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Application of response surface methodology for the optimization of oxidants in wheat flour

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Abstract

The objective of this study was to apply the statistical technique known as response surface methodology (RSM) to optimize the proportion between three oxidant compounds compromising lipoxygenase enzymes (x_1), benzoyl peroxide (x_2) and ascorbic acid (x_3) on wheat flour colour and some rheological characteristics of the dough. *P*, *L* and *W* parameters were obtained using a "*Chopin*" alveograph. Flour colour, characterized by brightness (*Lc*) and hue (*b*), was measured using a HunterLab colorimeter and was ranked visually by selected judges. Three regression models were fitted to the dependent variables *P*, *L* and Lc and showed non-significant lack of fit ($p > 0.114$) with an elevated determination coefficient (0.92, 0.84 and 0.94, respectively). A mixture containing 0.27% of x_1, 4.80 ppm of x_2 and 325 ppm of x_3 was obtained from the regression optimization and used for validating the models. These results suggest that, instead of the empirical methods, RSM can be applied to determine the optimal proportion between oxidant compounds normally used to improve colour and rheological parameters in wheat flour.

Keywords: Optimization; Dough; Lipoxygenase; Ascorbic acid; Wheat flour

1. Introduction

Wheat flour protein quantity and quality constitute principal factors responsible for bread-making functionality by providing visco-elasticity to dough (Dobraszczyk & Morgenstern, 2003; Tronsmo et al., 2003; Veraverbeke & Delcour, 2002). Prolamins are responsible for the viscosity and extensibility in a dough system whereas the glutelins provide elasticity. The prolamins and glutelins combine through covalent and non-covalent bonds to form the gluten complex, resulting in viscoelastic dough that has the ability to retain gas and produce a light baked product (Linsay & Skerritt, 1999; Veraverbeke & Delcour, 2002). During bread-making, the polypeptide chains present in the gluten develop a viscoelastic matrix able to support the pressure from the gas produced by the fermentation process (Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002). Studies have demonstrated that these characteristics mainly result from the disulphide bonds formed by the oxidation of gluten sulphydryl residues. This oxidation can lead to rearrangement of intra/inter chain disulphide bonds, resulting in better dough rheology (viscoelasticity), and improving stability of gluten matrix (Morel, Redl, & Guilbert, 2002). Peroxidation of lipid can also reduce its binding to gluten, resulting in more "free" polar lipids, beneficial for increased loaf volume and soft crumb (Xu, 2001). Natural aging during storage

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of flour is an expensive, time-consuming operation with no assurance of uniformity of product, probably because the low water content slows the reaction speed dramatically (Fortmann & Joiner, 1971; Nicolas & Drapron, 1983). In bread production, oxidizing agents are normally added to flour in order to improve dough strength with the consequence of higher bread volume (Grosch & Wieser, 1999). Among these are the fast-acting oxidants as KIO_3 and azodicarbonamide, and the slow-acting oxidants, as for example $KBrO_3$, ascorbic acid and lipoxygenase (LOX).

Higher flour extraction levels, (a high economic interest procedure), usually result in a pale yellow wheat flour colour, due to the carotenoids present in the aleurone layer (Fortmann & Joiner, 1971). This results in bakery products of a colour that is less commercially acceptable. Benzoyl peroxide is a free radical initiator, and it produces carotenoid oxidation by a typical free radical mechanism. (Saiz, Manrique, & Fritz, 2001). LOX is also a complementary bleaching agent to benzoyl peroxide. Soybeans are the richest and best known source of LOX (Faubion & Hoseney, 1981).Through full fat soy flour, LOX is recommended at 0.5–1.0% (flour basis) in bread formulations, but its effect on flavour can reduce its application to levels as low as 0.2%. In contrast to benzoyl peroxide, LOX action takes place in bread mixing because it needs water and oxygen to become active (Gélinas, Poitras, McKinnon, & Morin, 1998). As white colour is directly associated with high quality, the creamy yellow colour of the freshly milled wheat flour is generally considered an undesirable characteristic (Fortmann & Joiner, 1971). The oxidative agents may participate only in bleaching, only in dough improvement or in both reactions (Faubion & Hoseney, 1981; Hoseney, Rao, Faubion, & Sidhu, 1980).

Wheat mills determine the oxidant proportion to be added in the wheat flour, based empirically on rheological and colorimeter measures obtained from chemical analysis (ash, gluten), alveograph, farinograph or extensigraph. Thus, the objective of this study was to apply the statistical technique known as response surface methodology(RSM) to determine the optimal proportion between oxidant compounds instead of the empirical methods normally used to improve colour and some rheological parameters in wheat flour.

2. Materials and methods

2.1. Material

Freshly milled wheat flour, containing 11.9% of protein ($N \times 5.70$) and 0.63% of ash, with no additives, was obtained from Lapa Alimentos S.A. (Salvador, Brazil). 4.2 mg of iron (Ferrochel, Albitech Ltd.) and 150 μg of folic acid (Merck & Co, Inc.) were applied to 100 g of wheat flour according to Brazilian Nutritional Legislation (ANVISA, 2002). Benzoyl peroxide (La Lux 1 at 32%, Granotec do Brazil), maltodextrin (MorRex-1920, Corn Products Brazil), full fat soy flour not submitted to thermal treatment (FIS-25, Multisoy Ind. Com. Ltd.) and ascorbic acid (Cargill Food Ingredients Latin America) were used to prepare the mixtures.

