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IMPACT OF FIBER MIXTURE ON DOUGH AND CHAPATTI QUALITY USING D-OPTIMAL RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Demand for health oriented products such as sugar-free, low calorie and high fiber products are increasing. One such recent trend is to increase the fiber content in food products to overcome health problems such as hypertension, diabetes, and colon cancer, among others. Chapatti is an important staple food consumed by majority of the population in the Indian subcontinent hence it can be a very good vehicle for fiber fortification. Fiber from natural sources such as wheat, soy fiber and type III resistant starch (RS) were used to study their impact on rheological characteristics of whole wheat flour dough and chapatti singly and in associated mixtures at different levels. D-optimal response surface methodology mixture design was applied to a mixture containing three ingredients: x1, wheat fiber, x2, soy fiber and x3, type III RS. The variation selected to each variable was based on values which were optimized on the basis of sensory properties and textural properties of chapatti, where x1, x2, x3 were changed from 2.5 to 5 g/100 g of whole wheat flour. For each of the response variables, model summarized F-tests and lack of fit tests which were then analyzed for linear or quadratic models. Three-dimensional response surface plots were generated for all quality parameter. Calculation of optimal processing conditions for optimum stickiness, strength, tear force and extensibility of dough and chapatti were performed using a multiple response method called desirability. Addition of wheat, soy fiber and type III RS in wheat flour mixture decreased dough stickiness and improved dough strength. Dough containing wheat fiber (2.5%), soy fiber (5.0%) and type III RS (2.5%) yielded highly acceptable chapattis in terms of textural properties such as low tear force values and high extensibility.

Keywords: Chapatti, fiber, response surface methodology

INTRODUCTION

Cereal fiber, which have a great proportion of insoluble fiber, have physiological advantages such as the chewing mechanism, stimulation of intestine function and influence on intestine transit period (**Bollinger, 2000**). Health authorities, world-wide, recommend a decrease in the consumption of animal fats and proteins and an increase of cereal intake, which is an important source of dietary fiber, and, in most European countries, cereals constitute a major source of dietary fiber. In addition to these physiological properties, cereal fiber consisting mainly of cellulose have advantageous technological properties such as a high water and fat binding capacity and they are optimal ingredients for achieving high yields and reduced cost. One to three percent fiber in certain foods can also reduce lipid retention when these foods are fried (**Ang, 1993; Thebaudin *et al.* 1997**).

A wide variety of fiber from plant sources have been developed for use in various foods to provide more fiber, to improve the texture, color and aroma with a reduced energy of the final product (**Jeltema *et al.* 1983; Morrison *et al.* 2008; Sánchez -Alonso *et al.* 2007; Yanniotis *et al.* 2007**). Lemon and apple fiber have been reported to have relatively high water holding capacity and therefore used in cakes, breads and similar cereal products to improve the softness and the product yield with a reduced energy value of the product (**Chen *et al.* 1988**).The development of safer and healthier low-calorie products with acceptable functional and sensory characteristics, by employing the conventional processing equipment is of major industrial concern to fulfill consumers' expectations. The latest functional significance is of major interest in the performance of low-calorie staple foods, particularly chapatti. There are very few reports on effect of fiber addition on chapatti quality. Flour tortillas (soft tacos and tortillas, wraps, flat breads) with improved texture and nutritional characteristics were made using flour milled from specific barley cultivars with waxy starch characteristics and high levels of fiber (**Ames *et al.* 2003**). Therefore, to meet this requirement for dietary fiber, the development of enriched chapatti with a higher dietary fiber content could be a potential option. Chapatti can be enriched with dietary fiber, including wheat bran (**Ranhotra *et al.* 1990; Sidhu *et al.* 1999**), soy fiber, gums, such as guar gum and modified cellulose (**Pomeranz *et al.* 1977**), β-glucans (**Knuckles *et al.* 1997**). Both the expansion and structure of these products depend on starch gelatinization, which is affected by processing conditions and raw material composition. Increasing fiber content in the form of bran resulted in premature rupture of gas cells, which reduced overall expansion of bread in one study by **Mendonca *et al.* (2000)** and **Moore *et al.* (1990)**.The common experimental approach in most of the studies has been to investigate the effect of variables one at a time. However, the examination of several variables individually is laborious and time consuming. It results in large quantities of data that are difficult to interpret and, in addition, it fails to measure interaction effects. To overcome the limitations of this experimental approach, a process optimization technique that involves factorial designs and multiple regression techniques, called response surface methodology (RSM), can be used. The advantages of an RSM approach are that it examines variables simultaneously, it is less time consuming and more cost effective, and it explains any synergies between variables. Furthermore, the results may be illustrated graphically in easy to understand 2-D contour and 3-D response surface plots. When the mixture components are subject to the constraint that they must sum to one, there are standard mixture designs for fitting standard models, such as simplex-lattice designs and simplex-centroid designs. When mixture components are subject to additional constraints, such as a maximum and/or minimum value for each component, designs other than the standard mixture designs, referred to as constrained mixture designs or extreme-vertices designs, are appropriate. In mixture experiments, the measured response is assumed to depend only on the relative proportions of the ingredients or components in the mixture and not on the amount of the mixture. The amount of the mixture could also be studied as an additional factor in the experiment; however, this would be an example of mixture and process variables being treated together. Proportions of each variable must sum to 1. The main distinction between mixture experiments and independent variable experiments is that with the former, the input variables or components are non-negative proportionate amounts of the mixture, and if expressed as fractions of the mixture, they must sum to one. If for some reason, the sum of the component proportions is less than one, the variable proportions can be rewritten as scaled fractions so that the scaled fractions sum to one.

The purpose of the experiment in mixture design is to model the blending surfaces with some form of mathematical equation so that: predictions of the response for any mixture or combination of the ingredients can be made empirically, or some measure of the influence on the response of each component singly and in combination with other components can be obtained. The usual assumptions made for factorial experiments are also made for mixture experiments. In particular, it is assumed that the errors are independent and identically distributed with zero mean and common variance. Another assumption that is made, as with factorial designs, is that the true underlying response surface is continuous over the region being studied. D-optimal designs are often used when classical designs do not apply or work. D-optimal designs are one form of design provided by a computer algorithm. These types of computer-aided designs are particularly useful when classical designs do not apply. Unlike standard classical designs such as factorials and fractional factorials, D-optimal design matrices are usually not orthogonal and effect estimates are correlated. These designs are always an option regardless of model or resolution desired. These types of designs are always an option regardless of the type of model the experimenter wishes to fit (for example, first order, first order plus some interactions, full quadratic, cubic, etc.) or the objective specified for the experiment (for example, screening, response surface, etc.).

Planning a mixture experiment typically involves the following steps (**Cornell, 2000**):

1. Define the objectives of the experiment.
2. Select the mixture components and any other factors to be studied. Other factors may include process variables or the total amount of the mixture.
3. Identify any constraints on the mixture components or other factors in order to specify the experimental region.
4. Identify the response variable(s) to be measured.
5. Propose an appropriate model for modeling the response data as functions of the mixture components and other factors selected for the experiment.
6. Select an experimental design that is sufficient not only to fit the proposed model, but which allows a test of model adequacy as well.

Considering all these aspects this research aims at optimizing mixtures of fiber from different sources and their effect on chapatti dough and chapatti quality fresh as well as during storage to design low-calorie chapatti formulation. The gritty texture of fiber limits their application singly. Introducing fiber in chapattis will enhance the acceptance of fiber and at the same time will improve chapattis nutritional profile. Fiber from natural sources such as wheat, soy fiber and type III resistant starch (RS) were chosen for incorporation, as rich fiber sources. Impact of fiber added singly and in associated mixtures at different levels on the chapatti dough characteristics (dough stickiness, cohesiveness, strength) and chapatti quality parameters (tear force, extensibility, color) has been evaluated by response surface methodology mixture design.

MATERIALS AND METHODS

Materials

Branded whole-wheat flour (Ashirwad atta, ITC), double filtered groundnut oil (Dhara, Amul), and table salt (Tata Co.) were procured from the local market. Very purified fiber such as wheat & soy fiber (VITACEL®) consisting mainly of cellulose and hemicellulose as a functional ingredient were gifted by J. Rettenmaier & Söhne, Rosenberg, Germany. Type III resistant starch (Hi-Maize) was gifted by National Starch and Chemical Company, Mumbai, India. Preformed pouches (15 x 19 cm) of four layer laminate of PET 12µ, Al foil 12µ, Nylon 15µ, CPP 85µ was gifted by Paper Product Limited, Mumbai, India. All other chemicals used for the analysis were of analytical grade.

Methods

Dough preparation and evaluation of dough stickiness

Whole wheat flour and selected fibers at different level ranging from 5-15% (w/w of whole wheat flour) in the form of dry formulation were mixed thoroughly and dough was prepared. Dough stickiness was measured according to the method of **Hosney and Smegwish (1999)**.

One factor at a time optimization of fibers

In the present study the three fibers (wheat, soy and Type III RS) were optimized on the basis of one factor at a time method. Wheat, soy fiber and Type III RS were added at three different levels ranging from 5, 10 & 15 % on w/w basis of whole wheat flour. The dough was prepared and effect of level of fiber added on dough stickiness, dough strength and cohesiveness was studied.

Design of experiment

Once the level of fiber was optimized D-optimal mixture design was constructed using the software Design Expert Version 6.0.10 (Stat-Ease Corporation, Minneapolis, Minn., USA) and was used to analyze the results. Maximum and minimum predictor variable levels were chosen by carrying out preliminary trials as explained above. The sum of the factors were kept constant that is at 10.

Response surface methodology D-optimal mixture design

D-optimal mixture design applied to a mixture containing three ingredients: x_1- wheat fiber, x_2- soy fiber and x_3- type III RS. The variation selected to each variable was based on values which were optimized on the basis of sensory properties and textural properties, where x_1, x_2, x_3 were changed from 2.5 to 5 g/100 g of wheat flour. For each of the response variables, model summaries F-tests and lack of fit tests which were then analyzed for linear or quadratic models. The cubic model was aliased because there were not enough points for this type of model. From this information, the most accurate model was chosen via the sequential F-tests, lack-of-fit tests and other adequacy measures. Three-dimensional response surface plots were generated for each quality parameter. Calculation of optimal processing conditions for optimum tear force (g) and extensibility (mm) of chapatti was performed using a multiple response method called desirability (**Box and Wilson, 1951; Myers and Montgomery, 1995**). This optimization method incorporates desires and priorities for each of the variables. In this study, predictor variables were permitted to be at any level within the range of the design. Statistical experimental design was used to optimize the level of fiber in a mixture to obtain flour of mixed fibers from different sources, which was checked with respect to effect on dough stickiness and tear force and extensibility of chapatti.

For statistical calculations, the relationship between the coded values and actual values are described by the following equation:

$$X_i = \frac{A_i - A_0}{\Delta A}$$

Where, X_i is coded value of variable; A_i the actual value of variable; A_0 the actual value of the A_i at the center point; and ΔA the step change of variable. Chapatti tear force (g), extensibility (mm) (0, 1, 2 days) was taken as response variables.

Experiments were carried out in triplicate.

Quadratic equation for the variable was as follows

$$Y = \beta 0 + \Sigma \beta i\, Xi + \Sigma \beta ii\, Xi\, Xj + \Sigma i\, \Sigma j\, \beta ij\, Xi\, Xj$$

Y is the predicted response; β0 a constant; βi the linear coefficient; βii the squared coefficient; and βij the cross-product coefficient.

The above quadratic equation was used to build surfaces for the variables. The software Design Expert Version 6.0.10 was used to analyze the results. Three-dimensional mixtures plots of three factors versus tear force (g) and extensibility (mm) were obtained.

Preparation and evaluation of chapatti for tear force and extensibility and color (L a b).

In the present study chapattis were prepared and evaluated for tear force and extensibility according to the method of **Ghodke *et al.* (2009)**.

RESULTS AND DISCUSSION

Effect of fiber addition on dough and chappti

Table 1 shows the effect of fiber addition on the stickiness of chapatti dough with added fiber. Wheat, soy fibers and type III RS were added into whole wheat flour at three levels viz., 5, 10 and 15 % w/w of whole wheat flour. From Table 1 it can be observed that control dough showed dough stickiness of 33.36 g and with the addition of wheat fiber the stickiness was increased to 38.05 g at 5%. High water absorption attributed to decrease in the water binding capacity of gluten (**Dreese *et al.* 1982**) thus increased stickiness. The effect has been attributed to the hydroxyl groups in the fiber structure, which allows more water interactions through hydrogen bonding (**Guarda *et al.* 2004**).

Table 1- Effect of fiber addition on dough stickiness, dough strength and dough cohesiveness

Sample	Dough stickiness (g)	Dough strength (g)	Dough cohesiveness (mm/s)
Control	33.36±1.77	1.66±0.30	1.79±0.34
5g WF	38.05±3.32	2.82±0.88	2.07±0.59
10g WF	36.64±4.43	2.42±0.92	1.94±0.48
15g WF	32.19±2.41	2.79±0.46	1.88±0.46
5g SF	31.52±2.82	2.04±0.44	1.34±0.31
10g SF	29.05±1.87	1.65±0.29	1.14±0.39
15g SF	29.85±2.71	1.35±0.28	0.81±0.28
5g Type III RS	38.26±2.70	2.04±0.58	1.81±0.16
10g Type III RS	35.82±3.35	1.84±0.41	1.64±0.72
15g Type III RS	34.66±2.17	1.47±0.14	1.58±0.34

All the values are mean ± sd of three values; wf: wheat fiber; sf: soy fiber

Further addition of 10% and 15% wheat fiber, the dough stickiness was decreased due to the dilution of the gluten, as gluten fractions, have been shown to be important determinants of dough stickiness. Dough stickiness was reduced by addition of flour protein fractions concentrated in glutenin proteins (**Dhaliwal and Mac Ritchie, 1990**); gliadins have also been reported as responsible for dough adhesiveness (**Ram and Nigam, 1983**). When soy fiber was added the dough stickiness was decreased, this was due to the less water absorption of the soy fiber. These results could be explained by the interactions between fibers and gluten, as suggested by **Chen *et al.* (1988)**. With the addition of type III RS, dough stickiness was increased at 5% level but it decreased with increased level. Water absorption is generally accepted to be of main importance in dough stickiness (**Noguchi *et al.* 1976; Gaines, 1982; Dhaliwal *et al.* 1990; Heddleson *et al.* 1994)**. The higher the water absorption, the more sticky dough it gives (**Hlynka, 1970; Chen and Hoseney, 1995; Armero and Collar, 1997)**. Adding excess water to flour produces dough with better wetting properties. The dough surface is in better contact with the surface of a probe, giving higher surface adhesion. The dough strength observed in wheat fiber added dough was higher than the control, and in case of soy fiber the dough strength was decreased with the increased level of soy fiber, type III RS at 5% concentration resulted in small increase in dough strength but decreased with the increased concentration. Similar results were obtained by **Sudha *et al.* (2007)** who observed the weakening of the dough with the increased level of the fibers.

