PulpIng – Development of Pumpkin Pulp Formulation Using a Sustainable Integrated Strategy

PRIMA-Section 2 project (2019)

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Executive Summary

Background	Pumpkin by-products contain a rich array of bioactive compounds with various health benefits. These compounds possess antioxidant, antimicrobial, and anti-inflammatory properties, making them valuable for diverse applications in the food, pharmaceutical, and cosmetic industries. Optimized extraction methods ensure maximum recovery of these bioactive compounds, enhancing their potential for use in functional foods, nutraceuticals, and natural preservation systems. The utilization of Response Surface Methodology (RSM) holds significant importance in obtaining refined preserving compounds from pumpkin by-products. RSM is a powerful statistical tool that enables the optimization of multiple variables simultaneously while considering their interactions, allowing for the development of robust and efficient extraction processes. By employing RSM, it becomes possible to determine the optimal combination of extraction parameters, such as temperature, extraction time, and solvent concentration, which can maximize the selection of preserving compounds from pumpkin by-products. Moreover, RSM facilitates the construction of response surface models that provide valuable insights into the selective extraction process. These models allow for the prediction of the response variables within the experimental design space, enabling a deeper understanding of the optimal conditions required for obtaining refined preserving compounds. Furthermore, RSM aids in reducing the number of experiments needed for optimization by employing statistical techniques such as factorial designs, central composite designs, and Box-Behnken designs. This not only saves time and resources but also provides a more efficient and systematic approach to optimization. In the actual PulpIng project, the use of RSM in obtaining refined preserving compounds from pumpkin by-products enhances the efficiency, reproducibility, and scalability of the extraction process. Overall, this report presents a comprehensive analysis of the most suitable conditions for obtaining refined preserving compounds from pumpkin by-products. The aim is to identify the optimal parameters that can maximize the extraction of valuable compounds with preservation properties from pumpkin peels.
Objectives	The primary objectives of this report are as follows: a) To assess the impact of different extraction solvents on the recovery of preserving compounds from pumpkin peels. b) To identify the optimal conditions, such as solvent type, and extraction time, for maximizing the refinement of preserving peels compounds. c) To evaluate the potential antioxidant activity of the refined preserving compounds.
Methodology	***Preliminary fractionation.*** Peel extract fractionation is a process that involves the separation of different compounds present in the extract using various solvents. In this method a sequencial extraction is performed using solvents such as, methanol, n-hexane, dichloromethane, ethyl acetate, n-butanol and water. The extract is initially mixed with one of the solvents, and the mixture is vigorously shaken to allow the compounds to partition between the solvent and the aqueous phases based on their solubility. The separatory funnel is then used to separate the two immiscible phases based on their density differences. Water, as a polar solvent, is

particularly effective in extracting hydrophilic compounds, such as phenolic compounds and water-soluble vitamins. Butanol is selected for its ability to extract lipophilic compounds, including terpenoids. Methanol is a versatile solvent that can extract a wide range of compounds, both polar and non-polar ones, making it useful for comprehensive extraction. Ethyl acetate is employed specifically for extracting non-polar compounds, such as flavonoids and alkaloids. Dichloromethane is effective in extracting moderately polar compounds, while hexane is predominantly used to extract non-polar lipids and hydrocarbons.

Mixture design plan. In this study, we used a centered mixtures (simplex-centroid designs) plan. The X factors vary from 0 to 1 without constraints on the design space and represent the proportion of each solvent in the mixture. The points of designed experiments were represented as a triangle form. Each vertex of the triangle corresponds to one of the three solvents. The midpoints of the sides of the triangle consist of the binary combinations, and the central point of the triangle is assigned to the ternary combinations. As shown in Table 1, the complete experimental design consisted of 13 experiments performed in a standardized order.

