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Application of Box-Behnken Design to the Study of Fatty Acids and Antioxidant Activity from Enriched White Bread

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Este estudo avaliou a composição centesimal de ácidos graxos e a atividade antioxidante total de pães utilizando um design Box-Behnken composto de quinze experimentos e três fatores. Os pães estudados tiveram parte do seu tempo de cozimento alterado, assim como parte da sua farinha de trigo substituída por sementes de chia e folhas de cenoura. Os resultados de capacidade antioxidante de cada experimento foram superiores aos obtidos para um pão controle (495,33 μmol TEAC g^{-1} para ensaio DPPH e 413,85 μmol TEAC g^{-1} para ensaio FRAP), mostrando níveis de aumento de até 97 e 102% para os ensaios DPPH e FRAP, respectivamente. Os pães modificados também mostraram aumentos de até 216,69 e 18,88% na quantidade de ácido alfa-linolênico e na razão PUFA:SFA, respectivamente, em relação ao mesmo pão controle (este com 34,03 mg g^{-1} de ácido alfa-linolênico e razão de 2,86), bem como máxima redução de 70,01% na razão omega-3:omega-6 (15,59 para o pão controle).

This work determined the proximate/fatty acid composition and the antioxidant activity of breads using a Box-Behnken design composed of fifteen experiments and three factors. The studied breads had altered baking times and the wheat flour was partially substituted by chia seeds and carrot leaves. Total antioxidant capacity results were superior to those obtained from control bread (495.33 μmol TEAC g^{-1} for DPPH assays and 413.85 μmol TEAC g^{-1} for FRAP assays), showing increased levels of 97 and 102% for DPPH and FRAP assays, respectively. The addition of chia seeds and carrot leaves led to maximal alpha-linolenic acid increase of 216.69% and maximum PUFA:SFA ratio increase of 18.88% in relation to control bread (this with alpha-linolenic acid amount of 34.03 mg g^{-1} total lipid and a 2.86 ratio value), as well as a maximum omega-3:omega-6 ratio reduction of 70.01% (15.59 ratio value for control bread).

Keywords: bread, Box-Behnken design, fatty acids, antioxidants, response surface

Introduction

Bread is a food that forms part of the daily meals of many people worldwide, in Brazil its *per capita* consumption is 27 kg *per* year. Such popularity is attributed to its excellent flavor, low cost and high availability in bakeries. The bread market is undergoing a great expansion and there is a demand for new recipes and ingredients.[1] Bakery products are also an ideal matrix to add chemical compounds, which act in the prevention of nutrition-related diseases.[2,3] Among such compounds, those with an antioxidant mode of action and polyunsaturated fatty acids (PUFA) have received great attention lately.

In general, an antioxidant compound is any substance present in low concentrations when compared to an oxidizable substrate and effectively delays or inhibits its oxidation.[4] Currently, there is a great concern about the damage caused by oxidative stress, which can affect DNA leading to carcinogenic effects.[5] As a result, the importance of studying the antioxidant capacity of foods has been increasing.

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PUFA, especially from the omega-3 (n-3) group, are extensively featured in the scientific literature because they can lead to beneficial omega-3:omega-6 (n-6) ratios. An optimal n-6:n-3 ratio intake has been shown to prevent a series of human diseases such as coronary heart disease, cardiovascular disease, rheumatoid arthritis, depression, postpartum depression, cancers, diabetes and anti-inflammatory action.[6,7]

The incorporation of these compounds into bread alters its chemical composition substantially. To properly quantify such changes, a number of factors must be studied. However, this increase in the number of factors under study necessitates a significant number of experiments. To avoid this, the use of an alternative experimental design, such as the Box-Behnken design (BBD), could reduce the number of required experiments. BBD is a three-level fractional-factorial design consisting of three interlocked 2^2 factorial designs and a center point.[8]

By comparing the BBD with other response surface designs, like central composite and three-level full factorial design, it was demonstrated that BBD is slightly more efficient than the central composite design and much more efficient than the three-level full factorial designs. Another advantage of the BBD is that it does not contain points in which all factors are simultaneously at their highest or lowest levels. So, these designs are useful for the avoidance of experiments performed under extreme conditions, for which unsatisfactory results might occur.[8,9]

BBD has been applied in the optimization of several chemical and physical processes.[9,10] However, it has not been used to investigate how much the baking time and addition of foods rich in PUFAs and antioxidants change the chemical composition of bread. This work determined the proximate/fatty acid compositions and antioxidant activity of breads using a BBD design composed of fifteen experiments and three factors. The breads had part of their baking time changed, and the wheat flour was partially substituted by chia seeds and carrot leaves. Chia seeds (*Salvia hispanica* L.) were chosen for this work due to their remarkable n-3 fatty acid composition,[11] and carrot leaves (*Daucus carota* L.) were selected because they possess antioxidant compounds, like carotenoids and phenolics compounds.[12] Other statistical tools such as response surface methodology and analysis of variance were also employed.

Experimental

Sampling

Carrot leaves were acquired from retail stores, washed, sterilized with 200 ppm sodium hypochlorite solution for 15 min, and then dried in a laboratory oven (QUIMIS, Maringá, Brazil) with air circulation at 60 °C for 7 h. After this process, the dehydrated leaves were milled, passed through a 14 mesh (1.19 mm) sieve and stored at −18 °C under vacuum for later use. Chia seeds were also acquired from retail stores and underwent the same milling, sieving and storing processes as the dried carrot leaves.

