Wiley
Journal of Food Processing and Preservation
Volume 2024, Article ID 3594099, 13 pages
https://doi.org/10.1155/2024/3594099

WILEY

Research Article

Optimisation of Lutein Extraction From Gac (*Momordica cochinchinensis*) Fruit Peel and Evaluation of In Vitro Eye Protective Effects of the Extracts

Dao Nguyen[1], **Thilini Thrimawithana**[2], **Terry Piva**[2] and **Tien Huynh**[1]

[1]*School of Science, RMIT University, PO Box 71, Bundoora 3083, Australia*
[2]*School of Health and Biomedical Sciences, RMIT University, PO Box 71, Bundoora 3083, Australia*

Correspondence should be addressed to Tien Huynh; tien.huynh@rmit.edu.au

Received 14 November 2023; Revised 17 May 2024; Accepted 31 July 2024

Academic Editor: Maria Concetta Strano

Copyright © 2024 Dao Nguyen et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Lutein is a macular biopigment that can protect the retina from oxidative stress induced by chronic exposure to light and oxidants. The peel of gac fruit (*Momordica cochinchinensis*) contains high levels of lutein, but the extraction optimisation parameters are complex, requiring saponification, and the effects on ocular bioactivity are not well understood. This study is aimed at optimising parameters for lutein extraction validated using HPLC from gac peel to determine their protective effects on oxidatively stressed retinal cells. The factors investigated for optimisation were KOH concentration, time, and temperature using (1) single factor, (2) multiple factor response surface methodology, and (3) predictive modelling. The optimum conditions for lutein extraction using single factors were 9% KOH for 3 h at 60°C, multiple factors were 9% KOH for 3 h at 45°C, and predictive modelling was 18% KOH for 1.9 h at 39.9°C, with the maximum extraction of lutein at 12.37, 15.64, and 17.4 mg/g DW, respectively. The extracted lutein purity and chemical composition were compared using HPLC and FTIR and indicated high purity and similarities in all extracts including the commercially purified lutein. Lutein extracted exhibited benefits on oxidatively stressed retinal pigment epithelium (RPE) cells that reversed damage to baseline unstressed levels exceeding 80% viability, similar to purified lutein standards. The feasibility of gac lutein and extraction optimisation were discussed for commercial translational opportunities as a medicinal bioproduct with a focus on eye benefits.

Keywords: bioactivity; carotenoids; extraction; eye benefits; gac fruit; lutein; pigment; process optimisation

1. Introduction

Lutein is a xanthophyll, a key type of carotenoid synthesised by plants. Humans are unable to synthesise lutein and rely on its dietary intake. Lutein accumulates in the macula of the eye which is responsible for visual acuity. Numerous clinical studies have suggested that 10–20 mg of lutein ingested daily may prevent and manage eye diseases including age-related macular degeneration [1–6]. Dietary supplementation of lutein can also slow the progression of other eye disorders such as age-related cataracts [7], diabetic retinopathy [8], and retinopathy of prematurity [9, 10]. The main mechanisms of protective action of lutein on eye diseases were due to its capability to protect retinal pigment epithelium (RPE) cells against oxidative stress induced by chronic exposure to light and oxidants [11, 12]. Due to its benefits to eye health [13], the global market for lutein was $306 million in 2018 and is predicted to reach $396 million by 2024 [14]. At present, marigold petals (*Tagetes erecta* and *Tagetes patula*) are the primary commercial source of natural lutein for the nutraceutical markets. However, the high labour cost associated with the harvesting and processing of petals promotes the necessity of finding more cost-effective alternative sources [15].

Momordica cochinchinensis is a fruit well known in Southeast Asia by different vernacular names such as *mu bie* in Chinese, *phak khao* in Thai, *tabog-ok* in Filipino, and *gac* in Vietnamese [16]. Gac fruit peel accounts for 12.5%–16.2% of the fruit weight [17] and contains the highest levels of lutein in fruit [16, 18]. Different levels of lutein

in gac fruit peel range from 0.2 [19] to 52.0 mg/g [18] dry weight. This variation might be due to the differences in gac variety [20], growth condition [21, 22], fruit maturity at harvest [23], postharvest processing, and extraction methods [24]. Lutein in fruits and flowers is predominantly found in its esterified form, as these esters are more stable against oxidation [25]. Thus, to extract free lutein, saponification is used to remove the fatty acids bound to the OH group of lutein. Saponification is a key process for high lutein extraction and purity from natural sources, especially from plants containing high oils or fatty acids [26–28]. The saponification process is affected by factors such as temperature, time, and concentration of reagents, for example, KOH [29–31] that have impacted marigold flower petals [32], spinach leaves [29], and microalgae [30, 31, 33]. However, optimisation of lutein recovery, its purity, and retinal benefits from gac fruit peel have not yet been reported.

Response surface methodology (RSM) and predictive modelling are widely employed multivariate statistical techniques utilized in the optimization process to comprehensively characterize the effects of factors on the response and minimize the number of experiments required [29, 34]. Since the extraction of lutein is complex, RSM and predictive modelling may provide insight into more efficient processes for industrial translation. Hence, the aim of this study was to (1) optimise the saponification factors for lutein extraction from gac fruit peel using a single factor, multiple factor RSM, and predictive modelling and (2) examine the extracts obtained for lutein bioactivity on retinal cells. Results from this study will provide valuable information on the optimization of lutein extraction from gac and the potential benefits of gac lutein on eye health.

2. Materials and Methods

2.1. Chemicals. HPLC grade solvents (methanol, ethyl acetate, and acetonitrile), KOH, HCl, lutein standard (Batch BCCC1688), Cell Counting Kit-8 (CCK-8) viability assay, and 2,6-di-tert-butyl-4-methylphenol (BHT) were purchased from Sigma-Aldrich (Australia). Dulbecco's Modified Eagle's Medium (DMEM) low glucose (plus L-glutamine and sodium pyruvate), foetal bovine serum (FBS), cell culture antibiotics (5000 U penicillin and 5 mg streptomycin/mL), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2) (30% *v*/*v*), and trypLE were obtained from Thermo Fisher Scientific (Australia).

2.2. Plant Samples. Fully ripe gac fruits were collected in Dak Lak Province, Vietnam (12.7100° N, 108.2378° E) in November 2018. Identification was confirmed by indigenous locals, molecular studies, and voucher specimens deposited at the National Herbarium of Victoria (Accession MEL2472087). The fruit peel was collected, dried at 40°C for 12 h using a fan-forced dehydrating oven (Bosch, Germany), covered with aluminium, and transported to RMIT Melbourne in December 2018. The gac dried peel was stored in darkness at −20°C.

2.3. HPLC Analysis. The lutein concentration was determined using an Agilent HPLC 1260 system with OpenLab software, equipped with a UV detector set at 450 nm. A C18 column (4.6 mm × 250 mm, 5 μm) was used, and the method was run at a constant temperature (30°C) and a flow rate of 0.7 mL/min with an injection volume of 20 μL. The mobile phase consisted of methanol:acetonitrile:ethyl acetate (80:19:1, *v*/*v*/*v*). Compound identification was confirmed using retention time and UV spectra (450 nm) of the lutein standard. For quantification, lutein standard stock solution (800 μg/mL) was solubilized in ethyl acetate containing 0.1% BHT. Then a series of dilutions were prepared of the lutein stock solution in the mobile phase (methanol:acetonitrile:ethyl acetate, 80:19:1, *v*/*v*/*v*). Dilutions provided seven points with known concentrations (1.25, 2.5, 5, 10, 20, 40, and 80 μg/mL) to generate a calibration curve. The diluted standards were prepared on the same day as the absorbance measurements. The quantification method was validated for linearity, accuracy, limit of detection (LOD), and limit of quantification (LOQ) in accordance with ICH guideline Q2 (R2) [35].

The recovery rate of the lutein standard was also used to validate the accuracy of the HPLC method. Actual amounts of lutein standard (40, 50, and 60 μg/mL) were quantified (three replicates each), and then the recovery rates (percentage) were calculated using Equation (1).

$$\%\text{recovery} = \frac{\text{actual concentration of lutein standard quantified by HPLC}}{\text{added lutein standard concentration}} \quad (1)$$

The gac peel extracts were dissolved in ethyl acetate containing 0.1% BHT at a concentration of 800 μg/mL, then were diluted in the mobile phase (methanol:acetonitrile:ethyl acetate, 80:19:1, *v*/*v*/*v*) to a concentration of 40 μg/mL. The mixture was filtered through a 0.22 μm membrane filter prior to HPLC analysis. The analyses were performed in triplicate for all samples and lutein standards.

