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CONTENT

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CHEMOMETRIC APPROACH BASED ON FATTY ACID COMPOSITION AND $\delta^{13}C$ ANALYSIS FOR VERIFICATION OF ORGANIC RAW MILK FROM COWS WITH DIFFERENT DIET

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ABSTRACT

The growing interest in organic farming, which involves a significant percentage of "green grass" and hay in the animal diet and increasing the output of milk labeled "100% grass-fed," induces an urgent necessity for authentication such value-added products. The aim of this study was to develop the integrated approach for discrimination of organic milk from farms with silage-haylage and grass-hay cow diet. The research was based on the characterization of fatty acid composition and carbon stable isotopes ratios (δ13C), and included data processing with chemometric approach.

It has been shown that absolute value ranges of most fatty acids didn't allow to discriminate the milk from different organic farms, although the difference in certain fatty acids content between separate seasons has been proven to be statistically significant. This integrated approach with application of the principal components analysis envisaged that the analyzed parameters were normalized by the maximum value and the data matrix consisted of not only absolute values of the fatty acids content, but also additional derivative parameters (the sum of cis-, trans-isomers etc.). The approach proved the significance of content values of C16:1 trans-9, C18:1 trans-11, C18:3 cis-9,12,15, C18:2 trans-9,12 and conjugates of linoleic acid for milk samples discrimination. Thus, the analysis of the total data set of absolute and derivative normalized parameters by the method of principal components analysis allows to distinguish completely the organic milk with different share of green grass and hay in cows diet both the stalling and pasture periods.

1.Introduction

Increasing of consumers' interest in organic products leads to the growth of their producing. According to the Organic Milk Suppliers Cooperative (www.omsco.co.uk) the world production of organic milk will amount to ≈$13 billion by the end of 2019. Also, the interest in the so-called "pasture dairy management", which involves cows grazing on pastures in summer and feeding by hay with minimum usage of concentrates in winter, is renewed.

The certification requirements for organic dairy farms are different. Thus, the requirements for feedstuff on organic farms range from simple recommendations for pasturing (Japanese Organic Standard) to the certain values of the roughage percentages in the feed dry matter – from 60% (EU regulations, "Bioland") to 90% ("Biosuisse", Switzerland). Meanwhile, in the United States, the world biggest organic products market (Organic Milk Market Report, 2017) a number of organic manufactures has

approved own animal grass-fed cattle standard under the auspices of the American Grassfed Association. It establishes such requirements for the production of dairy and meat raw materials, so that the labelling of "100 % grassfed" of the corresponding dairy and meat products is legitimate. This certification exists in parallel with the additional certification for organic producers – "Pennsylvania Certified Organic 100% Grassfed certification" (www.paorganic.org/grassfed).

Besides, in the United Kingdom market (www.arlafoods.co.uk) organic milk from cows with grazing for more than 200 days per year is labelled as "free range" and in the Netherlands market organic milk and dairy products labelled as "meadow milk" from cows grazing at least for 120 days per year and 6 hours per day are available (www.weidemelk.nl). Accordingly, the price of such type of milk is much higher than the price of both organic and conventional ones. Thereby it is necessary to develop the approaches for authentication of such dairy products.

Cattle diet is one of the decisive factors which affects physical, chemical and biochemical parameters of milk, especially the milk fat composition. It is known that the higher percentage of fresh green grass and hay in the cow's diet leads to higher concentration of polyunsaturated fatty acids, in particular ω-3-acids and conjugates of linoleic acid (CLA) (Średnicka-Tober, 2016; Adler, 2013; Butler, 2008). However, it is usually difficult to distinguish the feed base of milk origin and dairy management type by the absolute values of fatty acids content in milk. Therefore, the combination of methods has recently been used to solve this problem.

Thus, the processing of triglyceride database of milk fat allowed to distinguish organic milk from farms certified according to European standards (organic, biodynamic) from conventional milk without labelling. However, the effectiveness of this approach decreased from ≈90% to 72% when analysing the samples has taken in early spring and summer (Capuano, 2013). Also, it was noted the complexity of identification of organic milk, "pasture" milk and milk from farms with biodynamic type of management only by triglyceride composition (Capuano, 2013).

The principal components analysis (PCA) of milk fatty acid composition discriminated the samples from farms with different types of cattle diet ("fresh-cut green grass", "pasture", organic, biodynamic and conventional). Reliable differences were obtained in summer for conventional and "fresh-cut green grass" milk. However, the differences in winter were less reliable (Capuano, 2014). Also, it was possible to discriminate organic and biodynamic milk from conventional by fatty acid composition. However, it was impossible to distinguish organic milk from biodynamic one by fatty acid composition both in summer and winter (Capuano, 2014).

It has also been shown that comparing the data of fatty acid and triglyceride composition gives the possibility to distinguish retail organic milk, conventional milk and retail milk from farms with the grazing duration at least 120 days per year. At the same time, this approach has not established the significant difference between the last and conventional types (Capuano, 2015). Thus, the absolute values of fatty acids content in milk could indicate the proportion of fresh green grass and hay in the cows' diet, but only in certain periods of the year in the cases of significant differences in feeding.

It is also possible to determine the composition of cattle diet by the ratio of stable isotopes $^{13}C/^{12}C$ ($\delta^{13}C$) in milk. Grass and hay on the one hand, and corn silage and grains on the other hand, are different in the type of plant photosynthesis (C_3 and C_4 respectively) and, accordingly, in the carbon isotopes profiles, which affects $\delta^{13}C$ values in milk. There is a positive correlation between the $\delta^{13}C$ values and the percentage of corn silage in the cows' diet (Camin, 2008; Zhukova, 2017). As a result, the $\delta^{13}C$ values in milk, as well as features of fatty acid composition, could reflect the proportion of grass in the cows' diet.

In addition, the lipids of green grass are characterized by the high content of unsaturated

fatty acids, which are localized in cells chloroplasts, so their composition depends on the type of photosynthesis. At low seasonal temperatures the plants with C_3-type photosynthesis dominate on pasture, and 50 – 75% of all fatty acids of green grass are linolenic acid and CLA (Elgersma, 2006; Elgersma, 2003; Dhiman, 1999, Kochubey, 1996). At the same time, in plants with C_4-type photosynthesis, which are predominantly distributed in warm climate, linolenic acid is less than 40% of all fatty acids (White, 2001). Also, in the pastures C14:0 and C16:0 acids dominate in the grass at the end of summer, and the C18:3*cis*-9,12,15(ω-3) and CLA content decreases as compared to the spring period and the first weeks of summer (Loor, 2002).

Thus, the analysis of correlation of fatty acid composition with $\delta^{13}C$ values could be the effective method for the authentication of dairy products and milk obtained from cows with a high content of grass in the diet. At present, there is a shortage of similar studies. The available results showed that the content of C18:3*cis*-9,12,15(ω3), which minimal value in organic milk products was 0.50%, correlated with $\delta^{13}C$ values, which maximal value was –26.5‰ (Molkentin, 2013). It was also noted that such an approach should take into account the region, feed, climatic conditions etc. (Molkentin, 2009; Petrov, 2016).

The purposes of our study were: 1) analysis of the fatty acid composition and ratios of carbon stable isotopes $^{13}C/^{12}C$ in milk from organic farms with different volumes of green grass and hay in the cattle diet; 2) multivariate analysis of milk fatty acid composition and $\delta^{13}C$ values database; 3) development of the integrated approach to the authentication of milk from grass-fed farms throughout the year.

2. Materials and methods

2.1. Sampling

The organic milk samples were taken from 2 organic farms in Zhytomyr region with silage- and haylage-based cattle diet (n = 30) (O1) and from 2 organic farms in Chernihiv region with grass- and hay-based cattle diet (O2) certified according to Council Regulation (EC) №834/2007 and Commission Regulation (EC) №889/2008 (n = 24). The research was carried out during the indoor period (November – April) and outdoor period (May – October) in 2015 – 2017. The samples of milk were taken from a tank on farms, transported in plastic bottles with a screw cap at +4ºC. Data on cow's feeding diet and daily consumption of dry matter (kg/cow) were received from farmers.

2.2. Instrumental analysis

For the milk fat fraction extraction, the milk sample was heated to 35°C in a water bath ("RVO-400", «Ingos, s.r.o. Czech Republic), stirred and cooled to 20±2°C. 100 ml of milk were mixed in a separating funnel, then were added 80 ml of ethanol, 20 ml of ammonium aqueous solution (14.0 mol/dm^3), 100 ml of diethyl ether, mixed vigorously for 1 min, then was added 100 ml of petroleum ether, gently mixed, then the liquid fraction was poured. Then was added 100 ml of 10% solution of sodium sulfate to the liquid fraction, stirred, filtered through a filter paper and evaporated on a rotary evaporator ("RVO-400", «Ingos, s.r.o. Czech Republic). This method was applied according to ISO 14156:2005.

The mixture of methyl esters of fatty acids were prepared by methanolysis of glycerides. 0.1 g of fat was transferred to a test tube, 5.0 ml of hexane was added, mixed thoroughly. 0.2 ml of sodium methylate solution (2 mol / dm 3) was added to the test tube, carefully stirred for 2 min, and filtered through a paper filter with added $Na_2HSO_4 \times H_2O$. This method was applied according to ISO 15884/IDF 182:2008

The fatty acid composition was analysed using the gas chromatograph Crystallux ("Analityka", Ukraine) with the flame ionization detector, SP 2556 column (Supelco, USA), 100 m × 0.25 mm I.D., 0.20 μm film layer, with the help of "Analityka" software (SPC "Analityka", Ukraine) for system monitoring and data processing. The mixture of 37 methyl esters of fatty acids FAME ("Sigma-Aldrich", USA) was used as the standard.

Parameters of the measurement: the initial temperature of the column was 60°C; isothermal period - 15 minutes; temperature rise to 186°C with rate of 10°C/min; isothermal period - 20 min; the temperature rise to the final column temperature - 220°C, with rate of 5 °C/min. The total analysis time was 120 minutes. The temperature of the detector was 260°C, the temperature of the vaporizer was 250°C. This method was applied according to ISO 15885/IDF 184:2008.

The ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) isotopes was analysed in milk fatty phase using mass spectrometer "MI-1201SG", ("Electron", Ukraine) at the Isotope Geochemistry Lab of the Institute of Environmental Geochemistry. The measurements were made by using the international standard polyethylene foil (PEF-1) and transferred to the international standard Vienna Pee Dee Belemnite (VPDB) according to Gerstenberg and Herrman, 1983. The isotopes ratio is given in ‰ by δ scale and calculated by equation (1):

$$\delta C = \frac{R_1 - R_2}{R_2} \cdot 1000‰ \text{ , (1)}$$

where C – Carbon, R_1 – $^{13}C/^{12}C$ ratio in test sample, R_2 – $^{13}C/^{12}C$ ratio in internal standard PEF-1.

2.3. Statistical analysis

The data statistical processing was carried out using the univariate dispersion analysis in MS Excel 2010. The multivariate data analysis was performed using PCA in PAST software (Hammer et al., 2001) at the Analytical Chemistry Department of the National University of Pharmacy.

3.Results and discussions

3.1. Analysis of cattle diet

Organic dairy farms regulate the composition of cattle diet according to the herd size, the possibilities of fodder procurement, the planned milk productivity and other factors. The analysis of livestock feeding diets has shown the differences depending on farming type (Table 1).

Table 1. Cows' diets on organic farms

Parameter	Organic farms with silage-haylage diet (O1)		Organic farms with grass-hay diet (O2)	
	Outdoor period	Indoor period	Outdoor period	Indoor period
Milk productivity of cows, kg/day*	22.0-23.0	22.0-23.0	11.0-12.0	8.0-9.0
Feed dry matter intake (DMI) per day, kg/cow*	18.1-24.0	20.3-25.4	8.0-9.0	13.5-14.0
Diet composition, % DMI*				
Green fodder (grass on pasture, fresh-cut grass)	11.9-20.5	-	88.0-93.0	-
Roughage (hay, straw)	14.3-23.0	8.5-23.7	-	21.8-24.3
Juicy feed, % DMI*				
- haylage	23.2-33.4	34.4-43.1	-	55.0-63.2
- corn silage	16.4-22.8	10.6-16.0	-	-
Concentrated feed (grains of cereals and beans; sunflower cake)	30.3-34.2	31.3-32.5	7.0-10.0	15.0-18.0

* data is presented in the form of range of values obtained during 2015 – 2017.

Thus, during the outdoor period, the diet on O1 farms with silage and haylage ration and herd size near 400 heads, was characterized by a greater feed variety. In structure of feed dry matter intake (DMI) a significant part was occupied by haylage, silage and hay, that meets the requirements of organic certification standard – not less than 60 % of DMI.

At the same time, the concentrated feed accounts for about one-third of DMI. On O2 farms with the grass and hay diet of cows and with the herd size of ≈115 heads the consumption of DMI comprises more than 90% of fresh grass of annual and perennial plants and concentrated fodder without corn silage addition.

During the indoor period on O1 farms the feed structure differed from the outdoor period by increasing the proportion of haylage and reducing the proportion of roughage. At the same time the values of other groups of feed were without significant differences. Rough, juicy and concentrated feeds without corn silage were used on O2 farms during the indoor period.

In addition, local climatic peculiarities of the farms affect diet forming and herd size. The farms with silage and haylage feeding type are located in Zhytomyr region, where the average temperature during the outdoor period is 21.7°C with the absolute maximum of 36.2°C, however, the rainfall is minor – 74.6 mm. The farms with grass and hay feeding type were located in Chernihiv region, where the average temperature during the outdoor period equals 22.3°C with the absolute maximum of 41.1°C, the rainfall is 66.3 mm. For comparison, in Switzerland and Germany during grazing period the average temperature is 25.3°C and 20.9°C respectively with the absolute maximum of 37.8°C and 36.0°C, however, rainfall is 183.2 mm and 86.2 mm, that causes the greater possibilities for cows feeding with fresh grass.

3.2. Fatty Acid Analysis

The analysis of fatty acids composition of milk from the farms during outdoor period has shown that absolute values of individual short chain fatty acids varied slightly (Table 2). However, the average annual content of C4:0 – C12:0 in milk from O1 farms was higher by 17.3% than in milk from O2 farms, C14:0 and C16:0 content – by 17.9% and 21.5% respectively. The average annual content of C18:0, on the contrary, was higher by 33.7% in milk from O2 farms than from O1 farms.

Variations between indoor and outdoor periods for milk from O1 and O2 farms within each farm were 1.9% and 5.5% for C4:0 – C12:0, 5.0% and 1.8% for C14:0, 4.5% and 7.5% for C16:0, respectively. Variations in C18:0 content between indoor and outdoor periods were 2.9% and 11.7% for O1 and O2 milk respectively (Table 2).

More significant differences have been revealed between O1 and O2 milk fatty acid composition. The content of C4:0 – C12:0 was higher by 21.1% and 14.8% for O1 milk in outdoor and indoor periods than for O2 milk; the C14:0 content was higher by 15.4% and 20.9% respectively, the C16:0 content was higher by 23.7% and 21.5% respectively than for milk from O2 farm (Table 2).