Bread production

A traditional white bread recipe was used,[13] but parts of the original wheat flour were substituted by different quantities of chia seeds and carrot leaves. The baking time was also investigated. A BBD design, generated by the Design-Expert 7.1 software (Stat-Ease Inc., USA) and composed of fifteen experiments, was employed to investigate the influence of these three factors (amount of chia seeds, carrot leaves and baking time) on total antioxidant capacity (TAC) and proximate/fatty acid compositions of bread. The parameters of the experiments can be observed in Table 1.

Table 1. Box-Behnken design layout with coded levels and actual values of variables

Experiment	X_1 - Chia seeds / (g *per* 100 g of wheat flour)	X_2 - Carrot leaves / (g *per* 100 g of wheat flour)	X_3 - Baking time / min
E1	1.0 (−1)	1.0 (−1)	70 (0)
E2	2.0 (1)	1.0 (−1)	70 (0)
E3	1.0 (−1)	2.0 (1)	70 (0)
E4	2.0 (1)	2.0 (1)	70 (0)
E5	1.0 (−1)	1.5 (0)	60 (−1)
E6	2.0 (1)	1.5 (0)	60 (−1)
E7	1.0 (−1)	1.5 (0)	80 (1)
E8	2.0 (1)	1.5 (0)	80 (1)
E9	1.5 (0)	1.0 (−1)	60 (−1)
E10	1.5 (0)	2.0 (1)	60 (−1)
E11	1.5 (0)	1.0 (−1)	80 (1)
E12	1.5 (0)	2.0 (1)	80 (1)
E13	1.5 (0)	1.5 (0)	70 (0)
E14	1.5 (0)	1.5 (0)	70 (0)
E15	1.5 (0)	1.5 (0)	70 (0)

Maximum and minimum levels were chosen by carrying out preliminary screening tests and according to economic aspects. The experiments were performed in a bread machine (Britânia brand, model Multi Pane) to make the enriched breads. A control bread, baked for 60 min (as specified in the original recipe)[13] and without the addition of chia seeds or carrot leaves, was also

produced to determine the incorporation efficiency of the ingredients under investigation in this food matrix. After the stipulated baking time, breads were allowed to cool to room temperature, then milled and stored at –18 °C under vacuum for later analysis.

Chemical analysis

All analyses were accomplished in three replicates. Total lipids (TL) in the bread samples were determined according to Bligh and Dyer.[14] The moisture content was determined using AOAC Official Method 930.15, ash content using AOAC Official Method 942.05 and crude protein (CP) was measured following AOAC Official Method 960.52,[15] using a factor of 5.70 to convert the percentage of nitrogen into protein percentage.

Fatty acid composition

Fatty acid methyl esters (FAME) were prepared by total lipid methylation following Hartman and Lago.[16] The methyl esters were separated by gas chromatography in a Varian 3380 (USA) gas chromatograph fitted with a flame ionization detector (FID) and a fused-silica capillary column (100 m × 0.25 mm id., 0.25 μm cyanopropyl CP-7420 select FAME). The injector and detector temperatures were 200 and 240 °C, respectively. The column temperature was maintained at 185 °C for 8 min, followed by a heating rate of 4 °C min^{-1} until 235 °C, which was maintained for 0.5 min. The ultra-pure gas flows were 1.2 mL min^{-1} carrier gas (hydrogen), 30 mL min^{-1} make-up gas (nitrogen), 350 mL min^{-1} synthetic air and 35 mL min^{-1} hydrogen flame gas, split injection ratio of 1:80.

To identify fatty acids, retention times were compared to those of standard methyl esters (Sigma, USA). Quantification of fatty acids was performed using tricosanoic acid methyl ester (Sigma, USA) as an internal standard, according to Joseph and Ackman.[17] Peak areas were determined using the software Star 5.0 (Varian, USA).

Antioxidant capacity analysis

For TAC analysis, all previously ground samples were passed through an 80 mesh (0.177 mm) sieve to avoid the influence of different particle sizes in Quencher procedures.[18] Solutions were prepared according to Serpen *et al.*[19] The stock solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained by dissolving 40 mg of DPPH in 200 mL of an ethanol/water mixture (50:50, v:v). The absorbance value of 0.75-0.80 was set by diluting the stock solution in approximately 800 mL of a

water/ethanol (50:50, v:v) mixture. The FRAP solution was prepared as described by Benzie and Strain,[20] diluting an aqueous solution of 10 mmol L^{-1} 2,4,6-tripyridyl-*s*-triazine (TPTZ) and 20 mmol L^{-1} ferric chloride in 300 mmol L^{-1} sodium acetate buffer (pH 3.6) at a ratio of 1:1:10 (v:v:v).

10 mg of each ground sample were weighed and placed into a centrifuge tube. For the DPPH and FRAP assays, 10 mL of the respective working solutions were added to start the reactions. All tubes were shaken for 1 h, centrifuged at 9200 g for 5 min, and then the absorbance of the supernatants were measured at 525 nm (for DPPH assay) and 593 nm (for FRAP assay).[19]

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard reference to convert the inhibition capability of each sample to the trolox equivalent antioxidant capacity (TEAC), as described by Serpen *et al.*[19]

Statistical analysis

All analyses were carried out in triplicate. The experimental results were analyzed through a response surface methodology generated by the Design-Expert 7.1 software (Stat-Ease Inc., USA). Model fit quality was evaluated by variance analysis (ANOVA) and determination coefficients. The basic model equation used to fit the data was:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \qquad (1)$$

where Y is the desired response, X_1, X_2 and X_3 are independent variables representing chia, carrot leaves and baking time, respectively. β_0 is a constant, β_1, β_2 and β_3 are the coefficients translating the linear weight of X_1, X_2 and X_3, respectively. β_{12}, β_{13} and β_{23} are the coefficients translating the interactions between the variables, and β_{11}, β_{22} and β_{33} are the coefficients translating the quadratic influence of X_1, X_2 and X_3.

