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Original Research Article

An Experimental Design Approach for the Optimization of Hesperidin Extraction from Orangette

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Abstract: The aim of this work is to study the development of a response surface methodology for the optimization of hesperidin extraction from orangette composition. The optimization of extraction parameters such as concentration, pH, temperature and extraction time were performed by the centered composite design (CCD) method. The analysis of variance showed a good fit of the model and the performance of the RSM method to improve hesperidin extraction, due to the fact that R2=0.927, R2Adjusted=0.825, R2Pred=0.602 and P<0.05. The optimal conditions determined for the extraction of hesperidin by the response surface methodology were 130 mg/ml for the solvent concentration, pH (12.67), temperature (62°C) and extraction time 3.45h for the extraction time with a theoretical yield is 67.44%. These results showed that the developed model is satisfactory and relevant for hesperidin extraction.

Keywords: Extraction, Optimization, Centered Composite Design, Orangette.

I. INTRODUCTION

Citrus fruits are an important source of vitamin C and polyphenol compounds [1]. These principal phenolic compounds include hydroxycinnamic acids (HCA) and the flavonoids, among which the flavanones are the most widespread [2].

Citrus flavanones, especially hesperidin, are known to have a wide range of therapeutic properties, it is an antioxidant that enhances the action of vitamin C to lower cholesterol levels. It is also known to have a pharmacological action as an anti-inflammatory agent, antihistamine, hypertensive, diuretic and antiviral[3–5]. Hesperidin also improves capillary health by reducing capillary permeability so that it can treat leg ulcers caused by poor blood circulation, in combination with diosmin. The possible anticancer activity of hesperidin could be explained by the inhibition of polyamine synthesis [6, 7].

Hesperidin was already a well-known bioflavonoid. Discovered in 1828 by LÊBRETON, in the crystalline state isolated from orange peels, it is part of various fruits of the hesperidia family. The structure of hesperidin is well known. It has a gross formula (C28H34O15).

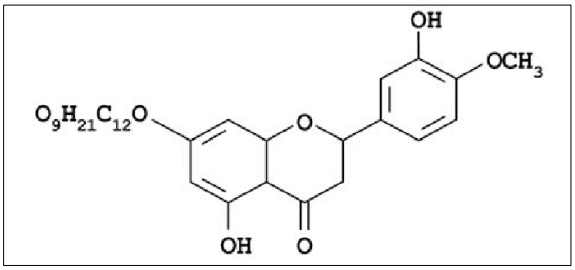

Figure 1: Chemical structure of hesperidin [8]

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The extraction of Hesperidin, from plant material, is a very important step in the isolation and identification of this compound. The crude, natural extract obtained are currently of great interest, and may be a promising source of new drugs [9].

One of the main problems of hesperidin extraction is the low solubility of hesperidin in water, which can limit the efficiency of the extraction process [9]. Hesperidin is a flavonoid compound that is found in various citrus fruits, and it is often extracted from the dried peel of citrus fruits. To overcome the low solubility problem, various solvents can be used for extraction, such as methanol, ethanol, and acetone [10]. However, the choice of solvent can also affect the selectivity and yield of the extraction process, as well as the potential toxicity of the extract. Other challenges that can affect hesperidin extraction include the complexity of the matrix (i.e., the plant material), the variability in the content of hesperidin among different citrus fruit varieties and geographic locations, and the potential degradation of hesperidin during the extraction process or storage [11].

Therefore, the improvement of the extraction efficiency becomes of major importance. Thus, the use of statistical techniques such as experimental design makes this improvement more and more possible. These methods, which allow obtaining a maximum of information from a minimum of experiments [12], consist in selecting and ordering the tests in order to identify the effects of the parameters involved on the response of the product. With the aim of a certain generalization of the results by means of an interpolation in the field of study in order to arrive at empirical laws [13].

Several authors have discussed the use of experimental designs in the optimization of hesperidin extraction conditions. Some have used other types of designs such as full factorial designs, and others have moved directly to optimization using surface response designs [14–16].