2.4. Single Factors for Saponification of Lutein. Dry gac peel powder (1 g) was mixed with ethyl acetate (20 mL) in a glass flask covered with aluminium foil to protect it from light. The mixture was stirred at room temperature (20°C) for 30 min and sonicated (Unisonics, Australia) for 15 min at 30°C. The mixture was allowed to cool down to room temperature (20°C) before saponification.

The single factors for saponification investigated were KOH concentrations (ranged 0%–21% at 60°C for 3 h), treatment times (ranged 0.5–13 h at 60°C and 10% KOH), and temperatures (ranged 20°C–90°C at 10% KOH for 3 h).

After saponification, the mixtures were allowed to cool to 20°C, before 60 mL of ethyl acetate was added to each sample. The mixture was spun at 3500 g for 10 min at 20°C in a Z206A centrifuge (Benchmark, Germany) and the supernatant was collected. The supernatant was adjusted to pH 7 using HCl before evaporation in a RVC2-33CD plus rotational vacuum concentrator (John Morris Scientific Pty Ltd, Australia) for 6 h at 45°C at 250 rpm. The dried extracts were stored at −20°C and protected from light.

2.5. Optimisation of Lutein Saponification Using RSM. Based on the results of the single factor optimisation, the influence of KOH concentration, saponification temperature, and saponification time and their responses (lutein yield and purity) was evaluated using RSM. A central composite rotatable design (CCRD) with three independent variables ($k = 3$) was performed to identify the relationship between the response functions and the input variable following a published method [36, 37]. Minitab Software Version 2021 was employed to design the RSM experiment. There were three factors consisting of five levels including $-\alpha$, -1, 0, $+1$, and $+\alpha$ (where α stands for "1.68719" determined by CCRD), and the analysis was performed in 20 runs which included six replicates at the centre point with the factors 9% KOH, 3 h, and 45°C (Figure 1). The interaction between these factors (KOH concentration, time, and temperature) and responses was evaluated using analysis of variance (ANOVA) within the regression model experimental data in Equation (2).

$$Y = \beta 0 + \sum_{0}^{i} \beta_i X_I + \sum_{0}^{i} \beta_{iI} X_i^2 + \sum_{0}^{i} \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response (lutein yield or purity), β_0 is the regression coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient between factors i and j, and X_i and X_j are the coded levels of independent factors (KOH concentration, saponification time, and saponification temperature).

Minitab Software 2021 version was used to analyse the significance and relationship between the three saponification factors (KOH concentration, time, and temperature) using ANOVA where $p < 0.05$. The effect was visualised using three-dimensional (3D) surface and two-dimensional (2D) contour plots and the significance of the selected factors and their interaction on lutein recovery and purification. Lutein purity was calculated using Equation (3).

$$\text{Purity}\,(\%) = \frac{\text{Lutein recovery}\,(\text{mg})}{\text{Extract mass}\,(\text{mg})} \times 100 \quad (3)$$

2.6. Validation of the Optimal Saponification Factors Predicted by RSM. To validate the accuracy of the optimisation, experiments were conducted at the predicted desirable saponification factors (KOH concentration, time, and temperature) determined by the quadratic regression model. Specifically, 1 g of dry gac peel powder was extracted using the same process as described before, and saponification was performed thrice in parallel at optimised KOH concentration, time, and temperature to validate the model. The lutein was quantified using HPLC. The error between predicted and actual lutein recovery was calculated using Equation (4).

$$\text{Prediction error}\,(\%) = \frac{\text{Measured lutein recovery}\,(\text{mg}) - \text{predicted lutein recovery}\,(\text{mg})}{\text{Measured lutein recovery}\,(\text{mg})} \times 100 \quad (4)$$

2.7. Fourier Transform Infrared Spectroscopy (FTIR). A Bruker Alpha spectrometer (Bruker, United States) was used to record the FTIR spectra of lutein standard and lutein extract from gac peel. The sample compartment was flushed with argon prior to each run. Each sample was dissolved as 100 μg/mL in a mixture of organic solvents (methanol:acetonitrile:ethyl acetate, 80:19:1 *v*/*v*/*v*). Then, 1 μL of each sample was spotted onto the FTIR instrument with a scanning range of 600–4000 cm^{-1}. The organic solvent peaks were subtracted from the original spectrum to obtain the final IR value and analysed.

2.8. Cell Culture. The effect of the saponified gac peel extracts (which contain different contents of lutein) on RPE cells exposed to oxidative stress was determined using the CCK-8 assay following the manufacturer's protocol (Sigma-Aldrich [Australia]) and method described in the previous study [38]. RPE cells (3×10^5 cells/well) were plated in a 96-well plate containing DMEM low glucose media plus 10% (*v*/*v*) FBS and 1% (*v*/*v*) penicillin and streptomycin. The cells were incubated at 37°C and 5% CO_2 in a humidified incubator until the cultures reached ~75% confluency. The cultures were serum-starved (2% FBS) in DMEM media for 12 h before treatment with 10 μg/mL of the saponified gac peel extracts or lutein standard for 48 h. Lutein extracts were dissolved in < 0.1% DMSO. The cells were then washed with fresh tissue culture media before being stressed with 500 μM H_2O_2 for 12 h at 37°C. After which, the cells were washed with PBS and incubated for 2 h at 37°C in 100 μL DMEM containing 10 μL CCK-8 solution for cell viability assessment. The absorbance was read at 450 nm on a CLARIOstar High-Performance Monochromator Multimode Microplate Reader (BMG LABTECH). The experiment was performed in triplicate. Viability was expressed as a percentage of the untreated control cells using Equation (5).

$$\text{Cell viability}\% = \frac{At - Ab}{Ac - Ab} \times 100 \quad (5)$$

where At was the absorbance of the treated cells, Ab was the absorbance of 10% CCK-8 reagent in DMEM media, and Ac was the absorbance of the untreated controls.

3. Results and Discussion

3.1. Validation of HPLC Detection. Lutein was identified and quantified using the known purified lutein standard. The calibration for the lutein standard was established using a

	Extract #	Saponification factors			Extracted		
		KOH (%)	Time (h)	Temperature (°C)	Total mass (mg/g)	Lutein yield (mg/g DW)*	% lutein
Single factor		9	3	60	17.87 ± 2.54^{de}	12.62 ± 1.23^{b}	69.21± 2.96^{c}
RSM multiple factor	1	3	1	25	31.38 ± 5.37^{b}	6.98 ± 1.49^{ef}	22.24 ± 3.67^{f}
	2	**9**	**3**	**45**	**18.91 ± 1.26^{de}**	**14.28 ± 2.41^{ab}**	**75.53 ± 5.01^{bc}**
	3	**9**	**3**	**45**	**20.02 ± 1.08^{d}**	**14.93 ± 1.08^{a}**	**74.56 ± 3.96^{bc}**
	4	3	5	65	6.19 ± 0.46^{gh}	2.75 ± 1.07^{gh}	12.32 ± 3.20^{g}
	5	9	3	78.63	1.82 ± 0.12^{i}	1.14 ± 0.35^{i}	62.63 ± 4.09^{d}
	6	3	5	25	10.58 ± 1.81^{fg}	2.49 ± 0.59^{h}	23.53 ± 3.92^{f}
	7	15	1	65	12.62 ± 1.96^{ef}	9.81 ± 1.36^{cd}	77.73 ± 5.94^{ab}
	8	15	5	25	9.30 ± 0.59^{gh}	5.96 ± 0.76^{fg}	64.10 ± 4.07^{cd}
	9	**9**	**3**	**45**	**19.44 ± 1.32^{de}**	**14.65 ± 1.23^{ab}**	**71.36 ± 5.19^{bc}**
	10	3	1	65	5.83 ± 0.47^{gh}	2.14 ± 1.43^{h}	13.70 ± 3.09^{g}
	11	1.09	3	45	57.23 ± 4.68^{a}	8.81 ± 1.83^{de}	13.39 ± 3.45^{g}
	12	**9**	**3**	**45**	**20.89 ± 1.07^{d}**	**15.64 ± 1.71^{a}**	**74.87 ± 3.83^{bc}**
	13	9	3	11.36	28.01 ± 3.21^{bc}	3.45 ± 0.95^{gh}	28.32 ± 2.89^{e}
	14	9	0.36	45	22.64 ± 2.92^{cd}	10.4 ± 1.17^{cd}	45.93 ± 5.84^{e}
	15	9	6.36	45	4.16 ± 1.19^{hi}	3.37 ± 0.57^{gh}	80.94 ± 3.68^{ab}
	16	19.09	3	45	17.22 ± 0.81^{de}	14.56 ± 2.18^{ab}	84.57 ± 4.03^{ab}
	17	15	5	65	6.33 ± 1.33^{gh}	5.68 ± 0.41^{fg}	89.77 ± 4.75^{a}
	18	15	1	25	20.38 ± 0.82^{d}	14.96 ± 1.07^{a}	73.39 ± 2.95^{bc}
	19	**9**	**3**	**45**	**19.84 ± 1.09^{d}**	**14.77 ± 1.04^{ab}**	**74.44 ± 4.11^{bc}**
	20	**9**	**3**	**45**	**18.49 ± 0.97^{de}**	**14.19 ± 1.52^{ab}**	**76.73 ± 3.97^{bc}**
Validated (predicted)		18	1.9	39.9	–	17.40 ± 0.00^{a}	–
Validated (actual)		18	1.9	39.9	21.68 ± 3.70 ^{d}	16.37 ± 0.97^{a}	75.5 ± 2.85^{bc}

