biology and life sciences forum

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Proceedings

Novel flours from *Neltuma affinis* fruit for improving technological quality and alveolar structure of gluten-free bread†

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† Presented at the 4th International Electronic Conference "Focus on Sustainable Food Systems: Current Trends and Advances"; Available online://foods2023.sciforum.net.

Abstract: The incorporation of alternative flours in the formulation of gluten-free (GF) baked goods has the potential to improve their nutritional and technological characteristics. In this sense, the addition of novel flours from the grinding of endocarp-seeds (ES), and exocarp-mesocarp (EM) of *Neltuma affinis* fruit in the design and formulation of GF bread was studied. The aim of this study was to evaluate the effects of different levels of ES, EM and water hydration (WH) on the alveolar structure, pH and colour of GF bread. A Box-Behnken response surface design was used. EM and ES were found to have a negative effect on pH. The alveolar structure of the bread was influenced by the level of hydration, with larger alveoli being observed in the bread crumbs at higher levels of hydration; and greater density at lower levels. The three factors studied showed a positive influence on the a* coordinate of colour of the crumb. In conclusion, the incorporation of alternative flours from *N. affinis* could provide a valuable approach to improve the nutritional profile and sustainability of an underappreciated species.

Keywords: formulation of bakery products; *Ñandubay*; alternative raw material; Box–Behnken experimental design

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Published: date

1. Introduction

Celiac disease and non-celiac gluten sensitivity are conditions that affect 1.4% of the world's population. Currently, a gluten-free diet (GF) is the only safe and effective treatment. The low fibre content of the GF diet is mainly attributed to the limited consumption of whole grains and the low fibre content in GF products which are mainly made from refined starches and/or flours. The introduction of high-fibre alternative flours in bakery products has the potential to improve both their technological and nutritional quality, having the latter an impact in consumer´s health. In addition, there is a global trend to guarantee sustainability in the development of new ingredients and/or additives, emphasizing the sustainable use of native resources, adding value to raw materials and taking advantage of the nutrients of available agro-industrial resources [1]. In this context, the addition of novel flours obtained from the grinding of the endocarp-seed (ES) and exocarp-mesocarp (EM) of the fruit of *Neltuma affinis*, an autochthonous Argentine species, was investigated in the design and formulation of GF bread. These flours are rich in fibre, protein and polyphenols. The aim of this study was to evaluate the effects of different

levels of ES, EM and water hydration (WH) on the alveolar structure, pH and colour of GF bread.

2. Materials and Methods

2.1. Materials

Fractions ES and EM were obtained from dry grinding *of N. affinis* fruits (see section 2.2). The rice flour was supplied by Cooperative Villa Elisa S.A. (Entre Ríos, Argentina). Corn starch (Maizena, Argentina), salt (Celusal, Argentina), sunflower oil (Natura, Argentina), sugar (Lesdesma, Argentina), hydroxypropylmethylcellulose (Methocel K4M, Dow Chemical, USA) and dehydrated yeast (LEVEX, Argentina) were purchase from the market.

2.2. Obtaining flour from the fruit of Neltuma affinis

The fruits of *N. affinis* were collected in the ecological reserve of Gualeguaychú, Entre Ríos, Argentina, spanning the months from November to April, precisely during their peak ripening phase. Subsequently, they underwent thorough a process of washing, disinfection, and drying using a plate dehydrator (FA 10-MZ, COBOS, Argentina) at 50°C for 4 hours. Following this, they were stored at -12°C until ready for use. The grinding process was carried out using a mill (HC-1000 Y, Arcano, China). In this procedure, portions of 100 grams of fruits were processed for 15 seconds. The resulting mixture was then sieved through various stainless steel meshes (A.S.T.M N° 5, 7, 10 and 20; Zonytest, Argentina), enabling the extraction of two distinct fractions: ES and EM flour, presenting a particle size smaller than 840 μm.

2.3. Process of making GF breads

Using a stand mixer (AEB-105, Alhias, China), according to the experimental design, different proportions of EM (0-20%), ES (0-20%), rice flour (0-50 %), corn starch (0-50%), salt (2%), sugar (5%), hydroxypropylmethylcellulose (0.5%), sunflower oil (6%), dehydrated yeast (3%) and water (70-160%) were mixed. This mixture of ingredients was kneaded for 2 minutes at the lowest speed setting (1-5). Subsequently, portions of dough weighing 30g for each formulation were placed into aluminium melds (40mm bottom diameter, 60mm top diameter and 60mm height) and fermented at 30°C and 90% relative humidity for the optimal fermentation time (OFT) of each formulation (see section 2.4). After fermentation time, the doughs were baked at 180°C for 30 minutes in an electric convection oven (Pauna, Beta 21L, Argentina). Finally, the loaves were cooled at room temperature for 1 hour and stored in polypropilene sealed bags for 24h until physicochemical meassurements were carried out to preven dehydration.

