.; Wang, X.; Drake, T.; et al. Oxygenated Carotenoid Lutein and Progression of Early Atherosclerosis: The Los Angeles Atherosclerosis Study. *Circulation* **2001**, *103*, 2922–2927. [CrossRef] [PubMed]
13. Hajizadeh-Sharafabad, F.; Ghoreishi, Z.; Maleki, V.; Tarighat-Esfanjani, A. Mechanistic Insights into the Effect of Lutein on Atherosclerosis, Vascular Dysfunction, and Related Risk Factors: A Systematic Review of in Vivo, Ex Vivo and in Vitro Studies. *Pharmacol. Res.* **2019**, *149*, 104477. [CrossRef] [PubMed]
14. Liu, Y.L.; Zhou, Z.Y.; Gao, M.; Ji, G.; Huang, C.; Fan, S.J. Therapeutic Effects of Herbal Formula Huangqisan on Metabolic Disorders via SREBF1, SCD1 and AMPK Signaling Pathway. *J. Integr. Med.* **2021**, *19*, 167–176. [CrossRef]
15. Jha, S.K.; Roy, P.; Chakrabarty, S. Nutraceuticals with Pharmaceutics: Its Importance and Their Applications. *Int. J. Drug Dev. Res.* **2021**, *13*, 1–10.
16. Fernández-Sevilla, J.M.; Acién Fernández, F.G.; Molina Grima, E. Biotechnological Production of Lutein and Its Applications. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 27–40. [CrossRef]
17. Li, J.; Engelberth, A.S. Quantification and Purification of Lutein and Zeaxanthin Recovered from Distillers Dried Grains with Solubles (DDGS). *Bioresour. Bioprocess.* **2018**, *5*, 1–10. [CrossRef]
18. Ruen-Ngam, D.; Shotipruk, A.; Pavasant, P.; Machmudah, S.; Goto, M. Selective Extraction of Lutein from Alcohol Treated Chlorella Vulgaris by Supercritical CO_2. *Chem. Eng. Technol.* **2012**, *35*, 255–260. [CrossRef]
19. Di Sanzo, G.; Mehariya, S.; Martino, M.; Larocca, V.; Casella, P.; Chianese, S.; Musmarra, D.; Balducchi, R.; Molino, A. Supercritical Carbon Dioxide Extraction of Astaxanthin, Lutein, and Fatty Acids from Haematococcus Pluvialis Microalgae. *Mar. Drugs* **2018**, *16*, 334. [CrossRef]
20. Fu, X.Q.; Zhang, G.L.; Deng, L.; Dang, Y.Y. Simultaneous Extraction and Enrichment of Polyphenol and Lutein from Marigold (*Tagetes erecta* L.) Flower by an Enzyme-Assisted Ethanol/Ammonium Sulfate System. *Food Funct.* **2019**, *10*, 266–276. [CrossRef]
21. Jalali-Jivan, M.; Abbasi, S. Novel Approach for Lutein Extraction: Food Grade Microemulsion Containing Soy Lecithin & Sunflower Oil. *Innov. Food Sci. Emerg. Technol.* **2020**, *66*, 102505. [CrossRef]
22. Boonnoun, P.; Tunyasitikun, P.; Clowutimon, W.; Shotipruk, A. Production of Free Lutein by Simultaneous Extraction and De-Esterification of Marigold Flowers in Liquefied Dimethyl Ether (DME)–KOH–EtOH Mixture. *Food Bioprod. Process.* **2017**, *106*, 193–200. [CrossRef]
23. Lakshmi, A.; Aishwarya, G.; Priya, S.; Rlc, S.; Raju, S.; Rao, V. Extraction and Biological Evaluation of Esterfied Lutein from Marigold Flower Petals. *J. Pharmacogn. Phytochem.* **2019**, *1*, 2523–6075.