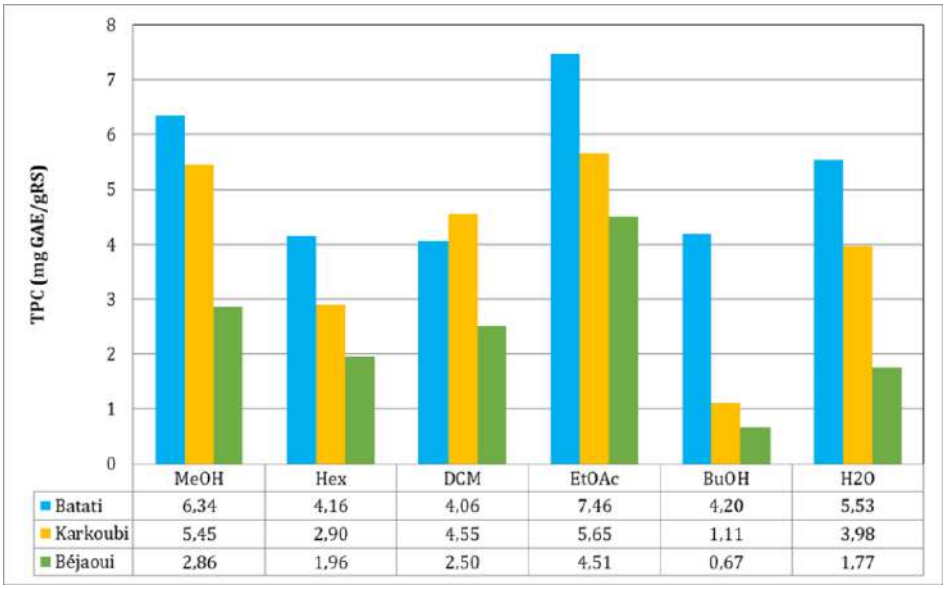
Table 1. Three-component axial screen matrix, with X1: methanol, X2: ethtyl acetat and X3: water.

No Exp	X1	X2	X3
1	1.0000	0.0000	0.0000
2	0.0000	1.0000	0.0000
3	0.0000	0.0000	1.0000
4	0.6667	0.3333	0.0000
5	0.3333	0.6667	0.0000
6	0.6667	0.0000	0.3333
7	0.3333	0.3333	0.3333
8	0.0000	0.6667	0.3333
9	0.3333	0.0000	0.6667
10	0.0000	0.3333	0.6667
11	0.6667	0.1667	0.1667
12	0.1667	0.6667	0.1667
13	0.1667	0.1667	0.6667

The measured responses are entered into the software NemrodW (LPRAI 2000) to define the optimal values for the proportions of ethyl acetate, methanol and water *via* three tools. First, the plotted curves were allowed to focus on the compromise areas among the three studied components. The target was to establish a surface curve reflecting the response changes. Secondly, the "Desirability" tool calculates the exact optimal combination respecting the percentage of compromise. This function, ranging from 0 to 1, allows to give a precise optimum setting. Finally, a test point on the obtained optimal mixture was applied experimentally.

Statistical analysis. For all tests, at least replicates were used. Means were compared using the Newman-Keuls (SNK) test at a level of $p < 0.5$ when significant differences were found by the statistical package SAS 9.1 (2002, 525).

Results and implications	The first step for the extraction refinement process involves the development of a linear gradient solvent system in order to fulfill compounds with a wide polarity range for the screening and separation of active molecules from pumpkin by-products. To find a gradient elution solvent system capable of separating compounds with a wide polarity range, various solvents were tested. In detail, common solvents used for liquid-liquid extraction (n-hexane, ethyl acetate, n-butanol, and water) were fixed, and several polarity modifier solvents (methanol, ethanol, and dichloromethane) were added. According to the results detailed in Figure 1, important variability can be observed when comparing the different solvent extracts as well as the three pumpkin varieties. Considering the solvent extractabilities, the ethyl acetate and the methanolic fractions seems to be richest ones in phenolic compounds, with a total polyphenol content reaching 7.5 and 6.3 mg GAE/gDR for Batati peels. The aqueous fraction contained also important TPC estimated at 5.5 mg GAE/gDR and was closely followed by hexane, dichloromethane and butanol fractions, with the highest TPC assessed around 4 mg GAE/gDR. When comparing the three pumpkin varieties it seems obvious that Batati peels contain the highest total phenolic contents, ranging from 7.4 to 4 mg GAE/gDR. For Karkoubi, TPC ranged from 5.6 to 1.1 mg GAE/gDR, while Bejaoui samples have a limited TPC of 4.5 mg GAE/gDR.