2.4. Determination of optimum fermentation times (OFT).

The OFT of each system was calculated using the Boltzmann sigmoid equation, according to the methodology reported by Ojeda et al. [2].

2.5. Physicochemical characterization of GF breads

The colour of crumb bread was assessed using a colorimeter (MiniScan EZ, HunterLaB, USA) under the D65 illuminator and with an observer angle of 10°. The results were expressed in the CIE Lab* colour space. Where, L* represent luminosity (ranging from white at 100 to black at 0), a* chromatic coordinate indicates the red- (positive) green (negative) component, and b* denotes denote the yellow- (positive) blue (negative) component.

The pH of the bread was determined using a pHmeter (SA270 ORION TM, USA) on a solution of bread and destilled water. The solution was prepared using a proportion of 1:10 and homogenized (MX-S vortex mixer, Dragon Lab, China) for 5 minutes.

The alveolar structure of crumb bread was analysed in slices of bread (10 mm of thickness) using a scanned image (HP, Ink Tank 315, China) with 1200 dpi of resolution. The images were processed using the Image J software (V 1.8.0, National Institutes of Health, USA) with the Otsu algorithm, following the methodology described by Genevois et al. [3]. The parameters reported are cell size (mm^2; CS) and cell density (number of alveoli/mm^2; CD)

All analytical determinations were performed in triplicate from independent samples.

2.6. Experimental design and statistical analysis

Utilizing a Box-Behnken experimental design, the effect of independent variables on the response variables was examined. The design was composed of three factors (ES; EM; WH) with three levels (-1; 0; +1) and three central points (0; 0; 0). The levels for each factor were as follows: ES and EM at 0, 10 and 20 g/100g, and WH at 70, 115 and 160 ml/100g. The experimental data was fitted to a second-degree polynomial function (Equation 1):

$$\Psi = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{11}x_{12} + B_{22}x_{22} + B_{33}x_{32} + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{23}x_2x_3. \quad (1)$$

Where Ψ is the dependent variable, x_1, x_2 and x_3 are the independent variables; B_0 is the value of the fitted response at the central point of the design; B_1, B_2 and B_3 are the linear regression coefficients; B_{11}, B_{22} and B_{33} are the quadratic regression coefficients; and B_{12}, B_{13} and B_{14} are the interaction coefficients [3].

The adequacy of the model was evaluated using the coefficient of determination (R^2>80%), adjusted R^2 (R^2_{adj} >70%), lack of fit test (p≥0.05) and the Durbin-Watson statistic (>1).

Statistical analysis was performed through ANOVA for a significance level (α) of 0.05 followed by a *post-hoc* LSD Fisher test to determined differences among mean values. All statistical analysis was performed using the Statgraphics Centurion XVI software (16.1.03. USA).

3. Results and Discussion

From the dry grinding of the *N. affinis* fruits two fractions were obtained: ES flour and EM flour, presenting a yield of 47.3% and 52.7%, respectively (Figure 1).

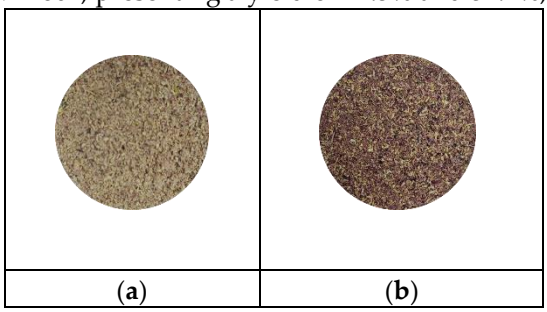

Figure 1. Images of *N. affinis* flour. (**a**) endocarp-seed flour; (**b**) exocarp-mesocarp flour.

The OFT values ranged from 10.49 to 66.36 minutes; systems with lower hydration levels exhibited shorter fermentation times.

Table 1 presents the results of colour, pH and alveolar structure for each system from the experimental design. The regression coefficients of the response variables adjusted to the model and statistical parameters for assessing adequacy of the model are stated in Table 2.

The crumb lightness values ranged from 33.83 to 77.16 in the three studied factors, presenting a significant positive effect (p<0.05) on this parameter the linear and quadratic terms of the EM fraction. Moreover, the formulations with higher proportions of *N. affinis*

flours presented lower L* values in comparison to the control bread (System 13). The chromatic coordinate a*, presented values from -1.38 to 19.90, and was fitted to the proposed model (R^2_{adj} 96.24). The linear terms of the three factors studied demonstrated a significant positive effect ($p<0.05$), being the coefficient of EM with the highest impact in the equation (Table 2). However, the interaction between ES and EM, and the quadratic term of EM, exerted an antagonistic effect on this parameter. In this context, Figure 2 illustrates the variation in colour among the different GF bread formulations. Those systems with higher amounts of EM showed the highest values in the chromatic coordinate a*, indicating a redness colour in the loaves due to the inherent colour characteristic of *N. affinis* flour.

