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CONTENT

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METAL CONCENTRATION AND LEAD-STRONTIUM ISOTOPE CHARACTERIZATION OF ITALIAN RIESLING

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ABSTRACT

Establishing the geographical origin of wines is an issue of major concern for countries around the world, in order to protect quality of wine in case of false statements regarding their geographical origin.The goal of this research was to assess the potential of elemental composition and isotopic signature of lead ($^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$ and $^{204}Pb/^{206}Pb$) and strontium ($^{87}Sr/^{86}Sr$) of wines from three Romanian vineyards, in order to highlight the reliable markers for Italian Riesling geographical origin of wines.In this study 33 white wines obtained from Italian Riesling cultivars were investigated. The wine samples were obtained from micro-wine production under conditions of 2006-2016 from Dealu Bujorului vineyard.The high concentration of K (176.09±3.09-514.03±0.72 mg/L), Mg (82.42±1.20-122.14±2.47 mg/L), Ca (30.39±1.50-77.45±1.97) and Fe were observed in the wine samples analysed. Heavy metals like Hg (16 µg/L), Pb (42.53 µg/L), As (13.02 µg/L) and Cd (0.08 µg/L) were found below acceptable limits.Concentration of Na (1 mg/L), Cu (1 mg/L), As (0.2 mg/L), Cd (0.01 mg/L), Zn (5 mg/L) and Pb (0.15 mg/L) metals in analysed wine samples were under Maximum Permissible Limits (MPL), respectively as published by the Organization of Vine and Wine. The variation of the $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ isotopic ratio of the investigated wine clearly demonstrated that these variables are suitable traces for wine geographical origin determination.

1. Introduction

The demand for high quality and safe food products is increasing all over the world and is promoted the development of strict control laws to certificate the authenticity and geographical origin, and to protect producers and consumers from different frauds. Among food products and alcoholic beverage, wine is one of the most studied and protected with very well-defined criteria for the certification of origin and provenance (Jackson, 2008). A complex characterization of wine chemical composition may provide key specific markers for wine origin differentiation and is the key requirements to obtaining DOC certification (Almeida *et al.*, 2003).

The sources of metals in commercial wines are endogenous and exogenous (Grindlay *et al.*,

2011). The endogenous metals are taken up through the grapevine root system from soil, and enrichment it can be observed during berry formation and grape maturation, affecting the finished wine composition. The amount of endogenous metals are characteristic for the type of soil in the vineyard, the climate conditions during the growing season of the grapes, grapevine varieties, and the stage maturity of the grapes (Pii *et al.*, 2017).

The metals of exogenous source are introduced during growth of grapes by the anthropogenic factors such as environmental pollution (soil contamination and irrigation water) (Fernandez-Calvino, 2008). The use of fungicides and pesticides, fertilisers, grape-growing approaches in viticulture management practices during the growing season of the grapes increases the amounts of metals such as K, Ca, Cd, Cu, Mn, Pb, and Zn in the finished wine (Lara *et al*., 2005).

The steps during the winemaking process often introduce exogenous elements such as Al, Cd, Cr, Cu, Fe, and Zn into the finished wine. Winemaking technology, can affect the final trace element composition of wine by the use of clarifying products such as bentonite, by the use of yeast hulls, by extended contact of wine with different equipment's made from aluminum, brass, glass, stainless steel and wood, and by the storage factors, all with an important role on the safety of wine consumption (Nicolini *et al.*, 2015).

White wine generally has lower concentrations of these metals (except possibly Cd and Pb) than red wines, because of to the white wine-making technology which uses fermentation without skins, and it is know that grape skins contain higher amount of metal than pulp (Orescanin *et al.*, 2003).

Organoleptic characteristics are very influenced by excessive intake of some metals in wines and it can lead to beverage toxicity (Lara *et al.*, 2005). Enrichment with certain metals may lead to detrimental effects on quality and physical properties, such as color, turbidity, precipitation, astringency due to the fact that metal species participate in oxidation-reduction reactions in wines (Catarino *et al.*, 2008).

Studies on red and white wines have highlighted the quantification of metals such as Na, Ca, As, Ni, Cu, Cr, Cd, Pb, Zn, Fe and others in wines from specific countries such as Spain (Núñez *et al.*, 2000), Italy (Nicolini *et al.*, 2015), Argentina (Azcarate *et al.*,2015), Turkey (Alkiș*et al.*, 2014), Romania (Bora *et al.*, 2018), Croatia (Leder *et al.*, 2015), Ukraine (Vystavna *et al.*,2014), Serbia (Kostić *et al.*, 2010) and/or focusing on the study of regional variability on mineral content from wine-producing areas and grape varieties with in a country.

Micronutrients such as Cu, Fe, Mn, Ni, and Zn are extremely useful to the life-cycle of the plants, animals, and also the human beings. These elements, due to some environmental physicochemical conditions can pass from conditions of micronutrients to that of toxic elements and may be considered as contaminating agents for the soil, water and also the air (Larcher *et al.*, 2003). Heavy metals, like Pb, Hg, As, and Cd, it know that they do not have any biological activity and, even at very low amounts, could have harmful effect on plants. Chemical elements (metals) with a concentration greater than 6 kg/dm³ can be considered to be heavy metals.

The chemical parameters used to discriminate the geographical origin and assessment of authenticity of wines are the specific content of organic constituents that vary regionally such as anthocyanins, flavanols and organic acids (Jaitz *et al.*, 2010), the stable isotope ratios of hydrogen, carbon, and oxygen (Buzek *et al.*,2017), and the elemental composition (Bora *et al.*, 2016; Bora *et al.*, 2018; Šelih *et al.*, 2014; Geana *et al.*, 2013) including rare earth elements (Gonzálvez *et al.*, 2009), $^{87}Sr/^{86}Sr$ isotope ratio (Geana *et al.*, 2017; Vinciguerra *et al.*, 2015), $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$ and $^{204}Pb/^{206}Pb$ isotope ratio (Bora *et al*., 2018) or a combination isotope ratio of light element (carbon and oxygen) and elemental analysis (Dutra *et al.*, 2011, Vorster *et al.*, 2010).

Several authors confirmed that $^{87}Sr/^{86}Sr$ ratio can be considered as a viable instrument for traceability and wine fingerprinting for Romanian PDO and GI wines (Geana *et al*., 2016) or Portuguese PDO wines, where soils were formed on distinct geological formations (Catarino *et al.*, 2016). Others author found that a strong relationship exists between the $^{87}Sr/^{86}Sr$ ratios of the wine and the grape, pointing out that the vinification technology does not alter the strontium reserve (Vinciguerra *et al.*, 2015).

Some studies prove that the Sr isotopes are a strong geochemical instrument for tracing the geographic authenticity and provenance of some wines. A wide variations of $^{87}Sr/^{86}Sr$ ratio was observed between wines from the different geographical regions, strengthen the link with the soil beneath the surface of the ground of the production territory (Marchionni *et al*., 2016).

Lead is found in the Earth's crust and it was reported to be emitted from anthropogenic activities such as burning process of fossil fuels, mining practices, paint industry, different batteries production, etc. (Izah *et al.*, 2017). The content of lead in wine and the amount in it may be explained by the natural sources as well as those related to the production processes during grape growing season and vinification technology (Pyrzynska, 2007; Stockley *et al.*, 2003).

The same as the $^{87}Sr/^{86}Sr$ isotope ratio, the variation of $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ isotope ratio, represent a powerful geological marker for wines geographical traceability and authentication of wine (Bora *et al.*, 2018).

Isotope ratio $^{206}Pb/^{207}Pb$ and $^{206}Pb/^{208}Pb$ are frequently used as tracers to make the difference between the source of lead, natural or anthropogenic. In Central Europe, the lead isotopic signatures of contamination sources recorded variation from relatively high $^{206}Pb/^{207}Pb$ ratios (natural Pb, charcoals, ashes-$^{206}Pb/^{207}Pb$=1.17-1.22) to low $^{206}Pb/^{207}Pb$ values (gasoline, petrol burning $^{206}Pb/^{207}Pb$=1.06-1.14) (Mihaljevic *et al*., 2006).

The aim of the present research was to determine the elemental composition and $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$ isotopic ration from Italian Riesling variety cultivated in Dealu Bujorului vineyard, in order to highlight the reliable markers for Italian Riesling geographical origin of wines.

2. Materials and methods

2.1.Study area

The Dealu Bujorului vineyard is located at 45°52′10″ N, 27°55′8″E, in the Galati County, Romania, at a distance of 56 km from Bârlad city and 55 km from Galati city. Although the region has a long tradition in culture of grapevine, Dealu Bujorului vineyard was developed with the establishment of Research and Development Station for Viticulture and Enology Bujoru (RDSVV Bujoru), from Târgu Bujoru city. In Dealu Bujorului vineyard predominant soil is levigated chernozem having a clayey sand texture with pH between 6.4 and 8.1. Although moisture deficit, natural conditions (ecoclimatic and ecopedological) offer viable ecosystem for the development of vineyard (Bora *et al.*, 2018).

The study area is spread on 6 ha and all the vines are planted at 2 m between rows and 1.2 m between vines. Grapevines were pruned according to the Guyot system and were grown on espelier.

2.2.Sample and microvinification process

The samples used in this experiment were obtained from the wines produced from Italian Riesling under the conditions of 2006-2016 years, from Dealu Bujorului. The wine samples resulted from micro-wine production. Micro-vine production was done according to the methodology described in (Bora *et al*., 2016). All wines were provided by the wineries as finished wines in 750 mL glass bottles with cork stoppers and were stored at 3-4 ^{0}C before analysis. One bottle was used for each sample, and three replicates were taken. All vines were planted since 1979, and the vine plantation was organized with 2.2x1 m distance between rows

and plants. Vines were pruned according to the Guyot system and were grown on speliers.

2.3.Reagents and solutions

Thirty elements (Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, V, U, Zn and Hg) were determined in order to assess their ability to discriminate wines by geographical origin. The analysis was made using multielement analysis and ICP-MS technique, after an appropriate dilution, using external standard calibration method. Each sample was analyzed in duplicate and each analysis was prepared from consisted of seven replicates. The calibration was performed using XXICertiPUR multielement standard, and from individual standard solution of Hg.

The working standards and the control sample were prepared daily from the intermediate standards that were prepared from the stock solution. The intermediate solutions stored in polyethylene bottles and glassware was cleaned by soaking in 10% v/v HNO_3 for 24 hours and rinsing at least ten times with ultrapure water. The accuracy of the methods was evaluated by replicate analyses of fortified samples (10 µL-10 mL concentrations) and the obtained values ranged between 0.8-13.1 percent, depending on the element. The global recovery for each element was estimated and the obtained values were between 84.6-100.9% (Geana *et al*., 2016).

For quality control purpose, blanks and triplicates samples (n=3) we analyzed during the procedure. The variation coefficient was under 5% and detection limits (ppb) were determined by the calibration curve method. Limit of detection (LoD) and Limit of quantification (LoQ) limits were calculated according to the next mathematical formulas: LoD=3SD/s and LoQ=10 SD/s (SD=estimation of the standard deviation of the regression line; s=slope of the calibration curve) (Table 1).

To verify the achieved accuracy and precision, ten NIST-SRM 987 and NIST-SRM 982 analysis results were pooled together with the calculated relative standard deviation presented in Table 2. Based on the obtained results, it was verified that, applying quadrupole ICP-MS, relative standard deviation and reproducibility of approximately 0.5% for $^{87}Sr/^{86}Sr$, $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ are feasible. The results were in agreement with those reported by (Ketterer *et al*., 1991; Barbaste *et al*., 2001; Geana *et al*., 2016; Almeida *et al*., 2016).

Table 1. Instrumental conditions for the determination of each element (ICP-MS)

Element	Correlation coefficient	LoD* (µg/L)	LoQ*** (µg/L)	BEC** (µg/L)	Element	Correlation coefficient	LoD* (µg/L)	LoQ*** (µg/L)	BEC** (µg/L)
Ag	0.9999	0.0450	0.1499	0.006	K	0.9999	2.1860	7.2794	31.728
Al	0.9999	0.0908	0.3024	5.282	Li	0.9999	0.0048	0.0160	0.020
As	0.9999	0.2335	0.7776	0.538	Mg	0.9999	2.7325	9.0992	9.099
Ba	0.9999	1.1075	3.6880	2.159	Mn	0.9999	0.0102	0.0340	0.085
Be	0.9999	0.0009	0.0030	0.002	Na	0.9999	3.9808	13.2561	32.121
Bi	0.9999	0.0067	0.0223	0.003	Ni	0.9999	0.0591	0.1968	0.091
Ca	0.9999	5.6649	18.8641	20.820	Pb	0.9999	0.0003	0.0010	0.002
Cd	0.9999	0.0202	0.0673	0.027	Rb	0.9999	0.0025	0.0083	0.008
Co	0.9999	0.0365	0.1215	0.152	Se	0.9999	0.5012	1.6690	0.920
Cr	0.9999	1.6630	5.5378	0.636	Sr	0.9999	0.1434	0.4775	0.955
Cs	0.9999	0.0001	0.0003	0.001	Tl	0.9999	0.0020	0.0067	0.002
Cu	0.9999	0.0402	0.1339	0.237	V	0.9999	1.2140	4.0426	4.263
Fe	0.9999	5.2102	17.3500	71.399	U	0.9999	0.0253	0.0842	0.005
Ga	0.9999	0.0105	0.0350	0.042	Zn	0.9999	0.3780	1.2587	5.401
In	0.9999	0.0030	0.0100	0.009	Hg	0.9999	0.0417	0.1379	0.128

*Detection limit; **Background equivalent concentration; ***Quantification limit.

Table 2. Strontium isotopic ration and Lead isotopic ration determination precision and accuracy based on the NIST SRM 987 (Strontium) and NIST SRM (Lead) 982 (n = 10)

Replicate	$^{87}Sr/^{86}Sr$ ([a])	RSD (%)	$^{207}Pb/^{206}Pb$ ([b])	RSD (%)	$^{208}Pb/^{206}Pb$ ([c])	RSD (%)	$^{204}Pb/^{206}Pb$ ([d])	RSD (%)
1	0.70493	0.31	0.46483	0.51	0.99891	0.67	0.00271	0.32
2	0.72046	0.45	0.47891	0.48	0.99452	0.61	0.00272	0.41
3	0.70325	0.63	0.46978	0.32	0.99794	0.55	0.00275	0.28
4	0.70634	0.48	0.47123	0.64	0.99688	0.64	0.00273	0.51
5	0.71478	0.36	0.46987	0.56	0.99726	0.48	0.00246	0.14
6	0.71245	0.59	0.46154	0.37	0.99647	0.56	0.00258	0.39
7	0.70987	0.46	0.47362	0.70	0.99969	0.34	0.00279	0.47
8	0.72326	0.42	0.45641	0.43	0.99744	0.58	0.00278	0.51
9	0.70845	0.68	0.41562	0.36	0.99576	0.59	0.00273	0.49
10	0.10789	0.47	0.45612	0.45	0.99874	0.61	0.00278	0.36
Average	0.71117	0.49	0.46179	0.48	0.99736	0.56	0.00270	0.41

2.4.Sample preparation for determination of metals from wine using ICP-MS.

For the determination of metals from wine samples were used an amount of 0.2 mL wine and adjust 8 mL (7 mL HNO_3 69%+1 mL H_2O_2), after 15-30 minutes the mineralization was performed using a microwave system Milestone START D Microwave Digestion System set in three steps: step I (time 10 min., temperature 200 ^{0}C), step II (time 15 min., temperature 200 ^{0}C) and step III (time 60 min., ventilation - temperature 35 ^{0}C). After mineralization, samples were filtered through a 0.45 mm filter and brought to a volume of 50 mL.

2.5.Instrumentation

The determination of metals was performed on mass spectrometer with inductively coupled plasma, (ICP-MS) iCAP Q Thermos scientific model, based polyatomic species before they reach the quadrupole mass spectrometer, using a PFA micro flow concentric nebulizer. The argon used was of 99.99% purity (Messer, Austria). The instrument was daily optimized to give maximum sensitivity for M^+ ions and the double ionization and oxides monitored by the means of the rations between Ba^{2+}/Ba^+ and Ce^{2+}/CeO^+, respectively, these always being less than 2%. The experimental conditions were: argon flow on nebulizer (0.84 L/min.), auxiliary gas flow 0.80 L/min., argon flow in plasma 15 L/min., lens voltage 7.31 V; RF power in plasma 1100 W, spray chamber temperature (2.51±1.00 ^{o}C).

Accuracy was calculated for the elements taken into consideration (0.5-5.0%).

2.6.Statistical analysis

The statistical interpretation of the results was performed using the Duncan test, SPSS Version 24 (SPSS Inc., Chicago, IL., USA). The statistical processing of the results was primarily performed in order to calculate the following statistical parameters: average and standard deviation. This data was interpreted with the analysis of variance (ANOVA) and the average separation was performed with the DUNCAN test at $p \leq 0.05$. Linear discriminant analysis (LDA) was performed in order to separate the wines by region and to identify the markers with a significant discrimination value (variables with Wilk's lambda near zero, p values <0.05 and higher F coefficients). Linear discriminant analysis (LDA) was performed using Microsoft Excel 2016 and XLSTAT Addinsoft version 15.5.03.3707.

3.Results and discussions

3.1.Physico-chemical analyzes of the Italian Riesling variety from Dealu Bujorului vineyard

The grape harvest was done late in the autumn, so the grapes were raisins with a reddish-yellow color depending on the degree of damage to the noble rot produced by *Botryotinia fuckeliana*. The fermentation process was slowly, lasted about one month, at 20 ^{0}C to ensure the reducing sugars concentration to establish for this type of wine. Total acidity was between 4.90 g/L $C_4H_6H_6$ (Italian Riesling from 2013) and 7.40 (Italian Riesling from 2006), this type of wine falls into the category of sweet reducing wines with a smell of berries with a taste of fried bread and floral shades with alcohol content higher then 12 (% vol. alcohol). The non-reducing extract was between 30.00 (g/L) (Italian Riesling from 2008) to 36 (g/L) (Italian Riesling from 2011) (Table 3).

Table 3. Qualitative characteristics of Italian Riesling from Dealu Bujorului Vineyard

Area	Variety	Years	Alcohol (% vol.)	Total Acidity (g/L $C_4H_6H_6$)	Volatile Acidity (g/L CH_3COOH)	Non-reducing extract (g/L)	Residual sugar (g/L)	Free SO_2 (g/L)	Total SO_2 (g/L)	Organoleptic Appreciation
Dealu Bujorului Vineyard	Italian Riesling	2006	14.00	7.40	1.50	33.00	88.40	22.00	93.61	91
		2007	13.54	6.38	0.89	31.20	65.04	31.02	114.45	95
		2008	14.00	6.50	0.66	30.00	46.60	24.00	132.21	94
		2009	12.00	7.10	0.63	33.00	67.90	21.00	136.94	96
		2010	13.70	5.50	0.72	30.00	36.80	25.00	146.00	94
		2011	12.10	6.70	0.84	36.00	36.80	29.00	218.47	91
		2012	13.12	5.78	0.87	35.00	45.12	31.02	158.47	95
		2013	13.00	4.90	0.79	34.00	77.20	25.12	145.23	91
		2014	13.40	5.40	0.57	34.00	40.70	21.06	210.23	96
		2015	14.02	6.21	0.99	33.56	55.02	23.20	168.45	95
		2016	14.06	6.54	0.36	31.40	54.05	21.45	178.56	91

3.2.Italian Riesling Mineral Content

In general, the elements in wine are present due to their extraction during the maceration period from grapes (Ivanova-Petropulos *et al.*,2013). These elements are present in grapes as results of their accumulation in the vine through the root from soil, or they could originate from the agents used in protecting the vine from diseases. During the maceration period, extracted elements are absorbed at the cell membrane of yeast, and afterward, their declines as a result of precipitation together with the yeast cell or precipitation in complexation reactions. By contact of wine with equipment, the addition of fining agents, or the changing of filters, during post-fermentation processes could increase the content of elements (Ivanova-Petropulos *et al.*,2013).

As we expected potassium was the most abundant element in all investigated white wine samples since this element is essential for the growth and the development of vine is a major component of fertilizes (Rodrigues *et al.*, 2011). The measured values ranged between 198.21±3.67 mg/L to 514.03±0.72 mg/L, these results agree with values reported in the literature (Iglesias *et al.*, 2007 average values of 819.61 mg/L; Álvarez *et al.*, 2012 average values of 865.30 mg/L). Magnesium and calcium were the second abundant element in this study. The magnesium concentration was higher in Italian Riesling from 2009 (122.14±2.47 mg/L) than in Italian Riesling from 2011 (82.42±1.20 mg/L), a decrease in

Mg concentration can be observed with the passage of time. While the Ca concentration ranged between 47.62±1.00 mg/L to 77.45±1.97 mg/L with an average value of 52.61 mg/L. The values obtained for Mg and Ca concentration in our selected wines were in good agreement with the results for Croatian wines (Vrček *et al.*, 2011 average values of 65.90 mg/L Ca and 98.20 mg/L Mg), Czech wines (Kment *et al.*, 2005) average values of 108.00 mg/L Ca and 75.40 mg/L Mg) and also Serbian wines (Ražić and Onjia, 2010 average values of 37 mg/L Ca and 95.73 mg/L Mg). On the other hand, our Ca and Mg contents were significantly higher than published data for Argentina wines (Lara *et al.*, 2005 average values of 12.50 mg/L) and also Belgium wines (Coetzee *et al.*, 2014 average values of 6.73 mg/L and 12.05 mg/L Mg) (Table 4).

The same as Mg concentration, the average content of Na differed among the year of wine production, the highest concentration of Na was recorder in Italian Riesling from 2015 (49.81±1.34 mg/L), with an average of 40.68 mg/L Na/L wine. These results are in agree with Ražić and Onjia, 2010 average values of 29.65 mg/L Na, Czech wines (Kment *et al.*, 2005) average values of 14.7 mg/L Na and Spanish (Iglesias *et al.*, 2007 average value of 37.19 mg/L Na) wines (Table 4).

The average values obtained for Li (10.07 mg/L), Cu (0.61 mg/L), Fe (2.47 mg/L), Mn (0.42 mg/L), Co (4.01 mg/L) and V (179.83 µg/L) were also present in amounts similar to previously published results (Pohl, 2007; Fabani *et al*., 2010; Di Paola-Naranjo *et al*., 2011; Ivanova-Petropulos *et al*., 2013; Avram *et al*., 2014; Catarino *et al*., 2014; Geana *et al*., 2017). These values obtained are similar with Macedonian wines (Ivanova-Petropulos *et al*., 2013), Spanish wines (Iglesias *et al*., 2007) and Czech (Kment *et al*., 2005) wines and have more vanadium than Belgian (Coetzee *et al*., 2014) ones. Regarding concentration of Li, Cu and Mn the highest concentration were obtained in Italian Riesling from 2010 (13.59±0.50 mg/L Li), Italian Riesling from 2009 (3.12±0.10 mg Cu/L) and Italian Riesling from 2011 (0.73±0.02 mg/L Mn). The highest concentration of Fe and Co were obtained at Italian Riesling from 2009 (3.12±0.10 mg/L Fe), Italian Riesling from 2010 (3.19±0.07 mg Fe/L), and in case of Co the highest concentration was registered at Italian Riesling from 2013 (6.81±0.58 mg/L Co) and 2014 (6.22±1.49 mg/L Co).

The content of trace elements (In, Sr, Ni, Rb, Se, Tl, U, Zn, Ag, Al, Be, Bi, Ba, Cr, Cs and Ga) found in Italian Riesling cultivated in Romanian are agreed with literature data (Kment *et al*., 2005; Filk et *et al*., 2011; Ivanova-Petropulos *et al*., 2013; Geana *et al*., 2013). Regarding Tl, Zn, Ba the highest concentration were at Italian Riesling from 2007, Se (Italian Riesling from 2008), Ni (Italian Riesling from 2009), U, Ag, Al, Be (Italian Riesling from 2010) and Sr, Bi (Italian Riesling from 2013). The results indicated that Italian Riesling cultivated in Romania are moderately rich in In, Sr, Ni, Rb, Se, Tl, U, Zn, Ag, Al, Be, Bi, Ba, Cr, Cs and Ga while white wines are moderately rich In, Sr, Zn, Al, Be, Bi and Ba.

Regarding the content of heavy metals (Hg, Pb, As and Cd) found in Italian Riesling cultivated in Romania, the results obtained are agreed with literature data (Avram *et al*., 2014; Đurđić *et al*., 2017). The As content was higher than in published data (Kment *et al*., 2005; Lara *et al*., 2005; Iglesias *et al*., 2007; Filk et *et al*., 2011; Alkiș *et al*., 2014), while the Pb content was lower than that in Czech (Kment *et al*., 2005) and Romanian wines (Geana *et al*., 2013) (Table 5).

Concentration of Na (1 mg/L), Cu (1 mg/L), As (0.2 mg/L), Cd (0.01 mg/L), Zn (5 mg/L) and Pb (0.15 mg/L) metals in

analysed wine samples were under Maximum Permissible Limits (MPL).

3.3. $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio from wine samples

Different scientific papers demonstrated that the lead and strontium isotopic values found in wines can be associated with those of vineyard soil (Voster *et al*., 2010; Di Paola-Naranjo *et al*., 2011; Almeida *et al*., 2016; Marchionni *et al*., 2016; Kristensen *et al*., 2016; Geana *et al*., 2017) and from this premise, we performed Sr isotope analyses of 90 bottled wines from there different geographical areas part of Romania in order to verify the possibility of using $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio in wine as reliable trace for geographical provenance.

Lead isotopic analysis of wines from Bordeaux (France) showed that lead in the wines changed over time to reflect the dominant source of atmospheric lead pollution in southern of France (Médina *et al*., 2000). Other studies have found that lead isotopic compositions in wine may not always reflect those of leaded petrol, but reflect the isotopic signature of local, dominant metallurgical industries (Larcher *et al*., 2003; Mihaljevič *et al*., 2006). These researches confirm atmospheric deposition as being the dominant contributor to the lead content and isotopic composition from wine. In Central Europe, Pb isotopic ratio, as signatures of pollution sources, ranges from relatively high $^{206}Pb/^{207}Pb$ ratios (natural Pb, coals, fly ashes, $^{206}Pb/^{207}Pb$=1.1700-1.2200) to low $^{206}Pb/^{207}Pb$ values (gasoline, petrol combustion, $^{206}Pb/^{207}Pb$=1.0600-1.1400) (Mihaljevič *et al*., 2006; Avram *et al*., 2014) (Table 6).

Regarding $^{206}Pb/^{207}Pb$ isotope ratios based on analyses it can be concluded that the vines grown in Dealu Bujorului (1.2686±0.0035 [0.2705])were affected by pollution produced by cars (automobile emissions) (if $^{206}Pb/^{207}Pb$=1.1000-1.1400 [automobile emissions]). The approximation of the Italian Riesling plantation over the intensely circulated roads could also be a possible explanation for these values. The values of $^{206}Pb/^{207}Pb$ isotope ratio are between the ranges from 1.1028 to 1.1781, values comparable with Avram *et al.*, 2014 (1.1100 to 1.2000 Romanian wines); Almeida *et al.*, 2016 (1.1440 to 1.1820 Brazilian wines).The abundance of the lead isotopes ^{204}Pb (non-radiogenic), ^{206}Pb, ^{207}Pb and ^{208}Pb (radiogenic) originated from the genesis of the substrate varies with geological ages.

The original composition of the rock upon its formation and consequently, with geographical areas (Shirahata*et al.*, 1980; Gulson*et al.*, 1981; Elbaz-Poulichet*et al.*, 1984), this property is useful in order to identify of the source of lead in a subjected wine sample provided that the measurements of the isotope ratio is precise and accurate.

The values of $^{208}Pb/^{206}Pb$ and $^{204}Pb/^{206}Pb$ isotope ratio are between the ranges from 2.1243 to 2.2185 ($^{208}Pb/^{206}Pb$) and 16.4968 to 16.6470 ($^{204}Pb/^{206}Pb$). The highest values of $^{208}Pb/^{206}Pb$ were registered in wine Italian Riesling wine from 2013 (2.2185±0.0067 [0.3134]) followed by the Italian Riesling from 2016 (2.2149±0.0037 [0.1762]). Regarding $^{204}Pb/^{206}Pb$ isotope ratio the highest values were registered to Italian Riesling from 2006 (16.6470±0.0130 [0.0781]) and Italian Riesling from 2011 (16.6385±0.0222 [0.1332]) while the Italian Riesling from 2010 recorded the lowest isotope ratio (16.4968±0.0169 [0.1022]).

Table 4. Total metal concentration of Italian Riesling

Area	Variety	Years	Total metal concentration									
			Ca (mg/L)	**Mg (mg/L)**	**K (mg/L)**	**Na (mg/L)**	**Li (mg/L)**	**Cu (mg/L)**	**Fe (mg/L)**	**Mn (mg/L)**	**Co(µg/L)**	**V(µg/L)**
			M.A.L	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**
			-	-	-	60 mg/L	-	1 mg/L	-	-	-	-
Dealu Bujorului Vineyard	Italian Riesling	**2006**	49.06±4.52^{d}	115.07±2.67^{b}	353.48±5.41^{c}	37.19±2.24^{c}	11.06±0.63^{c}	0.67±0.09^{abc}	2.27±0.21^{b}	0.60±0.21^{ab}	1.61±0.61^{e}	138.89±6.51^{e}
		2007	46.62±2.62^{d}	101.13±1.30^{e}	420.70±1.97^{b}	40.91±1.41^{cd}	9.58±0.57^{de}	0.80±0.05^{a}	1.56±0.28^{c}	0.52±0.08^{bc}	2.04±0.72^{de}	123.41±8.88^{f}
		2008	49.13±1.91^{d}	106.68±2.30^{d}	413.84±1.81^{b}	42.82±2.12^{b}	10.05±0.25^{d}	0.61±0.04^{b}	2.43±0.27^{b}	0.24±0.07^{de}	2.64±0.53^{de}	210.35±5.10^{b}
		2009	68.26±1.21^{b}	122.14±2.47^{a}	286.11±4.84^{d}	52.51±1.50^{a}	12.49±0.67^{b}	0.78±0.04^{ab}	3.12±0.10^{a}	0.40±0.05^{cd}	5.08±0.57^{b}	229.25±3.87^{a}
		2010	56.86±2.04^{c}	104.35±1.72^{de}	355.14±5.70^{c}	33.12±2.21^{d}	13.59±0.50^{a}	0.57±0.05^{b}	3.19±0.07^{a}	0.40±0.08^{cd}	4.78±0.30^{bc}	156.16±9.44^{d}
		2011	77.45±1.97^{a}	82.42±1.20^{g}	176.09±3.09^{i}	29.68±1.55^{d}	8.78±0.78^{efg}	0.63±0.08^{bc}	2.31±0.30^{b}	0.73±0.02^{a}	2.19±1.67^{de}	213.55±7.17^{b}
		2012	47.62±1.00^{d}	103.25±0.90^{de}	235.29±3.80^{f}	38.16±2.85^{c}	8.09±0.06^{g}	0.36±0.05^{d}	2.34±0.30^{b}	0.33±0.07^{de}	4.78±0.31^{bc}	176.19±8.12^{c}
		2013	57.23±0.53^{c}	94.46±2.56^{f}	514.03±0.72^{a}	42.92±2.01^{b}	9.49±0.64^{de}	0.61±0.10^{b}	1.22±0.15^{c}	0.23±0.02^{e}	6.81±0.58^{a}	176.76±6.97^{c}
		2014	30.39±1.50^{f}	113.40±3.17^{bc}	332.54±8.11^{d}	40.56±4.99^{cd}	10.03±0.34^{d}	0.61±0.20^{b}	2.09±0.44^{b}	0.57±0.03^{b}	6.22±1.49^{ab}	207.28±5.17^{b}
		2015	40.61±1.82^{e}	110.48±1.44^{c}	188.55±3.06^{h}	49.81±1.34^{a}	8.30±0.84^{fg}	0.35±0.06^{d}	3.41±0.23^{a}	0.32±0.09^{de}	4.73±0.71^{bc}	177.47±4.16^{c}
		2016	55.46±0.94^{c}	101.01±1.35^{e}	198.21±3.67^{g}	39.79±1.41^{cd}	9.32±0.58^{def}	0.68±0.05^{abc}	3.23±0.09^{a}	0.31±0.03^{de}	3.27±0.84^{cd}	168.80±5.67^{c}
Average			52.61	104.94	198.21	40.68	10.07	0.61	2.47	0.42	4.01	179.83
F.			111.734	82.861	1927.135	22.932	26.222	8.609	25.207	10.818	12.366	72.469
Sig.			$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$
Area	**Variety**	**Years**	**Total metal concentration**									
			Ag (µg/L)	**Al(µg/L)**	**As (µg/L)**	**Be (µg/L)**	**Bi (µg/L)**	**Cd (µg/L)**	**Ba (µg/L)**	**Cr (µg/L)**	**Cs (µg/L)**	**Ga (µg/L)**
			M.A.L	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**	**M.A.L**
			-	-	0.2 mg/L	-	-	0.01 mg/L	-	-	-	-
Dealu Bujorului Vineyard	Italian Riesling	**2006**	16.15±1.72^{c}	328.01±2.89^{b}	11.27±1.07^{ef}	0.17±0.02^{d}	LOQ^{h}	0.15±0.01^{a}	170.12±5.37^{ab}	317.57±6.69^{b}	5.55±0.67^{d}	2.99±0.55^{d}
		2007	12.28±2.02^{d}	245.15±9.62^{d}	13.90±0.51^{bc}	0.23±0.02^{c}	13.09±1.27^{de}	0.13±0.02 ^{a}	179.31±0.77^{a}	216.33±6.07^{f}	6.92±0.52^{bcd}	3.38±0.16^{d}
		2008	17.21±0.49^{c}	230.04±4.02^{e}	10.44±0.93^{f}	0.28±0.04^{b}	13.90±1.48^{cd}	0.16±0.01 ^{a}	172.18±8.52^{a}	323.95±2.49^{b}	7.73±0.71^{bc}	7.92±0.55^{bc}
		2009	21.77±2.44^{b}	190.01±5.29^{g}	13.24±1.02^{bc}	LOQ	8.26±1.21^{g}	LOQ^{b}	115.51±2.80^{e}	216.85±2.57^{f}	6.08±0.93^{cd}	7.27±0.53^{bc}
		2010	25.33±3.47^{a}	420.98±1.61^{a}	14.60±0.51^{b}	0.33±0.07^{a}	13.06±0.97^{de}	LOQ^{b}	127.02±5.19^{de}	246.32±10.02^{e}	11.04±1.39^{a}	7.62±1.19^{bc}
		2011	13.39±1.11^{d}	262.41±8.39^{c}	12.06±0.53^{cde}	0.27±0.03^{bc}	14.84±2.03^{cd}	LOQ^{b}	158.35±14.51^{bc}	212.08±1.42^{f}	8.67±0.79^{b}	6.29±1.58^{bc}
		2012	LOQ^{e}	320.58±2.20^{b}	16.37±0.77^{a}	0.14±0.02^{d}	17.35±0.94^{ab}	0.16±0.03 ^{a}	152.35±5.00^{c}	271.19±1.39^{c}	11.38±0.96^{a}	6.62±0.96^{c}
		2013	11.27±1.07^{d}	240.96±8.58^{d}	12.61±0.59^{de}	LOQ^{e}	19.44±1.63^{a}	0.15±0.02 ^{a}	130.72±2.29^{d}	214.75±4.17^{f}	12.31±2.07^{a}	8.29±0.55^{b}
		2014	21.780.47^{b}	192.69±1.69^{g}	14.31±0.97^{b}	0.16±0.02^{d}	16.12±1.38^{bc}	0.14±0.03 ^{a}	124.05±10.24^{de}	357.58±3.22^{a}	10.97±1.20^{a}	7.29±0.55^{bc}
		2015	LOQ^{e}	240.68±8.16^{d}	10.56±0.58^{f}	LOQ^{e}	10.36±0.82^{fg}	LOQ^{b}	129.68±3.89^{d}	212.16±3.17^{f}	6.75±0.77^{bcd}	9.94±0.29^{a}
		2016	LOQ^{e}	216.21±4.30^{f}	13.81±1.29^{bcd}	LOQ^{e}	11.30±1.14^{ef}	LOQ^{b}	154.33±6.85^{c}	257.42±3.33^{d}	10.97±1.20^{a}	8.28±1.52^{b}
Average			12.65	262.52	13.02	0.14	12.52	0.08	145.74	258.75	8.95	6.90
F.			99.971	406.426	14.451	62.440	50.812	74.112	29.766	368.163	14.995	16.298
Sig.			$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$

Average value ± standard deviation (n=3). Different letters are significantly different for $p \leq 0.05$ between varieties. The difference between any two values, followed by at least one common letter, is insignificant. MAL = Maximum allowed limit.LOQ - lower than the limit of quantification. LOQ for Ag: 0.1499 µg/L, LOQ for Be: 0.0030 µg/L, LOQ for Bi: 0.0067 µg/L, LOQ for Cd: 0.0673 µg/L.

Table 5. Total metal concentration of Italian Riesling

Area	Variety	Years	Total metal concentration									
			In (µg/L)	Sr (µg/L)	Ni (µg/L)	Rb (µg/L)	Se (µg/L)	Tl (µg/L)	U (µg/L)	Zn (µg/L)	Hg (µg/L)	Pb (µg/L)
			M.A.L	M.A.L	M.A.L	M.A.L	M.A.L	M.A.L	M.A.L	M.A.L	M.A.L	M.A.L
			-	-	-	-	-	-	-	5 mg/L	-	0.15 mg/L
Dealu Bujorului Vineyard	Italian Riesling	**2006**	10.22±1.92^{b}	177.78±5.96^{e}	750.87±12.43^{b}	1491.81±2.51^{d}	24.83±0.58^{bc}	2.44±0.10^{bc}	0.18±0.03^{b}	2141.57±9.50^{c}	0.18±0.03^{c}	56.31±1.15^{b}
		2007	10.07±2.20^{b}	149.23±6.18^{gh}	683.78±4.78^{c}	1643.82±17.76^{c}	22.68±1.70^{cd}	3.39±0.18^{a}	0.17±0.03^{b}	2526.26±8.66^{a}	0.19±0.03^{c}	70.62±7.36^{a}
		2008	11.07±1.34^{ab}	199.67±1.39^{bc}	643.76±10.82^{d}	1130.58±4.31^{g}	31.04±1.30^{a}	2.36±0.05^{bc}	0.17±0.01^{b}	1783.51±5.38^{f}	0.22±0.08^{c}	70.66±1.87^{a}
		2009	10.48±0.98^{ab}	166.15±11.67^{f}	927.76±9.73^{a}	1429.79±9.79^{e}	26.82±2.02^{b}	1.49±0.35^{de}	0.26±0.05^{a}	2135.25±10.85^{c}	0.39±0.04^{a}	42.65±1.46^{c}
		2010	12.79±0.74^{ab}	144.88±8.31^{h}	568.04±5.86^{e}	1861.19 ±5.74^{a}	25.92±1.58^{b}	2.06±0.15^{cd}	0.25±0.06^{ab}	2209.04±3.62^{b}	0.30±0.02^{b}	54.22±2.67^{b}
		2011	9.41±0.40^{b}	191.21±1.40^{cd}	670.55±8.70^{c}	1239.47±5.36^{f}	21.02±1.66^{d}	2.38±0.06^{bc}	0.20±0.02^{bc}	2136.24±8.08^{c}	LOQ^{d}	42.50±2.04^{c}
		2012	14.41±4.57^{a}	158.06±3.71^{fg}	345.92±9.11^{i}	1133.90±10.94^{g}	30.31±1.67^{a}	2.94±0.34^{ab}	LOQ^{c}	1977.16±11.53^{d}	0.17±0.02^{c}	22.16±1.01^{e}
		2013	12.71±3.14^{ab}	276.29±5.04^{a}	459.45±3.56^{f}	1780.45±4.51^{b}	19.81±1.34^{d}	2.45±0.54^{bc}	0.17±0.02^{b}	1872.53±3.52^{e}	0.20±0.07^{c}	29.84±1.56^{d}
		2014	10.67±1.53^{ab}	209.44±3.44^{b}	360.14±4.50^{f}	1651.54±2.85^{c}	20.41±0.80^{d}	2.74±0.78^{b}	LOQ^{c}	1985.09±4.72^{d}	LOQ^{d}	33.89±2.52^{d}
		2015	10.36±0.81^{b}	185.92±8.04^{de}	423.00±8.64^{g}	1654.61±0.52^{c}	24.15±2.71^{bc}	1.29±0.10^{e}	LOQ^{c}	2127.65±6.51^{c}	LOQ^{d}	22.99±2.02^{e}
		2016	9.68±1.39^{b}	133.04±7.85^{i}	324.99±5.44^{i}	1436.28±18.06^{e}	26.83±1.55^{b}	1.69±0.23^{de}	LOQ^{c}	2140.23±12.31^{c}	0.17±0.03^{c}	20.96±1.31^{e}
Average			11.08	181.06	559.84	1495.50	24.89	2.30	0.13	2094.05	0.16	42.53
F.			1.681	112.863	1725.853	2118.865	16.036	10.349	45.676	1667.945	32.444	127.826
Sig.			$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$	$p \leq 0.00$

Average value ± standard deviation (n=3). Different letters are significantly different for $p \leq 0.05$ between varieties. The difference between any two values, followed by at least one common letter, is insignificant. MAL = Maximum allowed limit. LOQ - lower than the limit of quantification. LOQ for In: 0.0100 µg/L, LOQ for U: 0.00842 µg/L,LOQ for Hg: 0.1379µg/L.

Table 6. $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$, isotope rations of Italian Riesling

Areas	Variety	Year	$^{207}Pb/^{206}Pb$	SD	RSD %	$^{208}Pb/^{206}Pb$	SD	RSD %	$^{204}Pb/^{206}Pb$	SD	RSD %	$^{87}Sr/^{86}Sr$	SD	RSD %	Pb (µg/L)	Sr (µg/L)
Dealu Bujorului Vineyard	Italian Riesling	**2006**	1.3148^{a}	0.0021	0.1572	2.1334^{a}	0.0020	0.0925	16.6470^{a}	0.0130	0.0781	0.7135^{b}	0.0018	0.2558	56.31±1.15^{b}	177.78±5.96^{e}
		2007	1.3151^{a}	0.0040	0.3057	2.1531^{a}	0.0030	0.1381	16.6163^{a}	0.0615	0.3699	0.7127^{b}	0.0005	0.0706	70.62±7.36^{a}	149.23±6.18^{gh}
		2008	1.3139^{a}	0.0028	0.2155	2.1368^{a}	0.0056	0.2609	16.6341^{a}	0.0251	0.1507	0.7194^{ab}	0.0059	0.8233	70.66±1.87^{a}	199.67±1.39^{bc}
		2009	1.1315^{d}	0.0018	0.1385	2.1282^{a}	0.0199	0.9356	16.6340^{a}	0.0199	0.1194	0.7147^{ab}	0.0036	0.5089	42.65±1.46^{c}	166.15±11.67^{f}
		2010	1.3056^{b}	0.0041	0.3102	2.1382^{a}	0.0073	0.3421	16.4968^{b}	0.0169	0.1022	0.7218^{ab}	0.0096	1.3305	54.22±2.67^{b}	144.88±8.31^{h}
		2011	1.3152^{a}	0.0081	0.6142	2.1293^{a}	0.0053	0.2495	16.6385^{a}	0.0222	0.1332	0.7196^{ab}	0.0067	0.9250	42.50±2.04^{c}	191.21±1.40^{cd}
		2012	1.3134^{a}	0.0010	0.0739	2.1535^{a}	0.0510	2.3687	16.6182^{a}	0.0377	0.2269	0.7164^{ab}	0.0046	0.6466	22.16±1.01^{e}	158.06±3.71^{fg}
		2013	1.3170^{a}	1.1731	0.0019	2.2185^{a}	0.0067	0.3134	16.6087^{a}	0.0737	0.4437	0.7208^{ab}	0.0050	0.7003	29.84±1.56^{d}	276.29±5.04^{a}
		2014	1.3131^{a}	0.0019	0.1714	2.1253^{a}	0.0006	0.0272	16.5201^{b}	0.0078	0.0472	0.7244^{a}	0.0025	0.3427	33.89±2.52^{d}	209.44±3.44^{b}
		2015	1.1363^{d}	0.0023	0.2015	2.1243^{a}	0.0023	0.1087	16.5195^{b}	0.0063	0.0379	0.7132^{b}	0.0017	0.2322	22.99±2.02^{e}	185.92±8.04^{de}
		2016	1.1767^{c}	0.0019	0.1619	2.2149^{a}	0.0037	0.1762	16.5126^{b}	0.0104	0.0630	0.7178^{ab}	0.0065	0.9102	20.96±1.31^{e}	133.04±7.85^{i}
Average			**1.2686**	**0.0035**	**0.2705**	**2.1341**	**0.0098**	**0.4557**	**16.5860**	**0.0268**	**0.1611**	**0.7177**	**0.0044**	**0.6133**	**42.53**	**181.06**
F			1025.862			1.147			9.190			9.056			127.826	112.863
Sig.			p ≤ 0.00			p = 0.00			p ≤ 0.00			p ≤ 0.00			p ≤ 0.00	p ≤ 0.00
Minimum Values			1.3131	0.0010	0.0019	2.1243	0.0006	0.0272	16.4968	0.0063	0.0472	0.7127	0.0005	0.0706	20.96±1.31	133.04±7.85
Maximum Values			1.3170	1.1731	0.3057	2.2185	0.0510	2.3687	16.6470	0.0737	0.4437	0.7244	0.0096	1.3305	70.66±1.87	276.29±5.04
Almeida *et al.*, 2016			1.1440		1.4000	2.1570		1.0000							76.00±13.00	
Avram *et al.*, 2014			1.1400	0.1000	8.7000	2.1000	0.1500	7.1000				0.7600	0.0100	1.3000	35.90	171.40
Mihaljevič *et al.*, 2006			1.7400	0.0030		2.0920	0.0070								11.11±5.28	
Barbaste *et al.*, 2001			1.1420	0.0020	0.1400	2.1250	0.0030	0.1600	17.6000	0.3000	1.7000				42.90±0.05	
Bora *et al.*, 2017			1.397	0.0007	0.0608	2.1069	0.0073	0.3446	16.6553	0.0299	0.1794	0.7189	0.0057	0.7869	42.63±1.52	234.92±9.80

SD – Standard deviation , RSD % relative standard deviation

The values of $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ isotope ratio obtained are comparable with Almeida *et al*., 2016 (2.0700 to 2.1570 Brazilian wines$^{208}Pb/^{206}Pb$; 16.6670 to 17.9960 Brazilian wines $^{204}Pb/^{206}Pb$) and also with Barbaste *et al*., 2001 (2.0990 to 2.1030 Italian wines$^{208}Pb/^{206}Pb$; 17.544 to 18.3210 Italian wines $^{204}Pb/^{206}Pb$).Concerning $^{87}Sr/^{86}Sr$, isotope ratio the values are between the ranges from 0.7127 to 0.7244, the highest values were registered to Italian Riesling from 2014 (0.7244±0.0025 [0.3427]) and Italian Riesling from 2010 (0.7218±0.0096 [1.3305]), lowest value of $^{87}Sr/^{86}Sr$ isotope ratio was recorded to Italian Riesling from 2007 (0.7127±0.0005 [0.0706]). The values of $^{87}Sr/^{86}Sr$ isotope ratio obtained are comparable with Geana *et al*., 2016 (0.71015 to 0.72311 Romanian wines); Avram *et al*., 2014 (0.7600 to 0.9300).

3.4.Combining multielement analysis and $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio for wine geographical discrimination

Elements like Mn, Cd, Li, Ba, Ca, Bi, Rb, Mg, Ag, Ni, Cr, Sr, Zn, Rb, Pb and Fe showed a high discriminatory power for geographic origin of Romanian wines. Additional new elements (Hg, Ag, As, Al, Tl, U), metal rations (K/Rb and Ca/Sr), and $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$ isotope ratio have been investigate in order to identify new tracers for geographical traceability of Romanian wines (Geana *et al.*, 2017; Bora *et al.*, 2018).

Multivariate chemometric method was applied for the differentiation of wines intro groups on the basis of their geographic origin. Stepwise linear discriminant analysis (LDA) was used to identify significant tracers for classification to the geographical discrimination of the wines samples. Bycross-validation, we established the optimal number of parameters required to obtain a robust model.

Based on the metal concentration the cross-validation technique provided a 59.85% percentage of predicted membership according to the wine geographic origin (F1=32.92% and F2=26.93%) (Figure 1). In case on the $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$ isotope ratio the cross-validation technique provided a 90.01% percentage of predicted membership according to the wine geographic origin (F1=46.97% and F2=43.04%) (Figure 2).

The linear correction revealed acceptable scores for the two defined discriminant factors (F1 and F2). $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio provided a 65.22% percentage of predicted membership according to the wine geographic origin (F1=62.08% and F2=33.14%) (Figure 3).

A significant differentiation of wines according to the geographic origin and vintage of wine obtained was carried out for wines samples, which demonstrates the importance of $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio for the geographical traceability of wines.

The differentiation of wines according to geographic origin based on the elemental profile and $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$ isotope ratio, in this case a 95.22% percentage of predicted membership according to the wine geographic origin was (F1=62.08% and F2=33.14%) (Figure 3b).

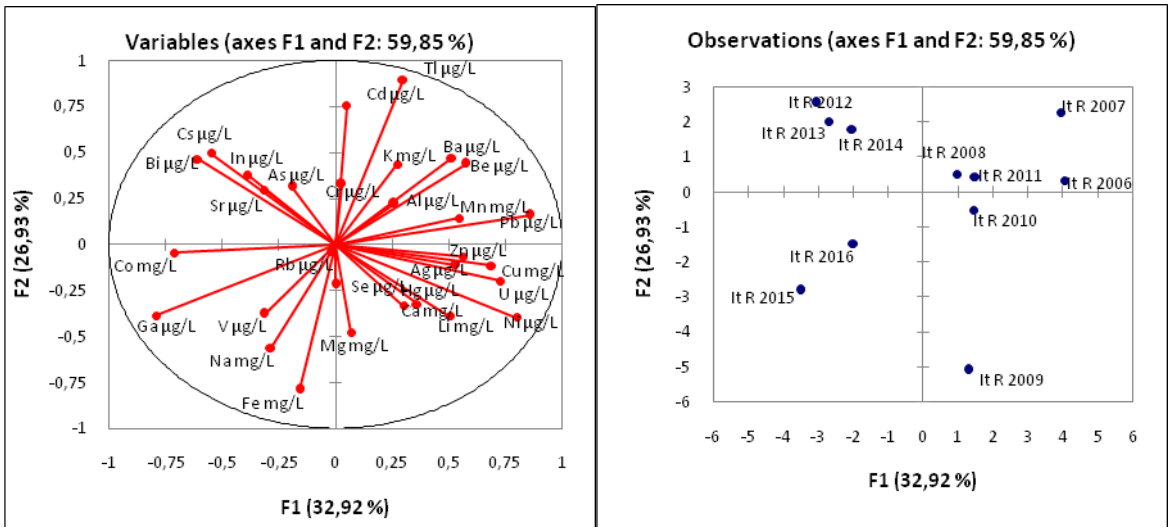

Figure 1. Correlation between analyzed parameters (metal concentration) and discriminant factors in analysis of Italian Riesling geographic origin

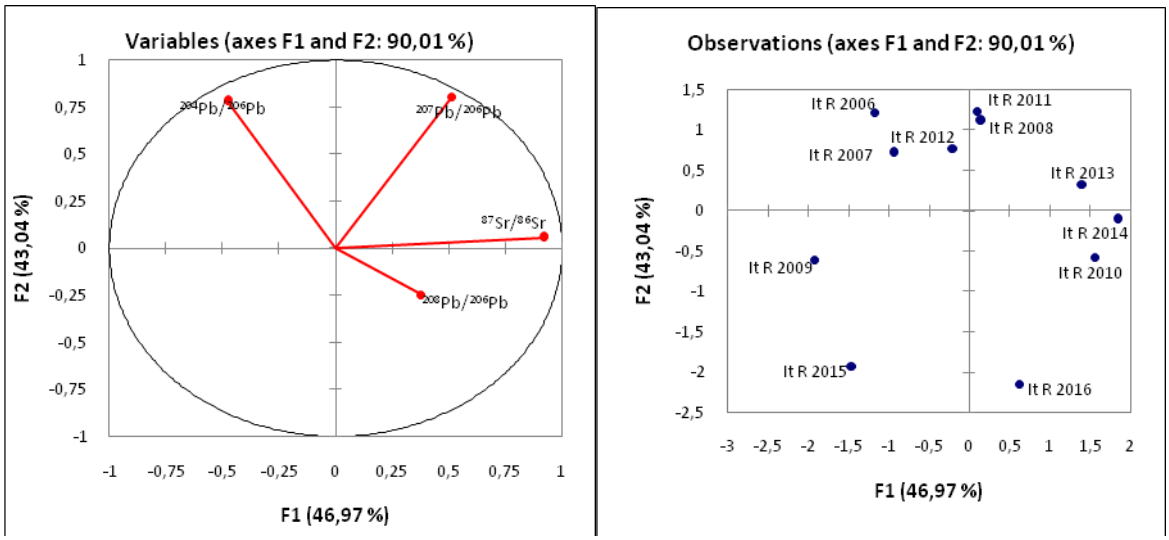

Figure 2. Correlation between analyzed parameters ($^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ isotope ratio) and discriminant factors in analysis of Italian Riesling geographic origin

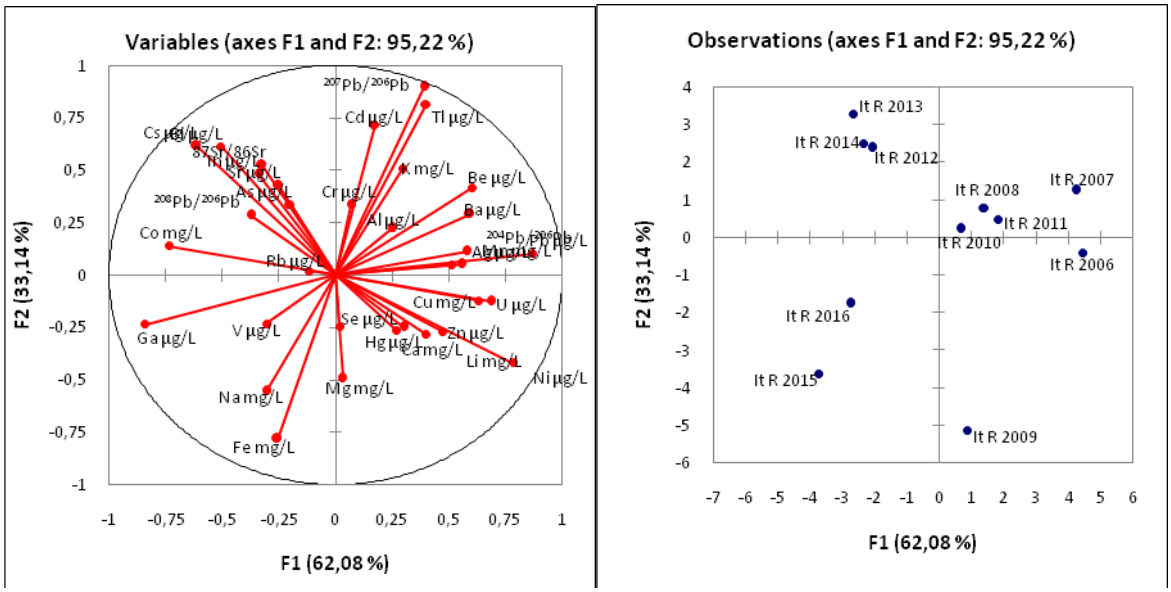

Figure 3. Correlation between analyzed parameters (multielement and $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ isotope ratio) and discriminant factors in analysis of Italian Riesling geographic origin

4. Conclusions

In this study the characterization of the Italian Riesling wine according to their elemental composition was performed. Potassium, calcium and magnesium were the most abundant elements in all investigated wines samples. Concentration of Na (1 mg/L), Cu (1 mg/L), As (0.2 mg/L), Cd (0.01 mg/L), Zn (5 mg/L) and Pb (0.15 mg/L) metals in analysed wine samples were under Maximum Permissible Limits (MPL), respectively as published by the Organization of Vine and Wine.

Based on the elemental profile of the Italian Riesling and $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$ isotope ratio, a relevant discrimination of wines according to their geographical origin was performed, the variation of the $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$, $^{87}Sr/^{86}Sr$ isotope ratio representing a strong geological marker for wines geographical traceability.

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PRODUCTION OF NATURAL CURED FRESH ORIENTAL SAUSAGE BY MEAT-ASSOCIATED LACTIC ACID BACTERIA: A POTENTIAL SOLUTION FOR NITRITE FREE AND LOW NITRITE MEAT PRODUCTS

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ABSTRACT
Nitrate and nitrite are used reliably in cured meat products to produce the characteristic red color and to control the growth of spoilage and pathogenic bacteria but safety of these additives was questioned. The main objective of this work was to develop natural additives that enhance the color and safety of sausage meat and to implement these additives as applicable alternatives to nitrite. Lactic acid bacteria (LAB) were isolated from natural fermented sausages then screened for their producing-ability of nitrate/nitrite reductase that depletes nitrate/nitrite as well as their antimicrobial activities against *Listeria* sp. Selected LAB isolates were screened mainly using parsley and celery juice in standard de Man, Rogosa and Sharpe (MRS) broth, then tested in sausage products. Sausages were subjected to visual and instrumental color assessment, pH and inhibition effect on *L. monocytogenes.* Some of produced sausages did score higher colorimetric a* (redness) value and sensory color scores and overall acceptability compared to controls with added nitrite,. Furthermore, 1-2 log cfu/g of *L. monocytogenes* were lower than the control sausage depending on the type of used protective LAB cultures. The pH values of sausages with LAB isolates and no nitrite were the lowest throughout processing which protected them against the development of the majority of pathogenic bacteria. This study showed that co-treatment of sausage products by natural source of nitrate/nitrite and LAB isolates producing nitrate and nitrite reductase has a great potential to substitute the nitrite in sausage production.

1. Introduction

In spite of modern advances in technology, the preservation of foods is still a debated issue, not only for developing countries (where implementation of food preservation technologies are clearly needed) but also for the industrialized world. Minimally-processed and naturally preserved foods are major challenges for the current food industry (Gálvez *et al.*, 2007). In many parts of the world, natural and organic foods have been experiencing an explosive market growth. Natural and organic processed meats have been a very significant part of that growth (Sebranek and Bacus, 2007). In the case of processed meat products that are typically cured by addition

of sodium/potassium nitrite or nitrate, the requirements for natural or organic do not permit addition of nitrite or nitrate. Nitrites are used in many countries as deliberate food curing additives to stabilize the color of cured meats (Wesley *et al.,* 1982) and protect against the risk of botulism (Tompkin *et al.,* 1978). However, addition of nitrite, and the associated likelihood of nitrosamine formation, has been a major concern to USDA and food processors (Havery and Fazio, 1985; Pensabene and Fidler, 1983).

The cured meat industry made substantial changes to the manufacturing process in the past 20 years to address some of the concerns about nitrite in cured meats. It has stopped using sodium nitrate (except for some specialty meats) in major meat processes and reduced the use of nitrite in the processing of cured meats. The industry also began using agents to help in blocking or inhibiting the formation of NOCs from nitrite. Such agents include ascorbate (vitamin C), erythorbate (chemically similar to vitamin C), and tocopherol (vitamin E) (Mirvish, 1986; Lathia and Blum, 1989). But no vitamin offers 100% protection. The only safe strategy is to avoid sodium nitrite completely.

The possibility of formation of the carcinogenic nitrosamines from nitrites used in cured meats prompted many investigators to search for nitrite substitutes (De Martinis and Freitas, 2003). In searching for a potential substitute for nitrite in meat products, one of the tested approaches is the microbial conversion of metmyoglobin into nitrosylmyoglobin (the cured-meat pigment). Li *et al.* (2013) have proofed the ability of *Staphylococcus xylosus* strain isolated from Chinese dried sausage to convert metmyoglobin into nitrosylmyoglobin in the absences of nitrite or nitrate which demonstrate a potential solution.

However nitrate did not have a direct role in the curing process and must be first reduced to nitrite to enter into curing reactions. Nitrate-to-nitrite reduction can be accomplished by microorganisms found in the natural flora of meat or by intentional addition of microorganisms with nitrate-reducing properties (Sanz *et al.,* 1997). The applied micro-organisms are mostly bacteria, in particular lactic acid bacteria, and they are designated as protective cultures (Lücke, 2000).

Another recent approach is increasingly directed towards utilization of a natural nitrate source and reducer (Sindelar *et al.,* 2007; Sindelar and Houser, 2010). Vegetables are well known to contain significant amounts of nitrate with much higher concentration in the form of juices and powders (Walker, 1990; Fujihara *et al.,* 2001). Considerable researches have been done on the utilization of celery juice powder and celery concentrate in the production of cured meat products (Sindelar *et al.,* 2007; Magrinya *et al.,* 2009).

Furthermore, the concept of biopreservation has been well developed for applications in fermented products since it is common to use bacteria as starter cultures in these products. However, the addition of micro-organisms to nonfermented products is a rather new concept (Vermeiren, 2006).

LAB are considered as food grade organisms that are safe to consume and have a long history of use in food and diverse strategies have been used LAB to develop healthier meat and meat products (Bredholt *et al.,* 2001).

In a model system of fresh oriental sausage, the objective of this study was to isolate LAB from Egyptian meat products and assess its ability to reproduce the characteristic pinkish cured color and to inhibit spoilage bacteria.

2. Materials and methods

2.1. Isolation of lactic acid bacteria and culture conditions

Bacterial isolates were isolated from commercially available home-made, raw fermented sausages by plating samples on MRS-agar (Merck, Darmstadt, Germany). Plates were incubated at different temperatures (15, 20, 30, 37ºC) for 24-48 h.

Gram-positive, catalase-negative, non-motile, rods or cocci isolates were maintained at -80˚C in glycerol stocks. Cultures were purified by successive subculturing on MRS-agar.

Isolates subsequently shown to express color enhancing properties and antimicrobial activities were further identified by physiological and biochemical tests, as described by Collins *et al.* (1989), Schillinger and Lucke (1987) and Stiles and Holzapfel (1997). Carbohydrate fermentation pattern were determined using API 50 CHL and API 20 kits (BioMe´rieux S.A., Marcy I'Etiole, France) according to the manufacturer's instructions. The isolates were then identified using the APILAB Plus software version 3.3.3 (BioMe´rieux).

2.2. Detection of nitrate and nitrite reductase activity

Nitrate reduction is determined on a semi solid and solid MRS medium supplemented with 0.2% potassium nitrate as described by Skerman 1967 and Balows *et al.* (1992). When visible growth is present, equal volumes (10 drops) of nitrate reagent A (0.8 g of sulphanilic acid in 100 mL of 5 N acetic acid) and reagent B (0.6 g of N-N-dimethyl-1- Naphthylamine in 100 mL of 5 N acetic acid) were added. Development of a red color indicates of the presence of nitrate reductase. If no color develops, zinc dust is added to the medium. Development of a red color after the addition of zinc confirms that nitrate is still present in the medium and that the organism cannot reduce nitrate. Absence of a red color after the addition of the zinc indicates that nitrate was reduced to nitrite which gets completely reduced to nitric oxide, nitrous oxide, or nitrogen, indicating that the organism has both the nitrate and nitrite reductases.

