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PAPER

CHANGES IN THE TEXTURE OF BUTTERNUT SQUASH FOLLOWING THERMAL TREATMENT

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ABSTRACT

Samples of butternut squash were heated in a convection steam oven at the temperature of 80°C and 100°C without any/or with addition of steam. The most significant changes of texture properties in the pulp were registered regarding its hardness and chewiness, while the alterations of its springiness and cohesiveness occurred within a smaller range. The decisive influence on changing the hardness and chewiness of butternut pulp was observed for the addition of steam, and, to a lower extent, for the time and temperature of treatment; in case of springiness the vital factor was the temperature of the process.

- Keywords: squash, thermal treatment, texture properties, convection steam oven -

INTRODUCTION

Pumpkin belongs to the family of *Cucurbitaceae*. Its edible part is the pulp of the fruit at different stages of ripeness, as well as its seeds (giant pumpkin and summer squash). The nutritional values of pumpkin fruit are high. This is determined primarily by a high content of carotenoids (from 2 to 10 mg·100 g^{-1}), which are characterized by antioxidant and anticancerous properties. Pumpkin fruit provides a good source of vitamins C, A and B, as well as minerals, such as potassium, phosphorus, calcium, magnesium, iron and selenium. They also contain organic acids (citric, malic and fumaric). Studies have revealed that the polysaccharides extracted from pumpkin have hypolipidemic activity. Additionally, pumpkin has low content of calories. Due to the presence of numerous, easily absorbed nutrients, it can be used as a component of slimming diets (CARVALHO *et al.*, 2012; NAWIRSKA-OLSZAŃSKA *et al.*, 2014; RAKACJEVA *et al.*, 2011; WOJDYLA *et al.*, 2007; ZHAO *et al.*, 2014).

Pumpkin pulp may be a healthy and valuable component of many dishes and fruit products. It is used to manufacture juices, baby foods and canned foods. A disadvantage of pumpkin, which may contribute to its low consumption, is its bland flavor and specific cucumber-like smell. This problem may be resolved by mixing pumpkin with other materials (e.g. cornel berries or quinces) in order to obtain food products of better sensory properties and chemical composition (NAWIRSKA-OLSZAŃSKA *et al.*, 2012). Pumpkin seeds are used in bakery, oil manufacturing and in pharmaceutical industry. Oil from pumpkin seeds contains valuable bioactive elements: squalene, unsaturated fatty acids, tocopherols (OBIEDZIŃSKA and WASZKIEWICZ-ROBAK, 2012).

Prior to consumption, pumpkin fruits are subjected to different types of treatment, most often thermal processing, during which their properties undergo changes (MAYOR *et al.*, 2011; CELAS-KA-GRZYWNA *et al.*, 2013), hence the aim of the study was to determine the changes in texture properties caused by thermal treatment in a convection steam oven with different parameters of the processes.

MATERIALS AND METHODS

Raw material

Research material was provided by butternut squash (*Cucurbita moschata* Duch.) originating from Portugal and purchased in London chain supermarkets. Butternut squash is an annual plant belonging to the gourd family (*Cucurbitaceae*), from Latin America. 100 g of butternut squash contains (after cooking): 0.9 g of protein, 7.4 g of carbohydrates, including 3.9 g sugars, 0.1 g of fat, 1.4 g of fiber, trace amounts of salt, 15 mg of (19% of RDA) vitamin C. Its caloric value is 156 kJ/37 kcal in 100 g. In the study we used ripe, healthy fruits, without any mechanical damage.

Treatment

The pumpkin was subjected to preliminary treatment: washing, peeling, removing the seeds. Such material was used to cut out samples for analyses. The pumpkin was sliced into 1-centimeter-thick slices. Next, cylinders of 2-centimeter diameter were cut out from the central part of the slices with the use of a calibrator. In this way cylinders were obtained of 1-centimeter height (h) and the diameter Φ = 2 cm. Six representative samples (cylinders) were selected for tests from each measurement series. Treatment was conducted in a convection steam oven (HOUNÖ CombiSlim CPE 2306 model, Randers, Denmark) at the temperature of 80 °C and 100 °C; 0, 20, 40, 60, 80 and 100% of steam added in relation to the initial humidity in the oven chamber; treatment time: 5, 10, 15, 20, 25 and 30 min.

For the temperature of 100 °C only the 0, 20, 40, 60% of steam addition were conducted. In case of steam addition of 80 and 100% resulted in structural changes going too far (overcooking), which made it impossible to carry out strength tests.

Strength tests

Immediately after thermal treatment warm samples were subjected to strength tests. Compression strength measurement for pumpkin samples was performed in the strength test machine, Zwick/Roell Z.5. (Zwick Roell Polska, Łódź, Poland). The material was subjected to double compression at the speed of head movement equal to 50 mm·min^{-1}. The process of compression was carried out at a stable deformation of the plates equal to 50% of their height, while the interval between the series was 5 s. The measurements were performed in 6 replications. On the basis of the measurements obtained in the form of texturegrams in the arrangement of two coordinates of strength and time, the following texture parameters were determined: hardness, springiness, chewiness and cohesiveness. After the tests, the results of the measurement were subjected to a statistical analysis. Namely, a double variance analysis was performed with the interaction for each of the analyzed properties with six variations for the temperature of 80°C and four variations for the temperature of 100°C.

Statistical analysis

Detailed comparisons of the mean values in pairs were performed on the basis of Tukey's multiple confidence intervals. Also, a com-

bined analysis of the four variations was performed for the temperatures of 80°C and 100°C, with the use of triple cross classification with interactions. Calculations were done in the SAS Enterprise Guide 5.1 software, adopting the significance level of 0.05 in all the statistical analyses.

RESULTS

The results regarding changes in the hardness of pumpkin pulp resulting from thermal treatment at the temperature of 80°C and 100°C during the time from 5 to 30 min at different levels of steam addition [%] are presented in Fig. 1(a,b). A significant impact of heating time and the amount of steam on the hardness of pumpkin hardness was observed.

In case of pumpkin heated at the temperature of 80°C, the hardness of the pulp was decreasing along with increasing the amount of steam added for all the analyzed heating time spans (Fig. 1a). On the other hand, the hardness of pumpkin pulp heated at the temperature of 100°C was decreasing along with the amount of steam added for all time spans adopted in the research program, except for the shortest period of 5 min (Fig. 1b). With this particular heating time and steam addition, an increase in pulp hardness was noted from 40 to 60%. Regardless of the adopted heating temperature, the lowest hardness was recorded for pumpkin pulp after heating it for 30 min. To analyze the effect of heating time and the amount of steam added during heating we used double cross classification with interaction. The analysis conducted suggests that heating time, the amount of steam added and the interaction between the heating time and the amount of steam added significantly differentiate pumpkin hardness. The results of a detailed comparative analysis of mean pumpkin hardness, based on Tukey›s multiple comparisons are presented in Table 1 for butternut squash heated at the temperature of 80°C, and 100°C.

The data in Table 1 suggest that the average hardness of pumpkin pulp heated at the temperature of 80°C for 5, 10 and 15 min does not differ significantly, and it is significantly higher than the hardness of pumpkin pulp heated for 20, 25 and 30 min. Analyzing the amount of steam added, the significantly highest value was recorded when no steam was added, while the significantly lowest value was with a 100% addition of steam. The analysis of Table 1 demonstrates that the average hardness of pumpkin pulp heated at the temperature of 100 °C for 5 min was significantly higher than the hardness of squash heated for a longer time. The significantly lowest hardness of pumpkin pulp was obtained for the heating periods of 20, 25 and 30 min. The significantly highest hardness of squash was noted when no steam was added. No significant differences in the mean hardness of pumpkin pulp were observed with adding 20, 40 and 60% of steam.

The results of studies on the springiness of pumpkin pulp heated at the temperature of 80 °C and 100°C depending on the amount of steam added [%] and the time of heating [min] are presented in Fig. 1(c,d).

The springiness of pumpkin pulp heated at the temperature of 80°C decreased when the amount of steam added increased from 0 to 40%, while a further increase in the amount of steam from 40% to 100% resulted in an increased value of springiness for the majority of heating periods. A different course of changes in springiness of squash heated during the shortest time of 5 min may be observed. The springiness of squash heated at the temperature of 100 °C increased along with the amount of steam added for the analyzed heating periods, except for the time of 15 min (Fig. 1d). For this particular period springiness decreased with increasing the

Table 1 - The results of Tukey's studentized range test for mean hardness values of pumpkin pulp heated at the temperature of 80°C and 100°C depending on heating time and the amount of steam added.

Temperature							
80ºC	Time [min]	5	10	15	20	25	30
	Mean values	205.92A	187.79A	185.51A	150.30B	129.10B	133.80B
	SD	27.07	25.19	26.02	24.20	19.01	25.58
	Steam [%]	0	20	40	60	80	100
	Mean values	442.17a	230.78b	118.33c	107.69c	68.39d	25.06e
	SD	12.05	14.06	7.38	8.82	3.82	1.09
100ºC	Time [min]	5	10	15	20	25	30
	Mean values	126.96A	86.85B	49.08BC	32.67CD	33.11D	67.10D
	SD	24.96	20.78	11.60	6.26	6.32	20.99
	Steam [%]	0	20	40	60	-	-
	Mean values	184.38a	33.74b	21.72b	23.99b	-	-
	SD	17.43	1.58	1.70	5.40	-	-

Means with the same letter are not significantly different at 0.05 significance level.

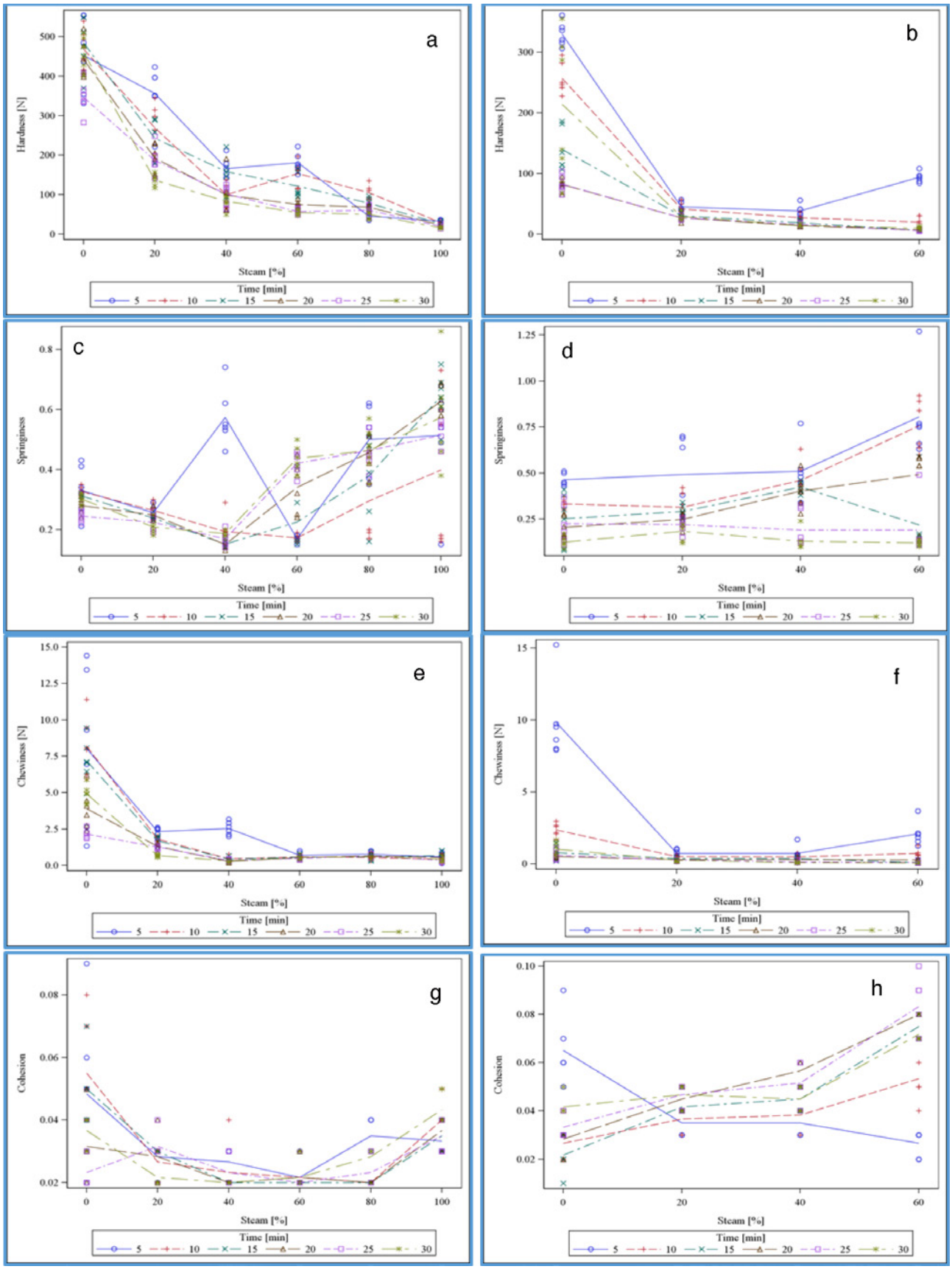


Fig. 1 - Analyzed properties of pumpkin pulp depending on amount of steam added and heating time: hardness of pumpkin pulp heated at the temperature of 80°C (a) and 100°C (b); springiness of pumpkin pulp heated at the temperature of 80°C (c) and 100°C (d); chewiness of pumpkin pulp heated at the temperature of 80°C (e) and 100°C (f); cohesiveness of pumpkin pulp heated at the temperature of 80°C (g) and 100°C (h).

Table 2 - The results of Tukey's studentized range test for mean springiness values of pumpkin pulp heated at the temperature of 80°C and 100°C depending on heating time and the amount of steam added.

Temperature							
80ºC	Time [min]	5	10	15	20	25	30
	Mean values	0.390A	0.275C	0.325BC	0.350AB	0.339AB	0.362AB
	SD	0.030	0.023	0.030	0.027	0.023	0.027
	Steam [%]	0	20	40	60	80	100
	Mean values	0.299c	0.239d	0.237d	0.294cd	0.426b	0.545a
	SD	0.008	0.005	0.027	0.021	0.020	0.028
100ºC	Time [min]	5	10	15	20	25	30
	Mean values	0.568A	0.467B	0.297CD	0.338C	0.207DE	0.140E
	SD	0.044	0.041	0.028	0.031	0.020	0.009
	Steam [%]	0	20	40	60	-	-
	Mean values	0.268c	0.292bc	0.353b	0.431a	-	-
	SD	0.022	0.022	0.028	0.053	-	-

Means with the same letter are not significantly different at 0.05 significance level.

amount of steam from 40 to 60%. The highest springiness of pulp was recorded for squash heated for the shortest time.

On the basis of double cross classification with interaction it can be concluded that the heating time, the amount of steam added and the interaction of heating time and the amount of steam added significantly differentiate the springiness of pumpkin pulp subjected to thermal treatment both at the temperature of 80°C and 100°C. The results of a detailed comparative analysis of mean springiness of pumpkin pulp on the basis of Tukey's studentized range test are presented in Table 2.

Analyzing the results presented in Table 2, it should be noted that the average springiness of pumpkin pulp heated at the temperature of 80°C for 5 min was the highest, yet it was not significantly higher than the mean springiness of squash for the heating time of 20, 25 and 30 min. The lowest mean springiness was obtained for the heating time of 10 min. On the other hand, the highest mean springiness of pumpkin pulp was obtained after adding 100% of steam, while the significantly lowest mean values of springiness were recorded in the situation when 20, 40 and 60% of steam was added.

Table 2 suggests that the mean springiness of squash heated at the temperature of 100°C for the time of 5 min was significantly highest at the level of significance of 0.05. The significantly lowest springiness of squash was obtained for the heating periods of 25 and 30 min. Analyzing the amount of steam added, the significantly highest springiness of squash was recorded with adding 60% of steam. No significant differences of mean squash springiness were noted when there was no steam addition or when 20% of steam was added.

The results of studies on the chewiness of squash heated at the temperature of 80°C and at 100°C depending on heating time and the amount of steam added are presented in Fig. 1(e,f). The chewiness of squash heated at the temperature of 80°C was decreasing when the amount of steam added increased from 0 to 20 and 40%, and when the addition of steam continued increasing no drop in chewiness was observed. The course of curves presented in Fig. 1f suggests that the chewiness of squash heated at 100°C for 5 min differed from the chewiness of squash heated for longer periods included in the research program. In case of the periods of 10, 15, 20, 25 and 30 min the chewiness of squash altered only slightly.

Double cross classification with interaction used to analyze the effect of heating time and the amount of steam added during heating on changing chewiness of butternut squash revealed that the heating time, the amount of steam added and the interaction of the heating time and the amount of steam added do not differentiate significantly the chewiness of squash. The results of a detailed comparative analysis of average chewiness of pumpkin pulp based on Tukey's multiple comparisons are presented in Table 3.

While analyzing the results presented in Table 3, it should be noted that the mean chewiness of squash heated at the temperature of 80°C for 5, 10 and 15 min was the highest and it was significantly higher than the mean chewiness of squash for the heating time of 20, 25 and 30 min. On the other hand, the significantly highest chewiness of squash was obtained when there was no steam addition. No significant differentiation in the chewiness of squash was observed when 15, 20, 25 and 30% of steam was added. The analysis of Table 3 demonstrates that the mean chewiness of squash heated at the temperature of 100°C for 5 min was significantly highest at the significance level of 0.05. The significantly lowest chewiness of squash was observed for the periods of 15, 20, 25 and 30 min. Analyzing the amount of steam, the significantly highest chewiness of squash was obtained when no steam

Table 3 - The results of Tukey's studentized range test for mean chewiness values of pumpkin pulp heated at the temperature of 80°C and 100°C depending on heating time and the amount of steam added.

Temperature							
80ºC	Time [min]	5	10	15	20	25	30
	Mean values	2.499A	2.005A	1.863AB	1.211BC	0.879C	1.249BC
	SD	0.557	0.492	0.420	0.228	0.114	0.281
	Steam [%]	0	20	40	60	80	100
	Mean values	5.7231a	1.5236b	0.7317c	0.5767c	0.6447c	0.5058c
	SD	0.5429	0.0946	0.1416	0.0233	0.0295	0.0372
100ºC	Time [min]	5	10	15	20	25	30
	Mean values	3.349A	1.023B	0.411C	0.356C	0.282C	0.363C
	SD	0.836	0.172	0.071	0.030	0.051	0.093
	Steam [%]	0	20	40	60	-	-
	Mean values	2.523a	0.408b	0.356b	0.569b	-	-
	SD	0.591	0.035	0.049	0.134		

Means with the same letter are not significantly different at 0.05 significance level.

was added. No significant differences of mean values of chewiness were noted when adding 20, 40 and 60% of steam.

The results of studies on the cohesiveness of squash heated at the temperature of 80 °C and at 100°C depending on heating time [min] and the amount of steam added [%] are presented in Fig. 1(g,h).

The cohesiveness of squash heated at the temperature of 80°C was changing depending on the amount of steam added and the course varied depending on the heating time (Fig. 1g). The highest cohesiveness was observed for squash to which no steam was added and when 100% of steam was supplied. In case of treatment at the temperature of 100°C for the heating periods of 10, 15, 20, 25 and 30 min the cohesiveness of squash generally increased along with the amount of steam added and it reached its highest values for 30% of steam (Fig. 1h). After heating for 5 min the cohesiveness of squash decreased along with increasing the amount of steam added.

The results of double cross classification with interaction used to analyze the effect of heating time and the amount of steam added during heating on changing cohesiveness of butternut squash revealed that the heating time, the amount of steam added and the interaction of the heating time and the amount of steam added significantly differentiate the cohesiveness of squash. A detailed comparative analysis of mean values of squash cohesiveness on the basis of Tukey's multiple comparisons revealed that the average cohesiveness of squash heated at the temperature of 80°C for 5 and 10 min was significantly higher, as compared with the mean cohesiveness of squash heated for 20 and 25 min (Table 4). The significantly highest cohesiveness of pumpkin pulp was recorded after heating with no steam addition and after adding 100% of steam. No significant differentiation of squash cohesiveness was observed after adding 15, 20, 25 and 30% of steam.

An analysis of Table 4 shows that the mean cohesiveness of squash heated at the temperature of 100°C for 20, 25 and 30 min was significantly

Table 4 - The results of Tukey's studentized range test for mean cohesiveness values of pumpkin pulp heated at the temperature of 80°C and 100°C depending on heating time and the amount of steam added.

Temperature							
80ºC	Time [min]	5	10	15	20	25	30
	Mean values	0.0322A	0.0311A	0.0286AB	0.0264B	0.0261B	0.0292AB
	SD	0.0022	0.0025	0.0020	0.0014	0.0011	0.0016
	Steam [%]	0	20	40	60	80	100
	Mean values	0.0408a	0.0278b	0.0222c	0.0211c	0.0244bc	0.0372a
	SD	0.0029	0.0009	0.0008	0.0005	0.0011	0.0010
100ºC	Time [min]	5	10	15	20	25	30
	Mean values	0.0404C	0.0388C	0.0458B	0.0525A	0.0538A	0.0513A
	SD	0.0034	0.0023	0.0041	0.0040	0.0041	0.0026
	Steam [%]	0	20	40	60	-	-
	Mean values	0.0361c	0.0419b	0.0453b	0.0650a	-	-
	SD	0.0027	0.0011	0.0015	0.0035	-	-

Means with the same letter are not significantly different at 0.05 significance level.

Table 5 - The results of Tukey's studentized range test for pairs of mean values for the three agents and analyzed properties.

Analyzed agent	Level of agent	Analyzed property			
		Hardness	Springiness	Chewiness	Cohesiveness
Temperature	80	224.74A	0.2674B	2.139A	0.0280B
	100	65.96B	0.3360A	0.964B	0.0471A
Steam	0	313.27A	0.2833B	4.123A	0.0385B
	20	132.26B	0.2657B	0.966B	0.0349C
	40	70.03C	0.2953B	0.544B	0.0338C
	60	65.84C	0.3625A	0.573B	0.0431A
Time	5	207.96A	0.4498A	3.3754A	0.035833A
	10	167.42B	0.3527B	1.8921B	0.035208A
	15	150.48B	0.2642CD	1.4421BC	0.037917A
	20	117.53CD	0.2960C	0.9350CD	0.038958A
	25	103.19D	0.2356DE	0.6769D	0.039167A
	30	125.53C	0.2119E	0.9869CD	0.038125A

Means with the same letter are not significantly different at 0.05 significance level.

highest at the significance level of 0.05. The significantly lowest mean cohesiveness of butternut squash was observed after 5 and 10 min of heating. Analyzing the amount of steam added, the significantly highest mean cohesiveness of squash was recorded after treatment with the addition of 100% of steam, while the significantly lowest values were noted when no steam was used.

The results of three-agent variance analyses for the studied properties, namely hardness, springiness, chewiness and cohesiveness provide a basis for claiming that all the agents analyzed in the present work, namely temperature, the amount of steam added, heating time and the interactions occurring between these agents significantly differentiate the studied qualities of butternut squash. The results of Tukey's multiple comparisons in pairs for the analyzed agents and for all the properties are presented in Table 5. These results show that there were significant differences concerning the analyzed properties of squash at the temperature of 80°C and 100°C. For hardness and chewiness the mean values of the properties at the temperature of 80°C were significantly higher, as compared with the mean values of the properties at the temperature of 100°C. In case of springiness and cohesiveness, it may be claimed that the mean value at the temperature of 100°C is significantly higher than the mean value at 80°C. Analyzing the comparisons of mean values for the properties with the use of different amounts of steam, it may be noted that the significantly highest mean hardness and chewiness were observed when no steam was added. On the other hand, the significantly highest springiness and cohesiveness were observed for the biggest amount of steam added, amounting to 60%. The most varied mean values of the studied properties were observed for the periods of treatment studied in the work. Only in case of the mean cohesiveness there were no significant differences for the pairs of mean values compared. The highest values of mean hardness, springiness and chewiness were noted for the shortest treatment time.

Structural and rheological properties determine behavior of the squash pulp under compression (SHIRMOHAMMADI *et al.*, 2014). Recognizing the mechanical properties of squash enables improvement of processing its pulp (SOSIŃSKA *et al.*, 2012). The texture of the squash pulp exhibits characteristics of chewiness and springiness, which can be modified by thermal treatment. Earlier studies concerning thermal treatment of pumpkin pulp in a convection steam oven revealed significant modifications in its texture properties (ŚLASKA-GRZYWNA *et al.*, 2013). It was noted that the most significant effect on changing hardness, springiness and chewiness of squash resulted from the amount of steam added, and to a lower degree from the time and temperature of treatment. Similarly, works by GONÇALVES *et al.* (2007) suggest a significant decrease in the firmness of squash during thermal treatment at the temperature of 75-95°C for 50 min. Prior to thermal treatment the firmness of squash was ca. 60 N, while after the treatment it did not exceed 10 N. Changes in the texture of pumpkin pulp following thermal treatment during its storage were studied by RATNAYAAKE *et al.* (2004). In their studies with the help of double-compression test they observed the most significant changes in the texture of pumpkin pulp in case of measuring its hardness and chewiness, while the changes were only slight in case of springiness and cohesiveness. The key factor affecting rheological qualities of vegetables is turgor (LIN TA-TE and PITT, 1986). Softening of tissues is related to the loss of turgor cells and their easier separation (GREVE *et al.*, 1994). Plant tissue is built of cells mutually linked by middle lamella. The cell wall is kept rigid due to hydrostatic pressure inside the cell, which normally amounts to 1-8 bar (0.1 - 0.8 MPa) (AGUILLERA *et al.* 1998). Cellulose present in the cell wall affects the rigidi-

ty and strength of the plant tissue, while pectins and hemicellulose present in middle lamella are responsible for its plasticity (LEWICKI and PAWLAK, 2003). Thermal treatment of vegetables results in structure alterations, tissue disintegration, enzyme inactivation, washing out soluble components, loss of firmness and, consequently, their softening (CRUZ *et al.* 2011; GALINDO *et al.*, 2005). According to researchers, both raw and cooked squash pulp provides numerous health benefits and can be used in prevention and treatment of certain diseases (CAILI *et al.*; 2006, NIEWCZAS *et al.*; 2005, STIRG, 1997). Studies suggest a possibility of selecting adequate parameters of thermal treatment helping to maintain the texture most required by consumers. Moreover, the research results will be useful for food producers, allowing them to select the optimal parameters of thermal treatment of squash pulp.

CONCLUSIONS

Thermal treatment in a convection steam oven results in statistically significant changes of all the studied parameters of squash texture, namely its hardness, springiness, chewiness and cohesiveness.

The range and dynamics of texture properties of butternut squash depends on the parameters of thermal treatment, such as temperature, amount of steam added and time.

The most significant range of modifications concerning texture qualities of squash were registered for its hardness and chewiness, while changes in its springiness and cohesiveness occurred to a smaller extent.

The decisive influence on changing the hardness and chewiness of squash was exerted by the addition of steam, while treatment time and temperature were less significant. In case of springiness the key agent was treatment time, while with cohesiveness it was the temperature of treatment.

Conducted studies will allow food producers to select the optimal parameters of thermal treatment of squash pulp for consumption purposes.

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PAPER

ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM *XI-GUA-MIAN* (FERMENTED WATERMELON), A TRADITIONAL FERMENTED FOOD IN TAIWAN

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ABSTRACT

Young watermelon fruit was peeled and pickled for fermentation to produce a unique fermented food named *xi-gua-mian* (fermented watermelon) in Taiwan. In this study, we investigated the LAB microflora in *xi-gua-mian*. A total of 176 LAB isolates were identified; 118 cultures were isolated from the *xi-gua-mian* sample collected from three different farmers markets and 58 from six young watermelon fruit samples. These isolates were characterized phenotypically and then divided into seven groups (A to G) by restriction fragment length polymorphism analysis, sequencing of 16S ribosomal DNA and other genotypic analysis. *Lactobacillus plantarum* was the most abundant LAB found in *xi-gua-mian* samples collected in southern Taiwan, Tainan City and *Pediococcus pentosaceus* was the most abundant LAB in northern Taiwan, Taoyuan County. We found that LAB stains are similar in samples collected in the same geographic region but significant variations were observed between samples collected among different regions. On the other hand, a greater LAB diversity was observed in the young watermelon fruit samples. In addition, 10 *Lactococcus lactis* subsp. *lactis* showed inhibitory activity against the indicator strain *L. sakei* subsp. *sakei* JCM 1157^T. This is the first report describing the distribution and varieties of LAB existing in the *xi-gua-mian* and the young watermelon fruits.

- Keywords: lactic acid bacteria, *xi-gua-mian*, fermented watermelon, Taiwan -

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a popular fruit in Taiwan. The farming area dedicated to watermelon production in Taiwan is reported to be largest among all fruits (LIN *et al.*, 2009). To have a better harvest, surplus fruits are eliminated and only one fruit is retained for every stock in the early phase of watermelon cultivation. In Taiwan, farmers use the eliminated young watermelon fruits to produce a unique fermented food named *xi-gua-mian* (fermented watermelon).

These immature watermelon fruits are peeled, cut, mixed with salt (NaCl) and then placed in a bucket. Salt is added to a final concentration of approximately 3-6%, and the bucket is sealed with heavy stones placed on the top of the cover. This process usually continues for 3 days and then the exuded water is drained. The bucket is sealed again with heavy stones and the fermentation process continues for at least 2 weeks at room temperature. Because of the contribution of the lactic acid bacteria (LAB), it has a special sour and sweet flavor. *Xi-gua-mian* is usually applied as a seasoning for various pork, seafood and poultry dishes in order to add a slightly acidic taste. Although the product is very popular, it has not been studied in detail.

Lactic acid bacteria (LAB) has been frequently found in various Taiwanese fermented foods such as *yan-tsai-shin* (fermented broccoli stems), *yan-jiang* (fermented ginger), *jiang-sun* (fermented bamboo shoot), *suan-tsai* (fermented mustard), *dochi* (fermented black beans), *jiang-gua* (fermented cucumbers), *yan-dong-gua* (fermented wax gourd) and *pobuzihi* (fermented cummingcordia) (CHANG *et al.*, 2011; CHEN *et al.*, 2006a, 2006b, 2010, 2012, 2013a, 2013b; LAN *et al.*, 2009). In these cited studies, various LAB species, such as *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis*, *Weissella cibaria* and *W. paramesenteroides*, were frequently found in the Taiwanese fermented products. However, there has been very little research reported on LAB distribution in fermented watermelon (*xi-gua-mian*).

One important attribute of LAB is the bacteriocin-producing abilities to inhibit food spoilage bacteria and many LAB strains isolated from the Taiwanese fermented foods were found to produce various bacteriocins. Some bacteriocins produced by these strains were further identified as novel bacteriocins in the later studies such as enterocin TW21, weissellicin L and enterocin T (CHANG *et al.*, 2013; CHEN *et al.*, 2013c; LIANG *et al.*, 2013).

The objectives of this study were to isolate LAB from the *xi-gua-mian*, to identify the isolates to the species level and to detect the antibacteri-

Table 1 - Analysis results and characteristics of isolates.

						16S rDNA RFLP groups						
						A	B	C	D	E	F	G
Sample No.	Location	pH	Salt con. (g/L)	Viable acid-producing cells (log CFU/mL)[α]	Lactic acid (g/L)	*L. plantarum*	*L. pentosus*	*P. pentosaceus*	*Lc. lactis* subsp. *lactis*	*Leu. mesenteroides*	*W. paramesenteroides*	*E. casslifiavus*
Fermented watermelon												
S1	Tainan	4.6	3.8	7.36±0.18	35.5	30	8					
S2	Tainan	3.9	3.8	6.77±0.17	95.0	36	4					
S3	Taoyuan	4.1	6.0	8.00±0.05	80.0			40				
Fresh watermelon												
W1	Hualien	–	–	1.84±0.09	–				7 (1[β])	1		2
W2	Hualien	–	–	3.77±0.01	–	2	1		7 (7)	2	1	
W3	Hualien	–	–	3.25±0.03	–	3	1		3 (2)	1	2	
W4	Tainan	–	–	3.04±0.06	–							10
W5	Tainan	–	–	3.51±0.08	–							12
W6	Chiayi	–	–	1.48±0.03	–				3			
Total						71	14	40	20	4	3	24

[α] The data are expressed as the mean±SD (n=3). [β] Number of BLIS-producing strains.
Abbreviations: L., *Lactobacillus*; P., *Pediococcus*; Lc., *Lactococcus*; Leu., *Leuconostoc*; W., *Weissella*; E., *Enterococcus*.

al activities of the isolates. Our results provide an example to understand the rich resources of LAB strains in the traditional Taiwanese fermented food.

MATERIALS AND METHODS

Xi-gua-mian and the young watermelon fruit samples

A total of 3 *xi-gua-mian* samples (S1-S3) were collected at three traditional farmers markets located in Tainan City and Taoyuan County (Table 1, Fig. 1A). In addition, six young watermelon fruit samples (W1-W6, approximately 8-15 cm in size) were collected from Hualien County, Tainan City and Chiayi County (Table 1, Fig. 1B). Samples were stored at 4°C and analyzed within 24 h of acquisition from the markets and the watermelon fields. The salt concentration and pH of *xi-gua-mian* juice was measured by using a model SK-5S salt meter (Sato Keiryoki, Tokyo, Japan) and a model B-112 compact pH meter (Horiba, Kyoto, Japan), respectively. Lactic acid in each *xi-gua-mian* samples was determined with a D-/L-Lactic Acid test kit (R-Biopharm AG, Darmstadt, Germany), according to the manufacturer's instructions.

Isolation of LAB

An initial analysis results showed that the *xi-gua-mian* samples S1 and S2 contained 3.8 % NaCl and sample S3 contained 6 % (Table 1). Therefore, MRS agar (Difco™ Lactobacilli MRS Broth; Sparks, MD, USA) containing 3 % NaCl was used for the isolation of LAB from *xi-gua-mian* samples S1 and S2. On the other hand, MRS agar containing 6 % NaCl was used for isolation from sample S3 and MRS agar without adding NaCl was used for isolation from young watermelon fruit samples. To distinguish acid-producing bacteria from other bacteria, 1% $CaCO_3$ was added to the MRS agar, and only colonies with a clear zone around them were selected (KOZAKI *et al.*, 1992). 0.5 g of crushed young watermelon fruit samples, and 0.5-mL aliquot of each *xi-gua-mian* juice samples were taken for LAB isolation. The isolation procedures of LAB were performed according to the methods described by CHEN *et al.* (2013a).

RFLP and sequence analysis of 16S rDNA

RFLP and sequence analysis of 16S rDNA were used to classify and identify the bacterial isolates. A colony PCR method described by SHEU *et al.* (2000) was performed in this study. PCR reactions were carried out using a Genomics *Taq* gene amplification PCR kit (Genomics, Taipei, Taiwan) and performed on a Gene Amp PCR System 9700 (PerkinElmer Corp., Boston, MA, USA) under the following conditions: 95°C for 3 min, 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 90 s, a final extension of 72°C for 10 min, and completion at 4°C (CHEN *et al.*, 2013b). 16S rDNA gene was amplified using the 16S rDNA universal primers 27F (5'- AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'- GGTTACCTTGTTACGACTT-3') (CHEN *et al.*, 2013b). RFLP analysis of 16S rDNA was also performed, as described by CHEN *et al.*, (2013b). In this study, three restriction enzymes, *Acc*II (CG/CG), *Hae*III (GG/CC) and *Alu*I (AG/CT) (Chen *et al.*, 2013b), were mainly used for grouping. For sequence analysis of 16S rDNA, the PCR products were purified and then sequenced with the following primer: 5´-GTCAATTCCTTTGAGTTT-3´ (920R). Sequence homologies were examined by comparing the obtained sequences with those in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using BLAST.

Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum*

A multiplex PCR assay with *recA* gene-derived primers was performed using the methods and conditions described by TORRIANI *et al.* (2001).

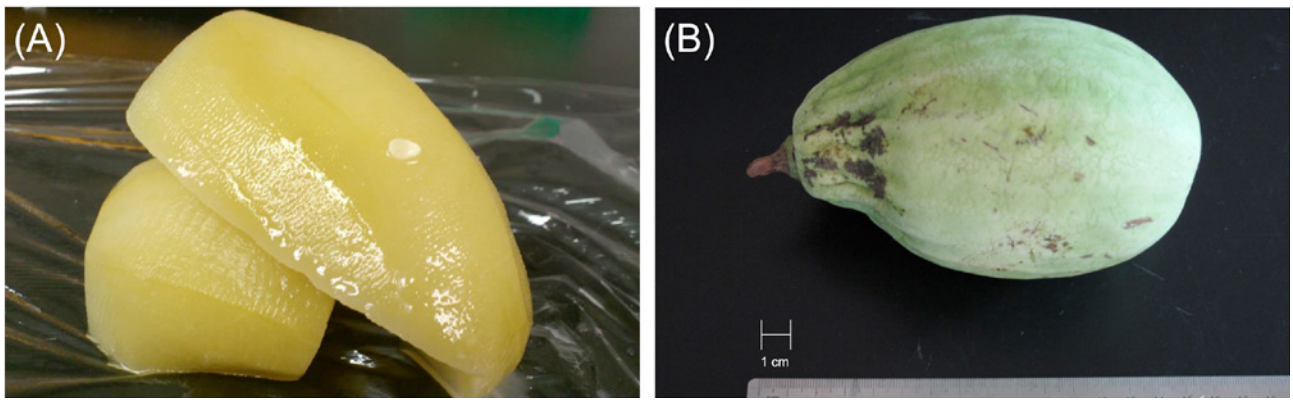


Fig. 1 - Pictures of (A) *xi-gua-mian* and (B) young watermelon fruit.

Differentiation of *Leuconostoc mesenteroides* and *Leu. pseudomesenteroides*

A rapid identification method described by JANG *et al.* (2003) was used to distinguish *Leuconostoc mesenteroides* and *Leu. pseudomesenteroides* isolates. Briefly, a PCR product of the isolate was amplified by using *Leuconostoc*-specific primers and then digested by using the restriction enzyme *Tsp509*I (/AATT) (JANG *et al.*, 2003). Restriction fragments were visualized on a 2% agarose gel in 1× TAE.

Differentiation of *W. paramesenteroides* and *W. hellenica*

In this study, isolate which showed high sequence homology to *W. paramesenteroides* and *W. hellenica* was further confirmed by using the restriction enzyme *Hha*I (GCG/C) described by CHEN *et al.* (2012).

Effect of NaCl on growth of isolates

Effect of NaCl on growth of isolates was assessed, as described by KOZAKI *et al.* (1992), by testing isolates for growth in MRS broth containing 0, 3 and 6% NaCl.

Detection of antibacterial activity

The agar well diffusion method as described by SRIONNUAL *et al.* (2007) was used to detect and determine the antibacterial activities of isolates. *Lactobacillus sakei* subsp. *sakei* JCM 1157^T was used as the indicator strain in this study. Antibacterial activity was further confirmed by pH adjustment and proteinase K treatment (SRIONNUAL *et al.*, 2007).

To determine whether nisin is the antibacterial substance, a PCR assay with the nisin-specific PCR primers, NISL: 5'-CGAGCATAATAAACGGC-3' and NISR: 5'-GGATAGTATCCATGTCTGAAC-3', described by VILLANI *et al.* (2001), were used for amplification in this study. In addition, a nisin Z producing strain, *Lc. lactis* subsp. *lactis* C101910 (YANAGIDA *et al.*, 2006) was used as the positive control and a non-BLIS (bacteriocin-like inhibitory substance) producing strain was used as the negative control.

RESULTS

In the *xi-gua-mian* samples collected from different markets, analyses of *xi-gua-mian* juice revealed different salt concentrations from 3.8 to 6.0% and lactic acid concentrations from 35.5 to 95.0 g/L (Table 1). The average number of viable acid-producing cells was 7.36±0.18, 6.77±0.17 and 8.00±0.05 log CFU/mL from the *xi-gua-mian* samples S1, S2 and S3, respectively (Table 1). The detailed analysis values of each sample are shown in Table 1 and a total of 118 acid-producing bacteria were isolated from these samples.

On the other hand, a total of 58 acid-producing bacteria were isolated from the young watermelon fruit samples. The number of viable acid producing cells on the six different young watermelon fruit samples was listed in Table 1.

The total 176 isolates were initially divided into six groups (R1-R6) according to cell morphology and the results of the 16S rDNA RFLP analysis. Of these isolated strains, 85 were placed in group R1, 40 in group R2, 20 in group R3, 4 in group R4, 3 in group R5, and 24 in group R6, according to RFLP patterns observed following digestion of their DNA with *Acc*II, *Hae*III, and *Alu*I. To identify the isolates, representative strains were randomly selected from each group, and 16S rDNA sequencing analysis was performed. The results identified group R1 isolates as *Lactobacillus plantarum*-related species, group R2 as *Pediococcus pentosaceus*, group R3 as *Lactococcus lactis* subsp. *lactis*, group R4 as *Leuconostoc mesenteroides*, group R5 as *Weissella paramesenteroides*, and group R6 as *Enterococcus cassliflavus*.

The identification of group R1 isolates was further verified using a multiplex PCR assay with *recA* gene-derived primers (TORRIANI *et al.*, 2001). An expected amplification band located at 318 bp and one at 218 bp (Fig. 2, lane 1 and 2) was respectively obtained from 71 and 14 isolates. Seventy-one isolates were therefore identified as *L. plantarum* and re-classified into group A. The remaining 14 isolates were identified as *L. pentosus* and re-classified into group B. All 4 isolates in group R4 were confirmed as *Leu. mesenteroides* based on *Tsp509*I digested fragments of the PCR product of *Leuconostoc*-specific primers and re-classified into group E (JANG *et al.*, 2003) (Fig. 2, lane 3; Table 1). Isolates in group R5 were further verified based on *Hha* I digested fragments of their 16S PCR product (CHEN *et al.*, 2012). All 3 strains were identified as *W. paramesenteroides* and re-classified into group F (Fig. 2, lane 4; Table 1). Following the re-classification of groups R1, R4 and R5, isolates in the remaining groups were also re-classified with a new code. The detailed distributions of LAB species are shown in Table 1.

Effect of NaCl on growth of all 176 isolates was estimated. All *P. pentosaceus*, *E. cassliflavus*, *L. plantarum*, *L. pentosus*, *W. paramesenteroides* and *Lc. lactis* subsp. *lactis* isolates grew well in MRS broth containing 0, 3 and 6 % NaCl except *Leu. mesenteroides* isolates. Growth of *Leu. mesenteroides* isolates was observed neither in 3 nor 6 % NaCl MRS broth.

Ten isolated *Lc. lactis* subsp. *lactis* strains showed antibacterial activity against *L. sakei* subsp. *sakei* JCM 1157^T (Table 1). The BLIS produced by all 10 strains maintained their antibacterial activities after neutralization (pH 6.5)

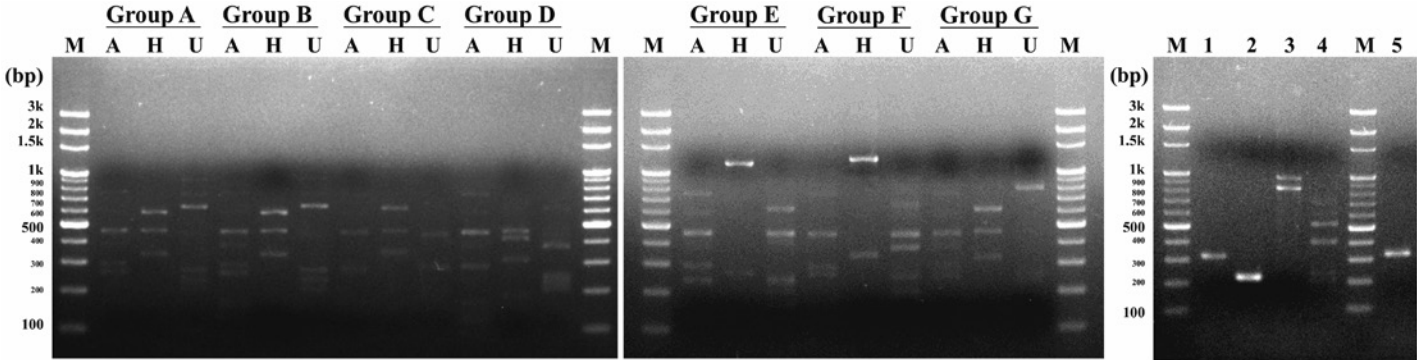


Fig. 2 - 16S rDNA RFLP patterns of *Acc*II, *Hae*III and *Alu*I digests from Groups A to G. Lane M, size marker; A, *Acc*II digested patterns; H, *Hae*III digested patterns; U, *Alu*I digested patterns; 1, amplification products obtained from the *recA* multiplex assay of *L. plantarum* isolates; 2, amplification products obtained from the *recA* multiplex assay of *L. pentosus* isolates; 3, *Tsp509*I digested patterns of *Leuconostoc*-specific PCR products from Group E isolates; 4, *Hha*I digested patterns of Group F isolates; 5, PCR products using nisin-specific primers.

but lost their antibacterial activities completely after treatment with proteinase K. In addition, nisin-specific primers were used to amplify a PCR fragment and identify the BLIS from these 10 strains. An expected amplification band located at 320 bp (Fig. 2, lane 5) was obtained from all *Lc. lactis* subsp. *lactis* isolates and the nisin Z producing strain, *Lc. lactis* subsp. *lactis* C101910 (YANAGIDA *et al.*, 2006). No amplification band was observed from the negative control strain.

DISCUSSION

In this study, LAB diversity in *xi-gua-mian* samples collected from different farmers markets and young watermelon fruits were studied. The final concentration of lactic acid and low pH values determined in the *xi-gua-mian* samples suggested that LAB contributed to the aroma and flavor development in *xi-gua-mian*.

The experimental data were treated according to critical values of Student's *t*-test. The viable acid-producing cell numbers between *xi-gua-mian* and fresh watermelon was significantly different ($p<0.0002$). We also found that the viable acid-producing cell numbers within geographical areas were different, but the statistical difference (standard deviation within *xi-gua-mian* group and fresh watermelon group was 0.61 and 0.93, respectively) was less than that between *xi-gua-mian* and fresh watermelon groups (4.56).

In addition, halotolerance of all isolates were assessed. All isolated strains grew well in MRS broth containing 3% and 6 % NaCl except *Leu. mesenteroides* isolates. Presence of NaCl in *xi-gua-mian* and isolation medium therefore might limited the growth of *Leu. mesenteroides*. Presence of *Leu. mesenteroides* was only observed in fresh watermelon fruits but not in *xi-gua-mian*. It is therefore considered that salt concentration has effect on diversity of LAB in the *xi-gua-mian*. Similar influence of NaCl concentrations on diversity of LAB in fermented foods has also been found in our previous studies (CHEN *et al.*, 2006a; 2006b).

Compared to the isolation results of *xi-gua-mian*, fewer viable acid-producing cell number were observed from the young watermelon fruit samples. It is presumably because the raw material always presents a lower number or microorganisms or the absence of salt in substratum used for the isolation from young watermelon fruit samples does not allow the selection of LAB. As in the case of *xi-gua-mian* samples, LAB stains are similar in samples collected in the same geographic region and diversities were observed between samples collected among different regions in the young watermelon fruit samples (Table 1). Different climate conditions were considered as the main factor, which may affect the distribution of LAB.

Although *xi-gua-mian* samples S1 and S2 were collected at different traditional farmers markets located in Tainan City, *L. plantarum* and *L. pentosus* were the most abundant LAB found in these two samples (Table 1). Different to the isolation results obtained in the Tainan City, *P. pentosaceus* was the most abundant LAB found in the sample collected in Taoyuan County (Table 1). Geographically, Tainan City is located in southern part of Taiwan that belongs to the tropics, while Taoyuan County is in northern subtropical regions. It is therefore considered that regional factors, such as climate conditions, raw materials for fermentation and fermentation methods, may affect the distribution of LAB.

Lactobacillus plantarum has been identified elsewhere as one of the most abundant LAB found in several Taiwanese fermented vegetables such as fermented bamboo shoots (*jiang-sun*), fermented cucumbers (*jiang-gua*), fermented broccoli stems (*yan-tsai-shin*) and fermented cummingcordia (*pobuzihi*) (CHEN *et al.*, 2010b, 2012, 2013a, 2013b). As well as *L. plantarum*, *P. pentosaceus* also have been previously found

as the most abundant LAB in the fermented mustard (*suan-tsai*) (CHEN *et al.*, 2006a). In addition, *L. plantarum* was found both in the partial samples of *xi-gua-mian* and the young watermelon fruits. It is therefore considered that *L. plantarum* found in *xi-gua-mian* may originate from the young watermelon fruits. To clarify these points, advanced analysis on more *xi-gua-mian* and young watermelon fruit samples will be necessary in the future.

The results of the antibacterial activity assay indicated that total 10 *Lc. lactis* subsp. *lactis* isolates showed inhibitory activities against *L. sakei* subsp. *sakei* JCM 1157^T. Complete inactivation of these BLIS produced by all 10 strains were observed after treating the cell-free supernatant with proteinase K, which indicates the proteinaceous nature of the active agents. When amplified with nisin-specific primers, the amplification band located at 320 bp indicated the existence of nisin-producing genes and BLIS from these 10 *Lc. lactis* subsp. *lactis* could be nisin-related variants (VILLANI *et al.*, 2001; ZENDO *et al.*, 2003). However, detailed information such as heat stability, their effect on enzymes, inhibition spectra, accurate molecular mass and amino acid sequences were not established in the current study.

Although LAB have been widely found in various fresh fruits, vegetables and various plant pickles, little information on the diversity of LAB associated with fermented watermelon or young watermelon fruits was obtained from previous studies. Future studies in our laboratory will characterize and identify the nisin-like BLIS, and we anticipate that the BLIS of LAB will be useful as food preservatives. The authors also hope that the results of this study can offer useful information for the improvement of *xi-gua-mian* production.

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PAPER

BEYOND THE USE OF FOOD SUPPLEMENTS: AN EMPIRICAL ANALYSIS IN ITALY

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ABSTRACT

This paper aims to profile Italian food supplements used by consumers based upon their psychometric patterns and demographic characteristics. The FTNS scale is used to assess empirically and evaluate the role of technophobic/technophilic consumer traits in determining the decision whether or not to consume supplements and vitamins and the frequency of their consumption. An ad-hoc survey was carried out in 2012 involving 400 residents of a metropolitan area in southern Italy. Our results show that women have a higher consumption frequency of dietary supplements, while age, BMI and education influence the propensity to consume. As regards food habits, the propensity to use dietary supplements is positively associated to the consumption of bread and pasta, red meat and pulses, and negatively with the consumption of fruit and cheese. Finally, the research supports the role of technophobic traits as consistent and significant determinants of the consumption frequency of dietary supplements.

- Keywords: consumption, neophilia, vitamins -

1. INTRODUCTION

Food supplements are edible products that include components proposed as a dietary enhancement (US Food and Drug Administration, 1994), regulated as food rather than drugs at least in the majority of developed countries. The dietary components might include vitamins, minerals, proteins (metabolites, enzymes and amino acids) and energy concentrate (energy bars).

Although by the end of the 1990s the use of dietary supplements was relatively frequent in industrialised countries, the consumption of such supplements has been further boosted in all the more affluent Western countries by new motivations associated to ageing populations and to continually changing lifestyles (BLENDON *et al.* 2001; GRAGAR, 2001; BABGALEH *et al.*, 2006; FELDMAN, 2014). In recent years, as regards general food consumption, ever more consumers have shifted from mere satisfaction of energy requirements to an attitude dictated by the need to promote well-being and reduce the risk of disease (MARQUES-VIDAL, 2004). In the US, the market for dietary supplements has grown dramatically in recent decades, recording an almost 80% increase from 1994 to 2000 (GREGER, 2001; BALLUZ *et al.*, 2005). In Italy, according to a recent survey by GfK Eurisko and Federsalus, for example, three out of every four individuals stated that they used at least one supplement for personal well-being in 2012. Wellness trends are generating new opportunities and challenges for companies in the vitamin and dietary supplement sector, and the producers of dietary supplements have substantially increased their investments and studies to ascertain the behaviour of consumers *vis-à-vis* food supplements. This analytical need is also emerging at scientific level with a view to understanding not only the consumption dynamics of such products but also the various underlying motivations (NICHTER and THOMPSON, 2006; O'CONNOR and WHITE, 2010; TAVANI *et al.*, 2014). In this regard, medical research and economic research are proceeding apace. The former aims to gain insights into the effects that food supplements have on the well-being of the individual, or the harmful effect of their excessive use to be able to better satisfy the demand for information and steer consumption of these products (GREGER, 2001); by contrast, economic research aims to analyze markets opportunities and challenges for new or existing products and the regulations for consumer information (RUSSO FRANCE and FITZGERALD BONE, 2005).

Furthermore, a large strand of research aims to profile consumers of supplements. In the UK, users of supplements are primarily female, vegetarian, less likely to drink alcohol, non-smoking, and more likely to engage in physical activities (KIRK *et al.*, 1999). Similar results were confirmed in the US (LYLE *et al.*, 1998; GREGER, 2001; ROCK, 2007). The increasing number of users is dictated by a strong aspiration towards better health (GREGER, 2001), by the need to protect one's state of health and at the same time lower the risk of disease, rather than only satisfy metabolic needs (ROBERFROID, 2000; GREGER, 2001; COX *et al.*, 2004; MARQUES-VIDAL, 2004).

Although health considerations appear to be a predominant incentive in choosing to use dietary supplements, the reasons for consuming such supplements are complex, combining social, psychological, educational and economic factors. A paradox has been pointed out (CONNER *et al.*, 2001): consumers of dietary supplements generally have higher recorded nutrient intakes from food sources alone than those who do not consume supplements. Although only a small percentage of individuals go beyond the line of reasonable consumption, those consuming excessively may sustain harmful effects (MEDEIROS *et al.*, 1999). This seemingly irrational behaviour provides the motivation to discard the neo-classical approach which analyses the consumer's rational choice as a utility maximisation process under budget constraint and graduate towards the use of analytical instruments that investigate individuals' cognitive and affective factors and their relations with consumption behaviour (VERNEAU *et al.*, 2014).

For instance, using a psychosocial model, the Protection Motivation theory, COX *et al.* (2004) analysed which characteristics of the product/message would impact on the motivations to purchase dietary supplements to prevent short-term memory loss. RUSSO FRANCE and FITZGERALD BONE (2005) examined the information environment in the dietary supplement industry. They analysed consumer product-specific as well as general beliefs about health, the supplement industry and the government. Their results suggest that information regarding a particular product can be overridden by the consumer's existing and distantly related beliefs.

Dietary supplements and vitamins represent a product category that falls between - and links – food and medicine, and therefore the perception of these products might also be influenced by the effect of risk. On this field, O'CONNOR and WHITE (2008) analysed consumers' willingness to trial functional foods and vitamin supplements. They found support for the Theory of Planned Behaviour (TPB) model in predicting people's willingness to trial functional food and vitamin supplement. The authors also suggested that non-users are influenced by the high-perceived risk associated with their use. According to the psychometric paradigm proposed by SLOVIC (1987), the more people are familiar and well informed about specific hazards, the lower is the perception of risk towards emerging technologies. In other words, the risk perception is affected by the knowledge of both

risks and benefits related to novel food technologies (FIFE-SCHAW and ROWE, 1996, SIEGRIST *et al.*, 2006). Thus, when knowledge is lacking, consumers' assessment of risks and benefits related to novel food products and emerging technologies is driven by heuristics (SIEGRIST *et al.*, 2008) and among them, trust and perceived naturalness have been identified as powerful factors (FREWER *et al.*, 2003; BRONFMAN *et al.*, 2008; CHRYSSOCHOIDIS *et al.*, 2009; EARLE and SIEGRIST, 2008; KJÆRNES, 2006 ROZIN *et al.*, 2004; STEPTOE *et al.*, 1995). SIEGRIST *et al.* (2008) have shown, for instance, that food products perceived as natural and healthy are more likely to be accepted by consumers.

Various psychometric scales have been developed and tested to study consumer acceptance towards new technology and, more generally, new food (GOLDSMITH and HOFACKER, 1991; PLINER and HOBDEN, 1992; EISER *et al.*, 2002; KIRK *et al.*, 2002; COX *et al.*, 2007; COX and EVANS, 2008). Among them, the Food Technology Neophobia Scale (FTNS) (Cox and Evans, 2008) has been judged to be a more suitable tool for assessing consumer fears of food technologies than an earlier food neophobia scale (FNS) (PLINER and HOBDEN, 1992) because of its specific focus on technology rather than food (MATIN *et al.*, 2012). The FTNS is a multidimensional scale which integrates the main drivers previously discussed, including naturalness, trust and perception of both risks and benefits of novel food technologies (COPPOLA *et al.*, 2014; VERNEAU *et al.*, 2014; COX *et al.*, 2010).

In this study, we adopted the FTNS to assess empirically and to evaluate the role of technophobic/technophilic traits in determining whether or not to consume supplements and vitamins and their consumption frequency. Moreover, to the best of our knowledge, the potential effect of the technophobia and technophilia traits in affecting this decision is still underexplored and this work is probably the first quantitative attempt to ascertain the determinants of supplement consumption in Italy.

While an empirical analysis of the motivations behind their use or non-use can be considered strategic for the industry sector to improve product penetration, assessment of the determinants of their consumption frequency is even more important in the policy debate on public health. Ascertaining the profile of consumers who are likely to use them excessively is an important step towards prevention.

The market for dietary supplements in Italy is one of the largest in Europe: it reached nearly 2 billion euros in 2013, showing an annual increase of around 3% (COUSYN *et al.*, 2013). The results show whether, and to what extent, the degree of consumer technophilia and hence the perception of risk for novelty and neophilia are associated, if at all, with dietary supplement consumption.

2. MATERIAL AND METHODS

2.1 The survey

An *ad-hoc*, face-to-face survey was conducted in 2012 with a convenient sample of 400 residents of the Naples metropolitan area (southern Italy). The questionnaire used for data collection comprised three sections.

Section a) includes socio-demographic characteristics and lifestyle factors including physical activities (such as gym and sport activities).

Section b) includes the consumption frequency scale for five categories of dietary supplements, namely mineral supplements, amino acid and/or protein supplement, vitamin supplement, beverages enriched with vitamins or minerals, and energy and protein bars. Several classifications of food supplements are proposed by the scientific literature, varying among those focusing on the product's functionality (RADIMER *et al.*, 2000), ingredients (SKEIE *et al.*, 2009), disease perspectives (MILLEN *et al.*, 2004) or consumer's point of view (TAVANI *et al.*, 2014). This paper adopts a market-driven classification, coherently with the European Commission directive (2002/46/EC) and with the Italian Legislation (Legislation Decree 169/2004), which characterizes food supplements as concentrated sources of nutrients for supplementing the intake in a normal diet (primarily vitamin, mineral salts and amino acids).

For each product, respondents were asked to select one of the following five options, labelled 0 "I do not consume the product", 1 "I seldom consume the product (no more than once a month)", 2 "I occasionally consume the product (no more than once a week), 3 "I consume the product frequently (more than once a week)" and 4 'I regularly eat the product (almost every day)". The above frequency scale of consumption can be considered an easily quantifiable measure of the phenomenon under observation in this study, namely individual consumption of dietary supplements. Furthermore, section b) comprises consumption frequency of all the other food categories in order to represent the whole consumption habits of the respondents.

Section c) comprises the Food Technology Neophobia Scale for investigating consumer attitudes to technology using the 13 items provided by COX and EVANS (2008). The FTNS translated into Italian were provided to the respondents who were called upon to express their degree of agreement-disagreement by using a Likert 7-point agree/disagree scale on their perception of new food technology, its uses, benefits and associated risks; the way they feel in new situations and behave when facing unknown circumstances; their food habits and the propensity to taste new food products.

2.2 The analytical framework

From an empirical point of view, individual consumption decisions on dietary supplements may be modelled through a two-stage process. In the first stage, individuals decide whether or not to consume supplements. In the second stage, the individuals shape their consumption habit by consumption decisions over time (CEMBALO *et al.*, 2014). The second stage decision is approximated here through a consumption frequency scale.

If the first stage could be of interest for marketing purposes, for identifying segments ready or "nearly" ready for the use of supplements, the second stage is particularly important for public health policy reasons, since it investigates the determinants of the use (including the excessive use) of dietary supplements. Both stages may depend on several individual factors involving interaction among the cognitive, social and cultural dimensions of consumption. In this paper we formally assess the extent to which neophobia-neophilia forces may influence such decisions. This study implements the Food Technology Neophobia Scale (FTNS) proposed by Cox and Evans (2008), allowing consumers with greater neophilia attitudes to be identified, potential early adopters of dietary supplements. Moreover, dietary supplement consumption decisions could depend on individual socio-characteristics and overall diet. Therefore other variables capturing these consumer characteristics and described in the previous section are included in the model.

Analytically, a two-step Heckman procedure (1979) can be used to analyse both stages of consumption decisions. As concerns the first stage, we assumed that observable characteristics of the consumers influence their consumption choices in terms of the probability that the consumer will use dietary supplements. Considering a sample of n observations indexed by i, the outcome $\{ y_i \}$ of whether or not to consume is a qualitative random variable taking in the presented case two levels: 0, 1.

$$y_i = \begin{cases} 1 & if \quad C_i^* > 0 \\ 0 & if \quad C_i^* \leq 0 \end{cases}$$

where C_i^* indicates consumption over time. Empirically, this relation can be analysed through Probit specification as

$$\pi_i = Prob(y_i = 1) = Prob(C_i^* > 0) = Prob(-u_{1i} < \mathbf{x'}_{1i}\boldsymbol{\beta}_1) = \Phi(\mathbf{x'}_{1i}\boldsymbol{\beta}_1) \qquad (1)$$

where π_i identifies the probability that the i-th respondent consumes the dietary supplement, u_{1i}, is the error term, (i.i.d.) ~ N(0,1), Φ is the cumulative density function of a standardized normal distribution, x'_{1i} is the set of k_1 consumer characteristics influencing the probability of consuming dietary supplements while β_1 are the respective parameters to be estimated.

As regards the second stage, consumption frequency, we may focus only on the "consumers" or the individuals who have decided to consume a dietary supplement at least once, ($y_1 = 1$). More specifically, a positive consumption over time, (C_i^*), is observed only if a consumer chooses to consume dietary supplements: $C^*_i > 0$. Formally, we can write the selection equation and the resultant outcome equation for C^* as follows:

$$\begin{aligned} &\mathbf{x'}_{1i}\boldsymbol{\beta}_1 + u_{1i} > 0 (selection\ equation) && i = 1, 2, \ldots, n_1 \\ &C_i^* = \mathbf{x'}_{2i}\boldsymbol{\beta}_2 + u_{2i} (outcome\ equation) && i = 1, 2, \ldots, n_2 \end{aligned} \qquad (1)$$

where

$$\begin{bmatrix} u_1 \\ u_2 \end{bmatrix} \sim N(\mathbf{0}, \boldsymbol{\Sigma}) \text{ and } \boldsymbol{\Sigma} = \begin{bmatrix} 1 & \cdot \\ \sigma_{12} & \sigma_2 \end{bmatrix}$$

Assuming that consumption frequency is influenced by a set of k_2 explanatory variables x_2, we wish to estimate β_2 parameters, under sample selection, with a potential source of inconsistency as:

$$E(C_i / \mathbf{x}_2, C_i^* > 0) = \mathbf{x'}_{2i}\boldsymbol{\beta}_2 + E(u_{2i} / C_i^* > 0) \qquad i = 1, 2, \ldots, n_2.$$

Because error terms have a bivariate normal distribution, the expectation $E(u2i \mid C^*i>0)$ is equal to $\sigma_{12}\lambda_i(x'_1 i\beta_1)$ where λ_i is known as the inverse of the Mills' ratio:

$$\lambda_i(\mathbf{x'}_{1i}\boldsymbol{\beta}_1) = \frac{\phi(\mathbf{x'}_{1i}\boldsymbol{\beta}_1)}{\Phi(\mathbf{x'}_{1i}\boldsymbol{\beta}_1)} \qquad i = 1, 2, \ldots, n_2$$

where $\phi(\bullet)$ is the probability density function of the standard normal distribution.

Following Heckman (1979) a consistent estimation of β_2 and σ_{12} can be obtained by augmenting the outcome equation with the inverse of the Mills' ratio obtained from the–estimates of the selection equation. In order to obtain a better identification of the Heckman model, we also impose exclusion restrictions (exclusion of at least one regressor being significant in the selection part, but not in explaining the outcome). The augmented equation was estimated by OLS using a linear functional form, while test statistics are based on Huber–White Sandwich estimation of variance.

The dependent variable of the outcome equation C_i^* is based on the stated frequency of consumption of the five categories of dietary supplements, and more precisely is defined as a linear additive aggregation of their stated frequency:

$$C_i^* = \sum_{d=1}^{5} S_{d,i}$$

where $S_{d,i}$ represents the stated frequency consumption score of the d-th dietary supplement for the i-th individual.

Table 1 - Stated frequency of consumption/use of dietary supplements.

	Mineral supplements	Protein supplements	Vitamin supplements	Enriched drinks	Energy bars
Never	56.25	83.7	53.53	39.95	75
Seldom	30.43	10.05	30.43	39.95	15.76
Monthly	9.24	2.72	10.05	14.4	7.34
Weekly	4.08	3.53	5.98	5.71	1.9
Daily	0	0	0	0	0

3. RESULTS

3.1 Descriptive results

Of the 400 respondents 9% failed to complete the survey or to answer key questions fully and thus, the final sample is based on 368 individuals. Socio-demographic information shows that the interviewees (165 male and 203 female; Italian frequency of female) were in the age range 17–70 years (32 ± 11 years; Italian average 43.0, Italian National Institute of Statistics 2011). Almost one third of consumers (30.7 %) fail to do any physical activity, while the others spend one hour per day on average. Just over half the interviewees have university degrees (Italian average 11.7%, Italian National Institute of Statistics 2011), while 7% had achieved minimum education levels (Italian average 21.7%, Italian National Institute of Statistics 2011). As regards the Body Mass Index (BMI), 66% of respondents were normal weight (BMI 18.5–24.9 kg/m^2) (Italian average 52.6%, World Health Organization 2005). These differences with the Italian population could be due to the specific context in which survey was carried out (residents of Naples metropolitan area).

Table 1 reports the stated frequency of consumption for the various categories of supplements: enriched drinks and vitamin supplements appear to be the supplements with a highest penetration rate in our sample, while energy bars and protein supplements report the lowest penetration rate. Around 50 % of respondents stated they used at least one vitamin supplement in the past; this percentage rises to 60 % when considering enriched drinks. Only 25 % and 15% of the respondents stated they had made previous use of energy bars and protein supplements, respectively. Overall, about 75% of the respondents declared to have consumed at least one category of food supplements in the past. This result is in agreement with data reported by Gfk-Eurisko and FederSalus.

Table 2 summarises the main characteristics of the respondents divided into two groups: consumers of dietary supplements and non-consumers.

From a preliminary analysis of the average values between the two groups, we found that consumers of dietary supplements seem younger than those not consuming dietary supplements. Among the different types of dietary supplements, the beverages enriched with vitamins or minerals are those most commonly consumed while amino acid and/or protein supplements are those least consumed (Table 2).

As regards dietary habits, consumers of dietary supplements compared to non-consumers show a higher consumption frequency for meats (both red and white meat), snacks and sugary drinks, while they show a lower consumption frequency for cheese, fruit and salad (Fig. 1).

For consumers of dietary supplements the consumption index (Fig. 2) assumes a mean value of 3.57 ± 2.5 in the range (1-13), while it necessarily assumes a value of zero for non-consumers.

Respondents general attitudes towards novel technology and how its benefits and risks are perceived are assessed by means of the 13 psychometric items of the FTNS (Table 3). Consumers of dietary supplements compared to non-consumers show a higher FTNS score. Cronbach's α of the scale is 0.83, indicating very good internal reliability. The mean level of agreement stated on a scale from 1 to 7 shows in the sample that the statements with the highest rates are *"There is no sense trying out high-tech food products because the ones I eat are already good enough"* together with *"New foods are no healthier than traditional foods"* and *"The benefits of new food technologies are often grossly overstated"*. These results seem in agreement with those of VERNEAU *et al*. (2014), highlighting that there is a great belief in Italian society in supporting natural foods, the Mediterranean diet along with the promotion of local and typical products. In turn, this outcome might reflect the opinion that innovation and manipulation in the food industry is somewhat futile, since traditional food products are often more highly appreciated and healthier.

By comparing the mean FTNS score for the entire sample (mean = 55.2, sd = 13.7, range 16-85) with those evaluated from other studies, it may be stated that our sample from the Naples metropolitan area presents less fear of food compared to the whole Italian population (mean =

Table 2 - Sample descriptive statistics according to consumption of dietary supplements.

Variable	Average	Std. Dev.	Min	Max	Average	Std. Dev.	Min	Max
	Non-consumers of dietary supplements				Dietary supplement consumers			
Socio-demographic characteristics								
Age (years)	38.94	13.85	20	71	30.05	9.92	17	70
Gender (2 male; 1 female)	1.61	0.49	1	2	1.53	0.50	1	2
BMI	24.04	3.23	18.14	34.29	23.06	3.72	17.02	40.14
Education classes[a]	3.51	0.69	1	4	3.44	0.62	1	4
Presence of children (1 yes; 0 no)	0.88	0.33	0	1	0.91	0.28	0	1
Physical activity (hours per week)	7.50	10.46	0	45	6.85	8.31	0	45
Income classes[b]	2.41	0.71	1	4	2.35	0.83	1	4
Dietary habitc								
Salads	2.41	0.78	0	4	2.29	0.89	0	4
Other vegetables	2.40	0.78	0	4	2.33	0.80	0	4
Fruit	3.16	0.97	0	4	2.85	1.09	0	4
Pulses	1.78	0.50	0	3	1.93	0.62	0	4
Milk & yogurt	2.50	1.11	0	4	2.51	1.11	0	4
Cheese	2.11	0.70	0	4	1.91	0.86	0	4
Red meat	1.83	0.54	0	3	1.98	0.48	0	4
White meat	2.00	0.54	0	3	2.09	0.56	0	4
Eggs	1.62	0.62	0	3	1.69	0.71	0	4
Bread and Pasta	2.87	0.72	0	4	2.99	0.75	0	4
Sugary drinks	1.23	1.03	0	4	1.48	1.14	0	4
Snacks	1.56	1.07	0	4	1.94	1.13	0	4
Wine & Beer	1.23	1.06	0	4	1.30	1.05	0	4
Dietary supplement consumption[c]								
Mineral supplements	0	0	0	0	0.79	0.85	0	3
Amino acid and/or protein supplement	0	0	0	0	0.34	0.75	0	3
Vitamin supplements	0	0	0	0	0.88	0.91	0	3
Beverages enriched with vitamins or minerals	0	0	0	0	1.10	0.84	0	3
Energy and protein bars	0	0	0	0	0.47	0.77	0	3

[a](1 primary school; 2 middle school; 3 high school; 4 university and higher);
[b](1 less than €1,000 per month; 2 €1,000-2,000; 3 €2,000-3,000; 4 more than €3,000);
[c](0 no consumption; 1 rare consumption, once a month; 2 frequent consumption, once a week; 3 very frequent consumption, more than once a week; 4 addictive consumption, every day).