Note: The three saponification factors (KOH percentage, temperature, and time) were tested at five levels with six replicates at the central level in 9% KOH for 3hat 45°C (bold). Single-factor and validated responses were compared to determine optimal extraction conditions. Responses were expressed as mean ± SE of three independent experiments, and each individual sample was run in triplicate ($n = 9$). Statistical significance was determined by one-way ANOVA with Tukey's test, where different superscript letters indicated statistical significance at $p \leq 0{:}05$ between different saponification factors. *Quantified by HPLC.

Figure 1: Optimisation of lutein extraction using response surface methodology design for three multiple saponification factors.

Table 1: Validation of the lutein standard quantified using HPLC.

Lutein standard (μg/mL)	Detected lutein (μg/mL)	Percent accuracy
40.00	39.42	98.54 ± 0.43
50.00	49.60	99.20 ± 0.31
60.00	59.35	98.92 ± 0.54

concentration curve. The LOD and LOQ for the lutein standard were 0.777 and 2.355 μg/mL, respectively. These values indicated that HPLC can detect lutein at a concentration as low as 0.777 μg/mL and can reliably quantify concentrations of 2.355 μg/mL or higher. The detection of the lutein standard was accurate, exceeding 98% (Table 1). The LOD, LOQ values, and recovery rates provide validation for the HPLC protocol employed in this study to accurately quantify lutein in the extracts [39].

3.2. Single Factor Analysis: KOH Concentration. The KOH concentration significantly affected the yield of lutein extracted from gac in a bell curve pattern ($p < 0.05$) (Figure 2(a)). The lowest lutein yield was 1 mg/g DW in the absence of KOH, and this was increased to a maximum of 12.63 mg/g DW with 9%–12% of KOH. Lutein in fruit peel extract is naturally complexed with fatty acids to form lutein esters [25] and requires further processing to extract free lutein forms. In the saponification process, ester bonds between lutein and fatty acids/lipids are cleaved in the presence of ethanolic alkali which releases the free form of lutein [30, 33]. At low concentrations of KOH (< 6%), the cleavage is incomplete due to insufficient KOH for cleavage of ester bonds, resulting in low lutein recovery yield from gac peel (Figure 2(a)). Optimum KOH concentrations are required as excessive cleavage can degrade lutein, particularly when concentrations surpass 12%, especially at elevated temperatures and during prolonged saponification periods [31, 40].

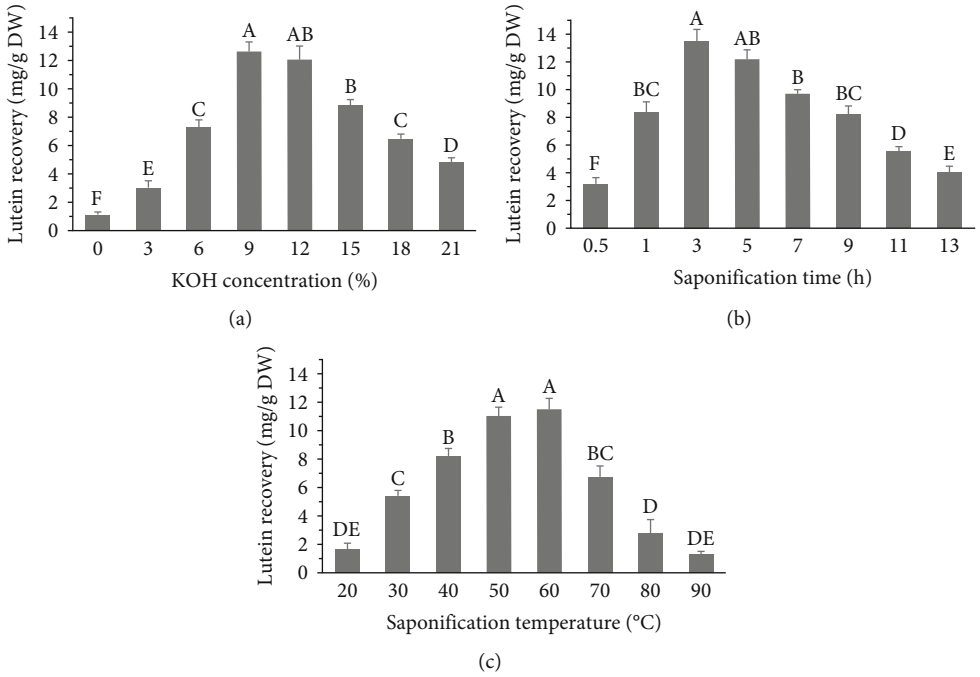


Figure 2: Single factor effects of (a) KOH concentration, (b) saponification time, and (c) saponification temperature on lutein extracted from gac fruit peel. The values were expressed as mean + SE of triplicates. Statistical significance was performed using one-way ANOVA with Tukey's test where different letters indicate statistical significance at $p \leq 0.05$. Treatments sharing the same letters indicate no statistical differences between treatments ($p > 0.05$).

3.3. Single Factor Analysis: Saponification Time. The saponification time impacts the transfer of active compounds from the substrate to the solvent via diffusion and permeation, thereby influencing both the saponification reaction and lutein recovery [19]. Saponification time also followed a significant bell curve pattern ($p < 0.05$), with 12.18 mg/g DW of free lutein being extracted after 3 h of incubation (Figure 2(b)). Inadequate extraction times may yield suboptimal extraction rates, leading to diminished yields. Conversely, protracted extraction durations may not proportionally enhance yields and can exacerbate the risk of degradation or loss of thermolabile compounds due to prolonged exposure to extraction conditions [19]. Furthermore, prolonged saponification times may contribute to solvent concentration reduction through evaporation, thereby diminishing the diffusion of lutein into the solvent and consequently reducing extraction yields, as observed in previous studies on microalgae and marigolds [32].

3.4. Single Factor Analysis: Saponification Temperature. The saponification temperature had a significant impact on the recovery of lutein from gac fruit peel ($p < 0.05$) (Figure 2(c)). The optimum temperature of 60°C resulted in 11.52 mg/g DW lutein. At extreme temperatures of 20°C and 90°C, there was less than 2 mg/g DW lutein extracted. Temperature is an important factor affecting the reaction rate. Raising temperature increases the average kinetic energy of the molecules, prompting them to move faster and collide with each other with more energy. Consequently, the rate of the reaction increases [41], and saponification is maximised at optimal temperatures [42]. Below this threshold, there is an insufficient level of molecule interaction to efficiently cleave esters bound to lutein, resulting in lower extraction yield [19]. However, temperatures beyond a certain threshold destroy the double bonds of polyunsaturated fatty acids, thereby reducing saponification [42]. Furthermore, lutein is a thermally sensitive compound that is significantly degraded at temperatures above 70°C. Therefore, at high temperature (above 70°C), the lutein extraction yield was also reduced [31, 43].

