Chapter 1
Graviola Fruit Extraction by Ionic Liquid Microwave-Assisted Extraction (IL-MAE)

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Abstract This chapter discusses the extraction method of Graviola fruit using ionic liquid microwave-assisted extraction (IL-MAE). Three parameters—irradiation power, solid/liquid (s/l) ratio, and extraction time—were investigated to obtain optimum extraction process resulting in a high yield and a low IC_{50} value for the anti-proliferation assay. The results showed that the extraction time of 1.74 min, the irradiation power of 300 W and the s/l ratio of 1:25 gave the highest extraction yield of 67.6%. Whereas the extraction time of 3 min instead of 2.98 min, the irradiation power of 700 W instead of 690 W, and the s/l ratio of 1:39 produced an average IC_{50} of 4.75 ± 0.36 µg/mL for MCF7 and 10.56 ± 2.04 µg/mL for HT29. The IL-MAE technique was found to be effective in the extraction of plant fruit as described in the case study.

Keywords Ionic liquid · Graviola · RSM · Microwave-assisted

1.1 Introduction

Ionic liquid (IL) is an organic liquid salt comprising of an organic cation matched with an organic or inorganic anion. ILs are generally perceived solvents owing to their phenomenal properties, such as poor power conductors, non-ionizing (e.g. non-polar), high viscosity, low combustibility, low vapor pressure, excellent thermal stability, wide liquid regions, and good solvating properties for polar and non-polar compounds (Huddleston et al., 2001).

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1.2 Ionic Liquid Microwave-Assisted Extraction (MAE)

The role of ILs is not limited to enhanced interactions between solute and solvent, which means increased solute solubility, but can be attributed to solvent-matrix interactions leading to changes in the permeability of the plant matrix. This is due to the disruption of the cell tissue and to the modification of the matrix permeability by the interaction of H bonding with the carbohydrates that form the cell walls. ILs could be considered as valuable solvent substituents for the extraction of value-added chemicals, especially when coupled with microwave-assisted extraction (MAE) and other methods known to disrupt targeted cell tissue that facilitate the whole process of extraction.

Higher yields and faster extraction of bio-compounds from biomass can be achieved through MAE processes. The pioneering work on the use of IL-MAE processes was first reported by Du and co-workers (2007), who demonstrated the effective utilization of aqueous IL solutions in MAE for the extraction of trans-resveratrol from *Rhizoma polygoni*.

1.2.1 Principle

Alupului and co-workers (2012) described the MAE mechanism involving three steps, starting from the separation of solutes from active matrix sites under increased pressure and temperature; then the distribution of solvent through sample matrix; finally, the release of solutes from the sample matrix to the solvent.

Summing up, almost all IL-based MAE processes reported to date are imidazolium-based ILs. The majority of the investigated ILs comprised of cations with short alkyl side chains length. Despite the lack of discussion on the mechanisms behind improved extraction yields, some authors have related the success of their extractions to the establishment of strong interactions, mainly hydrogen-bonding and $\pi-\pi$ interactions, between the ILs and the target bio-compounds. Various processing conditions, such as irradiation power, solid/liquid (s/l) ratio, and extraction time, chemical structure, and concentration of ILs, have been also addressed in previous studies. In general, high concentrations of ILs and low s/l ratios have promoted high extraction yields, whereas other parameters, such as irradiation power, have resulted in a lack of clear tendencies. Meanwhile, the use of tensioactive ILs promotes either an increase or a decrease in extraction yield, depending mostly on the hydrophilic-lipophilic ratio of the target bio-compound. There is therefore a need for more studies in this field, as many more conditions need to be explored, namely the size and type of IL aggregates (Ventura et al., 2017).

Table 1.1 Consumable used in this experiment

No.	Consumable	Usage
1	Whatman 3 mm filter paper	Filtration
2	Test tube	Sample collection

Table 1.2 Equipment used in this experiment

No.	Equipment	Usage
1	Microwave	Extraction
2	Freeze dryer	Drying sample

Table 1.3 Chemicals used in this experiment

No.	Chemicals	Usage
1	Ionic liquid solution ([C4MIM]Cl−, [C4MIM]BF4− and [C4MIM]PF6−)	Extraction
2	Graviola fruit	Extraction

1.2.2 Objective of Experiment

The objective of this procedure is to identify the most suitable condition for Graviola fruit extraction (GFE) using the IL-MAE method.

1.3 Materials

Tables 1.1, 1.2, and 1.3 show the materials used in this experiment.

1.4 Methodology

1.4.1 Statistical Optimization Set-Up

The use of statistical optimization experiments on various factors related to the production process is well documented. These techniques are necessary for the simultaneous analysis of various factors under study. Besides, they reduce the number of experiments involved, enhance data interpretation, and reduce the overall experimental time required. There are many software packages available for such data analysis. This study used Design-Expert v.7.0.8 as powerful software for determining the optimum extraction parameters of IL-MAE that can increase the extraction yield and the IC_{50} of IL-GFE toward cancer cell lines.

1.4.2 One-Factor-at-a-Time (OFAT) Experimental Design

The classical one-factor-at-a-time (OFAT) experimental design was employed to determine the possible optimum IL to maximize the yield of Graviola fruit extract. Previous literature has shown that several factors are affecting IL-MAE, such as extraction time, irradiation power, s/l ratio, and the type of IL used for extraction (Zhang et al., 2014). To increase the extraction efficiency of the three analytes, a series of the 1-butyl-3-methyl-imidazolium cation with various anions Cl−, BF4− and PF6− were compared, including [C4MIM]Cl−, [C4MIM]BF4−, [C4MIM]PF6− and deionized water, while other extraction parameters were kept constant at zero, i.e. extraction time (2 min), microwave irradiation power (450 W) and s/l ratio [1:20] (g/mL) (Zhang et al., 2014).