This study aims to set factors considered influential on the response studied. Thus, we were able to optimize these factors considered to influence the extraction of hesperidin by using the experimental designs of the response surface type, which are best recommended for optimizing the operational variables.

II. MATERIALS AND METHOD

- **Vegetable Material**

The orange peel, were harvested from the region of sidi yahia el gharb- Morocco, then dried and sorted according to their diameter measured with a caliper. For each diameter, an average of their weight was calculated. The samples were then finely crushed and sieved using a sieve with a whole diameter of 800µm. The final powder of each diameter was carefully stored in a dry place for its extraction.

- **Extraction of Hesperidin**

The working method was based on the solvent extraction of Hesperidin: which is the most used method for the extraction of phenolic compounds.

To extract Hesperidin from orange peel by solvent, we opted for the protocol described by Ruiz-Palomino *et al*., [15], with some modifications and applied to different diameters of orange peel: 10 g of orange peel powder of each diameter were macerated separately at room temperature with 25 ml of methanol and 4g of KOH (pH= 11) during 12 h. After complete maceration the mixture was filtered by large Buchner funnel. HCL acid was then added to the filtrate to change the pH to 3, and the solution was heated to 40 C for 50 min and left at room temperature for 24 h. The resulting hesperidin crystals are separated by filtration, rinsed with distilled water twice and oven dried for 30-1 h at 60 °C and weighed.

The extraction was repeated 3 times for each diameter. The results of these extractions were expressed as mean and standard deviation. Figure 1 represents the sequence of the phases of the hesperidin extraction processes.

1. Dry matter
2. Methanol & KOH
3. Alkalination of the medium
4. Extraction (12 hours)
5. Filtration
6. Acidification
7. Crystallization
8. Purification
9. Separation of crystals
10. Drying
11. Weighing

Figure 2: Hesperidin extraction protocol

- **Product Characterization and Identification**

Hesperidin is controlled according to the European pharmacopoeia monographs.

Table 1

Tests	Method	Specifications
Appearance and color		Amorphous powder, grey-beige to yellow ochre
Solubility	Eur. Ph. General notice	slightly soluble in water and in methanol
Identification by IR	Eur. Ph. 2.2.24	Conforms to the reference spectrum
Identification by HPLC	Eur. Ph. 2.2.29	Corresponds to the reference chromatogram.

- **Solubility Test**

The solubility of hesperidin was tested in water and in methanol according to the European Pharmacopoeia.

The solubility of hesperidin was also studied at different pH in a mixture of MeOH and KOH charge added gradually to reach the desired pH values (pH=6, pH=8, pH=10, pH=12 and pH=14). 5-10 mg of the extract was placed in test tubes and 5 ml of the mixture (MeOH/KOH) was added to each sample. Saturation was achieved in all solutions indicated by the appearance of a precipitate. The solutions were centrifuged, then 1 ml of the supernatants from each tube was diluted with 25 ml of the solvent, mixed by vortex, and the absorbance values of these solutions were measured at (290 nm). A calibration curve of OD versus concentration was previously plotted.

- **Identification Test**

The identification of flavonoids present in the powder of orangettes was carried out in two steps: an identification by spectroscopic methods (IR and UV-Visible) and an identification by HPLC analysis.

UV spectra were obtained in MeOH solvent with a double beam UV-visible spectrophotometer (Perkin Elmer Lambda 12) using methanol as blank.

IR spectra were obtained with a jasco 460 plus spectrophotometer. FTIR spectra of the compounds were recorded on a Perkin Elmer Spectrum FTIR system using potassium bromide pellets.

The analysis was performed at the Dioma: Drug Control Laboratory. The chromatographic conditions used to identify hespiridine were:

HPLC analysis for hesperidin was performed on an Agilent Infinity Lab LC Series 1260 Infinity II, equipped with a degasser, a binary pump, a column oven, and a Diode Array Detector (DAD) detector. Chromatographic separation was achieved using a Nucleosil C18 (100 mm × 4.6 mm i.d. x 3 µm particle size, porosity: 120 Ă) column. An isocratic mode elution consists of a methanol/purified water mixture (37/75), adjusted to pH 3.2 with phosphoric acid 1% and filtered through a 0.45-µm filter. Column temperature and flow rate were maintained at 50°C and 1.3 mL/min, respectively, while the eluent was monitored at 284nm of UV detection wavelength in 25min. The injection volume was 10 µL for each sample. Data acquisition and integration were carried out with Agilent OpenLAB CDS Workstation.