However, the C18:0 content in milk from O1 farms was lower by 24.5% and 43.4% respectively in outdoor and indoor periods than in milk from O2 farm.

Such differences can be explained by peculiar properties of fatty acids metabolism in ruminants. The C4:0 – C14:0 fatty acids are synthesized *de novo* in mammary gland, the synthesis of C16:0 occurs both *de novo* and from fatty acids of feed in the process of digestion in cows' small intestine, and the content of long chain acids depends on their content in feed (Mitani, 2016; Palmquist, 2006).

Table 2. Fatty acid composition of milk fat from cows with different diets, g/100g of fat

Fatty acid	Organic farms with silage-haylage diet (O1)						Organic farms with grass-hay diet (O2)						P-value
	Outdoor period		Indoor period		Average annual		Outdoor period		Indoor period		Average annual		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
C 4:0	4.000	0.228	3.947	0.419	3.961	0.321	3.779	0.401	4.123	0.398	3.988	0.426	>0.05
C 6:0	2.508	0.148	2.410	0.215	2.465	0.184	1.972	0.287	2.237	0.254	2.134	0.293	<0.00001
C 8:0	1.480	0.104	1.446	0.171	1.456	0.137	1.099	0.207	1.257	0.208	1.195	0.218	<0.00001
C 10:0	3.219	0.262	3.142	0.209	3.165	0.245	2.338	0.570	2.266	0.421	2.294	0.474	<0.00001
C 10:1	0.312	0.053	0.332	0.051	0.316	0.052	0.260	0.061	0.253	0.054	0.256	0.056	<0.001
C 11:0	0.057	0.029	0.048	0.027	0.053	0.028	0.038	0.024	0.035	0.011	0.037	0.017	<0.05
C 12: 0	3.542	0.303	3.521	0.198	3.521	0.260	2.451	0.535	2.443	0.454	2.446	0.475	<0.00001
C 13:0	0.189	0.038	0.199	0.020	0.194	0.032	0.218	0.039	0.245	0.051	0.234	0.047	<0.001
C 14:0	10.477	0.867	11.00	0.615	10.68	0.794	8.868	1.671	8.703	1.488	8.768	1.527	<0.00001
Σ *trans* C14:1	0.583	0.057	0.622	0.036	0.602	0.053	1.121	0.245	0.927	0.271	1.003	0.273	<0.00001
C 14:1 *cis*	0.985	0.095	0.932	0.057	0.959	0.086	0.819	0.300	0.845	0.169	0.835	0.223	<0.01
C 15:0	1.062	0.087	1.193	0.060	1.120	0.101	1.280	0.184	1.097	0.245	1.169	0.237	>0.05
C 15:1	0.366	0.046	0.397	0.036	0.379	0.044	0.448	0.054	0.436	0.105	0.441	0.087	<0.01
C 16:0	28.059	1.166	29.31	1.656	28.51	1.473	21.40	3.586	23.00	2.799	22.381	3.153	<0.00001
Σ *trans* C16:1	0.377	0.113	0.336	0.055	0.357	0.084	0.069	0.024	0.067	0.030	0.046	0.027	<0.00001
Σ *cis* C16:1	2.178	0.343	1.824	0.729	2.001	0.536	2.025	0.750	2.010	0.392	2.018	0.571	<0.01
C 17:0	0.697		0.740	0.054	0.716	0.081	0.779	0.102	0.743	0.236	0.757	0.193	>0.05
C 17:1 *cis*	0.240	0.093	0.233	0.021	0.237	0.019	0.224	0.076	0.254	0.054	0.242	0.064	>0.05
C 18:0	9.807	0.018	9.515	1.178	9.783	1.163	12.21	2.947	13.64	2.278	13.082	2.595	<0.00001
C 18:1n (1-9) t	0.531	1.187	0.479	0.128	0.511	0.114	0.612	0.211	0.638	0.182	0.627	0.189	<0.01
C 18:1n (10-13) t	1.394	0.102	1.369	0.334	1.394	0.324	3.255	0.701	1.941	0.633	2.455	0.919	<0.00001
C 18:1n9 *cis*	21.791	0.326	20.89	1.209	21.45	1.227	22.70	2.934	25.71	3.387	24.532	3.488	<0.0001
C 18:2n6 *trans*	0.351	1.162	0.276	0.077	0.324	0.113	0.610	0.184	0.321	0.136	0.434	0.210	<0.05
C 18:2n6 *cis*	2.502	0.129	2.251	0.226	2.403	0.250	2.194	1.485	2.132	0.459	2.156	0.963	>0.05
C 18:3n6	0.208	0.225	0.203	0.018	0.207	0.028	0.241	0.048	0.380	0.148	0.326	0.137	<0.0001
C 18:3n3	0.715	0.034	0.782	0.142	0.745	0.155	1.468	0.236	1.354	0.343	1.398	0.305	<0.00001
C20:0	0.155	0.162	0.155	0.021	0.155	0.019	0.178	0.015	0.240	0.030	0.209	0.023	<0.00001
Σ CLA	0.387	0.011	0.400	0.050	0.394	0.044	1.871	0.523	0.822	0.372	1.347	0.448	<0.00001
C 20:1	0.270	0.038	0.280	0.045	0.275	0.051	0.551	0.056	0.553	0.095	0.552	0.076	<0.05

C 22:0	0.037	0.056	0.041	0.021	0.039	0.018	0.036	0.021	0.049	0.022	0.043	0.022	>0.05
C20:3n6	0.021	0.014	0.033	0.018	0.027	0.017	0.021	0.015	0.037	0.011	0.029	0.013	>0.05
C22:1n9	0.051	0.015	0.074	0.026	0.063	0.024	0.048	0.036	0.066	0.035	0.057	0.036	>0.05
C23:0	0019	0.022	0.024	0.012	0.022	0.012	0.056	0.031	0.054	0.029	0.055	0.030	>0.05
C20:4n6	0.032	0.011	0.038	0.017	0.035	0.013	0.074	0.036	0.088	0.044	0.081	0.040	<0.05
C20:5n3	0.018	0.009	0.025	0.011	0.022	0.011	0.072	0.056	0.059	0.032	0.066	0.044	>0.05
C22:6n3	0.041	0.011	0.032	0.012	0.037	0.017	0.061	0.032	0.056	0.026	0.059	0.029	>0.05
Σ SFA	65.307		66.70		65.85		56.71		60.14		58.799		<0.00001
Σ UFA	32.769		31.19		32.17		37.62		38.02		37.862		<0.00001
Σ MUFA	29.077		27.77		28.58		32.13		33.70		33.084		<0.00001
Σ PUFA	4.275		4.041		4.193		6.611		5.249		5.781		<0.01
Σ n/i*	1.924		2.108		1.971		5.669		1.835		3.338		
Σ omega-3	0.774		0.839		0.804		1.601		1.469		1.523		<0.00001
Σ omega -6	3.501		3.202		3.389		5.010		3.780		4.258		<0.001
Omega 3/6	0.221		0.262		0.237		0.320		0.388		0.358		<0.00001

Σ SFA - the sum of saturated fatty acid; Σ UFA - the sum of unsaturated fatty acid; Σ MUFA - the sum of monounsaturated fatty acid; Σ PUFA - the sum of polyunsaturated fatty acid; Σ n/i – the sum of non-identified peaks.

Table 3. Ratio of carbon stable isotopes in organic milk fat phase from cows with different diets

Parameter	Values of $\delta^{13}C$ in milk fat phase, ‰												P-value
	Outdoor period				Indoor period				Average annual				
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	
Milk fat from cows with silage-haylage diet (O1)	-24,53	-28,00	-22,30	1,76	-24,85	-26,70	-22,90	1,32	-24,70	-28,00	-22,30	1,57	<0,00001
Milk fat from cows with grass-hay diet (O2)	-31,58	-32,90	-29,80	1,08	-29,31	-29,80	-28,60	0,33	-30,20	-32,90	-28,60	1,33	

In our study significantly differences ($P < 0.00001$) of the C16:0 content reflected the share of green grass and hay in the cattle diet. The diet based only on fresh grass and hay feeding leads to the energy feed deficiency and increasing the role of fat reserve and decreasing *de novo* synthesis. Correspondingly, this may reflected in the decreasing of the C16:0 content (Roca Fernandez, 2012). Thus, the C16:0 content can be considered as a marker for determination of the share of green grass and hay in cows' diet.

Our studies have shown that the total PUFA content was higher by 30.8% and 15.4% in outdoor and indoor periods respectively in milk from O2 farms than in milk from O1 farm. In particular, the average annual content of ω3-fatty acids was higher by 89.4% and ω6-fatty acids was lower by 25.64% in milk from O2 farms. The average annual ratio of ω3- and ω6-fatty acids was higher by 51.0% in milk from O2 farms than in milk from O1 farms.

The intensity of transformation processes of long chain fatty acids in cow body directly depends on intensity of feed biohydrogenation in rumen and amount of long chain fatty acids, especially PUFA, in livestock feed (Jenkins, T. C., 1993).

In mammary gland stearic acid (C18:0), which is the final product of biohydrogenation of feed PUFA, is the substrate for C18:1*trans*-11 *de novo* synthesis and its following transformation to C18:2*cis*-9,12 and CLA with the help of Δ9-desaturase. From 64% to 97% of CLA content in milk fat comes from C18:1*trans*-acids (Roca Fernandez et al., 2012; Palmquist, 2006; Kemp & Lander, 1984; Kemp et al., 1984). Thus, the large share of feed such as fresh grass on pasture or fresh-cut grass with high level of PUFA leads to increasing of vaccenic acid (C18:1*trans*-11) and conjugates of linoleic acid content in milk fat (Palmquist, 2006).

The average content of ΣC18:1*trans*-acids was 1.6 times higher in O2 milk compared to O1 milk. The differences between O2 and O1 milk in outdoor and indoor periods were 2.0 times and 1.4 times, respectively.

According to our results the average annual CLA content in O2 milk was 3.4 times higher as compared to O1 milk. The difference in CLA content between O1 and O2 milk during the indoor period was equal to 2.0 times, while during the outdoor period – 4.8 times.

The annual content of C18:2*cis*-9,12 was lower by 10.3% in O2 milk than in O1 milk; during indoor and outdoor period – by 5.3% and 12.3% respectively.

However, despite the high statistically significant difference between individual fatty acids of milk from different farms, the analysis of the absolute values ranges of the most of fatty acids does not allow to identify the milk origin. For example, the annual values of CLA content ranged from 0.327 g / 100 g to 0.506 g / 100 g of fat in milk from O1 farms and from 0.478 g / 100 g to 2.523 g / 100 g of fat in milk from O2 farms. During the indoor period the characteristic range of CLA for milk from O1 farms was 0.329 – 0.506 g / 100 g of fat, and for milk from O2 farms is 0.478 – 1.535 g / 100 g of fat. The most significant variations were observed during the outdoor period: for O1 milk the values ranged from 0.327 g / 100 g to 0.441 g / 100 g of fat, and for O2 milk – from 1.035 g / 100 g of fat to 2.523 g / 100 g of fat. It is possible that the CLA content of more than 1.000 g / 100 g of fat is distinctive only for organic milk from cows with the grass-fed diet.

Overlapping of the annual values for ΣC14:1*trans* has been detected: for O1 milk it was 0.489–0.670 g / 100 g of fat, and for O2 milk it was 0.663 – 1.650 g / 100 g of fat. During indoor period the values were 0.557 – 0.670 g / 100 g for O1 and 0.663 – 1.631 g / 100 g of fat for O2. However, during outdoor period they are clearly distinguished – 0.489 – 0.670 g / 100 g of fat for O1 milk and 0.811 – 1.650 g / 100 g of fat for O2 milk.

Annual ranges of the total content of C16:1*trans*-acids were not overlapped: in O1 and O2 milk were ranged 0.157 – 0.585 g / 100 g and 0.017 – 0.112 g / 100 g of fat respectively. It is also possible to suggest a hypothesis that the total C16:1*trans*-acids

content, which is not higher than 0.134 g / 100 g of fat, may be characteristic only for organic milk from farms with the grass-fed diet.

The ranges of annual values of the C18:3*cis*-6,9,12 content were overlapped: for O1 milk was 0.163 – 0.268 g / 100 g of fat and O2 milk was 0.174 – 0.857 g / 100 g of fat. However, during the indoor period the possibility for samples discriminate ion was higher: the ranges were 0.174 – 0.231 g / 100 g for O1 and 0.195 – 0.857 g / 100 g of fat O2 milk.

The similar situation was observed for the C6:0 during the outdoor period. Its values ranged from 2.251 to 2.870 g / 100 g for O1 milk and from 1.365 to 2.393 g / 100 g of fat for O2 milk. Also, the C8:0 content ranged from 1.171 to 1.733 g / 100 g in O1 milk and from 0.693 to 1.461 g / 100 g of fat O2 milk during the outdoor period.

3.3. $\delta^{13}C$ analysis and chemometric data processing

For the complete discrimination of organic milk it was proposed the integrated approach based on the application of PCA to obtained multidimensional data of fatty acid composition and stable isotopes ratios $^{13}C/^{12}C$ ($\delta^{13}C$) in milk fat. Such an integrated chemometric approach has allowed to discriminate the milk obtained from organic farms with silage/haylage and grass/hay feeding diets (Figure 1).

The analysis of $\delta^{13}C$ values in the fatty phase of milk has revealed statistically significant differences between the samples from the farms of both types (Table 3).

PCA has been carried out in several stages and in different variants. Thus, Figure 1a shows the data distribution plot by the first two principal components when analysing only absolute values of fatty acid composition of the milk samples. Such an analysis didn't allow to reliably distinguish milk from O1 and O2 farms. It was shown that the largest contribution into the first two principal components (which explained ≈92% of data variations) was ensured by the content of palmitic (C16:0) (0.69 in the loadings of PC1), oleic (C18:1*cis*-9) (-0.44 in the loadings of PC1), stearic (C18:0) (-0.45 in the loadings of PC1) and myristic (C14:0) (0.26 in the loadings score of PC1) acids. It should be noted that the influence of these parameters could not be considered as characteristic. This is explained by positively correlation of their relatively high content in milk fat and contribution significance PC1 and PC2. Contingently, they can be attributed to macroparameters of fatty acid composition.

The introduction of absolute $\delta^{13}C$ values into the data matrix (Figure 1b) greatly improved the samples discrimination – the zones overlapping on the plot become minimal. The loadings analysis has shown that this parameter has the largest contribution to PC1 (0.43) and PC2 (0.64). Unlike the previous distribution the significance of C16:0 (-0.64 in the loadings of PC1), C18:0 (0.40 in the loadings of PC1) and C14:0 (-0.23 in the loadings of PC1) content decreased, but C18:1*cis*-9 content increased (0.37 in the loadings of PC1).