1.4.3 Optimization of Extraction Conditions

The optimization experiments were designed using Design Expert software v.7 (Stat-Ease) to achieve the maximum extraction yield of Graviola fruit extract and the minimum IC_{50} toward cancer cell lines. Based on the Face-Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM), three independent variables, namely extraction time [A], irradiation power [B], and s/l ratio [C], with three levels were designed to increase response yield and IC_{50}. The center point (0), the lowest (−1), and the highest (+1) values for all factors were selected based on the previous literature (Table 1.4) (Maran et al., 2013).

The experimental design obtained from the FCCCD consists of 12 triplicates of experiments or runs. The experimental design used in this work is presented in Table 1.5.

All the experiments were carried out in triplicates. The percentage of extraction yield and half-maximal inhibition IC_{50} of IL-GFE toward cancer cell lines were considered to be the response (Y). The evaluation of the analysis of variance (ANOVA) was achieved by conducting a statistical analysis of the model. The relationship between the responses (dependent factors) and the experimental levels of each variable under study was described, and the fitted polynomial equation was expressed in the form of contour and response surface plots.

Table 1.4 Experimental design and levels of independent process variables

Independent variables	Symbol	Levels of independent variables		
		−1	0	+1
Extraction time (min)	A	1	2	3
Irradiation power (W)	B	300	500	700
Solid-liquid ratio (gm/L)	C	(1:20)	(1:30)	(1:40)

Table 1.5 Experimental design for optimization of extraction parameters using RSM

Run	Process variables			Responses	
	Factor 1 [A]	Factor 2 [B]	Factor 3 [C]	Extraction yield (%)	Cytotoxicity IC 50 (μg/mL)
	Time (min)	Power (W)	Solid-liquid ratio (g/mL)		
1	1.00	300.00	20.00		
2	2.00	500.00	20.00		
3	3.00	300.00	40.00		
4	3.00	500.00	30.00		
5	2.00	500.00	20.00		
6	1.00	500.00	30.00		
7	2.00	500.00	40.00		
8	3.00	700.00	20.00		
9	3.00	500.00	30.00		
10	2.00	500.00	40.00		
11	2.00	300.00	30.00		
12	2.00	500.00	30.00		
13	2.00	500.00	30.00		
14	1.00	700.00	40.00		
15	3.00	300.00	40.00		
16	2.00	300.00	30.00		
17	2.00	500.00	40.00		
18	2.00	500.00	30.00		
19	2.00	700.00	30.00		
20	1.00	300.00	20.00		
21	2.00	500.00	30.00		
22	1.00	700.00	40.00		
23	3.00	500.00	30.00		
24	3.00	700.00	20.00		
25	1.00	300.00	20.00		
26	2.00	500.00	20.00		
27	2.00	500.00	30.00		
28	2.00	700.00	30.00		
29	2.00	300.00	30.00		
30	1.00	500.00	30.00		
31	2.00	500.00	30.00		
32	3.00	700.00	20.00		
33	3.00	300.00	40.00		

(continued)

Table 1.5 (continued)

Run	Process variables			Responses	
	Factor 1 [A]	Factor 2 [B]	Factor 3 [C]	Extraction yield (%)	Cytotoxicity IC 50 (μg/mL)
	Time (min)	Power (W)	Solid-liquid ratio (g/mL)		
34	1.00	500.00	30.00		
35	1.00	700.00	40.00		
36	2.00	700.00	30.00		

1.4.4 Validation of the Experimental Model

The optimum result predicted by the model was validated concerning all three independent variables. Another set of extractions selected as predicted by the model of the Design Expert software were used to validate the maximum yield and minimum IC_{50} under defined conditions.

1.5 Results and Discussion

1.5.1 Extraction of Graviola Fruit Extract

IL-MAE was applied on Graviola fruit to increase the extraction yield and cytotoxicity of the extracted compounds against selected cancer cell lines. The IL-MAE method, using the optimum extraction parameters, produced 66.6% extraction yield as shown in Fig. 1.4 (Sect. 1.6.8). This yield was high for solvent used and short extraction time.

This result indicates the efficiency of ILs by modifying the permeability of the plant matrix through the interaction of H bonding with the carbohydrates that form the cell walls (Bogdanov, 2014), whereas microwave heating induces cell-wall disruption and accelerates diffusion through membranes that facilitates targeted analyte release (Guo et al., 2017). On the other hand, previous studies reported lower extraction yields from Graviola fruit using conventional extraction methods such as Soxhlet and maceration, which usually take many hours for the same extraction yield (Melot et al., 2009; Ragasa et al., 2012). According to Lu et al. (2008), the extraction yield from these conventional extraction methods is not economically beneficial because it is time-consuming and solvent-consuming.

1.5.2 The Best Ionic Liquid Solution by OFAT

Performance of 1-butyl-3-methyl-imidazolium cation with various anions (Cl−, BF4− and PF6−) in the GFE process using the OFAT experimental design was evaluated to observe the effects of various anions and deionized water on the extraction yield. The structure of ILs has a great influence on their physicochemical properties, which may have an impact on the extraction efficiency of targeted analytes (Zhang et al., 2011). In the series of ILs studied in this work, the Cl− anion showed a higher extraction yield than others for the examined sample, as shown in Fig. 1.1.