Nitrite reduction is determined in a semi solid MRS medium supplemented with 0.05% potassium nitrate. When there is visible growth, reagents A and B are added to the medium as for the nitrate test. Absence of a red color is positive for nitrite reduction, while development of a red color is a negative test result. At least 2 min were allowed for the color developing before considering the nitrate test negative. A non-inoculated nitrate control should be tested with reagents to determine whether the medium is nitrate-free and that the glassware and reagents have not been contaminated with nitrous oxide. The addition of too much zinc dust may result in a false negative reaction or just a fleeting color reaction.

2.3. Detection and quantification of antimicrobial activity

The antibacterial activities of LAB isolates was detected against 8 bacterial pathogens *viz. Bacillus cereus* ATCC 49064, *Clostridium perfringens* ATCC 13124, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19116, *Listeria ivanovii* Li4 (pVS2), *Salmonella enterica* ATCC 25566, *Yersinia enterocolitica* ATCC 23715, *Escherichia coli* as well as against one LAB strain (*L. sakei* NCDO 2714).

Strains were tested using spot-on-lawn method (Barefoot and Klenhammer, 1983). Further antibacterial activity quantification

in the liquid was determined by an adaptation of the critical two fold dilution method as described by Parente *et al.*, (1994) and Deraz *et al.*, (2005).

2.4. Determination of nitrate and nitrite content of parsley and celery juice

Parsley and celery samples from the same geographical area were collected to determine their average contents of both nitrate and nitrite and divided into three portions. The first portion was analysed at once, the second portion was stored in a refrigerator for 24 h before analysis, while the third portion was frozen at -20°C for 24 h before analysis.

An aliquot of 10 g of a well homogenized sample was blended for 5 min in a mixture of 70 mL H_2O and 12 mL 12% NaOH (pH 8). The suspension was then heated in water bath 50-60°C with occasional swirling until temperature reached ca. 50°C. After cooling, the suspensions were filtered through Whatman No 41 filter-papers. The first part of filtrate was discarded in order to overcome possible nitrate contamination from the filter-papers. Each sample was analyzed in duplicate and the result was expressed as mg/kg fresh weight.

The nitrate dosage was made through the spectrophotometric method by using phenol2,4disulphonic acid in basic medium (sodium hydroxide, 20%).Yellow nitro derivatives were obtained, that have absorption maxima at 420 nm (AOAC, 1975). The nitrites were determined according to Griess method (Griess, 1879).

The principle of this method is based on the reaction between nitrites and sulfanilic acid in acetic medium (1% w/v in 30% acetic acid) and N-(1-Naphthyl)ethylenediamine dihydrochloride (Marshall's reagent) to produce a purple-colored compound. It measures the intensity of the color of azoic compound that is formed after the diazotation reaction between sulphanilic acid and nitrites, followed by coupling the reaction with naphtylamine. After 20 min, the developed pink formed complex was measured at 520 nm wavelength. The calibration curves for nitrate and nitrite were linear for the studied concentration ranges.

2.5. Depletion of nitrate from natural sources by isolated LAB

Ability of LAB to deplete nitrate/nitrite from natural sources namely parsley and celery juices were determined during growth of putative color enhancing LAB and changes in concentration of nitrate/nitrite were monitored spectrophotometrically (AOAC, 1975; Griess, 1879). One mL of sterilized parsley and/or celery juices was added to 9 mL of MRS broth in test tubes.

LAB inoculums were inoculated separately into media, tubes and were incubated at 30°C for 4 days. The depletion of nitrate/nitrite were determined on 0,1,2,3 and 4^{th} day of fermentation. The consumption was calculated as the remaining amount of nitrate/nitrite (%) compared to control medium incubated without LAB. One hundred and three isolates from different meat products were exposed to this experiment.

2.6. Preparation of sausages

Two batches of oriental sausages were formulated including fat 21% using ground meat, 3.5 kg each. The first batch was prepared to test color enhancing capability while the second used to validate the impact of the protective effect of LAB isolates on sausage. Afterwards, the first batch was divided into portions, one added vegetable

juice (0.4%) and LAB culture at 7 log cfu/g sausages. The second treatment was considered as a positive control with 0.5% sodium nitrite. The second formulated sausage batch was subjected to two treatments, one with selected LAB isolate and one with sodium nitrite. To both treatments, *Listeria monocytogenes* ATCC 19116 (conducted with appropriate safeguards) was inoculated with a final concentration of 3 log cfu/g sausages.

All treatments and controls (C+) were manufactured using the same base formulation that included the following ingredients: 64.55% ground beef meat, 19.36% crashed ice (water), 7.75% wheat flour, 2.29% dry milk, 1.36% salt, 0.85% corn syrup (glucose), 3.24 onion flakes, 0.26% white paper, 0.14% starch.

The formulated lean meat was subsequently chopped with all the seasonings and stuffed in sheep natural casing (22-24 mm in diameter) which purchased as dry backed sheep casing from an industrial plant located in Dekhlea, Alexandria which provides natural casing to big companies of meat products.

After filling, the sausages were hung on rods for partial draining (a few hours). All patches of sausage were matured for 10 h at 15°C then kept at 20-22°C for 48 h. Two samples from each batch were taken during the processing (3 days) as follows: fresh prepared sausage and at the end of processing. Color-enhancing ability was determined visually and instrumentally.

Qualitative detection and enumeration of *L. monocytogenes* on the sausage samples were performed by plating on *L. monocytogenes* blood agar (LMBA) for 24 h at 37^{o}C.

2.7. Color measurements

Color was measured immediately after slicing the sausage lengthwise. Color measurements were conducted using a portable colorimeter (X-Rite, model SP 64, U.S.). The instrument was calibrated using a white standard plate (calibration plate CRA43) and the Hunter Lab color scale was used to measure color. The values, expressed as L^* (lightness), a^* (redness) and b^* (yellowness) units, were obtained from three different cut areas of each sausage. Data were recorded as means of three measurements from each treatment and control sausage.

2.8. Visual color evaluation

A ten-member descriptive panel was instructed to evaluate color intensity of the sausage samples. Panelists evaluated the samples for intensity of pink/red color, brown color, and uniformity of color according to Vermeiren (2006). A score above 5 indicated the sample being acceptable.

2.9. pH measurements

Aliquots of 10 g of homogenized sausage samples were blended with 90 mL of distilled deionized water. pH of the slurry was measured using an Orion 230 A Plus pH meter (Sensorex Co., CA, USA) calibrated with phosphate buffers of 4.0 and 7.0, according to the method of Sebranek *et al.* (2001). The measurement was carried out in triplicate.

3. Results and discussions

3.1. Isolation of lactic acid bacteria from commercial meat products

A total of 867 bacterial isolates in which 203 isolate with coccal shape and 664 in rod were collected. Bacterial isolates that Gram-

positive and catalase-negative, non-motile were considered as presumptive LAB.

Several criteria were then used in order to define the most reliable and appropriate one allowing a specific selection of 103 isolates belonging to the Lactic acid bacterial species. All selected isolates of LAB grew at conditions were used for meat fermentation at 15, 20 and 30°C in the presence of NaCl with a pH range from 5.0 to 5.5.

3.2. Detection of nitrate reductase activity of isolated LAB

The isolated LAB showed variable nitrate reductase activity. This variability has been observed by the detection of different values of nitrate reductase activity. Of the 103 strains of LAB tested, 26 isolates showed remarkable nitrate reductase activity. However, only 8 isolates was confirmed for both the nitrate and nitrite reductases. The appearance of red haloes or pink-red indicates the presence of nitrate reductase activity (Figure 1).

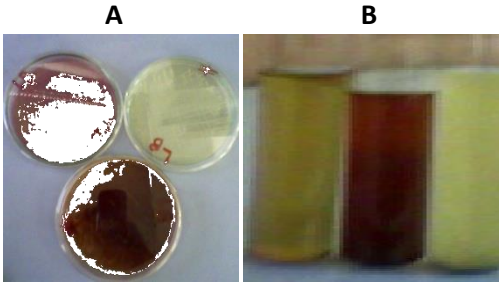

Figure 1. Confirmed nitrate reductase activity using (A) MRS agar plates and/or (B) semi solid MRS.

3.3. Detection of antimicrobial activity

To enhance the expected role of the isolated LAB with either nitrate and/or nitrite reductase, nitric oxide reductase and/or nitrous oxide reductase, the antibacterial properties towards both pathogenic and spoilage organisms that typically associated with meat product were evaluated.

As the aim of this experiment was to examine the selected LAB for their antibacterial capacity and a positive result, this is the detection of an inhibition zone, may due to the formation of various organic acids, carbon dioxide, bacteriocin, hydrogen peroxide, diacetyl and antibiotic production (Hutten *et al.,* 1995). In addition, this experiment did not target revealing the mechanism of inhibition and was meant only to select LAB isolates that possess remarkable antibacterial capacities.

Among 103 isolates, 58 LAB isolates showed antagonistic properties against at least one of the indicator bacteria. Of the 58 strains, 25 isolates showed antibacterial activity only against *Bacillus cereus* and one strain was only active against *L. monocytogenes. L. monocytogenes* is a significant food-borne pathogen in food industry especially in meat, poultry, and seafood products (Farber and Peterkin, 1991).

As shown in Table 1, the spectra of inhibition activity were different among the tested isolates. Isolates S4p8, Sm86, Sm96 showed the largest antimicrobial spectrum, exhibiting inhibitory activity against 5 pathogens followed by isolates S2p8, S3p13, S4m4, and S4m98 that inhibited 4 pathogens. The other indicators i.e *Clostridium perfringens* ATCC 13124 and *Lactobacillus sakei* NCDO 2714 were not inhibited by any LAB isolates. However, among the 58 tested isolates only 8 isolates could inhibit *L. monocytogenes* and were further tested to find out isolates with the strongest antilisterial effect in the processed meat. As shown in Table 1 only isolates S3Pl3, S4m9, S4P8, S4C18, S4m98, S4C11, Sm86 and Sm97 did reveal antilisterial activity.

The largest inhibition zones were observed towards *Yersinia enterocolitica* ss.

enterocolitica ATCC 23715 and *Salmonella enteric*, followed by *L. monocytogenes.*

Although LAB may be useful as protective cultures by lactic acid production and pH reduction which provide quite unfavorable conditions for pathogenic bacteria thereby preventing them from growing. However, several pathogenic microorganisms are able to survive in fermented sausages under certain conditions, especially during refrigerated storage.

Pathogenic strains of *Escherichia coli*, *L. monocytogenes* and *Yersinia enterocolitica* are inactivated better after the initial fermentation stage if stored at ambient rather than at refrigeration temperature which may increase the safety of these products (Lindqvist and Lindblad, 2009; Heir *et al.,* 2010). In our study, all batches of sausage were matured for 10 h at 15°C and then kept at 20-22°C for 2 days.

3.3. Selection of isolates with a strong antilisterial activity

To select the most promising isolates, quantitative determinations of antilisterial activity of 8 selected isolates were carried out (Table 2). To evaluate antilisterial activity production among the LAB isolates, cultures were assayed for their production using the two-fold serial dilution method.

Among tested isolates, only 2 isolates namely S4C18 and Sm97 showed strong activity of 25600 AU/ml against *L. monocytogenes* ATCC 19116. The rest of tested isolates had activity ranged from good and moderate to weak activity (Table 2).

Antibacterial production is influenced by many factors included LAB strain, growth temperature, pH and medium composition (Mataragas *et al.,* 2003). Because LAB strains are ‘generally recognized as safe (GRAS) in food production use of either their cell free supernatant or the LAB cultures for food preservation has received much interest (Cleveland *et al.*, 2001).

Moreover, food application benefits imparted by lactic acid bacteria are strain specific, and not species- or genus specific, those isolates were chosen as the most promising strains for more investigation for their antilisterial activity in the meat model system of the fresh oriental sausage.

3.4. Determination of nitrate and nitrite content of parsley and celery juice

Since the concentrations of nitrate in vegetables depend on biological properties of the plants such as, species, season, day light, temperature, type of soil, method of growth and fertilizers used (Tamme *et al.*, 2006), parsley and celery samples from the same geographical area were collected to determine their average content of both nitrate and nitrite.

The first part was analysed as soon as possible, the second part was stored under refrigerator condition for 24 h before analysis, and the third part was kept at -20 for 24 h then analysed. These different storage conditions were tested because under adverse post-harvest storage conditions, nitrite concentrations can increase in vegetables as a greater proportion of the nitrate is converted to nitrite as a result of bacterial contamination and endogenous nitrate reductase action.

Nitrite is quantitative determined using Griess method (Griess, 1879). This reaction was firstly described in 1879 and because of its simplicity and precision was intensively used to determine the nitrites from numerous biological samples (Sun *et al.*, 2003).

The main nitrate level were ranged from 900 to 1570 mg/kg depends on sample type and storage conditions. However, conversion of part of nitrate to nitrite was varied according to tested storage conditions. Fresh parsley samples contained nitrite ranged from 0 to 0.15 mg/mL.

However, samples stored under cooling for 24 h before analysis had higher nitrite concentration (8.7-8.9 mg/mL) compared to fresh samples.

Table 1. Antibacterial activity of lactic acid bacteria isolated from sausage using spot on lawn tests

Isolate	Indicator strains								
	L. Sakei	*L. innocua*	*L. monocytogen*	*L. Ivanova*	*Salmonella enteric*	*Bacillus cereus*	*Yersinia enterocolitica*	*Clostridium perfingens*	*E. coli*
S2P8	+	-	-	-	+	+	+	-	-
S2P11	-	-	-	-	+	+	+	-	-
S2P10	-	-	-	-	-	+	-	-	-
S2P9	-	-	-	-	+	+	-	-	-
S2P1	-	-	-	-	+	+	-	-	-
S2p3	-	-	-	-	-	+	+	-	-
S2P4	-	-	-	-	-	+	+	-	-
S3P5	-	-	-	-	-	+	+	-	-
S3P6	-	-	-	-	-	+	+	-	-
S3P13	-	-	+	-	+	+	+	-	-
S2P18	-	-	-	-	-	+	-	-	-
S3P19	-	-	-	-	-	+	-	-	-
S2P23	-	-	-	-	-	+	-	-	-
S3m1	-	-	-	-	-	+	+	-	-
S3m2	-	-	-	-	-	+	+	-	-
S3m3	-	-	-	-	-	+	+	-	-
S3m4	-	-	-	-	-	+	+	-	-
S3m9	-	-	-	-	-	+	-	-	-
S2m14	-	-	-	-	-	+	+	-	-
S3m15	-	-	-	-	-	+	+	-	-
S2m15	-	-	-	-	-	+	+	-	-
S3m16	-	-	-	-	-	+	-	-	-
S2m17	-	-	-	-	-	+	+	-	-
S3m17	-	-	-	-	-	+	-	-	-
S3m24	-	-	-	-	-	+	+	-	-
S3m29	-	-	-	-	-	+	+	-	-
S4m9	-	-	+	-	-	-	+	-	+
S4P8	-	+	+	-	-	+	+	-	+
S4m4	-	+	-	-	-	+	+	-	+
S4m3	-	-	-	-	-	+	-	-	-
S4C18	-	-	+	-	-	-	-	-	-
S4m52	-	+	-	-	-	+	+	-	-
S4m98	-	+	+	-	-	+	+	-	-
S4m96	-	+	-	-	-	+	+	-	-
S4m90	-	-	-	-	-	+	+	-	-
S4m84	-	-	-	-	-	+	+	-	-
S4C15	-	-	-	-	-	+	-	-	-
S4C83	-	-	-	-	-	+	-	-	-
S4C71	-	-	-	-	-	+	-	-	-
S4C79	-	-	-	-	-	+	-	-	-
S4C77	-	-	-	-	-	+	-	-	-
S4C75	-	-	-	-	-	+	-	-	-
S4P61	-	-	-	-	-	+	-	-	-
S4P63	-	-	-	-	-	+	-	-	-
S4C66	-	-	-	-	-	+	-	-	-
S4C11	-	-	+	-	-	+	+	-	-
S4C33	-	-	-	-	-	+	-	-	-
S4P58	-	-	-	-	-	+	-	-	-
S4C17	-	-	-	-	-	+	-	-	-
Sm 85	-	-	-	-	-	+	+	-	-
Sm86	-	+	+	+	-	+	+	-	-
Sm97	-	+	+	+	-	+	+	-	-
SP103	-	-	-	-	-	+	-	-	-
S4m48	-	-	-	-	-	+	-	-	-
Sm87	-	-	-	-	-	+	-	-	-

S4C53	-	-	-	-	-	+	+	-	-
S4C22	-	-	-	-	-	+	-	-	-
S4m9	-	-	-	-	-	+	-	-	-

Table 2. Quanification of the antilisterail activity of the most promising LAB isolates

Strain code	Test organisms	Inhibitory activity against *L. monosytogenes* (AU/ml)
S3P13	*L. monocytogenes; Salmonella enteric;Yersinia enterocolitica ss. enterocolitica; Bacillus cereus*	3200
S4m9	*L. monocytogenes; E. coli; Yersinia enterocolitica ss. Enterocolitica*	6400
S4p8	*L. monocytogenes; L. innocua; Bacillus cereus; E. coli-Yersinia enterocolitica ss. enterocolitica*	12800
S4C18	*L. monocytogenes*	25600
S4m98	*L. monocytogenes; L. innocua; Bacillus cereus; Yersinia enterocolitica ss. enterocolitica*	1600
S4C11	*L. monocytogenes; Bacillus cereus; Yersinia enterocolitica ss. Enterocolitica*	12800
Sm86	*L. monocytogenes; L. innocua; L. Ivanova- Bacillus cereus; Yersinia enterocolitica ss. enterocolitica*	800
Sm97	*L. monocytogenes; L. innocua; L. Ivanova; Bacillus cereus; Yersinia enterocolitica ss. enterocolitica*	25600

3.4. Depletion of nitrate from natural sources by isolated LAB

In this part of the study, natural sources of nitrate/nitrite namely parsley and celery juices were added to the growth medium and changes in concentration of nitrate/nitrite during growth of putative color enhancing LAB were monitored spectrophotometrically.

The tested isolates are divided into 3 main groups according to their abilities of assimilation of nitrate from natural parsley and celery: Group 1 (34 isolates), isolates with ability to utilize all NO_3 and converted it to NO_2 (Zero concentration of NO_3 with considerable amount of NO_2); Group 2 (9 isolates), isolates with ability to utilize all NO_3 and converted it to gaseous form (NO) (Zero concentration of both NO_3 and NO_2) and Group 3, this group contains the rest of the tested isolates which had moderate ability of conversion.

3.5. Primary stage of sausages production

The collected and isolated meat porn LAB were tested for their suitability to be candidate color enhancing cultures for sausage products. Furthermore, at this early stage of the work, it was of primary concern to study and evaluate the effect and behavior of the cultures on the sensory properties of a model product, imitating sausage products. Therefore, two different stages of sausage preparation were done to examine and scale down the final number of isolates applied to sausage formulated according to the local meat manufactures.

Beaker sausage (Sabel *et al.*, 1991) using two meat systems were first used. In this experiment, our batches of "beaker sausage" have only the main common sausage component including: fresh ground beef, glucose, NaCl. To some batches a high inoculum size of isolates were added with to assure domination of isolate (with natural source of nitrate mainly parsley or celery). To other batches sodium nitrite was added

only. All the isolates of the 1st group (34 isolates), the 2nd group (9 isolates) and the 3rd group (3 isolates) with an antibacterial activity (data not shown) were tested. Based on the obtained data, we have selected isolates with enhancing- color ability in beaker sausage (22 from group 1, 9 isolates from group 2 and nothing from group 3).

The most promised isolates based on the previous testes were explored in another set of batches (three batches). First batch contains permitted concentration of sodium nitrite at the level according to the Egyptian law (120 ppm) worked as a control. Second batch contains 0.4% celery juice with known nitrate concentration and tested LAB with a nitrate reductase enzyme (isolates selected from group1). The third batch contained isolates selected from group 2.

These batches were formulated according to local meat manufactures and have the following ingredients: ground meat, crashed ice, wheat flour, dry milk, salt, corn syrup, onion flakes, white pepper, starch and parsley with and without cultures of isolates or sodium nitrite. Batches were formulated by adding one big inoculums size and parsley concentrations of 0.4%.

Color development was followed for three days at 20 to 22ºC. Taken samples were evaluated for fixation of desirable color visually and instrumentally (data not shown). Based on the obtained results, 27 isolates were selected for further investigation.

3.6. Behavior of the selected putative color enhancing and protective cultures in formulated sausage products

The earlier experiments had resulted in the selection of the most promising color-enhancing LAB cultures. However, the action of LAB culture was just tested on a model product. Therefore, it was planned to validate the color-enhancing effect along their antagonistic activities on food-born pathogen *L. monocytogenes* simulating the industrial processing of sausage meat products.

Furthermore, large efforts were followed to understand the impact of the selected isolates cultures on the color properties of these products. We kept he major objective of our experiment is to establish the selected putative color-enhancing cultures and assured their positively capacity to enhance red meat color and not to negatively influencing the sensory properties. Furthermore, this experiment allowed comparisons of the selected 27 LAB with regard to their growth and acidifying capacity on the sausage products.

3.6.1. Sausage color evaluation

The instrumental and visual sausage color evaluation of different sausage batches are shown in Table 3 and Figure 2. The purpose of this evaluation was to determine the degree of difference on internal surface CIE L* (lightness), a* (redness), and b* (yellowness) values of three batches of prepared sausages with color enhancing LAB cultures compared to a reference sausage which was prepared with the addition of sodium nitrite.

The first batch of production included 10 sausage products. Color values and visual color preferences of 10 sausage samples showed that L* values of the nine sausages were almost the same or higher than the control. However, sausages produced with LAB isolates were perceived to have similar or less b* values compared to the one containing sodium nitrite. Similar results were observed by Magrinya *et al.* (2009) and Tsoukalas *et al.* (2011) for sausages produced with the utilization of celery powder or freeze-dried leek powder as a source for indirect addition of nitrate and nitrite. Sausages with celery or freeze-dried leek powder and added nitrite were yellower than the sausages produced without nitrite.

On the other hand, a decrease in CIE a∗ values (redness) was observed throughout the whole sausage products formulated with color-enhancing LAB isolates compared to control sample. Although the decrease of the redness values, three of sausage samples (S4m90, S4p60, S4m43) had higher scores and two (S4C35, S4C75) had similar score compare to control as judged by visual inspection. The color preference of the panel increased with increasing the values of lightness (L*) up to specific limit of redness values (a∗) with minimum value of 9.2.

The obtained CIE values of batch 2 sausage samples were not fully in agreement with CIE color measurement previously found for the sausages products of batch 1. Whereas the L* values for nine sausages out of ten were almost the same or lesser than control, b* values were similar or higher compared to the one containing sodium nitrite. Whereas the measurement of red color (a∗) showed that most sausages samples were redder than control. The redness difference was also noticeable visually. As the redness values increased, a higher acceptability score obtained with visual inspection.

For batch 3, most sausage samples had similar or higher values of both L* (lightness) and b* (yellowness) compare to control. However, all sausage samples had less values of a* (redness). That data were in a harmony with the lower scores for visual color preferences in comparison with control sausage sample.

The instrumental and visual sausage color evaluation showed the sausage samples S4m90, S4p60, S4m43 from batch one and S4C33, S4C67, S4C53, S4C18, S4P63, S4P58, S4m48 from batch two were the most preferred by the panel. These results were in agreement with the finding obtained by Zhu and Brewer (1998) where consumers were able to perceive a difference in a* value of 0.6 – 0.9 in fresh sausages.

3.6.2. pH values of sausage

The pH values of sausages with LAB isolates and no added nitrite were the lowest throughout processing (Table 3). The pH value of controls remained almost constant over the three days. As previously reported, the low pH values with the treatments is due to the higher LAB counts of these treatments compared to control (Tsoukalas *et al.,* 2011). Such low pH had a more negative effect on fermented sausage compared to fresh sausage.

At the end of ripening, the highest weight loss was observed fermented sausages with LAB and no added nitrite. This was due to the fact that their pH values were closer to the isoelectric point of meat proteins and, consequently, they had less ability to retain water (Tsoukalas *et al.,* 2011).

Sebranek (1979) discussed the importance of pH on the nitrite level and stated that a pH decrease as little as 0.2 pH units during product manufacture can result in doubling the rate of color formation and other curing reactions. Several pH differences greater than 0.2 units were observed which could explain the high acceptable color of produced sausages (Table 3).

Leistner and Roedel (1975) demonstrated that meat products are divided into three categories in term of shelf life stability, easily perishable, perishable and shelf-stable based on their pH and water activity values. Regarding pH; "easily perishable" meat products have a pH>5.2 and must be stored at or below +5°C. The "perishable" meat products have either a pH of 5.2–5.0 and must be stored at or below +10°C. The "shelf-stable" meat products have a pH <5.0; these products need no refrigeration and their shelf-life is often not

limited by bacteria but by chemical or physical spoilage, especially rancidity and discoloration. The lowering pH has an essential contribution in the inhibition of the undesirable microorganisms and accelerates proliferation of LAB in the sausages.

Table 3. Effect of various isolates of lactic acid bacteria on the color measurements, pH and overall acceptability of fresh oriental sausages

Batches	CIE color values				Visual color score	pH	
	L*	a*	b*	DE*		Day 0	Day 3
Batch 1							
C- NO2	46.26	11.93	13.67	11.56	6.5	6.05	5.9
S4m5	49.23	7.97	11.81	11.46	5.5	5.9	4.3
S4m10	48.74	8.78	12.28	11.1	5.6	5.9	4.2
S4m90	49.65	10.42	13.84	11.30	8.39	5.9	4.5
S4m96	45.99	8.97	12	11.86	5.4	5.8	4.4
S4p60	46.72	9.2	12.84	10.95	8.57	5.9	4.8
S4C35	50.11	8.45	12.97	10.88	6.78	5.7	4.3
S4C75	48.59	9.80	13.25	10.60	6.74	5.7	4.3
S4m43	46.69	11.01	12.89	11.58	8.79	5.8	4.9
S4C71	48.78	8.34	12.93	9.97	6.55	5.6	4.2
Batch 2							
C- NO2	52.62	9.68	12.56	11.36	6.0	6.1	6.0
S4C33	48.83	13.75	13.03	13.13	8.15	5.9	4.8
S4C67	44.56	13.01	12.61	13.45	8.21	6.0	5.1
S4C22	51.58	8.45	13.01	9.917	5.45	5.6	3.9
S4C53	48.1	13.86	14.39	11.65	8.14	6.1	4.7
S4C66	48.17	7.787	9.787	13.07	5.22	5.1	4.3
S4C18	49.59	10.17	11.42	11.89	8.23	5.6	3.9
S4P63	52.24	14.37	15.78	11.34	7.56	5.9	4.8
S4P58	48.25	12.32	13.25	11.61	7.55	5.9	5.7
S4m3	48.75	10.39	14.08	9.837	5.52	5.5	6.0
S4m48	50.56	9.547	11.8	13.09	7.64	5.8	3.9
Batch 3							
C- NO2	44.74	6.38	4.083	19.28	6.5	6.2	5.9
SP8	43.97	4.807	3.68	19.88	5.85	5.9	4.6
SP77	44.24	4.463	4.147	19.4	6.45	6.0	4.3
SP79	43.39	4.617	3.247	20.53	5.65	6.0	4,9
Sm11	44.88	3.843	5.52	18.07	5.8	5.8	4.5
Sm52	46.23	4.243	5.687	17.41	5.6	5.8	4.4
Sm86	46	4.25	5.353	17.78	5.6	5.8	4.4
Sm97	46.04	5.12	5.71	17.46	6.64	5.5	4.5
Sm98	46.17	5.127	5.253	17.78	5.6	5.6	4.4

3.6.3. Antilisterial impact on Sausage

Therefore to rule out the possibility that those isolates might have extra antilisterial impact and might be useful as biological control agents, an alternative to chemical preservatives in the sausages products. The fermented sausage formulation, described in the previous experiment, were prepared and inoculated with either S4C18 or Sm97 (7 log cfu/g sausages) to test the meat protective effect, mainly its antilisterial activity.

In accordance with the defined test method, the total amount of stuffing was divided into three experimental groups, as follows: a) one third of the stuffing was separated from the total weight and was

immediately filled into sheep natural casing (these sausages were a control group); b) in the rest of the stuffing, *L. monocytogenes* ATCC 19116 was inoculated to a final concentration of 3 log cfu/g stuffing.

Figure 2. Photographs showing different batches of meat fresh oriental sausages with inserted different color enhancing lactic acid bacterial isolates

After inoculation of *L. monocytogenes*, half of the stuffing was separated and filled into sheep natural casing (this group was a control sample reflecting the effectiveness of protective cultures; and c) into the remaining amount of the stuffing with *L. monocytogenes*, protective cultures of the selected isolates were added to achieve a final count of 7 cfu/g stuffing. After homogenization, the stuffing was filled in the same way into sheep natural casing. Samples for laboratory examinations were taken on days 0, 1 and 3. Three samples were collected at each step of sampling and

used for analysis to determine the listeria control or elimination effect of the of meat protective cultures used during the incubation.

L. monocytogenes was not detected in any sample of the control group of sausage in all stages of testing (days 0, 1 and 3) along the triplicate fermentations. The changes in the counts of the *L. monocytogenes* in the examined experimental groups were compared to samples of sausage inoculated only with *L. monocytogenes.*

One of the particularly important results in this study is those related to the changes in the number of inoculated *L. monocytogenes*. Based on these results, we get a straight answer about the impact of added LAB cultures on growth and the survival of this pathogen. The initial value that was common to all experimental samples (approximately 3 logs), were decreased 1-2 log cfu/g lower than the control sausage depending on the type of protective cultures. In anaerobically packaged cured meat product, recontamination with LAB occurs at low numbers and growth of *L. monocytogenes* is probable (Lücke, 2000). In general, high inoculua (10^6 -10^9 cfu/g) are needed to create an antagonistic effect (Nilsson *et al.*, 2005). Therefore, controlled growth of protective LAB in these meat products is a potential preservation strategy.

3.6. Identification of promising LAB in meat system

Based on the obtained data of color-enhancing ability and efficient antilisterial activity in the meat model system, a total of ten LAB isolates were chosen for further identification. The isolates were initially differentiated on the basis of their cultural and cellular morphological studies after which they were subjected to various physiological and biochemical tests. The isolates were Gram positive, rods, cocci, ovoid and produce no endospore and showed strong to moderate growth on MRS agar. The isolates were oxidase, catalase, gelatin, casein hydrolysis, Nitrate reduction. Some strains hydrolysed starch and they were facultative anaerobes and were fermentative rather than being oxidative in nature and continued by looking the formation of cell using 1000-magnified microscope.

After the preliminary characterization test, carbohydrate fermentation patterns of the ten isolates were tested using API 50 CHL and API 20 kits. The Results of identification using API kits and the determination of phenotypic rate using conventional method were showing that almost all selected isolates were LAB namely *Lactobacillus plantarum (*S4m43 and S4C67), *Lactobacillus curvatus (*S4C33), *Enterococcus Faecium (*S4m90 and S4P63), *Lactococcus lactis ssp lactic (*Sm97), *leuconostoc mesenteroides (*S4C53), *Pediococcus acidilactici (*S4C18), *Enterococcus faecalis (*S4m48) and *Aerococcus viridians (*S4p60).

Different recipes and ripening conditions may promote different types of LAB. When meat sausage is hygienically handled, the number of LAB on the surface is very low (<100/cm^2) at the time of stuffing (Kröckel, 2013). In normal circumstances, the populations of specific LAB present can be quite variable, even between products from the same processing plant. Therefore, the predominant microflora is influenced not only by the meat environment and its conditions of storage, but also by the type of strains present initially (Kröckel, 2013; Meat Technology Update, 2007).

4. Conclusions

Lack of functional meat products such as sausages in Egyptians markets, is a major problem. On the other hand, progressive consumption of nitrate/nitrite containing

sausages is a problem too, by child specially. However, this study showed that treatment combinations containing parsley/celery juice as indirect source of nitrate/nitrite and lactic acid bacterial isolates with ability to produce either nitrate or nitrite reductase starter were shown to be comparable to a sodium nitrite-added control for color, cured pigment, and sensory measurements.

Such treatments have the highest potential to reduce of consuming level of nitrate/nitrite and at the same time outstanding antibacterial activity against some of the meat spoilage and pathogenic bacteria of some of the isolated strains could reduce microbial load too. However, additional research is required to determine the effects of parsley/celery juice with LAB on the consumer acceptability of these treatment combinations and microbial shelf-life of the nitrite-free sausage.

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EFFECT OF SOME BIOGENIC AMINES ON PROTEIN CROSS-LINKING AND GEL FORMING ABILITY OF SURIMI FROM NILE TILAPIA INDUCED BY MICROBIAL TRANSGLUTAMINASE

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ABSTRACT

Impact of different biogenic amines including putrescine, histamine and tyramine at various levels (0, 2 and 5 mmol/kg) on gel properties of surimi from Nile tilapia added with microbial transglutaminase (MTGase) at 0.4 units/g was studied. All biogenic amines were able to act as an acyl acceptor in MTGase catalysed reaction, in which ammonia was released as by-product. The addition of biogenic amines into surimi had no impact on gel forming ability in the absence of MTGase. In the presence of MTGase (0.4 units/g), breaking force and water holding capacity of all gels increased, compared to those of gels without MTGase. Nevertheless, breaking force of gel containing MTGase was lowered in the presence of biogenic amine at a level of 5 mmol/kg. Polymerisation of myosin heavy chain in gel was enhanced when MTGase was added, but took place at a slightly lower degree when biogenic amines were present. Thus, biogenic amines, especially at high concentrations, decreased gel strengthening effect of MTGase in surimi.

1. Introduction

Biogenic amines have been found in various foods, particularly fish and fish products, cheese, meat and fermented foods (Ruiz-Capillas and Jimenez-Colmenero, 2004). They are low molecular-weight organic compounds, which are derived from the corresponding amino acids when the carboxylic group is removed by enzymatic reactions (Visciano et al., 2012; Zhai et al., 2012).

During storage and processing, food proteins can be degraded into free amino acids. When food is contaminated with bacteria containing decarboxylase, these free amino acids undergo decarboxylation and biogenic amines are produced (Ruiz-Capillas and Jimenez-Colmenero, 2004). In addition to the availability of free amino acids as precursors, the presence of microorganisms with

decarboxylases and favorable conditions for their growth and decarboxylation activity are required (Zarei et al., 2011; Zhai et al., 2012).

The concentrations of some biogenic amines (tyramine, putrescine, and histamine) normally increase during processing and storage of meat and fish (Ruiz-Capillas and Jimenez-Colmenero, 2004; Visciano et al., 2012). Özogul and Özogul (2006) reported that biogenic amine contents in sardines (Sardina pilchardus) increased with increasing storage time. The accumulation of histamine in humans causes health risks when the level is above 500 mg/kg. USFDA (2001) has also recommended that 100 mg/kg of tyramine and 1000 mg/kg of total biogenic amines should be the tolerance levels in fish.

Microbial transglutaminase (MTGase) has been widely used to induce the polymerisation of proteins, thereby increasing the gel strength of surimi (Benjakul et al., 2008; Duangmal and Taluengphol, 2010; Jiang et al., 2000). MTGase induces the formation of an ε-(γ-glutamyl) lysine cross-link in the proteins via acyl transfer between the ε-amino groups of a lysine residue and γ-carboxamide group of a glutamine residue (Motoki and Kumazawa, 2000). MTGase is also capable of catalysing acyl transfer reactions between the γ-carboxyamide of glutaminyl residues and various primary amines, diamine or polyamine in peptides or protein (acyl acceptors) (Motoki and Kumazawa, 2000). The amino groups of biogenic amines can function as acyl acceptor, and also other amines such as putrescine, ethylenediamine, spermine, spermidine and 3,3-iminobispropylamine have been reported to function as acyl acceptors (Lai et al., 2004; Punakivi et al., 2006; Schrode and Folk, 1978).

Nile tilapia is one of the important freshwater fish, contributing more than 40% to total aquaculture production in Thailand (Rawdkuen et al., 2009). Moreover, the low level of biogenic amines in tilapia has been reported, particularly after a long storage period (Kulawik et al., 2013; Sil et al., 2008). Since biogenic amines can serve as an acyl acceptor in acyl transfer reaction mediated by MTGase, the cross-linking between protein and protein by MTGase in surimi, especially produced from unfresh fish with high biogenic amines can be impeded. Nevertheless, there is no information regarding the effect of biogenic amines on gelling properties of surimi added with MTGase. Therefore, the aim of this study was to investigate the impact of some biogenic amines on gel forming ability of Nile tilapia surimi mediated by MTGase.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME), glycerol, histamine, glutaraldehyde, tyramine and putrescine were purchased from Sigma (St. Louis, MO, USA). Acrylamide, bisacrylamide and N,N,N',N'-tetra methylethylenediamine (TEMED) were procured from Fluka (Buchs, Switzerland). Microbial transglutaminase (MTGase) from Streptoverticillium mobaraense (TG-K) containing 1% of pure enzyme was supplied by Ajinomoto (Thailand) Co., Ltd. (Bangkok, Thailand).

2.2. Fish samples

Live Nile tilapia (*Oreochromis niloticus*) with the average size of 800-1000 g were obtained from a local market in Songkhla province, Thailand. After head-blowing, fish were transported in ice (fish/ice ratio of 1:2, w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 30 min. Fish were then washed and filleted manually. Subsequently, the fillets were deskinned and minced using a mincer with a hole diameter of 5 mm. Mince was stored in ice during preparation.

2.3. Effect of different biogenic amines on acyl transfer reaction in natural actomyosin mediated by MTGase

2.3.1. Preparation of natural actomyosin (NAM)

NAM was prepared according to the method of Benjakul et al. (1997). Fish mince (10 g) was homogenised in 100 ml of chilled

0.5 M NaCl, pH 7.0 using a homogeniser for 4 min at a speed of 11,000 rpm (IKA Labortechnik, Selangor, Malaysia). Overheating during extraction was avoided by keeping the sample in an iced container and each 20 s of homogenisation was followed by a 20 s rest interval. The homogenate was centrifuged at 5,000xg for 30 min at 4 °C using a refrigerated centrifuge (Avanti-JE Centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Three volumes of chilled water (0-4 °C) were added to precipitate NAM, which was then collected by centrifuging at 5,000xg for 20 min at 4 °C using a refrigerated centrifuge. The pellets were then dissolved by gradually stirring in an equal volume of chilled 0.45 M NaCl, pH 7.0 for 30 min at 4 °C.

2.3.2. MTGase mediated reaction in the presence of various biogenic amines

Ten ml of NAM solution (5 mg/ml) containing biogenic amines at different levels (0, 2, and 5 mM) were allowed to stand in ice for 1 h. The prepared solutions were preheated at 40 °C for 10 min in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany). Thereafter, 100 µl of MTGase was added into NAM to obtain a level of 20 units/g protein. The reaction mixtures were incubated at 40 °C for 30 min. The reaction was terminated by adding 10 ml of 15% (w/v) trichloroacetic acid. The resulting reaction mixtures were then subjected to the determination of ammonia content.

2.3.3. Determination of ammonia content

Ammonia content in reaction mixtures was determined according to the Standard Methods (APHA-AWWA/WPCF, 1998). Appropriately diluted sample (500 ml) was placed in 800-ml Borosilicate glass flask containing 25 ml of 0.125 M $Na_2B_4O_7$ and pH was adjusted to 9.5 using 6 N NaOH. The mixture was distilled and the distillate was collected in 50 ml of 2% boric acid containing the indicator (methyl red: methylene blue). The solution was then titrated with 0.02 N H_2SO_4 to reach the end-point. Ammonia content was expressed as mg/l using the following equation:

Ammonia content (mg/l) = (ml H_2SO_4 x 280)/ml of sample

2.4. Effect of MTGase on gel properties of surimi containing different biogenic amines at various levels

2.4.1. Preparation of surimi gel containing biogenic amines

The mince was then washed with cold water (5-8 °C) at a mince/water ratio of 1:3 (w/v). The mixture was stirred gently for 3 min and washed mince was filtered with a layer of nilon screen. The washing process was carried out three times. Finally, the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Grandiumpiant, Belluno, Italy) with a speed of 700 xg for 15 min. Washed mince was mixed thoroughly with 4% sorbitol and 4% sucrose as cryoprotectants. The resulting mixture was frozen at -18 °C using an air-blast freezer. The frozen surimi was kept at -20 °C until used. Before use, frozen surimi was tempered using a running water until temperature reached 0-2 °C.

To determine the effect of different biogenic amines (putrescine, tyramine and histidine) on gel forming ability of surimi, biogenic amines were added to the surimi to obtain the final concentrations of 0, 2, 5 mmol/kg and chopped for 3 min at 4 °C. The resulting mixture was allowed to stand for 1 h at 4 °C. The mixture was then added with 2.5% salt and the moisture content was adjusted to 82 % in the absence and presence of MTGase (0.4 units/g). The mixture was chopped for another 3 min to obtain the homogenous paste. The paste was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min. The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

2.4.2. Texture Analysis

Texture analysis of surimi gels was carried out using a Model TA-XT_2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (28 to 30 °C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

2.4.3. Determination of expressible moisture content and whiteness

Expressible moisture content and whiteness of surimi gels were determined according to the method of Benjakul et al. (2003).

2.4.4. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of surimi gels were analysed by SDS-PAGE according to the method of Laemmli (1970). To prepare the sample, 27 ml of 5% (w/v) SDS solution (85 °C) were added to the gel sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3,500xg for 20 min to remove undissolved debris. The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% β-ME. The samples (15 µg of protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid.

2.4.5. Microstructure analysis

The microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces (0.25 × 0.25 × 0.25 cm3) and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed samples were rinsed twice with distilled water. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100%. Samples were critical point dried (Balzers model CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on an FEI Quanta 400 SEM (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

2.5. Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). **3.**

3.Results and discussions

3.1. Effect of different biogenic amines on acyl transfer reaction in NAM mediated by MTGase

The effect of different biogenic amines including putrescine, histamine and tyramine at a concentration of 2 mM in NAM (5 mg/ml) as acyl acceptor in the MTGase catalysed reaction was determined by monitoring the formation of ammonia as shown in Table 1. MTGase can induce acyl transfer reaction, in which the acyl of γ-carboxamide groups of peptides is transferred to the ε-amino group of lysine residues and a variety of primary amines (acyl acceptor) (Folk and Finlayson, 1977; Gaspar and de Góes-Favoni, 2015; Motoki and Seguro, 1998). As the reactions mediated by MTGase

result in the formation of free ammonia, the measurement of ammonia can be used as a tool to monitor the overall transglutaminase reaction (Punakivi et al., 2006).

Table 1. Ammonia content in NAM formed via MTGase catalysed reaction in the absence and presence of different biogenic amines.

Samples	Ammonia content)mg/l(
NAM	ND**
NAM with MTGase	7.60±0.10a*
NAM+2 mM putrescine with MTGase	10.27±0.81b
NAM+2 mM histamine with MTGase	10.73±0.81b
NAM+2 mM tyramine with MTGase	9.80±0.81b

Values are given as mean ± SD (n=3)

* Different lowercase superscripts indicate the significant differences (P<0.05)

** ND: not detectable

When MTGase was added into NAM, ammonia was produced, indicating that cross-linking of NAM occurred intermolecularly or intramolecularly. Therefore, NAM could serve as the substrate for transglutaminase. Several studies have been reported that myofibrillar protein, especially myosin, is a good substrate for MTGase mediated reaction (Ahhmed et al., 2009; Chin et al., 2009; Ramirez-Suarez and Xiong, 2002). Ahhmed et al. (2009) reported that MTGase was able to catalyse the interconnections of myofibrillar protein extracted from beef and chicken, resulting in the significant increase in ε-(γ-glutamyl)lysine content. However, no ammonia was found in NAM in the absence of MTGase, indicating that no acyl transfer reaction took place. In the presence of biogenic amines at a level of 2 mM, the concentration of ammonia increased, compared to that without biogenic amines (P<0.05). Nevertheless, no difference in ammonia content was found between NAM added with different types of biogenic amines (P>0.05). Punakivi et al. (2006) reported the formation of ammonia in MTGase catalysed reaction using N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) as donor substrate and biogenic amines as acceptor. Cadaverine was the best acceptor substrate for MTGase with the highest concentration of ammonia produced (Punakivi et al., 2006). The similar concentration of ammonia produced from MTGase reaction having tyramine and histmine as acyl acceptor was found. Those biogenic amines were smaller in size in comparison with muscle proteins, where lysine was located. As a result, those biogenic amines could expose or localise themselves for acyl reaction more effectively than proteins. This was indicated by the higher ammonia content (Table 1). Therefore, putrescine, histamine and tyramine were shown to be the better acyl acceptor for MTGase reaction than NAM.

3.2. Effect of MTGase on gel properties of surimi in the presence of biogenic amines at different levels

3.2.1. Breaking force and deformation

Breaking force and deformation of gels from Nile tilapia surimi in the presence of biogenic amines (putrescine, histamine and tyramine) at 0, 2, and 5 mmol/kg without and with the addition of MTGase (0.4 units/g) are depicted in Fig. 1. Without MTGase addition, no difference in breaking force was found in gels without and with the addition of biogenic amines (P>0.05). Thus, biogenic amine ranging from 0 to 5 mmol/kg had no effect on gel forming ability of surimi from Nile tilapia.

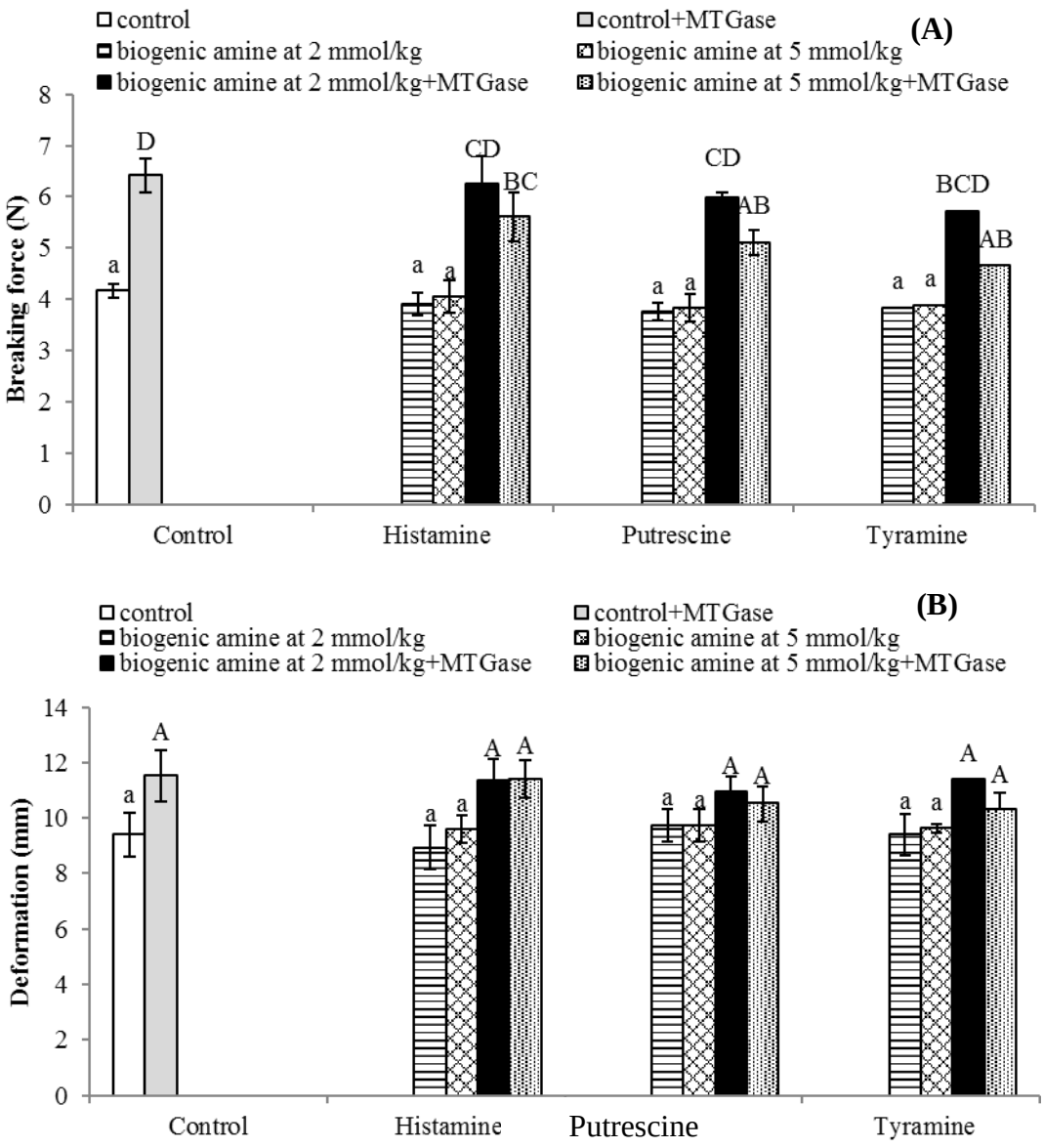


Figure 1. Breaking force (A) and deformation (B) of gels from Nile tilapia surimi containing different amines at various levels in the absence and presence of MTGase (0.4 units/g). Bars represent the standard deviation (n=3). Different uppercase or lowercase letters on the bars within the same MTGase level indicate significant difference (P<0.05). Lowercase letters on the bars indicate the significant difference (P<0.05).

When MTGase at a level of 0.4 units/g was incorporated into surimi gel, the increase in breaking force by 53.9% was observed, compared with that of the control gel (without MTGase and biogenic amines). MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins, in which ε-(γ-glutamyl) lysine cross-links between protein chains could be formed. As a result, the strength of gel matrix was increased. In the presence of biogenic amines at 2 mmol/kg, similar breaking force was noticeable,

compared to the control gel. It was suggested that the strong gel development was still achieved by addition of MTGase when biogenic amines were present at low level. These cross-links via covalent bonds altered the functional properties of food proteins (Gaspar and de Góes-Favoni, 2015). However, the strengthening effect of MTGase on surimi gel was lowered as biogenic amines at 5 mmol/kg were incorporated, regardless of type of biogenic amines. With the addition of MTGase, breaking force of gel from surimi added with tyramine at 5 mmol/kg had the increase by 19.7%, compared to that without MTGase, followed by gels containing putrescine and histamine, respectively. Apart from the cross-linking reaction, the other two reactions catalysed by transglutaminase are deamidation and amine incorporation (Motoki and Seguro, 1998). At high concentration, biogenic amines could function as a competitive substrate in the cross-linking reaction. Therefore, the presence of biogenic amines including histamine, tyramine and putrescine in surimi might prevent some intermolecular cross-linking between myofibrillar protein mediated by MTGase. Those biogenic amines could be found in unfresh fish used for fish mince or surimi production. This might be related with inferior setting phenomenon in poor quality surimi or the lower gel strengthening effect of MTGase for poor quality surimi.

For deformation, there was no marked difference amongst gels without and with biogenic amines (P>0.05). However, it was noted that deformation of gel slightly increased when MTGase was added (P<0.05). MTGase could induce the cross-linking of muscle protein. The interaction between those protein chains might yield the gel network with more elasticity as evidenced by the increased deformation. Although biogenic amines, particularly at higher level, lowered breaking force, they did not show the adverse effect on deformation of the resulting gels.

3.2.2. Expressible moisture content

Expressible moisture content of gels from surimi containing biogenic amines at different levels in the absence and presence of MTGase (0.4 units/g) is shown in Table 2.

Table 2. Expressible moisture content and whiteness of gels from Nile tilapia surimi containing different biogenic amines at various levels in the absence and presence of MTGase (0.4 units/g).

	Biogenic amine concentration)mmol/kg(	Expressible moisture content		Whiteness	
		Without MTGase	With MTGase	Without MTGase	With MTGase
Control		5.042±0.415[a]	4.470±0.286[a]	85.154±0.272[a]	85.365±0.440[a]
Putrescine	2	5.128±0.205[a]	4.966±0.364[a]	85.174±0.529[a]	85.530±0.602[a]
	5	5.175±0.233[a]	4.967±0.242[a]	85.351±0.381[a]	85.527±0.285[a]
Histamine	2	5.158±0.487[a]	4.664±0.300[a]	85.418±0.592[a]	85.629±0.259[a]
	5	5.176±0.382[a]	4.679±0.281[a]	85.080±0.732[a]	85.297±0.855[a]
Tyramine	2	5.154±0.373[a]	4.635±0.373[a]	85.096±0.747[a]	85.339±0.240[a]
	5	5.184±0.326[a]	4.712±0.252[a]	84.872±0.310[a]	85.271±0.497[a]

Values are given as mean ± SD (n=3).

* Different lowercase superscripts within the same column indicate the significant differences (P<0.05).

No difference in expressible moisture content of gels was noticeable when biogenic amines were incorporated in surimi, regardless of the types of biogenic amines. Non-significant decreases in expressible moisture content were observed in all gels, when MTGase was added (P>0.05). The lower expressible moisture content of gels suggested that more water was retained in the gel network (Niwa, 1992). This property depends on the structure of the muscle proteins that bind and interact with the water molecules in the gel network. It was noted that the addition of MTGase could increase the ability of gel in water holding as evidenced by the lowered expressible moisture content. The addition of MTGase could enhance the cross-linking of proteins to some degree, resulting in the formation of stronger network with the greater water holding capacity (Benjakul et al., 2008). With increasing water holding capacity, the better textural properties including stiffness, cohesion, chewability and elasticity of protein gels were obtained (Gaspar and de Góes-Favoni, 2015; Han et al., 2009; Min and Green, 2008). Although biogenic amines were able to serve as acyl acceptor for MTGase mediated reaction, the interconnected network between the main proteins, myosin heavy chain or actin, still took place. As a consequence, the network was able to imbibe water effectively. Therefore, biogenic amines at 2 or 5 mmol/kg showed no mark effect on water holding capacity of the resulting gels.

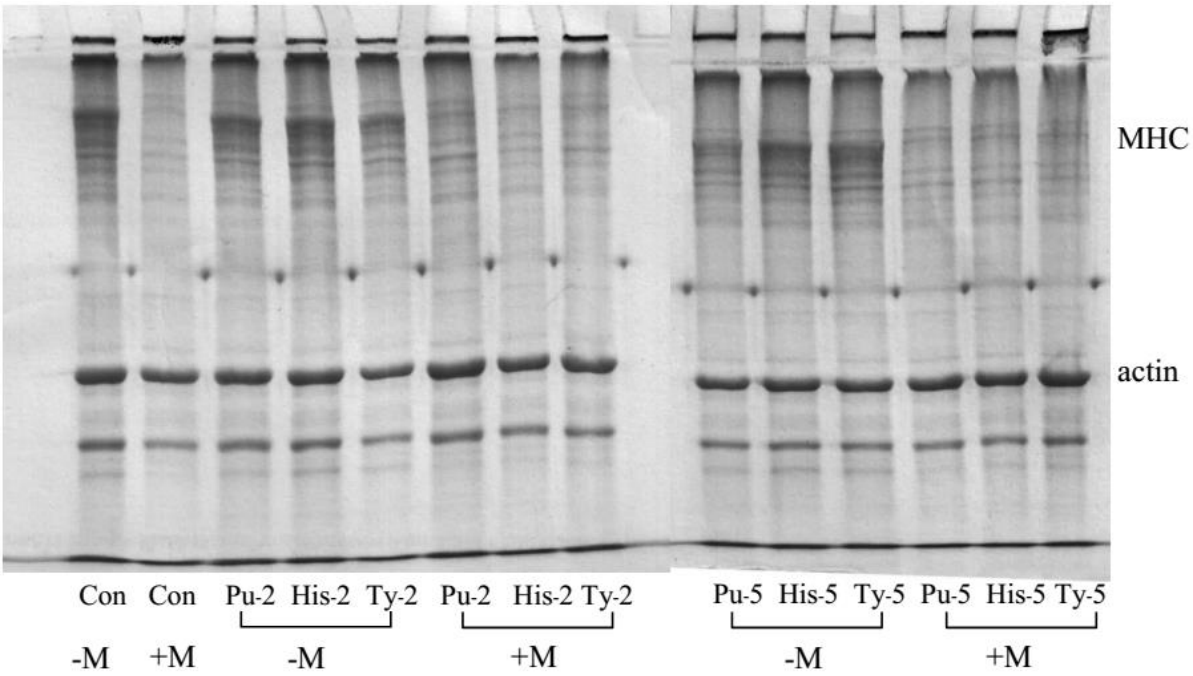
3.2.3. Whiteness

Whiteness of gels from surimi containing biogenic amines at different levels in the absence and presence of MTGase is shown in Table 2. Similar whiteness was observed amongst gels containing biogenic amines, irrespective of types and levels (P>0.05). Also, no differences in whiteness were found between gel added without and with MTGase addition. Kaewudom et al. (2012) also reported that the addition of MTGase at 1.2 units/g had no impact on whiteness of gels from threadfin bream surimi. Thus, the presence of putrescine, histamine and tyramine had no influence on the whiteness of gels from Nile tilapia surimi, irrespective of MTGase addition.

3.2.4. Protein pattern

Protein patterns of surimi gels containing biogenic amines at different levels in the absence and presence of MTGase are shown in Fig. 2. The control gels (without MTGase) contained myosin heavy chain (MHC) and actin as the major proteins. Similar pattern was noticeable between the control gel and those containing biogenic amines (2 and 5 mmol/kg). The result showed that biogenic amines had no pronounced impact on protein cross-linking.

When MTGase was added into the control gel, the intensity of MHC band decreased drastically, compared to that without MTGase addition. The disappearance of MHC indicated the formation of MHC cross-links mainly via the formation the ε-(γ-glutamyl)lysine isopeptide induced by MTGase. MHC has been known to serve as the major contributor for gel formation and determine gel property of fish protein (Nakahara et al., 1999). Although MHC band intensity decreased markedly, actin and tropomyosin were rarely changed in all samples. Thus, MHC appeared to be a preferable substrate for cross-linking induced by MTGase. Herrero et al. (2008) reported that the cross-links introduced by MTGase altered the structure of MHC, with a significant reduction in the content of the α-helix structure, and an increase in the β-sheet and percentage of pleats/folds, thereby allowing for the formation of high molecular weight polymers. These modifications improved textural properties, resulting in strong gels with a compact and ordered structural conformation (Gaspar and de Góes-Favoni, 2015).



noted that biogenic amines in surimi had no

Figure 2. Protein patterns of gels from Nile tilapia surimi containing different biogenic amines at various levels in the absence (-M) and presence (+M) of MTGase (0.4 units/g). Con: control (without amine); Pu: putrescine; His: histamine; Ty: tyramine; MHC: myosin heavy chain. Numbers designate the levels of biogenic amines added (mmol/kg); M: MTGase

When biogenic amine was incorporated into surimi in the presence of MTGase, MHC in gel was remained to some degree, compared to that found in the control gel. This confirmed that the cross-linking of muscle proteins was lowered when biogenic amines were present, resulting in the lowered protein-protein interaction. Lai et al. (2004) reported that the primary amine including putrescine and biotinylated pentylamine lowered the ability of transglutaminase in cross-linking glutamine monomers into high molecular weight complexes. The results were in accordance with the ability of biogenic amines as acyl acceptor in MTGase mediated reaction (Table 1).

3.2.5. Microstructure

Gels from Nile tilapia surimi showed the well-structured and fine matrix with highly interconnected strands. Similar structure was found between surimi gels without and with the addition of biogenic amines (Fig. 3). It was marked impact on microstructure of surimi gel. In the presence of MTGase, proteins could undergo the cross linking more effectively. Gel became slightly more compact, with a denser gel network and smaller voids as MTGase was added. Therefore, the addition of MTGase could induce the formation of stronger gel network as evidenced by a higher breaking force (Fig. 1) along with lowered expressible moisture content (Table 2). However, the control gel in the presence of MTGase showed the most compact network with the smallest voids, compared to gels containing biogenic amines, especially at 5 mmol/kg. MTGase induced the formation of isopeptide bonds between glutamine and lysine residues in proteins.

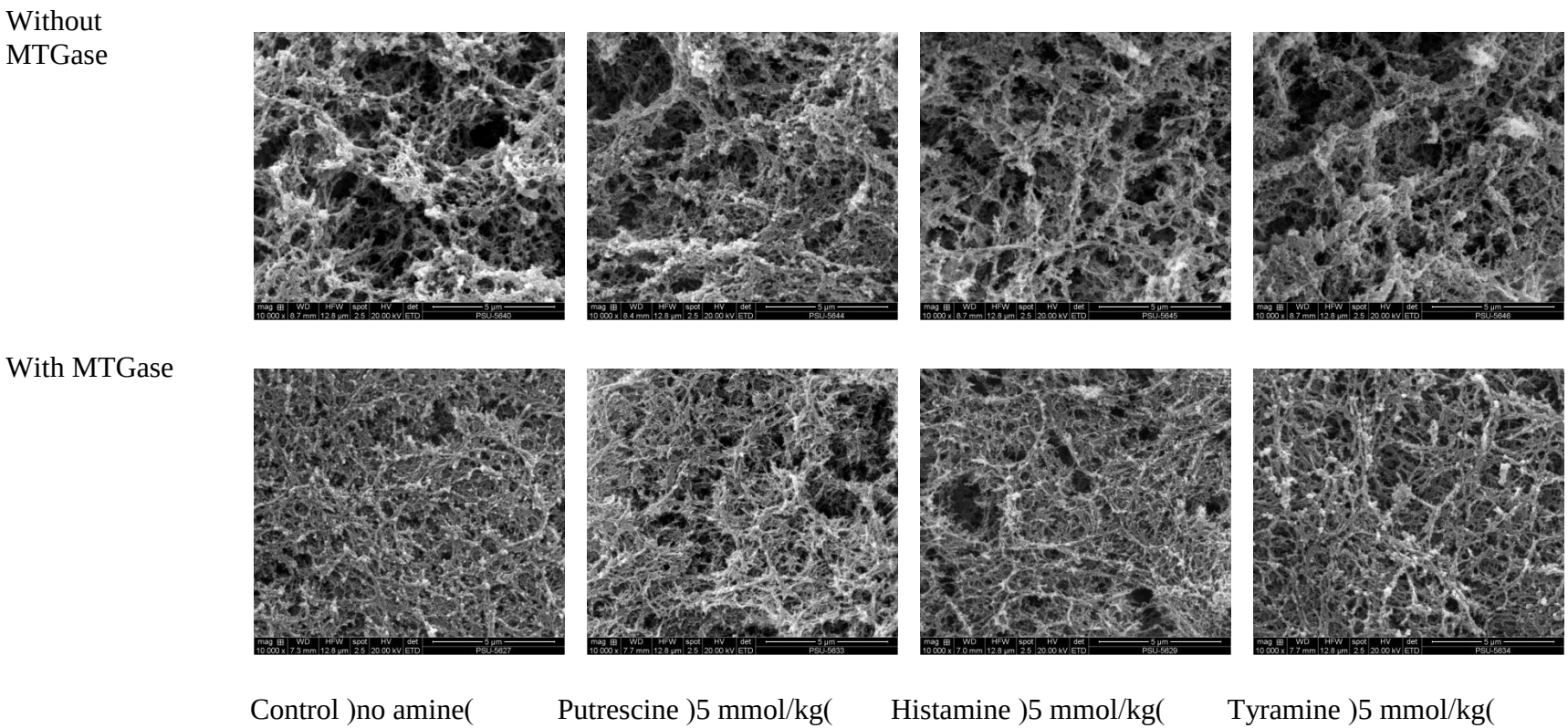


Figure 3. Electron microscopic image of Nile tilapia surimi gel added with different biogenic amines (5 mmol/kg) in the absence and presence of MTGase (0.4 units/g) (Magnification: 10,000×).