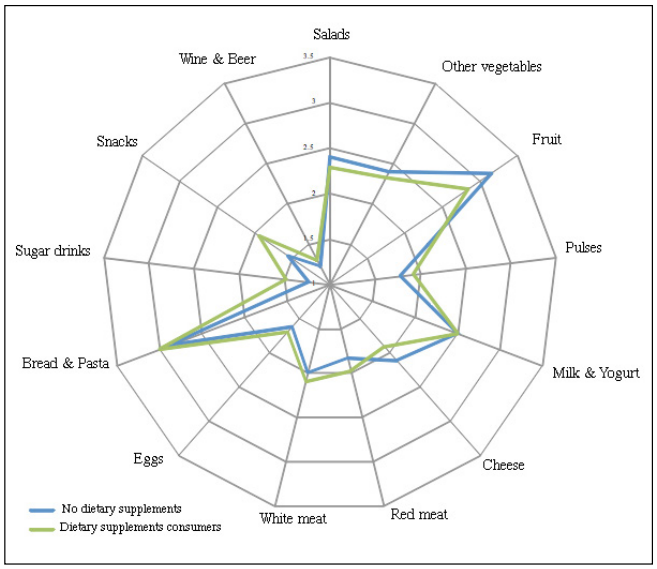


Fig. 1 - Comparison of dietary habits of respondents consuming dietary supplements and those not consuming.
Note: the scale range from 0 "I do not consume the product", to 4 'I regularly eat the product (almost every day)".

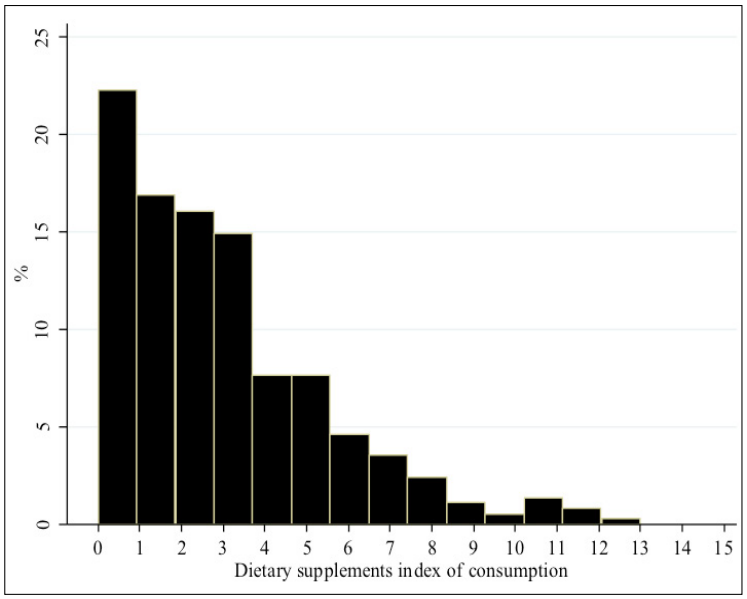


Fig. 2 - Frequency distribution of the dietary supplement index of consumption.

Table 3 - Descriptive statistics - Cox psychometric questions: item score on a 1 to 7 scale (1= strongly disagree; 7= strongly agree).

FTNS statements	Mean (Total Sample)	Std. Dev. (Total Sample)	Mean (Non-consumers of dietary supplements)	Mean (Dietary supplement consumers)
There is no sense trying out high-tech food products because the ones I eat are already good enough	4.8	1.8	5.38***	4.64
New food technologies are something I am uncertain about	4.2	2.0	4.48*	4.14
New foods are no healthier than traditional foods	4.6	2.0	4.73	4.53
The benefits of new food technologies are often grossly overstated	4.6	1.8	4.70	4.57
There are plenty of tasty foods around, so we do not need to use new food technologies to produce more	4.2	2.1	4.46*	4.14
New food technologies decrease the natural quality of food	4.3	1.9	4.51	4.22
New food technologies are unlikely to have long term negative health effects (R)	4.1	1.7	4.19	4.10
New food technologies give people more control over their food choices (R)	3.9	1.7	4.07*	3.80
New products using new food technologies can help people have a balanced diet (R)	4.2	1.4	4.28	4.11
New food technologies may have long-term negative environmental effects	4.0	1.8	4.21*	3.88
It can be risky to switch to new food technologies too quickly	4.2	1.8	4.45*	4.10
Society should not depend heavily on technologies to solve its food problems	4.6	1.9	4.77	4.56
The media usually provides a balanced and unbiased view of new food technologies (R)	3.7	1.9	3.75*	3.45

Note: Cronbach's α: 0.832; (**R**) means the item is reverse-coded.
*Difference across mean significant at the 10% level; ** at the 5% level; *** at the 1% level.

61, sd = 11.3, VERNEAU *et al*., 2014). That said, our results are comparable with those obtained with Canadians (mean = 58, MATIN *et al*., 2012) and Australians (mean = 54, EVANS *et al*., 2010).

3.2 Propensity to consume dietary supplements

The two-stage process of individual consumption decisions on dietary supplements is shown in Table 4.

Variables not significant at the $p < .10$ level in explaining any of the two stages are eliminated from the final models, starting with the least significant variable. On the left are the results of the first stage, indicating the individual determinants of the decision whether or not to consume dietary supplements. The results show that the propensity to consume dietary supplements depends on the age of the respondents (the latter is significantly associated with a lower propensity to consume dietary supplements)

Table 4 - I Stage and II Stage estimates.

	I Stage: Propensity to consume dietary supplements				II Stage: Consumption frequency decision			
	Coef. β1	Elasticity	Std. Dev.	p-value	Coef. β2	Elasticity	Std. Dev.	p-value
Socio-demographic								
Cons.	3.389		1.264	0.007	7.102		2.913	0.015
Age	-0.034	-0.353	0.007	0.000	-0.003	-0.044	0.036	0.924
Sex	-0.101	-0.051	0.182	0.579	-0.889	-0.541	0.370	0.016
Edu.	-0.284	-0.319	0.132	0.031	-0.284	-0.385	0.354	0.422
BMI	-0.043	-0.329	0.026	0.095	-0.118	-1.081	0.062	0.058
Food Habits								
Fruit	-0.136	-0.129	0.083	0.103	0.010	0.011	0.178	0.956
Pulses	0.274	0.170	0.148	0.063	0.436	0.325	0.330	0.186
Cheese	-0.263	-0.168	0.108	0.015	-0.451	-0.345	0.282	0.110
R. Meat	0.346	0.219	0.179	0.053	0.308	0.235	0.470	0.512
W. Meat	0.085	0.057	0.163	0.603	1.093	0.889	0.329	0.001
Bread& Pa	0.186	0.180	0.111	0.094	0.261	0.304	0.268	0.330
Attitudes								
FTNS	-0.191	-0.088	0.383	0.619	-2.083	-1.161	0.753	0.006

and on their BMI values (higher consumption of dietary supplements is associated with lower BMI levels). Respondent education also plays a major role in determining consumption decisions (DIAMANTOPOULOS *et al.*, 2003). Our estimate shows that less educated respondents are associated with a higher propensity to consume dietary supplements. Daily eating habits are a particularly significant factor affecting propensity to use supplements (KIM and KEEN, 2002). Our estimates show that intakes of carbohydrate and protein from the diet differ between those using or not using dietary supplements. Respondents who often consume refined cereals (bread and pasta), red meat and pulses show a higher propensity to use dietary supplements compared to those frequently consuming fruit and cheese. Finally, the attitudes of individuals to food technology measured through the FTNS do not appear to affect this propensity significantly.

3.3 Frequency of dietary supplement consumption

The results of the second stage on the determinants of the frequency of the supplements use is also reported on Table 4.

Studies in the US and Europe have shown that females, individuals in high socioeconomic categories, and individuals living in large cities are likely to use dietary supplements more often than others (SLESINSKI *et al.*, 1995; SCHELLHORN *et al.*, 1998). Our results confirm that women show a higher consumption frequency of dietary supplements. As regards the remaining socio-demographic characteristics, only the BMI value seems to influence (in inverse relation) the use of supplements. Although daily eating habits proved clearly associated to the propensity to use supplements, such habits almost entirely fail to explain respondents' consumption frequency. Only the consumption frequency of one food category over the 13 tested (white meat) is significantly associated to a higher use of supplements. Estimates show an elastic complementary relationship between the consumption of white meat and supplements intake. However, with the exception of age, BMI and the frequency of white meat consumption, the great majority of the socio-demographic variables collected and used in the analysis are unable to explain the consumption frequency of dietary supplements. In this case, analytical instruments that investigate individuals' cognitive and affective factors might help to profile the consumers of supplements.

The attitude of consumers to food technologies, as measured by the FTNS, effectively contributes to meeting this requirement. Specifically, respondents characterised by neophobia patterns and consequently showing low demand for novelty and neophilia are associated with a low consumption of dietary supplements. Furthermore, the estimated association between the FTNS and the index of supplements' frequency of consumption is quite strong (elasticity -1.161).

4. DISCUSSION AND CONCLUSIONS

Dietary supplements are a relatively new class of product that has gradually become established on the markets, especially in the US, but that is rapidly increasing its penetration also in European and Italian markets. However, this trend has generated several major issues and posed a number of challenges. For example, it has been shown that consumers have a certain difficulty in interpreting the claims of such products, both as regards functions and disease prevention. Moreover, general and specific beliefs systematically bias product-specific judgments regarding efficacy as well as scientific certainty. This leads to questions as to how such labels are interpreted by those at risk or affected by specific diseases.

Another important issue stems from the evidence that the use of dietary supplements is at least partially motivated by self-control of health (EISENBERG *et al.*, 1998; GREGER, 2001). This can lead many people to make inappropriate choices, for example, favouring the use of dietary supplements compared to proper varied nutrition, especially among those who are more vulnerable to pressure to use dietary supplements unnecessarily, despite the lack of evidence to suggest they are needed to meet dietary deficiency. It is this group, namely the more vulnerable, that needs to be able to make an informed choice so that their use of dietary supplements is connected to real rather than perceived need. This presents a paradox, because dietary supplements, which are used to enhance human health, have the potential to create distortions in eating habits, keep people from the objective of a healthy and complete diet, and may cause adverse reactions when used inappropriately and taken in excessive amounts. For these reasons, the study of consumer behaviour and analysis of the motivations that cause them to consume dietary supplements or otherwise is particularly important for both policy makers and industry.

While the major studies that have analysed supplement-taking behaviour are focused on motivational systems, primarily resorting to Protection Motivation theory (PMT) and Theory of Planned Behaviour (TPB) (CONNER *et al.*, 2001; COX *et al.*, 2004; O'CONNOR and WHITE, 2010), in our research we tested the role of attitude to food technologies as a predictor of the intention to consume dietary supplements. In particular, we analysed the role of food technology neophobia/neophilia, which has been extensively researched with reference to a great number of food products, technologies and attributes (ARVOLA *et*

al., 1999; HENRIQUES *et al.*, 2009; TOURILA *et al.*, 2001). Testing food technology neophobia/neophilia in the case of dietary supplements seems to be particularly useful because, at least from a marketing point of view, this class of product is difficult to classify, lying at the crossroads of food and drugs. Furthermore, this paper provides the first empirical attempt to profile dietary supplement consumers in Italy.

The study outlined three main results: first of all, our analysis shows two different patterns for users and non-users. Secondly, results show that being young, female, less educated and having a low BMI are factors that are associated to higher propensity to consume dietary supplements. As regards food habits, the propensity to use dietary supplements is positively associated to the consumption of refined cereals (bread and pasta), red meat and pulses. On the contrary, propensity to use dietary supplements is negatively related to consumption of fruit and cheese. The investigation of whether these factors are similar to those associated with dietary deficiency/excess goes beyond the scope of the current analysis and would require another study with that specific objective. Finally, the research supports the role of technophobic traits as important determinants of the consumption frequency of dietary supplements: consumers of dietary supplements compared to non-consumers show a higher FTNS score. In particular, non-consumers of dietary supplements endorse the notions that "*There is no sense trying out high-tech food products because the ones I eat are already good enough*", "*New food technologies are something I am uncertain about*" and "*It can be risky to switch to new food technologies too quickly*". On the other side, consumers of dietary supplements show higher trust levels than non-consumers, and they highlight the benefits of the new technologies, agreeing with the statements " *New food technologies give people more control over their food choices*" and "*The media usually provides a balanced and unbiased view of new food technologies*".

It would appear that a negative attitude to food technologies has the capacity to contain the consumption of dietary supplements within lower levels. Bearing in mind the potential challenges linked to the increasing consumption of dietary supplements, this finding may steer communication policies and information towards the specific group of people less affected by food technophobia. More generally, the results confirm the FTNS as a powerful tool to capture technophobic traits.

The findings of this study suggest that the introduction of some risk-based construct, namely the technophobic traits measured by means of the FTNS, might strengthen standard TPB models when health-related products like vitamins and dietary supplements are considered. However, surveying residents from a single metropolitan area may limit generalisability of the results to different areas.

Further studies could follow two different avenues. On the one hand, it could be useful to build up a modified structure of the TPB model, incorporating risk perception and food technophobia among general and specific-product attitudes in order to gain insights into supplement-taking behaviour. On the other, a greater effort is needed to make more thorough information available on the proper use of supplements. Indeed, it has observed that the propensity to consume dietary supplements is more commonly found among young people who are presumably also the category that has less need of supplements. This paradox, that people who least seem to need supplements are most likely to use them, has been called in the literature "the inverse supplement hypothesis" (KIRK *et al.*, 1999). It seems particularly true in the more affluent Western countries, like Italy, where a healthy and balanced diet should be adequate to ensure intake of all the main nutrients.

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PAPER

IDENTIFICATION OF NEOCHLOROGENIC ACID AS THE PREDOMINANT ANTIOXIDANT IN *POLYGONUM CUSPIDATUM* LEAVES

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ABSTRACT

To identify the predominant antioxidant compound in *Polygonum cuspidatum* leaves, the methanol extract of fresh samples were separated by liquid–liquid partitioning, octadecylsilyl Sep-pak® cartridge and high-performance liquid chromatography. The main active compound was identified as (1*R*,3*R*,4*S*,5*R*)-3-{[(2*E*)-3-(3,4-dihydroxyphenyl)-2-propenoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid (neochlorogenic acid) by nuclear magnetic resonance and liquid chromatography-mass spectroscopic analysis. Its content was found to be 2.31 mg/g of fresh leaves. As shown by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion scavenging assays, the contributions of neochlorogenic acid as an antioxidant were 16.5% and 36.5%, respectively, suggesting that neochlorogenic acid is the predominant antioxidant in *P. cuspidatum* leaves.

- Keywords: *Polygonum cuspidatum*, antioxidant, polyphenol, neochlorogenic acid -

INTRODUCTION

Polygonum cuspidatum, commonly known as Japanese knotweed, originated in East Asia and has spread widely to European and American countries where it has been listed as one of the most invasive plants. In some invaded areas it has become a severe environmental problem and governmental actions have been taken to thwart its spread (GREVSTAD *et al.*, 2013). However, the chemical and mechanical methods that have been used have not been successful in eliminating this plant, owing to its viability. Contrastingly, in other areas, *P. cuspidatum* has been used as medicine and consumed as a food. For example, in China its dried rhizomes are used in traditional Chinese medicine to treat inflammatory diseases, hepatitis, tumors, and diarrhea (CHEN *et al.*, 2013). It is also reported that the young stems of *P. cuspidatum* were consumed by native people of North America (CHEN *et al.*, 2013). In some areas of Japan, such as Kochi prefecture, the edible portions of young stems are pickled and cooked to be served as traditional dishes even today. The young leaves have also been recognized as edible (HASHIMOTO, 2003).

Over the past few decades the health-promoting effects of *P. cuspidatum* have attracted the attention of researchers and several bioactive compounds, particularly those with antioxidant activity, have been identified. Resveratrol, or *trans*-3,5,4′-trihydroxystilbene, also found in grape skins and wine, is abundant in the rhizomes of *P. cuspidatum*. Numerous health-promoting effects of resveratrol, including anticancer, anti-inflammatory, antiviral, and antifungal activities have been described (PENG *et al.*, 2013). Polydatin, a glycoside precursor to resveratrol, is also found in abundance in the rhizomes of *P. cuspidatum*. Polydatin has been linked with beneficial lipid-regulating, melanogenesis-inhibitory and hepatoprotective effects (PENG *et al.*, 2013; CHU *et al.*, 2005). Besides stilbene compounds, other antioxidants including anthraquinones, such as emodin and physcion, and flavonoids, such as catechin and quercetin, that possess health-promoting properties have also been found in the rhizomes of *P. cuspidatum* (CHEN *et al.* 2013; PENG *et al.*, 2003; CHU *et al.*, 2005).

Less research has been performed on the different parts of the plant. The rhizomes have been the most studied however the health-promoting effects of other parts of *P. cuspidatum* have not been studied. Although the stems and leaves are not as commonly used as the rhizomes, in a previous study we observed the antioxidant effect of the leaves was comparable to that of the rhizomes (KURITA *et al.*, 2014). Despite their high antioxidant capacity, only a few studies have been performed to identify antioxidant compounds in the leaves. In this study, we isolated and identified the predominant antioxidant compounds in the leaves of *P. cuspidatum*.

MATERIALS AND METHODS

Instruments

To determine 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, a TECAN CTS-R R-10 microplate reader (TECAN, Mannedorf, Switzerland) was used. High-performance liquid chromatography (HPLC) was performed with an LC-7100 pump, L-2300 column oven, and L-2420 UV VIS detector (Hitachi, Tokyo, Japan). Liquid chromatography-mass spectroscopy (LC-MS) was performed with a Waters ACQUITY UPLC system (Waters, Milford, USA) with a Cosmosil® $5C_{18}$ AR-II Column (150 × 4.6 mm i.d., particle size 5 μm, pore size 12 nm), (Nacalai Tesque Inc., Kyoto, Japan). The mobile phase of LC-MS included 20% MeOH, 1% acetic acid and 79% H_2O at a flow rate of 0.5 mL. Positive ion ESI with the capillary voltage at 3 kV was used. The source and desolvation temperatures were 150°C and 400°C, respectively, and the eluted compounds were detected at 254 nm. ^{1}H- and ^{13}C-NMR data for compound 1 were measured using a JEOL JNM-ECX500 (JEOL Resonance Inc., Tokyo, Japan) at 500 MHz. The letters (br.) s, d, t, q and m represent (broad)singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants are expressed in Hz. Specific rotation was determined by Horiba SEPA-500 (HORIBA Ltd., Kyoto, Japan), and the UV spectrum was measured with a Pharmacia Biotech Ultraspec 3000 UV/Visible Spectrophotometer (GE Healthcare UK Ltd., Buckinghamshire, UK). For the Folin–Ciocalteu method, UVmini-1240 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) was used for measurement.

Chemicals and reagents

All reagents used were of analytical grade or better. DPPH and HPLC-grade methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Neochlorogenic acid was obtained from Sigma Chemical Co. (St. Louis, USA), and chlorogenic acid was from MP Biomedicals, LCC (Santa Ana, USA). Phenol reagent solution for Folin–Ciocalteu assay was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Superoxide dismutase (SOD) Assay Kit-WST was purchased from Dojindo Laboratories (Kumamoto, Japan).

Isolation of antioxidants from *P. cuspidatum*

Sample materials were collected in Murotoshi, Kochi prefecture, Japan, in May 2013. The roots, stems, and leaves of *P. cuspidatum* were separated and extracted in an aqueous solution containing 80% methanol (MeOH) for 24 h, and the extraction was repeated twice. The extract was filtered using Minisart® RC 15 Syringe Filters made from regenerated cellulose

with a pore size of 0.45 μm (Sartorius Stedium, Göttingen, Germany). Twenty grams equivalents of fresh leaf weight (f.w.) were evaporated until dry under reduced pressure (1110 mg) and subjected to liquid–liquid partitioning. The residue of the MeOH extract was dissolved in 27.7 mL of water, and the solution was partitioned between hexane (19.6 mL × 3) and water and then between ethyl acetate (19.6 mL × 3) and water. The hexane (53.3 mg), ethyl acetate (93.2 mg), and water (960 mg) layers were collected. The water layer (1 g f.w. equivalent) was applied to a Sep-Pak® Plus C18 cartridge (Waters, Milford, USA), containing 360 mg of octadecylsilyl (ODS), and eluted with increasing concentrations of MeOH to obtain four fractions: 0% MeOH (25.6 mg), 20% MeOH (8 mg), 40% MeOH (3.6 mg), and 100% MeOH (trace amount) fractions. The ODS 20% MeOH fraction was further separated into six fractions by reverse-phase semipreparative HPLC (Cosmosil® $5C_{18}$ AR-II column, 250 × 10 mm i.d., particle size 5 μm, pore size 12 nm, Nacalai Tesque Inc.) and eluting with 20% MeOH containing 1% acetic acid at a flow rate of 3 mL/min and detected at 254 nm.

Compound 1 was isolated from fraction 2, and its structure is *(1*R*,3*R*,4*S*,5*R*)-3-{[(2*E*)-3-(3,4-dihydroxyphenyl)-2-propenoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid, neochlorogenic acid.* $[\alpha]_D^{20}$ + 12.00° (*c* = 0.02, MeOH). UV λ_{max} (MeOH) nm (ε): 238.5 (5383), 324.2 (8729). Positive-ion ESI-MS: *m/z* 355 $[M+H]^+$, 163 [M-quinic acid]$^+$. NMR spectral data were as follows. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.44 (d, 1H, *J* = 16.0 Hz, H_C-3), 7.00 (d, 1H, *J* = 2.5 Hz, H_C-2′), 6.93 (dd, 1H, *J* = 8.0, 2.5, Hz, H_C-6′), 6.75 (d, 1H, *J* = 8.0 Hz, H_C-5′), 6.21 (d, 1H, *J* = 16.0 Hz, H_C-2), 5.16 (dt, 1H, *J* = 3.5, 8.5 Hz, H_Q-3), 3.84 (dt, 1H, *J* = 7.5, 4.0 Hz, H_Q-5), 3.15 (m, 1H, H_Q-4), 2.00 (dd, 1H, *J* = 15.0, 4.0 Hz, H_Q-2′), 1.89 (dd, 1H, *J* = 15.0, 7.5Hz, H_Q-2), 1.83 (m, 2H, H_Q-6). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 176.1 (C_Q-7, s), 166.2 (C_C-1, s), 148.2 (C_C-4', s), 145.6 (C_C-3', s), 144.5 (C_C-3, d), 125.9 (C_C-1', s), 121.2 (C_C-6', d), 115.9 (C_C-5', d), 115.2 (C_C-2, d), 114.6 (C_C-2', d), 73.1 (C_Q-1, s), 71.6 (C_Q-5, d), 71.1 (C_Q-3, d), 67.2 (C_Q-4, d), 39.5 (C_Q-2, t), δ 35.2 (C_Q-6, t).

Structural determination of compound 1

The structure of compound 1 was established by independent injection and co-injection of fraction 2 with an authentic preparation in HPLC to confirm the retention times. The following conditions were used to identify the compound found in fraction 2: a Cosmosil® $5C_{18}$ AR-II column (150 × 4.6 mm i.d., particle size 5 μm, pore size 12 nm, Nacalai Tesque Inc.) was used with a mobile phase of 20% MeOH containing 1% acetic acid at a flow rate of 0.5 mL/min, and UV detection was set at 254 nm.

Determination of total phenolic content

The polyphenol content of *P. cuspidatum* leaves was determined by the Folin-Ciocalteu method as described by Singleton *et al.* with some modifications (SINGLETON *et al.*, 1999). In a test tube, 0.25 mL of sample solution, 0.1 mL of phenol reagent (1.8 N), and 0.25 mL of saturated sodium carbonate were added within 15 s and mixed. Then, 2.15 mL of water was added and mixed, followed by 1 h of incubation at room temperature. After incubation, the sample was measured at 725 nm. The measured value for the crude extract was expressed as gallic acid equivalent (GAE) per gram of the sample material.

DPPH radical scavenging activity assay

Antioxidant activity was measured using the DPPH method as described in our previous study (KURITA *et al.*, 2014). In a 96-well plate, 20 μL of sample solution, 80 μL of 0.1 M Tris-HCl buffer (pH 7.4), and 0.2 mM DPPH in ethanol solution were added and mixed. The mixture was incubated in the dark at room temperature for exactly 30 min. The radical scavenging rates of each sample and a control solution were measured at 517 nm. All experiments were performed in triplicate. The radical scavenging rate was calculated using following equation:

$$\text{Scavenging rate (\%)} = \\ = (A_{Control} - A_{Sample}) / A_{Control} \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is that of the sample. SC_{50}, which is the sample concentration at 50% of the scavenging ratio, was used to express the antioxidant capacity of each sample. To determine the contribution rate, SC_{50} was then converted to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) equivalent (TE) antioxidant capacity, TEAC, using the following equation (SHIMAMURA *et al.*, 2014):

$$\text{TEAC (mg TE/mg)} = \text{Trolox } SC_{50} \text{ (mg/mL)} / \\ / \text{Sample } SC_{50} \text{ (mg /mL)}$$

The contribution rate of the active compound was calculated using the following equation:

Contribution rate (%) = (TEAC of active compound × concentration of active compound in *P. cuspidatum*) / (TEAC of crude extract) × 100

Superoxide anion scavenging assay

A SOD Assay Kit-WST was used to determine the superoxide scavenging activity (SOSA) of each sample. The assay was performed according to the manufacturer's procedure. The resulting 50% inhibitory concentration (IC_{50}) was used to determine the SOSA, which was further used to evaluate the contribution of compound 1 to

the total antioxidative capacity. SOSA was defined using following equation:

$$\text{SOSA (unit/g)} = [1/IC_{50}\ (\text{mg/mL})] \times 0.02\ \text{mL} \times \times 1000\ \text{mg/g}$$

The contribution rate from SOSA was calculated using the following equation:

Contribution rate (%) = (SOSA of active compound × concentration of active compound in *P. cuspidatum*) / (SOSA of crude extract) × 100

RESULTS

Antioxidant capacities of different parts of *P. cuspidatum*

All results of DPPH radical scavenging activity assays had relative standard deviations (RSD) of < 5%. Among the MeOH extracts of the different parts of *P. cuspidatum*, the strongest activity was observed in the leaves (SC_{50}: 1.24 mg f.w./mL), followed by the rhizomes (SC_{50}: 1.63 mg f.w./mL) and stems (SC_{50}: 14.1 mg f.w./mL). This is consistent with our previous study which also found that the leaves and rhizomes showed almost equivalent antioxidant capacities (KURITA *et al.* 2014).

Fractionation and antioxidant activity of the leaf extract

The fractionated leaf extracts and antioxidant activities are shown in Fig. 1. Among all the layers, the water layer showed the highest activity (SC_{50}: 1.90 mg f.w./mL), followed by the ethyl acetate layer (SC_{50}: 13.2 mg f.w./mL). The separated hexane, ethyl acetate, and water layers were further combined for measurement. The combined sample yielded an SC_{50} of 1.8 mg f.w./mL, although some activity had been lost in the separation process. The combinations of the hexane and water layers (SC_{50}: 1.93 mg f.w./mL), and the ethyl acetate and water layers (SC_{50}: 1.69 mg f.w./mL) were measured and compared with the water layer only. The results suggest that the antioxidants were present mainly in the water layer because the activities of these combinations were close to that of the water layer only.

Antioxidant activities were observed in the ODS water and in the 20% and 40% MeOH fractions (Fig. 1). The ODS 20% MeOH fraction showed the highest activity, yielding an SC_{50} of 4.3 mg f.w./mL. All the fractions combined had an SC_{50} of 1.55 mg f.w./mL. When the ODS 20% MeOH fraction was combined with the second highest fraction, the ODS water fraction (SC_{50}: 5.56 mg f.w./mL), the SC_{50} of the combined sample was 1.68 mg f.w./mL. This suggests that the ODS water and the 20% MeOH fractions account for the majority of the antioxidant capacity of the water layer.

The ODS 20% MeOH fraction was further fractionated by reversed phase semipreparative HPLC, and the chromatogram is shown in Fig. 2. The highest antioxidant capacity was seen in fraction 6 (SC_{50}: 22.4 mg f.w./mL), followed by fraction 1 (SC_{50}: 32.4 mg f.w./mL) and fraction 2 (SC_{50}: 36.1 mg f.w./mL). A further HPLC analysis with multiple-wavelength detection using a SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan) detected no other distinct peaks in fraction 1 or 6. Fractions 1 and 6 were further separated to isolate and identify the compound; however, the antioxidant activity was dispersed during the process. In contrast fraction 2, which exhibited relatively high antioxidant activity, contained a major single peak at the retention time of 10.07 min. This major peak was assigned as compound 1, which was further purified. Compound

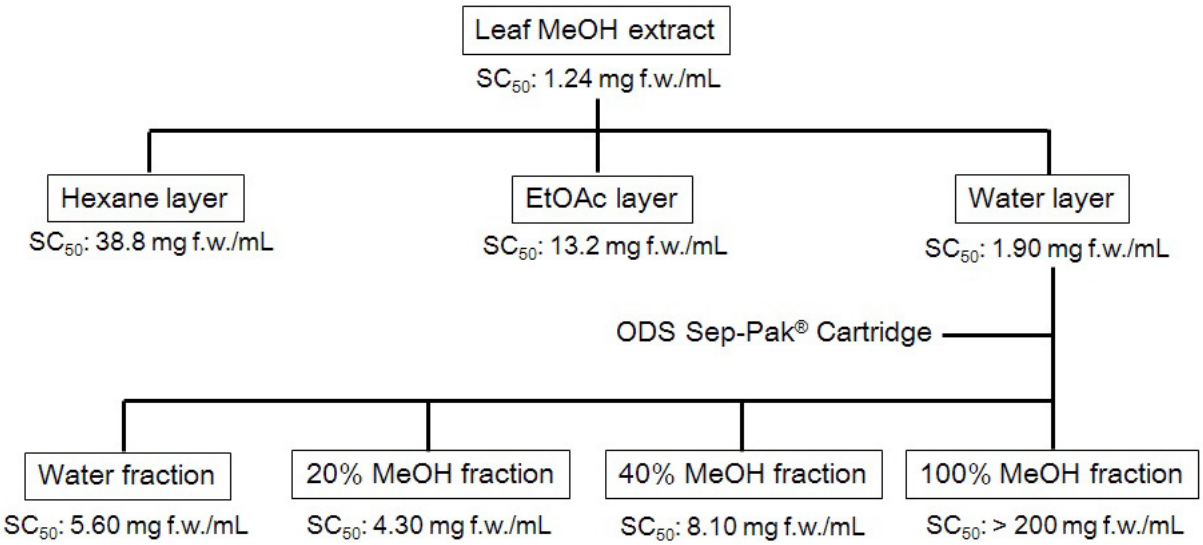


Fig. 1 - The separation process of the leaf extract and the antioxidant capacity of each fraction.

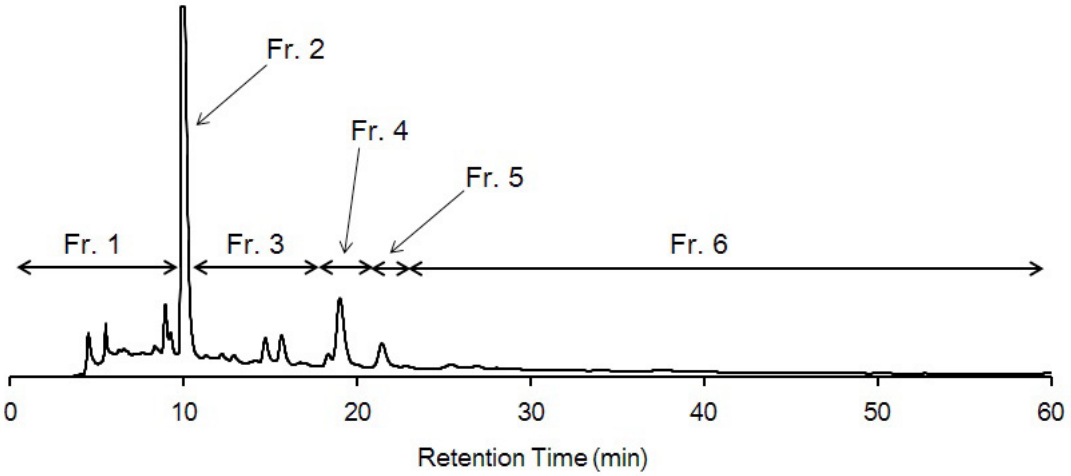


Fig. 2 - The chromatogram of ODS 20% MeOH fraction of the leaf extract. The SC_{50} of Fr. 1, 2, 3, and 6 were 32.4, 36.1, 62.4 and 22.4 mg f.w./mL, respectively. Fr. 4 and 5 was not determined since their SC_{50} were over 200 mg f.w./mL.

1 was present not only in the ODS 20% MeOH fraction but also in the ODS water fraction, which showed the second highest antioxidative activity among the ODS fractions. In the ODS water fraction, compound 1 was also found abundantly and accordingly was inferred to be the major compound in the water layer of the leaf extract.

Identification of compound 1

Compound 1 was found to have sixteen carbon atoms consisting of two methylene, eight methine, and six quaternary carbon atoms including two carbonyl groups (C_Q-7, δ 176.1 and C_C-1, δ 166.2) as a result of ^{13}C-NMR. This result was consistent with ^{1}H-NMR, which showed the presence of twelve hydrogen atoms in the spectrum. This compound contains a *trans*-form double bond (C_C-2, δ 115.2 and C_C-3, δ 144.5) signified by two hydrogen signals (δ 6.21 and δ 7.44) corresponding to a double bond where doublet with 16 Hz coupling constant. This double bond and a carbonyl group (C_C-1, δ176.1) were observed to be conjugated, consistent with these chemical shifts and the result from Heteronuclear Multiple Bond Correlation (HMBC). Because the observed six aromatic carbons in the ^{13}C-NMR spectrum corresponded to an ABX system at δ 6.75 (Hc-5′, d, J = 8 Hz), δ 6.93 (Hc-6′, dd, J = 2, 8 Hz), and δ 7.00 (Hc-2′, d, J = 2 Hz) in ^{1}H-NMR, compound 1 was found to contain a 1,2,4-trisubstituted benzene ring. For the abovementioned reasons, compound 1 was inferred to contain a caffeic acid moiety.

In the rest of the structure, three methine carbon atoms with oxygen atoms, one quaternary carbon, two methylene carbon atoms, and one carbonyl carbon were found. Two-dimensional NMR spectral data imply a six-membered ring substituted with four oxygen atoms. The methylene proton at δ 1.85 and the carbonyl carbon (C_Q-7, δ 176.1) were interrelated in HMBC spectroscopy. The other moiety was thus determined to be a quinic acid derivative.

The proton corresponding to the carbon of quinic acid (C_Q-3, δ 71.1) showed a downfield shift at 5.16 ppm, suggesting that this compound formed a caffeate ester. The molecular formula of a caffeoylquinic acid is $C_{16}H_{18}O_9$ and its molecular weight is calculated to be 354. Based on the ESI mass data (m/z 355 $[M+H]^+$), the molecular weight of compound 1 was found to be 354; therefore, compound 1 was assigned the molecular formula $C_{16}H_{18}O_9$. The data in the literature from ^{13}C-NMR and ^{1}H-NMR studies on chlorogenic acid (5-caffeoylquinic acid), cryptochlorogenic acid (4-caffeoylquinic acid) and neochlorogenic acid (3-caffeoylquinic acid) were compared with our observed data and most of the values for compound 1 matched with those of neochlorogenic acid (Fig. 3) (QIN *et al.*, 2006; HYUN *et al.*, 2010). The specific rotation value of compound 1 was also consistent with that of a neochlorogenic acid standard.

To determine the structure, fraction 2 was further analyzed using HPLC under the conditions described in *Determination of structure of compound 1*, and the result is shown in Fig. 4. The peak of compound 1 was observed at 8.1 min (Fig. 4a). The retention time of neochlorogenic acid was clearly different from that of chlorogenic acid; the neochlorogenic acid peak appeared at 8.04 min, whereas the peak of chlorogenic acid appeared at 16.67 min (Fig. 4b and 4c). Co-injection analysis showed that the peak of compound 1 was identical to that of neochlorogenic acid. Accordingly, compound 1 was assigned as neochlorogenic acid.

Quantification of neochlorogenic acid and its contribution to the whole leaf extract

The leaves of *P. cuspidatum* were freshly collected in Otoyo-cho in May 2014 to determine neochlorogenic acid content. One gram of fresh *P. cus-*

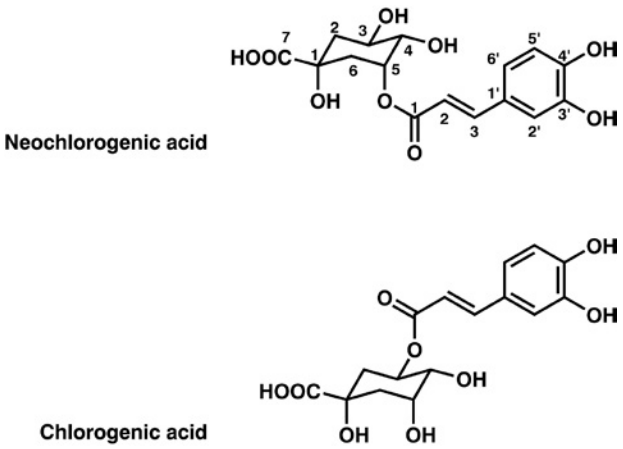


Fig. 3 - The structures of neochlorogenic acid and chlorogenic acid.

pidatum leaves contained 2.31 mg of neochlorogenic acid. By the Folin–Ciocalteu method, 17.9 mg GAE of phenolic compounds were found to be present in the fresh leaves; thus, neochlorogenic acid comprises 12.8% of the total polyphenol content. To evaluate the antioxidant capacity of neochlorogenic acid in *P. cuspidatum* two different assays, each measuring the sample's ability to quench reactive oxygen species in a different way, were performed. Antioxidant capacity cannot be evaluated by a single method because reactive oxygen species in the body do not always operate through the same mechanisms. The assays we used in this study were the DPPH radical scavenging (TEAC) and superoxide anion scavenging assays (SOSA). The TEAC values of the crude extract and neochlorogenic acid were 59.7 mg TE/g f.w. and 4.25 mg TE/mg, respectively, indicating the neochlorogenic acid contribution is 16.5%.

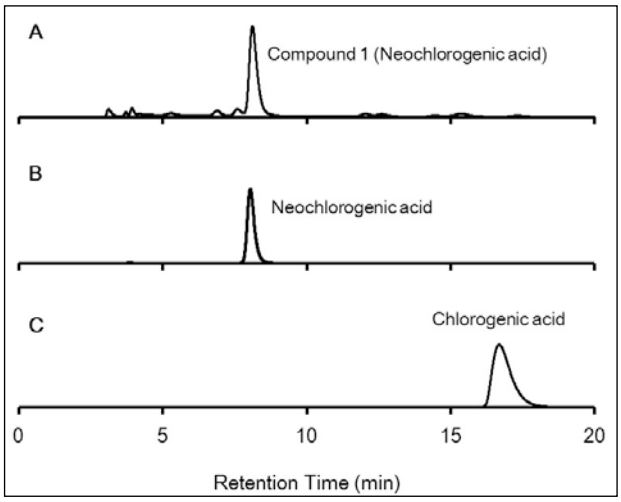


Fig. 4 - The chromatogram of the water layer of the leaf extract, neochlorogenic acid and chlorogenic acid.
In the water layer of leaf extract (A), compound 1 was observed at 8.11 min. Neochlorogenic acid (B) was found at 8.04 min whereas chlorogenic acid (C) was at 16.67 min.

However, by the superoxide anion scavenging assay, the SOSA values of the crude extract and neochlorogenic acid were 22.7 unit/g f.w. and 3.57 unit/mg, respectively, suggesting 36.5% of the antioxidant activity is by neochlorogenic acid. The disparate results may be explained by the different mechanisms of the two antioxidant activities (SHIMAMURA *et al.*, 2007). In the DPPH method, free radical scavenging activity is achieved by single electron transfer, and the assay simply measures the rate of free radical quenching. The superoxide anion scavenging assay, however, measures the sample's ability to scavenge superoxide anions produced by xanthine oxidase, thus evaluating the SOD-like activity of the sample. The superoxide anion further reduces 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) to produce formazan, which is detectable at a wavelength of 450 nm. Thus, the superoxide anion scavenging assay involves competition by the sample antioxidants with WST-1 in addition to the enzymatic reactions of xanthine oxidase. Taken together our results indicate that neochlorogenic acid in *P. cuspidatum* contributes a large part of its antioxidant activity, particularly as a superoxide anion scavenger.

In a study by Kirino *et al.* (2012) chlorogenic acid was reported as one of the major polyphenols in the leaves of *P. cuspidatum* (KIRINO *et al.*, 2012). The amount of chlorogenic acid was reported to be 0.36 mg/g of fresh leaves, which is only 1/6th of the neochlorogenic acid content observed in this study. In the chromatogram in Fig. 4, chlorogenic acid appeared to be a small peak in the water layer of the leaf extract. However, according to the data from Kirino *et al.*, the peak of chlorogenic acid was much more distinct than in our study. The contents of such antioxidants in *P. cuspidatum* may differ depending on its origin and harvest season, as we mentioned in a previous report (KURITA *et al.*, 2014). Stress factors such as sunlight and insects can influence antioxidant production levels as well.

Comparison of neochlorogenic acid contents in other food sources and its possible effects on human health

To the best of our knowledge, this is the first study to report the presence of neochlorogenic acid in *P. cuspidatum* leaves. Neochlorogenic acid is also found in Rosaceae fruits such as plums, cherries, and apples and Brassica vegetables such as broccoli and kale (BALLISTRERI *et al.*, 2013; KIM *et al.*, 2003; KAULMANN *et al.*, 2014). Among different kinds of sweet cherries, its content varied between 6.27–71.5 mg/100 g f.w. (BALLISTRERI *et al.*, 2013). Plums contain even higher amounts of up to 179 mg/100 g f.w., unsurprisingly neochlorogenic acid has been recognized as the predominant polyphenol in plums (KIM *et al.*, 2003). Brassica vegetables are also rich in the compound. Green vegetables such

as kale, broccoli, and Brussels sprouts contain 7.06, 5.61, and 4.59 mg/100 g f.w., respectively, of neochlorogenic acid (KAULMANN *et al.*, 2014). In comparison with these neochlorogenic-rich fruits and vegetables, the content was much higher in the leaves of *P. cuspidatum*, which yielded 231 mg of neochlorogenic acid per 100 g of fresh material. Our study suggests that the leaves of *P. cuspidatum* are a rich source of neochlorogenic acid.

Besides its antioxidant activity, neochlorogenic acid has been shown to exert health-promoting effects. As an antitumor agent, neochlorogenic acid has been found to suppress the growth of estrogen-independent MDA-MB-435 breast cancer cells (NORATTO *et al.*, 2009). This suppressive effect is selective for cancer cells and is more pronounced than that of chlorogenic acid. The compound has also been investigated in a weight-control study (SHIMODA *et al.*, 2006). In the study performed by Shimoda *et al.* (2006), experimental mice were fed a diet containing neochlorogenic acid (0.028% and 0.055%, respectively) extracted from green coffee beans for 6 days. The hepatic carnitine palmitoyltransferase activity of the experimental mice increased, indicating they had improved fat metabolism. These studies suggest that neochlorogenic acid could play a role in preventing chronic diseases and preserving healthy body weight when consumed in the diet. As a natural source of neochlorogenic acid, the leaves of *P. cuspidatum* may be used to improve human health in modern society.

CONCLUSIONS

For their medicinal effects the antioxidants in *P. cuspidatum* have been of interest to researchers, but other than the rhizomes the plant has not been extensively studied. The leaves possess high antioxidant activity and can be consumed in the diet as they currently are in Japan. Given the reports of health-promoting effects of neochlorogenic acid, our result that neochlorogenic acid is a main antioxidant in the leaves of *P. cuspidatum* may increase the utility of this hardy and prolific plant.

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PAPER

MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC ASPERGILLI-CONTAMINATED POULTRY AND ANIMAL FEEDSTUFF SAMPLES FROM THE WESTERN REGION OF SAUDI ARABIA

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ABSTRACT

The aflatoxigenic abilities of 64 and 17 isolates of *Aspergillus flavus* and *A. parasiticus* isolated from poultry and animal feedstuff samples collected from the western region of Saudi Arabia were studied. Thirty-three (51.6%) and 13 (76.5%) isolates of *A. flavus* and *A. parasiticus*, respectively, were aflatoxigenic. The ranges of aflatoxins in *A. flavus* and *A. parasiticus* isolates were 4.4-110 and 143.6-271.3 ppm (µg/g), respectively. *A. parasiticus* isolates generally produced a greater amount of aflatoxins than *A. flavus*. *A. flavus* isolates from poultry, cattle, and camel and cattle feeds produced aflatoxin amounts in the range 5.7-110, 4.4-19.0, and 7.0-28.5 ppm, respectively. From poultry feedstuff samples, *A. parasiticus* produced aflatoxins in the range 212.5-232.4 ppm. Some aflatoxin biosynthesis genes (*aflR*, *omt-1*, *ver-1*, and *nor-1*) were detected with variable frequencies in all *A. flavus* and *A. parasiticus* isolates. The genetic diversity among 64 isolates of *A. flavus* using internal transcribed spacer sequence results and the amplification of some aflatoxin biosynthesis genes revealed that the investigated isolates showed high heterogeneity.

- Keywords: *Aspergillus flavus,* Aflatoxin genes, Flourometer, molecular markers, genetic diversity -

INTRODUCTION

Aflatoxin contamination of agricultural commodities has gained global significance as a result of their deleterious effects on human and animal health as well as their importance in international trade. The contamination of foods by aflatoxigenic fungi, particularly in tropical countries, may occur during pre-harvesting, processing, transportation, and storage (ELLIS *et al.*, 1991; MANONMANI *et al.*, 2005). Regular monitoring of toxigenic mycobiota in agricultural-based feeds and foods is an essential pre-requisite in the development of strategies to control or prevent mycotoxin exposure of feed animals and human population. Studies on the prevalence of toxigenic mycobiota of animal/poultry feeds have been regularly and frequently reported, including studies from Brazil (OLIVEIRA *et al.*, 2006; ROSA *et al.*, 2006), Argentina (DALCERO *et al.*, 1997), Nigeria (OSHO *et al.*, 2007), Spain (ACCENSI *et al.*, 2004), and Pakistan (SALEEMI *et al.*, 2010).

The polymerase chain reaction (PCR) first described by SAKI *et al.* (1985) requires the presence of specific target sequences. When genes involved in the biosynthetic pathway are known, they represent a valuable target for the specific detection of toxigenic fungi. The first researchers to use this approach for the detection of toxigenic fungi were GEISEN *et al.* (1996) and SHAPIRO *et al.* (1996), describing a diagnostic PCR directed against DNA sequences in the aflatoxin biosynthetic gene cluster. However, when the genes responsible for mycotoxin production are unknown, other sequences can function as a target. Examples are rDNA sequences, genes, or anonymous DNA marker sequences. GEISEN (1998) and EDWARDS *et al.* (2002) reviewed available diagnostic PCRs for toxigenic fungi. The advantages of the PCR-based approach for the detection of toxigenic fungi compared with those of the classical mycological or chemical analysis is mainly the time aspect. For the chemical analysis of mycotoxins in food, elaborated protocols for sample preparation and expensive laboratory equipment are necessary. Classical mycological analysis requires the isolation and cultivation of the fungi on different media and at least one week of growth for their reliable identification. In addition, much expertise is required to recognize the species, particularly for the main genera of toxigenic fungi *Fusarium*, *Penicillium*, and *Aspergillus*. In contrast, DNA extraction from food samples and raw materials of food can be performed in a few minutes (KNOLL *et al.*, 2002a). Further, the use of modern thermocyclers can reduce analysis time to less than 1 h (KNOLL *et al.*, 2002b).

The aflatoxin biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region (YU *et al.*, 2004). *A. flavus*, *A. parasiticus*, and other *Aspergillus* section *Flavi* species share nearly identical sequences and conserved gene order in the cluster. In recent years, PCR detection of aflatoxin biosynthetic gene presence or expression has been used as a diagnostic tool for aflatoxigenic fungi in selected food commodities (GEISEN, 2007; GALLO *et al.*, 2012).

Aflatoxins are regarded as potent hepatocarcinogens and immunosuppressants, and there are reports showing that this group of mycotoxins poses the biggest threat to the poultry and livestock industry through low productivity and death (VAN EGMOND, 1989; CHUKWUKA *et al.*, 2010; PEDROSA and BORUTOVA, 2011). Therefore, the potential risks of aflatoxicosis in Saudi poultry and livestock must be clearly evaluated in order to ensure prompt legislative action and mitigation of aflatoxin contamination in feed. This study was designed to determine and evaluate the aflatoxin-producing potentials of *Aspergillus* section *Flavi* isolated from poultry and animal feedstuff samples collected from the western region of Saudi Arabia. Furthermore, the isolates were tested for the presence of four of the characterized aflatoxin biosynthetic genes in their genome in relation to aflatoxin production.

MATERIALS AND METHODS

Samples

Sixty-four *A. flavus* and 17 *A. parasiticus* isolates were used throughout this investigation. These isolates were retrieved from poultry and animal feedstuff samples collected from the western region of Saudi Arabia (Taif, Makkah, and Jeddah). The isolates were identified according to their morphological features as well as sequence results of internal transcribed spacer (ITS) regions. The sequence results were deposited in the GenBank.

Determination of total aflatoxin abilities of *Aspergillus* species isolates

The aflatoxin-producing abilities of the isolates were determined by cultivating the fungal strains in Czapek Yeast extract agar (BEN FREDJ *et al.*, 2009) medium for 5 days at 25±2°C. Total aflatoxins were extracted by grinding the moldy agar (20 g) in a Waring blender for 5 min with methanol (100 mL) containing 0.5% NaCl. The mixture was then filtered through a fluted filter paper (Whatman 2V, Whatmanplc, Middlesex, UK), and the filtrate was diluted (1:4) with water and re-filtered through a glass-fiber filter paper. Two milliliters of the glass-fiber filtrate were placed on AflaTest® WB SR Column (VICAM, Watertown, MA, USA) and allowed to elute at 1-2 drops/s. The columns were washed twice with 5 mL of water, and aflatoxin was eluted from

the column with 1 mL high performance liquid chromatography (HPLC)-grade methanol. A bromine developer (1 mL) was added to the methanol extract, and the total aflatoxin concentration was read in a recalibrated VICAMSeries-4 fluorometer set at 360 nm excitation and 450 nm emissions (LEWIS *et al.*, 2005).

Molecular detection of aflatoxin biosynthetic genes in Aflatoxigenic species of aspergilli

The isolation of DNA from mycelia was performed according to the method described by FARBER *et al.* (1997). Four published primer sets were used for the specific detection of *nor-1*, *ver-1*, *omt-A*, and *aflR* genes (CRISEO *et al.*, 2008). The 400, 537, 797, and 1032-bp fragments were amplified, respectively. A typical PCR was carried out under the following conditions: 5 μL of genomic DNA were used as a template (2 μg/mL), 0.5 U EuroTaq polymerase (Euroclone, Pero-Milan, Italy), 1 × reaction buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 7.5 pmol of each primer, in a total reaction volume of 50 μL. A total of 35 PCR cycles with the following temperature regimen were performed: 95°C, 1 min; 65°C, 30 s; 72°C, 30 s for the first cycle; and 94°C, 30 s; 65°C, 30 s; 72°C, 30 s for the 34 remaining cycles (CRISEO *et al.*, 2008). PCR products were separated on a 1.3% (wt/vol) agarose gel stained with ethidium bromide.

Statistical analysis of frequency of aflatoxin biosynthetic genes

Cluster analysis of data was performed by hierarchical cluster analysis (SPSS Software, SPSS Inc., USA; Norusis, 1993).

RESULTS AND DISCUSSION

Total aflatoxin potentials of *Aspergillus* species isolates

Thirty-three out of 64 (51.6%) and 13 out of 17 (76.5%) of *A. flavus* and *A. parasiticus* isolates were aflatoxigenic producers, respectively. The aflatoxin range in *A. flavus* and *A. parasiticus* isolates was 4.4-110 and 143.6-271.3 ppm (μg/g), respectively (Tables 1 and 2). DUTTA and DAS (2001) carried out a groundwork study, in which 256 feed samples collected from different parts of Northern India were analyzed for aflatoxigenic strains of *A. flavus/parasiticus* and for detection of AFB1. Out of 198 *A. flavus* and 15 *A. parasiticus* strains isolated, 76% and 86%, respectively, were found to be toxigenic. RAZZAGHI-ABYANEH *et al.* (2006) surveyed the distribution of *Aspergillus* section *Flavi* in cornfield soils in Iran and their results indicated that only 27.5% of *A. flavus* isolates were aflatoxigenic (B1 or B2 or both), and all the *A. parasiticus* isolates produced aflatoxins of both B (B1 and B2) and G (G1 and G2) types. PITT (1993) also, reported that *A. flavus* isolates produced B1 and B2 or both types, while *A. parasiticus* produced the four aflatoxin types. These results support the present findings indicating that the level of aflatoxin production by *A. parasiticus* was higher than that by *A. flavus* isolates (Tables 1 and 2). Further, KOEHLER *et al.* (1975) reported that *A. parasiticus* isolates generally produced a greater amount of aflatoxins than *A. flavus*.

The range of aflatoxin production by *A. flavus* isolated from poultry, cattle, and camel and cattle was 5.7-110, 4.4-9.0, and 7.0-28.5 ppm, respectively. From poultry feedstuff samples, *A. parasiticus* produced aflatoxins in the range 212.5-232.4 ppm. In Pakistan, SALEEMI *et al.* (2010) studied the mycoflora of poultry feed and mycotoxin-producing potential of *Aspergillus* species. They reported that the toxigenic fungi content among *Aspergillus* isolates was 73.58%, and that of aflatoxigenic isolates of *A. flavus* and *A. parasiticus* was 83.33% and 85.71%, respectively. Further, they recorded that, among toxigenic *A. flavus* isolates (10/12), six produced four aflatoxins (AFB1, AFB2, AFG1, and AFG2), two produced AFB1, AFB2, and AFG1, one produced AFB1, AFB2, and AFG2, and one produced AFB1 and AFB2. Among aflatoxigenic isolates of *A. parasiticus* (6/7), five produced four aflatoxins (AFB1, AFB2, AFG1, and AFG2) while one produced three (AFB1, AFB2, and AFG1).

The production range of aflatoxins from four isolates (TUH212, 221, 222, and 225) of *A. parasiticus* retrieved from cattle feed samples was 143.6-271.3 ppm. Further, two isolates (TUHT216 and 220) of *A. parasiticus* isolated from cattle and camel feed samples contained 195.5 and 211.2 ppm of aflatoxins (Table 1). Among isolates of *A. flavus* collected from Taif samples, TUHT53 showed the lowest aflatoxin potential (7.0 ppm) and TUHT44 showed the highest (106.8 ppm). Isolates TUHT185 and TUHT180 from feed samples collected from Jeddah showed the lowest (5.7 ppm) and highest (33.0 ppm) levels of aflatoxins, respectively (Table 1). For *A. flavus* isolates retrieved from feed samples collected from Makkah, TUHT117 and TUHT121 showed the lowest (5.0 ppm) and the highest (110 ppm) aflatoxin levels, respectively. The results shown in Table 2 indicated that, from *A. parasiticus* isolates, the lowest aflatoxin producer was TUHT212 (143.6 ppm), while the highest production was recorded in isolate TUHT222 (269.5 ppm). Data from different geographic areas demonstrated a great variability in the mycotoxin-producing potential of *A. flavus* and closely related species (HORN and DORNER, 1999). These results are in accordance with previous reports showing that these two species have the ability to produce both B and G aflatoxins (PITT and HOCKING, 1997; KUMEDA *et al.*, 2003; GHIASIAN *et al.*, 2004). In Al-

Table 1 - Total aflatoxins (PPM) and aflatoxigenic genes detected in 64 strains of *Aspergillus flavus* isolates collected from feedstuff samples.

Strains code	Source of isolation	Location	Total AFs (PPM)	aflR	omt-A	ver-1	nor-1
TUHT43	Poultry	Taif	N.D.	+	+	+	+
TUHT44	Poultry	Taif	106.8	+	+	+	+
TUHT46	Poultry	Taif	7.8	+	+	+	+
TUHT47	Poultry	Taif	N.D.	+	+	+	+
TUHT53	Poultry	Taif	7.0	+	+	+	+
TUHT59	Poultry	Taif	N.D.	+	-	+	+
TUHT63	Poultry	Taif	N.D.	-	+	+	+
TUHT84	Poultry	Taif	N.D.	-	-	-	+
TUHT85	Poultry	Taif	N.D.	+	-	-	-
TUHT86	Poultry	Taif	11.0	+	+	+	+
TUHT87	Poultry	Taif	N.D.	+	+	-	+
TUHT89	Cattle	Taif	N.D.	-	-	-	-
TUHT91	Cattle	Taif	10.0	+	+	+	+
TUHT92	Camel & cattle	Taif	N.D.	+	+	-	-
TUHT93	Poultry	Taif	16.0	+	+	+	+
TUHT94	Poultry	Taif	28.4	+	+	+	+
TUHT98	Camel & cattle	Taif	N.D.	+	-	-	-
TUHT99	Cattle	Taif	19.0	+	+	+	+
TUHT100	Poultry	Makkah	N.D.	-	+	-	-
TUHT104	Poultry	Makkah	N.D.	+	+	-	+
TUHT106	Poultry	Makkah	N.D.	-	+	-	+
TUHT107	Poultry	Makkah	8.9	+	+	+	+
TUHT108	Poultry	Makkah	N.D.	-	+	-	+
TUHT109	Poultry	Makkah	20.0	+	+	+	+
TUHT110	Poultry	Makkah	12.0	+	+	+	+
TUHT111	Cattle	Makkah	13.0	+	+	+	+
TUHT115	Camel & cattle	Makkah	N.D.	+	+	-	+
TUHT116	Cattle	Makkah	19.0	+	+	+	+
TUHT117	Cattle	Makkah	4.4	+	+	+	+
TUHT118	Cattle	Makkah	15.0	+	+	+	+
TUHT119	Camel & cattle	Makkah	N.D.	-	+	+	-
TUHT120	Camel & cattle	Makkah	28.5	+	+	+	+
TUHT121	Poultry	Makkah	110.0	+	+	+	+
TUHT123	Poultry	Makkah	N.D.	+	+	-	-
TUHT124	Poultry	Makkah	6.6	+	+	+	+
TUHT126	Poultry	Makkah	13.2	+	+	+	+
TUHT152	Poultry	Makkah	N.D.	+	-	-	-
TUHT154	Poultry	Makkah	8.5	+	+	+	+
TUHT155	Poultry	Makkah	N.D.	+	-	-	+
TUHT156	Poultry	Makkah	12.6	+	+	+	+
TUHT157	Cattle	Makkah	N.D.	+	-	-	-
TUHT158	Cattle	Makkah	7.3	+	+	+	+
TUHT160	Poultry	Jeddah	N.D.	-	+	-	+
TUHT112	Horses	Jeddah	N.D.	+	+	-	+
TUHT161	Poultry	Jeddah	N.D.	+	+	+	+
TUHT163	Poultry	Jeddah	N.D.	+	+	-	-
TUHT164	Poultry	Jeddah	N.D.	+	-	+	-
TUHT165	Camel & cattle	Jeddah	7.0	+	+	+	+
TUHT166	Camel & cattle	Jeddah	N.D.	-	+	-	+
TUHT168	Horses	Jeddah	6.1	+	+	+	+
TUHT172	Camel & cattle	Jeddah	N.D.	+	+	-	-
TUHT173	Camel & cattle	Jeddah	N.D.	+	+	+	+
TUHT174	Camel & cattle	Jeddah	7.5	+	+	+	+
TUHT176	Cattle	Jeddah	N.D.	-	+	+	-
TUHT177	Cattle	Jeddah	14.0	+	+	+	+
TUHT180	Poultry	Jeddah	33.0	+	+	+	+
TUHT181	Poultry	Jeddah	21.0	+	+	+	+
TUHT185	Poultry	Jeddah	5.7	+	+	+	+
TUHT186	Camel & cattle	Jeddah	8.0	+	+	+	+
TUHT187	Camel & cattle	Jeddah	N.D.	-	-	-	+
TUHT188	Camel & cattle	Jeddah	18.2	+	+	+	+
TUHT189	Camel & cattle	Jeddah	9.13	+	+	+	+
TUHT190	Camel & cattle	Jeddah	N.D.	+	-	-	+
TUHT193	Camel & cattle	Jeddah	14.3	+	+	+	+
Total			33	53	53	42	51

Table 2 - Total aflatoxins (PPM) and aflatoxigenic genes detected in *Aspergillus parasiticus* isolates collected from feedstuff samples.

Strains code	Source of isolation	Location	Total	aflR	omt-A	ver-1	nor-1
AFs (PPM)							
TUHT226	Cattle	Taif	N.D.	+	+	-	+
TUHT227	Cattle	Taif	N.D.	+	+	+	+
TUHT228	Cattle	Jeddah	N.D.	-	+	-	+
TUHT229	Poultry	Jeddah	N.D.	+	+	+	-
TUHT26	Poultry	Taif	212.7	+	+	+	+
TUHT211	Poultry	Taif	232.4	+	+	+	+
TUHT212	Cattle	Makkah	143.6	+	+	+	+
TUHT213	Poultry	Makkah	231.2	+	+	+	+
TUHT214	Poultry	Makkah	224.4	+	+	+	+
TUHT215	Poultry	Jeddah	212.5	+	+	+	+
TUHT216	Camel & cattle	Jeddah	195.4	+	+	+	+
TUHT219	Cattle	Taif	265.5	+	+	+	+
TUHT220	Camel & cattle	Taif	211.2	+	+	+	+
TUHT221	Cattle	Makkah	271.3	+	+	+	+
TUHT222	Cattle	Makkah	269.5	+	+	+	+
TUHT223	Poultry	Jeddah	227.2	+	+	+	+
TUHT225	Cattle	Jeddah	269.7	+	+	+	+
Total (17 Isolates)			**13**	**16**	**17**	**15**	**16**

geria, RIBA *et al.* (2010) determined the aflatoxin-producing capacity of 150 *A. flavus* isolates collected from wheat and its derivatives in 2004 and 2006, and the results showed that 72% of the strains produced aflatoxins. These strains produced amounts of AFB1 in the range 12.1-234.6 µg/g of CYA medium.

The results of the present study indicate that the aflatoxigenic species of *Aspergillus* vary in their aflatoxin potential according to the substrate and environmental factors. These results are in agreement with those reported by ABBAS *et al.* (2005).

Detection of some of aflatoxin biosynthesis genes in *Aspergillus* species

The production of aflatoxin involves a complex biosynthetic pathway consisting of at least 25 genes (YABE *et al.*, 1999; CRISEO *et al.*, 2001a, BHATNAGAR *et al.*, 2003; YU *et al.*, 2004; SCHERM *et al.*, 2005). All of the identified biosynthesis-related genes are located within a 75 kb DNA region in both *A. parasiticus* and *A. flavus*, and their relative positions in the cluster of both fungal species are similar (YU *et al.*, 2000; EHRLICH *et al.*, 2005). PCR was used for the detection of aflatoxigenic aspergilli based on the intermediated enzymes, including norsolorinic acid reductase encoding gene *nor-1*, the versicolorina dehydrogenase encoding gene *ver-1*, the sterigmatocystin 0-methyl transferase encoding gene *omt-1*, and the regulatory gene *aflR* (ERAMI *et al.*, 2009).

Representative aflatoxigenic and non-aflatoxigenic *A. flavus* and *A. parasiticus* isolates were subjected for detection of aflatoxin biosynthesis genes.

Detection of aflatoxin biosynthesis genes in *A. flavus* isolates

PCR was applied using four sets of primers for different genes involved in the aflatoxin biosynthetic pathway. Bands of fragments of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes were visualized at 1032 bp, 797 bp, 537 bp, and 400 bp, respectively (Fig. 1). All examined *A. flavus* isolates yielded different DNA banding patterns with a number of bands ranging from zero to four (Tables 1 and 2).

Table 1 outlines the total aflatoxin and aflatoxigenic genes (*aflR*, *omt-A*, *ver-1*, and *nor-1*) detected in 64 strains of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates collected from feedstuff samples. *A. flavus* isolates were represented by 35 isolates from poultry feed samples, 16 from camel and cattle feed, 11 from cattle feed, and two from horse feed. Thirty-eight out of 64 (59.4%) *A. flavus* isolates contained all four aflatoxin biosynthesis genes; among them 21 isolates were retrieved from poultry feedstuff samples, eight from camel and cattle feed, eight from cattle feed, and one from horse feed (Table 1). This result is in agreement with CRISEO *et al.* (2001a), who used specific PCR-based methods to prove that aflatoxigenic *A. flavus* isolates always contain the complete gene set.

Among the 38 isolates tht showed the presence of all four targeted genes, two isolates (TUHT43 and 47) were not aflatoxigenic. Therefore, this result indicated clearly that the presence of the four tested genes is not a sufficient marker for the differentiation between aflatoxigenic and non-aflatoxigenic isolates. Other studies (FLAHERTY and PAYNE, 1997; CHANG *et al.*, 1999a,b; 2000, CARY *et al.*, 2002; TAKAHSHI *et*

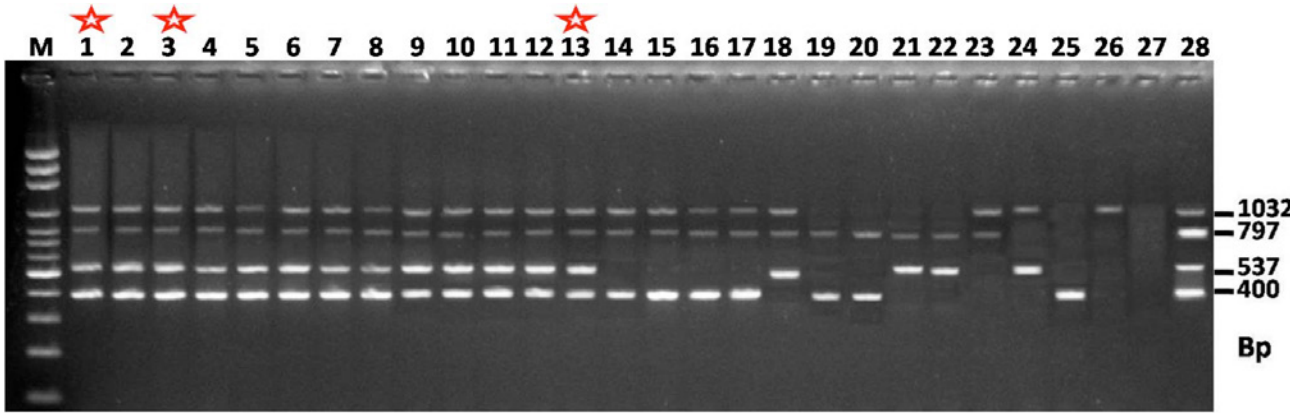


Fig. 1 - Aflatoxin biosynthesis genes amplifications. Lanes 1 - 7, *Aspegillus flavus* (TUHT 43, 44, 47, 91, 168, 188 and 219); Lanes 8 - 13, *A. parasiticus* (TUHT 26, 212, 216, 221, 223 and 227); lanes 14 -16, *A. flavus* (TUHT 87, 104, 115), lane 17 *A. parasiticus* (TUHT 226), lane 18 *A. parasiticus* (TUH 229); Lane 19, *A. flavus* (TUHT 160); Lane 20, *A. parasiticus* (TUHT 228); Lane 21 & 22, *A. flavus* (TUHT 119 & 176); Lane 23, *A. flavus* (TUHT 163); Lanes 24; *A. flavus* (TUHT 164); lane 25 *A. flavus* (TUHT84); lane 26 *A. flavus* (TUHT157); lane, 27 negative control and lane 28, positive control.
Asterisked lanes were non aflatoxigenic isolates.

al., 2002; EHRLICH *et al.*, 2003) have suggested that regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive and negative acting transcriptional regulatory factors affected by environmental and nutritional parameters. Furthermore, the lack of aflatoxin production apparently does not need to be related only to an incomplete pattern obtained in PCR-based detection. Different mutations may be responsible for the inactivation of aflatoxin biosynthetic pathway genes in other *A. flavus* strains (GEISEN, 1996).

Six isolates (9.4% of the tested isolates) with three gene amplicons were not aflatoxigenic (Table 3). From these, four, one, and one isolates were retrieved from poultry, camel and cattle, and horse feeds, respectively. Twelve isolates (18.8% of the tested isolates; six from poultry, five from camel and cattle, and one from cattle), contained two gene amplicons and seven isolates (10.9%) contained one gene amplicon (Table 3). On the other hand, one non-aflatoxigenic isolate (TUHT89) showed no bands, indicating a deletion of the targeted genes in this isolate. CRISEO *et al.* (2001a) proved that non-aflatoxigenic isolates of *A. flavus* were lacking one, two, three, or four PCR products, indicating that the genes do not exist in these strains or that the primer binding sites changed. Further, CRISEO *et al.* (2001b) reported that aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by the four genes *aflR*, *nor-1*, *ver-1*, and *omt-A*. GHERBAWY *et al.* (2012) reported on the presence of a complete set of these genes in seven aflatoxigenic isolates of *A. flavus* retrieved from date palm.

