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Optimisation of ultrasound-assisted extraction of carotenoids and antioxidant activity from *Citrus reticulata* Blanco peels (Wilking mandarin)

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ABSTRACT

A large amount of waste, especially the outer part of citrus fruits (peel), is generated after consuming the pulp and it remains unused. The valorisation of this waste by recovering its bioactive compounds seems interesting. The aim of this study was to find the optimal conditions using ultrasound-assisted extraction (UAE) that yield the highest carotenoid content and better antioxidant activity from *Citrus reticulata* Blanco peels.

Response surface methodology (RSM) through Box–Behnken experimental design was used to optimise the conditions for carotenoid extraction using UAE. Hexane concentration, temperature, and sonication time were selected as the main factors.

The results revealed that all independent variables affected the responses. The optimal UAE conditions for hexane concentration, temperature, and sonication time were 60.76%, 36.45 °C, and 37.32 min, respectively. The values of total carotenoid content (TCC) and total antioxidant activity (TAA) obtained by UAE were higher than those obtained by the maceration extraction method.

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It can be concluded that the medium and extraction parameters, including hexane concentration, temperature, and sonication time, significantly influenced the recovery of carotenoids and antioxidant activity. The optimisation study allowed determining the appropriate conditions to maximise both responses. Compared to conventional maceration, the UAE method was superior and more efficient for extracting carotenoids from *C. reticulata* Blanco peels.

KEYWORDS

optimisation, carotenoid, antioxidant activity, ultrasound-assisted extraction, *Citrus reticulata* peel

1. INTRODUCTION

The excessive quantities of food waste generated from fruits are considered a valuable source of many beneficial compounds (Ramadan and Farag, 2022). Citrus fruits are among the major processed fruits that generate large quantities of waste. Their peels provide an interesting raw material with valuable components, especially carotenoids (Chavan et al., 2018). Carotenoids have been used since ancient times as natural pigments and have attracted the attention of the scientific community and the agri-food industry regarding their benefits to human health, mainly their provitamin A and antioxidant activity (Zia-Ul-Haq et al., 2021). The recovery of these compounds from their original matrix using conventional and alternative extraction methods has mostly been performed. However, the long time, high solvent volume, and substantial energy consumption required by conventional methods make other alternative extraction methods more suitable to overcome the disadvantages of the traditional approach (Saini and Keum, 2018). Ultrasound-assisted extraction (UAE) is one of the alternative methods that is recognised as an effective and efficient food processing tool. However, the extraction of bioactive compounds using the UAE method can be influenced by various factors, including ultrasonic power, temperature, and time. Variation in these factors can significantly affect extraction yields, thus antioxidant properties (Saini and Keum, 2018). The response surface methodology (RSM) approach has proved its efficiency in carotenoid extraction. This statistical approach has been widely used to study complex processes and offers many advantages (Kadi et al., 2021).

The aim of this study is to evaluate the potential of UAE and optimise the extraction conditions using RSM to reach the maximum amount of carotenoid extraction and high antioxidant activity from *C. reticulata* Blanco peels.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

The solvents and chemicals used in this study were of analytical grade. β-Carotene, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, potassium phosphate monobasic, trichloroacetic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and ascorbic acid were purchased from Sigma-Aldrich. Hexane, ethanol, acetone, di-potassium hydrogen orthophosphate anhydrous,

potassium ferricyanide, and iron chloride ($FeCl_3$) were from Biochem Chemopharma. Potassium persulphate was provided by AnalaR NORMAPUR (VWR).

2.2. Plant materials

The citrus fruits (*C. reticulata* Blanco) were purchased from a local market in Bejaia City (Algeria) in February 2022. The fruits were of good quality and in the mature stage. The peels were separated from the pulp and cut into small pieces (0.5 cm^2) and dried in a forced-air oven (Nüve, FN 400, Ankara, Turkey) at 40 °C for approximately three days. The dried peels were then crushed into a fine powder using a laboratory grinder (IKA model-A11, Staufen, Baden-Wurttemberg, Germany). The powder was stored at 4 °C until further analysis.