However, the control gel added with MTGase showed the most compact network with the smallest voids, compared to gels containing biogenic amines, especially at 5 mmol/kg. MTGase induced the formation of isopeptide bonds between glutamine and lysine residues in proteins, thus introducing both inter- and intramolecular covalent cross-links (Motoki and Seguro, 1998). In the presence of biogenic amines, MTGase could induce cross-linking between glutamine and amines to a lower extent. Thus, protein-protein interaction was decreased. Nevertheless, in the presence of MTGase, there was no difference in microstructure of gel containing different biogenic amines.

4. Conclusions

Putrescine, histamine and tyramine were able to serve as the acyl acceptors in MTGase catalysed reaction. Biogenic amines had no profound effect on gelling properties of surimi in the absence of MTGase. However, biogenic amines, especially at high concentration, acted as the competitive substrates for MTGase, thereby lowering the cross-linking between muscle proteins. This led to the slight decrease in gel strength of resulting surimi gel. **5.**

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PROXIMATE AND MINERAL ANALYSIS OF A FEW EDIBLE FLOWERS USED IN COIMBATORE DISTRICT, TAMIL NADU

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ABSTRACT

Vegetables are any part of the plant such as root, tuber, bulb, stem, leaf, flower and seed. The local inhabitants of Coimbatore District mainly depend on wild indigenous vegetables around their locality for food. This paper deals about the proximate and mineral analysis of eight edible flowers used by the local people of Coimbatore District. Carbohydrate and crude fibre were maximum in the flowers of *Cucurbita maxima* (29.37±0.02% per g and 4.63±0.21% per g respectively), protein and moisture content were maximum in *Cassia auriculata* (22.95±0.01% per g and 91.83±0.54% per g respectively), lipid was maximum in *Benincasa hispida* (3.57±0.01% per g) and ash content was maximum in *Sesbania grandiflora* (1.76±0.04% per g). The contents of calcium was high in *B. hispida* (1.92±0.06 mg/ g), magnesium and potassium was high in *C. maxima* (0.98±0. mg/g and 0. 39±0.67 mg/g respectively).

1.Introduction

Among various kinds of plant, food plants received the earliest attention of mankind. Vegetables have been a part of human diet from time immemorial. Vegetables help to maintain healthy body weight. They are the store houses of vitamins, antioxidants, minerals and phytochemicals. Vegetables are low in calories and high in fibre and water content. According to the joint report of FAO/WHO minimum intake of 400g of vegetables and fruits was recommended to prevent the chronic diseases such as diabetes, cancer, obesity and heart disease and also to alleviate the micronutrient deficiencies in less developed countries.

Changing life style and globalization have favoured only few major vegetable crops which dominate in the production. As a result, some species of vegetable crops become lesser known. Lesser known vegetables are the local and traditional crops, whose distribution, biology, cultivation and uses are not well known. These wild food plants are consumed as food for their health benefits. They are the valuable sources of energy and micronutrients in the diet of the local communities. Flowers have been also used as food for their peculiar taste, colour and fragrance. In addition to this, they also have nutritional value. So far there are no reports on the flowers as vegetables.

Hence the present study was undertaken to determine the proximate and mineral analysis of a few flowers used as food in Coimbatore district. Usually the vegetative parts of any plant such as root, stem, leaf and fruits are used as vegetable. But it is quite interesting to note that the local communities of Coimbatore District use some of the flowers as food supplement during their season.

2. Materials and methods

2.1. Study area

Coimbatore District was located in the western part of Tamil Nadu located in between 10º 10' and 11º 30' North Latitude, 76º 40' and 77º 30' East Longitude, total area of 246.8 Km^2. It is surrounded by the Western Ghats in West and Nilgiri biosphere in North, Noyyal river in South. Black soil with red loamy soil were predominant. The minimum and maximum temperature in Coimbatore was between 35º C and 18º C.

2.1.1. Data collection method

In order to document the utilization of edible flowers in Coimbatore District, a survey was carried out during the August 2013-September 2014 among the indigenous local communities. They are not always confined to rural or remote areas, many of them were living in urban areas and continues to follow many traditional food habits.

The data collection of traditional knowledge of the lesser known edible flowers was recorded by making personnel visit and interview through questionnaire method. A brief discussion was made with them in their local language (Tamil) and the information on the edible flowers from the local communities was collected and compiled in the Table -1, providing their botanical name, local name, recipe and flowering season.

2.2. Plant collection

The flowers of *Azadirachta indica*, *Moringa oleifera*, *Cassia auriculata*, *Sesbania grandiflora*, *Sesbania sesban* and *Tamarindus indica*, *Benincasa hispida* and *Cucurbita maxima* were collected during their flowering season and identified using Flora of the Presidency of Madras (Gamble, 1847-1925).

2.3. Proximate analysis of the underutilized flowers

Fresh flowers were collected, washed in tap water and large sized flowers were cut in to small pieces and small flowers were used as such to determine the carbohydrate, protein, lipid, crude fibre, moisture and ash content.

2.3.1. Carbohydrate content (Hedge and Hofreiter, 1962)

The flowers were hydrolysed in boiling water bath for 3 hours with 2.5 N Hydrochloric acid and cooled at room temperature. It was neutralised with sodium carbonate and makeup the volume with distilled water, centrifuged for 5 minutes. The supernatant was taken with anthrone reagent and placed in boiling water bath for 8 minutes, cooled and optical density of the sample was read out in spectrophotometer at 630 nm. Glucose was used as a standard.

2.3.2. Protein content (Lowry et al., 1951)

Preparation of Reagent C- Reagent A (sodium hydroxide + sodium carbonate in distilled water) + Reagent B (sodium potassium tartrate + copper sulphate in distilled water)

The sample was ground using pestle and mortar with phosphate buffer, centrifuged for 5 minutes at 2000 rpm and the supernatant was collected. One millilitre of the sample was taken in test tube and 1N sodium hydroxide was added. After 5 minutes, Reagent C was added kept undisturbed for 5 minutes at room temperature. After that Folin Ciocalteau reagent was added, kept in water bath for 5-10 minutes, cooled and optical density of the sample was read out in spectrophotometer at 660 nm. Bovine serum albumin was used as a standard.

2.3.3. Lipid content (Bligh and Dyer, 1959)

Lipid content was determined gravimetrically by separating the water and solvent layers. The sample was homogenized with distilled water, chloroform and methanol. Then, it was centrifuged at 2000 rpm for 20 minutes. After centrifugation, the supernatant was transferred in to a separating funnel, undisturbed for 1 hour. The lipid layer determined by evaporating the solvent layer.

2.3.4. Crude fibre content (Maynard, 1970)

Subsequent acid and alkaline treatment results in hydrolytic degradation of cellulose and

lignin. The residue obtained was weighed, incinerated, cooled and weighed. Crude fibre was determined by the loss in weight.

The flower was defatted by petroleum ether and was boiled with dilute sulphuric acid for 30 minutes. The filtered residue was boiled with sodium hydroxide for 30 minutes. Again filtered and washed with dilute sulphuric acid, water and alcohol. Residue was weighed (W_1) and transferred to ashing dish and dried for 2 hours at 130◦ C, cooled and weighed (W_2). The residue was ignited at 600◦ C for 30 minutes, cooled in desiccator (W_3).

Crude fibre % = Loss in weight on ignition

$$\frac{(W_2 - W_1) - (W_3 - W_1) \times 100}{\text{Weight of the sample}}$$

2.3.5. Moisture content (AOAC, 2000)

Moisture content was determined by the difference in the weight of the flowers before and after drying in hot air oven. Empty dish and the lid was dried in oven and cooled in the desiccator. The empty dish and the lid were weighed. Three grams of the flowers was spreaded uniformly in the dish and placed on oven at 105◦C for 3 hours. After 3 hours the dish was cooled in desiccator and reweighed.

$$\text{Moisture content\%} = \frac{(W_1 - W_2) \times 100}{W_1}$$

W_1 = Weight (g) of the sample before drying;
W_2 = Weight (g) of the sample after drying

2.3.6. Ash content (AOAC, 2000)

Silica crucible and the lid were dried in oven and cooled in the desiccator. The empty silica crucible along with the lid were weighed. The sample was taken in the crucible and placed in the muffle furnace at 550◦C overnight. The crucible was cooled down with the lid in the desiccator and weighed.

$$\text{Ash \%} = \frac{\text{Weight of the ash}}{\text{Weight of the sample}} \times 100$$

2.4. Mineral analysis of the underutilized flowers

Mineral analysis was carried out by digesting the powdered sample with 10 ml of the mixture of nitric acid, sulphuric acid and perchloric acid. The digest was allowed to cool and makeup to 100 ml with deionised water and used for further analysis.

2.4.1. Calcium (Heald et al., 1965)

The digested sample was diluted with distilled water. The masking reagent (Cyanide solution, Hydroxylamine, Potassium ferrocyanide solution and Triethanolamine) and dilute sodium hydroxide was added drop wise to raise the pH to 12. After that, calcan indicator was added drop wise and titrated with standardized EDTA, end point was the colour change from red to blue.

2.4.2. Magnesium (Heald et al., 1965)

The digested sample was diluted with distilled water. Buffer solution and sodium tungstate solution was pipetted out in to the sample and pH was raised to 10. The contents were heated for 1 hour, cooled and filtered. To that precipitate, masking reagent (Cyanide solution, Hydroxylamine, Potassium ferrocyanide solution and Triethanolamine) was added and after the reaction takes place eriochrome black T indicator was added and titrated with standardized EDTA, end point was the colour change from red to permanent blue.

2.4.3. Potassium (Mutalik et al., 2011)

Potassium content was measured by using flame photometer. The sample was drawn into a non-luminous flame which gets ionised and the energy gets absorbed and light was emitted in a characteristic wavelength in the unexcited ground level. The intensity of the radiation emitted by the sample depends upon the concentration of the potassium. Pressure was maintained at 10 lbs/sq.inch and the sample was analysed. Potassium chloride was used as a standard.

ppm = milliEquivalent/litre of K^+ ion × atomic weight of potassium mg of potassium ion present in 100g of the sample = ppm × 100.

3.Results and discussions

3.1. Edible flowers along with thier local names and recipes

Vegetables play an important role in nourishing the ever-growing population due to their nutritive value. The habit of consuming variety of food has a traditional background. The benefits in consuming fresh vegetables are regulating cell proliferation, detoxifying carcinogens, protection against cancer. These vegetables contain vitamins, minerals and phytochemicals. The phytochemicals acts as antioxidants which has the ability to detoxify the carcinogens. The local communities of Coimbatore District, consume the flowers of *Azadirachta indica* (open flowers and buds), *Moringa oleifera* (open flowers and buds), *Cassia auriculata* (open flowers and buds), *Sesbania grandiflora* (buds), *Sesbania sesban* (open flowers and buds), *Tamarindus indica* (open flowers and buds), *Benincasa hispida* (petals of open flowers) and *Cucurbita maxima* (petals of open flowers), during their flowering season (Table-1). They were prepared as rasam, kootu, poriyal, thuvaiyal, idly, chutney, pachadi and soup etc.,

3.2. Proximate analysis of the edible flowers

Proximate analysis of the edible flowers was made to determine the carbohydrate, protein, lipid, moisture, ash and crude fibre content (Table-2).

Carbohydrate content ranges from 5.04 - 29.37% per g of the flowers. Maximum carbohydrate content was noted in *C. maxima* (29.37±0.02% per g) followed by *A. indica* (28.80±0.04% per g) and lower in *M. oleifera* (5.04±0.02% per g) respectively. *C. maxima* seeds helps in preventing the formation of kidney stones, by reducing the level chemical substances which can promote the stone formation (Suphakarn *et al*., 1987; Suphipat *et al*., 1993).

Protein content ranges from 6.12 - 22.95% per g of the flowers. It was maximum in *C. auriculata* (22.95±0.01% per g) followed by *S. sesban* (22.60±0.02% per g) and lower in *C. maxima* (6.12±0.01% per g) respectively. The tea prepared from *C. auriculata* leaves was used traditionally for curing chronic fever (Joshi *et al*., 2007).

Lipid content ranges from 0.22 - 3.57% per g of the flowers. It was maximum in *B. hispida* (3.57±0.01% per g) followed by *A. indica* (2.34±0.01% per g) and lower in *T. indica* (0.62±0.01% per g). In ayurveda, the fruits of *B. hispida* were used to treat peptic ulcer (Warrier *et al*., 1994). In ayurveda, the flowers of *A. indica* were used for the elimination of intestinal worms and for bile suppression (Biswas *et al*., 2002).

Moisture content ranges from 40.37 - 91.83% per g of the flowers. It was maximum in *C. auriculata* (91.83±0.54% per g) followed by *C. maxima* (91.37±0.08% per g), *B. hispida* (91.33±0.81% per g) and lower in *T. indica* (50.37±0.47% per g). The water content in vegetables enhance the food digestion and peristaltic movement on consumption.

Ash content was the measure of the mineral content of the vegetable, ranges from 0.23 - 1.76% per g of the flowers. It was maximum in *S. grandiflora* (1.76±0.04% per g) followed by *C. maxima* (1.50±0.07% per g) and lower in *M. oleifera* (0.23±0.04% per g). Flower extract of *S. grandiflora* used to treat night blindness (Amal, 2015).

Crude fibre content ranges from 0.23 - 4.63% per g of the flowers. It was maximum in *C. maxima* (4.63±0.21% per g) followed by *M. oleifera* (2.43±0.17% per g) and lower in *T. indica* (0.23±0.01% per g). The fibre content in vegetables has a positive impact in the digestion and constipation.

Mineral analysis of the edible flowers

The mineral elements are required in small amounts used by the body in many ways. They are the structural component of body tissues, maintains the acid-base balance, involved in contraction of muscles and transport of gases

(Murray *et al*., 2000). The mineral content of the edible flowers was present in Table 3.

Calcium content ranges from 0.08 - 1.92 mg/g of the flowers. Highest calcium content was observed in *B. hispida* (1.92 ±0.06 mg/g) followed by *M. oleifera* (0.52±0.34 mg/g) and lower in *S. sesban* (0.08±0.02 mg/g). Regular intake of the recommended calcium protects against osteoporosis.

Magnesium content ranges from 0.02- 0.98 mg/g of the flowers. It was found to be high in *C. maxima* (0.98±0.52 mg/g) followed by *C. auriculata* (0.67±0.76 mg/g) and lower in *S. sesban* (0.02±0.04 mg/g) respectively.

Potassium content ranges from 0.05 - 0.39 mg/g, was found to be high in *C. maxima* (0.39±0.67 mg/g) followed by *A. indica* (0.18±0.34 mg/g) and lower in *T. indica* (0.05±0.03 mg/g). Potassium, an important cation in regulating blood pressure, hence the consumption of potassium rich vegetables prevents hypertension (He and MacGregor, 2008).

Table 1. List of edible flowers, their local names and recipes

S.No	Plant species	Local Name	Recipes	Flowering season
1	*Azadirachta indica* A. Juss Meliaceae	Veppam	Prepared as veppam poo rasam, thuvaiyal and dried flowers were fried and eaten with salt and pepper	March – July
2	*Moringa oleifera* Lam. Moringaceae	Murungai	Cooked as poriyal	Throughout the year
3	*Cassia auriculata* Linn. Fabaceae	Avaran	It is prepared in the form of soup, idly	August – September
4	*Sesbania grandiflora* Linn. Fabaceae	Agathi	Cooked as poriyal	July – January
5	*Sesbania sesban* Linn. Fabaceae	Chithagathi	Cooked as mostly with eggs as omlettes	February-June
6	*Tamarindus indica* Linn. Fabaceae	Pulli	Cooked as rasam, pachadi and chutney	January – April
7	*Benincasa hispida* (Thunb.) Cogn. Cucurbitaceae	Vellaipoosani	Cooked as kootu	November – February
8	*Cucurbita maxima* Duch. Cucurbitaceae	Arasani	Cooked as kootu	November – February

Table 2. Proximate analysis of the edible flowers

S.No	Plant species	Carbohydrates %	Proteins %	Lipid %	Moisture content %	Ash content %	Crude fibre %
1	*Azadirachta indica*	28.80±0.04	13.04±0.02	2.34±0.01	54.33±0.63	0.33±0.04	0.23±0.04
2	*Benincasa hispida*	25.25±0.05	18.74±0.00	**3.57±0.01**	91.33±0.81	0.96±0.04	0.33±0.04
3	*Cassia auriculata*	13.55±0.05	**22.95±0.01**	1.18±0.01	**91.83±0.54**	1.40±0.07	1.23±0.07
4	*Cucurbita maxima*	**29.37±0.02**	6.12±0.01	1.43±0.00	91.37±0.08	1.50±0.07	**4.63±0.21**
5	*Moringa oleifera*	5.04±0.02	18.56±0.02	0.68±0.00	86.53±0.20	0.23±0.04	2.43±0.17
6	*Sesbania grandiflora*	18.46±0.01	6.13±0.03	1.77±0.00	76.64±0.20	**1.76±0.04**	1.00±0.07
7	*Sesbania sesban*	24.19±0.03	22.60±0.02	1.55±0.01	68.62±0.40	0.70±0.02	1.43±0.04
8	*Tamarindus indica*	9.29±0.02	16.80±0.02	0.22±0.01	40.37±0.47	1.16±0.02	0.43±0.01

Table 3. Mineral analysis of the edible flowers

S.No	Plant species	Calcium mg/g	Magnesium mg/g	Potassium mg/g
1	*Azadirachta indica*	0.12±0.03	0.02±0.03	0.18±0.34
2	*Benincasa hispida*	**1.92±0.06**	0.38±0.56	0.10±0.65
3	*Cassia auriculata*	0.16±0.20	0.67±0.76	0.15±0.67
4	*Cucurbita maxima*	0.40±0.40	**0.98±0.52**	**0.39±0.67**
5	*Moringa oleifera*	0.52±0.34	0.04±0.07	0.15±0.52
6	*Sesbania grandiflora*	0.16±0.04	0.14±0.79	0.10±0.78
7	*Sesbania sesban*	0.08±0.02	0.02±0.04	0.15±0.67
8	*Tamarindus indica*	0.12±0.06	0.07±0.05	0.05±0.03

4. Conclusions

The study revealed that the edible flowers are the good sources of macro and micronutrients in terms of proximate and mineral analysis. Hence regular intake of these flowers shall meet the nutritional requirement and maintain proper health.

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THE COIR SUBSTRATE FOR SOILLESS CULTURES, REUSED AS SOIL AMENDMENT (STUDY IN VITRO AND IN VIVO)

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ABSTRACT

The coir is a natural fibrous material and during its use as substrate in soilless cultures, is partially disintegrated, consisted by 56% of organic matter, and contain nutrient elements. The effects of addition of this residue on soil biological and chemical properties were studied by two ways, firstly with an incubation experiment of 15 weeks and secondly by a greenhouse tomato crop. **(In vitro experiment):** Into 50 g of soil of the studied greenhouse were added 0, 2.204, 4.410 and 6.610 g from the coir residue, containing 0, 1.25, 2.5 and 3.75 g of organic matter respectively. The treatment of soil with the larger rate showed a higher biodegradation of the total (SOM) about 35%. Nitrification was ensured, a significant increase in available forms of P, K, Zn, and Mn observed for all the treatments, salinity of soil did not show any significant increase and sodicity did not reached hazardous values for plants. **(In vivo experiment):** In a greenhouse with elevated soil salinity, 280 kg from the coir residue replacing basic and top dressing fertilization were applied in the area of 100 m^2, where tomato crop was established. The monitoring of soil fertilization was based on soil electrical conductivity, the (SOM) decomposed about 15%, soil salinity was maintained at tolerable levels, and tomatoes plants developed normally. The coir previously used as substrate in hydroponical cultivation is proved a valuable material for soil amendment in greenhouse crops, improving the soil chemical and biological properties, while constitutes a useful practice for environment protection.

1. Introduction

The amendment of cultivated soils with suitable organic materials, affects its chemical and physical properties (Koyama *et al.*, 2009; Herath *et al.*, 2013; Jayasinghe *et al.*, 2014). The use of organic materials as soil amendments is largely adopted, and that practice activates soil organic matter (SOM) role on biological status of soil fertility (Zhang *et al.*, 2012; Giacometti *et al.*, 2013; Gougoulias *et al.*, 2013; 2014; Gomez *et al.*, 2014; Pose *et al.*, 2015).

Furthermore, the soil amendments by using composts prepared with agricultural wastes, is an agricultural practice improving soil fertility and protecting environment simultaneously (Poulsen *et al.*, 2013; Ince *et al.*, 2015).

The coir is a natural fibrous material extracted from the bark of coconut, and shows increasing interest for its use as substrate in the hydroponical cultivated crops (Abad *et al.*, 2002; Nichols, 2013). After the coir use as substrate, it is partially disintegrated, but it is

still consisted 56% by organic matter, containing nutrient elements from its original chemical composition, or by retention of them during the use as hydroponical substrate. Studies have shown that the reuse of the coir substrate mixed with added organic fertilizers, improved the growth of the vegetables, as it compared with the use of the raw coir substrate (Lee *et al.,* 2015). However, in many cases, the reuse of the substrate it is not possible, by the danger of pathogens spreading, or by deterioration of many physical and chemical properties (Raviv, 2014). Few studies are reported about the application of coir in the soil, after its use as substrate in hydroponical cultivations (Koyama *et al.,* 2009), these results confirming particularly the improvement of soil porosity during its use as fertilizer. The purpose of the present work considers to evaluate the utility of coir residue as soil amendment, on biological and chemical soil properties, for an incubation experiment of 15 weeks and for a greenhouse tomato crop, after its use in two consecutive hydroponical crops (Gougoulias *et al.,* 2015).

The greenhouse soil is a particularly exploited material, where a system with strong potential of evapotranspiration (ET) is established, intensifying highly the accumulation of soluble nutrients elements in soil surface. The treatment of greenhouse soil with coir residue will be assessed if it ensures plant normal development.

2. Materials and methods

The purpose of this work is to evaluate the effects of incorporation of coir residue (Photo 1), on biological and chemical properties of tested soil, either in vitro (incubation experiment) or in vivo in a greenhouse tomato crop (case study, Photo 2); the crop experiment was established based on given data by the incubation experiment. The coir used previously as hydroponical substrate, was in pressurized block material from the company Forteco.

Photo 1. Coir after his use as substrate

Photo 2. Tomato fertilized with coir waste used as substrate

2.1. Experiment incubation

The incubation experiment was applied in the laboratory, where coir residues containing 56.73 % in organic matter (Table 1), were added to greenhouse soil samples, containing 0.5% organic matter; the coir residue added is originated from substrate used in two consecutive hydroponical crops. Then, in 50g of air-dried soil, were added 2.204, 4.410 and 6.610 g from the coir residues containing 1.25, 2.5 and 3.75 g of organic matter correspondingly. These treatments were maintained under the same conditions in an incubator.

Table 1. Chemical properties of soil and of the coir residue used for the incubation experiment (soil depth: 0-20 cm)

Property	Soil	Coir residue (DW)
Texture	Sandy Loam	-
pH (1:5 in water)	8.1 ± 0.03	4.90 ± 0.02
EC (dS m^{-1}), (1:5 in water)	0.5 ± 0.01	2.16 ± 0.02
Organic matter (%)	0.5± 0.01	56.73 ± 0.90
$CaCO_3$ (%)	8.6± 0.40	-
CEC (cmol kg^{-1})	19 ± 0.90	-
N -total (g kg^{-1})	1.59 ± 0.05	4.2 ± 0.30
$N\text{-}NH_4^+$ (mg kg^{-1})	22.2 ± 9.60	110.6 ± 12
$N\text{-}NO_3^-$ (mg kg^{-1})	131.3 ± 12	1771 ± 168
P –Olsen (mg kg^{-1})	16.2 ± 1.80	106.6 ± 10.50
P-organic (mg kg^{-1})	29 ± 2.60	149.0 ± 11
P -total (mg kg^{-1})	232 ± 16	868.5 ± 39
K-exchangeable (mg kg^{-1})	293 ± 7.20	296.4 ± 13
K-total (mg kg^{-1})	4910 ± 224	4224 ± 204
Na-total (mg kg^{-1})	488 ± 29	381.8 ± 37.20
Cu –total (mg kg-1)	54.6 ± 2.40	83.5 ± 4.20
Zn -total (mg kg-1)	62.4 ± 3.10	101.2 ± 4.98
Mn -total (mg kg-1)	637 ± 32	105.6 ± 5.80
Cu –DTPA (mg kg^{-1})	0.74 ± 0.04	31.5 ± 1.62
Zn -DTPA (mg kg^{-1})	0.38 ± 0.01	34.0 ± 1.90
Mn -DTPA (mg kg^{-1})	4.14 ± 0.30	32.2± 2.90

*CEC: cation exchange capacity; Data represent average means and SE deviation. n= (4).

In the incubator, the treatments were prepared in triplicates and kept at 28 ^oC for a period of 15 weeks. During the first three weeks of the incubation period, the moisture was maintained at two-thirds of field capacity, but for the next three weeks the soils were left to dry. This process was repeated until the end of the incubation period. According to (Wu and Brookes, 2005), the alternation of drying and rewetting soil samples enhances mineralization of both soil biomass organic matter and non-biomass organic matter. Drying and rewetting of soil is an important process in soil aggregation, metabolic activity of the soil microbial biomass, and in soil organic matter (SOM) decomposition (Mikha *et al.,* 2005; Gordon *et al.,* 2008).

2.2. Greenhouse tomato cultivation on soil (in vivo experiment, case study)

That experiment took place in the greenhouse of TEI of Thessalia, located in Larissa (Greece), in an area of 100 m^2, with Sandy Loam soil (Table 1). In order to respect the existed nutritional status in the soil of the disposed greenhouse, soil analysis and particularly soil electrical conductivity were taken into account. Soil salinity in greenhouses, depends not only from soil nutrient accumulation but also from water irrigation salts. In according to previous studies in this greenhouse cultivations, when values of soil electrical conductivity are higher than 0.4 dS m^{-1} in the extract (1part soil:5 parts H_2O), as the electrical conductivity in the irrigation water was in normal levels (0.51 dS m^{-1}), it is a criterion to omit the basic soil fertilization with mineral fertilizers, or to stop the application of top dressing fertilizers (Rhoades *et al.,* 1999; Gougoulias *et al.,* 2012). Then 280 kg of coir residue, used previously as substrate in a hydroponical cultivation, were added as unique basic fertilization, (Table 1). That added rate, correspond to 28 tons per Hectare of coir residue which, containing 15.88 tons of organic matter per Hectare correspondingly. After the incorporation of these residues, a seeding of tomato variety Sandin-F1 at 15/10/2012 was implemented, with 110 roots, and the cultivation lasted for 165 days. The control of soil salinity every week in soil depth of 0-15 cm during the cultivation period (Figure 1), showed that the contents of soil nutrients were satisfactory, and then top dressing applications would have been also omitted, (Gougoulias *et al.,* 2012). During the plant development, 86 days after the culture initiation, the contents in N, P and K in tomato leaves were also in normal levels (Table 2). The provisory elevation in soil of salinity values, during tomato cultivation could be due either to (SOM) biodegradation liberating ions, or to strong upward movement of soil moisture carrying soluble ions; the crop progressing alleviates this phenomenon by nutrients up taking, then at the end of the cultivation period, salinity reached the initial values and total production in greenhouse tomato reached a yield of 6.87 kg/m^2**.** For soil evaluation, samples were taking from 0-15 cm of depth every week from three places.

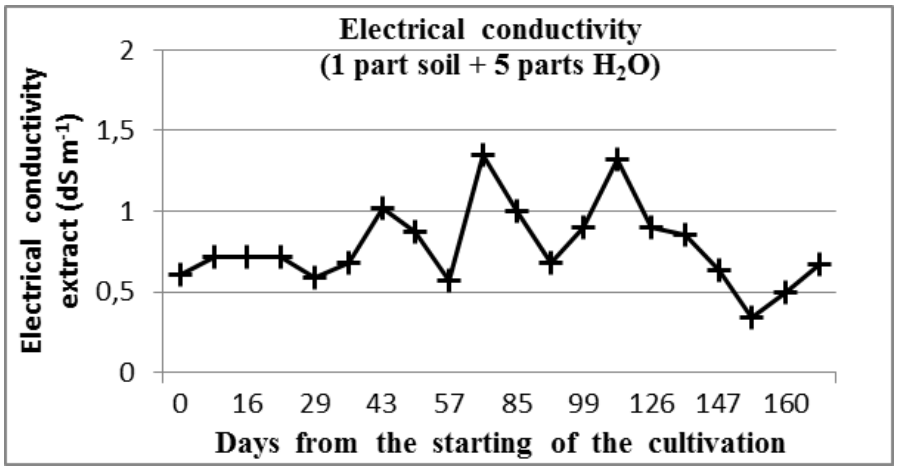

Figure 1. Soil electrical conductivity evolution, during cultivation period

Table 2. Foliar analysis of plants (DW)

	N	**P**	**K**
***Crop**	(%)		
Tomato	3.63	0.47	2.28

*86th days from the initiation of the cultivation

2.3. Methods of analysis

At the end of the incubation period and the crop harvesting, soil samples were analysed using the convenient methods which are referred by Page *et al.,* 1982 and Hesse *et al.,* 1972.

Organic matter was analyzed by chemical oxidation with 1 mol L^{-1} $K_2Cr_2O_7$ and titration of the remaining reagent with 0.5 mol L^{-1} $FeSO_4$.

Both ammonium and nitrate nitrogen were extracted with 0.5 mol L^{-1} $CaCl_2$ and estimated by distillation in the presence of MgO and Devarda's alloy, respectively.

Available P forms (Olsen P) was extracted with 0.5 mol L^{-1} $NaHCO_3$ and measured by spectroscopy. Organic phosphorus was measured after mineralization by combustion of the sample and subtraction of the mineral phosphorus amounts, which had previously been estimated in the laboratory. The mineral amounts were extracted with 1 mol L^{-1} H_2SO_4 and all forms were measured by spectroscopy.

Exchangeable forms of potassium ware extracted with 1 mol L^{-1} CH_3COONH_4 and measured by flame Photometer (Essex, UK).

Available forms of Mn, Zn, and Cu were extracted with DTPA (diethylene triamine pentaacetic acid 0.005 mol L^{-1} + $CaCl_2$ 0.01 mol L^{-1} + triethanolamine 0.1 mol L^{-1}) and measured by atomic absorption.

For the determination of total metals Mn, Cu and Zn, 1 g of wet material, was digested at 350 ^{0}C + 10 ml HNO_3 + 5 ml $HClO_4$. According to the method described by (Allen *et al.,* 1974 and Varian, 1989), the samples were analyzed by Atomic Absorption (Spectroscopy Varian Spectra AA 10 plus, Victoria, Australia), with the use of flame and air-acetylene mixture.

2.4. Statistical analysis

The experiment was repeated and the completely randomized design with four replications was used. Tukey's procedures were used to detect and separate the mean treatment differences at P = 0.05. Statistical analyses were performed by the statistical program MINITAB (Ryan *et al.,* 2005).

3. Results and discussion

3.1. Incubation experiment

The results of the laboratory experiment at the end of the incubation period, they showed a high rate of biodegradation of organic matter, more increasing by the application of the higher rates of coir residues (about 35%), while the lower rates and the control (soil), they showed a biodegradation of the organic matter about 10% (Figure 2). The nitrate forms predominate to ammonium forms in all treatments at the end of the incubation period, and then nitrification evolution after coir residues addition was not impeded.

In generally, significant increases in available forms of P, K, Zn, Mn & Cu were showed at the end of the incubation period, and these effects must attributed to soil biological activity, liberating mineral ions, (Figures 2, 3); available nutrients forms liberated, are originated either from (SOM) decomposition, or by soil microflora solubilising effects on insoluble soil compounds (Papadopoulos *et al.,* 1986).

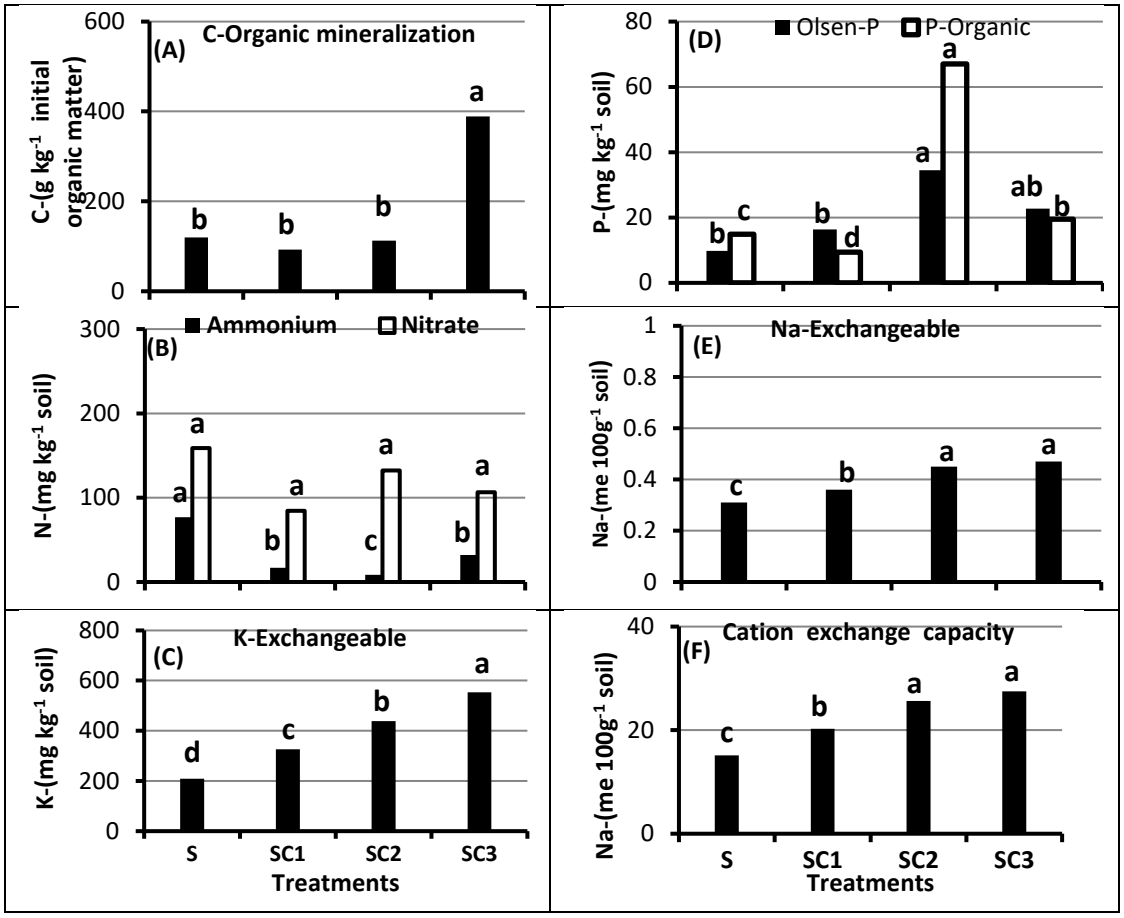

Figure 2. Effect of coir residue added rates on (A) soil organic-C mineralization; (B) soil mineral forms of N: ammonium and nitrate; (C) available K; (D) organic P and available; (E) exchangeable Na; (F) cation exchange capacity. Columns in each characteristic of each graph with the same letter do not differ significantly according to the Tukey's test (P=0.05). S, control (soil); SC1, SC2 or SC3, coir residue 2.204, 4.410, or 6.610 g per 50 g soil, respectively.

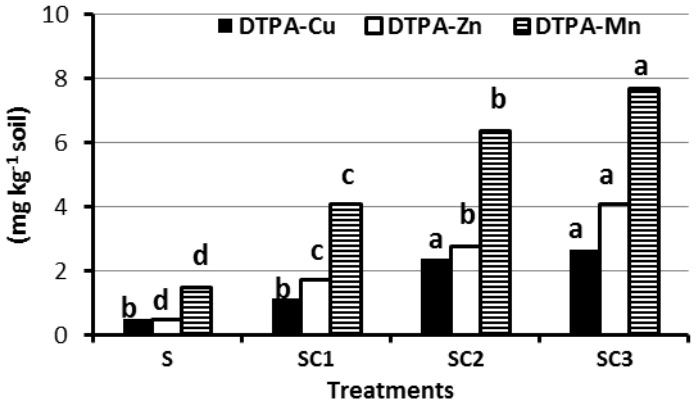

Figure 3. Effect of coir residue added rates on soil available forms of Cu, Zn and Mn. Columns in each characteristic of each graph with the same letter do not differ significantly according to the Tukey's test (P=0.05). S, control (soil); SC1, SC2 or SC3, coir residue 2.204, 4.410, or 6.610 g per 50 g soil, respectively.

The increase of exchangeable Na forms did not cause any sodicity risk for plants, while sodicity values are improved by significant increase of soil CEC values, after coir residue addition (Figure 2). Soil salinity did not increase significantly for all soil amendments with coir residue addition compared to the control, and pH showed also a very slight increase (Table 3). Finally, the application of coir residue in soil at the end of the incubation period, revealed an increase in total forms of Cu, Zn, Mn, and P, in comparison to the control (Table 3).

Table 3. Soil chemical properties at the end of the incubation period

***Treatments**	**Total forms (mg kg-1 soil)**				**(Extract 1: 5 in water)**	
	Cu	**Zn**	**Mn**	**P**	**EC (dS m^{-1})**	**pH**
S	11.07^{b}	36.18^{b}	654.23^{b}	129.48^{d}	0.43^{a}	6.8^{b}
SC1	19.87^{a}	59.95^{a}	696.56^{ab}	179.46^{c}	0.29^{b}	7.1^{a}
SC2	17.37^{ab}	56.87^{a}	652.00^{b}	330.36^{b}	0.43^{a}	7.1^{a}
SC3	22.22^{a}	54.61^{a}	721.41^{a}	363.10^{a}	0.44^{a}	7.0^{a}

*S: control (soil); SC1, SC2, or SC3 coir residue 2.204, 4.410, or 6.610 g per 50 g soil, respectively; EC: Electrical conductivity; Columns with the same letter do not differ significantly according to the Tukey's test (P=0.05).

Table 4. Chemical properties of the soil, before and after crop development

***property**	***SBACR**	***SAACR**	***SECP**
Texture	Sandy Loam		
Organic matter (%)	0.85 ± 0.02	2.50 ± 0.04	2.13 ± 0.03
pH (1:5 in water)	6.50 ±0.02	7.72 ± 0.02	7.78 ± 0.03
$CaCO_3$ (%)	4.20 ± 0.30	8.09 ± 0.87	7.80 ± 0.85
EC (1:5), (dS m^{-1})	0.60 ± 0.02	0.61 ± 0.03	0.67 ± 0.03
CEC (cmol kg^{-1})	26.0 ± 2.0	30.92 ± 2.2	30.05 ± 2.2
C/N	6.9	31.6	17.6
EXNa (mg kg^{-1})	390.2 ± 15	204.7	248.4
N-total (g kg^{-1})	1.02± 0.4	1.05 ± 0.3	0.88 ± 0.3
$N\text{-}NH_4^+$ (mg kg^{-1})	19.3± 5.2	435.7 ± 43.2	11.2 ± 4.5
$N\text{-}NO_3^-$ (mg kg^{-1})	283± 23	156.3 ± 16	167.5 ± 17.1
P-Olsen (mg kg^{-1})	21.5 ± 4.4	31.43 ± 4.8	19.15 ± 4.1
EXK (mg kg^{-1})	406 ± 13.5	405.6 ± 13.7	331.5 ± 10.7
Cu-total(mg kg^{-1})	37.1 ± 3.4	35.64 ± 3.6	30.43 ± 2.8
Zn-total (mg kg^{-1})	64.6 ± 3.8	68.45 ± 4.9	65.88 ± 3.3
Mn-total (mg kg^{-1})	724 ± 44	730.41 ± 49	678.3 ± 42
Cu-DTPA (mg kg^{-1})	1.1 ± 0.5	1.51 ± 0.2	1.94 ± 0.2
Zn-DTPA (mg kg^{-1})	1.05 ± 0.08	1.57 ± 0.2	2.0 1 ± 0.3
Mn-DTPA (mg kg^{-1})	10.2 ± 2.7	19.49 ± 3.3	13.10 ± 2.1

*CEC: cation exchange capacity; EXK: exchangeable-K; EXNa: exchangeable-Na; EC: electrical conductivity; SBACR: soil before the adding of the coir residue; SAACR: soil after the adding of the coir residue (seeding of tomatoes); SECP: soil at the end of the cultivation period; Data represent average means and SE deviation. (n) = 4

3.2. Greenhouse tomato cultivation

The incorporation of coir residue in soil for greenhouse tomato cultivation, resulted in significant increase of soil contents in organic matter, inorganic nitrogen, and available Mn forms (Table 4), while at the end of growing season, all these contents were decreased. Cation exchange capacity (CEC) increased, soil pH also increased but not at harmful levels, and salinity values did not show significant changes between the starting and the end of the cultivation period.

At the end of growing periods, despite the observed decomposition about 15%, sufficient organic contents remain in soils in order to cover needing for future. Table 5, shows the balance of available mineral nutrient elements in greenhouse soil at depth of 0-15 cm, during the cultivation period; in according to these data, the biodegradation of

the organic matter of coir residue added to the soil, would have contributed to a certain degree in enriching soil with mineral nutrients. Similar results of soil enrichment with nutrients, from incorporation in soil organic waste, they have been found and from previous studies (Weber *et al.*, 2007; Sarwar *et al.*, 2008; Koyama *et al.*, 2009; Lakhdar *et al.*, 2009; Diacono and Montemurro, 2010).

Table 5. Balance of available mineral nutrient elements in greenhouse soil at depth (0-15) cm

	Mineral-N	**Available-P_2O_5**	**Available-K_2O**	**EC (1:5), (dS m^{-1})**
	(kg/ha)			
*Start of growing period	634.83	103.41	1027.5	0.60
BF, (coir residues)	52.69	6.84	10	
End of crop period	375.27	92.11	838.95	0.67

*soil sample before application of coir residues; BF: basic fertilization.

4. Conclusions

In the study in vivo (greenhouse tomato cultivation), the coir residue addition was established combined with the omission of basic and top dressing fertilization for a soil already affected by residual effects of previous crops. That option has been showed as very reasonable act, in order to avoid by making worse the soil salinity status. Effectively, significant amounts of soil substances were transformed in available forms by the improving biological and chemical status of soil and the established tomatoes plants were developed under suitable soil conditions.

The coir residue previously used as substrate in hydroponical cultivation, it is a valuable material for soil amendment in greenhouse crops, improves soil chemical and biological properties, it does not make worse soil salinity, ensures satisfactory harvesting, while that recycling constitutes a useful practice for environment protection.

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EFFECT OF ASCORBIC ACID AND NISIN ON FRESH-CUT APPLES

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ABSTRACT
The objective of this research was to investigate the efficacy of ascorbic acid and nisin on quality of fresh-cut Fuji apples. Apple pieces were coated with sodium alginate (1% w/v), ascorbic acid (0-2% w/v) and nisin (0-75 ppm). Then, microbial growth, color, firmness, weight loss, and sensory qualities were measured during storage at 4°C. Coated samples had significantly lower aerobic microbial counts and fewer changes in color, texture and weight loss when compared to those of control samples. In sensory evaluation, the coated samples obtained the significantly higher acceptability scores than control samples at the same days of storage.

1. Introduction

Over half of all fruit consumed is stored fresh for in-home consumption and fruit is one of the most preferred snack foods since consumers concern more about their health and look for more foods with minimal preparation like fresh-cut fruit and premixed salad (Produce for Better Health Foundation, 2015). Nowadays, ready-to-eat fruits and vegetables, which are usually prepared for restaurants, retail markets, fast food restaurants, and dining commons (Watada and Qi, 1999), account for approximately 10% of all produce sales, with sales more than $10 billion per year (American Chemical Society, 2013). However, the production of fresh-cut, ready-to-eat fruit is challenging because cut fruit has a limited shelf-life when compared to whole fruit (Watada and Qi, 1999).

Apple is one of the most popular fruits consumed in 2014, second only to a banana (Produce for Better Health Foundation, 2015). Rocha and Morais (2003) found that the shelf-life of minimally processed apples (cv. Jonagored) was only three days due to surface browning which rapidly occurs when stored in the dark at 4 °C. Peeling and trimming remove natural protective skin of the fruit, which causes fast weight loss in fresh-cut apples. Beside the color change and weight loss, microbial spoilage, ethylene production, and increased respiration rate can lead to limited shelf-life of fresh-cut apples since wounding of apple tissue from peeling and slicing of fruit releases intracellular products that affect color, texture, and flavor of cut apples (Ahvenainen, 1996).

To enhance the quality and extend the shelf-life of minimally processed produce, an application of edible films and coatings is a potential method since this thin layer of edible material can work as a barrier which decreases respiration rate and water loss during storage (Guilbert *et al*., 1996). Edible coatings are applied on fresh-cut fruit to reduce decay and loss of humidity of fruit

pieces, delay ripening and color changes, improve appearance, and function as a carrier of antimicrobial agents, antioxidant agents, nutraceuticals, texture enhancers, flavors, and volatile precursors (Olivas and Barbosa-Cánovas, 2005). The application of edible films has greatly grown from only 10 companies in 1980s to more than 1,000 companies currently in the business, with more than $100 million sales a year (American Chemical Society, 2013).

Since varieties of minimal processed fruits have different specific characteristics, appropriate edible coatings must be developed and analyzed to find the appropriate coating for a specific fresh-cut fruit (Park, 1999). The main components for edible coating formulation for fresh fruits and vegetables are hydrocolloids, usually polysaccharides and proteins, and lipids (Olivas and Barbosa-Cánovas, 2005). Alginate can be used as a potential edible coating for fresh-cut apples because of its ability to form strong gels or insoluble polymers when cross-linked with multivalent metal cations such as calcium (Rhim, 2004). This polysaccharide is a polymer of D-mannuronic acid and L-guluronic acid, which is derived from a marine brown algae (*Phaeophyceae*) (Sime, 1990; Mancini and McHugh, 2000). Alginate coatings have been widely studied to prolong the shelf-life of minimally processed apples (Olivas *et al*., 2007; Rojas-Graü *et al*., 2007; Rojas-Graü *et al*., 2008).

Antibrowning activity of ascorbic acid has been studied in several minimally processed fruits and vegetables. It is a very useful antioxidant that inhibits browning reactions by ascorbyl formation, which scavenges the damaging radicals and reduces the o-quinones (Yamaguchi *et al*., 1999; Soliva-Fortuny and Martin-Belloso, 2003). Perez-Gago *et al.* (2006) found that ascorbic acid (1%) and cysteine (0.5%) in edible coatings prepared from whey protein concentrate and beeswax provided the most effective result in reducing enzymatic browning of fresh-cut Golden Delicious apples during storage at 4°C. Addition of ascorbic and citric acids to apple-based edible coatings also reduced browning of fresh-cut Granny Smith apples during storage at 5 ± 1°C (McHugh and Senesi, 2000). Moreover, apple slices coated with whey protein concentrate (5 g/100 ml) containing ascorbic acid (1 g/100 ml) and $CaCl_2$ (1 g/100 ml) showed the best results on microbial level and sensory quality when compared during storage in packed trays at 3°C for 2 weeks (Lee *et al*., 2003).

Nisin is a polypeptide with low molecular weight (3.3 kDa) synthesized by *Lactococcus lactis* subspecies *lactis*. It is an effective bacteriocin against Gram-positive bacteria, including *Bacillus cereus*, *Bacillus sporothermodurans*, and *Clostridium botulinum* (Thomas and Delves-Broughton, 2005; Carballo *et al*., 2012), as well as bacterial spores (Ray, 1992). Nisin has been added in the European food additive list with the assigned number E234 (European Commission, 1995). Nowadays, it is allowed in more than 50 countries all over the world (Aymerich *et al*., 2006). Nisin is mainly allowed in dairy products, salad dressings, vegetables and beverages (Delves-Broughton *et al*., 1996). In France, Italy, and Netherlands, nisin is permitted in cheese. In Russia, nisin (100 μg/g food) is permitted in vegetables, including raw, peeled, semi-preserved potatoes, cauliflower, and green peas. In Slovak Republic, nisin (12.5 μg/g food) is allowed in sterilized and pickled vegetables, as well as ready to eat meals (Thomas *et al*., 2000). In the United States, nisin has affirmed as generally recognized as safe (GRAS) and it is approved for inhibiting the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads and pasteurized cheese spread with fruits, vegetables, or meats (U.S. food

and Drug Administration, 2015). In Mexico and Peru, nisin is approved as a permitted additive in any food. In India, nisin is approved in coconut water with a permitted level at 5000 μg/g food. In Australia, nisin is permitted in cheese, processed cheese, and canned tomatoes (Cleveland *et al*., 2001).

Ukuku and Fett (2004) found that combination of nisin (50 μg/ml), sodium lactate (2%), and potassium sorbate (0.02%) was the most effective sanitizer treatment for reducing Salmonella on fresh-cut cantaloupe during 7 days of storage at 5°C. Recently, nisin-incorporated cellulose films have been reported as potential active packaging to enhance the safety of minimally processed mangoes against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, and *Alicyclobacillus acidoterrestris* during 12 days of storage at 5°C (Barbosa *et al*., 2013).

In this study, the efficacy of sodium alginate, incorporating with nisin and ascorbic acid, on quality attributes of minimally processed Fuji apples during storage at 4°C.

2. Materials and methods

2.1. Materials

Fuji apples (*Malus domestica*) were purchased from a local market in Bangkok, Thailand, stored at 4°C, and used within 24 h.

Food-grade sodium alginate (Modernist Pantry, Portsmouth, NH), nisin (Shandong Freda Biotechnology Co., Ltd., China) and ascorbic acid (Northeast General Pharmaceutical Factory, China) were used for the coating formulations. Food-grade calcium chloride (Qingdao Huadong Calcium Producing Co, Ltd., China) was used to induce the cross-linking reaction.

2.2. Sample preparation and treatment procesures

Sixteen aqueous solutions were prepared according the formulations in Table 1. The whole fruits were initially immersed in calcium hypochlorite solution (200 mg/L) for 2 min and rinsed with distilled water. Following good manufacturing practices, the fruits were peeled, cored, and cut into rectangular pieces (3 x 3 x 2 cm^3) using a sterilized hand-operated apple corer and knife. To block out the variation among apples, fresh-cut pieces were randomly selected and immediately immersed in 1 of the 16 solutions for 2 min, air-dried for 2 min, followed by immersing in aqueous solution of 2% w/v calcium chloride to gel for 2 min (except control), and then air-dried for 5 min.

Apple samples were then packaged in a resealable storage bag (18 x 20 cm^2) (Big C Supercenter Public Company Limited, Thailand) stored at 4°C for further analysis. The preparation process was independently repeated on 3 separate days as replication.

Table 1. Formulations of coatings used in the study.

Treatment No.	Ascorbic acid (% w/v)[b]	Nisin (ppm)[b]
1 (Control)[a]	0.0	0.0
2	1.0	0.0
3	1.5	0.0
4	2.0	0.0
5	0.0	25.0
6	0.0	50.0
7	0.0	75.0
8	1.0	25.0
9	1.0	50.0
10	1.0	75.0
11	1.5	25.0
12	1.5	50.0
13	1.5	75.0
14	2.0	25.0
15	2.0	50.0
16	2.0	75.0

[a]Control sample was coated with sterile water.

[b]Edible coating were prepared by mixing ascorbic acid and(or) nisin with 1% w/v sodium alginate.

2.3. Microbiological analysis

Total viable counts (TVC) were determined by the pour-plate method (AOAC, 2000) at 0, 3, 6, 9, 12, and 15 days of storage. 10 g of each sample was aseptically weighted and homogenized with 90 ml of sterile peptone water (Merck, KGaA, Germany) for 1 min using a stomacher (Funke-Gerber, Germany) at a speed of 230 rpm. The homogenized samples were serially diluted (1:10) in sterile peptone water. 1 ml of sample from serial dilutions was added to each duplicate sterile Petri dish. Plate Count Agar (PCA) was added to each Petri dish and samples were then incubated at 37±2 °C for 48 h. After incubation, colonies were counted, and microbiological data was expressed as logarithms of number of colony-forming units (logCFU/g). The best treatments screening from microbiological analysis were then evaluated for color, texture, and weight loss.

2.4. Color

Color values of the apple surface at 0, 5, 10, and 15 days of storage were directly determined with a colorimeter (Color Quest 45/0, Hunter Associates Laboratory, Inc., Reston, VA) at room temperature. The instrument was calibrated using a standard white and black reflector plates. Three apple pieces per treatment were measured and four readings were made in each replicate by changing the position of the sample. The results were expressed as CIE L*C*h* color space. L*, C*, and h* define the lightness, chroma and hue angle (°) respectively.

2.5. Texture profile analysis

Texture of apples was measured by compressing samples with Texture analyzer TA.XT2i (Stable Micro Systems Ltd., United Kingdom) as described by Olivas *et al.* (2007) at 0, 5, 10, and 15 days of storage.

2.6. Weight loss

Weight loss was assumed to correspond with water loss. It was measured at 5, 10, and 15 days of storage to determine the effectiveness of alginate coatings as moisture-barriers. The weight of three apple pieces in each treatment was monitored and the weight loss percent relative to initial weight was reported.

2.7. Sensory evaluation

Two sets of sensory evaluations were performed to determine the difference between coated and control samples and their acceptability among 30 partially trained panelists screening from juniors and seniors in the Department of Agro-Industrial, Food, and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Thailand.

In the first experiment, the best treatment (from microbiological and instrumental analysis) was selected and a difference-from-control test was performed to detect difference between coated and control samples at 0 and 10 days of storage when compare to a standard which was a fresh-cut apple piece without coating. The panelists were initially asked to evaluate and remember the standard, labeled with "S". Then, they were asked to rate the degree of difference in browning, firmness, and flavor between the standard and the samples. The scale in this test included 9 = extremely more than standard, 5 = no difference from standard, 1 = extremely less than standard.

In the second experiment, the coated and control samples at 0 and 10 days of storage were evaluated for overall acceptance using a nine-point hedonic scale, including 9 = like extremely, 5 = neither like nor dislike, 1 = dislike extremely. All samples in both experiments were labeled with random 3-digit codes and presented in random order.

2.8. Statistical analysis

All analyses were run in triplicate, except microbiological analyses, which were run in duplicate. Data were analyzed by analysis of variance (ANOVA) using IBM SPSS Statistics 21 (IBM Corporation, Armonk, NY). Duncan's multiple range test (DMRT) was used to determine significant differences among means (significance was defined at $p < 0.05$).

3. Results and discussions

3.1. Microbiological analysis

Samples from all treatments were analyzed for microbial growth during storage at 4°C. Total viable counts (TVC) of control and apples coated with 1% w/v sodium alginate incorporating with 2% w/v ascorbic acid and various concentrations of nisin during storage are presented in Fig.1. The same trend with significantly higher TVC *($p<0.05$)* occurred in the samples coated with other concentrations of ascorbic acid and nisin (data not shown). The initial number of bacteria in fresh cut apples was 1.17 logCFU/g, which indicated good quality of apples and appropriate sanitation used in this study. Less than 2 logCFU/g of total aerobic bacteria was also detected on fresh-cut Golden Delicious apples obtained from a local produce provider and processed under sterile conditions (Wang *et al*., 2007). As concentrations of ascorbic acid and nisin decreased, TVC increased with storage time *($p<0.05$)* (Fig. 1). Compared with the control, all treatments significantly inhibited the growth of bacteria in apple pieces during storage. All samples coated with nisin at any concentrations had less than 4 logCFU/g of TVC during 15 days of storage. The slowest growth of bacteria was found in the sample coated with 1% w/v sodium alginate incorporating with 2% w/v ascorbic acid and 75 ppm nisin.

Samples coated with solutions containing nisin showed slower growth of bacteria since nisin is effective against Gram-positive bacteria by forming pores at the cytoplasmic membrane. These pores disrupt the proton motive force and the pH equilibrium leading to leakage of ions, hydrolysis of ATP, and eventually cell death. Nisin can also inhibit cell wall biosynthesis by binding lipid II, a peptidoglycan precursor (Bauer and Dicks, 2005; de Arauz *et al*., 2009; Deegan *et al*., 2006). Recently, Barbosa *et al.* (2013) have reported that cellulose films incorporating with 25% nisin showed effective antimicrobial activity against *Staphylococcus aureus, Alicyclobacillus acidoterrestris, Listeria monocytogenes*, and *Bacillus cereus* on minimally processed mangoes during storage at 5±1°C without affecting their physicochemical and organoleptic characteristics. Beside its application as an edible film, Bari *et al.* (2005) found that 50 ppm nisin alone or in combination with 2% sodium lactate, 0.02% potassium sorbate, 0.02% phytic acid, and 10 mM citric acid as sanitizer treatments could improve the microbial safety against *Listeria monocytogenes* (2.20- to 4.35-log reductions) of fresh-cut cabbage, broccoli, and mung bean sprouts. A sanitizer treatment containing 50 ppm nisin and 2% sodium lactate has been recommended for inhibition of *Samonella* on fresh-cut cantaloupe during 7 days of storage at 5°C (Ukuku and Fett, 2004).

Ascorbic acid also has an antimicrobial effect against food pathogens by free radical formation during autoxidation and its oxygen absorption characteristic. An application of 0.2 or 0.4% ascorbic acid and 0.2% lactic acid has been suggested as a potential preservative to inhibit *E. coli* O157:H7 in laboratory medium (Brain Heart Infusion broth) and carrot juice during storage at 37°C for 8 h (Tajkarimi and Ibrahim, 2011).

3.2. Color

Since samples coated with 75 ppm of nisin showed the best results from microbiological analysis, they were chosen to determine their changes in color, texture, and weight loss.

Color is one of the most important quality attributes affecting acceptance of fresh and processed fruit and vegetables since consumers usually use this appearance factor as an indicator of freshness and flavor quality at the point of purchase (Pathare *et al*., 2013). Lightness (L*), chroma (C*), and hue angle (h*) of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage are presented in Fig. 2, 3, and 4 respectively. L* represents a measurement of luminosity. The higher the L* value, the lighter is the sample perceived by humans. C*, which is usually used as an indicator of color intensity, is a quantitative attribute of colorfulness. h*, a qualitative attribute of color, is used to indicate the difference of a color with reference to grey color with the same lightness. Red hue is defined as h* of 0° or 360° while yellow, green and blue hues are defined as h* of 90°, 180°, and 270° respectively (Pathare *et al*., 2013).

As concentrations of ascorbic acid decreased, L* and h* values decreased while C* values increased with storage time *($p<0.05$)* (Fig. 2 - 4). All samples treated with ascorbic acid showed significantly lower C* values, but higher L* and h* values *($p<0.05$)* when compared to the control. Range of h* values found in all samples (70° - 85°) represent red-yellow hue (0° - 90°) of samples (Pathare *et al*., 2013). These results indicated the lighter colors of samples coating with ascorbic acid. However, the samples treated with 1.5% w/v and 2% w/v ascorbic acid did not show significant difference in L*, C*, and h* values at the same day of storage *($p\geq0.05$)*.

Browning development in fresh-cut apples occurs due to broken tissue cells after cutting and releasing of enzymes, such as polyphenol oxidases (PPOs) (Garcia and Barret, 2002). Ascorbic is an antibrowning agent which reduces the o-quinones produced by the activity of PPOs, back to phenolic compounds (Soliva-Fortuny and Martin-Belloso, 2003). In the study of application of whey protein concentrate-based edible coatings on fresh-cut Golden Delicious apples, coatings prepared from whey protein concentrate (10% w/w), beewax (20% dry basis) and ascorbic acid (1% content) showed the most effective in reducing browning of apple pieces during storage at 5°C (Perez-Gago *et al*., 2006). Chiabrando and Giacalone (2012) have also reported that 1% w/v ascorbic acid and 1% w/v calcium chloride treatment was an effective inhibitor of PPO activity in minimally processed apples (Golden Delicious, Granny Smith, and Scarlet Spur) during storage at 4°C in darkness.

The control and sample coated with sodium alginate incorporating with nisin alone were not significantly different in L*, C*, and h* values at the same day of storage *($p \geq 0.05$)*. Therefore, coating with nisin alone did not affect the color of apple pieces. In the study of nisin-incorporated films on quality of minimally processed mangoes, nisin did not show any effect on color during storage at 5°C (Barbosa *et al*., 2013).

3.3. Texture profile analysis

Texture is another important factor that affects acceptability of consumers. Hardness or firmness is widely used as a texture parameter for whole and fresh-cut fruits (Harker *et al*., 1997). Hardness of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage are presented in Fig. 5. The hardness of control was significantly lower than coated

samples and significantly decreased with storage time *(p<0.05)* while hardness of coated samples remained constant with no significant difference among coated samples observed *(p≥0.05)*.

The similar results were also found in the study of alginate coatings (different coating formulations including alginate, alginate-acetylated monoglyceride-linoleic acid, and alginate-butter-linoleic acid) on minimally processed Gala apples. During storage, the firmness of uncoated control apples decreases due to cell wall degradation with the solubilization and depolymerization of pectin substances, as well as a reduction in turgor pressure. The edible coatings work as barriers to water vapor, preventing turgor loss in fresh-cut apples (Olivas and Barbosa-Cánovas, 2005).

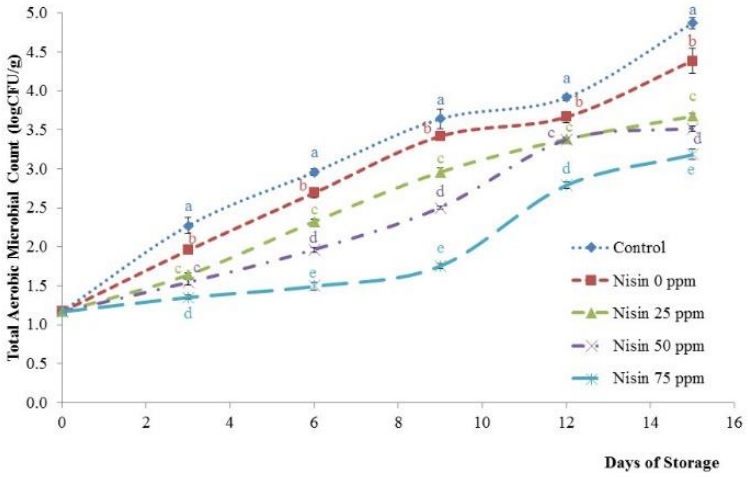

Figure 1. Total viable counts of control and apples coated with 1% w/v sodium alginate incorporating with 2% w/v ascorbic acid and various concentrations of nisin during storage at 4°C. Different letters (a, b, c, d, e) indicate significant difference at the same day of storage at *p<0.05* (n=3, error bars: standard deviations).