The frequencies of the four aflatoxin biosynthesis genes *aflR*, *omt-A*, *ver-1*, and *nor-1*, in the tested isolates were 53, 53, 42, and 51, respectively (Table 1). CRISEO *et al.* (2008) used 134 of non-aflatoxin producing strains of *A. flavus* isolated from food, feed, and officinal plants to study the different genes involved in the aflatoxin biosynthetic pathway. Their results indicated that the *nor-1* gene was the most representative (88%) of the four aflatoxin structural assayed genes, followed by *ver-1* and *omt-A*, which were found at the same frequency (70.1%). A lower incidence (61.9%) was observed for *aflR*. Further, CRISEO *et al.* (2008) demonstrated that a high number of aflatoxin non-producing strains (61.9%) contain the *aflR* gene. This could impair the use of *aflR* to identify aflatoxigenic aspergilli. Five out of ten *A. flavus* isolates were not aflatoxin producers (SCHERM *et al.*, 2005),

Table 3 - Origin and genetic patterns of 64 aflatoxigenic *Aspergillus flavus* isolates collected from feedstuff samples in this study. Values in brackets are percentages of the total samples analyzed.

Sample name	No isolates	Complete set	Three bands	Two bands	One band	Zero band
Poultry	35	21	4	6	4	-
Camel & cattle	16	8	1	5	2	-
Cattle	11	8	-	1	1	1
Horses	2	1	1	-	-	-
Total	**64 (100)**	**38 (59.4)**	**6 (9.4)**	**12 (18.8)**	**7 (10.9)**	**1 (1.6)**

indicating that the frequencies of occurrence of *aflR* and *omt-A*, *ver-1*, and *nor-1* genes were 8, 5, 9, and 5, respectively.

Detection of some of aflatoxin biosynthesis genes in *A. parasiticus* isolates

Seventeen *A. parasiticus* isolates collected from different feedstuff samples from various cities in Saudi Arabia were examined for the presence of aflatoxin biosynthesis genes using a specific primer set as mentioned above. The results indicated the presence of four bands for *aflR*, *omt-1*, *ver-1*, and *nor-1* genes at 1032 bp, 797 bp, 537 bp, and 400 bp, respectively (Fig. 1). All aflatoxigenic and non-aflatoxigenic isolates examined yielded different DNA banding patterns with the number of bands ranging from 2 to 4 (Tables 2 and 4).

Table 2 shows the total aflatoxin and aflatoxigenic genes detected in *A. parasiticus* isolates collected from three different feedstuff samples (poultry, camel and cattle, and cattle). Thirteen out of 17 *A. parasiticus* isolates were aflatoxigenic. The frequencies of occurrence of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes in *A. parasiticus* isolates were 16 (94.1%), 17 (100%), 15 (88.2%), and 16 (94.1%), respectively. Gherbawy *et al.* (2014) reported that *omt-A* was the most prevalent gene in *A. flavus* and *A. parasiticus* isolated from chili samples collected from Taif city (Saudi Arabia). Further, their results indicated that this gene was recovered from 27 out of 30 *A. flavus* isolates and two isolates of *A. parasiticus*, while *nor-1, aflR*, and *ver-1* genes were recovered from 25, 26, and 24 isolates of aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. Out of seven *A. parasiticus* isolates collected from poultry feedstuff samples, 6 (85.7%) contained four genes, while two (14.3%) showed the amplicons of three genes. The two *A. parasiticus* isolates collected from camel and cattle feedstuff samples showed a complete set of the targeted genes (Tables 2 and 4). Amplification of the four targeted genes in eight *A. parasiticus* isolates collected from cattle feedstuff samples showed that six (75%) had the four genes and one (12.5%) contained three genes (Tables 2 and 4). Further, one isolate contained two genes. GEISEN (1996) reported the presence of the abovementioned genes from two isolates of *A. parasiticus*. Additionally, SCHERM *et al.* (2005) indicated the presence of a complete set of genes (*aflR*, *omt-1*, *ver-1*, and *nor-1* genes) in three isolates of *A. parasiticus*.

The findings herein showed the presence of four targeted genes in all aflatoxigenic isolates of *A. parasiticus* and in one (TUHT229) non-aflatoxigenic isolate. Further, all non-aflatoxigenic isolates were missing one or more of the targeted genes. RASHID *et al.* (2008) studied the presence of *aflR*, *omt-1*, *ver-1*, and *nor-1* genes in 35 *A. parasiticus* isolates from stored wheat grains in Pakistan. Their results revealed that only one isolate showed the complete set of genes. Additionally, *omt-1*, *ver-1*, and *nor-1* genes appeared in 8, 10, and 13 isolates. Deletion of *aflR* in *A. parasiticus* abolishes the expression of other aflatoxin pathway genes (CARY *et al.*, 2000). Finally, the regulation of aflatoxin biosynthesis genes in *Aspergillus* spp. is affected by environmental and nutritional parameters (FLAHERTY and PAYNE, 1997; CHANG *et al.*, 2000; CARY *et al.*, 2002; TAKAHSHI *et al.*, 2002; EHRLICH *et al.*, 2003).

Genetic diversity among *A. flavus* strains isolated from feedstuff samples

Sixty-four aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* represented different sources of isolation and different locations were used in this part. Using the ITS region of rRNA sequencing results and amplification of some aflatoxin biosynthesis genes, the genetic diversity among those strains was studied.

Using ITS sequencing results of 64 isolates of *A. flavus*, a neighbor joining tree was constructed (Fig. 2). The population of *A. flavus* split into several clades and sub-clades; the bootstrap values for these clades and sub-clades ranged from 1 to 100, indicating a high heterogeneity in this population. Further, the clustering system did not correlate with the type of sample or its location. For example, *A. flavus* isolate TUTH157 (isolated from cattle feedstuff sample collected from Makkah) clustered together with isolate TUTH63 (isolated from poultry feedstuff sample collected from Taif) in one sub-clade with a 98 bootstrap value. Additionally, isolates TUTH154 and TUTH193 constituted one sub-clad with a 69 bootstrap value, although the first one was

Table 4 - Origin and genetic patterns aflatoxigenic *Aspergillus parasiticus* isolates collected from feedstuff samples. Values in brackets are percentages of the total samples analyzed.

Sample name	No isolates	Complete set	Three bands	Two bands
Poultry	7	6	1	-
Camel & cattle	2	2	-	-
Cattle	8	6	1	1
Total	**17 (100)**	**14 (82.4)**	**2 (11.8)**	**1 (5.9)**

isolated from poultry feedstuff samples from Makkah and the second from cattle and camel feedstuff samples from Jeddah (Fig. 2 and Table 1). Therefore, clustering according to the ITS sequencing results did not indicate any relationship among the isolate clustering system and their geographical distributions and even the sources of isolation. Aflatoxigenic isolates spread all over the constricted phylogentic tree without separation of the clades into toxigen-

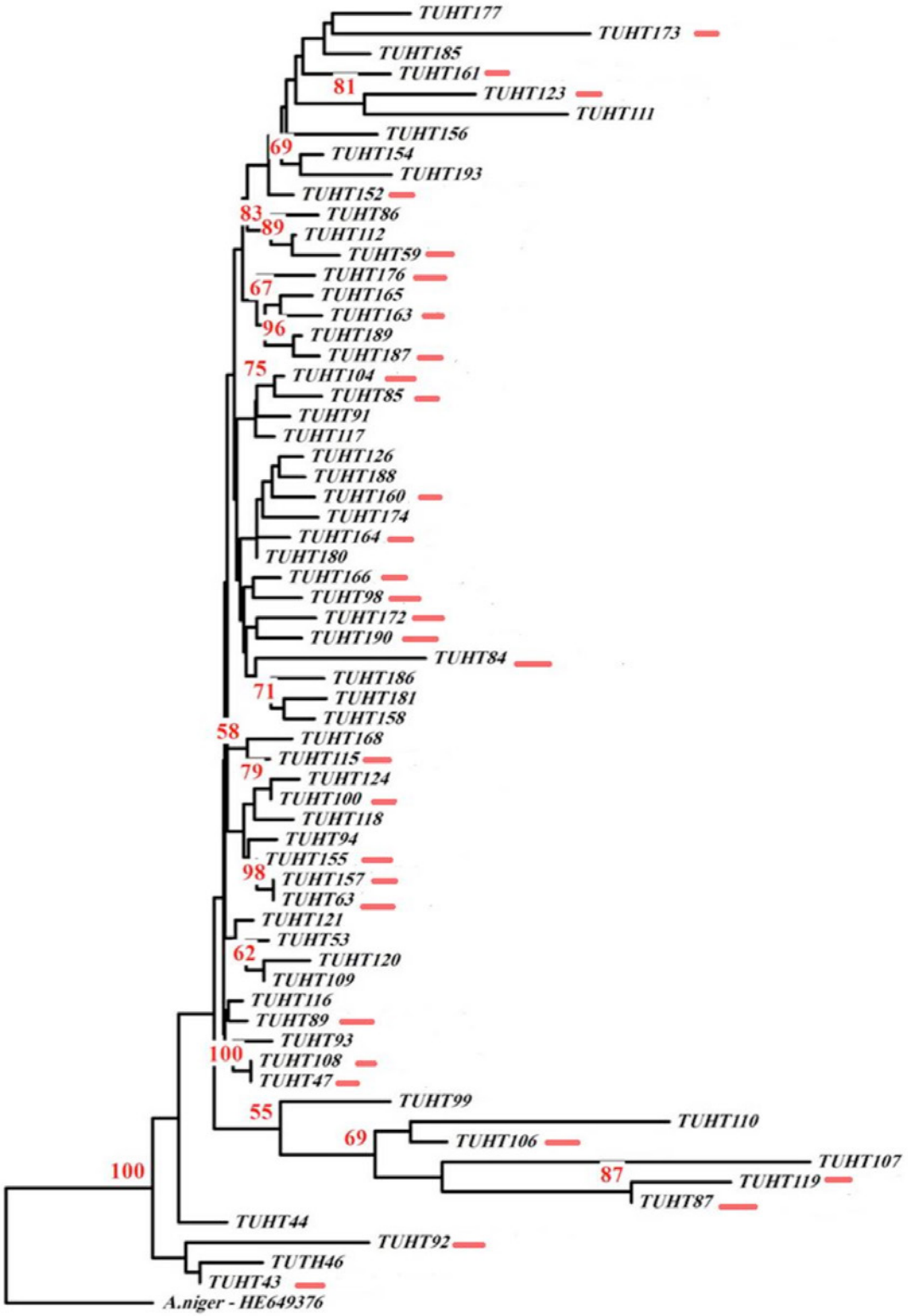


Fig. 2 - Phylogenetic tree based on the internal transcribed spacer (ITS) region of rRNA of aflatoxigenic and non aflatoxigenic 64 isolates of *Aspergillus flavus*. The tree was constructed by neighbor-joining algorithm using maximum composite likelihood model. Bootstrap factors less than 55 were not shown. The tree was rooted with *Aspergillus niger* [HE649376] as the out-group. Red rods indicated non aflatoxigenic isolates.

ic and non-toxigenic. For example, aflatoxigenic isolate TUHT189 clustered with non-aflatoxigenic isolate TUHT187 with a 96 bootstrap value (Fig. 2). Since the clustering system was based on ITS sequencing results, with non-functional spacers, there is no correlation between clustering system and toxin production. The present results show that isolates identified as *A. flavus* had a polyphyletic origin, supporting the genetic heterogeneity of *A. flavus* as previously dem-

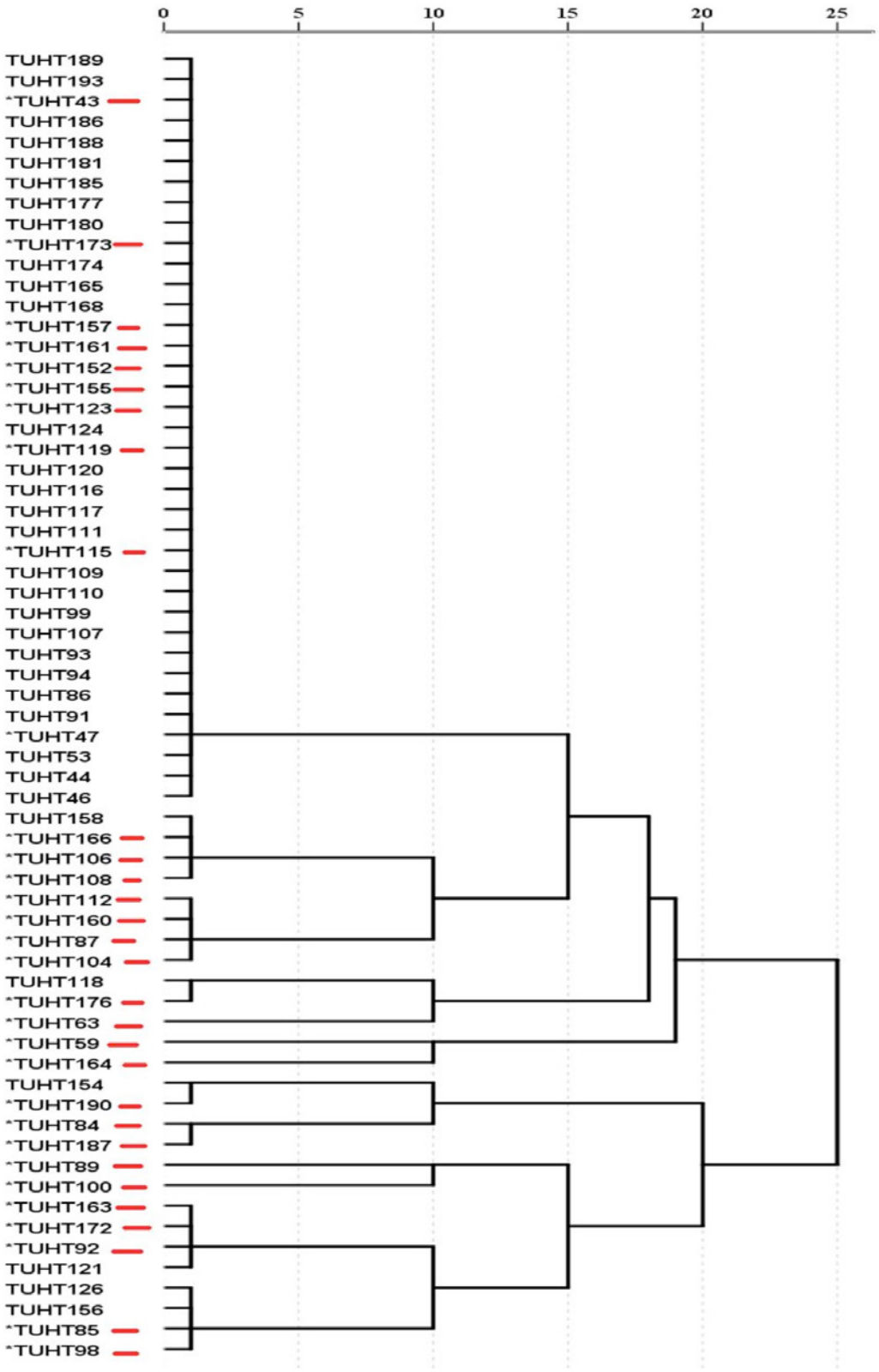


Fig. 3 - The hierarchical cluster analysis using average linkage between groups form *Aspergillus flavus* isolates based on amplification of aflatoxins biosynthesis genes. Red bars indicated non aflatoxigenic species.

onstrated by other studies (GEISER *et al.*, 1998, 2000; VAN DEN BROEK *et al.*, 2001; CHANG *et al.*, 2007; GONCALVES *et al.*, 2012).

The genetic diversity among *A. flavus* isolates was studied using the results of amplification of some aflatoxin biosynthesis genes. The results were subjected to hierarchical cluster analysis using average linkage between groups to construct a dendrogram showing the correlation between the isolates (Fig. 3). *A. flavus* isolates did not follow any rule in their clustering system. For example, aflatoxigenic isolates TUHT121 and TUHT126 (isolated from a poultry feedstuff sample collected from Makkah) and non-aflatoxigenic isolates TUHT85 (poultry feedstuff samples from Taif) and TUHT98 (camel and cattle feeds from Taif) were clustered together as shown in Fig. 3. On the contrary, TUHT112 (horse feeds from Jeddah), TUHT160 (poultry feeds from Jeddah), TUHT87 (poultry feeds from Taif), and TUHT104 (poultry feeds from Makkah) were non-aflatoxigenic isolates clustered together (Fig. 3). Generally, these results indicate that the presence or absence of PCR products for the targeted aflatoxin biosynthesis genes was not correlated with the type of feedstuff or the location of sample collection. Previous authors (GEISER *et al.*, 1998, 2000; MOORE *et al.*, 2009, GONCALVES *et al.*, 2012), found that the aflatoxin cluster genes were useful tools for phylogenetic studies in the section *Flavi*.

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PAPER

NUTRITIONAL EVALUATION OF WILD PLANT *CISSUS ROTUNDIFOLIA*

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ABSTRACT

This study aimed to evaluate the nutritional and antinutritional components of *Cissus rotundifolia* leaves. They contain an appreciable amount of protein (12.5%$_{db}$), fat (7.45%$_{db}$), crude fiber (8.34 %$_{db}$) and minerals (16.32%$_{db}$). The protein fraction contains a relatively high level of essential amino acids, which accounted for 44.3% of the total amino acids. The fat contains a high concentration of unsaturated fatty acids that comprises 55.1% of total fatty acids. The mineral profile is composed of macro- and microelements. The antinutritional factors oxalate, phytate, tannins and cyanogenic glycosides are present at very low concentrations. *Cissus rotundifolia* leaves can be considered a potential source of nutritional components for healthy food purposes.

- Keywords: antinutrients, nutrients, *Cissus rotundifolia*, evaluation -

INTRODUCTION

Wild edible plants are species of plants that grow freely in the wild habitat without any agricultural treatments and can be consumed as a food (BELUHAN and RANOGAJEC, 2010). These types of plants are consumed worldwide, from developing and developed nations alike, and provide nutrition and food security for poor rural communities in several regions across the world (SUNDRIYAL *et al.*, 2003; AFOLAYAN and JIMOH, 2009) while serving as a diet supplement in Japan, Europe and North American (CHEN and QIU, 2012; BURLINGAME, 2000; REDZIC, 2006). Wild edible plants are rich in minerals, vitamins, dietary fiber, fatty acids and amino acids (BARROS *et al.*, 2010; LUCZAJ, 2010). The nutritional values of these plant species are comparable to, or even exceed, the corresponding domesticated types of plants (BURLINGAME, 2000; TARDIO *et al.*, 2006; AFOLAYAN and JIMOH, 2009). Moreover, wild edible plants are considered a good source of phytochemicals for human therapeutics (PENNY *et al.*, 2002; MASUDA *et al.*, 2003; VARDAVAS *et al.*, 2006). However, the presence of antinutritional principles in some species of wild plants, such as phytic acid, tannins, saponins, alkaloids and oxalates, can limit their exploitation (GUIL *et al.*, 1997; GUPTA *et al.*, 2005; LACHUMY *et al.*, 2010). Previous studies have shown that the corresponding domesticated types of these plants contain similar levels of antinutritional factors (SHAD *et al.*, 2013). Moreover, some of the antinutritional factors have therapeutic potential; for example, phytic acid has been shown to have anticancer and antioxidant activity (JARIWALLA, 2001; SHAMSUDDIN, 2002). Thus, the compositional analysis and nutritional evaluation of such wild plants are necessary for understanding their impacts on consumer's health (GUIL *et al.*, 1997). *Cissus rotundifolia* (Forsk) Vahl. is a perennial, evergreen, climber, wild plant and is a species of Cissus belonging to the family of Vitaceae (grape family). It is known as a common Arabian wax cissus, Peruvian Grape Ivy, Venezuelan Tree bine and locally (in south Saudi Arabia) as Algalaf.

This wild plant is commonly used as food thickeners in rural Nigeria. Moreover, it was found to have many therapeutic effects as hypoglycemic (ONYECHI *et al.* 1998), hypolipidemic (BELL *et al.*1993). In addition, its extract exhibits antibacterial activity (ALZOREKY and NAKAHARA, 2003). *Cissus rotundifolia* grows extensively in the southern region of Saudi Arabia, and their leaves only are widely consumed after cooking by local people as leafy vegetables. Although it is commonly used to prepare various dishes according to traditional dietary culture of locals, its nutritional potential has not been assessed. Therefore, this study aimed to evaluate the nutritional and antinutritional components of *Cissus rotundifolia* leaves (CRL). These data would increase the awareness about the exploitation of this renewable natural resource as a food.

MATERIALS AND METHODS

Sample collection and preparation

The leaves of *Cissus rotundifolia* (20 kg) were collected from the Abha region in southern Saudi Arabia. The leaves were washed with distilled water, dried in a hot air oven at 50°C to a constant weight, ground to a fine powder and stored in airtight plastic bags at 4°C until analysis.

Proximate composition analysis

The moisture, ash, crude lipid, crude fiber and crude protein (N×6.25) contents were determined according to the standard methods of (AOAC, 2000).

Amino acids analysis

The defatted samples (0.2 g) were hydrolyzed with 6 N HCl (10 mL) in a sealed tube at 100°C for 24 hours. The hydrolyzates were completed to 25 mL with deionized water. Five ml of each hydrolyzate were evaporated until free from HCl vapor and dissolved in citrate buffer (CSOMOS and SIMON-SARKADI, 2002). The identification and determination of amino acids were conducted using the amino acid analyzer AAA-400 (INGOS, Czech Republic) equipped with an (OSTION LG ANB, INGOS) ion-exchange column (200 x 3.7 mm) and a flow photometer detector. The elution was carried out using a different pH gradient of sodium-citrate buffers. Chromatographic data processing including calculation of retention times and peak areas of separated amino acids were performed using AMIK software 3.0 (Czech Republic). A mixture of standard amino acids (INGOS, Czech Republic) was utilized as external standards.

Fatty acid analysis

The lipids were extracted according to the method outlined by EGAN *et al.* (1981) and GRESSLER *et al.* (2010). Briefly, 10 g of the sample was digested with 10 ml of hot concentrated HCl using a boiling water bath and vigorous stirring before the color of the content turned brown. The lipid was extracted by shaking with 30 ml of diethyl ether and was repeated three times. The solvent was evaporated and the total amount of lipid was gravimetrically estimated. The fatty acid was transmethylated into their corresponding methyl esters (RADWAN, 1978). The lipids (50 mg) were redissolved in 2 mL benzene, aliquots of 2 mL of methanolic sulfuric acid (1%, v/v) were added and the tubes were stoppered with nitrogen and kept in a water bath at 90°C for 90 min. Water (8 mL) was added, the methylated fatty acids were extracted with 5 ml petroleum ether and the mixture was evaporated to dryness. Two microliters of the

fatty acid methyl esters solution were injected into a HP (Hewlett Packard) 6890 GC, coupled with a splitless injector mode, a flame- ionization detector (FID) and a HP-5 column (5% diphenyl, 95% dimethyl polysiloxane, 30 m, 0.32 mm ID, 0.25 μm film thickness). The following operating conditions were used: injector temperature 220°C, oven temperature program: initial temperature 150°C for 2 min, raised to 200°C at a rate of 10 °C /min, then increased to 250°C at a rate of 5°C /min and held at 250°C for 9 min, detector temperature: 250 °C, carrier gas was nitrogen at a flow rate of 1 ml/min. The mixture of fatty acid standards was subjected to the same treatments of the samples and used to identify and quantify the fatty acids in the samples.

Mineral analysis

The samples were digested as described by AMIN *et al.* (2013). Briefly, leaf powder (0.5 g) was digested with 4 ml of concentrated nitric acid and 1 ml of perchloric acid, cooled and filtered with Whatman No.42 filter paper. The supernatant was completed to 50 ml with distilled water. The blanks were carried out using the same procedure. The mineral concentrations of the digested diluents were determined against a multielement standard solution (Campro Scientific, Berlin, Germany) using Inductively Coupled Plasma-Optical Emission Spectrophotometry ICP-OES (Varian 720-ES, Varian Inc, Palo Alto, CA, USA).

Determination of antinutrients

The content of oxalate was measured using the titrimetric method of SANCHEZ-ALONSO and LACHICA (1987). Phytic acid in leaves was quantified according to the method of LUCAS and MARKAKAS (1975). The spectrophotometric method described by SARKIYAKI and AGAR (2010) was used to estimate the amount of cyanogenic glycoside in leaves. The tannin content was estimated using spectrophotometric analysis according to the method of POLSHETTIWAR *et al.* (2007).

Statistical analysis

All measurements were achieved in triplicate and the results were expressed as the mean value ± standard deviation of three measurements, using SPSS 13.0 (SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Proximate compositions

The nutritional composition of the leaves (Table 1) was compared with those of the most widely consumed foods (wheat, rice and potato) throughout the world. This comparison is justified by the fact that in the countries of origin leaves are used in two forms: fresh and sundried powder. The latter one is consumed as a partial replacer of wheat flour, corn flour and rice, to overcome a deficient of these foods.

The determined nutrients of the leaves were superior to those of wheat, rice and potato. This emphasizes their value as a good source of nutrients. A relatively high ash content in the leaves was associated with the amount of mineral elements.

Amino acid composition and protein quality

By the amino acid analysis (Table 2) fifteen amino acids were identified in CRL protein fraction. Among the detected amino acids, eight of essential amino acids (EAAs), which amounted to 358.5 mg/g crude protein, was identified. This exceeded the value of EAAs that is recommended by FAO for adults (2013). The amount of EAAs comprised 44.3% of the total individual amino acids, which is a ratio similar to that reported for the domesticated vegetable kale leaves (LISIEWSKA *et al.*, 2011). The present analyses also indicated that the protein in CRL contained a considerable level (69.9 mg/g protein) of aromatic amino acids (AAA) (histidine, phenylalanine and tyrosine), which is much higher than the AAA scoring pattern recommended by FAO for adults (38 mg/g) (2013). Similar to previous studies performed on many domesticated vegetable species (LISIEWSKA *et al.*, 2011; KMIECIK *et al.*, 2009), glutamic acid was the major amino acid identified in CRL protein. Cysteine, methionine and tryptophan were excluded in this study because they were destroyed during acid hydrolysis. All individual EAAs in leaf proteins (Table 2) compared favorably with the corresponding amino acid reference that is recommended for adults by FAO (2013) except for histidine, which had a score slightly below what is recommended. Therefore, CRL can be considered a good source of balanced protein.

Table 1 - Proximate composition (g/ 100g) of CRL compared with wheat, rice and potato.

Constituent (%)[a]	CRL	Wheat[b]	Rice[b]	Potato[c]
Moisture	93.1±0.2	12.6	13.0	75.7
Crude protein (dry basis)	12.5±0.1	11.3	7.70	8.27
Crude fat (dry basis)	7.45±0.1	1.80	2.20	1.11
Crude fiber (dry basis)	8.34±0.2	13.2	2.20	9.94
Ash (dry basis)	16.3±0.2	1.70	1.20	3.98

[a]Values are expressed as the means ± SD of three separate determinations).
Source: [b]KOEHLER and WIESER (2013); [c]GUMUL *et al.* (2011)

Table 2 - Amino acid profile of CRL protein.

Amino acids	mg/g protein[a]	FAO Pattern 2013	% of total
Essential amino acids			
Histidine	16.4±0.2	15	2.03
Isoleucine	47.5±0.2	30	5.88
Leucine	96.6±0.4	59	11.9
Lysine	38.7±0.1	45	4.79
Phenylalanine	37.9±0.1		4.69
Threonine	23.7±0.2	23	2.94
Valine	69.9±0.6	39	8.65
Arginine	27.4±0.2		3.39
Non-essential amino acids			
Alanine	98.5±0.7		12.1
Aspartic acid	64.9 ± 0.1		8.03
Glutamic acid	127.3±0.6		15.7
Glycine	97.7±0.6		12.0
Proline	7.77±0.1		0.96
Serine	38.2±0.2		4.72
Tyrosine	15.5±0.1		1.92
Total EAAs[b]	358.5		
Total non- EAAs	450.0		
Total individual amino acids (mg/g protein)	808.5		
Total AAA[c]	69.9		
% of EAAs	44.3		
% of Non- EAAs	55.7		

[a]Values are expressed as the means ± SD of three separate determinations on dry weight basis;
[b]Essential amino acids;
[c]Aromatic amino acids (phenylalanine+ histidine +tyrosine).

Fatty acid profile of CRL

The data in Table 3 show that 12 fatty acids were determined in the leaf lipidic extract, four out of which are unsaturated fatty acids and comprised more than half (55.1%) of the total fatty acid content. This high level of unsaturated fatty acids makes the CRL of main health interest. Palmitic acid, oleic acid and linoleic acid were the three major components present in the leaves, representing 31.7%, 25.7% and 22.9% of the total individual fatty acids, respectively. Palmitic acid is commonly found in both animal and plant foods. WHO (2003), reported that, dietary intake of palmitic acid increases the risk of cardiovascular diseases. However, in moderation, palmitic acid may not be entirely bad, as it does display mild antioxidant and anti-atherosclerotic properties (CHO *et al.*, 2010). The high proportion of both oleic acid (omega-9 fatty acids) and linoleic acid (omega-6 fatty acids) in leaves raises the biological value; therefore, consuming the leaves could be healthy and meet a part of the essential fatty acids requirements. The data also show that the leaf lipids contain odd-numbered fatty acids (tridecylic, pentadecanoic and pentadecenoic acid) in its composition. Such fatty acids have been found in many daily consumed foods such as human milk (NISHIMURA *et al.*, 2013; KOLETZKO *et al.*, I988), ruminants milk (BREVIK *et al.*, 2005), fish (ATEŞ *et al.*, 2013), and commonly consumed vegetables (BATISTA *et al.*, 2011). Concerning the impact of odd-numbered fatty acids on health, MARTYSIAK-ZUROWSKA (2008) reported that there is no risk of presence of odd-numbered fatty acids in food as it is found in mother's milk and ruminant's milk.

Table 3 - Fatty acid composition of CRL.

Fatty Acid	FA (µg/g)[a]	% of total
Caprylic acid (C8:0)	7.56±0.3	0.23
Capric acid (C10:0)	12.4±0.2	0.38
Lauric acid (C12:0)	35.6±0.2	1.09
Tridecylic acid (C13:0)	63.1±0.1	1.93
Myristoleic acid (C14:1)	101.2±0.2	3.09
Myristic acid (C14:0)	39.8±0.2	1.21
Pentadecenoic acid (C15:1)	110.5±0.1	3.38
Pentadecanoic acid (C15:0)	92.8±0.2	2.83
Palmitic acid (C16:0)	1036.5±0.4	31.7
Linoleic acid (C18:2c)	750.2±0.1	22.9
Oleic acid (C18:1c)	841.5±0.5	25.7
Stearic acid (C18:0)	181±0.7	5.53
Total unsaturated fatty acids	1803.4	
Total saturated fatty acids	1468.8	
Total individual fatty acids	3272.1	
% of total unsaturated fatty acids	55.1	
% of total saturated fatty acids	44.9	

[a]Values are expressed as the means ± SD of three separate determinations on dry weight basis.

Mineral content of CRL

The contents of both macro- and microelements in leaves are presented in Table 4. Calcium, which is required for the formation of bone and neurological function (BRINI *et al.*, 2013), was the predominant element in leaves (15.1 mg/g). A modest consumption of 66.5 g of leaves per day would satisfy the adult daily requirement of calcium (1,000 mg/day), according to the Institute of Medicine (2011). Therefore, CRL could be a good source of calcium. Sodium was the second abundant element found in CRL, followed by potassium. Potassium and sodium play an important role in regulating blood pressure and body acid-base balance (CLAUSEN *et al.*, 2013; SIDDHURAJU *et al.*, 2001). An appreciable concentration of magnesium was determined in the leaves. Magnesium is needed to prevent heart disease and growth retardation (CHATURVEDI *et al.*, 2004). CRL could be considered a rich source of iron and an intake of 47.4 g of leaves could satisfy the recommended adult dietary intake (6 mg/day) of iron according to the Institute of Medicine (USA, 2001). Zinc, which is a component of many enzymes and a wide array of cellular and biochemical processes (KARCIOGLU, 1982; COLEMAN, 1992), is present in a moderate amount in leaves. Significant amounts of both copper and chromium, which are a component of many respiration enzymes and glucose tolerance factor, respectively (SANDS and SMITH, 2002; MERTZ, 1993), were observed in the leaves (FAILLA *et al.*, 2001; KELVAY, 2000).

Antinutritional factors

The edibility of any wild plant depends on the content of anti-nutritional factors. Analyses were carried out in CRL and results are shown in Table 5. The oxalate content was equal to 3.05mg/100 g, value lower than that reported (14.9 g/100 g) in common green leafy vegetable spinach (*Spinacia oleracia*) (YADAV and SEHGAL, 2003). The phytate level (0.76 mg/100 g) in leaves was found to be less compared with that reported in domesticated crops of *Solanum indicum* (695.8 mg/100 g, ABEROUMAND, 2012), lima beans (234 mg/100 g, EGBE and AKINYELE, 1990) and underutilized green leafy vegetables (0.92–13.06 mg/100 g, GUPTA *et al.*, 2005), indicating that the lower phytic acid content in CRL will provide a better bioavailability of minerals. The estimated tannin value in leaves is considerably lower compared with those (0.59 mg/100 g) reported in lima beans (*Phaseolus lunatus*) by EGBE and AKINYELE (1990). The detected level of cyanogenic glycosides (0.023 mg/100 g) can be consider inappreciable compared with those of lima beans (colored) (3120 mg HCN/kg) (SPEIJERS, 1993) and is much lower than the reported lethal dose (3.70 HCN mg/kg bw) for mouse (CONN, 1979). These results reveal that antinutritional factors exist in CRL, but at lower levels compared with many daily-consumed foods.

Table 4 - Mineral composition of CRL.

Mineral	Concentration[a]
Macroelements	*mg/g*
Calcium (Ca)	15.1±0.2
Magnesium (Mg)	3.55±0.1
Sodium (Na)	11.2±0.2
Potassium (K)	8.09±0.3
Microelements	*µg/g*
Iron (Fe)	126.6±3
Zinc (Zn)	51.6±0.3
Manganese (Mn)	31.3±0.6
Copper (Cu)	3.21±0.3
Chromium (Cr)	2.38±0.2

[a]Values are expressed as the means ± SD of three separate determinations on dry weight basis.

Table 5 - Antinutrients contents in CRL.

Compound	Content (mg/100g) [a]
Oxalate	3.05±0.1
Phytate	0.76±0.1
Tannins	0.26±0.1
Cyanogenic glycosides	0.023±0.0

[a]Values are expressed as the means ± SD of three separate determinations on dry weight basis.

CONCLUSIONS

The present study serves as a basis to encourage the local communities to exploit the nutritive potentials of the wild plant *Cissus rotundifolia.* Results of analyses demonstrated good nutritional qualities and CRL could, thus, contribute to overcome the nutritional deficiency especially in arid climates. Therefore, it is now imperative that a nutritional database of this wild plant is set up to retain the information for a better management and conservation of this natural resource and habitats related to it.

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PAPER

OPTMIZATION OF EXTRUSION PROCESS OF RICE FLOUR ENRICHED WITH PISTACHIO NUT FLOUR

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ABSTRACT

Response surface methodology deriving by superimposing individual contour plots, was used to investigate the optimum operating conditions for extrusion-cooking of rice flour enriched with pistachio nut flour. The highest barrel temperature (128°C) produced a stiff extrudates (high values of breaking strength *i.e.* 100 N/mm^2 and bulk density *i.e.* 2.2 g/mL). However, graphical optimization studies showed that the optimal operating conditions involved values of 16-17% water feed content and 70-95°C barrel temperature. This research points out the importance to study the biopolymer changes that occur during extrusion-cooking processing because of their huge effect on quality characteristics of extrudates.

- Keywords: pistachio nut flour, starch-lipid complexes, optimization response surface methodology, contour plot, breaking strength, bulk density -

1. INTRODUCTION

Nowadays, consumers prefer foods easy and convenient to eat (SCHWARTZ, 2009). Snacks and breakfast cereals are easy to carry, purchase and consume but they are essentially produced from starchy substances such as corn, rice, wheat (YASEEN and SHOUK, 2005) and therefore they could lack some important nutrients. Foods with poor nutritional value, lack in micronutrients such as vitamins, minerals, amino acids, fibers and high content of calories can be considered unhealthy. For this reasons, researches are focused on the improvement of nutritional characteristics by the addition of ingredients such as fruit, nuts, fibres, etc. Among nuts, pistachios could favourably be used thanks to their ability to lower the risk of cardiovascular diseases, to improve total cholesterol to HDL-C ratios, LDL cholesterol to HDL cholesterol ratios, and HDL cholesterol levels (KOCYIGIT *et al.,* 2006; SHERIDAN *et al.*, 2007; GEBAUER *et al.*, 2008). Consumption of pistachios was also found to increase antioxidant activity in the body (KOCYGIT, 2006) and to improve blood glucose levels (SARI *et al.,* 2010). A possible application of pistachio nut flour could be the production of extrudates in order to obtain snack foods with high nutritional and health value. DE PILLI *et al.*, (2011; 2012) studied the processing conditions that lead to the formation of starch-lipid complexes in a model system and in real food like extrudates made up of rice starch and pistachio nut flour, by differential scanning calorimeter (DSC). In addition, they evaluated the effects of starch-lipid complexes formation on system parameters, fat loss and the breaking strength of extrudates. The results of that work showed that the barrel temperature had a huge effect on system parameters in the real foods as the extraction of lipid fraction determined a decrease of friction force and therefore a decrease of mechanical energy input of processing (DE PILLI *et al.*, 2008a). Moreover, the formation of starch-lipid complexes in real food, was strongly dependent on water feed content, that consequently affected starch gelatinization. The highest fat loss and the hardest texture of extrudates made up of pistachio nut flour were obtained under processing conditions that favoured the maximum formation of starch lipid complexes. The main objective of those studies was to verify the efficacy of model system to describe the biopolymers changes that occur during processing of real food. However, the relationships between process variables and characteristics of the extrudates has not been studied in detail. Therefore, this study aimed to investigate the optimum operating conditions of extrusion and the effects of extrusion process variables on the characteristics of rice extrudates enriched with pistachio nut flour by using the response surface methodology (RSM). Furthermore, the regression models to predict the characteristics of the extruded material as a function of the process variables were also established.

2. MATERIALS AND METHODS

2.1 Raw materials

Rice starch (10.9% moisture) was provided by A.D.E.A. (Bursto Arsizio, Italy); pistachio nut flour was provided by Cartellone (Bronte, Italy); oleic acid was provided by Sigma-Aldrich (Milano, Italy).

The used pistachio nut flour had a moisture content of 4.8±0.2% and the following chemical composition (dry basis): protein (18.1±0.1%); lipid (49±0.5%); starch (3.3 ±1.5%); soluble sugars (4.5±0.2%); fiber (10.6±2%) and ash (9.7±0.1%).

The fat acid composition of lipid fraction of pistachio nut flour, determined according method proposed by RATNAYAKE *et al.* (2006) was: **C14:0** (0.09); **C16:0** (9.45); **C16:17** (0.86); **C17:0** (0.04); **C17:1** (0.07); **C18:0** (2.12); **C18:1** (70.17); **C18:2** (15.5); **C18:3** (0.32); **C20:0** (0.18); **C20:1** (0.48); **C22:0** (0.09); **C24:0** (0.04).

The chemical characteristics of tap water used for extrusion trials was: pH 7.7 $\pm$ 0.1, hardness (°f) 25.1±1.5, total dissolved solids dried at 180°C 645±38.5 mg/L and chloride content 54.6±0.4 mg/L.

The content of moisture, ash, protein and fat of flours were determined according to the 44-15A, 08-01, 46-10, 30-25 AACC International Approved Methods (2003).

2.2. Extrusion experiments

According to previous studies (DE PILLI *et al.*, 2011), the formula containing 75% rice starch and 25% pistachio nut flour was used. The extrusion experiments were carried out using a Thermo Prism PTW-24 (Thermo Haake PolyLab System, Germany) co-rotating twin-screw extruder. The screw geometrical features were the following: diameter 24 mm and length 672 mm (L/D = 28:1) and distance between shafts 19 mm. Fig. 1 reports the screw configuration used. During extrusion experiments, the screw speed was kept constant at 140 rpm, as well the flour feed rate was kept constant at 2.8 kg/h (dry weight). The flours were proportioned by volumetric gravity feeder. The extruder was divided into six zones, independent of each other for temperature control and adjustment. For all experiments, the first two zones were kept at 35 and 65°C respectively, whereas the last four zones were adjusted at the same temperature according to experimental plan (Table 1).

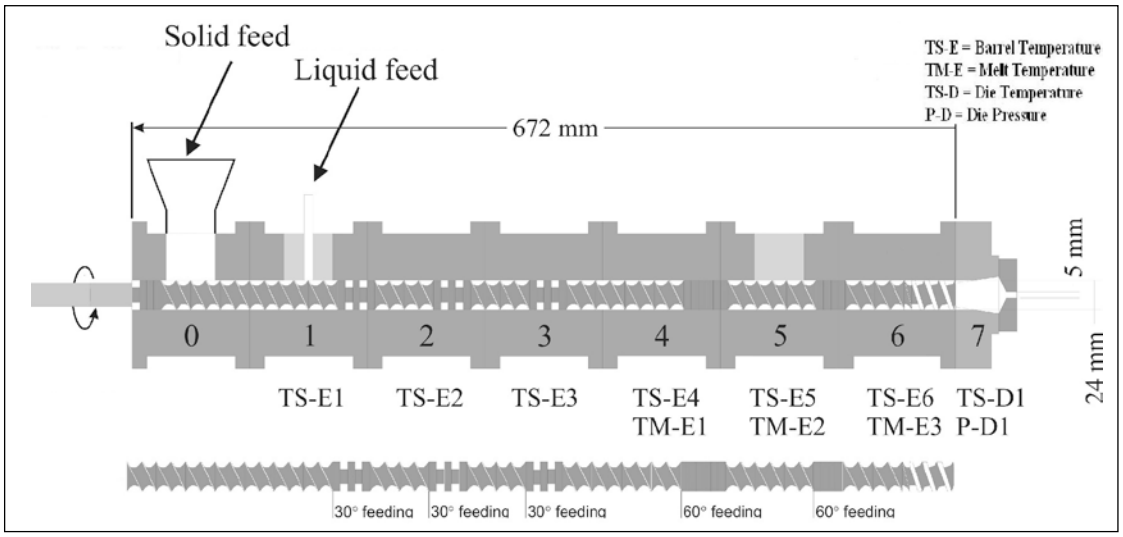


Fig. 1 - screw configuration used to extrude rice starch and pistachio nut flour.

The water was pumped to the first zone of the extruder and the delivery capacities of water pump were 7.5; 7; 6; 4.75; 4.25 L/h. These values were chosen to obtain the moisture feed content of dough indicated in the experimental plan (Table 1).

The die used had a spherical shape (diameter 300 mm) in which there was one circular hole with a diameter of 5 mm. At the exit of the die, the extrudates were manually cut into sticks (about 50 mm in length) using a knife. The extrudates were dried over night at 40°C in a vac-

Table 1 - Coded and actual values of variables (A) and the arrangement and responses of factorial design (B).

A)

Coded Level	Uncoded	
	Barrel temperatures of last four zones (X1) (°C)**	Water feed content (X2) (%)
+1.4	128	21.8
+1	120	21.0
0	100	19.0
-1	80	17.0
-1.4	72	16.2

**The first two zones were kept at 35° and 65°C respectively, whereas the last four zones were adjusted according to experimental plan.

B)

Treatments	Coded Level		Processing variables		Responses			
	X1	X2	X1 (°C)	X2 (%)	Y1 (%)	Y2 (nm)	Y3 (N/mm2)	Y4 (g/ml)
1	-1	-1	80	17.0	23.67±0.02	554±0.55	7.97±0.92	0,80±0.26
2	-1	1	80	21.0	23.00±0.06	556±0.80	14.21±3.12	0,81±0.45
3	1	-1	120	17.0	60.00±0.03	552±0.52	96.24±1.19	2,10±0.50
4	1	1	120	21.0	60.00±0.01	549±0.66	75.58±0.59	2,08±0.37
5	-1.4	0	72	19.0	20.00±0.07	559±1.17	7.58±1.40	0,80±0.26
6	1.4	0	128	19.0	70.00±0.01	552±0.41	99.04±0.19	2,11±0.11
7	0	-1.4	100	16.2	52.45±0.01	552±2.04	53.75±1.50	0,87±0.34
8	0	1.4	100	21.8	52.00±0.03	555±0.63	65.68±0.45	0,87±0.25
9	0	0	100	19.0	51.50±0.02	556±1.02	43.05±2.73	0,87±0.37
10	0	0	100	19.0	52.00±0.03	554±0.42	51.32±2.60	0,94±0.23
11	0	0	100	19.0	50.80±0.01	555±0.41	49.52±1.23	0,96±0.28

X1: barrel temperatures of last four zones (°C)**; X2:** water feed content (%)**; Y1**: complex index; **Y2**: λ_{max}; **Y3**: breaking strength; **Y4**: bulk density.

uum oven Salvis Vacucenter VC 50 (Salvis AG, Reussbühl/Lucerne, Switzerland). Samples used to determine complexing index and iodine spectrum of the soluble fractions of the extrudates were finely ground (particles < 300 microns) using a BUHLER ML 1204 mill (Germany). All the ground samples were defatted in a Soxtec fat-extractor with petroleum ether at 37°C (bp 34.6°C) for 155 min to remove uncomplexed lipids before submit samples to chemical analyses (BHATNAGAR and HANNA, 1994).

2.3 Experimental plan

The extrusion experiments were carried out at five temperature profiles (Table 1a) and percentages of water feed contents (21.8%, 21.0%, 19.0%, 17.0%, 16.2% expressed as percentage of dry basis). All extrusion experiments were performed at least in triplicate.

Coded and actual values of variables are shown in Table 1a, the factorial design of two variables (temperature profile and feed water content) and five levels of values were used according to Central Composite Design (CCD) (BOX *et al.*, 1978). This method was used to evaluate the single influences of the processing variables as well as their possible interactions. Eleven tests (Table 1b) with different combinations of process variable values were obtained.

2.4 Complexing Index

Complexing Index (CI) was determined using the method described by GURAYA *et al.* (1997). The iodine solution used for analysis was prepared by dissolving overnight 2 g of potassium iodine and 1.3 g of I_2 in 50 mL distilled water. Then the final volume was made to 100 mL using distilled water. A 5 g sample was mixed with 25 mL of distilled water in a test tube. The test tube was vortexed for 2 min and centrifuged for 15 min at 314.1 rad/s. The supernatant (500 ml) and distilled water (15 mL) were added to the iodine solution (2 mL). The tube was inverted several times and absorbance was measured at 690 nm through a UV/VIS spectrophotometer (Beckman DU 640, California). CI was calculated from the following equation (2):

$$CI(\%) = \frac{(Abs_{control} \quad Abs_{sample}) \quad 100}{Abs_{control}} \qquad (2)$$

The analysis was carried out in triplicate.

2.5 Iodine Spectra of Starch Samples

Starch samples were solubilised in l N NaOH as recommended by SCHOCH (1964). The absorbance spectra of starch-iodine complexes were measured using a spectrophotometer UV/VIS Perkinelmer Lambda 25 (Milan, Italy) from 400-700 nm, and wavelength of maximum absorption (λ_{max}) values were determined.

2.6. Breaking strength (N/mm^2)

A stable dynamometer Micro System TA-HDi Texture Analyser (ENCO s.r.l., Venezia, Italy) with a plunger was used for texture analysis. Extrudates were placed over two supports, 1.5 cm apart, and broken in the middle by a plunger that had a shape of a cone frustum (the thickness of contact surface with extrudate was 1 mm^2 and the speed was kept constant to 0.5 mm/s). Results were expressed as breaking strength (N/mm^2), *i.e.* the strength needed to break the extrudate. This index is related to microstructure of samples and it simulates the incisors impact at biting (VAN HECKE *et al.*, 1998). For each sample, at least ten repetitions were carried out.

2.7 Bulk Density (BD)

Bulk density was measured using a displacement method (YU *et al.* 2012). Extrudates were cut into strands of about 25 mm long and about 10 g strands were weighed (M, grams) and put in a 100 mL cylinder; then yellow millet particles were added to fill up the cylinder. The extrudates were taken out, and the volume of the yellow millet particles was measured (V, milliliters); ten measurements were performed to calculate the average. Bulk density (BD) was calculated as equation (3):

$$\text{Bulk density} = \frac{M}{100 \quad V}(g/ml) \qquad (3)$$

2.8 Statistical analysis

Data were submitted to statistical analysis using Statsoft, vers. 5.1 (Statsoft, Tulsa, USA) software. The analysis was carried out in two steps. The first involved a stepwise regression analysis to identify the relevant variables, and the second used a multiple regression analysis (Standard Least Square Fitting) to fit a second order mathematical model, according to the following polynomial equation:

$$y = B_0 + \Sigma Bi\chi i + \Sigma Bii\chi ii^2 + \Sigma Bij\chi i\chi j$$

where y is the dependent variable (complex index, iodine spectrum of the soluble fractions of the extrudates; breaking strength and bulk density of extrudates), B_0 is a constant value, χi and χj are the independent variables (barrel temperature and water feed content) in coded values and Bi, Bii and Bij are the regression coefficients of the model. This model allowed the effects of the linear (χi), quadratic (χi^2) and combined ($\chi i\chi j$) terms of the independent variables to be assessed on the dependent variable.

Variables with a significance lower than 95% (p>0.05) were left out of the equation. Iso-response surface were developed in order to describe both individual and interactive effects of the independent variables of the extrusion-cooking process on complex index, iodine spectrum of the soluble fractions of the extrudates; breaking strength and bulk density of extrudates.

Extrusion processing parameters were optimized by using the Design-expert version 8.07.1 (Stat-Ease Inc., Minneapolis, USA) through a conventional graphical method of RSM in order to obtain extrudates with acceptable properties. All the processing variables were kept within a range while the responses were either minimized (breaking strength and bulk density). Contour plots of all the responses were then superimposed, and the optimum region appeared. The contour plots were obtained by superimposing of contour plots from which one could determine the optimum process variables range (barrel temperature and water feed content) to obtain extrudates made up of rice and pistachio nut flour with specified properties.

3. RESULTS AND DISCUSSION

Fig. 2a shows the complexing index values as a function of barrel temperature and water feed content. The barrel temperature was the only processing variable that had a significant effect on complexing index. In particular, values of the complexing index increased with increasing of barrel temperature (Fig. 2a). This means that, in this case, the highest barrel temperatures did not involve the melting of starch-lipid complexes. It is possible to suppose that the presence of other components in the dough increase the melting temperature of starch-lipid complexes, that result then more protected by heating during processing. Moreover, the presence in lipid fraction of triglycerides, di-glycerides and fatty acids involves an increase of characteristic melting temperature of starch-oleic complexes (DE PILLI *et al.*, 2008b; 2011). To confirm the formation of starch-lipid complexes, values of λ_{max} for native starch extruded with and without pistachio nut flour were also determined. Rice flour, extruded without nut flour and with an amylose contents of 89 %, showed λ_{max} within 592-595 nm.

Fig. 2b shows that the increase of barrel temperature shifted λ_{max} from 595 nm towards the amylopectin side (520 nm), due to the decrease of available amylose that is bounded with lipids. These results are in agreement with those of complex index and confirm the formation of starch-lipid complexes (Fig. 2a).

In Fig. 3a is reported the break strength (BS) values as a function of barrel temperature and water feed content. Also in this case, the only variable that had a significant effect on microstructure of the extrudates was the barrel temperature. In particular, the extrudates obtained at the highest values of barrel temperature (128°C) opposed the highest resistance to break, while low values of break strength were obtained at the lowest barrel temperature (70°C) (Fig. 3a). The formation of starch-lipid complex-

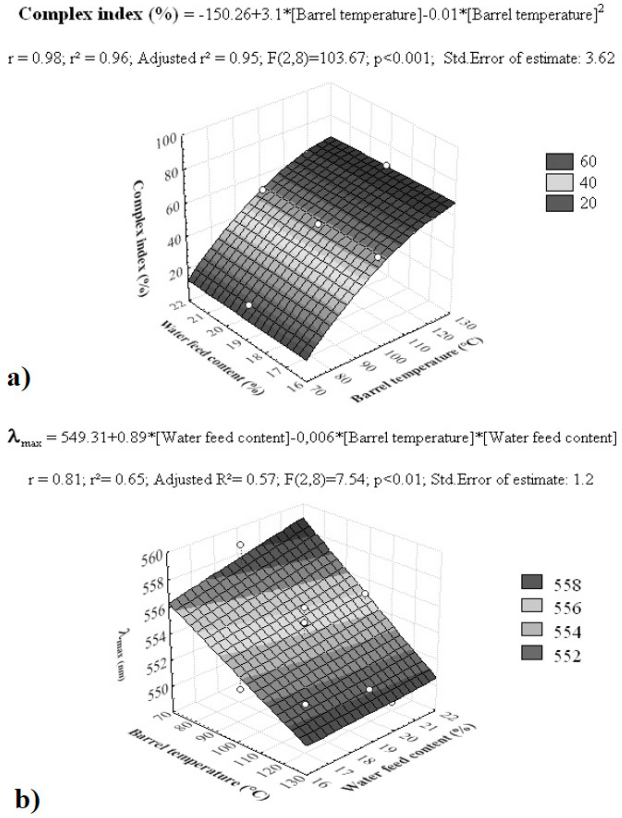


Fig. 2 - starch-lipid complex index (a) and λ_{max} values (b) of samples made up of rice starch and pistachio nut flour blend as a function of barrel temperatures and water feed content.

es obtained with the increase of barrel temperature could explain the high compactness of extrudates (BHATNAGAR and HANNA, 1994; DE PILLI *et al.*, 2008a,b).

Data of bulk density (BD) are in agreement with those of the break strength. In fact, the extrudates showed high values of bulk density at the highest barrel temperature (Fig. 3b). The increase of bulk density and break strength values of extrudates may be caused by an alteration in the ratio between free amylose and amylopectin. According to GUY and HORNE (1988), the elastic character of the molten extrudates creates a swell at the die that controls the overall phenomenon of expansion of the extrudates. LAUNAY and LISCH (1983) suggested that amylose–lipid complex formation was the key factor influencing the flow properties of starch pastes. When starch is extruded, expansion is dependent on the formation of a starch matrix that entraps the water vapor, resulting in the formation of bubbles (GUY and HORNE, 1988). It

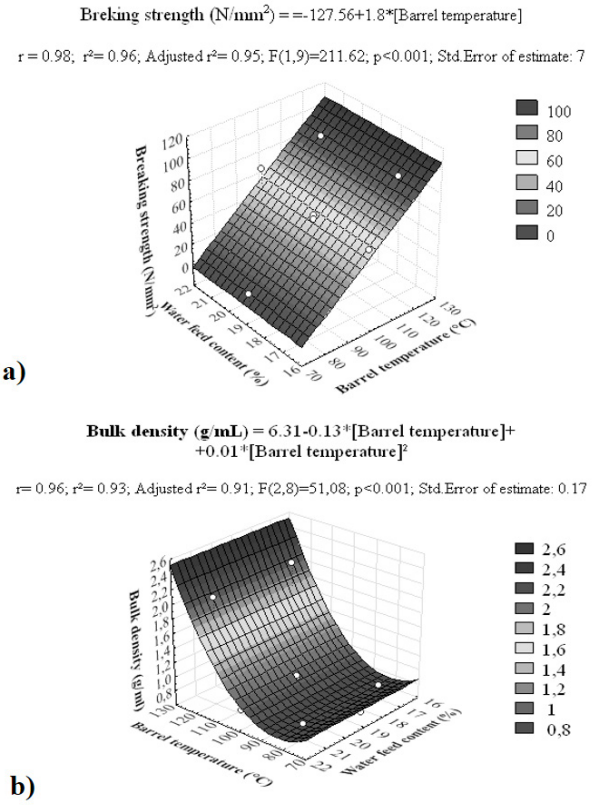


Fig. 3 - breaking strength (a) and bulk density values (b) of samples made up of rice starch and oleic acid blend as a function of barrel temperatures and water feed content.

is reasonable to speculate that the addition of lipids might have affected the character of this matrix (*i.e.*, the viscoelastic properties of molten extrudate) so that it could no longer hold water vapor, resulting in lower expansion and higher break strength. The increase of bulk density and break strength caused by decrease of swelling of starch can also be compared to that one of native starch upon gelatinization. Swelling is generally considered a property of amylopectin while amylose is considered a diluent. The amylose and native lipids contained in cereal starches may inhibit swelling under particular conditions when amylose–lipid complexes are likely to be formed (TESTER and MORRISON, 1990). According to KROG (1973), complex formation with the linear component of starches makes the structure more rigid and stabilizes the swollen granule against breakdown, resulting in restricted swelling. These statements are in agreement with DE PILLI *et al.* (2008b).

In this study, a conventional graphical method of multiresponse optimization technique was applied to obtain the combination of optimum process variable for the production of extrudates enriched with pistachio nut flour. To determine the extrudates with acceptable properties, main criteria of optimization constraints were related to bulk density (< 1.2 g/mL) and breaking strength (< 40 N/mm²). Superimposing the individual contour plots for the product response variables resulted in the identification of a region (shown by the blank space area) that satisfied all constraints as shown in Fig. 4. Superimposed contour plots indicated the ranges of variables that could be considered as the optimum range to obtain the best characteristics of extrudates in terms of bulk density and braking strength. The optimum ranges of variables obtained from the superimposed contours were 16–17 % water feed content and 70-95°C barrel temperature. Extrusion-cooking

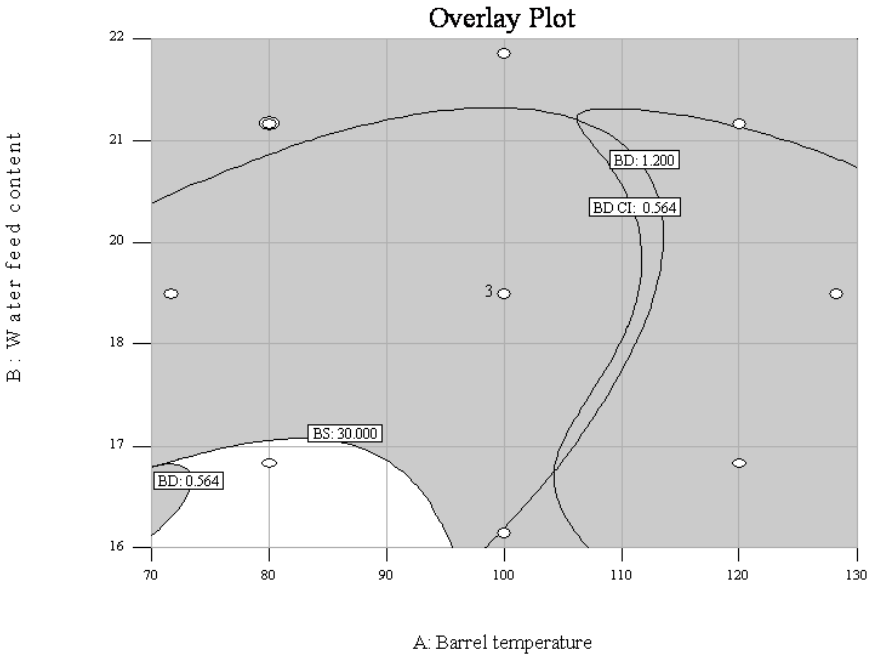


Fig. 4 - superimposed contours for the product responses affect by water feed content and barrel temperature. *BD: bulk density and BS: breaking strength.

was carried out for confirmation under the optimum process conditions and the responses were recorded (mean of five measurements). In particular, the following operating conditions were chosen: 72 °C barrel temperature and 16% water feed content. The values predicted by the software were ≤ 0.8 g/mL for bulk density and ≤ 20 N/mm^2 for breaking strength. The data obtained by extrusion experiments carried out at the same operating conditions were respectively 0.78 g/mL and 7.38 N/mm^2. The veracity of values of the responses predicted by the software was assessed with the help of a two-tailed, one-sample t-test. The results of the t-test indicated that the coefficient of variation was not greater than 5 %. Therefore, the developed model was suitable in representing the optimum operating conditions for this particular application.

4. CONCLUSIONS

The obtained results showed that the barrel temperature was the variable that has mainly affected the formation of starch-lipid complexes and structure of the extrudates. In particular, the worst characteristics of extrudates (hardness and bulk density of extruded products) were obtained at the highest temperature that corresponds to the maximum formation of starch-lipid complex.

Moreover, the model was found to be statistically valid and demonstrated adequate information regarding the behaviour of the responses upon variations of the process variables. Optimum process conditions and the corresponding predicted responses could be obtained with the help of the models. The predicted responses at the optimum conditions were not significantly different from the experimental values. According to the optimum conditions given for the variables, the process could be referred to standardization of industrial production of snack food made up of rice and pistachio nut flours with high qualitative characteristics.

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PAPER

RECOVERY AND BEHAVIOUR OF STRESSED *ESCHERICHIA COLI* O157:H7 CELLS ON ROCKET LEAF SURFACES INOCULATED BY DIFFERENT METHODS

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ABSTRACT

E. coli O157:H7 is an emerging public health concern worldwide because of its low infectious dose and ability to survive under adverse conditions. Tests were conducted to determine the ability of unstressed *E. coli* O157:H7 cells or those stressed by acid, cold, salt exposure or starvation to survive on the surfaces of rocket leaves after contamination by three methods (dip, spray or spot inoculation) and following storage at 10 or 25°C. *E. coli* O157:H7 numbers recovered from rocket leaves contaminated by the different techniques were in the order of dip > spot > spray inoculation. Numbers of stressed *E. coli* O157:H7 recovered after inoculation by all three methods increased significantly over 7d storage at 10 or 25°C, while unstressed *E. coli* O157:H7 only grew following dip inoculation. Exposure to adverse environmental conditions may increase the risk of *E. coli* O157:H7 survival and spread on leafy green vegetables.

- Keywords: *E. coli* O157:H7, environmental stress, leafy green vegetables, rocket leaves, inoculation method -

INTRODUCTION

Escherichia coli O157:H7 is a facultatively anaerobic, Gram-negative rod-shaped enteric bacterium which produces Shiga toxins 1 and/or 2 as important virulence factors and emerged as a foodborne pathogen in 1982. *E. coli* O157:H7 infection presents with numerous symptoms including abdominal pain, watery and bloody diarrhea, vomiting, mild fever, sometimes leading to hemorrhagic colitis and the hemolytic uremic syndrome (HUS) with renal tubular damage (WHO, 2011). SCALLAN *et al.* (2011) and THOMAS *et al.* (2013) reported that *E. coli* O157:H7 is associated with 63,153 and 12,827 foodborne illnesses every year in the US and Canada, respectively. *E. coli* O157:H7 illness outbreaks have been associated with a variety of foods including ground beef, spinach, lettuce, radishes, vegetable sprouts, fermented sausages, unpasteurized fruit juices, apple cider and raw milk (CHAURET, 2011). Annually, fresh produce is responsible for 45.9% of foodborne illnesses, 38.1% of hospitalizations and 22.9% of deaths caused by contaminated food in the US (PAINTER *et al.*, 2013). Furthermore, *E. coli* O157:H7 has been associated with repeated illness outbreaks resulting from the consumption leafy vegetables including lettuce, spinach, parsley and rocket leaves (OLAIMAT and HOLLEY, 2012; NYGARD *et al.*, 2008).

In recent years contamination of raw or minimally processed food products such as fresh produce with *E. coli* O157:H7 has become a concern worldwide because of its low infectious dose and ability to survive for long periods in the environment (CHAURET, 2011; DELAQUIS *et al.*, 2007). This contamination may occur on the surface of leafy vegetables due to the transfer of the pathogen from soil or water (DELAQUIS *et al.*, 2007). Survival of *E. coli* O157:H7 on these surfaces is affected by several factors including nutrient availability, competition with indigenous microflora, UV radiation and relative humidity (BRANDL, 2006). Fresh produce can be contaminated with foodborne pathogens during pre- or post-harvest processes (OLAIMAT and HOLLEY, 2012). Agricultural production and post-harvest environments may exert a variety of stresses on pathogens, which may affect their survival on the final product. In response, strategies have been developed by exposed pathogens to reduce the impact of this stress, including formation of aggregates in protective niches, localization in biofilms, and internalization within plant tissues. Bacteria have also been shown to respond to these stresses through genetic and/or physiological means including stress adaptation, development of cross-protection mechanisms, conversion to a viable but non-culturable (VBNC) state, through heterogeneous phenotypic expression, and by sheer genetic diversity (DINU *et al.*, 2009). Studies have shown that ability of *E. coli* O157:H7 to exhibit responses to sub-lethal environmental stresses, which may enable its survival under more severe conditions, enhance its resistance to subsequent processing conditions, and/or enhance its virulence. Therefore, understanding the effects of environmental stress caused by acid, cold, starvation and abnormal osmotic conditions on the survival of *E. coli* O157:H7 is important in order to assess and minimize the risk of foodborne illnesses caused by this organism (CHUNG *et al.*, 2006).

It has been reported that damaged produce supports the growth of foodborne pathogens, however, intact vegetables like lettuce, tomatoes, endive, carrots, cabbage, asparagus, broccoli and cauliflower may also permit the growth or survival of pathogens (OLAIMAT and HOLLEY, 2012). Several studies have investigated growth and survival of *E. coli* O157:H7 on the surfaces of leafy green vegetables including lettuce (LANG *et al.*, 2004b; MARKLAND *et al.*, 2013; MCEVOY *et al.*, 2009; CHANG and FANG, 2007), parsley (ISLAM *et al.*, 2004; LANG *et al.*, 2004b), spinach (MARKLAND *et al.*, 2013; LUO *et al.*, 2009), basil (MARKLAND *et al.*, 2013) and thale cress (COOLEY *et al.*, 2003). However, little information is available on the survival of stressed *E. coli* O157:H7 cells on the surfaces of leafy greens (MCEVOY *et al.*, 2009).

Since produce contamination may occur during pre- or post-harvest activities, different techniques have been used to reproduce contamination that may occur commercially. Spot inoculation, where a volume of inoculum containing a known cell density is applied at several locations on produce surfaces, can represent contamination that may occur from contact with soil, workers' hands, or equipment surfaces. Dip inoculation can represent contamination that may occur from run-off as well as irrigation, flume water use and water immersion which are common among industry practices. Additionally, spray inoculation can represent contamination that may result from aerosols (BEUCHAT *et al.*, 2003). Given the physical differences among each of the ways produce can become accidentally or deliberately contaminated, it became of interest to determine whether the experimental method used for fresh produce inoculation could influence inoculated pathogen survival or growth. Thus, the objective of the current study was to compare the recovery of unstressed or acid-, cold-, starved- and salt-stressed *E. coli* O157:H7 cells from the surfaces of rocket leaves inoculated by different methods and stored at 10° or 25°C.

MATERIALS AND METHODS

Preparation of bacterial strains and inocula

Four clinical isolates of *E. coli* O157:H7 (00:0304, 02:0627, 02:0628, and 02:3581) now in the Department of Nutrition and Food Technology, Jordan University of Science and Technology culture collection were stored individually at -80°C

in Trypticase Soy Broth (TSB; Oxoid Ltd., Basingstoke, UK) containing 20% (vol/vol) glycerol (Sigma-Aldrich, St. Louis, MO). Frozen stock cultures were activated by transferring one loopful from each culture to 10 mL of TSB and incubating at 37°C for 24 h. The strains were streaked on Sorbitol MacConkey Agar (SMAC) plates and stored at 4°C. One colony was transferred to 10 ml TSB and incubated at 37°C for 24 h. Equal volumes of each strain were mixed to prepare an *E. coli* O157:H7 cocktail which was centrifuged at 4,500 rpm for 20 min, the supernatant fluid was removed and the pellet was washed with sterile deionized water and then transferred to 10 mL of sterile deionized water. This suspension was diluted in sterile deionized water to achieve 10^8 CFU/mL.

Preparation of stressed *E. coli* O157:H7

Acid-stressed cells were prepared by transferring a loopful of each strain to 10 ml of TSB containing 10 g/L glucose and incubated at 37°C for 18 h where ~ 9 log CFU/mL was reached at a final pH 4.9 ± 0.1 (AL-NABULSI *et al.*, 2014; LEENANON and DRAKE, 2001). Equal volumes of each strain were mixed in sterile tubes to prepare a cocktail mixture containing equal numbers of each strain.

Salt-stressed cells were prepared by transferring a loopful from each strain into 10 ml of TSB supplemented with 0.65 M NaCl and incubated at 37°C for 18 h where ~ 9 log CFU/mL was reached. A cocktail was prepared containing equal numbers of each strain as described above and resuspended in 10 ml sterile deionized water (AL-NABULSI *et al.*, 2014; HAJMEER *et al.*, 2006).

Cold-stressed cells were prepared by inoculating a loopful of each strain into 10 ml of TSB at 37°C for 18 h where ~ 9 log CFU/mL was reached. A cocktail was prepared containing equal numbers of each strain as described above, was resuspended in 10 ml TSB and incubated for 7 d at 5°C (AL-NABULSI *et al.*, 2014; LEENANON and DRAKE, 2001).

Starved cells were prepared by inoculating a loopful of each strain into 10 mL of TSB which was incubated at 37°C for 18 h. A cocktail was prepared containing equal numbers of each strain as described above in saline solution (0.85% NaCl, pH 6.6) and incubated further for 48 h at 37°C (AL-NABULSI *et al.*, 2014; LEENANON and DRAKE, 2001).