Table 2 Effect of fiber addition on chapatti tear force (g) and extensibility (mm) over a period of 2 days storage at 37±2°C

Sample	0 Day		1 Day		2 Day	
	Tear force (g)	Extensibility (mm)	Tear force (g)	Extensibility (mm)	Tear force (g)	Extensibility (mm)
Control	316.45±35.46	5.51±0.59	358.61±23.47	1.66±0.38	447.64±83.38	1.22±0.14
5g WF	283.74±40.34	3.90±0.49	427.96±106.43	2.74±0.96	363.55±40.78	1.52±0.27
10g WF	261.18±85.63	3.68±1.27	365.24±43.50	1.86±0.34	356.04±16.67	1.50±0.10
15g WF	260.79±44.28	3.22±0.70	322.28±92.03	1.88±0.24	350.63±45.78	1.98±0.17
5g SF	238.09±28.11	4.82±0.92	374.59±43.17	1.93±0.15	445.33±125.52	1.55±0.33
10g SF	232.38±19.11	4.75±0.62	358.76±45.86	1.88±0.61	438.91±56.20	1.54±0.17
15g SF	213.52±30.60	3.66±0.38	330.79±67.63	1.81±0.22	413.47±56.40	1.23±0.26
5 Type III RS	345.24±60.60	5.06±1.22	503.02±98.82	2.05±0.44	492.86±20.21	1.49±0.19
10 Type III RS	335.45±67.74	4.91±1.44	590.15±72.20	1.45±0.19	492.00±64.57	2.62±2.95
15 Type III RS	334.93±56.31	4.16±0.89	661.17±2.72	1.61±0.33	438.38±27.21	1.31±0.28

Cohesiveness may be measured as the rate at which the material disintegrates under mechanical action. From Table .1 it can be seen that the cohesiveness of the dough samples were decreased with the increased level of fibers. This may likely be due to the interaction between polysaccharides and proteins from wheat flour as reported earlier by **Jones and Erlander (1967)**. The reduction in dough cohesiveness values due to various cereal fibers addition have been reported by (**Sudha *et al.* 2007; Piteira *et al.* 2006)**. The increase in dietary fiber content has been reported to cause several changes in wheat dough and bread: dough yield increases by 3-5%, the dough becomes shorter and moister and fermentation tolerance decreases (**Seibel, 1983**). Other dough properties e.g. kneading, handling properties, rising, fermentation, post-stiffening and stickiness can also be affected. The proportion of soluble and insoluble fiber influences the water absorption rate of the flour mixture (**Haseborg and Himmelstein, 1988**).

All the values are mean ± sd of three values; wf: wheat fiber; sf: soy fiber

Table 2 shows the effect of fiber addition on the chapatti tear force and extensibility. Tear force which indicates the force required to tear the chapatti. Decrease in tear force indicates the increase in softness of the chapatti whereas increase in tear force indicates the increase in hardness of chapatti. From Table 2 it can be observed that the softness was increased with the increased level of wheat fiber. Control chapatti showed 316.45 g tear force whereas with the addition of wheat fiber the softness of chapatti was increased; when soy fiber was added, softness was further increased. This might be due to the method of manufacturing for these two fibers is different and the difference in the composition of two, as the soy fiber manufacturing involves addition of small quantity of soy lecithin, and this lecithin which acts as an emulsifier thus acted as softening and natural antistaling agent (**Ranhotra, 1993**) in chapatti. Further, with the addition of type III RS chapatti hardness was increased; this was due to the high amylose content of the type III RS (www.hi-maize.com). Studies have shown that granular resistant starch provides better appearance, texture, and mouth feel than do conventional fiber sources and improves expansion and crispness in certain food applications (**Waring, 1998**). In a study conducted at the American Institute of Baking, NOVELOSE 240 starch was compared to various traditional fibers in a high- fiber sponge and dough formulation. Breads were supplemented with fiber or resistant starch to obtain a “high source” of fiber (5 g/50 g serving or 10% TDF). A blend in which resistant starch and oat fiber each contributed to half of the desired TDF was included to demonstrate how resistant starch can complement other fiber sources. Breads containing resistant starch were determined to have superior quality compared to those made with traditional fibers (**Waring, 1998**). Extensibility which indicates the chapatti elasticity, high extensibility values indicate fresh chapatti and with storage due to staling and retrogradation chapatti loses its extensibility (**Shaikh *et al.* 2007**) hence lower extensibility values indicates staled chapatti (**Shaikh *et al.* 2008**). From Table 2 it can be observed that with the fiber addition, extensibility of the chapatti was decreased due to the dilution of the gluten protein, whereas with the addition of type III RS; chapatti was found more extensible as can be seen from extensibility values. During two days storage of chapatti soy fiber shown to have an antistaling effect as can be seen from the lower tear force and high extension values as compared to control chapatti. Similar type of results were reported by **Seibel (1983)** who observed marked changes in loaf properties such as decreased bread volume and its elasticity. In another study by **Abdul-Hamid** and **Siew Luan (2000)**, addition of defatted rice bran as a source of dietary fiber in bread making resulted into reduced loaf volume and increased firmness of the bread.

Effect of fiber mixtures on dough stickiness, chapatti tear force and extensibility

Table 3- Experimental D-Optimal mixture design and responses for dough stickiness, strength & cohesiveness obtained from fiber mixture

Run order	Sample	WF (%)	SF (%)	Type III RS (%)	Dough stickiness (g)	Dough strength (g)	Dough cohesiveness (mm/s)
0.00	Control	0.00	0.00	0.00	33.36±1.77	1.66±0.30	1.79±0.34
1.00	RSM 1	2.92	2.92	4.17	30.50±1.44	1.62±0.29	1.30±0.34
2.00	RSM 2	3.75	2.50	3.75	30.05±2.67	1.92±0.47	1.21±0.39
3.00	RSM 3	2.50	3.75	3.75	26.81±2.39	1.24±0.53	0.73±0.28
4.00	RSM 4	2.50	2.50	5.00	30.87±1.08	2.65±0.46	1.75±0.49
5.00	RSM 5	4.17	2.92	2.92	29.74±3.58	1.99±0.50	1.50±0.53
6.00	RSM 6	2.50	2.50	5.00	31.70±0.90	2.85±0.70	1.99±0.51
7.00	RSM 7	2.92	4.17	2.92	31.09±2.25	2.20±0.26	1.42±0.48
8.00	RSM 8	3.75	2.50	3.75	30.28±1.88	2.10±0.49	1.42±0.46
9.00	RSM 9	2.50	5.00	2.50	30.66±1.36	2.45±0.52	1.63±0.49
10.00	RSM 10	3.33	3.33	3.33	30.89±1.33	1.47±0.36	1.07±0.20
11.00	RSM 11	5.00	2.50	2.50	35.35±1.53	2.38±0.54	1.46±0.39
12.00	RSM 12	2.50	5.00	2.50	32.34±1.54	2.63±0.51	1.77±0.60
13.00	RSM 13	5.00	2.50	2.50	34.68±1.70	2.45±0.38	2.14±0.48
14.00	RSM 14	3.75	3.75	2.50	35.64±2.08	2.18±0.68	1.70±0.36

All the values are mean ± sd of three values; wf: wheat fiber; sf: soy fiber

Table 3 shows the levels of selected predictor variables for the D-optimal mixture design and summarizes the experimental design for optimization of fiber mixtures. To examine the combined effect of three different components (independent variables) on dough stickiness, dough strength and dough cohesiveness, D-optimal mixture design of 1-14 experiments were performed with different combinations (Table 3). Table 4 also includes data showing effect of fiber mixture on chapatti tear force and extensibility (0, 1, 2 days) as a response. The application of RSM yielded following regression equation, which is an empirical relation between tear force 0 day (X_1), tear force 1 day (X_2), tear force 2 day (X_3) and the test variable (A- wheat fiber, B- soy fiber, C- type III RS) in coded units.

X_1: Tear force (0 D) = +338.69* A+256.19* B+348.32 * C-128.41 * A * B-178.31* A * C-6.75 * B * C

X_2: Tear force (1D) = +474.28*A+328.60* B+528.65*C-265.01* A* B-525.46*A*C-249.64*B*C

X_3: Tear force (2 D) = +496.40*A+380.93*B+554.75*C-95.01* A* B-316.85*A*C-52.40*B*C

Table 4- Experimental D-Optimal mixture design and responses for tear force (g), extensibility (mm) of chapatti (0, 1 & 2 day) prepared with fiber mixture addition

D-Optimal Mixture Design				0 Day		1 Day		2 Day	
Run order	WF (%)	SF (%)	Type III RS (%)	Tear force (g)	Extensibility (mm)	Tear force (g)	Extensibility (mm)	Tear force (g)	Extensibility (mm)
1.00	2.92	2.92	4.17	327.82±57.13	3.48±0.61	426.76±69.35	1.62±0.42	489.86±49.74	1.16±0.33
2.00	3.75	2.50	3.75	293.70±58.08	3.98±0.96	375.23±57.91	2.02±0.43	439.04±39.24	1.78±0.26
3.00	2.50	3.75	3.75	294.07±74.65	3.85±0.73	345.21±48.72	1.86±1.04	457.24±29.93	1.45±0.30
4.00	2.50	2.50	5.00	342.87±72.73	3.70±0.59	527.35±72.87	1.54±0.39	550.01±10.15	1.30±0.18
5.00	4.17	2.92	2.92	287.75±56.02	3.90±0.23	341.35±58.59	1.86±0.09	454.77±25.43	1.38±0.20
6.00	2.50	2.50	5.00	348.16±87.42	3.73±0.59	521.96±60.29	1.59±0.29	554.30±31.88	1.34±0.23
7.00	2.92	4.17	2.92	261.81±58.10	4.22±0.93	326.68±97.82	2.03±0.36	376.23±47.01	1.80±0.34
8.00	3.75	2.50	3.75	297.83±60.05	3.94±0.75	355.69±35.14	1.98±0.47	441.90±59.88	1.66±0.33
9.00	2.50	5.00	2.50	258.17±97.28	4.49±0.75	329.10±57.65	2.09±0.21	389.93±18.97	1.93±0.40
10.00	3.33	3.33	3.33	278.26±50.33	3.80±1.19	335.04±3.69	1.70±0.25	426.65±6.26	1.49±0.07
11.00	5.00	2.50	2.50	340.57±45.30	4.38±0.65	481.08±45.59	2.08±0.52	489.14±46.14	1.65±0.21
12.00	2.50	5.00	2.50	256.64±39.17	4.57±0.89	327.36±51.76	2.14±0.35	381.82±7.48	1.92±0.31
13.00	5.00	2.50	2.50	339.56±58.44	4.37±1.03	478.46±44.24	2.04±0.06	499.26±64.19	1.68±0.33
14.00	3.75	3.75	2.50	267.21±66.35	4.10±0.83	333.16±2.44	1.72±0.21	418.20±66.18	1.57±0.42

All the values are mean ± sd of three values; wf: wheat fiber; sf: soy fiber

The results of the second order response surface model fitting in the form of ANOVA for chapatti tear force (g) obtained by mixture design quadratic model is given in Table 5. The ANOVA of quadratic regression model demonstrates that the model is significant, as is evident from Fisher's F- test value being 40.57, 44.72 & 41.09 for tear force (0 day), tear force (1 day) and tear force (2 day) respectively, with a very low probability value for tear force 0, 1 & 2 days [(P model>F)= 0.0001]. The goodness of the fit of the model was checked by regression coefficient (R^2). In this case, the value of regression coefficient for tear force 0, 1 and 2 days was R^2 = 0.9621, 0.9655 & 0.9625 respectively. The R^2 value is always between 0 and 1 and the closer the R^2 is to 1.0, the stronger the model and the better it predicted the response (**Haaland, 1989**). For the tear force (0 day) response, mutual interaction of wheat fiber & type III RS (AC) (P<

0.0002) has the largest effect on tear force followed by wheat and soy fiber (AB) (P< 0.0048). Soy fiber, type III RS interaction was found to be insignificant ((P< 0.8439) (Table 5).

Table 5 - ANOVA for chapatti tear force (g) obtained by mixture design quadratic model

	Tear force (g) (0 day)				Tear force (g) (1 day)				Tear force (g) (2 day)			
Source	**SS***	**DF**	**F value**	**Prob > F**	**SS***	**DF***	**F value**	**Prob > F**	**SS***	**DF***	**F value**	**Prob > F**
Model	14434.53	5	40.57	< 0.0001	74602.06	5.00	44.72	< 0.0001	40050.96	5.00	41.09	< 0.0001
Linear Mixture	10418.34	2	73.21	< 0.0001	39326.33	2.00	58.94	< 0.0001	29885.96	2.00	76.66	< 0.0001
AB	1064.42	1	14.96	0.0048	4533.87	1.00	13.59	0.0062	582.75	1.00	2.99	0.1221
AC	2948.83	1	41.44	0.0002	25608.22	1.00	76.75	< 0.0001	9311.31	1.00	47.77	0.0001
BC	2.94	1	0.04	0.8439	4023.17	1.00	12.06	0.0084	177.23	1.00	0.91	0.3682
Residual	569.23	8			2669.12	8.00			1559.43	8.00		
Lack of fit	545.67	4	23.17	0.0050	2458.74	4.00	11.69	0.0177	1462.05	4.00	15.01	0.0112
R^2	0.9621				0.9655				0.9625			
Adj R^2	0.9383				0.9439				0.9391			

*ss- sum of square, df- degree of freedom, A - wheat fiber, B - soy fiber, C - type iii rs

Table 6 shows ANOVA for chapatti extensibility obtained by mixture design. For the extensibility (0 day) response, mutual interaction of wheat fiber & soy fiber (AB) (P< 0.0107) has the largest effect on extensibility followed by soy fiber & type III RS (BC) (P< 0.0219) and the interaction of wheat fiber and type III RS (AC) (P< 0.1856) showed to be insignificant.