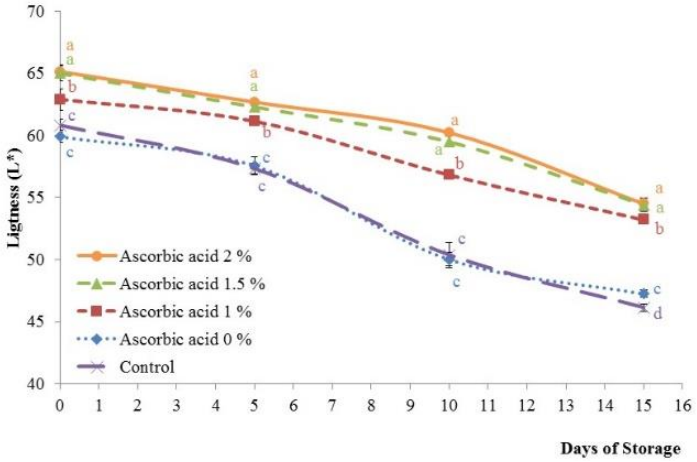

Figure 2. Lightness (L*) of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage at 4°C. Different letters (a, b, c, d) indicate significant difference at the same day of storage at *p<0.05* (n=3, error bars: standard deviations).

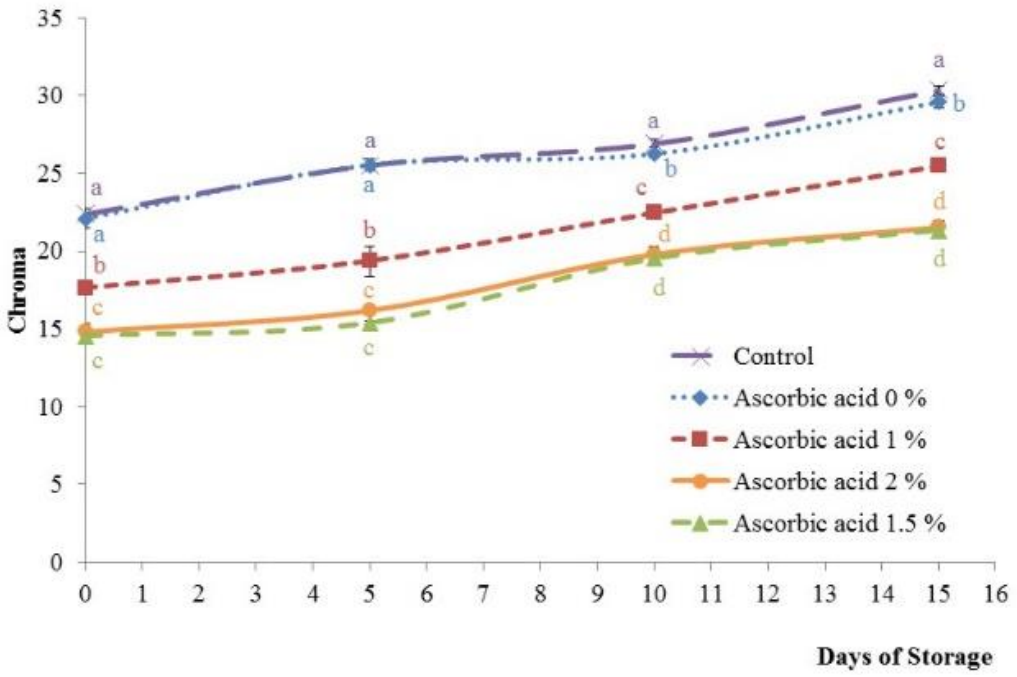

Figure 3. Chroma (C*) of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage at 4 °C. Different letters (a, b, c, d) indicate significant difference at the same day of storage at $p<0.05$ (n=3, error bars: standard deviations).

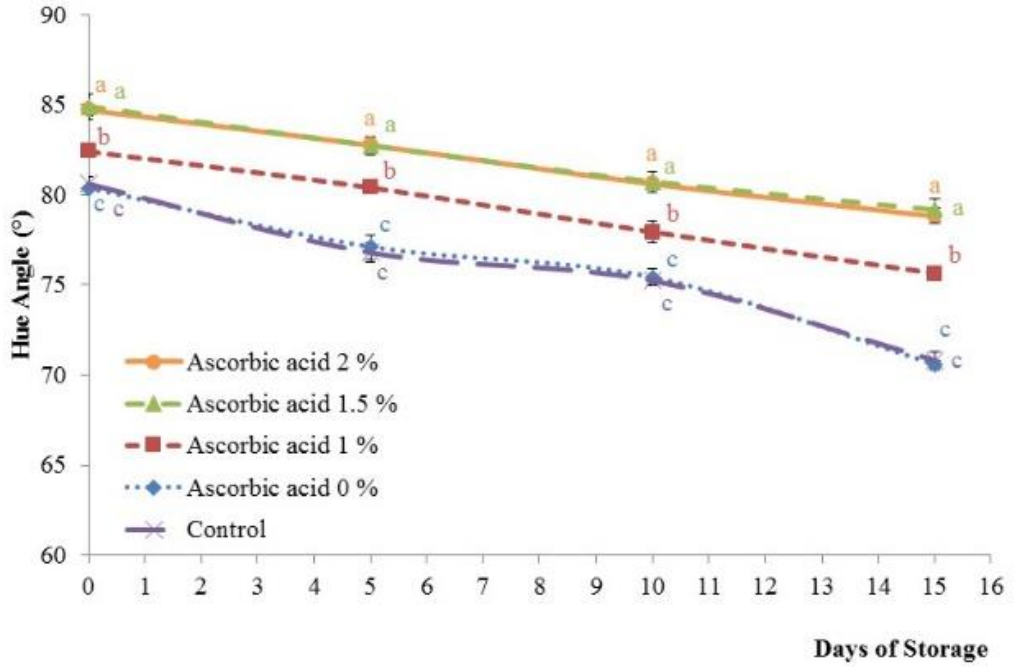

Figure 4. Hue angle (h*) of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage at 4

°C. Different letters (a, b, c) indicate significant difference at the same day of storage at $p<0.05$ (n=3, error bars: standard deviations).

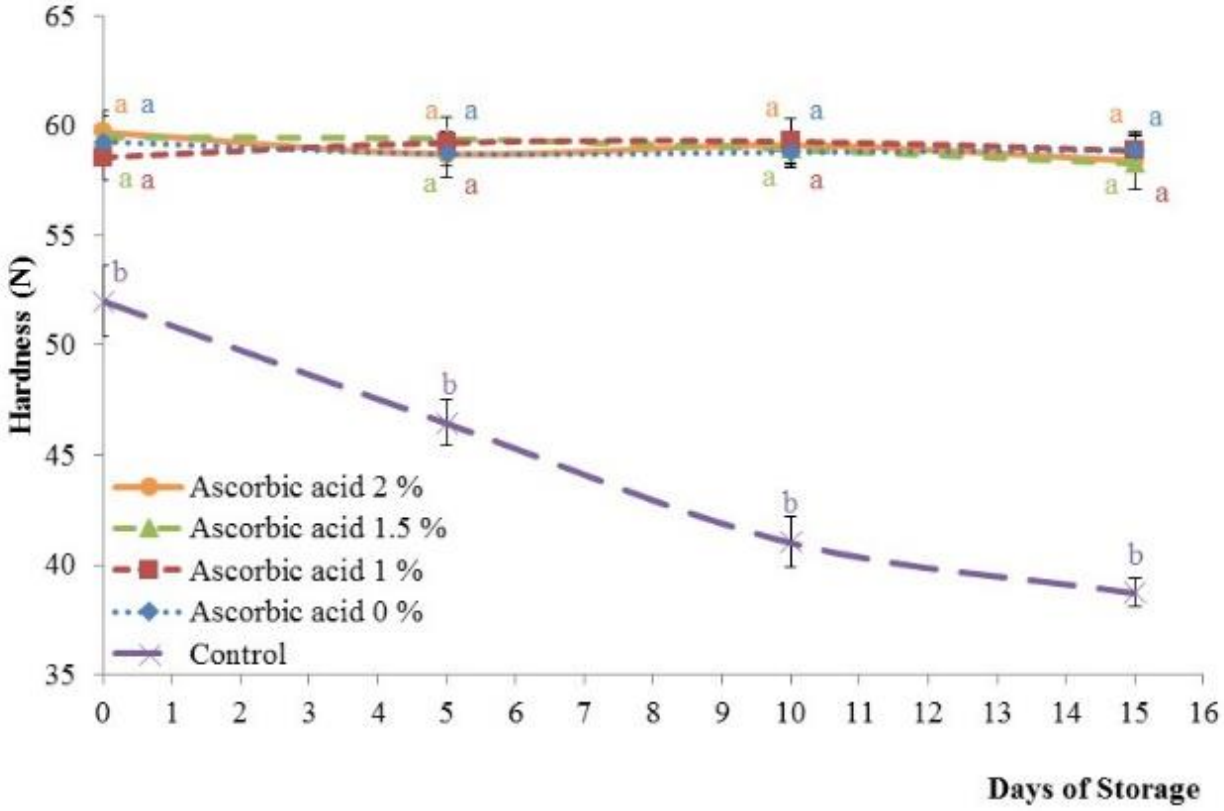

Figure 5. Hardness of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage at 4°C. Different letters (a, b) indicate significant difference at the same day of storage at $p<0.05$ (n=3, error bars: standard deviations).

3.4. Weight loss

Weight loss of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage are presented in Fig. 6. Weight loss of all samples significantly increased with storage time *($p<0.05$)*. The weight loss of control was significantly higher than coated samples *($p<0.05$)* while there was no significant difference among coated samples observed *($p\geq0.05$)*.

Olivas and Barbosa-Cánovas (2005) have reported the similar trend of weight loss in the study of alginate coatings on fresh-cut Gala apples. Weight loss occurs because the skinless tissue of apples is exposed to an environment with lower relative humidity. The edible coatings provide high relative humidity at the surface of fresh-cut apples, resulting in prevention of water loss. Besides, other types of edible coatings, such as calcium chloride, paraffin wax, and whey protein concentrate-based coating with beewax and ascorbic acid, were also reported as effective water vapor barriers of apple slices during storage (Hayat *et al.*, 2005; Perez-Gago *et al.*, 2006).

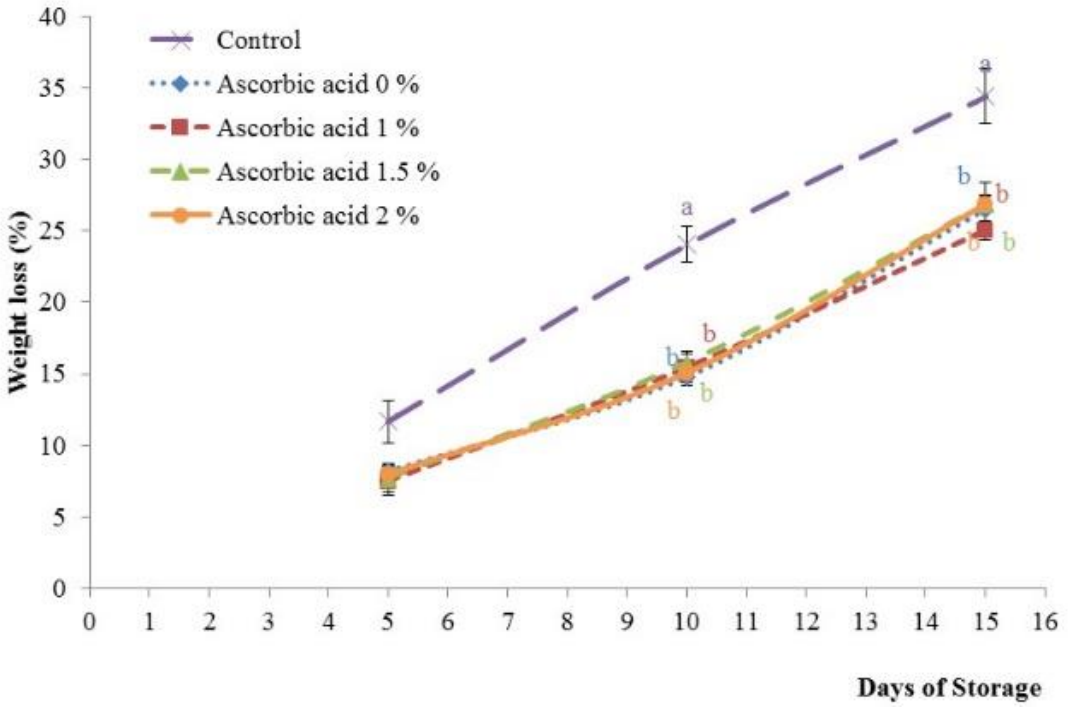

Figure 6. Weight loss of control and apples coated with 1% w/v sodium alginate incorporating with 75 ppm nisin and various concentrations of ascorbic acid during storage at 4 °C. Different letters (a, b) indicate significant difference at the same day of storage at $p<0.05$ (n=3, error bars: standard deviations).

3.5. Sensory evaluation

Since samples coated with 75 ppm of nisin showed the best results from microbiological analysis and samples coating with 1.5% w/v and 2% w/v were not significantly different in color, texture and weight loss. The apples coated with 1% w/v sodium alginate incorporating with 1.5% w/v ascorbic acid and 75 ppm nisin at 0 and 10 days of storage were selected for sensory evaluation compared to the control.

The difference-from-control test was conducted to evaluate whether the untrained panelists (n=30) could discriminate the coated sample from the fresh-cut uncoated apple, which was used as a standard. Scores from difference-from-control test of control and coated samples are presented in Fig. 7. In browning, all samples were significantly different *($p<0.05$).* The coated sample had the lowest score in browning at both 0 and 10 days of storage, which indicated that the coated sample was significantly lighter in color than the control at the same day of storage *($p<0.05$).* At the beginning of storage, both control and coated sample were not significantly different from the standard *($p\geq0.05$)* but they had considerably higher scores after 10 days of storage and were significantly different from the standard *($p<0.05$).* These results are also correlated with those from color by a colorimeter (Fig. 2-4). Recently, Perez-Gago *et al.* (2006) have reported that minimally processed apples treated with whey protein concentrate and ascorbic acid were ranked with the lowest browning in sensory evaluation.

Firmness in control also showed similar trend when compared to the hardness from texture analysis (Fig. 5). The control and standard had no significant difference in firmness *($p\geq0.05$)* while the coated sample

was significantly firmer than those at the beginning of storage *(p<0.05)*. After 10 days of storage, the firmness of both control and coated samples significantly decreased *(p<0.05)*; however, the coated sample was not significantly different from the standard *(p≥0.05)*. Similar to the texture analysis, the firmness of samples decreases during storage because cell wall degradation of pectin substances and a reduction in turgor pressure. The edible coatings work as barriers to water vapor, preventing turgor loss in fresh-cut apples (Olivas and Barbosa-Cánovas, 2005).

In the sensory evaluation on flavor, both control and coated sample were not significant different from the standard *(p≥0.05)* at the beginning of storage; however, panelists detected the significantly lower flavor in control at 10 days of storage *(p<0.05)*. Olivas and Barbosa-Cánovas (2005) have also observed that coated apples with alginate-based coatings had higher levels of volatiles when compared to the control during storage at 5°C.

The overall acceptance test was conducted to evaluate the level of acceptability on the coated sample among untrained panelists (n=30). Scores from overall acceptance test of control and coated samples are presented in Fig. 8. The higher the score is, the more panelists like the sample. At both 0 and 10 days of storage, the coated samples obtained significantly higher scores than the control *(p<0.05)*. At 10 days of storage, the control obtained the lowest score at approximately '3' or 'dislike moderately' while the coated sample still obtained score at approximately '6' or 'like slightly'. These results also correlated with those results from the difference-from-control-test (Fig. 7).

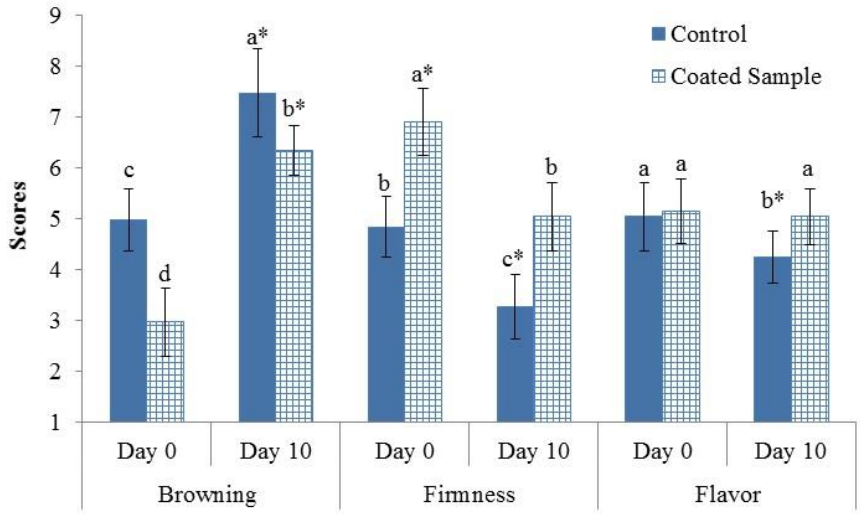

Figure 7. Scores from difference-from-control test of control and coated samples (1% w/v sodium alginate incorporating with 1.5% w/v ascorbic acid and 75 ppm nisin) at 0 and 10 days of storage at 4°C (9 = extremely more than standard, 8 = much more than standard, 7 = more than standard, 6 = slightly more than standard, 5 = no difference from standard, 4 = slightly less than standard, 3 = less than standard, 2 = much less than standard, 1 = extremely less than standard). Different letters (a, b, c, d) indicate significant difference between control and coated samples at

0 and 10 days of storage. (n=30, error bars: standard deviations). * indicates significant difference between samples and a standard (fresh cut apple) at $p<0.05$.

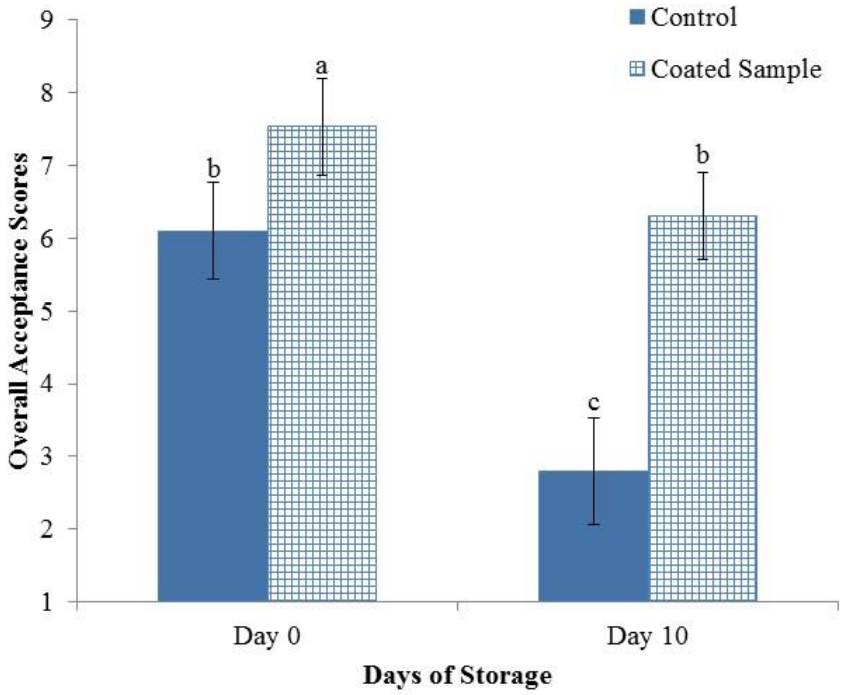

Figure 8. Overall acceptance scores of control and coated samples (1% w/v sodium alginate incorporating with 1.5% w/v ascorbic acid and 75 ppm nisin) at 0 and 10 days of storage at 4 °C (9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely). Different letters (a, b, c) indicate significant difference at $p<0.05$ (n=30, error bars: standard deviations).

4. Conclusions

The recommended concentrations of nisin and ascorbic acid to incorporate with 1 %w/v sodium alginate were 75 ppm and 1.5 % w/v respectively since this mixture of coating efficiently reduced the changes in microbial growth, color, hardness, and weight loss of samples while obtained the higher overall liking score when compared to the uncoated sample.

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FUNCTIONAL PROPERTIES OF PROBIOTIC ICE CREAM PRODUCED FROM GOAT'S MILK

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ABSTRACT

The aim of this study is to produce ice cream which is a delicious and healthy dairy product with increased health benefits by adding various functional properties. Accordingly, goat's milk, milk powder, sahlep, tagatose, and Litesse ultra and polydextrose (as prebiotics) and *Lactobacillus paracasei* subsp. *paracasei* and *Bifidobacterium longum* + *Bifidobacterium bifidum* combined culture (as probiotic cultures) and commercial raspberry and blackberry fruit sauces and fruit sauce prepared from frozen raspberry were used for production of ice cream. During ice cream production, rates of all ingredients were fixed except the fruit sauce. In the study, four types of ice cream were produced, namely control sample, sample containing commercial raspberry and blackberry sauces and sample containing raspberry sauce produced from frozen raspberries. The study was conducted as a single production containing two duplicates. Shelf-life of the samples were 120 days and some physicochemical and functional properties were examined on the 1st, 15th, 30th, 60th, 90th and 120th days of the storage and the results were evaluated. It was found that fruit sauce fortification had a significant effect on the fat, protein, titratable acidity, pH, ash, saccharose, melting rate, color (*L*, *a*, *b* values), total phenolics, anthocyanidin, flavonoid content and antioxidant capacity ($p<0.05$). In the light of the results obtained, it is believed that our study was important in terms of providing alternative and delicious functional products both for food industry and consumers.

1. Introduction

Probiotics are defined as selective viable microorganisms that have potential for improving the health and nutrition of the consumer following consumption with food due to their beneficial effects such as control of intestinal pathogens. Due to the interest in their potential beneficial health effects, many different types of products have been thought as carrier foods for probiotics (Ranadheera et al., 2013). Prebiotics are non-digestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favorable growth or activity of a limited number of indigenous bacteria. A food product containing both probiotics and prebiotics is called symbiotic. There is a relationship between probiotics and prebiotics. Prebiotic compounds are consumed by probiotics as carbon or energy sources in the colon. This situation causes an increase in probiotic count and reduction of pathogen microorganisms in the gut. This process forms the concept of a functional food.

Studies have shown that ice cream is much more suitable for containing probiotic bacteria compared to fermented dairy products. The pH of ice cream is higher than that of regular fermented milk, and it is significantly higher than those of other dairy products. This is important in regarding the fact that low pH may severely affect the survival of probiotic bacteria (Ranadheera, Evans, Adams, & Baines, 2012). Furthermore, ice cream is a well-accepted and liked milk product, therefore it is thought as an ideal vehicle for delivering probiotics (Alamprese et al., 2005; Turgut and Cakmakci, 2009; Ranadheera et al., 2013).

Goat's milk has beneficial effects on human health, physiological functions and on the nutrition of children and elderly people. Furthermore, fat is one of the most important components of goat milk. It is thought that it could be consumed without negative effects by people suffering cow milk allergy (Riberio and Riberio, 2010).

Goat's milk can be utilized to produce ice cream with a softer texture and desirable melting characteristics (Ribeiro and Ribeiro, 2010). One important point is that the incorporation of probiotics into ice cream does not affect the overall quality of the product (Ranadheera et al., 2013). On the other hand, the freezing and whipping processes involved in the ice cream production may lead to serious cell damage and consequent loss of probiotic viability. Development of probiotic ice cream can be technologically challenging due to instability of probiotics in frozen products (Ranadheera et al., 2013).

Berry fruits are one of the most important sources of phenolic compounds in our diets. Especially hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, catechins, and tannins, either hydrolyzable or condensed, are frequently present. Many of these compounds have a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory, and vasodilatory actions (Kahkonen et al., 2001). For example anthocyanins are absorbed as intact molecules in the stomach and have systemic activity, exerting antineoplastic, anticarcinogenic, antiatherogenic, antiviral and anti-inflammatory effects, as well as decreasing capillary permeability and fragility, inhibiting platelet aggregation and stimulating immune function (Limsuwan et al., 2014). Fruit extracts with phenolic compounds have numerous advantages for human health and in several studies it has been shown that they have high antioxidant potential (Kahkonen et al., 2001).

One of the biggest problems in probiotic dairy products is to provide that count of probiotic living bacteria as 10^7 CFU/ml. Therefore fat replacers such as polydextrose and litesse may be used to increase liveliness and provide the stability of probiotic microorganisms.

The aim of this study is to produce ice cream which is a delicious and healthy dairy product with increased health benefits by adding various functional properties. Accordingly, goat's milk, milk powder, sahlep, tagatose, and Litesse ultra and polydextrose (as prebiotics) and *Lactobacillus paracasei* subsp. *paracasei* and *Bifidobacterium longum* + *Bifidobacterium bifidum* combined culture (as probiotic cultures) and commercial raspberry and blackberry fruit sauces and fruit sauce prepared from frozen raspberry were used for production of ice cream. In the light of the results obtained, we conclusively believe that our study is of importance regarding both food industry and providing alternative and delicious functional products for consumers.

2. Materials and methods

2.1.Material

Raw goat's milk used in the production of ice cream were provided from Kay Sut Co. (Izmir-Turkey), skimmed milk powder (21% lactose and 36% protein) was provided from Pınar Sut Co. (Izmir-Turkey), and sahlep was provided from Ilkay Pastry and Sweets (Izmir-Turkey). In the production of ice cream

tagatose provided from Damhert Nutrition, Litesse® Ultra™ provided from Danisco and polydextrose provided from Sima Ltd. Co. (İstanbul-Turkey) were used. Raspberry and blackberry commercial fruit sauces were provided from Aromsa Food Flavor and Ingredients Co. (Kocaeli-Turkey). Sauce produced from frozen raspberry was prepared with Superfresh frozen raspberries.

2.2.Methods

Functional ice cream production

Ice cream samples were produced in a single production containing two duplicates. Ice cream samples were coded as explained in Table 1. Milk powder, sahlep, tagatose, Litesse and polydextrose were added to the goat's milk according to the mixture recipe for ice cream machine and then the mixture was pasteurized (at 90 ºC for 10 minutes). Then the mixtures were cooled to 37 ºC and inoculated with 3% culture. Inoculated mixtures were left to incubation at 37 ºC. The incubation was ended at pH 4.8-4.9 and the samples were taken to cold storage at +4 °C for 24 hours for maturation. The next day, following the maturation, the mixtures were divided into four groups and 10% fruit sauces were added to three of them before freezing. The control group, was not fortified with fruit sauce. Mixtures were frozen in batch type ice cream machine and packaged. Freezing process lasted for 9 minutes for each batch. First control group samples then fruit sauce fortified ice cream samples were produced. Fat, protein, titratable acidity, pH, ash, saccharose, melting rate, color (L, a, b values), total phenolics, anthocyanidin, flavonoid content and antioxidant capacity of the samples were performed on 1st, 15th, 30th, 60th, 90th, and 120th days of the storage. Production were conducted in Pilot Dairy Plant of Ege University Faculty of Agriculture, Department of Dairy Technology.

For the production of raspberry sauce raspberries were unfrozen, crushed and stirred on the laboratory hot plate for a short time. And then the raspberries were broken down by using a food processor and heated at 75°C for 10 minutes under control. Composition of 100 g commercial fruit sauces were given in Table 2.

Table 1. Ice cream sample codes

Sample Codes	Properties of the Coded Samples
K	Sample produced from freezing the fermented mixture exclusively.
F	Sample produced from addition of sauce prepared from frozen raspberries to the fermented mixture.
A	Sample produced from addition of commercial raspberry sauce to the fermented mixture.
B	Sample produced from addition of commercial blackberry sauce to the fermented mixture.

Table 2. Composition of commercial fruit sauces (100 g)

Properties	Values
Energy (kcal/kJ)	127 / 532
Fat (%)	0
Dry Matter (%)	31.65
Protein (%)	0.02

Physicochemical analyses

Dry matter analysis in raw goat's milk were performed by standard gravimetric method (Oysun, 1996), fat was determined with Gerber method (Oysun, 1996), protein content was determined with Kjeldahl method (AOAC, 1990), titratable acidity was determined with Soxhelet-Henkel method (Oysun, 1996) and pH was detected by using Hanna Microprocessor pH 211 digital pH meter (Coşkun, 2005). Phenolic content of fruit sauces was measured according to Folin-Ciocalteu colorimetric method (Cai et al., 2004), total anthocyanidin content was detected according to vanillin-HCl colorimetric method (Nakamura et al., 2003), total flavonoid content was determined with modified colorimetric method (Chun et al., 2003) and total antioxidant capacity was determined according to improved ABTS methods (Cai et al., 2004).

In the prepared ice cream mixtures, dry matter (Oysun, 1996), fat (TS 1330-Anon., 1992), titratable acidity (Yöney, 1968), pH (by using Hanna Microprocessor pH 211 digital pH meter) analyses were performed.

In ice cream samples, dry matter (Oysun, 1996), fat (TS 1330-Anon., 1992), protein (by using Leco FP-528 (USA) nitrogen detection device), titratable acidity (Yöney, 1968), pH (by using Hanna Microprocessor pH 211 digital pH meter), ash (Bradley et al., 1992), saccharose (IDF Standard 35A:1992), overrun (Metin, 2009), melting ratio (Gürsel and Karacabey, 1998), color (by using Konica Minolta CR-400 device), total phenolics by using Folin-Ciocalteu colorimetric method (Cai et al., 2004), total anthocyanidin content according to vanillin-HCl colorimetric method (Nakamura et al., 2003), total flavonoid content according to modified colorimetric method (Chun et al., 2003) and total antioxidant capacity values according to improved ABTS methods (Cai et al., 2004) were analyzed. Dry matter, fat, protein, ash, saccharose, total phenolics, anthocyanidin, analysis of flavonoid content and antioxidant capacity analyses were performed only on 1st day of the storage. All the other analyses were performed on the mentioned days of the storage.

Statistical Analysis

One-way analysis of variance (One-way Anova) was used for the determination of the differences between the ice cream samples and the effects of storage. Accordingly, statistical analysis software SPSS version 15.0 (SPSS Inc. Chicago, Illinois) was used. The significant data as a result of analysis of variance (ANOVA) were tested according to the Duncan multiple comparison test at $p < 0.05$ level.

3. Results and discussions

In this section, results for physicochemical and sensory properties on 1st, 15th, 30th, 60th, 90th and 120th days of the storage for each functional ice cream sample produced in a single production in two duplicates were given and evaluated.

Table 3. Total phenolic compounds (TPC) flavonoid content (FC) anthocyanidin (TA) and antioxidant capacity (TAOC) values of fruit sauces used in the production of functional ice cream.

	TPC	FC	TA	TAOC
F	4.28±0.07 X	6.22±0.35 X	1.64±0.01 X	6.91±0.00 X
A	6.22±0.01 Y	**29.61±0.15 Z**	4.60±0.19 Y	11.26±0.18 Y
B	**8.25±0.05 Z**	14.93±0.68 Y	**20.04±0.58 Z**	**14.26±0.12 Z**

X, Y, Z: Values with the different letters in the same column differ significantly (P<0.05).

Total phenolic contents were determined as mg gallic acid / g DM, total flavonoid content as rutin mg / g DM, total anthocyanidin content as catechin mg / g DM and total antioxidant capacity was determined as Trolox/100 g DM.

The composition of raw goat's milk used in the production of functional ice cream has an important effect on the quality of the end product. It was determined that the properties

of raw goat's milk used in the production were in compliance with the Turkish Food Codex Raw and Heat-treated Drinking Milk Communiqué (Communiqué no: 2000/6). As below phenolic properties of fruit sauces were shown (Table 3).

Analysis showed that raspberry sauce had the highest total flavonoid content (29.61 mg rutin/g DM). On the other hand, blackberry sauce had the highest total phenolic, anthocyanidin and antioxidant capacity values (8.25 mg of gallic acid / g DM, 20.04 mg of catechin / g DM and 14.26 mmol Trolox / 100 g DM). Changes in the average phenolic content (TPC), flavonoid content (TC), anthocyanidin (TA) and antioxidant capacity (TAOC) values of fruit sauces were analyzed with analysis of variance and Duncan test. Statistical analyses showed that the differences between the fruit sauces in terms of all properties were statistically significant ($p<0.05$).

Bilyk and Sapers (1986), Häkkinen et al. (2000), Kähkönen et al. (2001), and Kalt et al. (1999) reported that blackberry was more intense in terms of total phenolic compounds compared to raspberry. Garcia-Alonso (2004) also reported that raspberry contains higher in flavonoids compared to blackberry. It was found that blackberry was the rstudyichest fruit in all other contents except total flavonoid content. Study results were comparable with the related studies.

3.1.Properties of functional ice cream

Dry matter, fat, protein, titratable acidity, pH, ash, saccharose, overrun, melting rate, color, total phenolic content, anthocyanidin, flavonoid content, total antioxidant capacity, viscosity and texture analyses were performed. Dry matter, fat, protein, ash, saccharose, total phenolics, anthocyanidin, analysis of flavonoid content and antioxidant capacity analyses were performed once and only on 1st day of the storage.

3.2.Chemical composition of functional ice cream

Dry matter, fat, protein, ash and saccharose (%) values of functional ice cream samples were given in Table 4. As is seen in the results of the study, ice cream with the highest dry matter was A (27.22%), and the lowest dry matter was found in K (24.34%) sample. The higher dry matter content in ice cream samples fortified with fruit sauces compared to control sample is an expected result. Statistical analysis showed that the differences between the average dry matter values of functional ice cream samples were statistically not significant ($p>0.05$).

Table 4 Dry matter, fat, protein, ash and saccharose (%) values of functional ice cream samples.

	Dry Matter (%)	Fat (%)	Protein (%)	Ash (%)	Saccharose (%)
K	**24.34±0.74**	6.25±0.15 Y	**7.91±0.65 Y**	**1.26±0.02 Y**	**0.91±0.03 X**
F	26.00±0.26	**5.35±0.15 X**	7.65±0.36 Y	1.15±0.00 X	2.61±0.25 Y
A	**27.22±1.20**	**6.30±0.20 Y**	7.61±0.54 Y	1.23±0.02 Y	**4.50±0.23 Z**
B	26.96±1.01	6.25±0.15 Y	**5.25±0.00 X**	1.13±0.01 X	1.62±0.10 X

K: Ice cream produced from freezing the fermented mixture exclusively, F: Ice cream produced from addition of sauce prepared from frozen raspberries to the fermented mixture, A: Ice cream produced from addition of commercial raspberry sauce to the fermented mixture, B: Ice cream produced from addition of commercial blackberry sauce to the fermented mixture.

X, Y: Values with the different letters in the same column differ significantly ($P<0.05$).

Koçan and Koçak (2002), in their study on ice cream production fortified with emulsifiers at different ratios, reported the dry matter contents 32.55-32.63 %. Akın (2005), reported the dry matter contents of probiotic yoghurt ice creams between 30.71% and 37.01%. Korel et al. (2005) in their study on the chemical and microbiological quality of packaged and

unpackaged plain, cocoa and fruit (sour cherry, strawberry and lemon) ice cream available in Manisa market, reported that dry matter values varied between 60.58 - 71.00%. Dry matter values of fruit ice creams varied between 62.48% and 71.00%. Kesenkaş et al. (2013) produced ice cream by adding kefir and kefir culture to the cow, soy and mixture of these milk types. They reported that dry matter contents were between 26.5-27.50 %. Therefore it could be said that dry matter contents of the functional ice creams produced in our study are compatible with the other values in various studies.

The highest fat content was found in A (6.30%), while the lowest fat content was determined in F (5.35%) sample. Fat ratio of the A sample with the highest value was close to that of K sample. This was associated with the proportional decrease in fat percentage. According to the statistical analysis, fat content of F sample was significantly different from the other samples ($p<0.05$).

Alamprese (2002) reported that fat content and overrun were inversely related, and bacterial count, sugar and fat content had no effect on acidity although they affected pH and viscosity (Turgut, 2006). Antepüzümü (2005), in their study on ice cream production produced from mixture produced from goat's milk fortified with skim milk powder, sugar, cream and added different ratios of honey (20%, 30%, 40%, 50%) and glucose syrup (20%, 30%, 40%, 50%) reported that the fat contents of ice cream samples varied between 7.53% and 7.60%. Çeliker (2008), in their study, added hawthorn fruit syrup to two different ice cream samples at two different ratios (10-15%) and reported the fat ratios of ice cream samples varied between 5.05 - 6.25%. Aliyev (2006) reported that fat content of the kefir ice cream increased as the ratio of fruit pulp increased. In this study, it was stated that fat contents of ice cream samples with fruit sauce had close values to the control sample. Kesenkaş et al. (2013), in their study in which they produced ice cream by adding kefir and kefir culture to mixtures prepared from cow milk, soy milk and mixture of these milk types, reported the fat contents of the ice creams varied between 1.25 - 1.35 %. Ranadheera et al. (2013) investigated the effects of polypropylene and glass containers on the properties of chocolate ice creams produced from goat's milk by using *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* Bb-12, and a new probiotic bacterium *Propionibacterium jensenii* 702. In the mentioned study, fat ratios varied between 9.50-9.67%. Researches who carried out similar studies by using various fat substitutes had reported that ice creams with low fat ratios also had low viscosity values (Hatipoğlu, 2007). In our study it was aimed to produce functional ice creams produced in our study are comparable with the other studies.

The highest protein content was found in K (7.91%), while the lowest protein content was determined in B (5.25%) sample. According to the statistical analysis results, the difference between the protein contents of B sample and the other samples were statistically significant ($p<0.05$).

Protein contents of the ice cream samples containing raspberry sauces were close to control group samples. The lower protein content of samples containing blackberry sauce was associated with the lower protein content of blackberry sauce compared to other fruit sauces.

Aliyev (2006) reported that protein values of ice cream samples which contain kefir and blueberries were between 2.30-3.81%. Also Kesenkaş et al. (2013) stated that protein contents of ice cream samples, which produced from kefir and kefir culture added to ice cream mix prepared from cow, soy and mixture of them, were between 5.63 - 6.17 %. Durak (2006) and Aliyev (2006) reported that protein content of the ice cream samples decreased as the fruit pulp ratio increased. The researchers associated this result with the very low protein content of blueberries compared to the ice cream mixture. Korel et al. (2005) reported that

protein content of fruit ice creams were much lower than with and cocoa ice creams. The highest ash content was found in K (1.26%), while the lowest ash content was determined in B (1.13%) sample. Among the functional ice cream samples fortified with fruit sauce, A sample had higher ash value compared to other samples. According to the statistical evaluations, the differences between K and A sample; and F and B sample were statistically not significant ($p>0.05$).

Aliyev (2006), in their study on ice cream production containing kefir and blueberries, reported that the fruit pulp ratio had a significant effect on ash contents. In the study values varied between 0.59-0.91 %. Durak (2006), reported that the ash values of ice creams produced by adding yoghurt to the ice cream mixture varied between 0.59-0.92 %.

The highest saccharose ratio was found in A (4.50%), while the lowest saccharose was determined in K (0.91%) sample. Supporting this result, the measurements conducted before and after inversion showed that the highest values were found in A sample. The effect of fruit sauces on saccharose content was statistically significant ($p<0.05$). Saccharose values of K and B samples were close to each other.

3.3.Chemical properties of ice cream samples determined during storage

Titratable acidity (%), pH and overrun (%) values of functional ice cream samples were given in Table 5. Titratable acidity values of ice cream samples varied between 1.14% and 1.30% LA during 120 days of storage period. The highest acidity values were determined at the 15^{th} day in B and K samples (1.30% LA), and the lowest value was found in F sample (1.14% LA) at the 120^{th} day.

Changes in the average titratable acidity values of fruit sauces were analyzed with analysis of variance and Duncan tests. The changes in titratable acidity values were significant in F and A samples ($p<0.05$) while not significant in K and B samples ($p>0.05$). Also, fruit sauce use had a significant effect on the differences between the samples at the 120^{th} day ($p<0.05$) and F sample was different than the other products. In a general evaluation, acidity values increased in all samples at the 15^{th} day. In the further days of the storage, acidity values showed fluctuations with decreases and had the 1^{st} day values at the 120^{th} day of the storage. It can be said that blackberry sauce was the most influential fruit sauce on acidity.

Özcan and Kurdal (1997) reported that titratable acidity changed between 0.29-0.74% in lemon ice creams, 0.31-0.87% in sour cherry ice creams, and 0.11-0.41% in strawberry ice creams (Aliyev, 2006). Aliyev (2006) reported that acidity values of ice cream samples contain kefir and blueberries were between 0.19-0.79 %. Güven and Karaca (2002), in their study on fruity yoghurt ice cream and vanilla yoghurt ice cream containing different ratios of sugar (18%, 20% and 22%) and strawberry (15%, 20% and 25%), stated that acidity increased as the fruit ratios increased. Santos and Silva (2012), in their study on Mangaba fruit ice cream production by using fat substitutes (Selecta Light, Litesse and Dairy Lo) and sweeteners (Laktilol and Splenda), reported that acidity values varied between 5.26-6.40 % LA .

Increase in acidity in ice cream samples fortified with fruit, fruit pulp and fruit sauce/aroma was an expected result. Therefore, in our study, the increase in acidity can be associated with fruit sauce content, as well as the presence of probiotic bacteria.

pH values of ice cream samples varied between 3.99 and 4.96 during 120 days of storage period. The highest pH value was determined at the 120^{th} day in K sample (4.96), and the lowest value was found in F sample (3.99) at the 15^{th} day. Changes in pH values during the storage were statistically significant in all ice cream samples ($p<0.05$). Fruit sauces had a significant effect on 1^{st}, 30^{th}, 60^{th} and 90^{th} days of the storage between the ice cream samples ($p<0.05$).

Table 5. Titratable acidity (%), pH and overrun (%) values of functional ice cream samples

	Functional Ice Cream Samples				
	Days	K	F	A	B
Titratable Acidity (Lactic acid %)	1	1.19±0.00	1.16±0.00 a	1.18±0.01 a	1.18±0.01
	15	**1.30±0.05**	1.24±0.01 c	1.27±0.01 c	**1.30±0.11**
	30	1.17±0.01	1.17±0.00 ab	1.19±0.00 a	1.18±0.02
	60	1.22±0.01	1.21±0.00 bc	1.23±0.01 b	1.22±0.00
	90	1.28±0.05	1.24±0.03 c	1.29±0.00 c	1.25±0.00
	120	1.18±0.01 Y	**1.14±0.00 aX**	1.18±0.00 aY	1.18±0.00 Y
pH	1	4.56±0.00 bY	4.39±0.04 cX	4.56±0.01 bY	4.57±0.02 cY
	15	4.15±0.03 a	**3.99±0.04 a**	4.17±0.04 a	4.13±0.02 a
	30	4.58±0.02 bY	4.66±0.01 eY	4.57±0.01 bY	4.45±0.03 bX
	60	4.22±0.00 aX	4.25±0.02 bX	4.43±0.01 bY	4.48±0.00 bZ
	90	4.63±0.00 bT	4.54±0.00 dZ	4.48±0.00 bY	4.43±0.00 bX
	120	**4.96±0.03 c**	4.79±0.00 f	4.76±0.11 c	4.88±0.00 d
Overrun (%)	1	**40.97±1.97**	26.43±6.28	37.77±4.00	31.62±1.61
	15	**16.64±10.29**	29.58±4.81	20.89±4.95	25.86±7.43
	30	33.40±6.69	36.32±5.18	25.20±5.58	40.29±7.48
	60	25.05±12.67	25.36±1.23	24.62±0.56	26.76±0.86
	90	21.32±3.74	27.34±4.24	29.70±2.52	37.42±0.32
	120	31.36±8.35	35.02±9.16	33.89±11.14	33.13±4.61

X, Y, Z: Values with the different letters in the same row differ significantly (P<0.05).
a, b, c, d, e: Values with the different letters in the same column differ significantly (P<0.05).

In a general evaluation, pH values decreased in all samples on 15th day. In the further days of the storage, pH values showed fluctuations with the increases and decreases. At the end of the 120th day pH values reached the maximum levels.

Şimşek et al., (2006) reported that Vardar (2003) found the average pH values of probiotic fruit ice cream between pH 4.33-5.89. Coşkun (2005) reported that pH values of 25 plain and 25 strawberry ice cream offered to market in Tekirdag Province varied between pH 6.22 - 6.52. Aliyev (2006), in their study on ice cream production containing kefir and blueberries reported the pH values of the samples between pH 4.18 and 6.16. Hwang et al. (2009) were studied about grape wine sediments in ice cream production, reported that pH values varied between 6.32-7.14. Santos et al. (2012), in their study on ice cream production with Mangaba fruit by using fat substitutes (Selecta Light, Litesse and Dairy Lo) and sweeteners (Laktilol and Splenda), stated that pH values were between 4.50-4.94 and associated the decrease in pH with fruit.

Depending on the properties of ingredients added to the mixture and the freezer, a certain amount of air can be trapped inside the mixture. Therefore they can demonstrate a certain amount of overrun. Thus, ice cream becomes soft, homogeneous, mouth-soluble and easily edible. Overrun not only affected the structure of the ice cream, but also had an effect on the durability, yield and nutritional value (Kesenkaş et al., 2013). Overrun values were determined with the mentioned method. It was seen that overrun values of the ice cream samples showed very irregular fluctuations during storage. The highest overrun values was found in K sample at the 1st day of the storage (40.97%), while the lowest value was determined in K sample (16.64%) at the 15th day of the storage. Statistical analysis showed

that both storage and fruit sauces had no significant effect on overrun values of ice cream samples ($p>0.05$).

Turgut (2006), in the study on ice cream produced by adding cream at different ratios and different probiotic bacteria, reported that the highest overrun (44.55%) was samples containing 5% cream fortification and *B. bifidum,* and the lowest overrun (30.38%) was in samples containing 10% cream fortification and *L. acidophilus.* In the study, the effect of cream fortification was statistically significant ($p<0.01$). Aliyev (2006) stated that overrun values were between 18.55-32.74% in the study on ice cream production containing kefir and blueberries. Dervişoğlu and Yazıcı (2006), in their study on ice cream containing fruit fibers, determined the overrun values between 22.13-45.08%. Tokuç et al. (2008), in their study on probiotic ice cream production by using baby originated *Lactobacillus* spp., reported the overrun values between 89–90%. Hwang et al. (2009) in their study on using grape wine sediments in ice cream production, reported that overrun varied between 35.3-60.2 %. Temiz (1994) stated that overrun decreased in ice cream samples as the saccharose and fat free dry matter levels increased (Kır, 2007). Additionally, Christiansen et al. (1996) reported that the decrease in pH had a greater effect for the trapped air in the ice cream mixture, compared to dry matter and fat contents (Turgut, 2006). Goff (1997) reported that overrun and fat ratios were inversely proportional (Alamprese et. al., 2002). Gürsel and Karacabey (1998), similarly, reported that one of the negative effects on the overrun was excessive fat content. Güven and Karaca (2002) reported that the increase in sugar and fruit content had an effect on the increase in first dripping duration, overrun and viscosity values. Alamprese et al. (2002) in a study conducted on probiotic ice cream produced by using different ratios of sugar and fat, reported that overrun was inversely proportional with fat ratio and the effect of fat content on overrun was higher in ice creams with lower sugar concentrations. Akın et al., (2006) reported that the overrun increased in probiotic ice creams as inulin and sugar content increased. It was stated that low overrun may be associated with low fat ratio (Kesenkaş et al., 2013).

3.4.Total phenolics, anthocyanidin, flavonoid contents and antioxidant capacity

Phenolic content (TPC), flavonoid content (TC), anthocyanidin (TA) and antioxidant capacity (TAOC) values of functional ice cream samples were given in Table 6. Regarding total phenolic contents, the highest value was in B sample with 6.73 mg of gallic acid /g DM at the 1st day, and the lowest value was in K sample with 1.48 mg of gallic acid / g KM at the 120th day. Control sample having the lowest value was a certainly expected result. Ice cream samples containing commercial raspberry and blackberry sauces had higher values compared to samples containing raspberry sauce produced from frozen raspberries. Values have tended to decrease during storage. Total anthocyanidin values were between 0.28-17.98 catechin / g KM. The highest anthocyanidin value was determined on 1st day in B sample while the lowest value was found in K sample on 120th day. Anthocyanidin content of blackberry sauce was much higher compared to anthocyanidin content of the other fruit sauces.Flavonoid values varied between 0.71-12.69 mg rutin/g DM. The highest flavonoid value was determined at the 1st day in B sample while the lowest value was found in K sample at the 120th day. Blackberry fruit sauce has noticeably a higher flavonoid content than those of the other two sauces. The values decreased during storage.

Table 6 Phenolic content (TPC), flavonoid content (TC), anthocyanidin (TA) and antioxidant capacity (TAOC) values of functional ice cream samples

	Functional Ice Cream Samples				
	Days	K	F	A	B
Total phenolic compound contents (mg gallic acid / g DM).	1	3.19±0.04 eX	3.82±0.02 eY	5.24±0.02 fZ	**6.73±0.07 eT**
	15	2.62±0.04 dX	3.52±0.06 dY	5.09±0.00 eZ	6.54±0.04 eT
	30	2.33±0.09 cX	3.29±0.00 cY	4.90±0.01 dZ	6.31±0.02 dT
	60	2.19±0.09 bcX	3.13±0.03 bY	4.68±0.02 cZ	6.02±0.06 cT
	90	2.09±0.06 bX	3.03±0.04 bY	4.39±0.00 bZ	5.69±0.09 bT
	120	**1.48±0.03 aX**	2.87±0.06 aY	4.14±0.04 aZ	5.10±0.02 aT
Anthocyanidin values (mg catechin / g DM).	1	0.97±0.02 fX	1.26±0.04 eY	3.88±0.07 dZ	**17.98±0.07 eT**
	15	0.85±0.04 eX	1.10±0.01 deX	3.71±0.10 dY	17.80±0.21 deZ
	30	0.68±0.05 dX	0.96±0.05 cdX	3.42±0.02 cY	17.31±0.16 cdZ
	60	0.55±0.03 cX	0.82±0.04 bcY	3.22±0.03 bcZ	16.86±0.10 cT
	90	0.43±0.01 bX	0.64±0.10 abX	3.03±0.08 abY	15.54±0.18 bZ
	120	**0.28±0.00 aX**	0.51±0.09 aX	2.79±0.09 aY	14.11±0.03 aZ
Flavonoid values (mg rutin/ g DM)	1	1.37±0.08 dX	5.23±0.03 cZ	4.64±0.04 eY	**12.69±0.06 dT**
	15	1.24±0.05 cdX	5.05±0.04 bcZ	4.48±0.11 deY	12.37±0.01 dT
	30	1.20±0.00 cdX	4.82±0.18 bcY	4.30±0.11 cdY	12.12±0.18 cdZ
	60	1.12±0.00 bcX	4.57±0.09 bZ	4.09±0.08 bcY	11.46±0.14 bcT
	90	1.00±0.06 bX	3.95±0.22 aY	3.90±0.11 abY	10.99±0.10 bZ
	120	**0.71±0.05 aX**	3.62±0.29 aY	3.77±0.02 aY	10.19±0.41 aZ
Antioxidant capacity values (mmol Trolox/ 100 g DM)	1	1.77±0.02 cX	5.77±0.05 eY	8.51±0.02 eZ	**12.95±0.06 dT**
	15	1.57±0.00 bX	5.53±0.03 dY	8.33±0.04 dZ	12.74±0.05 cdT
	30	1.60±0.07 bcX	5.36±0.03 cY	8.26±0.05 cdZ	12.15±0.06 bcT
	60	1.46±0.05 abX	5.21±0.00 cY	8.13±0.03 cZ	11.78±0.05 bT
	90	1.36±0.06 aX	4.93±0.04 bY	7.86±0.06 bZ	11.09±0.10 aT
	120	**1.32±0.07 aX**	4.75±0.08 aY	7.42±0.05 aZ	10.46±0.41 aT

X, Y, Z, T: Values with the different letters in the same row differ significantly ($P<0.05$).
a, b, c, d, e, f: Values with the different letters in the same column differ significantly ($P<0.05$).

Total antioxidant capacity values were between 1.30 (K sample at the 120th day) and 12.95 (B sample at the 1st day) mmol Trolox/100 g DM. Antioxidant capacity of blackberry sauce was much higher than those of other sauces.

Both fruit sauce and storage have significant effects on total phenolic content, total anthocyanidin, flavonoid and total antioxidant capacity values ($p<0.05$).Hwang et al. (2009), in their study on using grape wine sediments in ice cream production, reported that total phenolic content was between 1.52-3.58 mg/mL and total phenolic content increased as the amount of sediment increased. It has been reported that total anthocyanidin content in ice cream containing 150 g/kg sediment was 120.2 g/mL. In the study, it was shown that sediment content had a significant effect on total phenolic content, anthocyanidin content and antioxidant activity. Also, it has been stated that anthocyanidin and polyphenolic compounds which remain very stable during ice cream process may have a role in the increase in antioxidant activity. Karaman (2009) reported that, in ice cream mixtures aromatized with tea and some herbal tea, total phenolic content increased as the tea and derivatives' content increased.

3.5.Melting rate

Melting rate values of functional ice cream samples were given in Table 7. K sample had a slightly higher melting rate compared to ice cream samples containing fruit sauces. This was associated with the dry matter content. Additionally, samples containing raspberry sauce had a higher resistance against melting compared to the others.

The effect of fruit sauce had a significant effect on melting rate only at the 60th day of the storage. Also, the changes in melting rate at the 60th minute in F sample during storage was significant ($p<0.05$).

Güven and Akın (1997), in their study on ice cream production in 5 different types produced with the addition of 0%, 1%, 2%, 3% and 4% milk powder, found the melting rate values 81.54%, 73.78%, 68.99%, 68.60% and 68.15%, respectively. Dağlı (2006) reported that melting was not observed in any of the ice cream samples in the first 6 minutes in the study on yoghurt ice cream production containing whey powder. It was also reported that the average melting rate in the 30th minute was 5% while this ratio reached to 90% at the 90th hour. It was found that other samples had a more rapid melting compared to the control sample.

Alamprese et al. (2002) reported that sugar and fat content had significant effects on melting properties and firmness (Turgut, 2006). Güven and Karaca (2002) reported that the increase in sugar and fruit content had an effect on the increase in first dripping duration, overrun and viscosity values. However, total melting duration decreased parallel to the increase in sugar and fruit content (Aliyev, 2006). Dervişoğlu et al. (2005), in their study on skim soy powder, reported that melting resistance and viscosity of ice cream mixture increased as the skim soy powder content in the mixture increased. Generally speaking, ice creams with high dry matter have a higher resistance against melting, whereas fruit ice creams with low dry matter content have shorter melting times. Conceptually, melting duration and melting rate are inversely proportional. In other words, ice cream melting in a shorter time have higher melting rates (Yeşilsu, 2006). Durak (2006) reported that the increase in fruit pulp content have a positive effect on the resistance against melting. It was reported that melting rate increased as the saccharose levels increased, on the other hand melting rate decreased as the skim milk dry matter content increased (Kır, 2007). Karaman (2011) reported that melting rate decreases while sahlep content used in ice cream is increased.

Table 7. Melting rate values of functional ice cream ice cream samples (%).

Sample Codes	Duration	Day 1	Day 15	Day 30	Day 60	Day 90	Day 120
K	30 minutes	20.00±14.63	19.62±12.87	5.07±2.60	8.10±0.76 Y	2.73±1.16	0.73±0.55
	60 minutes	23.31±13.48	26.60±13.00	11.86±2.05	10.76±1.79	13.24±2.77	9.54±0.71
	90 minutes	25.29±12.24	29.84±11.50	16.13±1.65	14.75±2.28	17.52±3.79	15.65±1.45
	120 minutes	**27.41±12.31**	**32.12±10.02**	**19.29±1.63**	**16.70±2.50**	**22.00±5.00**	**18.95±1.33**
F	30 minutes	2.42±0.44	5.26±3.58	2.45±1.18	0.89±0.28 X	2.76±0.47	0.71±0.66
	60 minutes	8.49±0.08 bc	6.15±2.73 ab	7.68±0.29 abc	4.15±0.72 a	11.69±0.10 c	4.01±0.02 a
	90 minutes	11.82±0.84	8.35±1.96	11.09±0.82	9.62±0.32	14.20±0.80	8.94±1.45
	120 minutes	**14.93±0.22**	**11.54±0.38**	**14.20±1.20**	**13.28±0.92**	**16.71±0.97**	**11.58±1.58**
A	30 minutes	10.51±2.88	1.20±0.41	6.23±3.09	2.47±1.18 X	1.65±0.90	2.11±0.94
	60 minutes	13.43±1.99	5.00±0.68	10.34±3.33	4.84±1.91	7.82±0.73	6.34±2.03
	90 minutes	16.06±1.74	8.75±1.24	14.43±3.57	9.11±2.35	12.19±0.16	11.90±1.81
	120 minutes	**18.20±1.70**	**11.01±1.76**	**17.19±3.39**	**12.48±2.33**	**15.52±0.00**	**15.42±1.70**
B	30 minutes	6.02±1.75	2.21±0.08	5.57±3.47	1.60±0.40 X	0.24±0.06	3.63±0.37
	60 minutes	9.49±0.89	2.95±0.66	8.06±4.76	3.17±0.57	5.37±0.64	6.69±0.20
	90 minutes	12.78±1.27	4.54±0.82	10.28±4.37	4.70±0.41	10.27±0.11	11.16±0.01
	120 minutes	**15.11±1.00**	**6.86±0.65**	**12.40±4.07**	**6.83±1.59**	**12.80±0.38**	**14.43±0.20**

X, Y: Values with the different letters in the same column differ significantly ($P<0.05$).

a, b, c: Values with the different letters in the same row differ significantly ($P<0.05$).

4. Conclusions

In our study, we aimed to produce ice cream which is a delicious and healthy dairy product with increased health benefits by adding various functional properties. Accordingly, goat's milk, tagatose, Litesse Ultra, polydextrose, *Lactobacillus paracasei* subsp. *paracasei*, *Bifidobacterium longum+Bifidobacterium bifidum* probiotic culture, raspberry and blackberry fruit sauces were used. It was thought that general fluctuations in analyses results were associated with fruit sauces. Therefore, it can be suggested that fruit sauces could be seedless and in a more homogeneous structure. In the light of the results obtained, we conclusively believe that our study is of importance regarding both food industry and providing alternative and delicious functional products for consumers, however further studies are required for the evaluation of compliance of our study with other grape-like fruits and milk types. Also, the effects of fat reduction and use of sweeteners other than natural origin sugar on glycemic index can be determined with the further studies.

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CHIA SEED (*SALVIA HISPANICA* L.): IT'S PLACE IN NUTRITION AND RELATION WITH HEALTH – A REVIEW

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ABSTRACT

Chia seed (*Salvia hispanica* L.) is getting attention with its high polyunsaturated fatty acid, dietary fiber content and antioxidant capacity in recent years. It is considered as a functional food or ingredient because of being a natural source of some bioactive substances such as polyphenol compounds, dietary fiber, and omega-3 fatty acid. In human consumption, it can be used alone as whole chia seed or for the preparation especially bakery products. It has high antioxidant properties and angiotensin converting enzyme inhibitory effects in vitro. It is considered that chia prevents from cardiovascular diseases, diabetes mellitus and obesity with its high omega-3 fatty acid and dietary fiber content. Furthermore previous studies demonstrated the fiber and protein fractions of chia seed can be used in food industry for many purposes. In this review, nutritional value of chia, usage in food industry and effects on health is examined.

1.Introduction

Chia seed is known since ancient ages and one of the main foods with corn, bean and amaranth that consumed frequently by Central Americans. Besides its consumption as food, it's been used for its medical properties in religious rituals by Aztec and Mayan civilizations (Coates, 2011). It's a flowering plant that botanically belongs to Lamiaceae family, Salvia specie originated in Mexico and Guatemala. Although there are plenty of chia plant, the most known, produced and related with health problems one is *Salvia hispanica* L (Álvarez-Chávez *et al.*, 2008; Muñoz *et al.*, 2013). Today, in addition to its origin Mexico; Bolivia, Paraguay, Argentina, Australia, Central America, Peru, Ecuador and Colombia are important producers (Ministry of Foreign Affairs, 2015). Chia seed has positive health effects effect because of its fatty acid composition, dietary fiber and bioactive components. As it can be used as a food or oil extract, it also can be used in many fields such as industrial food fortification, animal feed and in cosmetics (Mohd Ali *et al.*, 2012; Muñoz *et al.*, 2013).

2.Nutrient Composition

Dry matter content of chia seed changes between 91-96%. In terms of macronutrients; it contains 25-41% carbohydrate, 20-22% protein, 30-35% fat and 4-6% ash (European Union (EU), 2013). Also, it has a quite high dietary fiber changing between 18-41% (Reyes-Caudillo *et al.*, 2008; EU, 2013) Chia seed do not contain gluten and it has biologically active components that are antioxidant (Muñoz *et al.*, 2013). Ayerza (2013) reported that genotype and color differences in seed do not affect protein, fat, amino acid and antioxidant compositions.

However, seed's nutrient composition may vary from country to country, environmental conditions and ecological differences (Ayerza, 2009; Ayerza and Coates, 2009). According to U.S. Department of Agriculture (USDA) nutrient database, seed's nutritional comparison with other seed and cereals is given in Table 1(a);1(b) (USDA, 2016).