24. Prabhu, A.; Abdul, K.S.; Rekha, P.-D. Isolation and Purification of Lutein from Indian Spinach Basella Alba. *Res. J. Pharm. Technol.* **2015**, *8*, 1379. [CrossRef]
25. Derrien, M.; Badr, A.; Gosselin, A.; Desjardins, Y.; Angers, P. Optimization of a Green Process for the Extraction of Lutein and Chlorophyll from Spinach By-Products Using Response Surface Methodology (RSM). *LWT—Food Sci. Technol.* **2017**, *79*, 170–177. [CrossRef]
26. Dutra-Molino, J.V.; Araujo Feitosa, V.; de Lencastre-Novaes, L.C.; de Carvalho Santos-Ebinuma, V.; Moreni Lopes, A.; Faustino Jozala, A.; de Aráujo Viana Marques, D.; Pellegrini Malpiedi, L.; Pessoa, A. Biomolecules Extracted by ATPS: Practical Examples. *Rev. Mex. Ing. Quim.* **2014**, *13*, 359–377.
27. Kamal, I.; Besombes, C.; Allaf, K. One-Step Processes for in Situ Transesterification to Biodiesel and Lutein Extraction from Microalgae Phaeodactylum Using Instant Controlled Pressure Drop (DIC). *Green Process. Synth.* **2014**, *3*, 431–440. [CrossRef]
28. Dermiki, M.; Gordon, M.H.; Jauregi, P. Recovery of Astaxanthin Using Colloidal Gas Aphrons (CGA): A Mechanistic Study. *Sep. Purif. Technol.* **2009**, *65*, 54–64. [CrossRef]
29. Yau, Y.K.; Ooi, C.W.; Ng, E.P.; Lan, J.C.W.; Ling, T.C.; Show, P.L. Current Applications of Different Type of Aqueous Two-Phase Systems. *Bioresour. Bioprocess.* **2015**, *2*, 49. [CrossRef]
30. Craft', N.E.; Soares, J.H. Relative Solubility, Stability, and Absorptivity of Lutein and @-Carotene in Organic Solvents. *J. Agric. Food Chem.* **1992**, *40*, 431–434. [CrossRef]
31. Jalali Jivan, M.; Abbasi, S. Nano Based Lutein Extraction from Marigold Petals: Optimization Using Different Surfactants and Co-Surfactants. *Heliyon* **2019**, *5*, e01572. [CrossRef] [PubMed]
32. Khoo, K.S.; Chong, Y.M.; Chang, W.S.; Yap, J.M.; Foo, S.C.; Khoiroh, I.; Lau, P.L.; Chew, K.W.; Ooi, C.W.; Show, P.L. Permeabilization of *Chlorella Sorokiniana* and Extraction of Lutein by Distillable CO_2-Based Alkyl Carbamate Ionic Liquids. *Sep. Purif. Technol.* **2021**, *256*, 117471. [CrossRef]
33. Ba, D.; Boyaci, I.H. Modeling and Optimization i: Usability of Response Surface Methodology. *J. Food Eng.* **2007**, *78*, 836–845. [CrossRef]
34. Sharma, O.P.; Bhat, T.K. DPPH Antioxidant Assay Revisited. *Food Chem.* **2009**, *113*, 1202–1205. [CrossRef]

35. Ligor, M.; Trziszka, T.; Buszewski, B. Study of Antioxidant Activity of Biologically Active Compounds Isolated from Green Vegetables by Coupled Analytical Techniques. *Food Anal. Methods* **2013**, *6*, 630–636. [CrossRef]
36. Jiao, S.S.; Li, D.; Huang, Z.G.; Zhang, Z.S.; Bhandari, B.; Chen, X.D.; Mao, Z.H. Optimization of Supercritical Carbon Dioxide Extraction of Flaxseed Oil Using Response Surface Methodology. *Int. J. Food Eng.* **2008**, *4*, 4. [CrossRef]
37. Khanpit, V.V.; Mandavgane, S.A.; Tajane, S.P. Waste to Wealth-Recovery of Total Dietary Fibers from Waste Peel: A Generalized Model for Predicting Operating Parameters. *Biomass Convers. Biorefinery* **2021**, 1–10. [CrossRef]