It has been suggested that ILs with Cl− hydrophilic anion, could miscible in any proportion of water (Lu et al., 2008), thus yielding better extraction yields. A previous study conducted by Cláudio et al. (2013) reported that an aqueous solution of [C4MIM]Cl is the suitable extractive solvent of caffeine from *Paullinia cupana* seeds (guaraná) in which, under optimized extraction conditions (2.34 M [C4MIM]Cl, 70 °C, 30 min, 1:10 s/l ratio); the extraction efficiency increased by 50% compared to that obtained by Soxhlet extraction method using dichloromethane solvent. In another study, Jin and co-workers (2011) explored the ability of [C4MIM]Cl− to improve the release of phenolic aldehyde paeonol from the roots of *Cynanchum paniculatum*. The authors reported that the IL-assisted extraction in the optimum extraction conditions (70 °C, 8 h, 1:7.3 s/l ratio) produced a higher extraction yield than that of the Soxhlet extraction technique.

On the other hand [C4MIM][BF4] gave a lower extraction yield of Graviola fruit extract. A similar study was carried out using 1.5 M [C4MIM][BF4] and 1 M [C6MIM][BF4] to extract phenolic alkaloids liensinine, neferine, and isoliensinine from the *Nelumbo nucifera* seeds (Lu et al., 2008). The authors showed that the optimized extraction conditions of IL-MAE have increased the extraction efficiency

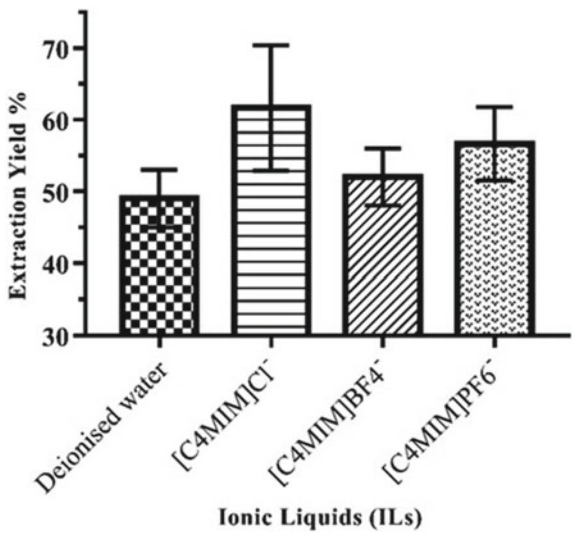

Fig. 1.1 Effect of different ionic liquids on extraction yield

by 20–50% compared to conventional heat reflux extraction (HRE) and MAE with 80% methanol, for a significantly reduced extraction time from 2 h (HRE) to 1.5 min (IL-MAE). However, a strong dependency has been observed between the s/l ratio and extraction efficiency; the efficiency was found to have decreased when the s/l ratio increased from 1:10 to 1:20. The increase of viscosity by increasing the chain length of the imidazolium cation was proposed as an explanation of this phenomenon.

Moreover, [C4MIM][PF6]—relatively hydrophobic—is only sparingly water-soluble and therefore gave lower extraction yield. It is hypothesized that the extraction yield is affected by the degree of hydrophobicity (Morais et al., 2017). Previously, Zhai et al. (2009) used pure [C4MIM][PF6] as a water substitute coupled with MAE for the extraction of essential oils from two commonly used cooking spices, known as *Cuminum cyminum* (cumin) and *Illicium verum* (star anise). In this study, ILs are found to be able to absorb microwave energy more easily than water allowing the appropriate extraction temperature to be reached approximately three times faster. Thus, IL-MAE has considerably shortened extraction time (15 min) compared to conventional hydrodistillation (180 min) to completely extract essential oils. Also, they reported that the use of IL-MAE has reduced the oxidation and hydrolyzation of the essential oil constituents.

1.5.3 Optimization of the IL-MAE Parameters by RSM

The regression analysis used ANOVA. The FCCCD under RSM was employed to investigate the optimal parameters of the three variables (extraction time, irradiation power, and s/l ratio) in order to maximize the extraction yield and minimize IC_{50} of the crude IL-GFE on MCF7 and HT29 cancer cell lines. The experimental results and the predicted yield along with IC_{50} obtained from the regression equations are presented for each run in Table 1.6. The second-order polynomial equation was fitted to the data by multiple regression procedures. Each run has a unique combination of factor levels and the responses: extraction yield (%) (Y1) and IC_{50} (μg/mL) of IL-GFE on MCF7 (Y2) and HT29 (Y3) cells. The model equations obtained are listed as Eqs. (1.1–1.3) for three-factor systems (process conditions).

$$\begin{aligned} \text{Y1 (Yield, \%)} = & 81.94 - 9.66\text{A} - 0.15\text{B} + 1.02\text{C} \\ & + 0.05\text{AB} + 0.48\text{AC} + 0.004\text{BC} \\ & - 1.66\text{A}^2 - 0.04\text{C}^2 - 0.001\text{ABC} \end{aligned} \tag{1.1}$$

$$\begin{aligned} \text{Y2 (MCF7} - \text{IC50)} = & 50.7 - 98.62\text{A} - 0.2\text{B} + 0.27\text{C} + 0.25\text{AB} \\ & + 3.64\text{AC} + 0.01\text{BC} - 8.88\text{A}^2 - 0.0002\text{B}^2 \\ & - 0.09\text{C}^2 - 0.007\text{ABC} \end{aligned} \tag{1.2}$$