Table 1(a). Comparison of nutrient composition of chia with other seeds and grain types (%) (USDA,2017)

Nutrients	**Chia seed**	**Flax seed**	**Soybean**	**Quinoa**
Energy (kcal)	486	534	446	368
Protein (g)	16.54	18.29	36.49	14.12
Total fat (g)	30.74	42.16	19.94	6.07
Carbohydrate (g)	42.12	28.88	30.16	64.16
Dietary fiber (g)	34.4	27.3	9.3	7.0
Saturated fatty acid (g)	3.33	3.663	2.884	0.706
Monounsaturated fatty acid (g)	2.309	7.527	4.404	1.613
Polyunsaturated fatty acid (g)	23.665	28.730	11.255	3.292
Omega-3* (g)	17.83	22.813	1.33	0.26
Omega-6** (g)	5.835	5.903	9.925	2.977

* α-linolenic acid (18:3), ** linoleic acid (18:2)

Table 1(b). Comparison of nutrient composition of chia with other seeds and grain types (%) (USDA,2017)

Nutrients	**Oat**	**Buckwheat**	**Wheat**	**Rice**	**Corn**
Energy (kcal)	389	343	339	370	386
Protein (g)	16.89	13.25	13.68	6.81	9.88
Total fat (g)	6.9	3.4	2.47	0.55	5.22
Carbohydrate (g)	66.27	71.5	71.13	81.68	74.93
Dietary fiber (g)	10.6	10.0	-	2.8	-
Saturated fatty acid (g)	1.217	0.741	0.454	0.111	0.820
Monounsaturated fatty acid (g)	2.178	1.04	0.344	0.200	1.483
Polyunsaturated fatty acid (g)	2.535	1.039	0.978	0.198	1.9
Omega-3* (g)	0.111	0.078	0.048	0.008	0.069
Omega-6** (g)	2.424	0.961	0.930	0.189	1.831

* α-linolenic acid (18:3), ** linoleic acid (18:2)

2.1.Fatty acid composition and stability

Chia seed has high polyunsaturated fatty acids and particularly cardiovascular protector effect ensure the relation with seed's fatty acid composition (Coates, 2011). Chia seed's dominant fatty acid component is essential omega-3 α-linolenic acid (ALA) and it has a respectable amount of other essential omega 6 linoleic acid (LA) within. According to studies, 60-65% of its fatty acid is ALA and 20% is LA (Álvarez-Chávez *et al.*, 2008; Ayerza, 2009; Ayerza and Coates, 2009). Thus, chia seed is more suitable due to its n-6/n-3 rate rather than another ALA source, flaxseed (Ciftci *et al.*, 2012). Nevertheless it should be remembered that n-6/n-3 rate of chia oil is unbalanced. This refers not to consume it as the only oil source in diet. On the other hand, "since the extent to which ALA is converted into physiologically essential long chain n-3 FA, in particular eicosapentaenoicacid (EPA, 20:5 n-3) and docosahexaenoicacid (DHA, 22:6 n−3), is very limited" (Schuchardt and Hahn, 2013), it is

better to say that for people who don't eat animal omega-3 sources, chia and other seeds can be a good substitute.

Furthermore, a higher content of ALA and LA results in low oxidative stability and shorter shelf life of the oil. However, in the dark, the combined addition of ascorbyl palmitate and tocopherol significantly reduced lipid oxidation and improved oil shelf life (at least up to 300 storage days) (Bodoira *et al.*, 2017). The addition of the different antioxidants increased the induction time of chia seed oil. The best effects were recorded in chia seed oil with the addition of ascorbyl palmitate, rosemary, and its blend with green tea extract (Ixtaina *et al.*, 2012). Besides adding antioxidants, there is new alternative to improve the chia oil stability like oil in water emulsions as delivery system (Julio *et al.*, 2015).

2.2.Dietary fiber content

For dietary fiber, chia seed is a quite good source. As its dominant fiber type is insoluble, it contains also soluble fiber (Mohd Ali *et al.*, 2012). Different seed types contain between 37-40 g/100 g dietary fiber that 33-35g/100 g is insoluble, 6-7 g/100 g is soluble fiber and most of the insoluble forms are cellulose, hemicelluloses and lignin; soluble are mostly mucilage (Reyes-Caudillo *et al.*, 2008). Chia seed is capable of contributing daily fiber intake effectively. Considering the recommendations for dietary fiber intake for adults (25-30 g/day), consumption of 15 g chia seed per day receives 20% of daily fiber intake approximately. Therefore, chia seed could contribute health particularly its fiber type and content. It is well known that dietary fiber is protective against many health problems from cardiovascular diseases to cancer (Marlett *et al.*, 2002).

2.3.Protein value

After the harvesting stage, the nutritional quality of the chia plant considerably decreases with an increase in the fibrous fractions and a dramatical decrease of the crude protein content (Peiretti and Gai, 2009). Still, chia seed's protein value is higher than many seed and traditional grains. Its gluten-free content provides advantage for especially celiac disease patients (Mohd Ali *et al.*, 2012). Furthermore, chia's protein fractions have angiotension converting enzyme (ACE) inhibitor effects *in vitro* (Salazar-Vega *et al.*, 2012; Segura-Campos *et al.*, 2013; Segura Campos *et al.*, 2013) suggesting that chia seed could have anti-hypertensive effects. However, regarding to essential amino acid content, chia seed should be consumed with lysine-rich foods due to its low quality protein and lack of lysine (Olivos-Lugo *et al.*, 2010). However in another study a balanced essential amino acid pattern was shown (Sandoval-Oliveros and Paredes-LóPez, 2012). This case shows that amino acid distribution may vary due to type of chia seed and where it is grown.

2.4.Mineral content and bioavailability

Chia seed's mineral composition varies between: 103-260 mg/100g for sodium; 726-826 mg/100g for potassium; 456-590 mg/100g for calcium; 9-12 mg/100g for iron; 77-449 mg/100g for magnesium; 604-919 mg/100g for phosphorus; Zn, 5-6.5 mg/100g for zinc; 1.7-1.9 for copper and 1.4-3.8 mg/100g for manganese in different studies (Chicco *et al.*, 2009; Jin *et al.*, 2012). It's observed that chia seeds have more calcium, phosphorus, magnesium, zinc, iron and copper than many seeds. However, fiber components of chia seed can affect bioavailability of these minerals (Capitani *et al.*, 2012). It is claimed that together with its ALA and fiber composition, chia seed's high mineral content provides beneficial effect on health (Chicco *et al.*, 2009). On the other hand, mineral composition of seed oil extract can vary depending on extraction method. Metals, especially copper and iron are not desired in oils because of their pro oxidant nature. That is why chia seed oils obtained by pressing or solvent extraction may/can be preferred (Ixtaina *et al.*, 2011).

2.5.Phytochemical content and antioxidant capacity

Total phytosterols in the oil ranged from 7 to 17 g/kg, and among sterols, β-sitosterol dominated and was found for up to 74% of the total unsaponified fraction. The content of β-sitosterol, stigmasterol and stigmastanol of chia seed is superior to that of peanut, rapeseed, safflower, sesame, and sunflower unrefined oils (Álvarez-Chávez *et al.*, 2008). Furthermore, tocopherol content of chia seed is 446 mg/kg and γ-tocopherol is the dominating isomer contributing 94.4% in chia to the total amount of tocopherols (Ciftci *et al.*, 2012). Considering chia seed's polyphenol content and antioxidant capacity, it's stated that main components of polyphenol are quercetin, kaempferol, myricetin, chlorogenic acid and caffeic acid (Reyes-Caudillo *et al.*, 2008; Marineli *et al.*, 2014; Martínez-Cruz and Paredes-López, 2014). Chia extracts have same peroxidation inhabitation capacity when compared to a commercial antioxidant product and thus, seeds' antioxidant capacity is quite high (Reyes-Caudillo *et al.*, 2008). Furthermore, chia's antioxidant capacity was found as same as coffee, tea and orange juice and higher than many grain products (Vazquez-Ovando *et al.*, 2009). Furthermore, chia seed or chia oil extract supplementation have increased the antioxidant capacity comparing to the control group in rat studies. Chia seed and chia oil extract diet groups have higher plasma glutathione, plasma catalase and glutathione peroxidase levels and glutathione reductase activity improvement on liver suggested it may help to prevent cardiovascular diseases with its antioxidant effects (Da Silva Marineli *et al.*, 2015a). In another study, improvement in superoxide dismutase and glutathione peroxidase expression has been recorded (Da Silva Marineli *et al.*, 2015b).

3.Usage in food industry

Chia seed's especially carbohydrate and protein characterization ensures its usage in many fields in the food industry. Chia seed's high fiber content is an appropriate component to be added to food due to its high water-holding capacity, emulsifier activity and stability (Vazquez-Ovando *et al.*, 2009). Chia gel has similar water-holding and fat-holding capacity, viscosity, emulsification activity and freeze-thaw stability with components that commonly used in food industry such as; guar gum and gelatin (Coorey *et al.*, 2014). For this reason it can be used as thickener, emulsifier substance and stabilizer in food. Moreover, adding chia flour to wheat flours, up to 15%, could be used to improve not only nutritional value, but also the technological properties (Verdú *et al.*, 2015). On the other hand, due to protein isolates produced from chia seed has good capacity of stability, water-holding and fat-holding capacity; it can be added to foods as functional component (Olivos-Lugo *et al.*, 2010). It's evinced that through the denaturation temperature of protein fractions is high (above 100°C); chia seed is suitable to be added to heat-processed food (Sandoval-Oliveros and Paredes-LóPez, 2012).

Chia seed may be considered as a functional food or ingredient due to its bioactive substances. Most common usage of seed is for bakery products. There can be some difficulties to keep the structure properly because of chia seed's gluten free nature. However, with using 25% of chia gel instead of eggs and oil, cake's color, taste, texture and overall acceptability has not been affected. Furthermore, it helped to decrease cholesterol content and increased the omega-3 fatty acid content according to control (Borneo *et al.*, 2010). There are other studies that show using chia in cakes provides omega-3 fatty acid enrichment (Pizarro *et al.*, 2013; Felisberto *et al.*, 2015). The best technological results were obtained for cakes produced with up to 15 g/100 g whole chia flour mixture (Pizarro *et al.*, 2013). Gluten-free bread produced from different levels of chia flour (no more than 10%) and tartary buckwheat has more protein, ALA, insoluble fiber when compared to gluten-free whole wheat bread (Costantini *et al.*, 2014). In another study, using chia and oat together to make cookies increase both omega-3 and β-glucan (Inglett *et al.*, 2014). Furthermore, chia

addition into bread provide a nutritional contribution in terms of increase of mineral and dietary fibre content (Švec *et al.*, 2016). Although usage of chia seeds enhance the protein, dietary fiber, antioxidants and mainly omega-3 fatty acids, ıt must be considered the formation of acrylamide and hydroxy methyl furfurol especially in bakery products like biscuits (Mesías *et al.*, 2016). Beside this, chia flour (partially defatted) can be added to meats in order to provide to enhange the omega-3 fatty acid value of meat (Souza *et al.*, 2015).

4. Health Effects

4.1. Animal studies

Oral chia seed supplementation on rats increases plasma ALA, EPA and DHA levels and improves omega 6/omega 3 ratio (Ayerza and Coates, 2007). Furthermore, chia oil extract and chia seed may help to improve cardiovascular health regardless of being milled or whole seed via hepatic lipid synthesis and secretion suppressing, hepatic and skeletal muscle fatty acid oxidation inducing nature of its long-chain omega-3 acid content (Lombardo and Chicco, 2006). Rats which had the same amount of ALA-including milled chia seed (15%) and chia oil extract (5%) have had reasonable decrease of total cholesterol, triglyceride and increase of HDL-C levels compared to corn oil (Ayerza and Coates, 2005). Similarly, whole chia seed supplementation to the rats' regimen provide decrease on triglyceride levels and increase HDL-C levels (Ayerza and Coates, 2007). Recently, chia seed provides development in vascular functions and it enhances vascular dilatation even in the high levels of cholesterol (Sierra *et al.*, 2015). These results suggest chia seed and oil extract can be alternatives for vegetarians or those who do not consume fish.

Consumption of chia seeds could contribute to glucose and energy metabolism in animals via reducing the plasma lipid levels and thus enhance peripheral insulin sensitivity (Chicco *et al.*, 2009; Poudyal *et al.*, 2012; Oliva *et al.*, 2013; Da Silva Marineli *et al.*, 2015b; Urrutia *et al.*, 2015). Chia seed supplementation to high sucrose diet postpones dyslipidemia and insulin resistance in short term (3 weeks). In addition, long term supplementation of chia decreases visceral adiposity in long term (2 months) (Chicco *et al.*, 2009), and decrease in hypertrophy in epididymal fat cells (Oliva *et al.*, 2013). Furthermore, 5% of chia seed supplementation to high carbohydrate and fat diet causes decrease in visceral adiposity, hepatic steatosis, cardiac and hepatic inflammation and fibrosis (Poudyal *et al.*, 2012). Chia seed and oil extract consumption could increase glucose tolerance and insulin sensitivity without influencing body weight and abdominal fat accumulation (Da Silva Marineli *et al.*, 2015b). Seeds that have high omega-3 fatty acid content can regulate lipogenesis on different tissues. When compared chia and flaxseed supplementation on adipose tissue formation and lipogenic gene expression, both seeds showed to inhibit acetyl CoA carboxylase 1, sterol-CoA desaturase and fatty acid desaturase 2 gene expressions in intramuscular adipose tissue; however, decrease of lipoprotein lipase and fatty acid desaturase 1 gene expressions was higher in chia seed supplement group (Urrutia *et al.*, 2015).

Anticarcinogenic effects of chia seed are limited. In a study the group takes chia oil extract have lower tumor weight and metastasis number compared to other oil type groups and have higher levels of apoptosis. This situation is explained by changes in PUFA distributions of neoplastic cell membranes of chia oil extract group and as EPA levels were increasing, arachidonic acid (AA) levels decreased (Espada *et al.*, 2007). There is a need for further studies to determine the mechanisms of chia seed's effects in other cancer types as well.

4.2.Human studies

Results of human clinical trials are summarized on the Table 2 (Vuksan *et al.*, 2007; Nieman *et al.*, 2009; Vuksan *et al.*, 2010; Guevara-Cruz *et al.*, 2012; Jin *et al.*, 2012; Brissette, 2013; Ho *et al.*, 2013; Toscano *et al.*, 2014; Nieman *et al.*, 2015). Regular consumption of chia seed may increase of

plasma omega-3 fraction levels due to its high omega-3 fatty acid content (Nieman *et al.*, 2009; Jin *et al.*, 2012). However, although *in vitro* and animal studies promise positive effects on blood pressure, the results of human studies are inconsistent (Vuksan *et al.*, 2007; Nieman *et al.*, 2009; Toscano *et al.*, 2014). Furthermore, consumption of chia may help to be reduce high-sensitivity C-reactive protein levels (Vuksan *et al.*, 2007), has positive effects on weight management (Guevara-Cruz *et al.*, 2012; Brissette, 2013) and postprandial glycemica (Vuksan *et al.*, 2010; Ho *et al.*, 2013). Also, for dietary interventions, considering the genetic variations may be beneficial. In a recent study, participants who have metabolic syndrome and *ABCA1* R230C gene variant in the group that has dietary intervention have more body weight loss (Guevara-Cruz *et al.*, 2012).

Table 2. Summary of clinical trials on human and primary findings

Authors/ year	**Duration of suplemantation**	**Subjects**	**Diet**	**Primary findings**
Vuksan *et al.*, 2007	12 weeks	Aged 18-75 Type 2 DM 11 male, 9 female	15 g/1000 kcal chia seed	- Decrease in systolic blood pressure, hs-CRP, HbA_1C and vonWillebrand factor - Increased plasma ALA and EPA levels
Nieman *et al.*, 2009	12 weeks	Aged 20-70 healthy overweight/obese 28 male, 48 female	25g whole chia seed or placebo flour two times per day	- No effect on body composition, inflammation, oxidative stress, blood pressure and lipoproteins - Increase in plasma ALA levels, no change in EPA, DHA levels
Vuksan *et al.*, 2010	Postprandial (15., 30., 45., 60., 90., 120. mins)	Healthy 6 male, 5 female	50 g carbohydrate including to diet that contains 0, 7, 15, 24 g chia seed	- Decrease in postprandial glycemia in a dose dependent manner -Decrease in appetite scores in different times with all doses
Guevara-Cruz *et al.*, 2012	2 months	Aged 20-60 healthy overweight/obese 67 individuals	Along with energy restriction soy protein, nopal, oat and chia seed including 235 kcal drink or placebo	-Decrease in body weight and waist circumference -Decrease in triglyceride, CRP, insulin and glucose intolerance -Reasonable decrease in body weight and increase in serum adiponectin concentrations of those who have *ABCA1* R230C gene variant with metabolic syndrome
Jin *et al.*, 2012	7 weeks	10 postmenopausal female	25 g milled chia seed	- Increase in ALA and EPA levels, no effect on DHA levels
Brissette, 2013	24 weeks	58 individuals with Type 2 DM	Chia seed or oat bran with	- More decrease of body weight, waist circumference

			energy restriction (amounts are unstated)	and hs-CRP levels in chia group -No difference of effects on glycaemic control and blood pressure
Ho *et al.*, 2013	Postprandial (15., 30., 45., 60., 90., 120. mins)	13 healthy individuals	0, 7, 15 or 25 g whole or milled chia seed in 50 g bread	- Decrease in blood glucose levels in a dose dependent manner whether it is milled or whole chia
Toscano *et al.*, 2014	12 weeks	26 hypertensive respondents aged 35-65	35 g/day chia flour or placebo	- Significant decreases between groups who took medical treatment against hypertension and who did not
Nieman *et al.*, 2015	Single dose	Aged 24-55, 16 male, 8 female athletes	7 kcal/kg chia seed oil in 500 mL water or placebo	- Increase in plasma ALA levels - No effect on running performance or inflammation

Chia seed supplement for athletes that perform more than 90 minutes can be used due to chia seed's omega-3 fatty acid, fiber, protein and antioxidant components (Illian *et al.*, 2011). On the other hand, 7 kcal/kg chia oil extract supplementation to the water of runners has no effect on performance instead an increase in plasma ALA levels (Nieman *et al.*, 2015). For sport nutrition more studies must conducted to determine accurate effects of chia.

5.Regulation of Usage and Safety

Chia seed's been classified as novel food in European Union Novel Food regulation number 258/97 in 1997 and European Commission did some revisions for usage in 2009. It's accepted that chia is an addible component to food and maximum 5% addition to bread may allowable (The Chia Company, 2011). Likewise, limited level of chia addition to bakery products has no negative effects on public health considering chia seed's nutrient components and consumption in EFSA panel report (EFSA, 2009). After increasing usage of chia in market, maximum 10% addition to bakery products, breakfast cereals, fruits, nuts and seed mixtures has been allowed by European Commission. It has been determined to inform the customers that packaged chia seed's consumption should not exceed 15 g per day (EU, 2013).

EFSA panel report notifies that the methodologies used until today are not sufficient to detect potential allergenicity of chia and toxicological findings on both human and animals are limited. However, widespread usage of chia in many countries provides supportive evidence for safety of chia seeds (EFSA, 2009). Recently, an IgE-mediative anaphylactic reaction is observed against chia's proteins (Jiménez *et al.*, 2015). So, it can be better to be careful for those who has sensitivity to certain foods like soy or nuts.

6. Conclusions

Chia seed has been used as a food since ancient ages and is becoming popular again today. It can be used as thickener, emulsifier, stabilizator in food industry due to its fiber components and protein fractions. Despite the fact that not being supported by all studies, through high omega-3 fatty acid, dietary fiber and polyphenol content, it can be preferred as a functional food in the prevention of particularly obesity, diabetes and cardiovascular diseases. Whilst it can be part of an adequate and balanced diet, considering chia seed as herbal gluten free omega-3 source, it can be an alternative to celiac

patients or vegetarians. Chia seed is approved as a safe food by European authorities and its usage, unless exceeding 15 g per day, is recommended consistent to the positive findings in conducted studies. It must be not forgotten that chia seed not a miracle but just be a good choice in diet in terms of valuable nutritional content.

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INFLUENCE OF CLARIFICATION TREATMENT ON OENOLOGICAL AND QUALITY CHARACTERISTICS ON YOUNG WHITE WINE SAUVIGNON

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ABSTRACT

Early conditioning treatments have been shown to have an effect of ameliorating properties without harmful effects on important quality characteristics. The aim of this study was to investigate the effects of some treatments on aroma compounds and also sensory and quality properties of young white wine.

The conditioning treatments influenced the physic-chemical parameters of the white wines; however, the results of the wine analysis indicate that there was a large variation in the concentration of free and total values of sulfur dioxide (SO2).

As for the content of volatile aromatic compounds, relative to the initial values, the additional and minor quantitative variations recorded during the treatments lead to higher or lower volatile compound contents (in some cases)

1.Introduction

The production of white wines is the natural vocation of most of our country's vineyards. Concerning the consumption of white wines, it can be appreciated that the taste of consumers is oriented towards dry wines, characterized by freshness and fruitfulness. A dry white wine that remembers the flavor and taste of grape is highly appreciated by consumers everywhere, these attributes being a guarantee of quality (Popescu T. et al., 1989).

Today, consumers expect all wine to be bright and clear, so clarification and stabilization have become important parts of the winemaking process. Clarification is accomplished by racking, fining, filtration and aging. Stabilization is done by racking, fining, filtration, chilling, ion exchange, aging and the use of special additives. Practically all white and blush wines require both clarification and stabilization treatments before they can be bottled.

Wine is an alcoholic beverage made up of water (80% to 85%), alcohols (mostly ethanol, 9% to 15%), and a variety of minor constituents (-3%) (Revi M. et al., 2014). Such minor constituents include organic acids, sugars, phenols, nitrogenous compounds, enzymes, vitamins, lipids, inorganic anions and cations, and a large number of volatile compounds. Of these, organic acids and phenols play a critical role by directly affecting product quality. Wine flavour is a complex mixture of taste and aroma attributes; the first is made up of a subtle balance of sweet (sugars), sour (organic acids),

and bitterIastringent (polyphenols) taste attributes; the second is made up of a large number of volatile compounds belonging to alcohol classes (lower and higher), organic esters, volatile organic acids, aldehydes, lactones, phenols, sulphur containing compounds, methoxy-pyrazines, norisoprenoids, ketones, and terpenes all of which contribute to wine aroma (Tores J. et al., 2004).

The production of wine with certain qualities of taste requires the mandatory presence of technological desiderata: the raw material with the qualities of composition and quality, the picking up of wine grapes and wine as much as possible from the grapes, and the protection of them by the lower possible doses sulphurous anhydride and some thermal fermentation regimes for must and keeping wine as convenient as possible (Stoica Felicia, 2006).

The quality and safety of food products are some of the most important factors influencing consumer choices in modern times (Considine K. M. et al., 2008). Today's consumers demand high-quality foods to be additive-free, fresh in flavour, microbiologically safe, and with an extended shelf life (Buzrul S., 2012). Since 1985, the US Food and Drug Administration (FDA) has required package labelling for most foods and beverages when sulphites are used as an additive. Due to its antiseptic and antioxidant properties, sulphur dioxide (S02) is one of the most versatile and efficient additives used in winemaking (Briones-Labarca, V. et al. 2017).

2.Materials and methods

2.1.Vineyard conditions and wine making

The grapes were collected in 2015 from Drăgășani Vineyard, stretching mainly to the West and North of Drăgășani town, parallel to the Olt River, over a length of about 65 km., being processed by Faculty of Horticulture Craiova, Department of Horticulture and Food science laboratory according to general wine making techniques. The Drăgășani vineyard, stretches between the Getic Subcarpathian Mountains and the Romanian Plain in the South and Southwest, lying between the parallel 44 ° 30'-44 ° 55 'north latitudes and the meridians 23 ° 55'-24 ° 15' eastern Longitidine (Stoica F. et al., 2017).

The wine used in this experiment was obtained from the Sauvignon grape variety by applying the following technology: healthy grapes, harvested to technological maturity, immediately processed; crushing and destemming of grapes; sulfating grapes and must with 50-60 mg / kg SO2; clarification of the must by decanting after 6-8 hours; conducting the alcoholic fermentation at temperatures of 18-22 ° C, first decantation of wine at 7-10 days after the end of the alcoholic fermentation.

2.2.Wine samples.

The young white wine Sauvignon used in this study was acquired from the Dragasani Winery's 2015 harvest in the Dragasani, Romania. The wine contained 30 mg/L of free sulphur dioxide (SO_2), but it did not contain bentonite. Once fermentation was completed, the white wine was packaged in 50-L glass pots and stored until the treatment in the dark. The treatment protocol was as follows: the bentonite dose used was 40g / HL, refrigeration was carried out at a temperature of -4.5 ° C and the pasteurization assumed that the wine was heated to 70 ° C for 10 minutes. All the wine analysis was realized in triplicate.

2.3.Physicochemical analysis

The values of the physico-chemical parameters, such as soluble solid, pH, total acidity, tartaric acid, and degree of alcohol were determined according to the Official Newspaper of the European Communities 1990. Total and free SO_2 were measured by the aspiration/oxidation method, also known as Rankin's method. Finally, determination of reducing sugar was by Fehling's method.

2.4.Chemicals and standard physicochemical analysis

Standards of volatile aroma compounds were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Dichloromethane (99.8%) and sodium sulphate (99%) were supplied by Kemika (Zagreb, Croatia). Pure deionised water was obtained from an Elix 3 purification system (Millipore, Bedford, MA, USA).

2.5.Analytical method

All samples were analyzed using gas chromatography and following the method used by the Laboratory of the Department of Horticulture and Food science and the laboratory of National Institute for Cryogenics and Isotopic Technologies (I.C.S.I. Rm. Valcea).

Sample preparation

Minor volatile compounds of the wine samples were liquid-liquid extracted and analyzed using gas-chromatography coupled with mass spectrometry (GC/MS). 100mL of wine sample was extracted 3 times with10/5/5 mL of dichloromethane, for 20 min, at 600 rpm. The organic extract was dried and concentrated to a volume of 1.5 mL. Carbonyl compounds were derivated with PFBOA (ortho-2,3,4,5,6-pentafluorobenzyl-ortho hydroxilamine), as described by Ţârdea C., 2007

GC/MS analysis

The volatile compounds concentration alcohols (1-propanol, 3-methyl-1-butanol, 1-hexanol, 1-henthanol and 1-octanol), esters (ethyl acetate, isobutyl acetate and isoamyl acetate) monoterpenes (nerol, geraniol and terpineol and linalol) and aldehydes (acetaldehyde and furfural) were determined using a gas chromatography system, a VARIAN 450 gas chromatograph GC-FID detector (flame ionization detection) with a set of 275^0C temperature for both the column TG-WAXMS 60 m, ID 0.32mm, film, 0.25mm, injector temperature 150°C, column temperature: 35°C, 3 min stand, climb to 20°C / min., up to 70 to 150°C with 27°/ min., stand 2 minutes, climb 200^0C, stand 2 minutes, climb to 240°C with 20°C/min. and stands 6 min. He carrier gas (1.2ml / min). Injection volume is 1 µL. The identification was made by comparing the retention times of standards from the calibration curve.

2.6.Statistical analysis

All parameters analized were assessed in triplicate, and the results were expressed as mean ± SD values of 3 observations. The mean values and standard deviation were calculated with the EXCEL program from Microsoft Office 2010 package.

3.Results and discussions

Clarifying treatments - the early conditioning of dry white wines (at the first decantation) proved to be a particularly favorable technological intervention for maintaining and enhancing the attributes of freshness and fruitiness. These treatments provide ideal conditions for the correct and ascendant evolution of quality attributes during the maturing of wines.

The data in Tables 1 and 2 highlight the dynamics of the main constituents of wine during complex conditioning.

The evolution of macro-constituents of wine generally shows significant decreases due to the elimination of the fractions responsible for instability. Fining refers to the deliberate addition of materials to a wine followed by the precipitation of components (Boulton et al., 1996). One agent, bentonite is commonly used to reduce protein content and aids in achieving a heat-stable wine. Thus, bentonization has the effect of deproteinization (Blade & Boulton, 1988) and the blue plating of metal removal from the wine. Heat treatment - pasteurization has many effects in wine: deproteinization, protective colloids formation, copper cassation prevention, dissolution of tartaric salts sub-microcrystals, biological and enzymatic stabilization. Refrigeration leads to the

precipitation and removal of crystallisable tartrates (potassium bitartrate and calcium tartrate), precipitation of crylable proteins. The total acidity shows a decrease of 0.32 g / L in the pasteurized variant and the volatile acidity of 0.06 g / l, in the same variant. Tartaric acid records the largest decrease, as expected, from refrigeration treatment. In terms of non-reducing extract and ash content, they decrease clearly in both chilled and pasteurized wines.

The effects of cold treatment from an organoleptic point of view are all the more important as the younger the wine. Thus, wines that was previously hard and rough, after refrigeration become more tasty because of decreasing acidity and tartrate precipitation.

Sulphur dioxide is an effective and low-cost additive for the preservation of wines and other food products (Coetzee & du Toit, 2015). Regarding the antioxidant protection, it is found that a 45-50 mg / L sulphite regime applied to the decanting of wine ensures this desideratum for the entire duration of the early conditioning process. SO2 content drops to all treatments applied to wine. The largest drops are recorded for cold and heat treatments applied to wine.

Since the early conditioning is intended to preserve the typicity of the wine, the effect of the treatments on the groups of compounds that contribute to the definition of the qualities - volatile compounds.

The data in Table 3 highlights the different but limited influence of treatments on the content of volatile compounds.

Table 1. Effect of different treatments on physicochemical parameters

Treatments	Alcohol degree %vol. X±SD	Tot.acidity g/L H_2SO_4	Volatile acidity g/L H_2SO_4	Tartaric acid g/L	Reducing sugar g/L	Non-reducing extract g/L	Ash g/L
C	11.85±0.08	4.50±0,01	0.44±0.01	4.62±0.15	1.4±0.21	21.91±1.00	1.78±0.09
BN	11.76±0.21	4.45±0.07	0.44±0.01	4.48±0.65	1.4±0.10	21.25±0.75	1.69±0.10
BG	11.69±0.30	4.44±0.07	0.43±0.02	4.45±0.65	1.4±0.10	20.79±0.77	1.68±0.13
RF	11.75±0.20	4.11±0.01	0.39±0.03	3.79±0.11	1.4±0.10	20.13±0.17	1.66±0.13
PS	11.67±0.11	4.20±0.12	0.38±0.03	4.13±0.05	1.4±0.10	19.93±0.37	1.65±0.12

C- untread-control, BN-bentonisation, BG-blue glue, RF-refrigeration, PS-pasteurization

Table 2. Effect of different treatments on free and total SO_2

Treatments	SO_2 Free – mg/L	SO_2 Total – mg/L
C	46.15±2.64	89.89±0.96
BN	32.71 ±1.64	78.36±0.09
BG	25,75±1.08	71.53±0.09
RF	21.78±0.32	68.36±0.04
PS	18.58±0,49	66.03±0.04

C- untread-control, BN-bentonisation, BG-blue glue, RF-refrigeration, PS-pasteurization

Table 3. Effect of different treatments on the volatile compounds

Compound mg/L	Untreated (control)	Bentonization	The blue glue	Refrigeration	Pasteurization
2-methyl-1-propanol	5.74±0.17	5.68±0.18	5.27±0.30	6.17±0.20	4.68±0.70
1- Hexanol	1.010±0.10	1.046±0.15	0.909±0.05	1.100±0.10	1.096±0.005
β- phenylethanol	64.86±1.19	64.16±1.90	63.20±1,25	66.90±2,50	70.53±1,00
Isoamyl alcohols	54.80±0.90	55.10±0.10	66.43±0.75	68.16±3.05	68.10±2.95
3-methyl-1-butanol acetate	0.619±0.02	0.549±0.03	0.655±0.007	0.494±0.13	0.398±0.07
Ethyl hexanoate	0.651±0.12	0.742±0.05	0.586±0.04	0.721±0.02	0.693±0.04
Hexyl acetate	0.389±0.009	0.414±0.01	0.348±0.03	0.340±0.02	0.393±0.02

Ethyl lactate	5.970±0.52	6.714±0.27	5.146±0.25	6.010±0.61	6.830±0.06
Ethyl octanoate	0.637±0.11	0.558±0.03	0.430±0.07	0.517±0.05	0.474±0.01
Ethyl decanoate	0.213±0.07	0.144±0.005	0.118±0.04	0.124±0.03	0.133±0.04
Ethyl dodecanoate	0.307±0.03	0.368±0.01	0.354±0.008	0.416±0.04	0.453±0.05

Relative to baseline values, the extra and minus quantitative variations recorded during treatments lead to higher or lower (in some cases) volatile compound contents. Evan if the final gains are due to the increase in the content in isoamyl alcohols, it is observed the maintenance in the initial limits and even a favorable evolution of some of the valuable components.

Bentonite can alter wine aroma constituents. Armada and Falqué (2007) found that Albariño wines fined with bentonite had a lower concentration of terpenes and C13-norisoprenoids (13%) and C6 alcohols (33%). These volatile compounds are responsible for the varietal aroma of Albariño wines and a decrease in concentration could diminish overall quality.

Also, bentonite treatment has a beneficial effect, further emphasizing the taste and smell attributes by eliminating foreign odors or excessive amounts of bitter, astringent phenols. Although it can not completely eliminate unwanted products, it manages to diminish their sensory negative impact. However, some authors (Sauvage FX et al., 2011, Lira E. et al., 2015) impute a diminishing of the aromatic and coloring compounds; the more important the higher the bentonite doses, the fact is also found in this case study.

Ethyl hexanoate was significantly highest in the bentonite and refrigerent treatments, while lowest in the blue glue treatment. Also, ethyl lactate has a same evolution. Ethyl decanoate was lower in all treatments while ethyl dodecanoate was significantly highest in all treatments.

Table 4. Odor thresholds (OT), concentrations (mg/L) in wine and aroma descriptors of volatile compounds found in Sauvignon wines (Sanborn M., 2008)

Volatile compounds	OT (mg/L)	Aroma descriptor
2-methyl-1-propanol	40	acid, fruit, floral
1- Hexanol	1.1	herbaceous, woody, alcohol
Isoamyl alcohols	30	whiskey, pungent
3-methyl-1-butanol acetate	1.5	banana, fruit, sweet
Ethyl hexanoate	0.08	fruit, pineapple, green-apple, banana
Hexyl acetate	-	sweet, fruity
Ethyl lactate	-	fruity, pineapple
Ethyl octanoate	0.58	fruit, floral, pear, sweet
Ethyl decanoate	0.5	brandy, oil, grape
Ethyl dodecanoate	-	mango, leafy, soapy

4. Conclusions

The production of dry white wines with superior qualities of taste implies the realization of a technological context from the aspect of all the factors involved, from the quality of the raw material to the conditioning and early bottling.

Early conditioning of dry white wines makes it possible to reduce the total SO2 content to relatively low levels, with the concurrent achievement of a corresponding free SO2 content.

Treatment with bentonite leads to a decrease in protein content, non-reducing extract and ash.

The volatile composition of Sauvignon, known for its fruity and floral bouquet, can respond favorably to clarification treatments.

From the organoleptic point of view, treatment with bentonite and refrigeration has the greatest impact. Thus, bentonite treatment has a beneficial effect, further emphasizing the taste and smell attributes by eliminating foreign odors or excessive amounts of bitter, astringent phenols.

The effects of cold treatment from an organoleptic point of view are all the more important as the younger the wine. Thus, wines that was previously hard and rough, after refrigeration become more tasty because of decreasing acidity and tartrate precipitation. Treatment with cold cause a slight shade of aging since oxygen dissolution step low temperature favoring the maturity of the wine.

Sauvignon's popularity to the Romania wine industry makes it a likely candidate for future processing research, such as fining.

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EVALUATION OF DOUGH RHEOLOGY AND QUALITY OF SUGAR-FREE BISCUITS: ISOMALT, MALTODEXTRIN, AND STEVIA

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ABSTRACT
In this study, the sucrose replacement by different concentrations as well as a combination of isomalt, maltodextrin, and stevia was approached to produce sugar-free biscuits. Some parameters of produced sugar-free biscuits such as dough rheology, texture, color, volume and density after baking under controlled conditions were investigated using instrumentally and sensory evaluation. The increment in the concentration of incorporated isomalt resulted in harder texture as well as lower volume. Prepared biscuits with 6% isomalt, 2.5% maltodextrin, and 0.06% stevia were reported as darker, and 3% isomalt, 2.5% maltodextrin, and 0.07% stevia were noted as lighter among the investigated samples. Incorporation of 6% isomalt, 2.5% maltodextrin, and 0.06% stevia resulted in the biscuits more comparable to elaborate one with sucrose, and with the highest acceptance level in sensory evaluations. However, the lowest level of quality biscuits was obtained using 0.06% stevia solely.

1.Introduction

Nowadays, the food industry has focused on the production of low-calorie foods due to the ongoing growth of attention to "healthy food." The significance of healthy food is a motivating consumer for a diet with less fat and sugar. However, in most foods, the elimination or reduction of any ingredient causes evident losses in overall acceptability. The consumption of high quantities of sugar has been linked to serious diseases, such as dental cavitation and obesity (O'Brien and Lemieux 1993). Moreover, Dietitians usually recommend the patients consuming foods contain fewer amounts of sugar, like biscuits (Drewnowski et al. 1998).

Therefore, removal of sugar to elaborate sugar-free biscuits is considered a primary task for the bakery industry. However, sugar cannot be easily substituted, especially in biscuit which owes its textural characteristics mostly due to relatively high sugar content. Moreover, sucrose plays a critical role in providing volume, texture, and sweetness in bakery products. Also, the presence of sugar leads to increasing the temperatures of egg protein denaturation, starch gelatinization and restricts gluten formation, therefore contributes to bulk and volume (Ngo and Taranto 1986; Shukla 1995). Consequently, the structural and sensory properties of biscuit would be affected by reducing the sucrose level (Frye and Setser 1991).

In recent years, some artificial and natural sweeteners, are recognized as sugar replacers (Gallagher et al. 2003; Lin et al. 2003; Neville

and Setser 1986; Newsome 1993). Stevioside, as a natural sweetener, is approximately 300 times sweeter than sucrose (Geuns 2003). Stevia which is demonstrated lots of beneficial effects in treating obesity, high blood pressure, and diabetes, has been approved by CODEX Alimentarius, and Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a confirmed ingredient in various types of products (India Stevia Association, Vishwanath and Waldshan 1991).

High-quality biscuit cannot be obtained with intense sweeteners solely. Therefore, the incorporation of bulking agents in biscuit formulation seems to be crucial (Deis 1994; Zoulias et al. 2000). Bulking agents like sugar alcohols as well as maltodextrins are expected to provide the required functional properties (Manisha et al. 2012). Being as a bulking agent for low-calorie baked goods, sugar alcohols, other advantages for human health in comparison with sucrose can be listed such as incomplete absorption and metabolism (Wennerholm et al. 1991), lower level of insulin (Finer 1991). Isomalt as a sugar alcohol is the only polyol derived from sucrose; by hydrogenation of isomaltulose. The principal components are the disaccharide alcohols 6-O-a-D-glucopyranosyl-D-sorbitol, and 1-O-a-D-glucopyranosyl-D-mannitol dehydrate (Ngo and Taranto 1986). According to previous investigations, the cookies contain polyols were markedly softer in comparison with prepared using the sucrose (Ronda et al. 2005; Zoulias et al. 2000). Using of sorbitol in biscuit formulation could lead to very soft texture. Moreover, suggested replacing a segment of sorbitol by isomalt to achieve a desirable quality (Peck 1994). Maltodextrins are produced from partial acidic or enzymatic hydrolysis of starch and mainly derived from corn, potato, wheat, and tapioca. Due to the small molecular size of amylose and amylopectin and their interactions, weak gels can be formed by maltodextrins. Maltodextrins could be used as texture modifiers, fat replacer and bulking agents (Chronakis 1998; Savitha et al. 2008).

This work aimed to evaluate the incorporation of isomalt, maltodextrin, and stevia alone or in combination as a sweetener system in sugar-free biscuits. The dough's rheological properties and the textural and physicochemical properties and sensory analysis of the biscuits were also investigated.

2.Materials and methods

2.1.Materials

White fine sugar, confectionary fat, skimmed milk powder, vanilla essence, sodium chloride, sodium bicarbonate, ammonium bicarbonate, lecithin, whole egg powder, and stevioside were procured from Kamvar Company, Esfahan, Iran. Wheat flour (14.1% moisture, 0.63% ash, 11.5% protein, 26.5% gluten) was obtained from Atlas Co, Esfahan, Iran, and isomalt and maltodextrin were purchased from Cargill, Berlin, Germany and Sepahan Pouran powder co, Esfahan, Iran, respectively (Table 1).

Table 1. Biscuit formulation

Ingredients	Control (g/100g flour)	Sugar-free biscuit (g/100g flour)
Wheat flour	100	100
Sugar	34	-
Confectionary fat	34	34
Milk powder	2	2
Vanilla essence	4	4
Sodium chloride	1	1
Sodium bicarbonate	0.4	0.4
Ammonium bicarbonate	0.1	0.1
Lecithin	2	2
Egg powder	1.8	1.8
Isomalt	-	3-6-9-12

Maltodextrin	-	2.5-5-7.5-10
Stevioside	-	0.06-0.08-0.1-0.14
Isomalt,Maltodextri,Stevia	-	6-2.5-0.06
Isomalt,Maltodextri,Stevia	-	3-2.5- 0.07
Isomalt,Maltodextri,Stevia	-	3- 5- 0.08
Isomalt,Maltodextri,Stevia	-	9- 5- 0.1
Isomalt,Maltodextri,Stevia	-	12- 7.5- 0.12
Isomalt,Maltodextri,Stevia	-	15- 10- 0.14

2.2.Experimental procedure

Table 2. The used treatments

Treatments	Treatment number
Isomalt 6%, Maltodextrin 2.5%, Stevia 0.06%	(T_1)
Isomalt 3%, Maltodextrin 2.5%, Stevia 0.07%	(T_2)
Isomalt 3%, Maltodextrin 5%, Stevia 0.08%	(T_3)
Isomalt 9%, Maltodextrin 5%, Stevia 0.1%	(T_4)
Isomalt 12%, Maltodextrin 7.5%, Stevia 0.12%	(T_5)
Isomalt 15%, Maltodextrin 10%, Stevia 0.14%	(T_6)
Isomalt 3%	(T_7)
Isomalt 15%	(T_8)
Maltodextrin 2.5%	(T_9)
Maltodextrin 10%	(T_{10})
Stevia 0.06%	(T_{11})
Stevia 0.14%	(T_{12})
100% Sucrose	Control

Biscuits were formulated with the aid of the research and development (R&D) department of the Kamvar Company, like a sugar-free bakery product factory in Esfahan, Iran (Table 2).

The reported formulations were obtained by trial and error. Confectionary fat was mixed with maltodextrin, isomalt, and stevia (depends on the formulation), in a mixer (Kenwood Chef A901, UK) for 15min at room temperature at high speed to form a cream and the other ingredients except wheat flour were then added to the cream and mixed for another 15min, finally, sifted wheat flour was added to the mixture to produce biscuit dough. The biscuits were baked in an air circulation oven (Memmert, Model UN750, Germany) at 255°C for 10 min.

Physical evaluations including volume, density, and color were performed on cooled samples. The rest of the samples were packaged in polypropylene bags for 24h and then texture, and sensory analyses were carried out. All the presented results were calculated from the average of two trials.

2.3.Analytical methods

2.3.1.Rheological properties of dough

2.3.1.1. Penetration test

The penetration of the dough was evaluated using a texture analyzer (Model LFRA 4500, Brookfield, USA). For the penetration test, 110 g of biscuit dough was weighed in the cell. The dough cell was placed on the base of the machine; then a 3 mm cylinder probe was moved down to the smooth surface, and the probe penetration test was commenced. The analyzer was set at a "return to start" cycle, a speed of 0.8 mm s^{-1} and a distance of 10 mm (Zoulias et al. 2000).

2.3.1.2. Texture profile analysis (TPA)

The dough was placed on the disks (2 cm diameter, 2 mm thickness) using a texture analyzer (Model LFRA 4500, Brookfield, USA) according to Bourne (1978). The analyzer was

set at a "texture profile analysis (compression)'' cycle, a speed of 1 mm s^{-1}, a distance of 10mm and a relaxation time of 2s between the two compressions. The following parameters were achieved: dough hardness (g), (height of compression peak); dough cohesiveness, the ratio of the areas of the two resistance peaks (A2/A1); Springiness is Length 2/ Length 1. Adhesiveness is the area of adhesion peak; Measurements were conducted twice, and the results are presented as the mean values (Zoulias et al. 2000).

2.3.2.Physicochemical properties of biscuits

The physical properties measured were color, volume, and density. The surface color of the biscuits was measured using a Minolta model CR-200 chromameter (Minolta Camera Co Ltd, Japan). The values of L* (Lightness), a* (redness-greenness) and b* (blueness-yellowness) were measured. The measurements were conducted in duplicate, and the results are presented as the mean values.

The volume of the biscuits was determined by rapeseed displacement method (**AACC Method 10-05.01**). The biscuits were weighed after removal from the pan, and the density was calculated by the ratio volume to weight.

For chemical properties, moisture content was determined using (**AACC Method 44-16.01**), ash content (**AACC Method 08-01.01**), protein content (**AACC Method 46-10.01**), and fat content (**AACC Method 30-10.01**), (AACC 2000).

2.3.3.Textural properties

2.3.3.1. Fracture Strength (Snap Test)

The 3-Point Bending Rig of Texture analyzer was used to determine fracture strength. The blade was brought down at a test speed: 0.8 mm/s, distance: 7 mm to contact the biscuit. The downward movement was continued till the biscuit breaks. The peak force was reported as fracture strength. Measurements were conducted twice, and the results are presented as the mean values (Singh et al. 2008).

2.3.4.Sensory analysis of biscuits

Sensory analysis of biscuits was conducted by 20 member's semi-trained panel. The examined sensory characteristics were sweetness, aftertaste, hardness, appearance and overall acceptance (combination of flavor, appearance, and texture). All five sensory attributes were rated on a 1–5 hedonic intensity scale where 1= lowest score and 5= highest score (Zoulias et al. 2000).

2.3.5.Statistical analysis

All tests were conducted at least in duplicates and average, and the standard deviation was calculated using Excel software (Office 2010). The experimental data were performed in a completely randomized design and were analyzed using Analysis of Variance (ANOVA) and expressed as mean±standard deviation. The significance of differences between the means was determined by Duncan new multiple range test ($p \leq 0.05$), and all statistical computations and analysis were carried out using SAS version 9.1.

3.Results and discussion

3.1.Effect of sugar replacement on dough rheology

The rheological properties of prepared dough were affected by the used sugar replacers as indicated in Table 3. The obtained maximum force by the probe penetration and TPA test of 3-Point Bending Rig of Texture analyzer dough prepared as treatment number 3 (T_3) were significantly greater than the others. In fact, due to the firmness of the T_3 dough, the sheeting was tough. Values of maximum force obtained by the TPA test, which represent hardness of the dough, not exactly followed the same order as probe penetration ones. According to probe penetration, T_4 dough presented the lowest hardness, followed by T_6, T_7, T_9, and T_{10}, while the T_3 and T_8 dough had significantly higher values. Also, the T_7, T_9, T_{11} and T_{12} presented nominal values of hardness as estimated by the TPA test, although a high maximum force was achieved by T_3. As can be seen, in both probe penetration and TPA tests, with increasing

isomalt from 3% to 15%, the harder dough was produced, while using isomalt up to 3% lead to softer dough according to the rheological tests. Increasing maltodextrin also leads to the harder dough with considering resulting of TPA test. It can be noted that due to the ability of maltodextrin in forming gels in the presence of water, maltodextrin would lead to decrease the elasticity of the dough (Savitha et al. 2008). TPA test was approached by Manohar and Rao to investigate the effect of sugar type and content on rheological properties of biscuit dough. According to this study using different types of sugars diminished hardness and consistency to the same extent (Manohar and Rao 1997), which is not exactly in agreement with results of the current study (Manohar and Rao 1997).

Table 3. Effect of sugar replacers on rheological properties of biscuit dough

Treat-ment number	Hardness force penetration (g)	Hardness force TPA (g)	Cohesiveness (A1/A2) g/s	Gumminess	Springiness	Adhesiveness (g/s)
(T_1)	1482.50 ± 0.535 c	1324.50 ± 26.823 f	0.24 ± 0b	317.36 ± 0.803 f	3.11 ± 0.007 edf	175.78 ± 4.468 c
(T_2)	1112.46 ± 1.364 d	1445.75 ± 1.062 e	0.23 ± 0.007 bd	348.98 ± 0.735 e	3.50 ± 0.084 b	165.91 ± 0.756 d
(T_3)	2083.50 ± 7.778 a	2151.75 ± 2.475 b	0.25 ± 0.007 a	557.31 ± 0.742 b	3.33 ± 0.021 bc	167.15 ± 0.742 d
(T_4)	453.86 ± 3.726 l	1558.50 ± 2.124 d	0.22 ± 0.028 ed	319.47 ± 1.449 f	2.90 ± 0.070 f	204.85 1.414 b
(T_5)	852.35 ± 3.033 f	1643.25 ± 1.060 c	0.23 ± 0.007 bd	370.55 ± 0.954 c	3.19 ± 0.007 dc	151.37 ± 0.735 f
(T_6)	660.50 ± 2.120 i	993.85 ± 1.901 h	0.25 ± 0a	244.64 ± 0.714 i	3.18 ± 0.007 dc	128.30 ± 0.558 h
(T_7)	586.25 ± 1.767 j	775 ± 2.122 k	0.24 ± 0.007 b	179.67 ± 0.240 k	2.99 ± 0.049 ef	102.26 ± 2.941 k
(T_8)	1725.40 ± 5.437 b	2671 ± 1.411 a	0.20 ± 0.007 e	575.67 ± 2.177 a	3.29 ± 0.197 dc	230.56 ± 0.615 a
(T_9)	681.87 ± 1.937 g	589.50 ± 1.416 l	0.23 ± 0.007 bd	144.83 ± 0.749 l	3.19 ± 0.219 dc	162.70 ± 0.586 e
(T_{10})	530 ± 1.414 k	1025.75 ± 1.064 g	0.25 ± 0.007 a	265.16 ± 0.360 h	3.33 ± 0.014 bc	175.49 ± 1.420 c
(T_{11})	351.30 ± 1.138 m	898 ± 1.858 j	0.23 ± 0.014 bd	281.61 ± 1.711 g	3.93 ± 0.035 a	144.89 ± 0.763 g
(T_{12})	672.07 ± 2.722 h	892.36 ± 1.616 j	0.23 ± 0.007 bd	207.37 ± 0.664 j	3.91 ± 0.063 a	121.59 ± 1.152 i
Control	992.14 ± 0.912 e	1643.93 ± 1.548 c	0.22 ± 0.007 ed	360.15 ± 1.195 d	3.16 ± 0.084 edc	113.86 ± 0.650 j

Mean values with the same superscript letter within the same column are not significantly different at P <0.05

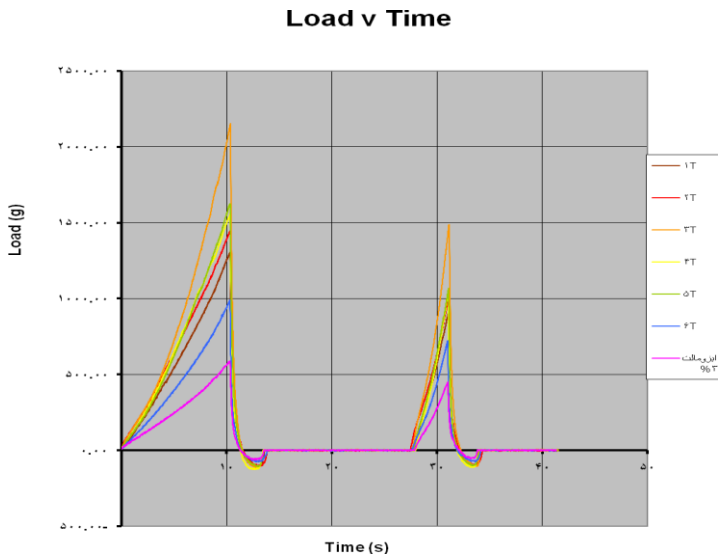

Figure 1.TPA graphs of sugar-free biscuit dough

The obtained results for the cohesiveness of dough did not show significant differences among all of the examined samples. As for gumminess, T_3 and T_8 doughs had the highest value, whereas T_7, had the lowest score among all samples due to its softer texture. T_7 and T_8 had the lowest and highest adhesiveness, respectively. Therefore, it is concluded that

increasing the amount of isomalt, influences all the rheological attributes. Moreover, T_4 and T_7 doughs had the lowest springiness while T_7 sample has the closest value to the control. On the other hand, supplementation of dough with stevioside did not affect the rheological properties of dough according to both conducted rheological tests, due to the small added quantity.

Figure 2. T_1 sample

Figure 3. T_6 sample

Figure 4. biscuits contain 12% Isomalt

Figure 5. biscuits contain 2.5% Maltodextrin

Figure 6. biscuits contain 3% Isomalt

Figure 7. biscuits contain 10% Maltodextrin

Figure 8 biscuits contain 0.06% Stevia

Figure 9. biscuits contain 0.14% Stevia

Figure 10. Control biscuits contain 100% sucrose

3.2.Effect of sugar replacement on biscuit properties

3.2.1.Physicochemical and textural properties of biscuits

Results of the volume and density quantifications of biscuits with different sweeteners are given in Table 4. While T_2 biscuits had volume values same as the control, T_5 and T_8 biscuits had volumes notably lower than the control. Due to the interactions between sweeteners, bulking agents, starch, and protein, which influence starch gelatinization and protein denaturation temperatures, could cause adverse changes in the thermosetting mechanism and heat transfer. Therefore the expansion of bubbles would be inadequate (Hicsasmaz et al. 2003; Neville and Setser 1986; Stauffer 1990).

Biscuits exhibit a substantial increment in the volume with an increase in maltodextrin from 2.5% (T_9) to 10% (T_{10}), due to the decrease in the viscosity of the biscuit dough. Increasing stevia from 0.06% (T_{11}) to 0.14% (T_{12}) would not affect the volume of the biscuits, due to the small concentration of added stevia. On the other hand, higher volume was observed for biscuits elaborated with 3% isomalt (T_7) in comparison with 15% isomalt (T_8). It seems that isomalt restricts biscuit spreadability more than sucrose; therefore, this fact could be associated with the higher firmness measured on biscuits manufactured with higher concentration (15%) of isomalt.

In the baking range, the solubility of isomalt is lower than sucrose as temperature increases (Mckemie 2006), so the viscosity and rate of flow of the respective doughs are expected not to be similar. Therefore it seems that the different sweeteners and bulking agents affect the setting time of dough, the gluten network properties, and glass transition. About the abovementioned results, it can be noted that the biscuits elaborated with combinations of isomalt, maltodextrin and stevia have volume values closer to sugar biscuits and using each of sugar replacers individually, would have adverse effects on volume.

Sugar-free biscuits with higher volume showed lower density than other samples (Table 4). However, while T4 biscuit had the maximum volume, at the same time, it had the lowest density due to the similarity of biscuits weight.

The color of the biscuits is one of the key features which affect the acceptability of the product by the consumer. Table 5; presents the L*, a*, and b* value. The a* and b* values of all samples did not show any significant differences. T_1 biscuits were significantly darker than the others while T_2 was lighter among the samples. Isomalt, as a polyol does not participate in Maillard reactions, as it is not reducing sugar, so would not affect the color of the biscuits. The addition of stevia had no significant effect on the color due to its high thermal resistant, while maltodextrin can promote browning reactions, due to its thermal degradation. As can be seen, in the T_2 to T_6 biscuits, by increasing the amount of isomalt and maltodextrin, the samples tend to get darker, and T_4, T_5, and T_8 biscuits have the closest lightness to the control. As can be seen in Table 5, T_4, T_5, T_6, T_8 and T12 biscuits had the closest a* to the control, while T_9 and T_{10} biscuits had closest b* to sugar samples.

Table 4. Volume, density and hardness of biscuits elaborated with different sugar replacers and with sucrose (control).

Treatment number	Volume (cm^3)	Density (g/cm^3)	Hardness (g)
(T_1)	18.19 ± 0^{f}	0.50 ± 0^{cd}	866.00 ± 6.05^{dec}
(T_2)	18.95 ± 0.07^{d}	0.45 ± 0^{ef}	664.80 ± 3.88^{e}
(T_3)	16.62 ± 0.10^{h}	0.58 ± 0.01^{b}	1069.50 ± 13.59^{dec}
(T_4)	21.70 ± 0.12^{a}	0.43 ± 0.01^{f}	1381.00 ± 13.54^{bc}
(T_5)	13.08 ± 0.04^{j}	0.67 ± 0^{a}	1689.30 ± 24.47^{b}
(T_6)	18.64 ± 0.19^{e}	0.52 ± 0.03^{c}	2340.50 ± 25.29^{a}
(T_7)	16.69 ± 0.09^{h}	0.53 ± 0.01^{c}	701.00 ± 33.94^{e}
(T_8)	13.10 ± 0.11^{j}	0.66 ± 0.01^{a}	1034.50 ± 20.30^{dec}
(T_9)	17.07 ± 0.07^{g}	0.52 ± 0^{c}	892.50 ± 17.67^{dec}

(T_{10})	19.79 ± 0.04 ^{b}	0.44 ± 0 ^{ef}	1311.30 ± 15.03 ^{dbc}
(T_{11})	16.16 ± 0.05 ^{i}	0.53 ± 0.01 ^{c}	805.30 ± 12.04 ^{de}
(T_{12})	19.38 ± 0.09 ^{c}	0.48 ± 0.01 ^{ed}	747.50 ± 17.37 ^{e}
Control	18.85 ± 0.06 ^{d}	0.52 ± 0.02 ^{c}	795.00 ± 18.96 ^{de}

Mean values with the same superscript letter within the same column are not significantly different at P <0.05

Table 5. L*, a* and b* parameters of biscuits elaborated with sugar (control) or with different sugar replacer

Treatment number	L*	a*	b*
(T_1)	40.40 ± 0.70^{f}	23.60 ± 0.27^{a}	48.35 ± 0.77^{g}
(T_2)	62.90 ± 0.56^{a}	12.95 ± 0.19^{d}	64.00 ± 0.07^{a}
(T_3)	55.60 ± 0.51^{cb}	17.95 ± 0.48^{cb}	58.65 ± 0.10^{bdc}
(T_4)	49.90 ± 0.50^{de}	21.00 ± 0.84^{cab}	54.10 ± 0.12^{fde}
(T_5)	48.30 ± 0.31^{de}	20.75 ± 0.31^{cab}	52.50 ± 0.04^{fge}
(T_6)	46.85 ± 0.35^{ef}	21.60 ± 0.82^{cab}	51.25 ± 0.19^{fg}
(T_7)	52.10 ± 0.21^{cd}	18.65 ± 0.77^{cb}	55.90 ± 0.09^{cde}
(T_8)	49.95 ± 0.75^{de}	19.50 ± 0.27^{cab}	54.00 ± 0.11^{ef}
(T_9)	56.90 ± 0.70^{b}	23.45 ± 0.88^{a}	60.45 ± 0.07^{cab}
(T_{10})	57.65 ± 0.20^{b}	22.25 ± 0.18^{ab}	60.50 ± 0.04^{cab}
(T_{11})	59.25 ± 0.06^{ab}	16.95 ± 0.06^{dc}	61.60 ± 0.05^{ab}
(T_{12})	59.10 ± 0.83^{ab}	20.50 ± 0.42^{cab}	62.40 ± 0.09^{ab}
Control	52.10 ± 0.42^{cd}	19.80 ± 0^{cab}	59.10 ± 0.06^{cb}

Mean values with the same superscript letter within the same column are not significantly different at P <0.05

Effect of total substitution of sugar by sweeteners, on the firmness of biscuits, is shown in Table 4. The hardness of the biscuits can be estimated from the peak force (Gaines 1991). T_6 biscuits showed significantly higher values of peak force, and consequently hardness than other samples. Olinger and Velasco (1991) and Peck (1994), found that sorbitol resulted in softer biscuits than sucrose. As can be seen, using 3% isomalt would lead to a soft biscuit, while increasing isomalt up to 15% would produce a notably hard one. Therefore, it can be noted that isomalt as a sugar alcohol, acts like sorbitol. T_2 and T_7 biscuits, elaborated with 3% isomalt (T_7), showed softer texture among the samples and had the closest texture to the control biscuits. With comparing T_7 and T_8 along with T_9 and T_{10}, it can be noted that increasing the amount of both isomalt and maltodextrin, significantly enhanced the hardness of the biscuits. Therefore, the hardness of T2 to T_6 biscuits, have an increasing trend from 664 to 2340g. Supplementation of the biscuits with stevioside did not affect the textural properties, probably because the added quantity was tiny. Therefore it can be concluded that the use of maltodextrin and isomalt individually, would lead to unacceptable texture while using an appropriate combination of these ingredients is beneficial for the suitable texture of biscuits.

As for moisture content, T_6, T_8 and T_{10} have the highest value among the samples. It can be attributed to their hard texture, and consequently more water requirement in their formulations.

As for ash content, as can be seen in Table 4, T_1 biscuits contain the highest ash while T_9 and T_{10} samples have the least value. Isomalt, Maltodextrin, and stevia are not expected to affect protein and fat content of biscuits, so, there is no significant difference between the samples, in the case of protein and fat content.

3.3.Sensory evaluation

Average results of sensory evaluation attributes of sugar-free biscuits are summarized in Table 7. About sweetness, the T_1, T_5 and T_6 biscuits were the closest to the control, while T_9 and T_{10} biscuits elaborated with 2.5% and 10% maltodextrin were the least sweet samples. These results are in agreement with the noted sweetener power of maltodextrin, isomalt, and stevia. It should be pointed out that maltodextrin does not taste sweet while stevia and isomalt can be regarded as sugar replacers. Concerning the

aftertaste, the hedonic panel did not find significant differences in preference regarding this attribute. Stevia can remain bitter aftertaste, while supplementation of biscuits with stevioside did not affect the after taste of the biscuits, probably because the added quantity was minuscule. Texture sensory evaluation was in high correlation with instrumental measurements. Depending on the sugar replacers used, significant differences in biscuit texture was obtained. T_1, T_2, and T_7 had significantly higher scores than the rest on the hedonic scale, while the lower score was achieved by T_6 biscuits, which were notably harder according to the panelists. Sensory analysis was in good agreement with instrumental evaluations. As can be seen in Table 6, using 3% isomalt would lead to a soft texture, while increasing the amount of isomalt, significantly harden the texture. Table 7 also shows average sensory scores for the overall acceptability of biscuits elaborated with the different sugar replacers. T_1 and T_5 biscuits were closest to the control, and more preferred by the panelists than the other samples. The panelists also assigned the lowest score in overall acceptability to T_{11} biscuits, which were mainly related to taste and texture. However, the most preferred samples, even more than the control, was given to T_1 biscuit, followed by T_5 which is in good agreement with instrumental evaluations.