Results and Discussion

Chemical analysis

The results of the proximate composition for each experiment are shown in Table 2. There was an increase in some parameters when compared to the control bread (which showed ash, CP and TL values of 1.60, 7.69 and 2.51%, respectively). The most significant TL increase (58.17%) was observed after the addition of chia seeds and carrot leaves to bread samples. In terms of TL content, the E3 and E4 runs produced the greatest concentrations, whilst

Table 2. Proximate composition of experiments

Experiment	X_1	X_2	X_3	Composition / (g *per* 100 g of bread)			
				Moisture	Ash	Crude protein	Total lipid
E1	−1	−1	0	36.76	1.66	7.40	2.88
E2	1	−1	0	34.07	1.79	7.63	2.98
E3	−1	1	0	35.15	1.73	7.48	3.50
E4	1	1	0	35.76	1.99	8.12	3.97
E5	−1	0	−1	36.16	1.73	7.44	3.16
E6	1	0	−1	37.28	1.86	7.86	3.29
E7	−1	0	1	33.00	1.69	7.43	3.19
E8	1	0	1	33.43	1.94	7.85	3.60
E9	0	−1	−1	35.36	1.68	7.48	2.91
E10	0	1	−1	35.66	1.89	7.76	3.05
E11	0	−1	1	35.22	1.73	7.47	3.02
E12	0	1	1	34.36	1.90	7.73	3.24
E13	0	0	0	33.89	1.85	7.60	3.35
E14	0	0	0	33.20	1.84	7.61	3.31
E15	0	0	0	33.57	1.84	7.60	3.28

E8 gave the lowest. It was observed that the increase in TL in the breads was proportional to the percentages of added chia seeds and carrot leaves. In relation to moisture, every experiment showed lower values regarding the obtained ones for control bread (38.02%), along with a difficulty in establishing a relation between results and X_1, X_2 and X_3 factors.

Total moisture values, which were measured at 105 °C, are not only related to water content, but with volatile compounds as well.[15] With an increase in the baking time, more of these compounds are expelled from the system due to evaporation, theoretically leading to concentration increases of other nutrients in the media.[21] However, during the early stages of the Maillard reactions occurring during bread baking, water is formed due to condensation of aldoses and amino compounds. More water is formed along with furfural of Schiff bases in the intermediate stages due to dehydration of 1-amino-1-deoxy-2-ketose which was originated by the Amadori rearrangement. These compounds are further consumed to form more amino compounds and melanoidins (high molecular weight brown compounds).[22] All these reactions occur at the same time during baking because melanoidins are formed with more frequency at bread crust, while at the center of it, the early and intermetidate stages of Maillard reaction are still occurring. Thus, the combination of all these factors together makes it difficult to predict how much the moisture varies. The same difficulty in prediction is observed for TL, probably because oxidized triacylglycerols can either remain unmodified or undergo a degradation reaction, increasing the amount of volatile oxidation compounds.[23]

An increase in the percentages of chia seeds and carrot leaves added to the bread led to a direct increase in the contents of ash and CP. Baking time did not have a high influence on the responses since the same amount of inorganic compounds (ash) was present in the bread for different baking time. The same logic can be used to explain the trend observed for the values of CP: as a bread bakes, its proteins undergo cross-linking and Maillard reactions, producing other compounds with lower solubility in water.[24] However, the Kjeldahl method only quantifies the total nitrogen from a sample, regardless of the molecule the nitrogen is bound to.

Fatty acid composition

Table 3 shows the fatty acid quantification from each experiment. The addition of chia seeds and carrot leaves led to maximal alpha-linolenic acid (18:3n-3) increase of 216.69% and maximum PUFA:SFA ratio increase of 18.88% in relation to control bread (which showed an alpha-linolenic acid amount of 34.03 mg g^{-1} TL and a 2.86 ratio value), as well as a maximum n-6:n-3 ratio reduction of 70.01% (15.59 ratio value for control bread). In most cases, decreases in linoleic (18:2n-6) and oleic (18:1n-9) acids were also observed in relation to control bread (which showed maximal linoleic and oleic acid values of 530.98 and 237.44 mg g^{-1} TL, respectively, for