Inoculation of leaf surfaces by spot, spray or dip methods

Rocket leaves were purchased from a supermarket in Irbid, Jordan on the day of each experiment. Damaged leaves were removed; intact leaves were washed with tap water and dried using a salad spinner. Unstressed and stressed *E. coli* O157:H7 cells were used to inoculate the rocket leaves to obtain an inoculum level of 7.0 log CFU/leaf. The following procedures were used for inoculation of rocket leaves: for spot inoculation, 50 µL cell suspension was added at different places on the surface of each leaf; for dip inoculation leaves were dipped in 100 mL of inocula prepared as described above for 1 min, and for spray inoculation 50 µL of inocula was sprayed on each leaf using a gas chromatography sample syringe connected to a nitrogen gas supply at 2 psi. Inoculated leaves were placed in a biosafety cabinet for 2 h to dry. After that, the leaves were incubated at 4°C for 22 h to allow to *E. coli* O157:H7 cells to attach to the leaf surfaces (LANG *et al.*, 2004), and samples were stored at 10 or 25°C for 7 d.

Microbiological analysis

The inoculated leaves were analyzed at 0.5, 1, 3, and 7 d after storage at 10 or 25°C. The leaves were transferred to a sterile stomacher bag, treated in a stomacher (Easy Mix, AES Laboratoire, France) for 2 min, serially diluted in 0.1 % peptone water and plated on Sorbitol MacConkey Agar supplemented with 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite (CT SMAC). The solidified CT SMAC (20 ml) had been overlaid with 10 ml TSA (thin agar layer format) to facilitate the growth of injured cells. Inoculated plates were incubated at 37°C for 18-24 h.

Statistical analysis

Data presented are means of three experiments with two replicates for each experiment (n=6). Values were analyzed by SPSS software, version 19 (IBM Inc., Armonk, NY) using a univariate general linear model. Differences were considered significant at $p \leq 0.05$.

RESULTS

Behaviour of unstressed *E. coli* O157:H7 recovered from leaf surfaces inoculated by spot, dip and spray methods

The initial number of *E. coli* O157:H7 applied to each leaf was ~ 7.0 log CFU/leaf by each of the three methods. However, significantly higher numbers of *E. coli* O157:H7 cells were recovered from the dip-inoculated rocket leaves (7.10 log CFU/leaf) compared to the spot- or spray-inoculated leaves (6.35-6.71 log CFU/leaf). Further, *E. coli* O157:H7 numbers recovered from the dip-inoculated leaves significantly increased and by 7 d reached 8.20 or 8.37 log CFU/leaf at 10 or 25°C, respectively. The spray and spot methods did not perform differently from each other, and numbers of the pathogen present on spray- and spot-inoculated leaves also increased during storage; however, changes (0.07-0.4 log CFU/leaf) were significantly smaller than with dip-inoculated samples (1.1-1.3 log CFU/leaf) (Table 1).

Table 1 - Viable count of unstressed *E. coli* O157:H7 cells on the surface of rocket leaves stored at 10° or 25°C after inoculation by three methods.

	Inoculation Method					
	10°C			25°C		
Day	Dip	Spot	Spray	Dip	Spot	Spray
0	7.10±0.75aB	6.71±0.41abAB	6.35±0.51aA	7.10±0.75aB	6.71±0.41abAB	6.35±0.51aA
0.5	7.95±0.08bB	6.74±0.52abA	6.43±0.21aA	7.10±0.44aC	6.71±0.32aB	6.24±0.29aA
1	7.98±0.08bC	7.19±0.58aB	6.43±0.20aA	7.67±0.59aB	7.21±0.62aA	6.35±0.76aA
3	7.92±0.85bB	6.50±0.74bA	6.59±0.83aA	7.72±0.08bB	6.81±0.61aA	6.73±0.46aA
7	8.20±0.44bB	6.78±0.48abA	6.72±0.63aA	8.37±0.11bB	6.89±0.46aA	6.78±0.54aA

Values in the same row at each temperature with the same uppercase letter are not significantly different ($p \geq 0.05$).
Values in the same column with the same lowercase letter are not significantly different ($p \geq 0.05$).

Behaviour of acid-stressed *E. coli* O157:H7 recovered from leaf surfaces inoculated by spot, dip and spray methods

The numbers of acid-stressed *E. coli* O157:H7 recovered from rocket leaves differed with the three methods and were ranked in the order of dip > spray > spot inoculation, although the numbers recovered following all inoculation methods were similar ($p > 0.05$). During storage, *E. coli* O157:H7 numbers recovered from dip-, spot- or spray-inoculated rocket leaves significantly increased, and increases by 7 d were 0.9 to 1.3 log CFU/leaf at 10°C and 1.2 to 1.6 log CFU/leaf at 25°C. Meanwhile, the highest *E coli* O157:H7 numbers present by 7 d storage at both temperatures were on rocket leaves inoculated by dipping (8.51-8.78 log CFU /leaf) and by spraying (7.79-7.93 log CFU /leaf) (Table 2).

Behaviour of cold-stressed *E. coli* O157:H7 recovered from leaf surfaces inoculated by spot, dip and spray methods

E. coli O157:H7 numbers recovered from the dip-inoculated leaves were significantly higher (7.53 log CFU/leaf) than those recovered from either the spot-inoculated (6.65 log CFU/leaf) or spray-inoculated leaves (6.48 log CFU/leaf). During storage, *E. coli* O157:H7 numbers recovered from rocket leaves, regardless of the inoculation method used, significantly increased (0.8-1.29 log CFU/leaf) at 10 and 25°C by 7 d (Table 3).

Behaviour of starvation-stressed *E. coli* O157:H7 recovered from leaf surfaces inoculated by spot, dip and spray methods

As with cold-stressed cells, numbers of starvation-stressed *E. coli* O157:H7 recovered from the dip-inoculated rocket leaves were significantly higher (7.50 log CFU/leaf) than those recovered from the spot- (6.64 log CFU/leaf) or spray-inoculated leaves (6.46 log CFU/leaf). During storage at 10 or 25°C, the numbers of *E. coli* O157:H7 cells recovered from rocket leaves inoculated using the three methods remained constant for 1 day, but after that there was a significant increase in their numbers (0.7-1.1 log CFU/leaf). Spot and spray inoculation methods had the same effect on *E. coli* O157:H7 numbers during storage; however dip inoculation enabled higher recoveries from the leaves at all storage intervals (Table 4).

Table 2 - Viable count of acid-stressed *E. coli* O157:H7 cells on the surface of rocket leaves stored at 10° or 25°C after inoculation by three methods.

	Inoculation Method					
	10°C			25°C		
Day	Dip	Spot	Spray	Dip	Spot	Spray
0	7.23±0.47aA	6.49±0.75aA	6.75±0.84aA	7.23±0.47aA	6.49±0.75aA	6.75±0.84aA
0.5	7.81±0.39bB	6.76±0.26aA	6.89±0.51aA	8.09±0.34aB	7.12±0.24aA	7.16±0.58aA
1	8.07±0.12bC	6.94±0.72abA	7.54±0.51bB	8.27±0.36abB	7.47±0.13abA	7.59±0.16abA
3	8.12±0.08bB	7.42±0.41bA	7.66±0.49bAB	8.50±0.18bcC	7.49±0.24abA	7.82±0.2bB
7	8.51±0.04cC	7.36±0.25bA	7.79±0.11bB	8.87±0.07cC	7.71±0.07bA	7.93±0.04bB

Values in the same row at each temperature with the same uppercase letter are not significantly different ($p \geq 0.05$).
Values in the same column with the same lowercase letter are not significantly different ($p \geq 0.05$).

Table 3 - Viable count of cold-stressed *E. coli* O157:H7 cells on the surface of rocket leaves stored at 10° or 25°C after inoculation by three methods.

	Inoculation Method					
	10°C			25°C		
Day	Dip	Spot	Spray	Dip	Spot	Spray
0	7.53±0.16aB	6.65±0.32aA	6.48±0.25aA	7.53±0.16aB	6.65±0.32aA	6.48±0.25aA
0.5	7.99±0.12bB	7.24±0.13bA	7.01±0.29bcA	7.53±0.51aB	6.90±0.57aA	6.76±0.48aA
1	7.81±0.68abB	7.23±0.15bA	6.69±0.66abA	7.86±0.10bB	7.06±0.57abA	6.97±0.51abA
3	8.15±0.06bcB	7.39±0.04bcA	7.34±0.03cA	8.35±0.04bC	7.52±0.06bcB	7.41±0.09bcA
7	8.43±0.09cB	7.45±0.08cA	7.38±0.09cA	8.39±0.05bC	7.94±0.07cB	7.62±0.07cA

Values in the same row at each temperature with the same uppercase letter are not significantly different ($p \geq 0.05$).
Values in the same column with the same lowercase letter are not significantly different ($p \geq 0.05$).

Behaviour of salt-stressed *E. coli* O157:H7 recovered from leaf surfaces inoculated by spot, dip and spray methods

Numbers of salt-stressed *E. coli* O157:H7 were also higher ($p < 0.05$) on dip-inoculated leaves than those of other treatments. Their numbers significantly increased on spray- or dip-inoculated leaves, and by 7 d reached 7.31 and 8.44 log CFU/leaf, respectively, at 10°C and 7.45 and 8.32 log CFU/leaf, respectively, at 25°C. However, there was no change in the numbers of *E. coli* O157:H7 recovered from spot-inoculated rocket leaves ($p > 0.05$). Increases during storage were 0.4 log CFU/leaf on samples spot-inoculated, but numbers on leaves from the other treatments increased 1.0 - 1.3 log CFU/leaf at both temperatures (Table 5).

Table 4 - Viable count of starvation-stressed *E. coli* O157:H7 cells on the surface of rocket leaves stored at 10° or 25°C after inoculation by three methods.

	Inoculation Method					
	10°C			25°C		
Day	Dip	Spot	Spray	Dip	Spot	Spray
0	7.50±0.19aB	6.64±0.30aA	6.46±0.27aA	7.50±0.19aB	6.64±.30aA	6.46±0.27aA
0.5	7.50±0.47aB	6.74±0.56aA	6.77±0.73abA	7.86±0.52aB	7.01±0.59aA	6.79±0.50aA
1	7.81±0.38aB	6.96±0.53abA	6.79±0.50abA	8.11±0.47abB	7.08±0.39aA	6.93±0.51aA
3	8.14±0.06bC	7.43±0.05cB	7.31±0.07bA	8.32±0.11bcB	7.54±0.07bA	7.44±.0.06bA
7	8.46±0.12bB	7.36±0.06bcA	7.21±0.43bA	8.51±0.06cB	7.54±0.07bA	7.54±0.08bA

Values in the same row at each temperature with the same uppercase letter are not significantly different ($p \geq 0.05$).
Values in the same column with the same lowercase letter are not significantly different ($p \geq 0.05$).

Table 5 - Viable count of salt-stressed *E. coli* O157:H7 cells on the surface of rocket leaves stored at 10° or 25°C after inoculation by three methods.

	Inoculation Method					
	10°C			25°C		
Day	Dip	Spot	Spray	Dip	Spot	Spray
0	7.10±0.91aA	6.94±0.38aA	6.45±0.26aA	7.10±0.81aA	6.94±0.38aA	6.45±0.26aA
0.5	7.81±0.37bB	6.86±0.49aA	6.82±0.55abA	7.86±0.52aB	6.99±0.60aA	6.81±0.52aA
1	7.86±0.38bcB	6.88±0.53aA	6.88±0.50abA	8.08±0.49aB	7.09±0.57aA	6.97±0.50aA
3	7.89±0.40bcB	7.02±0.50aA	6.94±0.52abA	8.13±0.51aB	7.20±0.53aA	7.30±0.52aA
7	8.44±0.07cB	7.36±0.15aA	7.31±0.17bA	8.32±0.11bB	7.38±0.44aA	7.45±0.06bA

Values in the same row at each temperature with the same uppercase letter are not significantly different ($p \geq 0.05$).
Values in the same column with the same lowercase letter are not significantly different ($p \geq 0.05$).

DISCUSSION

Different inoculation methods have been used experimentally to contaminate fresh produce in studies of the survival or inactivation of pathogens (AL-NABULSI *et al.*, 2014; LANG *et al.*, 2004 a,b; SINGH *et al.*, 2002). However, it is possible that the method chosen for inoculation may affect pathogen behaviours (survival, growth, injury, or death). In the present study three inoculation techniques (dip, spot and spray) were used and there was variability in the number of *E. coli* O157:H7 present on the leaves contaminated. It was found that dipping yielded larger numbers of unstressed or stressed *E. coli* O157:H7 cells on rocket leaves. This may have been because of the greater exposure of leaf surfaces, including cut surfaces where some cells could have been internalized. These results are similar to those obtained by LANG *et al.* (2004a) who showed that higher numbers of *E. coli* O157:H7 and *Salmonella* were recovered from dip-inoculated tomatoes compared to those spot- or spray–inoculated. In another study, LANG *et al.* (2004b) observed that applying the cell suspension to the surface of lettuce by dipping enhanced the internalization of bacteria at the cut edge and via stomata which can facilitate bacterial access to internal leaf tissue. The results of the current study also indicated that bacterial numbers recovered from spot-inoculated leaves were not significantly different from those recovered from those that were spray-inoculated. Similarly, LANG *et al.* (2004b) found that the number of *E. coli* O157:H7 and *Salmonella* recovered from lettuce when inoculated by spot or spray methods were similar. However, they recommended using the spot method as the standard for inoculation in studying the efficacy of sanitizers against pathogenic bacteria. SINGH *et al.* (2002) found that some sanitizers (thyme oil, aqueous chlorine dioxide, ozonated water) were less effective against *E. coli* O157:H7 on lettuce inoculated by dipping or sprinkling than by the spot or drop method. It should be noted that even when fresh produce was washed and sanitized using chemical agents such as chlorine, only a 1-2 log microbial reduction was achieved (OLAIMAT and HOLLEY, 2012).

Unstressed *E. coli* O157:H7 cells were able to grow when inoculated by dipping at 10 or 25°C, but cells inoculated by spraying or spotting survived without significant change in numbers at both temperatures over 7 d storage. These results are similar to those reported by CHANG and FANG (2007) who found that *E. coli* O157:H7 numbers on lettuce increased by 2.7 log CFU/g at 22°C, although they decreased by 1.4 log CFU/g at 4°C. FRANCIS and O'BEIRNE (2001) also reported that *E. coli* O157:H7 numbers increased by up to 2.5 log CFU/g after 12 d on spot-inoculated shredded lettuce, coleslaw and soybean sprouts at 8°C. LUO *et al.* (2010) found that storage of spray-inoculated lettuce at 5°C allowed *E. coli* O157:H7 to survive, but its growth was limited. At 12°C there was more than a 2.0 log CFU/g increase in *E. coli* O157:H7 numbers after 3 d storage. In contrast, MARKLAND *et al.* (2013) did not detect *E. coli* O157:H7 cells after 4 d on basil plants that were spray irrigated.

The behaviour of microorganisms in food products depends on the interaction of intrinsic and extrinsic factors such as temperature, pH, and water activity. Bacteria may encounter sub-lethal stresses in variety of food products, particularly minimally processed food such as fresh produce. Responses of bacteria to these stresses may enhance their survival under more severe conditions, enhance their resistance to subsequent processing conditions and perhaps enhance virulence. Thus, understanding the effects of environmental stress on the behaviour of pathogens is important in order to assess and minimize the risk of foodborne illness (CHUNG *et al.*, 2006). In *E. coli* O157:H7 exposure to stress can initiate several mechanisms to minimize the effects of the challenge. For example, the *rpoS* gene regulates expression of > 50 proteins that are involved in the general stress response. Also, heat and cold shock genes can play a major role in the level of expression of the response by stressed *E. coli* O157:H7. These mechanisms facilitate adaptation of *E. coli* O157:H7 to environmental change and increase its survival (CHAURET 2011). In the current study, numbers of stressed *E. coli* O157:H7 recovered by the three different methods increased significantly at 10° and 25°C. In contrast, MCEVOY *et al.* (2009) found that the behaviour of cold-stressed *E. coli* O157:H7 was similar to that of unstressed cells on fresh iceberg lettuce where the cold-stressed and unstressed cells grew significantly at 30°C, but survived without changes in their numbers at the non-permissive growth temperature of 5°C after 8 d. Several factors are likely to affect growth and survival of *E. coli* O157:H7 on fresh produce including its type (pH, surface smoothness, porosity, nutrient availability), physiological state, moisture, storage temperature > 7°C, and the identity of the bacterial strain. It was of interest from the present work that stressed cells of a 4 strain *E. coli* O157:H7 cocktail on Rocket leaves were able to increase in numbers during a week of storage at 10° and 25°C to similar extents, but unstressed cells did not.

In conclusion, it appears that the method used for bacterial inoculation of produce leaves can influence the levels of *E. coli* O157:H7 recovered from treated samples. The greatest uptake of cells from the inoculum occurred when leaves were dipped. Thus the importance of controlling the quality of water used in produce plant flumes and for produce immersion becomes apparent. Spot and spray inoculation yielded lower but similar levels of contamination. Thus produce contact with unclean equipment surfaces,

handling of produce in an unsanitary manner by employees and the occurrence of aerosols during processing can increase the bacterial burden that is likely to occur on the final product. Most importantly, when cells stressed by acidic pH, cold, starvation or salt exposure were inoculated on Rocket leaves, cells were able to grow slowly at both 10° and 25°C, whereas unstressed cells did not increase in numbers during 7 d storage. This unanticipated feature of *E. coli* O157:H7 may enhance its ability to be spread through shipments of produce during distribution, increasing risks associated with this foodborne pathogen.

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PAPER

INACTIVATION OF *ZYGOSACCHAROMYCES ROUXII* USING POWER ULTRASOUND AT DIFFERENT TEMPERATURES, PH AND WATER ACTIVITY CONDITIONS

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ABSTRACT

In this study, the effect of ultrasound treatments (20 kHz) combined with mild temperatures (thermo-sonication) on the inactivation of *Z. rouxii* was examined. Additionally, the effect of pH (4 and 7) and water activity (aw 0.99 and 0.94) of the sonication medium on yeast inactivation was determined. The $D_{(40\text{-}55)}$ values at a thermo-sonication amplitude of 80% were shorter than that obtained at 40%. Using thermo-sonication, particularly at a low aw, was associated with a significant synergistic effect for *Z. rouxii* inactivation ($p<0.05$). In most thermo-sonications at 50° and 55°C, the FDA requirement of a 5-log cycle reduction could be achieved (>5.7-log reductions in <0.2-0.2 min). Our findings show that sonication offers advantages in terms of reduced duration and temperature of pasteurization, without a reduction in structural and sensory quality particularly for fruit juices.

- Keywords: D value, inactivation, ultrasound, *Zygosaccharomyces rouxii* -

INTRODUCTION

The yeast *Zygosaccharomyces rouxii* represents a major cause of spoilage of foods and drinks that are packaged according to good manufacturing practices (GMP), including fruit juices, sauces, carbonated drinks, wine, salad dressings, and ketchups (JAMES and STRATFORD, 2003; PITT and HOCKING, 1985; LOUREIRO and MALFEITO-FERREIRA, 2003; FUGELSANG and EDWARDS, 2007; DEÁK, 2008). Typical physiological characteristics of *Z. rouxii* include tolerance to low-acidity preservatives, extreme osmotolerance, and the ability to adapt to high glucose concentrations, low water activity (aw) and thermal treatment (EMMERICH and RADLER, 1983; JAMES and STRATFORD, 2003; MARTORELL *et al.*, 2007). Thus, *Z. rouxii* is important to consider in examining spoilage during the processing of foods with low-acidity and high-sugar content.

The food industry most frequently uses traditional pasteurization methods such as low temperature long time (LTLT) and high temperature short time (HTST) to achieve shelf-life stability for fruit juices and drinks due to these methods' effectiveness and low cost. However, these procedures are associated with the loss of vitamins and volatile aromatic substances (KÖRMENDY, 2007; VASANTHA RUPASINGHE and LI JUAN YU, 2012). In addition to thermal pasteurization, other methods that are commonly utilized to prolong shelf-life include chemical preservatives such as potassium sorbate, sodium benzoate (VASANTHA RUPASINGHE and LI JUAN YU, 2012), citric acid and sulfur dioxide (WILEY, 1994; BATES *et al.*, 2001). Chemical preservatives used to prolong shelf-life may be associated with adverse health consequences in humans, depending on the characteristics of the consumer population and the frequency of consumption (ISMAN, 2000).

Although thermal treatment is the most common technique to inactivate microorganisms in food, there is an increased interest in the use of alternative food preservation methods as a response to consumer demand for food with conserved innate characteristics and no artificial preservatives (CORBO *et al.*, 2009; VASANTHA RUPASINGHE and LI JUAN YU, 2012; ALZAMORA *et al.*, 2003). Some of the non-thermal food preservation methods that may represent an alternative to thermal treatment include electric or magnetic fields, microwave radiation, ionizing radiation, high-intensity light pulses and high-hydrostatic pressure (CORBO *et al.*, 2009; DI BENEDETTO *et al.*, 2010). Additionally, power ultrasound (US) is a promising novel technology that minimizes the need for treatment, increases food quality, and conserves the characteristics and sensory qualities of the food. Power US is defined as the use of pressure waves between 20 and 100 kHz. The lethal effect of ultrasonic processing on microorganisms is achieved through the conversion of electrical energy to ultrasonic sound waves via the ultrasonic transducer and through the formation and collapse of vast numbers of small bubbles in each second during the propagation of ultrasonic waves within liquids. The quick formation and collapse of these bubbles (cavitation) creates very high local temperatures (5500°C) and pressures (50 MPa), which cause disruption of the cell wall and damage to the cell membrane and DNA (JIRANEK *et al.*, 2008; MANVELL, 1997; KNORR *et al.*, 2004; O'DONNELL *et al.*, 2010; CÁRCEL *et al.*, 2012; LEIGHTON, 1998; SORIA and VILLAMIEL, 2010). The duration and temperature of the procedure, the composition and volume of the liquid, and the form and dimensions of the microorganism are among the determinants of the antimicrobial efficiency of ultrasonic processes (BEVILACQUA *et al.*, 2013).

The possible areas of use for microbial inactivation by power US have been relatively well studied in the food industry. It has been reported that to achieve the FDA-required 5-log reduction in microorganisms, sonication should be used in combination with mild heat treatment and/or pressure (FDA, 2001; WALKLING-RIBEIRO *et al.*, 2009; BAUMANN *et al.*, 2005; D'AMICO *et al.*, 2006; UGARTE-ROMERO *et al.*, 2006; SALLEH-MACK and ROBERTS, 2007; TIWARI *et al.*, 2009). Many studies have reported the synergistic effect of the combination of non-thermal technologies and heat treatment on microbial inactivation (GUYOT *et al.*, 2007; LEE *et al.*, 2009; LEISTNER and GORRIS, 1995; RASO *et al.*, 1998; REDDY *et al.*, 2006; ROSS *et al.*, 2003). However, to our knowledge, there are no studies examining the combined effect of heat, pH and aw on *Z. rouxii* inactivation using US. Therefore, the aim of this research was to evaluate the effect of US with heat (thermo-sonication) on the inactivation of *Z. rouxii* at different pH and aw conditions. For this purpose, citrate buffer was chosen as the model medium, and the effect of thermo-sonication on *Z. rouxii* was tested under different pH and aw conditions. Thus, the optimum procedural parameters defined for *Z. rouxii* inactivation may be utilized as a model for the US-assisted pasteurization of real fruit juices and other drinks at mild temperature conditions.

MATERIALS AND METHODS

Maintenance of test strain

Zygosaccharomyces rouxii (NRRL Y-229) was obtained as a lyophilized culture from the ARS Culture Collection (Northern Regional Research Laboratory, United States Department of Agriculture, Midwest Area-National Center for Agricultural Utilization Research Microbial Genom-

ics & Bioprocessing Research Unit 1815 N University Street, Peoria, IL 61604). The culture tube was opened aseptically, the contents were transferred to a 2% Sabouraud Dextrose Broth (SDB, Merck, Germany), and the mixture was incubated for 48-72 h at 30°C. The stock cultures were then grown on Sabouraud Dextrose Agar (SDA, Merck, Germany) slants and stored at 4°C until use.

Preparation of yeast culture for inactivation studies

Z. rouxii subcultures were prepared by inoculating a test tube that contained 5 ml of sterile SDB with one single colony from a culture plate. The tubes were then incubated at 30°C for 48 h. Erlenmeyer flasks (250 mL) containing 50 mL of SDB were inoculated with this subculture. The flasks were incubated under agitation (130 rpm). The broth cultures were transferred to sterile centrifuge tubes, and pellets were obtained at 5500 rpm for 10 min. The pellets were then washed with saline water (0.85% NaCl) and resuspended in the same medium. *Z. rouxii* suspensions prepared in this way were used to inoculate sonication vessels at a final concentration of 10^8 CFU/mL.

Preparation of citrate buffer

All sonication and control group treatments in this study were applied in citrate buffer medium. Citrate buffer was prepared as two stock solutions (Stock Solution A: 0.1 M citric acid, $C_6H_8O_7.H_2O$ reagent, Carlo Erba, Italy; and Stock Solution B: 0.2 M di basic sodium phosphate, $Na_2HPO_4.2H_2O$, Merck, Germany). The final pH of the citrate buffer was measured using a pH meter (WTW InoLab 730, Germany).

Water activity (a_w) of the citrate buffer was adjusted to a_w 0.94 with glycerol (Merck, Germany). The aw values of the citrate buffer medium were measured at room temperature (23-25°C) with an AquaLab water activity meter (Decagon Devices, Inc., USA).

Combined treatments (Thermo-sonication treatments; TS-T)

Sonication was performed with a VC-750 Watt US generator and a Vibracell® WCX 750 (Sonics and Materials, CT, USA) model ultrasonic processor at a frequency of 20 kHz (maximum 124 μm amplitude). A solid sonication probe (13 mm in diameter) was used in all treatments. Levels of 40% (49.6 μm amplitude) and 80% ultrasonic power (99.2 μm amplitude) were applied in each case. Most of the sonication treatments were applied for 20 min. A 100 ml sterile water-jacketed vessel (Part No. 830-00010, Sonics and Materials, CT, USA) was used to hold the citrate buffer. The temperature of the citrate buffer in the vessel was controlled by a refrigerated circulating water bath (Polyscience-9102, IL, USA). The temperature of the medium in the vessel was monitored during the sonication process using the digital thermometer (Sonics and Materials, CT, USA) of the ultrasonic processor. The vessels and probes were sterilized at 121°C for 15 min before and after each experiment. The preparation of the sonication vessels and the sonication process are described below. Additionally, the experimental design of the combined treatments (TS-T at different medium conditions) and thermal treatments alone (T-T: control group treatments, at the same medium conditions) are summarized in Table 1.

(1) A total of 99 mL of citrate buffer was placed in a water-jacketed vessel.

(2) A sonication probe was immersed in the center of the vessel.

(3) The sonication procedure produces heat in a liquid medium; thus, to fix the temperature of the citrate buffer in the vessel at the target treatment temperature (40, 45, 50 or 55°C) during the sonication process, the temperature of the circulating water bath was adjusted to 7-10°C less than the target temperature. Then sonication was started.

(4) Immediately after reaching the target temperature, 1 mL of yeast suspension was added to produce a final concentration of 10^8 CFU/ml in the citrate buffer in the sonication vessel.

(5) At the beginning and during the treatment, 1 ml samples of citrate buffer samples were collected from the vessel and serially diluted in sterile saline water (1:10). If necessary, the sampling intervals and treatment times were adjusted (e.g., in the case of high temperature

Table 1 - Summary of the experimental design with thermo-sonication (TS-T) and thermal treatments (T-T) at different pH and aw levels.

Variables					
Treatment Temperatures	Sonication Levels	pH	aw	Treatments	
				TS-T	T-T
40°C, 45°C, 50°C and 55°C	-	4	0,99		+
			0,94		+
		7	0,99		+
			0,94		+
	40%	4	0,99	+	
			0,94	+	
		7	0,99	+	
			0,94	+	
	80%	4	0,99	+	
			0,94	+	
		7	0,99	+	
			0,94	+	

-: no sonication.

levels). Survival was determined using the drop-plate and spread-plate techniques. Aliquots of 0.02 ml (for drop-plate technique) or 0.1 mL (for spread-plate technique) were taken from the dilutions and plated on SDA. The plates were incubated at 30°C for 48 h, and counts of survivors in treated samples were conducted. All experiments were repeated at least two times.

Thermal Treatments (T-T) alone

The survival and growth of *Z. rouxii* was also determined in citrate buffer at different temperatures (40, 45, 50 or 55°C) and under different medium conditions (pH 4 and 7 and aw 0.99 and 0.94) without sonication. Treatments were performed in a shaking water bath (Memmert, Germany). The **T-T** process is described below and given in Table 1.

(i) A total of 99 ml of citrate buffer was placed in a flask.

(ii) To reach the target temperatures (40°, 45°, 50° and, 55°C), 99 mL of citrate buffer in flasks was pre-heated in a shaking water bath. The temperature of the citrate buffer in the flasks was monitored using a digital thermometer.

(iii) The citrate buffer reached the target temperature level.

(iv) One milliliter of yeast suspension was added to achieve a final concentration of 10^8 CFU/ml in the citrate buffer. This step corresponded to the beginning of the treatment time.

(v) During the treatment, 1 ml samples of the citrate buffer were collected from the flasks and serially diluted in saline water (1:10). The sampling intervals were 0, 1, 2, 4, 8, 12, 24 and 48 h. Viability counts were conducted as described above.

As shown in Table 1, 48 different (32 TS-T + 16 T-T) treatment conditions were studied to determine yeast inactivation, and each treatment was repeated in parallel at least two times.

Determination of D values

In this study, the inactivation of *Z. rouxii* was described using the first-order inactivation kinetic model. The D values were directly calculated from the k values (the slope of the inactivation curve) and the R^2 values.

First-Order Kinetic Model:

$$N / N_0 = e^{-kt} \text{ or } \ln N = \ln N_0 - kt$$

$$\log N = \log N_0 - k't$$

$$D = -\frac{1}{k'},$$

Where; No= initial cell number (CFU/mL),
t = treatment time (min),
N = number of the surviving cells (CFU/mL) after t minutes of treatment,
k = slope of inactivation curve (min^{-1}),
k´ = log of slope of inactivation curve (min^{-1}), and
D = decimal reduction time, or the time required for a 1-log cycle reduction in the microbial population.

Data were fitted to this model with a linear regression using the Microsoft Excel program. Additionally, log reductions (log CFU/mL) for each process were calculated using data of the initial and final yeast numbers in the vessel.

Viability of yeast cells in treated samples during storage

In this step of the study, we determined the growth of the survivors during storage at different temperatures (4° and 25°C). Two samples (10 mL) were taken from each treatment, with 5-log cycle reductions achieved; they were aseptically transferred into 10 mL double-strength glass bottles containing SDB and stored at 4° and 25°C for 60 d in the dark. During storage, 1 ml aliquots were taken predetermined intervals from each bottle and were then transferred into SDB; the tubes were incubated at 30°C for 3-5 days, and yeast growth was checked. The sampling intervals were 1, 7, 15, 30, 45 and 60 d.

Statistical analysis

Variance analysis was used to determine the effect of inactivation factors on D values. The plate-count data were logarithmically transformed for statistical analysis. The results ($\log_{10}$ CFU/ml) were subjected to an analysis of variance (SPSS Ver. 11.5, Chicago, Il, USA). For all experiments, a p value ≤0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

In the present study, the inactivating effect of ultrasound waves (20 kHz) on *Z. rouxii* was investigated in a model medium (citrate buffer). A total of 48 different experiments were performed to determine the effect of heat (40, 45, 50 and 55°C), pH (4 and 7), and aw (0.99 and 0.94) on ultrasonic inactivation (40 and 80% amplitude) of *Z. rouxii*. During the sonication procedure, periodical sampling from the sonication chamber was conducted to determine the number of viable cells of *Z. rouxii* (CFU/ml). A first-degree kinetics reaction was used to establish inactivation plots for *Z. rouxii* that were subsequently utilized to estimate the "D values" based on slope and R^2. Additionally, yeast reduction was determined based on a comparison of the cell numbers before and after the procedure. The difference in D values, as defined by the TS-T and T-T processes, were assessed using variance analysis.

Furthermore, the growth pattern of sublethally injured yeasts following TS-T and T-T processes were evaluated under different storage conditions (at 4° and 25°C for 60 d).

Inactivation of *Z. rouxii* at 40°C

An overall assessment of the results of all combined procedures at 40°C showed a smaller D value at 80% amplitude (0.94 aw and 0.99 aw; pH = 4 and pH = 7) than at 40% amplitude ($p<0.05$) (Fig. 1a). A generally reduced microbiological resistance to heat occurs in an acidic environment. However, in our study, the D_{40} values in the combined and thermal procedures at pH= 4 were statistically significantly higher than those at pH=7 ($p=0.012$).

The aw of the medium also had an impact on *Z. rouxii* inactivation. The D_{40} values estimated at 0.94 aw in all combined and thermal procedures were higher than those estimated at 0.99 w ($p<0.05$). Thus, a low aw was considered to give *Z. rouxii* a higher resistance to heat and sonication. Similar to this study, ALVAREZ *et al.* (2003) observed a 30-fold increase in the thermal decimal reduction time for *Salmonella enteriditis* by decreasing aw from 1 to 0.96, whereas only a two-fold increase was observed with mano-sonication, and a synergistic lethal effect with the combined use of heat and ultrasound was observed.

In our combined treatment procedures at 40°C, the reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 0.4-1.6 log CFU/mL and 0.8-3.6 log CFU/ml, respectively (Fig. 1b). In a study by BEVILACQUA *et al.* (2013), ultrasound was used to determine the reduction in several spoiling yeasts, including *Z. rouxii*, in fruit juices; similar to our observations, there was a maximum reduction of 1.7 log CFU/mL *Z. rouxii* in orange juice after sonication (40°C, 20 kHz, amplitude 60%, time 4 min, pulse 2 s).

According to the Hurdle concept, if the effect obtained via the combined use of two different inactivation factors is greater than the sum of the separate use of these methods, then a synergistic interaction is said to occur (LEISTNER and GORRIS, 1995). In the present study, treatment with a pH= 4 or 7 at 0.94 aw, with the combined use of ultrasound (40% and 80%) and heat, resulted in a significant synergistic interaction, although the D_{40} value was higher than that observed with an aw of 0.99. In control treatments performed at the same temperature, sonication at 0.94 (pH 4 and 7) and 0.99 aw (pH 4 and 7), the reduction in D_{40} values was, respectively 1/8-1/16 and 1/32-1/128 (Fig. 1a).

Inactivation of *Z. rouxii* at 45°C

The D_{45} values estimated for combined treatments at 0.94 aw were greater than those observed with 0.99 aw; however, the D_{45} values were lower than those obtained at 40°C (Fig. 2a). Overall, our results suggest that increased treatment temperatures resulted in increased yeast inactivation. Additionally, all sonications at 80% amplitude (0.94 aw and 0.99 aw; pH 4 and 7) had D values smaller than those found at 40% ($p<0.05$). The reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 0.5-2.0 log CFU/mL and 1.1-3.9 log CFU/mL, respectively (Fig. 2b).

In treatments at 0.99 aw (pH 4 and 7), a synergistic interaction for *Z. rouxii* inactivation was observed with the combined use of heat and ultrasound (40% and 80% amplitude). While synergy was present at 0.94 aw and pH values of 4 and 7 (40% and 80%), the D_{45} value was greater than that observed at 0.99 aw. Compared with control treatments at the same temperature and pH, the reductions in D_{45} obtained with the combined treatments at 0.94 and 0.99 aw were from 1/8-1/32 and 1/64-1/128, respectively (Fig. 2a). LOPEZ-MALO *et al.* (2005) assessed the sonication inactivation (20 kHz, 90 μm) of *Z. bailii* in 2% Sabouraud Glucose Broth with a pH of 3.5 and at three different aw (0.99, 0.97 and 0.95) and temperatures (45, 50 and 55°C) levels. Consistent with our findings, the D value at 45°C obtained with thermal treatment (TT) was significantly greater than that obtained with thermoultrasonication (TUT) ($p<0.05$). These authors found that at 45°C and at 0.99, 0.97, and 0.95 aw, the D value was reduced from 15.4 to 7.4, 26.8 to 8.6 and 43.5 to 12.9, respectively, with TT and TUT. Additionally, along with the reduction in aw, an increase in the D values was observed. Furthermore, a lower aw was associated with a greater synergistic effect in TUT. In the present study, the average D_{45} values of *Z. rouxii* for 0.99 and 0.94 aw at 45°C T-T (pH=4) were 98.66 and 140.1 min, respectively. In contrast, treatment TS-T under the same conditions (80% amplitude: 99.2 μm) resulted in D_{45} values of 0.58 and 4.33 min at 0.99 and 0.94 aw, respectively.

Inactivation of *Z. rouxii* at 50°C

In our sonication treatments, the minimum possible sampling interval from the sonication vessel was 20 s. Therefore, in some combined treatments, especially those conducted with high temperatures and high aw values (i.e., aw=0.99; 50 and 55°C), samples were taken after the first 20 s, and there were typically no viable yeast cells (for this reason, some D values in Figs. 3a and 4a are shown as <0.2 min). Additionally, yeast reductions are shown as >5.7-log CFU/mL because the maximum yeast reduction was determined as 5.7-log CFU/mL in this study (Figs. 3b and 4b).

Similar to our results obtained at 45°C, the estimated D_{50} for *Z. rouxii* at 50°C and 0.94

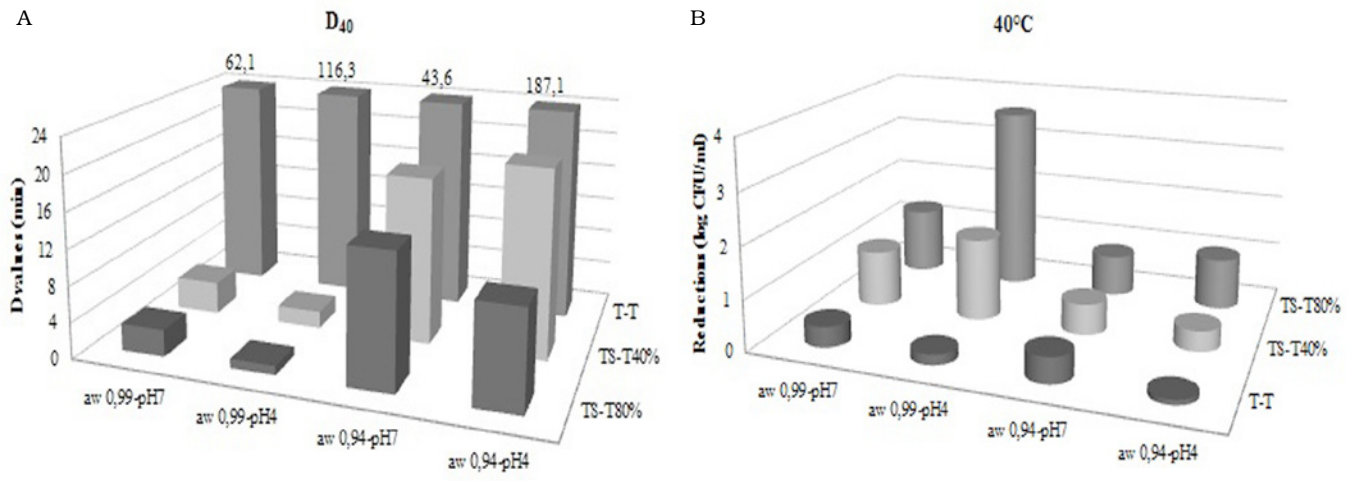


Fig. 1 - D_{40} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 40°C (B).

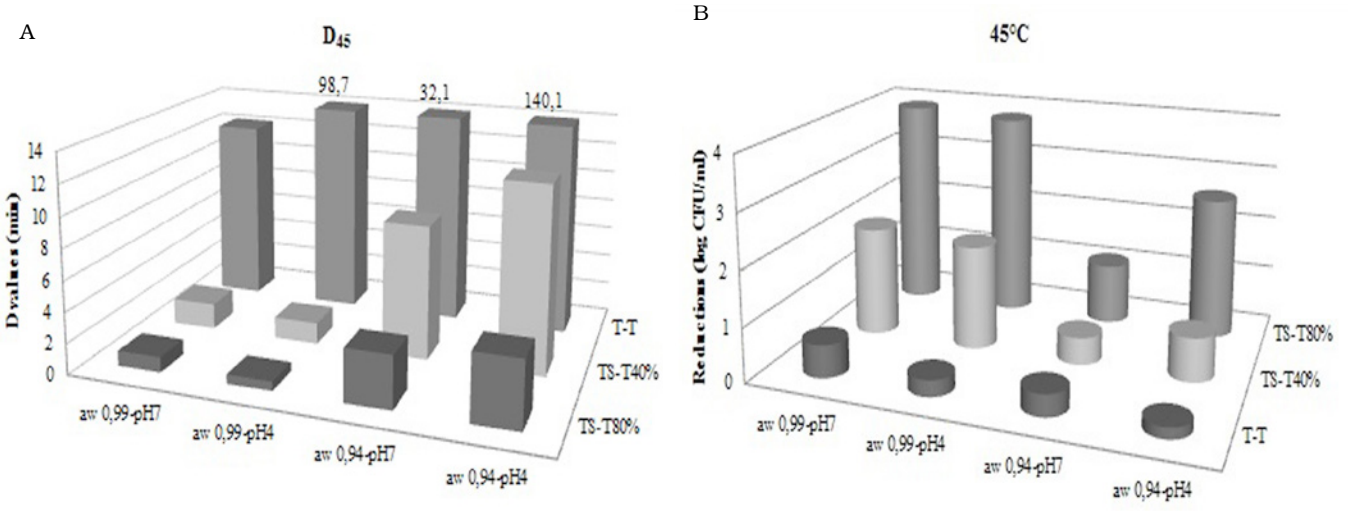


Fig. 2 - D_{45} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 45°C (B).

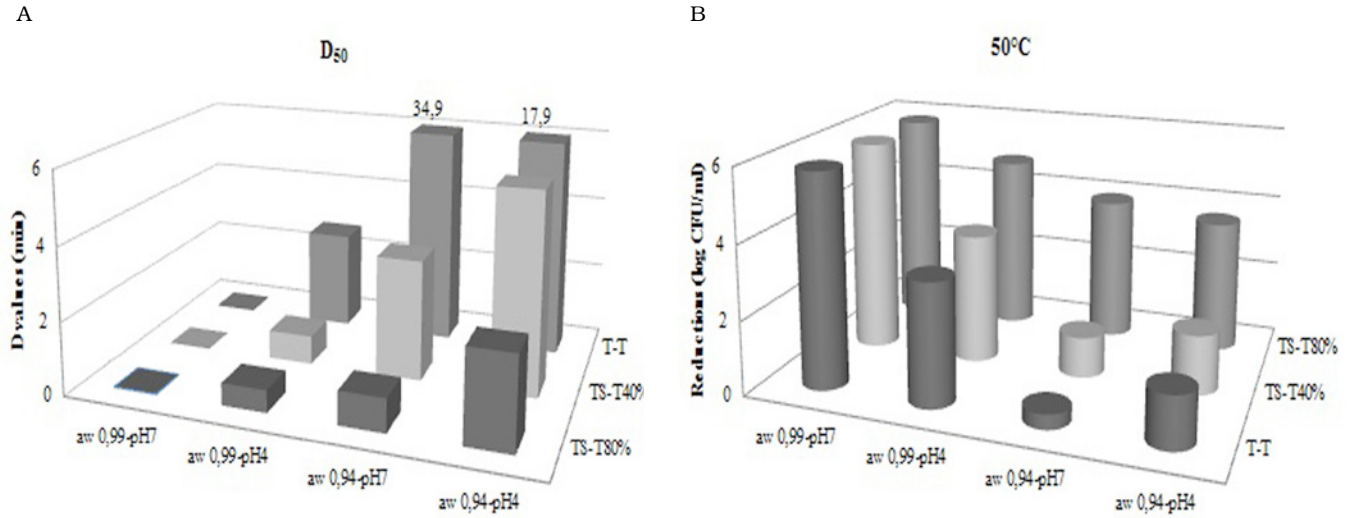


Fig. 3 - D_{50} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 50°C (B).

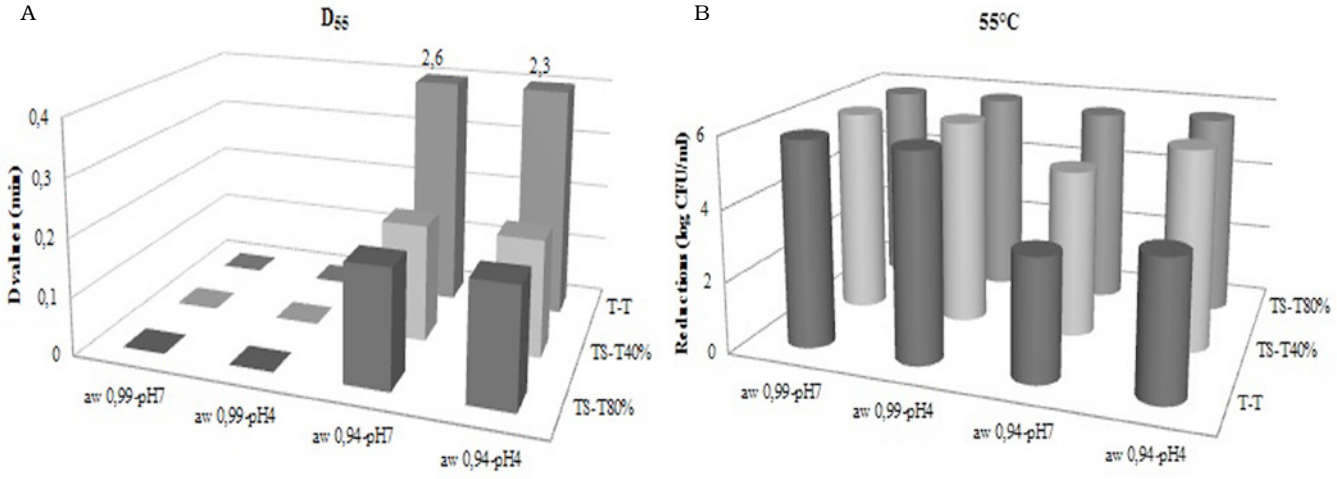


Fig. 4 - D_{55} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 55°C (B).

aw was greater than that observed at 0.99 aw (Fig. 3a). *Z. rouxii* showed a greater resistance to combined treatments at a pH of 4 than at a pH of 7, most likely because *Z. rouxii* is a yeast with good adaptation to lower pH values. The maximum D_{50} at 0.99 aw and pH 4 was 0.8 minutes, whereas the D_{50} value at pH 7 was <0.2 minutes. In treatments at 0.99 aw and pH 4, the combined use of ultrasound (40% and 80%) provided a significant synergistic interaction for *Z. rouxii* inactivation. However, no such synergy could be observed at 0.99 aw and pH 7 for the combined treatment. A synergistic effect could be observed at 0.94 aw, with pH values of 4 and 7, with sonication (40% and 80%), and with D_{50} values greater than that observed with 0.99 aw. Compared with controls under the same temperature conditions, the reduction in D_{50} in sonications of 0.94 aw (pH=4 and 7) and 0.99 aw (pH = 4 and 7) was 1/4-1/16 and 1/3-1/6, respectively. And the reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 1.1->5.7 log CFU/ml and 3.6->5.7 log CFU/ml, respectively (Fig. 3b).

Inactivation of *Z. rouxii* at 55°C

Compared with control treatments, the D_{55} values obtained with the combined treatments at 55°C and 0.99 aw suggested that the use of ultrasound did not result in a significant differences (p>0.05) in yeast inactivation and that heat was the primary determinant of inactivation.

In all combined treatments (at 0.94 aw), the D_{55} values for *Z. rouxii* were determined to be 0.2 minutes; at 0.99 aw, the D_{55} values were determined to be <0.2 minutes (Fig. 4a). Compared with controls at the same temperature levels, the reduction in D_{55} at sonications at 0.94 aw and pH levels of 4 and 7 was 1/8. In a similar study by GUERRERO *et al.* (2001), the inactivation of *S. cerevisiae* was examined at different amplitude levels (20 kHz, 71.4 and 107.10 μm), pH values (3 or 5.6), and temperatures (35°, 45°, and 55°C) in Sabouraud broth. In line with our findings, the D value at 55°C was lower that obtained at other temperature levels (i.e., 35° and 45°C) (p<0.05), whereas sonication, amplitude, and medium pH were not associated with a change in that reduction. However, in our study, the combined treatment with 0.94 aw resulted in an increased yeast inactivation (p<0.05), regardless of the pH and amplitude, and was associated with a synergistic effect. The D_{55} values obtained for all sonication procedures at 80% were lower than those obtained at 40%, although the differences were not statistically significant (p>0.05).

The reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 4.7->5.7 log CFU/mL and 5.6->5.7 log CFU/mL, respectively (Fig. 4b).

Viability of yeast cells in treated samples during storage

The growth during storage of sublethally injured yeast after combined treatments was tested. Samples were taken from treatments in which the 5-log cycle yeast reductions had been achieved (Figs. 3b and 4b) and stored for 60 d under different storage temperatures (4° and 25°C). As a result, none of the samples exhibited yeast growth during storage. This findings suggests that thermo-sonication is associated with irreversible cell damage. In a study by MARX *et al.* (2011) examining the effect of continuous and pulsed thermo-sonication (20 kHz frequency, at 60°C, 100% amplitude, for 30 min) on *S. cerevisiae* inactivation, the structural damage occurring in yeast cells after treatment was examined using scanning electron microscopy. They observed more broken cells using continuous rather than pulsed thermo-sonication treatments; however, they did not find any viable cells in their samples.

CONCLUSIONS

Compared with controls, all thermo-sonication procedures at 40, 45, 50 and 55°C resulted in a significant decrease in the D values (p=0.00) for *Z. rouxii* in our study. This finding shows a decreased resistance of *Z. rouxii* cells to heat together with the use of US.

The amplitude of the ultrasound waves was effective in the reduction of yeast cells, with lower D values obtained at the 80% amplitude than at 40%.

The use of US, particularly in medium with a low aw, resulted in significant synergistic effects for *Z. rouxii* inactivation. However, thermo-sonications performed at low aw (0.94) were associated with a more prolonged D value and a less marked reduction. Additionally, low aw was associated with the relative protection of yeast cells against thermo-sonication, particularly at lower temperatures.

Furthermore, as the sonication temperatures increased, the effects of amplitude, medium pH and aw on yeast reduction tended to weaken. Increased sonication temperatures (50° and 55°C) resulted in significant yeast inactivation (>5.7-log reductions). In most of the combined treatments at 50° and 55°C, the FDA requirement of a minimum of 5-log cycle reduction (within <0.2-0.2 min) could be met. However, although heat was the primary determinant of the yeast inactivation in combined treatments with high aw (55°C), the synergistic effect of US was more prominent than at 0.94 aw. The absence of yeast growth at 60 d that was observed in the samples obtained from the sonication chamber after combined treatments indicates that thermosonication was associated with irreversible yeast damage.

The findings of this study indicate that US combined with mild heat treatments (50° and 55°C) has the potential to inactivate *Z. rouxii* in fruit juices and beverages as an alternative to traditional pasteurization methods. Particularly for pasteurizing fruit juices to retain their structural and sensory qualities at higher temperatures, the use of US may offer certain advantages with respect to reducing the duration and temperature of the treatment.

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PAPER

EVALUATION OF FRUIT LEATHER MADE FROM TWO CULTIVARS OF PAPAYA

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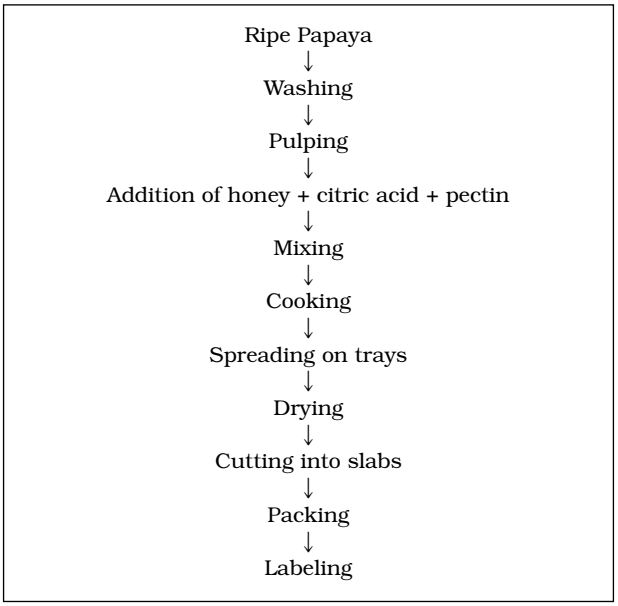
ABSTRACT

Two papaya cultivars were used to manufacture fruit leather. The objective of this study was to formulate papaya leather from locally grown papaya using natural ingredients like pectin, honey and citric acid. The fresh fruits were pureed and mix with natural ingredients, and dried in an oven at 60°C for 12 hours. The physicochemical properties and antioxidant activity were determined. The results showed that fruit leather made from Hongkong cultivar is significantly ($P<0.05$) higher in sensory parameters as well as physicochemical properties and antioxidant activity. The phenolics content and antioxidant activity increased by process of drying the fruit leather compared to fresh fruits in both papaya cultivars. Therefore, the consumer requirements for healthy and safe food products were respected.

- Keywords: fruit leather, antioxidant activity, phenolic content, sensory evaluation -

INTRODUCTION

Like numerous fruits and vegetables, papaya is a rich source of antioxidants. Antioxidants have a neutralising effect on free radicals, which are unstable molecules that can trigger a range of diseases, including cancers, cardiovascular and neurodegenerative diseases (PRIOR *et al.*, 1998). Naturally occurring antioxidants have been examined by (PRAKASH, 2010), who discerned that disease risk is reduced by such antioxidants as vitamin C, vitamin E, carotene, phenolic acids, phytate and phytoestrogens. Similarly, epidemiological research has emphasised the important role of antioxidants derived from fruits and vegetables in preventing degenerative processes (AMES *et al.*, 1993). Papaya fruit can be eaten fresh or as part of different processed foods, including baked products, beverages, cereals, confectionery, dairy snacks and sauces (USHBC, 2010). The demand for papaya as dried fruit is also high, alongside sultanas, peaches and apricots (LOHACHOOMPOL, 2007). Healthy products with dried papaya include breakfast cereals, energy bars and fruit snacks. In addition, there is a range of other papaya-based products, such as jam, jelly, papaya toffee, papaya bar, papaya squash, papaya soft drinks, papaya pulp powder, and others SWAMY and PREMNATH (2010). Fresh papaya is a seasonal fruit with a shelf life between one and two weeks. To respond to consumer demand, with fresh substitute is necessary to ensure year-round availability and drying is the most commonly used preservation method. As explained by (TESHOME, 2010), the drying process entails eliminating as much water as possible from the fresh fruit in order to inhibit enzyme and bacterial activity, thus halting decomposition. There are various types of drying processes, including sun drying, oven drying, cabinet drying, dehydrator drying and freeze drying. Based on food type, from 2 to 30% of water is left in the dried foods. In addition to prolonging product shelf life, water content reduction ensures that the product is stable from a microbiological perspective and minimises deteriorating chemical reactions. Fruit leathers are referred to the dried sheets of fruit pulp that taste sweet and have a soft, rubbery texture. Their production involves the dehydration of fruit puree to a leathery sheet (RAAB and OEHLER, 1999). In this regard, the study had two goals: (a) to use locally grown papaya to make fruit leathers based by using on natural ingredients like pectin, honey and citric acid, as well as to determine the cultivar most suitable for the production of papaya fruit leather; (b) to analyse the extent to which the drying process affects fresh and processed papaya in terms of antioxidant content, physic-chemical properties and sensory evaluation.



A process flow chart of papaya leather production.

MATERIALS AND METHODS

Samples collection and preparation

Papaya (*Carica papaya* L. cv. Hongkong and Eksotika) fruits at the mature stage of ripening were collected from Pusat Flora Cheras, Jabatan Pertanian, and Hulu Langat Semenyih in Selangor, Malaysia. The fruits were selected to ensure uniformity in size (800 g to 1000 g) and color as well as to ensure freedom from diseases and infection. The selected fruits were transferred on the same day to the University Kebangsaan Malaysia food laboratory, Bangi. The other three major ingredients used in the trials were honey (Polleney honey, Chaina), pectin (Germany) and citric acid (USA).

Procedure for making papaya fruit leather

For each cultivar, frozen papaya cultivars were thawed at 4^0C overnight in the fridge. Six hundred grams of thawed papaya cultivars were weighed. Honey 10% (v/v), 2% (v/v) of citric acid and % 6 (v/v) of pectin were weighed and mixed with papaya fruits. A Cascade blender model CE071BR (Japan) was used to mix all these ingredients for 2 minutes to make a puree. Cooking oil was lightly sprayed over trays made of stainless steel before 200 g of puree was spread uniformly over the trays with a metal spreader. The drying of the leather was done in the middle section of the cabinet dryer, which had been preheated to 60° ± 2°C. Throughout the drying interval, the dryness of the leather was closely monitored. Two batches were made for every cultivar each of them has three trays. The trays were dried for 12 hours for both papaya cultivars.

Physiochemical properties of papaya fruits

Moisture content was measured by drying sample at 105°C overnight in Memmert Oven (Germany). Titratable acidity (TA) was determined from 10 ml of sample diluted with 50 mL of water, titrated with 0.1 N NaOH and calculated as percent citric acid. Total soluble solids (TSS) were measured with an abbe refractometer at 20°C and pH was determined using pH meter using juice extracted directly from pulp.

Humidity content

The moisture content was determined by drying samples of approximately 1 g at 105°C in an forced air oven (Watson Victor Ltd, NZ) for 24 hours. The textural of papaya leather were conducted with a Stable Micro System TA-EZtest/ AGS-H- Japan).

Texture analyzer

The procedures for operating the texture analyzer were stated in the Standard Operating Procedure (SOP). The following parameters were determined: hardness (g/f). The pulp color was longitudinally determined on four points of each flat side of the fruit using a Minolta CR-300 colorimeter. The (L*) value represented the luminosity of the fruit, where 0 = black and 100 = white but the (a*) value ranged from the negative (green) to the positive (red) scale and the (b*) value ranged from negative (blue) to positive (yellow), (AOAC 1998).

Antioxidants extraction

Papaya were peeled, cut into 1 cm slices and crushed in a food processor to produce uniform slurries. The mixture was prepared fresh to preserve the extracted antioxidant compounds. In the extraction process, about 1 g of papaya slurries were weighed in universal bottles and 10 ml solvent was added. Solvents used were 50% aqueous methanol; samples (papaya slurries with solvents) were then homogenized using homogenizer (T 250, IKA, Germany) at 24,000 rpm for 1 min. All extracted samples were centrifuged by using tabletop centrifuge (MLX 210, Thermo-line, China) at 4750 *g* for 10 min. The supernatants were collected for further analysis.

Total phenol content (TPC)

Antioxidant activity was determined using TPC based on the method of (MUSA *et al.* 2011). Approximately 0.4 mL distilled water and 0.5 mL diluted Folin-Ciocalteu reagent were added to 100 µL papaya extracts. The samples (papaya extracts with Folin-Ciocalteu reagent) were set aside for 5 min before 1 mL 7.5% sodium carbonate (w/v) was added. The absorbances were taken at 765 nm wave length using a spectrophotometer after 2 h. The calibration curve of gallic acid (GA) was used for the estimation of sample activity capacity. The result was recorded in terms of mg of GA equivalents per 100 g of fresh sample (mg GA/100 g of FW).

Total flavonoid content (TFC)

The TF content was determined by the colorimetric method as described by (ABU BAKAR *et al.*, 2009). A total 0.5 mL of the extract was mixed with 2.25 mL of distilled water in a test tube, followed by the addition of 0.15 mL of 5% (w/v) $NaNO_2$ solution. After 6 min, 0.3 ml of a 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the reaction was allowed to stand for another 5 min before 1.0 ml of 1 M NaOH was added. The mixture was mixed well by vortexing, and the absorbance was measured immediately at 510 nm using a spectrophotometer (Epoch, Biotek, USA). The results were expressed as milligrams of quercetin equivalents (QE) per 100 g of fresh sample (mg QE/100 g of FW).

Ferric reducing antioxidant power (FRAP)

First, 300 mM acetate buffer FRAP reagent was prepared fresh as follows: pH 3.6 (3.1 g sodium acetate trihydrate plus 16 mL glacial acid made up to 1:1 with distilled water); 10 mM 2,4,6-tris (2-pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl; and 20 mM FeCl3·6H2O in the ratio of 10:1:1 to provide the working reagent. In addition, approximately 1 mL FRAP reagent was added to 100 µL papaya extracts, and the absorbances were taken at 595 nm wavelength using a spectrophotometer after 30 min. The calibration curve of Trolox was established to approximate sample activity capacity. The result was recorded as mg of Trolox equivalents (TEs) per 100 g of fresh sample mg (TE/100 g of FW) (MUSA *et al.*, 2011).

DPPH Radical scavenging activity

Based on the method of (Musa *et al.* 2011) the antioxidant activity was assessed using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging system. The stock solution was obtained by dissolving 40 mg DPPH in 100 mL methanol, which was stored at -20°C until further use. Approximately 350 mL stock solution was mixed with 350 mL methanol to obtain the absorbance of 0.70±0.01 unit at 516 nm wavelength by using a spectrophotometer (Epoch, Biotek, USA). In the dark, approximately 100 µL papaya extracts with 1 mL prepared methanolic DPPH solution was stored overnight for scavenging reaction. The percentage of DPPH scavenging activity was determined based on the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100,$$

where A is the absorbance.

ABTS assay

The ABTS radical cation (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was generated by the interaction of ABTS (250 μM) and $K_2S_2O_8$ (40 μM). After the addition of 990 μL of ABTS solution to 10 ml of fruit extract, the absorbance at 734 nm was monitored. The percentage decrease of the absorbance was calculated and plotted as a function of the concentration of the extracts and Trolox for the standard reference data (Özgen *et al.* 2006). The following formula was used:

$$\text{Percentage (\%) of reduction power} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100,$$

where A is the absorbance.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted according to (HUANG *et al.*, 2002). The ORAC assay was carried out on a fluorescence microplate reader (FLUOstar Omega, BMG LABTECH, Multi-Detection Microplate Reader, Germany). Peroxyl radicals were generated by AAPH, and fluorescence microplate reader was used at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Trolox was used as standard (50, 25, 12.5, 6.25, 3.12 mM). Proper dilutions of papaya extracts were made with ORAC buffer (potassium phosphate buffer, pH 7.4). For each ORAC run, a micro plate was prepared containing 25uμ of Trolox standards, buffer control, and sample dilutions, as well as 150ul of fluorescein (FL) solution. All ORAC analyses were performed at 37^{0}C with a 20 min incubation and 60 min run time. After the incubation, 25ul of AAPH was added to each well for a final volume of 200 uL. The results were calculated using the differences of areas under the FL decay curves between the blank and a sample and were expressed as micromole Trolox Equivalents per gram of sample (μmol TE/g).

Sensory evaluation

A consumer acceptability sensory trial was conducted at University Kebangsaan Malaysia in the sensory evaluation laboratory. Panellists comprised 30 volunteers who were staff or students at the University. Each panellist was asked to taste two samples, one from both cultivar (2 x 2 cm square). Attributes selected for the papaya fruit leather were colour, sweetness, sourness, flavour, texture and overall appearance. In this study, the hedonic scale was implemented; on a scale of 1 to 7 there were tabulations of scores, where 1 indicates "extremely dislike" and 7 represents "extremely like" (AMINAH, 2004). For reliability purposes, distilled water was given to the panelists for them to rinse the mouths between evaluations.

Statistical analysis

Data were expressed as the means values ± standard deviation. Mean of minimum three measurements were compared by analysis of variance (ANOVA). Significant differences between means were determined by Duncan ($P<0.05$). Correlation analysis was performed using Pearson's. The software used was SPSS ver.19. (BRYMAN and CRAMER, 2012).

RESULTS AND DISCUSSION

Physicochemical properties of papaya

The pH, titratable acidity and TSS for the two papaya cultivars are shown in Table 1. The cultivars exhibited considerable differences in terms of pH ($P<0.05$). The Hongkong cultivar had a higher level of pH (5.47), while the Eksotika cultivar had a lower pH (5.34). In comparison to fresh fruit, drying caused a substantial decline in the pH of all fruit leathers ($P<0.05$). Furthermore, there were significant discrepancies between the average pH of the Hongkong cultivar (3.93) and that of the Eksotika cultivar (3.82). Likewise, (HARSIMRAT, 1998) demonstrated that acidity has a positive effect on shelf life. By contrast, (BABALOLA *et al.*, 2002) found that papaya leather stored in a cool environment for 30 days had a higher pH compared to other samples. Similar findings were obtained in the case of pineapple leather (PHIMPHARIAN *et al.* 2011), mango leather (AZEREDO *et al.*, 2006) papaya and guava leathers (BABALOLA *et al.*, 2006). The present study revealed that the titratable acidity differed substantially between the cul-

Table 1 - Effect of processing on pH, TA and TSS of two papaya cultivars. Result showed mean ± standard deviation.

Cultivars	pH		TA		TSS	
	Fresh	Leather	Fresh	Leather	Fresh	Leather
Hongkong	5.47±0.02^a	3.93±0.01^b	0.15±0.01^b	1.63±0.02^a	11.74±0.52^b	68.50±0.57^a
Eksotika	5.34±0.03^a	3.82±0.01^b	0.17±0.02^b	1.48±0.03^a	12.46±0.14^b	70.50±0.60^a

$^{a-b}$ Mean with different letters within each raw are significantly different ($P< 0.05$).

tivars of both fresh and dried papaya. Hongkong and Eksotika had the highest average total acid content in fresh fruit, with 0.15% and 0.17%, respectively, of citric acid (reference). In the case of all fruit leathers, drying determines a considerable increase in the titratable acidity. As shown in Table 1, the highest acidity among fruit leathers was shown by the cultivar Hongkong (1.63%), while the lowest was exhibited by Eksotika (1.48%). In keeping with BEAUDRY *et al.* (1992), the results of this study indicated that the titratable acidity (TA) of fresh papaya is between 0.3 ± 0.1 to 0.7 ± 0.1% of citric acid equivalent. The addition of 4% of citric acid to the fruit leather puree increased the TA of the two papaya leather cultivars. In addition, drying contributes to concentration in the fruit's natural acidity, leading to a significant increase in the acidity of the fruit leather. Among all cultivars, Eksotika displayed the highest levels of titratable acid (0.17%). However, after drying, TA was highest in leathers with concentrations between 1.48 and 1.63 %. The high levels of acidity in fruit leather not only inhibit bacterial growth, but also protect the colour and flavour of the fruit. Hence, in terms of processing or manufacturing, papaya cultivars with high acidity should be used. This study also found that Hongkong cultivars is most appropriate for fruit leather production. VAIDYA *et al.*, (2007) reported the acidity of fruit leather made from kiwifruit which was found to be 3.8% but the reason for the high acidity was not discussed. Vega Galvez *et al.* (2009) reported an acidity of 2.2 ± 0.12% (monohydrated citric acid), pH of 2.7 ± 0.09, and soluble solids of 15.0 ± 0.07 °Brix in dried O'Neil blueberries. The two cultivars also differed greatly with regard to TSS ($P<0.05$). The TSS of Hongkong cultivar fresh fruit was 11.74 while Eksotika fruit cultivar was 12.46. Although there is a substantial increase in the °Brix of all fruit leathers after drying, with a TSS of 68.50 and 70.50 respectively for both papaya cultivars (Hongkong and Eksotika). The two cultivars did not display noticeable discrepancies. All processed papaya leathers had higher TSS than fresh fruit. The higher levels of °Brix in fruit leathers compared to fresh fruit, particularly sweet fruit, had already been noted in earlier research. For example, the addition of ingredients, such as pectin, glucose, syrup, and sugar, to raw pineapple puree increased the TSS of the latter between 66.4 and 75.3 °Brix. The pineapple fruit leather had a final TSS of between 82.4 and 86.9 after drying (PHIMPHARIAN *et al.*, 2011). In the case of kiwi fruit, (VAIDYA *et al.*, 2007) observed that the addition of 15% sugar increased its °Brix, which became even higher (68 °Brix) after the fruit was dried in a cabinet drier for 15 hours at a temperature of 45° ± 2° C. The present study used honey as an added sweetener. In this research, creamed honey (83 °Brix) was added as a sweetener. The °Brix found in creamed honey was similar to that found by ANUPAMA *et al.* (2003). The °Brix of blueberry fruit leathers increased due to the addition of 15% of honey. The high °Brix of blended papaya leather was explained by (KUMAR *et al.*, 2008) in terms of a high carbohydrate content, making it a good energy source.

Humidity content of fresh fruit

As illustrated in Fig. 1, the moisture content of two papaya leathers were 14.31% and 15.42%, (Hongkong and Eksotika) respectively. The fruit leathers from different cultivars exhibited discrepancies with respect to moisture content levels ($P<0.05$). In this study, given the relative reduced moisture content (22%-24%), all cultivar leathers can be classified as concentrated or intermediate moisture foods. Although the product thickness may be one of the reasons for high moisture content in this product. Nevertheless, the final product thickness was decreased from 4 mm to 1 mm. Based on their research on hot air drying of grape leather, MASKAN *et al.* (2002)

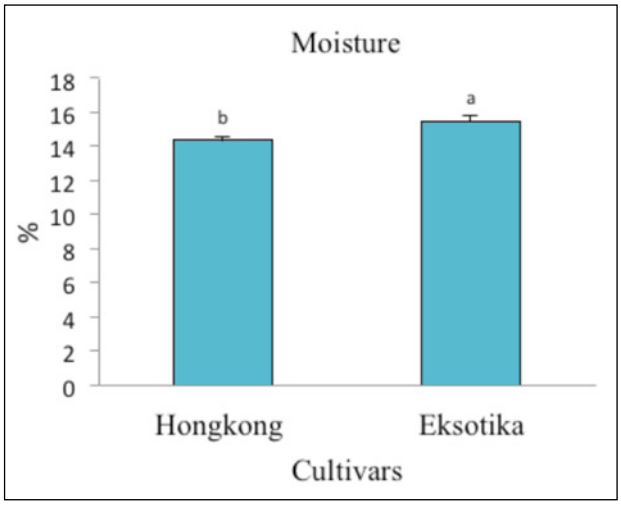


Fig. 1 - Effect of processing on moisture of two papaya cultivars.
[a-b] Mean with different letters are significantly different ($P < 0.05$).

argued that drying of the product surface occurs rapidly at high temperatures, particularly in the case of thinner samples. The moisture content of jackfruit leather, papaya leather and blended papaya leather was determined to be 11-17% (CHE MAN *et al.*, 1992), 12-13% (CHAN and CAVALETTO, 1978) and 20.80% (KUMAR *et al.*, 2008), respectively. However, IRWANDI *et al.* (1998) emphasized that, despite suppressing bacterial development and extending shelf life, a low moisture content of fruit leathers may have an adverse effect on texture quality. HUANG and HSEIH (2005) found that increasing the pectin concentration (from 1 to 1.5%) affected the hardness of the sample and decreased moisture content and aw of pineapple fruit leathers had an a_w value of < 0.55. Similarly, PHIMPHARIAN *et*

al. (2011) reported that both moisture content and water activity were influenced by the pectin concentration. What is more, the pectin concentration also affected aw on pear fruit leather.

