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

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Executive Summary

Background	Stabilizing phenolic compounds is crucial due to their limitations in solubility and stability. Phenolic compounds possess beneficial properties such as antioxidant and anti-inflammatory effects, but their limited aqueous solubility hampers their bioavailability. By encapsulating them, their solubility and stability can be significantly improved, leading to enhanced absorption and bioavailability. Additionally, encapsulation protect phenolic compounds from degradation caused by factors like heat, light, and oxidation, thereby preserving their structural integrity and activity. Encapsulation systems also offer formulation flexibility, allowing customization for controlled release, tissue targeting, and compatibility with other ingredients. Moreover, encapsulation provide improved stability during storage, preventing aggregation or separation of phenolic compounds and maintaining their desired concentration and activity. Meanwhile, comparing different combinations of enpsulating components through experimental design is important for several reasons. Firstly, it allows for the identification of the optimal formulation that provides the desired characteristics and stability for a particular application. Secondly, it helps understand the impact of different phase combinations on the solubility, encapsulation efficiency, and release properties of active compounds. Furthermore, it ensured compatibility and preventing instability. Thus, since encapsulation procedures could be quite expensive for the companies, the stability of the free form extract was also studied and evaluated to guarantee its stability throught its application in the punkim pulp.
Objectives	The encapsulation of squash-refined extract into an emulsion-based delivery system will be optimized using an experimental design plan. For this purpose, different technologies of encapsulation will be adopted including the use of Ultrathorax, sonication and magnetic stirring apparatus. Different ingredients will be screened mainly coating materials ratio and compositions. The obtained emulsions will be compared based on their total phenolic contents as well as their antioxidant activities. In addition, the free extract obtained from de Butternut Squash peels (Task 2.2) was evaluated to verify its stability over time stored with heat.
Methodology	Squash landraces Batati (NGBTUN 746) genotype was studied for their peels. The encapsulation of the refined peels extract was optimised as detailed bellow. ***Encapsulation protocol.*** Maltodextrin (X1) and gum arabic (X2) were used as coating materials in different ratios ranging from 0 to 0.8. 10 g of coating material was dissolved in 90 g of hot distilled water (40°C) to form a 10% coating material solution. The solution was mixed using magnetic stirring for 1 hour and then stored at 4°C for 24 hours to complete hydration. Once the coating solutions were prepared, the coating material solution was mixed with the concentrated phenolic extract (X3) and homogenized using magnetic stirring for 60 minutes at 60°C, followed by ultra-turax stirring for 5 minutes at 11,000 rpm. To ensure complete homogenization, the samples were subjected to sonication for 5 minutes.

Mixture design plan. To evaluate the impact of different rates of maltodextrin and arabic gum on phenolic compounds and their antioxidant activities, a comprehensive factorial design (3^2) was employed. The design consisted of five replicates at the center point to generate surface plots and estimate the pure error of multiple regression models, resulting in a total of 13 sample preparations performed in a standard order.

Table 1. Three components axial screen matrix, with X1: Maltodextrin, X2: Arabic gum and X3: extract concentration ranging from 0.2 to 0.5.

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 inserted into the software NemrodW (LPRAI 2000). The obtained experimental data were then fitted to the selected regression model to understand the relationship between each factor and the various responses. The significance of these correlations was assessed using t-statistic at a 95% confidence interval. Non-significant terms (P-value > 0.05) were removed from the initial equation, and the data were refitted to the refined model. The quality of the mathematical models was evaluated using response surface methodology (RSM) and analysis of variance (ANOVA) based on the F-test, probability values (P-values) for lack-of-fit, the percentage of total explained variance (R^2), and the adjusted determination coefficient (R^2_{adj}). These measures provided insights into the variability in the observed response values, which could be attributed to the experimental factors and their linear and quadratic interactions. To maximize the polyphenol content and antioxidant activity, a simultaneous optimization of the desirability function was conducted.

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).