2.2. Experimental design

Table 1 presents the experimental design "*centroid simplex*" applied to a mixture containing three ingredients: x_1, lipoxygenase content in full fat soy flour (LOX), x_2, benzoyl peroxide (PBZ) and x_3, ascorbic acid (ASC). Maltodextrin was used to keep the total solids constant in the formulation. The variation selected to each variable was based on values allowed by the legislation (ANVISA, 1999; FDA, 2003), where x_1 (LOX) changed from 0 to 1 g/100 g of wheat flour, x_2 (PBZ) from 0 to 60 ppm and x_3 (ASC) from 0 to 500 ppm. The tests started 72 h after the addition of the mixtures to allow for a complete action of benzoyl peroxide. The characteristics of flour and dough-processing conditions were kept absolutely constant in this study. The assay was performed within one month of milling.

2.3. Ingredients' nutritional composition

The moisture of the wheat flour was determined by drying the sample at 130 °C to constant weight, according to

Table 1
Experimental design "centroid simplex"

Mixtures	Proportion of each compound in the mixture	Values of each compound (g) in 100 g of mixture[a]				
	(x_1,x_2,x_3)	LOX (x_1)	PBZ (x_2)	ASC (x_3)	MD[b]	Total[c]
Mix 1	(1, 0, 0)	100.00	0.00	0.00	0.00	100.00
Mix 2	(0, 1, 0)	0.00	0.60	0.00	99.40	100.00
Mix 3	(0, 0, 1)	0.00	0.00	5.00	95.00	100.00
Mix 4	(1/2, 1/2, 0)	50.00	0.30	0.00	49.70	100.00
Mix 5	(1/2, 0, 1/2)	50.00	0.00	2.50	47.50	100.00
Mix 6	(0, 1/2, 1/2)	0.00	0.30	2.50	97.20	100.00
Mix 7	(1/3, 1/3, 1/3)	33.30	0.20	1.67	64.83	100.00
CONT	(0, 0, 0)	–	–	–	100.00	100.00

[a] LOX is the full fat soy flour used as lipoxygenase source, PBZ is the benzoyl peroxide and ASC is the ascorbic acid.
[b] Maltodextrin used to keep the total volume constant.
[c] One gram of the total mixture was added to 100 g of the wheat flour.

the AACC method (44-15 A). Soy flour moisture was determined by the AOAC method (955.04) at 105 °C. Nitrogen content was determined by using the microKjeldahl method the AACC (46-11 A) and was multiplied by a factor of 5.70 and 6.25 to determine protein in wheat and soy flour, respectively. The ash content was determined by AACC method (08-01). The wheat flour lipids were extracted by AOAC (940.22) while soy flour lipids were determined by AOAC (1995). Carbohydrate content was obtained by difference (AACC, 2000; AOAC, 1995). Tests were done in triplicate.

2.4. Lipoxygenase (LOX) activity in soy flour

The LOX activity in full fat soy flour was determined according to Axelrod, Cheesbrought, and Laakso (1981), with some alterations proposed by Oliveira et al. (1998). Sodium linoleate (10 mM) was used as substrate and prepared adding 156 μl of linoleic acid (Sigma L1376, St. Louis, MO, USA) and 180 μl of Tween 20 to 40 ml of O_2 free water. The solution was homogenized avoiding air bubbles. 2 N NaOH was added to yield a clear solution, bringing the volume to 50 ml. The substrate solution was divided into 1 ml portions in small screw-cap vials, flushed with N_2 before closing, and kept frozen at −80 °C until needed. Full fat soy and wheat flour (20 mg) were mixed with 1.2 ml of extraction buffer (Tris 60 mM, $CaCl_2$ 15 mM, and saccharose 13%, pH 8.2) taking care to avoid significant heating and then centrifuged at 13,000 rpm for 20 min at 4 °C. LOX1 activity was determined by addition of 2.5 μl of supernatant (flour extract containing the enzyme) to 6.0 μl of substrate and 1.0 ml of sodium 0.1 M borate buffer ($Na_2B_4O_7 \cdot 10H_2O$, pH 9.5). After each addition, the mixture was stirred inside a quartz cuvette. Absorbance at 234 nm was recorded each 10 s after the enzyme addition up to 15 min, using a SHIMADZU UV1240 (Shimadzu Corporation, Tokyo, Japan) spectrophotometer. LOX3 activity was determined by the addition of 35 μl of substrate and 1.0 ml of 0.2 M sodium phosphate buffer (1.973 g/100 ml of $NaH_2PO_4 \cdot H_2O$ and 1.014 g/100 ml of $Na_2HPO_4 \cdot 2H_2O$; pH 6.8) to 15 μl of supernatant, the absorbance being measured at 280 nm, following the same procedure described above. A cubic regression was fitted to each sample using the triplicate values. Their 1st derivative was calculated to identify the maximum point of the curve and the respective absorbance value was obtained by substitution of this point (expressed as time in minutes) in the respective cubic regression (Pinchuk & Lichtenberg, 2002). This value corresponded to the absorbance at the "steady-state". One unit of LOX1 activity was considered to cause an increase in A_{234} of 0.001 per min at pH 9.0 at 25 °C when linoleic acid was the substrate. The same was applied to LOX3, considering A_{280} and pH 6.8. The specific activity was expressed by the unit value/mg of protein. The Lowry method was used to quantify the protein extract during the enzymatic activity determination (Lowry, Rosebrough, Farr, & Randall, 1951).