Texture of papaya leather

The papaya leather cultivars in Hongkong and Eksotika had a range texture (Fig. 2) of 490.48 - 483.60 respectively. A possible cause for the high texture was the pectin, which generated a firm gel structure followed by a tough texture. The high texture due to the 6% pectin concentration used in this study was combined with the reduced moisture content of the papaya leather. Reduced moisture content and harder texture are the outcome of higher temperatures and extended drying periods (CHE MAN, 1995) and (OKILYA *et al.*, 2010). A comparison was difficult to achieve due not only to the different genetic structure of this fruit (BABALOLA *et al.*, 2002), but also to extra ingredients that influenced the texture quality. GUJRAL and KHANNA (2002) found that

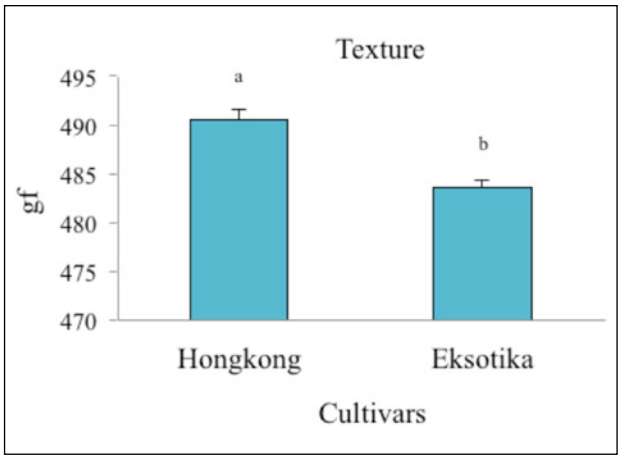


Fig. 2 - Effect of processing on texture of two papaya cultivars.
[a-b] Mean with different letters are significantly different ($P < 0.05$).

the tensile force in the mango leather was reduced by increased levels of sucrose (ranging from 4.5% to 9%). The texture decreased even more when skim milk powder was added, in comparison to soy protein concentrate. In this study, it is probable that the texture or extensibility of papaya leathers was affected by the pectin, honey and citric acid that were added. HUANG and HSIEH (2005) obtained a hardness value for pear fruit leathers of between 4420 and 13200 g (18 formulations with various water, pectin and corn syrup ratios). There were also differences in terms of ingredients, while the texture of the leathers may have been influenced by water absorption and the protein content of the fruit (BABALOLA *et al.*, 2002). By contrast to the results of HUANG and HSIEH (2005), the values obtained in this study were considerably lower.

Colour measurements

The final fruit leather product (Table 2) was lighter in colour (L* mean value of 32.10 and 30.70) less than the fresh fruit (L* mean value of 48.52 and 45.43). All cultivars decreased in brightness (L*) indicating that fresh papaya had a lighter colour compared to the fruit leathers. This was expected as the drying and addition of pectin, honey and citric acid to papaya puree can have significant effects on the colour of the papaya fruit. For example, citric acid is a strong acid and the addition of citric acid in the papaya puree may have impacted the stability of the anthocyanins. Anthocyanins are highly unstable and very susceptible to degradation. Anthocyanins are oxidised in the absence of oxidase enzymes and subsequent condensation reactions can lead to brown pigment formation (Singleton, 1987). This reaction may have led to colour changes in the papaya fruit leather but the addition of citric acid was necessary in the production of papaya fruit leather as it protected the natural colour and helped destroy bacteria during drying. Pectin concentration has also been found to affect the colour of the product as the absorbance intensity was decreased in the production of jam, which suggested a relationship between pectin and anthocyanin degradation (DERVISI *et al.*, 2001). Significant increases in L* values after drying were also observed by YANG and ATALLAH (1985). The authors suggested that in both forced air and micro-convection dried papaya increased L* values indicated a higher loss of anthocyanin from thermal degradation. However, for a* values a significant decrease was found in both papaya cultivars after drying, which may be due to anthocyanin oxidation as well as heat degradation during dehydration. In this study, papaya fruit leather also showed lower a* (13. 21 and 11.61) than fresh fruit (from 27.24 and 20.34) for two papaya cultivars Hongkong and Eksotika respectively. The b* values in C. papaya (Hongkong) were higher (10.25) compared to C. papaya (Eksotika). The extra added ingredients and the drying process had an effect on the b* value. The leather cultivars in Hongkong and Eksotika had an average b* value of 10.25 and 8.84 respectively. Similar results were also observed in previous studies with jackfruit leather (CHE MAN and SIN, 1997; OKILYA *et al.*, 2010) and blended papaya leather (KUMAR *et al.*, 2010). After drying, these fruit leathers became darker. This was especially prevalent in light coloured fruit leather (RAAB and OEHLER, 1999). Other factors that can also affect papaya anthocyanins are: pH, storage, temperature, light, light, oxygen, concentration and structure of anthocyanins, other flavonoids, protein and miner-

Table 2 - Effect of processing on colour of two papaya cultivars. Result showed mean ± standard deviation.

Cultivars	*L**		*a**		*b**	
	Fresh	Leather	Fresh	Leather	Fresh	Leather
Hongkong	48.52±1.02[a]	32.1±0.71[b]	27.24±0.51[a]	13.21±0.20[b]	31.71±0.50[a]	10.25±0.41[b]
Eksotika	45.42±1.13[a]	30.7±1.01[b]	20.34±0.42[a]	11.61±0.18[b]	29.41±0.15[a]	8.84±0.65[b]

[a-b] Mean with different letters within each raw are significantly different (P< 0.05).

Table 3 - The effect of processing on the total phenolics content and total flavonoids content of two papaya cultivars. Result showed mean ± standard deviation.

Phenolics	Fresh		Leather	
	Hongkong	Eksotika	Hongkong	Eksotika
TPC	49.61±1.03 [a]	62.59±1.09 [b]	104.71±2.50 [b]	121.4±1.79 [a]
TFC	40.01±1.26[b]	45.40±0.82 [a]	91.43±1.54 [b]	108.78±1.77 [a]

[a-d] Mean with different letters within each raw are significantly different (P< 0.05).

als. These factors were associated with colour changes in papaya fruit. In this study, during the development of fruit leather the interactions between heat and the food ingredients may have significantly affected the anthocyanins' stability and this could have resulted in the colour change of the fruit. Ingredients such as honey contain antioxidants as well as hydrogen peroxide, which may cause degradation of anthocyanins by oxidation mechanism or by indirect oxidation (LOHACHOOMPOL, 2007). Also, it is noted that under high concentrations of oxygen and ascorbic acid increased pigmentation loss occurred which resulted in change to the colour of papaya. Other major factors mentioned by IRWANDI *et al.* (1998) that influenced the colour of fruit leathers were: processing condition, storage time and temperature.

Phenolics content and antioxidant activity

A comparison between fresh fruit and fruit leathers in terms of the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity (FRAP, DPPH, ABTS and ORAC) were illustrated in Tables 3 and.4. The fresh fruit showed different trends with regard to total phenolic content and total flavonoid content. The TPC and TFC were higher in Eksotika (62.59 mg GAE/100g DW and 45.40 mg QE/100g DW, respectively) than in Hongkong (49.61 mg GAE/100 g DW and 40.01 mg QE/100g DW, respectively). Also an antioxidant activity (FRAP, DDPH, ABTS and ORAC) was higher in Eksotika (197.41 mg TE/100g DW, 71.48%, 73.89% and 13.62 μmol TE/g DW, respectively) than in Hongkong (127.74 mg TE/100g DW, 49.62%, 61.84% and 11.50 μmol TE/g DW, respectively).

Antioxidant capacity and phenolic concentration were found to differ according to the types of papaya cultivars. The reason for this may be differences in regions, climate, as well as in the solvents employed for extraction. Furthermore, antioxidant activity may also be affected by lipid composition, antioxidant concentration, temperature, pH, oxygen, and water. Compared to earlier research carried out by (CONNOR *et al.*, 2002), (EHLENFELDT and PRIOR, 2001) and (PRIOR *et al.*, 1998), this study reported higher levels of antioxidant activity. Although dried fruit leathers showed comparable trends, fruit leather had higher TPC, TFC and antioxidant activity (FRAP, DPPH, ABTS, and ORAC) than fresh fruit. Drying determines increase in the levels of antioxidant activity and phenolics in both cultivars. However, Eksotika exhibited a greater increase in antioxidant activity than Hongkong. The fruit leathers showed different trends with regard to total phenolic content and total flavonoid content. TPC and TFC were higher in Eksotika (121.41 mg GAE/100g DW and 108.78

Table 4 - The effect of processing on antioxidant activity (FRAP, DPPH, ABTS and ORAC) of two papaya cultivars. Result showed mean ± standard deviation.

Antioxidant activity	Fresh		Leather	
	Hongkong	Eksotika	Hongkong	Eksotika
FRAP	127.74±1.88[b]	197.41±2.50[a]	231.51±3.87[b]	284.32±1.10[a]
DPPH	49.62±108[b]	71.48±0.87[a]	76.11±0.13[b]	89.47±102[a]
ABTS	61.84±0.86[b]	73.89±1.79[a]	84.97±0.60[b]	92.12±1.52[a]
ORAC	11.50±0.72[b]	13.62±0.96[a]	29.54±0.24[b]	34.40±1.91[a]

[a-d] Mean with different letters within each raw are significantly different (P< 0.05).

mg QE/100g DW, respectively) than in Hongkong (104.71 mg GAE/100 g DW and 91.43 mg QE/100g DW, respectively). Also, an antioxidant activity (FRAP, DDPH, ABTS and ORAC) was higher in Eksotika (284.32 mg TE/100g DW, 89.47%, 92.12% and 34.40 μmol TE/g DW, respectively) than in Hongkong (231.51mg TE/100g DW, 76.11%, 84.97% and 29,54 μmol TE/g DW, respectively).

As previously shown, in both cultivars, drying causes increase in phenolics and antioxidant activity within the range of 50% to 53%. This increase is due to loss of moisture from the samples and thus reflected in the weight, leading to an increased concentration, as well the addition of honey and lemon contributed to the increase in phenols and antioxidants.

Total phenolic contents assay is known to overestimate the content of phenolic compounds, because other agents present in food, such as carotenoids, amino acids, sugars and vitamin C, can interfere (BAHORUN *et al.*, 2004; LUXIMONRAMMA *et al.*, 2003). Furthermore there may be a contribution of millard reaction products to the total phenolic and antioxidant activity (ZHUANG and SUN, 2011). Oxidation produces free radicals which are taken up by the vitamins and polyphenols. Reports that the antioxidant activity of partially oxidised polyphenols is higher compared to that of non-oxidised phenols have prompted further research. As highlighted by GARAU *et al.* (2007), there are other factors that may contribute to a reduced antioxidant activity; these include extended drying intervals. Despite the use of identical cultivars, it is difficult to generate a comparison between the antioxidant activity results of this study and those of earlier ones, due to differences in the assays, extraction techniques and standards (TE, GAE) employed. Moreover, apart from fruit quality, antioxidant activity is also influenced by factors such as geography, environment, climate and harvesting practices. The analysis of the impact of drying on total phenolic content revealed that, in contrast to fresh fruit cultivars, there was a reduction in total phenolic content. Thermal deterioration is the likely cause for the increase in the total phenolic content of the two cultivars. Furthermore, DI SCALA *et al.* (2011) specified that the total phenolic content may also decline due to dehydration, during which polyphenols bind to other compounds, such as proteins, or their chemical structure undergoes changes that existing techniques are unable to extract or identify. In the present study, although the increase was significant, papaya fruit leathers exhibited higher antioxidant activity and phenolics content than fresh fruit.

Table 5 - Correlation coefficients of antioxidants activities of different papaya cultivars.

Correlation coefficient (R2)	FRAP	DPPH	ABTS	ORAC
TPC	0.95	0.80	0.87	0.98
TFC	0.92	0.86	0.85	0.98

Correlation of TPC and TFC with FRAP, DPPH, ABTS and ORAC assays

A correlation analysis among phenolic compounds (TPC and TFC) assays, and antioxidant activity (FRAP, DPPH and ABTS) was performed regardless of the extraction cultivars. A high correlation (Table 5) was found between TPC, TFC and antioxidant activity (FPAP, DPPH ABTS and ORAC) for both cultivars (Hongkong and Eksotika). Thus, it can reasonably be concluded that in the extract, antioxidant activity is related to the active component. Findings of researches

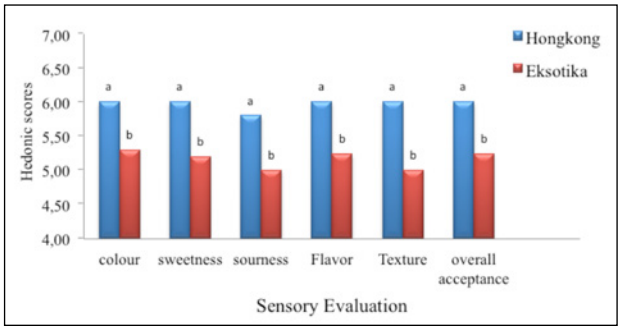


Fig. 3 - The effect of processing on texture of two papaya cultivars.
[a-b] Mean with different letters are significantly different ($P < 0.05$)

of correlation analyses among TPC, TFC, and antioxidant activities (FRAP, DPPH, and ABTS) are high (MAHATTANATAWEE *et al.*, 2006). There have been significant effects on the antioxidant activities of papaya fruit.

Sensory evaluation

The statistical analysis of the sensory evaluation was conducted on the basis of 30 responses. The average scores for six properties of all fruit leathers are presented in Fig. 3. A score of 1 signified 'dislike extremely', while a score of 7 signified 'like extremely'. The cultivars differed significantly ($P<0.05$) in terms of colour, sweetness, sourness, flavour, texture, and general product acceptance. Of the two types of cultivars, Eksotika achieved the lowest acceptability scores of colour. This implied the importance attributed to the visual appearance of the product. There was a greater preference for the papaya leather cultivar Hongkong, due to its reddish colour, than for the dark coloured Eksotika. Surprisingly, the results for fresh papaya were different. In a study undertaken by (SAFTNER *et al.*, 2008), the highest scores among all cultivars

were obtained by the Highbush cultivars Coville and Hannah's Choice for the intense blue colour, acceptable appearance, colour, fruit size, sweet/tart balance, flavour and overall eating quality. In a different study, GUJRAL and KHANNA (2002) increased the sucrose level in order to enhance the colour, flavour and texture of mango leather. Such approaches should be applied in the case of blueberry fruit leathers as well, to improve the darker colour of certain varieties of blueberry. Similarly, the colour of the papaya leather could be enhanced by adding other colourless fruit (DERVISI *et al.*, 2001).

The fruit leathers obtained an average sweetness score of 6.00. This meant that the panelist 'liked' the product sweetness and thus it was necessary to add honey. However, as warned by KUMAR *et al.* (2008), the overall taste rating may decline due to an excessive increase in the amount of sugar. The sourness of the papaya leather was 'moderately liked' by a panelist, with an average score of 5.6. Moisture content and duration of drying have an impact on the texture of fruit leather. The moisture content is reduced and the texture is hardened by higher temperatures and extended drying intervals (OKILYA *et al.*, 2010). Furthermore, the texture quality of the end-product may also be affected by the addition of flavour and colour-enhancing ingredients like pectin, honey, sugars, nuts, salt and other fruits (RAAB and OEHLER, 1999). Eksotika cultivars obtained a lower flavour score (5.2) than Hongkong (6.0). OKILYA *et al.* (2010) explained that the amount of sugar within the fresh pulp affects how the fruit leather tastes. The taste of papaya fruit leather was enhanced in this study by adding honey and citric acid. Similarly, KUMAR *et al.* (2008) noted that papaya and guava fruit leather were affected by the addition of extra ingredients. Compared to individual scoring, the overall score for sensory attributes was considerably improved by the addition of 60% papaya and 40% guava, the nutritional and textural quality of the fruit leather remained unaffected. It was necessary to make this addition in order to enhance the low scent of the papaya fruit, which constituted a major obstacle to the commercial use of this fruit. As specified by (Raab and Oehler 1999), the taste of fruit leather could also be improved by using additional ingredients like leaf oregano and garlic salt. The blueberry fruit leather achieved an average overall score of 5.0, indicating that the panelists 'moderately liked' it. Theoretically, the overall reception of all sensory attributes of the papaya fruit leather was the reason for its overall acceptability. The Hongkong cultivar obtained an overall acceptable score of 6.00 making it the best liked fruit leather. The colour, appearance, sweetness, sourness, texture and flavour of the cultivar determined the preference of the panelists for it. Furthermore, Hongkong received an overall acceptability score of 6 out of 7. On the other hand, Eksotika obtained the lowest score, being 'moderately liked' by the panelists for its colour, general appearance and flavor.

CONCLUSIONS

Fruit leather was successfully developed from two different papaya cultivars using three additional ingredients - honey, pectin and citric acid. This processed product was intended to preserve or enhance the nutritional value and sensory quality of the papaya fruit. The moisture content of the fruit leather derived from the two cultivars was reduced (14.31% and 15.42%), signifying that they were safe from a bacterial viewpoint and could be classified as an intermediate moisture food. The phenolics content and antioxidant activity were increased using the process of drying the fruit leather. Substantial discrepancies in colour, sweetness, sourness, texture, flavour and overall acceptability were reflected in the consumer sensory assessment. 'Like moderately' was the average overall acceptability score. However, according to the results of the sensory evaluation, panelists expressed a low preference for the Eksotika fruit leather, which received a 'moderately liked' score. The results of the present study have great significance for producers of papaya leathers. Among the main factors that determined the acceptability of the fruit leathers were colour, sweetness, sourness, texture and flavour. The end-product can be considered natural, as only small amounts of honey, citric acid and pectin were added in this study. Hence, the consumer requirements for healthy and safe food products were respected

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PAPER

VARIATION IN PHYSICO-CHEMICAL/ANALYTICAL CHARACTERISTICS OF OIL AMONG DIFFERENT FLAXSEED (*LINUM USITTATISSIMUM* L.) CULTIVARS

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ABSTRACT

The present study evaluates and compares the proximate parameters of flaxseed, as well as the physicochemical characteristics of the extracted flaxseed oils of locally grown eight cultivars. The oil, protein, fiber and ash content of the seeds ranged from32.56-39.98%, 16.02-18.50%, 23.30-26.88 and 3.20-3.60%, respectively showing considerable variation among cultivars. The quality attributes such as unsaponifiable matter, peroxide value, acid value, *para*-anisidine value, conjugated dienes and trienes as well as tocopherols content of the tested flaxseed oils varied significantly ($p<0.05$) among cultivars. The major tocopherol was γ-tocopherol (173.7 to 257.9 mg/L) followed by relatively low quantities of α-tocopherol (8-12 mg/L), while δ-tocopherol was not detected. α-Linolenic acid was found to be the principal fatty acid in the range of 44.51 to 54.87%, while the second major fatty acid present in the oils was oleic acid (21.05 to 30.96%). The variation in the characteristics of oils among different cultivars observed during present investigation might be attributed to difference in genetic makeup and harvesting regimes of the flax plants.

- Keywords: flaxseed, folch method, tocopherols, phenolic antioxidants, fatty acids, GC-MS -

INTRODUCTION

Flax (*Linum usitatissimum* L.) is a multi-purpose and economically important oilseed crop. Flaxseeds, which contain approximately 36 to 40% oil, have long been used in human diet and animal feed (TOURE and XUEMING, 2010). By virtue of the presence of physiologically active components, which provide health benefits beyond basic nutrition, flaxseed is often grouped as "functional food" (HASLER *et al.,* 2000). Historically, the oil extracted from flaxseeds, has been used as a basic component in the preparation of paints or polymers, linoleum, varnishes, inks and cosmetics (El-BELTAGI *et al.,* 2007; ZHANG *et al.,* 2008; JHALA and HALL, 2010). However, during the past decade, there has been an increasing interest in the use of flaxseed oil to improve human health status due to its high nutraceutical potential (OOMAH, 2001; CHOO *et al.,* 2007). The potential health benefits of flaxseed oil including reduction in serum cholesterol levels and decreased incidence of diabetes, breast and colon cancer can be ascribed to the presence of high-value antioxidants, tocopherols, lignans and essential fatty acids (Muir and Westcott, 2003; HOSSEINIAN *et al.,* 2006; CHOO *et al.,* 2007; TOURE and XUEMING, 2010). Flaxseed oil is one of the richest sources of unsaturated fatty acid, especially, linolenic acid (C18:3) with amount in the range of 50-60% of the total fatty acids present (FLACHOWSKY *et al.,* 1997).

The agronomic conditions, such as the soil characteristics, agro-climatic conditions and the cultivar influence the unsaturated fatty acids composition in flaxseed (DUAN *et al.,* 2003). Moreover, studies indicate that the oil content, fatty acids profile and other physicochemical properties vary in the flaxseed crops grown in different parts of the world (TAYLOR and MORRICE, 1991; WAKJIRA *et al.,* 2004; HALL *et al.,* 2006). Traditionally, flaxseed has been grown in the Asian subcontinent for its oil; however in Pakistan its applications have been limited to industrial uses. The present study mainly focused on the evaluation and comparison of proximate parameters of flaxseed, as well as the physicochemical characteristics (such as the refractive index, density, iodine value, acid value, peroxide value, *para*-anisidine value etc.) and the composition of tocopherols and fatty acids of flaxseed oil from different flax cultivars (*Linum usitatissimum* L.) indigenous to Pakistan to assess their nutritional value.

EXPERIMENTAL

Seeds and chemicals/reagents

Commercially available hybrid varieties (Chandni, LS-108, LS-105, LS-99 and LS-29) of flaxseed used in this study were obtained from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan and LS-33, LS-31 and LS-13 were obtained from National Agriculture Research Center (NARC) in Islamabad, Pakistan. Three different seed samples for each of the flaxseed variety were collected (8×3=24). All the reagents (analytical and HPLC grade) used were from Merck (Darmstadt, Germany) or Sigma–Aldrich (Buchs, Switzerland). Pure standards of tocopherols (α-tocopherol, γ-tocopherol, δ-tocopherol), and fatty acid methyl esters were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Extraction of oil

Seeds of different flaxseed cultivars were crushed with a domestic electric grinder. The oil from the seeds was extracted using the Folch method (FOLCH *et al.,* 1957). After oil extraction, the solvent was removed under vacuum in a rotary evaporator (Eyela, Rotary Vacuum Evaporator N.N. Series equipped with an aspirator and a digital water bath SB-651, 33 Japan) at 45°C. The extracted oil was then stored in a refrigerator at 4°C until used for analyses.

Analysis of oil seed residues

Proximate analyses of oilseed residues (meals), left after oil extraction, were completed according to standard methods. Protein contents (N-6.25) were determined according to AOAC method 954.01 (AOAC, 1990), using a Kjeldahl apparatus. The fiber contents were determined employing ISO method 5983 (ISO, 1981). Briefly, 2 g of finely ground defatted sample was taken and boiled with 250 mL of 0.255 *M* H_2SO_4, followed by the filtration and washing of insoluble residues. The residues were then boiled with 250 mL of 0.313 *M* NaOH, filtered, washed, and dried. The dried residues were weighed and burnt at 600°C using a muffle furnace (Eyela, TMF-2100, Tokyo, Japan) and the loss of mass was determined gravimetrically. Ash contents were determined by following ISO method 749 (ISO, 1977). Two grams of meal was carbonized by heating on a gas flame and then ashed in an electric muffle furnace at 600°C, until a constant mass was achieved.

Physicochemical properties of oil

The extracted oils were analyzed for density, refractive index, peroxide value (PV), acid value, iodine value (IV), saponification value, and unsaponifiable matter following AOCS methods Cc10a-25, Cc7-25, Cd1-25, Cd8-53, F-9a-44, Cd3-25, and Ca61-40, respectively (AOCS, 1997). The determinations of conjugated dienes (CD) and conjugated trienes (CT) were made using a Hitachi U-2001 spectrophotometer. The oil samples were diluted with isooctane and ab-

sorbance values recorded at 232 and 268 nm for conjugated dienes (CD) and conjugated trienes (CT), respectively. Specific extinctions were determined following the IUPAC method II D.23 (IUPAC, 1987). The *para*-anisidine value of the flaxseed oil samples was monitored according to IUPAC method II D.26 (IUPAC, 1987). The oil samples diluted with isooctane were reacted with *p*-anisidine solution (0.25% w/v) in acetic acid for 10 min and the absorbance of the resulting colored solution recorded at 350 nm using a spectrophotometer.

Tocopherols

For tocopherol analysis, an HPLC method was adopted from YAQOOB *et al.* (2010), with some modifications. A Waters Alliance 2695 HPLC system equipped with YMC-Pack ODS AM-303, C 18 column (250mm x 4.6mm x 5μm) and Agilent series 1050 diode array detector, (UV 295 nm) was used. The temperature of the column was maintained at 30°C. The chromatographic separation was performed by isocratic elution with a mixture of acetonitirile and isopropanol (40:60 v/v) at a flow rate of 1 mL/min (Pressure 120 bar). Briefly, flaxseed oil (1 g) was accurately weighed into a 5 mL sample vial wrapped in aluminum foil to prevent photo-oxidation. The oil was dissolved in 5 mL acetonitrile before injection. Samples were injected into the column through an injection loop (20 μL). Tocopherols were identified by comparing the retention times of the unknowns with those of pure standards of α-, γ-, and δ-tocopherols. Acquisition of data was made using Agilent Chem-station software. The samples were prepared and analyzed separately in triplicate.

Fatty acids profile

Fatty acid methyl esters (FAMEs) were prepared by IUPAC standard method 2.301 (IUPAC, 1987) which involved the trans-esterification of fatty acids with methanol under base –catalyzed conditions. Briefly, 0.2 g of oil was placed in 10 mL capped vials; 5mL of redistilled methanol was added followed by the addition of a pellet of KOH. The content of the vials were heated at 60°C in a heating mantle until the droplets of fats disappeared. Upon cooling, the reaction mixture was gently transferred to a separating funnel. Small amount of n-hexane was added. Separating funnel was shaken gently. The upper hexane layer was recovered and washed with distilled water. This hexane solution was dried over anhydrous sodium sulfate, filtered and used for gas chromatographic analysis.

FAMEs were separated on an Agilent 5890 series II GC fitted with a 7673B auto sampler and a capillary column (30 m × 0.25 mm × 0.25 μm) with a DB-WAX (film thickness 0.20 μm) stationary phase and a flame ionizing detector (FID). Helium was used as carrier gas at a flow rate of 1.5 mL/min. Other conditions were as follow: injection volume 1μL, split mode (split ratio1:100), injector temperature, 280°C, initial oven temperature, 170°C; hold up 2 min, 170-240°C (ramp rate 2°C/min) hold up 10 min, detector temperature 260 °C. FAMEs were identified by comparing their relative and absolute retention times with those of authentic standards. The FA composition was reported as a relative percentage of the total peak area. The internal standard used was nonadecanoic acid. All of the quantifications were done by Agilent Chem-station software.

Statistical analysis

Three different seed samples for each of the variety were taken and analyzed individually in triplicate and data reported as mean ± SD (n= 3× 3 =9). An analysis of variance (ANOVA) was performed using Minitab 2000 Version 16.1 statistical software (Minitab Inc. State College, PA, USA). Significant differences ($P<0.05$) of means were calculated using Duncan's multiple range tests.

RESULTS AND DISCUSSION

Proximate analysis of seeds

The data obtained for the proximate analysis of flaxseeds of eight different cultivars grown in Pakistan is presented in Table 1. Oil contents varied from 33.25 to 38.38% indicating a significant difference among cultivars selected ($p<0.05$). The variety Chandani had the highest oil yield whereas LS-13 contained the lowest. In another study from Pakistan, Anwar *et al.*, (2013) investigated the oil yield for soxhlet-extracted flaxseed to be 42.80%; such variation in oil yield may be linked to the different extraction method used.

The oil content of Pakistani flaxseeds are comparable to those grown in Canada, North America and Egypt, i.e. 36%, 31.9 to 37.8% and 36-39%, respectively (HETTIARACHCHY *et al.,* 1990; OOMAH and MAZZA 1998; El-BELTAGI *et al.,* 2007). However, the present oil contents from Pakistani flaxseed cultivars were lower than those reported for Polish flaxseed cultivars, *i.e.*, 41.4% (KOZLOWSKA, 1989) but higher than Ethiopian flaxseed cultivars, 29.1-35.9% (WAKJIRA *et al.*, 2004). Such variations in flaxseed oil content with in the countries might be linked to varietal and agro climatic conditions of the regions.

The moisture, crude protein, fiber and ash contents of different cultivars of Pakistani flaxseed ranged from 5.98 to 6.22%, 16.02 to 18.50%, 23.30 to 26.80% and 3.21 to 3.60%, respectively. There was no significant difference

Table 1 - Proximate analysis of Pakistani flaxseed (*Linum usittatissimum.* L) cultivars.

Variety	Parameters				
	Oil content (%)	Moisture content (%)	Protein content (%)	Fiber content (%)	Ash content (%)
Chandni	38.38±1.80^{ab}	6.02±0.11^{a}	18.50±0.56^{ab}	26.80±1.22^{a}	3.60±0.12^{ab}
LS-108	36.38±1.52^{de}	6.12±0.13^{ab}	18.00±0.45^{d}	26.80±0.98^{a}	3.55±0.11^{d}
LS-105	37.01±1.46^{e}	6.22±0.15^{ab}	17.98±0.63^{cd}	26.00±1.06^{ab}	3.50±0.11^{cd}
LS-99	38.02±1.60^{c}	6.10±0.13^{a}	17.86±0.48^{bc}	25.5±1.14^{ab}	3.50±0.13^{bc}
LS-33	35.30±1.35^{f}	5.99±0.12^{ab}	16.02±0.62^{a}	23.50±0.88^{b}	3.21±0.12^{a}
LS-31	34.98±1.47^{a}	5.98±0.10^{ab}	16.62±0.54^{a}	23.3±1.16^{b}	3.26±0.14^{a}
LS-29	38.00±1.59^{d}	6.02±0.14^{a}	17.99±0.39^{e}	25.75±1.06^{ab}	3.58±0.14^{e}
LS-13	33.25±1.31^{bc}	6.00±0.11^{b}	17.00±0.52^{ab}	23.68±1.14^{b}	3.40±0.16^{ab}

Values (mean ± SD) are average of triplicate samples of each cultivar, analyzed individually in triplicate (n = 1 x 3 x 3), (P<0.05). Different letters in superscript indicate significant differences.

Table 2 - Physico-chemical characteristics of oil extracted from Pakistani flaxseed (*Linum usittatissimum.* L.) cultivars.

Parameters	Varieties							
	Chandni	LS-108	LS-105	LS-99	LS-33	LS-31	LS-29	LS-13
Refractive index (40°C)	1.4729±0.009^{a}	1.4728±0.006^{a}	1.4707±0.005^{a}	1.4737±0.007^{a}	1.4732±0.005^{a}	1.4734±0.006^{a}	1.4706±0.009^{a}	1.4728±0.007^{a}
Density g/mL (25°C)	0.928±0.18^{a}	0.929±0.12^{a}	0.9278±0.14^{a}	0.928±0.14^{a}	0.928±0.16^{a}	0.929±0.15^{a}	0.928±0.19^{a}	0.9279±0.21^{a}
Iodine Value g of I/100 g of oil	198±3.96^{a}	199±4.26^{a}	195.9±3.24^{a}	201±4.39^{a}	195±3.85^{a}	196±4.96^{a}	197±3.68^{a}	199±3.88^{a}
Unsap matter (%)	2.20±0.04^{c}	2.60±0.04^{a}	2.20±0.02^{c}	2.00±0.02^{d}	2.4±0.03^{b}	1.96±0.05^{d}	2.0±0.02^{d}	1.80±0.04^{e}
Saponification value mg of KOH/100 g of oil	189±2.78^{a}	187±3.25^{a}	185.9±3.70^{a}	186±3.46^{a}	185±4.58^{a}	185.86±3.72^{a}	187±3.94^{a}	184±4.71^{a}
FFA (% as oleic acid)	1.399±0.03^{d}	1.579±0.04^{c}	1.624±0.02b^{c}	1.399±0.03^{d}	1.725±0.05^{a}	1.721±0.04^{ab}	1.447±0.02^{d}	1.732±0.05^{a}
Peroxide value (meq/kg of oil)	1.00±0.02^{c}	1.20±0.03^{ab}	1.18±0.02^{ab}	1.00±0.04^{c}	1.14±0.03^{b}	1.20±0.02^{a}	1.22±0.03^{ab}	1.14±0.05^{b}
$^{1cm}\mathcal{E}_{1\%}$ (λ 232)	5.07±0.20^{ab}	4.70±0.15^{b}	4.81±0.28^{b}	4.79±0.19^{b}	4.80±0.22^{b}	4.75±0.14^{b}	5.55±0.22^{a}	4.76±0.18^{b}
$^{1cm}\mathcal{E}_{1\%}$ (λ 268)	1.80±0.08^{ab}	2.00±0.09^{a}	1.90±0.08^{a}	1.60±0.04^{cd}	1.50±0.05^{d}	1.80±0.07^{ab}	1.70±0.05^{bc}	1.40±0.06b^{cd}
Para-anisidine value	1.13±0.05^{c}	1.41±0.05^{ab}	1.40±0.06^{ab}	1.29±0.05^{bc}	1.36±0.05^{ab}	1.42±0.06^{ab}	1.48±0.05^{a}	1.32±0.04^{ab}

Values (mean ± SD) are average of triplicate samples of each cultivar, analyzed individually in triplicate (n = 1 x 3 x 3), (p<0.05).
Different letters in superscript indicate significant differences.

in moisture content for flaxseeds between different cultivars (p>0.05). However, crude protein, fiber and ash contents were notably different among the cultivars of flaxseeds (p<0.05). Depending on the cultivar and growing conditions, flaxseed has been reported to contain an average of 23% to 34% protein, 4% ash and 5% fiber (MUIR and WESTCOTT, 2003). Our results are comparable to the previous reports on flaxseed cultivars grown in different regions of the world. Protein contents of Polish and North American cultivars were reported to be greater than 20%, while Canadian cultivars had protein generally less than 20% (OOMAH and MAZZA, 1998; CHOO *et al.,* 2007). Crude fiber content in different flaxseed residues has been reported to be in the range of 7-10% (GUTIERREZ *et al.,* 2010). The difference in oil, crude protein, and fiber and ash contents of flax seed of different cultivars might be attributed to differences in growing conditions and genetic makeup of flax plants (OOMAH and MAZZA 1993; DUAN *et al.,* 2003).

Physico-chemical properties of oil

The physico-chemical parameters determined for oils extracted from eight different cultivars of flaxseeds are presented in Table 2. The results indicated that the refractive index (40°C) and density (25°C) ranged from 1.4706 to 1.4737 and 0.9278 to 0.929 mg/mL, respectively, with non-significant (p>0.05) difference among cultivars Our findings are consistent with the previous reports in which the refractive index of flaxseed oil at 20°C was reported to be 1.475, while the density of flaxseed oil at 25°C was 0.925 to 0.935 (PRZYBYLSKI, 2005). The density of flaxseed oil is greater than most other vegetable oils, and this might be attributed to the greater content of linolenic acid (GREEN and MARSHALL, 1984).

The iodine value, unsaponifiable matter, saponification number and acid value are characteristic for flaxseed oils that contain a large percentage of polyunsaturated fatty acids. The iodine values for the tested oils ranged from 195 to 199 g of I_2/100 g of oil with non-significant

(p>0.05) among cultivars. Iodine value for flaxseed oil has been reported to vary between 180 to 203 g of I_2/100g of oil (PRZYBYLSKI, 2005). LONG *et al.* (2011) reported iodine value of flaxseed oil to be 162 I_2/100g. The saponification value and unsaponifiable matter of the tested flaxseed oils ranged from 184 to 189 mg of KOH/100g of oil and 1.8 to 2.6%, respectively. Saponification values did not differ significantly (p>0.05) whereas the unsaponifiable matter varied significantly within the oils of different cultivars (p<0.05). In previous reports, the percentage of unsaponifiable matter in flaxseed oil was in the range of 0.1 to 1.7% for raw oil, and up to 0.6% for refined flaxseed oil (ESKINETAL, 1996; CHOO *et al.,* 2007). TEH and BIRCH (2013) reported the unsaponifiable value to be 0.4% for cold pressed flaxseed oil.

Free fatty acids (FFA) are produced by the hydrolysis of triglycerides (LAFONTAN and LANGIN, 2009). The FFA content of the tested flaxseed oils ranged from 1.40 to 1.73%, as oleic acid. The FFA content varied significantly within different flaxseed cultivars (p<0.05). In a previous report, FFA value for flaxseed oil was reported to be 0.1 to 2.0% (PRZYBYLSKI, 2005). LONG *et al.* (2011) reported the FFA in the flaxseed oil extracted by enzymatic extraction and solvent extraction to be 1.5 and 1.1%, respectively. FFA value for the cold pressed flaxseed oil was reported to be 0.75% (TEH and BIRCH, 2013). FFA in most of the freshly extracted crude vegetable oils is normally below 1.0%. These hydrolytic products are mainly formed as result of chemical hydrolysis (due to presence of moisture in seeds) or enzymatic hydrolysis. A low value of oil FFA is an indication that the seeds have been preserved under proper storage conditions with good state.

The peroxide value for the flaxseed oils of different cultivars ranged from 1.0 to 1.22 meq/kg of oil that is well below the limit for peroxide value. CHOO *et al.*, (2007) reported the peroxide value ranging from 0.5 to 2.9meq/Kg of cold-pressed flaxseed oil sold in New Zealand. Peroxide value for the enzymatic, solvent extracted flaxseed oils was reported to be 1.2 and 1.0 meq/kg of oil, respectively (LONG *et al.* 2011) while that for cold pressed flaxseed oil was reported to be 2.04 meq/kg of oil (TEH and BIRCH, 2013). Peroxide value is an indicator of primary oxidation products; the extent of these products may range up to 10-15 meq/kg of oil (CHOO *et al.,* 2007).

The *p*-anisidine value of the oils extracted from different cultivars of flaxseed ranged from 1.13 to 1.48(p<0.05). The values are higher than those investigated by CHOO *et al.* (2007) who reported the *p*-anisidine value to be in the range of 0.36 to 0.4. However, the present values were comparable to the values of enzymatically and solvent extracted flaxseed oil reported previously, 1.2 and 1.0, respectively (LONG *et al.,* 2011). Conjugated dienes and trienes produced as a result of secondary oxidation of polyunsaturated fatty acids can be determined by measuring the absorbance at 232 and 272 nm, respectively (Frankel, 2005). The specific extinction at 232 and 272 nm of different flaxseed oils ranged from 4.70 to 5.55 and 1.40 to 2.00, respectively. CHOO *et al.*, (2007) reported absorbencies at 232 and 270 nm of 1.7 to 2.75 and 0.2 to 0.4, respectively for cold pressed flaxseed oil. TEH and BIRCH (2013) reported absorbencies at 232 and 272 nm for the cold pressed flaxseed oil to be 2.02 and 0.02, respectively which is very low as compared to our present results. Our results are comparable to those reported previously (REED *et al.*, 2001).

Tocopherol content

The tocopherol contents of the different flaxseed oils are shown in Table 3. Gamma (γ)-tocopherol was the main tocopherol in flaxseed oils, with contribution of approximately 90% of the total tocopherols. The γ-tocopherol content ranged from 173.7 to 257.9 mg/kg of oil and significantly differed in different cultivars (p<0.05). Alpha (α) tocopherol was the other tocopherol found in the oils (~10%) while delta tocopherol was not detected. The contents of α tocopherol varied from 39 to 18.7 (mg/kg of oil) showing a significant difference among different cultivars (p<0.05). The difference in the contents of tocopherols might be due to the varying ge-

Table 3 - Tocopherol contents (mg/kg) of oil extracted from Pakistani flaxseed (*Linum usittatissimum.* L.) cultivars.

Tocopherol	Cultivars							
	Chandni	LS-108	LS-105	LS-99	LS-33	LS-31	LS-29	LS-13
γ-tocopherol	204.0±5.0^{bc}	217.8±4.8^{b}	179.6±5.8^{de}	173.7±5.5^{e}	201.3±6.2^{c}	192.2±4.9^{cd}	257.9±5.6^{a}	190.8±4.3^{a}
α-tocopherol	24.9±0.2^{e}	20.4±0.2^{f}	30.2±0.2^{c}	18.4±0.3^{g}	27.2±0.3^{d}	20.9±0.2^{f}	38.5±0.3^{a}	32.8±0.3^{b}
δ-tocopherol	ND	ND	ND	ND	ND	ND	ND	ND
Total tocopherols	228.9	238.2	209.8	192.1	228.5	213.1	296.4	223.6

Values (mean ± SD) are average of triplicate samples of each cultivar, analyzed individually in triplicate (n = 1 x 3 x 3), (P<0.05). Different letters in superscript indicate significant differences. ND = not detected.

Table 4 - Fatty acid composition (g/100g of FA) of oil extracted from Pakistani flaxseed (*Linum usittatissimum.* L) cultivars.

Fatty acid (FA)	Varieties							
	Chandni	LS-108	LS-105	LS-99	LS-33	LS-31	LS-29	LS-13
C 16:0	5.95±0.07^{cd}	5.94±0.07^{b}	5.8±0.06^{b}	6.14±0.07^{a}	6.11±0.19^{bc}	6.52±0.01^{bc}	6.27±0.01^{d}	6.21±0.03^{cd}
C 18:0	4.63±0.04^{c}	4.70±0.12^{d}	4.41±0.03^{cd}	4.97±0.08^{bc}	4.83±0.06^{ab}	4.68±0.06^{a}	4.58±0.01^{d}	4.4±0.04^{bc}
C 18:1 (n-9)	30.46±0.63^{ab}	28.86±0.53^{de}	26.25±0.35^{e}	30.96±0.47^{c}	21.05±0.45^{f}	28.33±0.56^{a}	24.09±0.58^{d}	26.75±0.59^{bc}
C 18:1 (n-7)	1.69±0.58^{a}	1.64±0.51^{a}	1.61±0.54^{a}	1.53±0.41^{a}	1.46±0.44^{a}	1.83±0.64^{a}	1.69±0.49^{a}	1.8±0.63^{a}
C 18:2 (n-6)	11.26±0.04^{ab}	11.18±0.01^{d}	7.63±0.74^{cd}	11.88±0.21^{bc}	11.68±0.8^{a}	10.5±0.01^{a}	10.08±0.1^{e}	9.42±0.13^{ab}
C 18:3 (n-3)	46.02±0.10^{f}	47.67±0.11^{d}	54.29±0.44^{c}	44.51±0.12^{e}	54.87±0.01^{a}	48.13±0.14^{g}	53.29±0.02^{b}	51.41±0.08^{e}

Values (mean ± SD) are average of triplicate samples of each cultivar, analyzed individually in triplicate (n = 1 x 3 x 3), (P<0.05). Different letters in superscript indicate significant differences.

netic makeup and growing conditions of different cultivars (OOMAH and MAZZA, 1997). Our results are consistent with previous reports in which gamma tocopherol was reported as the predominant tocopherol in flaxseed oils (GREEN and MARSHALL, 1984; HERCHI *et al.* 2011). The γ- and α-tocopherol content of the Pakistani cultivars is considerably greater than that in the flaxseed oil varieties of American, Canadian, New Zealand and Turkish origin which contain an average127, 93, 140 and 146 (mg/kg of oil) of tocopherols, respectively (BUDINETAL.,1995; OOMAH and MAZZA, 1997; CHOO *et al.,* 2007; BOZAN and TEMELLI, 2008).

The tocopherol contents of Pakistani flaxseed cultivars were comparable to those reported for Egyptian cultivars (210 mg/kg of oil) (EL-BELTAGI *et al.*, 2007). TEH and BIRCH (2013)reported the contents of γ-tocopherol to be 370 (mg/kg of oil) in cold pressed flaxseed oil, which is considerably higher the present finding.

Fatty acid profile

The fatty acid profile showed a significant variation in the contents of fatty acids within the oils of different flaxseed cultivars (p<0.05) as shown in Table 4. The amount of total unsaturated fatty acids in flaxseed oils of the selected cultivars was observed to be in the range of 88.79 to 89.78% while the amount of total saturated fatty acids ranged from 10.21 to 11.20 % with non-significant (p>0.05) varation among cultivars. One distinct feature of flaxseed oil is the presence of high amount of linolenic acid. In the current study the quantities of linolenic acid were observed to be 44.51 to 54.87%, for different cultivars (p<0.05).

The results are comparable to the previous reports for American and Egyptian flaxseed varieties with 45 to 52% and 46 to 50% alpha linolenic (ALA) acid, respectively (DECLERCQ *et al.,* 1992; El-BELTAGI *et al.,* 2007). For Ethiopian flaxseed cultivars the ALA contents were 52% (WAKJIRA *et al.,* 2004). However, the ALA contents of the Pakistani cultivars were less than those reported for the flaxseed cultivars grown in New Zealand and Canada, i.e. 59.65 and 59%, respectively (HETTIARACHCHY *et al.,* 1990; CHOO *et al.,* 2007). Moreover, BOZAN and TAMELLI (2008) reported ALA levels to be 56.5 to 61% for flaxseed from Turkish origin, i.e. greater than our findings.

The trends for FA results in the present study are also in agreement with the reports that with an increase in ALA in flaxseed oil, there is a corresponding decrease in oleic acid (CHOO *et al.* 2007). The flaxseed cultivar LS-33 had the highest contents of ALA (54.87%) and lowest amount of oleic acid (21.05%), while LS-99 contained the lowest amount of alpha linolenic acid (44.51%) and the highest amount of oleic acid (30.96%). Overall, the amount of linolenic acid ranged from 44.51 to 54.87%, while that of oleic acid ranged from 21.06 to 30.96% for different cultivars of flaxseed grown in Pakistan.

CONCLUSIONS

The oil yield considerably varied among the selected flaxseed cultivars. Similarly, the significant differences for most of the physico-chemical/analytical characteristics among the tested oils were recorded. Such variations in oil quality characteristics might be linked to different genetic makeup of the cultivars as well as to their variable harvesting conditions. Overall, the flaxseed cultivar Chandi, LS-99 and LS-29 had relatively higher oil yield; the cultivar NS-29, LS-108, Chandi and LS-33 exhibited greater amount of tocopherols whereas those of LS-33, LS-29 and LS-105 were rich in alpha linolenic acid (ALA) among others. The findings of this comparative study can be useful for selection of economically and nutritionally important flaxseed cultivars, especially, as ingredient for functional foods and nutraceuticals.

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PAPER

DIFFERENTIAL METHOD TO DETERMINE THERMAL DEGRADATION KINETICS OF CHLOROPHYLL IN VIRGIN OLIVE OIL

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ABSTRACT

Differential method is presented to study thermal degradation kinetics of chlorophyll in virgin olive oil. The oil samples, naturally containing 20.0 mg/kg chlorophyll were stored at 150°, 160°, 170°, 180°, 190° and 200°C until the time at which chlorophyll contents had reduced to the certain amounts. The concentration gradually decreased as heating time increased. A half order equation was found as the best model for the present experimental data. Differential method with graphic and substitution methods was compared for the determination of the rate constant and the half-time. The rate constants and half life at 150°C were determined in the range of 0.20-0.22 and 12.14-13.12 for the thermal process of chlorophyll in virgin olive oil, respectively. The reaction rates increased approximately 1.1 times with increment of every 10°C from temperature of 150°C. Conversely, the half lifes decreased 0.9 times for increment of every 10°C. The activation energies were approximately 24 J/kg for differential method, and 22 J/kg for graphic and substitution methods.

- Keywords: chlorophyll, kinetics, thermal degradation, virgin olive oil -

INTRODUCTION

Chlorophylls are responsible for the green color of all vegetables and fruits. Animal tissues can't synthesis chlorophylls, though animal cells can chemically modify them for assimilation. These compounds should be supplied from food (GIUFFRIDA *et al.*, 2007). Chlorophyll and its various derivatives have been used in traditional medicine and for therapeutic purposes for many years and perhaps have the potential role of these pigments in the prevention of human cancers that has drawn more recent attention (FERRUZZI and BLAKESLEE, 2007).

The color of olive oil is principally related to its perceived quality, and therefore to its acceptability. The economic importance of the appearance of the oils is unquestionable. The color of virgin olive oil is due to the natural pigments chlorophylls, and carotenoids (MINGUEZ MOSQUERA *et al.*, 1994).

Olive oil contains originally the chlorophylls a and b. Chlorophyll a, pheophytin a, is typically found in higher amounts than chlorophyll b. The distribution and content of chlorophyll in olive oil are dependent on a number of factors including species, agroclimactic conditions, pre- and postharvest treatment, and type and degree of food processing (MINGUEZ-MOSQUERA *et al.*, 1990, GANDUL-ROJAS *et al.*, 1996, GIUFFRIDA *et al.*, 2007, CRIADO *et al.*, 2008, CERRETANI *et al.*, 2008, GIUFFRIDA *et al.*, 2011).

The grades of oil extracted from the olive fruit are classified as virgin, lampante, refined and olive pomace oil. Virgin oil is produced by the use of mechanical means only, with no chemical treatment or heat. Virgin oil includes both virgin olive oil (VOO) and extra-virgin olive oil (EVOO) products, depending on quality. Therefore, virgin olive oil should be preferably added as the final seasoning in fresh salads, soups, or more elaborated dishes (CARLA *et al.*, 2013) but olive oil like other vegetable oils is used in several cooking processes such as deep-frying, pan-frying, roasting, microwave cooking, etc. (WATERMAN and LOCKWOOD, 2007; BOSKOU, 2009). Each thermal processing type has particular characteristics as depending on process temperature and time. CARLA *et al.* (2013) summarized several works related to olive oil that used as the cooking base, grouped the works by real and simulated cooking method and showed the analytical parameters chosen by the authors to evaluate olive oil performance. For example, in frying process the both methods were tested with several olive oil commercial grades, at temperatures ranging from 170°C to 180°C in real frying, and from 160°C to 190°C in simulated frying, i.e. being the olive oil heated without any food. Some authors also compared the effects of adding fresh oil between frying sessions in the oil performance. In the previous studies it was made on thermal stability of olive oil. The studies on thermal stability of olive include the thermal decomposition of commercial vegetable oils of some of their thermal properties (DWECK *et al.*, 2004), the thermal degradation study of four unsaturated or saturated esterified C18 fatty acids with glycerol (VECCHIO *et al.*, 2008), stability of olive oil during heating (BERASATEGI *et al.*, 2012), the heat-oxidation stability of binary blends made with palm oil and several extra virgin olive oils (DE LEONARDIS and MACCIOLA, 2012) and effects of the main virgin olive oil antioxidants under mild temperature conditions (MANCEBO-CAMPOS *et al.*, 2014) but, virgin olive oil contains minor constitutes together with triglycerides, the thermal effect on chlorophyll stability and degradation in olive oil has not been studied extensively.

Kinetic modelling recently gaining increasing interest in food science gives the possibility of controlling changes in foods such as to control food quality during processing and shelf life (NIAMNUY *et al.*, 2012; GOULA, 2013; GRAUWET *et al.*, 2014; REMINI *et al.*, 2015). Microbiological changes which are called as predictive microbiology have been worked up to recent years but it can also be applied for biological, chemical and physical changes. The rate of a reaction and its temperature dependence, the occurrence of such a reaction can be predicted and controlled under specified conditions. The difficulties in kinetic modelling are choosing the right model for a reaction. For example; one of the difficulties is that too few data points are available to decide for the correct order. In general, researchers in food science have limited themselves often to simple reaction kinetics. i.e. it is trying to fit a zero-, first- or second order model to their data (VAN BOEKEL, 1996; VAN BOEKEL, 1999).

The present study focused on the determination of rate order and characterization of the Arrhenius parameters governing the thermal degradation reactions of chlorophyll in virgin olive oil by using the differential method and was to compare it with other two rate order determination methods.

2. MATERIAL AND METHODS

2.1. Materials

Olive oil from olive fruits harvested in 20012 - 2013 season were obtained from a local olive oil plant (Demirkol Ltd., Kahramanmaras, Turkey). Working principle of the plant is that olives are stored in the hopper of olive elevator and transported to washing machine. First leaves of olives are removed by leaf remover. Then olives are washed without giving harms to its pulp in the olive washing unit. Olives are transported to crusher by crusher elevator. Olives are crushed and become semi paste in crusher. Semi paste olives are mixed to obtain oil in malaxers. Crushed olive is fed into the decanter without water through a

pulp pump. Input product comes out of decanter as oil and pomace with black water. The characteristic of the olive oil are as follows: free acidity, 0.49 % oleic acid; peroxide value, 5.22 meq O_2/kg; K_{232} and K_{270} extinction coefficients, 1.89 and 0.15; respectively, according to the analytical methods described in European Regulation EEC 2568/91 (EEC, 1991) and chlorophyll content, 20.0 mg/kg (POKORNY *et al.*, 1998). The oil samples (25 ml each) were transferred into 50 mL glass bottles. The bottles were sealed with teflon-coated rubber seals and aluminum caps and stored at 150°, 160°, 170°, 180°, 190° and 200°C under dark condition in a forced air oven. Chlorophyll content was measured with 2-h intervals from initial time until the time at which chlorophyll contents had reduced to 1 mg/kg All samples were prepared in duplicate.

2.2. Determination of chlorophyll content in olive oil

The chlorophyll content of olive oil was analyzed using the method described by POKORNY *et al.* (1998). The sample was measured at 630 nm, 670 nm and 710 nm in a 10 mm spectrophotometer cell against air, instead of a reference cell. The method is suitable for the determination of quantities of chlorophyll pigments higher than 1 mg/kg. The following equation was used for determining the chlorophyll content

$$[C] = 345.3(A_{670} - 0.5A_{630} - 0.5A_{710})/L$$

Where: [C] = content of chlorophyll pigments in mg of *pheophytin a* in 1 kg of oil,
A = absorbance at the respective wavelength (nm),
L = thickness of the spectrophotometer cell (mm).

2.3. Kinetic theory

Differential method was used for determination of the degradation rate order and the rate constant of chlorophylls in olive oil.

It was expressed the concentration at any temperature as a function of time in a power series, with constants a, b, c by deriving from the experimental concentration-time data

$$[C] = at^2 + bt + c$$

Where concentration ([C]) and time (t) were expressed in mg/kg and in hour.

Rate of reaction in *mg/kgh* (*v*) was estimated from the following equation;

$$v = -\frac{d[C]}{dt}$$

The most simple general rate equation was used for a single reactant at concentration [C]:

$$v = k_n [C]^n$$

Where *n* = rate order, k_n = rate constant at order *n*.

By taking the logarithm of the above equation to base e it follows that:

$$\ln v = n\ln [C] + \ln k_n$$

Rate order and rate constants at different temperatures were determined by plotting graph

ln v versus ln [C]

The half-life value ($t_{1/2}$) of chlorophyll degradation was calculated using the equation given below after founding rate order and rate constants:

$$t_{1/2} = 0.597\ [C_0]^{1/2}/k_{1/2};\ [C_0] =$$

initial concentration of chlorophy

$Lnk_{1/2}$ was plotted versus 1/T to determine Arrhenius parameters (A and E_a) by taking the logarithm of Arrhenius equation; $k_{1/2}$ = Aexp(-E_a /RT) to base e; where E_a is the activation energy (J/kg), A is the pre-exponential factor or Arrhenius constant, R* is the specific gas constant for pheophytin a (9.543 J/kg K), and T is the absolute temperature (K).

Differential method using for determination of the rate constant was compared with substitution and graphic methods.

In substitution method the k value at a temperature was calculated by substituting initial concentration, concentration at any time and time values into the following half order rate equation:

$$k_{1/2} = 2/t\times([C_0]^{1/2} - [C]^{1/2}) \text{ for } n = ½$$

In graphic method the above equation was rearranged as $[C]^{1/2} = [C_0]^{1/2} - (k_{1/2}/2)\times t$ and $[C]^{1/2}$was plotted versus t to determine $k_{1/2}$ value (the plot not shown).

3. RESULTS AND DISCUSSION

Virgin olive oil is a food matrix contains triglyceride having a high percentage of monounsaturated fatty acids and also other minor constituents such as the phenols, chlorophyll and carotenoids fundamental in contributing to specific characteristics of virgin olive oil. Therefore the kinetic study and characterization of the Arrhenius parameters related with the thermal degradation reactions of chlorophyll in VOO were performed in an oil matrix system to establish mathematical models enabling the prediction of the degradation of this pigment during VOO thermal processing.

Changes with respect to the time in chlorophyll concentration in oil matrix during thermal

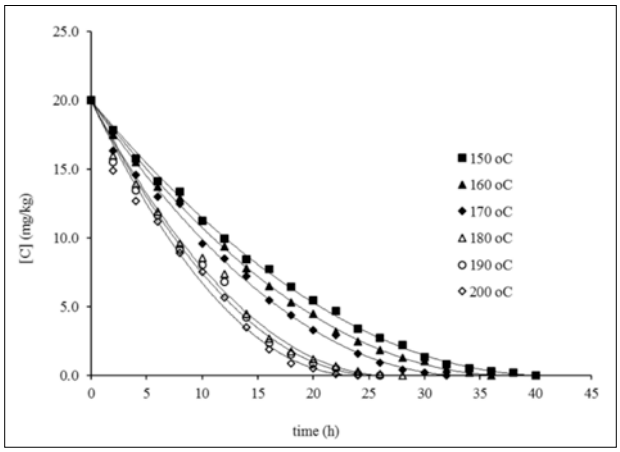


Fig 1 - Changes with respect to the time in chlorophyll concentration in oil matrix during thermal processing.

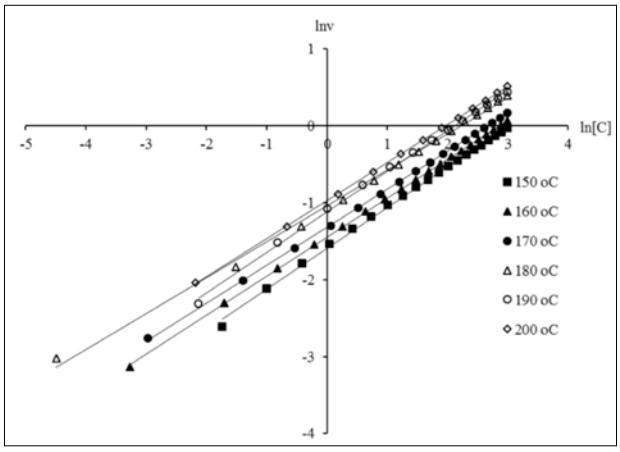


Fig 2 - lnv - ln[C] plot to estimate the rate order and rate constants at different temperatures.

Table 1 - Best fit equations for the concentration-time data.

T (oC)	[C]=at2-bt+c			R²
	a	b	c	
150	0.012	0.970	20.0	0.999
160	0.014	1.066		0.998
170	0.018	1.183		0.993
180	0.028	1.489		0.993
190	0.030	1.558		0.991
200	0.035	1.678		0.987

[C]: Chlorophyll concentration; t: time; a, b and c: function coefficients.

Table 2 - Best fit equations for lnk - ln[C] data.

T (oC)	lnv=n×ln[C]+lnkn		R²
	n	lnkn	
150	0.53	-1.59	0.998
160	0.51	-1.45	0.999
170	0.49	-1.32	0.999
180	0.47	-1.04	0.997
190	0.53	-1.11	0.998
200	0.50	-0.97	0.999

[C]: Chlorophyll concentration; v: reaction rate; k: reaction constant; n: reaction order.

processing, expressed in mg/kg, were shown in Fig. 1. The chlorophyll concentrations gradually decreased while heating times increased. The experimental data was transferred to Sigmaplot (version 12.0) program and trial and error method was applied to find the best fit curve equation on the data. The chlorophyll concentration at any temperature was expressed as a function of time. The best fit mathematical equations for the changes in the experimental data with the reaction time were selected to verify the rates of reaction at any temperature. The equations and their constants are shown in Table 1. The initial concentration of chlorophyll was arbitrarily set at 20.0 units. The reaction mechanism for chlorophyll degradation kinetics was assumed as a simple reaction type;

Pheophytin a → colorless products

where k_n = rate constant for n order

The rates of reaction were obtained by taking derivatives of the concentrations with respect to time. So lnv versus ln[C] was plotted to estimate the rate order and rate constants at different temperatures (Fig. 2).

Table 2 shows the best fit equations for lnk - ln[C] data. After estimating rate order as half order reaction it was calculated coefficients of the best equations for it (Table 3). An assumption had been made for order of reaction of thermal chlorophyll degradation in a lot of previous studies on the processes of different food matrices such as fermentation of pickles coleslaw and olives (MINGUEZ-MOSQUERA *et al.*, 1992, MINGUEZ-MOSQUERA *et al.*, 1994; HEATON *et al.*, 1996) or thermal processing of spinach (CANJURA *et al.*, 1991; YONGXI *et al.*, 2000) and also such as the visual green color a degradation (STEET and TONG, 1996; WEEMEAS *et al.*, 1999; AHMED *et al.*, 2002; THRON *et al.*, 2001; AHMED *et al.*, 2004; APARICIO-RUIZ *et al.*, 2011, AHMED *et al.*, 2013; MERCALI *et al.*, 2014; DONG *et al.*, 2014), and kinetics studies had been gone on assuming an order of 1. But VAN BOEKEL (2009) reported that the best model for the decomposition of chlorophyll is not only first-order equa-

Table 3 - Best fit equations for half-order rate.

T (oC)	[C]1/2=[C0]1/2 - k1/2/2×t		R²
	k1/2	[C0]1/2	
150	0.21	4.47	0.998
160	0.24		
170	0.26		
180	0.33		
190	0.34		
200	0.38		

tion, but also could be half-order equation; for example, applying nonlinear regression to the data of SCHWARTZ and VON ELBE (1983), the best order n is 0.5 ± 0.5 for chlorophyll a and 0.6 ± 0.4 for chlorophyll b (± 95% confidence interval). Thus, the present data similar to the data of VAN BOEKEL (2009). It was compared the differential method with the other two methods; substitution and graphic method. The results obtained from the three different methods are shown in Table 4. It was found that the rate constants and half-lifes at each temperature determined by three methods were close together. The reaction rates increased approximately 1.1 times with increment of every 10°C from temperature of 150°C. But, in general the reaction rate doubles for each 10°C increase in temperature (APARICIO-RUIZ *et al.*, 2010). However, CLARK (2009) reports that this approximation (about the rate of a reaction doubling for a 10 degree rise in temperature) only works for reactions with activation energies of about 50 kJ/mol fairly close to room temperature, and the rate constant goes on increasing as the temperature rise up, but the rate of increase falls off quite rapidly at higher temperatures. The half-life of a reaction is defined as the time at which the concentration of component A is at half its initial value. It provides a highly detailed description of how fast a reaction is occurring. In the present work, the half-life decreased 0.9 times for each 10 °C increase in temperature.

The activation parameters were determined for the thermal process of chlorophyll in virgin olive oil in the range between 150°C and 200°C. The resulting logarithmic plot is shown in Fig. 3. The estimated values used in the Arrhenius Equation for chlorophyll degradation reaction during heating by using three methods is shown in Table 5. The Ea determined by graphic method (22.43 J/kg) was the same value found in substitution method whilst the value in differential method was 24.05 J/kg. Average activation energies for chlorophyll with respect to first order reaction were reported to be in range of 14.8 and 15.3 kcal/mol in the different temperatures and pH range (RYAN-STONEHAM and TONG, 2000; KOCA *et al.*, 2006). If a compound has low activation energies it is highly sensitive to temperature (JAISWAL *et al.*, 2012)

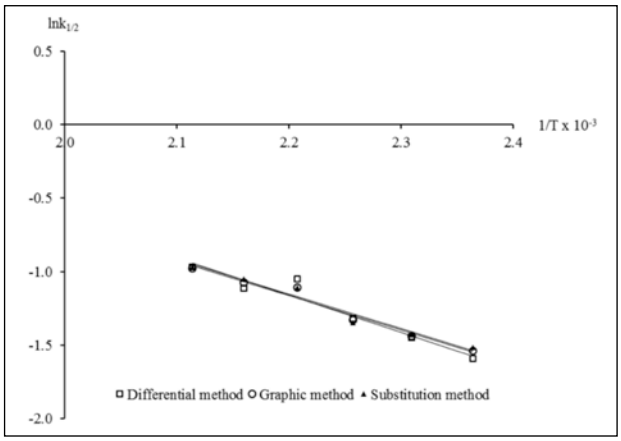


Fig 3 - lnk versus 1/T plot for the thermal process of chlorophyll in virgin olive oil.

Table 4 - Comparison of methods used for determination of the rate constant and the half-life.

T (oC)	rate constant (k1/2)			half-life (t1/2)		
	D	G	S	D	G	S
150	0.20	0.21	0.22	13.12	12.44	12.14
160	0.24	0.24	0.24	11.36	11.22	11.12
170	0.27	0.26	0.26	9.96	10.09	10.27
180	0.35	0.33	0.33	7.55	8.12	8.09
190	0.33	0.34	0.35	8.12	7.79	7.63
200	0.38	0.38	0.38	7.04	7.11	7.03

D: differential method. G: graphic method. S: substitution method.

4. CONCLUSIONS

Thermal processing played an important role for degradation of chlorophyll in virgin olive oil during heating in high temperature range. The kinetics of degradations of chlorophyll in oily food matrices was studied by using differential method. The rate order of chlorophyll degradation reaction was determined as half order reaction that are not yet reported in literature from the experimental results. The degradation reaction of chlorophyll in many of previous studies have been fitted first-order kinetic model by assumption. Use of half-order reaction model for chlorophyll degradation should be encouraged by further studies.

Table 5 - Arrhenius constant, and activation energy for chlorophyll.

Method	k=Aexp(-Ea/R*T)			Best fit equations for lnk1/2 vs 1/T data	
	A	Ea (J/kg)	R*(J/kg.K)		R^2
D	79.84	24.05	9.543	lnk1/2= -2.52x103(1/T) + 4.38	0.93
G	55.70	22.43		lnk1/2= -2.35x103(1/T) + 4.02	0.97
S	55.15	22.43		lnk1/2= -2.35x103(1/T) + 4.01	0.97

D: differential method. G: graphic method. S: substitution method.

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PAPER

PHYSICOCHEMICAL, MICROBIOLOGICAL AND COLOUR ATTRIBUTES OF HORSE SALAMI ESTABLISHED DURING THE RIPENING PERIOD

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ABSTRACT

Changes in physicochemical, colour, textural, microbiological and sensory attributes occurring during the processing of Horse Salami and established on manufacturing days 0, 7, 14, 21, 28, 42, 60, 90 were studied. Significant changes ($P<0.05$) in physicochemical parameters attributable to moisture loss, as well as changes in colour and textural properties were observed during the fermentation and ripening stage. Proteolysis and lipolysis, coming as a result of endogenous enzymatic activity and high lactic acid bacteria and staphylococci counts, contributed to specific organoleptic properties of the final product. Sensorial profiling showed a significant ($P<0.05$) acid taste, lactic acid odour and flavour intensity, and low fat/lean ratio and smokiness and saltiness values. Final Horse Salami products were microbiologically safe, the dominant microbial population thereby being *Lactobacillus plantarum*, *Lactococcus lactis* ssp. *lactis*, *Enterococcus faecium* and *Staphylococcus xylosus*.

- Keywords: CIE L*, a*, b*, horse Salami, microbiological aspect, physicochemical properties, Sensory attributes, texture profile analysis -

INTRODUCTION

Horse Salami, an indigenous Croatian meat product, is a dry fermented sausage made of horse meat supplemented with pork fatback, salt and spices. In Croatia, the tradition of Horse Salami production is kept by the Italian minority populating the eastern part of the Country (in specific, the western Slavonian region). In the past, this product had been known as "the dish of the poor"; nowadays, it represents a highly appreciated autochthonous Croatian meat product having a great potential to become a PGI (*Protected Geographical Indications*) & PDO (*Protected Designation of Origin*). Although horse meat has a high nutrition, as well as a high mineral value (due to its vitamin B and iron content, respectively) (BADIANI *et al.*, 1997; FRANCO *et al.*,, 2011), human consumption is negligible in comparison with other conventional types of meat like pork, beef or chicken (LOMBARDI *et al.*, 2005). Horse meat used for the production of Horse Salami is obtained from horses slaughtered at the end of their (5-year or longer) lifecycle. The meat has no appreciable organoleptic qualities. Its original colour is deep red larded with yellow fat, while the meat is tough to chew due to the connective tissue maturation (LITWINCZUK *et al.*, 2008; TATEO *et al.*, 2008).

Horse Salami has specific sensorial properties (smell and taste) attributable mainly to drying and smoking, but also to ripening, as well as to enzymatic, lactic acid bacteria and mould activity. The recipe is 130 years old and the sole difference in final products coming from various producers boils down to the difference in mass fraction of fatback used in the Salami preparation (ranging from 12 to 15%). The production of the traditional Horse Salami mainly takes place on small farms; we are therefore talking a small-scale production seasonal in its nature, fluctuating on a year-by-year basis dependent on weather conditions. In light of the foregoing, standardization of the Horse Salami production becomes imperative. Dry sausages produced in various European countries, mainly Spain and Italy, have been extensively studied for their physicochemical composition, colour and textural properties (CASIRAGHI, *et al.*, 1996; GIMENO *et al.*, 2000; BRUNA *et al.*, 2001; SPAZIANI *et al.*, 2009). However, scientific information on this Croatian indigenous dry sausage, which would efficiently contribute to its characterization and production standardisation, is virtually non-existent.