The peak for hesperidin in the sample was confirmed by comparing the full UV spectra absorptions and retention time (t_r) of the target peak with those of hesperidin standard. The hesperidin content was quantified by interpolating the peak area into calibration curve.

Reference Solution: Dissolve around 45 mg hesperidin reference substance exactly weighed in dimethylsulfoxide R and make up to 25 ml with the same solvent.

Test Solution: Dissolve around 45 mg hesperidin exactly weighed in dimethylsulfoxide R and make up to 25 ml with the same solvent.

- **Optimization of Hesperidin Extraction Using Response Surface Methodology**

In this study, an RSM experimental design was created to outline the relationships between the variables and concentration and simultaneously evaluate the optimal level of the variables: solvent concentration (X1), pH (X2), extraction time (X3), and temperature (X4), in regard to hesperidin concentration response. We opted for the response surface method (RSM) based on a centeral composite design with four variables and five levels. The number of experiments conducted was given by the following formula: $2^k + 2k + N0$, k being the number of variables. Thus, the total number is $16 + 2\times4 + 1 = 25$ experiments to be performed in order to optimize the responses and estimate the mathematical model (table 3). A DCC can be depicted as a cube, for which each axis represents a variable. The coded and actual values of each independent variable in the RSM design are listed in Table 2.

The optimization study was conducted on the factors found to be influential by the screening study conducted by *Lefebvre et al.,* [11]. Thus, we have the factors and their variation ranges as follows: The concentration of the organic solvent (methanol) between 20% and 100% with a variation step equal to 40. The choice of this experimental range is based on data from the literature [11]. The heating temperature in the oven was between 25°C and 75°C, with a variation step equal

to 25°C. The pH is between 10.5 and 14 with a variation step equal to 1.75. As the extraction is not instantaneous, many publications describe a progressive loading of the base to avoid excessive initial pH. The extraction time is between 30 minutes and 6.5 hours. The chosen extraction time was related to literature data [18]. The response studied is the yield of hesperidin which is translated by the formula given by Falleh *et al*., [17]:

$$R(\%) = \frac{Mext}{Mech} \times 100$$

Where: R is the yield in %; Mext is the mass of the extract after evaporation of the solvent in mg and Mech is the dry mass of the plant sample in mg.

Table 1: Coded and actual levels of independent variables for hesperidin extraction

Level	Code	Value			
		X1: Solvent concentration(mg/ml)	X2: pH	X3: Extraction time (h)	X4: Temperature (°C)
Low	-1	20	10.5	0.5	25
Center	0	60	12.25	3.5	50
High	+1	100	14	6.5	75

Table 3: Structure of design matrix and extraction yield of hesperidin as a response for each experiment

Run number	X1	X2	X3	X4	Yield (%)
1	-1	-1	-1	-1	0.01
2	1	-1	-1	-1	0.02
3	-1	1	-1	-1	0.05
4	1	1	-1	-1	41.20
5	-1	-1	1	-1	29.15
6	1	-1	1	-1	0.90
7	-1	1	1	-1	0.05
8	1	1	1	-1	21.24
9	-1	-1	-1	1	4.50
10	1	-1	-1	1	0.10
11	-1	1	-1	1	0.02
12	1	1	-1	1	27.50
13	-1	-1	1	1	68.80
14	1	-1	1	1	58.70
15	-1	1	1	1	0.20
16	1	1	1	1	37.80
17	-1	0	0	0	38.50
18	1	0	0	0	60.05
19	0	-1	0	0	22.08
20	0	1	0	0	21.00
21	0	0	-1	0	19.00
22	0	0	1	0	52.00
23	0	0	0	-1	42.00
24	0	0	0	1	59.00
25	0	0	0	0	68.90