38. Farías-Campomanes, A.M.; Rostagno, M.A.; Coaquira-Quispe, J.J.; Meireles, M.A.A. Supercritical Fluid Extraction of Polyphenols from Lees: Overall Extraction Curve, Kinetic Data and Composition of the Extracts. *Bioresour. Bioprocess.* **2015**, *2*, 1–13. [CrossRef]
39. Melo, R.P.F.; Barros Neto, E.L.; Moura, M.C.P.A.; Castro Dantas, T.N.; Dantas Neto, A.A.; Oliveira, H.N.M. Removal of Reactive Blue 19 Using Nonionic Surfactant in Cloud Point Extraction. *Sep. Purif. Technol.* **2014**, *138*, 71–76. [CrossRef]
40. Tanhaei, B.; Pourafshari Chenar, M.; Saghatoleslami, N.; Hesampour, M.; Kallioinen, M.; Sillanpää, M.; Mänttäri, M. Removal of Nickel Ions from Aqueous Solution by Micellar-Enhanced Ultrafiltration, Using Mixed Anionic-Non-Ionic Surfactants. *Sep. Purif. Technol.* **2014**, *138*, 169–176. [CrossRef]
41. Goswami, A.; Nath, J.; Purkait, M.K. Cloud Point Extraction of Nitrobenzene Using TX-100. *Sep. Sci. Technol.* **2011**, *46*, 744–753. [CrossRef]
42. Macías-Sánchez, M.D.; Fernandez-Sevilla, J.M.; Fernández, F.G.A.; García, M.C.C.; Grima, E.M. Supercritical Fluid Extraction of Carotenoids from Scenedesmus Almeriensis. *Food Chem.* **2010**, *123*, 928–935. [CrossRef]
43. Fu, X.Q.; Ma, N.; Sun, W.P.; Dang, Y.Y. Microwave and Enzyme Co-Assisted Aqueous Two-Phase Extraction of Polyphenol and Lutein from Marigold (*Tagetes erecta* L.) Flower. *Ind. Crops Prod.* **2018**, *123*, 296–302. [CrossRef]
44. Jalali-Jivan, M.; Abbasi, S.; Fathi-Achachlouei, B. Lutein Extraction by Microemulsion Technique: Evaluation of Stability versus Thermal Processing and Environmental Stresses. *LWT* **2021**, *149*, 111839. [CrossRef]
45. Goto, M.; Kanda, H.; Wahyudiono; Machmudah, S. Extraction of Carotenoids and Lipids from Algae by Supercritical CO2 and Subcritical Dimethyl Ether. *J. Supercrit. Fluids* **2015**, *96*, 245–251. [CrossRef]
46. Mezzomo, N.; Ferreira, S.R.S. Carotenoids Functionality, Sources, and Processing by Supercritical Technology: A Review. *J. Chem.* **2016**, *2016*, 1–16. [CrossRef]
47. Hung, K.; Chen, B.; Yu, L.E. Cloud-Point Extraction of Selected Polycyclic Aromatic Hydrocarbons by Nonionic Surfactants. *Sep. Purif. Technol.* **2007**, *57*, 1–10. [CrossRef]
48. Kanda, H.; Wahyudiono; MacHmudah, S.; Goto, M. Direct Extraction of Lutein from Wet Macroalgae by Liquefied Dimethyl Ether without Any Pretreatment. *ACS Omega* **2020**, *5*, 24005–24010. [CrossRef]
49. Sindhu, E.R.; Preethi, K.C.; Kuttan, R. Antioxidant Activity of Carotenoid Lutein in Vitro and in Vivo. *Indian J. Exp. Biol.* **2010**, *48*, 843–848.
50. Wang, M.; Tsao, R.; Zhang, S.; Dong, Z.; Yang, R.; Gong, J.; Pei, Y. Antioxidant Activity, Mutagenicity/Anti-Mutagenicity, and Clastogenicity/Anti-Clastogenicity of Lutein from Marigold Flowers. *Food Chem. Toxicol.* **2006**, *44*, 1522–1529. [CrossRef]

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