At the second stage the data of fatty acid composition and $\delta^{13}C$ values in fatty phase were normalized (by the maximum value for each parameter).

Such an approach allowed to minimize the main problem of PCA – the scale, i. e., to equalize the impact significance of macroindicators and minor components on the results of chemometric analysis. As a result of such actions the distribution picture has been improved, but not completely (Figure 1c). Also, the loadings analysis has shown another contribution of parameters to the principal components structure. While PC1 explained only ≈40% of data variations, PC2 and PC3 had the equal value of 10%.

It should be noted that using just this approach the significance of such acids as C16:1*trans*-9 (-0.42 in the loadings of PC1), C18:1*trans*-11 (0.28 in the loadings of PC1), C18:3*cis*-9,12,15 (0.30 in the loadings of PC1), C18:2*trans*-9,12 (0.20 in the loadings of PC1) and CLA (0.31 in the loadings of PC1) were established. These acids are characteristic for milk fat composition and especially for milk from cows with grass and hay diet (Elgersma, 2006). The effect of other UFA content on the

possibility of distinguishing the milk samples from different farms was also quite high. At the same time the significance of $\delta^{13}C$ influence decreased (0.14 in the loadings of PC1).

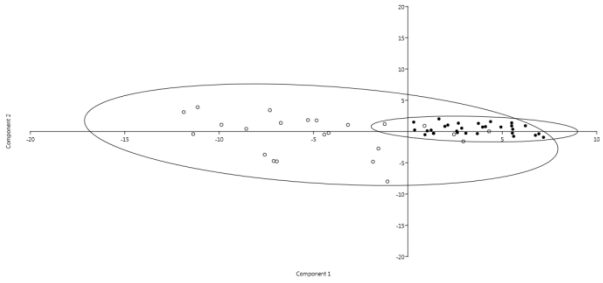

Figure 1a. PC1 and PC2, fatty acids composition, absolute values

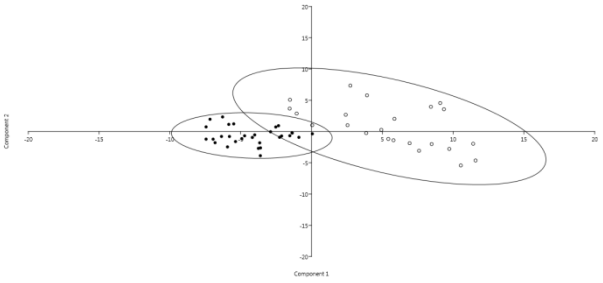

Figure 1b. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute values

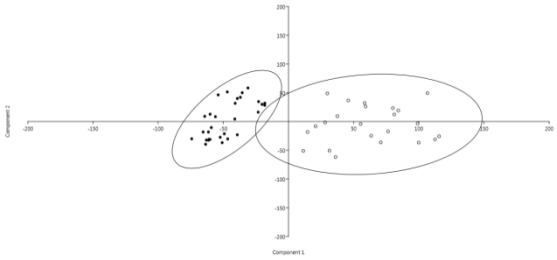

Figure 1c. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute normalized values

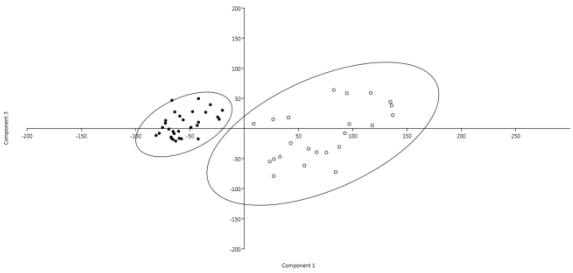

Figure 1d. PC1 and PC3, fatty acids composition and $\delta^{13}C$, absolute and derivative indicators, normalized values

Figure 1. The effect of the integrated approach on discrimination of organic milk from farms with different cows' diets by PCA: ○ – farms with silage-haylage diet; ● – farms with grass- hay diet.

Taking into account the results of chemometric analysis of normalized data we have introduced the additional derivative parameters describing the milk samples composition. Such parameters are the total *cis*-isomers and *trans*-isomers of UFA content, total UFA content, total MUFA content, total PUFA content, total dienes and trienes content, C18:0 content, total C18 UFA content, the ratio of ΣC18 UFA and Σ*trans*-isomers, the ratio of ΣC18 UFA and Σtrienes; the ratio of ΣC18 UFA and CLA content.

At the third stage the calculated data set of derived parameters has been added to the matrix of absolute values of fatty acid composition and $\delta^{13}C$. Then the matrix normalization has been done (in the same way as at the previous stage) followed by its processing by PCA. This analysis of such complex database completely divided two data groups on the plot (Figure 1d). While PC1 explains only ≈45% of variations, and PC2 and PC3 still have an equal value of 10%.

It should be noted that it is rather problematic to allocate the parameters with the greatest contribution to the principal components in the loadings matrix. The most of UFA have high contributions, however, the contribution of C16:1*trans*-9 (-0.35 in the loadings of PC1), vaccenic (C18:1*trans*-11) (0.25 in the loadings of PC1), linolenic (C18:3*cis*-9,12,15) (0.27 in the loadings of PC1), linolalidine (C18:2*trans*-9,12) (0.17 in the loadings of PC1) acids and CLA(0.28 in the loadings of PC1) remained maximal.

Among calculated derivative parameters the contribution into the structure of the first, second

and third components is maximal for such parameters as the total *trans*-isomers content (0.18 in the loadings of PC1), total trienes content (0.23 in the loadings of PC1), total unsaturated C18 UFA (0.11 in the loadings of PC1), the ratio of ΣC18 UFA/CLA content (-0.29 in the loadings of PC1) and far exceeds the contribution significance of $\delta^{13}C$ values (0.12 in the loadings of PC1).

4. Conclusions

The method of milk examination, including the organic one, to detect the presence of grass share in cows' diet has been proposed. This method is based on the integrated approach, which includes the analysis of fatty acid composition, the ratios of carbon stable isotopes in milk fat phase followed by the calculation of the set of derivative parameters, the normalization of the absolute values of these parameters (by the maximum value) and the processing of obtained data matrix by PCA. Among the calculated derivative parameters the contribution into the structure of the first, second and third components is maximal for such parameters as the total *trans*-isomers content, total trienes content, total unsaturated C18 UFA, the ratio of ΣC18 UFA and CLA content. The proposed approach has allowed to distinguish organic milk from farms with different percentage of green grass and hay in cattle diet during indoor and outdoor periods. It could be a potential tool for confirming organic milk authenticity, especially labelled as "grass fed".

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EFFECTIVENESS OF VACCUM IMPREGNATION TREATMENT AND VACCUM FRYING ON STRUCTURAL, NUTRITIONAL AND SENSORY PROPERTIES OF CALCIUM FORTIFIED POTATO CHIPS

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ABSTRACT

The effectiveness of VI technology on the mineral fortification, structural differences, nutritional and sensory properties of calcium fortified potato chips was evaluated. Furthermore, vacuum frying was also performed to achieve low oil absorption for potato chips. Vacuum pressure, blanching time, calcium concentration, were the significant variables affecting quality attributes and mineral fortification. Targeted 25% RDI for calcium fortification could be achieved at an optimized condition of 1.33 min (blanching time), 1.20% (calcium concentration), 40mm Hg (vacuum pressure), 12.01 min (restoration time). Restricted changes were recorded for hardness (455N), color ($\Delta E = 68.54 \pm 1.93$) and sensory score (8.1) compared to the control. Structural observation reflected that although there was a deposition of calcium on the cell wall periphery, but the granule appearance remained the same. Additionally, Vacuum fried calcium fortified potato chips have shown low oil uptake percentage. Thus, VI treatment and vacuum frying proved to be the most effective treatment in maintaining the overall quality of potato chips.

1.Introduction

Potato chips account 85% of total salty snack food market consumed worldwide. Potato chips are popular for their unique characteristics such as flavor, and crispy texture. Potato based snacks have experienced an increased rate of growth over the last 20 years because snacks have become part of the main meal as well as between-meal nourishments. These processed foods though taste very good and are very lucrative but pose various health problems by means of - under nutrition causing nutrition deficiency and over nutrition leading to obesity, hypertension, hyperlipidemia, diabetes, cancer, and so forth. Nowadays, under nutrition especially micronutrient deficiencies are very much relevant among (Black et al., 2008) school-aged children. Worldwide, an inadequate consumption of calcium over an extended period has been found to induce calcium deficiency risk among 3.5 billion people (Kumssa et al., 2014). About calcium, per se, the recommended intake for adolescents and adults is in the range of 1000-1300 mg per day (Food and Nutrition board, 2011). However, dietary calcium intake has been reported to be low in children (Bhatia, 2008) and adults (Harinarayan, 2007) in India. Low

calcium intake has been reported to be associated with osteoporosis, hypertension and many more disorders (Life extension update, 2010). Most of the people fulfill their calcium demand from dairy products. However, concerns like lactose intolerance, dietary fat, cholesterol and other related allergies, among some individuals, have led to switch their preferences from dairy to non-dairy products. Thus, the difference between recommended and actual calcium intake compel the manufacturers to market an increasing number and variety of calcium–fortified products (Konar et al., 2015).

Food fortification, among various innovative strategies, is one of the techniques that has been harmlessly and adequately used in order to counteract micronutrient deficiencies. Among the technologies used in the development of food fortification, Vacuum Impregnation (VI) has been identified as a new processing technique based on the process of diffusion (Tiwari and Thakur, 2016). It consists of two steps, at first most air and part or all of the native solution are extracted from the porous spaces of the food (vacuum) and second replaced by an external solution when vacuum-treated samples are dipped in fortificant solution for predefined time (impregnation) (Tiwari and Thakur, 2017). It has dual advantages, firstly, it will partially dehydrate the sample and secondly, it enables to introduce controlled quantities of solute into the food particles (Torreggiani and Bertolo, 2001). Concerning VI treatment for potatoes, reports like zinc enrichment of potato tuber (Erihemu et al., 2015); ascorbic acid enrichment of whole potato tuber (Hironika et al. 2011); and calcium and zinc fortification of potato chips (Joshi et al., 2016); calcium fortification of potato chips (Tiwari et al., 2018) has been found. In light of this observation, fortification of potato chips, because of their porous matrix (1%) and wide acceptability, with calcium will be one of the effective alternatives to overcome the problem of recommended and actual Calcium intake difference.

However, potato chips have also been reported to have an oil content ranging from 35 to 45g/100g (wet basis) and which is a major factor affecting consumer acceptance for oil–fried products nowadays (Dueik and Bouchon, 2011). Concerning various health negative impacts, fat content of potato chips is an important parameter to be controlled during processing. Consequently, there is a demand for healthier potato-based snacks that offers the same desired organoleptic characteristics of commercially available potato chips while lowering down the fat absorption. Therefore, Vacuum frying, as an option for fried potatoes with low oil content and desired texture and flavor characteristics (Garayo and Moreira, 2002) was also taken into consideration.

Thus, in the current study, authors attempted to fortify potato chips with calcium through VI by investigating the effects of vacuum pressure, blanching time, calcium concentration, and restoration time on calcium impregnation of potato chips. Furthermore, effect of VI and Vacuum frying on quality attributes *viz* structure (SEM observation), color, fat content, physicochemical properties and sensory analysis, were also examined concerning desired organoleptic properties of commercially available potato chips.

2. Materials and methods

2.1. Variety Selection

Kufri chipsona-1 variety of Potatoes fresh harvest (*Solanum tuberosum* L.) was chosen and procured from CPRI Regional Station Modipurum, Uttar Pradesh, India.

2.2. Fortificants and carrier

Food grade Calcium chloride was purchased from Titen Biotech Limited,

Delhi, India for fortification purpose. Potato chips were used as a carrier.

The standard RDI values (www.lenntech.com/recommended-daily-intake.htm 2015) of mineral were based on 2000 calorie intake (4 to 17 years of age). The research work has been planned in such a manner that the targeted level (25% RDI for calcium) of calcium for potato chips can be achieved by consuming 30g serving in accordance with Recommended Amount Customarily Consumed (RACC).

2.3. Experimental Design

Process optimization and product development was done using Response Surface Methodology (RSM). The combined effect of four independent variables i.e. calcium concentration, blanching time, vacuum pressure, restoration time coded as X_1, X_2, X_3, X_4, respectively on impregnation level of calcium in potato chips was evaluated through four-factor three level Box-Behnken design (Table 1). Optimization was done based on calcium content, hardness and overall acceptability in all experimental samples. A 27 runs (trials) were done thrice and the respective responses were observed (Table 2).

The response function was partitioned into three different components as - linear, quadratic, and interactive (Equation -1).
Where Y - response;

X_1, X_2 - input variables;

β_0 - intercept;

β_1 - linear coefficient;

β_{ii} - quadratic coefficients;

β_{ij}- interaction coefficients.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_1 + \sum_{i=1}^{k} \beta_{ii} X2 + \sum_{i>1}^{k} \beta_{ij} X_i X_j \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

2.4. Vacuum Impregnation Process

In a closed chamber, raw potato chips, after blanching at 72^0C for predefined time, were encircled on perforated base and vacuum was created for a predefined time. When the constant level of vacuum (15 mm Hg) was attained for a pre-defined period, raw chips were right away dipped to impregnation/fortificant solution of particular concentrations (1:4 (w/v)) for the predetermined restoration time as per RSM design (Table 2).

2.5. Vacuum Frying

The experiments were performed using a vacuum fryer available at ARAS lab, Ghaziabad, U.P. The fryer consists of a heating element. Inside the vessel, there was a basket and centrifuging system (de-oiling system) with a maximum rotational speed of 750rpm (63g units). In the vessel, with a vacuum capacity of 5.37kpa, vacuum was achieved by a dual seal vacuum pump (Model 1402 Welch Scientific Co., Skokie, IL). The frying process consists of loading 12 potato slices (about 35g) into the basked closing the lid and depressurizing the vessel. After the pressure in the vessel reached 5.37kpa at 120°C for 40 min., 20 min/ each hours, basket was sub-merged into the oil. After 6 minutes of frying, the basket was raised, and the centrifuging system was applied for 405 at maximum speed (750rpm). Then, the vessel was pressurized, and the potato chips were allowed to cool down at ambient temperature before storing them in polyethylene bags inside of desiccators for further examination.

2.6. Physicochemical analysis

2.6.1. Compositional Analysis

The following analysis were performed in triplicate: moisture content; ash; crude protein; carbohydrate content following standard methodology by Rangana (2007) and Larmond (1977).

2.6.2. Mineral content estimation

Calcium content of control, commercial, and experimental potato chips were estimated by Atomic Absorption Spectrophotometer (Shinadzu AA700) using wet ashing procedure as described by Raghuramulu et al. (2003)

2.6.3. Oil Content

The total lipid extraction and purification of oil content was estimated by standard methodology by Tarmizi and Niranjan (2010). This method consists of an initial extraction with a mixture of 1:2:0.8 (v/v/v) in chloroform, methanol and water. Then, this mixture was adjusted to 2:2:1.8 (v/v/v) to continue further extraction. In this way, the chloroform layer contains the purified oil. The oil content was expressed as kg oil/kg dry solid.