Table 3. Fatty acid quantification and the n-6:n-3 and PUFA:SFA ratios

Experiment	X_1	X_2	X_3	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	PUFA[a]	SFA[b]	n-6[c]:n-3[d]	PUFA:SFA
								(mg *per* g of total lipid)				
E1	−1	−1	0	149.44	38.94	228.33	509.84	59.90	569.74	188.38	8.51	3.02
E2	1	−1	0	130.24	35.38	195.48	437.81	90.84	528.65	165.62	4.82	3.19
E3	−1	1	0	122.53	32.10	186.88	447.71	51.48	499.19	154.63	8.70	3.23
E4	1	1	0	134.57	34.51	221.20	489.73	85.45	575.18	169.08	5.73	3.40
E5	−1	0	−1	171.58	43.53	260.51	594.27	77.09	671.36	215.11	7.71	3.12
E6	1	0	−1	150.74	37.49	217.37	485.62	107.43	593.05	188.22	4.52	3.15
E7	−1	0	1	154.06	38.28	230.93	528.89	73.33	602.22	192.35	7.21	3.13
E8	1	0	1	146.94	33.55	203.76	473.32	104.02	577.34	180.49	4.55	3.20
E9	0	−1	−1	163.21	38.93	219.01	571.50	100.61	672.10	202.14	5.68	3.32
E10	0	1	−1	128.96	30.67	185.30	417.84	81.76	499.61	159.63	5.11	3.13
E11	0	−1	1	153.82	37.37	223.64	507.22	98.29	605.51	191.19	5.16	3.17
E12	0	1	1	151.06	39.23	217.50	492.01	92.19	584.20	190.29	5.34	3.07
E13	0	0	0	144.61	37.47	219.06	503.86	95.38	599.24	182.09	5.28	3.29
E14	0	0	0	147.41	37.94	223.37	511.11	94.67	609.13	185.35	5.21	3.29
E15	0	0	0	150.42	40.87	229.80	511.32	93.23	604.55	191.29	5.48	3.16

[a]PUFA: sum of polyunsaturated fatty acids. [b]SFA: sum of saturated fatty acids. [c]n-6: omega 6 fatty acid. [d]n-3: omega 3 fatty acid.

control bread). According to Perini *et al.*,[25] linoleic and α-linolenic fatty acids are strictly essential because they are not produced by human *de novo* synthesis and must be provided by an adequate diet. The ingestion of 18:1n-9, instead of saturated fatty acids (SFA), helps to reduce the low density lipoprotein levels in blood and, therefore, cardiovascular diseases occur with a lower probability.[26] To indicate whether a particular food is healthy, the UK Health Department recommends that the value of the PUFA:SFA ratio must be above 0.45.[27] The PUFA:SFA ratios of all the bread examined in this study were within these recommended values. Simopoulos[28] found that the optimal n-6:n-3 fatty acid ratio for human ingestion varies from 1 to 2. This conclusion was based on other medical research, which evaluated the relationship of the ingestion of essential fatty acids to the healing rate of patients with chronic diseases. Based on these findings, we observed that the breads with high percentages of chia seeds were the closest to the ideal ratio as stipulated above.

The chemical and physical changes that might occur to a lipid depend on its composition and treatment conditions. If the amount of oxygen in media is scarce, thermolytic reactions will occur. For SFAs, acrolein, oxopropyl esters, diacylglycerols and other compounds originating from non-oxidative decomposition are formed at 180 °C under low oxygen conditions. Unsaturated fatty acids might be dimerized or polymerized *via* Diels-Alder-type reactions.[29] However, whilst kneading the dough during the bread making process, a considerable amount of air is incorporated into the system, thus it is unclear how much thermolytic reactions might affect the SFA concentrations.

In the presence of oxygen, aldehydes are formed as the major oxidative products of fatty acid oxidation. SFAs and their esters possess greater stability than their unsaturated counterparts. However, at a temperature of 150 °C or higher, they undergo oxidation, producing a series of carboxylic acids, *n*-alkanes, lactones and alkenes.[29]

Thus, as mentioned in the chemical analysis section, part of the oxidized triacylglycerols can suffer degradation reactions leading to volatile compounds that can escape to the atmosphere. But such oxidation can be avoided to a certain degree by substances with antioxidant activity, like Maillard reaction products (MRPs) and phenolic compounds.[30] All of these possibilities make it difficult to predict how the fatty acid composition of breads might change with simultaneous changes of X_1, X_2 and X_3 factors.

Antioxidant capacity analysis

Table 4 shows the results of the TAC assays from each experiment. The experiments that showed the greatest TAC values through the DPPH assay were E3, E4 and E12. In the FRAP assay, the E3, E4 and E10 experiments showed the highest TAC values. It can be seen, that all these experiments were the ones with the maximum percentage of carrot leaves added. The lowest TAC values from the DPPH and FRAP assays were detected for the experiment E9, which was performed with the lowest quantity of carrot
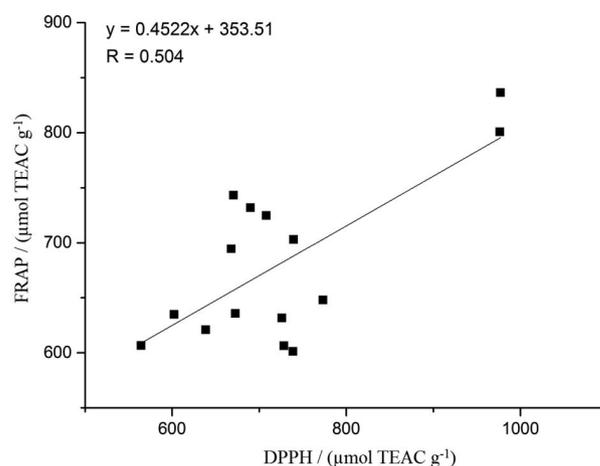
Table 4. Results of antioxidant capacity by DPPH and FRAP assays

Experiment	X_1	X_2	X_3	Antioxidant capacity / (μmol TEAC g^{-1})	
				DPPH	FRAP
E1	−1	−1	0	728.34	606.30
E2	1	−1	0	689.93	731.88
E3	−1	1	0	976.98	836.43
E4	1	1	0	976.25	800.80
E5	−1	0	−1	738.77	601.36
E6	1	0	−1	726.09	631.51
E7	−1	0	1	708.04	724.85
E8	1	0	1	739.46	703.10
E9	0	−1	−1	564.51	606.64
E10	0	1	−1	670.53	743.21
E11	0	−1	1	667.87	694.45
E12	0	1	1	773.04	648.04
E13	0	0	0	638.72	620.97
E14	0	0	0	602.32	634.93
E15	0	0	0	672.56	635.61

Figure 1. Correlation graphic of DPPH and FRAP values.

leaves. These results show that there exists a possible positive relationship between an increase percentage of carrot leaves and TAC of bread.