Therefore, the aim of this study was to investigate, for the first time ever, physicochemical composition, microbiological and sensorial attributes of the dry-fermented sausage known as Horse Salami and the changes occurring during 90 days of its manufacturing. Investigations also included instrumental measurements of colour and texture of the studied Salami on certain processing days, as well as the isolation and identification of autochthonous microbial population and gathering of other data needed for microbiological safety evaluation of the final product.

MATERIAL AND METHODS

The manufacturing process

Samples of traditional Horse Salami (24 units) were manufactured in a small-scale facility in the western Slavonian region (the Eastern Croatia). All samples were prepared using traditional procedures that made no use of additives such as starter cultures supplemented with nitrites, nitrates or ascorbic acid (namely, the production of traditional Croatian meat products does not involve the use of additives). Such a traditional production takes about 3 months (90 days). Horse Salami is made of meat of older (5+ years), worn-out horses, mainly of the Hrvatski Posavac breed. After slaughtering, fat and connective tissue are carefully removed from the horse meat. This is especially important when it comes to fat, because horse fat has a particularly unpleasant smell and taste. The meat is then grinded using a grinding plate having holes measuring 6 mm in their diameter and left to rest overnight (12 hours at the minimum) in a special container equipped with a decantation hole. Grinded horse meat is then mixed with pig fatback represented in the amount of 12%. Before its mixing with the horse meat, the fatback is grinded using a grinding plate having holes measuring 10 mm in their diameter. The mixture of meat and fat is then mixed with salt added in the amount of 2.2%, red paprika powder added in the amount of 0.2%, hot red paprika powder added in the amount of 0.3%, garlic added in the amount of 0.2%, and black pepper added in the amount of 0.3%. In the subsequent course, the mixture gets to be stuffed into a horse small intestine (roughly 50 cm long and 50 mm wide in diameter) or into collagen casings (of the same dimensions). Thereafter, the Horse Salami is smoked on a dry hard wood (hornbeam, beech and its sawdust) every few days (for 2-3 hours) for the total of four weeks. At this stage, the temperature and relative humidity should be kept at 18 to 20°C and 70 to 90%, respectively. After smoking, the Horse Salami is left to ripen. This stage is the longest and should take about two months, throughout which period the Salami should be kept in a dark room at the temperature ranging from 14° to 17°C, with the relative humidity ranging from 70 to 80%. After that, Horse Salami is ready for consumption. Within this study frame, samples of Horse Salami were taken on the processing days 0, 7, 14, 21, 28, 42, 60 and 90. In total, 24 samples were produced; at each processing stage, three samples were taken for the analyses.

ANALYTICAL METHODS

Physicochemical parameters

Before the analysis, the Sausage samples were homogenised using a knife mill Gridomix GM 200 (Retsh, Germany) and prepared according to ISO 3100-1:1975.

Water content was determined gravimetrically (ISO 1442:1997) at 103°C (Epsa 2000 Bari, Croatia), while the ash content was established according to ISO 936:1998, by virtue of burning the samples at 550 °C (LV9/11/P320 Nobertherm, Germany). Total protein content was determined using the Kjeldahl method (ISO 937:1978) that made use of an Unit 8 Basic digestion block (Foss, Sweden) and a Kjeltec 8400 automated distillation & titration device (Foss, Sweden). The total fat content was determined using the Soxhlet method (ISO 1443:1973), which involves digestion of a sample in acidic environment followed by fat extraction with petroleum ether using a Soxtherm 2000 Automatic device (Gerhardt, Germany). The determination of collagen content was performed through the analysis of hydroxyproline according to ISO 3496:1994 that made use of a spectrophotometer (Hach DR/4000U, Germany). Sodium chloride content was determined using the internal titration method (TRAJKOVIĆ *et al.*, 1983). In this analysis, 2 g of each sample were homogenized with sand and 3 mL of water. The content was transferred into a 100 mL-volumetric flask, stirred and placed for 15 min into a water bath at 100 °C. After cooling, the flask was filled with water up to the mark and filtered. An aliquot (25 mL) of the filtrate was transferred into an Erlenmeyer flask containing a few drops of K_2CrO_4 indicator (62 g/100 mL of water) and titrated with 0.1 M-$AgNO_3$ until a persistent reddish colour was obtained. Sodium chloride content was calculated based on the expenditure of titration reagent and its concentration.

pH values were determined in a homogenate diluted with distilled water (1:10, p/v) using pH/Ion 510 – Bench pH/Ion/mV Meter (Eutech Instruments Pte Ltd/ Oakton Instruments, USA) according to the pH/Ion 510 Instruction Manual. Water activity (a_w) was determined at the room temperature (20°±2°C) using a Rotronic Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland). All chemicals used for analyses of physicochemical parameters were of an analytical grade. For each sample, three independent measurements were made.

Instrumental determination of colour

Instrumental colour measurements (those of L*, a*, and b* values) were performed using a Hunter-Lab Mini ScanXE (A60-1010-615 Model Colorimeter, Hunter-Lab, Reston, VA, USA). The instrument was standardized on each occasion using a white ceramic plate (L_0 = 93.01, a_0 = -1.11, and b_0 = 1.30). The CIELAB space values (L*, a* and b*) (CIE, 1976) correspond to lightness, greenness (-a*), redness (a*), blueness (-b*) or yellowness (b*). The colour measurements performed on the Horse Salami took place at the room temperature (20°±2°C). Each sample was cut in slices and colour-measured at ten different spots.

Texture Profile Analysis

Texture Profile Analysis (TPA) was performed using a TA.XT2i SMS Stable Micro Systems Texture Analyzer (Stable Microsystems Ltd, Surrey, England) equipped with a P/75 aluminium cylindrical probe. This involved cutting the samples into 1.5 cm-thick slices and their double compression so as to downsize them to 40% of their original thickness. Force-time curves were recorded at the across-head speed of 5 mms^{-1} and at the same recording speed. The following parameters were quantified (BOURNE, 1978): hardness (kg), i.e. the maximum force required to compress the sample; springiness (ratio), i.e. the ability of the sample to recover its original form after the cessation of the deforming force; cohesiveness (ratio), i.e. the extent to which the sample could be deformed prior to rupture; chewiness (kg), i.e. labour required to masticate the sample before swallowing, which represents the product of hardness multiplied by cohesiveness and springiness; and finally resilience (ratio), so as to determine how well the product "fights to regain its original position". These parameters were obtained using the Texture Expert for Windows (Version 1.0) Stable Micro Systems. With each sample, eight determinations of texture parameters were made.

Microbiological analysis

After aseptically removing and discarding the casing, 10 g of the product were recovered in an aseptic manner, homogenized in 90 ml of the sterile 0.5%-saline solution and serially diluted before their planting on a non-selective (peptone yeast extract glucose agar, Biolife, Milano, Italy), PCA-agar (standard plate count agar) (Biolife, Milano, Italy) and the following selective media: MRS-agar (Biolife, Milano, Italy) intended for lactic acid bacteria growth and Baird-Parker agar (Merck, Darmstadt, Germany) intended for staphylococci growth. The plates were incubated under conditions specified in Table 1.

Isolation and identification of microbial population in the final product

Classical microbiological and biochemical (API) methods (Table 1) were used for the isolation and identification of the natural microbial population in the traditionally produced Horse

Table 1 - Classical microbiological and biochemical (API) methods of isolation and identification of microbial population applied in the Horse Salami analyses.

Microorganism	Nutrient media	Incubation conditions	API test
Salmonella sp.	RP-broth, XLD (Biolife, Italy)	37°C 24-48 h	API 20 E V4.1
Enterobacteriaceae	VRBG (Biolife, Italy)	37°C 24 h	API 20 E V4.1
Staphylococcus aureus	BP (Biolife, Italy)	37°C 48 h	API Staph V4.1
Coagulase negative staphylococci (CNS)	BP (Biolife, Italy)	37°C 48 h	API Staph V4.1
Sulphite reducing clostridia	Sulphite agar (Biolife, Italy)	37°C 72 h	-
Listeria monocytogenes	Fraser broth Palcam agar (Biolife, Italy)	37°C 24 h	API Listeria V1.2
Lactic acid bacteria	MRS agar (Biolife, Italy)	30°C 48-72 h	API 50 CHL V5.1 API 20 STREP V7.0
Yeasts	Sabouraud agar (Biolife, Italy)	25°C 48-72 h	API 20 C AUX V4.0 Yeasts

salami (i.e. in the final product obtained after 90 production days). Ten grams of the sample were homogenized in 90 mL of sterile 0.5% saline solution and serially diluted before planting on a non-selective medium (peptone yeast extract glucose agar, Biolife, Milano, Italy) and selective media under conditions specified in Table 1. Colonies randomly taken from selected plates were identified on the basis of their morphology, Gram-staining, cell morphology and catalase reaction. The identity of bacteria species was further confirmed using the API identification kits (BioMérieux, France).

Sensorial analysis

The final Horse Salami product (obtained after 90 days) was subjected to a quantitative descriptive analysis performed by a panel of seven (3 male and 4 female) trained experts according to ISO 6658:2005 standard. The panellists had completed a preliminary three session-training in order to familiarize themselves with the samples under investigation. Fourteen attributes were examined and rated on a 5-point scale, "1" thereby standing for "poorly perceived or absent" and "5" standing for "intensely perceived". During these three training sessions, the descriptors to be targeted by the analysis were agreed upon. The latter included as follows: 2 external attributes (appearance, hardness), 4 attributes descriptive of a slice (fat/lean ratio, easy peeling capability, colour intensity, sliceability), 5 attributes descriptive of perceptions during mastication (flavour intensity, juiciness, smokiness, acid taste, saltiness) and 3 attributes descriptive of the product smell (spice odour, lactic acid odour, mould odour). The Sausage samples were coded using a three-digit code and presented in form of oblique slices approximately 0.4 cm thick. Water was provided to clean the panellists' palate between analyses.

Data analysis

Differences between the average values of the same physicochemical, colour, texture, microbiological and sensory parameters were analyzed using the analysis of variance (ANOVA) and the Fisher's least significant difference test (LSD), with statistical significance being set at $P<0.05$. Moisture, fat, protein, collagen and NaCl content, pH, a_w, colour and textural parameters were subjected to correlation analysis (Pearson´s correlation test) so as to determine their possible statistically meaningful relationships. Statistical analysis was carried out using Statistica Ver. 8.0 StatSoft Inc. Tulsa, OK, USA.

RESULTS AND DISCUSSION

Physicochemical parameters

Basic chemical composition, salt (NaCl) content, pH values and water activity (a_w) of the Horse Salami, established at various processing stages, are given in Table 2. The average initial moisture content of the Horse Salami found to be 61.91% had significantly decreased (P<0.05) as the processing went on due to smoking and dry-ripening typical of dry fermented sausages (LIZASO *et al.*, 1999; PEREZ-ALVAREZ *et al.*, 1999; SALGADO *et al.*, 2005; SALGADO *et al.*, 2006; LORENZO *et al.*, 2012). Higher moisture losses were observed in the first 21 processing days and on day 28, which is characteristic for this type of product (< 40%) and dry sausages in general (PLEADIN *et al.*, 2014). Further ripening leads to additional moisture content reduction, so that the lowest value (28.51%) was determined on manufacturing day 90. In 2012, LORENZO and co-workers reported higher initial and final moisture values for the foal salchichon. This can be explained by the fact that horse meat has a lower water content as compared to foal meat (LITWINCZUK *et al.*, 2008; LANZA *et al.*, 2009; TATEO *et al.*, 2008), as well as by the longer ripening period of the Horse Salami. The final moisture content was also lower than in similar dry sausages coming from Spain (GIMENO *et al.*, 2000; RUBIO *et al.*, 2007; LORENZO *et al.*, 2012), which can also be attributed to a longer ripening period of the Horse Salami.

The highest amount of proteins (30.53%) was determined on day 90. The results are consistent with the published literature data, which show that due to prolonged drying and ripening (weight loss of up to 50%) and a high share of lean meat used in stuffing preparation, moisture and protein content in ripened dry-fermented sausages tend to be similar (30-40%), indicating a high nutritional value of the final product (PLEADIN *et al.*, 2014).

The average fat content of the Horse Salami had increased significantly (P<0.05) from day 1 to day 90 (from 13.84 to 28.54%), in proportion to the duration of the Horse Salami ripening process and dehydration, i.e. the continuous reduction of water content in the product; the same goes for the protein and collagen content (Table 2). Fat as a substantial component of fermented sausages has multiple functions; it represents a concentrated energy source (9 kcal/g) and the source of essential fatty acids and fat-soluble vitamins (MELA, 1990). Furthermore, it is contributing to the fullness of flavour, texture and softness of the product, all of the aforementioned being relevant for the quality and acceptability of the product in question (OLIVARES *et al.*, 2010). Hydrolysis and oxidation of fatty acids that occur during the ripening process largely contribute to the taste of fermented sausages (ORDONEZ *et al.*, 1999). The final fat content was lower, while the final protein content turned out to be higher than in Spanish and Italian dry fermented sausages (DELLAGLIO *et al.*, 1996; RUBIO *et al.*, 2008).

The average initial ash content was 3.13% and had increased significantly (P<0.05), reaching the ultimate value of 5.72%, whereas water activity (a_w) (Table 2) had decreased significantly (P<0.05) during the smoking and dry-ripening period (from 0.96 to 0.78). Changes in mass fraction of individual basic constituents and water activity decrease seen after 90 days of Horse Salami production (Table 2) are mostly caused by the drying process, i.e. the loss of water occurring during ripening.

Changes in pH values seen during the processing of the Horse Salami are presented in Table 2. pH value had decreased during the first 21 days of processing (from 5.58 to 4.71), possibly as a result of the presence of organic acid produced by bacteria (LUCKE, 1994). This pH drop is typical of most dry fermented sausage (PEREZ-ALVAREZ *et al.*, 1999; GIMENO *et al.*, 2000; LIZASO *et al.*, 1999; MUGUERZA *et al.*, 2002; BOZKURT and BAYRAM, 2006; VAN SCHALKWYK *et al.*, 2011). At the final processing stage, pH values increased to 4.94, possibly due to the liberation of peptides, amino acid and ammonia re-

Table 2 - Basic chemical composition, salt content, a_w and pH of the Horse Salami established during the manufacturing process.

	Processing time (days)							
	0	**7**	**14**	**21**	**28**	**42**	**60**	**90**
Moisture (%)	61.91a±0.06	55.67b±0.01	48.92c±0.09	43.22d±0.06	37.57e±0.01	35.16f±0.04	31.61g±0.06	28.51h±0.02
Fat (%)	13.84h±0.03	15.83f±0.03	17.71e±0.01	18.55d±0.02	18.59d±0.04	20.59c±0.02	25.45b±0.06	28.54a±0.12
Protein (%)	17.05h±0.04	22.48g±0.01	23.73f±0.01	24.36e±0.08	27.62d±0.04	27.95c±0.05	29.34b±0.23	30.53a±0.05
Collagen (%)	0.63e±0.11	1.19d ±0.11	1.56cd±0.07	2.05c±0.21	2.06c±0.09	2.82b±0.15	2.84b±0.12	3.93a±0.10
Ash (%)	3.13g±0.02	3.73f±0.06	4.56e±0.04	4.87d±0.01	4.94d±0.01	5.36c±0.05	5.45b±0.06	5.72a±0.01
Salt (NaCl) (%)	2.30g±0.04	2.71f±0.05	3.29e±0.03	3.63d±0.05	3.75c±0.05	3.81c±0.05	4.24b±0.02	4.51a±0.03
aw	0.96a±0.01	0.93ab±0.02	0.91b±0.01	0.88c±0.01	0.87c±0.03	0.86c±0.04	0.86c±0.01	0.78d±0.01
pH	5.58a±0.03	4.99b±0.05	4.74f±0.10	4.71g±0.16	4.72fg±0.08	4.76f±0.06	4.81d±0.10	4.93c±0.07

Values are means ±SD obtained with three measurements. Values displayed in the same row and tagged with different letters (a-h) are significantly different (P<0.05).

sulting from a proteolityc reaction (SPAZIANI *et al*, 2009). The final pH was lower than in most dry fermented sausages (5.2 to 5.8) (BOVER-CID *et al.*, 2001; RUBIO *et al.*, 2007; ROSERIO *et al.*, 2010), which can be explained by horse meat properties in terms of higher glycogen content as compared to pork, beef and foal meat (LAWRIE and LEDWARD, 2006).

The salt content of the Horse Salami had significantly increased during processing (P<0.05) (Table 2). Literature sources have reported the average mass fraction of salt in dry sausage stuffing to range from 2.0% to 2.6%, and that in final products to range from 3.3% to 4.3% (OCKERMAN and BASU, 2007; STAHNKE and TJENER, 2007). In this study, mass fraction of salt (NaCl) established during the Horse Salami manufacturing process ranged from 2.31% to 4.51%.

Instrumental colour properties

The CIELAB space (L*, a* and b*) values of the Horse Salami were significantly affected (P<0.05) by the length of smoking and ripening period (Table 3). Lower lightness L* values seen with an increased length of processing are probably related to the dark colour of the Horse Salami coming as a consequence of browning. A similar decrease in L* values during ripening was reported by BOZKURT and BAYRAM (2006) for Turkish sucuk, and by LORENZO *et al.* (2012) for foal salchichon.

Redness (a*) had significantly (P<0.05) decreased at all processing stages. Similar lower a* values were seen during the ripening of Spanish pork dry sausages and foal salchichon, as reported by PEREZ-ALVAREZ *et al.* (1999) and LORENZO *et al.* (2012). Lowering of a* values can possibly be explained by total or partial denaturation of nitrosomyoglobin coming as a result of lactic acid production. L* and a* values lower than those reported by LORENZO *et al.* (2012) can probably be related to the nature of horse meat, which is darker and redder than foal (TATEO *et al.*, 2008)

Yellowness (b*) had decreased from 20.32 to 9.11 and had varied significantly (P<0.05) during the production process. The decrease in b* values seen with the prolongation of the processing time was also reported by other authors (PEREZ-ALVAREZ *et al.*, 1999; LORENZO *et al.*, 2012) and explained by the decrease in concentration of oxymyoglobin coming as a result of oxygen consumption executed by microorganisms.

Texture Profile Analysis

Texture Profile Analysis (TPA) parameters of the Horse Salami established during the smoking and dry ripening period are presented in Table 4. Average hardness values had significantly increased (P<0.05) from 0.32 to 20.54 kg as the processing went by. This can be related to the coagulation of muscle protein coming as a result of low pH values and sausage drying (BOZKURT and BAYRAM, 2006).

Springiness and cohesiveness had significantly decreased (P<0.05) during the processing (from 0.76 to 0.64 and from 0.67 to 0.43, respectively). Springiness is related to elastic proper-

Table 3 - Colour parameters of the Horse Salami established during the manufacturing process.

	Processing time (days)							
	0	7	14	21	28	42	60	90
L*	46.67a±1.08	43.01b±1.05	41.81c±0.37	40.54d±0.30	38.75e±0.35	33.77f±1.27	33.28f±0.92	31.28g±0.89
a*	17.71ab±0.40	17.29ab±2.03	18.54a±3.44	16.16ab±2.65	15.49b±0.39	12.07c±0.62	10.86cd±01.06	8.15d±0.69
b*	20.32a±1.49	18.01bc±2.52	17.98bc±2.63	16.51c±2.22	13.39d±0.43	13.14d±0.80	12.14d±2.04	9.11e±0.58

Values are means ±SD obtained with ten measurements. Values displayed in the same row and tagged with different letters (a-g) are significantly different (P<0.05).

Table 4 - Parameters obtained by virtue of Textural Profile Analysis (TPA) of the Horse Salami during the manufacturing process.

	Processing time (days)							
	0	7	14	21	28	42	60	90
Hardness (kg)	0.32h±0.01	3.67g±0.19	4.61f±0.09	6.19e±0.21	9.91d±0.11	14.94c±0.25	17.58b±0.81	20.54a±0.92
Springiness	0.76a±0.03	0.62def±0.03	0.68bc±0.01	0.73ab±0.02	0.58f±0.01	0.61ef±0.02	0.66cd±0.01	0.64cde±0.03
Cohesiveness	0.67a±0.04	0.51c±0.04	0.48cd±0.01	0.65ab±0.12	0.46cd±0.01	0.46cd±0.01	0.43d±0.03	0.43d±0.03
Gumminess (kg)	0.26f±0.01	1.87e ±0.15	2.26e±0.07	4.02d±0.50	4.56d±0.06	6.87c±0.13	7.73b±0.50	8.83a±0.54
Chewiness (kg)	0.20g±0.02	1.16f±0.09	1.54e±0.04	2.94d±0.40	2.64d±0.04	4.19c±0.10	5.10b±0.49	5.65a±0.55
Resilience	0.19a±0.04	0.15bcd±0.02	0.16bc±0.03	0.18ab±0.05	0.15bcd±0.01	0.12e±0.01	0.13de±0.02	0.14cde±0.01

Values are means ±SD obtained with eight measurements. Values displayed in the same row and tagged with different letters (a-h) are significantly different (P<0.05).

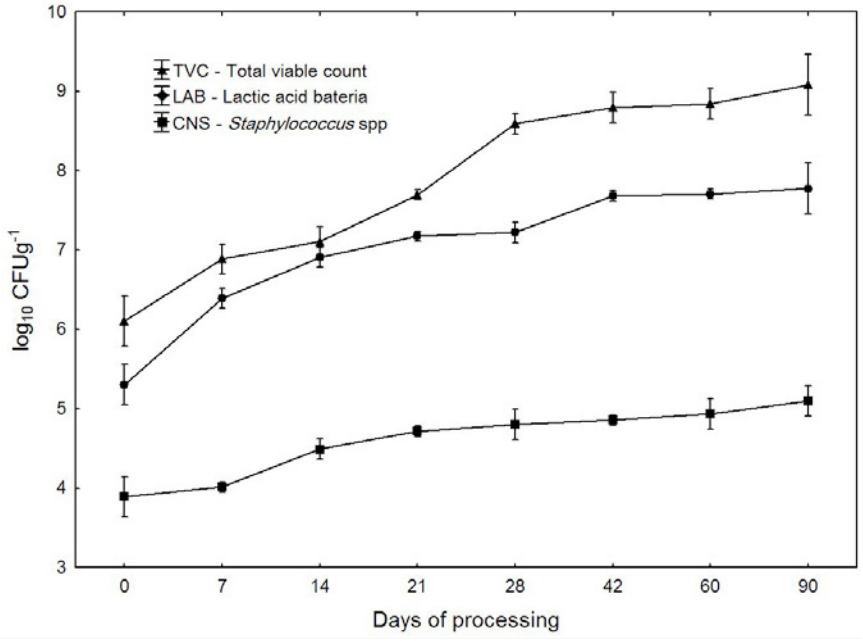


Fig. 1 - Changes in microbial counts seen during the processing of the Horse Salami (mean±standard deviation obtained with three samples).

ties, so that the decrease in this textural property of the Horse Salami is most likely to be related to the removal of water (BOZKURT and BAYRAM, 2006).

Increases in gumminess and chewiness values (from 0.26 to 8.83 and from 0.20 to 5.65, respectively) seen during the Horse Salami processing were statistically significant ($P<0.05$). Increase in chewiness values indicates that the Horse Salami becomes tougher during the ripening period (SZCZESNIAK, 2002), possibly due to moisture loss.

Resilience values established at the beginning and at the end of the processing were 0.19 and 0.14, respectively. Significant changes in resilience during smoking and ripening failed to be observed ($P>0.05$) (Table 4).

Microbial counts

Microbial flora changes seen during manufacturing are shown in Fig. 1. The initial bacterial counts were 6.09 log CFU g^{-1} for total viable count (TVC), 5.29 log CFU g^{-1} for lactic bacteria (LAB), and 3.88 log CFU g^{-1} for *Staphylococcus* spp, respectively. Relatively low bacterial counts in the Salami stuffing indicate a good hygienic quality of the raw materials. TVC, LAB and *Staphylococcus* spp counts had significantly increased during the ripening period ($P<0.05$). This increase in bacterial count is typical of most naturally dry fermented European sausages (KOZAČINSKI *et al.*, 2008). At the end of the Horse Salami production process, the mean values were 9.10, 7.79 and 5.10 log CFU g^{-1}, respectively.

As reported by many studies, microorganisms most represented during the ripening of cured sausages and meat products are LAB (LIZASO *et al.*, 1999; SAMELIS and GEORGIADOU, 2000), whose counts tend to remain stable throughout the ripening period. Within the frame of this study, high LAB counts had been found during the first 28 ripening days, which can be related to a substantial pH drop witnessed during that period (Table 2). LAB inhibit the growth of pathogenic and spoilage bacteria by virtue of formation of lactic acid, acetic acid and possibly bacteriocins (LUCKE, 2000).

Isolation and identification of microbial population

Native sausage products are of a higher quality than those obtained by virtue of controlled fermentation with the addition of industrial starters (LEBERT *et al.*, 2007). Many authors support the view that indigenous microflora or microorganisms present in traditional sausages originate from raw materials or the manufacturing environment (MAURIELLO *et al.*, 2004; RANTSIOU *et al.*, 2005). This microbiota is commonly referred to as "the house flora" (GARCIA-VARONA *et al.*, 2000).

Therefore, in this study, the isolation and identification of autochthonous microbial population inhabiting the Horse Salami was performed. The results of a microbiological analysis (Table 5) showed the dominant microflora to be the lactic acid bacteria strain termed *Lactobacillus plantarum, Lactococcus lactis* ssp. *lactis*, and *Enterococcus faecium* while the most represented coagulase-negative staphylococci strain was *S. xylosus*. The yeast *Candida famata/Debaryomyces hansenii* was found as well, which is in agreement with the results of NIELSEN *et al.* (2008), who stated that halophilic yeasts most frequently isolated from fermented meat prod-

Table 5 - Biochemical (API) results of the final product obtained after 90 days of manufacturing.

Microorganism	Values log CFU g^{-1}±SD	API test
Salmonella sp.	-	-
Enterobacteriaceae	-	-
Staphylococcus aureus	-	-
CNS (Coagulase negative staphylococci)	5.10±1.5	*S. xylosus*
Sulphite reducing clostridia	-	-
Listeria sp.	-	*Listeria grayi*
Lactic acid bacteria	7.79±1.3	L. *lactis* ssp. *lactis, Lactobacillus plantarum, Enterococcus faecium*
Yeasts	3.25±1.2	*Candida famata/Debaryomyces hansenii*

ucts are *Debaromyces hansenii*, *Candida famata*, *Candida zeylanoides*, *Trichosporon* sp., *Cryptococcus* sp. and *Rhodotorula* sp. Yeasts also play an important role in the maturation of sausages, since their lipolytic and proteolytic activity contributes to the development of sensory characteristics of fermented sausages (KOVAČEVIĆ, 2001; ALAGIĆ *et al.*, 2008).

In the Horse Salami samples, bacteria of the *Salmonella* genus, *Enterobacteriaceae*, sulphite-reducing Clostridia, *L. monocytogenes* or *S. aureus* were not found; however, API biochemical tests uncovered the presence the *Listeria grayi* bacterium which is non-pathogenic (Table 5). Issues sometimes emerging with this type of fermented meat product are short shelf-life and poor hygienic surroundings, but the sausages produced in this investigation were proven to be microbiologically safe. It should be pointed out that biochemical (API) tests gave very good results (identification of one species with ID > 98,2-99.9 %).

The isolated lactic acid bacteria *L. lactis* ssp. *lactis*. *L. plantarum and E. faecium* could be used as starter cultures for meat products. *L. plantarum* as an autochthonous meat microflora is widely spread in nature (SALAMA *et al.*, 1995; AYAD *et al.*, 2001), *L. lactis* ssp. *lactis* in fermented sausage has rarely been reported so far and therefore further studies must to include detail molecular identification of isolated strains because API identification is not 100% precisely. The interest in exploring the potential of new strains isolated from different natural ecosystems to the effect of aroma compounds production has recently increased (AYAD *et al.*, 2001; FRECE *et al.*, 2009; BABIĆ *et al.*, 2011, FRECE *et al.*, 2014). Metabolic properties of the *L. plantarum, E. faecium* and *L. lactis* species have both direct and indirect influence on organoleptic, nutritional and hygienic quality of fermented products. More and more research is focused on the isolation and identification of autochthonous functional starter cultures with the aim of developing new functional meat products that will be recognised and labelled as autochthonous to the region in which they are produced (BABIĆ *et al.*, 2011, FRECE *et al.*, 2014, FRECE *et al.*, 2014 a,b). Therefore, *L. plantarum, E. faecium, L. lactis* and *S. xylosus* as potential functional autochthonous starter cultures will be thoroughly investigated in the future. Further studies will be carried out to detail phenotypic, genotypic and physiological characterization of isolated strains of staphylococci and LAB.

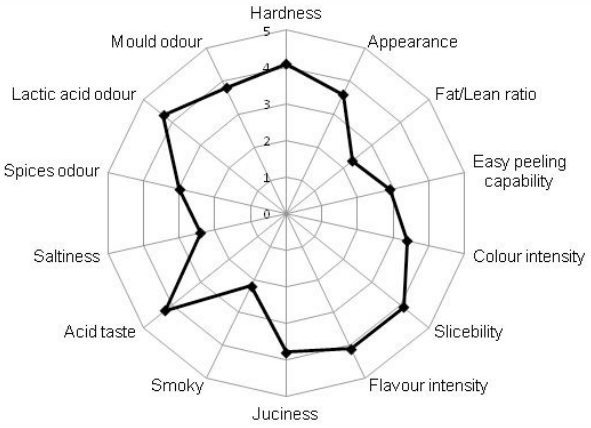


Fig. 2 - Mean values of sensory properties of the final Horse Salami.

Sensory characteristics

Complex interaction between physicochemical, biochemical and microbiological processes, playing a role in formation of chemical compounds, and the modification of molecules responsible for the texture and appearance of the final product also determine its sensory characteristics.

Average scores given by the panellists at the end of the Horse Salami manufacturing process are shown in Fig. 2. As for the external attributes, the Horse Salami scored highly when it comes to hardness (4.10±0.71) and low when it comes to appearance (3.60±0.43). It was highly rated for its sliceability, but low-rated when it comes to its colour intensity, fat/ lean ratio and easy peeling capacity. After slicing, the highest scores were obtained for the fat distribution (4.78±0.67), while the fat/lean ratio scored low (2.22±0.44).

Regarding the attributes that describe perceptions during mastication, Horse Salami was highly rated for its flavour intensity (4.10±0.44), juiciness (4.24±0.21) and acid taste (3.79±0.17), and low-rated for its saltiness and smokiness (2.41±0.31 and 2.20±0.19).

During the fermentation of dry sausages, LAB produce lactic acid (MATEO *et al.*, 1996) responsible for the sour taste (LOTONG *et al.*, 2000) and odour of the product, while mould odour is to be associated with 1-octen-3-ol, which spreads a typical mushroom odour (MEYNIER *et al.*, 1998). In the present study, all three attributes scored highly (lactic acid taste 4.24±0.18; lactic acid odour 4.31±0.22; mould odour 3.82±0.15).

As for the smell descriptors, lactic acid (4.3±0.22) and mould odour (3.8±0.15) were dominant, while the spice odour scored low (3.00±0.28).

Correlation between the parameters

Instrumental colour parameters of the Horse Salami, established during its processing, were significantly inversely correlated (P<0.05) to the protein, fat, ash, collagen and salt content. Moisture content and a_w values exhibited a significant direct correlation (P<0.05) to the instrumental colour parameters (Table 6). Relationships between the moisture, protein, fat, ash, collagen and salt content and a_w on one hand, and hardness, gumminess and chewiness on the other, were also significant (P<0.05) (that between moisture and a_w being an inverse one). Pearson's correlation coefficients indicated that springiness and resilience are not significantly (P>0.05) correlated to the basic chemical composition, salt content and a_w (Table 6).

CONCLUSIONS

This study investigated into the changes in physicochemical, colour, textural, microbiological and sensorial properties of the Horse Salami as an indigenous Croatian dry fermented sausage. During 90 days of manufacturing, major changes in physicochemical, colour and textural properties took place during the fermentation and ripening stage, pointing to proteolysis and lipolysis phenomena coming as a result of endogenous enzymatic activity, as well as to high lactic acid bacteria and staphylococci counts contributing to the specific organoleptic attributes of the final product. Sensorial profiling of the final Horse Salami showed a significant acid taste, lactic acid odour and flavour intensity, and low fat/lean ratio, smokiness and saltiness values. The final product was proven to be microbiologically safe, the dominant microbial population being *L. lactis* ssp. *lactis, L. plantarum, E. faecium* and *S. xylosus*.

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Table 6 - Pearson´s correlation coefficients established between basic chemical composition, salt content, aw, texture and instrumental colour parameters.

	Hardness (kg)	Springiness	Cohesiveness	Gumminess (kg)	Chewiness (kg)	Resilience	L*	a*	b*
Moisture (%)	-0.95**	0.19	0.68**	-0.96**	-0.94**	0.48	0.95**	0.89**	0.95**
Fat (%)	0.95**	0.058	-0.64	0.94**	0.95**	-0.35	-0.91**	-0.95**	-0.93**
Protein (%)	0.96**	-0.28	-0.77**	0.95**	0.93**	-0.51	-0.95**	-0.90**	-0.97**
Collagen (%)	0.95**	-0.09	-0.59	0.95**	0.94**	-0.37	-0.94**	-0.96**	-0.96**
Ash (%)	0.92**	-0.10	-0.61	0.94**	0.93**	-0.44	-0.95**	-0.87**	-0.91**
Salt (NaCl) (%)	0.94**	-0.03	-0.62	0.94**	0.95**	-0.33	-0.92**	-0.90**	-0.95**
a_w	-0.86**	0.12	0.51	-0.86**	-0.84**	0.20	0.84**	0.89**	0.94**

Values marked with ** are statistically significant (P<0.05).

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PAPER

CONSUMER FAIR PRICES FOR LESS PESTICIDE IN POTATO

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ABSTRACT

This study estimates Turkish citizens' willingness to pay (WTP) for reduced pesticides on potatoes. These estimates rely on data collected from 393 persons covering all regions in Turkey through an online survey during the period from June 22 - July 21, 2014. The average WTP was found to be about TL 1.68 for all observations including zero bids and TL 2.91 excluding zero bids. The results of the probit model show that cosmetic defects, free-pesticide potatoes with insect damages, age, and gender were identified by the model to have significant impacts on the probability of WTP.

- Keywords: dichotomous contingent valuation, food safety, organic potatoes, probit model, reduced pesticides -

INTRODUCTION

Pesticides are defined by the European Commission (EC) (2009) as substances or mixtures of substances including chemical compounds intended for killing, destroying, or mitigating any pest. The use of pesticides has tragically and rapidly increased since 1960's due to the green revolution (CARVALHO, 2006). As explained by HOPPIN *et al.* (2007), pesticides could cause some respiratory diseases to farmers. Similarly, ALAVANJA *et al.* (2004) stated that indirect exposures which occur by way of drinking water, food or air happen more frequently than direct exposures occurring to individuals who apply pesticides in agriculture.

The consumption level of pesticides in Turkey increased to 54,000 tonnes in 2002 but during the last decade the level notably decreased to 40,000 tonnes (MFAL, 2012). The amount of pesticides used in Turkey seems quite low when compared with countries such as Germany and France in Europe according to the FAO statistics.

Stated and revealed preferences are the methods that are often used to measure the WTP of consumers. As stated by EBERLE and HAYDEN (1991), each individual`s valuation of a non-market good is reflected through a direct questionnaire approach. Thus, our research is mainly based on the Contingent Valuation Method (CVM) and food safety issues through the responses which come from an online survey which covers the whole of Turkey. The food safety issue plays a crucial role for both policy makers and consumers, with fast dissemination of information through social network. As underlined by ROWELL (2004), food safety and sustainable food supply are on the agenda of developed countries to develop diets that are fundamentally affordable and health-enhancing.

The overall objective of this study is to assess Turkish consumers' attitudes towards purchasing reduced pesticides that are guaranteed not to be risky to human health. The specific objectives are: determine consumers' attitudes and concerns toward pesticide use in potatoes and ascertain consumers' willingness to pay higher bid amounts for reduced pesticides in potatoes by ensuring no pesticide residues, and estimate consumers' mean WTP for reduced pesticides potatoes.

There are several reasons why the potato product is chosen. Firstly, potato is one of the most consumed vegetables in Turkey and its consumption increases yearly even if its price increases, this is according to the data extracted from the database of the Turkish Statistic Institute (TURKSTAT). Second, it is a traditional food that has a wide usage with different vegetables. Last but not least is the over-use of pesticides used on potatoes and pesticide residues in it (BIRINCI and UZUNDUMLU, 2009; AYAZ and YURTTAGUL, 2008).

Following the Introduction, methodology will be covered in detail in section 1. Within the framework of the methodology, there is discussion of: sample size, and data analysis covering both questionnaire design and descriptive statistics including the socio-economic characteristics of the respondents and consumer preferences with respect to health risks and why the CVM is used. The second section will comprehensively focus on the econometric results and their interpretations. Regarding the econometric results, descriptive statistics and regression analysis will be included, and also, the assumptions made to perform the study is included. The paper ends with a brief Conclusion.

MATERIALS AND METHODS

The online survey as mentioned in the previous sections randomly covered all of Turkey through the social network. Surveys' results in Table 1 clearly demonstrate that the rate of participation in survey in the North East region (NE) is proportionally higher than other regions while some regions such as Aegean region (AEG) and the South East region (SE) has a lower participation rate considering their population. A high rate of responses in some regions might be explained with a fast spreading of surveys linked with the help of respondents.

The analysis was based on applying the CVM that is defined as "any approach to valuation of a commodity which relies upon individual responses to contingent circumstances posited in an artificially structured market" (SELLER *et al.*, 1985). This method was first proposed by CIRIACY-WANTRUP in 1947 in order to estimate the benefits of the prevention of soil erosion (KONTOLEON *et al.*, 2005; CAMERON, 1992). The CVM, which is basically based on a survey-based methodology for eliciting consumers' valuations of non-market goods and services, has been widely applied by researchers and policy makers in health economics and food safety for several decades and received considerable attention in the literature. It was stated by JEAN *et al.* (1995) that benefit estimates that are comparable to estimates from market-based approach can be produced by the CVM. There are a number of studies which have been used in surveys with discrete answers that have been analysed with logit and probit techniques (BUZBY *et al.*, 1995; AKGUNGOR *et al.*, 2001; GARMING and WAIBEL, 2006; KALOGERAS *et al.*, 2009).

Determining sample size

The sample size is defined by considering the current Turkish population and calculated according to the formula provided by FINK (2003):

$$n = \frac{N}{(1 + N * p^2)}$$

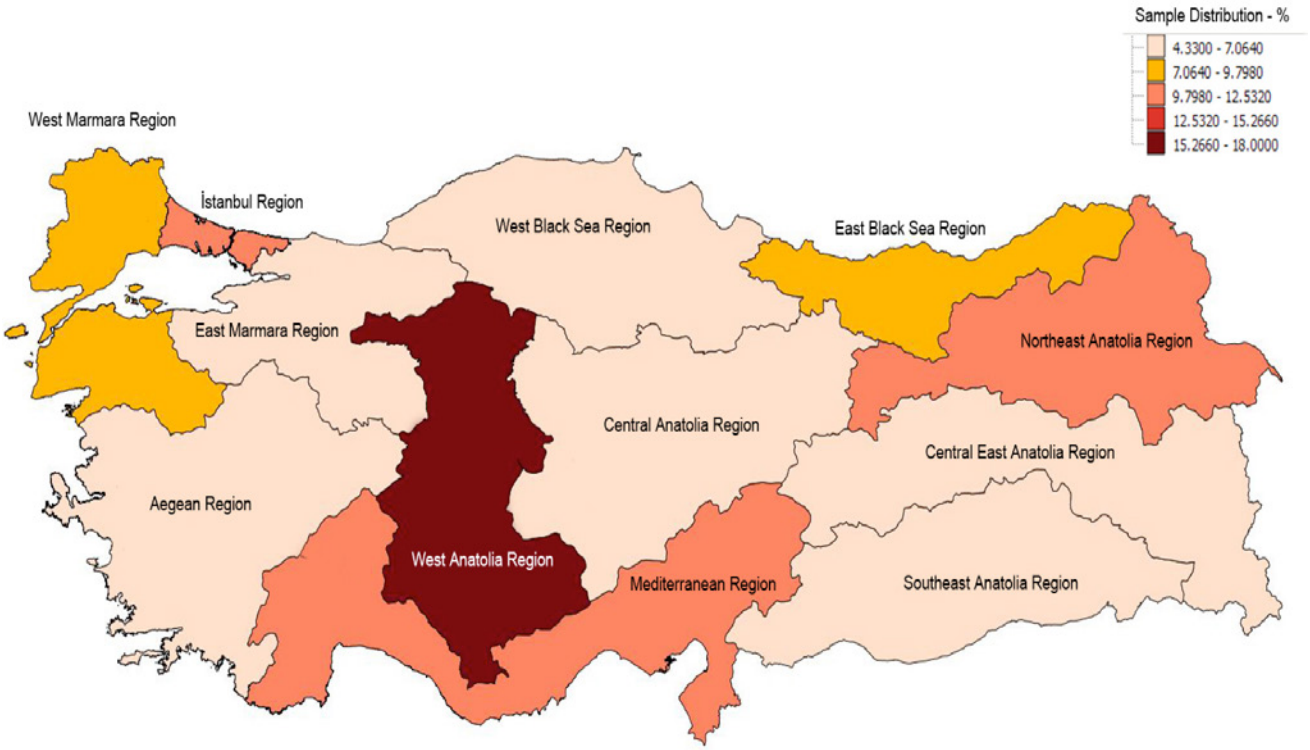


Table 1 - Percentages of Survey and Turkish Population at the level of NUTS 1 Regions (Source: Primary data extracted from survey).

Where n is the sample size determined, N is the population size, p is level of precision. The sample size is 400 at 95% confidence level and a 5% margin of error. But 393 samples were used after the first elimination due to the incompleteness.

Survey and data generation

Before moving further through online survey, the first draft was shared with 10 Turkish consumers by using face to face interview method in order that the perspective of a consumer side is truly reflected in the format of questions. After receiving some positive and negative feedback, the questionnaire form was finally rearranged in a short and clearer way as the first draft shared with consumers was found slightly longer and unclear instructions. Particularly, open-ended questions were not preferred by these consumers. Instead, options were included in some of the questions. Also, the answer choices were re-organized according to the consumer`s expectations.

Following pre-test with Turkish consumers, the link to the online survey was shared with Turkish consumers via the social networks such as in general e-mails, Facebook, LinkedIn and forums, and in particular regional development agency network covering all Turkey for one month as from June 22 until July 21, 2014. The survey mainly comprised of three parts. The first part covered the questions to elicit perceptions that are related to pesticide residues. The consumers were asked about their perceptions of pesticide residues in potatoes as well as the cosmetic defects. The question on cosmetic defects intended to measure whether or not the consumers would be willing to purchase fresh produce with insect damage, such as worm holes or irregular shape of the potatoes. The second part included WTP questions. The survey asked consumers the maximum WTP for reduced pesticide residues in potatoes. Socioeconomic questions were inserted in the third part. For simplicity, the survey was designed to simulate consumers' potato purchasing behaviour of their respective households under alternative prices on reduced pesticides in potatoes. The scenario was built on the consumers that were provided with a label that guarantees that the potatoes were tested and certified that they do not contain pesticide residues harmful to human health by assuming no change in quality. By doing so, we were able to see if the consumer`s WTP is enough to justify these increased costs of production with a reduction in pesticide use.

Regression Models of the CVM

Probit and logit which are known as non-linear functions of unknown coefficients in literature are widely applied in binary choice models. Though both models may give similar results, there are slight differences because of

the tail of observations. AMEMIYA (1981) expressed that the samples with heavier tails are more appropriate for logit models. A similar stance was made by CAKMAKYAPAN and GOKTAS (2013). They observed that logit model is generally preferred for large sample sizes (500 and 1000) and probit model is usually for smaller sample sizes. So, probit model will ultimately be employed for estimations because of the sample size. Alternatively, tobit model will be applied to measure WTP amounts that are obtained through single bounded dichotomous questions since the endogenous variable includes zero values.

Probit model

The Probit model is defined by WOOLDRIDGE (2006) as:

$$Zn=Xn\beta+un.$$

Where β is a vector of parameters including the intercept term; xn is a vector of covariates; u is the error term which either has the standard logistic distribution or the standard normal distribution. In either case, u is symmetrically distributed about zero. Zn is the unobservable amount that respondents are willing to pay for the reduced pesticides in potatoes.

WTPi is the observed dichotomous variable stating whether the individual pays or not. It can be defined as follow:

$$WTPn=0 \text{ if } WTPn^*\leq 0$$
$$WTPn=1 \text{ if } WTPn^*>0$$

As it is indicated by WOOLDRIDGE (2006), the main goal in binary responses is to explain the effects of x on the response that follows the probability P(y=1|x).

$$P(WTP=1|x)=P(WTPn^*>0|x)=P[e>-(\beta 0+x\beta|x]=$$
$$=1-G[-(\beta 0+x\beta 0]=G(\beta 0+x\beta).$$

The direction of the effect of xj on E(WTP*|x)= β0+xβ and on E(WTP|x)=P(y=1|x)=G(β0+x β) is similar to each other.

It is not possible to apply OLS due to the nonlinear nature of E(y|x). Maximum likelihood methods thus must be used in order to estimate limited dependent variable models. The maximum likelihood can be written as follows (WOOLDRIDGE, 2006);

$$f(WTP|xi;\beta)=[G(xi\beta)]y[1-G(xi\beta)]1-y, \text{ WTP-0,1,}$$

It can easily be seen that when y=1 results in G(x, β) and when y=0, we get 1- G(xiβ). The function of log likelihood for observation is a function of the parameters and the data (xi, yi)

$$li(\beta)=WTPilog[G(xi\beta)]+(1-WTPi)log[1-G(xi\beta)].$$

Tobit model

The general formulation of the Tobit model can be expressed in the following way (GREENE, 2000; WOOLDRIDGE, 2006);

$$WTPn^* =Xi\beta+ui.$$
$$WTP=0 \text{ if } yn^* \leq 0.$$
$$WTP=WTP^* \text{ if } WTPn^*<0$$

E[WTPn*|xnβ] is xn`β. Wherefore, the nth individual, Xn is a vector of explanatory variables, ui is a random disturbance term, and β is a parameter vector common for each individual. By assuming the random error is independent and normally distributed among respondents, the expected WTP for an observation drawn at random from the population is

$$E[WTP|xn]= \varphi(Xn`\beta/\sigma)+ xn`\beta+\sigma\lambda n)$$

Where φ (Xn`β/σ)/Φ(Xn`β/σ);

Where φ represents the normal distribution function and σ represents the standard deviation. Moreover, the expected value of WTP for observations above zero, which will be called E(WTP*), is simply Xβ plus the expected value of the truncated normal error terms. The expected WTP can be expressed as

$$E(WTP)= \varphi(X\beta/\sigma)E(WTP^*)$$

WOOLDRIDGE (2006) points out that the function of the tobit model which is based on maximum likelihood estimation can be shown as:

$$\text{Ln L } (\beta, \sigma)= \sum_{i=1}^{N} WTPn \sum_{i=1}^{N} WTPn(WTPn=0)$$
$$\ln[1-G(xn\ \beta/\sigma)]+(WTPn>0)\ln\{(1/\sigma)g$$
$$[(WTPn-xn\ \beta)/\sigma]\}$$

Where G(.) is the standard normal cumulative distribution function; g(.) is the standard normal density function; and σ refers the standard deviation of the error term. By maximising the log-likelihood function, the Tobit estimator $\dot{\beta}\dot{\beta}$ is obtained.

RESULTS AND CONCLUSIONS

As indicated in Table 2, 63.10 % (248) of the 393 respondents that were considered in the study are males, and 36.90 % (145) are females, which represents all of Turkey. It is also shown that 54.20 % (213) of the surveyed respondents are 31-45 years old, followed by individuals of 18-30 and 46-64 years old, representing 38.17 % (150) and 7.38 % (29) of the sample respectively. The educational attainment of the respondents is in favour of higher level of education, 53.94 % (212) acquired a university degree followed by 42.24 % (166) of post graduate degree. When comparing the above figures with

the data of TURKSTAT as in Table 3, our sample has higher income and education levels, and a higher percentage of males.

Regarding working status, a great majority of respondents (70.74 %) are employed in the public sector, while only 18.32 % and 4.33 % of the respondents work in the private sector and are unemployed respectively. Taking into consideration income level of respondents, it was found that the middle income group was overwhelmingly predominant. Respondents from low, medium and high income level consisted of roughly 12 %, 66 % and 32 % respectively. The average size of the household of respondents is 3 individuals per household and their age distribution reflected 31-45 years old.

Table 2 - Characteristics of the sample.

Sample Size:393	**Freq.**	**%**
Gender	393	100
Male	248	63,10
Female	145	36.90
Age	393	100
18-30	150	38.17
31-45	213	54.20
46-64	29	7.38
>64	1	0.25
Employment Status	393	100
Public sector	278	70.74
Private sector	72	18.32
Retired	5	1.27
Unemployed	17	4.33
Housewife	5	1.27
Student	13	3.31
NGO	3	0.76
Education	393	100
Pri&High School	15	3.82
Graduate	212	53.94
Post Graduate	166	42.24
Household Size	393	100
1 person	47	11.96
2 people	63	16.03
3 people	123	31.30
4 people	107	27.23
>4 people	53	13.49
Monthly Income (1 TL=£0,28)	393	100
849 TL or less	16	4.07
850 TL – 1449 TL	29	7.38
1500 TL – 2149 TL	43	10.94
2150 TL – 2799 TL	69	17.56
2800 TL – 3449 TL	44	11.20
3500 TL – 4149 TL	64	16.28
4150 TL or more	128	32.57
Place of residence during the first 15 years of life	393	
100		
City or suburb	251	63.87
Small town	96	24.43
Village	46	11.70

Table 3 - Comparison of Sample Sociodemographics Versus Turkey's Population.

Sociodemographies	**Sample**	**Turkey's Population***
Female (%)	36.9	49.8
Household Size	3.1	3.7
Graduates (%)	96.2	12.0
Median Income (TL)	3150	1838
Median age	40	31

*Elaborated from data extracted from TURKSTAT.

Table 4 fundamentally indicates the basic preferences stated by Turkish consumers for pesticides and food safety issues. Survey results showed that approximately 75 % of respondents have no idea about the pesticides and their harmful effects whereas only 20 % indicated limited knowledge about pesticides. Respondents aged 46-64 showed a higher degree of knowledge about pesticides.

A great majority of those having pesticide knowledge specified mass media as a source of knowledge on pesticides. When a cross check question about the pesticides in potatoes was later asked, more than 50 % of respondents again indicated no idea about it; while 32 % of those have an opinion of "there are pesticide, hormone and other chemicals that are harmful for health". Regular shapes of potatoes are predominantly remarked by respondents (around 56 % of respondents). A similar viewpoint comes from another question to observe how cosmetic defects are important for individuals. More than 86 % of respondents pointed out that they are not willing to pay for potatoes with insect damages even though they are pesticide-free produce. This finding might be interpreted that for those who are willing to pay more for pesticide-free products, suppliers should ensure that they can be provided with satisfactory quality standards. OTT and MALIGAYA (1989) quoted in WEAVER *et al.* (1992) found that 88 % of the respondents would be unwilling to accept those defects. Apart from cosmetic defects, Independent science-based advice is one of the most important critical issues in food safety in the European Union. European Food Safety Authority (EFSA) as an independent body is responsible for carrying out risk assessment from risk management (EFSA, 2014). Conflict of interest inevitably appears when the same institutions both control and monitor the same findings. This is a crucial issue for Turkey as well. Therefore, a question was asked to observe the respondents' opinions on "Who should carry out food safety control?". The least frequent responses for this question are municipalities and public agents with roughly 4% and 12% respectively. The majority, 37%, of respondents preferred having an independent laboratory certification for more fair and transparent food safety control.

Based on the data in Tables 1, 2, and 4, respondents aged 31 to 45 and having Master and PhD. degrees were found to be more willing-to-accept insect damage in reduced pesticides in potatoes than those aged 46 and older, and those having non-college and college degrees respectively. Males, lower income households and college graduates were found to be less willing to accept cosmetic defects in reduced pesticides in potatoes than were females, high income households and non-college graduates respectively. Finally, the survey results show that respondents considering pesticides in potatoes that are harmful for health and having no idea about it were found to be more willing-to-pay than were those considering no harmful pesticides in potatoes and having no idea about pesticides respectively. This matter was comprehensively argued by RAVENSWAAY (1990). She mainly discussed that people with college degrees might be less concerned than those with non-college degrees since reaching knowledge for them is less costly than others. They are, as a result of this deduction, least willing-to-pay for the safe food.

Additionally, it is possible to make regional comparisons at the level of NUTS 1 regions. Respondents from South East Region (SE), Middle Eastern Anatolia (ME) and West Marmara Region (WMAR) were found to be less willing to pay for extra payment per kg for pesticide-free potatoes than were other regions, while respondents from IST (Istanbul) and East Marmara Region (EMAR) which are largely industrialised parts of Turkey were found to be more willing to pay for it than were other regions. Despite this result, it does not make sense at all to have any correlation between income and willingness to pay for the price increase per kg for potatoes has no major effect on individuals' incomes. This is supported by BUNTE *et al.* (2010). They showed that any reduction in organic prices for some products such as potato has no considerable effect on demand.

Respondents from SE and EMAR were found

Table 4 - Pesticide concerns and purchasing preferences of Turkish consumers.

Source of Concern	**Freq.**	**%**
Remember a serious incident	1	1.05
Heard concern expressed over one or more of mass media	48	50.53
Heard concern expressed by NGO`s	4	4.21
Heard concern expressed by Public agents	7	7.37
Other	35	36.84
Opinion about the pesticides in potatoes	**Freq.**	**%**
There is no pesticide, hormone and other chemicals	17	4.33
There are pesticide, hormone and other chemicals, but residues are not risky for health	33	8.40
There are pesticide, hormone and other chemicals that are harmful for health	127	32.32
No idea	216	54.96
Purchasing preferences	**Freq.**	**%**
No preservative including pesticide and hormones	21	5.34
Taste	78	19.85
Price	71	18.07
Regular shape	220	55.98
Brand	3	0.76
Purchasing place of potatoes	**Freq.**	**%**
Open-air market	151	38.42
Greengrocer	46	11.70
Supermarket/Hypermarket/Shopping centre	174	44.27
Villagers	15	3.82
Others	7	1.78
Importance of cosmetic defects	**Freq.**	**%**
Not important	0	0.00
Less important	53	13.49
More important	268	68.19
Highly important	72	18.32
Food safety control	**Freq.**	**%**
Municipalities	16	4.07
Public agents	50	12.72
Universities	66	16.79
Independent agents	139	37.37
Producer Unions	13	3.31
Consumer Unions	109	27.74

Table 5 - Collinearity diagnostic.

Variable	VIF	1/VIF
Working Condition	1.38	0.72475
Income	1.38	0.725141
Education	1.15	0.871934
Age	1.12	0.893866
Insect Damage	1.06	0.943308
Living in a village	1.06	0.945987
Reason for Health	1.04	0.96083
Harmful pesticides	1.04	0.960983
Cosmetic Defects	1.02	0.981626
Mean VIF	**1.14**	

to be more willing-to-accept insect damage on pesticide-free produce than were other regions and respondents from West Black Sea (WBS), and AEG are less willing to accept insect damage on reduced pesticides in potatoes than were other regions.

Variance Inflation Factor (VIF) should not ideally exceed rule of 4, rule of 10 in literature. If it exceeds the rule of thumb, it is regarded as casting doubts on the estimations of regression analysis. As attentively viewed from the results given in table 5, the VIF values among independent variables change between 1.02 and 1.38 and mean VIF value is 1.14, which has sufficiently concrete evidence that there is no serious multi-collinearity in the model.

Table 6 exhibits the estimation results provided from the ordered probit model. As is illustrated, cosmetic defects for consumer preferences, free-pesticide potatoes with insect damages, indicating reasons of health for WTP questions, age, and gender were identified by the model to have significant impacts on the probability to WTP while spending the first 15 years in a village was found to negatively impact the probability to WTP. However, income and education were not found to have a significant impact, positively or negatively, on the probability to WTP.

Being female increases the probability of WTP by 21% as revealed in most of the studies (HENSON, 1996; GILL *et al.*, 2000; LOUREIRO *et al.*, 2002; KONTOLEON *et al.*, 2005; SUNDSTROM and ANDERSSON, 2009).

This can easily be explained as women are more sensitive to food safety problems than men. Also, those indicating health reasons for WTP question were found to increase the probability to WTP by 43%. On the contrary, KALOGERAS *et al.* (2009) found that health aspect does not significantly influence the probability of WTP. Similar effects were observed on cosmetic defects and age. Considering cosmetic defects as an important feature for their purchasing preferences raises the probability to WTP by 12%. In much the same way, the age of our model had a positive impact (by 10%) on WTP as in most of the studies (MISRA *et al.*, 1991; KONTOLEON *et al.*, 2005; DETTMANN and DIMITRI, 2010). Contrariwise, the age of the consumers were found to have a negative effect

Table 6 - The Probit Model.

Dependent Variable: WTP Variable	Coefficient	Standard error	Marginal effect	Standard error
Constant	-2.70513***	0.739544	-	-
Knowledge	-0.25266	0.180227	-0.09928	0.07116
Cosmetic Defects	0.319988**	0.128319	0.124498**	0.0499
Insect Damage	0.41714*	0.223001	0.154209**	0.07671
Harmful pesticide	0.242785	0.159825	0.093258	0.06043
Reason for Health	1.151586***	0.149467	0.434584***	0.05094
Age	0.267368**	0.122852	0.104025**	0.04781
Working Condition	0.078549	0.063429	0.030561	0.02469
Gender	0.556782***	0.152852	0.210075***	0.05503
Education Level	0.046347	0.36927	0.018112	0.14491
Income	0.038651	0.131314	0.015038	0.05109
Living in a village	-0.39952*	0.219629	-0.15794*	0.0866

***Indicates significance at 1% level,
**at 5% level,
*at 10% level.

Probit regression	Number of obs = 393
	LR chi2(11) = 93.65
	Prob > chi2 = 0.0000
Log likelihood = -220.82775	Pseudo R2 = 0.1749

166 left-censored observations at pay <=0;
227 uncensored observations;
0 right-censored observations.

on the WTP for organic potatoes by LOUREIRO and HINE (2002) and reduced pesticides in tomatoes by AKGUNGOR *et al.* (2007). Additionally, spending the first fifteen years in a village reduces the probability to WTP by 16%, ceteris paribus. The interpretation could be made that those people who spent their first fifteen years in a village might have a lower level of education, thus, less knowledge of pesticide impacts and less sensitiveness to the topic.

Table 7 summarizes the results of the Tobit model concerning their marginal effects. Individuals who considered cosmetic defects as important features for potato preferences, who are female, and who were indicating health reasons for WTP questions have higher WTP. To put it in context, considering cosmetic defects as important features for potato preferences raises the WTP amount by TL 0.3[1], and similarly, being female raises the WTP amount by TL 0,4 respectively, ceteris paribus. Respondents who spent their first fifteen years in a village have significantly lower WTP.

The mean WTP amount was estimated for the reduced pesticides in potatoes in Turkey on the basis of CVM study. The survey covering all of Turkey showed that respondents, representing different geographical areas, on an average are willing to pay extra TL2.90 if the zero respondents corresponding to approximately 42% are not included in the models. If it was included, the mean would be extra TL1.67. These absolute numbers can be given in percentages as 48% and 83% price premium for reduced pesticides in potatoes per kg, respectively. The average market price for potato was found as TL 3.50 based on the virtual Turkish super-market prices for those dates. The estimations could be likely interpreted that demand for organic food among Turkish consumers is growing. In a similar study, GIL *et al.* (2000) presented that Spanish consumers living in Navarra and Madrid would be willing to pay 17 % and 5.6 % more for organic potatoes, respectively. This big gap between Turkish and Spanish consumers can be explained mainly by the organic markets in Turkey that are not sufficiently saturated yet.

A similar result was found by AKGUNGOR *et al.* (2007) that Turkish consumers would be willing to pay 36% price premium for organic products or certified products. Also, WEAVER *et al.* (1992) found that 26% of respondents in Pennsylvania were willing to pay more than 15% for organic tomatoes. As seen from the values and percentages, there are no extreme prices that are accepted by consumers. This situation was argued by RAWENSWAAY (1990) that consumers would be willing to pay modest amounts to reduce perceived health risks in food.

Two important caveats can be placed on any discussion drawn from the survey results. First, actual WTP cannot be observed as it is solely based on stated preferences. Second is the homogenous distribution of individuals with respect to income and education. In spite of the fact that education and income are found to be significant factors for many WTP studies, no relationship was found in our model.

The first one seems more important while income has a minor impact on an individual's budget as indicated by BUNTE *et al* (2010). However, there is no consensus in literature in-

Table 7 - The Tobit Model.

Dependent Variable:MWTP **Variable**	**Coefficient**	**Standard error**	**Marginal effect**	**Standard error**
Constant	-5.15815***	1.344697	-	-
Knowledge	-0.27364	0.328352	-0.1237628	0.14583
Cosmetic Defects	0.681142***	0.228425	0.3135761***	0.10497
Insect Damage	0.607647	0.373106	0.2955922	0.19146
Harmful pesticide	0.361183	0.284776	0.1690033	0.13527
Reason for Health	2.334047***	0.288372	0.9972112***	0.11239
Age	0.529826**	0.220929	0.243915**	0.10148
Working Condition	0.166313	0.112667	0.076565	0.05181
Gender	0.903691***	0.263868	0.4291193***	0.12871
Education Level	0.379784	0.655299	0.1673579	0.2761
Income	0.028448	0.233872	0.0130964	0.10767
Living in a village	-0.87384**	0.41634	-0.3699325**	0.16148

***Indicates significance at 1% level,
**at 5% level,
*at 10% level.

Tobit regression	Number of obs = 393
	LR chi2(11) = 98.80
	Prob > chi2 = 0.0000
Log likelihood = -627.37228	Pseudo R2 = 0.0730

dicating a certain effect of education on WTP amount. Though DETTMAN and DIMITRI (2010) found a positive relation between education and WTP for organic products, MISRA *et al.* (1991); BUZBY *et al.* (1995); THOMPSON and KIDWELL (1998); BORCELETTI and NARDELLA (2000) and SUNDSTROM and ANDERSSON (2009) found a negative relation. It was also affirmed by Van RAVENSWAAY (1995) that the people with higher education level may be less concerned about pesticides because they might be better able to reach reliable information. These results might help to affirm why there is no significant impact of education on WTP in our model considering an outstandingly high rate of educated respondents.

Lastly, survey results show that the respondents overwhelmingly indicate that they have no idea about the level of pesticide residues used in the food. Roughly 32% of respondents considered that there are serious pesticide residues in potatoes, which are harmful to human health. An interesting finding from the survey results comes from the question "who should be responsible for controlling and monitoring of residues in food". Approximately 37.4% of respondents were in favour of independent laboratories while only 12.7 % went for public agents as an answer to this question. This clearly demonstrates that there is a high demand from consumers' side to independent agents for neutral decisions rather than public institutions.

As a result, this study stresses the consumer attitudes for pesticides in potatoes by employing CVM and single-bounded probit and tobit models. One of the drawbacks of the survey is based on the stated preferences rather than revealed preferences. The respondents might answer the questions with overestimation if compared with real situations. It would thus be better as a future research agenda to conduct another study in order to observe if similar results were truly provided by respondents.

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[1] Turkish Lira equals roughly € 0.34.

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APPENDICES

Appendix 1. Survey Instrument

Appendix 1.1. Hypothetical Scenario

Health risks resulting from pesticide use have made food safety a priority issue on the public policy agenda in developed countries. A research made in U.S showed that pesticide residues were rated a serious risk by 68 of respondents attending in a survey. Pesticides can cause many types of health problems in humans. "Pesticides have been linked to a wide range of human health hazards, ranging from short-term impacts such as headaches and nausea to chronic impacts like cancer, reproductive harm, and endocrine disruption (Toxic Action Center1)".
EC Directive 2009/128/EC determined the sustainable use of pesticides to reduce health risks resulted from pesticides. Therefore, EU countries minimise or ban the use of pesticides for health reasons. Turkey as a candidate country for EU membership has to harmonize her own legislations and directives.
The amount of pesticide use in Turkey has gradually increased since 2009 and it was over 40,000 ton in 2011 according to the data taken by the Ministry of Food, Agriculture and Livestock of Turkey. Particularly, potato is one of the most consumed vegetables which seriously include pesticide residues in Turkey. The scenario it is proposed for this survey is a price increase for reduced pesticides in potatoes per kg.
The research project is aimed at evaluating your opinion of reduced pesticides in potatoes. Reduced pesticides are in general valued for one or more of the following attributes: better taste, food safety, health, freshness, environment preservation and local production. Good Agricultural Practices are "practices that address environmental, economic and social sustainability for on-farm processes, and result in safe and quality food and non-food agricultural products" (FAO, 2003).
More precisely the main aim of this study is to find out what would persuade you to buy reduced pesticides in potatoes. On this basis the questionnaire tries to find out your opinion of the quality and availability of the reduced pesticides in potatoes in Turkey and the price that you would be willing to pay for these reduced pesticides in products.
Finally, for the purposes of the study you are required to give truthful answers and we recommend that you think carefully about the scenario previously mentioned, your disposable income and health concerns during the questionnaire survey. Furthermore, you should notice that this survey is completely anonymous and confidential. However, if you desire a copy of the final study, you should provide an email address so it can be sent to you.

Appendix 1.2. Questionnaire

Questions about qualifying candidates
1) Please indicate your current place of residence.
2) Please indicate whether you participate in the decisions regarding the payments in your household.
a) Yes
b) No

Questions about perceptions for food
3) Please indicate whether or not you have an idea regarding level of pesticides and hormones in potatoes, if you indicate choice a, please go question 5.
a) No idea
b) Little information
c) Sufficient information
c) All information in detail
4) Please indicate your recalling of pesticide information as related to level of concern for human health.
a) Remember a serious incident
b) Heard concern expressed over one or more of mass media
c) Heard concern expressed by NGO`s
d) Heard concern expressed by Public agents
e) Other
5) Please indicate the most important feature of potato for your purchasing preferences.
a) No preservative including pesticide and hormones
b) Taste
c) Price
d) Regular shape
e) Brand
6) Please indicate how cosmetic defects are important for your purchasing preferences in pesticide free products. Cosmetic defects refer growth cracks and knobby or irregular growth.
a) Not important
b) Less important
d) More important
c) Highly important
7) Please indicate if you accept potatoes with insect damage, such as worm holes in pesticide free products.
a) Yes
b) No
8) Please indicate your opinion about the pesticides, hormones, and other chemicals for potatoes.
a) There is no pesticide, hormone and other chemicals
b) There are pesticide, hormone and other chemicals, but residues are not risky for health
c) There are pesticide, hormone and other chemicals that are harmful for health
d) No idea
9) Please indicate what you generally do in order to alleviate your concern over pesticide dangerous in the potatoes.
a) Nothing
b) Washing it with plenty of water
c) Consuming by peeling off it
d) Cooking
e) Other (Please specify)
10) Please indicate whether or not fresh fruit and vegetables are as healthy as it was in the past with respect to health safety.
a) Never healthy
b) Still healthy
c) Better healthy
d) No idea

Questions about willingness-to-pay

At this stage, you should consider that the payment vehicle for the reduced pesticide in potato will lead to increases in potato prices if you favour the reduced pesticides in potato. Moreover, we strongly recommend you to consider your disposable income, health concerns, and possible positive and negative consequences of the reduced pesticide in potato when making your decision.

11) Would you be willing to pay extra 2 TL/per kg for reduced pesticides in potato? If answer is yes, please go to question 12, otherwise go to question 18.
a) Yes
b) No
12) Would you be willing to pay extra 2.5 TL/per kg for reduced pesticides in potato?
a) Yes
b) No
If answer is no, please go to question 17.
13) Would you be willing to pay extra 3 TL/per kg for reduced pesticides in potato?
a) Yes
b) No
If answer is no, please go to question 17.
14) Would you be willing to pay extra 3.5 TL/per kg for reduced pesticides in potato?
a) Yes
b) No
If answer is no, please go to question 17.
15) Would you be willing to pay extra 4 TL/per kg for reduced pesticides in potato?
a) Yes
b) No
If answer is no, please go to question 17.
16) Would you be willing to pay above 4 TL/per kg for reduced pesticides in potato? Also please indicate how much you would be willing to pay.
a) Yes (Please specify): TL
b) No
How much:.....

[1] http://www.toxicsaction.org/problems-and-solutions/pesticides

17) Would you please indicate the reason for the expressed amount?
a) More healthy
b) A reasonable price for my budget
c) More tasty
d) Protecting environment
e) Protecting local producers
f) Other (Please specify)

Questions about social and economic factors
18) Regarding your age, which of the following would you select?
a) 17 or less
b) 18-30
c) 31-45
d) 46-64
e) 65 or more
19) Regarding your working condition, which of the following would you select?
a) Public sector
b) Private sector
c) Retired
d) Unemployed
e) Housewife
f) Student
g) Farmer
h) NGO
20) Regarding your gender, which of the following would you select?
a) Male
b) Female
21) Regarding your marital status, which of the following would you select?
a) Married
b) Single
22) Regarding your family composition, which of the following would you select?
a) Have children
b) Do not have children
23) Regarding the size of your household, which of the following would you select?
a) One person
b) Two persons
c) Three persons
d) Four persons
e) More than four persons
24) Regarding your education level, which of the following would you select?
a) Primary school graduate
b) Secondary school graduate
c) High school graduate
d) Bachelor's degree graduate
e) Master's degree graduate
f) Ph.D. 's degree graduate
g) Other:.....
25) Regarding your monthly income, which of the following would you select?
a) 849 TL or less
b) 850 TL – 1449 TL
c) 1500 TL – 2149 TL
d) 2150 TL – 2799 TL
e) 2800 TL – 3449 TL
f) 3500 TL – 4149 TL
g) 4150 TL or more
26) Please indicate the place of residence during the first 15 years of life?
a) City or suburb
b) Small town
c) Farm
27) Please indicate the place you are currently living?
a) Less than 3 years
b) 3-5 years
c) 6-10 years
d) 11-20 years
e) More than 20 years
28) Please indicate from where do you generally purchase potatoes?
a) Open-air market
b) Greengrocer
c) Supermarket/Hypermarket/Shopping center
d) Villagers
e) Others
29) Please indicate your preference about which agent should ideally and fairly be responsible for food safety control?
a) Municipalities
b) Public agents
c) Universities
d) Independent agents
e) Producer Unions
f) Consumer Unions

Thank you for your time!