Table 1.6 FCCCD experimental design and result of the responses

Run	Extraction condition			Y_1: yield (%)		Y_2: IC_{50} of IL-MAE on MCF7 cells		Y_3: IC_{50} of IL-MAE on HT29	
	A: extraction time (min)	B: irradiation power (W)	C: solid—liquid ratio (1 g/mL)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	3	700	20	56.66±0.58	57.23	15.40±1.25	15.65	14.95±2.44	14.76
2	3	300	40	54.66± 0.58	55.05	14.42±1.71	13.88	39.09±8.92	39.01
3	1	700	40	52.00±1.00	52.45	19.16±0.68	19.55	24.66±1.22	24.51
4	1	300	20	65.66±1.15	66.03	10.21±1.71	10.23	11.69±1.27	11.66
5	1	500	30	58.66±1.15	59.11	22.87±0.59	23.03	29.8±0.59	29.72
6	3	500	30	59.00±1.00	59.36	13.66±0.78	13.31	12.17±0.48	12.01
7	2	300	30	66.33±1.53	66.38	14.70±0.31	14.45	28.87±1.83	27.95
8	2	700	30	55.33±1.53	55.42	22.54±0.08	22.85	11.85±0.88	10.79
9	2	500	20	59.00±1.00	59.48	8.12±0.33	8.22	12.99±2.06	12.89
10	2	500	40	53.00±1.00	53.32	28.30±1.41	28.00	12.46±1.32	12.32
11	2	500	30	60.66±0.58	60.90	26.81±0.77	27.05	18.46±1.65	19.37
12	2	500	30	61.00±1.00	60.90	27.50±1.8	27.05	18.79±1.39	19.37

$$\begin{aligned} \text{Y3 (HT29} - \text{IC50)} = & -51.4 - 36.6\text{A} + 0.0002\text{B} + 4.3\text{C} \\ & + 0.08\text{AB} + 1.52\text{AC} + 0.001\text{BC} + 1.49\text{A}^2 \\ & - 0.07\text{C}^2 - 0.004\text{ABC} \end{aligned} \quad (1.3)$$

where Y is the predicted responses; Y1 is the extraction yield of Graviola fruit; Y2 is the IC50 of IL-MAE on MCF7 cell; Y3 is the IC50 of IL-MAE on HT29 cell; whereas A, B and C are the coded values for extraction time, irradiation power, and s/l ratio, respectively.

Based on the results of the experimental design; the yield of Graviola fruit extract ranged between 52 and 66.33%. In which the yield (%) was maximum at a low value of microwave irradiation power (B), mid-value of extraction time (A), and mid-value of s/l ratio (C). The yield was minimum at a low value of extraction time (A), and a high value of microwave irradiation power (B), and a high value of s/l ratio (C). The values of B and C have a significant effect on extraction efficiency (Table 1.6).

On the other hand, IC_{50} values of IL-GFE ranged between 8.12 and 28.3 µg/mL when MCF7 cells were treated under different IL-MAE conditions, and IC_{50} values of IL-GFE ranged from 10.79 to 39.01 µg/mL when HT29 cells were treated with different IL-MAE conditions. The IC_{50} value of IL-GFE on MCF7 cell was the lowest when the lowest value of the s/l ratio (C) and mid values of extraction time (A) and irradiation power (B) were applied; whereas IC_{50} was maximal at the highest value of s/l ratio (C) and mid values of extraction time (A) and irradiation power (B). Besides, the IC_{50} value of IL-GFE on HT29 was minimal when the highest value of irradiation power (B) and mid values of extraction time (A) and s/l ratio (C); whereas the IC50 value was maximum when treated on HT29 at the highest values of extraction time (A) and s/l ratio (C), and lowest value of irradiation power (B).

1.6 Discussion

1.6.1 Analysis of Variance

The F-test and p-value evaluated the statistical significance of the regression model. ANOVA for the fitted polynomial models of GFE yield is presented in Table 4.2, whereas the IC50 for MCF7 and HT29 are presented, respectively in Tables 1.9 and 1.10. The Quadratic model was applied in this case because of its high coefficient of determination (R^2) and its ability to enhance model terms.

1.6.2 Analysis of Variance of the Extraction Yield

The level of significance and adequacy of the generated model was assessed by ANOVA, considering the *p*-value. Generally, the model terms with *P*-value less than 0.05 are significant. The *p*-value < 0.0001 and *F*-value of 52.4 indicate that the model term is highly significant (Table 1.7).

Besides, the *p*-value is used to check the significance of the individual coefficient and their corresponding interactions between the variables. Additionally, the response showed that the linear coefficients of power (B) and s/l ratio (C), interaction terms—AC (contact time and s/l ratio), BC (contact power and s/l ratio), ABC (contact time, power and s/l ratio)—and the quadratic coefficients—A2 and C2—were also significant ($p < 0.05$) and considerably affected the extraction yield. The very small *p*-values ($p < 0.05$) indicated that the extraction power and s/l ratio were correlated with the extraction yield. The adequate precision of the model can be verified using the lack of fit. The result of ANOVA showed that LOF is not significant ($P > 0.05$) with a *p*-value of 1.00 relative to the pure error of extraction yield.