Stability. To verify the stability of the free extract obtained from de Butternut Squash peels, it was incubated in an oven at 60 °C for 21 days, being evaluated at 0, 7, 14, and 21 days of storage. Samples were assessed for antioxidant capacity

	through DPPH free radical scavenger (DPPH), reducing power (RP) and total phenolic content (TPC). For the RP, it was evaluated considering the ability of the extracts to reduce $Fe3^{+}$, being the results expressed as IC_{50}, referring to the extract concentration necessary to inhibit the iron reduction by 50%, in µg/mL. The TPC was assessed by the Folin–Ciocalteu (F–C) methodology, and results were expressed in gallic acid equivalents, in milligrams per gram of extract (mg/g). And, the DPPH assay, that measures the radical scavenging activity of the 2,2-Diphenyl-1-picrylhydrazyl free radical, was expressed as IC_{50}, that is the extract concentration necessary to inhibit the oxidation by 50%, in µg/mL. 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	***Comparative study.*** An experimental design, mixture design type, was elaborated to encapsulate Batati peels refined extracts. Maintaining the highest level of bioactive compounds in the product is of primary interest in the encapsulation process. Besides, biological activity of the products might also be interested. Main results on the runned thirteen experiences were detailed in table 2.

Table 2. Three components axial screen matrix and the values of the experimental responses for total phenolic content (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	22	33.46
2	0.0000	1.0000	0.0000	37.24	58.63
3	0.0000	0.0000	1.0000	24	51.39
4	0.6667	0.3333	0.0000	51.11	74.54
5	0.3333	0.6667	0.0000	28.22	46.04
6	0.6667	0.0000	0.3333	34.8	46.62
7	0.3333	0.3333	0.3333	52.38	67.59
8	0.0000	0.6667	0.3333	35.57	60.51
9	0.3333	0.0000	0.6667	30	50.81
10	0.0000	0.3333	0.6667	25	40.4
11	0.6667	0.1667	0.1667	38.05	58.91
12	0.1667	0.6667	0.1667	32.84	54.14
13	0.1667	0.1667	0.6667	39.82	61.81

Based on the information presented in table 2, it has been observed that the three factors under investigation, namely Maltodextrin (X1), gum arabic (X2), and concentrated phenolic extract (X3), exert a significant influence on both the phenolic content and antiradical activity. Upon comparing the total phenolic content (TPC) of the 13 encapsulated extracts that were studied, a considerable degree of variability becomes evident, as TPC values ranged from 22 to 52 mg GAE/g DR in experiment 1 and 7, respectively. This signifies that the choice of factors employed during the

encapsulation process plays a pivotal role in determining the phenolic content of the extracts.

Furthermore, it is noteworthy that the antiradical activity of the encapsulated extracts appears to be highly sensitive to the ratio and compositions of the coating materials utilized. Notably, the efficiency of the extracts in scavenging DPPH radicals varied significantly across different systems. For instance, the system incorporating the lowest concentration of extract in conjunction with maltodextrin and Arabic gum demonstrated a remarkably high DPPH scavenging ability efficiency, reaching an impressive 74.5%. Conversely, in the system that contained the lowest extract concentration without the presence of Arabic gum, the scavenging ability was limited to only 33%.

The data from table 2 underscore the significant impact of the studied factors (Maltodextrin, gum arabic, and concentrated phenolic extract) on both the phenolic content and antiradical activity of the encapsulated extracts. Moreover, the results highlight the substantial variability in total phenolic content and the sensitivity of antiradical activity to the composition and ratio of coating materials utilized. This suggests that careful consideration of these factors is crucial in order to optimize the encapsulation process and enhance the functional properties of the encapsulated extracts.

Mixture design plan. The use of maltodextrin and arabic gum was one of the main factors of the encapsulation process. It was effective to the maintenance of bioactive compounds content and activity in the final product. The mixture compounds effects on encapsulating polyohenols (Table 3) showed that individual factor coefficients were significant parameters in since their *p*-values were statistically lower than 0.05, especially the refined extract concentration. Whereas for the antiradical activity, individual factor exhibited a highly significant antioxidant effects while their interactions were not significant.