On the other hand, the chromatic coordinate b* ranged values from 6.83 to 20.05. In this parameter, both fractions of *N. affinis* flours demonstrated a significant antagonistic effect, with ES having a positive impact and EM yielding a negative influence. Furthermore, the quadratic terms of EM and WH had a positive effect, whereas the ES-EM interaction and the quadratic term of ES negatively affected this coordinate. Correspondingly, formulations with higher proportions of ES presented greater lightness (L) and higher values in the chromatic coordinate b*, suggesting a more yellowish tone (Figure 2).

Figure 2. Image of GF breads sliced with substitution of endocarp-seed flour or exocarp-mesocarp flour from *N. affinis*, and different hydration levels from experimental design. * represents central point of experimental design; ** represent control bread.

The pH value was negatively affected by the EM fraction. The pH exhibited a negative correlation with the percentages of ES and EM in the GF bread formulations. The control formulation displayed the highest pH value (Table 2). This phenomenon can be attributed to the particular composition of EM, as well as due to the denaturation of proteins during cooking and the release of acidic compounds during fermentation, which leads to a decrease in the pH values of the samples [3].

Regarding alveolar structure, WH had a notable impact in GF bread formulation with the addition of *N. affinis* flours. A significant positive effect was observed on CS; meanwhile a negative effect was presented in CD. Additionally, the interaction between ES-WH demonstrated a significant positive effect on CD. As shown in Figure 2, the systems with lower hydration levels corresponded to a crumb with smaller alveolar sizes. A higher hydration level could potentially disrupt the alveolar structure through the collapse of gas bubbles, resulting in larger cell sizes and less uniform crumb. Conversely, very low hydration levels might impede the development of gas bubbles, leading to denser and more uniform crumb [3; 4]. These results agree with those found by Tsatsaragkou et al. [5], who report that water significantly influences the structural properties of GF breads enriched with carob seed flour; where increased water content leads to an open cell structure.

Table 1. Experimental design with the independent and dependent variables of the GF bread formulations.

Systems	Independent Variables	Dependent Variables

	ES[1]	EM[2]	WH[3]	L[4]	a*[5]	b*[6]	pH	CS[7]	CD[8]
1*	10	10	115	44.31 ± 0.57^{d}	16.33 ± 0.36^{fg}	11.52 ± 0.28^{f}	4.21 ± 0.02^{abc}	0.81 ± 0.07^{cde}	0.16 ± 0.01^{bc}
2	20	0	115	52.81 ± 0.59^{g}	9.43 ± 0.81^{c}	20.05 ± 0.70^{i}	4.39 ± 0.02^{cd}	0.87 ± 0.11^{e}	0.08 ± 0.01^{a}
3	0	10	160	46.65 ± 0.75^{ef}	13.43 ± 0.35^{d}	7.63 ± 0.25^{abc}	4.31 ± 0.02^{bc}	0.77 ± 0.01^{cde}	0.07 ± 0.03^{ab}
4	20	10	160	44.34 ± 1.14^{d}	17.37 ± 0.16^{h}	13.05 ± 0.43^{g}	4.10 ± 0.00^{abc}	0.72 ± 0.04^{bcd}	0.15 ± 0.05^{bc}
5	10	20	160	36.48 ± 1.06^{ab}	19.57 ± 0.80^{i}	8.17 ± 0.55^{bcd}	4.03 ± 0.06^{ab}	0.69 ± 0.03^{bc}	0.14 ± 0.01^{abc}
6	10	0	160	65.05 ± 0.02^{h}	4.97 ± 0.13^{b}	18.14 ± 0.23^{h}	4.81 ± 0.02^{6}	0.85 ± 0.02^{de}	0.12 ± 0.03^{abc}
7*	10	10	115	44.12 ± 0.43^{d}	16.15 ± 0.31^{fg}	11.24 ± 0.54^{f}	4.20 ± 0.00^{abc}	0.77 ± 0.10^{cde}	0.14 ± 0.04^{abc}
8	0	20	115	36.72 ± 0.79^{b}	19.90 ± 0.28^{i}	6.83 ± 0.25^{a}	4.08 ± 0.02^{c}	0.63 ± 0.04^{b}	0.15 ± 0.01^{abc}
9	20	20	115	33.83 ± 0.77^{a}	19.64 ± 0.40^{i}	10.04 ± 0.44^{e}	3.99 ± 0.02^{a}	0.71 ± 0.08^{bc}	0.18 ± 0.02^{c}
10	10	20	70	41.60 ± 0.34^{c}	16.54 ± 0.17^{g}	8.41 ± 0.25^{cd}	4.03 ± 0.06^{ab}	0.25 ± 0.09^{a}	1.26 ± 0.06^{f}
11	0	10	70	48.74 ± 0.67^{f}	13.69 ± 0.25^{d}	8.72 ± 0.10^{d}	4.31 ± 0.04^{bc}	0.27 ± 0.01^{b}	1.16 ± 0.10^{e}
12	10	0	70	68.82 ± 0.01^{i}	4.99 ± 0.32^{b}	19.44 ± 0.33^{i}	4.82 ± 0.04^{e}	0.21 ± 0.01^{a}	1.23 ± 0.03^{f}
13	0	0	115	77.16 ± 2.39^{j}	-1.38 ± 0.05^{a}	7.43 ± 0.55^{ab}	5.29 ± 0.02^{f}	0.62 ± 0.11^{b}	0.13 ± 0.02^{abc}
14	20	10	70	45.90 ± 0.54^{d}	15.17 ± 1.05^{e}	13.28 ± 0.69^{g}	4.18 ± 0.04^{abc}	0.27 ± 0.02^{a}	1.06 ± 0.05^{d}
15*	10	10	115	49.95± 0.96^{d}	15.66 ± 0.26^{ef}	10.98 ± 0.63^{f}	4.40 ± 0.28^{de}	0.72 ± 0.09^{bcd}	0.13 ± 0.02^{abc}