Table 1.7 ANOVA for yield (%) fitted quadratic model of extraction conditions

Source	Sum of squares	Degree of freedom	Mean squares	*F*-value	*P*-value
Model	658.28	9	73.14	52.4	<0.0001*
A-extraction time	0.17	1	0.17	0.12	0.7325
B-iradiation power	187.04	1	187.04	134	<0.0001**
C-solide-liquid ratio	54	1	54	38.69	<0.0001**
AB	3.06	1	3.06	2.19	0.1506
AC	27.56	1	27.56	19.75	0.0001**
BC	11.67	1	11.67	8.36	0.0076
A^2	11.11	1	11.11	7.96	0.009
C^2	81	1	81	58.03	<0.0001**
ABC	17.5	1	17.5	12.54	0.0015*
Residual	36.29	26	1.4	–	–
Lack of fit	0.75	1	0.75	0.53	0.4744NS
Pure error	35.54	25	1.42	–	–
Cor total	694.58	35	–	–	–

* $P < 0.05$ means the model terms are significant
** $P < 0.01$ means the model terms are highly significant
NS: Not significant

Table 1.8 The analysis of the model fitting

Elements	Values	Elements	Values
Standard deviation (SD)	1.02	R^2 (R-Squared)	0.9617
Mean	58.50	Adjusted R^2	0.9485
C.V.	1.74	Predicted R^2	0.9244
PRESS	52.96	Adeq precision	26.770

1.6.3 Test for Significance of the Regression

Following the response surface regression processes, R^2 was calculated to confirm the significance of the model and to compare the actual and adjusted R^2. The closer the actual R^2 to the adjusted R^2, the more significant the model terms are. The actual and adjusted R^2 are shown in Table 1.7. If R^2 is closer to 1, the correlation between the observed and the predicted values are better. The higher values of R^2 (0.96) and the closest to the adjusted R^2 (0.948) for extraction yield also indicated the efficacy of the model, which suggests that the model equation could account for 96.17 and 94.85% variation. Thus, when the R^2 value is closer to 1, the model is better fitted (Table 1.8).

Adequate precision compares the range of the predicted values with the average prediction errors at the design points. Additionally, the signal-to-noise ratio was measured in which values of more than 4 are considered desirable (Ma et al., 2011). Adequate precision of 26.77 for extraction yield indicates an appropriate signal and the generated model can be used to navigate the design space. The coefficient of variation (CV) is the ratio of the standard error and the lower the CV value the reliability of the experiment achieved. The model is commonly considered to be reasonably reproducible if its CV is less than 10% (Ma et al., 2011). A low CV value of 1.74 for extraction yield suggested an excellent precision and reliability of the experiment.

1.6.4 The Interaction Response Effects

The three-dimensional (3D) response surface plots demonstrated the interaction effects of the independent variables on the dependent one. Also, surface graphs were used to select the optimum conditions to maximize the response. Figure 1.2 represents the interactions between the experimental levels of two tested variables and their impact on the response while the third variable was kept constant at zero. Various shapes of the contour plots indicate various interactions between different variables. Elliptical contours appeared when there was a typical interaction between variables (Zhang et al., 2011).

Figure 1.2a illustrates the impact of extraction time and irradiation power and their interaction on the extraction yield when fixing the s/l ratio at 1:30. The yield

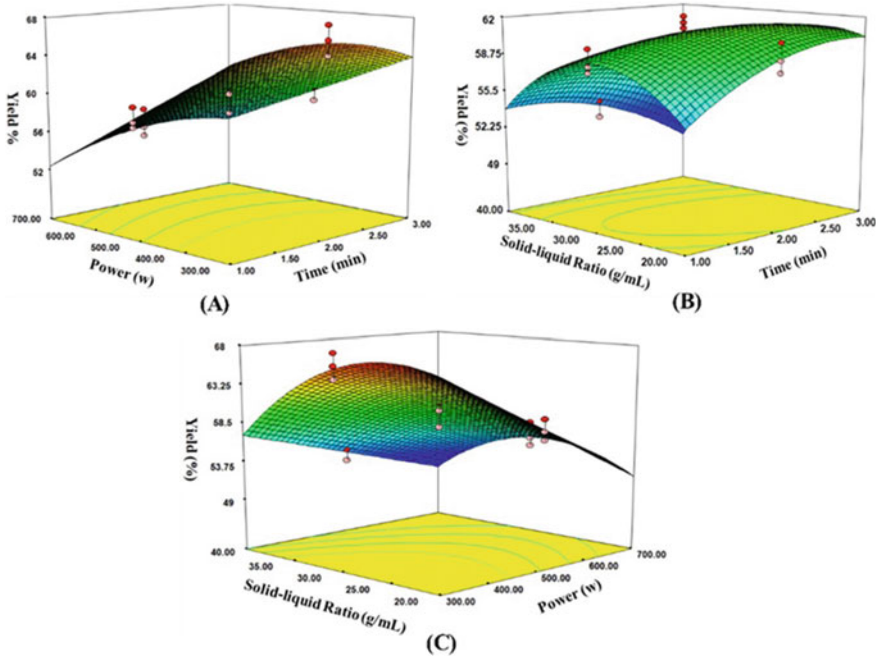

Fig. 1.2 3D response surface plots show the extraction parameters effect of IL-MAE on the yield of Graviola fruit. **a** The effect of extraction time vs. irradiation power on the yield; **b** The effect of extraction time vs. solid–liquid ratio on the yield; **c** The effect of irradiation power vs solid–liquid ratio on the yield

of Graviola fruit extract increased with the increase of extraction time from 1 to 2 min and then decreased. Microwave irradiation takes a certain amount of time to cause cell wall disruption and release the target component. However, the prolonged heating time led to a decrease in extraction yield because of thermal degradation and polymerization of the Graviola fruit sample (Guo et al., 2017). On the other hand, the increase in irradiation power from 300 to 700 W decreased the extraction yield. The irradiation power influences interactions and equilibrium rates and controls the analyte partitions between the sample and extraction phase (Ma et al., 2010). The elliptical contour shape of the response surface curve suggests a slight interaction between the two variables.