Table 6 - ANOVA for chapatti extensibility (mm) obtained by mixture design

	Extensibility (mm) (0 day)				Extensibility (mm) (1 day)				Extensibility (mm) (2 day)			
Source	**SS***	**DF**	**F value**	**Prob > F**	**SS***	**DF***	**F value**	**Prob > F**	**SS***	**DF***	**F value**	**Prob > F**
Model	1.2848	5	18.50	0.0003	0.4896	5	11.91	0.0015	0.4896	5	11.91	0.0015
Linear Mixture	0.9597	2	34.54	0.0001	0.3017	2	18.35	0.0010	0.3017	2	18.35	0.0010
AB	0.1524	1	10.97	0.0107	0.1518	1	18.47	0.0026	0.1518	1	18.47	0.0026
AC	0.0291	1	2.10	0.1856	0.0322	1	3.91	0.0833	0.0322	1	3.91	0.0833
BC	0.1118	1	8.05	0.0219	0.0005	1	0.06	0.8116	0.0005	1	0.06	0.8116
Residual	0.1111	8			0.0658	8			0.0658	8		
Lack of fit	0.1066	4	23.69	0.0048	0.0617	4	15.04	0.0112	0.0617	4	15.04	0.0112
R^2	0.9204				0.8816				0.8816			
Adj R-Squared	0.8706				0.8075				0.8075			

*ss- sum of square, DF- degree of freedom, A - wheat fiber, B - soy fiber, C - type III RS

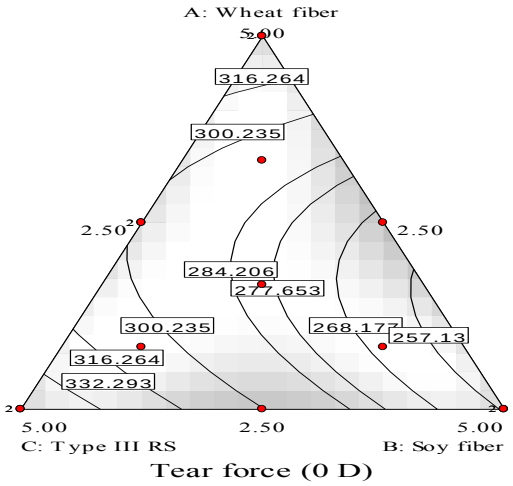

Fig. 1 (a)

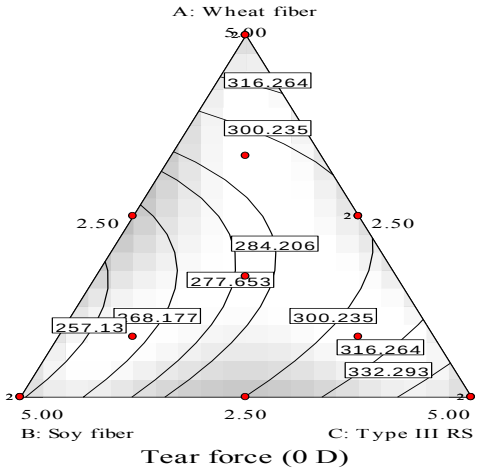

Fig.1 (b)

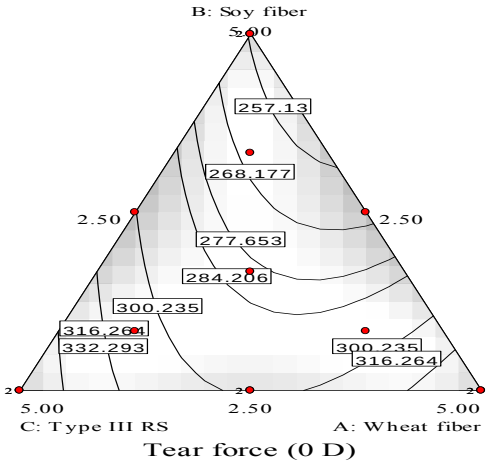

Fig.1 (c)

Figure 1 Contour plot of chapatti tear force (0 Day); Fig. 1 (a) the effect of wheat fiber and soy fiber; Fig.1 (b) the effect of wheat fiber and type III RS Fig.1 (c) the effect of soy fiber and type III RS on tear force (0 Day). Other variables are held at zero level.

The contour plots are generally the graphical representations of the regression equation from which the values of tear force (0, 1, 2 day) and for different mixtures of variables respectively can be predicted. Each contour curve represents an infinite number of combinations of two least variables with the other maintained at zero level. The maximum predicted value is indicated by the surface confined in smallest ellipse in the contour diagram. Figures 1a- 1c, shows the triangle contour plot for chapatti tear force (0 Day). Figure 1a shows interaction between wheat fiber and soy fiber. As soy fiber content in the mixture increased the softness of chapatti was also increased. The increased wheat fiber content also resulted in decreased softness of the chapatti while addition of type III RS resulted in increased hardness of the chapatti.

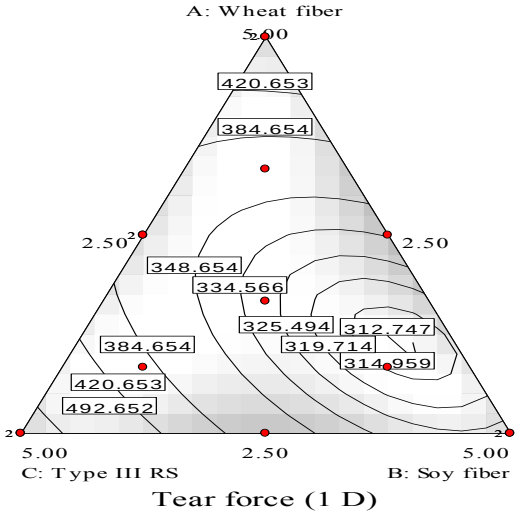

Fig. 2 (a)

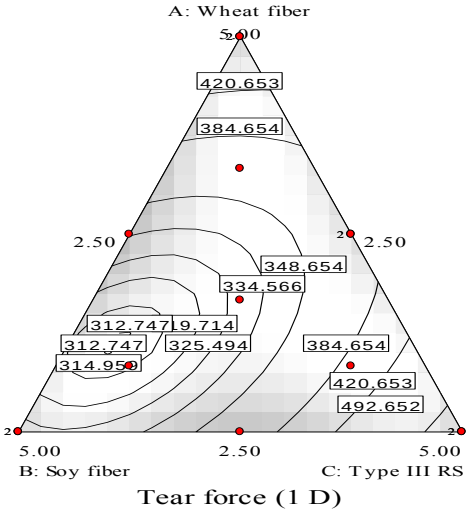

Fig. 2(b)

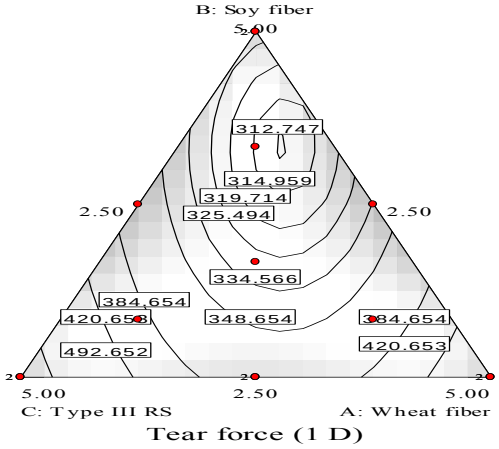

Fig.2 (c)

Figure 2 Contour plot of chapatti tear force (1 Day) Fig. 2 (a) the effect of wheat fiber and soy fiber; Fig.2 (b) the effect of wheat fiber and type III RS Fig.2 (c) the effect of soy fiber and type III RS on tear force (1 Day). Other variables are held at zero level.

Figure 2a depicts "mound shaped" surface at the corner. The contour line indicates that chapatti tear force was dependent on soy fiber and wheat fiber content. Figure 2b shows similar type of contour as Figure 2a and it can be seen that highest tear force values occurred at high soy fiber (5.00%) addition and at low wheat fiber (2.50%) addition. Figure .2c shows similar contour as Figure 2b for tear force values, whereas the response surface is more dependent on the soy fiber content.

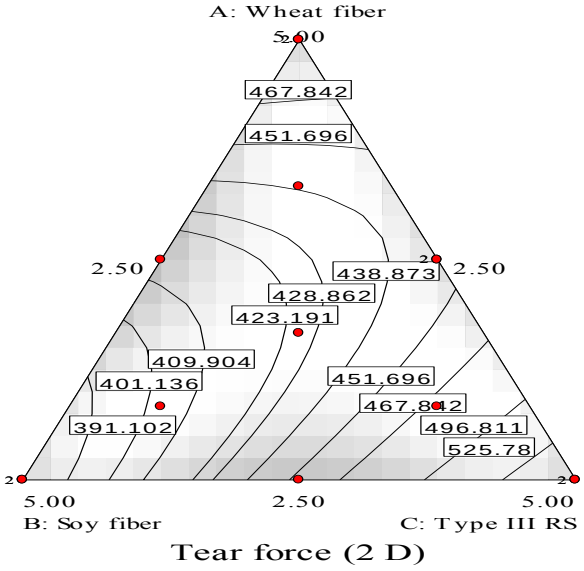

Fig . 3(a)

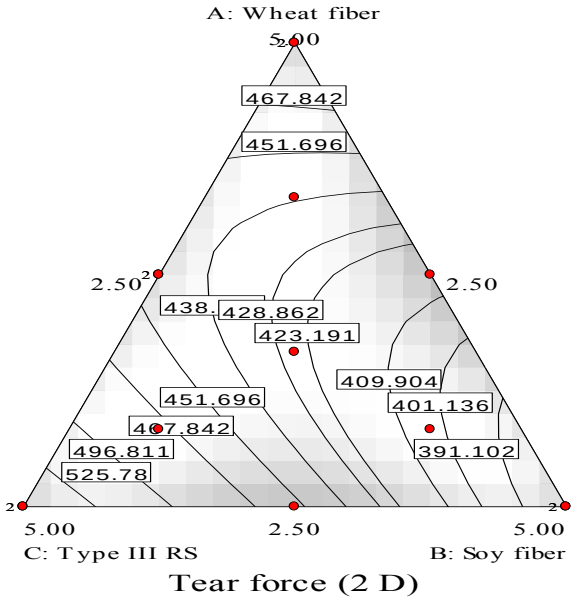

Fig. 3 (b)

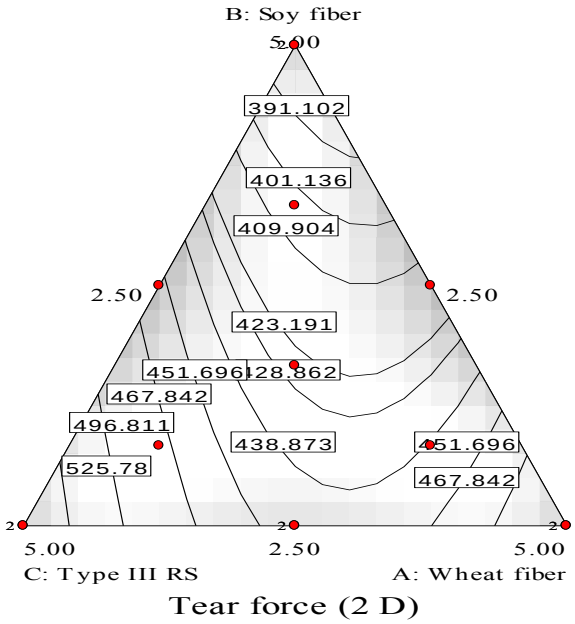

Fig. 3 (c)

Figure 3 Contour plot of chapatti tear force (2 Day) Fig. 3 (a) the effect of wheat fiber and soy fiber; Fig.3 (b) the effect of wheat fiber and type III RS Fig.3 (c) the effect of soy fiber and type III RS on tear force (2 Day). Other variables are held at zero level.

Figures 3a- 3c show ternary response & contour plot of calculated tear force (2 day). Figure 3a illustrates contour plot of chapatti tear force after two days storage from the interaction between wheat fiber and soy fiber while keeping the other at 0 levels. A linear increase in chapatti hardness was observed when soy fiber was decreased from 5% to 2.50 %. When wheat fiber was increased from 2.5% to 4.17% a linear decrease in the hardness was observed. Figure 3b depicts the effect of wheat fiber and type III RS on tear force (2 Day). Wheat fiber at 2.92% and soy fiber at 4.17% resulted in retention of softness of chapatti. Similar pattern was observed with respective to Figure 3c.

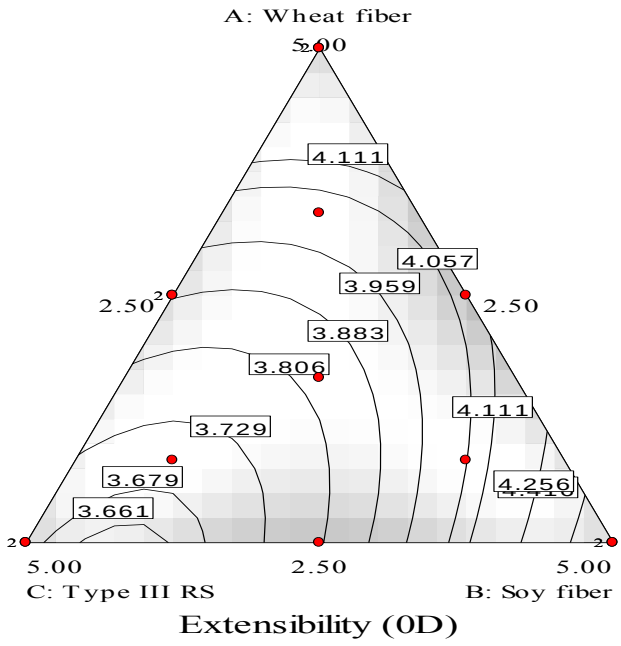

Fig 4 (a)

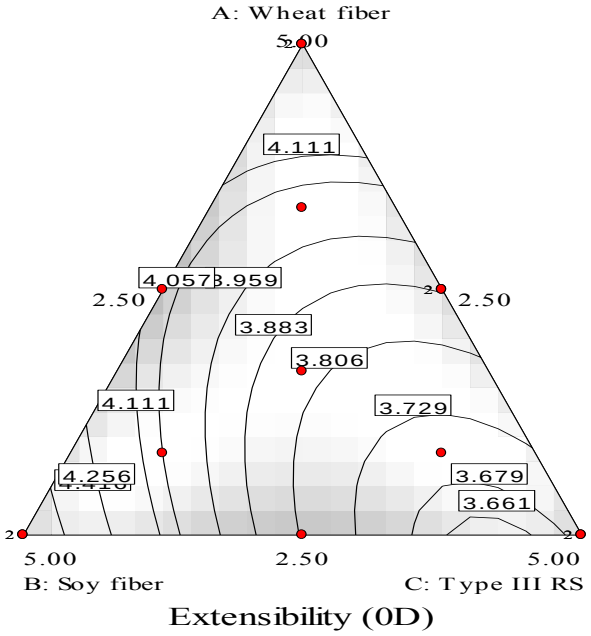

Fig 4 (b)

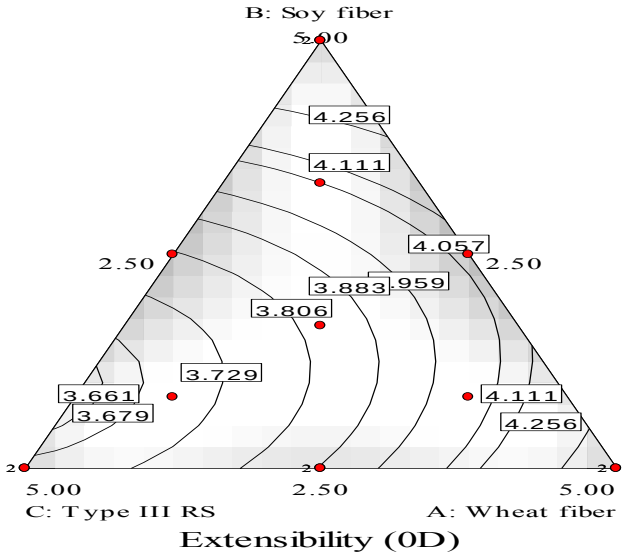

Fig 4 (c)

Figure 4 Contour plot of chapatti extensibility (0 Day); Fig. 4 (a) the effect of wheat fiber and soy fiber; Fig.4 (b) the effect of wheat fiber and type III RS Fig.4 (c) the effect of soy fiber and type III RS on extensibility (0 Day). Other variables are held at zero level.