3.5. Optimisation of Saponification Factors Using RSM

3.5.1. Fitting the Models. The optimal results from the single saponification factors (KOH, time, and temperature) using traditional incremental experiments were used to design the RSM prediction on Minitab Software Version 2021, with the three factors coded as X_1 for KOH concentration, X_2 for saponification time, and X_3 for saponification temperature. The three factors consist of five levels including $-\alpha$, -1, 0, $+1$, and $+\alpha$ (where 0 stands for the central point, -1 and $+1$ stand for the lowest and highest value based on the actual selection from the single optimisation, and α stands for "1.68719" determined by CCRD). The value range for each factor was 1.09%–19.9% for X_1 (KOH concentration), 0.36–6.36 h for X_2 (saponification time), and 11.36°C–78.63°C for X_3 (saponification temperature) (Figure 1). In total, 20 experiments were generated and conducted using different combinations of these factors to gain a better insight into their effects and interactions on lutein recovery. The interaction

Table 2: Analysis of variance (ANOVA) for response surface methodology quadratic model for the optimisation of lutein recovery from gac fruit peel.

	Source of variation	Sum of squares	Df	Mean square	*F* value	*p* value	Significance
Single factor	Model	555.875	9	61.764	116.91	0.0001	Significant
	X_1—KOH (%)	34.277	1	34.277	64.88	0.0001	Significant
	X_2—Saponification time	9.892	1	9.892	18.72	0.0001	Significant
	X_3—Saponification temperature	165.605	1	165.605	313.46	0.001	Significant
RSM multiple factor	$X_1 * X_1$	16.654	1	16.654	31.52	0.0001	Significant
	$X_2 * X_2$	110.740	1	110.740	209.61	0.0001	Significant
	$X_3 * X_3$	299.797	1	299.797	567.47	0.0001	Significant
	$X_1 * X_2$	10.695	1	10.695	20.24	0.001	Significant
	$X_1 * X_3$	0.090	1	0.090	0.17	0.688	Not significant
	$X_2 * X_3$	12.425	1	12.425	23.52	0.001	Significant
	Lack of fit	3.914	5	0.783	2.86	0.137	Not significant
	Pure error	1.369	5	0.274			
	Residual	7.796	10	1.014			
	Cor total	561.158	19				
	$R^2 = 0.9806$	Adj $R^2 = 0.9721$		Pred $R^2 = 0.9535$		C.V (%) = 7.79	

between the independent factors and lutein recovery was evaluated by fitting Equation (6) to the data from the experiments using multiple regression analysis.

$$\begin{aligned}\text{Lutein recovery (mg/g)} = {} & -12.41 + 1.205\,X_1 + 0.937\,X_2 \\ & + 0.8996\,X_3 + 0.01558\,X_2 * X_3 \\ & - 0.0482\,X_1 * X_2 - 0.00089\,X_1 * X_3 \\ & - 0.02986\,X_1^2 - 0.1733\,X_2^2 \\ & - 0.011403\,X_3^2 \end{aligned} \tag{6}$$

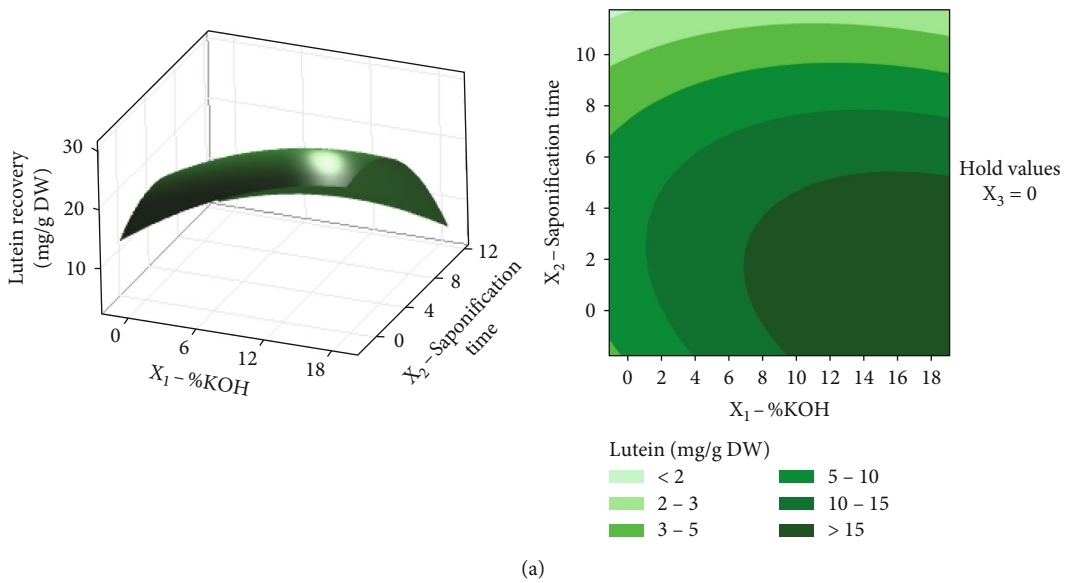
All three parameters significantly influenced lutein recovery (F value = 116.91, $p < 0.001$) with some but not all factors significantly interacting (Table 2). The lack of fit was not significant ($p > 0.05$), indicating the model's best fitness and its ability to predict the outcomes [36]. Moreover, the high R^2 value (0.98) and the low difference between the R^2 value and adjusted R^2 (0.009) (Table 2) indicated that this regression model can be employed to predict optimal lutein recovery from gac fruit peel [36, 37].

3.5.2. Multiple Factor Analysis on Lutein Extraction Using RSM. Lutein extracted varied between 1.14 and 15.64 mg/g DW (Table 2), with all three factors significant ($p < 0.001$) and with cross-interactions except KOH percentage and temperature (Table 2). The optimum lutein extraction was 9% KOH for 3 h at 45°C with over 14 mg/g DW extracted (Figure 1), but there were other factorial combinations that also produced high lutein yields, and this could be considered for industrial applications with reduced time or temperature limitations. For example, extraction with 15% KOH for 1 h at 25°C (#18) produced the maximum at 14.96 mg/g DW lutein, saving time and heating costs that might offset the higher concentration of KOH required. In general, other lower lutein recovery was at random KOH concentrations, at high temperatures (> 65°C) for long extraction times (≥ 5 h) (Figure 1). This suggests that elevated temperatures play a crucial role in causing a decline in lutein content within the extraction mass.

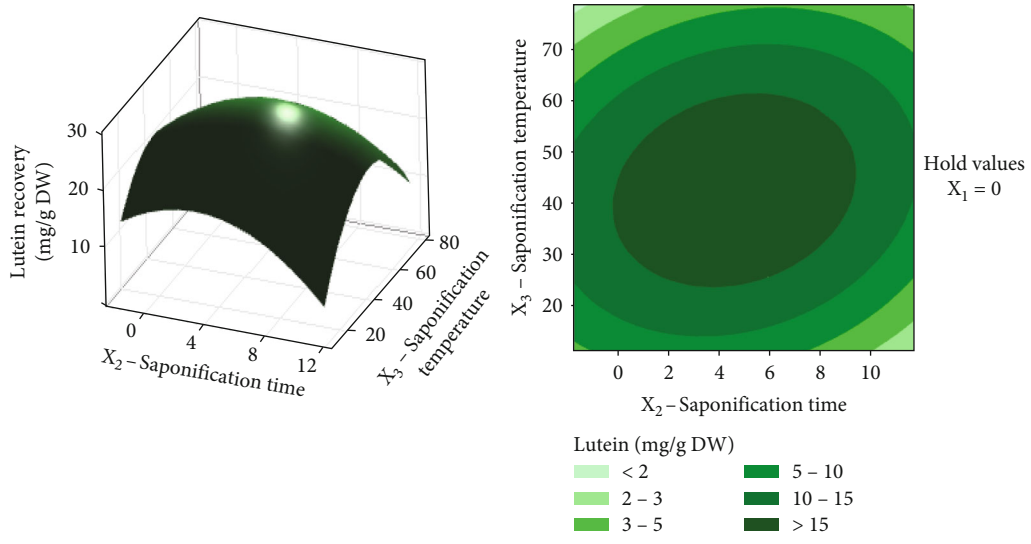
The optimal saponification temperature for extracting lutein from gac peel aligned with that for other plant sources such as marigold petals [28, 32], spinach [28], and microalgae [30, 31]. However, optimal KOH concentrations varied depending on the lutein source. High chlorophyll content in plants like spinach and microalgae necessitated KOH concentrations of over 50% for lutein extraction, as KOH is crucial for chlorophyll removal to achieve high-purity lutein [29–31]. For lutein extractions from gac peel, the optimal KOH concentration was 18%, similar to that for marigold petals [28, 32]. This lower optimal KOH concentration for gac peel and marigold petals likely stems from their lower chlorophyll content compared to green materials like spinach leaves and microalgae.