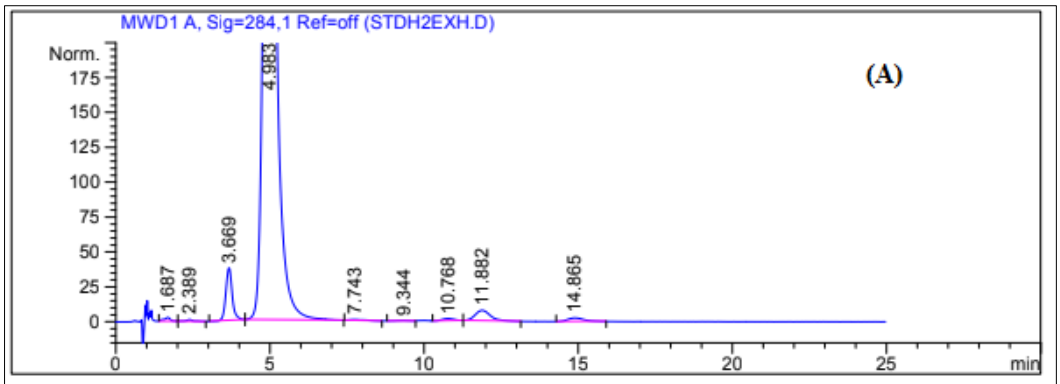
A polynomial model constructed from data analysis by multiple regressions with the least-square method was performed to represent the response as a function of independent variables.

Data Analysis

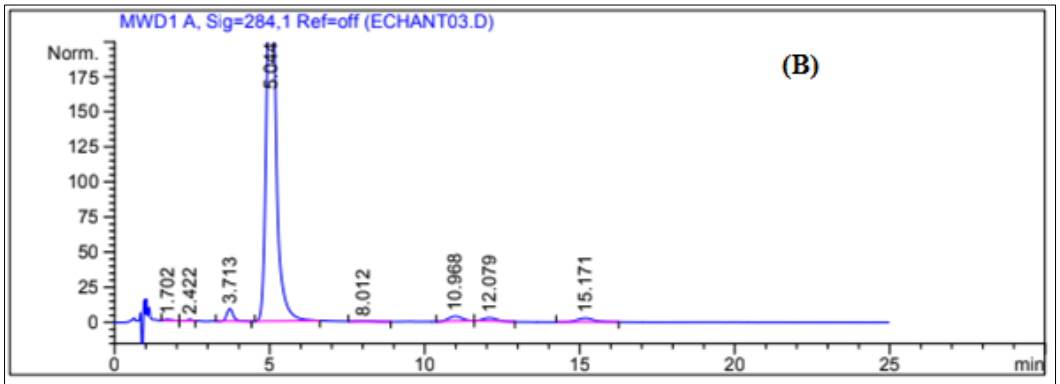
Nemrowd software was used to generate experimental design, statistical data analysis (regression analysis and analysis of variance (ANOVA)), and surface response model visualization.

III. RESULTS AND DISCUSSION

To develop hesperidin as a functional product ingredient, efficient production of hesperidin is required. Hesperidin is known to be a major compound in orangets. HPLC analysis has clearly shown the presence of hesperidin in the total extract of C. citrus peels (Fig. 2).



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Area %	Name
1	1.687	BB	0.2006	39.35528	0.1648	?
2	2.389	BBA	0.2111	18.39730	0.0771	?
3	3.669	PB	0.2189	536.98718	2.2491	ISONARINGINE
4	4.983	BBA	0.3028	2.28683e4	95.7813	HESPERIDINE
5	7.743	BBA	0.4435	21.74404	0.0911	?
6	9.344	BB	0.3230	7.24111	0.0303	?
7	10.768	BB	0.3531	37.96936	0.1590	?
8	11.882	BBA	0.5064	254.00279	1.0639	NEOPONCERINE
9	14.865	BBA	0.5118	91.53379	0.3834	HESPERITINE



Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Area %	Name
1	1.702	BB	0.2011	12.33262	0.1822	?
2	2.422	BB	0.1832	13.49485	0.1994	?
3	3.713	BBA	0.2358	134.28658	1.9841	ISONARINGINE
4	5.044	BBA	0.3053	6305.59814	93.1664	HESPERIDINE
5	8.012	PBA	0.4478	12.38581	0.1830	?
6	10.968	PB	0.3834	112.04886	1.6555	?
7	12.079	BBA	0.3823	73.94489	1.0925	NEOPONCERINE
8	15.171	PBA	0.4871	104.01306	1.5368	HESPERITINE

Figure 3: HPLC chromatogram of hesperidin (A) and HPLC chromatogram of orangette extract (B).