Figure 1.2b showed the response surface graph of extraction time and s/l ratio and their interactions on the extraction yield when fixing the power at 500 W. The yield of Graviola fruit increased with an increase in extraction time from 1 to 2 min and s/l ratio from 1:20 to 1:25 and then the yield decreased. Smaller solvent volumes would make the target extraction incomplete, while a large quantity of solvent could make the extraction process more complicated in addition to unnecessary waste (Ma et al., 2010). The 3D response surface plots show an elliptical contour shape indicating well defined operating conditions and a significant interaction effect between the two variables.

Figure 1.2c illustrated the response surface graph of the effect of irradiation power and s/l ratio and their interactions on the extraction yield while fixing the extraction time at 2 min. The yield of Graviola fruit extract increased with an increase in s/l ratio from 1:20 to 1:25 and then decreased. However, the yield decreased when the irradiation power increased from 300 to 700 W. The response plot showed an imperfect elliptical contour shape and the interaction between the two variables could be improved if the power was reduced.

The model equation was used to predict the optimum extraction conditions to maximize the yield of Graviola fruit extract. The optimum conditions suggested by the model for achieving an elevated yield are as follows: 1.74 min extraction time, 300 W irradiation power, and 1:25 s/l ratio to achieve 67.6% extraction yield. Compared to other studies, the extraction yield obtained in this study using IL-MAE was higher than that obtained in earlier research using conventional extraction methods (Bonneau et al., 2017; Melot et al., 2009). The results suggest that the MAE mechanism was based on a cell-wall explosion, which was investigated by Paré and Belanger (1993).

1.6.5 Validation of the Model

In order to validate the developed model and verify the optimum results, another set of extractions was carried out. Due to the limitation of the instruments, the chosen optimum parameters were 2 min extraction time instead of 1.74 min, 300 W irradiation power, and 1:25 s/l ratio (g/mL). The results of validation experiments produced an average yield of 66.6 ± 2.52%. These values are close to the predicted value of 67.64% and indicate the adequacy of the optimization model.

1.6.6 Analysis of Variance of the IC_{50}

The F-test and p-value evaluated the statistical significance of the regression model. The ANOVA for the fitted polynomial models of the IC_{50} of IL-GFE on MCF7 and HT29, respectively, are shown in Tables 1.9 and 1.10. The polynomial model was chosen because of its high R^2 and its ability to improve the model terms.

The adequacy and level of significance of the generated model were assessed through ANOVA by considering the p-value. Model F-value of 128.33 and p-value of < 0.0001 for MCF7, and F-value of 35.43 and p-value < 0.0001 for HT29 indicate that the model is highly significant (Tables 1.9 and 1.10). The p-value is used to check for the significance of the individual coefficient and their corresponding interactions between the variables. Additionally, the response revealed that linear coefficients, all interaction terms, and quadratic coefficients were significant ($p < 0.05$) and had

Table 1.9 ANOVA for the IC_{50} of IL-GFE on MCF7 cells fitted quadratic model of extraction conditions

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*-value
Model	1566.09	10	156.61	128.33	<0.0001**
A-TIME	127.05	1	127.05	104.11	<0.0001**
B-POWER	92.12	1	92.12	75.49	<0.0001**
C-RATIO	611.05	1	611.05	500.72	<0.0001**
AB	262.39	1	262.39	215.01	<0.0001**
AC	8.25	1	8.25	6.76	0.0154*
BC	88.89	1	88.89	72.84	<0.0001**
A^2	236.74	1	236.74	194.00	<0.0001**
B^2	218.28	1	218.28	178.87	<0.0001**
C^2	239.95	1	239.95	196.63	<0.0001**
ABC	156.78	1	156.78	128.47	<0.0001**
Pure Error	30.51	25	1.22	–	–
Cor Total	1596.60	35	–	–	–

*P<0.05 means the model terms are significant
**P<0.01 means the model terms are highly significant

Table 1.10 ANOVA for the IC_{50} of IL-GFE on HT29 cells fitted quadratic model of extraction conditions

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*-value
Model	2666.16	9	296.24	35.43	<0.0001**
A-TIME	466.40	1	466.40	55.78	<0.0001**
B-POWER	435.03	1	435.03	52.03	<0.0001**
C-RATIO	0.43	1	0.43	0.051	0.8230
AB	364.36	1	364.36	43.58	<0.0001**
AC	130.91	1	130.91	15.66	0.0005**
BC	701.28	1	701.28	83.88	<0.0001**
A^2	8.94	1	8.94	1.07	0.3106
C^2	183.06	1	183.06	21.90	<0.0001**
ABC	140.31	1	140.31	16.78	0.0004**
Residual	217.38	26	8.36	–	–
Lack of fit	9.01	1	9.01	1.08	0.3083
Pure error	208.37	25	8.33	–	–
Cor total	2883.54	35	–	–	–

*P<0.05 means the model terms are significant
**P<0.01 means the model terms are highly significant