2.6.4. Colorimetric measurements

With the use of a Hunter color meter (Hunter Associates Lab Inc (Model No: LabScan XE, USA) fortified potato chips was measured for any change in color parameters (Hunter L, a, b). The color values were expressed as L ranged from L00 (black) to L0100 (white), -a (greenness) to + a (redness), and –b (blueness) to + b (yellowness). The instrument was calibrated before the experiments with a white ceramic plate (X = 79.31, Y = 84.11, Z = 88.94). The potato chips covering the entire surface area of 1 cm pore, were scanned at three different locations to determine the average L*, a*, and b* values during the experiment. In addition, the total color change (ΔE) Equation (2) was also calculated from the Hunter L*, a*, b* scale and used to describe the total color change during fortification of potato chips.

$$\Delta E=\sqrt{(\Delta L*)^2+(\Delta a*)^2+ (\Delta b*)^2} \quad \ldots\ldots\ldots\ldots (2)$$

where $\Delta L*$ = L*sample-L*standard; $\Delta a*$ = a*sample-a*standard; $\Delta b*$ = b*sample-b*standard.

2.7. Structural Changes through SEM

By examining the calcium distribution in potato chips and in turn effect on granule appearances through SEM (Scanning Electron microscopy - EVO 18, Zeiss model) fortified potato chips and control samples were evaluated for their structural differences, The sample size of 1x 1cm was placed on sputter coated with gold-palladium plating (of surface thickness 15 to 28nm) at 3 millibar vacuum and 15 million volt current for the time of 180 seconds. After time attainment, SEM observation were captured to evaluate the structural differences before and after fortification

2.8. Consumer Evaluation

The consumer sample population were selected from nearby areas of sec 125 Noida, UP, India, who were 18–55 years of age and of various socioeconomic back- grounds. Only those were selected to participate in the study, who consumed chips at least once in a month. A target of 100 participants were selected. Individual tables with written instructions and ballots were provided to consumers. Samples coded with random three-digit numbers and control potato chips were served. The consumers were asked to record the acceptability and intensity scores for overall impression, overall flavor, overall texture, overall color (9-point scale with 9 =like extremely and 1 = dislike extremely); hardness (9-point intensity scale with 9= extremely tough and 1 = extremely soft); and crunchiness (9-point intensity scale with 9 = extremely crunchiness and 1= not at all crunchy).

2.9. Statistical analysis

For the statistical analysis, Analysis of variance (ANOVA) and regression

calculation using SAS (version 11) were used. All analytical measurements were taken in triplicate for each treatment. Data were analyzed using the SAS Version 5.0 software package. Means were separated using least significance difference analysis at $P \leq 0.05$.

3.Results and discussions

Using VI technique it had been possible to raise the calcium level in potato chips from 93.3mg to 800 mg/100 under the parameters of the study. The regression analysis indicated that the calcium content in potato chips was a function of vacuum pressure, restoration time, blanching time, and calcium content in impregnation solution.

3.1. Effect of process variable on calcium impregnation

Figure 1a (i) showed that blanching time was a quadratic function for calcium impregnation in chips i.e. with the increase in blanching time, calcium concentration in potato chips increase initially but decreased later. Maximum level of impregnation achieved at the blanching time of 1.6 min. It was because 1.4 min was the blanching time, sufficient to enhance the desired porosity for an effective and efficient targeted impregnation level. Current findings are in line with the reports of Alzamora et al. (2005), that Blanching treatment often produces profound structural alterations (swelling of cell walls, disruption of membranes, etc.) which affects mass transport phenomena and thus resulting in the extensive uptake of solute inside the cytoplasm of parenchyma cells. However, when blanching time was increased from 1.5 to 2 min a sharp decrease was also observed. Such negative effect of prolonged blanching was also observed by Bellary and Rastogi (2014) on banana slices due to gelatinization of starch. The potato slices were also prone for starch gelatinization.

From Fig. 1a (i) it was also observed that the effect of calcium ion concentration was more direct i.e. as the calcium concentration increased in the solution, impregnation level or calcium content in the chips also increased linear manner. This linear behavior of fortificant concentration was also observed by Joshi et al. (2016).

Figure 1a (ii) reflected that with increase in restoration time, calcium impregnation increased initially in linear fashion followed by decrease in level of calcium impregnation i.e. quadratic function in later stage. Highest level of impregnation achieved at restoration time of 15 min. It was due to the fact that Restoration time of 15 min was sufficient enough to incorporate the desired level of calcium to the potato chips. In favor of the findings Palou and Welti-Chanes (2003) reported that with the help of restoration time, when a porous tissue is immersed in a PAC concentrated solution under vacuum conditions, air is extracted from the pores and then, when atmospheric pressure is restored, the impregnation solution penetrates the intercellular spaces by capillary action and by the pressure gradient (i.e. the Hydrodynamic mechanism, HDM) that are imposed to the system, helping incorporation of PACs without exposing the food structure to the eventual stress.

As far as the whole model is concerned, the model has been significant ($p \leq 0.05$) having F value 8.43 and R-square 0.907. The value of adequate precision which measures signal to noise ratio was 10.8 ($\geq$4.0). This shows that the model can be used to navigate the design space.

3.2. Fortification Confirmation

The developed potato chips using optimized conditions (calcium chloride concentration, blanching time, vacuum level and rest period were at1.20%, 1.33 min, 40mm Hg, 12.01 min respectively) can be considered as rich source of calcium with

acceptable sensory scores (≥8.1 on 9.0-point Hedonic scale vs. 8.0 of control preparation) (Fig. 2).

3.3. Physicochemical analysis

3.3.1. Compositional Analysis

Proximate analysis of optimized fortified chips are shown in Table 3. As observed, there were no significant difference between the proximate composition of fortified potato chips and control potato chips except in ash and moisture content. Moisture content was found to be low in calcium fortified potato chips compared with control chips. This was due to the application of VI technique which has been reported to be associated with lowering the moisture content of the food products. In favor of the findings, Zhao and Xie (2004) reported that VI removes most of the water from the product thereby reducing energy consumption. Moreover, the dehydration of samples results in decrease in the cellular respiration rate and, consequently, an increase in the shelf-life of the processed fruit, both of which are more marked if calcium is added to the sample (Moraga et al., 2008).

Fortified samples were also reported to have high ash content in comparison with control potato chips. This was due to mineral fortification which led to higher ash content

3.3.1.1. Oil Content

Current experimental results showed that oil uptake for that for vacuum fried chips were low (30%) compared to atmospheric frying (42%). Oil reduction may be due to the lower vapour pressure of water during vacuum frying and the lower temperatures reaching during the process, as opposed to atmospheric frying, where important structural changes, which increase oil absorption. Thus, developed product can be a good alternative for nutritious foods.

3.3.2. Mineral estimation

Mineral estimation reflected that an optimized calcium fortified potato chips (800 mg/100g) had 8.57 times and 5.17 times higher calcium content in comparison to its control (93.3 mg/100g) and commercial (154.65 mg/100g) counterparts respectively.

3.3.3. Color Analysis

The L*, a* and b* of control potato chips were L*: 64.60± 1.831; a*: −2.09± 0.273; b*: 17.51 ± 1.315 respectively. Lightness (L value) is utmost important color parameter for fried foods and is usually used as a quality control determinant (Mariscal et al., 2008). Results indicated that lightness increased as the calcium fortificant concentration increased. In favor of the findings, Saftner et al. (2003) found treatments with calcium inhibited color changes and development of tissue translucency in honeydew chunks. Additionally, calcium can also help to keep longer the fresh-like appearance of minimally processed fruits and vegetables by controlling the development of browning. Control of the flesh browning has been observed in fruits in different studies, e.g. in peaches (Manganaris et al., 2007) and pineapple (Hewajulige et al., 2003).

Redness (a value) is an undesirable quality factor in fried foods (Krokida et al. 2001). Redness of potato chips was not found to be affected significantly with mineral fortification. Blanching at 72^0C, selection of low reducing sugar variety, elevated temperature storage (10^0C-12^0C) and medium temperature frying (Nourian et al., 2003) were the prominent reasons for unchanged redness color parameter. In the similar way, yellowness (b value) varied the least ($p \geq 0.05$) showing no significant effect of iron fortification on potato chips.

Thus, total color of fortified potato chips at an optimized condition (3%) can be described as light yellow (L*: 67.54; a*: 2.15; b*: 18.01) which was quite similar to

commercial preparation i.e. fried potato chips (L*: 68.60; a*: 1.09; b*: 17.51). Improved colour, was the result of combined application of VI and blanching, because both have been reported to retain or improve the natural colour of the food product (Pedreschiet al., 2004: Alzamora et al., 2000)

3.4. Structural Changes through SEM

There were no significant difference observed between fortified potato chips cell structure and control potato chips cell structure as shown in figure 3(a) and 3(b), displayed that at 100x magnification. Even under magnified view at 500x (Figure 3(c) and 3(d)), it was observed that though there was some deposition of calcium on fortified fries cell periphery but granule appearance remained the same. SEM images revealed that VI can be considered as a tool in the development of new value added fruit or Vegetable products without disrupting their cellular structure, while conveniently modifying their original composition (Tiwari and Thakur, 2016). Current experiments were in line with the results of Gras et al. (2003) that many calcium ions of VI products existed only in the ICAS, not inside of cells. In the present study also, the calcium salts were also kept in the ICAS without disrupting internal cells. Moreover, with increase in calcium concentration, firmness also get increased. Current experimental results are in agreement with those reported by Luna-Guzman and Barrett (2000) and Saftner et al. (2003) that calcium chloride treatment improves the firmness and the quality of freshly cut cantaloupes and honeydew, respectively. This was the reason for better mouth-feel of calcium fortified potato chips compared with control potato chips.

3.5. Consumer Evaluation

Table 4 summarizes the consumer sensory evaluation of the developed fortified potato product. The hedonic scales results showed that the only significant difference ($P \leq 0.05$) which occurred was with 9% ISP for overall flavor. No significant difference in overall impression of chips fortified with calcium using VI technique was observed. Moreover, chips fortified with calcium enhanced the overall texture. Similar observations were found in freshly cut cantaloupes and honeydew as reported by Luna-Guzman and Barrett (2000) and Saftner et al. (2003).

Table 1. Independent variables and their coded and actual values used for optimization

Independent variable	**Units**	**Symbol**	**Code Level**		
			-1	0	1
Calcium salt concentration	%	X_1	1	3	5
Blanching time	Min	X_2	0	1	2
Vacuum pressure	Min	X_3	25	40	55
Restoration time	Min	X_4	10	15	20

Table 2. The Box-Behnken design and experiment data for mineral impregnation in potato chips

Treatments	Independent Variable				Dependent Variable	
	X_1 Calcium (%)	X_2 Blanching Time (min)	X_3 Vacuum pressure (mm Hg)	X_4 Restoration period (min)	Calcium (mg/100g)	Hardness (Newton)
1	1	1	40	15	615.71	447.96

2	5	1	40	15	2449.94	591.01
3	1	2	40	15	1812.54	342.17
4	5	2	40	15	2471.74	505.20
5	3	0	25	10	1326.64	625.92
6	3	0	55	10	1574.44	407.99
7	3	0	25	20	1469.94	501.44
8	3	0	55	20	1861.10	536.94
9	3	0	40	15	1800.34	561.15
10	1	0	40	10	232.24	360.39
11	5	0	40	10	802.74	447.96
12	1	0	40	20	266.64	415.75
13	5	0	40	20	1848.94	443.96
14	3	1	25	15	1395.54	545.52
15	3	2	25	15	1220.64	312.00
16	3	1	55	15	1427.14	543.64
17	3	2	55	15	1879.0	412.22
18	3	0	40	15	1598.74	348.99
19	1	0	25	15	297.44	562.79
20	5	0	25	15	1950.94	466.88
21	1	0	55	15	215.24	452.54
22	5	0	55	15	1147.74	476.40
23	3	1	40	10	1504.84	321.01
24	3	2	40	10	1483.0	425.74
25	3	1	40	20	1820.74	407.05
26	3	2	40	20	1443.2	533.29
27	3	0	40	15	1696.84	448.66

Table 3. Physicochemical analysis of an optimized fortified potato chips

Properties	**Control**	**Mineral (Calcium) Addition**		
		1%	3%	5%
Proximate Composition				
Protein (g/100g)	7.6± 0.1 [a]	7.4± 0.1 [b]	7.5± 0.1 [c]	7.5 ± 0.0 [d]
Fat (g/100g)	18.98± 0.0 [a]	15.24 ± 0.1 a	15.35 ± 0.0 a	15.38 ± 0.1 a
Ash (g/100g)	3.74 ± 0.0 [a]	4.7 ± 0.0 [a]	5.9 ± 0.0 [a]	6.8 ± 0.0 [b]

Moisture (g/100g)	3.45 ± 0.1 [a]	2.01 ± 0.1 [b]	2.07 ± 0.0 [c]	2.04 ± 0.1 [d]
Carbohydrates (g/100g)	54.5 [a]	54.6 [a]	54.6 [a]	54.6 [a]
Energy (cal/100g)	452.78 [a]	439.65 [a]	439.62 [a]	439.60 [a]
Minerals				
Calcium (mg/100g)	98.3 ± 0.03 [a]	525 ± 1.12 [b]	816 ± 1.67 [c]	999 ± 3.23 [d]
Color				
Value L*	67.60 ±2.11	68.05 ±1.21	68.54 ±0.18	68.89 ±0.08
Value a*	−2.07 ±0.01	-1.06 ±0.11	-1.09±1.01	-1.07 ±1.41
Value b*	17.51 ±0.61	16.98 ±0.19	16.38 ±1.61	16.46 ±0.34

Table 4. Effect of calcium fortification on sensory evaluation of the potato chips

Potato Chips	Overall Taste	Overall Flavor	Overall Color	Hardness	Crispiness	Overall acceptability
Control Chips	7.96±1.09[a]	8.07 ± 1.11[a]	7.95±1.31[a]	8.20±1.17[a]	8.11±1.04[a]	8.05 ± 1.01[a]
Fortified Chips	7.58±1.14[a]	7.38±1.10[b]	8.56±1.05[b]	8.01±1.07[a]	8.06±1.01[a]	8.12 ± 0.93[b]

[a-l]Values with the different letters within the same column are significantly different at $p < 0.1$. Mean ±SD (n=56). 1-9 scale: 1 = dislike extremely. 9 = like extremely.