Figure 4a represents ternary response & contour plot of chapatti extensibility (0 day) with the interaction of the effect of wheat fiber and soy fiber. Extensibility of the chapatti was increased as type III RS addition was increased; this was due to the overall increased hardness of chapatti due to the addition of type III RS which contained 97% amylose and amylose has been reported as main reason for staling in starch containing products. As can be seen from **c**ontour plot of chapatti extensibility (0 Day); the effect of wheat fiber and soy fiber on extensibility chapatti (Figure 4a**)** with the addition of soy fiber and wheat fiber extensibility was increased and, with the addition of type III RS; extensibility was decreased (Figure 4b). Figure 4bc showed similar trend.

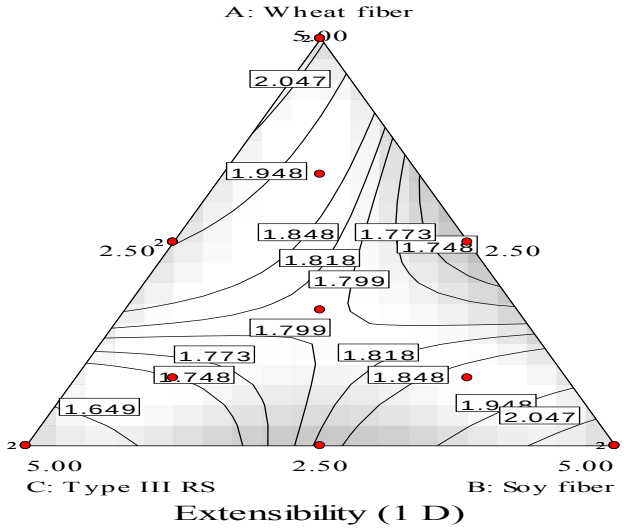

Fig. 5a

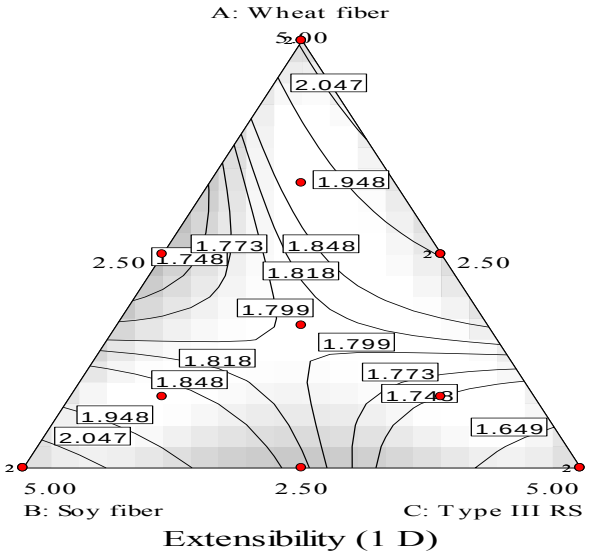

Fig. 5b

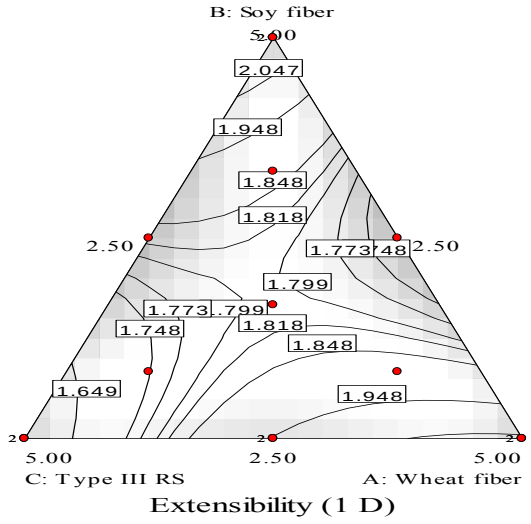

Fig. 5c

Figure 5 Contour plot of chapatti extensibility (1 Day); Fig. 5(a) the effect of wheat fiber and soy fiber; Fig.5 (b) the effect of wheat fiber and type III RS Fig. 5(c) the effect of soy fiber and type III RS on extensibility (1 Day). Other variables are held at zero level

In figures 5a- 5c show that one asymmetric saddling at the center of contour plot, indicating a rather complex relationship between the independent and dependant variable. Saddle point is indicated as the absence of a unique maximum or minimum. Higher level of soy fiber added in chapatti resulted in retention of extension values even after two days of storage, indicating soy fiber as an antistaling agent which prevented the loss in elasticity due to retrogradation.

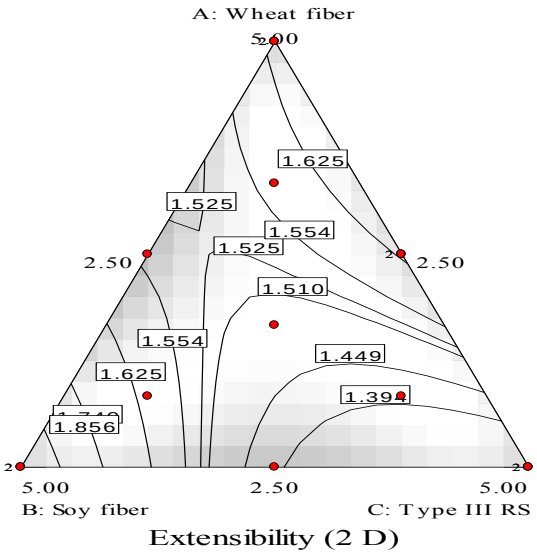

Fig. 6a

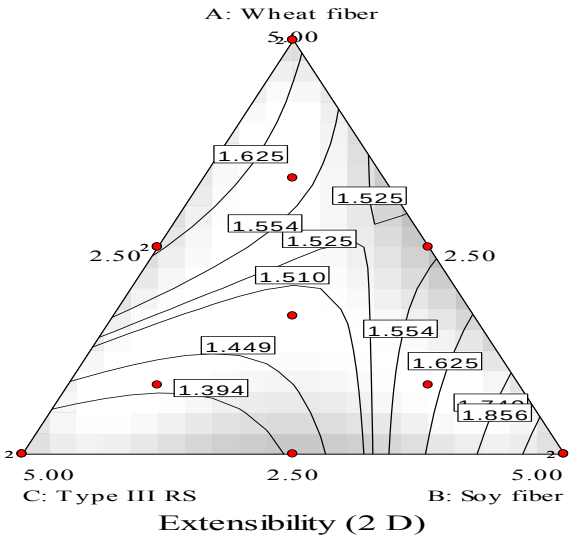

Fig. 6b

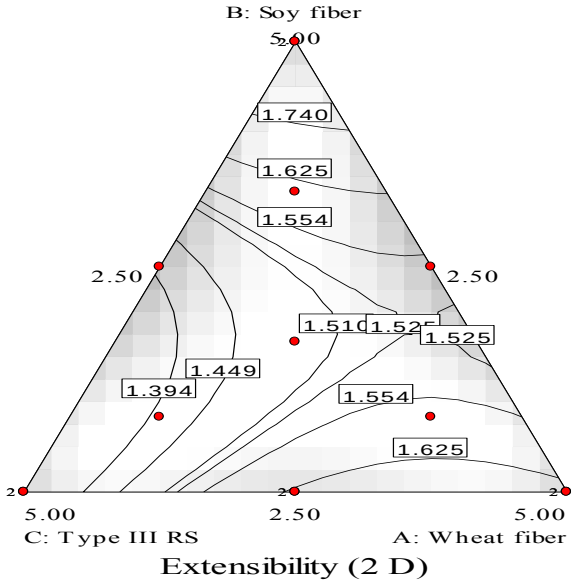

Fig. 6c

Figure 6 Contour plot of chapatti extensibility (2 Day); Fig. 6(a) the effect of wheat fiber and soy fiber; Fig.6 (b) the effect of wheat fiber and type III RS Fig. 6(c) the effect of soy fiber and type III RS on extensibility (2 Day). Other variables are held at zero leve.

Figures 6a - 6c depict the complex relationship between the independent and dependent variable. The decreased extensibility in chapatti indicated loss in freshness of chapatti (**Ghodke *et al*, 2009**) on 2 day of storage. **Pomeranz *et al* (1977)** observed the deleterious effects of fiber addition on bread dough structure and suggested that it could be due to the dilution of the gluten network, which in turn impairs gas retention rather than gas production. This was detected in a microscopic examination in which a major difference between the crumb structure of control and fiber containing breads was detected (**Pomeranz et *al.* 1977**). The crumb structure of wheat breads was composed of thin sheets and filaments which were essentially absent in fiber -enriched breads. According to **Gan *et al.* (1992)** the bran materials in expanded dough appear to disrupt the starch gluten matrix and also restrict and force gas cells to expand in a particular dimension. This greatly distorts the gas cell structure and may contribute to the resultant crumb morphology which is an important element of crumb texture. Supplementation of baked products with dietary fiber requires changes in processing techniques for production of baked goods to achieve good consumer quality.

CONCLUSION

From present study it can be observed that addition of wheat, soy and type III RS fibers to whole wheat flour affected the rheological characteristics of the dough in various ways. The addition of these fibers in mixture decreased the dough stickiness and improved dough strength. This is beneficial from the industry point of view where the energy required is reduced due to reduced stickiness. Dough containing wheat fiber (2.5%), soy fiber (5.0%) and type III RS (2.5%) yielded highly acceptable chapattis in terms of textural properties. In the present study fibers, when added singly positively affected the quality of dough as well as chapatti; but when used in combination further improved the textural properties of chapatti hence the acceptability. These studies have demonstrated the potential for developing fiber -rich chapatti in order to increase the dietary fiber intake.

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MODIFICATION OF PLA FOIL SURFACE BY ETHYLCELLULOSE AND ESSENTIAL OILS

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ABSTRACT

The increasing consumer demand for safety and long-term products motivates the packaging industry to produce antimicrobial packaging. The task of the antimicrobial packaging is not only the inhibition of growth of the pathogenic microflora, but also the maintenance of sensory characteristics of the product for a long time. The aim of the study was to evaluate antimicrobial properties of modified PLA foils against Gram-positive and Gram-negative bacteria. Biodegradable PLA foils were covered 10% ethylcellulose (EC) as carrier and commercial essential oils from fennel, rosemary and caraway as active substances. Antimicrobial properties were tested against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). The study was conducted using the American method ASTM E 2180-01. The results of experiments showed that modified essential oils PLA foils reduced the amount of Gram-positive and Gram-negative bacteria. The best antibacterial properties would have PLA foil coating 10% EC with 50 mg/dm^2 of fennel essential oil.

Keywords: *Escherichia coli, Staphylococcus aureus*, PLA, essential oils

INTRODUCTION

Packages materials made from synthetic polymers are commonly used in the packaging industry. They show excellent properties such as high liquid and vapor barrier (especially in the case of composite materials) or good mechanical properties. However, one of the drawbacks is the lack of susceptibility to biodegradation **(Szumigaj *et al.*, 2008)**. Therefore, potentially dangerous plastics are very often being replaced with eco-friendly materials. One of the most popular representatives of biopolymers is a polylactide (PLA), which is decomposed into water and carbon dioxide. This polymer can be easily processed, and it is not toxic to higher organisms **(Nowak & Pająk, 2010)**. Unfortunately, the PLA also has many drawbacks **(Nampoothiri *et al.*, 2010)**. It is known that due to the high gas permeability, the polymer is often used for packaging food products with short time validity and flowers **(Ambrosio-Martin *et al.*, 2014)**. A solution of this problem may be covering PLA with coatings that improve the barrier properties against gases. The coatings may also have antimicrobial properties, which will increase the attractiveness of the PLA as a packaging material. The development of natural antimicrobial coatings, leading to active packaging, may be uncompromising achievement, significantly affecting the quality of the packaged goods, among other foodstuffs and flowers. Coatings containing active substances can be divided into those that migrate into the packaged product and those that do not **(LaCoste *et al.*, 2005)**. The active compounds should inhibit the growth of microorganisms responsible for spoilage of packaged products and pathogenic microorganisms. The task of the active packaging is not only inhibiting the growth of pathogenic microflora, but also maintaining the sensory characteristics of the product for a long time.
Active packaging containing essential oils and spices extracts have antimicrobial properties against many bacteria and fungi **(Sahari & Asgari, 2013)**. Their natural components include antimicrobial phenolic compounds, aldehydes, ketones, alcohols, ethers and hydrocarbons **(Hyldgaard *et al.*, 2012; Kalemba & Kunicka, 2003)**. The suitable concentration of the essential oil inhibiting microbial growth influences the quality of active packaging. The packaging containing essential oils as active ingredients exhibit a broad range of applications in the food industry **(Sadaka *et al.*, 2014)**.
Essential oils such as rosemary (*Rosmarinus officinalis* L.), caraway (*Carum carvi* L.) and fennel (*Foeniculum vulgare* Mill.) have shown antibacterial and antifungal activity **(Begum *et al.*, 2008; Diao *et al.*, 2014; Jalali-Heravi *et al.*, 2011)**. Rosemary oil also possesses analgesic, anti-inflammatory, anti-oxidative, anti-tumor, anti-ulcerogenic and hepatoprotective properties **(Minaiyan *et al.*, 2011)**. The volatile oils from *C. carvi* have also been used as an anti-ulcerogenic, anti-tumor, anti-proliferative and anti-hyperglycemic agent **(Thippeswamy *et al.*, 2013)**. Fennel essential oil has shown anti-oxidant, cytotoxic, anti-inflammatory, hypotensive, hepatoprotective, anti-thrombotic and anti-mutagenic activity **(Rahimi & Ardekani, 2013)**. These oils are used in many industries and in natural medicine. The antimicrobial properties of essential oils are strictly connected with their chemical composition. The rosemary essential oil composition was dominated by 1.8-cineole and camphor, followed by α-pinene, β-pinene and β-cariophyllene **(Aouadi *et al.*, 2014)**. Caraway fruits contain a several components. Carvone, limonene, germacrene D and transdihydrocarvone are the main components available in their essential oil **(Darougheh *et al.*, 2014)**. Important compounds of the fennel essential oil are mainly *trans*-anethole, estragole and fenchone. Also, fennel oil contains trace amounts of other compounds including α-pinene, limonene, β-pinene, β-myrcene, and p-cymene **(Gori *et al.*, 2012)**.
Staphylococcus aureus and *Escherichia coli* are two opportunistic pathogens that are responsible for moderate to severe and life-threatening infections. *S. aureus* is also a worldwide cause of food-borne infections **(Fleurot *et al.*, 2014)**. These bacteria produce toxins which are responsible for staphylococcal food poisoning **(Argudin *et al.*, 2010)**. *E. coli* is present in human and animal intestine. Pathogenic strains are responsible for intestinal disorders e.g. diarrhea and extraintestinal infections in both humans and animals including urinary tract infections (UTI), septicemia and meningitis **(Jafari *et al.*, 2012)**.
The aim of the study was to examine the antibacterial activities of modified PLA foil surface by 10% ethylcellulose (EC) and commercial essential oils (rosemary, caraway and fennel) against representatives Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Antibacterial properties was evaluated according to the European Norm **ASTM E2180-07 (2012)** for polymeric materials.