The relationship between lutein recovery and the independent factors was visualised using 3D surface and 2D counterplots (Figure 3). The interactions of KOH concentration and saponification time ($X_1 * X_2$), saponification time and temperature ($X_2 * X_3$), and KOH concentration and temperature ($X_2 * X_3$) showed a concave response surface model that identified optimal areas of lutein recovery (Figure 3).

The interaction between saponification temperature and time had a significant impact on lutein extraction ($p < 0.001$), and optimum lutein extraction was achieved with combinations of factors yielding > 15 mg/g DW (Figure 3). The actual highest lutein extraction was 15.64 mg/g obtained at 45°C in 3 h, but when saponification temperatures were > 65°C for

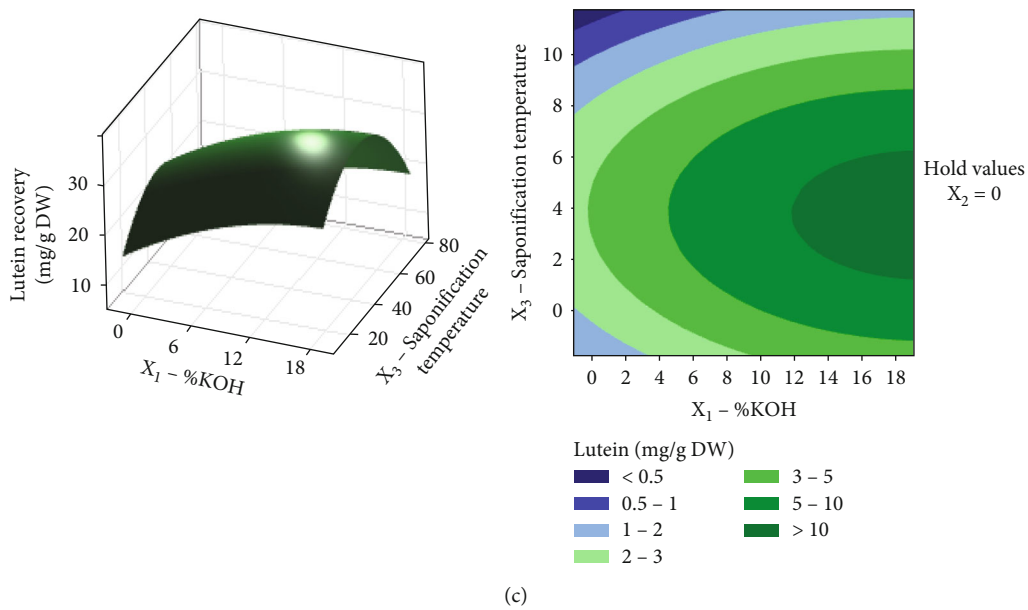


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: 3D surface response and 2D contour plots displaying the correlation of independent factors on the extraction of lutein from gac fruit peel. (a) KOH concentration and saponification time, (b) saponification time and saponification temperature, and (c) KOH concentration and saponification temperature.

longer than 5 h, the amount extracted was significantly lower (Figure 1). This was in agreement with other studies on the extraction of lutein from marigold flowers [28] and spinach [29]. The optimum temperature of 45°C is more commercially viable by economically reducing energy costs during extraction. Given the time that is also reduced, this could be significant cost savings with similar lutein recovered and justifies the necessity to use RSM optimisation.

3.5.3. Validation of the Optimal Factors Predicted by RSM. The optimum conditions for lutein recovery validated from RSM predictions were 18.0% KOH for 1.9 h at 39.9°C and a predicted maximum lutein extraction of 17.4 mg lutein/g DW (Figure 1). The actual lutein extracted at this condition was 16.37 mg/g DW with 75.5% lutein recovery, which was 1.03 mg/g less than the validated predicted extraction. The percentage error between the actual and predicted lutein extraction was 5.56% (Figure 1) but was not significantly different. This error value was less than 10%; thus, the model equation is robust and can be applied for extracting lutein from gac fruit peel [39, 44], and the benefit is flexibility for the industry to adjust according to needs and available resources.

This validated response from RSM modelling reflected the interaction between the three variables to optimise lutein recovery with increased KOH concentration but reduced saponification time and temperature. However, the costs involved with doubling the KOH concentration may offset the benefits of reduced time and temperature, given that energy costs are more affordable (AU$0.10 kWh) than KOH (AU$5/kg) in Vietnam, where the source of most gac fruits is. More importantly, the environmental and health concerns with KOH need to be carefully considered [45], particularly if the end use of lutein is medicinal and needs further refinement to remove high KOH residues.

3.6. Effects on the Lutein Yield and Biochemical Characterisation. The lutein content in the extraction mass was converted to the percent lutein extracted to indicate the extraction efficiency of the factors used. The highest value was almost 90% lutein, achieved with 15% KOH concentration for 5 h at 65°C (Extract #17). However, the actual lutein yield was suboptimal, with only 5.68 mg/g DW of gac peel. This low amount might be due to lutein degradation caused by the high alkali concentration and elevated temperature during the extraction period, as reported in previous studies on marigold petals [27, 32] and microalgae [31, 46]. This produced almost a third of the optimum lutein yield from Extract #12 with 15.64 mg/g DW.

When the lutein yield and percent lutein extraction efficiency were compared in HPLC chromatograms, the higher percent lutein extracted had higher lutein levels with high purity (Figure 4). The extract with the highest percent lutein extracted (89.77%) (Figure 4(b)) exhibited a peak similar to the lutein standard (Figure 4(a)), along with minor peaks observed at retention times ranging from 2.80 to 13.11 min. As the percent lutein decreased, there was a concomitant increase in the number of unidentified peaks and concentrations (Figures 4(c) and 4(d)). These prominent peaks might indicate the presence of esterized-bound lutein that remained incompletely eliminated by fatty acids during specific saponification conditions, as shown in previous studies [19].

Furthermore, both the highest purity extract (Figure 5(b)) and the lutein standard (Figure 5(a)) exhibited comparable FTIR spectral patterns. Notably, the lutein standard and the 89.77% lutein extract displayed a distinct and broad transmittance band between 3180 and 3520 cm^{-1}, which corresponded to the characteristic OH stretching vibrations of free lutein. This observation suggests that the saponification reaction effectively eliminated the fatty acids that were bound to the OH group of lutein. The 45.93% lutein extract also exhibited