Table 6. Effect of sugar replacers on chemical properties (%) of biscuits

Treatment number	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	pH
(T_1)	2.05 ± 0.07^{d}	1.51± 0.01^{a}	7.05 ± 0.07^{e}	21.55 ± 0.32^{a}	7.67± 0.03 ^{e}
(T_2)	3.45 ± 0.07^{b}	0.99 ± 0^{b}	7.30 ± 0.14^{de}	21.43 ± 0.15^{a}	8.40 ± 0.02^{a}
(T_3)	2.55 ± 0.07^{c}	1.00 ± 0^{b}	7.36 ±0.19^{dc}	21.06 ± 0.08^{b}	7.90 ± 0.01^{d}
(T_4)	3.54 ± 0.05^{b}	1.00 ± 0^{b}	7.47 ±0.07^{dcb}	21.11 ± 0.02^{a}	7.88 ± 0^{d}
(T_5)	2.10 ± 0.01^{d}	0.99 ± 0^{b}	7.30 ± 0^{dec}	21.61 ± 0.06^{a}	7.50 ± 0^{f}
(T_6)	3.97 ± 0.03^{a}	0.89 ± 0.01^{c}	7.49 ± 0.09^{bdc}	21.02 ± 0.07^{b}	7.08 ± 0.02^{g}
(T_7)	2.55 ± 0^{c}	0.99 ± 0 ^{b}	7.67 ± 0.18^{ab}	21.93 ± 0.13 ^{a}	8.42 ± 0.01^{a}
(T_8)	3.90 ± 0.03^{a}	0.84 ± 0^{d}	7.36 ± 0.18^{dc}	21.32 ± 0.09^{a}	8.07 ± 0.05^{c}
(T_9)	3.53 ± 0.04^{b}	0.51 ± 0.01^{e}	7.56 ± 0^{cb}	21.38 ± 0.04^{a}	7.76 ± 0.05^{e}
(T_{10})	3.95 ±0.07^{a}	0.50 ± 0^{e}	7.45 ± 0.14^{bdc}	21.46 ± 0.01^{a}	7.41 ± 0^{f}
(T_{11})	2.52 ±0.03^{c}	0.99 ± 0 ^{b}	7.83 ±0.02^{a}	21.09 ± 0.01^{b}	8.19 ± 0^{b}
(T_{12})	2.50 ± 0.07^{c}	0.99 ± 0 ^{b}	7.63 ± 0.14^{ab}	21.18 ± 0.05^{b}	7.89 ± 0.12^{d}
Control	2.00 ± 0^{d}	0.99 ± 0^{b}	7.37 ± 0.07^{dc}	21.23 ± 0.09^{a}	7.76 ± 0.02^{e}

Mean values with the same superscript letter within the same column are not significantly different at P <0.05

Table 7. Sensory evaluation of biscuits prepared with different sugar replacers

Treatment number	Sweetness	Aftertaste	Hardness	Appearance	Overall Acceptability
(T_1)	4.95 ± 0.22^{a}	4.90 ± 0.30 ^{ab}	4.90 ± 0.30 ^{a}	4.90 ± 0.30^{a}	4.90 ± 0.30 ^{a}
(T_2)	4.05 ± 0.39^{b}	4.90 ± 0.30 ^{ab}	4.90 ± 0.30 ^{a}	2.97 ± 0.30^{d}	3.25 ± 0.44 ^{c}
(T_3)	3.45 ± 0.75^{c}	4.10 ± 0.30 ^{d}	3.10 ± 0.35 ^{d}	3.90 ± 0.30^{c}	3.95 ± 0.51 ^{b}
(T_4)	3.90 ± 0.64^{b}	4.10 ± 0.30 ^{d}	3.05 ± 0.51 ^{d}	4.25 ± 0.44^{b}	3.95 ± 0.51 ^{b}
(T_5)	4.70 ± 0.57^{a}	4.90 ± 0.30 ^{ab}	1.85 ± 0.48 ^{e}	4.90 ± 0.30^{a}	4.65 ± 0.67 ^{a}
(T_6)	4.70 ± 0.57^{a}	4.90 ± 0.30 ^{ab}	1.10 ± 0.30 ^{f}	4.90 ± 0.30^{a}	3.95 ± 0.51 ^{b}
(T_7)	2.10 ± 0.30^{e}	4.70 ± 0.92 ^{bc}	4.95 ± 0.22 ^{a}	3.95 ± 0.51^{c}	3.90 ± 0.30 ^{b}
(T_8)	2.10 ± 0.30^{e}	4.90 ± 0.30 ^{ab}	4.10 ± 0.30 ^{b}	4.10 ± 0.30^{cb}	3.90 ± 0.30 ^{b}
(T_9)	1.05 ± 0.22^{g}	4.05 ± 0.51 ^{d}	3.10 ± 0.30 ^{cd}	2.45 ± 0.75^{e}	1.75 ± 0.44 ^{e}
(T_{10})	1.00 ± 0.22^{g}	4.10 ± 0.30 ^{d}	2.10 ± 0.30 ^{e}	3.90 ± 0.64 ^{c}	1.75 ± 0.44 ^{e}
(T_{11})	1.50 ± 0.51^{f}	4.50 ± 0.51 ^{c}	3.35 ± 0.67 ^{c}	1.50 ± 0.51 ^{f}	1.25 ± 0.44 ^{f}
(T_{12})	2.50 ± 0.51^{d}	4.50 ± 0.51 ^{c}	3.35 ± 0.67 ^{c}	2.90 ± 0.30 ^{d}	2.25 ± 0.44 ^{d}
Control	4.95 ± 0.22^{a}	5.00 ± 0.00 ^{a}	4.90 ± 0.30 ^{a}	4.90 ± 0.30 ^{a}	4.90 ± 0.30 ^{a}

Mean values with the same superscript letter within the same column are not significantly different at P <0.05

The cookies formulated with isomaltulose were significantly harder than those elaborated with sucrose. Sucrose is more soluble than isomalt. Accordingly, the dough contains isomalt was firm and not sticky. The harder dough could not let more expansion during baking, so its density remained higher, which reserved evaporation adequately to cause the upper value for water retention by the baked cookie elaborated with sucrose. Curley and hoseney (1984) showed that the cookies made with 0% dissolved sucrose were harder than the one with, 100% dissolved sucrose. They concluded that the volume of total solution in cookie dough influenced its grade of softness. It can be noted that both sugar type and particle size can affect on sugar functionality in cookie baking which is associated with the extent of the boost in gelatinization temperature of starch (Slade and Levine 1987).

Sucrose addition causes a reduction in biscuit resistance. Though, the penetration curve of biscuits made with polyols was higher than the sucrose-reduced biscuits. The biscuits with reduced sucrose content were more resistant to rupture. These findings are in agreement with previous studies (Sai Manohar and Rao 1999; Pareyt et al. 2009). The present authors proved the there is a competition between sucrose and gluten for water absorption in previous trials. With the addition of sugar, the gluten network formed was damaged, which was reported by other authors (Pareyt and Delcour 2008) as well. One of the influences of sucrose in the final product is that a fragile network is formed and biscuits break simply. With decreasing the amount of sucrose, the gluten network converts to a stronger one, consequently lead to growing breaking strength. Furthermore, Maache-Rezzoug et al. (1998) confirm that sucrose dissolves protein and starch molecules, resulting in a brittle product. The results are in agreement with Baltsavias (1999).

4.Conclusions

The production of sugar-free biscuits with very similar characteristics to ordinary biscuits contains sugar, and good consumer acceptance was achieved in this study. Among the proposed formation, the treatment contains 6% isomalt, 2.5% maltodextrin, and 0.06% stevia (T_1) was the best formula, since the resulting biscuits gave sensory evaluation scores very similar to those of control, even surpassing it in some attributes such as taste, aftertaste, and outward appearance. Despite the fact that the sugar replacers are tested, were not participating in Maillard reactions, T_1 biscuits had golden brown color even better than those manufactured with sucrose which can promote these browning reactions, directly or as consequence of thermal degradation. In this sense, despite the unacceptable results obtained in general with other treatments, T_5 biscuits also can, to some extent, be comparable to the control. It can be noted that either of isomalt, maltodextrin, and stevia individually, does not have the potential of producing sugar-free biscuits. Therefore, the addition of appropriate amounts of isomalt, maltodextrin, and stevia to correct the appearance, texture, and sweetness of sugar-free biscuits could replace sucrose to give products with acceptable properties.

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EXPERIMENTAL INVESTIGATION OF A MULTI-UNIT SQUARE PEF TREATMENT CHAMBER FOR FOOD PROCESSING

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ABSTRACT
The objective of this paper is dedicated to the experimental analysis of a square pulsed electric field (PEF) treatment chamber comprising two and three parallel units constituted by three and five alternate HV-Ground electrodes respectively. The chamber made of Plexiglas has a square paralelipedic shape of dimensions 6x6x10 cm^3, in which are placed vertical and parallel stainless steel electrodes, of dimensions 10x6 cm^2. This chamber has been compared to the classic one-unit model constituted of two electrodes, one HV and one ground electrodes both placed on the side walls of the chamber. The experimental analysis was made using a spark-gap switch pulse generator, by measuring the mass of PEF pretreated extracted juice from red beet, the amount of betanine and the consumed energy. The obtained results have shown that the proposed models constituted of two and three units leads to better yields than the similar classic model constituted of only one treatment unit. Indeed, much higher increase rate values of extraction up to 40% with an energy saving up to 48% of both juice mass and betanine were obtained with the multi-units chambers with smaller energy consumption.

1. Introduction

During the last two decades, the pulsed electric field (PEF) treatment was found to be useful for enhancing the pressing, drying, extraction and diffusion processes (Barsotti, 1998; Angersbach, 2000; Vorobiev, 2005; Vorobiev, 2006). PEF technology is based on a pulsing power delivered to the product placed between a set of electrodes confining the treatment gap of the PEF chamber (Bellebna, 2017; Bermaki, 2017). The basic idea of the treatment chamber is to keep the treated product inside during pulsing, although the uniformity of the process is highly dependent on the characteristic design of the treatment chamber (Maged, 2012; Amiali, 2006; Adedeji, 2008).

Food product is placed in the treatment chamber, either in a static or continuous design, where two electrodes are connected together with a non-conductive material to avoid electrical flow from one to the other.

Parallel plates, wire-cylinder, rod–rod, rod–plate, and coaxial (concentric) cylinders are all potential electrode configurations. Parallel plates and coaxial electrode configurations have been used in most of the studies reported. Parallel plates produce uniform distribution of electric field strength and are simple in design. Coaxial electrodes on the other hand, provide smooth and uniform product flow and are attractive for industrial applications.

Uniform electric fields can be achieved by parallel plate electrodes with a gap sufficiently smaller than the electrode surface dimension. Disk-shaped, round-edged electrodes can minimize electric field enhancement and reduce the possibility of dielectric breakdown of fluid foods.

A large number of studies have been performed with parallel plate system in batchwise and later in continuous flow operation. Batch chambers provide many advantages for laboratory use: small volumes of treated media are required and the treatment temperature is easy to maintain by cooling the electrodes and by slow repetition rate (Toepfl, 2005; Toepfl, 2007). The continuous co-field PEF treatment chamber is constituted of two hollow cylindrical electrodes separated by an insulator to form a tube through which the product flows. Although in this configuration the product flow as well as the electric field lines are perpendicular, the field distribution in such model is not uniform.

The application of pulsed electric field (PEF) in industry is a major challenge today. Industry requires a continuously increasing processing rate and a simultaneously decreasing energy consumption rate. In all treatment chamber (TC) models used in industry and in research laboratories, the product to be treated is placed in a single-volume unit delimited between two electrodes. This study experimentally analyses the effectiveness of a new TC configuration consisting of two or three parallel PEF-treatment units and containing several alternate ground and HV electrodes for enhancing PEF treatment and reducing energy consumption.

2. Materials and methods

2.1. The experimental setup

The experimental setup used in the present work is composed of a number of components, comprising a high DC voltage source, an energy storage capacitor, a spark gap switch and a treatment chamber (Fig.1). A DC high voltage (Spellman 40 kV, 9 mA) charges the bank of capacitors until producing the spark gap's breakdown, causing an abrupt voltage (shock) applied to the load (treatment chamber where the sample is disposed). The storage element is composed of three sets of five series capacitors (2 µF, 2 kV), with the possibility to reach a maximum voltage of 10 kV and a total capacitance of 1.2 µF (Fig.2).

2.2. Treatment chamber models

Three square paralelipedic treatment chambers of same dimensions 6x6x10 cm^3comprising one, two and three treatment units were used, constituted of two, three and five identical parallel stainless steel electrodes respectively. Each chamber is made of Plexiglas in which are placed identical vertical and parallel stainless steel electrodes, of dimensions 6x10x2 cm^3.

The three TC, having the same maximum volume of 360 ml, are described below (Fig.3):

The model TC1 is constituted of one unit and comprises two electrodes: one HV electrode and one ground electrode both placed on the side walls of the chamber.

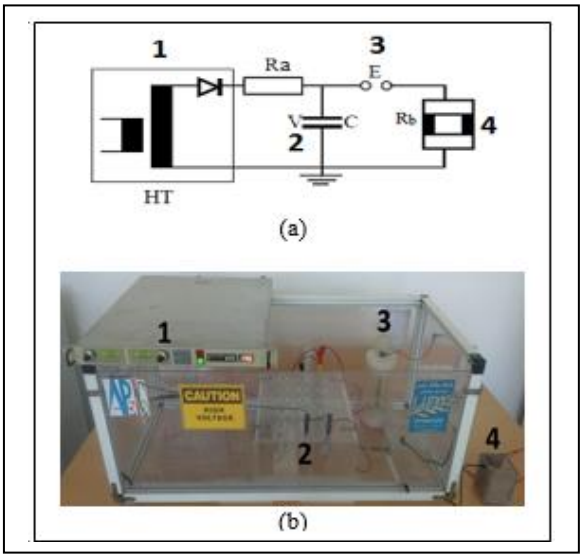

Figure1. The pulse generator
a) Descriptive schematic of the setup, b) The photography of the setup1- HV DC power supply, 2-Set of capacitors, 3- Spark gap switch, 4-Treatment chamber

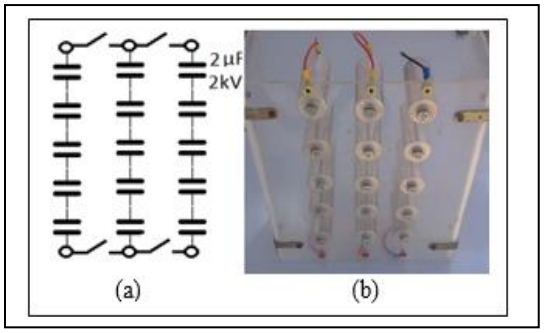

Figure2. The bank of capacitors.
a) *Descriptive schematic; b) P*hotography of the capacitors bank

The model TC2 is constituted of two parallel units comprising three electrodes: one central HV electrode and two side ground electrodes.

The model TC3 is constituted of three parallel units comprising five alternate HV and ground electrodes: two HV electrodes and three ground electrodes.

Fresh red beets, of average mass 60 g each one, were used. After sorting and cleaning operations, a homogenous mash was obtained using a domestic food processor.

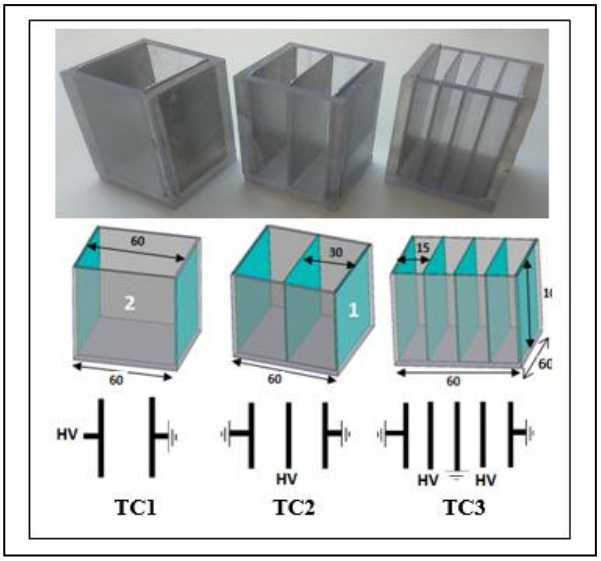

Figure3. The treatment chambers
1. Stainless steel electrodes;
2. Plexiglas(dimensions in mm)

Before each experiment, the mash was properly mixed to obtain a homogenous mixture. Right after PEF treatment, a hydraulic pressing machine (Mega, 15 tons) was used for the juice extraction. The filled extraction chamber was pressed until a defined pressure of 100 kg/cm^2, and was then held at this pressure for 5 min. For all the experiments, the same sample mass m=80 g was used.

2.3. Experimental procedure

The extracted juice filtered through a stainless steel sieve, was collected in a plastic collector placed under the extraction chamber and then analyzed by measuring both its mass using an electronic balance of 0.1 mg precision and the betanine concentration by measuring the absorbance of beet juice using a spectrophotometer (Optizen 200 plus,Mecasys Co, Ltd) at λ= 530 nm.

An experimental investigation was performed to compare the PEF treatment efficiency between the three models. For each model, the influence of the applied voltage (V, kV), the pulses number (n) and the pulse duration (T, µs) was analysed. The pulse duration is controlled by the appropriate value of the capacitance C (Table 1) deduced from the corresponding voltage wave shape (Fig.4).

Table 1. Values of the pulse duration according to the corresponding capacitance

C (µF)	0.2	0.4	0.5	0.8	1.2
T (µs)	8	20	24	40	60

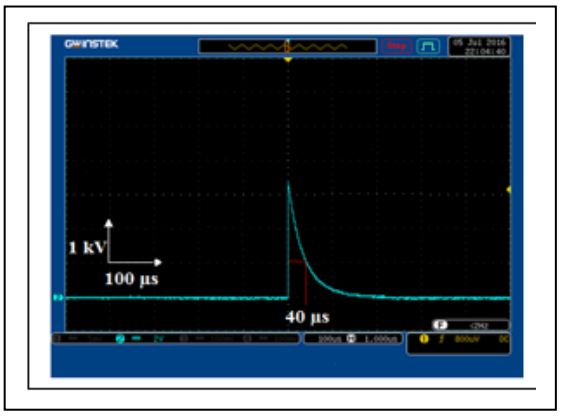

Figure 4. Example of a pulse shape of duration T= 40 µs obtained with C=0.8 µF

Moreover, the mass of extracted juice m (g), the amount of betanine expressed in terms of Absorbance A and the energy $W= 1/2\ nCV^2$ were considered significant to be considered for the evaluation of the PEF-treatment efficiency.

2.4. Experimental design methodology

The methodology of the experimental design makes it possible to determine the number of experiments to be achieved, according to a well-defined objective, to simultaneously study several factors, to reduce dispersion related to measurements, to appreciate the effects of coupling between factors and, finally, to evaluate the respective influence of the factors and their interactions (G. Taguchi, 1987; L. Eriksson, 2000). Before starting the experiments, it is necessary to set the best and suitable design, which can model the process with the most possible precision. In this work, the composite centered-faces design (CCF), which gives quadratic models, was adopted (Hicks, 1990).

MODDE5.0 (Umetrics AB, Umea, Sweden) which is a Windows program for the creation and evaluation of experimental design is used (MODDE5.0, 1990). The program assists the user in interpretation of the results and the prediction of the responses. The optimization stage of an experimental procedure should enable the identification of the "set point," i.e., the values of the control factors for which the response of the process is a maximum, is a minimum, or approaches a target (Rezouga, 2009).

For PEF treatment process, the maximization of the juice mass and the absorbance as well as the minimization of the energy could be the chosen criterion of evaluation.

The response surface method (RSM) was employed for set point identification in this paper. The central composite face-centered (CCF) design is most commonly used with the RSM, as it supports quadratic polynomial models (Medles, 2007). With such models, the response y of the process is expressed as a function of e factors u_i (i = 1... e) as:

$$y = f(u_i) = C_0 + \sum C_i U_i + \sum C_{i,j} U_i U_j + \sum C_{ii} U_i^2 \quad (1)$$

A normalized centered value can be defined for each factor as follows:

$$x_i = (U_i - U_{ic})/\Delta U_i = U_i^* \quad (2)$$

Where:

$$U_{ic} = (U_{i\,max} + U_{i\,min}) / 2 \quad (3)$$

$$\Delta U_i = (U_{i\,max} - U_{i\,min}) / 2 \quad (4)$$

With these notations, the response function becomes

$$y = f(x_i) = a_0 + \sum a_i x_i + \sum a_{i,j} x_i x_j + \sum a_{ii} x_i^2 \quad (5)$$

Where x_i can take any value between -1 (for the minimum input value u_{imin}) and +1 (for the maximum input value u_i max). For the three factors considered in this paper, i.e., $x1 = V*$, $x_2 = n*$ and $x_3 = T*$, the quadratic model is

$$y = a_0 + a_1V^* + a_2n^* + a_3T^* + a_{1,2}V^*n^* + a_{1,3}V^*T^* + a_{2,3}n^*T^* + a_{11}V^{*2} + a_{22}n^{*2} + a_{33}T^{*2} \quad (6)$$

3. Results and discussion

3.1. Screening experiments

Screening experiments are designed here to identify the variation domain of the three factors, which are called "one-factor-at-a-time" experiments. The optimization stage of an experimental procedure should enable the determination of factor values for which the amount of juice and pigment extraction is a maximum and the consumed power is a minimum (Nemmich, 2015; Qian, 2015).

In all the experiments carried out in this section, for each TC model, one factor was varied while the two others were kept constant. Thus, tables 2, 3 and 4 represent the variation of the PEF treatment efficiency, in terms of extracted juice mass m, absorbance A and consumed energy W, according to V, n and T, respectively.

As expected, the mass of extracted juice and the quantity of betanine obtained with PEF-treated samples increase according to the applied voltage for the three chambers. Beyond a determined value of the voltage (5 kV for TC1, 4 kV for TC2 and 3 kV for TC3), the effect of the PEF treatment is inversed due to "oxidation" of the product caused by the surplus of energy.

According to Jaeger (Jaeger, 2012), high-intensity treatment may soften the plant tissues to an extent that is unfavourable for dejuicing; excessive softening causes the compaction and closure of capillaries in the press cake. Similarly, variation of the extracted juice mass with the applied field intensity has also been reported in other studies in which the extraction yield is relatively low at higher values than at lower values of electric field intensity (Puértolas, 2010; Loginova, 2011; Bai, 2013; Brito, 2012). In a previous study, the authors explained that the decrease in the extraction yield could be caused by irreversible permeabilization that occurs at higher electric fields (Guderjan, 2005).

However, the treatment is more efficient for models TC2 and TC3 compared with the classic chamber TC1 comprising two electrodes only. While for the model TC1, the optimal treatment was obtained for V = 7 kV (m= 31.9 g, A= 0.394 and W=980 J), for the multi-unit chambers greater values of m and A were obtained with smaller energy. Thus, for models TC2 and TC3, the optimal treatment was obtained for V = 6 kV (m= 34.2 g, A= 0.452 and W=720 J), and V= 5 kV (m= 33.4 g, A= 0.474 and W=500 J) respectively.

Table 2. Extracted juice mass m, absorbance A and Energy W, as functions of the applied voltage V (kV), for the three chambers (n=50, T= 40 µs)

V (kV)	TC1		TC2		TC3		
	m(g)	A	m(g)	A	m(g)	A	W (J)
1	16.6	0.237	17.2	0.346	19.3	0.390	20
2	19.3	0.253	21.5	0.381	25	0.432	80
3	21	0.302	27.7	0.4	30.4	0.453	180
4	25.4	0.346	28.2	0.427	33	0.470	320
5	28.0	0.356	30.5	0.446	33.4	0.474	500
6	30.1	0.382	34.2	0.452	30	0.386	720
7	31.9	0.394	29.8	0.362	28.4	0.332	980
8	29.8	0.345	28.2	0.325	19.3	0.390	1280

Energy saving when using multi-units treatment chambers compared to the model TC1 was estimated by the following relation:

$W_{saving} = (W_{TC1} - W_{TCi}) / W_{TC1}$ (7)

W_{TC1}: Energy consumed by model TC1

W_{TCi}: Energy consumed by model TC2 or TC3.

In comparison with the model TC1, an energy saving of 26% and 48 % were achieved with models TC2 and TC3 respectively.

The advantage of the multi-units chambers is that for the same voltage the electric field is increased compared to the "one-unit" chamber, due to the lower value of the inter-electrodes gap. For example, when a same voltage of 6 kV is applied, the electric field of TC1, TC2 and TC3 is equal to 1, 2 and 4 kV/cm respectively.

Furthermore, as seen in Table 3, the mass of extracted juice and the quantity of betanine obtained with PEF-treated samples increase with pulses number, for the three chambers. The treatment is more efficient for models TC2 and TC3 compared with the classic chamber TC1. While for the model TC1, the optimal treatment was obtained for n= 100 pulses (m= 36.7 g, A= 0.359 and W=360 J), for the multi-unit chambers greater values of m and A were obtained with less pulses. Therefore for models TC2 and TC3, the optimal treatment was obtained for n= 80 (m= 36.2 g, A= 0.476 and W=288 J), and n= 60 (m= 36.2 g, A= 0.606 and W=216 J) respectively. An energy saving of 20% and 40% was achieved for models TC2 and TC3 respectively.

On the other hand, the mass m and absorbance A, obtained with PEF-treated samples increase with the pulse width, for the three chambers, and decreases beyond a determined value (Table 4). As for the previous factors, the treatment is more efficient for multi-units models compared with the classic chamber. For the model TC1, the optimal treatment was obtained for T= 60 µS (m = 31.4 g &A= 0.302), however for the models TC2 and TC3 greater values were obtained with smaller pulse width. The optimal treatment was obtained for T= 40µS with both models (m= 31.4 g &A=

0.331 with TC2; m= 32.7 g &A= 0.342 with TC3). For this latter, a more efficiency treatment was obtained for T= 40 µs (m= 36.2 g &A = 0.496).

Table 3. Extracted juice mass m, absorbance A and Energy Was functions of the pulses number n, for three chambers (V = 3 kV, T = 40 µs)

n	TC1		TC2		TC3		
	m (g)	A	m(g)	A	m (g)	A	W (J)
20	24.6	0.247	28.9	0.291	28.8	0.324	72
40	28.1	0.331	30.8	0.336	31.8	0.333	144
60	29.6	0.327	32.4	0.382	36.2	0.606	216
80	32.0	0.328	36.2	0.476	31.4	0.418	288
100	36.7	0.359	28.6	0.450	28.5	0.450	360
120	28.6	0.304	27.1	0.321	25	0.414	432

The results obtained in this section served to the definition of the variation domain of V, n, and T for each treatment chamber (Table 5) in view of the modeling step for the set-point identification.

Table 4. Extracted juice mass m, absorbance A and Energy Was functions of the pulse width T, for the three chambers (V=3 kV, n=60).

T (µS)	TC1		TC2		TC3		
	m (g)	A	m (g)	A	m (g)	A	W (J)
8	21.7	0.256	23.5	0.263	24.6	0.297	54
20	24.7	0.264	29.4	0.266	32.7	0.342	108
40	27.6	0.269	31.4	0.331	36.2	0.496	216
60	31.4	0.302	28.1	0.288	30.8	0.441	324

Table 5. Variation limits of input factors for each model of treatment chamber

	V(kV)		n		T (µs)	
	V_{min}	V_{max}	n_{min}	n_{max}	T_{min}	T_{max}
TC1	6	8	80	120	20	60
TC2	5	7	60	100	20	60
TC3	4	6	40	80	20	60

3.2. Set point identification

The identification of the set point V_0, n_0, and T_0 for each treatment chamber model was carried out by performing a central-faced composite design; the two levels "max" and "min" are the limits established in the previous section for each of the three control variables (V_{min}, V_{max}), (n_{min}, n_{max}), and (T_{min}, T_{max}). The central point: V_c, n_c, and T_c was calculated as follows:

Model TC1:

$V_c = (V_{max} + V_{min})/2 = (8 + 6)/2 = 7$ kV

$n_c = (n_{max} + n_{min})/2 = (120 + 80)/2 = 100$

$T_c = (T_{max} + T_{min})/2 = (20 + 60)/2 = 40$ µs

Model TC2:

$V_c = (V_{max} + V_{min})/2 = (7 + 5)/2 = 6$ kV

$n_c = (n_{max} + n_{min})/2 = (100 + 60)/2 = 80$

$T_c = (T_{max} + T_{min})/2 = (20 + 60)/2 = 40$ µs

Model TC3:

$V_c = (V_{max} + V_{min})/2 = (6 + 4)/2 = 5$ kV

$n_c = (n_{max} + n_{min})/2 = (80 + 40)/2 = 60$

$T_c = (T_{max} + T_{min})/2 = (20 + 60)/2 = 40$ µs

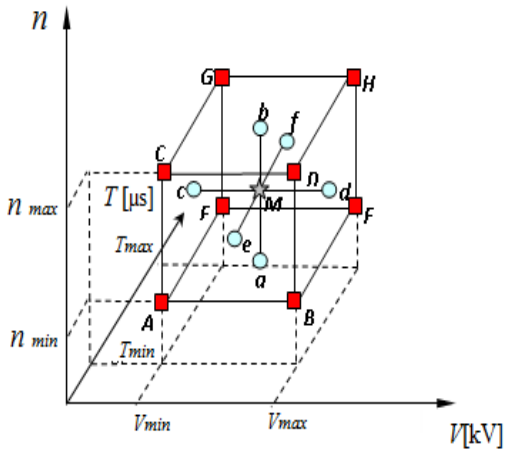

Figure 5. Diagram of experiments of a CCF design of 3 factors.

Figure 5 shows experiments to be carried out with a CCF design of 3 factors. It comprises 8 experiments located at the extremities of the cube (square points A, B…H), 6 experiments located in

the centres of the cube faces (round points a, b…f) and 3 identical experiments done in the central point M (star point). Thus, a CCF design with 3 factors includes 17 experiments.

chambers, obtained according to equation 6, related to the responses considered for optimization, which are the extracted juice mass, the absorption of betanine and the energy consumption, given by software MODDE 5.0 are represented in Table 9. Moreover, the program calculates two significant statistical criteria, which make it possible to validate (or not) the mathematical model, symbolized by R^2 and Q^2. The former is called the goodness of fit, and is a measure of how well the model can be made to fit the raw data; it varies between 0 and 1, where 1 indicates a perfect model and 0 no model at all. The latter is called goodness of prediction, and estimates the predictive power of the model. Like R^2, Q^2 has the upper bound 1, but its lower limit is $-\infty$. For a model to pass the diagnostic test, both parameters should be high.

Table 6. Results of the CCF experimental design (Model TC1)

Exp No	V (kV)	n	T (µs)	m (g)	A	W (J)
1	6	80	20	24.6	0.2	576
2	8	80	20	28.1	0.31	1024
3	6	120	20	25.3	0.285	864
4	8	120	20	27.8	0.34	1536
5	6	80	60	26.4	0.295	1728
6	8	80	60	28.6	0.358	3072
7	6	120	60	29	0.355	2592
8	8	120	60	30.1	0.385	4608
9	6	100	40	28.2	0.312	1440
10	8	100	40	30.2	0.342	2560
11	7	80	40	29.3	0.291	1568
12	7	120	40	31.3	0.33	2352
13	7	100	20	29.1	0.245	980
14	7	100	60	30.2	0.292	2940
15	7	100	40	32.1	0.294	1960
16	7	100	40	31.8	0.294	1960
17	7	100	40	31.9	0.294	1960

Table 7: Results of the CCF experimental design (ModelTC2)

Exp No	V (kV)	n	T (µs)	m (g)	A	W (J)
1	5	60	20	30.3	0.36	300
2	7	60	20	36.7	0.373	588
3	5	100	20	30.7	0.477	500
4	7	100	20	33.3	0.337	980
5	5	60	60	31.8	0.437	900
6	7	60	60	38.2	0.422	1764
7	5	100	60	31.8	0.477	1500
8	7	100	60	33.6	0.344	2940
9	5	80	40	32.5	0.504	800
10	7	80	40	37.1	0.428	1568
11	6	60	40	34.7	0.48	864
12	6	100	40	33.6	0.448	1440
13	6	80	20	34.1	0.434	576
14	6	80	60	35.6	0.464	1728
15	6	80	40	36.7	0.486	1152
16	6	80	40	36.4	0.46	1152
17	6	80	40	37	0.49	1152

Table 8. Results of the CCF experimental design (Model TC3)

Exp No	V (kV)	n	T (µs)	m (g)	A	W (J)
1	4	40	20	39.6	0.513	32
2	6	40	20	36.6	0.481	48
3	4	80	20	40.8	0.585	64
4	6	80	20	34.7	0.445	96
5	4	40	60	40.8	0.545	96
6	6	40	60	38.7	0.53	144
7	4	80	60	39.9	0.585	192
8	6	80	60	36.2	0.452	288
9	4	60	40	41.3	0.612	96
10	6	60	40	37.2	0.491	144
11	5	40	40	41.3	0.588	80
12	5	80	40	40.3	0.556	160
13	5	60	20	40.5	0.542	60
14	5	60	60	40.7	0.572	180
15	5	60	40	41.3	0.594	120
16	5	60	40	40.9	0.588	120
17	5	60	40	40.7	0.598	120

The coefficients "a_1" related to the voltage V are negative for models TC2 and TC3 indicating that smaller values of V are recommended for these chambers. On the other hand, coefficients "a_1" are greater than "a_2" and "a_3" related to the pulses number n and pulse Width T respectively, which point out that the effect of the electric field is more significant in PEF treatment.

Furthermore, among the three analyzed factors, the pulse width affect significantly the The experiments results obtained with the CCF design are given in Tables 6, 7 and 8 for TC1, TC2 and TC3 models respectively. The mathematical models of the three treatment energy W consumed during treatment; the coefficients "a_3" related to the energy W are much higher than "a_1" and "a_2". In addition, according to the smaller values of coefficients "$a_{1,2}$", "$a_{1,3}$" and "$a_{2,3}$" related to the interactions between the three factors, no significant effect exits between them. The software also offers the possibility to identify the optimal values of the factors which should give the highest amount of extracted juice mass and betanine absorption for the smallest energy consumption. It contains an optimization routine that is capable of simultaneously processing several responses, affected by different weighting coefficients.

MODDE.05 has an optimizer tool for the identification of the set-point corresponding to the optimal values of factors obtained by maximizing the process outcome and minimizing the power (Fig.6). "iter" is the number of iterations and "log (D) is the Log of overall distance to the target; the value of Log (D) equals zero when all responses are between Target and Limit. The smaller Log (D), the better is the result. Log (D) becomes negative when the values of all responses are still closer to the Target (Modde.05, 1999). The set-points proposed by the optimizer tool for the three treatment chamber models are summarized in Table 10.

Table 9. The coefficients of the mathematical models for each treatment chamber

Coefficients	TC1			TC2			TC3		
	m (g)	A	W(J)	M	A	W	m	A	W
a_0 (Cte)	31,43	0,29	1960	36,04	0,48	1152	41,03	0,59	120
a_1 (V)	1,13	0,028	560	2,18	-0,035	384	-1,9	-0,044	24
a_2 (n)	0,64	0,024	398,4	-0,87	0,0011	294,4	-0,51	-0,0034	40
a_3 (T)	0,94	0,03	996	0,59	0,016	588,8	0,41	0,012	60
$a_{1,2}$ (V*n)	-0,26	-0,011	112	-1,05	-0,034	96	-0,59	-0,028	8
$a_{1,3}$ (V*T)	-0,33	-0,009	280	-0,1	-0,0027	192	0,41	0,003	12
$a_{2,3}$ (n*T)	0,46	-0,0035	200	-0,2	-0,015	148	-0,34	-0,01	20
$a_{1,1}$(V^2)	-1,86	0,032	40	-0,75	-0,02	32	-1,84	-0,033	5,66e-6
$a_{2,2}$ (n^2)	-0,76	0,016	5,56e-5	-1,40	-0,022	7,44e-5	-0,28	-0,012	5,85e-6
$a_{3,3}$ (T^2)	-1,41	-0,026	0,00023	-0,70	-0,037	0,0002	-0,49	-0,027	9,665e-6

	Factor	Role	Va	Low Lin	High Li
1	Applied Volta	Free		6	8
2	Pulses numbe	Free		80	120
3	Pulse duration	Free		20	60

	Response	Criteria	Weight	Min	Target	Max
1	Mass of juice	Maximize	1	31,0916	31,7816	
2	Absorbance	Maximize	1	0,367644	0,384324	
3	Energy consumpti	Minimize	1		442,16	833,04

Iteration: 217 Iteration slider:

	1	2	3	4	5	6	7	8
	Applied Voltage	Pulses number	Pulse duration	Mass of juice	Absorbance	Energy consumption	iter	log(D)
1	6	120	20,2816	25,5009	0,2876	823,298	153	1,5926
2	8	80	20	27,9969	0,3068	973,596	154	1,2516
3	6,0108	119,943	36,0264	28,2418	0,3398	1549,52	217	1,1406
4	7,7437	119,999	43,0604	31,0797	0,3678	3095,19	117	1,2049
5	7,7023	120	42,3462	31,1296	0,3647	3013,47	192	1,1814
6	7,7437	119,999	43,0604	31,0797	0,3678	3095,19	117	1,2049
7	7,7422	119,999	44,0041	31,1136	0,3682	3160,21	98	1,2238
8	7,7023	120	42,3462	31,1296	0,3647	3013,47	192	1,1814

Figure 6. Results of the optimization routine of MODDE 5.0 for maximization of juice mass and betanine extraction and minimization of the energy consumption (example of the model TC1)

As seen in Table 10, the obtained results confirm definitely the superiority of the three-unit treatment chamber model TC3. While the mass m of the extracted juice increases from 28.24 (TC1) to 40.9 (TC3) corresponding to a variation rate of 39%, the energy W decreases in the same time with a rate of 95%. respectively in comparison with the one unit model. The set-point identification analysis has confirmed definitely the superiority of the multi-units models. A treatment chamber with more parallel units should give much better results with less energy.

Table 10. The optimal values of the factors

	V(kV)	n	T(µs)	m (g)	A	W(J)
TC1	6	120	36	28.24	0.34	1550
TC2	6.63	66	42.9	37.6	0.46	1256
TC3	4	72	23.63	40.9	0.59	67

4. Conclusions

A comparative experimental analysis was performed between three different treatment chambers constituted of one (TC1), two (TC2) and three (TC3) parallel units. Although, the volume of the chambers is the same, however the one comprising three units has given the best results in terms of extracted juice mass, amount of betanine and energy consumption. For the same voltage, thus for the same energy, the electric field of the multi-units models (TC2 and TC3) is two and three times higher

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A COMPARISON BETWEEN THE EFFECTS OF CONVENTIONAL EXTRACTION METHODS ON THE QUANTITY AND QUALITY OF THE CHEMICAL COMPOSITION OF THE ESSENTIAL OILS OF *THYMUS DAENENSIS* AND *FERULAGO ANGULATA* (SCHLECHT.) BOISS

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ABSTRACT
The components of essential oils extracted from two thyme species were determined by the hydrodistillation (HD) and microwave-assisted extraction (MAE) after ultrasound pretreatment, and to examine the effects of these methods on the quantity and quality of the two essential oils. Scanning electron microscopy (SEM) was used to charechterized the effect of HD and MAE processes on thyme leaves. The process variables included the plant to water ratios of 1:10 and 1:20, the microwave power of 330, 660 and 990 w, and the ultrasound pretreatment at the indirect state (at 20 and 45°C) was considered as the affecting factor for the extraction of the essential oils. Application of microwave and the rise in its power led to an significant improvment on the yield and quality of essential oils. It also reduced the time needed for the extraction curve to be constant from 4 h to 1 h. A total of 23 and 25 constituents were identified in *Thymus daenensis* and *Ferulago angulata* (Schlecht.) Boiss essential oils, respectively. GC-MS analysis showed thymol (69%) and β-phellandrene (35%) had the largest contents in *Thymus daenensis* and *Ferulago angulata* (Schlecht.) Boiss essential oils, respectively. SEM of thyme leaves undergone HD and MAE showed rupture of cellular wall and gland containing essential oils with MAE. Ultrasound pretreatment at various temperatures, utilization of microwave at different powers and the change in the plant to water ratio did not have any considerable effects on the refractive index, antioxidant activity and specific gravity of the two essential oils.

1.Introduction

Application of plants as medicine has long been taken into account by the practitioners of traditional medicine for disease management. The history of medical science in Iran dates back to the Aryan era, nearly 7000 B.C. In other civilizations such as the Egyptian civilization (5000 B.C.) medicinal plants were also applied for disease treatment. When investigating plant species, the word “Persica” is frequently seen in the scientific name of

many species, which reveals that the species is of Persian origin. 7500-8000 plant species exist in Iran among which approximately more than 200 species have medicinal and economical values. At the same time, more than hundreds of plants are employed in traditional medicine (Golmakani and Rezaei, 2008).

Thyme, an aromatic herb from the Lamiaceae family, is a valuable medicinal plant and popular in traditional and modern medicine. So far, a variety of 215 and 14 thyme species have been identified in the whole world and Iran, respectively (Jamzadeh, 1995). Since thymol is its major component, this herb is widely used in food, pharmaceutical and cosmetic industries. Furthermore, the aqueous, aqueous-alcoholic and propylene glycolic extracts of thyme are used in the manufacture of shampoos, lotions and ointments (Omidbeigi, 2001). The antifungal, antiparasitic and antibacterial effects of this plant as well as its therapeutic impacts on asthma, chronic dry cough and bronchitis have been proved.

Ferulago angulata (Schlecht.) Boiss is a valuable plant native to western Iran, which is a member of the Apiaceae family. It has been distributed in Iran, Turkey, Syria, Lebanon and Iraq. In Iran, it grows in northeast and northwest heights and more widely in the region of central Zagros. *F. angulata* (Schlecht.) Boiss is a medicinal plant and has long been employed as a pain killer and digester. It has been used for curing intestinal worms and hemorrhoids as well. In recent years, some researchers have examined the nutraceuticals of this plant (Javidnia et al., 2006). For instance, researchers investigated the constituents of the essential oil of its aerial twigs (Rezazade et al., 2002). In that research, a total of 33 compounds were identified constituting 89.7% of the whole compounds including monoterpene (77.1%) and sesquiterpene (12.6%). The main compounds included α-pinene (17.3%), bornyl acetate (14.45%) and cis-ocimene (14.4%). The antioxidant and antibacterial effects of this plant have been examined as well (Javidnia et al., 2006). In the case of *T. daenensis*, Sajjadi and Khatamsaz (2003) reported thymol (73.9%), carvacrol (6.7%), p-cymene (4.6%), b-bisabolene (1.5%), terpinen- 4-ol (1.4%), borneol (1.1%) and spathulenol (1.0%) as the main constituents. MAE is a novel environmentally-friendly extraction method with a shorter extraction time and less solvent consumption. It is an easy and quick way of separating the remains of systemic fungicides from wooden plant tissues. The conventional solid-liquid extraction technics (soxhlet) have two principle disadvantages: 1) a large volume of organic solvents is required which could result in the pollution of the sample and the solvent loss, caused by evaporation, during extraction. 2) a perfect extraction required several hours or even several days of performing the process. On the other hand, MAE does not have these disadvantages (Armstrong, 1999). The more rapid extraction of pollutants from environmental samples through MAE than through the conventional ones reveals that the former is a valid and appropriate technic for such an application. This technic can be a suitable substitute for the conventional ones because of its shorter extraction time and less energy consumption (Camel, 2000).

2. Materials and methods

2.1. Supply and drying of *Thymus daenensis* and *F angulata* (Schlecht.) Boiss

Fresh aerial parts of both *T. daenensis* and *F. angulata* (Schlecht.) Boiss were supplied in spring from the mountainous city of Dena, Kohkiluye and Boyerahmad province, Iran. The plant samples were confirmed by the research center of medicinal plants of Yasouj University, Yasouj, Iran. After cleaning and separating the damaged parts through screening, both samples were shadow-dried. After complete drying, the samples were packaged and prepared for essential oil extraction.

2.2. Hydrodistillation

Dried plant materials were powdered and the conventional hydrodistillation was done. In this, 100 g of each powdered sample were weighted separately and added to 1000 ml distilled water in a round bottom flask following by heating to boil and then the temperature was reduced to 60 °C and kept for 3 h. The oils were dried over

anhydrous sodium sulfate and kept in amber vials at 4 °C until further analysis (Rota et al., 2004).

2.3. Microwave-assisted extraction (MAE)

MAE is similar to Hydrodistillation. The only difference between these two methods is the thermal energy source. An electric heater is used in Hydrodistillation, while the microwave power is used in MAE for heating the sample and water.

In this method, the glass container of the sample and water was placed in the microwave oven. However, the condenser, collector and the reflux column were located outside the oven. The sample size was equal to 60 g in all experiments and the plant to water ratio was equal to 1:10 and 1:20. The microwave power was equal to 30% (330 w), 60% (660 w) and 90% (990 w). according to preliminary experiments, the time required for complete MAE (at the lowest power) was at most 2 h. As a result, this value was considered as the MAE time. The essential oil percentage (w/w) was determined at 10-min intervals during the first half an hour of extraction, and at 15-min intervals thereafter. All the experiments were duplicated.

2.4. Essential oil extraction using the indirect ultrasound pretreatment

In this method, 60 g the plant sample and 500 ml water were poured into a volumetric flask and sealed. After that, the flask was put in an ultrasonic bath. The applied power was half of the maximum output power of the apparatus. The bath temperature varied from 20 to 45°C. The blank pretreatment was also applied. It was performed at 20°C without the application of ultrasound. Immediately after the indirect ultrasound pretreatment, Hydrodistillation and MAE were carried out. All experiments were conducted in duplicate.

2.5.Identification the chemical composition of the essential oils

The dried essential oils prepared through Hydrodistillation and MAE were diluted with 1 ml n-hexane. After complete mixing, 1 µl of the resulting mixture was injected into a 7890A gas chromatograph coupled with mass spectrometer (Agilent Technologies, Wallbronn, Germany) equipped with BP-5 capillary column (length, 30 m; internal diameter, 0.25 mm, film thickness: 0.25µm). The ionization energy was equal to 70 ev. The ionization method was EI and the ionization source temperature was 220°C. The scanning range of the spectra varied from 50 to 550 nm and Chemstation was the applied software. The chemical compounds were identified with the aid of the retention times of the components of the resulting spectrum and comparing them with those present in reference books (Adams, 2007; Swigar, 1981) and those of the standard compounds present in the MS computer library (WILEY275.L). In this way, the chemical constituents of the two essential oils were identified and after plotting their contents, comparison was done according to the areas under the peaks.

2.6. The physical and chemical tests of the essential oils

The extracted essential oils were separated at 0.5-, 1-, 2-, 3- and 4- hour intervals. They were then dried with anhydrous sodium sulfate and weighed. 1000 µl of each sample was weighed using a digital balance and the specific gravity of each essential oil was calculated through dividing its mass (kg) by its volume (m^3). The refractive indices of the samples were measured based on the national standard No. 13575 of institute of standards and industrial research of Iran (ISIRI). The antioxidant activity of the samples was determined (Burits and Bucar, 2000). All of the experiments were done in duplicate.

2.7. Scanning electron microscopy (SEM)

The samples of the dried, MAE- and HD-treated thyme leaves were observed under a scanning electron microscope (S-360, Cambridge, England). Initially, the coating process with a thin layer of gold under vacuum was done and then the images were captured at an accelerating voltage of 20.0 kV.

2.8. Statistical analysis

A completely randomized block design (CRBD) was employed for the design of experiments and data analysis. Duncan`s multiple range test was applied for mean comparison at 95% confidence level ($p<0.05$). SPSS 22.0 was used for the design the experiments and data analysis and all of the graphs were plotted using Excel 2013.

3.Results and discussions

3.1. Comparison of the extraction yield of the essential oils

As can be seen in figures 1 and 2, at the plant to water ratio of 1:20, the extraction kinetics of Hydrodistillation was different from that of MAE for both of the essential oils. In MAE, the process reached its constant state in less than 2 h, while in Hydrodistillation, it became constant after about 4 h. In addition, the results revealed that the extraction initiation time was equal to 30 min for hydrodistillation, whereas it was 24, 12 and 6 min for the microwave power of 30, 60 and 90%, respectively in the case of the both essential oils. As the microwave power increased, the extraction time decreased for both plants as with the rise in the microwave power from 30 to 60%, extraction initiation time and the extraction completion time declined. Furthermore, the time required for the extraction curve to be constant was reduced. It could be declared that changing the extraction method had a positive effect on the extraction yield and reduced the extraction time. Moreover, the results indicated that the extraction yield of *T. daenensis* and *F. angulata* (Schlecht.) Boiss essential oils were in order 3.84 and 2.62% at 90% microwave power.

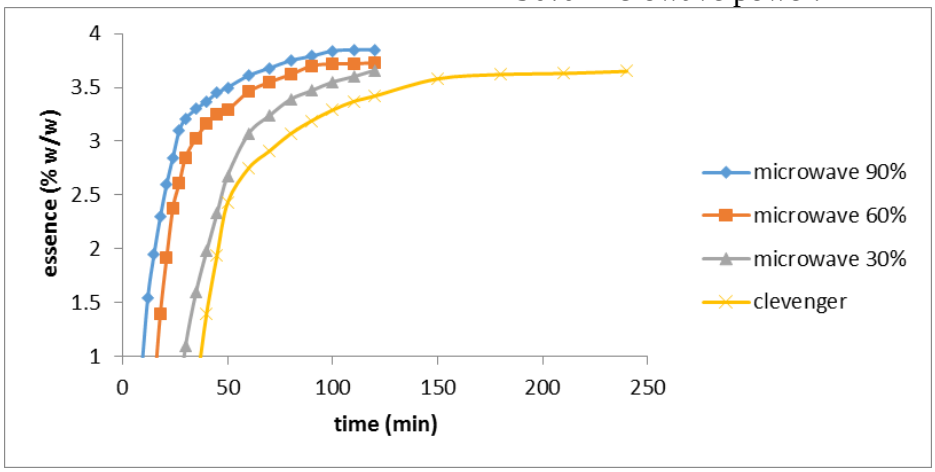

Figure 1. Extraction of *Thymus daenensis* essential oils through Hydrodistillation and microwave-assisted extraction (MAE) at the plant to water ratio of 1:20.

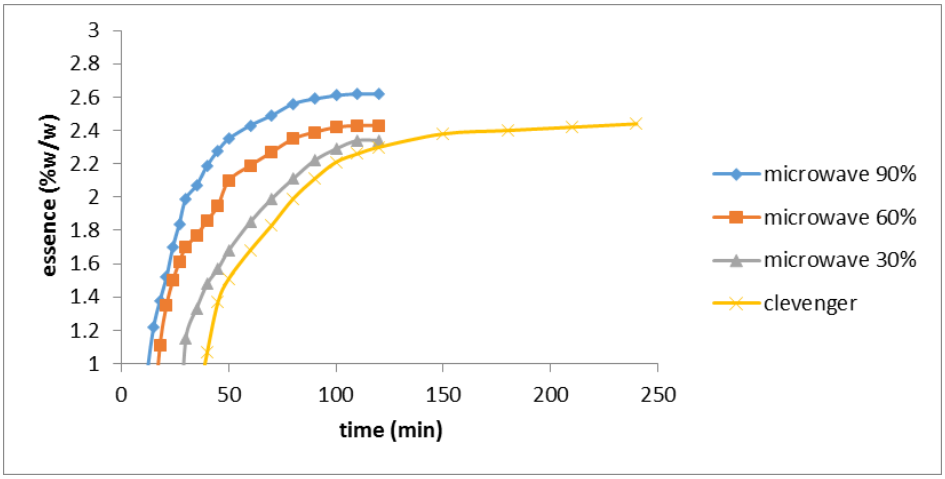

Figure 2. Extraction of *Ferulago angulata* (Schlecht.) Boiss essential oils through Hydrodistillation and microwave-assisted extraction (MAE) at the plant to water ratio of 1:20.

Microwave causes the fast breakdown of cell walls, thus enhancing the extraction yield in a short time. This high yield is due to the penetration of microwave into the cells. In addition, this method reduces water consumption as well as saving time and energy. As a result, it decreases the costs and improves the extraction efficiency. The loss of a large content of volatile compounds during long extraction times could be the reason behind the low extraction yield at low microwave powers (Bayramoglu et al., 2008). In similar study, it was revealed that the extraction yield of cubeba Litsea essential oil through Hydrodistillation was much lower than that through MAE. The rise in the extraction yield is induced by the high penetration power of microwave into the plant cells leading to a higher extraction yield (Zhang et al., 2011). Some authors carried out a research on the Hydrodistillation and MAE of lavender essential oil (Chemat et al., 2006). They concluded that MAE was better than Hydrodistillation in terms of energy consumption, extraction time (10 min compared with 90 min), extraction yield, and the purity and quality of the product. Other researchers who worked on eucalyptus, argued that the highest essential oil extraction yield belonged to MAE. They also claimed that high microwave powers and short extraction times increase the extraction rate (Ouedrago et al., 2009; Safaei-ghomi et al., 2009).

3.2. The effect of the plant to water ratio on the extraction yield

As shown in figures 1 to 4, in both methods, the decrease in the plant to water ratio form 20:1 to 10:1 brought about a reduction in the extraction initiation time, the extraction completion time and the time required for the extraction curve to get constant. As depicted in figures 3 and 4, the extraction initiation time was equal to 24 min in Hydrodistillation. It was also equal to 18, 8 and 4 min at the microwave powers of 30, 60 and 90%, respectively. In all cases, the extraction initiation time was lower in 10:1 ratio than in 20:1 one. Additionally, at both ratios, MAE was better than Hydrodistillation. Moreover, as the microwave power increased, the extraction completion time decreased.

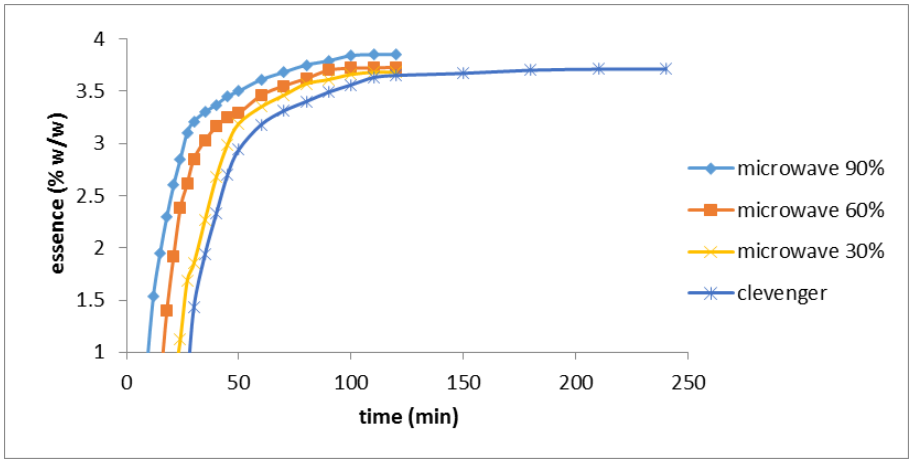

Figure 3. Extraction of *Thymus daenensis* essential oil through hydrodistillation and microwave-assisted extraction (MAE) at the plant to water ratio of 1:10.

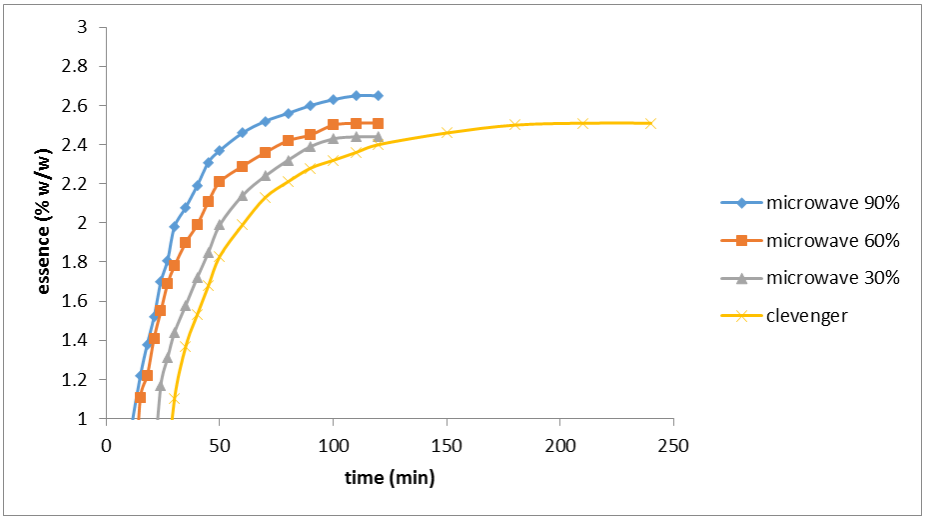

Figure 4. Extraction of *Ferulago angulata* (Schlecht.) Boiss essential oil through hydrodistillation and microwave-assisted extraction (MAE) at the plant to water ratio of 1:10.

3.3. The effect of indirect ultrasound pretreatment on the extraction yield

The effect of indirect ultrasound pretreatment before hydrodistillation and MAE was also investigated on the extraction yield of the two essential oils. This process was conducted at 20 and 45°C for 1 h. The blank sample was kept at 20°C for 1 h with no sonication and then underwent hydrodistillation and MAE at 30 and 60% microwave powers. As illustrated in figures 5-8, indirect ultrasound pretreatment at 20 and 45°C did not have any significant effects on the extraction initiation time and completion times of both essential oils. At the same time, the extraction initiation and completion times declined slightly after sonication at 45°C which could be attributed to the higher sonication temperature. The results demonstrated that after ultrasound pretreatment, extraction initiation time, extraction completion time and the time required for the extraction curve to be constant decreased more in MAE as compared with hydrodistillation. Furthermore, extraction initiation and completion times were reduced with the rise in the microwave power from 30 to 60%. As observed in figures 5 and 7, indirect ultrasound pretreatment, even at 45°C,

did not have a noteworthy effect on the extraction time. Therefore, application of ultrasonic bath only increases the costs and is not effective on reducing the extraction time. As can be seen in figures 6 and 8, there was not a considerable difference between the blank (macerated sample at 20°C) and the sample indirectly sonicated at 20°C. The effects of ultrasound include the more pronounced penetration of solvent into the cells as well as improving mass transfer. Indeed, ultrasound causes the destruction of cell walls and facilitates the release of the cell contents. Consequently, efficient destruction of cell walls and effective mass transfer are the major factors enhancing the yield of ultrasound-assisted extraction (Mason et al., 1996).

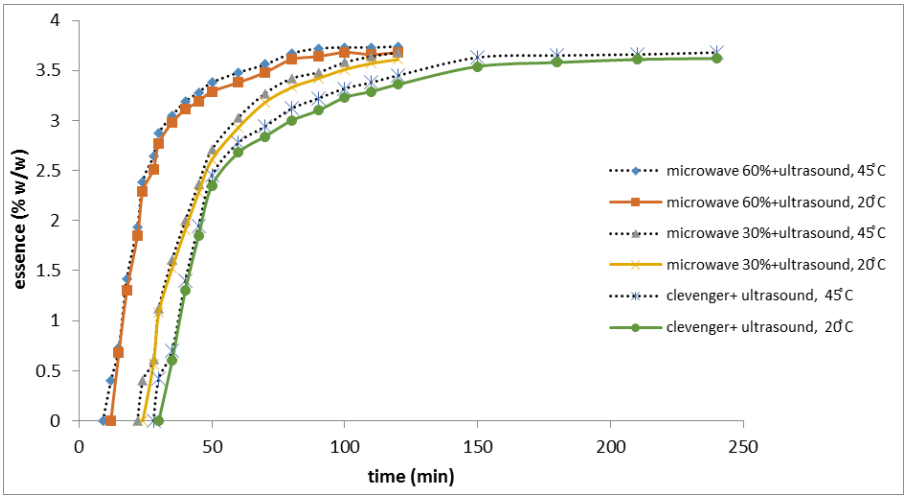

Figure 5. The effect of ultrasound pretreatment at different temperatures on the extraction of *Thymus daenensis* essential oil through hydrodistillation and microwave-assisted extraction (MAE)

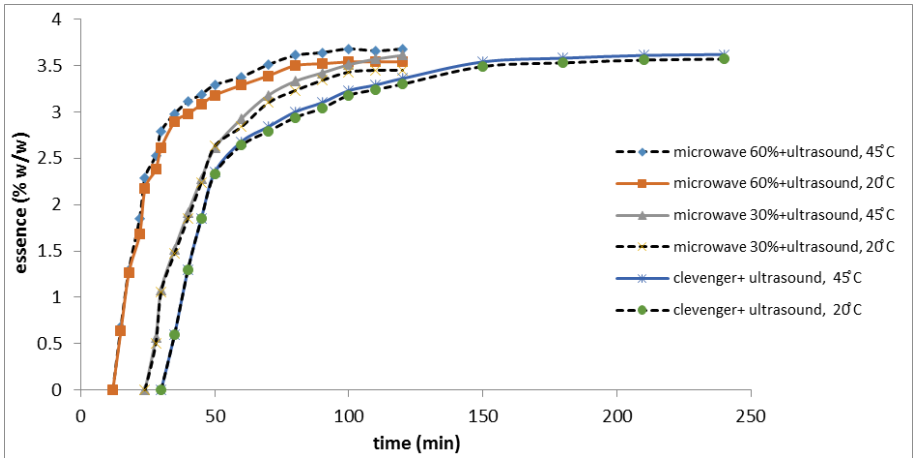

Figure 6. The effect of ultrasound and soaking pretreatments on the extraction of *Thymus daenensis* essential oil through hydrodistillation and microwave-assisted extraction (MAE)

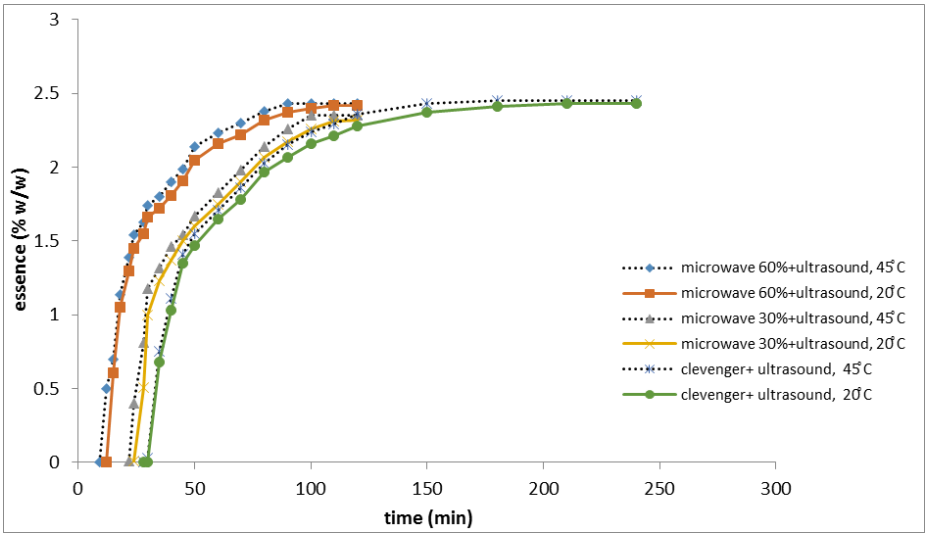

Figure 7. The effect of ultrasound pretreatment at different temperatures on the extraction of *Ferulago angulata* (Schlecht.) Boiss essential oil through hydrodistillation and microwave-assisted extraction (MAE)

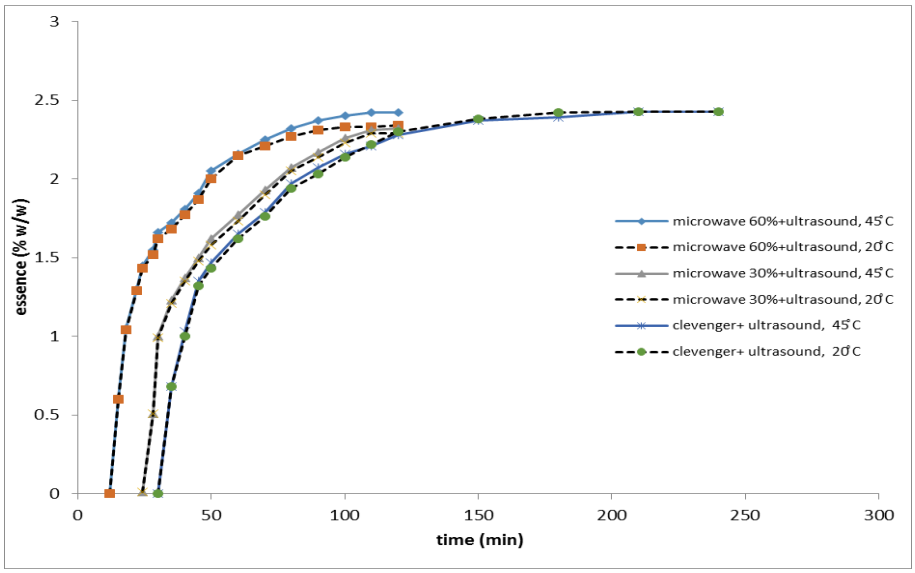

Figure 8. The effect of ultrasound and soaking pretreatments on the extraction of *Ferulago angulata* (Schlecht.) Boiss essential oil through hydrodistillation and microwave-assisted extraction (MAE)

Researchers cited that ultrasound was an appropriate pretreatment to increase the extraction yield of almond, apricot and rice bran oils (Sharma and Gupta, 2004). Ultrasound elevates the extraction kinetics and improves the extract quality. The ultrasound-assisted extraction rate of limonene was two times higher than that of the conventional extraction (Chemat et al., 2004).