MATERIAL AND METHODS

Bacterial strains, media and growth condition

S. aureus (DSMZ 346) and *E. coli* (DSMZ 1576) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (GmbH, Germany).

E. coli were cultivated on MacConkey agar (Merck KGaA, Germany) at 37°C for 24 h, *S. aureus* on trypticase soy agar (TSA) (Merck KGaA, Germany) containing 10% sodium chloride (Sigma-Aldrich, Poland) at 37°C for 24 h.

PLA foil and essential oils

PLA foil used in the study was embossed in Center of Bioimmobilisation and Innovative Packaging Materials with a thickness of 50 µm. The foil was cut into sheets of A4, coated with essential oil and sterilized under a UV lamp after 1 h on each side. Rosemary oil (*R. officinalis* L.), caraway oil (*C. carvi* L.) and fennel oil (*F. vulgare* Mill.) used in this study were obtained from Vera-Nord Company, Poland (commercial producers of plant essential oils and aromatic substances). Quality of the oils was ascertained to be more than 98% pure. The oils exhibited a strong and characteristic odor.

Coating PLA foil

The essential oils were poured into beakers and weighed. To obtain relative concentrations of the extracts, 10% EC dissolved in 96% ethyl alcohol (Sigma-Aldrich, Poland) was added. To each beaker 0.5% (w/w) Tween 20 (Scharlau Chemie S.A., Spain) was added and stirred until a uniform distribution of the oils in EC. 5% solutions of essential oils were prepared in the carrier – EC. Coater (Unicoater 409, Erichsen, Germany) equipped with a roller with a diameter of 1.27 mm wire was used to apply coating to the sheets of PLA foil. The foils were then dried for 15 min at 55°C. Layer of suitable thickness was obtained. The content of active substance in the coating to the foil surface was calculated using equation from **ASTM E 2180-07 (2012)**:

$$A = X\,[\%] \times G\,[\mu m] \times 0{,}01 \times 1000\,[mg/dm^2] \qquad /1/$$

where A is the content of active substance in the coating relative to the foil surface, X is the percentage of the active substance in an amount applied to the foil sheet, and G is the thickness of the deposited foil.
It was prepared 5 tested foils: 3 coated different oils and 2 controls, which was cut for 6 squares (3x3 cm). Controls were: pure PLA (foil nr 1*) and PLA + 10% EC (foil nr 2*).

Attenuated total reflectance FT-IR PLA foil

Examination of the foil surface structure was performed with using infrared spectrometry with Fourier transform by attenuated total reflectance (ATR FT-IR). Two samples were used: pure PLA foil and PLA foil coated with 10% EC. Infrared spectra were studied in the wavelength range of the radiation 4000-600 cm^{-1} with a number of four scans per sample. The tests were performed on both inner and outer side of PLA foil. In order to compare the spectra obtained with the spectrum of reference materials substance spectra library was searched.
The presence of the coating was also observed with a scanning electron microscope (SEM) Vega 3 LMU (Tescan, Czech Republic).

Antibacterial activity of coated PLA foils

The antimicrobial properties of coated PLA foil were studied with using reference method **ASTM E 2180-07 (2012)**, which is exploited for the establishment of a research activity antimicrobial agent(s) incorporated into the polymeric or hydrophobic materials.
From reference bacterial strains cultivated 24 h at 37°C on agar media (MacConkey - *E. coli,* TSA - *S. aureus*) solutions 1.5 x 10^8 cells/ml of 0.9% NaCl concentration were prepared with using McFarland scale turbidity. 1 ml of this suspensions was added to 100 ml of 0.3% TSA medium at a temp. of 45°C to obtain 1.5 x 10^6 cells/ml suspension. Next 1 ml of 1.5 x 10^6 cells/ml bacterial suspension was gently inoculated on the earlier prepared foil samples, which were placed on Petri dishes with 90 mm diameter. The Petri dishes were inserted into the climate chamber at 37°C and humidity of 90%. After 24 h, the samples were transferred from plates to falcons containing 30 ml of trypticase soy broth (TSB) (Merck KGaA, Germany) and stirred for 1 min. Next have taken 200 µl of TSB suspension, prepared serial dilutions, inoculated on Petri dishes with TSA and incubated for 24 h at 37°C. After incubation colonies were counted using colony counter (Ika2002 POL-EKO, Poland) and their average values (of three repeats) were tabulated. Countable colonies were found in dilution of 10^{-3} for *S. aureus* and 10^{-6} for *E. coli*.

Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation of at least three experiments were determined using Microsoft Excel 2010 (Micrososoft Corporation, USA).

RESULTS AND DISCUSSION

In recent decades, interest in essential oils, which are used for centuries in natural medicine, increased. Many essential oils are claimed to possess antimicrobial activity and they have been used for the prevention and treatment of many infectious diseases as alternative remedies. It is known that the efficacy of the essential oil depends on many environmental and genetic factors. It has been proven that the main factors responsible for the diverse chemical composition of essential oils are climatic conditions, geographic origin, time of collection, distillation conditions, correct farming practices and part of the plant from which oil is extracted **(Aćimović *et al.*, 2014; Fernández *et al.*, 2014; Msaada *et al.*, 2007**). In our study commercial essential oils derived from rosemary, caraway and fennel were used. These oils did not have a precise characteristic all the factors influencing on bactericidal activity but they are used in aromatherapy, aromatization of premises, rubbing, sauna, bath or shower.

Content of active substances in coated PLA foils

The content of active substances was calculated using Eqn. 1. Foils nr 3*-5*, 100 µm thickness (G) were coated with 5% of each active substances (X). The content of active substance in the coating relative to the foil surface was 50 mg/dm^2 (A) - Table 1.

Table 1 The content of active substance coated PLA foils

Number of foil	The diameter of the wire [mm]	X [%]	G [µm]	A [mg/dm^2]
1*	-	-	-	-
2*	1.27	-	100	-
3*	1.27	5	100	50
4*	1.27	5	100	50
5*	1.27	5	100	50

Legend: 1* – pure PLA foil (control sample), 2* – PLA foil + 10% EC (control sample), 3* – PLA foil + 10% EC + 5% caraway oil, 4* – PLA foil + 10% EC + 5% rosemary oil, 5* – PLA foil + 10% EC + 5% fennel oil

Analysis of modified PLA foils using ATR FT-IR

Spectroscopy analysis using ATR FT-IR was performed on both sides of two samples of PLA foils: pure and coated with 10% EC. Experiments have shown that both the pure PLA foil and modified 10% EC do not have impurities that could adversely affect the microorganisms. Results of the obtained spectrum shown no significant differences in the peaks. Thus proving that pure PLA foil did not contain substances that could indicate the pollution (Figure 1). Experiments have also confirmed that the spectrum of PLA foil with 10% EC content coincided with the spectrum of pure PLA foil (Figure 2). Therefore, also been proving that 10% EC coating on the surface of PLA foil did not cause adverse chemical changes of the biopolymer. Analysis of the obtained spectra of pure PLA foil and the uncoated surface of the modified PLA foil with using a library of spectra showed 99% similarity. The spectrum of 10% EC modified PLA foil was characterized by the greatest degree of match to the spectrum of the standard (pure PLA foil) equal to 94% (Figure 3).
SEM analysis of coated and pure PLA foil confirmed the presence of the coating film on the surface of PLA foil (Figure 4), proving the fact that the EC, due to good adhesion to the foil surface, was properly selected carrier. Shell did not undergo separation from the surface of the PLA and did not break.

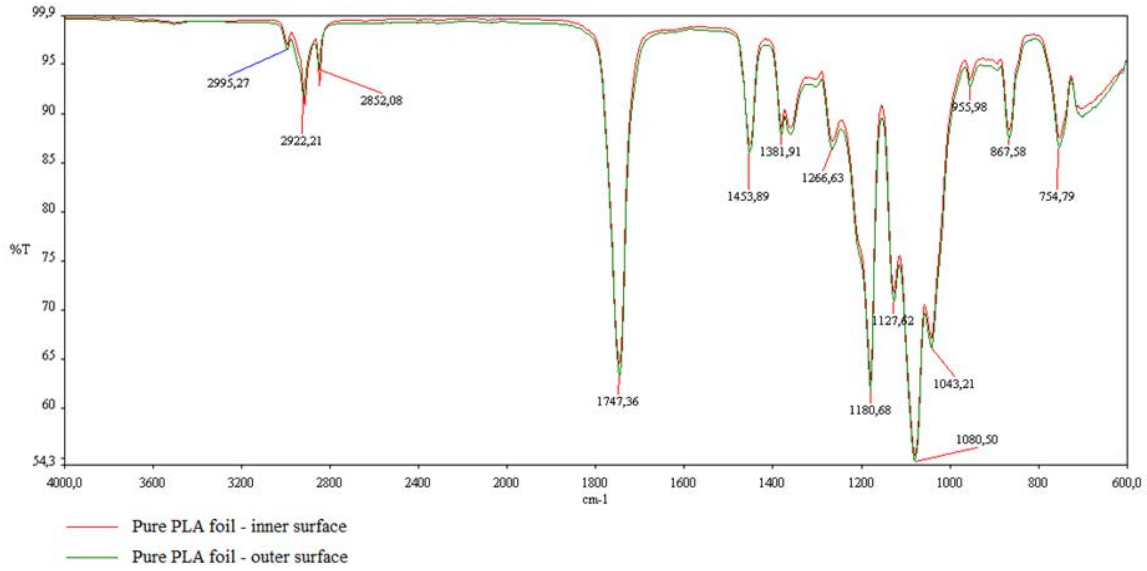

Figure 1 ATR FT-IR spectra of pure PLA foil measured on the two surface sides

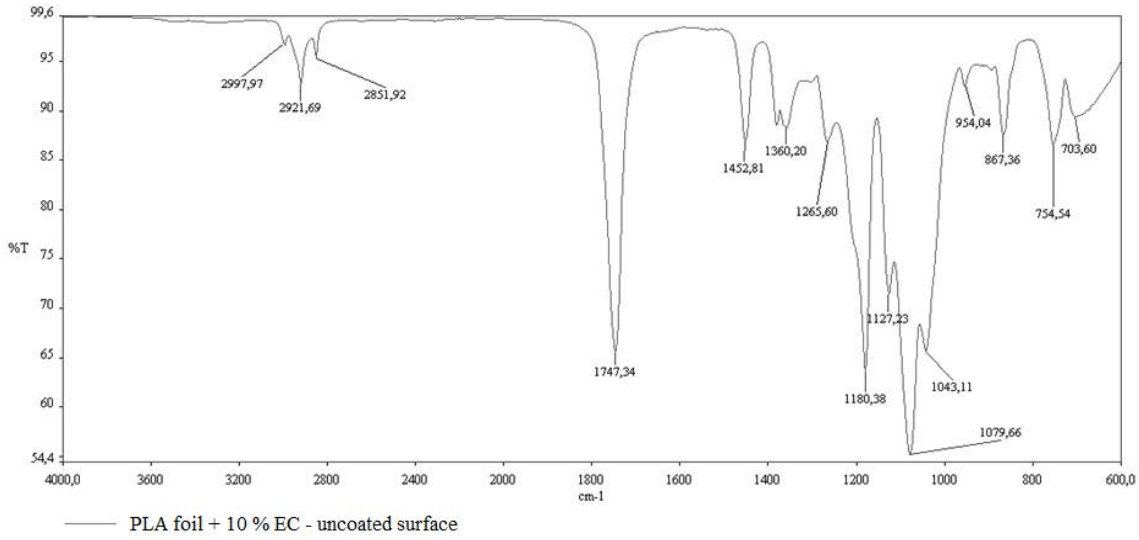

Figure 2 ATR FT-IR spectra of the modified PLA foil with 10% EC - uncoated surface

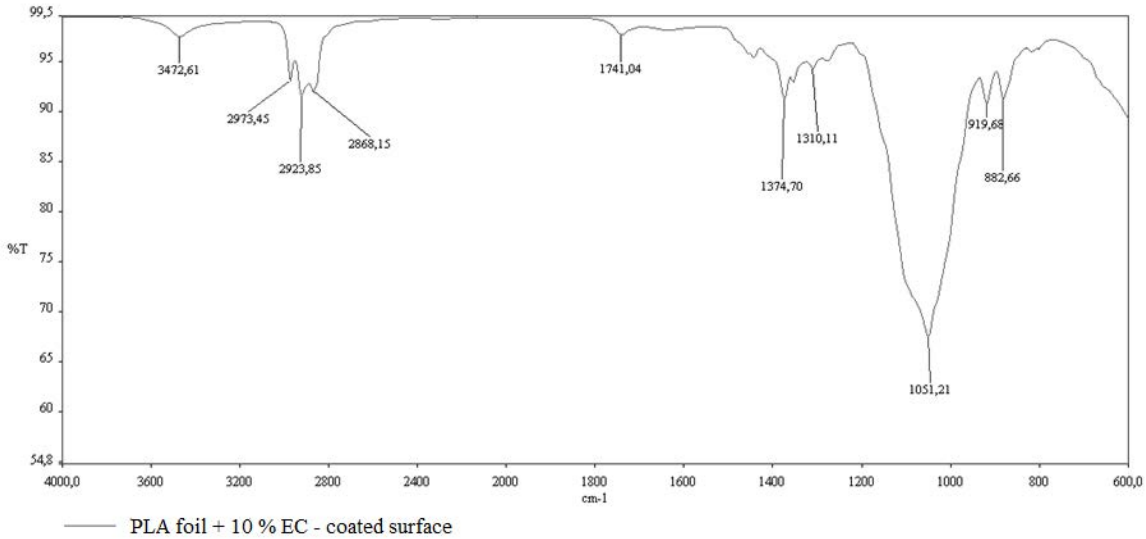

Figure 3 ATR FT-IR spectra of the modified PLA foil with 10% EC - coated surface

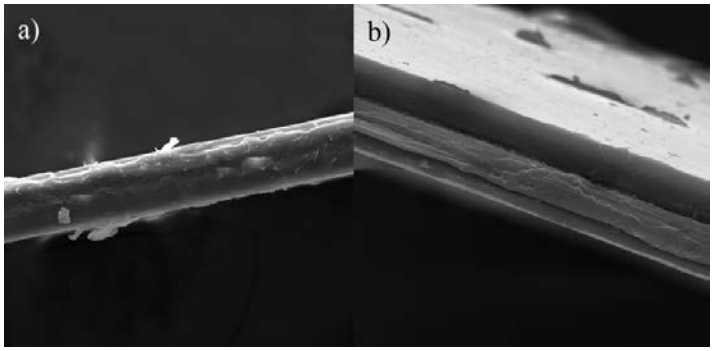

Figure 4 SEM picture of PLA foil surface: a) control, b) coated 10% EC

The antimicrobial properties of the coated PLA foils

According to **Jamshidian *et al.* (2010)** the innovative strength of PLA antimicrobial packaging has a direct impact on consumer health by creating safer and more wholesome packaged foods. **Lopez-Rubio *et al.* (2006)** claims that the most common and considered for novel bioactive packaging include antimicrobials, vitamins, phytochemicals, prebiotics, marine oils, and immobilized enzymes. In our study coating with 10% EC as a carrier and commercial essential oils (caraway oil, rosemary oil and fennel oil) as active substance were produced. It was found that 10% EC had a minor effect on the growth of *S. aureus*. The decrease of bacterial cells was 8.53% due to the effect of the pure PLA foil (Table 2, Figure 5). The study showed that foils containing active essential oils have similar antibacterial properties against *S. aureus*. The best properties reducing Gram-positive bacteria had fennel oil (85.36%), next rosemary oil (84.74%) and caraway oil (81.08%) - no significant differences (Table 2, Figure 5).