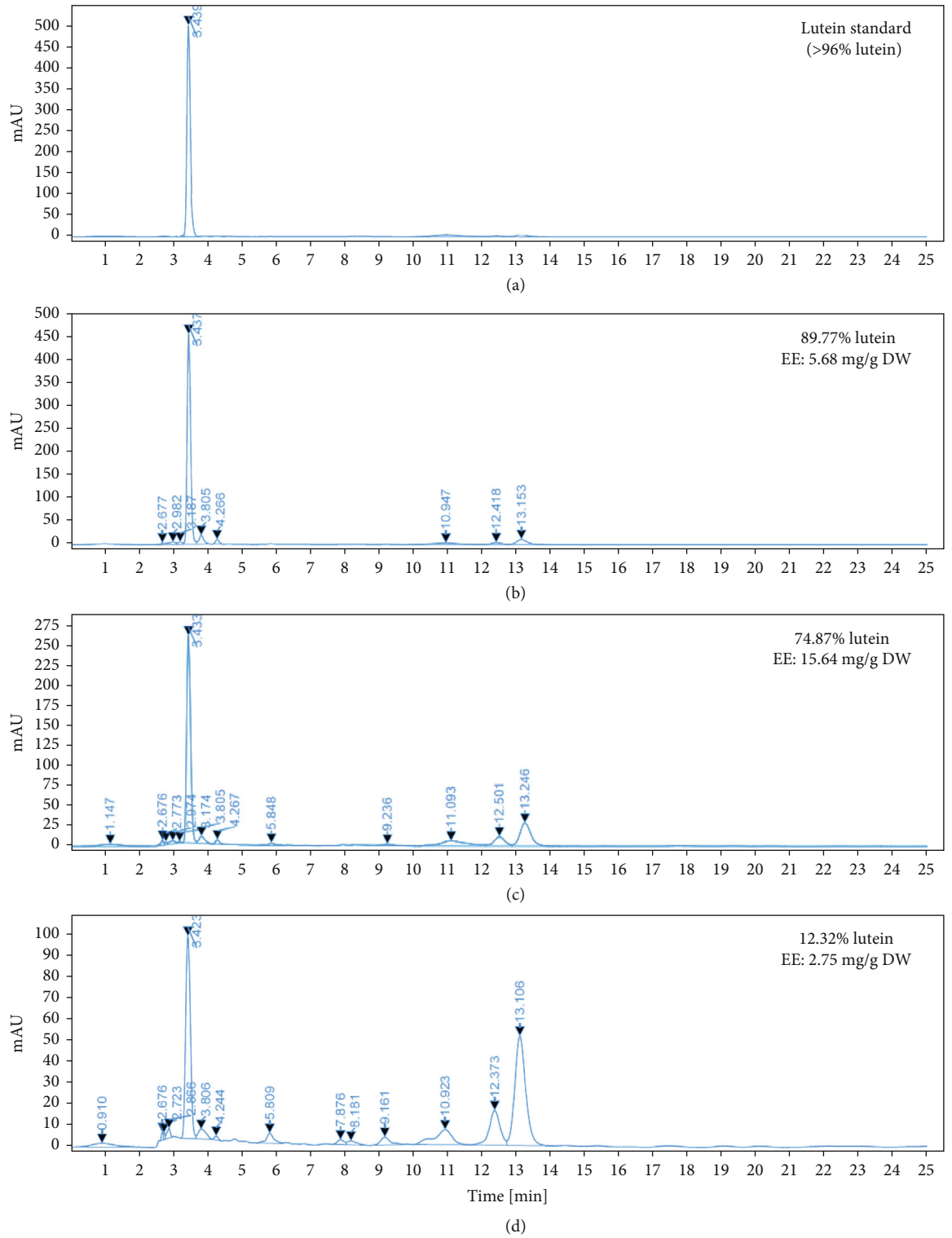


Figure 4: (a) HPLC chromatogram of the lutein standard (40 μg/mL) and (b–d) lutein extracts (40 μg/mL) from gac peel with lutein content (percentage) and extraction efficiency (mg/g DW). EE = extraction efficiency.

OH vibrations, albeit with a lower signal intensity compared to the lutein standard (Figure 5(c)), indicating that the removal of fatty acid binding to the OH group was not entirely complete [47]. However, in the case of the extract with low purity (12.32% lutein), the stretching pattern was indistinct (Figure 5(d)), implying that the OH group remained nonfree and a significant portion of lutein molecules in the extract remained in an esterized form [47].

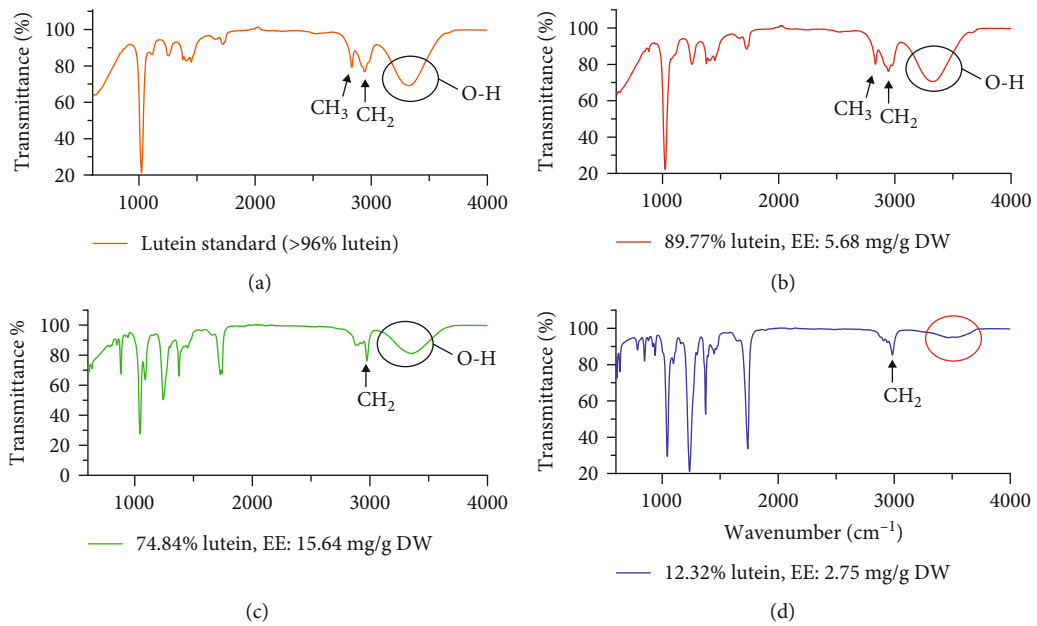


Figure 5: Fourier transform infrared spectroscopy (FTIR) spectra of the (a) lutein standard and (b–d) lutein extracts from gac peel with lutein content (percentage) and extraction efficiency (mg/g DW). EE = extraction efficiency.

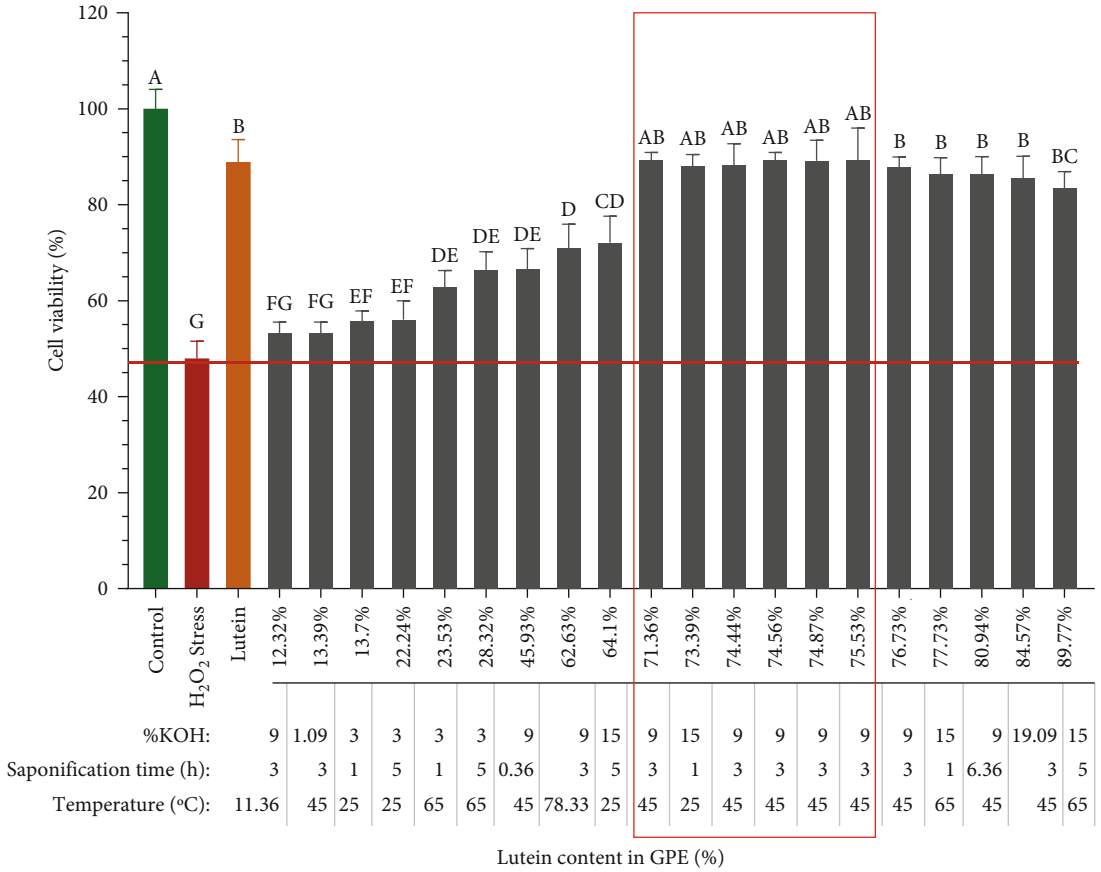


Figure 6: Protective effects of gac fruit peel extracts from RSM modelling (Figure 1), ordered according to percent lutein extraction efficiency on retina cell viability exposed to H_2O_2 stress. The RPE cells were treated with 10 μg/mL GPE for 48 h before stress with 500 μM H_2O_2 for 12 h. Cell viability was expressed as a percentage of the untreated controls (represented as 100%) and shown as the mean + SEM of three replicate values performed in triplicate. Statistical significance was determined by one-way ANOVA with Tukey's test where different letters indicated statistical significance at $p \leq 0.05$ between different treatments. The red box indicated the highest retinal benefits.