2.3. Optimisation of UAE of carotenoids from citrus fruit peels

2.3.1. Sequential methodology. In order to optimise the extraction of carotenoids from citrus fruit peels and select the parameters that influenced total carotenoid content (TCC), the extraction parameters were tested in a sequential procedure. These parameters included hexane concentration in the hexane/acetone mixture (30%, 50%, 70%, 90%, and 100%), temperature (20 °C, 25 °C, 30 °C, 35 °C, and 40 °C), and sonication time (5 min, 15 min, 25 min, 35 min, and 45 min).

2.3.2. Response surface methodology. The experimental optimisation design was performed according to Ndayishimiye and Chun (2017). In this approach, the effects of independent variables, namely hexane concentration, temperature, and sonication time, on two responses, TCC and antioxidant activity measured with $ABTS^{\bullet+}$ radical scavenging assay, from *C. reticulata* Blanco peels were investigated.

The total carotenoid content was determined using the method published by Machmudah and Goto (2013), and the results were expressed as micrograms of β-carotene equivalent per gram of dry matter (dry powder). The maceration and ultrasound-assisted extraction were carried out according to Yan et al. (2015). Maceration was applied as a reference conventional method in order to evaluate the performance of the UAE procedure.

2.3.3. Total antioxidant activity of extracts. For antioxidant characterisation of the optimal extract obtained through UAE and the extract performed by maceration using the same extraction conditions as UAE, the TAA was investigated using three tests, the ABTS and DPPH radical scavenging assays and ferric reducing power (FRP). The assays were performed according to Re et al. (1999), Song et al. (2013), and Oyaizu (1986), respectively. The results of ABTS and DPPH assays were expressed as micromoles of Trolox equivalent per gram of dry matter (μmol TE/g DM). For the FRP assay, the results were expressed as micromoles of ascorbic acid equivalent per gram of dry matter (μmol AAE/g DM).

2.3.4. Statistical analysis. For the sequential methodology data, the statistical analysis was carried out using one-way analysis of variance (ANOVA) following Tukey's post-hoc test. The data collected for model validation and comparison between the UAE and maceration methods were analysed using Student's paired *t*-test. The statistical analysis of optimisation results and graphical construction were carried out using JMP14 software, and significance was considered for *P*-values less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Sequential methodology

The single-factor approach was used to determine the effect of extraction parameters on TCC and to establish the range of influential parameters for the RSM study. The results of the sequential carotenoid extraction procedure are presented in Fig. 1.

The impact of hexane concentration (30%, 50%, 70%, 90%, and 100%), temperature (20 °C, 30 °C, and 40 °C), and sonication time (5 min, 15 min, 25 min, 35 min, and 45 min) on TCC was observed across all levels of each factor.

3.2. Response surface methodology

Under the tested experimental conditions, the highest TCC value (1169.36 μg β-CE/g DM) was observed using 65% hexane for solvent concentration, 40 °C for temperature, and 45 min for sonication time. However, the highest ABTS value (4.33 μmol TE/g DM) was obtained under 65% hexane, 30 °C, and 25 min. The optimal conditions obtained by the model resolution through maximised desirability, considering the highest values for both TCC and ABTS test, were 60.76% hexane, 36.45 °C, and 37.32 min. The results obtained in this study showed higher carotenoid yields when compared to those obtained by the microwave method for *Citrus clementina* peels (Kadi et al., 2021) and those obtained by the ultrasound method for *Citrus sinensis* peels (Montero-Calderon et al., 2019). This difference is probably due to several factors, especially the difference in citrus variety and extraction method. The highest carotenoid yield obtained under optimal conditions, which is higher than 20 μg per gram of food, is considered a very-high content of carotenoids in human food (Zia-Ul-Haq et al., 2021). To the best of our knowledge, there are very few studies that have reported on the optimisation of the antioxidant activity of carotenoids using RSM, and this investigation appears to be one of the few studies describing this aspect.