Table 2 Influence of coatings containing essential oils on the number of *S. aureus* cells

Number of foil	*X* [%]	*G* [µm]	*A* [mg/dm^2]	The amount of bacterial cells [cfu/ml]
1*	-		-	27.33 ± 4.65
2*	-	100	-	25 ± 4.36
3*	5	100	50	5.17 ± 1.26
4*	5	100	50	4.17 ± 1.04
5*	5	100	50	4 ± 0.5

Legend: *1 – pure PLA foil (control sample), 2* – PLA foil + 10% EC (control sample), 3* – PLA foil + 10% EC + 5% caraway oil, 4* – PLA foil + 10% EC + 5% rosemary oil, 5* – PLA foil + 10% EC + 5% fennel oil

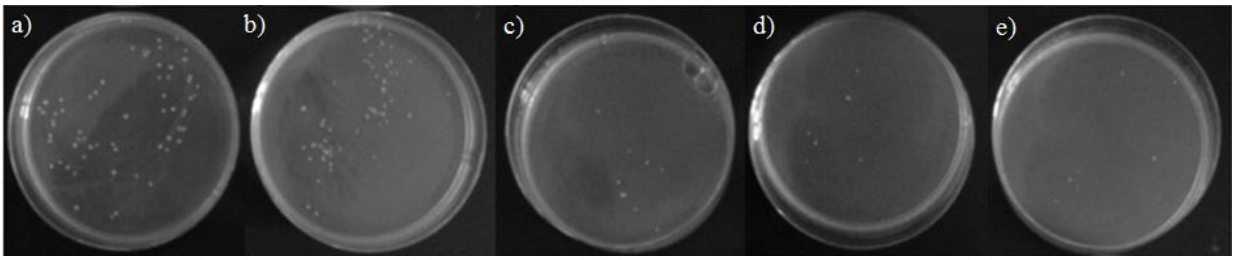

Figure 5 Influence of coatings PLA foil containing: a) pure PLA foil, b) 10% EC, c) 10% EC + 5% caraway oil, d) 10% EC + 5% rosemary oil, e) 10% EC + 5% fennel oil to reduce the number of *S. aureus* cells

The antibacterial effect of the active foils to *E. coli* was weaker. 10% EC reduces the amount of bacterial cells by 18.48% in comparison to pure foil (Table 3, Figure 6). Foils containing active essential oils have similar antibacterial properties against *E. coli*. The reduction of the number of microbial cells was 70% for fennel oil, 69.01% for rosemary oil and 66.5% for caraway oil (Table 3, Figure 6).

Table 3 Influence of coatings containing essential oils on the number of *E. coli* cells

Number of foil	*X* [%]	*G* [µm]	*A* [mg/dm^2]	The amount of bacterial cells [cfu/ml]
1	-		-	33.33 ± 3.25
2	-	100	-	27.17 ± 2.02
3	5	100	50	11.17 ± 2.57
4	5	100	50	10.33 ± 3.25
5	5	100	50	10 ± 1.32

Legend: 1* – pure PLA foil (control sample), 2* – PLA foil + 10% EC (control sample), 3* – PLA foil + 10% EC + 5% caraway oil, 4* – PLA foil + 10% EC + 5% rosemary oil, 5* – PLA foil + 10% EC + 5% fennel oil

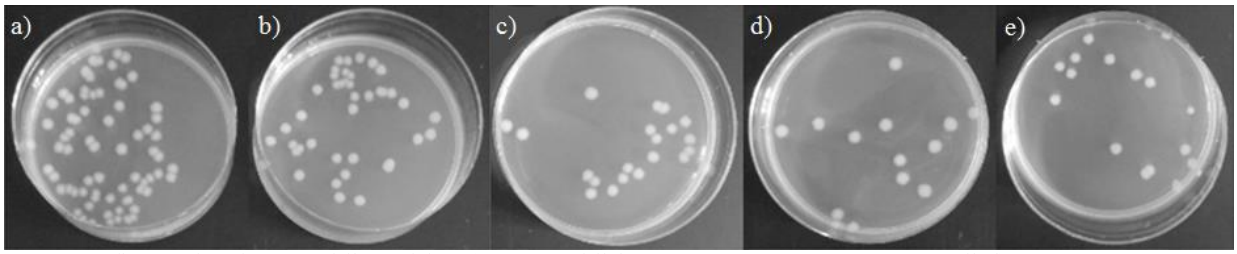

Figure 6 Influence of coatings PLA foil containing: a) pure PLA foil, b) 10% EC, c) 10% EC + 5% caraway oil, d) 10% EC + 5% rosemary oil, e) 10% EC + 5% fennel oil to reduce the number of *E. coli* cells

The topic of the antimicrobial properties of PLA foil containing active substances have been analyzed by other authors as well. The thermoplastic starch chitosan diffusion process to the substrate in conjunction with polylactide was studied **(Bie *et al.*, 2013)**. The authors investigated the effect of analyzed substance mixtures on the growth of *E. coli* and *S. aureus*. It has been shown that the addition of thermoplastic starch in the matrix enhanced the hydrophilicity of PLA blend, which preferably affects the diffusion of chitosan to the medium. Starch improves dynamic contact angle of the active substance. In addition, a blend consisting of 36% thermoplastic starch, 54% PLA and 10% of chitosan significantly reduced the number of cells of *E. coli* and *S. aureus*. Other scientists demonstrated antimicrobial properties of PLA foil coated cinnamaldehyde, which is unsaturated aldehyde naturally present in essential oils **(Makwana *et al.*, 2014)**. They have found reduced the number of *E. coli* and *Bacillus cereus*. The Gram-positive bacilli were more sensitive to the active oil substances than Gram-negative rods. These results correspond with our results, which proved that also more sensitive to the active essential oils coating on the surface of PLA foil were Gram-positive bacteria. Also **Burt & Reinders (2003)** claim that the cell wall of Gram-negative bacteria is more resistant to the toxic effects of essential oils than Gram-positive bacteria. The structure of the Gram-positive bacteria cell wall allows hydrophobic molecules to easily penetrate to the cells **(Nazzaro *et al.*, 2013)**.

The study performed by **Erdohan *et al*. (2013)** shown that olive leaf extract (*Olea europaea*) located in the coating on the surface of PLA foil has antibacterial activity against *S. aureus*. The authors found that the increase of active substance on PLA foil from 0.9 mg to 5.4 mg increases a zone of bacterial growth inhibition from 9.1 mm to 16.2 mm. **Seydim & Sarikus (2006)** proved that whey protein based edible foils incorporated with oregano, rosemary and garlic essential oils had antimicrobial activity against *S. aureus, Salmonella* Enteritidis, *Listeria monocytogenes* and *E. coli*. **López *et al.* (2007)** shown

the **antimicrobial** activity of polypropylene and polyethylene/ethylene vinyl alcohol copolymer incorporating **essential oil** of cinnamon, oregano, clove or cinnamon fortified with cinnamaldehyde against a wide range of microorganisms.

CONCLUSION

Our results confirmed that commercial essential oils coated modified 10% EC PLA foils cause significant growth inhibiting effects on Gram-positive (*S. aureus)* and Gram-negative (*E. coli*) bacteria and have similar antimicrobial properties as essential oils obtained from plants in the laboratory condition. In this study we used a standard strain of *S. aureus* DSMZ 346 (known as strain P-209) and standard strain of *E. coli* DSMZ 1576 (known as Crooks). The efficiency of rosemary, caraway and fennel essential oils against the representatives of most important human pathogens provides a scientific ground for future. Experiments indicate that 10% EC is an effective carrier of active substances used to create antimicrobial coatings. In summary the packaging materials produced using such coatings could protect food against undesirable microorganisms including pathogens. The best antibacterial properties would have PLA foil coating 10% EC with 50 mg/dm^2 fennel essential oil.

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HEALTH SAFETY OF EDIBLE WILD MUSHROOMS COLLECTED FROM THE INDUSTRIAL AREA

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ABSTRACT

The health risk associated with consumption of edible mushrooms derived from area surrounding the metallurgical plants was assessed. Three species (*Xerocomus badius* (Fr.), *Suillus luteus* (L.) and *Leccinum scabrum (Bull.) Grey*) of mushrooms have been studied. Samples were collected at increasing distances (0.2, 5, 10, and 15km) from the border of an industrial area. Determination of the content of 13 elements (Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, Pb, Tl, Zn and Se) using ICP-OES method with prior microwave mineralization was made. Among studied metals only Al concentration in mushrooms was distance-dependent. The content of Al in tested samples ranged from 2.8 – 39.6 mg.kg^{-1} dry matter and significantly ($P<0.05$) decreased with increasing distance from the industrial plant. High levels of Fe, Ni and Cu were observed in the mushrooms collected near the aluminum foundry (up to 5 km). For other studied elements (K, Mg, Zn, Mn and Se) no effect of pollutants emitted by foundry for their level in mushrooms was observed.

Keywords: Mushrooms, aluminium, pollution, food safety

INTRODUCTION

Mushrooms as food accompanied mankind from immemorial time **(Patel and Goyal, 2012)**. However, despite the presence of many nutrients in mushrooms **(Rudawska and Leski, 2005; Sas-Golak *et al.*, 2011)**, scientists are divided about their positive impact on the human health. For some of them, mushrooms have been reported to be a therapeutic foods **(Mattila *et al.*, 2000; Mahendra *et al.*, 2005)**, useful in preventing diseases such as hypertension **(Mujić *et al.*, 2011)**, hypercholesterolemia **(Alberts *et al.*, 1989)**, cardiovascular diseases **(Guillamóna *et al.*, 2010)** or cancer (**Rajewska and Bałasińska, 2004; Patel and Goyal, 2012)**. Moreover, their functional characteristics are mainly related to the chemical composition **(Manzi *et al.*, 2001)**.
On the other hand, it was found that different species of mushrooms may accumulate toxic elements (**García *et al.*, 1998; Isildak *et al.*, 2004; Árvay *et al.*, 2014**). So that, consumption of large amounts of fungi can be associated with the risk of high heavy metals intake **(Cocchia *et al.* 2006)**. Due to this ability fungi can be used as bioindicators of environmental contamination with toxic metals **(García *et al.*, 1998; Stihi*et al.*, 2011).** Until now, various studies have shown that accumulation of heavy metals in mushrooms is dependent on: the species of mushrooms, the age of mushrooms as well as mycelium, the source of pollution with heavy metals as well as the distance to this source (**Kalač and Svoboda, 2000).**
The aim of the study was to assess the health risk associated with consumption of edible mushrooms derived from area surrounding the metallurgical plants.

MATERIALS AND METHODS

Material

Three species: *Xerocomus badius* (n=6), *Suillus luteus* (n=5) and *Leccinum scabrum* (n=4) of wild edible mushrooms have been studied (**Table 1**). Samples were collected in forests surrounding two aluminum foundry localized closely to Stalowa Wola Smelter in South-Eastern Poland in 2014. Young mushrooms were collected at various distances from the border of an industrial area in order 0.2, 1, 5, 10, and 15km in the same direction of wind. Selecting points of the harvesting were dependent on the location of forest areas.

Table 1 Points of harvesting analyzed species of fungi

Point	Sample No.	Species	Distance from contaminationsource
A	1	*Xerocomus badius*	up to 200 m
	2	*Leccinum scabrum*	
	3	*Xerocomus badius*	
	4	*Suillus luteus*	
B	5	*Leccinum scabrum*	5 km
	6	*Xerocomus badius*	
	7	*Xerocomus badius*	
	8	*Suillus luteus*	
C	9	*Suillus leteus*	10 km
	10	*Xerocomus badius*	
	11	*Leccinum scabrum*	
D	12	*Suillus luteus*	15 km
	13	*Xerocomus badius*	
	14	*Leccinum scabrum*	
	15	*Suillus luteus*	

Methods

The fruiting bodies of mushrooms were first manually cleaned from leaves, needles and soil and dried in warm room during 2 weeks. Dry material was milled and stored in laboratory until analysis. The solid samples (1g) were mineralized with 8 mL of nitric acid (65% pure- basic, POCh Gliwice, Poland) using microwave mineralization (Ultrawave, Milestone Ethos-One, Italy) during 30 min.The clear solution volume was made up to 50 mL for each sample using deionised water. The quantitative (mg.kg^{-1}d.m.) determination of 13elements (Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, Pb, Tl, Zn and Se) was carried out by Optical Emission Spectrometry with Inductively Induced Plasma (ICP-OES) using ThermoiCAP 6500 (Thermo Fisher Scientific Inc., USA).

Statistical analysis

Statistical calculations were performed using StatSoft Statistica, 9.0. Normality of distribution was checked with Shapiro-Wilk test. As a significant differences between the meansat $p < 0.05$ values were considered. For the verification

nonparametric Kruskal-Wallis test was used. Pearson's correlation coefficients to assess metal-metal interaction were calculated.

RESULTS AND DISCUSSION

Among studied elements, for Al, Ca, Cu, Ni and Fe the higher contamination of mushrooms harvested in the immediate vicinity of the foundry was observed (**Table 2)**. Moreover, between these elements high correlation (r>0.6) appeared (**Table 3**). For other studied elements the dependency of their concentrations in mushrooms on the distance from metallurgic plant was no occurred.