3.7. Protective Effects of Lutein on RPE Cells Against H_2O_2 Stress. Lutein is a macula pigment and confers protection by reducing oxidative and inflammatory stress [6]. Based on RSM modelling (Figure 1), the 20 GPE were tested for their protective abilities on retinal pigment epithelial cells. The retinal cell viability when stressed with H_2O_2 was reduced by half, but this was restored to almost the baseline control unstressed levels with 10 μg/mL of GPE containing > 71% lutein extraction efficiency (Figure 6). GPE comprising as little as 13.7% of lutein significantly protected RPE cells against H_2O_2 stress ($p < 0.05$). Extracts at the central point (red box) containing 71%–76% lutein (with lutein yield ranging from 14.19 to 15.64 mg/g DW) exhibited the highest retinal protection which was similar to the unstressed controls and better than the pure lutein standard ($p < 0.05$). These high-performing extracts were mostly from the RMS optimum predictions (five out of six) and supported its robustness to use for optimal lutein extraction and bioactivity efficacy. These GPE that contain only 71%–76% lutein could also have other bioactive compounds such as lycopene and β-carotene [22] and phytochemicals [48] that may exert a synergistic effect against H_2O_2 stress in RPE cells, as seen by the greater protection conferred by GPE when compared to lutein alone [11]. Given that a cocktail of beneficial active phytocompounds exist from gac fruit naturally, and these can be extracted with high yield (GPE #17 containing 89.77% lutein) in conditions that use less resources and energy than purified commercial lutein, it makes it feasible for upscaling and mass production.

4. Conclusion

This study is the first to optimise lutein extraction from gac fruit beyond single-factor analysis and incorporate RSM to generate a prediction formula. The extraction of lutein was affected by all three factors (percent KOH, saponification time, and temperature), and the extraction was optimised for maximum retinal benefits, particularly against oxidative stress (H_2O_2). Based on the results, it is recommended that an industrial upscale of lutein extraction be conducted at the RSM central point, 9% KOH for 3 h at 45°C. The extract obtained at the RSM central point conferred significant benefits to retinal health and likely contained mixtures of bioactives such as other carotenoids and peptides [19, 22]. The gac peel extract was more beneficial than synthetic lutein standards, suggesting high bioavailability and synergistic activity of phytochemicals in the extract. The gac peel used in this study is a waste byproduct from the agriculture industry and presents opportunities to upcycle. Since the input costs of gac waste can produce medical-grade bioactives such as lutein for eye benefits, there is significant feasibility for the commercialisation of gac peel as an alternative rich source of lutein.

Data Availability Statement

The data supporting the findings in this study is available from the corresponding author upon request.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Dao Nguyen: conceptualisation, methodology, investigation, data curation, visualization, writing–original draft. Tien Huynh: conceptualisation, methodology, supervision, writing–review and editing. Thilini Thrimawithana: methodology, supervision, writing–review and editing. Terry Piva: supervision, writing–review and editing.

Funding

Open access publishing facilitated by the RMIT University, as part of the Wiley - RMIT University agreement via the Council of Australian University Librarians.

Acknowledgments

Open access publishing facilitated by the RMIT University, as part of the Wiley - RMIT University agreement via the Council of Australian University Librarians.

References

[1] R. A. Bone, J. T. Landrum, and A. Mukherjee, "Efficacy of diacetate esters of macular carotenoids: effect of supplementation on macular pigment," *Journal of Nutrition and Metabolism*, vol. 2018, Article ID 4632081, 2018.

[2] M. L. Peng, H. F. Chiu, H. Chou et al., "Influence/impact of lutein complex (marigold flower and wolfberry) on visual function with early age-related macular degeneration subjects: a randomized clinical trial," *Journal of Functional Foods*, vol. 24, pp. 122–130, 2016.

[3] M. Sawa, T. Shunto, I. Nishiyama et al., "Effects of lutein supplementation in Japanese patients with unilateral age-related macular degeneration: the Sakai lutein study," *Scientific Reports*, vol. 10, no. 1, p. 5958, 2020.

[4] J. Landrum, R. Bone, V. Mendez, A. Valenciaga, and D. Babino, "Comparison of dietary supplementation with lutein diacetate and lutein: a pilot study of the effects on serum and macular pigment," *Acta Biochimica Polonica*, vol. 59, no. 1, pp. 167–169, 2012.

[5] E. Yonova-Doing, P. G. Hysi, C. Venturini et al., "Candidate gene study of macular response to supplemental lutein and zeaxanthin," *Experimental Eye Research*, vol. 115, pp. 172–177, 2013.

[6] D. Nguyen, T. Thrimawithana, T. J. Piva, D. Grando, and T. Huynh, "Benefits of plant carotenoids against age-related macular degeneration," *Journal of Functional Foods*, vol. 106, p. 105597, 2023.

[7] T. A. Ciulla and B. R. Hammond, "Macular pigment density and aging, assessed in the normal elderly and those with cataracts and age-related macular degeneration," *American Journal of Ophthalmology*, vol. 138, no. 4, pp. 582–587, 2004.

[8] M. M. Moschos, M. Dettoraki, M. Tsatsos, G. Kitsos, and C. Kalogeropoulos, "Effect of carotenoids dietary supplementation on macular function in diabetic patients," *Eye Vision*, vol. 4, no. 1, p. 23, 2017.

[9] C. T. C. Romagnoli, S. Persichilli, J. Gervasoni, C. Zuppi, G. Barone, and E. Zecca, "Lutein absorption in premature infants," *European Journal of Clinical Nutrition*, vol. 64, no. 7, pp. 760-761, 2010.

[10] C. L. I. Dani, F. Favelli, S. Frosini et al., "Lutein and zeaxanthin supplementation in preterm infants to prevent retinopathy of prematurity: a randomized controlled study," *The Journal of Maternal-Fetal & Neonatal Medicine*, vol. 25, no. 5, pp. 523–527, 2012.

[11] H. H. Leung, J. M. Galano, C. Crauste, T. Durand, and J. C. Lee, "Combination of lutein and zeaxanthin, and DHA regulated polyunsaturated fatty acid oxidation in H2O2-stressed retinal cells," *Neurochemical Research*, vol. 45, no. 5, pp. 1007–1019, 2020.

[12] A. Pintea, D. O. Rugină, R. Pop, A. Bunea, and C. Socaciu, "Xanthophylls protect against induced oxidation in cultured human retinal pigment epithelial cells," *Journal of Food Composition and Analysis*, vol. 24, no. 6, pp. 830–836, 2011.

[13] E. Y. Chew, T. E. Clemons, J. P. SanGiovanni et al., "Secondary analyses of the effects of lutein/zeaxanthin on age-related macular degeneration progression: AREDS2 report no. 3," *JAMA Ophthalmology*, vol. 132, no. 2, pp. 142–149, 2014.

[14] Andrews, "Lutein market by form, production process and region - global forecast to 2028," 2019, https://www.marketsandmarkets.com/Market-Reports/lutein-market-69753879.html.

[15] J. Mares, "Lutein and zeaxanthin isomers in eye health and disease," *Annual Review of Nutrition*, vol. 36, no. 1, pp. 571–602, 2016.

[16] H. V. Chuyen, M. H. Nguyen, P. D. Roach, J. B. Golding, and S. E. Parks, "Gac fruit (*Momordica cochinchinensis Spreng.*): a rich source of bioactive compounds and its potential health benefits," *International Journal of Food Science and Technology*, vol. 50, no. 3, pp. 567–577, 2015.