Optimization of the Extraction Process

The extraction yields of hesperidin under designed experiments are given in Table 3. As shown in Table 2, the maximum extraction yields were 68.80% and 68.90 resulted from run experiments of 13 and 25, respectively. The minimum extraction yield was obtained at run no. 1 when both temperature and time values were set at the level of -1. The lower amounts of yield are observed for the experiments in which their time, concentration of solvent or temperature was adjusted at their lowest level (run experiments 1, 2, 3, 6, 7, 9, 10 and 15). The statistical analysis of the studied factors on the extraction yield of hesperidin is presented in Table 4. The P-value can determine the significance of the coefficients and also provides insight into the mutual interactions between independent variables. Model terms having $P<0.05$ were significant.

Table 4: Estimation and statistics of coefficients

Name	Coefficient	STD	P-value %
X0	50.453	4.4206409	< 0.01 ***
X1	5.902	2.4131185	3.45 *
X2	-1.956	2.4131185	43.7
X3	9.802	2.4131185	0.228 **
X4	6.778	2.4131185	1.85 *
X1-1	1.897	6.4153547	77.4
X2-2	-25.838	6.4153547	0.241 **
X3-3	-11.878	6.4153547	9.4
X4-4	3.122	6.4153547	63.7
X1-2	10.635	2.5594987	0.196 **
X1-3	-2.737	2.5594987	31.0
X2-3	-10.150	2.5594987	0.266 **
X1-4	1.030	2.5594987	69.6
X2-4	-6.190	2.5594987	3.62 *
X3-4	7.708	2.5594987	1.31 *
Regression	-	-	0.0660 ***
R^2	0.927		
R^2_{adj}	0.825		
R^2_{pred}	0.602		

According to the results obtained from ANOVA analysis table (Table 3), the model has an F-value (9.0666) and a low p-value (<0.05), indicating the validation of the model that can be used for optimization of extraction parameters. Next, the coefficient of determination R2 was determined from the experimental values is 0.927. The R2 Adjusted and R2 Predicted values were recorded as 0.825 and 0.602, respectively. These values are close to 1. This means that the model is statistically validated [17, 18]. The purpose of this statistical test is to determine whether there are coefficients that are not influential, i.e., that have no effect on each of the responses. If there are one or more coefficients that are not influential on all responses, they can be removed from the mathematical model in order to simplify it and improve its quality (Table 4).

As shown in Table 4, tree linear terms of solvent concentration (X1, P=3.45% <5%), extraction time (X3, P=1.85% <5%), and the temperature (X1, P=0.228 % <5%). As well as, the interaction term of them X1-2(P=0.196 % <5%), X2-4 (P=0.266% <5%), X3-4 (P=1.31% <5%), and X2-3 (P=3.62% <5%) were statistically significant with 95% confidence. while tree quadratic terms of X12, X32 and X42 (P-value>0.05) were not significant.

In addition to these results, the temperature factor (X4) has shown a high influence on the yield, as well as the concentration factor (X1) and the extraction time (X3) have a positive influence on the response. On the other hand, the pH factor (X2) had no influence on the extraction yield. In general, multiple regression analysis of hesperidin determined the following second-order polynomial equation, where X1, X2 X3 and X4 are the coded values.

As can be seen in Figure 2, which represents the correlation between the predicted and experimental data, the R2 value is 0.92 demonstrating that the experimental model had a good predictive ability for Hesperidin.