3.2.1. Model fitting. The RSM approach was used to evaluate the effect of independent variables (hexane concentration, temperature, and sonication time) on dependent variables (TCC and ABTS). The various statistical parameters are summarised in Table 1. The coefficient of determination R^2 was 0.99 for TCC and 0.97 for ABTS, implying that 99% and 97% of the variations, respectively, were explained by the models. The high values of R^2 (R^2 close to 1) given by the two models mean that the calculated and measured responses were very close, which reflects the good quality and accuracy of the developed models.

The adjusted R^2 and R^2 for each model were close, which indicates the precision of the elaborated models. The high *F*-value and low *P*-value for the two mathematical regression models (TCC and ABTS) indicate that they are statistically significant (Atkinson and Donev, 1992). In addition, the error of adjustment was checked through the lack of fit estimated by the *P*-value, which was higher than 0.05 (*P*-value > 0.05) for both models. The coefficient of variation (CV%) for the TCC and ABTS models was less than 5 and 10, respectively. The low CV% value found in the present study indicates the reliability of the model. Equations (1) and (2) express the relationship between the independent variables and the dependent variables for each model.

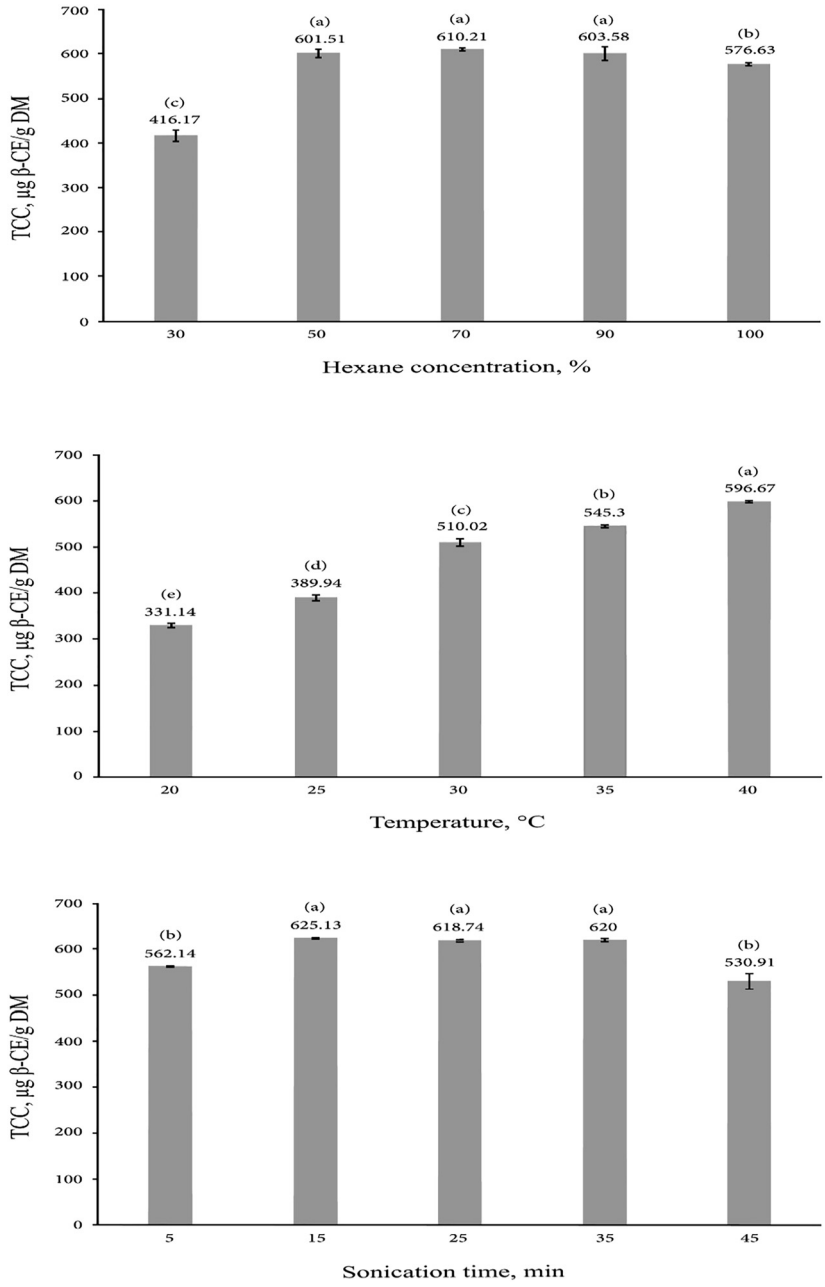

Fig. 1. Results of single-factor experiments of carotenoids with ultrasound-assisted extraction. Results of each parameter with different letters are statistically different (ANOVA-Tukey, $P < 0.05$, a > b > c > d > e). β-CE: β-carotene equivalent; TCC: total carotenoid content; DM: dry matter

Table 1. Analysis of variance of TCC and ABTS models for carotenoids extraction from *C. reticulata* Blanco peels

Factors	DF	Sum of squares	Mean square	*F*-value	*P*-value	R^2	R^2_{Adj}	CV%
TCC								
Model	9	44987.73	499984.20	81.03	<0.0001	0.99	0.98	2.91
Lack of fit	3	2885.58	961.86	9.67	0.10			
Pure error	2	198.90	99.45					
Residual	5	3084.48	616.90					
Cor. total	14	452942.20						