Table 2 The concentration of elements [mg. kg^{-1}d.m.] in mushrooms collected in the increasing distance from the aluminum foundry (A<B<C<D) Mean±standard deviation and coefficient of variation V [%] were shown.

Element	Distance from the metallurgic plant			
	0.2 km (A) (n=4)	5 km (B) (n=4)	10 km (C) (n=3)	15 km (D) (n=4)
Al	28.7 ± 9.3[a]	17.8 ± 8.2[a]	7.3 ± 5.1[b]	8.6 ± 1.0[b]
	32.4%	45.9%	70.4%	11.5
Ca	94.4 ± 20.0[a]	126.3 ± 53.2[a]	11.7 ± 20.2[b]	8.3 ± 1.4[b]
	21.2%	42.1%	`173.2%	17.3%
Cd	2.1 ± 2.3	3.8 ± 3.0	3.6 ± 3.6	1.0 ± 0.8
	108.3%	78.3%	98.4%	78.6%
Cu	117.8 ± 73.4	170.5 ± 100.4	44.9 ± 18.9	68.1 ± 48.3
	62.3%	58.9%	42.0%	70.9%
Fe	198.8 ± 57.9[a]	185.6 ± 59.9[a]	35.0 ± 18.0[b]	36.7 ± 22.7[b]
	29.1%	32.3%	51.5%	61.9%
K	29737.5 ± 8727.4	34925.0 ± 9717.6	31521.7 ± 6714.4	28271.7 ± 4752.2
	29.3%	27.8%	21.3%	16.8%
Mg	916.3 ± 187.9	1036.9 ± 222.3	921.7 ± 176.5	920.8 ± 50.1
	20.5%	21.4%	19.2	5.4
Mn	24.9 ± 7.1	41.9 ± 29.8	13.0 ± 1.3	17.6 ± 6.0
	28.7%	71.3%	10.2%	33.9%
Ni	2.6 ± 1.9	3.0 ± 1.2	0.2 ± 0.3	0.1 ± 0.1
	73.6%	41.4%	118.4%	173.2%
Pb	0.7 ± 0.3	0.9 ± 0.5	0.7 ± 0.3	0.8 ± 0.9
	47.9%	51.0%	44.3%	113.1%
Zn	125.1 ± 62.9	143.9 ± 46.6	128.3 ± 34.7	157.5 ± 55.2
	50.3%	32.4%	27.0%	35.0%
Tl	3.7 ± 1.3	3.4 ± 1.2	2.4 ± 2.0	2.6 ± 1.4
	35.4%	35.3%	81.9%	54.5%
Se	0.7 ± 0.5	0.8 ± 0.5	0.9 ± 1.5	0.8 ± 1.0
	73%	65.8%	159.5%	132%

a, b samples statistically different

With increasing distance from the plant, the Al content in mushrooms statistically ($P<0.05$) decreases in a species-specific manner (**Figure 1**). Samples *Leccinum scabrum* in the vicinity of the factory show a higher concentration of aluminum than *Suillus luteus* or *Xerocomus badius*. The highest value 39.6 mg.kg^{-1}d.m. was recorded in the case of sample 2 (*Leccinum scabrum*), while the average for the distance A was 28.7 ± 9.3 mg.kg^{-1}d.m. The mean level for sample collecting in point D amounted 8.6 mg.kg^{-1}d.m (**Table 2**). The lowest content 2.8 mg.kg^{-1}d.m. was recorded in the sample No. 10 (*Xerocomus badius*). Obtained results showed that the aluminum content in tested samples in comparison to those available in literature was relatively low. Tests carried out on *Xerocomus badius* from the western Poland showed aluminum content in levels from 22.20 ± 4.57 to 28.08 ± 5.81 mg.kg^{-1}d.m. **(Mleczek *et al.*, 2013)**, which are comparable to the average level of this element reported in mushrooms from tested area. Studies conducted by **Rudawska and Leski (2005)** showed significantly higher Al concentration in mushrooms as compared to our findings. The biggest differences were found for *Leccinum scabrum*, where the aluminum content in the cited work amounted to 365 ± 62.5 mg.kg^{-1}d.m., which is a value almost 10 times higher than in own results.

Table 3 The Pearson correlation coefficient (r) between the concentration of analyzed elements in mushrooms

	Al	*Ca*	*Cd*	*Cu*	*Fe*	*K*	*Mg*	*Mn*	*Ni*	*Pb*	*Se*	*Tl*	*Zn*
Al	1												
Ca	0,769	1											
Cd	-0,02	0,407	1										
Cu	0,643	*0,965*	0,317	1									
Fe	*0,917*	*0,961*	0,245	0,884	1								
K	0,079	0,651	0,893	0,651	0,442	1							
Mg	0,106	0,715	0,59	0,816	0,493	0,889	1						
Mn	0,495	*0,919*	0,393	*0,983*	0,79	0,739	*0,907*	1					
Ni	0,839	*0,992*	0,367	*0,932*	*0,986*	0,58	0,624	0,866	1				
Pb	-0,15	0,403	0,126	0,619	0,185	0,528	0,84	0,728	0,294	1			
Se	-0,77	-0,63	0,448	0,038	-0,73	0,119	-0,2	-0,57	-0,66	-0,29	1		
Tl	*0,967*	0,882	0,02	0,809	*0,972*	0,225	0,328	0,688	*0,924*	0,103	-0,85	1	
Zn	-0,48	-0,22	-0,47	0,038	-0,35	-0,15	0,248	0,132	-0,31	0,732	-0,18	-0,3	1

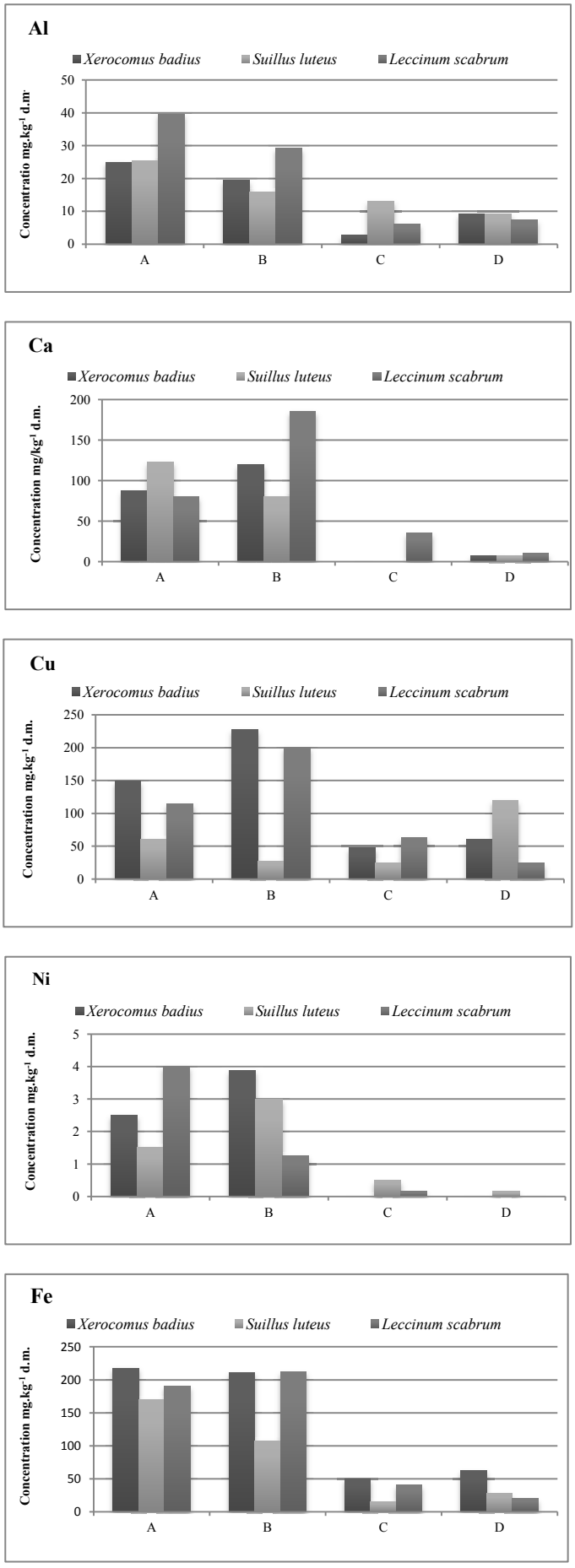

Figure 1 Changes in tested elements content in the fungi samples correlated with increasing distance from the point of emission

Tested samples contain significant amounts of copper, iron, nickel and calcium at a distance up to 5 km inclusive (**Figure 1**). The concentrations of these elements often exceed the values presented in the literature and were significantly higher than mean levels for samples collected from further distance. The average copper content in the samples from a distance of 5 km was 144.1± 86.2 mg.kg^{-1}d.m. The highest content was characterized by *Xerocomus badius* (188.8 ± 80.7 mg.kg^{-1}d.m.), and the lowest by *Suillus luteus* (43.6± 22.9 mg.kg^{-1}d.m). However, literature show that in the clear area of Europe, concentrations of copper in *Xerocomus badius* are in the range 25-75 mg.kg^{-1}d.m. **(Kalač, 2010)**. For *Leccinum scabrum* from eastern Poland, the concentrations of copper in the hat of mushroom was determinated as 24 ± 7 mg.kg^{-1}d.m. **(Bielawski and Falandysz, 2008).** For other elements (K, Mg, Zn, Mn, Se) the impact of pollutants emitted by foundry for their concentrations in the studied fungi were not observed. The level of macronutrients such as K and Mg was 20 000 - 40 000 mg.kg^{-1}d.m. and 800 - 1 800 mg.kg^{-1}d.m., respectively. Similar results were obtained by **Rudawska and Leski (2005)**, who recorded the concentration of K and Mg 21 300 mg.kg^{-1}d.m. and 400 mg.kg^{-1}d.m. for *Leccinum scabrum,* 29 700 mg.kg^{-1}d.m. and 800 mg.kg^{-1}d.m. for *Suillus luteus* and 34 900 mg.kg^{-1}d.m. and 900 mg.kg^{-1}d.m. for *Xerocomus badius*, respectively. In our study the observed level of selenium was significantly higher in the case of *Suillus luteus*. The highest concentration of this element (2.6 m.kg^{-1}d.m.) was recorded in sample of *Suillus luteus* collected 10 km away from polluted area indicating no effect of plants on the level of this element in mushrooms.

The concentration of lead in the tested samples ranged from 0.25 mg.kg^{-1}d.m. (*Xerocomus badius*, 15 km) to 1.825 mg.kg^{-1}d.m. (*Suillus leteus,* 15 km). The results show any relationship between the level of lead and the distance from the plant or species of fungi. The concentration of cadmium in the tested samples was in the range of 0.175 mg/kg d.m. (*Suillus luteus*, 200 m) to 7.5 mg.kg^{-1}d.m. (*Leccinum scabrum*, 10 km). Concentrations of lead in the tested samples are within the limits set for the samples from uncontaminated areas **(Kalač, 2010)**. However, some samples of *Xerocomus badius* and *Leccinum scabrum* have exceeded the maximum levels of cadmium from uncontaminated areas reported in the literature **(Kalač, 2010)**. The presented results clearly indicate a reduced content of cadmium in *Suillusluteus* in comparison to other species. A similar levels of cadmium have been found by other authors in Poland **(Falandysz *et al., 1993;* Falandysz and Chojnacka, 2007)** and Europe (**Kalač, 2010).**

The studied mushrooms *Xerocomus badius*, *Suillus leteus* and *Leccinum scabrum* contain minerals required in human diet, such as Ca, Cu, Fe, K, Mg, Mn, Zn and Se as well as toxic elements, such as Al, Cd, Pb and Tl. The level of toxic elements was lower that minerals. Since for some metals, their concentration in mushrooms harvested in the immediate vicinity of plants are even several times higher, it appears that mushrooms harvested within about 5 km should not be used for human consumption. What is true, calculations show that even excessive intake (100 g) of fresh mushrooms per week is not associated with a risk of exceeding the allowable limits of weekly intake (calculated as % of PTWI) recommended by **WHO/FAO (2011)**, which amounted for Al (0.1-0.41), Cu (0.18-0.70), Fe (0.09-0.51), Zn (0.26-0.32), Pb (2.11-2.90) and Cd (5.86-21.71% of PTWI). The results shown that high accumulation of cadmium in the analyzed fungi harvested near metallurgical plant may pose health risk especially in the case of enhanced consumption. Consequently, secure zone in which mushrooms will be free from this heavy metal is located within a radius> 15 km from the aggregation of metallurgical plants.

CONCLUSION

It was confirmed that heavy metal emission by metallurgic plants caused environmental pollution which resulted in enhanced accumulation of some metals in fruiting bodies of mushrooms. The high accumulation existed mainly in the distance up to 5 km from the border of plant. However, in most cases the average consumption of polluted mushrooms did not pose the risk for human health, excluding cadmium intake. As expected, metal uptake seems to be species dependent: *Leccinum scabrum* accumulated more Al and *Xerocomus badius* acumulate a large amount of Cu. The least susceptible to pollution turned out to be *Suillus luteus,* which was simultaneously rich in Se. Those some species are the most susceptible biomarkers of environmental pollutionwith heavy metals.

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EFFECT OF ULTRASOUND AND THERMAL TREATMENT ON PECTIN METHYLESTERASE ACTIVITY IN PAPAYA (*Carica papaya*) JUICE

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ABSTRACT

Among the pectic enzymes present in fruits and vegetables, pectin methylesterase (PME) is usually related to the loss of quality and it causes adverse effects on finished products. In this research, the kinetic of ultrasound and thermal treatments are evaluated in the PME activity in papaya juice. The results showed that the ultrasound treatment caused an increase in the catalytic activity up to 52%. After a while, the catalytic activity decreased in 27% indicating that the ultrasound was not effective in the enzymatic inactivation, whereas the thermal treatment inactivated 71% of the PME. However, these results open perspectives to evaluate the effect of ultrasound and enhance the catalytic activity of enzymes of industrial interest.