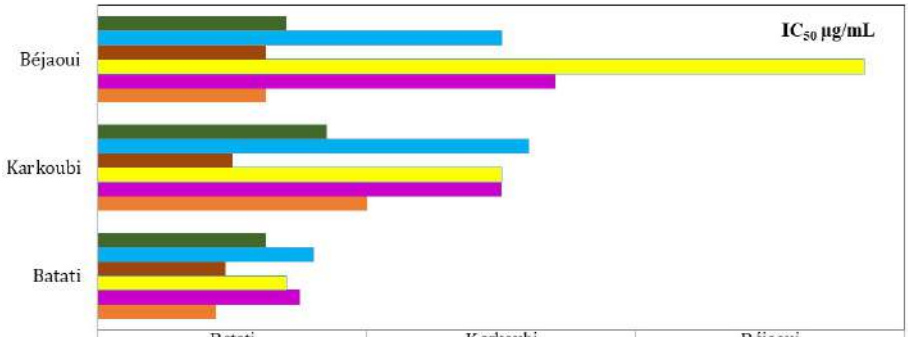


	MeOH	Hex	DCM	EtOAc	BuOH	H2O
Batati	6,34	4,16	4,06	7,46	4,20	5,53
Karkoubi	5,45	2,90	4,55	5,65	1,11	3,98
Béjaoui	2,86	1,96	2,50	4,51	0,67	1,77

Figure 1. Total polyphenol content (TPC expressed in mg GAE/gDR) extracted by linear gradient solvent from crude Batati, Karkoubi and Bejaoui peels extracts. MeOH: methanol fraction; Hex: hexane fraction; DCH: dichloromethane fraction; EtOAc: ethyl acetate fraction; BuOH: butanol fraction; H_2O: aqueous fraction.

Regarding the phenolic profile, the results concerning the antiradical activities of the fractions showed significant variability among the different solvent extracts as well as among the three varieties (Figure 2). Considering the solvent efficiencies and in accordance with phenolic contents results, the best antiradical activity was detected in the methanolic fraction with an IC_{50} value equal to 175 µg/mL closely followed by the ethyl acetate fraction with an IC_{50} value of 190 µg/mL. The other IC_{50} fraction were significantly higher and even reached 1140 µg/mL in the dichloromethane fraction. Considering the varieties variability, Batati peels, that contained the highest total phenolic content, exhibited the best antiradical activity expressed by the lowest IC_{50} values that were comprised between 175 and 320

µg/mL. For Karkoubi, the IC_{50} values ranged from 200 to 640 µg/mL, while the best IC_{50} value in Bejaoui samples was equal to 250 µg/mL and the least efficient one was estimated at 1140 µg/mL.



	Batati	Karkoubi	Béjaoui
H2O	250	340	280
BuOH	320	640	600
EtOAc	190	200	250
DCM	280	600	1140
Hex	300	600	680
MeOH	175	400	250

Figure 2. Antiradical activity, expressed as inhibition concentration at 50% (IC_{50}) in extract fractions from Batati, Karkoubi and Bejaoui peels. MeOH: methanol fraction; Hex: hexane fraction; DCH: dichloromethane fraction; EtOAc: ethyl acetate fraction; BuOH: butanol fraction; H_2O: aqueous fraction.

This preliminary study allowed the selection of methanol, ethyl acetate and water as the main efficient solvents in extracting antioxidant molecules. Based on these results, an experimental design, mixture design type, was elaborated to refine the obtained extracts. Starting with Batati genotype (NGBTUN 746), the main results in the experiments performed were detailed in Table 2.

Table 2. Three-component axial screen matrix and the values of the experimental responses for total phenolic contents (Y1, expressed in mg GAE/g DR) and antiradical activity (Y2 expressed in inhibition percentage).

N° Exp	X1	X2	X3	Y1	Y2
1	1.0000	0.0000	0.0000	14.44	53.32
2	0.0000	1.0000	0.0000	17.08	29.57
3	0.0000	0.0000	1.0000	12.64	37.98
4	0.6667	0.3333	0.0000	15.92	66.09
5	0.3333	0.6667	0.0000	16.10	55.23
6	0.6667	0.0000	0.3333	12.53	27.89
7	0.3333	0.3333	0.3333	15.85	49.62
8	0.0000	0.6667	0.3333	15.30	44.93
9	0.3333	0.0000	0.6667	11.02	31.59
10	0.0000	0.3333	0.6667	14.50	47.61
11	0.6667	0.1667	0.1667	15.20	58.95
12	0.1667	0.6667	0.1667	16.20	50.88
13	0.1667	0.1667	0.6667	14.64	35.40

Moreover, the mixture compounds effects on extracting polyphenols (Table 3) showed that individual solvent coefficients were highly significant parameters since their *p*-values were statistically lower than 0.01. Whereas for the antiradical activity, individual solvents as well as their combination exhibited significant synergistic antioxidant effects.