ABTS								
Model	9	10.14	1.13	21.29	0.0018	0.97	0.93	7.72
Lack of fit	3	0.20	0.07	2.25	0.32			
Pure error	2	0.06	0.03					
Residual	5	0.26	0.05					
Cor. total	14	10.40						

R^2: Coefficient of determination; R^2_{Adj}: Adjusted coefficient of determination; CV%: Coefficient of variation; DF: Degree of freedom; TCC: Total carotenoid content; ABTS: $ABTS^+$ free radical scavenging

$$Y_{TCC} = 960.57 - 45.09X_1 + 98.13X_2 + 25.93X_3 - 270.28X_1^2 + 119.72X_2^2 - 51.36X_3^2 \quad (1)$$

$$Y_{TAA} = 1034.59 - 138.23X_1 + 88.98X_3 - 280.54X_1^2 - 204.52X_2^2 \quad (2)$$

where Y is the response, and X_1, X_2, and X_3 are the independent variables: hexane concentration, temperature, and sonication time, respectively.

3.2.2. Effect of variables. Figure 2 shows the sorted parameter estimates for each model (TCC and ABTS). The *t*-ratio value and *P*-value of linear, quadratic, and interaction effects are given. Typically, the further the *t*-ratio value is from 0, the closer the *P*-value is to 0 ($P < 0.05$). This implies the significance of the considered term of the mathematical model, and *vice versa* (Goupy and Creighton, 2006). In the TCC model, all linear and quadratic effects were significant ($P < 0.05$). For the ABTS assay, all linear and quadratic terms of hexane concentration, the quadratic term of temperature, and the linear term of sonication time had significant effects ($P < 0.05$). However, the interaction effects of both TCC and ABTS models were not significant ($P > 0.05$).

Our results were consistent with other studies using UAE for carotenoid extraction. These studies reported that all linear and quadratic effects were significant (Goula et al., 2017; Civan and Kumcuoglu, 2019). In addition, Ordóñez-Santos et al. (2015) and Yan et al. (2015) found that interaction effects were not significant. These findings suggest that each parameter has a significant effect on the responses (TCC and ABTS), while the lack of significant interaction terms indicates that any change in one parameter does not influence other parameters.