Appendix 2. Summary and descriptions of variables

Variable	Obs	Mean	Std. Dev.	Min	Max
pay393	1.676845	1.621334	0	6	
bid 393	0.5776081	0.4945699	0	1	
Knowl	393	0.2417303	0.4286774	0	1
Cosm_Def	393	3.048346	0.5626138	2	4
Insect_Dam	393	0.1399491	0.3473765	0	1
Harmfulpes	393	0.3231552	0.4682776	0	1
Age393	2.697201	0.6123035	2	5	
Work_Cond	393	1.608142	1.289334	1	8
Gender	393	0.3689567	0.4831373	0	1
Marital	393	0.4707379	0.4997793	0	1
Hav_Child	393	0.4274809	0.4953436	0	1
Household	393	3.142494	1.197383	1	5
livingincity	393	0.6386768	0.4809963	0	1
livingindist	393	.2442748	0.4302041	0	1
livinvilage	393	0.1170483	0.3218877	0	1
Educ	393	0.956743	0.2036944	0	1
Income	393	2.223919	0.6273835	1	3
livinvilage	393	0.1170483	0.3218877	0	1

Appendix 3. Multicollinearity analysis

	Knowl	Cosm_Def	Insect_Dam	Harmfulpes	ReasonHealth	Age	Work_Cond	Gender	Educdumy	Income	livinvilage
Knowl	1.0000										
Cosm_Def	-0.0380	1.0000									
Insect_Dam	0.2519***	-0.0739	1.0000								
Harmfulpes	0.3469***	-0.0401	0.1604***	1.0000							
ReasonHealth	-0.0888	-0.0306	-0.0828	0.0709	1.0000						
Age	0.1435***	-0.0092	-0.0281	0.0129	-0.0121	1.0000					
Work_Cond	0.0057	0.0016	0.1114**	-0.0179	(-)0.1438***	-0.0667	1.0000				
Gender	-0.0129	(-)0.1127*	0.0716	0.0129	0.0402	(-)0.1647*	0.0853**	1.0000			
Educdumy	0.0032	-0.0262	-0.0224	-0.0135	0.0508	-0.0439	(-)0.327***	0.0707	1.0000		
Income	-0.0216	-0.0018	(-)0.109*	0.0309	0.0623	0.2566***	(-)0.4558***	(-)0.2059*	0.2357***	1.0000	
livinvilage	0.1457***	0.0955*	0.0356	0.0531	-0.0762	0.1803***	-0.0306	-0.0816	-0.0393	0.0467	1.0000

Appendix 4. Regression analysis

Source	SS	df	MS	Number of obs =	393
				F(9, 383)	10.79
Model	14.56733	9	1.61859267	Prob > F	0
Residual	57.46829	383	.150047753	R-squared	0.2022
				Adj R-squared	0.1835
Total	72.03562	392	.183764345	Root MSE	0.38736

Knowl	Coef.	Std. Err.	t	P>t	[95 % Conf.	Interval]
Cosm_Def	-0.01601	.0350985	-0.46	0.649	-0.0850178	0.053002
Insect_Dam	0.233844	.0579889	4.03	0.000	0.1198276	0.34786
Harmfulpes	0.290287	.0426197	6.81	0.000	0.2064893	0.374085
ReasonHealth	-0.07965	.0416259	-1.91	0.056	-0.1614897	0.002198
Age	0.100169	.0337963	2.96	0.003	0.0337199	0.166619
Work_Cond	-0.01019	.0178243	-0.57	0.568	-0.0452324	0.024859
Educdumy	0.06667	.102861	0.65	0.517	-0.1355734	0.268913
Income	-0.04631	.0366208	-1.26	0.207	-0.1183125	0.025693
livinvilage	0.126524	.062492	2.02	0.044	0.0036537	0.249395
_cons	-0.01413	.1961847	-0.07	0.943	-0.3998678	0.3716

Appendix 5. Covariance matrix of coefficients of regress model

e(V)	Cosm_Def	Insect_Dam	Harmfulpes	ReasonHealth	Age	Work_Cond	Educdumy	Income	livinvillage	_cons
Cosm_Def	0.0012319									
Insect_Dam	0.0001513	0.00336271								
Harmfulpes	4.661E-05	-0.00041276	0.00181644							
ReasonHealth	3.774E-05	0.0001939	-0.0001506	0.00173271						
Age	3.531E-05	0.00002271	6.14E-06	0.00001231	0.0011422					
Work_Cond	4.64E-07	-0.0000685	8.00E-06	0.00008935	-2.13E-05	0.00031771				
Educdumy	8.484E-05	-0.00015164	0.00011349	-9.37E-06	0.0003194	0.0004571	0.0105804			
Income	-1.32E-06	0.00014854	-5.893E-05	9.25E-06	-0.000321	0.00025825	-0.000478	0.00134108		
livinvilage	-0.0002205	-0.00013419	-0.0001421	0.00020032	-0.000363	0.00004616	0.000235	7.79E-06	0.0039053	
_cons	-0.0039642	-0.00104393	-0.0005647	-0.00142089	-0.002716	-0.00152239	-0.010951	-0.0020787	0.0008133	0.0384884

Appendix 6. Correlation matrix of coefficients of regress model

e(V)	Cosm_Def	Insect_Dam	Harmfulpes	Reason_health	Age	Work_Cond	Educdumy	Income	livinvillage	_cons
Cosm_Def	1									
Insect_Dam	0.0743	1								
Harmfulpes	0.0312	-0.167	1							
ReasonHealth	0.0258	0.0803	-0.0849	1						
Age	0.0298	0.0116	0.0043	0.0087	1					
Work_Cond	0.0007	-0.0663	0.0105	0.1204	-0.0354	1				
Educdumy	0.0235	-0.0254	0.0259	-0.0022	0.0919	0.2493	1			
Income	-0.001	0.0699	-0.0378	0.0061	-0.2594	0.3956	-0.127	1		
livinvilage	-0.1005	-0.037	-0.0534	0.077	-0.1719	0.0414	0.0366	0.0034	1	
_cons	-0.5757	-0.0918	-0.0675	-0.174	-0.4096	-0.4354	-0.5427	-0.2893	0.0663	1

Appendix 7. Logit model

WTP	Coef.	Std. Err.
Knowl	-0.4106674	0.3074221
Cosm_Def	0.5257642**	0.2167355
Insect_Dam	0.6643928***	0.3706913
Harmfulpes	0.4106739	0.269297
ReasonHealth	1.886197***	0.2523839
Age	0.4439607**	0.2078363
Work_Cond	0.1279345	0.1060791
Gender	0.9363477***	0.2617843
Educdumy	0.0939652	0.6196589
Income	0.0539704	0.2209946
livinvilage	(-)0.681425*	0.3660309
_cons	-4.444088	1.238931
Number of obs=393		
LR chi2 (11)=93.33		
Prob>chi2=0.0000		
Pseudo R2=0.1743		

***Indicates significance at 1% level, **at 5% level, *at 10% level.

Appendix 8. Statistic values of WTP before and after trimming outlier

Percentiles		Smallest	pay		Percentiles		Smallest	Trimmed data (5%)	
1%	0	0			1%	0	0	pay	
5%	0	0			5%	0	0		
10%	0	0	Obs	393	10%	0	0	Obs	374
25%	0	0	Sum of Wgt.	393	25%	0	0	Sum of W	374
50%	2		Mean	1.676845	50%	2		Mean	1.497326
		Largest	Std. Dev.	1.621334			Largest	Std. Dev.	1.444224
75%	2.5	6			75%	2.5	4		
90%	4	6	Variance	2.628723	90%	4	4	Variance	2.085784
95%	4	6	Skewness	0.424015	95%	4	4	Skewness	0.2272442
99%	4	6	Kurtosis	2.16069	99%	4	4	Kurtosis	1.633528

PAPER

OPTIMIZATION OF MICROWAVE-ASSISTED DRYING OF JERUSALEM ARTICHOKES (*HELIANTHUS TUBEROSUS* L.) BY RESPONSE SURFACE METHODOLOGY AND GENETIC ALGORITHM

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ABSTRACT

The objective of the present study was to investigate microwave-assisted drying of Jerusalem artichoke tubers to determine the effects of the processing conditions. Drying time (DT) and effective moisture diffusivity (EMD) were determined to evaluate the drying process in terms of dehydration performance, whereas the rehydration ratio (RhR) was considered as a significant quality index. A pretreatment of soaking in a NaCl solution was applied before all trials. The output power of the microwave oven, slice thickness and NaCl concentration of the pretreatment solution were the three investigated parameters. The drying process was accelerated by altering the conditions while obtaining a higher quality product. For optimization of the drying process, response surface methodology (RSM) and genetic algorithms (GA) were used. Model adequacy was evaluated for each corresponding mathematical expression developed for interested responses by RSM. The residual of the model obtained by GA was compared to that of the RSM model. The GA was successful in high-performance prediction and produced results similar to those of RSM. The analysis and results of the present study show that both RSM and GA models can be used in cohesion to gain insight into the bioprocessing system.

- Keywords: Jerusalem artichoke, microwave-assisted drying, effective moisture diffusivity, response surface methodology, genetic algorithm -

1. INTRODUCTION

The Jerusalem artichoke (Helianthus tuberosus L.) has been gaining increasing attention due to the potential use of this plant as a feedstock for the synthesis of new products and the awareness of its significant health benefits. The storage form of carbon in Jerusalem artichokes, inulin, makes this plant attractive compared to the majority of crops that store carbon as starch (KAYS and NOTTINGHAM, 2008; VAN LOO *et al.*, 1995; WATHERHOUSE and CHATTERTON, 1993). In spite of its high potential usage in the food industry, consumption of this plant as a raw material is limited due to changes during its postharvest period (CABEZAS *et al.*, 2002; MODLER *et al.*, 1993; TAKEUCHI and NAGASHIMA, 2011). Therefore, increasing the Jerusalem artichoke shelf-life by processing is of prime importance, and dehydration of its tubers should also be considered in this regard. Various drying technologies have been extensively used as a preservation technique in the food industry. Specific technologies, such as microwave-assisted drying, for grains, crops and foods have been well documented (AL-HARAHSHEH *et al.*, 2009; GIRI and PRASAD, 2007; SHARMA and PRASAD, 2001).

The main reasons to consider the use of microwave energy are to accelerate the drying process, improve product quality, and reduce costs (AL-HARAHSHEH et al., 2009; GIRI and PRASAD, 2007; MCLOUGHLIN *et al.*, 2003). However, additional effort is required to standardize microwave technology in the drying process. For this reason, microwave-assisted drying requires investigation in terms of the underlying physical phenomena, such as the mechanism of molecular transfer. Effective moisture diffusivity is one of the parameters used to evaluate the drying of food materials from the point of view of intramolecular mass transfer, since transfer of water molecules throughout the solid matrix is generally a rate-controlling step in drying processes (DADALI *et al.*, 2007). Another significant step is to optimize processing variables according to desired targets including faster and more efficient processing and improved product quality. Response surface methodology (RSM) is a statistical procedure frequently used for process optimization. It uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate problems. The equations describe the effect of the test variables on the responses, determine interrelationships among test variables and represent the combined effect of all test variables in the response. This approach enables an experimenter to efficiently explore a process or system. In recent years, other optimization techniques have also been developed and adapted to food processes. In process engineering design, genetic algorithms (GAs) are considered a novel technique (GOLDBERG, 2001). For highly complex and nonlinear processes, researchers have reported successful GA applications in analyzing the osmotic dehydration of kiwifruit (FATHI *et al.*, 2011a) and carrot slices (MOHEBBI *et al.*, 2011a), and plant oil extraction from cloves by supercritical CO_2 (HATAMI *et al.*, 2010).

To our knowledge, there are no reported studies on the microwave-assisted drying of Jerusalem artichokes as well as its optimization in terms of drying performance and quality characteristics. Therefore, the objective of this study was to investigate and optimize the processing conditions of microwave-assisted drying of artichoke tubers. Additionally, GA was conducted to evaluate its performance in the optimization of the proposed drying technique.

2. MATERIALS AND METHODS

2.1 Preparation of samples

Fresh Jerusalem artichoke tubers were purchased from the local market and stored at 4°C. The tubers were peeled and sliced at a specified thickness by using a lab-scale slicer on which the thickness was adjusted in the range of 1-10 mm. All slices had the same projected area (30*40 mm, wide*length) to avoid its effect on drying due to any change; the slice thicknesses for each trial were changed as presented in Table 1. Microwave output power was another process variable that was examined at three levels (100, 200, and 300 W), as shown in Table 1. The third variable was the concentration of the pretreatment solution. The experimental design was planned such that there were some trials (run order of trials was 1, 9, 15, and 16; Table 1) excluding the NaCl in pretreatment, and in the remaining trials the slices were treated with NaCl solution (Table 1) to determine the effect of salt on the drying characteristics of interest. The pretreatment was carried out with NaCl solutions of specified concentrations (Table 1) at 25°C with controlled agitation for a period of 2 h. After pretreatment, the samples were removed and rinsed with distilled water to remove the solute that had adhered to the surface and then dried in a microwave oven at the output power specified in Table 1. In the case of samples that were not subjected to pretreatment, aliquots of 50 g of tuber slices were directly dried in the microwave oven (details provided below), whereas pretreated samples were weighted as 50 g after immersion in NaCl solution for 2 h (Table 1). The initial moisture content of Jerusalem artichokes was determined by placing the tubers in a conventional oven at 105°C until no further change in weight of the sample was observed. The average moisture content of fresh Jerusalem artichoke tubers was 81.77 ± 0.89%. The moisture content of any pretreated J. artichoke slice did not vary significantly; even with a 2% (w/v) NaCl

Table 1 - Experimental design of microwave drying and corresponding responses.

Standard order	Run order	Power (W)	Thickness (mm)	NaCl conc. (g/100 mL)	Drying time (min)	Effective diffusivity*10^{-8} (m^2/s)	Rehydration ratio
9	1	200	2	0	6	0.79	3.27
16	2	200	4	1	8	1.61	2.76
13	3	200	4	1	9	1.58	3.18
2	4	300	2	1	4	0.77	4.47
14	5	200	4	1	14	1.50	3.71
4	6	300	6	1	5	7.62	3.19
3	7	100	6	1	96	0.42	4.28
15	8	200	4	1	12	1.31	3.18
5	9	100	4	0	75	0.26	3.98
12	10	200	6	2	11	3.37	2.97
7	11	100	4	2	83	0.24	3.57
11	12	200	2	2	5	0.68	5.29
1	13	100	2	1	33	0.11	4.45
8	14	300	4	2	4	4.60	3.57
10	15	200	6	0	9	3.77	4.03
6	16	300	4	0	7	3.32	3.19

concentration in the pretreatment solution. This may be due in part to the low temperature level and short duration of the pretreatments.

2.2 Drying equipment and experimental method

A programmable domestic microwave oven (Samsung-MW71E, Malaysia) with a maximum output power of 800 W and wavelength of 2,450 MHz was used for drying. Aliquots of 50 g of pretreated or fresh tuber slices were spread on a glass dish (dried and weighed before use) as a single layer and placed on the center of the turntable of the microwave cavity. Drying was performed for each trial at the microwave output power levels specified in Table 1. Moisture loss was measured periodically (60-s intervals) by taking out and weighing the dish on a digital balance. The drying process continued until the desired moisture content was attained (< 10%, w/w). Trials were carried out according to the experimental design including the processing conditions and run order for each trial (Table 1). The rehydration ratio (RhR) was also determined for J. artichoke slices dried according to each trial specified in Table 1. The RhR is an important quality parameter to evaluate the drying process in terms of product quality. Dried slices were immersed in warm water (50°C) and their weight gain was monitored until it stabilized. The RhR was calculated as a ratio of net weight gain to initial sample amount.

2.3 Theoretical approach to effective moisture diffusivity

The effective moisture diffusivity (EMD) was determined to obtain information about the mechanism of moisture transfer and complexity of the drying process. It was defined by Fick's second law with the assumption that diffusion is the only physical mechanism to control the transfer of water molecules to the surface. Artichoke slices prepared at different thicknesses were assumed to be an infinite slab, since other directions were large enough compared to the thickness. Thus, moisture movement was only throughout thickness. Fick's second law for moisture movement was solved with the following assumptions:

- the particle was homogenous and isotropic
- the material characteristics were constant, and the shrinkage was negligible
- mass transfer was in one direction
- moisture was initially uniformly distributed throughout the mass of a sample
- the pressure variations were negligible
- evaporation occurred only at the surface
- surface diffusion was ended, so the moisture equilibrium arises on the surface
- effective moisture diffusivity was constant versus moisture content during drying
- resistance to mass transfer at the surface was negligible compared to the internal resistance of the sample
- mass transfer was represented by a diffusional mechanism

The following analytical solution of Fick's second law proposed by CRANK (1975) was used to calculate the effective moisture diffusivity.

$$MR = \frac{M_t - M_e}{M_i - M_e} = \frac{8}{\pi^2} \sum_{i=0}^{\infty} \frac{1}{(2i+1)^2} \exp\left(- \frac{(2i+1)^2 \cdot D_{eff} \cdot \pi^2}{4L^2} \cdot t \right) \quad \text{Eq. (1);}$$

where D_{eff} is the effective moisture diffusivity (m^2s^{-1}), L is the half thickness (drying from both sides) of slab (m), MR was the fractional mois-

ture ratio, t was the drying time (s). M_t was the moisture content of the material at any time, t; M_i was the initial moisture content of the material before drying; and M_e was the equilibrium moisture content of a dehydrated artichoke slice, all moisture content values were in dry basis.

For long-term drying, only the first term of Eq.(1) was used to explain the drying procedure. The equilibrium moisture content (M_e) was assumed to be zero for microwave-assisted drying. The final equation to calculate the EMD was as follows:

$$MR = \frac{M_t}{M_i} = \frac{8}{\pi^2}\exp\left(-\frac{D_{eff}\cdot\pi^2\cdot t}{4L^2}\right) \quad \text{Eq. (2);}$$

Further simplification of Eq. (2) resulted in a straight-line equation as Eq. (3);

$$\ln(MR) = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{D_{eff}\cdot\pi^2}{4L^2}\cdot t\right) \quad \text{Eq. (3);}$$

The effective moisture diffusivity was calculated by fitting Eq. (3) to the curve of ln(MR) vs. time (Fig. 1), and the results are presented in Table 1.

2.4 Experimental design

Drying time (Z_1), effective moisture diffusivity (Z_2), and rehydration ratio (Z_3) were the responses used to optimize the process variables by response surface methodology (RSM). A Box-Behnken design was employed in this regard. Independent process variables (X_1, X_2, and X_3) were microwave output power, slice thickness, and concentration of the pretreatment solution (NaCl); each was specified at three levels with 16 runs including four replicates at the central point. The ranges and levels of independent variables are presented in Table 1. Minitab (Minitab 15.1.0.0) was used to analyze the experimental data, which were fitted to a second-order polynomial regression model including the coefficients of linear, quadratic and two factors interaction effects. The proposed model was as follows:

$$Z = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii}X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}X_iX_j \quad \text{Eq. (4)}$$

where Z was the response of the equation, b_0 was the constant coefficient, β_i was the linear coefficient (main effect), β_{ii} was the quadratic coefficient, and β_{ij} was the two factors interaction coefficient. The surfaces of the predicted responses were plotted by Sigma Plot (v. 8.02; 2002) (SPSS Inc. Chicago, IL, USA). The values of R^2, adjusted-R^2, and lack-of-fit of models were evaluated to check the model adequacies.

2.5 Optimization by genetic algorithm

The genetic algorithm (GA) is a global search algorithm, which is designed to mimic Charles Darwin's principle of "survival of the fittest" to solve complex optimization problems without falling into local optima (GOLDBERG, 2001; MOHEBBI *et al.*, 2011b; MORIMOTO, 2006). MATLAB version 2010b (MathWorks, Inc.) was used to optimize the interested responses of microwave-assisted drying of Jerusalem artichoke tubers as a function of process conditions by the GA.

3. RESULTS AND DISCUSSION

This study was designed to evaluate microwave-assisted drying of Jerusalem artichokes and to optimize the process using response

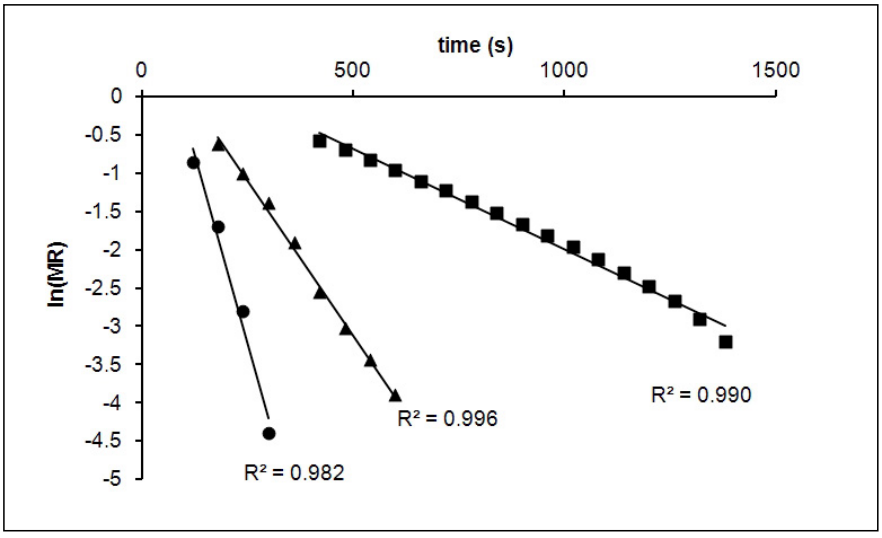


Fig. 1 - Linear relation between ln(MR) and drying time for the slice thickness of 2 mm treated with 1% NaCl and dried at 100 W output power (■), the slice thickness of 2 mm without treatment and dried at 200 W output power (●), the slice thickness of 4 mm treated with 1% NaCl and dried at 200 W output power (▲) and the fitted proposed model line (—).

Table 2 - Regression coefficients of predicted models for the investigated responses of microwave assisted drying of J. artichoke.

Variable[a]	Coefficient		
	Drying time	Effective Moisture Diffusivity	Rehydration Ratio
β_0	130.625*** [e]	4.055999*	5.918046***
β_1	-1.285***	-0.02718*	-0.01159* [c]
β_2	16.562***	-1.60884**	-0.79208** [d]
β_3	0.875^{ns} [b]	-	1.029874*
β_{11}	0.003***	0.0000339^{ns}	3.22E-05**
β_{22}	-	0.096813^{ns}	0.15846***
β_{33}	-	-	0.114851^{ns}
β_{12}	-0.062***	0.008183***	-0.00139*
β_{13}	-	-	0.001977*
β_{23}	-	-	-0.38475***
model	***	***	***
linear	***	**	**
quadratic	***	ns	***
cross-product	***	***	***
R^2	0.98	0.98	0.98
Adj-R^2	0.97	0.96	0.94
Lack-of-fit	0.101	0.312	0.085

[a] Polynomial model $Z = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j$ adjusted by backward elimination at the level of 0.05% with the lack-of-fit test, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient. [b] ns, not significant ($p > 0.05$); [c] *, significant at $p \leq 0.05$; [d] **, significant at $p \leq 0.01$; [e] ***, significant at $p \leq 0.001$.

surface methodology (RSM) and genetic algorithms (GA). Drying of J. artichoke tubers resulted in good performance with high quality product in terms of drying time (DT), effective moisture diffusivity (EMD), and rehydration ratio (RhR). Models developed by RSM and GA displayed similar performances to predict the experimental results determined for each interested response.

Multiple linear regression analysis of the experimental data yielded second-order polynomial models for predicting DT, EMD, and RhR. Analysis of variance (ANOVA) was conducted to determine significant effects of process variables on each response and to fit second-order polynomial models to the experimental data. Regression equation coefficients of the proposed models and statistical significance of all main effects calculated for each response were obtained. The effects that were not significant ($p > 0.05$) were stepped down from models without damaging the model hierarchy (Table 2). The ANOVA table also showed that the lack of fit was not significant for all response surface models at a 95% confidence level. On the other hand, R^2 and *Adj-*R^2 were calculated to check the model adequacy as *lack-of-fit* > 0.05; $R^2 \geq 0.98$; and *Adj-*$R^2 \geq 0.94$ (Table 2).

3.1 Drying time

Drying time (DT) is important because it is an index of the drying performance. A reduction in drying time means less energy requirement for the process. Table 2 shows that both microwave power and slice thickness significantly affected DT to decrease the moisture content of slices to less than 10% ($p \leq 0.05$), whereas a change in the salt (NaCl) concentration of the pretreatment solution was not an important factor ($p > 0.05$). The microwave-assisted drying process, which reduced the moisture content of Jerusalem artichoke to less than 10%, took 4-96 min varying based on the process variables. The DT decreased as microwave output power increased due to higher energy transfer for unit process time (Fig. 2). A similar microwave power effect on DT was reported previously (AL-HARAHSHEH *et al.*, 2009; SOYSAL, 2004; SUMNU *et al.*, 2005). The favorable influence of output power on DT may be attributed to the heating mechanism of microwave technology causing high internal pressure and concentration gradients, which increases the flow of liquid throughout the food (AL-HARAHSHEH *et al.*, 2009; SUMNU *et al.*, 2005; WANG and SHENG, 2006). The second factor that had a significant effect on DT values was slice thickness (Fig. 2). However, an increase in DT is not desirable from an economical point of view, and there was a positive relationship between slice thickness and DT (Table 2 and Fig. 2). Drying time to decrease moisture content under a target level (< 10%) increased with thicker slices, especially when a low output power was set (Fig. 2). A similar result related to the effect of slice thickness on DT was obtained by GIRI and PRASAD (2007) studying the drying kinetics and rehydration characteristics of mushrooms that were processed in microwaves.

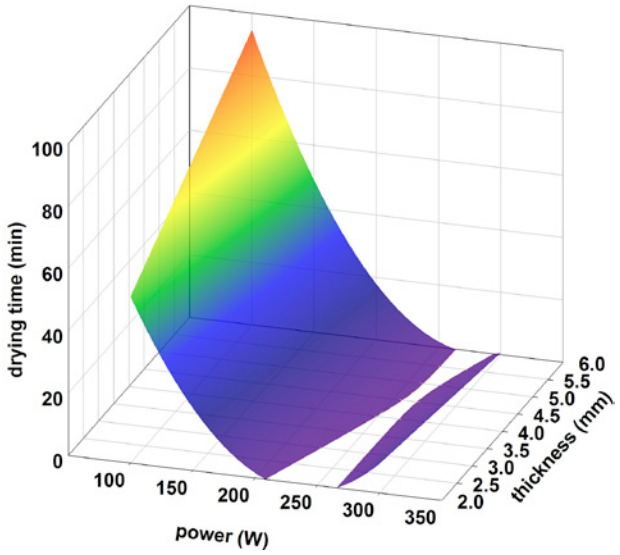


Fig. 2 - Response surface for the effects of power and slice thickness treated with 1% NaCl solution on drying time of Jerusalem artichoke slices.

3.2 Effective moisture diffusivity

Increasing the effective moisture diffusivity (EMD) is desirable in a microwave-assisted drying process, since this technique is expected to create awareness and an improvement in process performance is one of the novelties. The EMD was calculated and used as an index of the rate of the drying process (Table 1). The mass transfer of water molecules in potato matrix dried using different techniques has been previously studied. For microwave application on potatoes, the calculated diffusivities were reported in the range of $1.91*10^{-8}$ $m^2.s^{-1}$ to $3.73*10^{-8}$ $m^2.s^{-1}$ (MCMINN *et al.*, 2003), which were comparable with EMDs ($0.11*10^{-8}$ $m^2.s^{-1}$ to $7.62*10^{-8}$ $m^2.s^{-1}$ depending on processing conditions) of Jerusalem artichoke slices dried in a microwave oven. According to the results of the ANOVA of EMD, the output power and slice thickness are two important factors affecting the EMD of the drying process ($p \leq 0.05$) (Table 2). The EMD remained almost constant with changing slice thickness (2-6 mm) at an output power of 100 W (Fig. 3). Similarly, changing the output power (100-300 W) did not significantly affect the EMD of 2-mm thick tubers. However, there was a significant interaction between both factors (microwave output power and thickness) ($p \leq 0.05$), and the EMD increased when higher values of slice thickness and output power were selected (Fig. 3). DATTA and RAKESH (2013) reported that microwave heating is superior compared to conventional heating, since significant internal evaporation inside the microwave-heated material leads to additional mechanisms of moisture transport that enhance moisture loss during heating. Thus, an increase in microwave power results in more energy transfer to the food material during drying and as a result more internal evaporation resulting in a higher EMD.

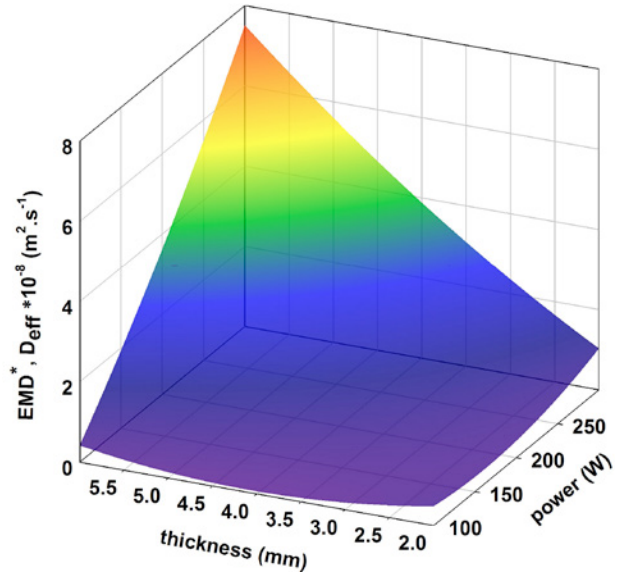


Fig. 3 - Response surface for the effects of power and slice thickness on EMD of Jerusalem artichoke slices irrespective of pretreatment.
* effective moisture diffusivity

3.3 Rehydration ratio

Rehydration ratio (RhR) is a widely used quality index for dried products. Rehydration values provide information about the changes in physical and chemical properties of a dried sample attributed to drying and treatments preceding dehydration (MASKAN, 2000). To investigate the effect of drying conditions on final product quality, the RhR of dried tuber slices were determined (Table 1). The effects of drying conditions on RhR were analyzed by ANOVA and showed that all processing conditions were effective on the rehydration capacity of microwave-assisted dried Jerusalem artichoke slices except for the quadratic term of NaCl concentration of the pretreatment solution ($p \leq 0.05$) (Table 2). Figures 4, 5, and 6 display the change of RhR with output power, slice thickness, and NaCl concentration. The RhR of dried samples at an output power around 250 W was smaller than that measured for slices dried at any other power level, when tuber slices were dried without pretreatment. On the other hand, a minimum RhR value was measured for J. artichoke pretreated slices dried at an output power of less than 250 W, and tuber slices dried at 200 W had the lowest RhR when they were treated with the highest concentration (2%) of NaCl solution (Fig. 4). This negative effect of increasing output power on RhR results from quick sample shrinkage due to rapid water loss depending on the internal temperature. The reason for the change in the effect of high output power with the NaCl concentration of the pretreatment solution may

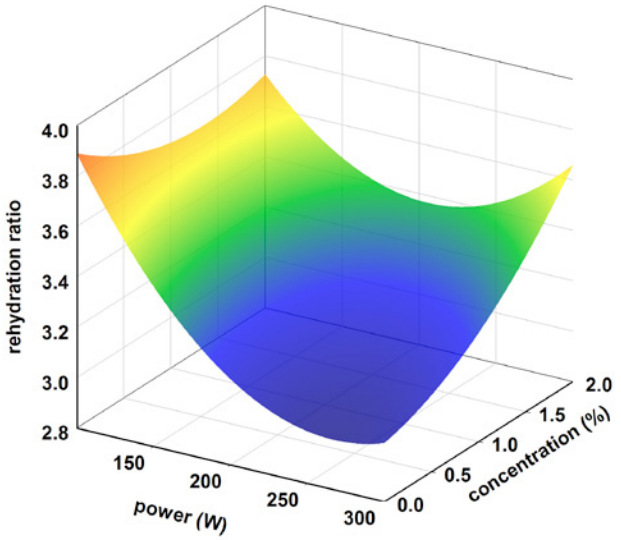


Fig. 4 - Response surface for the effects of power and slice thickness treated with 1% NaCl solution on the rehydration ratio of Jerusalem artichoke slices.

result from partial water loss occurring during pretreatment, although the change in the final moisture content of dried slices pretreated with NaCl solution was not significant compared to the water content of fresh tuber slices (data not shown). In other words, microwave-assisted drying finalized in a shorter period for samples with less moisture content compared to fresh ones. Thus, the internal temperature of a sample never reaches to its level seen at drying of the sample without pretreatment, which means less shrinkage and high RhR. These results are consistent with the changes in RhR with microwave power also observed by WANG and XI (2005). Slice thickness was another factor that had a significant effect on RhR values. Change in RhR was plotted as a function of slice thickness vs. NaCl concentration and slice thickness vs. output power as shown in Figures 5 and 6, respectively. The RhR of the dried products decreased with an increase in slice thickness. The effect of NaCl concentration on this trend was significant when low slice thickness values were conducted (Fig. 5). The RhR increased with increasing NaCl concentration of pretreatment solution when thinner slices were analyzed (Fig. 5). A decrease in RhR was also detected with increasing thickness under the effect of power (Fig. 6). Thickness effects may result from greater volumetric heating, which generates higher pressure inside the Jerusalem artichoke tuber, resulting in boiling and bubbling of the samples and reduced RhRs of the dried products (WANG and XI, 2005).

3.4 Optimal responses

An optimization procedure by RSM was conducted for all responses as a function of processing conditions. The EMD and RhR were maximized, since higher values of these responses means faster drying and better product quality, respectively. The DT response was minimized because a short process length is preferred due to economical considerations. As a consequence of the optimization procedures for these three drying characteristics, the following operating conditions were found to be optimal: power of 235 W; slice thickness of 5.95 mm; and NaCl concentration of 0.081.

3.5 Genetic algorithms

The GAs were used to select the best subset of variables and to build predictive regression models in order to study the relationships between the results obtained from the experimental trials (DT, EMD, RhR) and the pro-

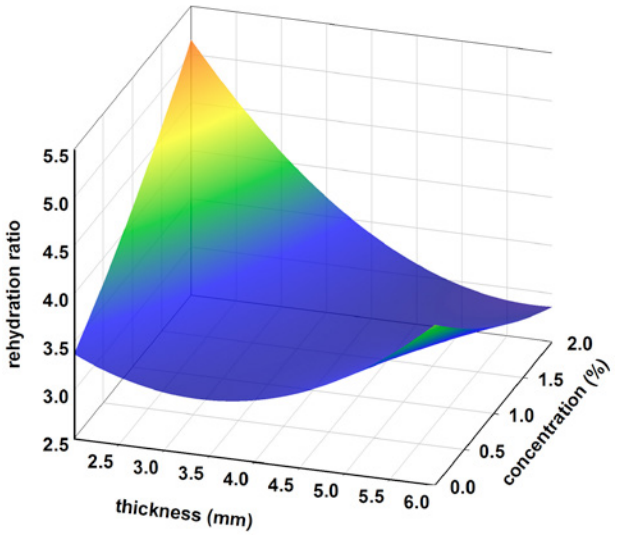


Fig. 5 - Response surface for the effects of power and concentration (slice thickness of 4 mm) on the rehydration ratio of Jerusalem artichoke slices.

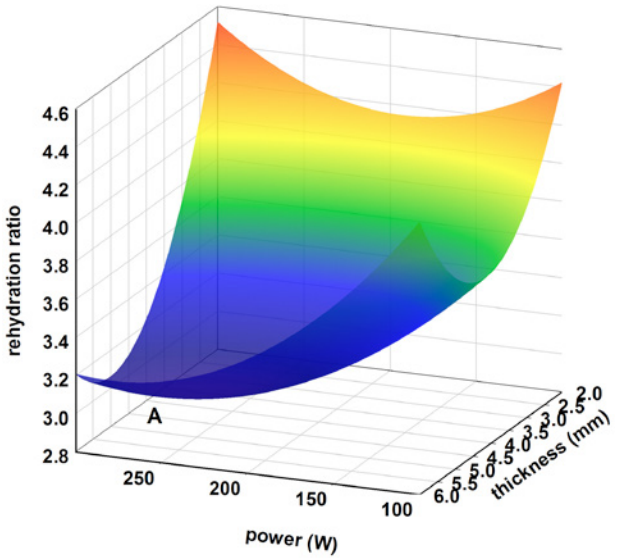


Fig. 6 - Response surface for the effects thickness and concentration (power of 200W) on the rehydration ratio of Jerusalem artichoke slices.

Table 3 - Model coefficients of proposed second order polynomial model[a] obtained by GA.

Variable[a]	Coefficient		
	Drying time	Effective Diffusivity	Rehydration Ratio
β_0	-0.500	0.522	-0.143
β_1	0.118	0.439	-0.267
β_2	0.035	0.024	0.107
β_3	0.488	0.065	0.190
β_{11}	0.253	0.065	0.481
β_{22}	-0.129	0.065	0.024
β_{33}	-0.335	0.439	-0.309
β_{12}	0.047	0.024	0.107
β_{13}	0.076	0.060	-0.600
β_{23}	-0.500	-0.558	0.600

[a] Polynomial model $Z = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient. Independent process variables (X_1, X_2, and X_3) were microwave power, slice thickness, and concentration of pretreatment solution (NaCl).

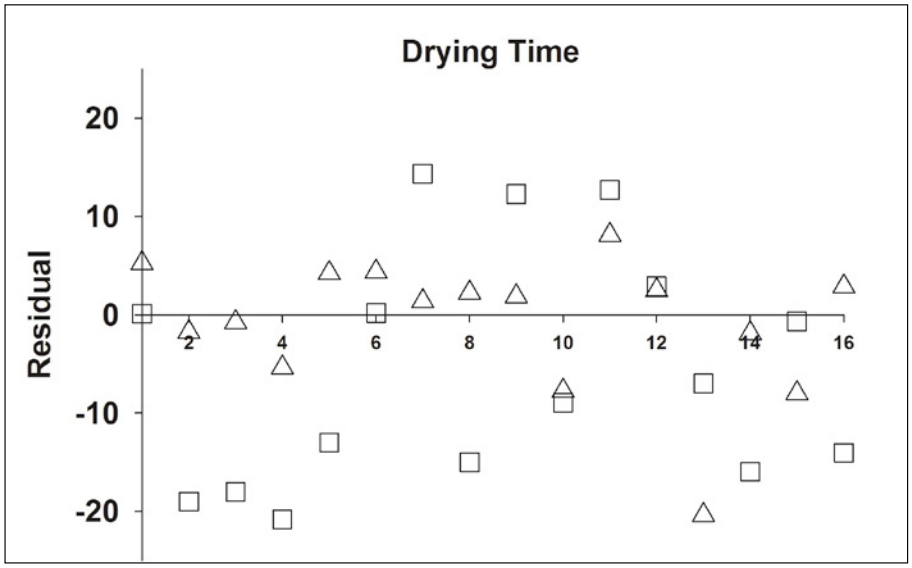


Fig. 7 - Residuals between experimental results and predicted responses by RSM (Δ) and GA (□) models calculated for each trials of drying time in experimental design.

cess parameters. The coefficients of regression models corresponding to DT, EMD, and RhR are presented in Table 3. The residual is an index of model performance where a smaller residual indicates better prediction performance. Thus, residuals between experimental results and predicted values by RSM and GA are shown in Figures 7-9 for each response. Models produced by GA display a similar performance in prediction of EMD and RhR values as those produced by RSM. Figure 7 shows smaller residuals of DT values predicted by models using RSM than GA. Although a performance decrease was seen in the prediction of DT values by GA, this procedure presented in this work can be applied for optimization in microwave-assisted drying of food materials as a rapid and non-destructive inspection method. GAs have been reported as a novel approach in the osmotic drying of kiwifruit by FATHI *et al.* (2011b). Similarly, MOHEBBAT *et al.* (2011) reported genetic algorithms as a method with a high potential for optimization in all food processes.

4. CONCLUSIONS

The experimental results and their analysis demonstrate the possibility of using this innovative method based on microwave technology for the drying of Jerusalem artichoke tubers. To the best of our knowledge, this is the first study on the microwave-assisted drying of Jerusalem artichoke tubers and optimization of process parameters using RSM and GA procedures. The results of the present work demonstrate the feasibility of the DT, EMD,

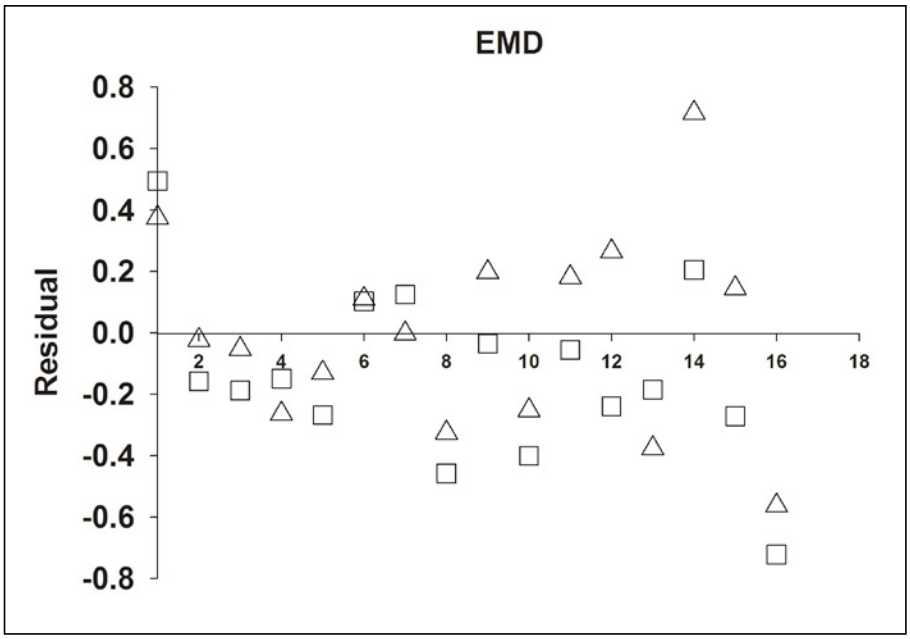


Fig. 8 - Residuals between experimental results and predicted responses by RSM (Δ) and GA (□) models calculated for each trials of effective moisture diffusivity in experimental design.

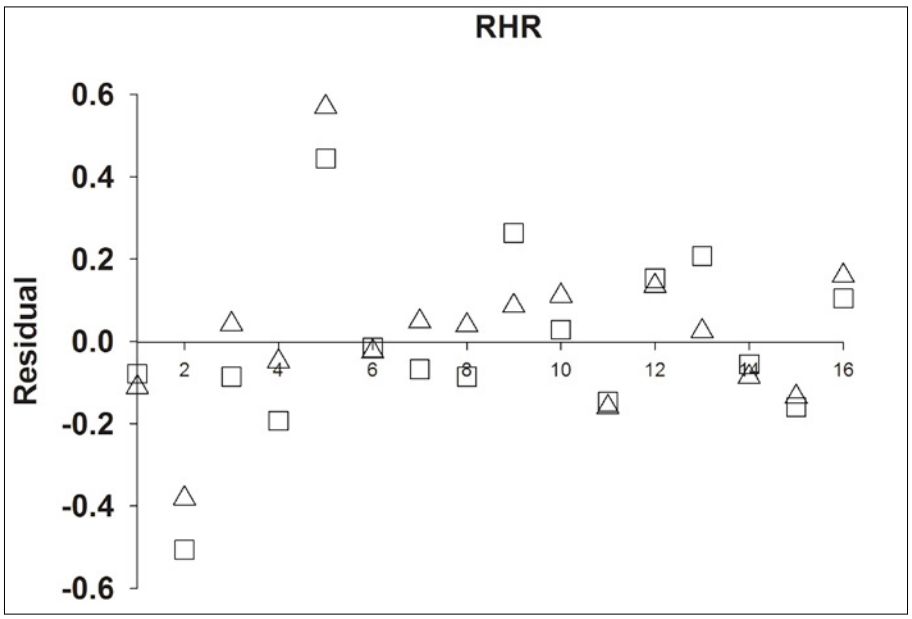


Fig. 9 - Residuals between experimental results and predicted responses by RSM (Δ) and GA (□) models calculated for each trials of rehydration ratio in experimental design.

and RhR determinations for accurate prediction. The performance of RSM with respect to R^2, adj-R^2 and lack-of-fit values was acceptable. The GA and RSM methods produced similar models of performance for microwave-assisted drying of artichoke tubers. The analysis and results from this present study imply that both RSM and GA models can be used in cohesion to gain complete insight into the bioprocessing system.

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PAPER

MEAT PRODUCTION TRAITS OF LOCAL KARAYAKA SHEEP IN TURKEY 1. THE MEAT QUALITY CHARACTERISTIC OF LAMBS

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ABSTRACT

This study is an investigation into the meat quality parameters of Karayaka lambs at different slaughter weights (SWs). The single-born Karayaka male lambs (n=30) selected for this study were an average live-weight of 20 kg and weaned at 2.5-3 months of age. The animals with pre-specified SWs were divided into slaughter weight (SW) groups (30, 35, 40, 45 and 50 kg) using a fully randomized design. To determine the *M. longissimus dorsi et thoracis* (LD) muscle meat quality characteristics, six lambs from each weight group were slaughtered. Results revealed significant differences among the slaughter groups with regard to pH, color parameters (L*-lightness, a*-redness, b* -yellowness), cooking loss (CL), drip loss (DL), moisture (M), crude protein (CP) and intramuscular fat (IF) ratios. Increasing water holding capacities (WHCs) and hardness values were observed with increasing SW. Significant differences were also observed among the slaughter groups with regard to total monounsaturated fatty acid + total polyunsaturated fatty acid/total saturated fatty acid ratios and total cholesterol content.

- Keywords: Karayaka sheep, fatty acid composition, lambs, meat quality, slaughter weight -

INTRODUCTION

Mutton is a significant protein source for humans. Although Turkey is among those countries with rapidly increasing population, there has been an approximately 48% decrease in the country's sheep stocks in recent decades. According to the latest statistics, around 13.7% of Turkish red meat production comes from sheep-raising (TUIK, 2014). Such a ratio clearly indicates the significance of mutton in red meat production of Turkey. To meet animal protein requirements, and to provide a healthy and balanced nutrition, especially for children, but also for all ages, the quality and amount of red meat per unit animal definitely requires improvement. The link between beef, mutton and an increased risk of cardiovascular disease has repeatedly been the focus of concern (WOOD *et al.*, 1999; NUERNBERG *et al.*, 2008). Beef and mutton are regarded as having a higher saturated fatty acid content and cholesterol level than other red meat and poultry (KARACA and KOR, 2007). However, conjugate linoleic acid, a derivative of linoleic acid of unsaturated fatty acids, has anti-carcinogenic and beneficial effects on human health, such as decreasing body fatty acids and improving immunity. Previous research has revealed that lamb has higher rates of this fatty acid than other meat sources (INANÇ, 2006; KURBAN and MEHMETOĞLU, 2006). Along with ever developing and changing consumer demand, there is a need for studies about fatty acids and the cholesterol contents of muttons of local sheep breeds and such studies will unquestionably provide a great contribution to the preservation of local breeds and gene source. In lambs, meat quality is significantly affected by genotypes (ESENBUĞA *et al.*, 2001; PURCHAS *et al.*, 2002; MARTÍNEZ-CEREZO *et al.*, 2005), slaughter weights (SWs) (JEREMIAH *et al.*, 1998; PURCHAS *et al.*, 2002; MARTÍNEZ-CEREZO *et al.*, 2005), gender (DRANSFIELD *et al.*, 1990), pre-slaughter stress (TEIXEIRA *et al.*, 2005), carcass cooling ratio (TEIXEIRA *et al.*, 2005), raising system (VELASCO *et al.*, 2004; CARRASCO *et al.*, 2009) and maturation duration (TEIXEIRA *et al.*, 2005).

Karayaka sheep have low fertility (52-103%) (AKÇAPINAR *et al.*, 2002; AKSOY, 2008), milk production (40-45 kg) and live weight (35-50 kg) (SÖNMEZ *et al.*, 2009), while the quality of meat traits is better than that of other local breeds such as Red Karaman, Anatolian Merino and Awassi. Karayaka sheep constitute about 4-5% of the total Turkish sheep population and are extensively reared in the Black Sea Region of Turkey (ULUTAS *et al.*, 2008).

The present study was conducted to determine the meat quality traits of Karayaka lambs with different SWs.

MATERIAL AND METHODS

The present research was conducted in the sheep barns of the Agricultural Research Farm of Gaziosmanpaşa University (2011-HADYEK-046 numbered local ethics committee approval). Singleton-born Karayaka male lambs (n = 30) with an average live-weight of 20 kg and weaned at 2.5-3 months of age were considered for the study. The SWs and age of lambs at slaughter were 30 kg and 104.8±4.83 days; 35 kg and 119.2±4.29 days; 40 kg and 135.8±1.87 days; 45 kg and 154.6±1.99 days; 50 kg and 163.6±3.26 days, respectively. The animals with pre-specified SWs were divided into SW groups in a fully randomized design. Lambs housed together in 5 × 8 meter pens. Before the initiation of fattening, the lambs were disinfected against internal and external parasites. Following an initial one-week feeding adaptation period, the actual fattening was commenced and lambs were fed until they reach SWs of 30, 35, 40, 45 and 50 kg. Six lambs were slaughtered from each weight group. Lamb fattening feed (concentrated feed) and lentil straw (coarse fodder) were used as the feed material. During the fattening period, lamb-fattening feed was supplied *ad libitum* and coarse fodder was supplied at a ratio of 100 g/head/day. The nutrient contents of the concentrated feed and coarse fodder are provided in Table 1. Fresh water and licking stones were continuously supplied to animals during the experiments. The lambs with the desired SWs were taken into private pens. The animals were not fed for 12 hours prior to slaughter; they were then were transported for 10 minutes to a local licensed abattoir. After holding them in the paddock of the slaughterhouse for two hours, they were slaughtered following the standard commercial slaughter procedures (TSI, 1987). The lambs were brought to slaughter within ±1 kg of the expected SWs. After slaughter, the carcasses were kept at +4°C for 24 h and then the *M. longissimus dorsi et thoracis* (LD) muscles were isolated for meat quality analyses. Sufficient samples taken from these muscles were vacuumed and stored at +4 °C for analysis, at -20 °C for mois-

Table 1 - The chemical composition of concentrated feed and coarse fodder.

Nutrient content	Concentrated feed	Lentil straw
Dry matter (%)	92.00	91.30
Crude protein (%)	20.63	5.78
ADF (%)	26.39	55.59
NDF (%)	37.96	56.29
Crude fat (%)	2.60	1.49
Crude ash (%)	10.40	9.60
Metabolic energy (kcal/kg)	2658	2012

ADF:Acid Detergent Fiber
NDF:Neutral Detergent Fiber

ture (M), crude ash (CA), crude protein (CP), intramuscular fat (IF) and at -80 °C for defrosting and cooking loss (CL), texture, fatty acid composition and cholesterol analyses. The pH of the LD muscle samples was measured at the 45th minute and 24th hour after slaughter with a meat pH meter (Testo 205, Germany). Measurements were taken from three different locations of the samples and an average of those three measurements was taken as the pH value of that sample (RAMÍREZ and CAVA, 2007).

Meat color measurements were performed on the LD at the level of the 12th and 13th ribs, one and 24 hours after slaughter with a Konica Minolta CR-400 (Japan) spectro-colorimeter. Commission International de l'Eclairage (CIE) (1976) standards were used for the measurements (CIE, 1986). The color parameters (L*-lightness, a*-redness, b*-yellowness) were measured from five different sections of each sample. A data set was created by taking the average of measurements for each of the three parameters (ÖNENÇ *et al.*, 1999a,b). Then C (chroma = $(a^{*2}+b^{*2})^{1/2}$) and H° (hue = $\tan^{-1}(b^*/a^*)$ values were calculated (ÖNENÇ, 2003).

Water holding capacities (WHCs) were measured in accordance with the press method developed by Grau and Hamm (1956). A 25 g meat sample was taken from each main sample and ground in an Aura Type 103 (Turkey) brand mini chopper. Then, 1 g of chopped sample was placed in between two filter papers (Whatman 1 Qualitative Circles 125mm Ø Cat No: 1001 125); glass plates were placed above and below the filter papers and a 2.250 kg weight was placed on them. After five minutes, samples were taken out the filter papers and re-weighed (BARTON-GADE *et al.*, 1993). Then, WHC was calculated, using the equation of "WHC (%) = ((Initial sample weight – Pressed sample weight) / Initial sample weight) x 100".

To determine drip loss (DL), 20-25 g samples were taken from LD muscle and vacuumed into plastic bags. The vacuumed samples were stored at 4°C. The samples were then taken out of the vacuum bags three and seven days later, dried without any pressure, and reweighed. The ratio of the difference between the initial and final weights was calculated to find DL% after three and seven days (BOND and WARNER, 2007).

To determine the CL, 40-50 g samples were taken from the LD muscle, placed into vacuum bags and cooked in a water bath (70°C) for 40 min. The samples were then placed under a running tap for 30 minutes to lower the sample temperature to 25°C (MITCHAOTHAI *et al.*, 2006). Then the samples were taken out of the bags, blotted without any added pressure and reweighed. The CL was calculated using the equation of "CL (%) = ((Initial sample weight – Cooked sample weight) / Initial sample weight) x 100.

Textural characteristics were determined at room temperature, using the P36/R probe of a Texture Analyzer (TA.XP Plus - Stable Micro Systems, Godalming, UK) (MARTİNEZ *et al.*, 2004). Sample dimensions were arranged into 1x1x1 cm (cubic) cubes and before, during and after, probe speeds were respectively set as 1, 5 and 5 mm/s.

The M, CP and CA contents of the LD muscle samples were determined in accordance with AOAC (1990). The IF contents were determined, according to the heat extraction method with an Ankom (XT10, Spain) Extractor device (OKEUDO *et al.*, 2007).

The extraction of lipids for fatty acid analysis was performed with chloroform/methanol (2:1), as described by FOLCH *et al.* (1957). Triglycerides in the cold-extracted lipids were converted into fatty acid methyl esters, in accordance with AOCS (1993). The fatty acid composition of the samples were determined using a Perkin Elmer Clarus 500 (USA) gas chromatography device, equipped with a FID (Flame Ionization Detector) detector and a Thermo Scientific Tr 70 Capillary column (30 m x 0,25 mm and 0,25 µ film thickness). Helium (1 mL/min) was used as a carrier gas. Split ratio was set as 1/50, operational temperature for injection block as 250°C and for detector as 260°C. The temperature increase rate was 1°C/min, to increase the column temperature from 140°C to 180°C and 2°C from 180°C to 200°C. Samples were kept at a final temperature of 200°C for eight minutes. A Supelco 37 FAME mix (C4-C24) (Bellefonte, PA, USA) was used as the standard by which to define the fatty acids. The results were expressed in % methyl esters.

About 0.3-0.5 g of lipid samples was taken from the lipid, cold-extracted from the LD muscle, and the samples were placed into closed glass tubes. Then, 0.3 mL 33% KOH and 3 mL 95% ethyl alcohol solution was added, and the mixture roughly mixed and saponificated in a water bath at 60°C for 15 min. The tubes were cooled down, 10 mL hexane and 3 mL of distilled water was added and the roughly mixed samples were then kept for 10 minutes for phase separation. To determine cholesterol content, a 1 mL sample was removed from the hexane fraction into a test tube. The hexane was removed using nitrogen gas. A $FeCl_3$ stock solution was prepared with 840 mg $FeCl_3$ and 10 mL concentrated glacial acetic acid, and 1 mL of this stock solution was increased to 100 mL with a concentrated glacial acetic acid, to prepare the $FeCl_3$ working solution. Later on, the 1.5 mL $FeCl_3$ working solution was added to test tube and the resulting solution was roughly mixed. After 15 minutes, 1 mL of concentrated sulphuric acid was added and the samples were mixed in a tube mixer for 1 min. The tubes were placed in the dark for 45 min. The absorbance values of the resulting purple color were read at 560 nm wavelength of a UNICAM UV/Vis model spectrophotometer. Cholesterol standard curves were cre-

Table 2 - Meat quality characteristics of *M. longissimus dorsi et thoracis* (LD).

Traits	Slaughter weight (kg)					MSE	P
	30	35	40	45	50		
pH_{45m}	6.15^{c}	6.10^{c}	6.31^{b}	6.14^{c}	6.46^{a}	0.01	***
pH_{24h}	5.55^{c}	5.60^{c}	5.75^{ab}	5.70^{b}	5.80^{a}	0.01	***
Color $_{60m}$							
L*	33.99^{a}	33.90^{ab}	33.23^{b}	33.59^{ab}	32.10^{c}	0.10	***
a*	12.55^{a}	12.25^{a}	10.47^{b}	10.27^{b}	10.49^{b}	0.08	***
b*	3.15^{a}	3.04^{a}	1.30^{b}	1.07^{b}	0.94^{b}	0.07	***
C*	12.94^{a}	12.64^{a}	10.57^{b}	10.33^{b}	10.53^{b}	0.09	***
H°	14.38^{a}	13.41^{a}	6.50^{b}	5.90^{b}	5.34^{b}	0.29	***
Color $_{24h}$							
L*	41.04^{a}	39.70^{ab}	39.68^{ab}	39.58^{ab}	38.60^{b}	0.22	*
a*	13.27^{d}	14.35^{ab}	14.12^{bc}	13.75^{c}	14.61^{a}	0.06	***
b*	5.03^{a}	5.35^{a}	4.08^{b}	4.18^{b}	5.02^{a}	0.06	***
C*	14.21^{b}	15.36^{a}	14.64^{b}	14.39^{b}	15.37^{a}	0.07	***
H°	20.83^{a}	20.21^{a}	16.25^{c}	16.55^{c}	18.79^{b}	0.20	***
Drip loss (%)							
3rd day	8.10^{a}	8.71^{a}	7.15^{b}	9.67^{a}	9.94^{a}	0.20	***
7th day	12.22^{ab}	11.73^{ab}	9.35^{c}	13.20^{a}	10.94^{b}	0.24	***
Cooking loss (%)	28.25^{a}	27.23^{a}	26.11^{ab}	25.03^{b}	24.73^{b}	0.29	**
WHC (%)	34.37^{d}	36.20^{c}	36.28^{c}	37.74^{b}	39.15^{a}	0.21	***
Texture (kg/cm^2)	4.51	4.91	5.18	5.96	7.29	0.35	-

WHC: Water Holding Capacity; MSE: Mean Standard Error
-: Non-significant, *: P<0.05, **: P<0.01, ***: P<0.001
Means within a row with different letters differ significantly (P<0.05)

Table 3 - Compositional properties of *M. longissimus dorsi et thoracis* (LD) (%).

Traits	Slaughter weight (kg)					MSE	P
	30	35	40	45	50		
Moisture	75.92^{ab}	75.08^{cd}	76.18^{a}	74.46^{d}	75.33^{bc}	0.12	***
Protein	20.14^{ab}	20.82^{a}	20.13^{ab}	20.68^{a}	19.85^{b}	0.11	*
IF	2.59^{b}	2.67^{b}	2.41^{b}	3.44^{a}	2.98^{ab}	0.08	**
Ash	1.08	1.06	1.07	1.08	1.06	0.01	-

IF: Intramuscular Fat; MSE: Mean Standard Error
-: Non-significant, *: P<0.05, **: P<0.01, ***: P<0.001
Means within a row with different letters differ significantly (P<0.05)

ated and the cholesterol content of the samples was expressed as mg cholesterol/100 g sample (RUDEL and MORRIS, 1973).

Statistical analyses were performed using SPSS (1999) software. The Duncan's test was used to determine differences among the means (DÜZGÜNEŞ *et al.*, 1987).

RESULTS AND DISCUSSION

Mean values, for the meat quality traits of the LD muscles of Karayaka lambs with different SWs, are shown in Table 2, the compositional nutrient content in Table 3 and, fatty acid composition and cholesterol contents in Table 4.

Meat pH values have distinctive impacts on meat quality traits, such as color, WHC and texture. Therefore, the pH plays a significant role in the quality assessment of meat (KARACA, 2010). In the present study, pH measurements were performed 45 minutes (pH_{45m}) and 24 hours (pH_{24h}) after the slaughter. In both measurement times, the differences in muscle pH values of the slaughter groups were found to be significant (P<0.001; Table 2). Similar to the current findings, the significant effects of SWs on final pH values were reported in previous studies (BERIAIN *et al.*, 2000; YAKAN and ÜNAL, 2010); however, others reported insignificant effects (MARTÍNEZ-CEREZO *et al.*, 2005). Increasing pH_{24h} values were observed in this study with increas-

ing SWs and the relevant values varied between 5.55 – 5.80. Based on the assumption that a final pH value above 5.8 is considered undesirable, it can be said that the final pH ranges were both appropriate and inside normal range (YAKAN and ÜNAL, 2010).

WHC is closely related to pH and therefore it is considered as a significant parameter for meat quality assessments (YAKAN, 2008). The differences between WHCs of the slaughter groups were also found to be significant (P<0.001; Table 2). Increasing WHC values were observed with increasing SWs. LAWRIE and LEDWARD (2006) reported increasing WHCs with increasing pH values. However, current findings were contrary to those reports. Cold-induced contraction might have such effects on WHC. Such contractions have higher impacts on carcasses with high pH levels. Cold carcass contractions result in decreasing intra-myofibril spaces and water release from the meat (KARACA, 2010). The WHCs of lambs fed with concentrated feed were reported as between 9.76 - 28.27 (BERIAIN *et al.*, 2000; EKIZ *et al.*, 2009; YAKAN and ÜNAL, 2010).

Consumers commonly assess the meat they buy based on fattiness, general appearance and color; regarding light colored meat as that of young animals, which they prefer to buy (SAÑUDO *et al.*, 2007). In the present study, the color parameters L*, a* and b* were measured over hot carcasses (60 minutes after slaughter) and cold carcasses (24 hours after slaughter) of LD muscle samples and significant differences were observed between slaughter groups with regard to L*, a*, b*, C* and H° values, in both measurement periods (P<0.05; Table 2). Similar to the current findings, BERIAIN *et al.* (2000) and MARTINEZ-CEREZO *et al.* (2005) reported significant effects of SWs on the color parameters. The decreasing L* values observed in this study were concomitant with increasing SWs. The a* values recorded at 24h in 50 kg group was higher than those recorded in 40 and 45 kg groups and similar to those recorded in 35 kg group. BERIAIN *et al.* (2000) carried out a study on Lacha and Rasa Aragonesa lambs with different SWs (12, 24 and 36 kg) and reported decreasing L* values and increasing a* values with increasing SWs. In other studies carried out with local lamb breeds, fed by concentrated feeds, L* values (24 hours after slaughter) were reported as between 37.91 – 42.72; a* values as between

Table 4 - Cholesterol content (mg/100 g meat) and fatty acid composition (%) of lipids of *M. longissimus dorsi et thoracis* (LD).

Traits	Slaughter weight (kg)					MSE	P
	30	35	40	45	50		
C8:0	0.210	0.170	0.172	0.156	0.154	0.01	-
C10:0	0.310	0.258	0.220	0.222	0.242	0.01	-
C11:0	7.188	6.156	6.123	5.664	5.530	0.14	-
C12:0	0.415	0.224	0.172	0.138	0.142	0.03	-
C14:0	2.927^{a}	2.940^{a}	2.725^{ab}	2.530^{ab}	2.380^{b}	0.07	*
C14:1	0.215	0.134	0.090	0.110	0.122	0.01	-
C15:0	0.300	0.204	0.165	0.104	0.200	0.02	-
C16:0	23.135	23.206	23.493	23.914	24.326	0.21	-
C16:1	1.055	1.278	0.928	1.234	0.808	0.11	-
C17:0	0.750	0.968	0.717	0.818	0.808	0.07	-
C17:1	0.550	0.695	0.537	0.612	0.584	0.05	-
C18:0	13.792^{b}	14.374^{b}	14.350^{b}	13.964^{b}	16.306^{a}	0.25	**
C18:1	37.867	39.622	39.980	43.132	40.294	0.59	-
C18:2 (n-6)	7.572^{a}	6.790^{ab}	7.380^{a}	5.334^{b}	5.878^{ab}	0.28	*
C18:3 (n-6)	0.028	0.098	0.012	0.008	0.014	0.01	-
C18:3 (n-3)	0.023	0.030	0.005	0.012	0.010	0.01	-
C20:0	0.140	0.060	0.065	0.042	0.036	0.01	-
C20:1	0.247	0.244	0.213	0.138	0.178	0.03	-
C20:3 (n-3)	0.180	0.102	0.060	0.058	0.102	0.01	-
C21:0	0.188	0.026	0.015	0.034	0.016	0.02	-
C22:1	2.725^{a}	2.442^{a}	2.527^{a}	1.722^{b}	1.832^{b}	0.11	**
ΣSFA	48.947^{ab}	48.586^{b}	48.217^{b}	47.586^{b}	50.140^{a}	0.21	**
ΣMUFA	42.660^{b}	44.414^{b}	44.275^{b}	46.948^{a}	43.818^{b}	0.38	*
ΣPUFA	7.779^{a}	6.980^{ab}	7.449^{a}	5.362^{b}	5.979^{ab}	0.27	*
(ΣMUFA+ΣPUFA)/ΣSFA	1.033^{bc}	1.059^{abc}	1.073^{ab}	1.102^{a}	0.995^{c}	0.09	*
ΣPUFA/ΣSFA	0.159^{a}	0.144^{ab}	0.154^{abc}	0.113^{c}	0.119^{bc}	0.05	*
Total cholesterol	199.799^{b}	194.143^{bc}	162.044^{d}	224.326^{a}	190.381^{c}	3.03	***

MSE: Mean Standard Error

-: Non-significant, *: P<0.05, **: P<0.01, ***: P<0.001; Means within a row with different letters differ significantly (P<0.05); SFA: Saturated Fatty Acid; MUFA: Mono-Unsaturated Fatty Acid; PUFA: Poly-Unsaturated Fatty Acid

16.08 – 21.26 and b* values as between 5.10 – 8.45 (EKIZ et. al., 2009; ESENBUĞA et. al., 2009; KARACA, 2010; YAKAN and ÜNAL, 2010).

Various researchers have shown carcass weight as the most significant factor indicating lamb carcass and meat quality (DİAZ *et al.*, 2002; VERGARA *et al.*, 1999). PEÑA *et al.* (2005) reported darkened meat color with increasing lamb carcass weights. Similar to the current findings, SAÑUDO *et al.* (2000) reported decreasing a* values and increasing L* values with decreasing carcass fat ratios.

Texture is another factor affecting meat quality. Consumers specify meat hardness as a significant quality indicator (KARACA, 2010). SHACKELFORD *et al.* (1991) reported that consumers and taste panelists indicated meats with a hardness value over 5.5 kg/cm^2 as hard meats. For Karayaka lambs in the present study, except for the SW groups of 45 kg (5.96 kg/cm^2) and 50 kg (7.29 kg/cm^2), the hardness values were within the limits specified by SHACKELFORD *et al.* (1991). Although not significant (P>0.05), increasing hardness values were observed in this study with increasing SWs (Table 2). The hardness value of entire SW groups of Karayaka lambs were lower than the values reported by ESENBUĞA *et al.* (2001) for Awassi and Red Karaman. The hardness value of 40 kg SW group of the present study were higher than those reported by EKIZ *et al.* (2009) for Merino, Ramlıç, Kivircik lambs (40-41 kg SW); and by PERLO *et al.* (2008), for Corriedale lambs (41 kg SW). Some other researchers reported the hardness values of lambs fed with concentrated feeds (24-30 SW) as between 3.35-4.01 kg/cm^2 (SANTOS-SILVA *et al.*, 2002a; EKIZ *et al.*, 2009; YAKAN and ÜNAL, 2010).

With regard to DL on 3rd and 7th days, the differences between the slaughter groups were found to be significant (P<0.001; Table 2). Increasing SWs resulted in increasing DLs on the third day. The highest DL on 3rd and 7th days was observed in the 50 kg (9.94%) and 45 kg (13.20%) weight groups.

The CL values decreased with increasing SWs (P<0.01; Table 2). Although GÖKALP *et al.* (1993) indicated lower CL values for high WHC meats, contrary results were observed in this study. EKIZ *et al.* (2009) slaughtered Merino, Ramlıc and Kivircik lambs fed with concentrated feeds at 40-41 kg weights and observed the CL, respectively as 27.14, 25.57 and 29.54%. The CL for 40 kg SW of the present study (26.11%) was higher than the value determined by EKIZ *et al.* (2009) for the same-weight Ramlıc lambs, and lower than Merino and Kivircik lambs. The CL determined for 30 kg SW groups of Karayaka lambs was similar to that reported by the same researchers for 26 kg Imroz lambs (28.91%) and higher than the value reported by Chios lambs (27.81%).

While the differences between SW groups were found to be significant with regard to CP and M contents (P<0.05), the differences in CA contents of the groups were insignificant (P>0.05; Table 3).

