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REPORTS WITH THE MATHEMATICAL MODELS (OBTAINED BY RSM) OF THE DEPENDENT VARIABLES USED IN THE OPTIMIZATION OF THE EXTRACTION OF THE PRESERVING COMPOUNDS

DELIVERABLE 2.4

PulpIng

Developing of **Pu**mpkin Pu**lp** Formulation using a Sustainable **In**te**g**rated Strategy

Index

Document Information

Deliverable Number	2.4
Deliverable name	Reports with the mathematical models (obtained by RSM) of the dependent variables used in the optimization of the extraction of the preserving compounds
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1. Summary

PulpIng project aims at the development of a high-quality pumpkin pulp product enriched and preserved by added-value compounds obtained from pumpkin by-products, fostering an integrative and sustainable strategy. Obtaining extracts with high preservative capacity from pumpkin by-products, more specifically the seeds, peel and fibers, is the main goal of the WP2 – "Sustainable recovery of compounds with preserving capacity from pumpkin by-products". This report regards the deliverable D 2.4 – "Reports with the mathematical models (obtained by RSM) of the dependent variables used in the optimization of the extraction of the preserving compounds" of the WP2, that comprises the optimization study by RSM to describe the best extraction conditions, fostering sustainability and efficiency in extraction procedures.

2. Description of work

Pumpkin by-products selected as the most promising in obtaining preservative compounds in Task 2.1 were studied for the extraction optimization by comparing different methodologies and responses. Samples were treated through conventional procedures of maceration and an alternative method assisted by ultrasounds. The relevant independent variables of time, temperature or power, and solvent were evaluated. Antioxidant methods of reducing power, total phenolic content, and yield were the dependent variables (responses).

2.1. Goal

To achieve the optimal extraction conditions, using eco-friendly and easy-to-perform methodologies, based on innovative technologies, and green extraction solvents. The most relevant variables were selected, to simplify processes and make them more sustainable in terms of energy, labor and solvent consumption.

3. Optimization study – IPB Contribution

3.1. Methodology

The most promising bioresidues and varieties for obtaining extracts rich in preservative compounds were described in the Delivarable 2.1, namely the peels of the variety Butternut Squash (BS) from Portugal, and of the varieties 'Landrace from the region of Trikala - Turbinate' (Ri2), 'Voutirato' (Ri16), and 'Local landrace "Leuka Melitis" - Round' (Ri17) from Greece. These selected samples were treated in a study of extraction optimization, in order to achieve the

most suitable extraction conditions to obtain the preserving compounds from pumpkin by-products.

For that purpose, the samples (BS, Ri2, Ri16, and Ri17) were subject to two different extraction types, namely maceration (MAC) and ultrasound assisted extraction (UAE). For each, an optimization procedure was performed, in which three variables of the extraction procedure were chosen, and 17 individual extractions were performed. The optimization procedure used the response surface methodology (RSM) based on the Box-Behnken experimental design. The optimized responses were dry residue (DR, R_1), reducing power (RP, R_2) and total phenolic content (TP, R_3), as schematized in the **Figure 1**. In some cases, not all responses could be optimized. The function used for DR and TP was "Maximize", in order to the combination of the factor interval to render the highest possible amount, while the RP used the function "Minimize", due to this assay being expressed as EC_{50}, namely the least amount of extract that can quench 50% of free radicals.

For the MAC, the extraction time (X_1), extraction temperature (X_2) and ethanol percentage (X_3) were the chosen parameters to vary, while the chosen parameters for UAE were % of ultrasonic power (X_1) fixed at 500 Watts, extraction time (X_2) and ethanol percentage (X_3). The intervals of variation are detailed in **Table 1**. The solid/liquid ration was fixed in 25 g/L. The rational for the intervals of variation of each factor were sourced from literature.

Table 1. Variation of each factor for the MAC and UAE extractions.

	Maceration (MAC)			Ultrasound Assisted Extraction (UAE)		
	X_1 – Extraction Time (min)	X_2 – Temperature (ºC)	X_3 – Ethanol (%)	X_1 – Power % (watts)*	X_2 – Extraction Time (min)	X_3 – Ethanol (%)
Run1	67.5	30	0	20	32.5	0
Run2	120	55	100	20	60	50
Run3	67.5	55	50	80	5	50
Run4	67.5	55	50	50	5	0
Run5	67.5	80	0	50	60	0
Run6	67.5	80	100	50	32.5	50
Run7	15	80	50	50	32.5	50
Run8	15	55	100	80	32.5	0
Run9	67.5	55	50	50	32.5	50
Run10	15	30	50	50	32.5	50
Run11	67.5	30	100	80	60	50
Run12	120	55	0	50	5	100
Run13	67.5	55	50	20	32.5	100
Run14	120	30	50	20	5	50
Run15	120	80	50	50	32.5	50
Run16	67.5	55	50	50	60	100
Run17	15	55	0	80	32.5	100

*20% of power = 100 Watts; 50% of power = 250 Watts; 80% of power = 400 Watts.

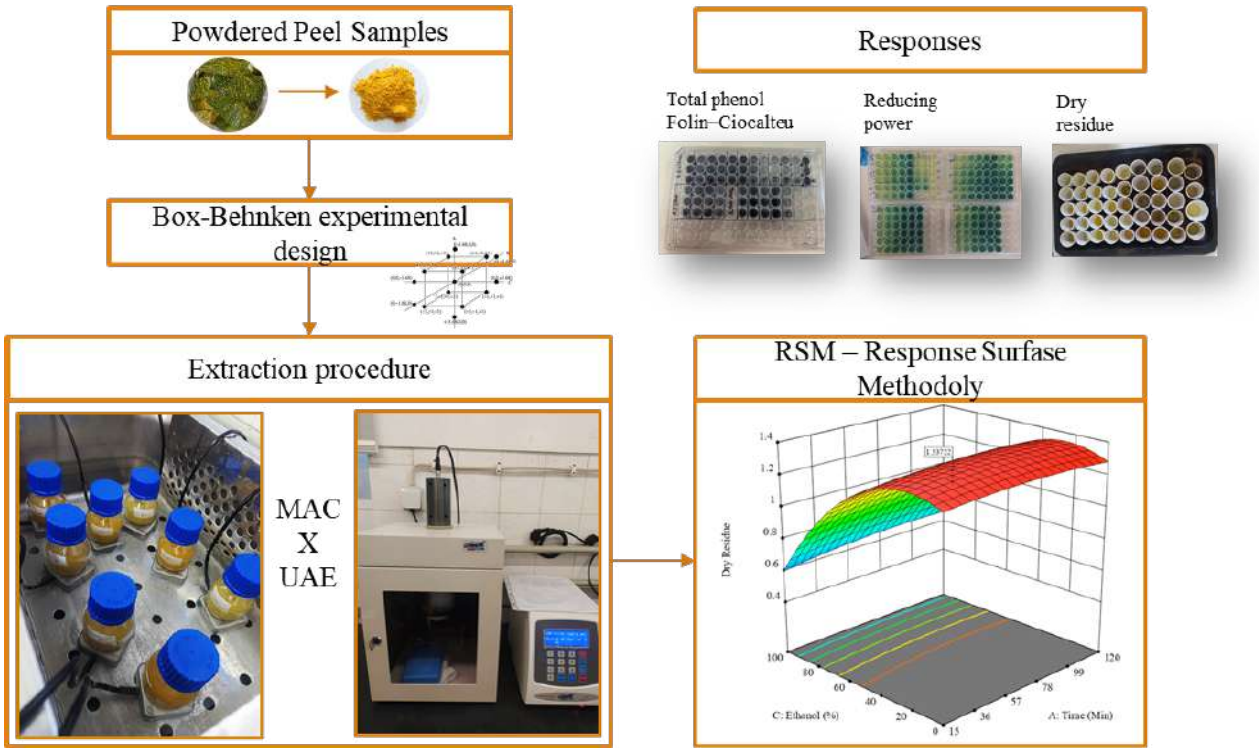


Figure 1. Scheme of steps to optimize conditions that maximize extraction responses.