Table 3. Mixture compounds effects.

Total polyphenol content					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	14.473	2.30	0.799	18.12	***
b2	16.485	2.30	0.799	20.64	***
b3	12.526	2.30	0.799	15.69	***
b12	4.369	2.25	3.528	1.24	25.5%
b13	-4.783	2.25	3.528	-1.36	21.6%
b23	4.120	2.25	3.528	1.17	28.1%
Antiradical activity					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	53.91	2.30	4.022	13.4	***
b2	28.97	2.30	4.022	7.2	***
b3	37.4	2.30	4.022	9.3	***
b12	95.07	2.25	17.770	5.35	**
b13	-68.16	2.25	17.770	-3.84	**
b23	57.22	2.25	17.770	3.22	*

Consequently, the predictive mathematical models, representing the response in terms of the three constituents, is represented by the following equations:

For TPC: Y TPC = 14,47 * X1 + 16,48 * X2 + 12,52 * X3

For antioxidant activity: Y DPPH = 53,91 * X1 + 28,97 * X2 + 37,40 * X3 + 95,07 * (X1*X2)-68,16* (X1*X3) + 57,22 * (X2*X3)

These equations were transposed into isoprene curves as exhibited in Figure 3.

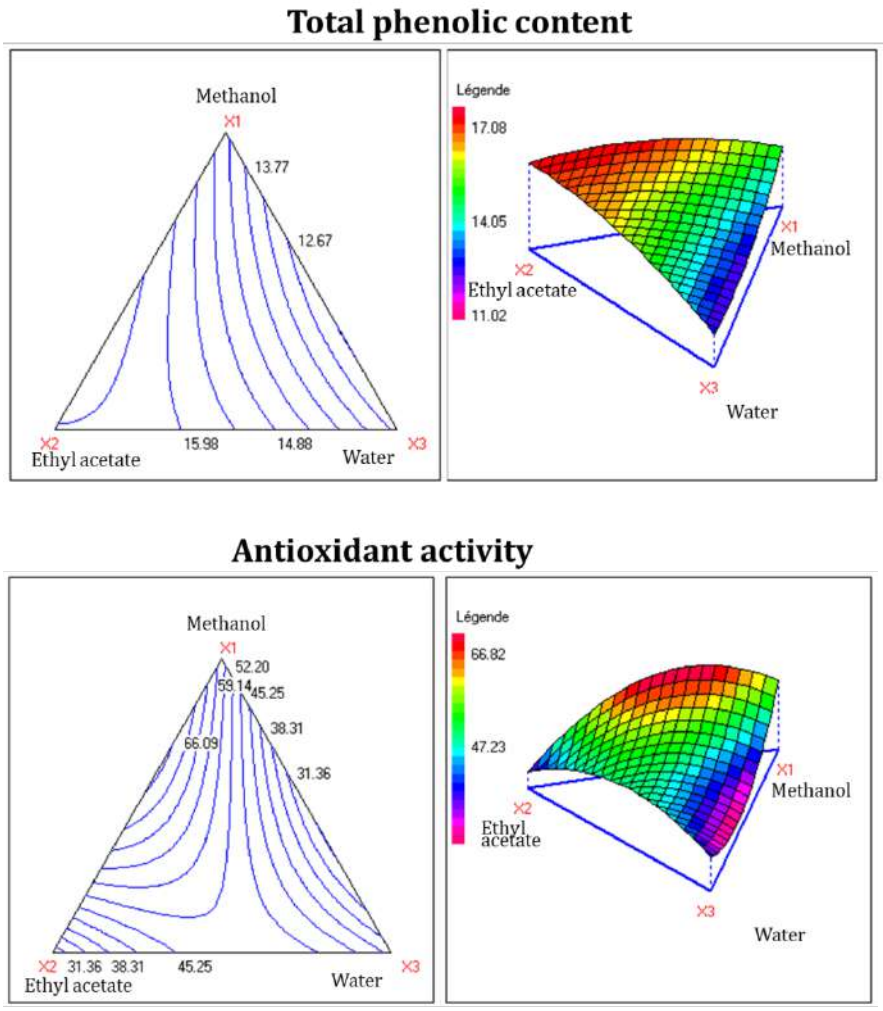


Figure 3. Isoresponses and mixture plot.