Table 3. Mixture compounds effects

	Total phenolic content				
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	41.204	29.3	11.817	3.49	*
b2	30.848	29.3	11.817	2.61	*
b3	187.002	246.7	38.279	4.89	**
b12	51.012	2.11	18.192	2.8	*
b13	-297.878	199.53	93.683	-3.18	*
b23	-227.759	199.53	93.683	-2.43	*

Antiradical activity					
	Coefficient	**F.Inflation**	**Ecart-Type**	**t.exp.**	**Signif. %**
b1	29.858	29.3	8.148	3.66	**
b2	49.408	29.3	8.148	6.06	***
b3	157.296	246.77	26.396	5.96	***
b12	11.672	2.11	12.545	0.93	38.6%
b13	-134.566	199.53	64.599	-2.08	7.4%
b23	-117.775	199.53	64.599	-1.82	10.9%

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

For TPC: Y TPC = 41,2 * X1 + 30,84 * X2 + 187,0 * X3 + 51,01 * (X1*X2) - 297,87* (X1*X3) -227,75* (X2*X3)

For antioxidant activity: Y DPPH = 29,85 * X1 + 49,40 * X2 + 157,29 * X3

These equations were transposed into isoprenic curves as exhibited in figure 1.

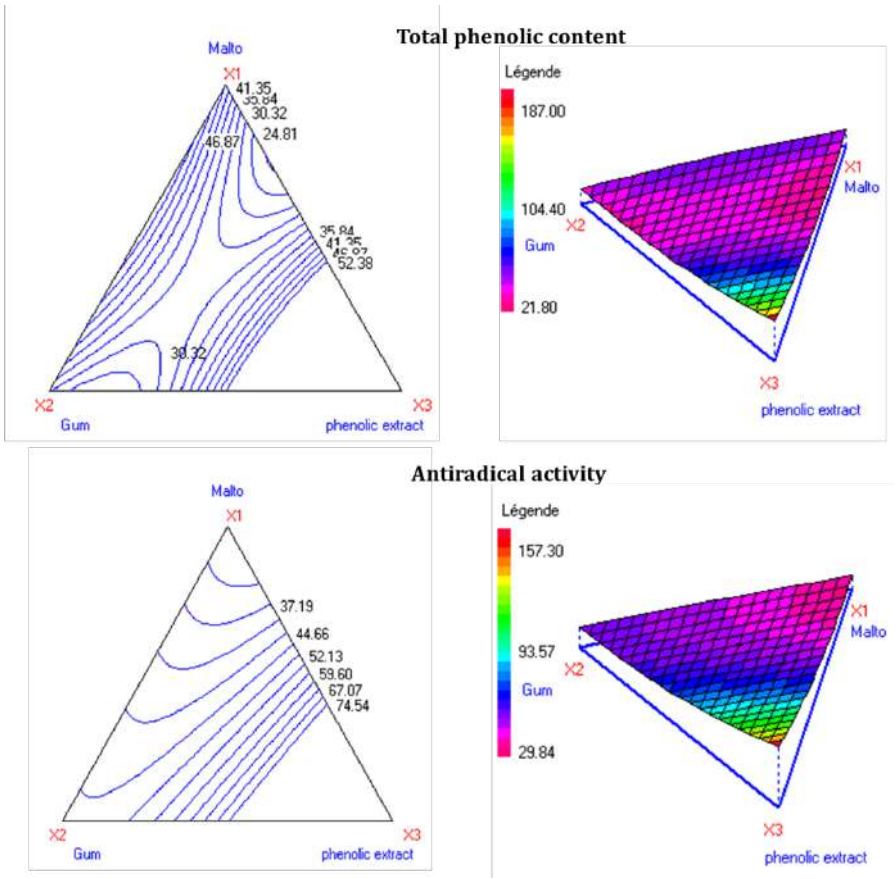

Figure 1. Isoresponses and mixture plot.