3.1. Results

3.2.1. Maceration Extraction (MAC)

The MAC consists of placing the powder sample in contact with solvent at a certain temperature and time, under agitation. It is a conventional method typically used on an industrial scale, given its simplicity. For these, 0.5 g of sample were extracted in 20 mL of solvent (ethanol/water), in the different conditions previously described in **Table 1**. MAC was performed using a water bath with agitation through a Cimarec™ magnetic stirrer under a fixed speed (500 rpm, Thermo Scientific).

Peels of 'Landrace from the region of Trikala - Turbinate' (Ri2)

Considering the MAC extraction of sample Ri2, **Table 2** shows the responses (R) of each run, values with which the response surface methodology was applied to, using Design Expert.

Table 2. Responses for the MAC extraction for sample Ri2.

	Maceration		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R_3 – Total Phenols (mg/g)
Run1	1.268	118	132
Run2	0.688	169	126
Run3	1.295	329	142.21
Run4	1.286	313	112
Run5	1.267	401	98
Run6	0.754	830	163
Run7	1.283	924	124
Run8	0.583	274	108.3
Run9	1.202	345	103
Run10	1.135	85	98
Run11	0.488	131	128
Run12	1.302	401	104
Run13	1.293	334	107
Run14	1.127	261	138
Run15	1.286	416	120.7
Run16	1.284	366	117
Run17	1.264	332	47.53

For R_1 (dry residue) of sample Ri2, extracted through MAC, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.984 and the following coded equation is described in Eq. (1).

$$R_1 = 1.27 + 0.0171X_1 + 0.0714X_2 - 0.3232X_3 + 0.0027X_1X_2 + 0.0169X_1X_3 - 0.0666X_2X_3 - 0.0245X_1^2 - 0.0396X_2^2 - 0.2882X_3^2 \quad \text{Eq. (1)}$$

Thus, the optimal values that maximize the amount of dry residue were set at 40 minutes, 70 ºC and 19% of ethanol, which are previewed to render 1.35 g/100g dw. The first row of **Table 3** shows the 3D response charts for R_1 at the optimal points. Overall temperature and time did not have much influence in the optimization of the response, due to very low variation, while amounts of ethanol beyond 40% seem to reduce the dry residue yield.

For R_2 (RP) of sample Ri2, a quadratic function was obtained, although two runs were eliminated due to being outliers, which allowed for a non-significant lack of fit and an adequate fit of the model, with an adjusted R^2 of 0.997. The coded equation was is follows in Eq. (2).

$$R_2 = 337.4 - 83X_1 + 247X_2 + 110.5X_3 - 171X_1X_2 - 226.5X_1X_3 + 104X_2X_3 + 41.05X_1^2 + 43.05X_2^2 - 10.45X_3^2 \quad \text{Eq. (2)}$$

For this response, the minimize function was chosen, thus obtaining an optimal point of 30 minutes, 31 ºC and 29% of ethanol, rendering a previewed EC_{50} of 27 µg/mL. The RSM charts can be found on the second line of **Table 3**, showing the lowest values colored in dark blue. The two most important factors were extraction time and solvent percentage.

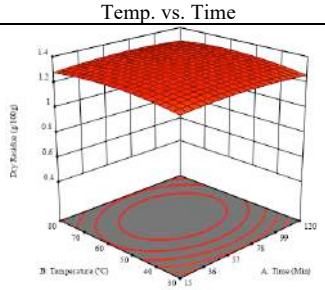
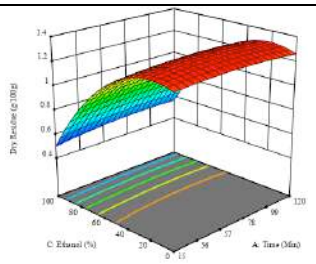
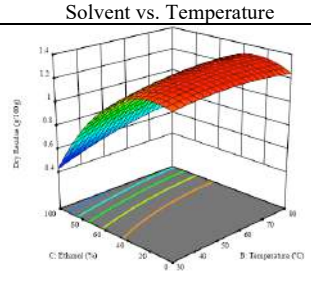
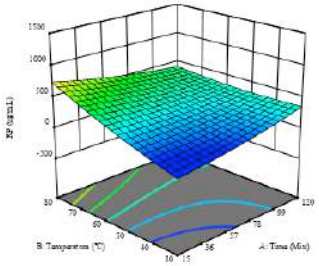
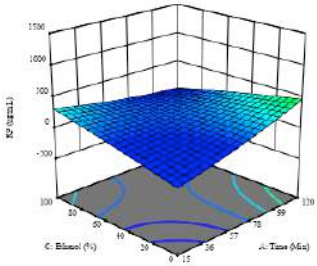
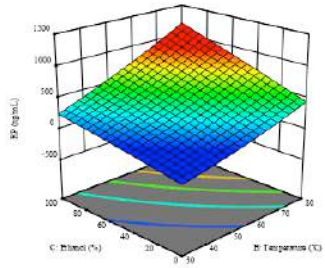
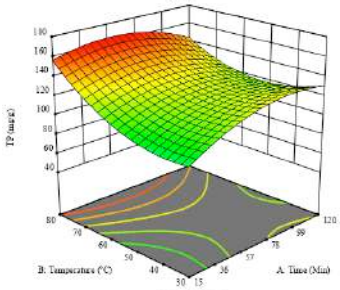
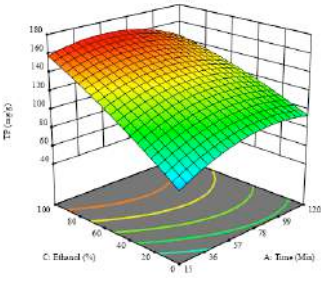
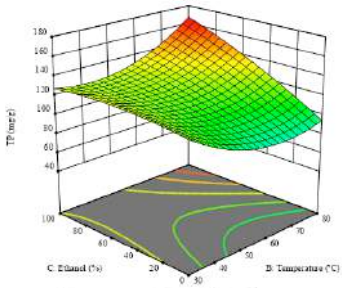
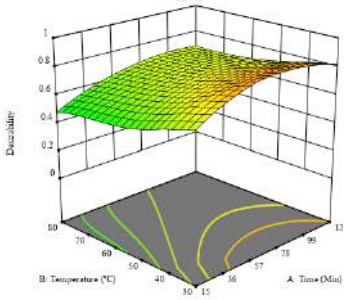
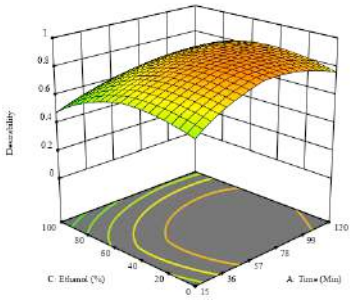
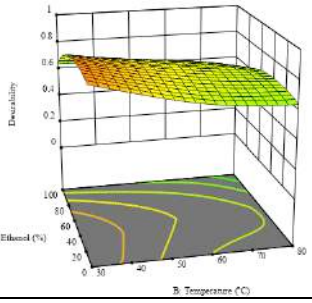
Finally, the total phenols (R_3) rendered a quadratic function with an adjusted R^2 of 0.7203 and a coded equation showed in Eq. (3).

$$R_3 = 111.24 + 13.861X_1 + 1.21X_2 + 17.97X_3 + 10.83X_1X_2 + 9.69X_1X_3 + 17.25X_2X_3 - 14.93X_1^2 + 18.86X_2^2 - 4.85X_3^2$$ Eq. (3)

The optimal point, which maximized the amount of total phenols is 62 minutes, 80 ºC and 97% of ethanol, which are previewed to render 165 mg/g. The optimal values are shown in the third row of **Table 3**. Overall, the most important factors were temperature and ethanol percentage, although higher values for temperature should be considered due to a preview of higher yields at temperatures over 80 ºC.