3.2.3. Prediction of optimal parameters. The prediction profilers were used to explain how changes in variable values affect the responses. With regards to the hexane concentration factor, it was found to have a high and proportional effect on the TCC and ABTS values until their maximum values reached 61% and 56% hexane, respectively, after which a decrease in their values was observed (Fig. 3A and B). The same effect was observed for both TCC and ABTS,

Sorted Parameter Estimates

Term (A)	Estimate	Std Error	t Ratio	Prob > |t|
Hexane [C] (%) x Hexane [C] (%)	-270.28	12.93	-20.91	<0.0001*
Temperature (°C) (20, 40)	98.13	8.78	11.17	0.0001*
Temperature (°C) x Temperature (°C)	119.72	12.93	9.26	0.0002*
Hexane [C] (%) (30, 100)	-45.09	8.78	-5.13	0.0037*
Time (min) x Time (min)	-51.36	12.93	-3.97	0.01*
Time (min) (5,45)	25.93	8.78	2.95	0.03*
Hexane [C] (%) x Temperature (°C)	-15.22	12.42	-1.23	0.27
Temperature (°C) x Time (min)	11.92	12.42	0.96	0.38
Hexane [C] (%) x Time (min)	-10.86	12.42	-0.87	0.42

Term (B)	Estimate	Std Error	t Ratio	Prob > |t|
Hexane [C] (%) x Hexane [C] (%)	-280.54	29.74	-9.43	0.0002*
Temperature (°C) x Temperature (°C)	-204.52	29.74	-6.88	0.0010*
Hexane [C] (%) (30,100)	-138.23	20.21	-6.84	0.0010*
Time (min) (5,45)	88.98	20.21	4.40	0.0070*
Time (min) x Time (min)	-56.86	29.74	-1.91	0.11
Temperature (°C) (20,40)	-10.87	20.21	-0.54	0.61
Temperature (°C) x Time (min)	11.93	28.55	0.42	0.69
Hexane [C] (%) x Time (min)	9.65	28.58	0.34	0.75
Hexane [C] (%) x Temperature (°C)	4.13	28.58	0.14	0.89

Fig. 2. Sorted parameter estimates for TCC (A) and ABTS (B) models. Term (A): linear, quadratic and interaction terms of TCC model. Term (B): linear, quadratic and interaction terms of ABTS model [C]: Concentration, Std Error: Standard error, Prob > |t|: *P*-value of terms, *: The term is significant ($\alpha = 0.05$)

indicating a noticeable coordination between these two responses. The observed results can be explained by the nature of carotenoids present in the matrix and the polarity of the solvent used. As reported by Saini and Keum (2018), acetone and hexane are commonly used for the extraction of polar and non-polar carotenoids, respectively. Mixing these two solvents can lead to high carotenoid content and antioxidant activity.

The temperature of the extraction had an increased effect on carotenoid recovery; the highest content was obtained under the temperature of 40 °C (Fig. 3A). This seems reasonable because increasing temperature leads to more efficient recovery of the substances due to the destruction of the matrix cell wall and the increase in the diffusion coefficient of the solvent into the matrix (Herrero et al., 2005). Similar to TCC, an important effect of temperature on ABTS was noticed (Fig. 3B). A high activity of the extract was found at around 30 °C; however, additional heat over 30 °C led to low activity. According to Chemat et al. (2017), when using UAE, the beneficial effects were observed at low temperatures (less than 30 °C).

Regarding the effect of sonication time (Fig. 3A and B), TCC and ABTS increased slightly from 0 min until about 33 and 40 min, respectively. Afterwards, a slight decrease without significance was recorded for TCC up to 45 min. Indeed, no change in the response (TCC and ABTS) occurred. This is probably due to complete extraction of carotenoids, where any further increase in sonication time does not lead to any further improvement in extraction. From the results, it can be concluded that there is no need to extend the extraction time further to increase the recovery of carotenoids from the peels of *C. reticulata* Blanco.