The same trend is not observed after increasing the amount of chia seeds in bread, which did not result in a great change in the final TAC data when compared to carrot leaves. Nevertheless, the TAC results of every experiment were superior to those obtained from the control bread (495.33 μmol TEAC g^{-1} for the DPPH assay and 413.85 μmol TEAC g^{-1} for the FRAP assay), showing an increase in levels of up to 97 and 102% from the DPPH and FRAP assays, respectively. These results showed that the simultaneous incorporation of chia seeds and carrot leaves positively changed the TAC levels of bread.

DPPH and FRAP assays (Figure 1) showed a relatively low correlation (r = 0.504). Despite these methods possessing the same reaction mechanism (electron transference), they are influenced by pH and the solvent of the reaction media.[31] In this study, even with the direct procedure (Quencher) which is performed without extraction steps, there is still solvent in the reaction media, required for solubilization of the radicals used in every assay. In the DPPH method, a 50:50 (v:v) mixture of ethanol and water is responsible for the radical solubilization, whilst in the FRAP procedure radicals are solubilized in water. Furthermore, in the FRAP assay, pH is controlled at 3.6, unlike the DPPH assay. Some or all of these factors may have been responsible for the low correlation values obtained between the assays.

As can be observed, it was difficult to establish a pattern with simultaneous variation of the X_1, X_2 and X_3 factors. Although carotenoids from carrot leaves are very heat stable even after prolonged heat treatments, the phenolic compounds from the leaves can be consumed as reactants in Maillard reactions. But polyphenols with an intermediate-oxidation state can scavenge radicals with greater efficiency than the non-oxidized ones, as they can donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or support an unpaired electron through delocalization around the π-electron system with more ease. It should be noted that during the baking process, MRPs with pro-oxidant activity are formed in the early stages of the Maillard reaction prior to the Amadori rearrangement. However, as the reaction advances and food-browning becomes more intense, melanoidins with antioxidant activity are formed. The rate of formation of all these compounds depends on the intensity and/or duration of heat treatment.[30]

A series of events that might occur during the mixing process of the food matrices is redox reaction between natural antioxidant compounds and lipid oxidation products. They have almost unpredictable consequences on overall antioxidant properties and stability of food. Besides, MRPs can also help in prevention of lipid oxidation.[30] Thus, the simultaneous effects which happen between antioxidant compounds, MRPs and lipids were probably the reason why a defined trend regarding simultaneous modification of X_1, X_2 and X_3 factors was not achieved.

ANOVA and response surface methodology

Further chemometric analyses of the results were hampered, probably due to the high chemical complexity of this food matrix (bread). However, this was not the case for ash, CP, n-3 and n-6:n-3 data. Thus, these four

responses were analyzed by ANOVA and response surface methodology. Multiple regression analysis was employed on the data and between the models which were suggested by the software (linear, two factor interaction (2FI), quadratic and cubic). The quadratic model was selected as the most suitable, because it has a high significance order and it is not aliased.[32] The adjusted models which were obtained for ash, CP, n-3 fatty acids and n-6:n-3 ratios, as well as their ANOVA parameters, are listed in Table 5. ANOVA showed that the lack of fit obtained for the models was insignificant, meaning they were suitable for the evaluation of response surfaces.

The coefficient of determination (R^2) is the proportion of variation, in a determined response, which is attributed to the model instead of random errors. A well-adjusted model must not possess an R^2 value lower than 0.80. When R^2 is close to 1, this means that the empirical model is suitable for the obtained data.[32]

Table 6 shows that the R^2 values for the response variables were greater than 0.80, indicating the good quality of the obtained models. R^2 values ranged from 0.9852 to 0.995. Adding a variable to the model will always increase the R^2 value, independently of its statistic significance, however. Thus, a high R^2 value does not mean that, in every case, the model to which it corresponds is suitable. Thus, it is better to use an adjusted R^2 (adj-R^2) of over 0.90 to evaluate adequacy of a model. The obtained adj-R^2 values were above 0.95 for every response. This is very important since a high adj-R^2 value means that insignificant terms were not included in the model.[32]