The final row of **Table 3** shows the charts of the Desirability function, in which all three responses are considered, allowing for the determination of the optimal point of all three responses. For this, the time was set at 75 minutes, 30 ºC and 24% of ethanol, rendering a dry residue of 1.28 g/100g, and EC_{50} of 158 µg/mL for RP and 136 mg/g of total phenols. This function allows for an equilibrium of each response that individually might be reduced, but considering all three responses together, the conditions will be suitable and optimized for all.

Table 3. Response 3D charts of the MAC extracted Ri2 sample at the optimal points.

Sample		Temp. vs. Time	Solvent vs. Time	Solvent vs. Temperature
Ri2	R_1 – DR			
	R_2 – RP			
	R_3 – DPPH			
	Desirability			

Peels of 'Voutirato' (Ri16)

Considering the MAC extraction of sample Ri16, **Table 4** shows the responses (R) of each run, values with which the response surface methodology was applied.

Table 4. Responses for the MAC extraction for sample Ri16.

	Maceration		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R_3 – Total Phenols (mg/g)
Run1	1.3801	244	120
Run2	0.7489	101	77
Run3	1.2842	304	131
Run4	1.4146	405	116
Run5	1.8918	612	91
Run6	0.8541	351	127
Run7	1.4090	345	88
Run8	0.7306	348	99,11
Run9	1.3097	416	96
Run10	1.2939	128	113
Run11	0.5727	143	124
Run12	1.3882	504	101
Run13	1.3857	388	89
Run14	1.3159	263	113
Run15	1.5026	470	97
Run16	1.3279	359	104
Run17	1.3183	-	132

For R_1 (dry residue) of sample Ri16, extracted through MAC, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.975, although one run had to be eliminated due to being an outlier. The coded equation is expressed in Eq. (4).

$$R_1 = 1.34 + 0.0255X_1 + 0.0675X_2 - 0.33147X_3 + 0.0179X_1X_2 - 0.0129X_1X_3 + 0.0811X_2X_3 + 0.0232X_1^2 - 0.0127X_2^2 - 0.3211X_3^2 \quad \text{Eq. (4)}$$

Thus, the optimal values that maximize the amount of dry residue were set at 120 minutes, 73 ºC and 24% of ethanol, which are previewed to render 1.515 g/100g. The first row of **Table 5** shows the 3D response charts for R_1 at the optimal points. Temperature and extraction time had low effect in the variation of dry residue, and once again, the ethanol content was determinant, as was in sample Ri2.

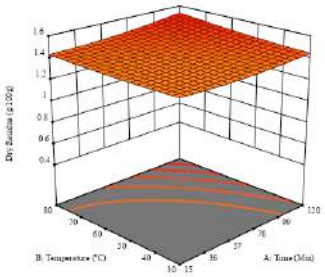
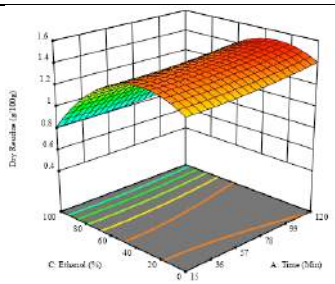
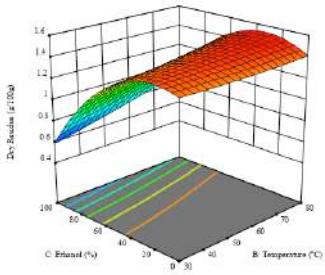
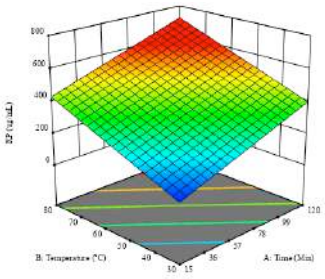
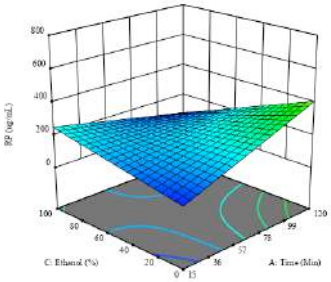
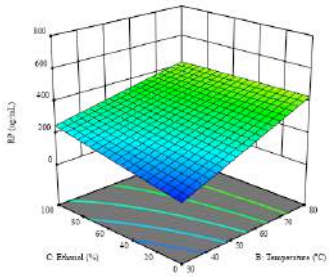
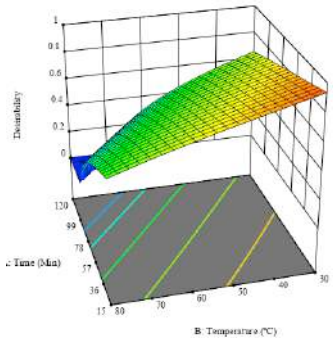
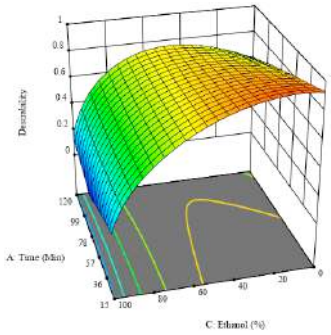
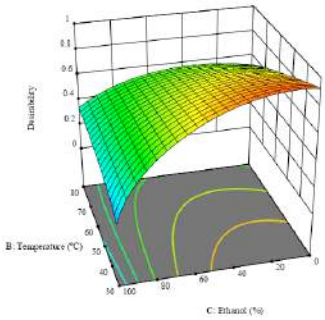
Regarding R_2, (RP) for MAC, the model chosen was a 2-factor interaction (2FI) with and adjusted R^2 of 0.79 and non-significant lack of fit for the 16 runs (one run did not show any antioxidant activity). The coded equation obtained is presented in Eq. (5).

$$R_2 = 332.03 + 31.68X_1 + 125X_2 - 85.07X_3 - 2.5X_1X_2 - 121.86X_1X_3 - 40X_2X_3 \quad \text{Eq. (5)}$$

The minimize function resulted in an optimal point of 15 minutes, 30 ºC and only 2% of ethanol, with a previewed EC_{50} of 100 µg/mL. The second row of **Table 5** shows the 3D charts for R_2. Considering the coded equation, the factor with highest influence was temperature, followed by time, revealing that lower EC_{50} values are favored by lower temperature and shorter extraction times. An adequate modeling was not possible for TP of sample Ri16, thus, the third row of **Table**

5 represents the desirability of R_1 and R_2, which rendered a preview of 1.4 g/100g of dry residue and 112 µg/mL of RP at 15 minutes, 30 ºC and 10% of ethanol.

Table 5. Response 3D charts of the MAC extracted Ri16 sample at the optimal points.

Sample		Temp. vs. Time	Solvent vs. Time	Solvent vs. Temperature
Ri16	R_1 – DR			
	R_2 – RP			
	Desirability			

Peels of 'Local landrace "Leuka Melitis" - Round' (Ri17)

Considering the MAC extraction of sample Ri17, **Table 6** shows the responses (R) of each run, values with which the response surface methodology was applied.

Table 6. Responses for the MAC extraction for sample Ri17.