2.5. Alveograph testing

Alveograph measurements were obtained, under conditions of constant dough water content and mixing times, using the standard Method 54-30 (AACC, 2000). The following alveograph parameters were automatically recorded by a computer software programme: maximum over-pressure needed to blow the dough bubble (P index of resistance to extension), average abscissa at bubble rupture (L index of dough extensibility), and deformation energy (W index of dough strength). Two curves were considered for each sample and the analysis were conducted in the Chopin model MA95 alveograph at the temperature of 18–22 °C, UR 65 ± 15% with 2.5% of saline solution.

2.6. Wheat flour colour evaluation

The wheat flour colour was sensorially evaluated by a "ranking test for difference" with 18 selected judgers using a *Macbeth Spectral light* with daylight (6770 °K). The eight mixtures (Mix1 to Mix7 plus CONT) were coded with three numbers and presented in a randomized design block. The diffusion vision angle of 45° was applied in relation to incident light under light-grey base. The judges ranked the sample from [1] most white to [8] most yellow.

Flour colour was also analyzed using a colorimeter (model Color Quest XE, Hunter Associates Laboratory Inc., Reston, Virginia, USA). In the Hunter-Lab colorimeter, the colour of a sample is denoted by the three dimensions L, a and b, corresponding to the XYZ CIE lab system. The L value gives a measure of the lightness of the product colour from 100 for perfect white to zero for black, as the eye would evaluate it. The "redness/greenness" and "yellowness/blueness" are denoted by a and b values, respectively. In this study, the code "Lc" was used instead of "L", because the latter was applied to rheological measurement for dough extensibility.

2.7. Equations modelling

The experimental results obtained by the sensory and instrumental determinations were applied to obtain the regression models. Based on experimental design (Table 1), the cubic model could be fitted in case the lacks of fit of the quadratic or linear models were statistically significant ($p < 0.05$)

$$\hat{y}_i = b_1^* x_1 + b_2^* x_2 + b_3^* x_3 + b_{12}^* x_1 x_2 + b_{13}^* x_1 x_3 + b_{23}^* x_2 x_3 + b_{123}^* x_1 x_2 x_3,$$

where y_i, evaluated response; b_i, coefficients estimated by the least square method; x_i, dependent variables, being $1 \geqslant x_i \geqslant 0$ e$\Sigma x_i = 1.0$ (Eriksson, Johansson, & Wikstrom, 1998).

Quality of the models fitness was evaluated by ANOVA, in which the repetition supplied the freedom degree to obtain the pure error. Calculations and graphics were performed by STATISCA v.6 (StatSoft Inc., Tulsa, USA).

The simultaneous optimization was obtained by Derringer and Suich (1980) methodology and the values were further applied in validating the models, using the same experimental procedure as used at the beginning of this study.

2.8. Statistical analysis

In addition to the statistical procedures described above to model the polynomial equations, all results were primarily submitted to the normality test and variance homogeneity. The treatments were treated by ANOVA, followed by the Tukey HSD test, considering significant p values <0.05. When, the Levene test was significant, no parametric statistical procedures were used (Kruskal–Wallis ANOVA by ranks). The sensory results from ranking test were submitted to Friedman ANOVA according to Meilgaard, Civille, and Carr (1999). Sensory and instrumental colour results were correlated by linear regression.

3. Results and discussion

3.1. Lipoxygenase activity and nutritional composition

Fig. 1 presents LOX1 activity in full fat soy flour (27.7 ± 0.89 units/μg protein). No LOX1 activity was found in wheat flour. On the other hand (Fig. 2), wheat flour presented a higher LOX3 activity (9.72 ± 0.84 units/μg protein) than soy flour (1.00 ± 0.83 units/μg protein). The nutritional composition of the wheat and soy flour and the wheat flour treated with 1% of the different mixtures according to the experimental design are presented in Table 2. Differences in the protein water interaction are related to the source of protein, degree of protein denaturation and aminoacid composition (Wang & Zayas, 1991). Soy flour increased the moisture in treatments where it was applied at a higher proportion, maybe because of the high water absorption capacity presented by the soy protein (Hettiarachchy & Kalapathy, 1998).

3.2. Polynomial models

Two models were fitted for P and L index as W index did not present significant variation between the wheat flours.

$$\hat{y}_P = \underset{(\pm 1.93)}{107.27x_1} + \underset{(\pm 1.93)}{78.27x_2} + \underset{(\pm 2.11)}{98.84x_3} + \underset{(\pm 9.69)}{34.36x_1x_2} + \underset{(\pm 9.69)}{26.36x_2x_3}$$

$$y_L = \underset{(\pm 1.90)}{45.77x_1} + \underset{(\pm 1.90)}{66.77x_2} + \underset{(\pm 1.90)}{44.97x_3}$$

y_P and y_L represent the estimated value for the P and L parameters when the values x_i change inside the experimental range ($1 \geqslant x_i \geqslant 0$ and $\Sigma x_i = 1.0$), b_i represents the model coefficients estimated by the least square method and the values between parenthese are the standard errors of coefficients.