IF in 50 kg was similar to IF observed in all the other SW groups. The highest value was observed in the 45 kg (3.44%) groups and the lowest value was seen in the 40 kg (2.41%) SW groups. YAKAN (2008) reported a decreasing IF content in Bafra lambs with increasing SWs, with the highest value for 30 kg (4.20%) and the lowest value for 40 kg (2.80%) weight groups. The CP ratios for the Karayaka lambs in the present study were similar to the values determined by previous researchers for local, crossbred and heritage breeds of lamb (BERIAIN *et al.*, 2000; MACIT *et al.*, 2003; PERLO *et al.*, 2008; ESENBUĞA *et al.*, 2009). The LD muscle M contents of Karayaka lambs of the present study (74-76%) were similar to values reported by the other researchers for the same muscle (73-76%) (BERIAIN *et al.*, 2000; PERLO *et al.*, 2008; ESENBUĞA *et al.*, 2009).

In ruminants, almost all of the fats are localized as triglycerides in adipose, and fatty acids are localized as C16 and C18. In general, more than 80% of the fatty acids are composed of C14:0 (myristic acid); C16:0 (palmitic acid), C18:0 (stearic acid) and C18:1 (oleic acid) (KARACA, 2010). The order of those primary fatty acids in the present study was observed as C18:1, C16:0 and C18:0 in all SW groups and only the differences in the C14:0 and C18:0 fatty acids were found to be significant (P<0.05; Table 4). With regard to unsaturated fatty acids, the differences in C18:2 (n-6) (linoleic acid) and C22:1 (erucic acid) fatty acids of the weight groups were found to be significant (P<0.05). On the other hand, differences in the monounsaturated fatty acid contents of the groups were insignificant (P>0.05). The differences between the SW groups were also found to be significant, with regard to total monounsaturated fatty acids, total polyunsaturated fatty acids, total unsaturated fatty acid/total saturated fatty acid ratios and total polyunsaturated fatty acid/total saturated fatty acid ratios (P<0.05). The highest total saturated fatty acid content was observed in the 50 kg (50.14%) and the lowest in the 45 kg (47.58%) SW group. In general, a decreasing total of saturated fatty acid contents were observed with increasing SWs. While such decreases comply with the findings of some previous research (DÍAZ *et al.*, 2005; ORIANI *et al.*, 2005; YAKAN and ÜNAL, 2010), they differed from an other study (SANTOS-SILVA *et al.*, 2002b). The total unsaturated fatty acid / total saturated fatty acid ratios of Karayaka lambs of the present study varied between 0.99-1.10. Such values were reported in previous studies as between 0.09 – 0.95 for the lambs fed with concentrated feeds (ROWE *et al.*, 1999; DÍAZ *et al.*, 2002; KARABACAK, 2007). The total unsaturated fatty acid / total saturated fatty acid ratios of the Karayaka lambs in the present study were higher than the other studies. Such differences were mainly due to differences in genotype and the age of slaughter, since

genotype, age of slaughter, gender and type of fat stores, and the anatomic location of muscles and fats are major factors affecting the fatty acid composition of meat.

The differences in cholesterol levels of the SW groups were found to be significant (P<0.001; Table 4). The highest total cholesterol level was observed in the 45 kg (224.32 mg/100 g meat), the lowest value in the 40 kg (162.04 mg/100 g meat) weight groups. YAKAN and UNAL (2010) carried out a study on Bafra lambs and reported the highest total cholesterol levels for the 45 kg (63.00 mg/100 g meat) and the lowest levels for the 35 kg (53.80 mg/100 g meat) SW groups. BUNCH *et al.* (2004) reported the total cholesterol level of Wool lambs with 46-54 kg SWs and fed with concentrated feed, as 117 mg/100 g meat; as 73 mg/100 g meat for Callpyge Wool x St. Croix lambs; 50 mg/100 g meat for Callpyge Wool x Wool lambs; 149 mg/100 g meat for Dorper x Wool lambs and 131mg/100 g meat for Dorper x St. Croix lambs. Similarly, SALVATORI *et al.* (2004) reported the total cholesterol level of extensively fed Ile de France x Parliarola and Gentile di Puglia Sopravissana lambs respectively as 63.0 and 60.3 mg/100 g meat. In another study carried out on Corriedale and Corriedale crossbreds, the total cholesterol level was reported as 62.03 for the lambs fed with concentrated feeds and as 57.76 mg/100 g meat for range-fed lambs (ROWE *et al.*, 1999). The total cholesterol values of the LD muscle of the Karayaka lambs in the present study were higher than thoses values reported by ROWE *et al.* (1999), SALVATORI *et al.* (2004) and BUNCH *et al.* (2004).

CONCLUSIONS

In conclusion, with regard to meat quality parameters, except for CA and hardness, the differences in entire traits of LD muscle of the different SW groups of Karayaka lambs of the present study were found to be significant. Increasing SWs resulted in increasing WHC and hardness values, and decreasing CL values, but the differences between the hardness values of the samples were not found to be significant. Among the fatty acids, except for C14:0, C18:0, C18:2 (n-6) and C22:1, differences in the entire fatty acid contents of SW groups were found to be insignificant.

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PAPER

EFFECTS OF GARLIC EXTRACT ON COLOR, LIPID OXIDATION AND OXIDATIVE BREAKDOWN PRODUCTS IN RAW GROUND BEEF DURING REFRIGERATED STORAGE

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ABSTRACT

The study aims to investigate the effects of garlic extracts on color, lipid oxidation, and oxidative breakdown products in raw ground beef during refrigerated storage. The two treatments were: control group (C, with no addition) and experiment group (D, 50 mg garlic extracts added to 100 g beef). Adding garlic extracts significant increased a* value ($P_A \leq 0.05$), and significant decreased TBARS and PV values ($P_A \leq 0.05$). The pH and –SH value of D group had a decreasing tendency (P_A=0.0522) and an increasing tendency (P_A=0.0636) respectively compared to C group. Garlic extracts protected phospholipids, fatty acids and polypeptides from oxidation. The results indicate that garlic extracts have the antioxidant activity, helping maintain the meat color, inhibiting lipid oxidation and protein degradation of raw ground beef during refrigerated storage.

- Keywords: garlic extracts, color, lipid oxidation, oxidative breakdown products, raw ground beef -

INTRODUCTION

Lipid oxidation is one of the primary mechanisms of quality deterioration in meat and meat products. The adverse changes in quality are manifested in flavor, color, texture and nutritive value, and the possible production of toxic compounds (CLAUDIA *et al.*, 2014). Beef and its products are rich in protein and lipids, which make them most suitable for consumer. However, beef contains high level of unsaturated fatty acids which are prone to oxidation (TICHIVANGANA and MORRISSEY, 1985). To prevent or reduce lipid oxidation, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), trihydroxybutyrophenone (THBP), propyl gallate (PG), nordihydroguaiaretic acid (NDGA) and ethoxyquin have been applied in meat products (TRINDADEA *et al.* 2014). However, their application had been restricted because of their potential health risks and toxicity (SUN and FUKUHARA, 1997). Natural antioxidants can be used as alternatives to the synthetic antioxidants because of their safety and equivalent or greater effect on inhibition of lipid oxidation (ANDREW *et al.*, 2014).

Garlic (*Allium sativum*) has been a favorite additive to enhance the flavor of food as well as herbal medicine for many years in various cultures. It is well known that garlic has antimicrobial, antiprotozoal, antimutagenic, antiplatelet and antihyperlipidemic properties. Garlic holds a unique position for therapeutic potential due to the antioxidant activity by scavenging reactive oxygen species (ROS), enhancing the cellular antioxidant enzymes, and increasing glutathione in the cells. Numerous studies have demonstrated that garlic exhibits cardioprotective (RAHMAN and LOWE, 2006), liver-protective (WANG *et al.*, 1998), beneficial effects in diseases such as ischemic-reperfusion arrhythmias and infarction (RIETZ *et al.*, 1993), ischemic heart disease (ARORA *et al.*, 1981), hypertension (FOUSHEE *et al.*, 1982), hyperlipidemia (ERNST *et al.*, 1985), as well as prevent the processes of cancer (TANAKA *et al.*, 2006) and aging (LI *et al.*, 2012).

A number of researchers reported that garlic and different garlic extracts have antioxidant activity contributed by organosulfur compounds, flavonoids and phenolic compounds (BOREK, 2001; OTUNOLA and AFOLAYAN, 2013). RAHMAN *et al.* (2012) researched antioxidant properties of raw garlic extract using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging methods. CAO *et al.* (2013) had studied the effects of garlic on quality and shelf life of stewed-pork during refrigerated storage. However, less attention has been assigned to garlic extracts used as an antioxidant in beef, especially in identifying the oxidative breakdown products so far. The objective of the present study was to determine the effect of garlic extracts on pH, color stability, lipid oxidation, and oxidative breakdown products of raw ground beef during refrigerated storage in order to provide a scientific basis for using garlic extracts as natural antioxidants to maintain the meat quality, extend shelf-life and prevent economic loss.

MATERIALS AND METHODS

Materials

Garlic extracts (10:1 of garlic: garlic extracts, three percent allicin content) was purchased from Yuanshen Bio-Tech Ltd. (Xian, China). Glutathione were obtained from Sigma Aldrich Inc. (St. Louis, MO, USA). Ethanol, chloroform, methanol, ammonium thiocyanate, iron (II) chloride, trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), cumene hydroperoxide, tetraethoxypropane and other reagent were 'AnalaR' grade from China Medicine (Group) Beijing Chemical Reagent Corporation (Beijing, China). Water for ultra-performance liquid chromatography quadrupole time of flight (UPLC-QTOF) was purified with a Milli-Q Gradient A10 system (Millipore, Beijing, China). Formic acid and acetonitrile were HPLC-grade (Fisher Scientific, New Jersey, USA).

Three Simmental crossed cattle (620±15kg, 18 months) were slaughtered at a local commercial abattoir (Jinweifuren Co. Ltd., Beijing, China). Transport, slaughtering, or invasive procedures on live animals involving in this study were handled in strict accordance to the guidelines approved by the Animal Welfare Committee of China Agricultural University (Permit Number: DK1008). After ageing at 4°C for 72 h, the *longissimus dorsi* (LD) were excised from 12^{th} and 13^{th} rib of left half side carcass. The muscles were minced twice through a 5 mm plate of meat mincer (model JYS-A800, Joyoung, China) after removing the connective tissue and visible fat. The contents of moisture, protein and fat of the ground beef were 73.14%, 22.26%, and 2.83% respectively.

Treatments

The meat sample of each cattle was formed into two patties (100 g portions) using a meat former and assigned to the following two treatments:: control group (C, 100 g ground beef with no addition) and experiment group (D, 50 mg garlic extracts added to 100 g ground beef). To eliminate the influence of microorganism and ensure thorough mixed, garlic extracts dissolved in 10 mL of a distilled water and ethanol mixture (1:1, v/v) and then mixed with the muscles of the experiment group. The same volume of distilled water and ethanol mixture (with no added ingredients) was added to the control group.

Meat samples were put into a constant temperature incubator (MJX-320, Jiangnan, Ningbo, China) at 4 °C for 13 days. Meat samples (three replicates) were collected in 1.5 mL centrifuge tube at storage times of 1, 3, 5, 7, 10, 13 days and were frozen rapidly in liquid nitrogen for subsequent analysis.

Analysis of pH and color

A pH meter (pH Spear, Eutech Instruments, USA) was used to measure the pH of the ground beef. The color of the ground beef was determined by portable colorimeter (CR400/410, Minolta, Japan). The specifications of the colorimeter are light source: pulsed xenon lamp; Illuminant: C, D65; illumination area: Φ8/Φ11; Inter instrument agreement: ΔE*ab within 0.6; repeatability: within ΔE*ab0.07 standard deviation. The color results were calculated based on L*, a*, b* (lightness, redness, and yellowness respectively) in the CIELAB space. A white plate (CIE L*= 97.83, a*=-0.43, b*=1.98) was used for calibration.

Analysis of lipid oxidation

Lipid oxidation was evaluated by the peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) according to the method of RICHARDS *et al.* (2003) (RICHARDS and DETTMANN, 2003) and free thiol groups (-SH) was analyzed following the method proposed by EGELANDSDAL *et al.* (2011) with minor modifications.

Determination of PV: Approximately 0.3 g of sample was homogenized for 30 s with 5 mL of cold chloroform/methanol (1:1) in a 50 mL glass tube using a refiner (FJ-200, Jintan, China). Subsequently, the glass tube was rinsed for 30 s again with 5 mL of cold chloroform/methanol (1:1). The homogenate and wash solution were then combined in a 25 mL glass test tube. Adding 3.08 mL of 0.5% NaCl then mixed for 10 s with a vortex (G560E, Scientific Industries Inc., New York, USA). Next, the mixture was centrifuged at 1821g (DL-6000B, Shanghai Anting, China) for 6 min at 4°C. Two milliliters of the lower chloroform layer was removed and transferred to a tube by a glass syringe before 1.3 mL chloroform/methanol (1:1) was added to the 2 mL sample. Then 25 μL of 3.94 mol/L ammonium thiocyanate and 25 μL of 18 mmol/L iron (II) chloride were added to the tube, mixing thoroughly after each addition. Finally, the sample was incubated at room temperature for 20 min and the absorbance at 500 nm was measured using a spectrophotometer (UV1102, Shanghai Tianmei, China). A standard curve was constructed using cumene hydroperoxide and the PV in the sample was expressed as μmol/kg.

Determination of TBARS: mixing 50% trichloroacetic acid (TCA) with 1.3 % thiobarbituric acid (TBA) on the day of use and then heating to 65°C. Approximately 0.12 g of the muscle sample was added to 1.2 mL the TCA - TBA mixture and mixed via a vortex (G560E, Scientific Industries Inc., New York, USA) and then heated at 45°C for 60 min using a vapor-bathing constant temperature vibrator (HQ45, Chinese Academy Of Sciences, Wuhan, China) before the sample was centrifuged at 12,000 g for 5 min (Mikro 200R, Hettich, Germany). The absorbance of the supernatants at 532 nm was determined. A standard curve was prepared using tetraethoxypropane, and the concentration of TBARS in the samples was expressed as μmol/kg.

Determination of -SH: approximately 0.1 g of the muscle sample was weighed into a 10 mL centrifuge tube then 1700 μL phosphate-buffered saline (pH=8.2) and 100 μL DTNB were added. The mixture was heated at 45 °C away from light for 60 min using a vapor-bathing constant temperature vibrator (MJX-320, Jingbo Jiangnan, China). Eight milliliters of methanol was added to the tube which was vortexed for 30 s before centrifuging at 1821g for 20 min at 4°C. Subsequently, 1 mL supernate was transferred to a new centrifuge tube, diluted with 3 mL methanol and then the absorbance was measured at 412 nm. Glutathione was used for generating a standard curve and concentration of -SH in samples was expressed as mmol/kg.

Analysis of oxidative breakdown products

Sample preparation: 50 mg muscle sample from each treatment at 13 days was weighed into a 2 mL Eppendorf tube. The first step: 1.5 mL cold water/methanol (1:1) and 0.5 g 1 mm Zirmil ceramic beads were added to the tube and then mixed thoroughly using a tissue homogenizer (Precellys 24, Bertin, France) for two cycles at 6500 Hz, 40 s for each cycle. The mixture was centrifuged at 12,000 g (Mikro 200R) for 10 min at 4°C. Supernate (400 μL) was collected in a new Eppendorf tube and stored at 4°C for further use. The second Step: the residual sediment from step one was extracted by 1.5 mL cold chloroform/methanol (3:1) again with the same procedure of homogenization and centrifugation and the same volume of supernate collected in the Eppendorf tube. The mixed samples from two steps, were then concentrated in a centrifugal concentration meter (ZLS-1, Herexi, Hunan, China). Water/methanol (9:1; 120 μL) was used to dissolve the samples with vortex oscillation for 40 s. Supernate (100 μL) was collected in a lining tube for subsequent UPLC-QTOF analysis.

UPLC analysis: The substances in the 6 μL extracted sample were separated using a UPLC system (Acquity UPLC/XEVO G2 Q ToF, Waters) after being loaded onto a high-resolution and high-

performance UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm, Waters). The column temperature was 50 °C and the flow rate was 0.3 mL/min with solvent A (water + 0.1 % formic acid) and solvent B (acetonitrile + 0.1% formic acid). The elution program consist of 98:2 (A: B) for 1 min, increasing gradually to 100 % B by 16 min, then 100 % B held for 2 min, a ramp to 98:2 (A: B) within 10 s, and returned to 98:2 (A: B) for 2 min (re-equilibration of the column) before the loading of the next sample.

Q-TOF MS conditions: The separated components from the UPLC were subsequently analyzed by quadrupole time of flight mass spectrometry (Q-TOF MS) equipment (Acquity UPLC/XEVO G2 Q ToF, Waters) operated using a negative electrode for electrospray ionization. The settings were as following: data acquired in a full scan mode (mass: charge ratio (m/z) 50-1200) at a rate of 2 spectra/s; capillary and sampling cone voltages at 2500 and 35 V, respectively; desolvation temperature at 350°C; desolvation gas flow at 720 L/h; cone gas flow at 50 L/h; source temperature at 105°C. All gas paths used nitrogen and data were collected using Masslynx 4.1 data management software (Waters, USA). The final data was expressed by the ID number, relative m/z values, retention times and ion intensity.

STATISTICAL ANALYSIS

This experiment was assigned two factors with two treatments and seven storage time levels.s. The mixed model procedure of SAS 9.0 (SAS Institute Inc.) was used to analyze the effects of garlic extracts and storage time. The following statistical model was used for analysis:

$$y_{ik} = \mu + \alpha_i + \beta_k + (\alpha\beta)_{ik} + e_{ik}$$

where y_{ik} is an observed value for TBARS, PV, -SH, pH, L*, a*, and b*, taken from sample receiving treatment i at time k;μis the overall mean; α_i is the fixed effect of treatment i; β_k is the fixed effect of time k; $(\alpha\beta)_{ik}$ is the interaction between treatment and storage time; and e_{ik} is the residual value.

The variation tendency of pH and color, lipid oxidation with different storage times was analyzed by the contrast model of SAS 9.0. The means that were significantly different were analyzed using the t-test and different storage times were analyzed using Duncan's multiple comparison tests. A level of $P \leq 0.05$ was considered significant and $0.05 < P < 0.1$ was considered tendency.

Differences in oxidative breakdown products among treatments were analyzed with principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) using SIMCA-P software (Umetrics, Ume, Sweden). The different oxidative breakdown products between two treatments were analyzed in terms of ion intensity using the t-test. Metabolites that met the criterion of $P \leq 0.05$ of the t-test and a fold change > 1.5 were selected as different oxidative breakdown products whose formulae were searched in metlin database.

Table 1 - The effects of garlic extracts and storage time on physical meat quality, lipid oxidation in raw ground beef during refrigerated storage.

Items		TBARS	PV	-SH	pH	L*	a*	b*
Treatments	C	22.41^{a}	46.56^{a}	6.62	5.86	51.22	13.50^{b}	14.07^{b}
	D	19.59^{b}	30.22^{b}	7.10	5.85	51.35	14.49^{a}	14.59^{a}
	SEM	0.6121	9.7158	0.3015	0.0027	0.2020	0.2086	0.1422
Times	1	10.25^{c}	23.78^{b}	7.16^{ab}	5.91^{a}	51.05^{b}	18.05^{a}	14.71^{a}
	3	13.50^{b}	28.94^{b}	8.14^{a}	5.84^{b}	50.13^{c}	15.68^{b}	13.52^{b}
	5	26.92^{a}	42.11^{ab}	7.23^{ab}	5.84^{b}	51.96^{a}	15.44^{b}	14.76^{a}
	7	24.20^{a}	69.32^{a}	7.12^{ab}	5.83^{b}	51.99^{a}	13.98^{c}	15.02^{a}
	10	26.07^{a}	44.82^{ab}	6.03^{bc}	5.82^{c}	51.01^{b}	10.18^{d}	13.55^{b}
	13	25.08^{a}	21.39^{b}	5.45^{c}	5.91^{a}	51.57^{ab}	10.65^{d}	14.43^{a}
	SEM	0.6121	9.7158	0.3015	0.0027	0.2020	0.2086	0.1422
P_A		<0.001	0.0500	0.0636	0.0522	0.4248	<0.001	0.0001
P_B	L	<0.001	0.5139	<.0001	0.1356	0.0123	<0.001	0.5835
	Q	<0.001	0.0085	0.0238	<.0001	0.0852	0.9871	0.8118
P_{AB}		0.0383	0.9684	0.1200	0.0035	0.5571	0.0034	0.0152

Means in the same column with different superscripts are significantly different (P <0.05).C-control group, D-garlic extracts group. P_A is the P value of treatments; P_B is the P value of storage times; L stands for the linear effects of storage times; Q stands for the quadratic effects of storage times. P_{AB} is the P value interaction between treatments and storage times.

RESULTS

The effects of garlic extracts and storage time on physical meat quality, lipid oxidation in raw ground beef during refrigerated storage

According to Table 1, L* and a* value presented an increasing and decreasing linear change respectively (P_BL<0.05), TBARS, PV, -SH, and pH had a quadratic change over storage time (P_BQ<0.05). More details, TBARS value significant increased in the first five storage days (P_B<0.05), and then no significant changes in the residual storage time. PV and -SH value were firstly increased and then decreased with the highest value at seventh day and third day respectively of storage time. On the contrary, pH was firstly decreased and then increased with the lowest value at tenth day of storage time, ranging from 5.82 to 5.91. However, storage time had neither linear nor quadratic effect on b* value in raw ground beef during refrigerated storage.

As shown in Table 1, Adding garlic extracts could significant increased ($P_A \leq 0.05$) a* and b* values. The pH of D group had a decreasing tendency compared to C group (P_A=0.0522). There was no significantly different on L* value between treatments (P_A=0.4248). TBARS and PV values of D group were significant lower ($P_A \leq 0.05$), and –SH tended to increase (P_A=0.0636) than C group in raw ground beef during refrigerated storage.

Because table 1 could not show the effects of garlic extracts on every storage time, the significant indicators a*, PV, and TBARS values ($P_A \leq 0.05$) influenced by garlic extracts were further analyzed on every storage time. Fig. 1 showed that a* value was significant increased following the addition of garlic extracts on days 3, 7 and 13 during refrigerated storage relative to the control($P < 0.05$). Adding garlic extracts had a tendency to reduce the PV values on days 5 and 13 ($0.05<P < 0.1$), and significantly reduced the PV value on 10 days of storage time as compared to control ($P < 0.05$) (Fig. 2). Fig. 3 showed that a decreasing tendency of TBARS value in E group was found compared to C group at the storage time 3, 7, and 10 days ($0.05<P < 0.1$). The TBARS value was significantly ($P < 0.05$) lower in D group than C group at the end of storage time.

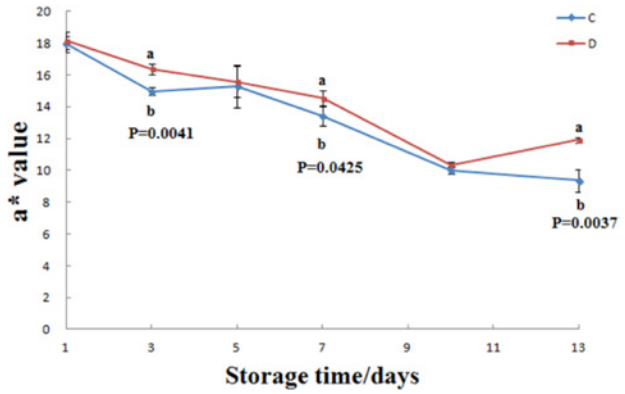


Fig. 1 - Effects of garlic extract on a* value at different storage time of raw ground beef.

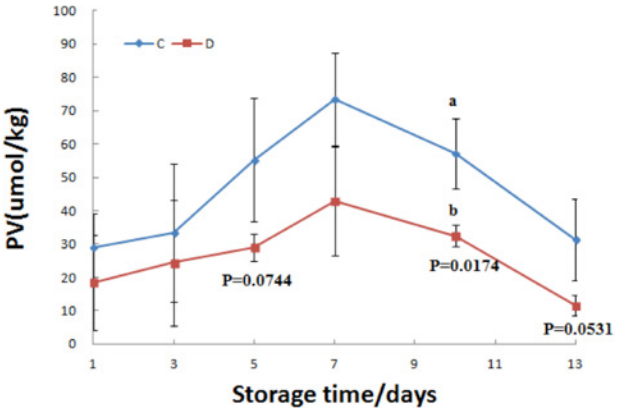


Fig. 2 - Effects of garlic extract on PV at different storage time of raw ground beef.

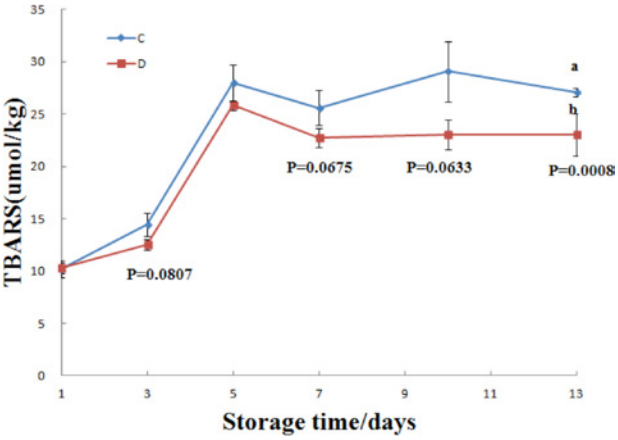


Fig. 3 - Effects of garlic extract on TBARS at different storage time of raw ground beef.

Effects of garlic extract on oxidative breakdown products

PCA score plot of different treatments at the 13 days storage time of raw ground beef was shown in Fig. 4. These results show an easily visible separation of two different groups C and D marked with black and red colors. Fig. 5 showed the loading scores plot from OPLS-DA between C group and D group. Each numbered point represented one breakdown product, which the further away from the center, the more likely that breakdown products between two groups are different.

According to the results from OPLS-DA analysis and original data, the different breakdown products were confirmed between C and D group and shown in Table 2. There were total nine different breakdown products significantly higher in D group than C group ($P < 0.05$) and could been divided into three categories, including phospholipids, fatty acids and polypeptides. PC was belongs to phospholipids. Fatty acids contained malic acid, 16-hydroxy-9E-hexadecenoic acid and 9-hydroxy-10E-octadecen-12-ynoic acid. Polypeptides were made up with Gln-Ile-Asn-Leu, Arg-Pro-Lys-Arg, Met-His-Gln –Asn, Thr-Lys-Lys-Thr, and Gly-Arg-Cys.

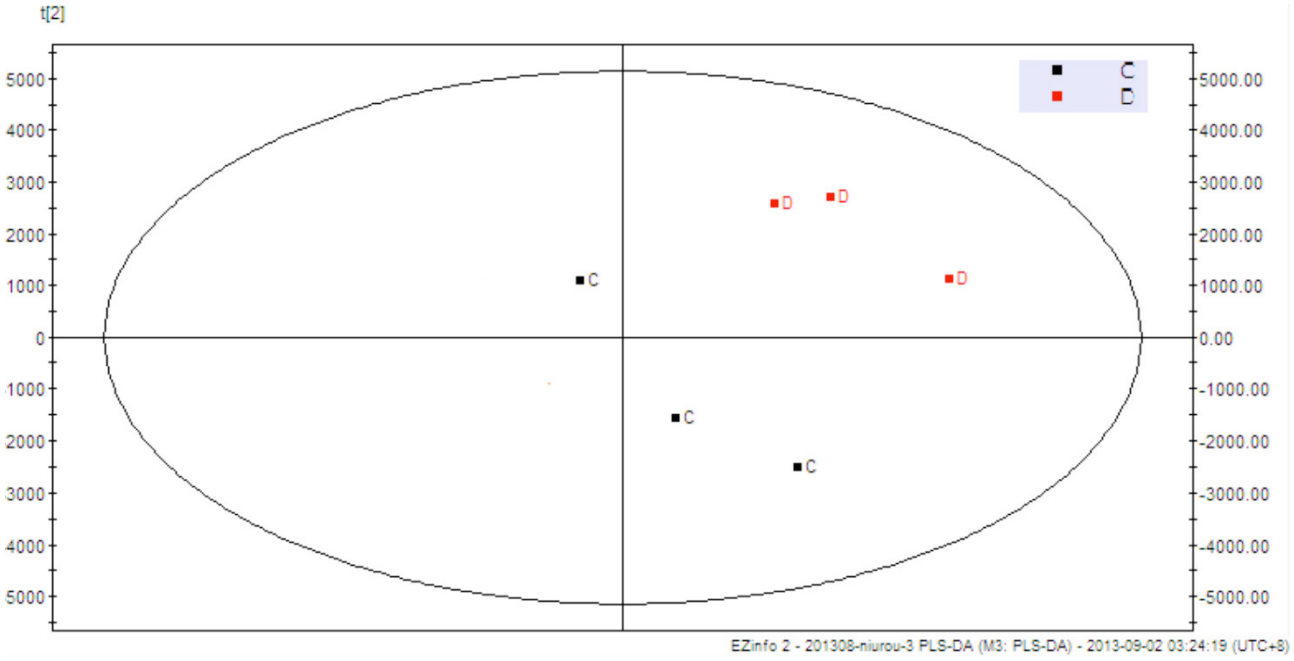


Fig. 4 - PCA score plot of different treatments at the end of storage time in raw ground beef.

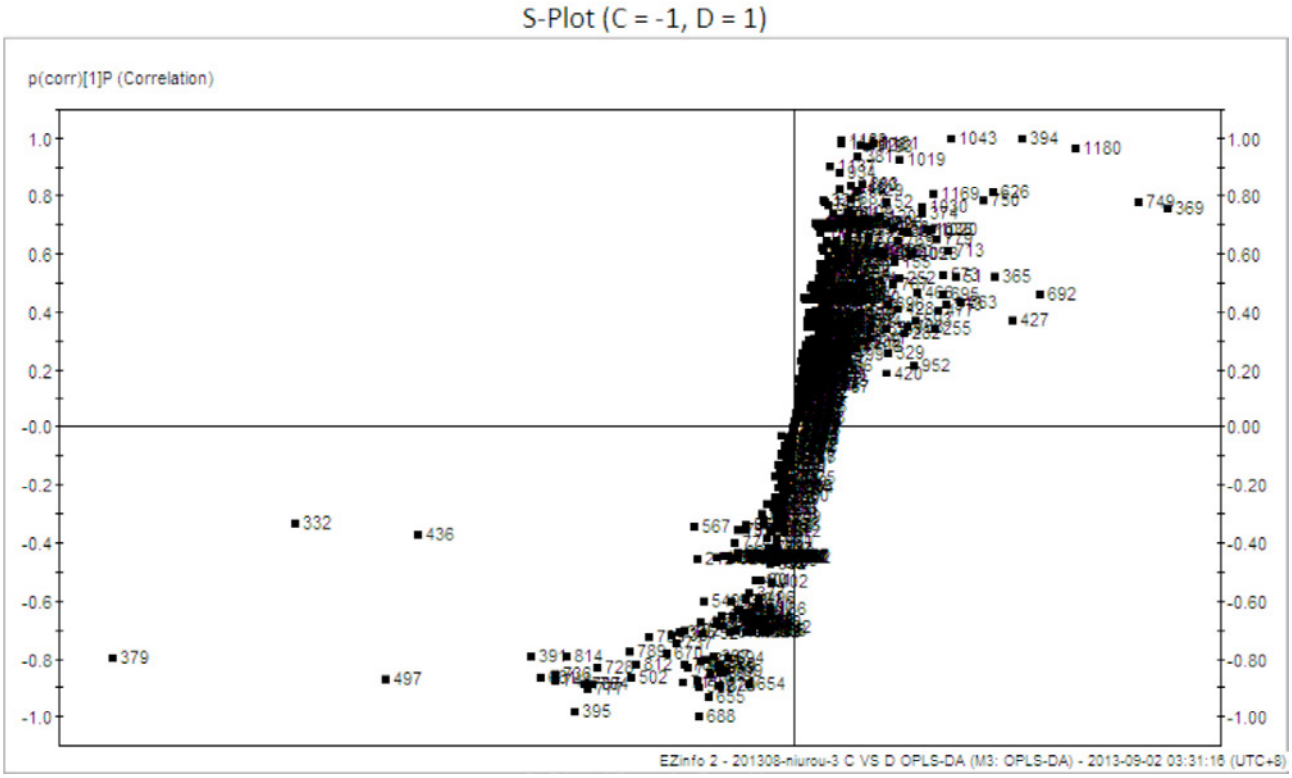


Fig. 5 - Loading scores plot from OPLS-DA between C group and D group at the end of storage time in raw ground beef.

Table 2 - Different breakdown products between C and D treatments at the end of storage time (n=3).

Products	Formula	[M-H] calculated	[M-H] observed	Masserror	*P*	Fold change
PC(15:1(9Z)/0:0)	$C_{23}H_{46}NO_7P$	478.2939	478.2930	1.88	0.0099	+∞
Malic acid	$C_4H_6O_5$	133.0142	133.0142	0	<0.001	+∞
16-hydroxy-9E-hexadecenoic acid	$C_{16}H_{30}O_3$	269.2122	269.2119	1.11	0.0034	10.54
9-hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	293.2122	293.2110	4.09	0.0166	2.35
Gln- Ile-Asn-Leu	$C_{21}H_{38}N_6O_7$	485.2729	485.2773	9.07	0.0047	+∞
Arg-Pro-Lys-Arg	$C_{23}H_{45}N_{11}O_5$	554.3532	554.3462	12.63	0.0094	+∞
Met-His-Gln-Asn	$C_{20}H_{32}N_8O_7S$	527.2042	527.2073	5.88	0.0159	+∞
Thr-Lys-Lys-Thr	$C_{20}H_{40}N_6O_7$	475.2886	475.2886	0	0.0196	+∞
Gly-Arg-Cys	$C_{11}H_{22}N_6O_4S_1$	333.1351	333.1349	0.60	0.0494	+∞

$[M-H]^-$, protonated molecular ion; Mass error expressed as ppm.
Fold change, which was based on the original data, was defined as the fold difference in the observed concentrations between C and D groups. A positive number for a fold change indicates that the value of D group was greater than the C group, whereas the opposite is indicated by a negative number.

DISCUSSIONS

pH and color

The pH of fresh meat is an important indicator to determine the freshness of meat, which can be influenced by the accumulation of organic acids and amines during refrigerated storage. Organic acids, produced by gram positive bacteria and released by the decomposition of lipids oxidative, decrease the pH of meats, whereas the rise of pH was due to the decomposition of alkaline ammonia, which induced by gram negative bacteria (LEFEBVRE *et al.*, 1994). The pH value with a quadratic change over storage time in this study because the result of the interaction of these two effects. The results pH value was inconsistent with CAO *et al.* (2013) found that all the samples gradually increased in stewed-pork during storage at 4°C for 12 days (CAO *et al.*, 2013). The reason of different results was mainly caused by adding ethanol to eliminate the influence of microorganism in the present study.

The color of foods is one of the major determinants of its appeal to consumers and consequently, sales of the product. The color of meat and meat products is influenced by the percentage of red appeared deoxymyoglobin, metmyoglobin and oxymyoglobin in muscle, which would predispose the meat to a faster browning rate (HUNT *et al.*, 1999). The result of decreased a* value over storage time in the present study highly agreed with FERNANDEZ-LOPEZ *et al.* (2005) (FERNANDEZ-LOPEZ *et al.*, 2005) mainly was caused by the formation of metmyoglobin during storage. Several authors have studied the effect of different antioxidants on the color of meat and meat products and have reported that the antioxidants could retard the decrease of a * values during storage (FERNANDEZ-LOPEZ *et al.*, 2005; SANCHEZ-ESCALANTE *et al.*, 2001; KIM *et al.*, 2013). In agreement with these studies, garlic extracts also could retard the decrease of a * values compared to the control group during storage. Garlic extracts could stabilize the redness in raw ground beef during refrigerated storage due to its antioxidant properties preventing the oxidation of oxymyoglobin.

Lipid oxidation

Lipid oxidation is a critical and undesirable phenomenon for meat and meat productions since the undesirable rancid off-flavours and potentially toxic products, leading to the qualitative deterioration. Peroxides are the primary products of lipid oxidation which is generated by oxygen attacking on the double bond in fatty acids. Therefore, peroxide value (PV) is usually as an indicator to clarify the extent of oxidation (MOTTRAM, 1998). Peroxide is very reactive and actually degraded into secondary oxidation products during the storage of lipid-containing foods (JUNTACHOTE *et al.*, 2007). Therefore, PV value increased and thereafter decreased during storage in present study was caused by the peroxide formation and degradation. Similar results of PV fluctuated over storage time were reported by TEETS *et al* (2008) (TEETS and WERE, 2008).

The TBARS value is an index of secondary lipid oxidation and can be used to monitor lipid oxidation in meat samples during storage (QIN *et al.*, 2013). The secondary lipid oxidation products mainly aldehydes (or carbonyls), which contribute to off-flavors in oxidized meat and meat products, result from the degradation of lipid hydroperoxides formed during the oxidation process of polyunsaturated fatty acids(FERNANDEZ *et al.*, 1997). XIE *et al.* (2012) reported that the TBARS value was increased with the storage time at 4°C for 3 days in beef of five cattle breeds (XIE *et al.*, 2012). The results of this study showed that a similar trend, which the TBARS value significant increased in the first five storage days.

CAO *et al* (2013) found that stewed-pork treated with extracts of ginger, onion, garlic had lower PV and TBARS value compared to the control. PARK *et al.* (2010) reported that all extrac-

tion solvents of garlic could reduce the TBARS value in fresh pork patties during refrigerated storage (PARK and CHIN, 2010). SALLAM *et al* (2004) observed that garlic extracts had a marked effect in reducing PV and TBARS values of chicken sausage (SALLAM *et al.*, 2004). In agreement with earlier studies, the same results of decreased PV and TBARS values in the present study indicate that garlic extracts are effective at delaying lipid peroxidation in raw ground beef during refrigerated storage. According to YIN *et al.* (2003), four garlic-derived organosulfur compounds, diallyl sulfide (DAS), diallyl disulfide (DADS), s -ethyl cysteine (SEC), n-acetyl cysteine (NAC), significantly delayed oxymyoglobin and lipid oxidations in ground beef (YIN and CHENG, 2003). OTUNOLA *et al.* (2013) found that garlic extracts have high flavonoids and phenolics contents and high antioxidant activities (OTUNOLA and AFOLAYAN, 2013). Therefore, the ability of garlic extracts to inhibit lipid oxidation is probably related to their antioxidant activity contribute to organosulfur compounds, flavonoids and phenolics compounds.

Free thiol (-SH) was used to stabilize primary oxidation products (SISTA *et al.*, 2000). The increase of -SH value at the first three storage days was probably caused by oxidized glutathione (GSSH) being transformed into glutathione (GSH) under the action of glutathione reductase. On the contrast, transforming hydrogen peroxide into water under the action of glutathion peroxidase results in the decrease of –SH value after three days during storage time. The decrease –SH value in beef over storage time was observed by SULLIVANA *et al.* (2012) (ZAKRYS-WALIWANDER *et al.*, 2012).

GSH is considered an abundant antioxidant within the cell and is essential for regulation of intracellular redox status (IZIGOV *et al.*, 2011). Allicin is formed by alliin enzymatically modified under the action of alliinase in garlic (OKADA *et al.*, 2005). According to the study of KIM *et al.* (1997), Allicin is the main antioxidative component of freshly crushed garlic cloves (KIM *et al.*, 1997). HOREV AZARIA *et al.* (2009) reported that Allicin could directly raising glutathione content in the cell and indirectly increasing glutathione by allicin derivatives such as S-allylmercaptoglu tathione and S-allylmerc aptocysteine (HOREV-AZARIA *et al.*, 2009). Therefore, the existence of Allicin could explain the results that adding gailic extracts increase the –SH value.

Breakdown products

In the present study, garlic extracts could protect the phospholipids, unsaturated fatty acids and polypeptides from oxidation. Phospholipids, which constitute the lipid bilayer defining the outer confines of a cell, are the primary substrates for lipid oxidation and membrane components in close contact with the catalysts of lipid oxidation, which are located in the aqueous phase of the muscle cell (PULFER and MURPHY, 2003). Skeletal muscle was susceptible to oxidative due to the membrane lipid systems that were high in unsaturated fatty acids (CHAN and DECKER, 1994) (CHAN and DECKER, 1994). XIE *et al.* (2012) found that higher unsaturated fatty acids content in Qinchuan cattle lead to more easily lipid oxidation (XIE *et al.*, 2012). In addition, GRUEN *et al.* (2001) revealed that increase of oxidation can enhance protein degradation (GRUNE *et al.*, 2001). Therefore, the results indicate that garlic extracts could help to stabilize the muscle membrane and reduce the degradation of fat and protein due to its antioxidant.

CONCLUSIONS

The results of this study clearly revealed that the effects of garlic extract on color, lipid oxidation and oxidative breakdown products of raw ground beef during refrigerated storage. The results of higher a * value and lower PV, TBARS values indicate that garlic extracts have the antioxidant activity, helping maintain the beef color, inhibiting lipid oxidation. In addition, our experiments provide new and important information regarding the effects of garlic extracts on inhibiting lipid oxidation and protein degradation through protecting phospholipids, unsaturated fatty acids and polypeptides in raw ground beef during refrigerated storage. Overall, garlic extracts could be used as natural antioxidants to maintain the meat quality, extend shelf-life and prevent economic loss.

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PAPER

EFFECTIVENESS OF SANITIZING AGENTS IN INACTIVATING *ESCHERICHIA COLI* (ATCC 25922) IN FOOD CUTTING BOARD SURFACES. REMOVAL *E. COLI* USING DIFFERENT SANITIZERS

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ABSTRACT

The objective of this study was to investigate *Escherichia coli* adhesion on new and used polyethylene cutting board surface and evaluate it's removal using different sanitizer (peracetic acid, chlorhexidine, sodium hypochlorite and organic acids). Results indicated that the number of adherent cells increased with time in both surfaces evaluated. Evaluating the sanitizer action, 0.5% peracetic acid was more effective in removal *E. coli* than chlorhexidine and organic acids at same concentration in both surfaces. Peracetic acid and sodium hypochlorite also showed effectiveness at concentrations of 0.2% and 0.5% on new surfaces, respectively. 0.8% of chlorhexidine and 2.0% of organic acids showed similar effectiveness in the removal *E. coli* on new and used surfaces, respectively. These results suggest that peracetic acid is considerable promise sanitizer for application in surfaces of the food processing industry.

Keywords: removal, peracetic acid, cutting boards, *Escherichia coli*

INTRODUCTION

E. coli is a gram-negative bacteria that present surface layer organizations of the type fimbriae, exopolysaccharides (EPS) or flagella, that favor the adherence to materials or host cell surfaces motility and pathogenicity. Food can become contaminated with *E. coli* when animals are slaughtered or processed, even if precautions are taken and also when it is handled by a person infected with *E. coli*, or from cross-contamination (BEUMER and KUSUMANINGRUM, 2003). Food residues left on food processing or handling equipment may provide a niche of microorganisms that can rapidly grow. The growth of pathogenic bacteria can result in cross-contamination from food processing surfaces such as cutting boards to food products (MONTVILLE *et al.*, 2012).

In the food industry, good manufacturing, hygienic production and regular cleaning and disinfection procedures are very important, since food safety and quality are determined by the efficacy of sanitizer agents (KROLASIK *et al.*, 2010). Bacteria have the ability to adhere to any surface including, but not limited to, glass, stainless steel, polypropylene, rubber and wood (COQUET *et al.*, 2002; TEIXEIRA *et al.*, 2008). To prevent bacterial attachment on surfaces the choosing an appropriate sanitizer is very important for achieving a satisfactory end result in microbiological indexes. Many sanitizers have been broadly used across many industries to reduce pathogenic bacterial contamination in food products or on kitchen utensils, because these compounds have been shown to effectively inactivate foodborne pathogenic bacteria (CABEÇA *et al.*, 2012; FRANK, 2003; ROSSONI and GAYLARDE, 2000). Therefore, more studies into the bactericidal properties of sanitizers at different concentrations and contact times are required to define the correct application.

Many researchers have examined materials employed in manufacturing of foods contact surfaces such as stainless steel (CABEÇA *et al.*, 2012; FRANK, 2003; KROLASIK *et al.*, 2010; ROSSONI and GAYLARDE, 2000; RYU and BEUCHAT, 2005), but few reports bacterial removal on commercial polyethylene cutting boards used in industrial food preparation have been published to date.

The objective of this study was to evaluate the *E. coli* adhesion on new and used cutting board surfaces and removal with different sanitizers used in food industry (peracetic acid, chlorhexidine, sodium hypochlorite and organic acids). For each sanitizer tested, different concentrations were evaluated over 72 h, determining the sanitizer's effectiveness on new and used polyethylene cutting boards.

MATERIAL AND METHODS

Surface material

The food processing surfaces evaluated in this study was new and used polyethylene cutting board, white high-density polyethylene (HDPE plastic). The boards were obtained from cutting room of a slaughter unit, where the used surfaces had around of 45 days of handle. Surfaces materials with 1.0 cm x 1.0 cm plates were cut, cleaned by brushing employing liquid detergent and water, and rinsed with distilled water. They were immersed in 70% ethanol, for 1 h, to fat removal, and again rinsed with distilled water and air dried. The surfaces were exposed to ultraviolet light 254 nm for 1 h to sanitize them, as described by PARIZZI (1999), before deposition of any bacterial cultures.

Adhesion of *Escherichia coli* on food processing surfaces

Bacterial strains were obtained from Seattle, USA, 1946 (American Type Culture Collection; Rockville, MD, USA). For the study of adherence was used an *E. coli* (ATCC 25922) strain, grown previously in Luria Bertani broth - LB (tryptone 10.0 g L^{-1}, yeast extract 5.0 g L^{-1}, NaCl 5.0 g L^{-1}) and incubated at 35°C (±2) for 24 h. *E. coli* was chosen as indicator organism, commonly present in industrial food plants.

The cleaned surfaces were immersed, at 25°C, in Erlenmeyer containing 100 mL of LB supplemented with a suspension of bacterial cells in order to obtain a count of 10^3 CFU mL^{-1}. The sterilized surface, for each time, was immersed in these Erlenmeyer with sterilized forceps and incubated at 35°C in LB broth. The quantities of adhered cells per square centimeter were evaluated for 72 h of contact time (0.1, 1, 3, 6, 12, 24, 48, and 72 h) on new and used surface. The initial time (0 h) corresponds to the analysis performed immediately after the immersion of the surfaces in the Erlenmeyer containing the medium culture and the bacterial suspension. Triplicates were performed for each treatment.

After the incubation, the surfaces were withdrawn from the bacterial suspension *E. coli* and transferred to tubes, containing 10 mL of peptone water 0.1% (p/v) for 1 min, to remove planktonic cells. Subsequently, immersed in tubes containing 5 mL of the same diluent solution and vortex for 1 min, to remove sessile cells (PARIZZI, 1999). The contact areas were swabbed and the adhered microorganisms in the swabs were transferred to tubes, containing 10 mL of peptone water 0.1% (p/v) sterilized at 121°C, for 15 min. The tube was stirred using a vortex for 10 s to release the bacteria from the swab. Next, 1 mL of solution was carefully plated on LB agar, incubated at 35-37°C for 24 h, to colony counting.

Efficiency of different sanitizers against *Escherichia coli* on food processing surfaces

The sanitizers used in this study were chosen to represent those used in the food industry. The following sanitizers were used: peracetic acid 15% (Johnson Diversey, São Paulo-SP, Brazil), chlorohexidine 20% (AD Foods Industry Ltda, Laguna-SC, Brazil), sodium hypochlorite 10% (CSM Chemical Products Ltda, Chapecó-SC, Brazil) and organic acids (formulated with lactic acid-30%, citric acid-3%, ascorbic acid-3%, and salts of fatty acids-7% in water). For each sanitizer, different concentrations (0, 0.2, 0.5, 0.8 and 2.0%) were investigated for 10 min of exposure, to evaluate their efficiency in removal the adhered cells. These agents were diluted in sterilized distilled water according to the supplier's instructions. After this treatment, the surfaces were immersed (separately) in 10 mL of sterilized water, for 1 min and repeated twice to removal the excess of sanitizer.

The counts of bacterial adhesion and inactivation by sanitizers were carried out using swab on cutting boards, evaluated through the standard plate count method. Then, plated on LB agar, incubated at 35-37°C for 24 h to colony counting. All determinations were performed in triplicate and the results expressed in terms of mean values (PARIZZI *et al.*, 2004).

Statistical analysis

Descriptive analyses, including the mean value and variability (standard deviation) and graphic displays were performed. Results obtained in experimental design described previously were performed considering a 95% confidence level ($p<0.05$) by the Tukey's test, using the software Statistica 8.0 (StatSoft Inc®, USA).

RESULTS

E. coli adhesion in food processing surfaces

Fig. 1 show the number of *E. coli* adhered on new and used cutting board surfaces with different contact times. Numbers of *E. coli* were estimated and expressed as $\log_{10}$ colony forming units per cm² (log CFU cm^{-2}). A fast adhesion of *E. coli* on both surfaces studied were observed for up to 12h, becoming constant after 24h on used surfaces, when the maximum population reached (6.92 log CFU cm^{-2}). A significant difference ($p<0.05$) was observed in the intensity of adhesion between the surfaces until 24h.

Effect of different sanitizers for inactivating *Escherichia coli*

Figs. 2, 3, 4 and 5 show the *data's* of inactivation *E. coli* on new and used cutting boards

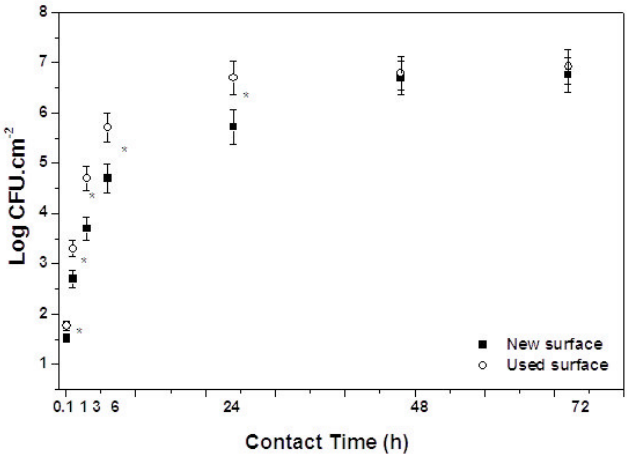


Fig. 1 - Counts of *E. coli* on new and used cutting board surfaces without the presence of sanitizers, over 72 h of contact time. Bars represent the standard errors of the mean from triplicate experiments and * simbolize significant differece ($p<0.05$).

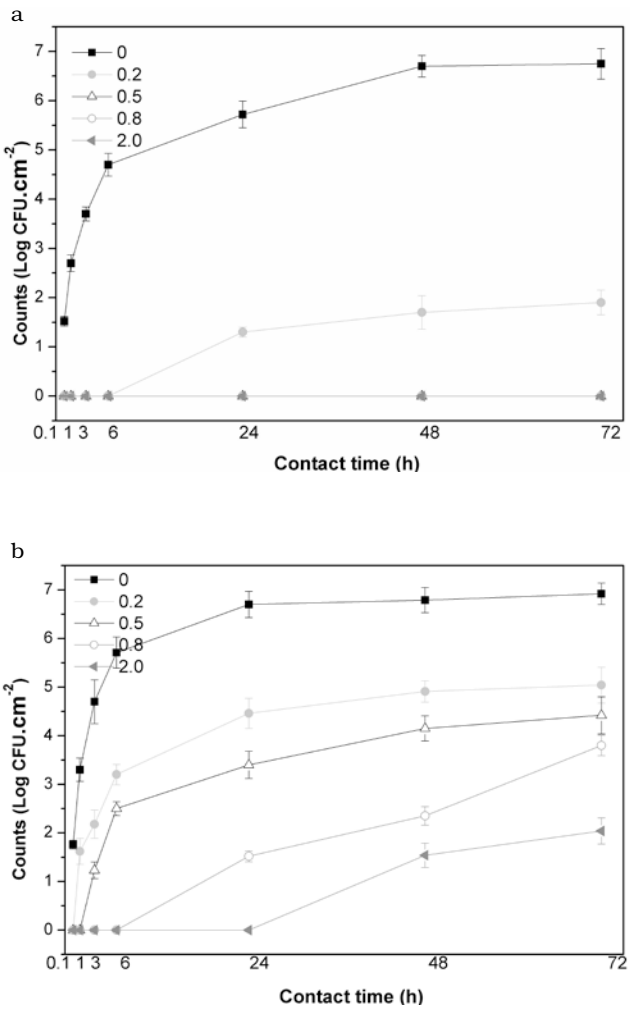


Fig. 2 - The efficacy of different concentrations of peracetic acid (0, 0.2, 0.5, 0.8 and 2.0%) on the reduction of *E. coli* on: (a) new and (b) used cutting board surfaces, over 72 h. Bars represent the standard errors of the mean from triplicate experiments.

with different concentrations (0, 0.2, 0.5, 0.8 and 2.0%) of peracetic acid, chlorhexidine, organic acid and sodium hypochlorite sanitizers, respectively, over 72 h of contact time.

Fig. 2 a and b demonstrates that the bacteria exhibited a significant decrease in the survival rate of viable cells after treatment with peracetic acid. The concentration of 0.5% peracetic acid indicated by the supplier was completely effective for inactivating *E. coli* at all times investigated on new surfaces, while 0.2% peracetic acid was effective for up to 6 h, and able to reduce the number of adhered cells of 4.4 and 5.0 log for 48 and 72 h, respectively (Fig. 2a).

In Fig. 3 a is possible to observe that only the highest concentration of chlorhexidine (2.0%) was completely effective for inactivating *E. coli* on new surfaces, for 72 h. In used surfaces this

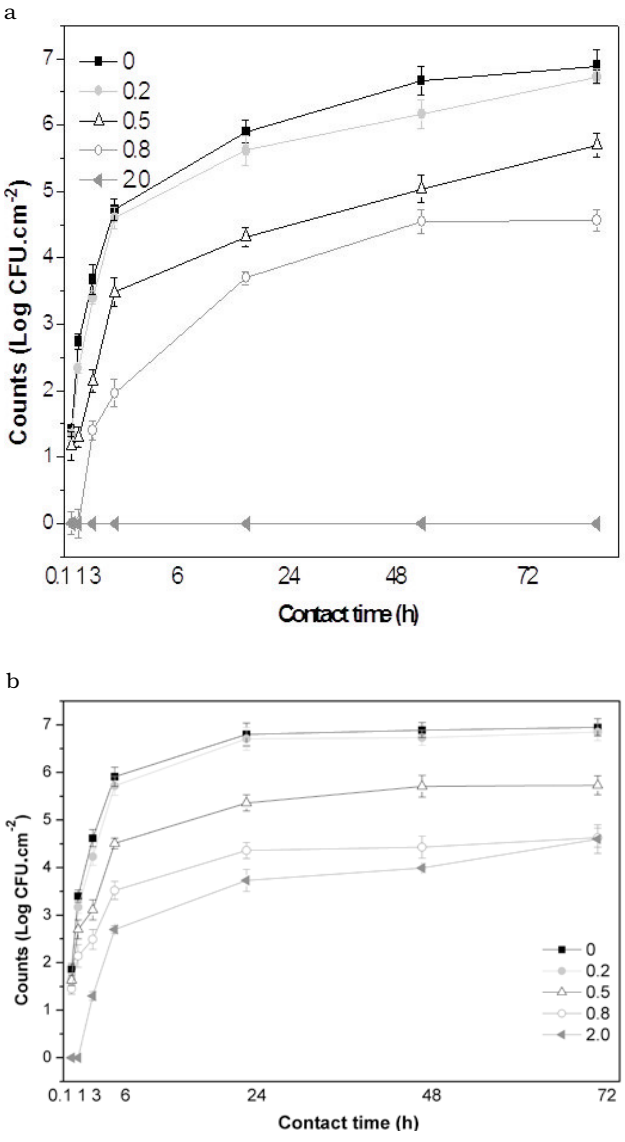


Fig. 3 - The efficacy of different concentrations of chlorhexidine (0, 0.2, 0.5, 0.8 and 2.0%) on the reduction of *E. coli* on: (a) new and (b) used cutting board surfaces, over 72 h. Bars represent the standard errors of the mean from triplicate experiments.

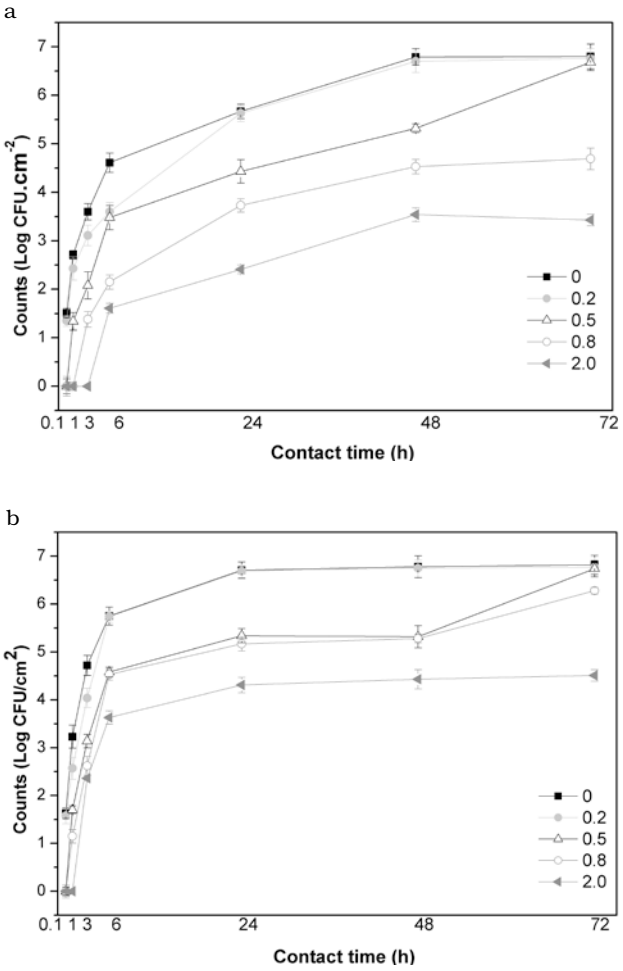


Fig. 4 - The efficacy of different concentrations of organic acid (0, 0.2, 0.5, 0.8 and 2.0%) on the reduction of *E. coli* on: (a) new and (b) used cutting board surfaces, over 72 h. Bars represent the standard errors of the mean from triplicate experiments.

sanitizer (2.0%) was effective until 1h of contact (Fig. 3 b), and reduce around 2.5 log CFU cm^{-2} of cells after 72 h. In this way, chlorhexidine sanitization had a better effect on removal attached cell on new surfaces.

For 0.8% chlorhexidine was observed completely *E. coli* removed on new boards with 1h of contact, but not was effective on the used boards. Consequently, the lower concentrations investigated (0.2 and 0.5%) not show complete inactivation. On used cutting board, all concentrations of sanitizer studied not inactive bacteria after 1 h of contact.

According to the suppliers, also organic acids are suggested in a concentration of 0.5%. In this way, this concentration showed efficiency only for 10 min, on both surfaces evaluated. Higher concentrations, 0.8 and 2.0%, were effective for removing *E. coli* up to 1 and 3 h of contact on new surfaces, respectively (Fig. 4a). The results also indicated that the amount of adherent cells reduced 2.4 log with 2.0% organic acid and was efficient for 1h on used surfaces (Fig. 4b). This

low efficiency of organic acids can be explained by the fact that the compounds are in a dissociated form at the product application moment and dilute the sanitizer, so a higher dissociation leads to lower efficiency (BELTRAME *et al.*, 2012).

Fig. 5 demonstrates the efficiency of sodium hypochlorite against *E. coli.* The concentration (0.5%) indicated by the supplier was able to remove bacteria cells, at all exposure times, on new surface (Fig 5a). On the other hand, to obtain the same effect, on the used surface, a concentration of 2.0% was required (Fig 5b).

Effectiveness correlation between different sanitizers

The sanitation in food surfaces, including cutting boards is critical for the control of microbial contamination of foods and is a significant concern of food preparation and processing industries and public health agencies. In this way, to compare the efficacy of sanitizers (peracetic acid, chlorhexidine, sodium hypochlorite and organic acids) used in the food industries was evaluated a concentration of 0.5%, after 3 h of contact, on new and used surfaces for *E. coli* removal (Fig. 6). The concentration of 0.5% correspond the minimum recommended by the supplier and 3 h of contact is the maximum time (practiced by the food industry) for disinfecting surfaces used.

Comparing the sanitizers, the peracetic acid was completely effective in removing *E. coli* on new and used surfaces ($p<0.05$), as well as for sodium hypochlorite only new surfaces. It was found that chlorhexidine and organic acids exhibit reductions on new and used cutting boards (Fig. 6), without significant difference between the sanitizers ($p>0.05$), but less effectively than other sanitizers evaluated in this work ($p<0.05$).

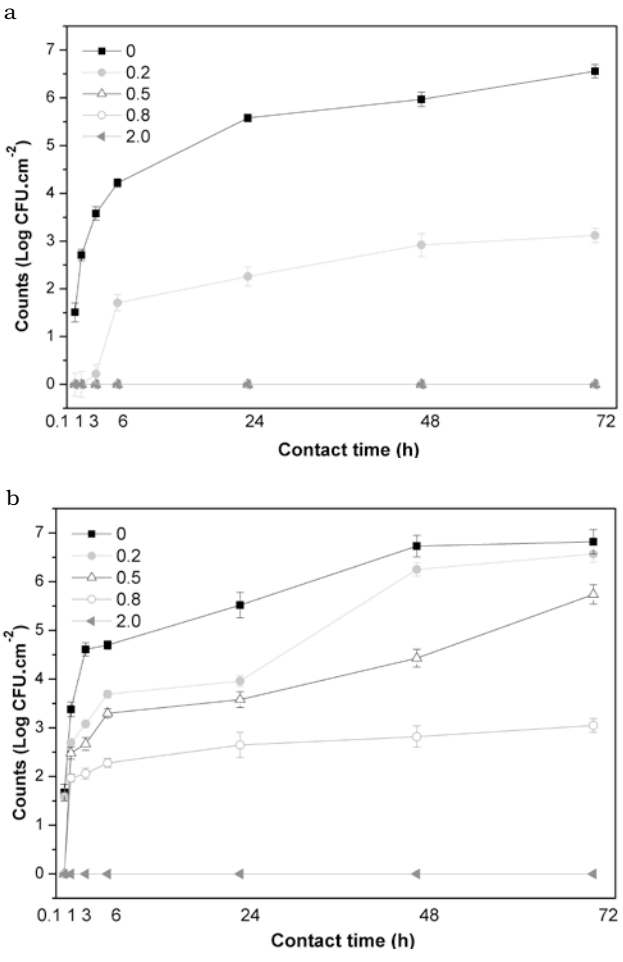


Fig. 5 - The efficacy of different concentrations of sodium hypochlorite (0, 0.2, 0.5, 0.8 and 2.0%) on the reduction of *E. coli* on: (a) new and (b) used cutting board surfaces, over 72 h. Bars represent the standard errors of the mean from triplicate experiments.

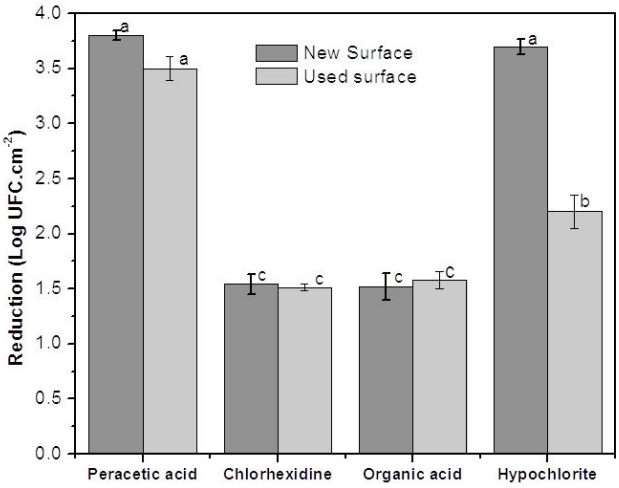


Fig. 6 - The efficacy of different sanitizers (concentration of 0.5%), over 3 h, on the reduction *E. coli* from new and used cutting board surfaces. Means (± standard deviations) followed by the same letters represents no significant difference at 5% level (Tukey's test) between the sanitizers and surfaces.

DISCUSSION

The differences of adhesion on cutting boards could be due microbiological, physical and chemical parameters related to the polyethylene. Particularly, in this study can be verify that the used surfaces have higher counts until 24 h (Fig. 1), possibly due to the surface characteristics, which visually present more cracks and wear by 45 days of use in the slaughter unit. The surface topography has been widely studied, since microorganisms adhere more easily in fissures or cracks, and can resist cleaning and disinfecting procedures (HILBERT *et al.*, 2003; PARIZZI *et al.*, 2004). Thus, macroscopic and microscopic characteristics are crucial for microbial adhesion, reflected in the food contamination by spoilage or pathogenic microorganisms (VADILLO-RODRÍGUEZ *et al.*, 2004). After 48 h the number of adherent cells remained constant over time in both surfaces. This was

also observed in surface reaches saturation level with greater numbers of planktonic cells and not result in greater number of adherent cells (HOOD and ZOTTOLA, 1997).

The results of bacteria removal demonstrate that from 48 h of contact (in used surface), even with concentrations 4 times superior than recommended by suppliers, peracetic acid was not effective. This suggests that the attachment increase during the contact time. Similar results was found by other researcher (ADETUNJI and ISOLA, 2011). MILLER *et al.* (1996) evaluated the potential of water for removal *E. coli* 0157:H7 from polyethylene cutting boards, and the microorganism was incubated for 0 to 30 h, at 37°C, to determine their inhibitory potential. The authors observed an increase in bacteria cells on the boards during the first 30 min of contact, and the water removed 2.3 log CFU cm^{-2} from the surface.

CABEÇA *et al.* (2012) carried out a study of disinfection on stainless steel surfaces, using biguanide and peracetic acid, and verified that they were able to reduce *E. coli* cells adhered of 2.2 and 2.1 log CFU cm^{-2} for 10 min, respectively, with a concentration of 0.5% (w/v). In the present work was possible reduce 3.5 log CFU cm^{-2} after for 3 h, at the same concentration of peracetic acid on new and used polyethylene cutting boards. Peracetic acid disinfectant activity is based on the release of active oxygen. It disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and transports through dislocation or rupture of cell walls. It may also be effective on outer membrane lipoproteins, facilitating action against Gram-negative bacteria. Intracellular peracetic acid can also oxidize essential enzymes. Thus, vital biochemical pathways, transported through the membrane and intracellular solute levels of are damaged, and alterations in the DNA molecule (KITIS, 2004).

In this study, all concentrations of chlorhexidine not were effective for the removal of bacteria after 1 h. This low activity may be due mechanism action, rapid absorption of bacterial cells, resulting in several cytological modifications that affect permeability and optical properties. Studies have shown that chlorhexidine reacts with the cell from lipophobic groups, causing a disorientation of the lipoprotein membrane and generating a change in osmotic barrier function (KUDAVIDANAGE *et al.*, 2009). Chlorhexidine is a cationic molecule with a wide antimicrobial spectrum against both Gram-positive and Gram-negative bacteria (MOHAMMADI and ABBOTT, 2009). This group of biguanides differs from other cationic biocides that interact only superficially with the lipid bilayer altering fluidity through cation displacement and head group bridging (GILBERT and MOORE, 2005). In a study performed by HOUARI and DI MARTINO (2007) the authors verified that chlorhexidine diacetate (Fluka) was able to inhibit the biofilm formation of different bacteria such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* at conventional in-use concentrations.

Second Patel (2005), the bacteria resistance to antimicrobial agents begins at the attachment phase and increase with the biofilm age. Although, bacteria in biofilms are surrounded by an extracellular matrix that might physically restrict the diffusion of antimicrobial agents, this does not seem to be a predominant mechanism of biofilm-associated antimicrobial resistance.

Another indication of high counts are the surface roughness and hydrophobicity that can significantly affect the attachment, formation places for microorganism's accommodation and permanent adhesion. MOVASSAGH *et al.* (2010), showed counts of 7.69 log UFC cm^{-2} for *E. coli* O_{111} on polyethylene surfaces. Second the authors, bacteria encountered in food processing environments can be very hardy and difficult to remove. Bacterial attachment and subsequent survival involved interactions between a bacterial cell, surface and surrounding microenvironment.

The removal bacteria by sodium hypochlorite can be associated with water forms hypochlorous acid, which contains active chlorine (a strong oxidizing agent). Chlorine exerts its antibacterial action by irreversible oxidation of a sulfhydryl group of essential enzymes to microorganisms, disabling metabolic functions of the bacterial cell (POGGIO *et al.*, 2012). Sodium hypochlorite may also have a deleterious effect on the bacterial DNA, involving the formation of chlorinated derivatives of nucleotide bases. Furthermore, it has been reported that sodium hypochlorite can induce disruption of the bacterial membrane (MC DONNEL and RUSSEL, 1999).

Organic acids have an inhibitory action in the undissociated form, from 100 to 600 times greater than the dissociated form. Undissociated organic acid can permeate the cell membrane by diffusion and release protons in the cytoplasm of the cell. The influx of protons induces acidification of the cytoplasm and dissipates the membrane proton potential (KITKO *et al.*, 2009). This inhibits the transport mechanism for the substrate, energy generation and synthesis of macromolecules (STOPFORTH *et al.*, 2003).

CONCLUSIONS

In both surfaces studied it was observed a fast adhesion of *E. coli* and present lower counts in new surface when compared with used. The biofilm formed on used polyethylene cutting boards reduces significantly the action of sanitizers. Among the sanitizers evaluated, peracetic acid was the most efficient for reducing *E. coli* counts.

On the new cutting boards concentration of 0.5% peracetic acid was effective in eliminating *E. coli* adhesion during 72 h evaluated and un-

til 1 h in used surface. Hypochlorite, chlorhexidine and organic acids demonstrated similar effects until 1h, reducing the total adhesion with 0.8 and 2.0% on new and used cutting boards, respectively, although 2.0% sodium hypochlorite has been effective for total removal until 72 h. The order of efficacy in removing *E. coli* was as follows: peracetic acid, sodium hypochlorite, chlorhexidine and organic acids.

The results of the study showed the importance of hygiene procedures on surfaces that come into contact with food. It was found that biofilm formation can occur over a short time, which emphasizes the need for good cleaning procedures during food processing.

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