	Maceration		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R_3 – Total Phenols (mg/g)
Run1	0.9072	34	105
Run2	0.3582	202	107
Run3	0.9366	1333	84.88
Run4	0.8919	664	80
Run5	0.9540	1013	94
Run6	0.4007	561	125
Run7	0.9490	608	115
Run8	0.2785	455	106.8
Run9	0.8825	550	78
Run10	0.8385	148	83
Run11	0.1692	82	134
Run12	0.9422	551	87
Run13	0.8675	498	67.5
Run14	1.0039	477	64
Run15	1.0592	741	75.7
Run16	1.0111	597	70
Run17	0.9194	1066	65.19

For R_1 (dry residue) of sample Ri17, extracted through MAC, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.966. The coded equation is shown in Eq. (6).

$$R_1 = 0.9179 + 0.0473X_1 + 0.0555X_2 - 0.3145X_3 - 0.0138X_1X_2 + 0.0145X_1X_3 + 0.0462X_2X_3 + 0.0308X_1^2 - 0.0140X_2^2 - 0.3241X_3^2 \quad \text{Eq. (6)}$$

Thus, the optimal values that maximize the amount of dry residue were set at 98 minutes, 79 ºC and 27% of ethanol, which are previewed to render 1.06 g/100g of dry residue. In **Table 7**, the first row shows the 3D charts of R_1 for sample Ri17, showing that like the previous samples, for the dry residue (R_1), the temperature and time had low influence in improving the yield, being the amount of ethanol the factor that markedly made this response vary. R_2, for reducing power did not allow for a satisfactory modelling, and thus was not added.

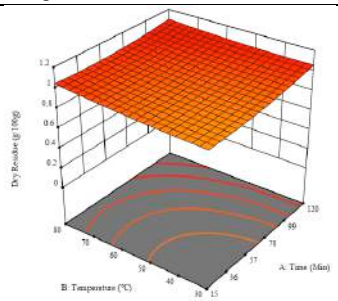
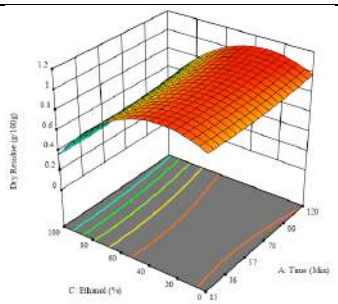
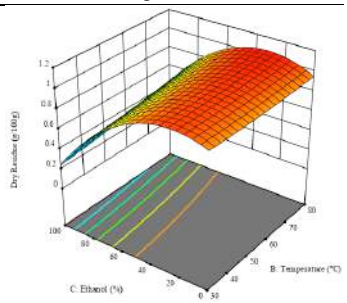
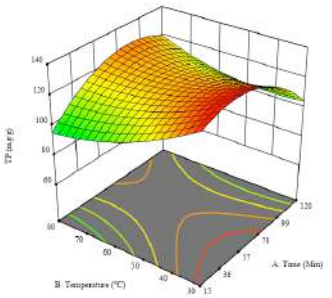
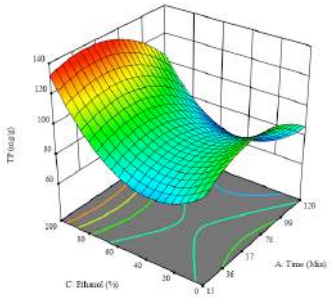
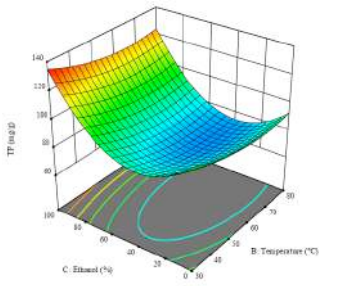
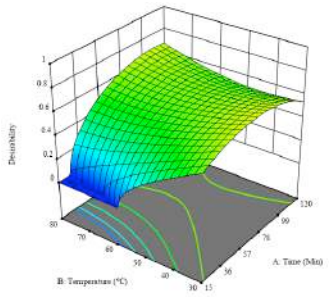
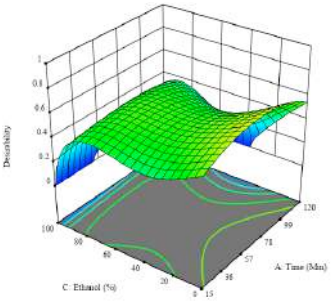
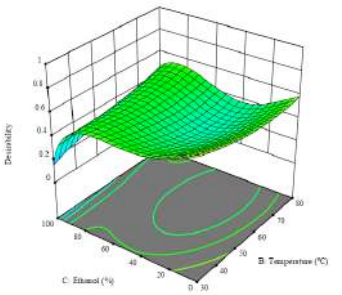
Considering R_3, the values allowed for a significant model and non-significant lack of fit after removing one outlier. The coded equation was as follows in Eq. (7).

$$R_3 = 76.08 + 4.46X_1 - 6.04X_2 + 15.20X_3 + 12.93X_1X_25.4X_1X_3 + 0.5X_2X_3 - 16.33X_1^2 + 6.68X_2^2 + 31.75X_3^2 \quad \text{Eq. (7)}$$

For this response, the factor with highest influence was also the percentage of ethanol, which can be seen graphically in the second row of **Table 7**. The optimal point after applying the maximize function was set at 68 minutes, 30 ºC and 100% of ethanol, which is previewed to render 135 mg/g of total phenols. Considering the Desirability function for these two responses (R_1 and R_3) of sample Ri17 extracted through MAC, the optimal point was set at 67 minutes, 30 ºC, and 0%

of ethanol, in which a dry residue of 0.9 g/100 and 106 mg/g of total phenols are expected. The 3D charts of the Desirability function are in the third row of **Table 7**.

Table 7. Response 3D charts of the MAC extracted Ri17 sample at the optimal points.

Sample		Temp. vs. Time	Solvent vs. Time	Solvent vs. Temperature
Ri17	R_1 – DR			
	R_3 – TP			
	Desirability			

Peel of 'Butternut Squash'

Considering the MAC extraction of sample BS,

Table 8 shows the responses (R) of each run, values with which the response surface methodology was applied.

The final sample extracted through MAC, BS, rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.758. The coded equation is presented in Eq. (8).

$$R_1 = 1.36 + 0.1203X_1 + 0.0748X_2 - 0.3590X_3 + 0.0267X_1X_2 - 0.0635X_1X_3 + 0.0878X_2X_3 - 0.0946X_1^2 + 0.0817X_2^2 - 0.37021X_3^2 \quad \text{Eq. (8)}$$

Table 8. Responses for the MAC extraction for sample BS.