For the colour parameters, it was possible only to fit a model for Lc, as the b and sensory data did not achieve the basic assumptions for the variance homogeneity necessary to evaluate the polynomial models.

$$y_{Lc} = \underset{(\pm 0.03)}{91.44x_1} + \underset{(\pm 0.03)}{92.16x_2} + \underset{(\pm 0.03)}{1.80x_3} + \underset{(\pm 0.15)}{0.80x_1x_2} + \underset{(\pm 0.15)}{0.90x_1x_3} + \underset{(\pm 0.15)}{0.72x_2x_3} + \underset{(\pm 1.09)}{2.61x_1x_2x_3}$$

The analysis of variance and determination of the fitness quality of the three models proposed in this study are showed in Table 3 and the response surfaces presented in Figs. 3–5. It can be observed that 92%, 84% and 94% of P, L and Lc variation were explained by the respective models and the lack of fit was not significant ($p < 0.05$).

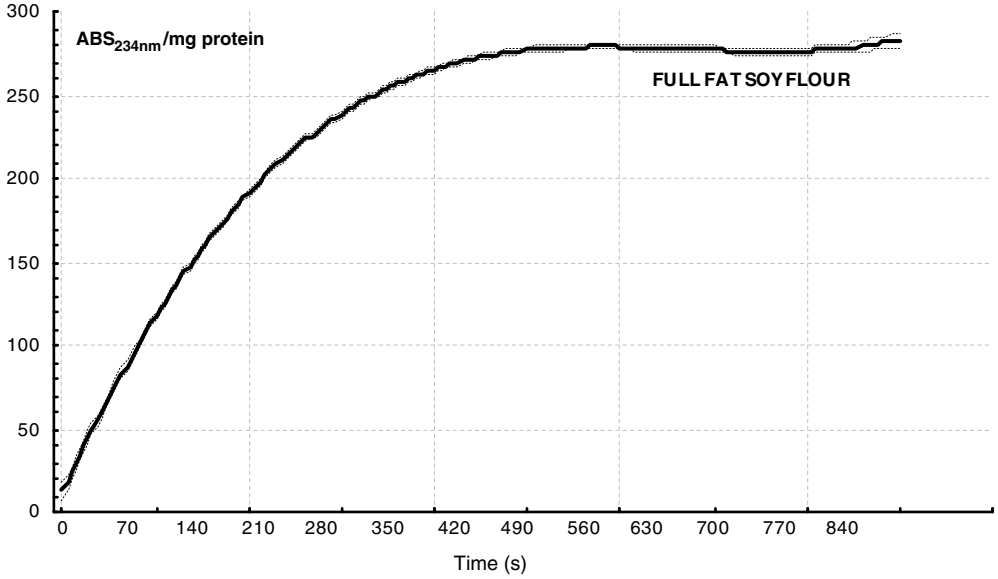

Fig. 1. Spectrophotometric monitoring of the activity of lipoxygenase 1-induced linoleic acid oxidation at 234 nm (LOX 1).

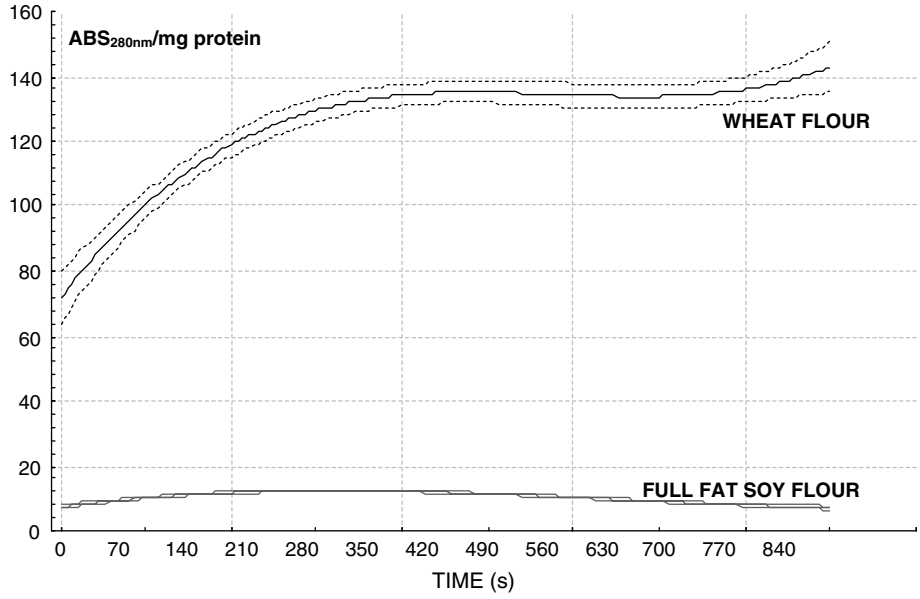

Fig. 2. Spectrophotometric monitoring of the activity of lipoxygenase 3-induced linoleic acid oxidation at 280 nm (LOX 3).