*replicates of the central point of the experimental design.[1]Endocarp-seed flour of the fruit of *N. affinis*, expressed as % in the GF bread formulation; [2]Exocarp-mesocarp flour of the fruit of *N. affinis*, expressed as % in the GF bread formulation; [3]Water hydration, expressed as % in the GF bread formulation; [4]Bread crumb lightness (white: 100; black: 0); [5]Chromatic coordinate that represents the variation between red and green of the bread crumb; [6]Chromatic coordinate that represents the variation between yellow and blue of the bread crumb; [7]Cell size, expressed as mm^2; [8]Cell density, expressed as alveoli/mm^2.

Different letters for the same parameter indicate significant differences ($p<0.05$) with a confidence level of 95%

Table 2. Regression coefficients of the adjustment to the second degree polynomial model of the response variables for the formulation of GF bread.

	Regression coefficients					
	L	**a***	**b***	**pH**	**CS**	**CD**
Constants	12807.6000	-3.1608	17.7197	4.9975	-1.6697	4.9270
A: ES	-237.9520*	0.3975*	0.8113*	-0.0289	0.0162	-0.0054
B:EM	9.4891*	1.5690*	-0.5028*	-0.0978*	0.0203	-0.0061
C: WC	-208.0390*	0.0688*	-0.1251	0.0035	0.0335*	-0.0705*
AA	-6.5632*	-0.0038	-0.0153*	-0.0001	-0.0002	-0.0004*
AB	0.0779*	-0.0277*	-0.0235*	0.0020	-0.0004	0.0002

AC	2.6773*	0.0013	0.0004	-0.0001	-0.0001	0.0001*
BB	6.6280*	-0.0381*	0.0137*	0.0018	-0.0003	0.0003*
BC	-1.5656*	0.0016*	0.0005	0.0001	-0.0001	-0.0001
CC	0.9190*	-0.0003	0.0004*	-0.0001	-0.0001*	0.0002*
R^2	49.9705	**98.6595**	93.3162	**95.7408**	**96.8661**	**99.7637**
R^2_{adj}	0.0000	**96.2467**	81.2853	**88.0742**	**91.2250**	**99.3383**
Lack of fit	0.0000	**0.0489**	0.0120	**0.4393**	**0.2394**	**0.0900**
DW	2.5591	**2.3528**	2.4612	**1.3950**	**1.8717**	**2.1902**

* significant coefficients of the model ($p \leq 0.05$).

4. Conclusions

The level of water hydration and the addition of flours from the grinding of *N. affinis* fruit in the formulation of GF bread give as result loaves with colours and honeycombed crumbs, qualities that appeal to consumers of GF products. The ES factor exhibited a positive impact on the b* colour coordinate. Meanwhile the EM factor had a positive and negative effect on a* coordinate and pH, respectively. Furthermore, WH had a positive effect on cell size, but conversely, a negative impact on cell density of bread crumb.

In conclusion, the addition of ES and EM with the appropriate WH is very promising as an alternative approach to produce GF bread with improved technological characteristics. The use of alternative flours in GF bread formulation, such as that derived from *N. affinis*, could serve as a valuable tool to improve the nutritional profile and sustainability of an underestimated species.

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