All together, the adopted software concluded that the targeted limit can be achieved with 91% desirability using a mixture consisting of

23.8% Maltodextrin+ 27.7% Arabic gum+48.5% refined extract

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

Table 4. Eperimental vatlidation of the obtained formula.

	Predicted values	Experimental values
TPC	47.46	46.01
DPPH test	66.54	64.85

Stability results. The results are presented in table 5.

Table 5. Changes in the antioxidant activity and the total phenolic content of the free extract during the storage time at 60 °C.

Days of storage	DPPH (ug/mL)	TPC (mg/g)	RP (ug/mL)
0	1515 ± 46 [a]	219 ± 7 [d]	1103 ± 25 [a]
7	159 ± 4 [b]	324 ± 8 [c]	300 ± 15 [b]
14	109 ± 2 [b,c]	460 ± 12 [b]	264 ± 7 [c,d]
21	89 ± 8 [c]	567 ± 2 [a]	242 ± 21 [d]

Means of the same column followed by different letters are statistically different according to Student's t-test at $p < 0.05$.

During storage at high temperatures, the extract not only remained bioactive but also had an increase in its antioxidant properties and total phenolic compounds. The IC_{50} decreased in almost 17 times for DPPH and 5 times for RP from time 0 to time 21, while the total phenolic content increased from 219 ± 7 to 567 ± 2 mg/g, at respective times.

Also, the antimicrobial activity was also tested, against five gram-negative bacteria, three gram-positive bacteria, and two fungi, being determined the minimum inhibitory concentration (MIC) and the minimum bactericidal (MBC) or minimum fungicidal (MFC) concentrations, up to the maximum tested concentration of 10 mg/mL.

As seen in table 6, the antibacterial activity improved between 0 and 7 days: the extract presented activity against *Enterobacter cloacae* (MIC 10 mg/mL) at day 7, while didn't present at day 0; also, the MIC decreased from 10 to 5 mg/mL against *Salmonella enterica*, *Bacillus cereus*, and *Staphylococcus aureus*; and, the activity against *Yersinia enterocolitica* and *Listeria monocytogenes* remained (MIC 5 mg/mL). While between day 7 and day 14, the antibacterial activity slightly decreased, presenting MIC values of 10 mg/mL against the same 6 bacteria inhibited on day 0.

Table 6. Changes in the antibacterial activity changes of the free extract during the storage time at 60 °C.

	0		7		14		21		Streptomicin 1 mg/mL		Methicilin 1 mg/mL		Ampicillin 10 mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria														
Enterobacter cloacae	>10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	10	>10	10	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	10	>10	5	>10	10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	5	>10	5	>10	10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria														
Bacillus cereus	10	>10	5	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	5	>10	5	>10	10	>10	10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	10	>10	5	>10	10	>10	10	>10	0.007	0.007	0.007	0.007	0.15	0.15

On the other hand, as shown in table 7, the antifungal activity decreased during the experiment, going from protected against the two fungi tested, for one fungus in day 7, and no activity up to 10 mg/mL to days 14 and 21.

Table 7. Changes in the antifungal activity of the free extract during the storage time at 60 °C.

	0		**7**		**14**		**21**		**Ketoconazole**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus brasiliensis	5	>10	10	>10	>10	>10	>10	>10	0.06	0.125
Aspergillus fumigatus	10	>10	>10	>10	>10	>10	>10	>10	0.5	1

This great effect of the temperature in the improvement of the bioactivities can be resulting of a compound change. Similar results were seen in papaya, in which the fermentation processes increased the TPC and antioxidant properties (Leitão, et al., 2023).

Reference

Leitão, M., Ferreira, B., Guedes, B., Moreira, D., García, P. A., Barreiros, L., & Correia, P. (2023). Screening of Antioxidant Effect of Spontaneous and Bioinoculated with Gluconobacter oxydans Fermented Papaya: A Comparative Study. Fermentation, 9(2), 124.