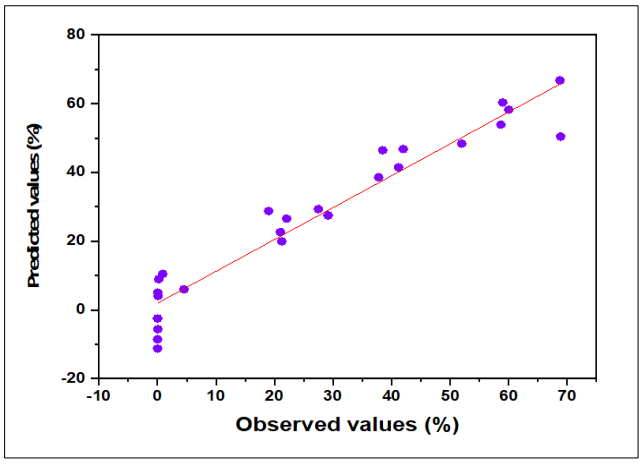


Figure 4: Quality adjustment between actual and predicted values.

Determination of Optimal Conditions and Model Validation

The aim of this work was to maximize the extraction yield of hesperidin from the range of extraction parameters. Based on the practical results obtained and statistical analysis, the optimal conditions were obtained when the concentration, pH, time and temperature were 130.223ml, 12.676, 3.458h and 62.217°C, respectively. To determine the validation of the model and to determine the optimal conditions, the actual and predicted values were compared within the 95% confidence interval (Table 5).

Table 5: Coordinates of the maximum and the predicted optimal conditions of the factors

Variable	Value	parameter	Value
X1	1.755596	Concentration	130.2238
X2	0.243799	pH	12.6766
X3	-0.013707	Temps	3.4589
X4	0.488711	Temperature	62.2178

Table 6: Characteristics of the maximum

Response	Response	Value	di %	weight	min di %	max di %
Y1	Yield	67.4472	99.99	1	0.00	99.99
	Desirability		99.99		0.00	99.99

In addition, the desirability functions under the optimal conditions were calculated to be 0.99 (Table 6). These results confirm that mathematical models with good correlation could be used for optimization of hesperidin extraction.

3D graphs were used to investigate the effect of each factor on the response, i.e., the amount of hesperidin extraction. Extraction time, concentration, temperature and pH have a significant effect on the yield of hesperidin compounds as explained in each graph. Each graph is representative of the effect of two independent factors on the response of the dependent variable and its maximum response.