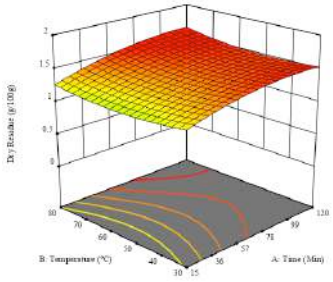
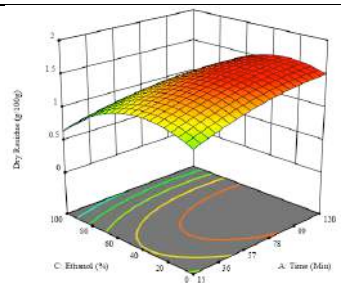
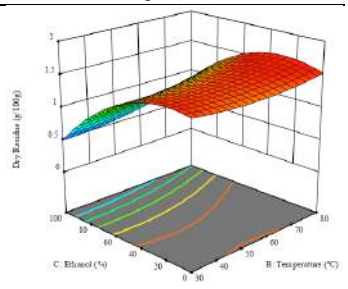
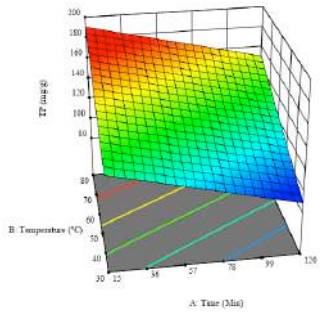
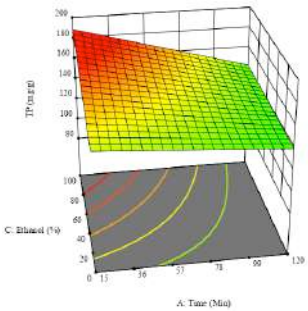
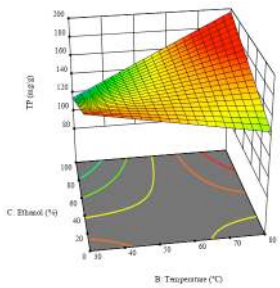
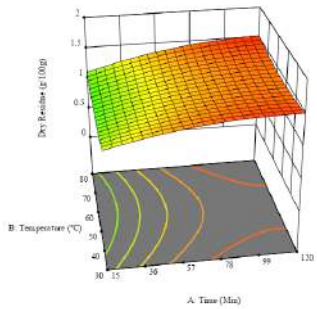
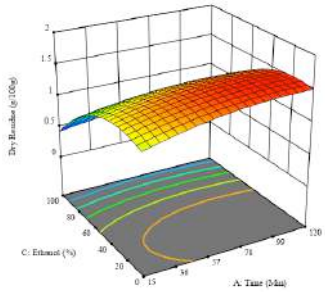
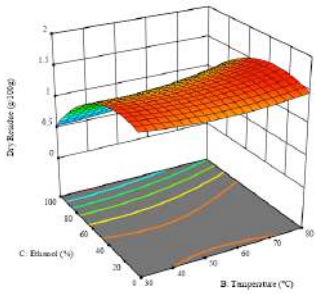
	Maceration		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R_3 – Total Phenols (mg/g)
Run1	1.4345	76	167
Run2	0.7602	196	112
Run3	1.4211	1417	161
Run4	1.4260	355	146
Run5	1.5479	327	138
Run6	0.8797	542	70
Run7	1.3095	330	177
Run8	0.4338	443	160
Run9	1.3908	243	147
Run10	1.3529	78	152
Run11	0.4153	178	95
Run12	1.4793	271	152
Run13	1.1361	519	122
Run14	1.3270	247	123
Run15	1.3906	1354	150
Run16	1.4150	419	195
Run17	0.8989	668	166

Thus, the optimal values that maximize the amount of dry residue were set at 114 minutes, 76 ºC and 20% of ethanol, which are previewed to render 1.60 g/100g of dry residue. In **Table 9**, the first row shows the 3D charts of R_1 for sample BS which show a very similar profile to the R_1 of the other samples, namely the defining factor being ethanol percentage. R_2 could not be optimized due to fitting problems. R_3 optimization achieved a significant 2FI model after the removal of 2 outliers and an R^2 of 0.775, being the coded equation as follows in Eq. (9)

$$R_3 = 164.64 - 14.75X_1 + 13.29X_2 - 9.71X_3 + 0.5X_1X_2 - 8.5X_1X_3 + 28.07X_2X_3 \quad \text{Eq. (9)}$$

Time seemed to be the most important factor for R_3, meaning that lower extraction times seemed to show higher total phenolics. In terms of the optimal points to maximize TF, they are located at 19 minutes, 77 ºC and 72% of ethanol, which are previewed to render EC_{50} of 187 µg/mL 187. The Desirability 3D charts are shown below R_2, being the optimal point at 84 minutes, 30 ºC and 0% ethanol, rendering 1.49 g/100g of dry residue and 169 mg/g of total phenols.

Table 9. Response 3D charts of the MAC extracted BS sample at the optimal points.

Sample		Temp. vs. Time	Solvent vs. Time	Solvent vs. Temperature
But	R_1 – DR			
	R_3 – TP			
	Desirability			

3.2.2. Ultrasound assisted extraction (UAE)

UAE is based on cavitation for cell destruction and release of the compounds in the solvent, in order to reduce variables such as time, temperature and amount of solvent. The UAE was performed using an ultrasonic device (QSonica sonicators, model CL-334) at a fixed frequency (40 kHz). The condenser flasks were always positioned at the same distance from the transducer without additional agitation. Power and time were controlled from the instrument panel. To control the temperature, i.e. to avoid overheating, an ice bath was used. The powdered peel (0.75 g) was placed in a beaker with 30 mL solvent (ethanol/water) and extracted under different conditions previously described in **Table 1**.

Peels of 'Landrace from the region of Trikala - Turbinate' (Ri2)

For sample Ri2, **Table 10** shows the responses (R) of each run, values with which the response surface methodology was applied to using Design Expert.

Table 10. Responses for the UAE extraction for sample Ri2.

Ultrasound Assisted Extraction			
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R_3 – Total Phenols (mg/g)
Run1	1.0956	25	163
Run2	0.9058	38	178
Run3	1.6383	206	142
Run4	1.2494	319	170
Run5	1.2364	304	127
Run6	1.1203	191	127
Run7	1.0374	856	139
Run8	1.4696	323	143
Run9	1.0878	262	133
Run10	1.0857	93	144
Run11	1.2304	35	133
Run12	0.2360	317	180
Run13	0.3102	316	144
Run14	0.9322	197	134
Run15	1.3657	575	106
Run16	0.2539	267	153
Run17	0.5238	117	96

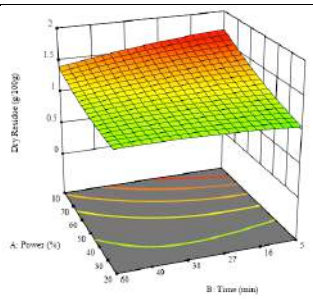
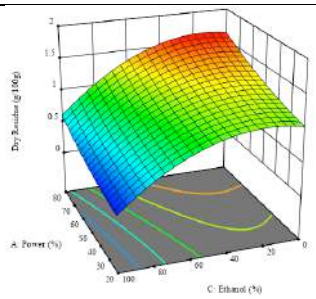
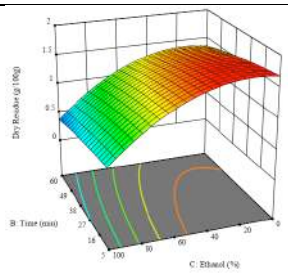
For R_1 (dry residue) of sample Ri2, extracted through UAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.899. The coded equation is shown in Eq. (10).

$$R_1 = 1.14 - 0.2023X_1 - 0.0537X_2 - 0.4659X_3 - 0.0954X_1X_2 - 0.0401X_1X_3 + 0.0077X_2X_3 - 0.0716X_1^2 - 0.0343X_2^2 - 0.3612X_3^2 \quad \text{Eq. (10)}$$

The optimal points for R_1 were set at 80% power, 18 minutes and 16% ethanol, which are expected to render 1.6 g/100g of dry residue. The 3D charts are shown on **Table 11**, in which it is clear that ethanol content shows a higher influence in the amount of total phenols, while the ultrasonic intensity has a slight influence. R_2 and R_3 could not be optimized for sample Ri2.

Table 11. Response 3D charts of the UAE extracted Ri2 sample at the optimal points.

Sample	Power vs. Time	Power vs. Solvent	Time vs. Solvent

Ri2	R_1 – DR			

Peels of 'Voutirato' (Ri16)

For sample Ri16, **Table 12** shows the responses (R) of each run, values with which the response surface methodology.