Table 2
Nutritional composition of the wheat and soy flour and wheat flours prepared according to the experimental design

Assay[A]	(x_1, x_2, x_3)	Moisture[B]	Protein	Lipid[C]	Ash	Carbohydrate
Soy flour	–	5.25 ± 0.10	40.6 ± 0.83	23.47 ± 0.57	5.03 ± 0.09	25.6
Wheat flour	–	13.84 ± 0.37	11.9 ± 0.57	1.42 ± 0.09	0.63 ± 0.04	72.2
Mix 1	(1, 0, 0)	$14.61^{b} \pm 0.05$	11.16 ± 0.04	1.64	0.67 ± 0.05	71.7
Mix 2	(0, 1, 0)	$12.91^{a} \pm 0.86$	11.1 ± 0.30	1.42	0.67 ± 0.05	73.9
Mix 3	(0, 0, 1)	$12.96^{a} \pm 0.16$	11.7 ± 0.91	1.42	0.57 ± 0.00	73.4
Mix 4	(1/2, 1/2, 0)	$14.32^{ab} \pm 0.17$	11.1 ± 0.40	1.53	0.76 ± 0.07	72.3
Mix 5	(1/2, 0, 1/2)	$13.93^{ab} \pm 0.10$	11.7 ± 0.74	1.53	0.77 ± 0.09	72.0
Mix 6	(0, 1/2, 1/2)	$13.68^{ab} \pm 0.23$	11.1 ± 0.19	1.42	0.74 ± 0.05	73.1
Mix 7	(1/3, 1/3, 1/3)	$13.72^{ab} \pm 0.40$	11.0 ± 0.13	1.49	0.65 ± 0.07	73.2
CONT	(0, 0, 0)	$13.75^{ab} \pm 0.54$	11.2 ± 0.02	1.42	0.63 ± 0.14	73.0
p[D]	–	*0.030527*	*0.619380*	–	*0.344291*	–

[A] Values are g/100 g expressed by mean ± SD ($n = 3$).
[B] Values followed by the same letter in the column are not significantly different ($p < 0.05$).
[C] Values estimated according to the mixture composition compounds.
[D] Probability value (p) for the difference between the eight mixtures, obtained by one-way ANOVA and Tukey HSD test.

3.3. Rheological properties of dough

The empirical rheological measurements, which use large deformations, generally give good correlations with bread-making quality (Tronsmo et al., 2003). Methods based on biaxial extension, for example, the alveograph, can be considered the most appropriate for measuring rheological properties of dough. The major advantage of these tests is that the deformation closely resembles practical conditions experienced by the cell walls around the expanding gas cells within the dough (Dobraszczyk & Morgenstern, 2003). During proofing and baking, the growth of gas bubbles determines the expansion of the dough and therefore the ultimate volume and texture of baked product (He & Hoseney, 1991).

The W index (Table 4), that is normally used to estimate the dough behaviour during the baking process, did not show any variation among the mixtures. This result could be due to the high variation observed in Mix1 and also because the W index was obtained by the area under the

Table 3
Analysis of variance of the models obtained by the response surface methodology applied to the experimental data

Parameters[a]	*DF*	*p*	*Df* (lack of fit)	*p* (lack of fit)	R^2
P	4	0.000010	2	0.321871	$R^2 = 0.9461$; $R^2_{Adj} = 0.9222$
L	2	0.000015	4	0.252447	$R^2 = 0.8668$; $R^2_{Adj} = 0.8426$
Lc	6	0.000000	0	–	$R^2 = 0.9525$; $R^2_{Adj} = 0.9366$

R^2 is the variation proportion explained by the polynomial model.
[a] *P* represents resistance to dough extension, *L* dough extensibility and *Lc* lightness.

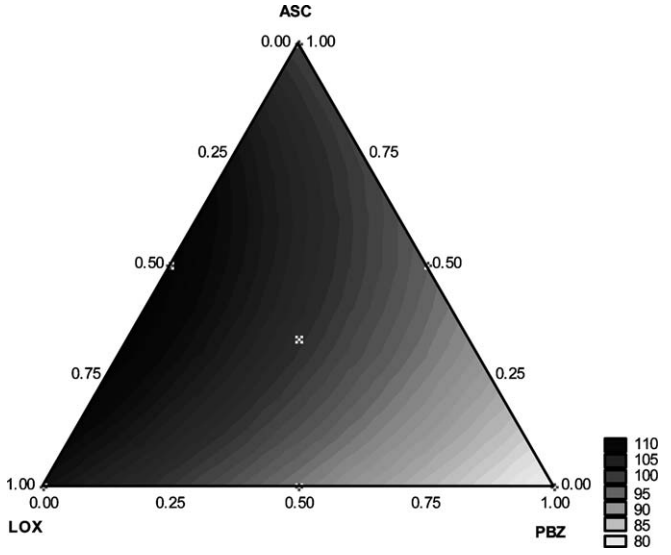

Fig. 3. Response surfaces obtained by the polynomial model fitted to dough extensibility (L).