3.4. The effect of the different extraction methods on the extraction initiation time

As can be seen in figures 9 and 10, the extraction initiation time of hydrodistillation was significantly ($p<0.05$) longer than that of MAE for both of the plants. Additionally, as the microwave power increased, this time declined. In both methods, the extraction initiation time was reduced at the plant to water ratio of 1:10. Similarly under this condition, with a rise in the

microwave power, the extraction initiation time decreased significantly ($p<0.05$). The reason behind this fact is the reduction in the water present, and consequently in the time required to elevate its temperature. Likewise, after indirect ultrasound pretreatment, the extraction initiation time of hydrodistillation was significantly ($p<0.05$) longer than that of MAE. Furthermore, when the microwave power increased, this time decreased. In the case of all treatments, the extraction initiation time was not change significantly except for the one sonicated at 45°C. This could be due to the higher temperature of the plant-water mixture when placed in the microwave oven.

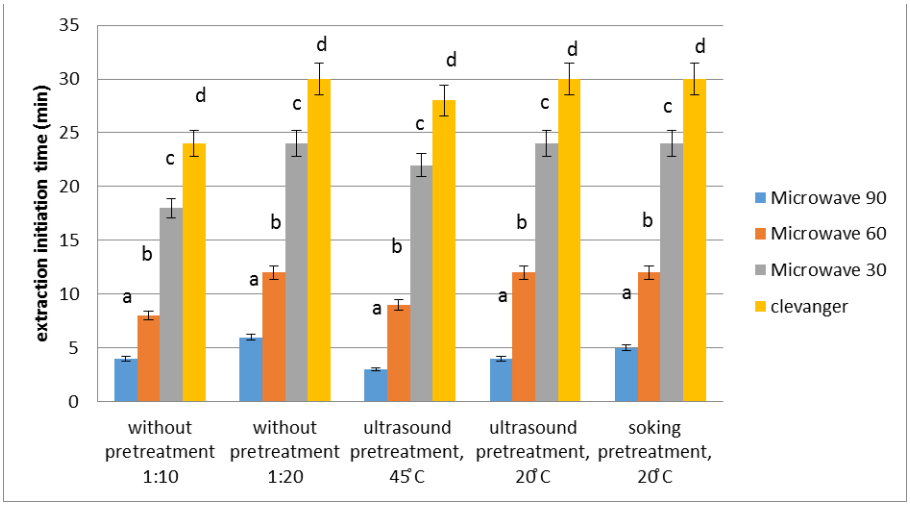

Figure 9. The effect of ultrasound pretreatment and different extraction technics on the extraction initiation time of *Thymus daenensis*.

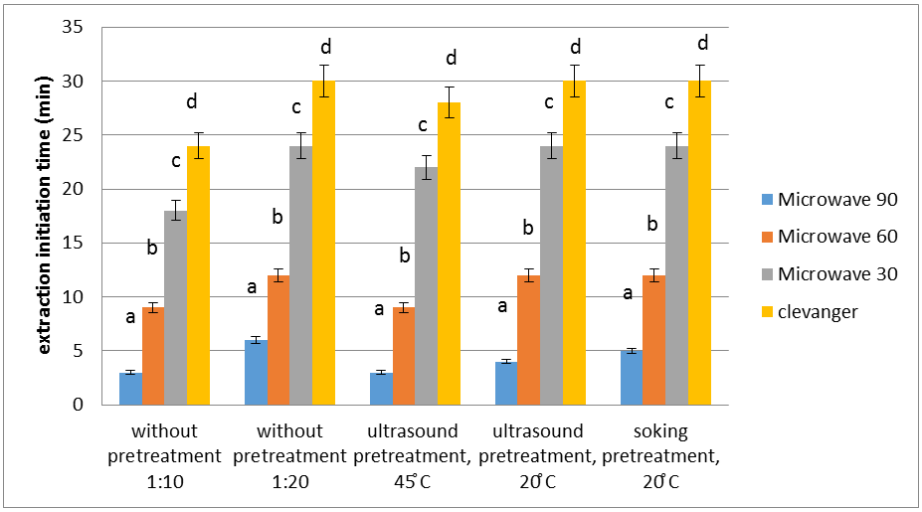

Figure 10. The effect of ultrasound pretreatment and different extraction technics on the extraction initiation time of *Ferulago angulata* (Schlecht.) Boiss

3.5. The effect of the different extraction methods on the specific gravities of the essential oils

The specific gravity values of the two essential oils are presented in Table 1. It was found out that the ultrasound pretreatment, the two extraction methods, the different microwave powers as well as the various plant to water ratios did not have considerable impacts on the specific gravities of the two essential oils.

3.6. The effect of the different extraction methods on the refractive indices of the essential oils

As shown in Table 2, the ultrasound pretreatment, the two extraction technics, the various microwave powers and the different plant to water ratios did not significantly affect the refractive indices of the two essential oils.

Table 1. The effect of ultrasound pretreatment and the two extraction methods on the specific gravities of *Thymus daenensis* and *Ferulago angulata* (Schlecht.) Boiss

Specie	Extraction condition	Extraction condition			
		Microwave 90%	Microwave 60%	Microwave 30%	clevenger
Thymus daenensis	Without pretreatment,1:10	0.938 ± 0.01[a]	0.940 ±0.03[a]	0.937 ± 0.02[a]	0.937 ±0.02[a]
	Without pretreatment, 1:20	0.941 ± 0.01[a]	0.938 ± 0.01[a]	0.938 ±0.04[a]	0.944 ±0.01[a]
	Ultrasound pretreatment, 45℃	0.943 ± 0.02[a]	0.945 ± 0.01[a]	0.942 ±0.01[a]	0.941 ±0.00[a]
	Ultrasound pretreatment, 20℃	0.935 ± 0.01[a]	0.937 ± 0.03[a]	0.939 ±0.01[a]	0.940 ±0.02[a]
	soking pretreatment, 20℃	0.941 ± 0.03[a]	0.944 ± 0.02[a]	0.943 ±0.02[a]	0.938 ±0.01[a]
Ferulago angulata (Schlecht.) Boiss	without pretreatment 1:10	0.921 ± 0.02[a]	0925 ± 0.04[a]	0.928 ±0.03[a]	0.919 ±0.01[a]
	without pretreatment 1:20	0.926 ± 0.02[a]	0.918 ± 0.02[a]	0.926 ±0.01[a]	0.917 ±0.02[a]
	ultrasound pretreatment, 45℃	0.923 ± 0.01[a]	0.922 ± 0.01[a]	0.927 ±0.02[a]	0.918 ±0.01[a]
	ultrasound pretreatment, 20℃	0.917 ± 0.03[a]	0.925 ± 0.01[a]	0.921 ±0.02[a]	0.920 ±0.00[a]
	soking pretreatment, 20℃	0.914 ± 0.02[a]	0.919 ± 0.02[a]	0.923 ±0.01[a]	0.927 ±0.02[a]

Table 2. The effect of ultrasound pretreatment and the two extraction methods on the refractive indices of *Thymus daenensis* and *Ferulago angulata* (Schlecht.) Boiss

Specie	Extraction condition	Extraction condition			
		Microwave 90%	Microwave 60%	Microwave 30%	Clevenger
Thymus daenensis	Without pretreatment,1:10	1.506 ± 0.01[a]	1.506 ± 0.01[a]	1.507 ± 0.02[a]	1.506 ± 0.00[a]
	Without pretreatment, 1:20	1.507 ± 0.00[a]	1.506 ± 0.01[a]	1.506 ± 0.01[a]	1.506 ± 0.01[a]
	Ultrasound pretreatment, 45℃	1.506 ± 0.00[a]	1.506 ± 0.01[a]	1.506 ± 0.00[a]	1.506 ± 0.01[a]
	Ultrasound pretreatment, 20℃	1.506 ± 0.01[a]	1.506 ± 0.00[a]	1.507 ± 0.01[a]	1.506 ± 0.00[a]
	soking pretreatment, 20℃	1.507 ± 0.01[a]	1.506 ± 0.00[a]	1.506 ± 0.01[a]	1.506 ± 0.02[a]
Ferulago angulata (Schlecht.) Boiss	without pretreatment 1:10	1.503 ± 0.00[a]	1.503 ± 0.00[a]	1.503 ± 0.00[a]	1.502 ± 0.01[a]
	without pretreatment 1:20	1.503 ± 0.01[a]	1.502 ± 0.02[a]	1.506 ± 0.00[a]	1.503 ± 0.02[a]
	ultrasound pretreatment, 45℃	1.503 ± 0.00[a]	1.503 ± 0.02[a]	1.506 ± 0.01[a]	1.503 ± 0.02[a]
	ultrasound pretreatment, 20℃	1.502 ± 0.01[a]	1.503 ± 0.01[a]	1.506 ± 0.02[a]	1.503 ± 0.01[a]
	soking pretreatment, 20℃	1.503 ± 0.01[a]	1.502 ± 0.00[a]	1.506 ± 0.01[a]	1.502 ± 0.01[a]

3.7. Qualitative and quantitative determination of the constituents of the essential oils

T. daenensis and *F. angulata* (Schlecht.) Boiss essential oils extracted through hydrodistillation and 90% MAE were assessed through GC-MS. As summarized in Tables 3 and 4, a total of 23 and 25 compounds were identified in *T. daenensis* and *F. angulata* (Schlecht.) Boiss essential oils, respectively. These compounds constitute more than 94 and 93% of *T. daenensis* and F angulata (Schlecht.) Boiss essential oils, respectively. As indicated in Table 3, thymol was the major compound of *T. daenensis* essential oil constituting about 69% of the entire essential oil followed by p-cymene. The results also showed that β-phellandrene was the major component of *F. angulata* (Schlecht.) Boiss essential oil (Table 4) which took up about 35% of the whole essential oil. After this compound, α-Phellandrene had the second highest percentage. At the same time, a comparison between the effect of two extraction methods on the constituents of the two essential oils demonstrated that the extraction method did not have a significant effect ($p<0.05$) on the percentage of the extracted compounds.

Table 3. The compounds present in *Thymus daenensis* essential oil extracted through hydrodistillation and MAE

No.	Compound	Retention index	Relative peak area (%)	
			Microwave, 90%	Clevenger
1	α-Phellandrene	1011	0.05 ± 0.01[a]	0.05 ± 0.01[a]
2	Δ-3-Carene	1013	0.06 ± 0.01 [a]	0.05 ± 0.01[a]
3	Linalool	1079	0.1 ± 0.02[a]	0.09 ± 0.02[a]
4	Aromadendrene	1439	0.12 ± 0.04[a]	0.12 ± 0.03[a]
5	α-Humulene	1453	0.12 ± 0.03[a]	0.13 ± 0.04[a]
6	γ-Cadinene	1549	0.19 ± 0.06[a]	0.17 ± 0.05[a]
7	Spathulenol	1561	0.20 ± 0.03[a]	0.15 ± 0.03[a]
8	α-Pinene	930	0.20 ± 0.06[a]	0.16 ± 0.04[a]
9	β-Pinene	962	0.22 ± 0.05[a]	0.20 ± 0.06[a]
10	Elemol	1550	0.25 ± 0.05[a]	0.21 ± 0.04[a]
11	β-Bisabolene	1506	0.27 ± 0.07[a]	0.22 ±0.05[a]
12	Caryophyllene oxide	1590	0.39 ± 0.09[a]	0.34 ± 0.07[a]
13	α-Thujene	925	0.8 ± 0.11[a]	0.73 ± 0.08[a]
14	Tricyclene	927	0.91 ± 0.13[a]	0.86 ± 0.11[a]
15	α-Terpinene	1025	1.03 ± 0.12[a]	0.97 ± 0.10[a]
16	Limonene	1031	1.05 ± 0.16[a]	1.10 ± 0.15[a]
17	Myrcene	926	1.15 ± 0.18[a]	1.19 ± 0.16[a]
18	Carvacrol	1308	1.25 ± 0.17[a]	1.18 ±0.19[a]
19	γ-Terpinene	1057	2.50 ± 0.24[a]	2.42 ± 0.22[a]
20	Methyl carvacrol	1240	3.20 ± 0.27[a]	3.62 ± 0.25[a]
21	β-Caryophyllene	1413	3.90 ± 0.28[a]	3.80 ± 0.24[a]
22	p-Cymene	1019	6.60 ± 0.36[a]	6.49 ± 0.39[a]
23	Thymol	1297	69.73 ± 2.69[a]	69.29 ± 2.68[a]
Total peak area (%)			94.31	93.19

3.8. The effect of the different extraction methods on the antioxidant activities of the essential oils

The ability to donate the hydrogen atom or electron as a result of the presence of some pure compounds, is determined through measuring the discoloration of the purple color of DPPH methanolic solution. In this method, the free radical scavenging ability of the essential oils revealed their high antioxidant activity. The in vitro study using DPPH showed that essential oil had a considerable capability of inhibiting lipid oxidation and can be used as an antioxidant in high-fat food products. However, research has shown that such methods of antioxidant activity evaluation are weakly correlated with the ability of these compounds to prevent lipid oxidation because the in vitro assays do not consider factors such as the physical location of the antioxidant and the environmental conditions (Decker et al., 2005; Chen and Spiro, 1995).

A comparison between the effects of the extraction methods on the antioxidant activity of the two essential oils (figures 11 and 12) showed that the extraction method did not have significant effect ($p<0.05$) on the antioxidant activities of the *T. daenensis* and *F. angulata* (Schlecht.) Boiss essential oils. This reveals that the extraction method did not have a negative impact on the quality and quantity of the antioxidant compounds present in two the essential oils.

Table 4. The compounds present in *Ferulago angulata* (Schlecht.) Boiss essential oil extracted through hydrodistillation and MAE

No.	Compound	Retention index	Relative peak area (%)	
			Microwave, 90%	Clevenger
1	Hexanal	790	0.06 ± 0.01[a]	0.06 ± 0.01[a]
2	Nonane	781	0.07 ± 0.01[a]	0.06 ± 0.01[a]
3	Toluene	902	0.10 ± 0.01[a]	0.12 ± 0.02[a]
4	Methyl,2-methylbutyrate	748	0.15 ± 0.03[a]	0.15 ± 0.02[a]
5	&-Cadinene	1516	0.15 ± 0.03[a]	0.17 ± 0.04[a]
6	Geranyl propionate	1447	0.20 ± 0.04[a]	0.19 ± 0.03[a]
7	β-Bourbonene	1366	0.20 ± 0.04[a]	0.18 ± 0.04[a]
8	α-Cubebene	1351	0.27 ± 0.06[a]	0.21 ± 0.05[a]
9	Decanal	1188	0.55 ±0.08[a]	0.49 ± 0.09[a]
10	Spathulenol	1570	0.67 ±0.09[a]	0.65 ± 0.07[a]
11	Germacrene D	1482	0.74 ± 0.09[a]	0.69 ± 0.10[a]
12	Bornyl acetate	1273	0.90 ± 0.06[a]	0.93 ± 0.07[a]
13	Camphene	954	1.15 ±0.11[a]	1.10 ± 0.12[a]
14	Terpinen-4-ol	1173	1.35 ± 0.18[a]	1.34 ± 0.16[a]
15	(Z)-β-Farnesene	1449	1.50 ± 0.14[a]	1.45 ± 0.13[a]
16	ɤ-Terpinene	1059	1.63 ± 0.19[a]	1.66 ± 0.21[a]
17	Sabinene	965	2.05 ± 0.20[a]	2.01 ± 0.23[a]
18	α-Pinene	933	2.17 ± 0.24[a]	2.08 ± 0.29[a]
19	Methyl eugenol	1372	3.95 ± 0.32[a]	3.95 ± 0.33[a]
20	Allo-ocimene'	1120	4.77 ± 0.37[a]	4.70 ± 0.31[a]
21	Terpinolene	1078	5.12 ± 0.33[a]	5.17 ± 0.28[a]
22	β-Pinene	977	7.20 ± 0.45[a]	7.23 ± 0.41[a]
23	Thymol	1273	8.45 ± 0.45[a]	8.45 ± 0.47[a]

24	α-Phellandrene	997	15.10 ± 0.97[a]	15.17 ± 1.01[a]
25	β-Phellandrene	1030	35.16 ± 1.82[a]	35.02 ± 1.74[a]
Total peak area (%)			93.66	93.23

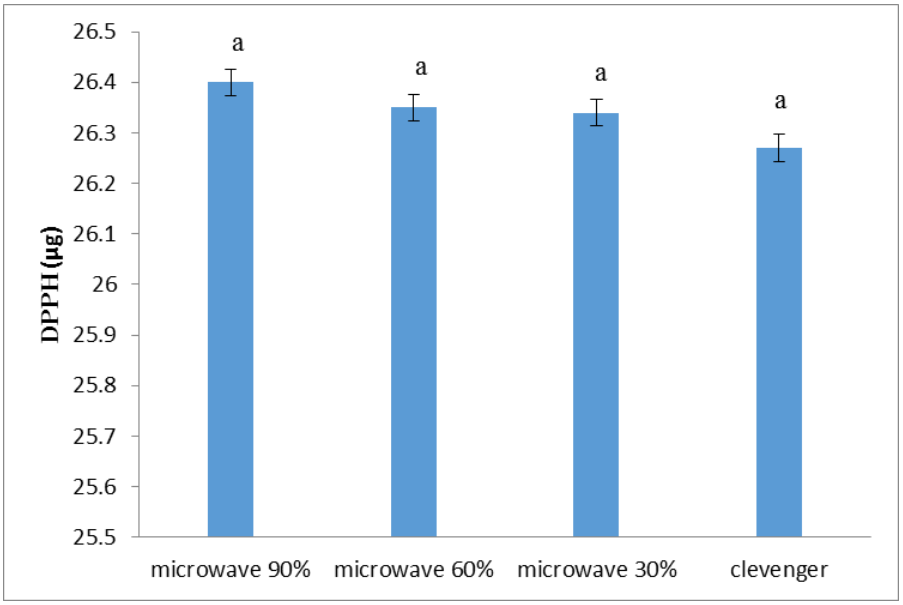

Figure 11. The effect of hydrodistillation and MAE on the antioxidant activity of *Thymus daenensis* essential oil

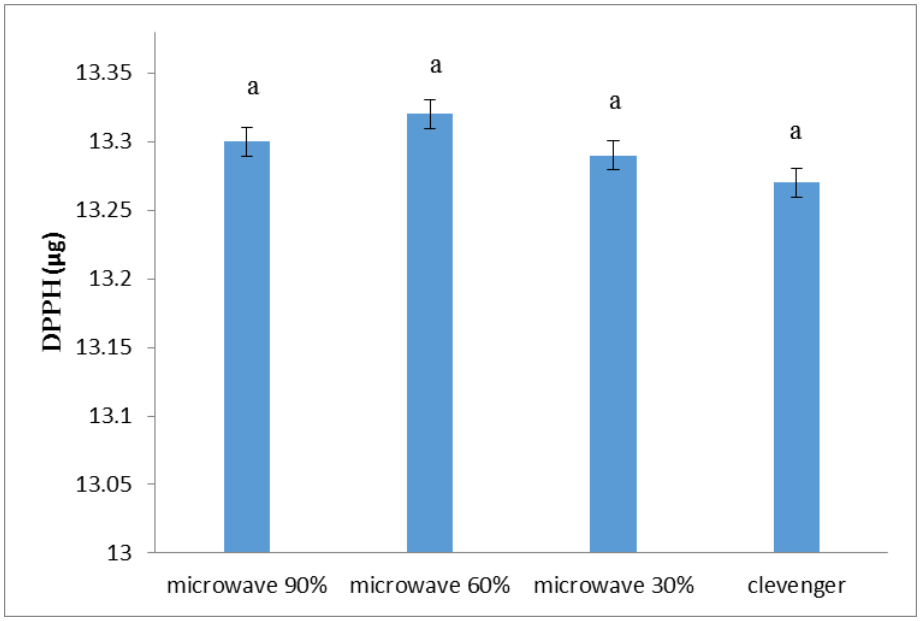

Figure 12. The effect of hydrodistillation and MAE on the antioxidant activity of *Ferulago angulata* (Schlecht.) Boiss essential oil

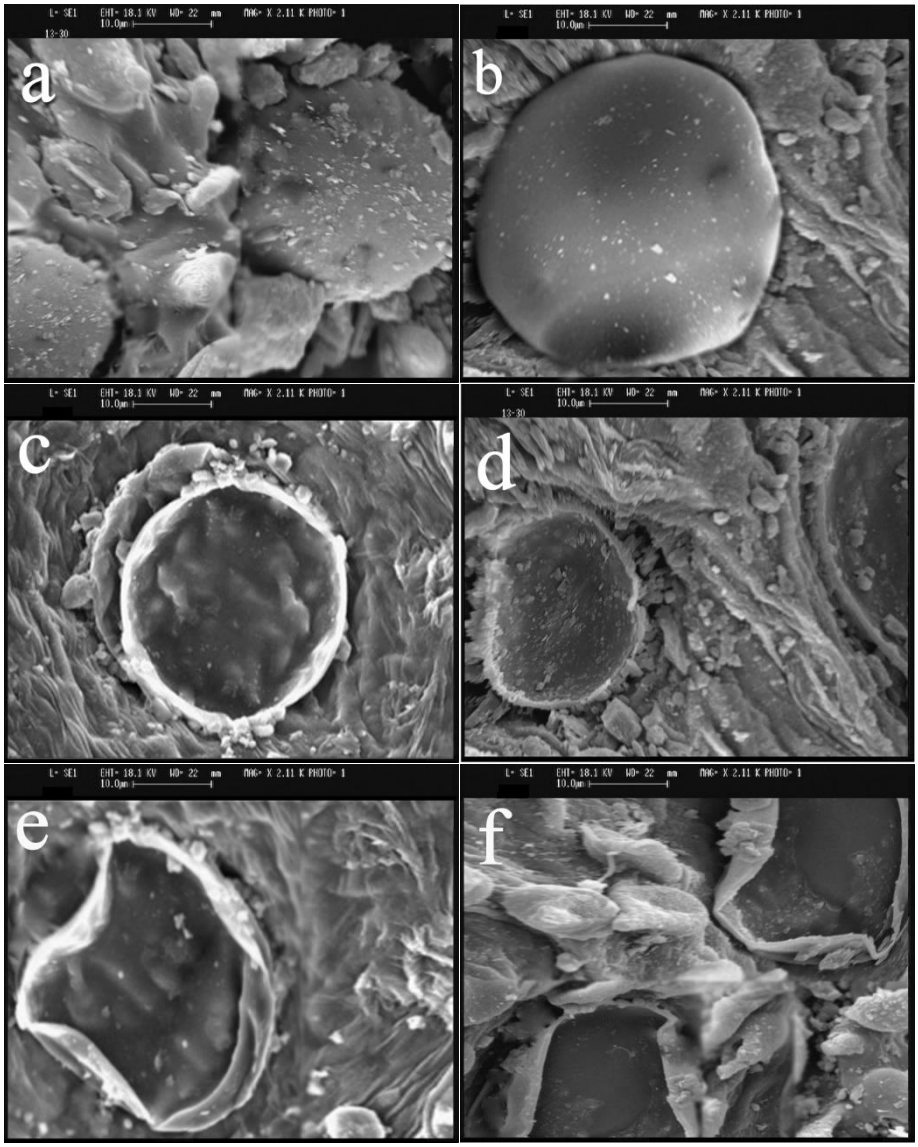

Figure 13. micrograph of thyme leaves: (a) *Ferulago angulata* (Schlecht.) Boiss, untreated*; (b) Thymus daenensis* untreated; (c) *Ferulago angulata* (Schlecht.) Boiss MAE treated; (d) *Thymus daenensis* MAE treated; (e) *Ferulago angulata* (Schlecht.) Boiss HD treated and (f) *Thymus daenensis* HD treated and

3.9. SEM analysis

After extraction, the structural changes in the leaves were investigated through scanning electron microscopy. The micrographs of the untreated (i.e., before the extraction) and treated (MAE and HD) sample are shown in fig. 13. It could be found that differnt extraction methods had similar impacts on both the leaves of *T. daenensis* and *F. angulata* (Schlecht.) Boiss, but a different effect was observed between the methods. Researchers claimed that different recognizable physical changes in the plant material occured due to the application of various extraction methods (Bousbia et al., 2009). The time needed for destroying the glands with MAE is significantly lower than with HD. It could be concluded from this trend that MAE ruptured the thyme glandular walls more rapidly and more efficiently. On the other hand, visual assessment of the micrographs of

untreated and treated of both types of thyme leaves showed the significant effect of the extraction methods on their structure. As shown in fig. 13b and 13c, the essential oils of HD-treated glands were compeletly discharched but thier appearances were wrinkled but the glands of the MAE-treated samples (fig. 13d and 13e) are smoothe in surface. This result could be related to the heat transfer rate difference between MAE and HD. Authers illustrated that heat production in MAE, due to the use of three types of heat transfer within the sample, is faster than in HD that used two types of them (Ferhat et al., 2006). In microwave heating, subjecting the glands to an acute thermal stress followed by increasing the pressure within the glands beyond their expansion capasity causes a faster rupture of the cell walls than other methods (Golmakani and Rezaei, 2008b). Similar results were reported for the extraction of rosemary leaves in hexane through microwave (Paré and Bélanger,1997; hen and Spiro, 1995).

4. Conclusions

The results showed that the application of microwave and the increase in its power resulted in a reduction in the extraction initiation and the extraction completion times. In addition, it decreased the time needed for the extraction curve to be constant. Nevertheless, microwave did not have a significant effect on the specific gravities, refractive indices, constituents and the antioxidant activities of *T. daenensis* and *F. angulata* (Schlecht.) Boiss essential oils.

Utilization of indirect ultrasound pretreatment did not significantly influence the qualitative and quantitative composition of *T daenensis* essential oil. Thyme leaves micrograph showed that MAE cause a suddenly rupture of the cellular walls followed by a higher extraction efficiency at a shorter time. In conclusion, it could be declared that given the importance of energy and the use of medicinal plants, MAE can dramatically reduce energy consumption and extraction time.

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DOUGH REOLOGY AND PROPERTIES OF GLUTEN-FREE RICE BREADS AS AFFECTED BY ADDITION OF HYDROCOLLOIDS AND EMULSIFIERS

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ABSTRACT

The demand for gluten-free products is rising since an increasing number of people are suffering from celiac disease or other allergic reactions/intolerances to gluten consumption. In order to mimic the viscoelastic properties of gluten and to increase the dough gas-retaining ability, the use of hydrocolloids and emulsifiers in gluten-free bread formulations is required. In this study, addition of different hydrocolloids (agar gum, guar gum, hydroxylpropyl methylcellulose [HPMC], pectin, citrus fiber, pectin + guar gum and pectin + HPMC mixture) at 2% (w/w) and emulsifiers (diacetyl tartaric acid ester of monoglycerides [DATEM] and glyceryl monostearate [GMS]) at 0.5% (w/w) in gluten-free formulations based on rice flour and potato starch was carried out in order to investigate their effects on dough rheological properties and bread quality. Rice dough containing no hydrocolloid and emulsifier were used as control formulations. Hydrocolloids improved the water absorbtion capacity and dough consistency as assayed by farinography and determined positive effects on the texture of the breads, by increasing the specific volume and crumb porosity. An increment of the crumb porosity, moisture content and elasticity was recorded at the addition of emulsifiers. The highest bread specific volume and crumb porosity were obtained for bread samples containing pectin+HPMC and pectin+guar gum mixtures blend with DATEM while GMS addition determined the highest increase of the crumb elasticity and moisture content.

1.Introduction

Celiac disease is a chronic malabsorption disorder caused by the ingestion of cereal proteins, namely the gliadin fraction of wheat and the prolamins of rye, barley and possibly oats (Gallagher et al., 2004), leading to the inflammation of the small intestine and to the malabsorption of important nutrients like calcium, iron, folic acid and fat soluble vitamins (Gujral et al., 2004). It is currently considered that this gluten-sensitive entheropathy culminating in the intestinal mucosal damage can be caused by genetic predisposition, environmental factors and immunologically-based inflammation (Demirkesen et al., 2010; Gallagher et al., 2004). Screening studies show a high prevalence of celiac disease among both healthy children and adults and a high ratio of known to undiagnosed cases (Fasano and Catassi, 2001).

The only effective treatment for this disease is to follow a strict gluten-free diet throughout the life-span, which, in time results in clinical and mucosal recovery and risk reduction of malignant complications (Mariotti et al., 2009). This requires the patient to avoid consumption of any bread, cereal or other food made with wheat, rye, barley, triticale, dinkel, kamut and oat flour or ingredients, and by-products made from those grains (Gallagher et al., 2004). The apparent or real increase in celiac disease, or other allergic reactions/intolerances to gluten consumption led to a rising demand for gluten-free products, mainly in the countries where cereal poducts, especially breads, are the basic components of the diet (Moore et al., 2006; Lazaridou et al., 2007).

A variety of breads have been created as replacements for traditional wheat breads trying to provide alternative gluten-free products. One of the most suitable cereal flour for preparing gluten-free products is the rice flour, due to its unique attributes such as bland taste, colorless and hypoallergenic properties, low level of protein, sodium, fat, fiber and high content of easily digested carbohydrates (McCarthy et al., 2005; Demirkesen et al., 2010).

However, the absence of gluten represents a major technological challenge, as it is the essential structure-building protein, providing some unique functional properties. Gluten is responsible for the protein-starch interaction that gives specific viscoelastic properties in bread dough and for the stabilization and retention of the gas cells during the proofing and baking process (Ahlborn et al., 2005). Therefore, ingredients, like starches of different origin, dairy proteins, other non-gluten proteins, gums, hydrocolloids, and their combinations, that have the ability to mimic the properties of gluten, are generally required for the production of gluten-free breads (Moore et al., 2006; Mariotti et al., 2009).

Due to their structure forming properties, gums and hydrocolloids are essential ingredients in gluten-free bread formulations for improving the dough consistency and gas retaining capacity, the bread texture and appearance and for shelf-life extension of the final products. Several studies have been conducted on the effects of hydroxypropyl methylcellulose (HPMC), carboxymethylcellulose (CMC), agarose, sodium alginate, konjac gum, guar gum, k-carrageenan, xanthan gum, Psyllium fiber, sugar beet fibre and oat β-glucan on dough rheology and bread quality parameters in gluten-free formulations (Haque and Morris, 1994; Kadan et al., 2001; Rosell et al., 2001; Guarda et al., 2004; Moore et al., 2004; McCarthy et al., 2005; Lazaridou et al., 2007; Cappa et al., 2013).

In addition to hydrocolloids, emulsifiers are widely used in commercial wheat bread formulas to improve the dough strength and increase gas cell stabilization in the dough by forming liquid lamellar films surrounding the gas cells. Emulsifiers also contribute to retarding starch retrogradation by inhibiting the migration of water through interaction with starch molecules (Eduardo et al., 2014). Several studies reported on the effects of emulsifiers on the specific volume, staling and self-life of gluten-free breads. Generally, these studies demonstrated that the addition of emulsifiers at their optimum level enhanced the quality of the gluten-free breads (Nunes et al., 2009; Onyango et al., 2009; Purhagen et al., 2012).

The aim of the present work was to analyze the effects of the inclusion of different hydrocolloids and gums (agar gum [AG], guar gum [GG], pectin [P], citrus fiber [CF] and hydroxypropyl methylcellulose [HPMC]) and emulsifiers (diacetyl tartaric acid ester of monoglycerides [DATEM] and glyceryl monostearat [GMS]) into a gluten-free formulation based on rice flour and potato starch, on the rheological properties of

gluten-free doughs and on some breads quality characteristics.

2. Materials and methods

2.1. Materials

Commercial rice flour obtained from Solaris Plant S.R.L. (Romania) and potato starch from Soia Produkt S.R.L. (Romania) were used in the present study for all bread formulations. The rice flour had moisture, protein and lipid content of 12.8, 6.5 and 2.8% respectively. Fresh yeast (Lesaffre, France), sunflower oil (Expur S.A., Romania), dry milk (JP Food Company S.R.L., Romania), salt, and sugar were bought from local markets. Hydroxypropyl methylcellulose (HPMC) Methocel K4Mm was obtained from Dow Chemical Company (Michigan, USA). Agar gum, guar gum, pectin, and citrus fiber (Citri-Fi 100FG, Fibrestar, Inc., Willmar, MN) were obtained from Enzymes and Derivates S.A. (Romania). Diacetyl tartaric acid ester of monoglycerides (DATEM 2005K) and glyceryl monostearate (RADIAMULS® 2910) were from EDR Ingredients (Romania).

2.2. Dough rheological properties

The effects of different hydrocolloids and emulsifiers on dough mixing properties were investigated using Farinograph®-AT (Brabender® GmbH&Co KG, Duisburg, Germany). A 300 g of flour and starch basis and each hydrocolloid or hydrocolloid mixture at 2% level (w/w) were loaded in the farinograph. The selected emulsifiers were added at 0.5% level (w/w).

Powders were pre-mixed for 5 min and kneading was carried out for 15 min. Assays were carried out at 30 °C. The parameters obtained from the farinogram curves included development time in minutes (DT, time to reach the maximum consistency), water absorbtion capacity (WAC) of flour blends in percentages, dough consistency at the DT (C) and degree of softening (DS, 10 min after beginning), both in FU (Farinograph Units).

2.3. Bread making procedure

Basic dough recipe on 100 g rice flour and starch basis consisted of 60% rice flour, 40% potato starch, 10% milk powder, 5% vegetable oil, 5% yeast, 5% sugar and 2% salt. The amount of water (30 ^{o}C) added to the rice dough was 150% (rice flour and starch basis). Hydrocolloids (agar gum, guar gum, pectin, citrus fiber, HPMC, pectin+guar gum and pectin+HPMC) were added at 2% w/w while emulsifiers (DATEM and GMS) at 0.5% w/w (rice flour and starch basis). The samples from basic dough recipe without any hydrocolloid and emulsifier were used as control.

Before adding into the dough mixture, the hydrocolloid was dispersed in half of the water to be used in the dough formulation using a high speed homogenizer. During preparation of the bread, dry ingredients (rice flour, sugar and salt) were mixed thoroughly, and then the vegetable oil was added. Finally hydrocolloid suspension and water were added slowly and mixed for 3 min using a mixer (Kitchen Aid, 5K45SS, USA). After complete mixing, the dough sample was placed in a greased 70 × 120 × 220 mm pan covered with a warm, damp cloth and proofed for 40 min at room temperature. Following proofing, the samples were baked at 200 ^{o}C for 30 min in an electrical convection oven (Brio-Inox, Gierre, Milano).

2.4. Bread quality evaluation

Analyses of breads were performed 24 hours after baking. Three loaves from each treatment were tested. For evaluation of fresh bread quality, the height, weight, specific volume, titratable acidity, moisture content, bread crumb porosity and elasticity were determined according to the Romanian official methods (STAS 91-2007).

The bread specific volume was expressed as the ratio of volume to weight of baked bread (cm^3/100 g). Crumb moisture was determined by air oven gravimetric method AACC 44-15A.

Bread crumb porosity (%) was determined by the ratio of pores volume to total volume of a known volume of crumb, knowing its mass and density. A cylindrical piece of crumb was cut from a 60 mm slice obtained from the middle of the loaf by a cylindrical sharpened brass perforator (internal diameter 45 mm) and weighed. The slices were obtained by cutting the bread transversely using a cutter to obtain uniform slices of 60 mm thickness. To obtain an average porosity, three cylinders, from three different areas, were analysed.

For determination of bread crumb elasticity, a piece of bread crumb was cut as previously described and pressed to the half of its height for 1 min using a screw-driven pressing device which consisted of a fixed and a mobile plate and a ruler. Then, the pressure was removed and after 1 min the height of the compressed piece of crumb was measured. The elasticity values were obtained as the ratio (given as percentage) between the height after compression and recovery, and the initial height. Measurements of all the quality parameters were performed in three replicates.

3. Results and discussions

The rheological behavior of rice dough samples was analyzed through the farinograph parameters. The effect of hydrocolloid addition to the flour blend used in the gluten-free formulations is shown in Figure 1. As anticipated on the basis of previous findings, addition of hydrocolloids substantially increased the water binding which determined the increase of the moisture content in the final product (Figures 1 and 2).

Among the rice dough samples containing different hydrocolloids (without emulsifier), the highest water absorbtion capacity values were obtained for HPMC containing samples, results that agree with the data reported by Guarda et al. (2004) on wheat dough. This effect has been attributed to the hydroxyl groups in the hydrocolloid structure which allow more water interactions through hydrogen bonding (Rosell et al., 2001).

The addition of hydrocolloids increased the dough consistency. This is consistent with previous findings showing that addition of hydrocolloids increases the viscosity of any product containing water system by entrapping the water molecule in the hydrocolloid gel matrix, thus improving the consistency of product by water phase management (Mudgil et al., 2011).

HPMC at 2% brought about the highest increase in the dough consistency followed by HPMC-pectin mixture, pectin, guar gum, citrus fiber and agar gum.

An increase in the water absorbtion capacity of the rice flour dough by various hydrocolloids has been reported earlier (Rosell et al., 2001; Guarda et al., 2004; Lazaridou et al., 2007).

The development time, required by the dough to reach the maximum consistency, decreased at hydrocolloid addition, with the exception of pectin, agar gum and citrus fiber that increased the development time (Figure 1). The addition of pectin and citrus fiber determined also the highest softening of the rice flour dough. Lazaridou et al. (2007) found that the development time increased at the addition of hydrocolloids with the exception of guar gum and HPMC that decreased the time to reach the dough consistency of 500 BU.

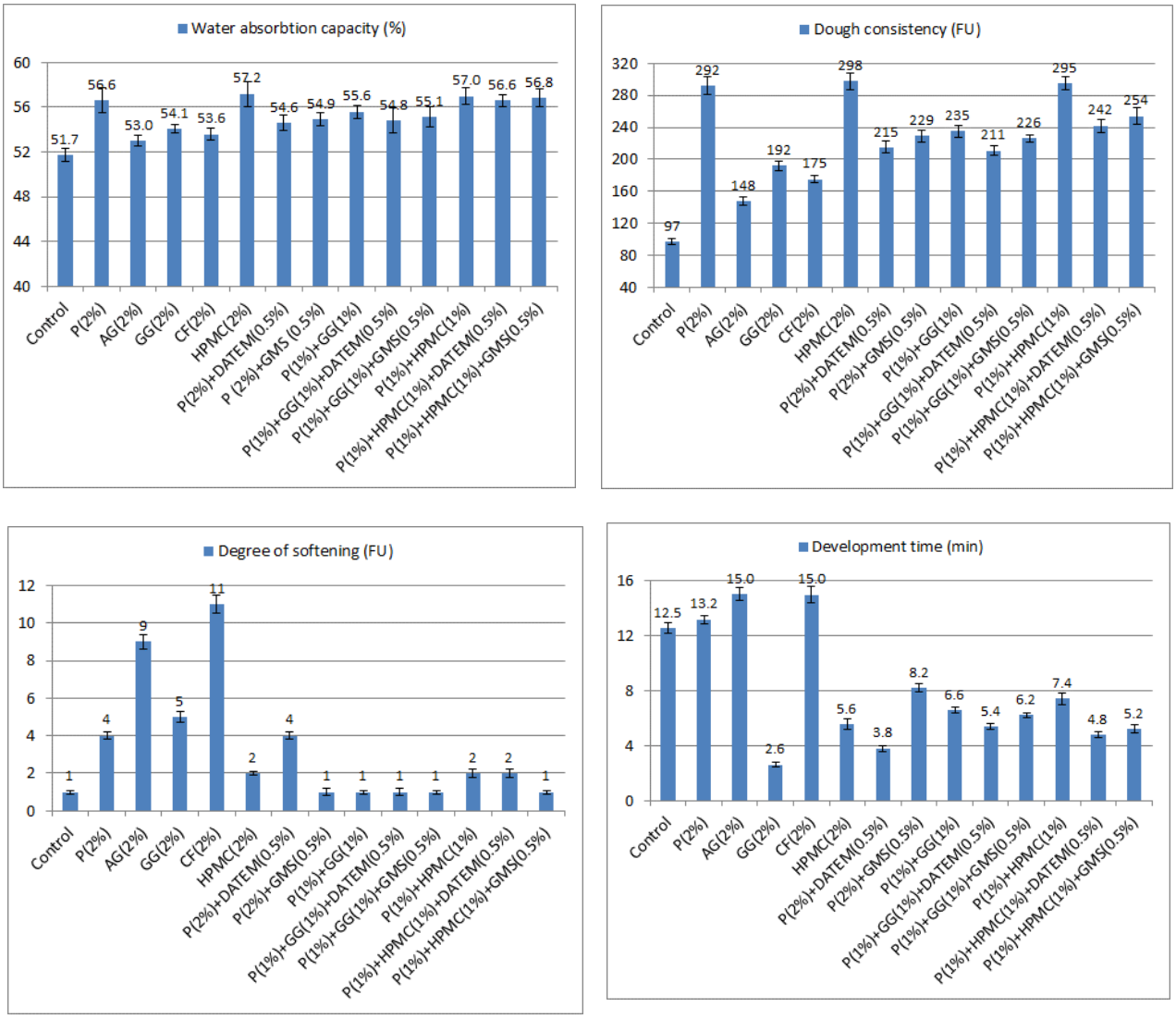

Figure 1. Farinograph parameters of rice gluten-free doughs as affected by hydrocolloids and emulsifiers

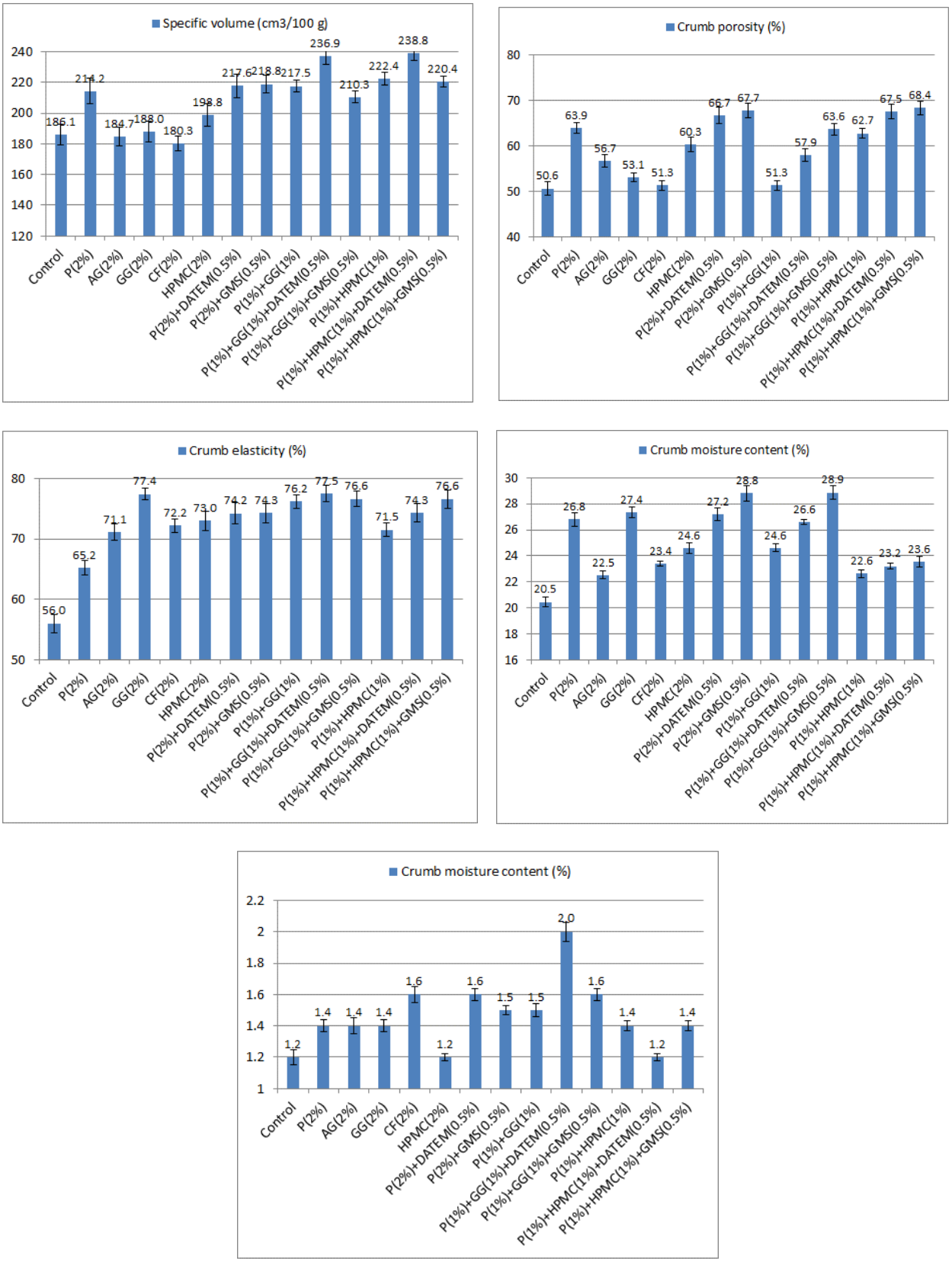

Figure 2. Bread properties as affected by hydrocolloids and emulsifiers

Addition of emulsifiers caused a decrease in the consistency and water absorption capacity values of rice dough samples (Figure 1).

The baked bread samples were evaluated in terms of height, volume, specific volume, crumb porosity, crumb elasticity, crumb moisture content and acidity as shown in Figure 2.

The addition of hydrocolloids generally determined positive effects on the texture of the breads, by increasing the specific volume and crumb porosity. Numerous previous studies have shown that hydrocolloids can improve the volume and texture of gluten-free breads (Cato et al., 2004; Lopez et al., 2004; Ribotta et al., 2005; Ahlborn et al., 2005; Lazaridou et al., 2007).

The highest increase of specific volume was observed for pectin followed by HPMC.

Bell (1990) attributed the effectiveness of the modified polysaccharide derivatives such as carboxymethyl cellulose, hydroxy-propyl methylcellulose, and methylcellulose to their hydrophobic groups which induce an increased interfacial activity within the dough system during proofing and to their ability to form a «thermal gel», thus increasing gas retention through baking and consequently leading to a better loaf volume.

The addition of hydrocolloids in gluten-free breads at 2% concentration increased the bread crumb elasticity compared to the control formulation, results in agreement with those reported by Lazaridou et al. (2007).

Without emulsifier, the HPMC-pectin mixture determined the highest specific volume (222.39 cm^3/100 g) after baking. This could be probably related to the higher consistency of the dough (Figure 1), that allowed the creation of the film-like structure, fundamental to obtain a good technological quality of the bread. Although the bread samples obtained with pectin-guar gum mixure had a lower specific volume and porosity than those obtained with HPMC-pectin mixture, they were characterized by a higher elasticity and crumb moisture content. This is in good agreement with previous findings showing that fibres that are high in soluble fibre (such as pectin) can positively influence the softness of the crumb by helping to retain moisture (Cappa et al., 2013).

As a result of the fibres ability in binding water and holding it through the baking process, the addition of hydrocolloids determined an increase of the crumb moisture content relative to the control. In this study DATEM and GMS determined the increase of the bread hight, specific volume, crumb porosity and elasticity. Furthermore, the addition of emulsifiers brings an increase of the crumb acidity. The positive effects of emulsifiers have been demonstrated by Ribotta et al. (2003) in wheat breads or by Demirkesen et al. (2010) in rice gluten-free breads. They showed that addition of emulsifiers clearly improved the volume of the breads by allowing the entrapment of air bubbles in dough and providing stability to the dough mixture during baking.

The bread samples supplemented with DATEM had higher specific volume values compared to GMS added breads, regardless of hydrocolloid used.

The addition of the emulsifiers increased the crumb elasticity as compared to the corresponding gluten-free formulations without emulsifiers. The highest crumb elasticity was obtained for rice dough samples containing GMS.

4. Conclusions

Addition of hydrocolloids and emulsifiers in rice flour based gluten-free formulations were evaluated using dough rheological measurements and baking tests.

Results showed that addition of hydrocolloids substantially increased the water absorption capacity and the dough

consistency while an opposite effect was noted with the addition of emulsifiers. HPMC, pectin and HPMC-pectin mixture were observed to be the most effective hydrocolloids in improving dough rheological properties.

Baking tests showed that hydrocolloids determined positive effects on the texture of the breads, by increasing the specific volume and crumb porosity. Without emulsifier, the HPMC-pectin mixture determined the highest specific volume and crumb porosity after baking while the bread samples obtained with pectin-guar gum mixture presented a higher elasticity and crumb moisture content.

Emulsifiers contributed to the improvement of the bread texture by increasing the specific volume, crumb porosity and elasticity, with DATEM being more effective than GMS.

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THERMODYNAMIC ANALYSIS OF DRYING POTATO CUBES IN A FLUIDIZED BED DRYER

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ABSTRACT
In this study, wasted exergy and energy were analyzed in the laboratory for the drying of potatoes using a fluidized bed dryer. Drying was performed at the inlet temperatures of 45, 50 and 55 °C and the air velocity of 3.2, 6.8 and 9.1 $m.s^{-1}$ and bed depth of 1.5, 2.2 and 3 cm. The effects of temperature, air velocity and bed depth on energy consumed and the waste of exergy were investigated. The results showed that with increasing temperature and velocity and depth of the bed of samples, consumed energy and wasted exergy were increased. The lowest energy consumption was obtained at a depth of 1.5 cm, a velocity of 3.2 m/s and a temperature of 45 ° C, and the maximum energy consumption was obtained at a depth of 3, a velocity of 9.1 m/s and the temperature of 55 °C. Also, the minimum wasted exergy was at a depth of 1.5 cm, a velocity of 3.2 m/s and a temperature of 45 ° C and the maximum exergy was a depth of 3, a velocity of 9.1 m/s and the temperature of 55 °C.

1.Introduction

Potato is the most important crops among the dicotyledon that is presently ranked fourth in the world after wheat, rice and corn. This plant's adaptation to climatic conditions of different regions is in a way that potato is now produced in more than 140 countries. It is a useful matter for powering because this plant is less affected by pests compared with the grain and its growing is easier (Aprajeeta *et al*., 2015; Ruhanian and Movagharnejad, 2016). A large number of industrial products of potatoes are divided into two categories of dried potatoes products from baked potatoes and dried potatoes products from fresh potatoes(Lisinska and Leszczynski, 1989) . Preservation of food through drying is one of the oldest and the most widespread method that can be used to enhance the strength of the food. Drying food is removing the moisture so that the product can be stored for a long time and be protected against corruption(Min *et al.*, 2005) . Drying by reducing the microbial enzyme activity and reducing the speed of chemical reactions, increases shelf life of the product and by reducing the weight and volume of materials, packaging, facilitates transportation and storage of products and decreases the cost of these procedures. In the case of drying, in addition to preventing the loss, the marketing can be controlled at sensitive times and required potatoes of a lot of consumers (such as barracks, restaurants, etc.) can be delivered as dried form(Salimi Hizadji *et al*., 2010) . The power consumption of dryers is variable from the values less than 5% for the chemical industrial processes to 35% for paper-making operations. The main cost for dryers is in their performance rather than the initial cost of investment. Therefore, new ideas in

methods of drying and dryer design are required to optimize energy consumption and operating conditions(Chen et al., 1996) . According to studies carried out on various dryers at different temperatures by other researchers, it has been considered that air velocity and temperature of drying have a significant effect on the texture of foods. Considering the thermal efficiency of the processes of drying, fluid bed dryers are widely used for drying certain foods because of high rates of heat and mass transfer and high speed of drying. Fluid bed dryers in chemistry, metallurgy and pharmaceutical industries have many applications(Izadifar and Mowla, 2003) (Kachro *et al.*, 1995)(Ratti and Mujumdar, 1995).In designing a system, exergy analysis method provides valuable information on proper selection of design components and applicable methods. These information are much more effective for determining design and operation costs, energy storage, fuel switching capability and pollution(Elkhodiry *et al.*, 2015)(Afzal *et al.*, 1999) (Abbasi Souraki and Mowla, 2008). The fluid bed dryer are used for crops like rice, maize, wheat, sorghum, tea, pollen, fruits and vegetables (Mwithiga and Olwal, 2005)(Dimattia *et al*., 1996)(Prasad *et al.,* 1989).Aghbashlo et al. (2011) in their research concluded that the input energy values and specific energy requirement for thin-drying of berberis fruit were found to be in the range of0.643348–35.20032 (kWh) and 20.9355–1110.0700 (kWh/kg) from 50Cto70C with drying air veloc-ities of 0.5–2 m/s, respectively (Nazghelichi *et al*., 2011). Kouchakzadeh *et al*. (2011) found that the average power consumption and heat loss in their tests were 62.13 and 18.99 kW, respectively and the ratio of heat loss on power consumption showed that the effi-ciency of practical pistachios flat plate dryer was about 69.4% (Kouchakzadeh and Tavakoli, 2011). Sarker, (2013) showed that in reducing paddy moisture from 22% to 23% (wb) down to around12.5% (wb) using drying temperature of 41–42C, IBDs consumed around 10% higher thermal energy and around 20% lesser electrical energy while head yield rice yield was reduced by 1–4%(Sarker et al., 2015). Nikbakht et al. (2014) in assessment of the specific energy requirement in various dryers concluded that the highest and lowest values belonged to the vacuum and microwave methods with 318.42 and 4.32 MJ/kgW, respectively(Nikbakht *et al.*, 2014)

In another study, a fluidized bed dryer with a capacity of 22 tons per hour was designed to dry paddies, and the calculations showed that lesser exergy losses lead to an increase in exergy efficiency. This demonstrated that using the energy in air is effective for the drying process. The exergy balance analysis carried out in this research demonstrated that only 31–37% of exergy was used for drying rice. This illustrated the purposes of the remaining exergy usage. Exergy usage should be increased by providing enough insulators in the dryer body and by recycling exhaust air; this can be examined in terms of economic efficiency.(Sarker e*t al., 2015)*In the energy and exergy analysis of drying native cassava starch in a tray dryer, it was concluded that for starch with contents of 0.76% ash, 0.85% crude protein, 0.16% crude fat, negligible amounts of fiber, an average granule size of 14.1 mm, pH of 5.88, 23.45% amylose content, and a degree of crystallinity of 22.34%, energy utilization and its ratio increased from 1.93 to 5.51 J/s and 0.65 to 0.6 as the drying temperature was increased from 40 to 60 ℃ (Aviara *et al.*, 2014).The aim of the present study is to present a thermodynamic analysis of potatoes drying in different inlet temperatures, air velocity and bed depth in fluidized bed dryer and to determine the best conditions for minimum consumed energy and wasted exergy during the drying process.

2. Materials and methods

2.1. Material preparation

Freshly harvested potatoes were purchased from a local market and stored in a laboratory refrigerator at 5℃. At the beginning of each test, potatoes were washed, peeled, and cut manually using cubic device with dimensions of 0.6× 0.6 cm and a height of 0.5 cm. The drying experiment was conducted using a laboratory fluidized bed dryer made in the Department of Mechanical Bio-systems of the University of Agricultural Sciences and Natural Resources of Gorgan, Iran.

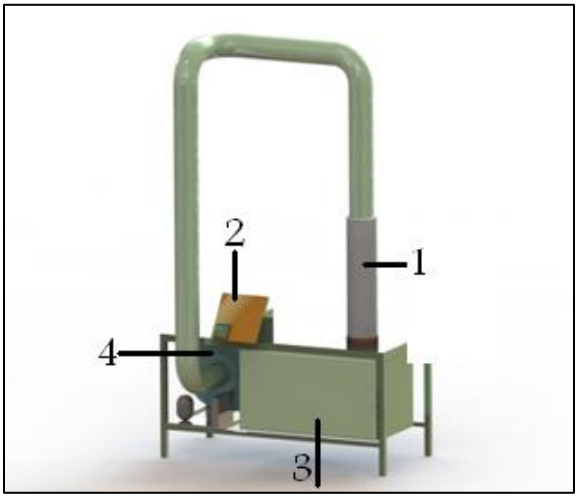

Figure1. Schematic illustration of testing apparatus
1- Fluidizing chamber 2- heater control 3- heaters 4- fan

2.2. Experimental procedure

To supply the required air flow, a centrifugal blower with a 3hp CDF90L_2 three-phase electric motor (KAIJIELI) was used. For outlet temperature measurement, an ST_941 standard multi-meter with an accuracy of ±0.1℃ was employed; to measure dryer wind speed, an anemometer (LUTRON, AM-2416) with an accuracy of 0.1m/s was utilized. The dryer contained an automated temperature controller with an accuracy of ±1℃ (Figure 1). Samples were weighed every 5 minutes using a Dj 2000A weigh scale (Shinko electric scale), which had an accuracy of 0.01 g. During drying, the outlet air temperature of the dryer and the airflow ratio were recorded at 5 minute intervals.

Samples were weighed at the beginning, and after the dryer reached to the desired temperature, potatoes were placed inside the drying cabinet. The experiment was performed at temperatures of 45, 50, and 55℃, bed depth of 1.5, 2.2 and 3 cm and speeds of 3.2, 6.8, and 9.1 m $s^{-1}$the potatoes had a cubical shape with dimensions of 0.6× 0.6 cm and a height of 0.5 cm. Each treatment was repeated three times and the test was performed at a temperature of 30℃ with a relative moisture content of 50%. Variance analysis was carried out using a factorial experiment and a completely randomized design via SAS software (9.1.3 portable).

2.3. Determination of moisture content

To determine the moisture content of the samples, they were placed inside an oven at a temperature of 105℃; the final weights were measured after 24 hours. Having determined the initial and final weights, and using eq. 1, the initial moisture content could be obtained (Martynenko and Zheng, 2016):

$$M=\frac{m_1-m_2}{m_1}. \quad (1)$$

The initial moisture content of the potatoes was 78.81 %.

2.4. Analysis of consumed energy

In this research, energy utilization was expressed using the first law of thermodynamics, as follows (Azadbakht *et al*., 2017):

$$Eu = \dot{m}_{da} \times (h_{dai} - h_{dao}). \quad (2)$$

The air mass flow rate was obtained using eq. 3(Aghbashlo *et al.*, 2008):

$$\dot{m}_{da} = \rho_a \times v_a \times A_{dc}. \quad (3)$$

Dryer air enthalpy was obtained using eq. 4(Corzo *et al*., 2008):

$$h_{da} = C_{pda} \times (T - T_\infty) + h_{fg}. \quad (4)$$

Inlet and outlet air-specific heat capacities were calculated with eq. 5(Corzo *et al*., 2008):

$$C_{pda} = 1.004 + 1.88 \times w. \quad (5)$$

During energy and exergy analysis of the potatoes drying process, eq. 6 was used for transformation of relative moisture content to the air moisture content ratio (kg water/kg dry air)(Topic, 2007):

$$w = 0.622 \times \frac{\varphi \times P_{vs}}{P - P_{vs}}. \quad (6)$$

The inlet and outlet air moisture content ratio was obtained using eq. 7(Azadbakht *et al*., 2017) :

$$w_{dao} = w_{dai} + \frac{\dot{m}_v}{\dot{m}_{da}}. \quad (7)$$

Moreover, $\dot{m}_v$ was calculated using following equation (Nazghelichi *et al*., 2010):

$$\dot{m}_v = \frac{w_t - w_{t+\Delta t}}{\Delta t}. \quad (8)$$

2.5. Analysis of exergy

Equation 9 was employed to calculate exergy, representing functional exergy equation with a steady flow(Midilli and Kucuk, 2003):

$$Ex = \dot{m}_{da} \times C_{pda} \times ((T - T_\infty) - T_\infty \times Ln\frac{T}{T_\infty}). \quad (9)$$

Wasted exergy in the drying chamber were obtained using eq. 10 (Prommas *et al*., 2010):

$$Ex_l = Ex_i - Ex_o. \quad (10)$$

3.Results and discussions

3.1. Consumed Energy

Analysis of variance of changes of velocity, temperature and depth on the energy consumption of potatoes in a fluid bed dryer has been shown in Table 1. Table 1 shows the changes in velocity, temperature and depth on consumed energy were effective at probability level of 1%. And the interactions of velocity and temperature, velocity and depth, and temperature and depth on energy consumption were significant at probability level of 1%. Therefore, the average was compared using LSD test and the results has been given in Figure 2 and 3.

Table 1. Variance analysis energy consumption under different speed, depth and temperature in fluidized bed dryer

Source of variation	Degrees of freedom	Sum of squar	Mean Square	F Value
Speed	2	3.114	1.557	20.61**
Depth	2	10.892	5.446	72.08**
Temperature	2	18.909	9.454	125.13**
Speed×Temperature	4	2.027	0.506	6.71**
Speed×Depth	4	3.994	0.998	13.22**
Depth× Temperature	4	5.236	1.309	17.33**

** and *Significant difference at 1% level (p <0.01) and at 5% level (p <0.05) respectively, ns not significant difference

3.1.1.Interaction of Depth and Temperature

Figure 2 shows the effect of depth at different temperatures on energy consumption that the highest and lowest energy consumption are 2.3266 kJ/s and 0.4370 kJ/s at temperatures of 55 and 45 and a depth of 3 and 2.2 cm, respectively.