Table 12. Responses for the UAE extraction for sample Ri16.

	Ultrasound Assisted Extraction		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R3 – Total Phenols (mg/g)
Run1	1.405	147	131
Run2	1.281	100	90
Run3	1.873	495	107
Run4	1.409	337	132
Run5	0.649	295	86
Run6	1.275	341	97
Run7	1.224	420	96
Run8	1.535	340	97
Run9	1.278	316	99
Run10	1.207	159	86
Run11	1.371	103	99
Run12	0.459	346	117
Run13	0.406	369	147
Run14	1.177	377	97
Run15	1.27	408	57
Run16	0.706	502	126
Run17	0.667	267	100

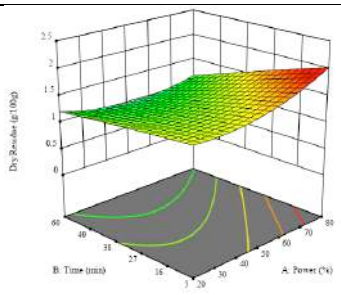
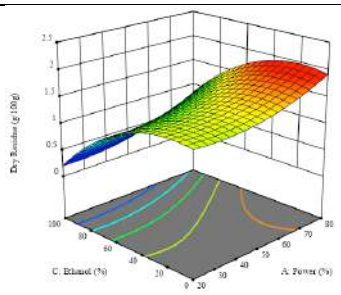
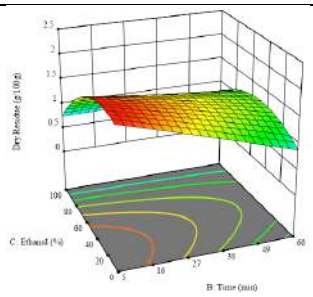
For R_1 (dry residue) of sample Ri16, extracted through UAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.8748. The coded equation obtained is described in Eq. (11).

$$R_1 = 1.25 - 0.1471X_1 - 0.1139X_2 - 0.3450X_3 - 0.1515X_1X_2 - 0.0328X_1X_3 + 0.2518X_2X_3 + 0.1861X_1^2 - 0.0114X_2^2 - 0.4336X_3^2 \quad \text{Eq. (11)}$$

The model placed the optimal points at 76% of power intensity, 7 minutes and 17% of ethanol while predicting a dry residue of 1.9 g/100g. **Table 13** shows the 3D charts for sample Ri16,

revealing that to some extent all three parameters influenced the optimal point of dry residue. Higher power, lower ethanol content and sorter time extractions seem to improve the obtention of higher quantities of residue. R_2 analysis only rendered a linear model and thus was not considered for optimization studies, while R_3 did not produce satisfactory results regarding lack-of fit and thus was also not considered.

Table 13. Response 3D charts of the UAE extracted Ri16 sample at the optimal points.

Sample		Power vs. Time	Power vs. Solvent	Time vs. Solvent
Ri16	R_1 – DR			

Peels of 'Local landrace "Leuka Melitis" - Round' (Ri17)

For sample Ri17, **Table 14** shows the responses (R) of each run, values with which the response surface methodology was applied.

Table 14. Responses for the UAE extraction for sample Ri17.

	Ultrasound Assisted Extraction		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R3 – Total Phenols (mg/g)
Run1	0.9210	215	91
Run2	0.8410	157	100
Run3	1.2430	423	70
Run4	0.9890	703	111
Run5	0.9130	607	64
Run6	0.9040	461	81
Run7	0.7990	484	81
Run8	1.0060	762	108
Run9	0.8820	736	75
Run10	0.6770	235	75
Run11	0.8740	173	73
Run12	0.1700	348	113
Run13	0.1280	-	113
Run14	0.6340	621	274
Run15	0.7080	628	74
Run16	0.0840	-	181

Run17	0.6770	1528	37

For R_1 (dry residue) of sample Ri17, extracted through UAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.8970. The coded equation is presented in Eq. (12).

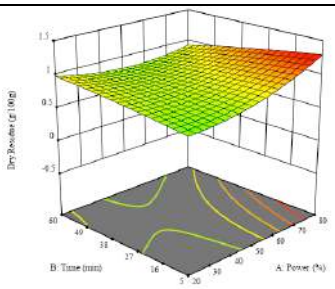
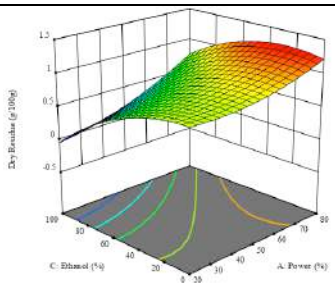
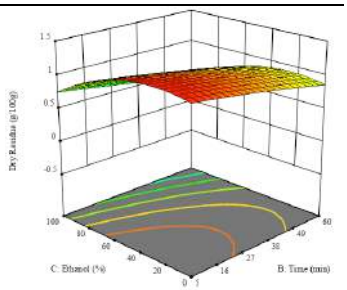
$$R_1 = 0.7940 + 0.1595X_1 - 0.0405X_2 - 0.3463X_3 - 0.1440X_1X_2 + 0.1160X_1X_3 - 0.0025X_2X_3 + 0.1240X_1^2 - 0.02X_2^2 - 0.2350X_3^2 \quad \text{Eq. (12)}$$

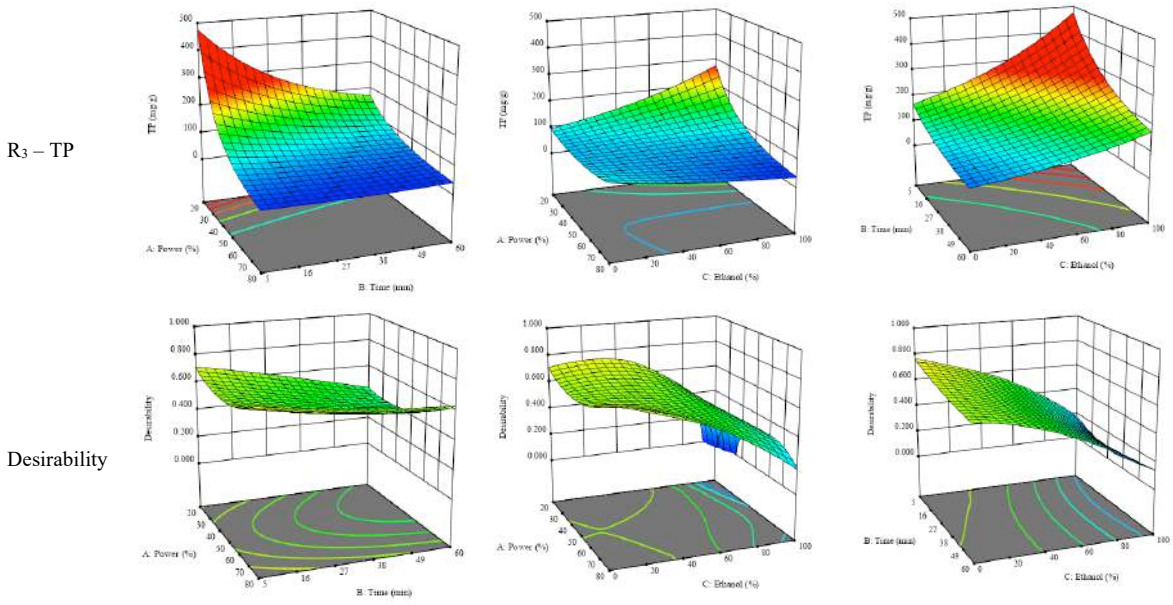
By interpreting the coded equation, the factor with highest influence seems to be the intensity of the ultrasonic waves, observed by the high values of the optimal point, which was set at 79% of ultrasound power, 9 minutes and 31% of ethanol, which should render 1.25 g/100g of dry residue. The 3D charts show a similar trend, being displayed in the first row of **Table 15**, in which higher power intensities render higher yields in dry residue while lower extraction time also seems to favor yields. R_2 did not allow for an optimization procedure. Regarding R_3, the values allowed for a quadratic model with a natural log transformation and ignoring two outliers. The model showed a significant fit and non-significant lack thereof with an adjusted R^2 of 0.9870. The coded equation was as follows in Eq. (13).