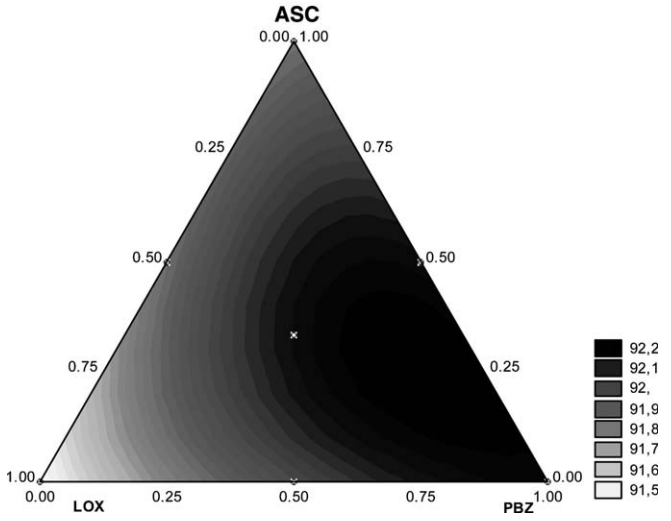

Fig. 4. Response surfaces obtained by the polynomial model fitted to dough resistance to extension (maximum over-pressure needed to blow the dough bubble ($P = h \times 1.1$)).

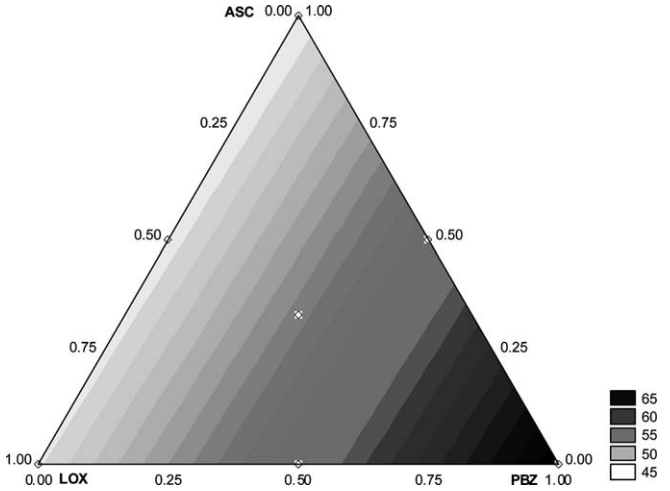

Fig. 5. Response surfaces obtained by the polynomial model fitted to lightness.

Table 4
Wheat flour alveograph parameters

Assay[A]	P[B]	L[B]	W[B]
Mix 1	106.00 ± 2.83^{bc}	45.50 ± 4.95^{a}	196.00 ± 16.97
Mix 2	77.00 ± 0.00^{d}	68.00 ± 4.24^{c}	187.00 ± 5.66
Mix 3	99.00 ± 4.24^{ab}	47.00 ± 0.004^{ab}	206.00 ± 8.49
Mix 4	95.00 ± 2.834^{ab}	58.50 ± 3.544^{bc}	215.50 ± 6.36
Mix 5	111.00 ± 1.41^{c}	46.00 ± 1.41^{a}	219.50 ± 3.54
Mix 6	94.50 ± 2.12^{a}	53.50 ± 0.714^{ab}	212.50 ± 4.95
Mix 7	103.00 ± 4.244^{abc}	49.00 ± 1.414^{ab}	214.00 ± 5.66
p[C]	*0.000136*	*0.000888*	*0.050210*

[A] The rheological parameters observed in control wheat flour (wheat flour without any Mix addition) were: P (89.00 ± 5.66), L (54.00 ± 1.41) and W (187.00 ± 7.78).
[B] Values are expressed by mean $\pm$ SD ($n = 3$). Means followed by the same letter within a column are not significantly different ($p < 0.05$).
[C] Probability value (p) for the difference between the seven treatments.

P/L curve in the alveogram. Tronsmo et al. (2003) demonstrated that gluten extensibility and elasticity were negatively correlated to each other ($r = -0.90$); that is, flours of strong protein quality showed high elasticity and low extensibility.

The dough extensibility (Table 4) was increased by the PBZ (Mix2 and Mix4). PBZ caused a significant increase in dough extensibility, followed by a significant reduction on dough elasticity (Figs. 3 and 4). According to Visschers and de Jongh (2005), cysteine residues in proteins occur as the free sulphydryl form (CSH) or the oxidized cystine (CSSC), forming SS bonds. The sulphydryl residues can display a strong chemical reactivity toward a number of compounds, reactivity being directly related to pK_a (around 8.3), which implies that the reactivity is greatly reduced under mild acid conditions. It has been demonstrated that for bread-making, chemical reactivity of cysteine containing proteins serves the stabilization gas cells in the gluten network matrix (Morel et al., 2002). The main stable metabolite in the decomposition of PBZ is benzoic acid (Saiz et al., 2001). Possibly, the benzoic acid formed by PBZ reaction with carotenoids present in wheat flour was enough to produce mild acid conditions, decreasing the dough elasticity. As the benzoic acid and pH dough were not determined in this study, it is not possible to be conclusive.