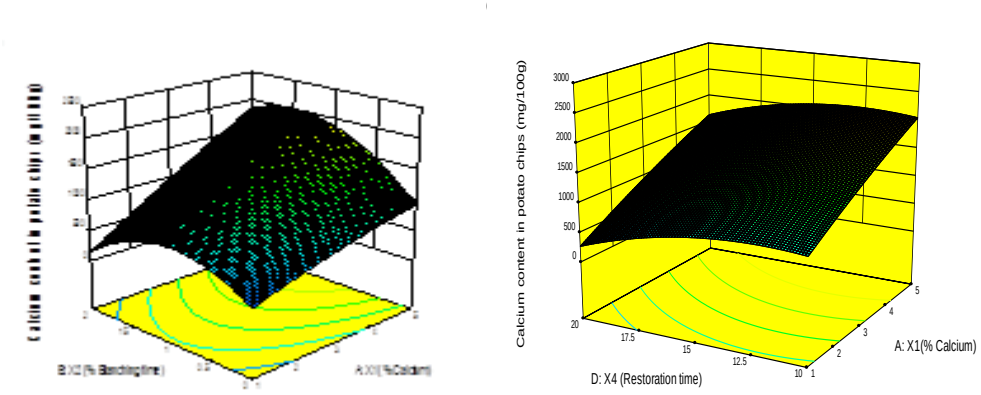

Figure 1a. Effect of i) blanching time and calcium concentration on calcium impregnation ii) restoration time and calcium concentration on calcium impregnation

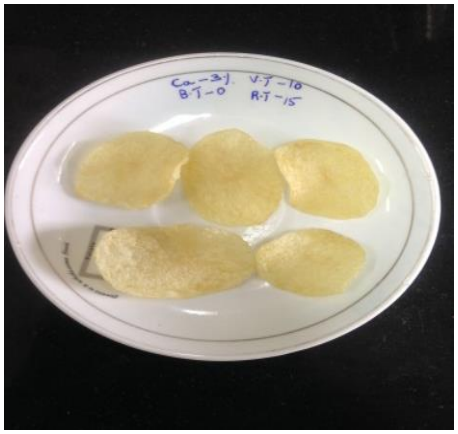

Figure 2. Calcium fortified chips

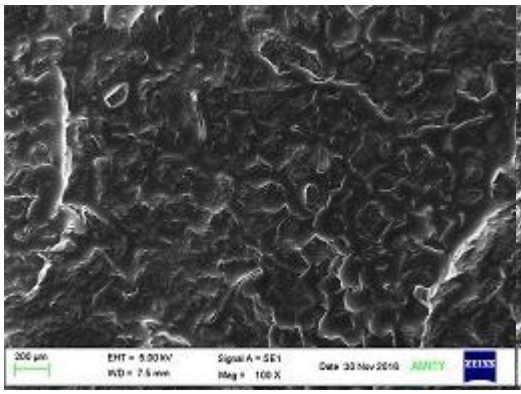

Figure 3a. Control Chips (100x)

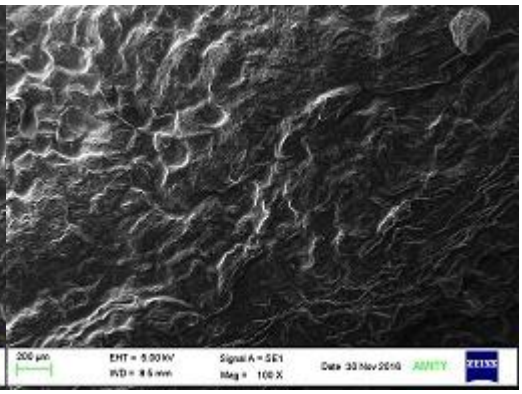

Figure 3b. Calcium fortified chips (100x)

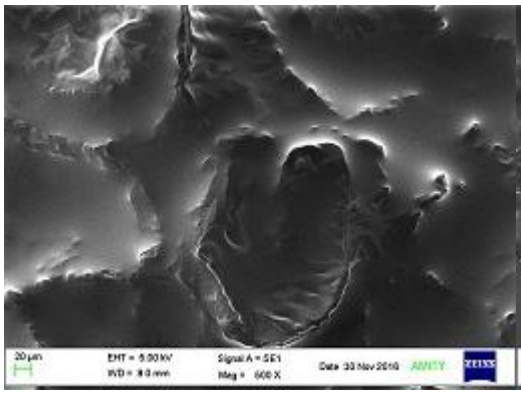

Figure 3c. Control chips (500x)

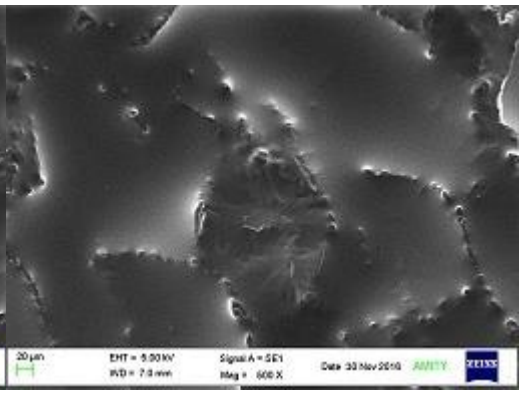

Figure 3d. Calcium fortified chips (500x)

Figure 3. Structural effect of calcium impregnation in comparison with control

4. Conclusions

Impregnation treatment promoted significant changes in calcium concentrations, especially at 40 mm Hg vacuum pressure for 15 min restoration time. The Ca^{2+} amount incorporated into the potato chips would satisfy about 25% of the adequate Intake for school aged children.

SEM observation gave further insight in the cell structure and showed that although there was deposition of mineral on cell wall periphery, yet granule appearance remained the same. The descriptive structure attributes and consumer attributes demonstrated that fortification enhanced the chips texture by improving the firmness. In addition, low oil uptake percentage was found for vacuum fried iron fortified potato fries. Thus, calcium fortified potato chips were at par with commercial potato chips and can be proclaimed as healthy alternative to calcium intake.

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CLASSIFICATION OF THE ENERGY AND EXERGY OF MICROWAVE DRYERS IN DRYING KIWI USING ARTIFICIAL NEURAL NETWORKS

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ABSTRACT

This investigation uses the artificial neural network model to classify the energy and exergy of the kiwi drying process in a microwave dryer. In this experiment, classification was carried out separately for various pre-treatments and microwave powers using three pretreatments (oven, ohmic, and control treatments) and microwave power values (360, 600, and 900W), and the artificial neural network model. Classification was done using 5 different input data groups. The first group included the overall data (energy efficiency, special energy loss, exergy efficiency, and exergy loss), while the second to fifth groups included the data on the exergy efficiency, special energy loss, energy efficiency and special exergy loss in the order mentioned, which served as the classification inputs. Considering the results, the best R and Percent Correct values for the oven (Percent Correct=90 – R=0.709) and ohmic (Percent Correct=83.33– R=0.846) pretreatments were obtained. The values of this parameters were also calculated for the control (Percent Correct=71.43 – R=0.843), the 360W power (Percent Correct=92.86 – R=0.9975), the 600W power (Percent Correct=100 – R=0.9124), and the 900W power (Percent Correct=100 – R=0.9685). The overall data was used in the classification phase. In addition, the maximum correctly detected data for the oven, ohmic, and pretreatment was 18 (20 items), 15 (18 items), and 5 (7 items), respectively. The maximum correctly detected data for the 360W power, 600W power, and 900W power levels was 13 (14 items), 15 (15 items), and 16 (16 items), respectively. In sum, the neural network using the overall data input displayed acceptable efficiency in classifying the energy and exergy data of the kiwi drying process in microwave dryers

1. Introduction

Artificial neural networks (ANNs) have been widely used in different fields of agriculture like economic, energy and environmental modeling as well as to extend the field of statistical methods, in the Last few decades. A big advantage of ANNs over statistical methods is that they require no assumptions about the form of a fitting function. Instead, the network is trained with experimental data to find the relationship; so they are becoming very popular estimating tools and are

known to be efficient and less time-consuming in modeling of complex systems compared to other mathematical models such as regression(B. Khoshnevisan, Sh. Rafiee, M. Omid 2013) The concept of Artificial Neural Networks (ANN) was developed about fifty years ago, but it has been used for practical applications for approximately the last twenty years. Artificial Neural Networks are one of the two major fields of Artificial Intelligence (AI) with the other one being Expert Systems. ANN try to mimic the human brain learning process and are able to learn key information patterns in a multidimensional information domain (Mavromatidis et al. 2013). Artificial Neural Network (ANN) models are developed for each system to provide the energy baseline, which is modelled as a dependency between the energy consumption and suitable explanatory variables. The tool has two diagnostic levels. The first level broadly evaluates the systems performance, in terms of energy consumption, while the second level applies more rigorous criteria for fault detection of supermarket subsystem(Mavromatidis et al. 2013). Neural networks have become ubiquitous in applications ranging from computer vision to speech recognition and natural language processing. While these large neural networks are very powerful, their size consumes considerable storage, memory bandwidth, and computational resources (Han et al. 2015).

The classification problem is the problem of assigning an object into one of predefined classes based on a number of features or attributes extracted from the object. In machine learning, classification is categorized as a supervised learning method. A classifier is constructed based on a training set with known class labels(Siswantoro et al. 2016). A well trained network learns from the pre-seen experimental dataset (training data) and generalizes this learning beyond to the unseen data which is called 'prediction'. Furthermore, artificial neural networks (ANNs) are able to model non-linear behaviors and complex processes. This is highly important considering the drying applications in which the nature is seriously non-linear and simple modeling methods fail. Although ANN methods are frequently reported on drying fruits and vegetables (Nazghelichi et al. 2011, Nikbakht et al. 2014). Artificial neural networks have been used in the past years for modeling many processes in food engineering. Behroozi Khazaeia et al. (2013) used neural networks to 9 model and control the drying process of grapes(Behroozi Khazaei et al. 2013). Aghbashlo et al. (2012) used artificial neural networks to predict exergetic performance of the spray drying process for fish oil and skimmed milk powder(Aghbashlo et al. 2012). Kerdpiboon et al. (2006) used artificial neural network analysis to predict shrinkage and rehydration of dried carrots(Kerdpiboon et al. 2006). Hernández-Pérez et al. (2004) proposed a predictive model for heat and mass transfer using artificial neural networks to obtain on-line prediction of temperature and moisture kinetics during the drying of cassava and mango(Hernández-Pérez et al. 2004)(Guiné et al. 2015).The purpose of this investigation was to classify the amount of energy and exergy of the microwave dryer for the input of the grid with different potentials and pretreatment (ohmic, oven, and control samples) using the neural network, And is artificial neural network able to detect the amount of energy and extrusion for pre-treated and unprocessed products?. Also The sensitivity coefficient of the data was also analyzed using the neural network to determine which network the input was most sensitive to classification.

2. Materials and methods

2.1. Sample preparation

Newly-harvested kiwi fruit were purchased from the local store in Gorgan city of Iran, and were kept in the laboratory at 10 ° C. At the beginning of each experiment, the kiwi was washed and the slices were cut in circular in a thickness of 5 mm and they were weighted. Then, samples were placed in an oven with Temperature at 100 ° C for 3, 5 and 7 min to be

pretreated. Also samples were placed in an ohmic heating with voltage 80 for 3, 5 and 7 min to be pretreated. Drying process was performed in a microwave dryer in the Bio System Mechanics Department of Gorgan University of Agricultural Sciences and Natural Resources Figure 1.

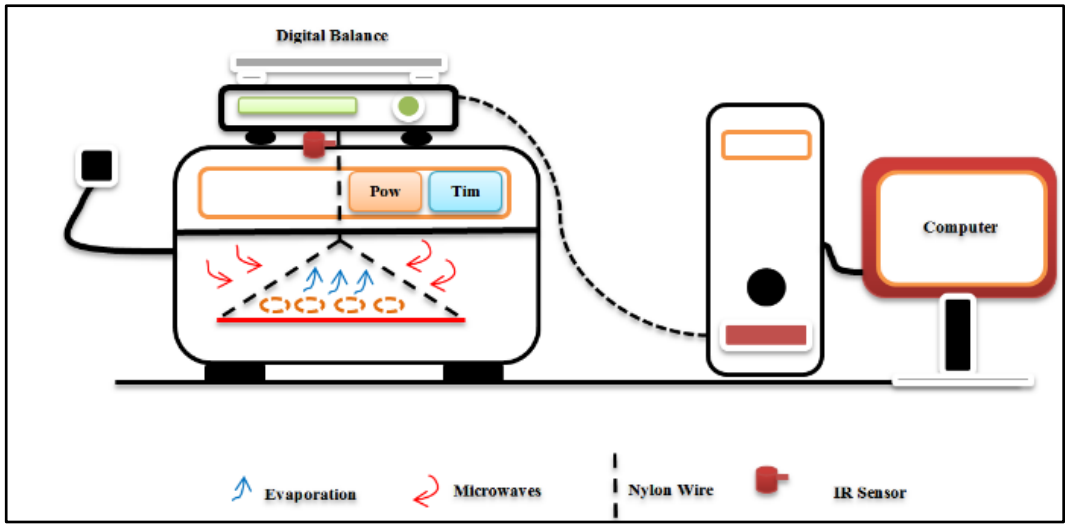


Figure 1. Diagram of microwave drying system

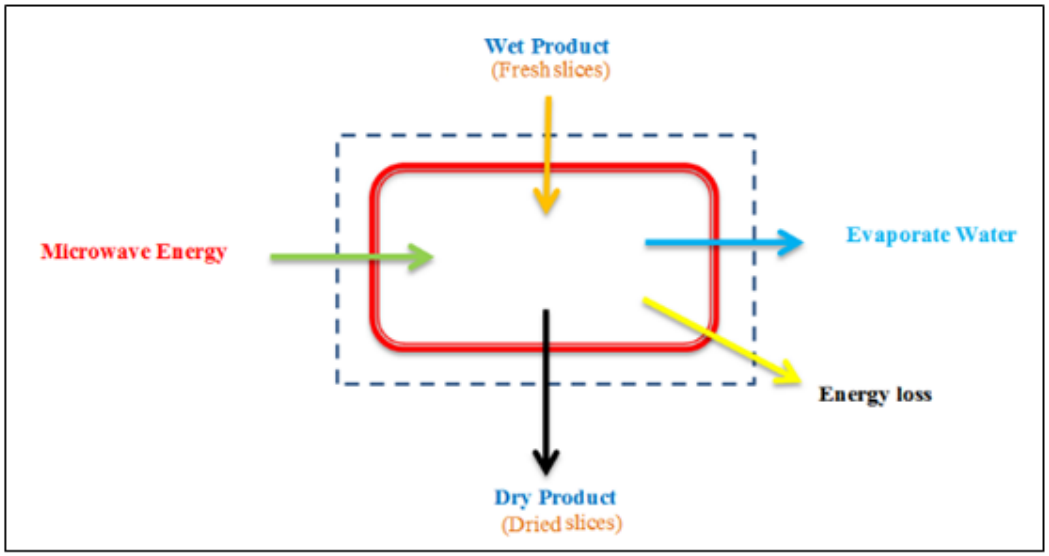


Figure 2. Volume control of microwave system

2.2. Experiment method

Slices were pretreated and placed in containers and dried at three powers of 360, 600 and 900 W. The weight of kiwi was measured using a 0.01 mg precision scale. The weight of each sample was measured and recorded at a time interval of 1 minute to reach constant moisture. For each of the treatments, the experiments were repeated three times. The experiment was conducted at a temperature of 20 ° C and relative humidity of 79%. The moisture content of kiwi was also calculated using equation (1) (Yogendrasasidhar & Pydi Setty 2018).