All together, the adopted software concluded that the targeted limit can be achieved with 99% desirability using a mixture consisting of:

53.4% methanol+ 45.8%

ethyl acetate+0.8% water

The experimental validation of this formula is detailed in Table 4.

Table 4. Experimental validation of the obtained formula.

	Predicted values	Experimental values
TPC	16,44	15,60
DPPH test	65,54	64,14

The same methodology was also applied to the two other Tunisian genotypes: Karkoubi (NGBTUN748) and Bejaoui (NGBTUN751). Main results of the two experimental designs were resumed in Tables 5, 6 and 7 as well as in Figure 4.

Table 5. Values of the experimental responses for total phenolic contents (Y1, expressed in mg GAE/g DR) and antiradical activity (Y2 expressed in inhibition percentage) for Karkoubi (NGBTUN748) and Bejaoui (NGBTUN751) genotypes.

	Karkoubi		**Bejaoui**	
N° Exp	Y1	Y2	Y1	Y2
1	15.42	48.82	14.59	56.17
2	27.50	71.32	12.31	22.46
3	10.35	28.44	10.00	34.47
4	12.92	32.54	15.97	72.08
5	19.12	63.06	14.00	50.96
6	18.96	58.97	9.62	30.56
7	16.85	59.60	9.23	51.97
8	16.30	51.52	8.47	48.07
9	10.67	30.25	6.04	36.93
10	23.41	65.52	7.49	48.07
11	15.48	55.50	11.13	55.73
12	15.92	54.10	10.50	52.70
13	16.16	46.97	10.00	53.71

Table 6 A. Mixture compound effects for Karkoubi genotype

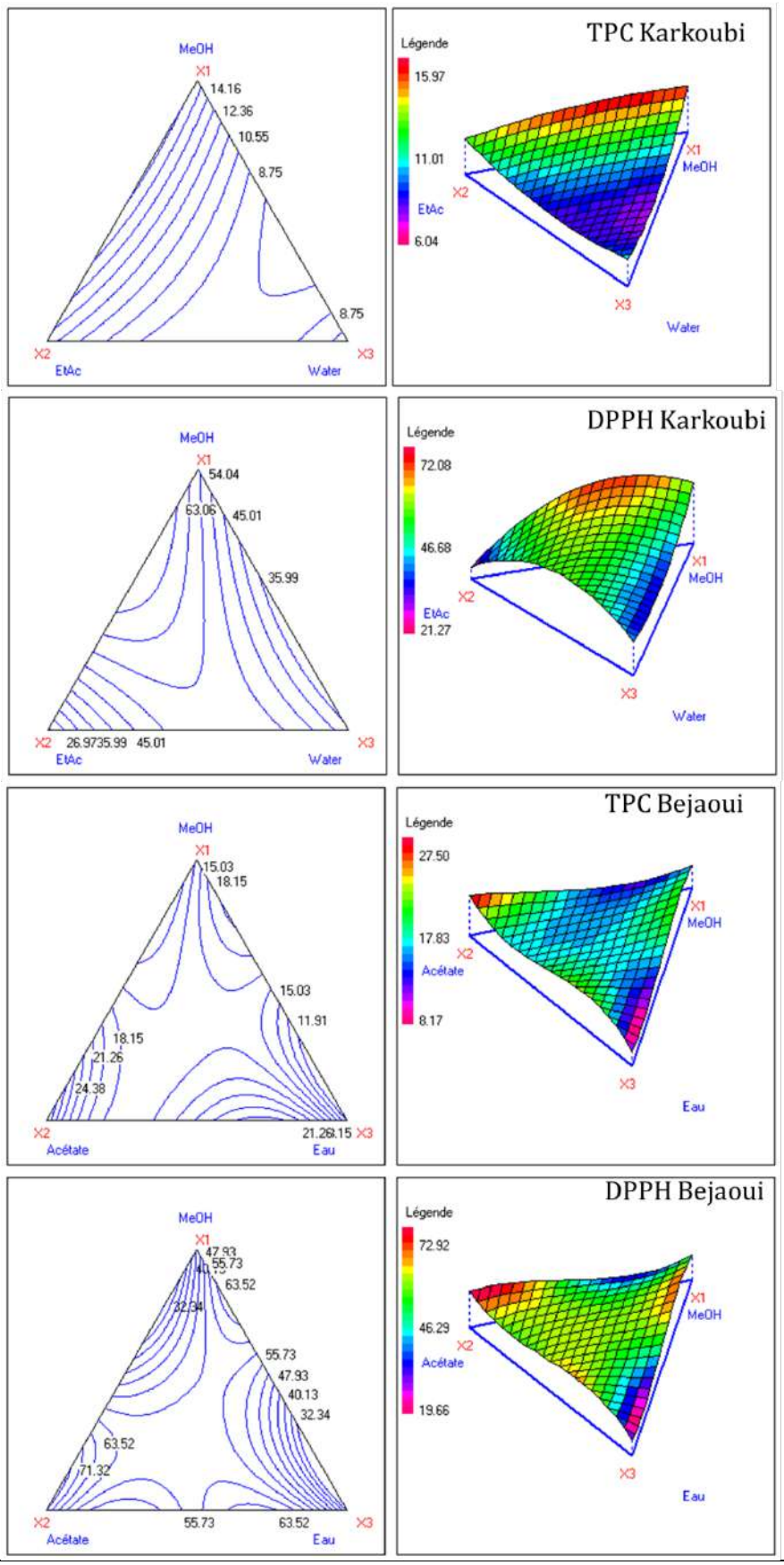
Total polyphenol content					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	14.78	2.30	1.013	14.6	***
b2	11.94	2.30	1.013	11.79	***
b3	10.14	2.30	1.013	10.02	***
b12	6.44	2.25	4.475	1.44	19.2%
b13	-19.53	2.25	4.475	-4.37	**
b23	-12.31	2.25	4.475	-2.75	*
Antiradical activity					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	54.77	2.30	5.39	10.15	***
b2	21.27	2.30	5.39	3.94	**
b3	37.14	2.30	5.39	6.88	***
b12	107.64	2.25	23.84	4.51	**
b13	-47.89	2.25	23.84	-2.01	8.3%
b23	93.58	2.25	23.84	3.92	**