3.2.4. Validation of models. To pronounce on the validation and acceptability of the models, a comparison between the predicted values calculated through established mathematical models

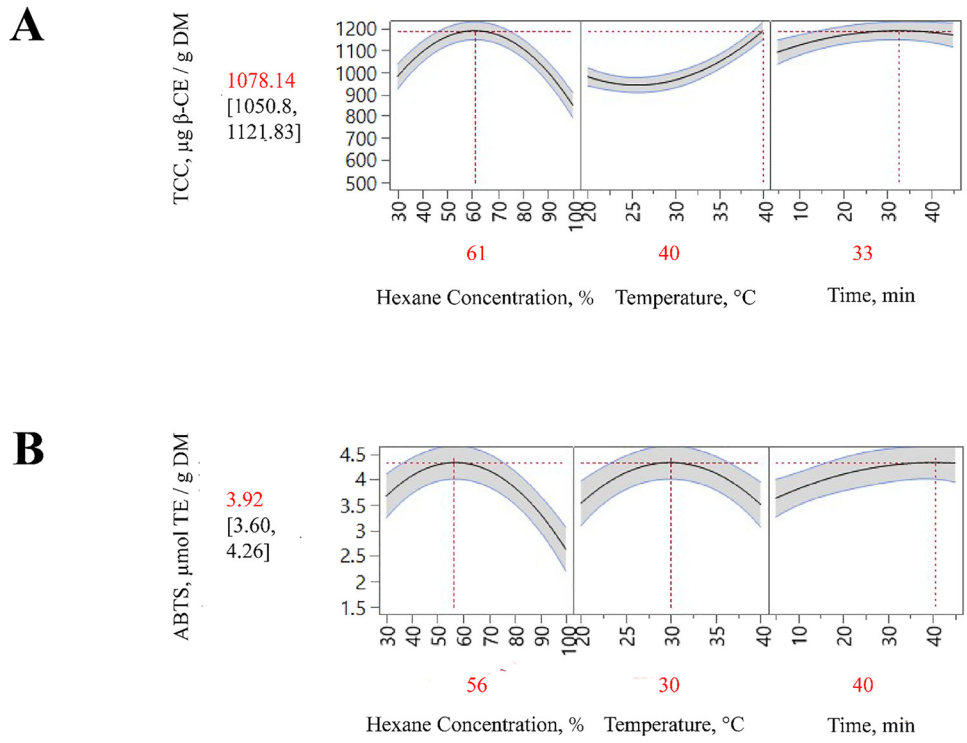

Fig. 3. Prediction profilers for TCC (A) and ABTS (B) models. TCC: Total carotenoid content; ABTS: $ABTS^{+}$ free radical scavenging; β-CE: β-carotene equivalent; TE: Trolox equivalent; DM: dry matter

and the measured values obtained under optimal conditions was carried out. The data of Table 2 indicate that the measured and predicted values obtained for both models (TCC and ABTS) were similar, manifesting the validity and suitability of developed models for the extraction of carotenoids from *C. reticulata* Blanco peels.

Table 2. Optimal conditions of carotenoids extraction and predicted and experimental values of TCC and ABTS tests

Factors	Optimal values	Adjusted optimal values	TCC (µg β-CE/g DM)		ABTS (µmol TE/g DM)	
			Predicted value	Experimental value	Predicted value	Experimental value
Hexane concentration (%)	60.76	61	1078.14[a]	1065.40 ± 28.03[a]	3.92[a]	3.78 ± 0.61[a]
Temperature (°C)	36.45	36				
Sonication time (min)	37.32	37				

β-CE: β-Carotene equivalent; TE: Trolox Equivalent; TCC: Total Carotenoid Content; ABTS: $ABTS^{+}$ free radical scavenging; DM: Dry matter

[a]: The same letter assigned indicates the statistical similarity of results (Student-*t* test, $P > 0.05$).