$$MC = \frac{W - We}{W} \quad (1)$$

2.3. Energy analysis

The mass and energy survival in the microwave dryers' chamber is shown in Figure 2. The general relation of mass moisture survival is calculated using Equation (2) (Darvishi et al. 2016).

$$\sum m_{in} = \sum m_{out} \quad (2)$$

According to Equation 3, the initial mass of the sample is equal to the amount of water vapor removed and the rate of dried sample mass.

$$m_o = m_{ew} + m_p \quad (3)$$

The mass of evaporated water is obtained using Equation 4 (Darvishi et al. 2014).

$$m_{wt} = m_d(M_0 - M_t) \quad (4)$$

The protected energy of the sensible heat, latent heat, and the thermal source of the microwave were calculated using Equation 5 and the input energy of the dryer was calculated using Equation 6 (Jindarat et al. 2011). In equation 5, the lost energy is$P_{ref} + P_{tra}$. Equation 6 shows the amount of input energy of the microwave. This formula is composed of three parts, including absorbed energy, reflected energy, and passed energy. In equation (6) equals to the absorbed energy of product.

$$P_{in} = P_{abs} + P_{ref} + P_{tra} \quad (5)$$

$$\mathrm{P_{in}} \times \mathrm{t} = \left(\left(\mathrm{mC_pT}\right)_{\mathrm{dp}} - \left(\mathrm{mC_pT}\right)_{\mathrm{wp}}\right) + \lambda_K m_w + E_{ref} + E_{tra} \quad (6)$$

The latent heat of the kiwi samples is calculated using Equation 7 (Abdelmotaleb et al. 2009).

$$\frac{\lambda_K}{\lambda_{wf}} = 1 + 23\exp(-40M_t) \quad (7)$$

The latent heat of free water evaporation has been calculated by Broker et al and using Equation 8 (Darvishi 2017).

$$\lambda_{wf} = 2503 - 2.386(T - 273) \quad (8)$$

The thermal capacity is a function of the moisture content and can be calculated through Equation 9 (Brooker et al. 1992).

$$C_P = 840 + 3350 \times \left(\frac{M_t}{1 + M_t}\right) \quad (9)$$

The thermal efficiency of the dryer is calculated using Equation 10 (Soysal et al. 2006).

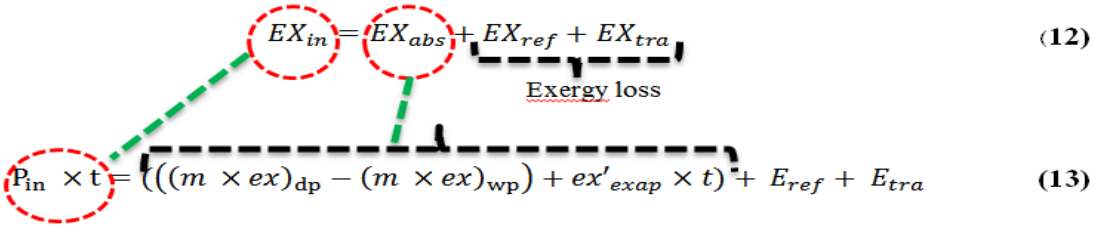
$$\eta_{en} = \frac{energy\ absorption}{P_{in} \times t} \quad (10)$$

The specific energy loss was measured using Equation 11 (Darvishi et al. 2014)

$$E_{loss} = \frac{E_{in} - E_{abs}}{m_w}\ or\ E_{loss} = (1 - \eta_{en}) \times \frac{P_{in} \times t}{m_w} \quad (11)$$

2.4. Exergy analysis

The general exergy equilibrium in the microwave chamber was stated as follows (Darvishi et al. 2016)



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The amount of exergy transmitted due to evaporation in the drying chamber was calculated using Equation 14 (Sarker et al. 2015)

$$ex'_{exap} = (1 - \frac{T_0}{T_p}) \times m_{wv}\lambda_{wp} \quad \textbf{(14)}$$

In formula 14, mwv is calculated using formula 15 (Darvishi et al. 2016)

$$m_{wv} = \frac{m_{t+\Delta t} + m_{wv}\lambda_{wp}}{\Delta t} \quad \textbf{(15)}$$

Specific exergy loss was calculated using formula 16 (Darvishi et al. 2014):

$$ex = C_p[(T - T_0) - T_0 \ln(\frac{T}{T_0})] \quad \textbf{(16)}$$

Exergy efficiency for each dryer system as the exergy rate used in drying the product to the exergy of drying source supplied to the system is calculated by the Equation 17 (Dincer & Sahin 2004)

$$\eta_{en} = \frac{exergy\ absorption}{P_{in} \times t} \times 100 \quad \textbf{(17)}$$

The specific exergy loss was calculated using Equation 18 (Darvishi 2017).

$$EX_{loss} = \frac{EX_{in} - EX_{abs}}{m_w} \quad \textbf{(18)}$$

In this research, the source of temperature and pressure in the environment was 20 ° C and 101325 Pascal, respectively. After calculating the energy and exergy, all the data is sorted in Excel.

2.5. Artificial Neural Network Modeling

In this research, the artificial multilayer perceptron (MLP) neural network was used for systematization energy and exergy of the microwave dryer to classification pre-treatment (oven and ohmic) and power microwave by one hidden layer and 5 neurons using the NeuroSolution 6 software. Hyperbolic tangent linear activation functions (Equation 19), which are the most common type of activation functions, were used in the in hidden input and output layer. In this paper, the Levenberg-Marquardt algorithm was used to learn the network. Additionally, 70% of the data were used for training, 15% of them were used for network evaluation (Validating Data), and 15% of the data were used for testing the network (Testing data) (Table 2). Five repetitions were considered to achieve the minimum error rate and maximum network stability as a mean of 4000 Epoch for the network. Error was estimated using algorithm with back propagation error.

The inputs for the neural network are divided into the following modes:

1. Energy efficiency, Specific energy loss, Exergy efficiency, Specific exergy loss (total data) were considered as network inputs
2. Energy efficiency was considered as network inputs
3. Specific energy loss was considered as network inputs
4. Exergy efficiency was considered as network inputs
5. Specific exergy loss was considered as network inputs

The classification for data pre-treatment (Oven, Ohmic and control) and power microwave (360 , 600 and 900 W) were based on the inputs above. Five repetitions were considered to achieve the minimum error rate and maximum network stability as a mean of 4000 Epoch for the network. Error was

estimated using algorithm with back propagation error. Statistical parameters including RMS, Root Mean Square Error (RMSE), R2, and Mean Absolute Error (MAE), NMSE were calculated for inputs and relationships were calculated using the formulas shown in Table 1.

Table 1. Neural Network Relationships

Formula	Formula Number	Reference
Tanh = $\frac{e^{x}-e^{-x}}{e^{x}+e^{-x}}$	(19)	(B. Khoshnevisan, Sh. Rafiee, M. Omid 2013)
$R^2 = 1-\frac{\sum_{i=1}^{n}(P_i-O_i)^2}{(P_i-O)^2}$	(20)	(Azadbakht et al. 2016)
$R = \sqrt{1-\frac{\sum_{i=1}^{n}(P_i-O_i)^2}{(P_i-O)^2}}$	(21)	
$RMSE = \sqrt{\sum_{i=1}^{n}\frac{(P_i-O_i)^2}{n}}$	(22)	(B. Khoshnevisan, Sh. Rafiee, M. Omid 2013)
$MAE = \frac{\sum_{i=1}^{n}\lvert P_i-O_i\rvert}{n}$	(23)	(Azadbakht et al. 2017)

Table 2. Optimization values for artificial neural network parameters

Input	Number of hidden layers	Learning rule	Type of activation function	The number of One hidden layer neurons	Testing data %	Training data %	Cross Validation%
total data	1	Levenberg Marquardt	Hyperbolic tangent	5	15%	70%	15%
Energy efficiency	1	Levenberg Marquardt	Hyperbolic tangent	5	15%	70%	15%
Specific energy loss	1	Levenberg Marquardt	Hyperbolic tangent	5	15%	70%	15%
Exergy efficiency	1	Levenberg Marquardt	Hyperbolic tangent	5	15%	70%	15%
Specific exergy loss	1	Levenberg Marquardt	Hyperbolic tangent	5	15%	70%	15%

3.Results and discussions

3.1. Classification based on pre-treatment of ohmic, oven and control

Table (3) shows the MSE, RMSE, NMSE, R-MAE, and percent correct values. According to this table, the best MSE, NMSE, R-MAE, and percent correct values were associated with the oven and ohmic pretreatments in the classification process. All of the energy efficiency, special energy use, exergy efficiency, and special exergy use values constituted the input. The best oven pretreatment

values were MSE $_{Train}$ =0.123, RMSE $_{Train}$ =0.350, NMSE $_{Train}$ =0.497, MAE $_{Train}$=0.248, R $_{Train}$=0.709, and Percent Correct $_{Train}$=90. Moreover, the best ohmic pretreatment values were MSE $_{Train}$=0.068, RMSE $_{Train}$=0.260, NMSE $_{Train}$=0.258, MAE $_{Train}$=0.164, R $_{Train}$=0.846, and Percent Correct $_{Train}$=83.33. Finally, the best Percent Correct $_{Test}$ value was obtained through the network input that was composed of the energy efficiency, exergy efficiency, and exergy loss data. The best MSE $_{Test}$, RMSE $_{Test}$, NMSE $_{Test}$, and MAE $_{Test}$ values were obtained by means of the network that used the overall data as the input. As regards the ohmic pretreatment, the best Percent Correct $_{Test}$ value was obtained through the network that used the exergy loss as the input. The best MSE $_{Test}$, RMSE $_{Test}$, NMSE $_{Test}$, and MAE $_{Test}$ values were also similar to the oven results. The R_{Train} values of the oven pretreatment corresponding to the energy efficiency, special energy loss, exergy efficiency, and special exergy loss were 0.061, 0.31, 0.49, and 0.44, respectively. These values were 0.182, 0.501, 0.567, and 0.501 for the ohmic pretreatment in the order mentioned. These values did not suit the classification. The Percent Correct $_{Train}$ values corresponding to the energy efficiency, special energy loss, exergy efficiency, and special exergy loss were 84.21, 68.42, 82.35, and 50 using the oven pretreatment and 20, 77.78, 47.37, and 81.81 using the ohmic pretreatment in the order mentioned. The best MSE, RMSE, and R-MAE values for testing the network with the oven and ohmic pretreatments were obtained when the network carried out the classification using the overall data as the input. Table (3) shows the "Test" values of this network.

Table 3. Error values in predicting experimental data using optimal artificial neural network

	Total input					
Performance	**Oven**		**Ohmic**		**Control**	
	Train	Test	Train	Test	Train	Test
MSE	0.123	0.187	0.068	0.106	0.038	0.111
RMSE	0.3507	0.4324	0.2608	0.3256	0.1949	0.3332
NMSE	0.497	0.757	0.285	0.476	0.292	0.643
MAE	0.248	0.343	0.164	0.229	0.080	0.170
R	0.709	0.537	0.846	0.738	0.843	0.636
Percent Correct	90	75	83.33	33.33	71.43	50
	Energy efficiency					
MSE	0.271	0.349	0.259	0.237	0.091	0.019
RMSE	0.52058	0.59076	0.50892	0.48683	0.30166	0.13784
NMSE	1.110	1.412	1.050	0.960	0.787	0
MAE	0.480	0.564	0.456	0.452	0.195	0.121
R	0.061	-0.326	0.182	0.305	0.466	0
Percent Correct	84.21	100.00	20.00	0.00	16.67	0
	Specific energy loss					
MSE	0.231	0.272	0.190	0.219	0.114	0.067
RMSE	0.4806	0.5215	0.4359	0.4680	0.3376	0.2588
NMSE	0.949	1.100	0.793	0.887	0.781	0.679
MAE	0.419	0.479	0.322	0.393	0.247	0.157
R	0.310	0.016	0.501	0.479	0.481	0.633
Percent Correct	68.421	25	77.778	100	12.500	0

	Exergy efficiency					
MSE	0.200	0.417	0.168	0.371	0.120	0.011
RMSE	0.4472	0.6458	0.4099	0.6091	0.3464	0.1049
NMSE	0.852	1.689	0.687	1.501	0.748	0
MAE	0.373	0.607	0.322	0.561	0.186	0.079
R	0.490	-0.428	0.565	0.239	0.601	0
Percent Correct	82.35	100	47.37	40	22.22	0
	Specific exergy loss					
MSE	0.199	0.377	0.187	0.177	0.014	0.381
RMSE	0.4461	0.6140	0.4324	0.4207	0.1183	0.6173
NMSE	0.806	2.181	0.749	1.022	0.232	1.545
MAE	0.403	0.574	0.374	0.322	0.057	0.452
R	0.441	-0.083	0.501	0.199	0.878	0.406
Percent Correct	50	100	81.81	50	66.66	20

The results from the classification conducted using the neural network, the oven and ohmic pretreatments and the control sample are shown in Table (4). According to the results from the Train classification, when the overall data was used as the input, the network displayed an acceptable capacity to distinguish the classified data pretreated by the oven and ohmic from the control data in the classification process. Moreover, the neural network was more potent in classifying the oven data than the ohmic data, resulting in the accurate classification of 18 data items out of the 20 data items. However, the ohmic results were 15 data items out of the total 18 data items, 5 of the 7 control treatment data items were classified accurately. As seen in Table (4), when only the energy efficiency was used as the classification input, the classification did not succeed. In other words, of the 20 ohmic data items, only 4 data items were identified for classification while 16 data items were wrongly classified for the oven. Concerning the ohmic pretreatment data, of the 19 data items, 16 and 3 data items were classified wrongly for the oven and ohmic pretreatments, respectively. Moreover, concerning the control data, , only 2 of the 7 input data items were classified correctly, reflecting the incapacity of the energy efficiency (as the input) to identify the data for classification purposes. The data was mostly classified for the oven. As regards the special energy loss, 16 and 3 data items of the 19 input data items for the oven were classified correctly and wrongly for the ohmic, respectively. As for the ohmic, 14 and 4 data items of the 18 data items were classified correctly and wrongly for the oven, respectively. Moreover, as for exergy efficiency, of the 17 data items, 14 and 3 data items were classified correctly and wrongly for the oven, respectively. As for the ohmic, 9 and 10 data items of the 19 data items were classified accurately and wrongly, respectively. Out of the 19 data items for the control treatment, only 2 data items were classified correctly. According to these results, the classification of the ohmic and oven data did not match the control data, while not all of the inputs mistook the oven and ohmic data for the control data. Concerning the data classification using the exergy loss as the network input, out of the 19 oven data items, 10 and 9 data items were classified correctly and wrongly, respectively. Moreover, 18 and 4 data items of the 22 ohmic data items were classified accurately and wrongly (for the oven), respectively. Table (2) shows the “Test” data detected and classified for the network of concern.