[17] D. Nguyen and T. Huynh, "Diversity in nutrition and bioactivity of gac fruit," in *Gac Fruit: Advances in Cultivation, Utilization, Health Benefits and Processing Technologies*, M. H. Nguyen and C. T. Kha, Eds., CABI, 2022.

[18] J. Kubola and S. Siriamornpun, "Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of Thai gac (*Momordica cochinchinensis* Spreng)," *Food Chemistry*, vol. 127, no. 3, pp. 1138–1145, 2011.

[19] H. V. Chuyen, P. D. Roach, J. B. Golding, S. E. Parks, and M. H. Nguyen, "Optimisation of extraction conditions for recovering carotenoids and antioxidant capacity from gac peel using response surface methodology," *International Journal of Food Science and Technology*, vol. 52, no. 4, pp. 972–980, 2017.

[20] D. Wimalasiri, T. Piva, S. Urban, and T. Huynh, "Morphological and genetic diversity of *Momordica cochinchinenesis* (Cucurbitaceae) in Vietnam and Thailand," *Genetic Resources and Crop Evolution*, vol. 63, no. 1, pp. 19–33, 2016.

[21] N. Nanta, P. Songsri, B. Suriharn, and K. Lomthaisong, "Seasonal variation in lycopene and beta-carotene content in *Momordica cochinchinensis* (LOUR) Spreng (gac fruit) genotypes," *Pakistan Journal of Botany*, vol. 52, no. 1, pp. 235–241, 2020.

[22] D. Wimalasiri, T. Piva, and T. Huynh, "Diversity in nutrition and bioactivity of Momordica cochinchinensis," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 6, no. 3, p. 378, 2016.

[23] X. T. Tran, S. E. Parks, P. D. Roach, J. B. Golding, and M. H. Nguyen, "Effects of maturity on physicochemical properties of gac fruit (*Momordica cochinchinensis* Spreng.)," *Food Science & Nutrition*, vol. 4, no. 2, pp. 305–314, 2016.

[24] T. C. Kha, M. H. Nguyen, and P. D. Roach, "Effects of spray drying conditions on the physicochemical and antioxidant properties of the gac (*Momordica cochinchinensis*) fruit aril powder," *Journal of Food Engineering*, vol. 98, no. 3, pp. 385–392, 2010.

[25] L. Etzbach, A. Pfeiffer, F. Weber, and A. Schieber, "Characterization of carotenoid profiles in goldenberry (*Physalis peruviana* L.) fruits at various ripening stages and in different plant tissues by HPLC-DAD-APCI-MS(n)," *Food Chemistry*, vol. 245, pp. 508–517, 2018.

[26] M. Butnariu, "Methods of analysis (extraction, separation, identification and quantification) of carotenoids from natural products," *Journal of Ecosystem & Ecography*, vol. 6, no. 2, pp. 1–19, 2016.

[27] X. Q. Fu, N. Ma, W. P. Sun, and Y. Y. Dang, "Microwave and enzyme co-assisted aqueous two-phase extraction of polyphenol and lutein from marigold (*Tagetes erecta* L.) flower," *Industrial Crops and Products*, vol. 123, pp. 296–302, 2018.

[28] T. Wang, J. Han, Y. Tian et al., "Combined process of reaction, extraction, and purification of lutein in marigold flower by isopropanol–KOH aqueous two-phase system," *Separation Science and Technology*, vol. 51, pp. 1–9, 2016.

[29] M. Derrien, A. Badr, A. Gosselin, Y. Desjardins, and P. Angers, "Optimization of a green process for the extraction of lutein and chlorophyll from spinach by-products using response surface methodology (RSM)," *LWT*, vol. 79, pp. 170–177, 2017.

[30] M. C. Chan, S. H. Ho, D. J. Lee, C. Y. Chen, C. C. Huang, and J. S. Chang, "Characterization, extraction and purification of lutein produced by an indigenous microalga *Scenedesmus obliquus* CNW-N," *Biochemical Engineering Journal*, vol. 78, pp. 24–31, 2013.

[31] J. T. M. Leema, P. T. Jothy, and G. Dharani, "Rapid green microwave assisted extraction of lutein from *Chlorella sorokiniana* (NIOT-2) - process optimization," *Food Chemistry*, vol. 372, article 131151, 2022.

[32] S. Manzoor, R. Rashid, B. Prasad Panda, V. Sharma, and M. Azhar, "Green extraction of lutein from marigold flower petals, process optimization and its potential to improve the oxidative stability of sunflower oil," *Ultrasonics Sonochemistry*, vol. 85, article 105994, 2022.

[33] M. C. Ceron-Garcia, C. V. Gonzalez-Lopez, J. Camacho-Rodriguez, L. Lopez-Rosales, F. Garcia-Camacho, and E. Molina-Grima, "Maximizing carotenoid extraction from microalgae used as food additives and determined by liquid chromatography (HPLC)," *Food Chemistry*, vol. 257, pp. 316–324, 2018.

[34] A. Altemimi, D. A. Lightfoot, M. Kinsel, and D. G. Watson, "Employing response surface methodology for the optimization of ultrasound assisted extraction of lutein and β-carotene from spinach," *Molecules*, vol. 20, no. 4, pp. 6611–6625, 2015.

[35] ICH Harmonised guideline, *Validation of analytical procedures Q2 (R2)*, FDA, 2022.

[36] M. A. Bezerra, R. E. Santelli, E. P. Oliveira, L. S. Villar, and L. A. Escaleira, "Response surface methodology (RSM) as a tool for optimization in analytical chemistry," *Talanta*, vol. 76, no. 5, pp. 965–977, 2008.

[37] D. Basß, "Modeling and optimization I: usability of response surface methodology," *Food*, vol. 78, no. 3, pp. 836–845, 2007.

[38] D. Nguyen, J. Holien, C. Dekiwadia, T. Thrimawithana, T. J. Piva, and T. Huynh, "*Momordica cochinchinensis* (gấc) seed extracts induce apoptosis and necrosis in melanoma cells," *Pharmaceuticals*, vol. 16, no. 100, 2023.

[39] P. K. Sahu, N. R. Ramisetti, T. Cecchi, S. Swain, C. S. Patro, and J. Panda, "An overview of experimental designs in HPLC method development and validation," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 147, pp. 590–611, 2018.

[40] M. Gong, Y. Wang, and A. Bassi, "Process analysis and modeling of a single-step lutein extraction method for wet microalgae," *Biotechnology and Bioprocess Engineering*, vol. 101, no. 22, pp. 8089–8099, 2017.

[41] I. W. M. Smith, "The temperature-dependence of elementary reaction rates: beyond Arrhenius," *Chemical Society Reviews*, vol. 37, no. 4, pp. 812–826, 2008.

[42] P. N. Babu and P. Binnal, "Production of high purity biodiesel through direct saponification of wet biomass of Chlorella protothecoides in a low cost microwave reactor: kinetic and thermodynamic studies," *Korean Journal of Chemical Engineering*, vol. 34, no. 4, pp. 1027–1036, 2017.

[43] S. Nam, C. H. Lee, S. M. Shim, D. U. Lee, and S. Lee, "Functional characterization of marigold powder as a food ingredient for lutein-fortified fresh noodles," *Applied Sciences*, vol. 11, no. 2, p. 861, 2021.

[44] C. F. Wu and K. H. Ful, "A residual-stress model for the milling of aluminum alloy (2014-T6)," *Journal of Materials Processing Technology*, vol. 51, pp. 87–105, 1995.

[45] J. A. Young and J. K. Taylor, "Potassium hydroxide," *Journal of Chemical Education*, vol. 82, no. 9, p. 1304, 2005.

[46] K. Lee and K. Yoo, "Optimization of lutein recovery from Tetraselmis suecica by response surface methodology," *Biomolecules*, vol. 11, no. 2, p. 182, 2021.

[47] P. K. Bolla, V. Gote, M. Singh, M. Patel, B. A. Clark, and J. Renukuntla, "Lutein-loaded, biotin-decorated polymeric nanoparticles enhance lutein uptake in retinal cells," *Pharmaceutics*, vol. 12, no. 9, p. 798, 2020.

[48] A. V. Le, T. T. Huynh, S. E. Parks, M. H. Nguyen, and P. D. Roach, "Bioactive composition, antioxidant activity, and anticancer potential of freeze-dried extracts from defatted gac (*Momordica cochinchinensis* Spreng) seeds," *Medicine*, vol. 5, no. 3, p. 104, 2018.