Table 5. ANOVA and quadratic models for the obtained responses

			Ash		
Source	Sum of squares	DF	Mean square	*F*-value	*p*-value prob > *F*
Model	0.14139257	9	0.015710286	52.8050496	0.0002
X_1	0.075602161	1	0.075602161	254.112242	< 0.0001
X_2	0.051424245	1	0.051424245	172.845987	< 0.0001
X_3	0.001159211	1	0.001159211	3.89631413	0.1054
X_1X_2	0.00373321	1	0.00373321	12.5479794	0.0165
X_1X_3	0.004349403	1	0.004349403	14.6191114	0.0123
X_2X_3	0.00028224	1	0.00028224	0.94865858	0.3748
X_1^2	0.001798603	1	0.001798603	6.04542342	0.0573
X_2^2	0.002651139	1	0.002651139	8.9109457	0.0306
X_3^2	0.00109498	1	0.00109498	3.68042175	0.1132
Residual	0.001487574	5	0.000297515		
Lack of fit	0.001433648	3	0.000477883	17.7234207	0.0539
Pure rrror	5.39267×10^{-5}	2	2.69633×10^{-5}		
Cor total	0.142880144	14			
			Crude Protein		
Source	Sum of squares	DF	Mean square	*F*-value	*p*-value prob > *F*
Model	0.569751667	9	0.063305741	926.425474	< 0.0001
X_1	0.3655125	1	0.3655125	5348.96341	< 0.0001
X_2	0.1540125	1	0.1540125	2253.84146	< 0.0001
X_3	0.00045	1	0.00045	6.58536585	0.0503
X_1X_2	0.042025	1	0.042025	615	< 0.0001
X_1X_3	0	1	0	0	1.0000
X_2X_3	1×10^{-4}	1	1×10^{-4}	1.46341463	0.2805
X_1^2	0.007339103	1	0.007339103	107.401501	0.0001
X_2^2	0.000339103	1	0.000339103	4.96247655	0.0764
X_3^2	3.14103×10^{-5}	1	3.14103×10^{-5}	0.45966229	0.5279
Residual	0.000341667	5	6.83333×10^{-5}		
Lack of fit	0.000275	3	9.16667×10^{-5}	2.75	0.2779
Pure rrror	6.66667×10^{-5}	2	3.33333×10^{-5}		
Cor total	0.570093333	14			

Table 5. continuation

			n-3		
Source	Sum of squares	DF	Mean square	*F*-value	*p*-value prob > F
Model	3490.525466	9	387.8361629	39.5831337	0.0004
X_1	1982.270163	1	1982.270163	202.313431	< 0.0001
X_2	187.739556	1	187.739556	19.1609774	0.0072
X_3	0.111339819	1	0.111339819	0.01136351	0.9193
X_1X_2	2.297182979	1	2.297182979	0.2344539	0.6487
X_1X_3	0.030947516	1	0.030947516	0.00315855	0.9574
X_2X_3	40.60328383	1	40.60328383	4.14403134	0.0974
X_1^2	588.6656013	1	588.6656013	60.0800839	0.0006
X_2^2	360.6064342	1	360.6064342	36.8040273	0.0018
X_3^2	277.1224763	1	277.1224763	28.2835308	0.0031
Residual	48.99007813	5	9.798015626		
Lack of fit	46.58392366	3	15.52797455	12.9068809	0.0728
Pure rrror	2.40615447	2	1.203077235		
Cor total	3539.515545	14			
			n-6/n-3		
Source	Sum of squares	DF	Mean square	*F*-value	*p*-value prob > F
Model	26,68768434	9	2.965298261	36.8830749	0.0005
X_1	19.55207024	1	19.55207024	243.193233	< 0.0001
X_2	0.06181048	1	0.06181048	0.76881324	0.4207
X_3	0.072228111	1	0.072228111	0.89839017	0.3867
X_1X_2	0.131852811	1	0.131852811	1.64001618	0.2565
X_1X_3	0.069213073	1	0.069213073	0.86088843	0.3961
X_2X_3	0.139333562	1	0.139333562	1.73306351	0.2451
X_1^2	4.832155901	1	4.832155901	60.1034879	0.0006
X_2^2	0.809305348	1	0.809305348	10.0663296	0.0247
X_3^2	0.826655202	1	0.826655202	10.2821312	0.0238
Residual	0.401986313	5	0.080397263		
Lack of fit	0.362573589	3	0.120857863	6.13293627	0.1434
Pure rrror	0.039412724	2	0.019706362		
Cor total	27.08967066	14			
			Models and Responses		

Ash = $1.84257 + 0.097212X_1 + 0.080175X_2 + 0.012038X_3 + 0.030550X_1X_2 + 0.032975X_1X_3 - 8.40000\text{E}^{-3}X_2X_3 - 0.022071X_1^2 - 0.026796X_2^2 - 0.017221X_3^2$

Crude protein = $7.60333 + 0.21375X_1 + 0.13875X_2 - 7.50000\text{E}^{-3}X_3 + 0.10250X_1X_2 - 2.60209\text{E}^{-4}X_1X_3 - 5.00000\text{E}^{-3}X_2X_3 + 0.044583X_1^2 + 0.0095833X_2^2 - 2.91667\text{E}^{-3}X_3^2$

n-3 = $94.43027 + 15.74115X_1 - 4.84432X_2 + 0.11797X_3 + 0.75782X_1X_2 + 0.087960X_1X_3 + 3.18604X_2X_3 - 12.62657X_1^2 - 9.88252X_2^2 + 8.66337X_3^2$

n-6/n-3 = $5.32715 - 1.56333X_1 + 0.087899X_2 - 0.095018X_3 + 0.18156X_1X_2 + 0.13154X_1X_3 + 0.18664X_2X_3 + 1.14399X_1^2 + 0.46817X_2^2 - 0.47317 X_3^2$

[a]DF: degrees of freedom.