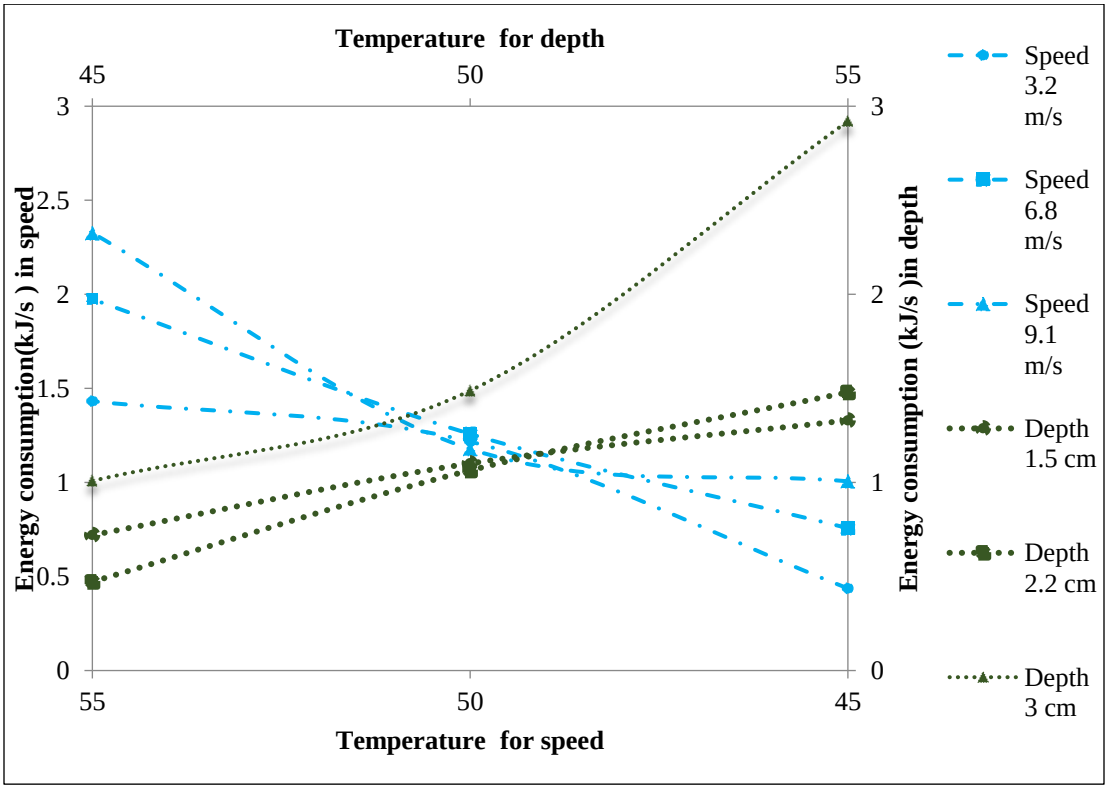

Figure 2. The effects of depth and speed in different temperature of dryer on energy consumption

According to this Figure, the energy consumption has been increased by increasing the depth. Moreover, at a constant depth energy consumption has been increased with increasing temperature. Because higher temperatures cause more decrease in moisture. Because by increasing the depth, the number of layers in the bed dryer is increased and volume of inside of chamber has been increased that causes humidity exiting from the mass more difficult and increases the amount of required energy drying time. In other words, higher temperatures cause more reduction in mass and humidity. This also causes an increase in energy consumption. These results are similar with the results of study of AhmadiChenarbon et al. (2011) on Hypericum perforatum .(AhmadiChenarbon *et al*., 2011)

3.1.2. Interaction of Air Velocity and Temp erature

According to Figure 2, the highest and lowest energy consumption are 2.3266 kJ/s and 0.4370 kJ/s at temperatures of 55 and 45 and a depth of 9.1 and 3.2 cm, respectively. According to this Figure, with an increase in velocity of hot air, energy consumption has been increased. In addition, at a constant velocity, energy consumption has been also increased by increasing the temperature. This is because the increase in temperature and inlet air raises energy consumption and inlet enthalpy and an increase in enthalpy also transfers heat and mass, and this transfer causes an increase in energy consumption. These results are similar to the results of AhmadiChenarbon et al. (2011) on Hypericum perforatum and Motevali et al (2012) on Effects of microwave pretreatment on the energy and exergy utilization in thin-layer drying of sour pomegranate arils.(AhmadiChenarbon *et al.*, 2011)(Motevali and Minaei, 2012) .

3.1.3. Interaction of Air Velocity and Depth

Figure 3 shows the effect of velocity at different depth on energy consumption. The highest and lowest energy consumption are 2.2894 kJ/s and 0. 7034 kJ/s at a depth of 3 and velocity of 9.1 m/s and a depth of 1.5 and velocity of 3.2 m/s, respectively. According to Figure 3, the energy consumption has been increased by increasing the depth. With the increase in air velocity, energy consumption has been increased. At a constant speed by increasing the depth, the energy consumption has been enhanced. This is because some of the energy of hot air of dryer that moves towards the top of the fluid bed chamber, is absorbed by product in the chamber, so the less amount of hot air reaches to the top of the cylindrical chamber. It can be said that as with increasing depth, the mount of product has been increased and by increasing the number of particles, the less amount of hot air reaches to the top parts and the required energy for reducing humidity has been increased up to a certain amount and energy consumption is increased. Nazghelichi et al. (2013) have also reported same results.(Nazghelichi *et al.*, 2013)

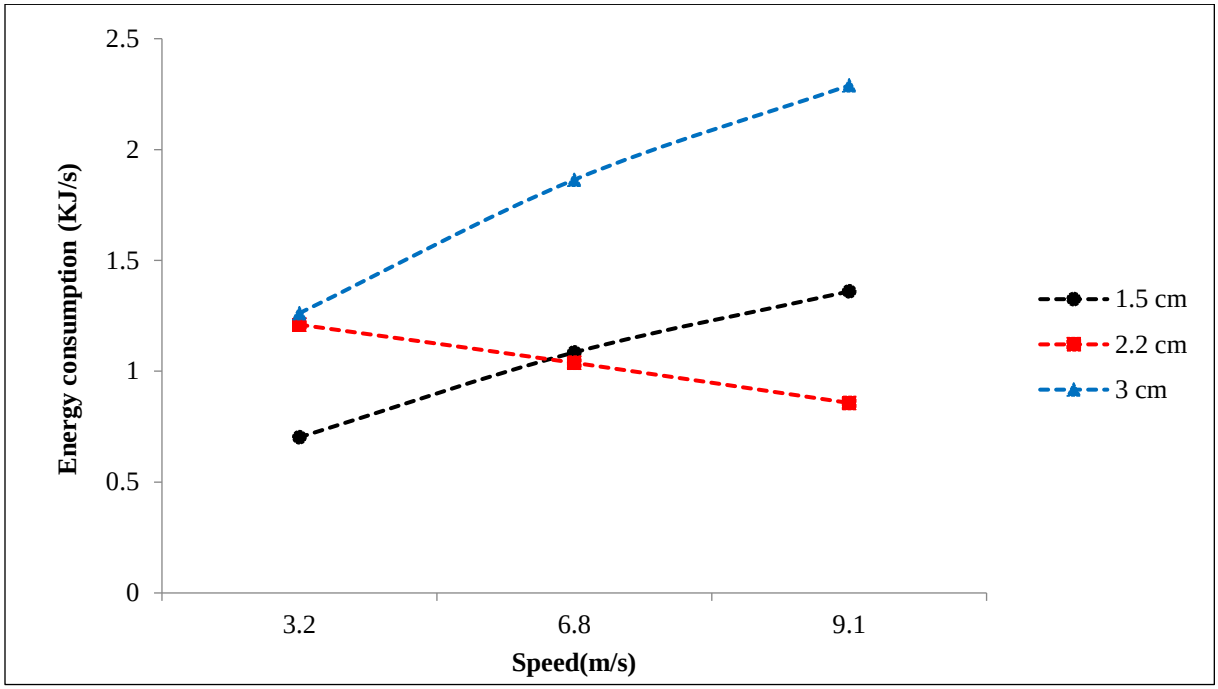

Figure 3 . Effects of depth in different speed of dryer on energy consumption

3.2. Wasted Exergy

Analysis of variance of changes in velocity, temperature and depth on the amount of wasted exergy of potatoes in a fluid bed dryer has been shown in Table 2. As Table 2 shows, changes in velocity, temperature and depth on wasted exergy are effective at probability level of 1%. Moreover, the interactions of velocity and temperature, velocity and depth, and depth and temperature on exergy consumption are significant at probability level of 1%. Therefore, the average was compared using LSD test and the results has been given in Figure 4 and 5.

Table 2. Variance analysis wasted exergy under different speed, depth and temperature in fluidized bed dryer

Source of variation	Degrees of freedom	Sum of Square	Mean Square	F Value
Speed	2	0.664	0.3321	27.46**
Depth	2	1.213	0.606	50.15**
Temperature	2	3.301	1.650	136.39**
Speed×Temperature	4	0.446	0.1115	9.22**
Speed×Depth	4	0.444	0.1112	9.19**
Depth× Temperature	4	0.629	0.157	13.01**

** and *Significant difference at 1% level (p <0.01) and at 5% level (p <0.05) respectively, ns not significant difference

3.2.1. Interaction of Depth and Temperature

Figure 4 shows the effect of depth at different temperatures on wasted exergy shows that the highest and lowest energy consumption are 1.063 kJ/s and 0.14895 kJ/s at temperatures of 55 and 45 and a depth of 3 and 2.2 cm, respectively. Figure 4 shows that the amount of wasted exergy has been increased with increasing depth. At a constant depth, wasted exergy has been also increased with increasing temperature. It can be said that with increasing temperature, parameters such as mass, heat and friction have been increased and with increasing them, the amounts of wasted exergy has been also increased. Alexander KaragÜzel et al. (2012) studied on peas and beans in a fluid bed dryer and reported the same results .(Karagüzel *et al.*, 2012)

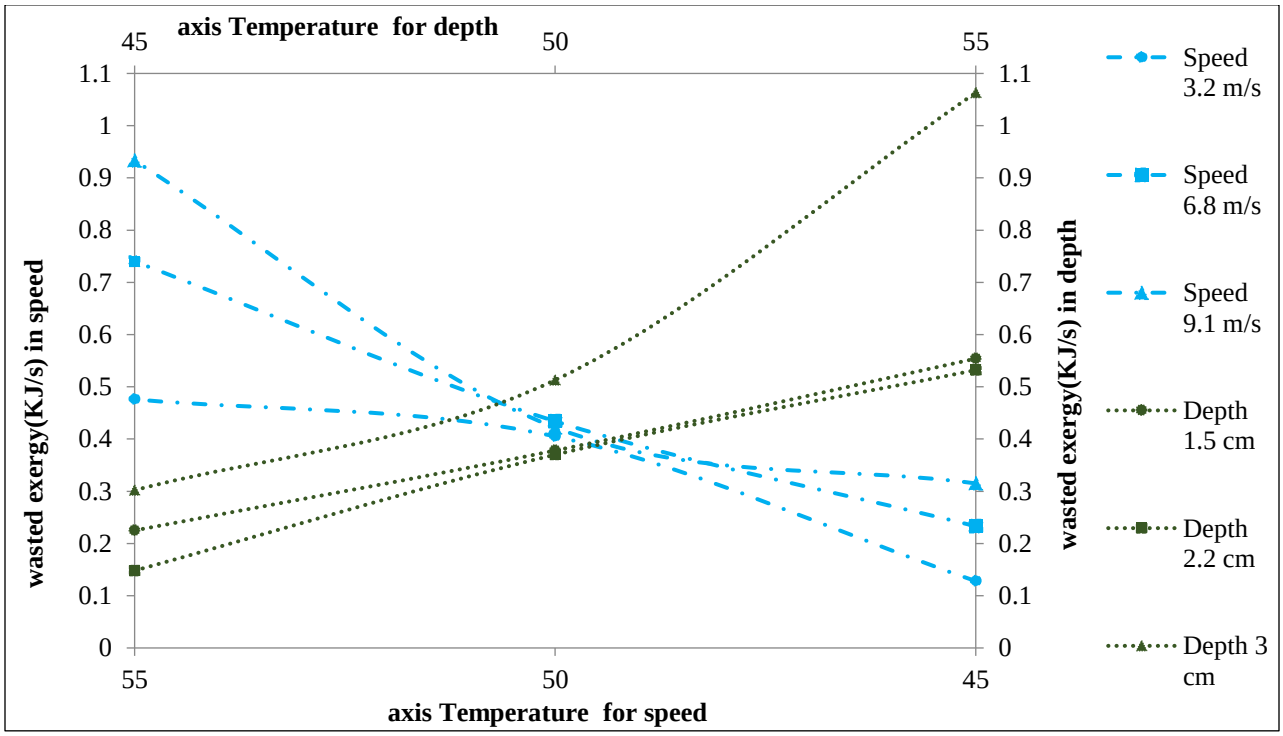

Figure 4 . Effects of depth and speed in different temperature of dryer on wasted exergy

3.2.2. Interaction of Air Velocity and Temperature

Figure 4 shows the effect of velocity at various temperatures on wasted exergy. The maximum and minimum wasted exergy are 0.933 and 0.128 kJ/s at temperatures of 55 and 45 ° C and velocities of 9.1 and 3.2 m/s, respectively. According to Figure 4, with increasing the velocity and temperature, the wasted exergy has been increased. The temperature of the top air at the inlet of dryer and this exergy increases evaporation of moisture and exergy consumption and thus wasted exergy is increased. This is in accordance with the results of Akpinar. (2004) .(Akpinar, 2004)

3.2.3. Interaction of Air Velocity and Depth

Figure 5 shows the effect of velocity at various depths on wasted exergy. The maximum and minimum wasted exergy are 0.8318 KJ/s and 0.25927 KJ/s in depths of 3 and 1.5 cm, and velocity of 9.1 and 3.2 m/s, respectively. Wasted exergy was increased with increasing depth and velocity. The reason is that with increasing depth, more work is needed for drying samples and more consumption of exergy is required to supply the needed work, therefore waste of exergy is increased. As mentioned with increasing air velocity of dryer, inlet flow rate is increases and because flow rate has a direct relationship with exergy, so by increasing its amount, wasted exergy was also increased. And this is in accordance with the results of Akpinar et al. (2005) about drying potatoes slices in the cyclone type dryer (Akpinar *et al.*, 2005).

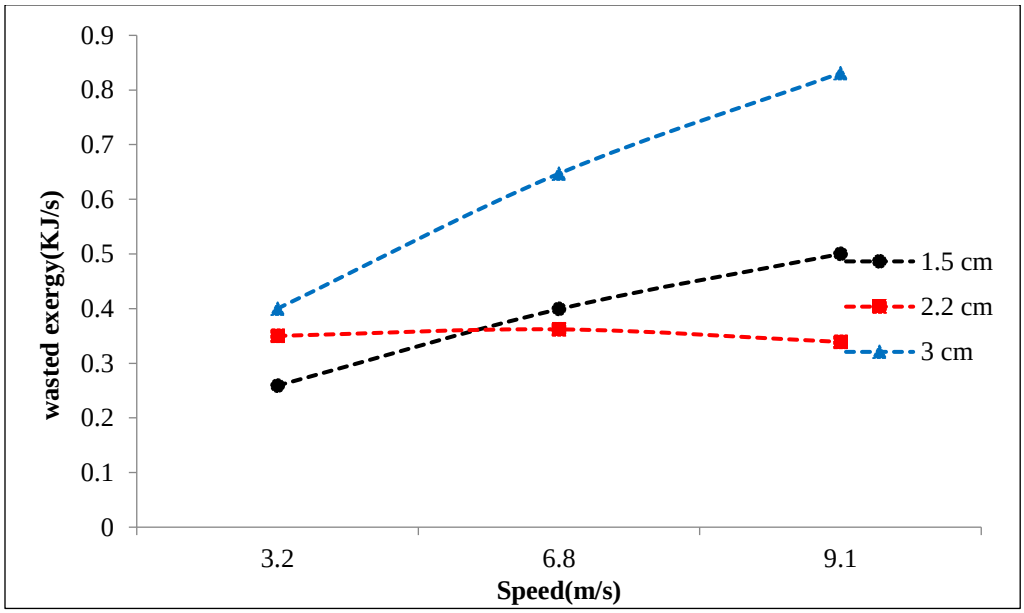

Figure 5 . Effects of depth in different speed of dryer on wasted exergy

4. Conclusions

In the present study, the effect of three parameters of temperature, velocity and depth of the bed of samples on consumed energy and wasted exergy were studied. The results showed that with increasing temperature and velocity and depth of the bed of samples, consumed energy is increased.

The lowest energy consumption was obtained at a depth of 1.5 cm, a velocity of 3.2 m/s and a temperature of 45 ° C, and the maximum energy consumption was obtained at a depth of 3, a velocity of 9.1 m/s and the temperature of 55 °C.

In the case of wasted exergy, it was determined that with increasing temperature and velocity and depth of the bed of samples, wasted exergy is increased.

The minimum wasted exergy was at a depth of 1.5 cm, a velocity of 3.2 m/s and a temperature of 45 ° C and the maximum exergy was a depth of 3, a velocity of 9.1 m/s and the temperature of 55 °C.

According to the results obtained from testing the parameters of velocity, temperature and depth as well as the interaction of these three factors, these factors effect on energy consumption at level

of 1%.

According to the results of experiments, the lowest consumed energy and wasted exergy are at a depth of 1.5 cm, the velocity of 3.2 m/s and a temperature of 45 °C.

Thus, a lower drying temperature and slower velocity for drying the potato by fluid bed method are recommended.

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EFFECTS OF OLIVE LEAF EXTRACT ON THE OXIDATION STABILITY AND MICROBIOLOGICAL QUALITY OF DRY FERMENTED SAUSAGE (SUCUK)

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ABSTRACT

The effects of olive leaf extract (OLE; 0, 125, 250 and 500 ppm) on the physical, chemical and microbiological properties (pH, free fatty acids, thiobarbituric acid, colour, total aerobic mesophilic bacteria, lactic acid bacteria, *Enterobacteriaceae*, mould and yeast) of Turkish dry fermented sausage were determined. Sausage production was carried out using beef, beef fat, sheep tail fat, garlic and various spices. Sausages that had been ripened were packed in vacuum and stored at 4ºC for 60 days. The analyses were carried out periodically during ripening (0, 6 and 12th days) and storage (30 and 60th days). OLE reduced the level of free fatty acid and thiobarbituric acid at the last days of ripening and during the storage. Moreover, OLE reduced count of lactic acid bacteria and total aerobic mesophilic bacteria during the storage. The addition of OLE was not cause significant differences on the pH and colour values of sausage.

1. Introduction

Sucuk, a traditional fermented dry product, is one of the popular meat products in Turkey. Sucuk and similar products are manufactured in many countries like especially Middle East and Europe (Bozkurt and Erkmen, 2002). Sucuk, is known as a dried fermented meat product, is usually produce using beef, beef fat, sheap tail fat, salt, garlic, sugar, nitrite, nitrate and various spices (Aksu and Kaya, 2004, Gökalp et al., 1999, Kılıç, 2009). Many biochemical reactions such as glycolysis, lipolysis and proteolysis occur during the fermantation and ripening of sausage. These reactions play an important role in the formation of aroma, taste, colour and sensory properties of the final product during the fermentation and ripening (Gökalp et al., 1998, Gökalp et al., 1999).

Meat and meat products are known to be highly susceptible to lipid oxidation and contamination of foodborne pathogenic microorganisms (Hayes et al., 2010). Synthetic preservatives are widely used to control of spoiling and food borne pathogen microorganisms in meat products. However, there are serious concern that synthetic preservatives have toxic effects (Alirezalu et al., 2016). Furthermore, it has been reported that extracts such as tyhme and sesame oil, green tea extract to have stronger antioxidant activity than synthetic antioxidants and they can be used to increase the quality properties of products such as sausage (Bozkurt, 2006, Bozkurt, 2007). In recent years, studies on antimicrobial plant extracts such as tyhme, sage, olive leaf extract gained speed due to

presence on the GRAS list (Erdohan and Turhan, 2012).

Olive leaf is a waste product which arises during the processes such as olive picking, gathering and oil processing. The olive leaf has many compounds, which are known to have many biological activities such as antioxidants, antimicrobials and antifungals. (Harp, 2011). The OLE which has a dark brown and bitter taste obtained from leaves of olive tree (*Olea europaea* L., Oleaceae), is quite rich in terms of components called olive biophenols. The most important bioactive component of OLE is oleuropein (Sudjana et al., 2009). Other biophenols, such as hydroxytyrosol, thiazole, caffeic acid, routine, vanillin, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside are also presence other than oleuropein (Lee and Lee, 2010). Oleuropein is believed to be responsible for the curative effects of the OLE extract (Salem et al., 2015). Studies have reported that OLE reduces blood pressure in animals, regulates cardiac arrhythmia and prevents intestinal spasms (Benavente-Garcia et al., 2000, Pereira et al., 2007).

There are some studies (Hayes et al., 2010, Gök and Bor, 2012, Ahmed et al., 2014, Khiddir, 2015) in the literature about the use of OLE in meat products. Hayes et al. (2010) reported that use of OLE in beef meatball limited lipid oxidation and total number of viable microorganisms. The effect of some plant extract on shelf life and quality characteristics of meatball during storage were investigated by Gök and Bor (2012) and they were stated that OLE had higher antioxidant activity than usage other plant extract. Ahmed et al. (2014) investigated effect of OLE on microbial load of shrimps and found that OLE could be used as antibacterial in shrimp production. Khiddir (2015) conducted a study to determine the antimicrobial and antioxidant properties of OLE on fresh fish and stated that OLE could be used to improve the shelf life and quality characteristics of meat and meat products. It is observed that OLE can be used as antimicrobial and antioxidant agent in meat and some meat products in consideration of literature. However, any study that used OLE in the production of Turkish fermented sausage was not found. In this study, the effects of OLE on the physical, chemical and microbiological properties of sausage were investigated during ripening and storage periods.

2. Materials and methods

A day after slaughtering, three-year-olds beef, beef fat and sheep tail fat were obtained from a local butcher (Adıyaman, Turkey). OLE which was produced as a commercial product was supplied from Nurbal Gıda San. Tic. Ltd. Şti. (Istanbul, Turkey).

2.1. Sausage preparation

The beef (71%), spices (3.6%), garlic (2%), salt (%1.9), sugar (0.5%), OLE (0, 125, 250 and 500 ppm) and sodium nitrite (150ppm) were mixed and minced in a cutter (Arı Makina, Turkey). Then beef fat (10.5%) and sheep tail fat (10.5%) were added to this mixture during cutting and mixing process. Each batches of sausage batter stuffed into collagen casings (Naturin Darm, Germany) of 40 mm diameter. A 10% potassium sorbate solution was sprayed on each sample. Samples were ripened for 12 days in a climatic cabin (Qualitec, Biosan, Konya). Ripening temperature was started from 23 °C and then decreased 1°C for every day until 6th day. The relative humidity was 60±3% in the first 5 hours for equilibration. Then relative humudity was increased to 90±3% and decreased 1 unit for every day. At the end of the 12 days, samples packed with vacuum by using polyethylene bag and stored at refrigerated condition (4 °C) for 60 days

2.2. Determination of the pH

The sample was grinded with a blender and ten grams of sample was homogenized in 100 mL distilled water. Then pH was measured using a pH-meter (Hanna 2215, USA) (Ockerman, 1985).

2.3. Determination of thiobarbituric acid (TBA) and free fatty acid (FFA) values

TBA (mg malonaldehyde/kg sausage) was determined according to Tarladgis *et al.* (1960). FFA was determined according to the titration method as outlined Egan *et al.* (1981). 25 ml of chloroform and 10 g ground sample in the presence of anhydrous sodium sulphate was then mixed for 5 min, and then filtered with filter paper. The free fatty acids were titrated with 0.1 N NaOH. Free fatty acid was expressed as g oleic acid/100 g fat. Fat was determined as reported by AOAC (2000).

$$\text{Free fatty acid (\%)} = (S \times N \times F) \times 28.2 / W \quad (1)$$

where S is the volume of titration (ml), N is the normality of the sodium hydroxide solution, F is the factor of the sodium hydroxide solution and W is the fat weight (g) in the sample.

2.4. Microbiological analysis

A 15 g sample was aseptically taken from each sample and homogenised in 135 ml of sterile salt solution (0.85% NaCl; Merck). Violet Red Bile Glucose Agar (VRBGA; Merck) was used to Enterobacteriaceae incubated at 30 °C for 24 h. Total aerobic mesophilic bacteria was determined on Plate Count Agar (PCA; Merck) incubated at 37°C for 48 h (APHA, 1992), while Lactic acid bacteria (LAB) was determined on De Man Rogosa Sharpe Agar (MRS; OXOID) incubated at 30°C for 72 h. and the count of mould-yeast was determined on Dichloran Rose Bengal Chloramphenicol Agar (DRBCA; Merck) incubated at 25°C for 5 days.

2.5. Instrumental colour analysis

The colour values of the samples were measured by using a colorimeter (Minolta CR-400, Japan). Before each measurement, colorimeter was calibrated against a standardisation plate. The colour values were measured according to CIELAB systems as *L* (lightness), *a* (redness) and *b* (yellowness) values. The mean of seven measurements were taken for each *L*, *a* and *b* values.

2.6. Statistical analysis

The study was done in two replications. Two samples were produced for each treatment, and the measurements were repeated twice for each sample. The data were subjected to analysis of variance (ANOVA). The results were expressed as mean ± standard deviation (SD). The differences among the samples were compared using Duncan's multiple-range tests. A probability value of $p<0.05$ was considered significant.

3.Results and discussions

3.1. Effect of OLE on the pH of the sausage

The pH, which has a critical significance for these type products, has significant effects on the properties of the dry fermented sausage (Gökalp et al., 1999). The addition of OLE on the pH values of the sausage was not cause a significant difference. However, the effect of ripening and storage period on pH values was found to be significant. As summarized in Table 1, pH values decreased during ripening and storage periods. Lactic acid bacteria produce lactic acid by the breakdown carbohydrates in dry fermented sausages (Pérez-Alvarez et al., 1999). From the first day of ripening, it is seen that pH values decreases with the reason of lactic acid formation. The reduction in pH during the ripening period is very important in terms of the desired flavour, colour development and inhibition of unwanted microorganisms in the

dry fermented sausage (Bozkurt, 2006, Pehlivanoğlu et al., 2015).

Table 1. The effects of ripening and storage on pH values of the sausage.*

OLE (ppm)	Ripening (day)			Storage (day)	
	0	6	12	30	60
0	5.86±0.01 ^{A}	4.95±0.09 ^{B}	4.72±0.18 ^{BC}	4.70±0.08 ^{BC}	4.61±0.05 ^{C}
125	5.82±0.03 ^{A}	4.97±0.18 ^{B}	4.70±0.18 ^{B}	4.69±0.10 ^{B}	4.63±0.11 ^{B}
250	5.81±0.02 ^{A}	5.01±0.03 ^{B}	4.72±0.05 ^{C}	4.69±0.11 ^{C}	4.60±0.05 ^{C}
500	5.81±0.05 ^{A}	4.95±0.09 ^{B}	4.75±0.05 ^{C}	4.71±0.04 ^{CD}	4.57±0.03 ^{D}

OLE: Olive leaf extract

* The effects of OLE on the pH value of the sausage was not significant ($p>0.05$).

$^{A-D}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).

3.2. Effect of OLE on microbial quality of sausage

The effects of OLE on the counts of *Enterobacteriaceae* and yeast-mold were not found to be significant on the 0th day of ripening period. However, they were not detected on the 6th day and afterwards. Kurt and Zorba (2010) found *Enterobacteriaceae* and yeast-mold in dry fermented sausages only first days of ripening. It can be said that this situation related to *Enterobacteriaceae* is caused by a decrease in water activity and an increase in acidity. However, mould and yeast was affected by potassium sorbate applied to sausage casings. Kaban and Kaya (2009), Yalınkılıç et al. (2012) reported that *Enterobacteriacea* is sensitive to water activity and acidity. Similar results have been obtained in the study conducted by Kurt (2016) and this researcher stated that drying and increasing acidity in sausages had significant effects on *Enterobacteriaceae*.

The effects of OLE on the count of total aerobic mesophilic bacteria was not significant during ripening period, whereas it was found to be significant during storage period. The addition of 250 and 500 ppm OLE reduced the count of total aerobic mesophilic bacteria of samples on the 30th day of storage (Table 2). Moreover, 500 ppm OLE caused a significant difference in the count of total aerobic mesophilic bacteria of sausages on the 60th day of storage. The decrease in the count of total aerobic mesophilic bacteria can be related to the antimicrobial properties of phenolic compounds present in the olive leaf extract (Basmacıoğlu-Malayoğlu and Aktaş, 2011). Gök and Bor (2012) reported that addition of OLE to meatballs reduced the count of total aerobic mesophilic bacteria. Ahmed et al. (2014) reported that the number of total aerobic mesophilic bacteria in shrimps treated with OLE solutions at different concentrations decreased with increasing OLE concentration. Our results are similar to other studies (Aksu and Kaya, 2004, Gök et al., 2011) in the literature which vegetable additives are used in fermented sausage production. Gök et al. (2011) investigated the effects of some herbal antioxidants such as rosemary and rosemary extract on the quality characteristics of dry fermented sausage and they stated that these natural additives reduced the count of total aerobic mesophilic bacteria of sausage. Another study (Aksu and Kaya, 2004) stated that usage *urtica dioica* in dry fermented sausage caused a decrease in the number of total aerobic mesophilic bacteria.

The effects of ripening and storage periods on the number of total aerobic mesophilic bacteria were found to be significant (Table 2). The number of total aerobic mesophilic

bacteria increased during ripening in all samples, however decreased during storage period. Some researchers (Bozkurt and Erkmen, 2002, Gök et al., 2011) reported that the number of total aerobic mesophilic bacteria in the dry fermented sausage increased on the first days of ripening and decreased during the last days of ripening period. Fernández-López et al. (2008) reported that the number of total aerobic mesophilic bacteria in Spanish-type dry fermented sausage increased during the ripening. Kurt (2016) reported that the count of total aerobic mesophilic bacteria of sausages increased during ripening and decreased during storage. This researcher stated that fermentation conditions are effective in the increase in the number of total aerobic mesophilic bacteria in the ripening period. On the other hand, the decrease in the count of total aerobic mesophilic bacteria during the storage period has been associated with vacuum packaging and refrigerated storage.

Table 2. The effects of OLE on the count of total aerobic mesophilic bacteria (TAMB) and lactic acid bacteria (LAB) during ripening and storage stage.*

OLE (ppm)	Ripening (day)			Storage (day)	
	0	6	12	30	60
TAMB (log cfu/g)					
0	3.84±0.06 ^{D}	7.89±0.09 ^{C}	8.12±0.01 ^{A}	8.07±0.00 ^{aAB}	7.96±0.01 ^{aBC}
125	3.83±0.01 ^{B}	7.84±0.17 ^{A}	8.00±0.01 ^{A}	8.03±0.04 ^{aA}	7.97±0.03 ^{aA}
250	3.81±0.08 ^{B}	7.75±0.21 ^{A}	7.99±0.01 ^{A}	7.95±0.01 ^{abA}	7.90±0.04 ^{aA}
500	3.83±0.06 ^{B}	7.71±0.12 ^{A}	7.92±0.01 ^{A}	7.76±0.08 ^{bA}	7.69±0.08 ^{bA}
LAB (log cfu/g)					
0	3.42±0.05 ^{C}	8.38±0.08 ^{A}	8.40±0.05 ^{A}	8.34±0.05 ^{AB}	8.21±0.04^{bA}
125	3.43±0.10 ^{B}	8.27±0.01 ^{A}	8.34±0.05 ^{A}	8.28±0.08 ^{A}	8.22±0.07 ^{aA}
250	3.45±0.05 ^{C}	8.34±0.07 ^{A}	8.29±0.02 ^{A}	8.22±0.07 ^{AB}	8.11±0.03 ^{abB}
500	3.39±0.04 ^{C}	8.32±0.09 ^{A}	8.20±0.10 ^{AB}	8.10±0.12 ^{AB}	7.95±0.06 ^{bB}

* There is no significant difference between the groups of columns without lowercase letters.
$^{A-D}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).
$^{a-b}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).
OLE: Olive leaf extract

The effects of OLE on the lactic acid bacteria was not significant in the ripening period of sausage, however was found to be significant in the storage period. As shown in Table 2, 250 and 500 ppm OLE caused a significant reduction in the count of lactic acid bacteria on the 60th day of storage. The reduction in the number of lactic acid bacteria is thought to be related to the antimicrobial properties of the phenolic compounds present in OLE (Lee and Lee, 2010, Basmacıoğlu-Malayoğlu and Aktaş, 2011). Lactic acid bacteria causes dropping pH in the fermentation of sausage, furthermore, they have beneficial effects such as inhibition of some undesirable microorganisms, formation of taste and aroma (Çon and Gökalp, 1998, Yaman et al., 1998, Ordóñez et al., 1999, Dalmis, 2007, Kurt and Zorba, 2010). For this reason, the antimicrobial effect of OLE on the lactic acid bacteria is important during storage period instead of the ripening period. Our results agree with the results of the study by Kurt (2016). Kurt (2016) reported that grape seed flour reduced the number of lactic acid bacteria of dry fermented sausage during the storage period. This effect has been

associated with antimicrobial properties of grape flour.

The number of lactic acid bacteria increased in the ripening period and decreased during the storage period (Table 2). This change which has taken place during ripening and storage processes in the count of lactic acid bacteria was similar to the previous studies (Bozkurt and Erkmen, 2007, Kaban, 2013). Bozkurt and Erkmen (2007) stated that the number of lactic acid bacteria in fermented sausage production increased during the first ten days of ripening and decreased during the storage period. Kaban (2013) stated that lactic acid bacteria in sausages constitute predominant microflora during the fermentation.

3.3. Effects of OLE on the free fatty acid (FFA) and thiobarbituric acid (TBA) values of sausages

The effect of OLE on the FFA of sausages was found to be significant. As shown in Table 3, 500 ppm OLE caused a decrease in the FFA value of sausage on the 12th day of ripening and during storage. The reduction in FFA value can be explained by the inhibition of bacteria with lipolytic activity as a result of the antimicrobial properties of OLE (Harp, 2011). Gök (2006) stated that the use of rosemary extract in sausage production limits the formation of FFA and this effect may lead to the bacteriostatic effect of antioxidant components present in the rosemary extract on bacteria with lipolytic activity.

FFA values of the samples increased during the ripening and storage periods. Lorenzo and Franco (2012) stated that the FFA value of Spanish type sausage was increased during ripening by lipolytic activity. Coşkuner et al. (2010) reported that the FFA value of dry fermented sausages increased during the storage stage. Karslıoğlu et al., (2014) stated that FFA value increased during the ripening and storage period in turkey meat sausage. Lipids, one of the major components of fermented sausage production, are exposed to two reactions, including lipolysis and oxidation during the ripening period. These reactions, which occur in lipids, have a significant effect on the sensory properties of sausage (Lizaso et al., 1999). Lipids are degraded by lipolysis with endogenous and microbial lipases, and free fatty acids occur in fermented sausage production (Ordóñez et al., 1999, Dalmış, 2007, Karslıoğlu et al., 2014).

Another important indicator of changes in oxidation stability as well as FFA is the TBA values. The effect of OLE addition on the TBA values of sausage was found to be significant in ripening and storage periods (Table 3). 250 and 500 ppm OLE provided a significant reduction in the TBA value of sausages on the 12th day of ripening and during the storage period. The decrease in TBA value might be due to the antioxidant effect of OLE (Hayes et al., 2011). In a study (Hayes et al., 2010) in which OLE was added to beef meatball, OLE was reported to limit lipid oxidation. Gök and Bor (2012) investigated the effects of some plant extracts on the shelf life and quality characteristics of meatball and reported that OLE had a significant antioxidant activity. Alirezalu et al. (2016) stated that addition of OLE limits lipid oxidation in Frankurter type sausages and there is an important relationship between antioxidant activities and phenolic content of plant extracts.

TBA values generally show an increase during the ripening and storage in sausage type fermented meat products (Dalmış, 2007). As a matter of fact, the effect of ripening and storage periods on the TBA value of sausage was found to be significant. As shown in Table 3, TBA values increased during the ripening and on the 30^{th} day of storage. However, significant difference among TBA values was not found on the 30^{th} and 60^{th} days of storage. Previous studies (Kayaardı and Gök, 2003, Kurt, 2006, Kurt,

2016) reported an increase in TBA values during the ripening of sausage. Despite the increase in TBA in our study, the TBA content of samples during the ripening and storage was below the acceptable limit (0.7-1.0 mg/kg) (Ockerman, 1976).

Table 3. The effects of OLE on free fatty acid and thiobarbituric acid values of sausage during ripening and storage.*

OLE (ppm)	Ripening (day)			Storage (day)	
	0	6	12	30	60
FFA (% oleic acid)					
0	1.25±0.02 ^{D}	3.05±0.23 ^{C}	3.88±0.08 ^{aB}	4.09±0.02 ^{aAB}	4.30±0.04 ^{aA}
125	1.26±0.03 ^{C}	3.02±0.25 ^{B}	3.89±0.00 ^{aA}	4.06±0.02 ^{aA}	4.19±0.08 ^{aA}
250	1.26±0.01 ^{D}	3.01±0.25 ^{C}	3.80±0.09 ^{aB}	3.96±0.05 ^{aAB}	4.17±0.07 ^{aA}
500	1.28±0.04 ^{D}	2.98±0.21 ^{C}	3.56±0.05 ^{bB}	3.70±0.10 ^{bAB}	3.94±0.05 ^{bA}
TBA (mg molanaldehyde/kg)					
0	0.107±0.003 ^{C}	0.128±0.005 ^{B}	0.197±0.009 ^{aA}	0.212±0.009 ^{aA}	0.207±0.005 ^{aA}
125	0.109±0.000 ^{C}	0.122±0.003 ^{C}	0.190±0.002 ^{aB}	0.209±0.008 ^{aA}	0.207±0.011 ^{aA}
250	0.107±0.003 ^{B}	0.119±0.008 ^{B}	0.182±0.009 ^{abA}	0.192±0.001 ^{abA}	0.190±0.008 ^{abA}
500	0.104±0.006 ^{B}	0.111±0.008 ^{B}	0.170±0.001 ^{bA}	0.182±0.009 ^{bA}	0.178±0.003 ^{bA}

* There is no significant difference between the groups of columns without lowercase letters.
$^{A-D}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).
$^{a-b}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).
OLE: Olive leaf extract

3.4. The effect of OLE on colour values of sausage

It is known as the colour of the food is an important factor in the consumer's preference. The results of colour analysis show that the effects of OLE on *L, a, b* values of sausages were not significant. However, the effects of ripening and storage periods on the *L, a, b* values of the sausage were significant (Table 4). In all sample groups, the *L* (brightness) value decreased during the ripening period. The differences in *L* values of the samples containing 125 and 250 ppm OLE were not found to be significant at the storage, while the L values of samples containing 0 and 500 ppm in 30 day storage were increased. Bozkurt (2006) reported that the *L* values of sausages decreased during the ripening phase. It is stated that the decrease in the *L* value represents the dark colour formation (Bozkurt, 2006, Lorenzo and Franco, 2012). Some researchers (Dalmış, 2007) have stated that the brightness of meat products depends on proteins and some factors such as pH, moisture content. When the pH-value falls below the isoelectric point of proteins, the proteins become denatured and their light absorption properties change (Dalmış, 2007).

The effects of ripening and storage on *a* (redness) values were found to be significant in all sample groups, except for *a* value of sausage which added 500 ppm OLE. *a* value of sausages decreased during the ripening period. It was observed that the a value was increased in sausages which added 0, 125 and 250 ppm OLE during the first 30 days of storage and no significant change in *a* value was found in all sample groups after 30th day. Previous studies (Bozkurt, 2006, Bozkurt, 2007, Kargozari et al., 2014) reported to decrease the *a* value of sausage at

the end of ripening. Decrease in *a* value during ripening period may be related to the partial or complete denaturation of the myoglobin, oxymyoglobin and nitrosomyoglobin resulting from lactic acid formation (Lorenzo and Franco, 2012, Kargozari et al., 2014).

While the effect of ripening and storage stages on *b* value of sausage was found to be significant in all sample groups, but no significant change was observed during the storage period in b value of sausage which added 125 ppm OLE. A decrease in b values of all sample groups was observed during the ripening period. At the storage period, b value of sausages which added 0, 250 and 500 ppm OLE to sausages increased during the first 30 days, but no increase in the b value of these sausages was found on the following days. It is observed that b value decreases during the ripening period as reported in the literature (Gök, 2006, Karabacak and Bozkurt, 2008). Pérez-Alvarez et al. (1999) reported that the b value of Spanish-type fermented sausage decreased during the fermentation stage. The decrease in b value during the fermentation stage might be to the decrease in oxymyoglobin due to the consumption of oxygen by microorganisms.

Table 4. Effects of ripening and storage on the colour values of sausage.[*]

OLE (ppm)	Ripening (day)			Storage (day)	
	0	6	12	30	60
L					
0	29.18±0.47 ^{A}	24.85±0.49 ^{B}	23.04±0.62 ^{C}	24.35±0.09 ^{B}	24.35±0.09 ^{B}
125	29.87±0.03 ^{A}	24.88±1.22 ^{B}	23.29±1.38 ^{B}	24.13±0.14 ^{B}	23.92±0.45 ^{B}
250	29.60±0.52 ^{A}	25.25±0.44 ^{B}	23.84±1.00 ^{B}	24.29±0.29 ^{B}	24.43±0.49 ^{B}
500	29.44±0.21 ^{A}	25.19±0.21 ^{B}	23.60±1.02 ^{C}	24.19±0.01 ^{BC}	24.26±0.10 ^{BC}
a					
0	6.10±0.07 ^{A}	3.51±0.01 ^{C}	2.83±0.19 ^{D}	4.30±0.15 ^{B}	4.29±0.09 ^{B}
125	6.30±0.19 ^{A}	3.31±0.33 ^{C}	2.75±0.30 ^{D}	4.01±0.21 ^{B}	4.04±0.20 ^{B}
250	6.30±0.01 ^{A}	3.64±0.23 ^{B}	2.85±0.20 ^{C}	3.70±0.30 ^{B}	3.73±0.32 ^{B}
500	6.35±0.25 ^{A}	3.51±0.09 ^{B}	3.10±0.03 ^{B}	3.64±0.47 ^{B}	3.68±0.38 ^{B}
b					
0	6.19±0.16 ^{A}	3.47±0.40 ^{B}	2.70±0.08 ^{C}	3.87±0.12 ^{B}	3.90±0.26 ^{B}
125	6.76±0.24 ^{A}	3.34±0.62 ^{B}	3.17±0.00 ^{B}	3.81±0.17 ^{B}	3.80±0.14 ^{B}
250	6.44±0.26 ^{A}	3.88±0.09 ^{B}	3.17±0.48 ^{C}	3.89±0.07 ^{B}	3.86±0.19 ^{B}
500	6.49±0.22 ^{A}	3.78±0.02 ^{B}	3.09±0.45 ^{C}	3.84±0.16 ^{B}	3.93±0.18 ^{B}

[*] There is no significant difference between the groups of columns without lowercase letters.

$^{A-D}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).

$^{a-b}$ Different uppercase letters in a row show significant differences between the groups ($p<0.05$).

OLE: Olive leaf extract

4. Conclusions

Addition of olive leaf extract in dry fermented sausage production was effective in inhibiting the formation of free fatty acid and thiobarbituric acid during ripening and storage periods. In addition, OLE had the antimicrobial effects on sausages during storage period. The addition of OLE did not cause any significant

difference on the pH and color values of the sausage. The results show that OLE can be used as an alternative natural additive for dry fermented sausages, especially in terms of oxidative stability.

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EFFECT OF COMMERCIAL PROBIOTIC *LACTOBACILLUS* ON FATTY ACIDS PROFILE AND NUTRITIONAL VALUE OF *CYPRINUS CARPIO* IN IRAN

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ABSTRACT

The purpose of this study was to evaluate the effect of commercial probiotic *Lactobacillus* on fatty acids profile of common carp fish (*Cyprinus carpio*). Three treatments contain zero (control), 10^3 $CFUg^{-1}$ and 10^6 $CFUg^{-1}$ probiotic *Lactobacillus* which were grown. The fish fatty acid profile was measured by Murph method. The results showed that the use of commercial probiotic *Lactobacillus* causes to change fish fatty acid profile, so that the amount of saturated fatty acids significantly was decreased with feed probiotic. The highest unsaturated fatty acids (MUFA) were observed in treatment A which was higher than the control treatment. For unsaturated fatty acids level (HUFA), was no significant difference between the control and other treatments ($p< 0.05$), while for control treatment was significantly higher than fish feed with the probiotics. The ω-3 fatty acids content for diet contain probiotics was higher than the control treatment. The ω-6 fatty acids in experimental treatments compared to the control treatment were decreased but there was no significant difference.

1. Introduction

Human nutritional needs, especially the need for nutrients have led to the domestication of animals and humans have long been an increase in efforts to decentralize their education. The aquatic animals such as seafood had a significant contribution to the rapid growth in population growth slowed considerably in recent years (Ebrahimi, 2005). Good health depends on maintaining sustainable aquaculture and aquatic organisms and ideal conditions for maximum growth of farmed aquatic animals and also reduce the cost of completing the process. The researchers and breeders are constantly seeking new and better ways to achieve the goals are aquaculture. Probiotics were used to stimulate the growth of other organisms are (Fuller, 1992). In this century means widespread probiotics as live microorganisms which improve the properties of the natural micro flora via oral administration host and beneficial effects on consumer health leave a defined. Probiotics contain live microorganisms and specific products or product in sufficient number to flora through adoption or colonization on the part of the host body and thus exert beneficial effects on the health of their host (Scherezenmeir and Verse, 2001). Most probiotics are lactic acid bacteria to humans

and land and marine animals (Gatesoupe, 1999; Vine *et al.,* 2006; Kesarcodi- Watson *et al.*, 2008). More probiotics so far as factors biological control in aquaculture have been introduced same lactic acid bacteria, Pseudomonas, Bacillus (Verschuere *et al.*, 2000). In many resources as some of the lactic acid bacteria such as Lactobacillus positive effects on growth, survival and nutritional factors of seafood (Phianphak *et al.,* 1999; Abraham *et al.,* 2008). Add probiotics to the diet of fish, enhances the activity of digestive enzymes and stimulates appetite which fish nutritive value(Irianto and Austin, 2002), host intestinal microbial balance, the useful substances such as vitamins, minerals and certain enzymes and stimulate and increase the efficiency of the immune system, growth, development levels, (Gatesoupe, 1998; Kim and Austin., 2006; Merrifield *et al.,* 2009; Son *et al.,* 2009; Lim *et al.,* 2005) and also increase water quality and increased survival of the fishes (Panigrahi, 2007). Lipids play an important role in maintaining human health. The importance of fish in this regard, because it is the most important source of omega-3 fatty acids is very high. (Zibaee Nezhad *et al.*, 2008). The most important feature of aquaculture in terms of nutritional value, in addition to having a significant amount of protein, plenty of unsaturated fatty acids in their adipose tissue. The number of links varies between one and six ties and the higher chain length and number of double bonds, increases the nutritional value of products (Stansby, 1990). Human beings are capable of synthesizing fatty acids with more than 4 long-chain precursors of short-chain double bonds (Sargent et al., 1989), but docosahexaenoic acid and eicosapentaenoic acid in the human food sources are necessary.

Nutritional importance of fish in the world is known that among them we can reduce blood triglyceride, blood pressure and glucose metabolism changes noted (Aro *et al.,* 2005). Unsaturated fatty acids in preventing and reducing the risks of heart disease, increased growth of children, the combined structure of the brain, retina, testis and sperm, to facilitate the delivery and development of fetal neural are valuable (Hedayati fard *et al.,* 2000; Aggelousis *et al.,* 1991; Stansby *et al.,* 1990;). Oleic acid, linoleic acid, alpha-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic fatty acids are the most important in nutrition (Ackman, 1995). Now carp fish is one of the most important farmed species in Iran and the world. Because of the unique characteristics of this species in aquaculture industry is cultivated. Efforts to reduce feed conversion and growth as well as increased resistance to diseases of the most important research objectives associated with this species in the world (Alishahi *et al.,* 2011). Despite these studies, only a few studies have examined the effect of bacteria on fatty acids. Plante *et al.,* (2007) showed that changing microbial environment using probiotic bacteria Artherobacter sp. In addition Tapia- Paniagua and *et al.,* (2014) proved that a positive correlation between the level of probiotic bacteria Shewanella spp and fatty acids linolenic acid and linoleic acid in fish there Solea senegalensis. In another study conducted in 2010 by Garcia Delabanda et al., Solea senegalensis fed cod liver fatty acids and probiotics Pdp11 Pdp 13 compared to the control group showed no significant difference. The amount of fat and fatty acids in fish is not a fixed value (Zlatanos and Laskaridis., 2007). Koi fish due to the economical and tasty meat in most countries is of particular importance (Vosoughi and Mstjyr, 2005). Much research on the feeding of fish, particularly carp, common carp has been done, but optimization can lead to improved nutritional status and quality of this fish. The use of dietary supplements such as probiotics, probiotic and synbiotic to enhance growth and feed efficiency is one of the ideas put forward in this regard (Hosseinifar *et al.,* 2010). Various commercial companies to produce different categories of probiotic bacteria and yeast have even necessarily for any other farmed fish should be bred doses of a variety of practical tests. Aim of this study was to determine fatty

acids profile of common carp *(Cyprinus carpio)* was affected feed with commercial probiotic *Lactobacillus.*

2. Materials and Methods

This study was carried out in Behbahan Khatam Alanbia University of Technology at 2013 in Department of Fisheries, for period 56 days. 9 fiberglass 300-liter tanks were used. After sterilization, storage volume of 250 liters of water and the temperature of tanks were fixed. Physico-chemical parameters (temperature, dissolved oxygen, pH) were same in the tanks. Water physico-chemical conditions used in this study were: Temperature: 24±1; Dissolved oxygen: 7.5 milligrams per liter, pH: 7.5±0.3 and replacing the water daily with volume 30%. Photoperiod to 12 hours of light and 12 hours of darkness. 243 fishes with an average weight of 78.5 ± 39.53 grams by fiberglass tanks of carp fish farms. The number of fishes in each tank were 27 pieces. Fishes randomly to three treatments include: levels of 0 (control or C), 10^3 $CFUg^{-1}$ (A) and 10^6 $CFUg^{-1}$ (B) probiotic *Lactobacillus* were divided in three replications. During the period of adaptation, fishes were fed with control diet and then with experimental diets. Fish feed on a daily basis by 2% of body weight. In this study, diet in Kermanshah were produced cooperative so that its chemical composition is presented in Table 1.

Table 1. Analysis of the used diet

Composition	**(%)**
Moisture	7.42
Crude protein	36.19
Crude fat	17.61
Ash	7.27

In order to provide food diet contains commercial probiotic *Lactobacillus*, concentrations 0.5 and 1.5g / kg were carried out for treatments 10^6 $CFUg^{-1}$ and 10^3 $CFUg^{-1}$. Probiotic *Lactobacillus* used in this study, the company of America Primalac has been supplied 10^8 bacteria per gram of probiotic. A sufficient amount of probiotics were combined in distilled water, and then was added to the basal diet. Then the material was passed through a meat grinder with a diameter of 2 mm springs and metal strings and poured out onto the tray in the oven at 40 °C for 2 hr. (Oji fard *et al.,* 2000). The food pellets packed in bags were stored in the fridge. At the end of 56 days of training, in order to empty the digestive tract, fishes feed was stopped for 48 hours, from 6 farmed fish tanks was removed randomly. Fish peeled, isolated muscle tissue and fatty acid profiles and chemicals were stored in the freezer (Ng *et al.,* 2003).

2.1. Determination of fatty acid profile

For determination of fatty acid profile, frozen fish fillets were sent to the laboratory of animal nutrition in Tarbiat Modarres University. For analysis of fatty acids of sample, the method (Murph, 1993) was performed. The types and amounts of fatty acids by gas chromatography (GC unit, Unicam4600, USA) were determined. Equipped with sensors, flame ionization capillary column with dimensions of 30 meter in 0.22 micro meter and the helium was used as carrier gas. Column oven temperature and the temperature was scheduled to be set the first time 140 ° C for 5 minutes. Temperature of 160 to 180°C over a period of 9 minutes and reached 180 to 200 degrees Celsius and the temperature remained until the end. The time of recognition accuracy was 25 minutes. Detector temperature of 300°C, the temperature of 240 °C and pressure injection site was 70BPX column.

3. Results and discussion

The results of the effect of commercial probiotic *Lactobacillus* of on common carp fillet fatty acid profile were shown in Table 2.

According to Table 2, it can be seen that the amount of saturated fatty acids in fish fed with diets contain probiotics, found significant differences with treatment C ($p<0.05$). The highest percentage of saturated fatty acids in treatment C and the lowest in treatment A were observed. The amount of unsaturated fatty

acids with a double bond (MUFA) no significant difference between treatment C and the other treatments ($p< 0.05$), but the difference between treatment C significantly in fish feed by probiotics was lower.

Table 2. Fatty acid profiles of common carp fillets during was affected probiotic lactobacillus of trade business (%)

Fatty acids	Treatment C(Control)	Treatment A	Treatment B
C14:0	0.806±0.05 [a]	0.786±0.10 [a]	0.804±0.03 [a]
C16:0	16.84±0.2 [a]	16.31±0.4 [a]	17.05±0.5 [b]
C17:0	3.95±3.31 [a]	0.25±0.01 [b]	0.20±0.05 [c]
C18:0	2.34±2.17 [a]	4.83±0.20 [b]	4.63±0.32 [b]
C20:0	1.70±0.16 [b]	0.93±0.49 [a]	0.95±0.46 [a]
Total SFA	24.64±0.60 [b]	23.14±0.23 [b]	23.19±1.05 [b]
C16:1	3.33±2.75 [b]	5.76±0.31 [a]	5.95±0.22 [a]
C17:1	0.22±0.06 [a]	0.23±0.04 [b]	0.19±0.03 [c]
C18:1	25.89±21.88 [b]	47.91±1.40 [a]	47.61±3.12 [a]
C20:1	0.77±0.60 [c]	0.16±0.06 [a]	0.10±0.04 [b]
C22:1	0.37±0.04 [c]	0.38±0.03 [a]	0.34±0.07 [b]
Total MUFA	30.59±23.92 [b]	54.44±1.78 [a]	54.20±0.29 [a]
C18:2(n-6)	28.44±13.52 [b]	15.94±1.41 [a]	15.57±3.20 [a]
C18:3(n-3)	10.44±10.01 [b]	0.41±0.25 [a]	0.40±0.02 [a]
C18:3(n-3)	0.83±0.48 [b]	1.47±0.16 [a]	1.42±0.28 [a]
C20:2	0.18±0.06 [b]	0.23±0.00 [a]	0.25±0.03 [a]
C20:3(n-9)	0.32±0.20 [a]	0.21±0.01 [b]	0.25±0.05 [ab]
C20:3(n-3)	0.46±0.08 [a]	0.46±0.00 [a]	0.43±0.02 [a]
C20:4(n-6)ARA	1.25±0.10 [b]	1.13±0.08 [a]	1.13±0.04 [a]
C20:5(n-3)EPA	0.29±0.02 [a]	0.20±0.04 [b]	0.26±0.08 [c]
C22:5(n-6)	0.19±0.01 [a]	0.21±0.02 [b]	0.17±0.02 [c]
C22:5(n-3)	0.17±0.02 [a]	0.20±0.03 [b]	0.14±0.03 [c]
C22:6(n-3)DHA	2.06±0.17 [a]	1.82±0.25 [b]	1.94±0.25 [c]
Total PUFA	44.66±23.31[b]	22.32±2.02 [a]	22.01±3.84 [a]
ω3	3.82±0.27 [b]	4.17±0.49 [a]	4.21±0.61 [a]
ω6	34.52±23.56 [b]	17.94±1.55 [a]	17.47±3.29 [a]
PUFA/SFA	1.79±0.90 [b]	0.96±0.10 [a]	0.94±0.21 [a]
n3/n6	0.12±0.10 [b]	0.22±0.02 [a]	0.24±0.04 [a]
DHA/EPA	7.19±1.1[a]	9.04±0.76[b]	7.9±1.59 [c]

The same letters in each column showed no significant difference. ($P <0.05$)

For unsaturated fatty acids with more than one double bond (HUFA), there was no significant difference between the control treatment and the other treatments ($p< 0.05$), while the control treatment was significantly higher than in fish fed with probiotics which was greater. Obtained results showed, the percentage of omega-3 fatty acids found in fish fillets under different treatments did not show any significant differences, however, the highest in the treated samples B but treatment C was the lowest. There are no significant differences between Omega-6 fatty acids in treatments ($p< 0.05$). The lowest level found in

treatment B and the highest rate in the control treatment.

According to the results, there was no significant difference ($p<0.05$) for the ratio of polyunsaturated to saturated fatty acids and unsaturated fatty acids with together in treatments so that the highest found in the control treatment and the lowest found in the B treatment. The ratio between omega-3 fatty acids to omega-6 fatty acids was no significantly different between in control and experimental treatments, but the highest ratio in treatment B and the lowest rate was observed in the control treatment. The results of this study showed there was no significant differences between treatments for ratio between the fatty acids DHA/EPA ($p< 0.05$), however, the highest ratio found in treatment A and lowest were shown in the B treatment.

Food additives are one of the ways which provide necessary nutrients to support the growth and development of fish, which could increase the health, resistance. Thus, in recent years a lot of research has been carried out on the use of food additives that improve the health of fish and shrimp. Food additives such as probiotics are useful. The mechanism of probiotics action in the gastrointestinal tract to improve absorption of fish food, production of extracellular enzymes and vitamins.

Statistical analysis of fatty acid profile of common carp fillets at the end of the growing period showed the lowest level of saturated fatty acids (SFA) in treatments compared to the control treatment so that there was a significant difference. According to some studies, the main role of saturated fatty acids to increases the risk of cardiovascular diseases which only 3 fatty acids play this role which include myristic acid, lauric acid and palmitic acid (Mahan *et al.*, 2008) . In this study, treatments with probiotics reduced the amounts of myristic acid, palmitic fatty acid than the control group. Palmitic acid was the most abundant of saturated fatty acid in the experimental fish. Research has shown that all species of fish that have been studied, these saturated fatty acids, found the highest value among (Ghosh et al, 2007; Ghosh et al., 2004). Plante and colleagues (2006) showed changes using Artherobacter sp. weight and body fat of Haddock fish fry which has increased fatty acids. The results of this experiment showed fillet composition of fatty acids in carp diets by adding commercial probiotics dramatically were changed. According to a study Sargent (2002) and Millamena (1996) composition of fatty acids in the fish life is a function of the fatty acid in foods and the most important environmental factor affecting the composition of fatty acids in fish. Most of monounsaturated fatty acids (MUFA) were observed in group A was higher than the control group, which the difference was not significantly between treatments A and B.

The results of this study also showed that oleic acid, was the major fatty acid MUFA in treatments. The oleic acid (C18:1) in A treatment found significantly higher level than other treatments. Gutierrez and Silvia (1993) stated that the most abundant fatty acid in fish, found monounsaturated, oleic acid, which agreed with the obtained results. According to the results of this study, by adding commercial probiotics lactobacillus in common carp diet, the unsaturated fatty acids and polyunsaturated (PUFA) significantly were decreased. The highest amount of alpha-linolenic (C18:3(ω-3) found in treatment A fillet with 1.47±0.16 and the lowest amount found in control treatment with 0.83±0.48 respectively, so that, there was significant differences between treatments. The control group found lowest percentage of linoleic acid (C18: 2 (n-6), but statistically there was no significant difference between A and B treatments ($p< 0.05$). A survey that was conducted by Tapia-Paniagua et al., (2014) showed that a positive correlation between the level of probiotic bacteria and fatty acids of linolenic acid and linoleic acid in *Solea senegalensis* fish that contrasts with the results of this research. The reasons for this subject can be species of fish, type of probiotics, nutrition and also noted differences in climatic conditions for fish. In the present study, omega-3 fatty acids in diet contains probiotics was higher than the control treatment. The lowest

ω-6 fatty acids found for treatment B. Study of fatty acids, omega-3 to omega-6 ratios and PUFA / SFA is important for nutritional perspective. Pigott and Tucker (1990) stated that the ratio n3/n6 can be the best indicator to measure the nutritional value of fish oil. The ratio of omega 3 to omega-6 in the human diet by lowering plasma lipids prevent heart disease and reduce the risk of cancer (Kinsella *et al.,* 1990). The amount of omega-3 to omega-6 fatty acids ratio recommended by nutritionists more than 1: 4 (Valencia *et al.,* 2006). In this study, this ratio found 0.12±0.10 compared to the control treatment and were obtained in treatments A and B, respectively 0.22±0.02 and 0.24±0.04, which found significantly more than the control group. The minimum recommended amount of PUFA/SFA fatty acids is the 0.45 times (HMSO, 1994). In the present study, PUFA to SFA ratio in the control treatment was higher than other treatments but, in all three treatments was higher than the ratio of 0.45. Although the control treatment in ratio of PUFA / SFA fatty acids are far better than the treatments contain probiotics, but that does not mean that the fish no longer be denied treatment.

In general, the fishes polyunsaturated fatty acids such as omega-3, DHA and EPA have an important role in human health. The presence of these fatty acids in farmed fish tissue in human health is very important. In this study, the addition of commercial probiotic *Lactobacillus,* increased content of omega-3 fatty acids DHA, EPA and omega-6 fatty acids of carp body. The ratio of DHA / EPA in treatment B was higher than other treatments, but there was no significant difference between them.

4. Conclusions

It can be concluded that the commercial probiotic *Lactobacillus* improved the fatty acid profile of common carp fillet, which it can increase the nutritional value of fish. According to the results of this study 10^3 treated samples found a higher nutritional value compared to the other treatments.

5.References

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Innovative packaging system for extending the shelf life of food during storage

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Article in brief

Technical University of Cluj Napoca-North University Center of Baia Mare Romania is the consortium coordinator of the European Project entitled: Active GRAphene based FOOD packaging systems for a modern society (http://chimie-biologie.ubm.ro/grafood/), aiming to develop an innovative packaging system for the food storage in safety conditions.

The consortium is consisting in two universities (Technical University of Cluj Napoca-North University Center of Baia Mare Romania, University of Camerino Italy), an institute of research (National Institute of Chemistry Ljubljana Slovenia) and three companies (Ceprohart Braila Romania, Synbiotec Italy and Andaltec Spain). The GRAFOOD project will run until 2020.

This initiative seeks to introduce nanomaterials such as graphene in food packaging, in order to reduce the amount of wasted food by increasing the shelf life.

Researchers have proposed creating an active paper-based and polylactic acid (PLA) modified with probiotic-activated graphene oxide and with nano-Ag-TiO_2.

The initiative has a budget of 760 000 euros, funded by the European M-ERANET 2016 program, and the research team from Technical University of Cluj Napoca-North University Center of Baia Mare Romania will be responsible for preparation and characterization of the active compound, for developing and validating the active food packaging prototype and for the Romanian and European patent.

The goal is to transfer the benefits of nanomaterials, such as silver, titania, carbon, to the food industry.. These are materials with high functional properties, successfully applied in many areas, so, the consortium team will work together to bring their benefits to both consumers and the food industry.

The participation of Technical University of Cluj Napoca-North University Center of Baia Mare Romania in this European project is the second step in the European strategy undertaken for developing of innovative solutions for food packaging. First project entitled: Smart functions of packages containing nano-structured materials in food preservation (Smartpack) MNT-ERANET program aimed to obtain smart packaging systems based on nanostructured composites containing TiO_2 for food storage and was run in the period 2012-2015.