Keywords: Ultrasound, pectin methylesterase, inactivation, papaya

INTRODUCTION

Fruit consumption is important in public health care because it reduces the risk of various cancers and it also helps to reduce obesity (**de Bruijn, 2010**). Therefore, the development of new technologies, to obtain minimal processed foods, retaining their nutritional and organoleptic qualities, are important issues in food the industries.
Pectin methylesterase (PME, EC3.1.1.11) is usually found in the cell-wall of plants, phytopathogenic fungi and bacteria; it catalyzes the hydrolysis of the methylester bonds, releasing methanol and pectin with free carboxyl groups; PME also catalyzes the enzymatic de-esterification of pectin, decreasing its degree of methylation (DM). Therefore, the inactivation of endogenous PME in fruits is important to avoid a production of low methoxyl pectins (DM < 50%), which could result in a decrease of the quality of fruit texture. On the other hand, in low-acid fruits, the presence of divalent ions such as calcium, interconnect pairs of carboxyl groups of different low methoxyl pectin chains, forming aggregation of pepectic substances, phenomena know as cloud loss of fruit juices (**Croak and Corredig, 2006**).
Mainly, the inactivation of PME is conducted using thermal process, with a negative impact on the product attributes. Prolonged treatment at high temperatures, such as those needed to sterilize low-acid vegetables can result in a partial or total pectin depolymerization, resulting in a texture degradation of fruits (**Croak and Corredig, 2006**). Several works inactivating fruit PMEs in combination with or whitout thermal processes such as high pressure, high intensity pulsed electric field, have been reported elsewhere (**Castro *et al.*, 2006; Espachs-Barroso *et al.*, 2006; Guiavarc'h *et al.*, 2005; Polydera *et al.*, 2004; Velázquez-Estrada, *et al.*, 2012; Wilińska *et al.*, 2008**). However, a few studies have been reported combining the thermal process and the ultrasound (**Raviyan, *et al.*, 2005; Terefe *et al.*, 2009; Tiwari *et al.*, 2009**). The mechanism of enzyme inactivation by ultrasound treatment involves the formation, growth, and collapse of tiny gas bubbles or cavities in a liquid where the ultrasound waves travel through it (**Raviyan *et al.*, 2005**). Most of the data regarding the inactivation of PME using ultrasound processes are conducted for tomato (**Raviyan *et al.*, 2005; Plaza *et al.*, 2007**), apple juice (**Abid *et al.*, 2014**), carrots (**Gamboa-Santos *et al.*, 2012**), orange juice (**Tiwari *et al.*, 2009**), mango juice (**Santhirasegaram *et al.*, 2013**), and cantaloupe melon juice (**Fonteles *et al.*, 2012**); from experiments conducted in various fruits, the researcher indicate that ultrasound has advantageous because of the reduction PME activity is in a short times and it does not have significant effects on vitamins content, pH, sugars and phenolic compounds. In this context, the present study focuses on the kinetics of inactivation of PME by ultrasound and thermal treatment, in papaya juice.

MATERIALS AND METHODS

Fruit samples and sample preparation

The papaya fruits, were purchased in a wholesale food market in the city of Tingo María, Huánuco Perú. After, we proceeded according to the flowchart show in Figure 1. The operations are described below:
The fruit was washed with distilled water (GFL, D-30938 Burgwedel, Type 2004), removing all impurities attached on its surface. After it was performed peeled with stainless steel knives, by cutting lengthwise, seeds were separated with a stainless steel spoon. The pulp was obtained with the juicer (DeLonghi Duo System, Model: ROBOdiet).
After 5 mL of papaya pulp were packaged in glass tubes with screw caps. In this stage the pH was analyzed (OAKTON pH 1100), °Brix (Labor MIN) and the titratable acidity to obtain the index of maturity of the product.

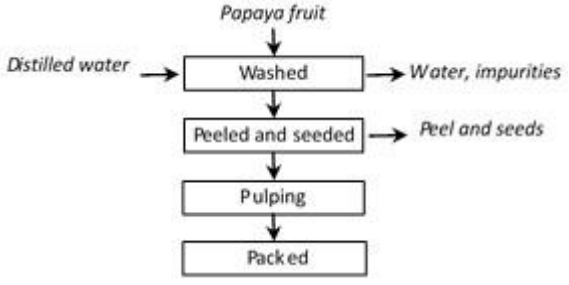

Figure 1 Flowchart of operations for obtain papaya juice

Ultrasonic treatment

The treatment was performed by placing the papaya pulp (5 mL) in glass tubes, within the ultrasound equipment (JAC Ultrasonic Lab. Companion Model: 1002) at different times (1, 2, 3, 4, 5, 6, 8 and 10 min at 40 kHz) the temperature of water in the ultrasound equipment was maintained at 27°C. Then the tubes were immersed in a container containing crushed ice to stop the enzymatic reaction in the papaya juice. The samples were kept in the ice bath to measure the activity of the pectin methylesterase.

Thermal treatment

The glass tubes containing papaya pulp, were placed in a maria bath (GEMMYCO, Model: YCW-010E) in total immersion, then the maria bath was scheduled with a maximum temperature of 75.5 °C.
After that, samples were taken (papaya pulp tubes) at different times of thermal treatment (to intervals of 4 min for 56 min) and immediately were immersed in a container containing crushed ice in order to stop the inactivation of PME by heat. Then, the pectin methylesterase activity was assessed.

Measurement the activity of the pectin methylesterase (PME)

Activity of pectin methylesterase (PME) was performed according to the method reported by **Stoforos *et al*. (2002)**. In a titration vessel 42 mL of aqueous suspension of pectin 0.5% (pH 7.0), was added, then 15 mL of NaCl (0.5 M) plus 2.8 mL deionized water. Then adjusted to pH 7.5 with NaOH (0.01 N), the hydrolysis reaction was initiated by adding 200 µL of the juice obtained from the papaya pulp (previously filtered to vacuum with Wattman paper No. 40). This was continuously titrated with NaOH (0.01 N) for 30 min, recording the volume consumed during titration at 27±0.4 °C, all samples were measured in triplicate. The enzymatic activity (AE), was calculated as follows:

$$AE = \frac{dV_{NaOH}}{dt} \cdot \frac{N_{NaOH}}{V_{sample}}$$,

Eq. 1

Where **AE**: [µeq H^+/(min. mL)], $\mathbf{dV_{NaOH}/dt}$ means, $\mathbf{N_{NaOH}}$ is the Normality of Sodium Hydroxide and $\mathbf{V_{sample}}$ the volume used in the sample in mL.

Statistical analysis
Statistical analyzes were performed using Statistica V8, the inhibition of activity PME subjected to an analysis of variance (ANOVA), with a level of 0.05.

RESULTS AND DISCUSSION

State of ripeness
Fruit ripening in papaya (*Carica papaya*) varies widely in terms of skin color changes, pulp firmness and shelf life. However, yellow color in the fruit skin (Figure 2) has been used as a harvest index criterion to assure adequate ripening and maximum shelf life (**Basulto *et al*., 2009**). The maturity index is a measurement that can be used to determine if a fruit is mature (**Fawole and Opara, 2013**). The contents of titratable acids (TA) and hence the sugar/acid ratio (TSS/TA) is highly correlated with the maturity index (**Reichel *et al*., 2010**). It is known that the acidity in the papaya fruit increases during maturation, because increases its content of vitamin C, also increases in total soluble solids content (**Serry, 2011**). In the Table 1 are shows the results of evaluations conducted for the papaya pulp.

Figure 2 Fruit ripe of papaya (*carica papaya*)

Table 1 Analysis of pH, º Brix, acidity titratable and maturity index ripe papaya pulp

Analysis in papaya pulp[1]	Means
pH	5,80 ± 0,20
% Total soluble solids (TSS)	11,00 ± 0,96
% Acidity titratable[γ] (TA)	2,18 ± 0,34
Maturity index (TSS/TA)	5,10 ± 0,50

[1]Data expressed as mean ± standard deviation, n=3. [γ] Expressed in % citric acid

The maturity index (TSS/TA), by which we measured the physiological maturity of the fruit ripens, was 5.10±0.50. That indicates the stage of development of the fruit, is usually associated with consumer acceptation (**Blankenship *et al*., 1997**).

Kinetic of inactivation by ultrasound and thermal treatment of PME

Pectin methylesterase (PME; E.C.3.1.1.11) is one of the enzymes present in many fruits and it usually bring negative consequences on the characteristics of the fruit (**Yeom *et al*., 2002**), it is an endogenous pectic pectin enzyme found presents in many fruits that de-esterifies the methyl group of pectin and converts it into low methoxy pectin or pectic acids (**Giner *et al*., 2000**). In this experiment, we evaluated the effect of ultrasound and the thermal treatment with respect to the time for inactivating the PME.
Regarding the results of the effects of ultrasound treatments on enzyme activities of PME in papaya juice is show in the Figure 3, the inhibition of PME by ultrasound were kept at the constant water temperature of 27°C and, the ultrasound power (40 kHz), inactivation kinetics shown two phases; one phase comprises the activity increasing of the PME from 2 to 4 minutes, until the initial enzyme activity was 3.8 µeqH^+/(min mL) at 4 min showed that the maximum activity was 5.8 µeqH^+/(min mL) which corresponds to an increase of 52%, this behavior is probably due by the cavitation bubbles that enhance the activity of the enzyme (**Subhedar and Gogate, 2014**), producing changes in the enzyme conformation mainly attributed to Trp, Tyr and Phe residues, particularly to Trp residue, could induce molecular unfolding of protein, destroying hydrophobic interactions of protein molecules, causing that hydrophobic groups and regions inside the molecules are exposed to the outside (**Jia *et al*., 2010**). Subsequently, from minute 4 to 5, it was observed a decreasing in the enzyme activity up to 2.6 µeqH^+/(min mL) equivalent to 27% compared with the initial enzyme activity. Ultrasound can rupture the weak linkages like hydrogen bonds or Van der Waals interactions and bring conformational changes in the protein structure (**Bashari *et al*., 2013**) and probably destroying hydrophobic interactions of protein molecules (**Gülseren *et al*., 2007**). These events cause the enzymatic inactivation (**López and Burgos, 1995; Cullen *et al*., 2012**).

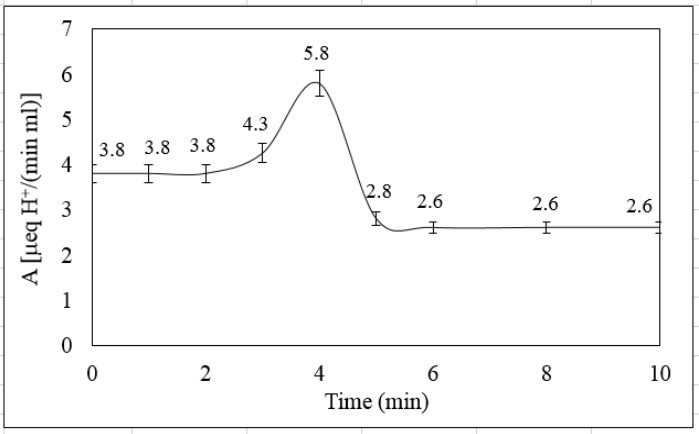

Figure 3 Kinetic of inactivation of PME in papaya juice by ultrasound (40 KHz)

Similar results were obtained by **Gamboa-Santos *et al*. (2012)**, who evaluated the inactivation of PME at 20 kHz, in carrots ground and sliced. They reported a decrease of the PME activity of 49.0 ± 3.0% treated at 35 °C by 60 min while the carrot pieces obtained in maximum decrease to 53.5 ± 2.1% at 70 °C for 15 min. Meanwhile **Wu *et al*. (2008)**, they inactivated PME by thermosonication in tomato juice (24 kHz) at different times and temperatures, as a result for inactivation of 90% PME were required 41.8, 11.7 and 4.3 minutes to 60 °C, 65 °C and 70 °C, respectively. An important aspect concerning the short PME inhibition refers **de Assis *et al*. (2000),** who describe that this behavior may be related to the presence of isoenzymes, or possible aggregates which form during the process of sonication and that is added to protect the enzyme decreasing the damage to the protein structure. It is also known that the active site of the PME is situated in the outer part of the β-helix of the PME **(Giovane *et al*., 2004)**. The results obtained demonstrate the relevance of ultrasound applications in the

increasing and decreasing of activity of the PME.

On the other hand, the Figure 4 showed that the kinetics inhibition of PME by effect of temperature in function of time. The increasing temperature and time produced the reduction of PME activity. Arising from this, the mathematical model was obtained that explain the inhibition of PME activity (Equation 2).

$$AE = 7.1 - 0.15T + 0.09t; R^2 = 0.99 \quad \text{Eq. 2}$$

Where **T** represents the Temperature in (ºC) and **t** is the time in unities of (min).

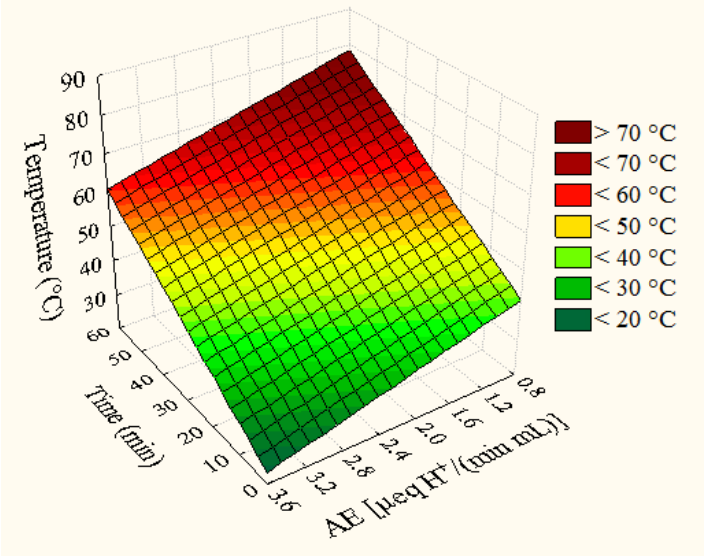

Figure 4 Enzymatic activity in papaya juice as function of time and temperature of thermal treatment

The effect of temperature, generated by the water bath, was effective in reducing the enzymatic activity of the PME, we obtained 71% inactivation at 56 minutes 75.5 °C, at atmospheric pressure. **Cano *et al*. (1997)**, reported a maximum inactivation of PME (25% reduction of the initial activity) for the treatment of orange juice to 200 MPa and 30 °C the tested range was from 50 to 400 MPa and 20 to 60 °C. Meanwhile, **Stoforos *et al.* (2002)** evaluated the inhibition of PME in tomato pulp, applying heat (60-75 °C) and pressure of 0-800 MPa, obtaining greater inactivation at 75 °C and atmospheric pressure, also reported an increase of inactivation at higher pressures that 700 MPa at 75 °C, and demonstrated that the loss of enzyme activity may occur via two different mechanisms, one mechanism may be associated with the temperature and the other with the pressure induced. **Ly-Nguyen *et al.* (2002)**, extracted and purified PME from carrots and evaluated the effect of heat treatment, after 10 min and 65 °C of treatment the PME activity was not detected. While **de Assis *et al*. (2000)** required 98 min and 110 °C to inactivate the PME in acerola. Our results obtained include greater time and temperature (56 min, 75.5 °C) to reduce by 71% the activity of the PME. It is clear that among the various sources of obtaining PME, the inactivation have different temperatures **(de Assis *et al*, 2000; Wu *et al*, 2008).**

CONCLUSION

The effect of increasing temperature over time, caused the reduction in high percentage of the PME activity, however the kinetics of sonication has a positive effect on PME activity because partly increased the activity to obtain 52% in 4 min of treatment, subsequently the effect of inactivation decreased 27% compared with the untreated sample at initial time. The increase in enzyme activity by the ultrasound open perspectives for increases the capacity of catalysis in other enzymes of industrial interest.

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