Table 4. Correct and incorrect values for each network's input data

	Total input train					
Output / Desired	**control**		**ohmic**		**oven**	
	Test	**Train**	**Test**	**Train**	**Test**	**Train**
oven	1	2	2	3	3	18
ohmic	0	0	1	15	1	2
control	1	5	0	0	0	0
	Energy efficiency					
oven	0	5	4	16	5	16
ohmic	0	0	0	4	0	3
control	0	1	0	0	0	0
	Specific energy loss					
oven	1	7	0	4	1	13
ohmic	0	0	4	14	3	6
control	0	1	0	0	0	0
	Exergy efficiency					
oven	0	7	3	10	4	14
ohmic	0	0	2	9	0	3
control	0	2	0	0	0	0
	Specific exergy loss					
oven	4	1	4	4	2	10
ohmic	0	0	0	18	0	9
control	1	2	1	0	0	1

Table (5) shows the learning results of the neural network. According to this table, the best learning results in the training phase were obtained when the classification was carried out using the oveall network inputs, and thus it performed the classification in Run=1 and Epoch=3999. Given the RUN and Epoch values of the neural network with all inputs it could be stated that the neural network classified the data satisfactorily at a good speed when all of the input data was selected. As for cross validation, the best network with energy loss was simulated, which performed classification for RUN=4 and Epoch=76. Based on these values it is concluded that the data was not properly assessed for the classification purposes.

Table 5. Some of the best neural network topologies to predict test values

	Cross Validation		**Training**	
	Run	**Epoch**	**Run**	**Epoch**
Total input data	4	87	1	3999
Energy efficiency	5	2177	2	2000
Specific energy loss	4	76	3	4000
Exergy efficiency	4	234	2	3999
Specific exergy loss	5	38	5	2897

Figures (3) and (4) show the Test and Training sensitivity coefficients of the network. As seen in Figure (3), the highest sensitivity coefficient was obtained in the testing using all inputs. As for the oven pretreatment, ohmic pretreatment, and control treatment the highest sensitivity coefficient was obtained with the overall data of energy efficiency, energy efficiency, and exergy loss, respectively. Figure (4) also suggests that the Training sensitivity coefficients of the oven pretreatment, ohmic pretreatment, and control treatment were obtained using the overall inputs of special exergy loss, energy efficiency, and exergy loss, respectively.

3.2. Classification based on the input power of the microwave

Table (6) shows the MSE, RMSE, NMSE, R-MAE, and percent correct values. According to this table, the best MSE, NMSE, R-MAE, and percent correct values were associated with the microwave power in the classification process, all of the energy efficiency, special energy loss, exergy efficiency, and special exergy loss values constituted the input. The best 360 W power values were Train =0.00243، RMSE Train =0.0493 ، NMSE Train =0.0113 ،0.0458= Train MAE ، 0.9975 = R Train ، =92.86 Train Percent CorrectMoreover, the best 600 W powe values were Train =0.0403 MSE، RMSE Train =0.0.2009 ، NMSE Train =0.1816 ،0.0878= Train MAE ، 0.912 = R Train ، =100 Train Percent Correct, also the best 900 W power values were = 0.0153 MSE Train، RMSE Train =0.124 ، NMSE Train =0.067 ،0.0679= Train MAE ، 0.9685 = R Train ، =100 Train Percent Correct .Finally, the best Percent Correct Test value was obtained through the network input that was composed of the specific exergy loss data. The best MSE Test, RMSE Test, NMSE Test, and MAE Test values were obtained by means of the network that used the exergy efficiencyas the input. As regards the ohmic pretreatment, the best Percent Correct Test value was obtained through the network that used the exergy loss as the input. The best MSE Test, RMSE Test, NMSE Test, and MAE Test values were also similar to the oven results. The RTrain values of the oven pretreatment corresponding to the energy efficiency, special energy loss, exergy efficiency, and special exergy loss were 0.061, 0.31, 0.49, and 0.44, respectively. These values were 0.182, 0.501, 0.567, and 0.501 for the ohmic pretreatment in the order mentioned. These values did not suit the classification. The R Train values corresponding to the energy efficiency, special energy loss, exergy efficiency, and special exergy loss were 0.73, 0.45, 0.71 and 0.53 using the 360 W and 0.52, 0.42, 0.43 and 0.58 using the 600 W in the order mentioned, and for 900 W, best value was 0.69, 0.46, 0.57 and 0.60, That this amount can not be suitable for classification . Table (6) shows the "Test" values of this network.

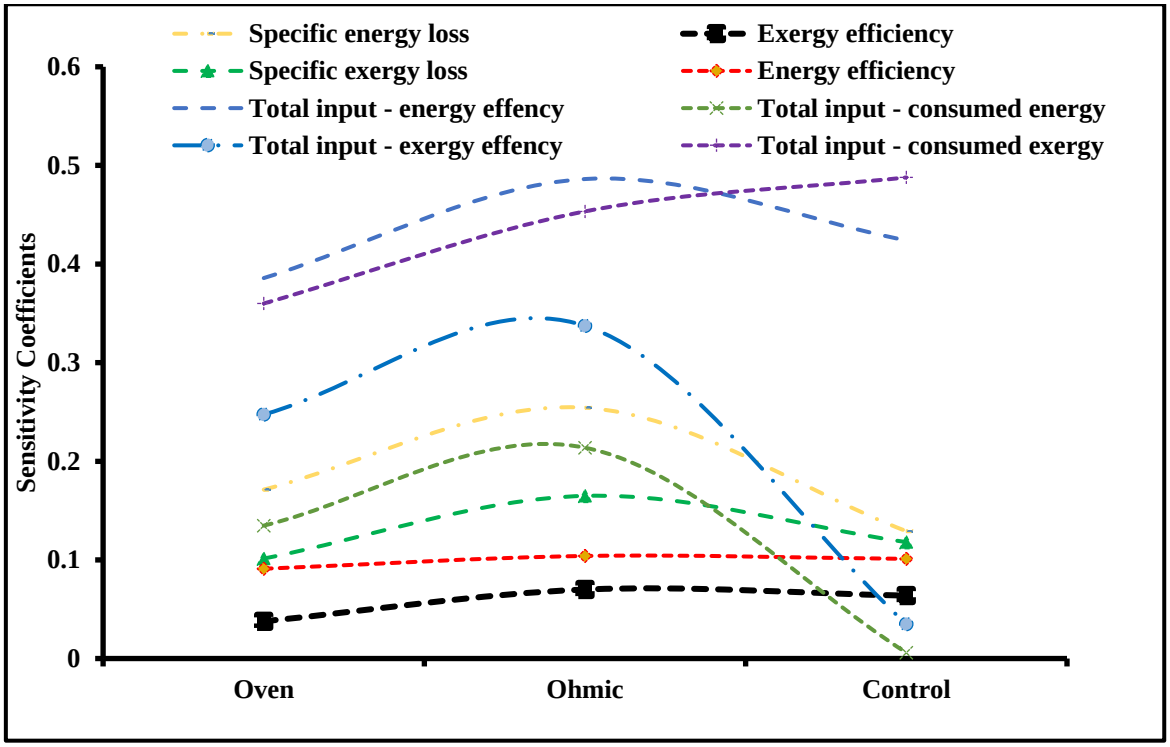

Figure 3. Artificial Neural Network Test sensitivity coefficients

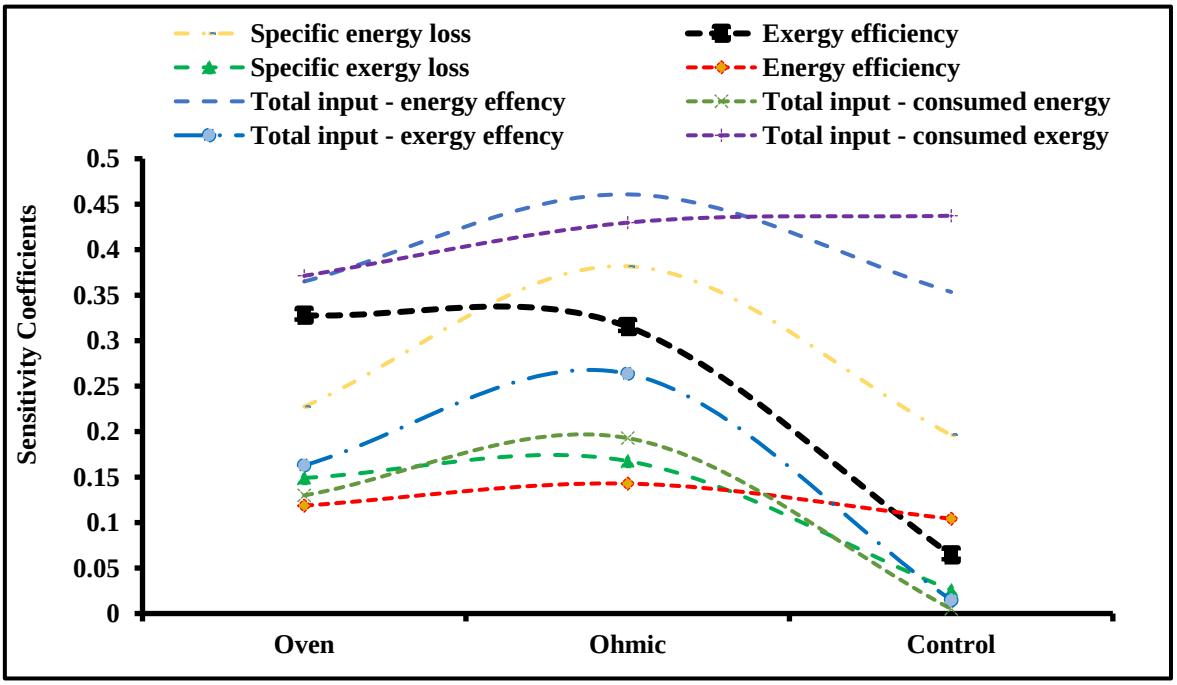

Figure 4. Artificial Neural Network Train sensitivity coefficients

Table 6. Error values in predicting experimental data using optimal artificial neural network

	Total input data					
Performance	**360 W**		**600 W**		**900 W**	
	Train	**Test**	**Train**	**Test**	**Train**	**Test**
MSE	0.002437	0.12654	0.040361	0.17813	0.015376	0.24952
RMSE	0.049366	0.355725	0.2009	0.422054	0.124	0.49952
NMSE	0.011369	0.51247	0.181623	0.80157	0.067106	1.44368
MAE	0.045817	0.16661	0.087845	0.25175	0.067987	0.28026
R	0.9975	0.7905	0.9124	0.6703	0.9685	0.3460
Percent Correct	92.86	75	100.00	100	100	50
	Energy efficiency					
MSE	0.08987	0.1544	0.14406	0.2470	0.12513	0.2727
RMSE	0.299783	0.392938	0.379552	0.496991	0.353737	0.522207
NMSE	0.45955	0.6947	0.67218	1.0002	0.51293	1.5776
MAE	0.18729	0.2175	0.29344	0.3742	0.26264	0.3899
R	0.73613	0.6154	0.57258	0.4120	0.69871	0.3132
Percent Correct	58.33	67	64.29	50	89.47	50
	Specific energy loss					
MSE	0.1542	0.268	0.1853	0.297	0.1835	0.251
RMSE	0.392683	0.517687	0.430465	0.544977	0.428369	0.500999
NMSE	0.7886	1.207	0.8089	1.718	0.7807	1.017
MAE	0.3132	0.414	0.3788	0.480	0.3723	0.464
R	0.4597	-0.106	0.4378	-0.053	0.4686	0.167
Percent Correct	41.67	0	75.00	50	41.18	0
	Exergy efficiency					
MSE	0.1052	0.2934	0.1837	0.2492	0.1523	0.0763
RMSE	0.392683	0.517687	0.430465	0.544977	0.428369	0.500999
NMSE	0.4911	1.1883	0.8265	1.1215	0.6649	0.4415
MAE	0.2175	0.4104	0.3691	0.3964	0.3080	0.1730
R	0.7135	0.2689	0.4166	0.1289	0.5789	0.7497
Percent Correct	64.3	75	80.0	33	37.5	50
	Specific exergy loss					
MSE	0.1576	0.2620	0.1344	0.2956	0.1499	0.2875
RMSE	0.396989	0.511859	0.366606	0.543691	0.387169	0.53619
NMSE	0.7093	1.0610	0.6542	1.7105	0.6377	1.2936
MAE	0.3260	0.3979	0.2766	0.3917	0.3176	0.4012
R	0.5395	0.3503	0.5883	-0.0581	0.6039	0.4255
Percent Correct	40	25	46	0	100	100

The results from the classification conducted using the neural network, the microwave power's 360 W, 600W and 900 w are shown in Table (7). According to the results from the Train classification, when the overall data was used as the input, the network displayed an acceptable capacity to distinguish the classified data, Moreover, the neural network was more potent in classifying the oven600W and 900 W power data than the 360 W power data. Resulting in the accurate classification of 18 data items out of the 20 data items. However, the ohmic results were 15 data items out of the total 18 data items, 5 of the 7 control treatment data items were classified accurately. As seen in Table (7), when only the energy efficiency was used as the classification input, the classification did not succeed, In other words, of the 12 360 W data items, only 7 data items were identified for classification while 5 data items were wrongly classified for the 900 W. the 600 W power data, of the 14 data only 9 data items were identified for classification while 5 data items were wrongly. For 900 W power Classified from 19 data, 17 data are correct and 2 data are wrong.As regards the special energy loss, 5 and 7 data items of the 12 input data items for the 360 W power were classified correctly and wrongly, respectively, As for the 600 W power, 12 and 4 data items of the 16 data items were classified correctly and wrongly for, respectively, Moreover, as for exergy efficiency, of the 14 data items, 9 and 5 data items were classified correctly and wrongly for the 360 W power, respectively. As for the 600 W power, 12 and 3 data items of the 15 data items were classified accurately and wrongly, respectively. For 900 W power Classified from 16 data, 6 data are correct and 10 data are wrong.. Also for the data classification using the special exergy loss as the network input, out of the 15 360 W power data items, 6 and 9 data items were classified correctly and wrongly, respectively. Moreover, 6 and 7 data items of the 13 600 W power data items were classified accurately and wrongly, respectively. Table (7) shows the "Test" data detected and classified for the network of concern. For 900 W power Classified from 17 data, 17 data are correct and 0 data are wrong.