Table 6. Parameters for the obtained quadratic models

	Ash	Crude protein	n-3	n-6:n-3
Mean	1.81	8.37	87.05	5.93
Standard deviation	0.017	8.440×10^{-3}	3.13	0.28
R^2	0.9896	0.9995	0.9862	0.9852
Adjusted R^2	0.9708	0.9985	0.9612	0.9585
Coefficient of variation / %	0.95	0.10	3.60	4.78

The coefficient of variation (CV) describes the extension of data dispersion. As a general rule, CV must not be above 10%. None of the CV values obtained in this work exceeded this limit, representing a good precision and reliability in the experiments which were conducted.[32]

Figure 2 shows the response surface graphs for X_1 and X_2 factors against the obtained responses. Baking time was fixed at 70 min. The values for ash and CP increased with the simultaneous incorporation of chia seeds and carrot leaves.

(a)

(b)

(c)

(d)
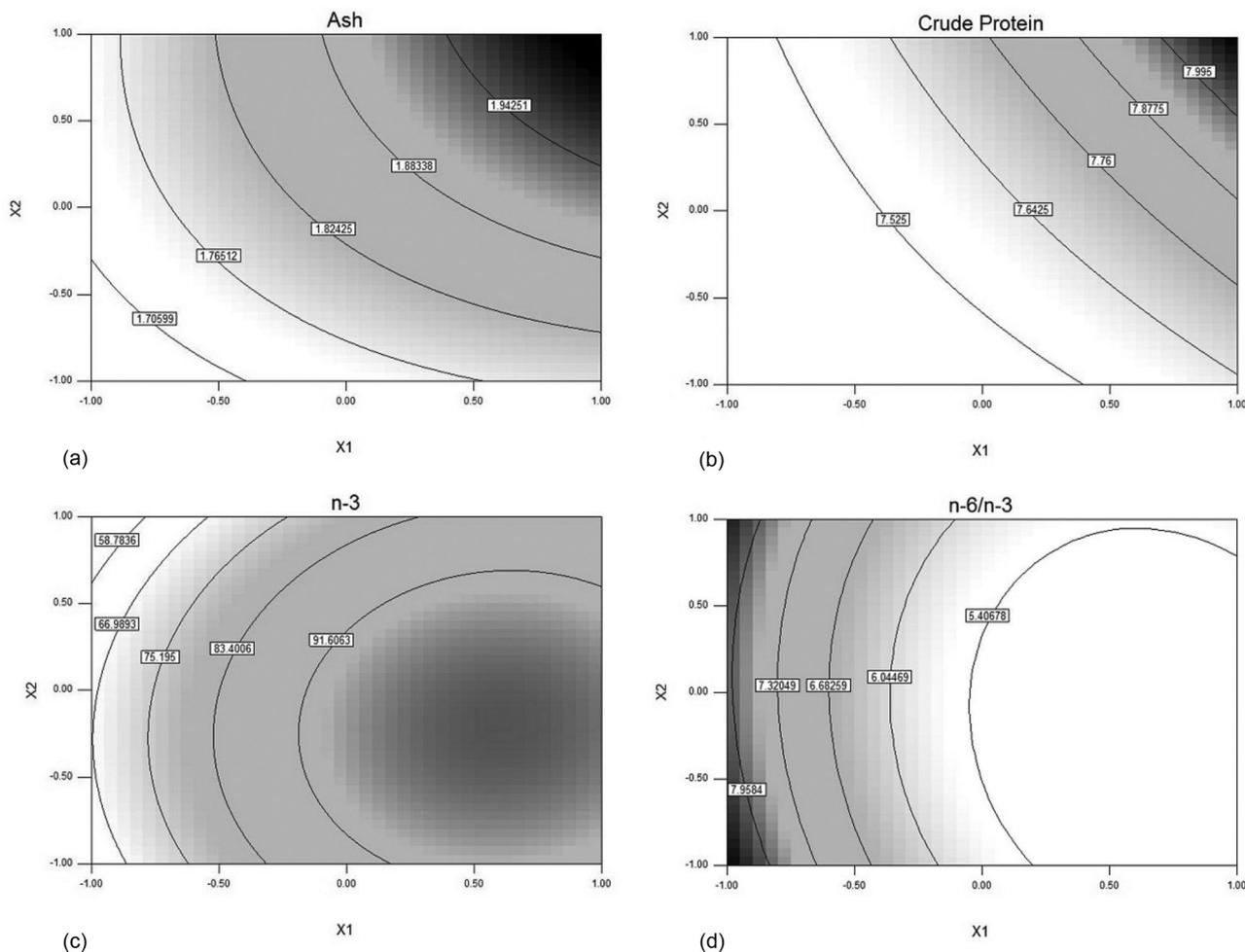
Figure 2. Contour charts showing the effects of the variables to the values of (a) ash, (b) crude protein, (c) n-3 and (d) n-6:n-3.

From the n-3 response surface, it is clear that X_1 (chia seeds) was the factor which greatly affected total concentration of this fatty acid, while for the n-6:n-3 ratio, the best values were obtained with the highest X_1 percentage. It is important to note that, unlike for the other responses, n-6:n-3 ratio values must be as low as possible.

Conclusions

The BBD, allied with FRAP, DPPH and GC-FID methods, confirmed that, despite the deleterious effects of a high temperature baking process, there was a considerable incorporation of n-3 fatty acids and antioxidant compounds into a recipe of traditional white bread. However, probably due to a series of physicochemical phenomena which occurred simultaneously, defined trends regarding simultaneous modification of X_1, X_2 and X_3 factors were not achieved for the majority of responses, except for ash, CP, n-3 and n-6:n-3 data. Further chemometric analysis showed that the devised empirical models were suitable for

the obtained data, for the evaluation of response surface and that chia seeds had a greater influence on responses than carrot leaves.