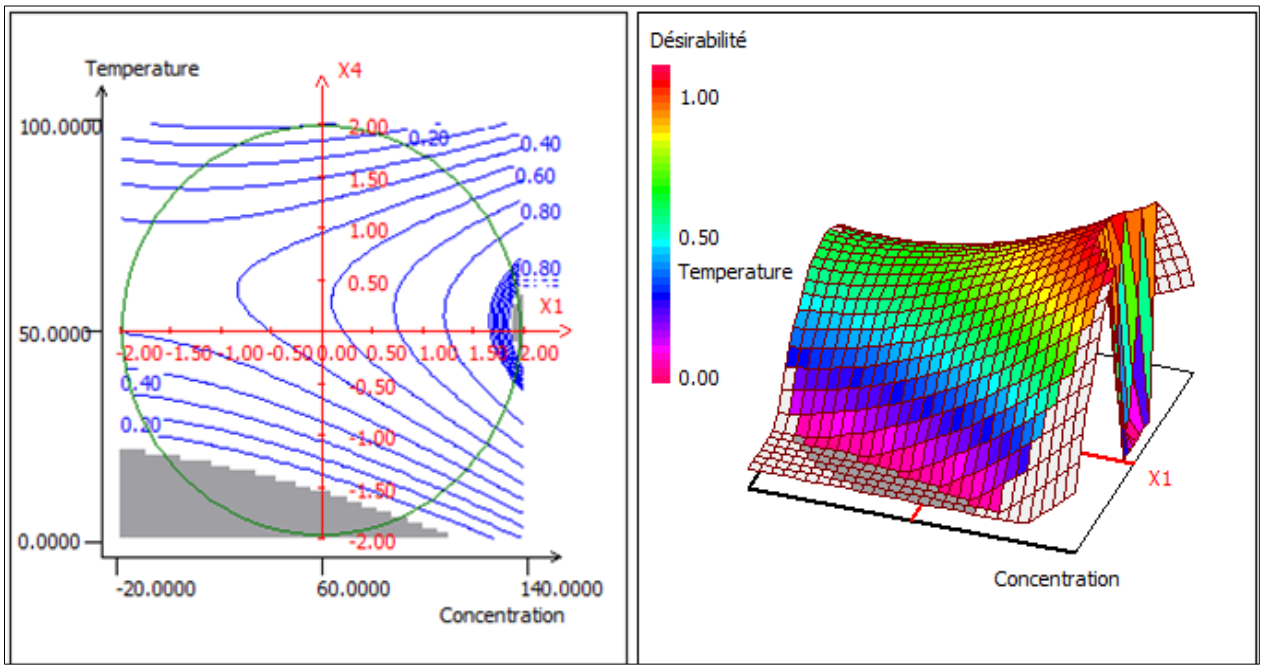


Figure 5: Desirability variation in the design: Solvent concentration, temperature (fixed factors: pH = 12.6766 - Time = 3.4589 h)

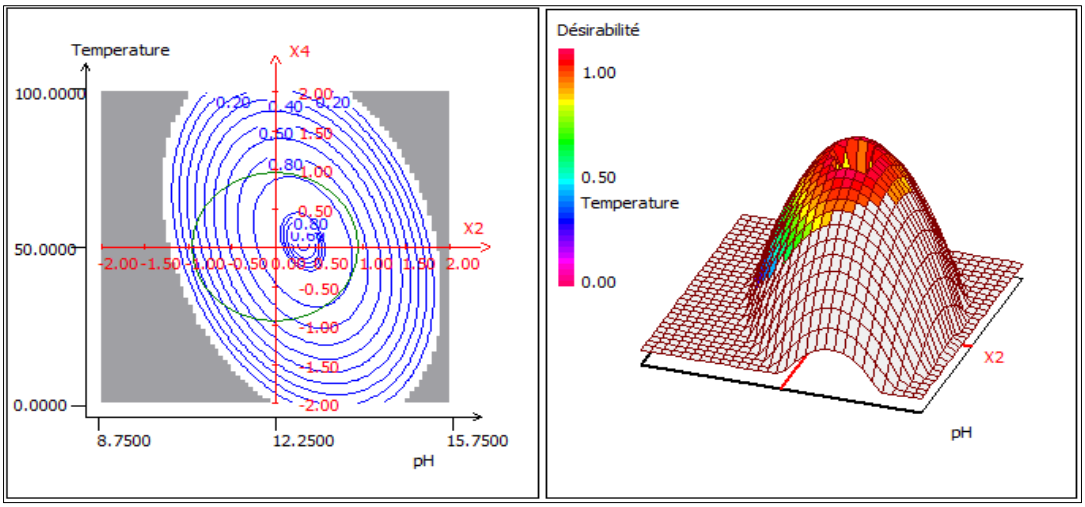


Figure 6: Variation of desirability in the design: pH, temperature (fixed factors solvent concentration = 130.2238 ml - Time = 3.4589 h)