$$R_3 = 4.35 - 0.4320X_1 - 0.2536X_2 + 0.0063X_3 + 0.2625X_1X_2 - 0.5298X_1X_3 + 0.0095X_2X_3 + 0.2490X_1^2 + 0.1029X_2^2 + 0.0042X_3^2 \quad \text{Eq. (13)}$$

In the case of R_3, the factor with higher influence was the percentage of ethanol, judging by the coded values of the equation. The optimal point was set at 20% power intensity, 29 minutes and 100% ethanol, which is expected to yield 307 mg/g of total phenols, beyond what was achieved in the variation intervals of the factors. The second row of **Table 15** shows the 3D charts for this response, showing the lower needs of ultrasonic intensity to promote higher total phenols while high yields of ethanol and longer extraction time seem to promote these bioactive molecules.

Table 15. Response 3D charts of the UAE extracted Ri17 sample at the optimal points.

Sample		Power vs. Time	Power vs. Solvent	Time vs. Solvent
Ri216	R_1 – DR			



The Desirability function pointed towards an optimum of 80% of ultrasound, 5 minutes of extraction time and 0% of ethanol, which would render 1.12 g/100g of dry residue and 120 mg/g of total phenols. The corresponding 3D charts are in the final row of **Table 15**.

Peel of 'Butternut Squash'

For sample BS, **Table 16** shows the responses (R) of each run, values with which the response surface methodology was applied.

Table 16. Responses for the UAE extraction for sample BS.

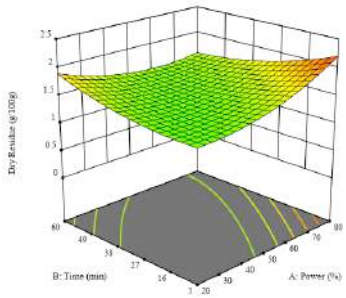
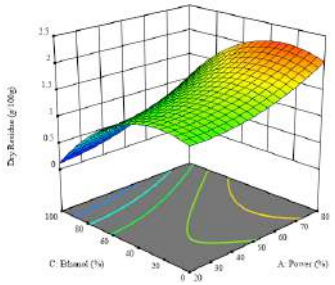
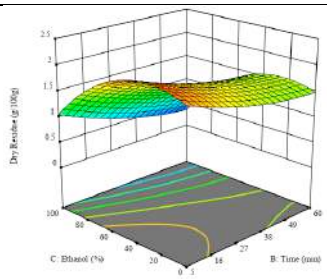
	Ultrasound Assisted Extraction		
	R_1 – Dry Residue (g/100g)	R_2 – Reducing Power (µg/mL)	R3 – Total Phenols (mg/g)
Run1	1.509	114	398
Run2	1.468	95	323
Run3	2.301	208	672
Run4	1.437	247	400
Run5	1.669	230	246
Run6	1.716	211	339
Run7	0.704	199	569
Run8	1.493	290	127
Run9	1.278	372	170
Run10	1.439	199	166
Run11	1.411	116	139
Run12	0.208	-	79
Run13	0.299	523	122
Run14	1.331	589	191
Run15	1.425	388	40
Run16	0.268	506	119
Run17	0.55	738	94

For R_1 (dry residue) of sample BS, extracted through UAE, the analysis of the 17 runs rendered a quadratic function with a significant model, a non-significant lack of fit, an adjusted R^2 of 0.6745. The coded equation obtained is described in Eq. (14)

$$R_1 = 1.31 + 0.1435X_1 - 0.0576X_2 - 0.5979X_3 - 0.2568X_1X_2 + 0.0667X_1X_3 - 0.0430X_2X_3 + 0.1913X_1^2 - 0.1241X_2^2 - 0.5410X_3^2 \quad \text{Eq. (14)}$$

Once again, similarly to Ri17, the ultrasonic intensity was the factor with highest influence, rendering an optimal point at 80%, 5 minutes and 27% of ethanol, rendering 2.29 g/100g of dry residue. The 3D charts can be seen in the first row of **Table 17**, confirming what was sought for the optimal point, namely the higher influence of ethanol percentage in the total yield in dry residue. R_2 and R_3 only allowed for linear models, not allowing to obtain satisfactory R^2 and thus were not optimized.

Table 17. Response 3D charts of the UAE extracted BS sample at the optimal points.

Sample		Power vs. Time	Power vs. Solvent	Time vs. Solvent
Ri216	R_1 – DR			

3.2.3 Global results

Overall, in terms of optimization, the maceration extractions seemed to be the best candidates for optimization and the ones with more robust results. It is clear that the amount of ethanol showed higher influence in the yields of dry residue for the maceration extractions, while temperature and time also showed some influence when obtaining better EC_{50} results for RP. Considering TP, the influence of total phenols is quite case specific.

The UAE were somewhat harder to obtain satisfactory values for optimization, but, when possible, once again, the percentage of ethanol seemed to be a determinant factor in the variation of yields.

4. Optimization study – CBBC Contribution

CBBC started on the optimization of the natural preservative by defining the optimum conditions for their extraction. With this respect, Response surface methodology (RSM) is a technique for optimizing a process that includes sophisticated calculations or laborious experiments. This

method creates an appropriate experimental design that incorporates all of the independent variables and makes use of the data input from the experiment to arrive at a conclusion. A regression analysis, that is well-designed, is based on the controlled values of independent variables. Hence the importance of the preliminary study. The preliminary study allows screening of appropriate independent variables and determining their optimum experimental domain for an appropriate experimental RSM design. For the current study, three factors were selected: temperature of extraction, time of extraction and alcohol percentage. Details are listed in **Table 18**.

Table 18. Factor domains selected for the preliminary study.

Percentage of Ethanol	Extraction time	Extraction temperature
10%	5 min	30°C
20%	15 min	40°C
30%	30 min	50°C
40%	60 min	60°C
50%	120 min	-

Three organs from the selected varieties (Karkoubi and Bejaui) were assessed for the preliminary study, seeds, peels and fiber were freeze-dried and ground in a Mettler AE 200 blender. The yield of each organ is represented in **Figure 2**.

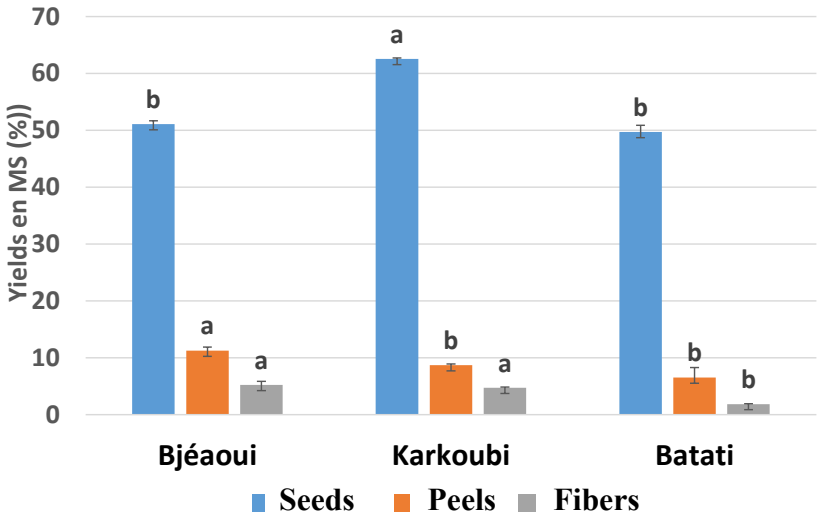


Figure 2. Yield in percentage of dry weight from seeds, peels and fiber of Bejaoui, Karkoubi and Batati samples. 1). Means ± SD of three replicates from the same tissue (seeds, peels and fibers) followed by the same letter are not significantly different at $P < 0.05$.