Dough extension resistance, which represents the dough elasticity (Table 4), was significantly increased in the presence of the mixture containing 250 ppm of ASC plus 0.5% of LOX (Mix5), the mixture containing 0.3% of LOX plus 167 ppm of ASC and 20 ppm of PBZ (Mix7) and 1.0% of LOX (Mix1), and significantly reduced when maximum PBZ concentration was applied (Mix2). Mix5, which represents the mixture containing 250 ppm of ASC plus 0.5% of LOX, showed a significantly higher P index (111 ± 1.41)

than Mix3 (99 ± 4.24) which contains 500 ppm of ASC. This result suggests that LOX could have improved the ASC effect on dough elasticity (Fig. 4).

LOX and ASC affect the dough extension resistance in different ways. The reaction sequence is initiated when flour with ASC is mixed with dough. L-threo-ASC is quickly oxidized to L-threo-DASC (dehydroascorbic acid) by ascorbate acid oxidase (EC1.10.3.3) naturally present in wheat, although non-enzymatic oxidation by gaseous oxygen can also take place (Cherdkiatgumchai & Grant, 1986; Koehler, 2003a). The hypothesis proposed by Grosch and Wieser (1999) is based on rapid oxidation of endogenous glutathione reduced (GSH) to glutathione disulphide (GSSG) catalyzed by the enzyme GSH-DH dehydroascorbate reductase (EC1.8.5.1) using L-threo-DASC as co-substrate. SS/SH interchange of GSSG with gluten proteins, leading to a rapid blocking of SH groups on gluten. GSH released in the latter reaction is oxidized again by DASC. According to this reaction sequence, the improved action of ASC is due to the fact that most of the GSH present in flour is oxidized to GSSG so rapidly that the SH/SS interchange reaction of GSH with intermolecular bonds of gluten molecules are minimized. These reactions would depolymerise gluten proteins and weaken the dough. It has been shown that endogenous GSH reacts almost exclusively with intermolecular SS bonds, which are responsible for aggregative properties of glutenin low molecular weight subunits. Recent research conduced by Koehler (2003b) confirmed the Grosch and Wieser (1999) hypothesis, by the identification of mixed disulphides formed by reaction of GSSG and gluten proteins with free thiol groups. GSH level increases strongly with the ash content, which correspond to the flour extraction grade of the flour, because GSH is mainly located in the germ and aleurone cells and correlates negatively with the flour storage time (Grosch & Wieser, 1999).

Lipoxygenase (EC1.13.11.12 linoleate: oxygen oxidoreductase) is a dioxygenase that catalyses, as a primary reaction, the hydroperoxidation, by molecular oxygen, of linoleic acid and other polyunsaturated lipids that contain a *cis*,*cis*-1,4 pentadiene moiety. From soybeans, three isozymes have been isolated: lipoxygenases 1, 2 and 3, which have been categorized into two classes. Class I is characterized by high pH optima of around 9.0 and formation of large amounts of 13L-hydroperoxides (LOX1), and Class II has pH optima of around 7.0 and equal formation of 9D and 13L-hydroperoxides (LOX2 and LOX3) (Axelrod et al., 1981; Kumar, Rani, Tindwani, & Jain, 2003). The mechanism by which LOX acts as a dough improver requires both free lipid and oxygen. The model takes as its basis, the long-held view that soy flour imparts its improving effect in dough by oxidizing sulphydryls in gluten protein. This model is based on a decrease in flour sulphydryl groups, seen when LOX is added (Faubion & Hoseney, 1981). The reaction is initiated when one molecule of hydroperoxide interacts with the inactive native enzyme (Fe^{2+}LOX) and yields an active enzyme (Fe^{+3}LOX), presumably via a single electron transfer from iron to oxygen. Active enzyme (Fe^{+3}LOX) is reduced by the abstraction of a hydrogen from the 1,4 diene system of the linoleic acid substrate and a base abstracts a proton, forming a Fe^{+2}LOX-R$^{\cdot}$ radical compound. Molecular O_2 reacts with the fatty acid radical (Fe^{+2}LOX$\cdots$ROO$^{\cdot}$), followed by a single electron transfer from iron to the peroxy radical, regenerating the active enzyme (Fe^{+3}LOX+ $^{\cdot}$ROO^{-}), producing a peroxide anion, which receives the proton from the base (probably water), yielding two optically active hydroperoxide of linoleic acid (9D^{-}ROOH and 13L^{-}ROOH) (Faubion & Hoseney, 1981; Prigge et al., 1997). These lipid peroxides are able to react with a variety of proteins and amino acids, including the sulphydryl residues in the gluten proteins (Faubion & Hoseney, 1981). The results suggested that 0.5% and 1.0% of LOX addition had the same effect on dough elasticity when in the presence of 250 ppm of ASC or 30 ppm of PBZ. Mix5, containing 250 ppm of ASC, resulted in a higher P index than that with 500 ppm when 0.5% of LOX was present in the mixture, suggesting that the iron present in the LOX molecule (Fe^{+3}LOX) could contribute to the ASC oxidation, beside its direct action on the SH residues in gluten. However, more studies are needed to clarify this mechanism.