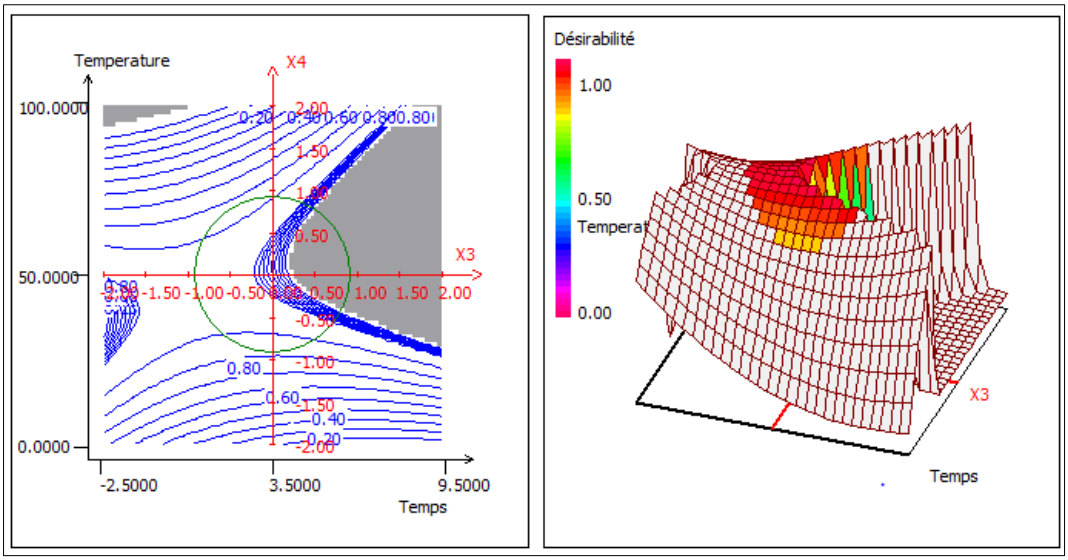


Figure 7: Variation of the desirability in the design: time, temperature (fixed factors: solvent concentration = 130.2238 ml - pH = 12.6766)

The overall analysis of the results obtained from the response surfaces and effect plots show that the highest extraction yield was found at high temperature 62°C with the increase in the concentration of solvent used. The extraction time (3.45min) had a significant factor in the extraction of hesperidin from the orange peel. On the other hand, the pH factor had a slight effect on the extraction yield, moreover, the interaction between extraction time and extraction temperature produced a significant effect. On the other hand, the interaction between (pH-time) as well as (pH-temperature) produced a negative effect.

It is very interesting to note that the optimal extraction conditions have been described by several authors in the literature based on the experimental designs concerning hesperidin. As an example Nipornrama *et al*., [19], found the yield extracted amounts of 64.4 and 61.5 mg/g from the skin of Citrus reticulata Blanco by the WATER and MEM processes, respectively, using acetone-water (80:20 v/v) and 40 min as extraction time.

Then, another author Esther Gómez-Mejía, *et al*., [20], were extracted citrus peel waste based on microwave-assisted extraction (MAE). The optimized conditions using RSM were 15min extraction time, 40-60 (v/v) EtOH-H2O and 90°C extraction temperature. And finally, author Tsuyoshi Inoue *et al*., 2010 were extracted hesperidin from thinned Citrus unshiu fruit peels by microwave-assisted extraction [21]. The yield of hesperidin reached 58.6 mg/g, with the ratio of DMSO: methanol (1: 1, v/v), the temperature was 140°C and the extraction time was 8 min using the surface response methodology. Under these optimal conditions, 86.8% (47.7 mg / g) of hesperidin.

The hesperidin extraction using experimental design will depend on the specific experimental design used and the results obtained. However, generally

speaking, an experimental design can help to systematically evaluate different factors that could affect the extraction of hesperidin from plant materials, such as the type of solvent, extraction time, temperature, and solvent-to-solid ratio. Based on the results obtained, it may be possible to identify the optimal conditions for the extraction of hesperidin, which can help to improve the efficiency and yield of the extraction process. Additionally, experimental design can provide insights into the interactions between different factors and their impact on the extraction process.

Overall, the use of experimental design in hesperidin extraction can provide a systematic and efficient approach to optimize the extraction process, which can be useful for various applications in the food, pharmaceutical, and cosmetic industries.

IV. CONCLUSION

In general, experimental design can help optimize the extraction process by identifying the key variables that affect the yield of hesperidin and determining the optimal conditions for extraction. Factors such as solvent concentration, extraction time, temperature, and pH can be optimized using experimental design techniques such as response surface methodology. The optimal conditions determined in this study for the extraction of hesperidin using the RSM(centered composite design) were 130 mg/ml for the solvent concentration, pH (12.67), temperature (62°C), and a time extraction of 3.45h wich give a theoretical yield of 67.44%.

By optimizing these extraction conditions, it would be possible to improve the yield of hesperidin from orangette, which has potential health benefits due to its antioxidant and anti-inflammatory properties. However, further studies are needed to confirm the efficacy of the extraction process and to determine the optimal conditions for large-scale production.

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