Table 3. Comparison of total carotenoid contents and antioxidant activity of *C. reticulata* Blanco peels by UAE and maceration methods

Extraction method	Hexane concentration (%)	Temperature (°C)	Time (min)	TCC (μg β-CE/g DM)	TAA		
					ABTS (μmol TE/g DM)	DPPH (μmol TE/g DM)	FRP (μmol AAE/g DM)
UAE	61	36	60	1050.00 ± 16.21[a]	3.84 ± 0.42[a]	3.79 ± 0.06[a]	10.47 ± 0.28[a]
Maceration	61	36	120	926.49 ± 3.42[b]	2.55 ± 0.13[b]	2.83 ± 0.24[b]	7.81 ± 0.32[b]

UAE: Ultrasound-Assisted Extraction; β-CE: β-Carotene Equivalent; TE: Trolox Equivalent; AAE: Ascorbic Acid Equivalent; TCC: Total Carotenoid Content; TAA: Total Antioxidant Activity; DM: Dry matter

[a, b]: Different letters indicate statistically significant differences (Student's *t*-test, $P < 0.05$; a > b)

3.3. Comparison of UAE and maceration

To evaluate the effectiveness of UAE for carotenoid extraction from *C. reticulata* Blanco peels, it was compared with maceration extraction under the same optimal conditions obtained by the RSM study, and the results are presented in Table 3. To establish an effective comparison, the procedure started by setting the conditions at the same levels of hexane concentration and temperature. Then, several extraction cycles were carried out to deplete carotenoids in the powder. There was a correspondence in the results of TCC and TAA. A high amount of TCC was confirmed by high antioxidant activity, which was confirmed by the three TAA methods (ABTS, DPPH, and FRP).

High activity was observed in all three TAA assays, which can be explained by the high reducing power of iron and high free radical scavenging capacity of carotenoids. The results of this comparison revealed that UAE provided higher extraction efficiency in a shorter time in terms of TCC and antioxidant capacity compared to maceration, which resulted in a slightly lower yield in a prolonged extraction time. This can be explained by the use of sound waves to break down cell walls, making the extraction of components faster than in conventional maceration (Zannou et al., 2022). This characteristic can be considered the main advantage of UAE for carotenoid extraction from *C. reticulata* Blanco peels. Our results are higher compared to the carotenoid value obtained from *Citrus sinensis* under optimal conditions of UAE (6.3 ± 0.1 μg β-carotene/g) (Montero-Calderon et al., 2019). In the investigation of Ordóñez-Santos et al. (2021), the carotenoid content reached 1450 μg β-carotene/g of dry sample using the ultrasonic extraction from mandarin peel, which was higher than our results. The difference in the total carotenoid content of citrus fruits depends on many parameters such as species, variety, and geographical environment.

4. CONCLUSIONS

In this study, we propose using the RSM approach to optimise the conditions for extracting carotenoids from Wilking peels using an alternative extraction method (UAE). Under optimal conditions revealed by the models, the predicted values were confirmed experimentally and validated statistically. All parameters investigated in the present study had an impact on carotenoid recovery and their antioxidant potential. Hexane concentration and extraction temperature are the primary factors influencing both the yield of carotenoid extraction and the antioxidant activity of the extracted compounds. Hexane concentration and temperature showed a high influence, whereas sonication time had a slight impact. Compared to maceration, the UAE method provided slightly higher TCC and TAA; its main advantage was the shorter time required, reflecting the positive effects of UAE on carotenoid extraction and their antioxidant activity. Considering its time-saving potential, it can be concluded that the use of ultrasonic-assisted extraction along with response surface methodology is an effective approach for recovering carotenoids with high antioxidant capacity from the peels of *C. reticulata* Blanco.

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