Table 1.11 The analysis of the model fitting

Statistical analysis	MCF7	HT29
R-Squared	0.98	0.92
Adjusted *R*-Squared	0.97	0.90
Coefficient of variation (CV)	5.93	14.71
Adequate Precision	33.05	18.75

remarkable effects on the IC_{50} of MCF7 cell lines. Also, the response revealed that the linear coefficients A (time) and B (power), all interaction terms, and the quadratic coefficient C (s/l ratio) were significant ($p < 0.05$) and had remarkable effects on the IC_{50} of HT29 cell lines. The very small *p*-values ($p < 0.05$) indicated that the extraction time, power, and s/l ratio were significantly correlated with the IC_{50} of MCF7 cell lines. The result of ANOVA for HT29 showed that the lack of fit is not significant ($p > 0.05$) with the *p*-value of 0.3083, which is relative to the pure error for the IC_{50} of the cell.

1.6.7 Test for Significance of the Regression

Following the response surface regression processes, R^2 was calculated to reaffirm the significance of the model and to compare the actual and adjusted R^2. The actual and adjusted R^2 are shown in Table 1.11. The higher values of R^2 (0.98) nearest to the adjusted R^2 (0.97) for IC_{50} of MCF7 and the value of R^2 (0.92) nearest to the adjusted R^2 (0.90) for IC_{50} of HT29 also indicated the efficacy of the model, suggesting that the model equation could account for 98.09 and 97.32% variation for MCF7 and 92.46 and 90.85% variation for HT29. Thus, when the R^2 value is closer to 1.0, the model is better fitted.

Adequate precision compares the range of the predicted values with the average prediction errors at the design points. Additionally, it measures the signal-to-noise ratio where values greater than four are considered optimal. Adequate precision of 33.05 for MCF7 and 18.75 for HT29 indicates an adequate signal and the generated model can be used to navigate the design space. CV is the ratio of the standard error and the lower the CV value, the higher the reliability of the experiment achieved. The model is commonly considered to be reasonably reproducible if its CV is less than 10% (Zhang et al., 2014). A relatively low CV value of 5.93 for MCF7 and 14.71 for HT29 suggested a good precision and reliability of the experiment.

1.6.8 The Interaction Response Effects

The 3D response surface plots were drawn to demonstrate the interaction effects of the independent variables on the dependent variable. Also, surface graphs are used

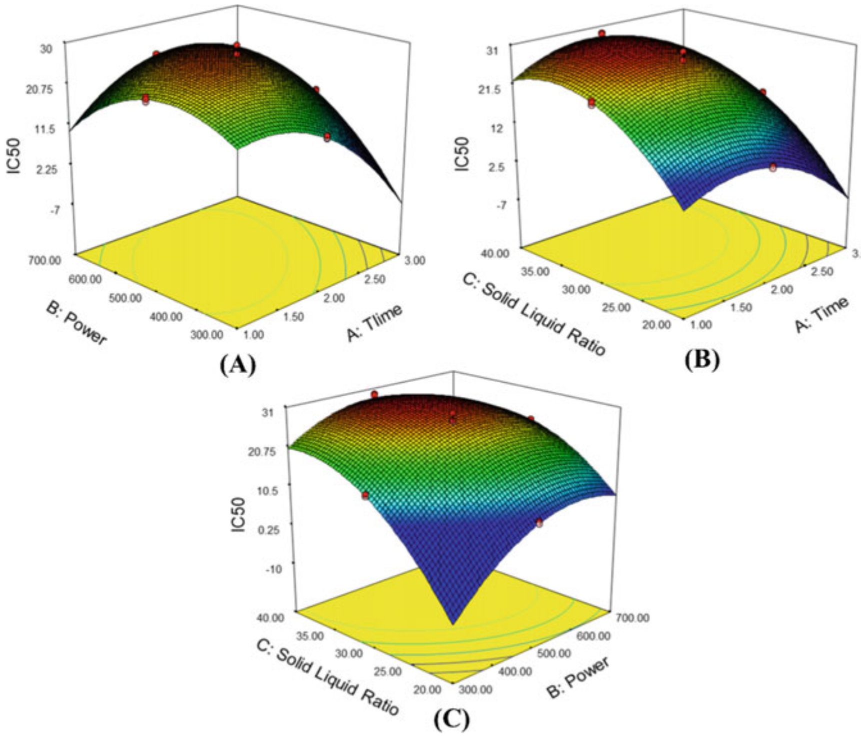

Fig. 1.3 3D response surface plots show the extraction parameters (IL-MAE) on the IC_{50} of MCF7 cell line. **a** The effect of extraction time vs irradiation power on the IC_{50} of MCF7; **b** The effect of extraction time vs solid–liquid ratio on the IC_{50} of MCF7; **c** The effect of irradiation power vs solid–liquid ratio on the IC_{50} of MCF7

to select the optimum conditions to minimize the response (IC_{50}). Figures 1.3 and 1.4 represent the interactions between the experimental levels of the two measured variables and their effect on the response while the third variable was kept constant at zero. Elliptical contours were obtained when there was a perfect interaction between the independent variable (Zhang et al., 2011).

Figures 1.3a and 1.4a illustrated the effect of extraction time and irradiation power and their interaction on IC_{50} while the s/l ratio (1:30) remained constant. The IC_{50} of IL-GFE on MCF7 cells decreased with the increase of time from 1 to 3 min and increased with the increase of power from 300 to 500 W then decreased. On the other hand, the IC_{50} of IL-GFE on HT29 cells decreased with an increase of extraction time from 1 to 3 min and an increase of irradiation power from 300 to 700 W. The elliptical contour shape of the response surface curve showed a slight interaction between these variables.