Considering Bejaoui samples, results of each organ are presented in **Figure 3**.

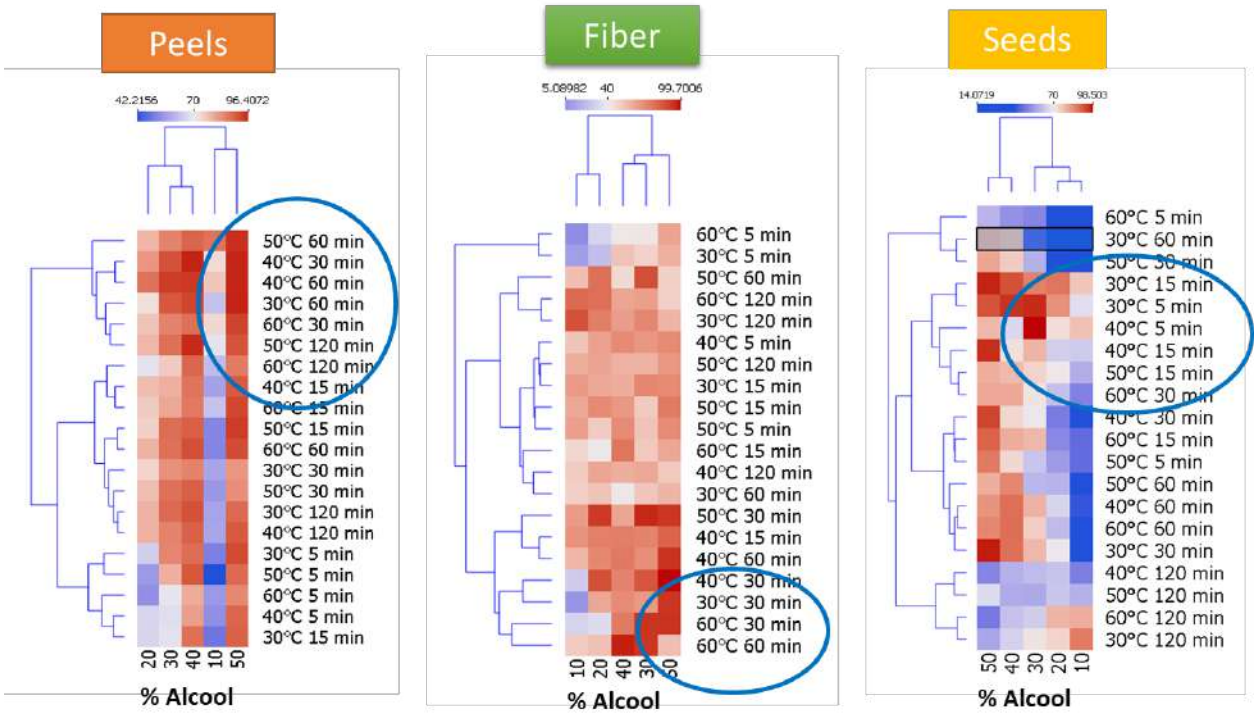


Figure 3 Preliminary study results concerning time of extraction, temperature of extraction and alcohol percentage for the seeds, peels and fiber of Bejaoui variety.

Analysis of **Figure 3** highlighted significant differences between the three organs as the extraction conditions varies greatly depending on the organs nature. Accordingly, it may be concluded from figure the optimum range of extraction that were detailed in **Table 18**.

Table 19. Summary of optimal conditions for extraction of natural preservatives from pumpkin variety Bejaoui (fibers, peels, and seeds).

	Alcohol percentage	Temperature	Time
Fibers	50%	30°C - 40°C	30 min
Peels	50%	40°C - 50°C	30 min – 60 min
Seeds	30%	30°C - 40°C	5 min – 15 min

The same protocol was applied to Karkoubi samples and mains results were presented in **Table 20** and **Figure 4**.

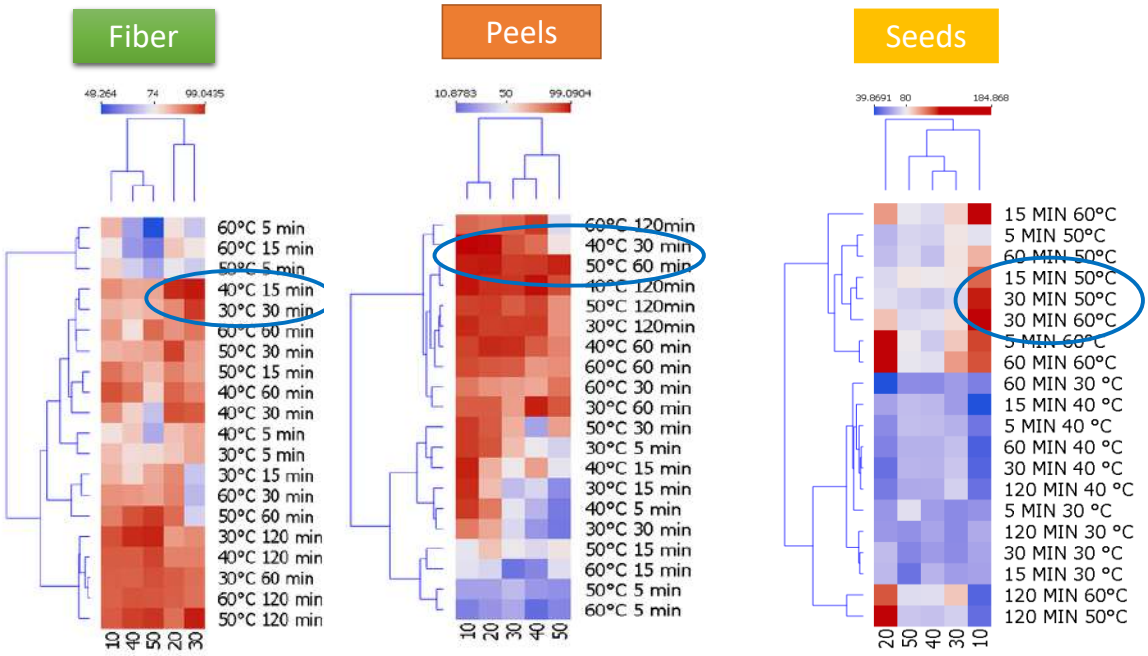


Figure 4. Preliminary study results concerning time of extraction, temperature of extraction and alcohol percentage for the seeds, peels and fiber of Karkoubi variety.

Table 20. Summary of optimal conditions for extraction of natural preservatives from pumpkin variety Karkoubi (fibers, peels and seeds).

	Alcohol percentage	Temperature	Time
Fibers	30%	30°C - 40°C	5 min – 15 min
Peels	10% - 20 %	40°C - 50°C	30 min – 60 min
Seeds	10%	50°C - 60°C	15 min – 30 min

5. Prospection

Once obtained the mathematical models (by RSM) of the dependent variables used in the optimization of the extraction of the preserving compounds, the next steps are to evaluate the scaling up processes (Deliverable 2.5) and the economic and technology involved (Deliverable 2.6).