3.4. Wheat flour colour

The instrumental (Hunter Lab) and sensory results of wheat flour colour evaluation are shown in Table 5. The mixture containing full fat soy flour (LOX) showed significantly lower lightness (*Lc*) than the other mixtures. Fig. 5 shows that the lightness increases with the increase of PBZ in a linear way. The maximum concentration of PBZ (60 ppm, Mix2) promoted a significant *b* (yellowness/blueness) reduction in the wheat flour. The sensory results showed a linear and significant ($p = 0.034$; $r^2 = 0.46$) correlation with the instrumental *b* parameter, indicating a higher human visual perception for ''yellowness/blueness'' than for lightness.

Table 5
Wheat flour instrumental and sensory colours

Assay	L^{A}	B^{A}	Sensory score[B]
Mix 1	91.4 ± 0.06^{d}	11.2 ± 0.05^{a}	7.28 ± 1.78^{c}
Mix 2	92.2 ± 0.03^{a}	10.2 ± 0.01^{c}	1.17 ± 0.51^{a}
Mix 3	91.8 ± 0.08^{c}	11.2 ± 0.06^{a}	4.17 ± 1.89^{b}
Mix 4	92.0 ± 0.06^{c}	11.2 ± 0.01^{a}	4.61 ± 2.00^{b}
Mix 5	91.8 ± 0.06^{b}	11.3 ± 0.05^{a}	5.00 ± 1.68^{b}
Mix 6	92.1 ± 0.04^{a}	10.6 ± 0.13^{a}	4.22 ± 2.37^{b}
Mix 7	92.1 ± 0.03^{a}	11.1 ± 0.05^{a}	4.44 ± 2.36^{b}
CONT	$92.1 \pm 0.03^{a,c}$	11.1 ± 0.03^{a}	8.06 ± 1.43^{c}
p^{C}	*<0.000001*	*0.010100*	–

[A] Means of three determinations. Means followed by the same letter within a column are not significantly different ($p < 0.05$).
[B] Sensory analysis results were treated by Friedman ANOVA.
[C] Probability value (*p*) for the difference between the eight treatments.

Table 6
Comparison of the estimated values obtained by the polynomial models and the values measured experimentally

Parameters	Estimated value[a]	Observed value[a]	p[b]	Relative error (%)
Lc	91.96 (91.90–92.00)	92.00 (91.68–92.31)	0.999802	0.04
P	106.87 (103.55–110.20)	108.50 (89.44–127.56)	0.761864	1.48
L	46.93 (44.25–49.61)	44.50 (25.44–63.56)	0.555512	5.58

[a] Values expressed by mean (95% CI).
[b] Probability values by χ^2 test.

Freshly milled flour has enough pigment to cause the typical yellowish colour. Wheat flour contains ≅3.0 mg/kg of carotenoids, that being the highest quantity represented by a xanthophyll known as lutein (Saiz et al., 2001). Xanthophyll and xanthophyll esters have no vitamin A potency (Fortmann & Joiner, 1971). Flour bleaching primarily involves a disruption of the conjugated double bond system of carotenoids to a less conjugated colourless system (Saiz et al., 2001). PBZ can react with flour carotenoids (mainly lutein), forming a resonance stabilized peroxyl radical carotenoid, although it could also be trapped, generating a stable adduct (Pinzino, Capocchi, Galleschi, Saviozzi, & Zandomeneghi, 1999). Otherwise, PBZ bleached the xanthophylls present in the wheat flour and bleached the carotenoids present in the full fat soy flour.

3.5. Optimization and model validation

Besides explaining the behaviour of variables by the contour curves, the models fitted in this study could also be applied for optimization. Thus, the latter procedure was conducted in order to maximize the three responses (*P*, *L* and *Lc*) simultaneously. The final result for this optimization suggested that a mixture containing 0.27% of full fat soy flour (LOX), 4.80 ppm of benzoyl peroxide (PBZ) and 325 ppm of ascorbic acid (ASC) in flour could be a good mixture of these three oxidant compounds in order to achieve the maximum dough resistance, extensibility and whiter flour. This new mixture was submitted to the same experimental analytical procedures applied as at the beginning of this study and the estimated and observed results are presented in Table 6. There was no significant difference between the estimated and observed values, suggesting a good fit between the models to the experimental data. The optimized mixture (*Lc* of 92.0 ± 0.13) was as white as the control (*Lc* of 92.1 ± 0.03) and the resistance to extension of the gluten structure presented by the optimized mixture ($P = 108.5 \pm 2.12$) indicated a 22% increase in comparison to the control assay ($P = 89.0 \pm 5.70$).

4. Conclusions

Response surface methodology was demonstrated to be an efficient statistical tool, able to model lightness characteristics of wheat flour by an instrumental procedure (colorimeter), dough extensibility and resistance to extension as a function of the proportion of oxidants compounds added to wheat flour. These results also suggested that lipoxygenase, obtained from full fat soy flour, may improve the ascorbic acid effect on dough elasticity. The advantage of the RSM application over the empirical methods usually applied in many wheat mills, blenders and bakeries, is that the optimum proportion between the oxidants compounds can be fitted for each type of wheat flour, proceeding with an experimental design containing only seven assays.

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