Figures 1.3b and 1.4b showed the response surface graph of extraction time and s/l ratio and their interactions with the IC_{50} when power was kept constant at 500 W. The IC_{50} of IL-GFE on MCF7 cells increased with an increase of time from 1 to

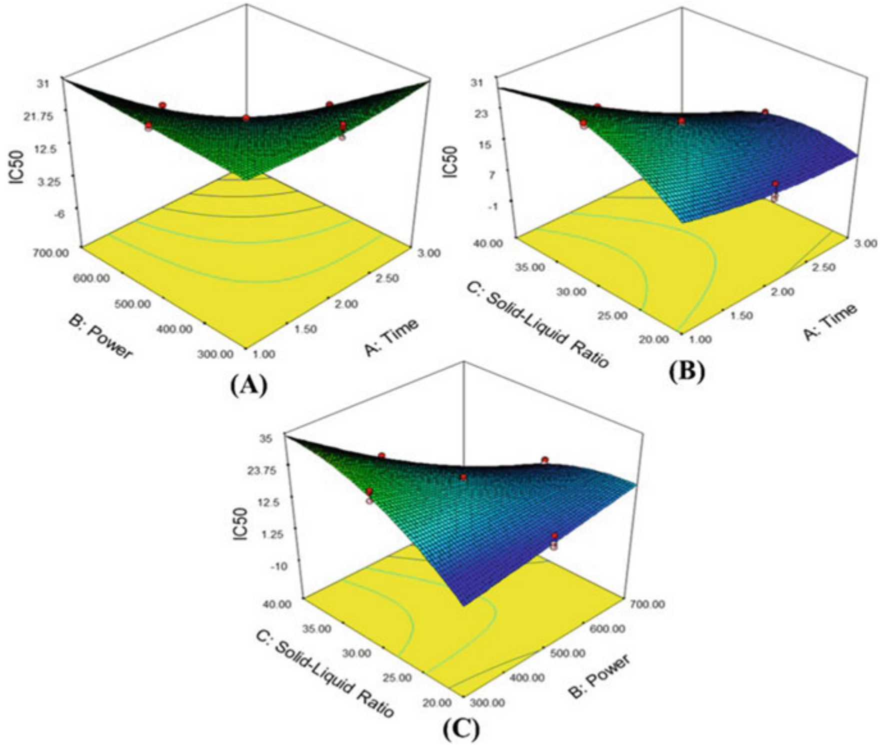

Fig. 1.4 3D response surface plots show the extraction parameters (IL-MAE) on the IC_{50} of the HT29 cell line. **a** The effect of extraction time vs irradiation power on the IC_{50} of HT29; **b** The effect of extraction time vs solid–liquid ratio on the IC_{50} of HT29; **c** The effect of irradiation power vs solid–liquid ratio on the IC_{50} of HT29

2 min and the s/l ratio increased from 1:20 to 1:35, then IC_{50} reduced. On the other hand, the IC_{50} of IL-GFE on HT29 cells increased with an increase of time (1–2 min) and s/l ratio (1:20–1:30), then IC_{50} reduced. The 3D response surface plots of MCF7 cells showed an elliptical contour shape indicating well defined operating conditions and a significant interaction effect between the two variables.

Figures 1.3c and 1.4c illustrated the response surface graph of the effect of irradiation power and s/l ratio and their interactions on the IC_{50} when extraction time was kept constant at 2 min. The IC_{50} of IL-GFE on MCF7 and HT29 increased with the increase of power (300–500 W) and s/l ratio (1:20–1:30), and then IC_{50} decreased. The response plot of HT29 showed an imperfect elliptical contour shape and the interaction between the two variables can be improved if the power is reduced.

The model equation was used to predict optimum extraction conditions. The optimum conditions suggested by the model to obtain the lowest IC_{50} are as follows: extraction time of 2.98 min, an irradiation power of 690 W and s/l ratio of 1:39; IC_{50} of 3.41 μg/mL for MCF7 and 6.61 μg/mL for HT29 was achieved under optimum

Table 1.12 Results from the validation of the model

	Extraction time (min)	Irradiation power (W)	Solid-liquid ratio (g/ml)	MCF7 IC_{50} (μg/mL)	HT29 IC_{50} (μg/mL)
Predicted	2.98	690	(1:39)	3.41	6.61
Actual	3	700	(1:39)	4.75 ± 0.36	10.56 ± 2.04

conditions. These results confirm the previous study on the anti-proliferative activity of isolated compounds from Graviola fruit against human prostate cancer PC-3 cells (Sun et al., 2016).

1.6.9 Validation of the Model

To validate the developed model and verify the optimum results, other sets of cytotoxicity assays were carried out. Due to the instrumental limitation, the optimum extraction conditions were set to the following: extraction time of 3 min instead of 2.98 min, irradiation power of 700 W instead of 690 W, and s/l ratio of 1:39. The results from validation experiments yielded an average IC_{50} of 4.75 ± 0.36 μg/mL for MCF7 and 10.56 ± 2.04 μg/mL for HT29. These values are close to the predicted value indicating the adequacy of the optimization model (Table 1.12).

1.7 Conclusion

The IL-MAE technique presented was effective in extracting plant fruit as described in the case study.

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