Table 6 B. Mixture compound effects for Bejaoui genotype

Total polyphenol content					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	15.35	2.71	0.842	18.23	***
b2	27.41	2.71	0.842	32.57	***
b3	10.27	2.71	0.842	12.21	***
b12	-24.63	2.72	3.763	-6.55	**
b13	8.39	2.72	3.763	2.23	11.1%
b23	4.11	2.72	3.763	1.09	35.6%

Antiradical activity					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	48.409	2.71	4.185	11.57	***
b2	71.30	2.71	4.185	17.04	***
b3	28.32	2.71	4.185	6.77	**
b12	-54.62	2.72	18.706	-2.92	6.0%
b13	29.09	2.72	18.706	1.56	21.7%
b23	34.86	2.72	18.706	1.86	15.9%

Figure 4. Isoresponses and mixture plot for Karkoubi (NGBTUN748) and Bejaoui (NGBTUN751)



Taken the data together, the adopted software concluded that the refined extract can be performed with 99 desirability for Karkoubi (NGBTUN748) using a mixture composed of:

64.4% methanol+ 34.4% ethyl acetate+1.1% water

while the desired refined extract for Bejaoui genotype is predicted to be achieve with 87.4% desirability through a mixture containing:

87.3 % methanol+ 6.5 % ethyl acetate+ 6.2% water

The experimental validation data of these formulas are detailed in Table 7.

Table 7. Experimental validation of the two obtained formulas.

	Karkoubi (NGBTUN748)	
	Predicted values	**Experimental values**
TPC	15	16.17
DPPH test	66.94	62.24
	Bejaoui (NGBTUN751)	
	Predicted values	**Experimental values**
TPC	16.06	16.17
DPPH test	49.88	50.47

Once the experimental validation confirmed the mathemathical model, all the refined extracts obtained were assessed for their phenolic composition (using a HPLC apparatus) along with an assessment of their biological activities such as antibacterial activity and cytotoxicity level (presented in Deliverable 3.2) for all samples performed.

The methodology described within this deliverable, with the most suitable consitions will be applied to the selected extracts obtained in WP2.