Acknowledgments

References

1. Esteller, M. S.; Yoshimoto, R. M. O.; Amaral, R. L.; Lannes, S. C. S.; *Food Sci. Technol.* **2004**, *24*, 602.

2. Day, L.; Seymour, R. B.; Pitts, K. F.; Konczak, I.; Lundin, L.; *Trends Food Sci. Technol.* **2009**, *20*, 388.

3. Rocha, L. S.; Santiago, R. A. C.; *Food Sci. Technol.* **2009**, *29*, 820.

4. Sies, H.; Stahl, W.; *Am. J. Clin. Nutr.* **1995**, *62*, 1315.

5. Klaunig, J. E.; Wang, Z.; Pu, X.; Zhou, S.; *Toxicol. Appl. Pharmacol.* **2011**, *254*, 86.

6. Navarro, R. D.; Navarro, F. K. S. P.; Ribeiro Filho, O. P.; Ferreira, W. M.; Pereira, M. M.; Seixas Filho, J. T.; *Food Chem.* **2012**, *134*, 215.

7. Visentainer, J. V.; Souza, N. E.; Matsushita, M.; Hayashi, C.; Franco, M. R. B.; *Food Chem.* **2005**, *90*, 557.

8. Ferreira, S. L.; Bruns, R. E.; Ferreira, H. S.; Matos, G. D.; David, J. M.; Brandao, G. C.; da Silva, E. G.; Portugal, L. A.; dos Reis, P. S.; Souza, A. S.; dos Santos, W. N.; *Anal. Chim. Acta.* **2007**, *597*, 179.

9. Khajvand, T.; Chaichi, M. J.; Nazari, O.; Golchoubian, H.; *J. Lumin.* **2011**, *131*, 838.

10. Tekindal, M. A.; Bayrak, H.; Ozkaya, B.; Genc, Y.; *Turk. J. Field Crops* **2012**, *17*, 115.

11. Uribe, J. A. R.; Perez, J. I. N.; Kauil, H. C.; Rubio, G. R.; Alcocer, C. G.; *J. Supercrit. Fluids.* **2011**, *56*, 174.

12. Almeida-Muradian, L. B.; Popp, V.; Farias, M. P.; *Food Sci. Technol.* **1997**, *17*, 120.

13. http://www.britania.com.br/arquivos/britaniashop/manuais/9064301000.pdf, accessed in April 2013.

14. Bligh, E. G.; Dyer, W. J.; *Can. J. Biochem. Physiol.* **1959**, *37*, 911.

15. AOAC; *Official Methods of Analysis*, 15th ed., Association of Official Analytical Chemists: Arlington, 1990.

16. Hartman, L.; Lago, R. C. A.; *Lab. Pract.* **1973**, *22*, 475.

17. Joseph, J. D.; Ackman, R. G.; *J. AOAC Int.* **1992**, *75*, 488.

18. Gökmen, V.; Serpen, A.; Fogliano, V.; *Trends Food Sci. Technol.* **2009**, *20*, 278.

19. Serpen, A.; Gokmen, V.; Fogliano, V.; *Meat Sci.* **2012**, *90*, 60.

20. Benzie, I. F. F.; Strain, J. J.; *Anal. Biochem.* **1996**, *239*, 70.

21. Türkkan, A. U.; Cakli, S.; Kilinc, B.; *Food Bioprod. Process.* **2008**, *86*, 163.

22. Purlis, E.; *J. Food Eng.* **2010**, *99*, 239.

23. Caponio, F.; Summo, C.; Pasqualone, A.; Bilancia, M. T.; *J. Cereal Sci.* **2008**, *48*, 407.

24. Singh, H.; *Food Chem.* **2005**, *90*, 247.

25. Perini, J. A. L.; Stevanato, F. B.; Sargi, S. C.; Visentainer, J. E. L.; Dalalio, M. M. O.; Matsushita, M.; Souza, N. E.; Visentainer, J. V.; *Braz. J. Nutr.* **2010**, *23*, 1075.

26. Elmadfa, I.; Kornsteiner, M.; *Ann. Nutr. Metab.* **2009**, *55*, 56.

27. Department of Health; *Nutritional Aspects of Cardiovascular Disease. Report of the Cardiovascular Review Group of the Committee on Medical Aspects of Food Policy _ Report on Health and Social Objects*, vol. 46; Her Majesty's Stationery Office (HMSO): London, UK, 1994.

28. Simopoulos, A. P.; *Mol. Neurobiol.* **2011**, *44*, 203.

29. Nawar, W. W.; *J. Chem. Educ.* **1984**, *61*, 299.

30. Nicoli, M. C.; Anese, M.; Parpinel, M.; *Trends Food Sci. Technol.* **1999**, *10*, 94.

31. Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S. E.; Bektasoglu, B.; Berker, K. I.; Özyurt, D.; *Molecules* **2007**, *12*, 1496.

32. Neto, B. B.; Scarminio, I. S.; Bruns, R. E.; *Como Fazer Experimentos: Pesquisa e Desenvolvimento na Ciência e na Indústria*; Editora da Unicamp: Campinas, Brasil, 2001.