Table 7. Correct and incorrect values for each network's input data

	Total input data					
Output / Desired	**360 W**		**600 W**		**900 W**	
	Train	**Test**	**Train**	**Test**	**Train**	**Test**
360 W	13	3	0	0	0	0
600 W	1	1	15	3	0	1
900 W	0	0	0	0	16	1
	Energy efficiency					
360 W	7	2	1	0	0	0
600 W	3	0	9	2	2	1
900 W	2	1	4	2	17	1
	Specific energy loss					
360 W	5	0	1	0	1	0
600 W	6	1	12	1	9	4
900 W	1	2	3	1	7	0
	Exergy efficiency					
360 W	9	3	1	2	0	0

600 W	5	1	12	1	10	1
900 W	0	0	2	0	6	1
	Specific exergy loss					
360 W	6	1	0	0	0	0
600 W	1	1	6	0	0	0
900 W	8	2	7	2	17	3

Table 8. Some of the best neural network topologies to predict test values

	Cross Validation		***Training***	
	Run	Epoch	Run	Epoch
Total input data	4	63	2	132
Energy efficiency	3	1	2	339
Specific energy loss	5	22	5	122
Exergy efficiency	4	4	3	133
Specific exergy loss	3	42	5	121

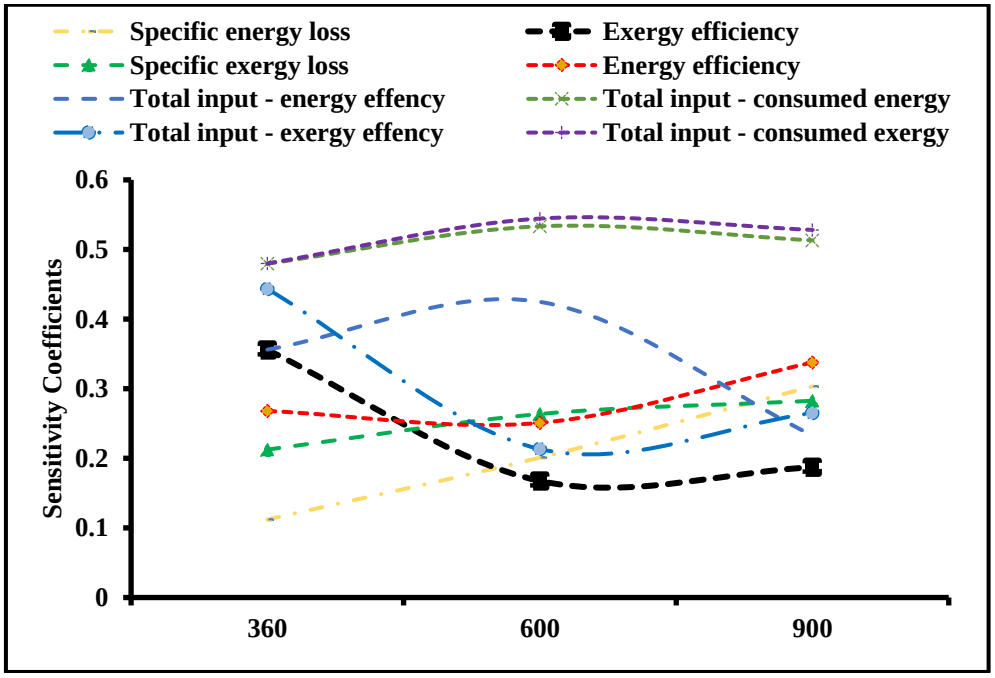

Figure 5. Artificial Neural Network Test sensitivity coefficients

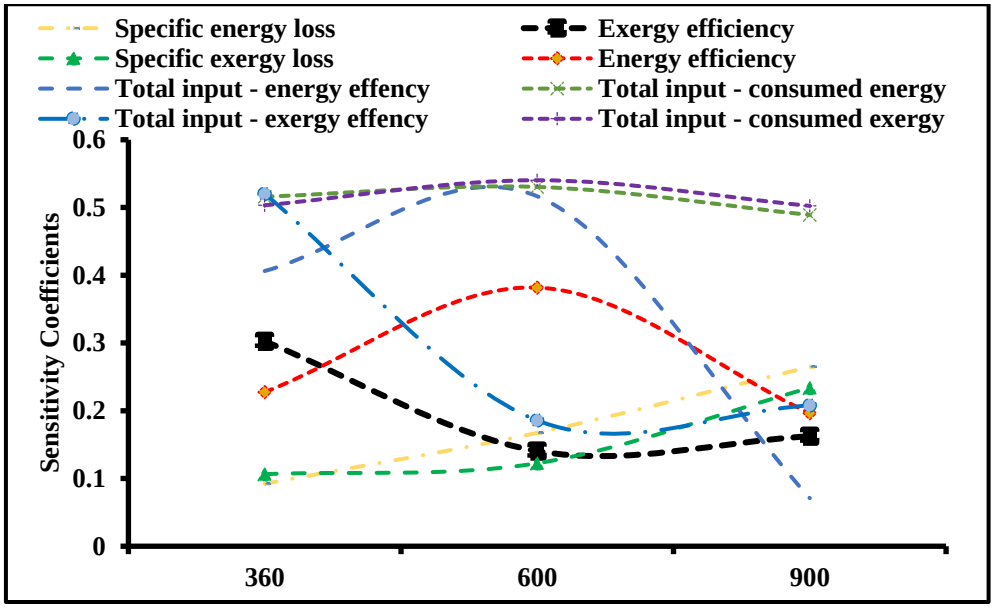

Figure 6. Artificial Neural Network Train sensitivity coefficients

Table (8) shows the learning results of the neural network. According to this table, the best learning results in the training phase were obtained when the classification was carried out using the oveall network inputs, and thus it performed the classification in Run=12 and Epoch=132. Given the RUN and Epoch values of the neural network with all inputs it could be stated that the neural network classified the data satisfactorily at a good speed when all of the input data was selected. As for cross validation, the best network with energy efficiency was simulated, which performed classification for RUN=3 and Epoch=761. Based on these values it is concluded that the data was not properly assessed for the classification purposes.

Figures (5) and (6) show the Test and Training sensitivity coefficients of the network. As seen in Figure (5), the highest sensitivity coefficient was obtained in the testing using all inputs, As for the power 360, 600 and 900 W the highest sensitivity coefficient was obtained with the overall data (Specific exergy loss). Figure (6) also suggests that the Training sensitivity coefficients of the 360 W was obtained using the overall inputs (special energy loss) and for 600 W and 900 W was obtained using the overall inputs (special exergy loss)

4. Conclusions

According to the results, the neural network classifies the energy and exergy more effectively when there are more input items. The best network suiting the energy and exergy data was the network using the energy efficiency, special energy loss, exergy efficiency, and special exergy loss data (i.e. overall data) as the input. In this state, the neural network detected the pretreatment and microwave power data with acceptable precision and using the overall data as the input improved the classification precision. Moreover, when the overall data served as the input, the neural network staged the ability to learn better and faster than the other states, and it trained the network with fewer RUNs than the cases with fewer inputs. The sensitivity coefficient of the classification also indicated that when the neural network was trained using the overall data as the input, the sensitivity coefficient observed in the network testing and training phases was larger. In sum, the neural network displayed an acceptable capacity to classify the pretreatment and

microwave power data for the classification of energy and exergy of the kiwi drying process.

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CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM PANTELLERIA ISLAND AUTOCHTHONOUS AND NATURALIZED SPICES AND EVALUATION OF THEIR INDIVIDUAL AND COMBINED ANTIMICROBIAL ACTIVITIES

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ABSTRACT

In this study, the antimicrobial activity of the essential oils (EOs) from *Origanum majorana* L. and *Rosmarinus officinalis* L. growing in Pantelleria (Sicily, Italy) were tested alone and in combination against some prokaryotic and eukaryotic food-borne pathogens. The chemical composition of the EOs as well as the minimum inhibitory concentrations (MIC) against the most sensitive strains were also determined. Both EOs showed interesting antimicrobial effects against all bacteria and yeasts tested. MIC was in the range 1.25–2.50 µl/ml. Interestingly, *O. majorana* was particularly rich in thymol acetate, while carvacrol was present at very low percentages. Also *R. officinalis* EOs composition was different from rosemary collected in different areas, as being particularly rich in caryophyllene. Furthermore, the antimicrobial activity of the combination of *O. majorana* and *R. officinalis* EOs indicated their potential as food biopreservatives.

1. Introduction

Spices are widely used in different countries of the Southern Europe and North Africa because of their aromatic, nutritional and antioxidant properties (Pezzani *et al.*, 2017). Among the aromatic plants, marjoram and rosemary belong to the Labiatae family (*Lamiaceae*) and play a key role in the Mediterranean cuisine and diet (Gurbuz *et al.*, 2016). Almost 75% of the *Origanum* species are restricted to the eastern Mediterranean area; eleven species are present in Greece, five of which are found in Crete (Aligiannis *et al.*, 2001). The presence of *Origanum* also occurs commonly throughout Asia, Europe, and northern Africa (Han *et al.*, 2017). Members of this genus are extensively used in the flavoring and preservation of foodstuffs and alcoholic beverages (La Pergola *et al.*, 2017). Similarly, rosemary is used worldwide for its antimicrobial and antioxidant activities, along with anti-inflammatory and anti-tumoral properties (Bajalan *et al.*, 2017).

Recently, there is a growing interest in industry to replace synthetic chemicals by natural products extracted from aromatic plants showing bioactive properties. Among

them, *Rosmarinum officinalis* is considered one of the most important sources of both volatile and non-volatile bioactive compounds (Ojeda-Sana *et al.*, 2013). Within the same family, the essential oils (EOs) extracted from wild and cultivated Sicilian *Origanum majorana* have been considered natural antimicrobials (Tuttolomondo *et al.*, 2013). In addition, they could represent a successful approach to contain the rising of bacterial resistance to synthetic antimicrobial compounds (Pezzani *et al.*, 2017).

However, the biological properties of EOs depend on their chemical composition, which is genetically determined and influenced by the geographical origin, ecological conditions, growth stage and extraction method (Gaglio *et al.*, 2017). Recently, a wide range of biological interactions between the various components of EOs is acquiring attention for food preservation purposes. EOs mixtures show the advantage that reduce the negative impact on food sensory properties due to addition of large amounts of EOs from a single given species often required to contrast microbial development (Nikkhah *et al.*, 2017). Several aspects of EOs combination have been studied (de Rapper *et al.*, 2016) and reviewed (Kohiyama *et al.*, 2015). However, only a few studies have been carried out on the combined effects of EOs (Nguefack *et al.*, 2012; Nikkhah *et al.*, 2017). Recently, Baj and co-workers tested the antioxidant properties of mixtures of EOs from *Ocimum basilicum* L., *Origanum majorana* L. and *Rosmarinus officinalis* L.

However the antimicrobial activity of the mixtures have not been investigated (Baj *et al.*, 2018). To this purpose, no exhaustive reports are available on the EOs composition and the combined biological activity of *O. majorana* and *R. officinalis* from Pantelleria (TP, Sicily). Due to its position in the Mediterranean Sea, this island hosts aromatic plant species characterized by distinct chemical profiles of EOs. Secondary metabolite profiling may allow to acquire information on the origin, autochthonous and healthy properties of the studied spices.

Therefore, the present study was aimed to investigate on the *in vitro* antimicrobial properties of the EOs extracted from marjoram and rosemary species growing in Pantelleria Island, alone or in combination, against several worldwide food-borne microorganisms and to correlate the biological activities to their chemical composition.

2. Materials and methods

2.1. Plant material and extraction of EOs

Origanum majorana (65 Kg) and *Rosmarinus officinalis* (65 Kg) grown respectively, naturalized and wild in Pantelleria (Sicily, Italy) were collected from Nikà area (36°75' N, 11°98' E) and Satarìa area (36°78' N, 11°95' E), respectively, in spring 2015. *Origanum majorana* (synonymous *Majorana hortensis* Moench) is explicitly indicated as cultivated in Pantelleria (Giardina *et al.*, 2007) from ancient time and where now it is naturalized. In Italy it is considered archeophyte (a plant species which is non-native to a geographical region, but which was introduced in "ancient" times) (Celesti-Grapow and Accogli, 2010).

Rosmarinus officinalis is an important element of the Pantelleria vegetation (Gianguzzi, 1999). Plant specimens were deposited at Herbarium Mediterraneum Panormitanum [PAL], Italy, /Origanum majorana/109617 and /Rosmarinus officinalis/109618 and in Herbarium SAF at Department of Agricultural Food and Forest Sciences (N. SAF 54pl and 55pl) (n = 10 per species). The plants were kept in dry and cool conditions until extraction of EOs carried out by steam distillation through a 60-l stainless steel extractor (Cucuzza Inox Impianti S.A.A., Grammichele, Italy).

2.2. Chemical analysis of the EOs

EOs from *O. majorana* and *R. officinalis* were analyzed by gas chromatography (GC)

and mass spectrometry (MS) technique in order to determine their chemical profiles. To this purpose, a GC/MS system consisting of a GC instrument (Agilent 6890; Palo Alto, CA, USA) and a mass selective detector (Agilent 5975 c; Santa Clara, CA, USA) was used.

The column set was a capillary column Carbowax (30-m length, 0.25-mm internal diameter and 0.25-lm film thickness; Supelco, Milan, Italy). The operating conditions were as follows: 1 µl of EOs was injected in the split ratio (1:50) mode at a temperature of 250 °C. GC/ MS instrument operated at 70 eV in the EI mode over the m/z range 30 – 550. Helium carrier gas flow was at 1 ml/min and the temperature of the oven was programmed from 40 to 230 °C at 4 °C/min and then held isothermal for 50 min; the injector temperature and the transfer line were set at 250 °C. All measurements were carried out in triplicate.

The identification of the chemical compounds was achieved by matching the fragmentation patterns of the experimental mass spectra with the commercial library NIST05. The relative proportions of the individual components were expressed as percent peak areas normalization, with all relative response factors being taken as one.

2.3. Microbial strains

In order to test the inhibitory properties of *O. majorana* and *R. officinalis* EOs, several bacterial and yeast strains of food origin and belonging to the culture collection of the Agricultural Microbiology Unit of the Department of Agricultural, Food and Forest Science – University of Palermo (Italy) – were used as indicators. Among prokaryotes, *Acinetobacter guillouiae, Bacillus cereus, Serratia grimesii, Hafnia halvei, Hafnia paralvei, Enterobacterludwigii, Listeria monocytogenes, Raoultella ornithinolytica, Stenotrophomonas maltophilia, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus warneri* species were used. The strains *Lactobacillus sakei* LMG 2313 and *Listeria innocua* 4202 were also included as being highly sensitive. The eukaryotes strains included *Aureobasidium pullulans, Candida intermedia, Candida parapsilosis, Candida zailades, Cryptococcus curvatus, Pichia fermentans, Rhodotorula glutinis, Rhodotorula mucilaginosa, Saccaromyces cerevisiae.*

All bacteria were subcultured in Brain Heart Infusion (BHI) broth (Oxoid, Milan, Italy) incubated at 37 °C for 24 h, with the exception of *Lb. sakei* LMG 2313 and *B. cereus* that were cultured in de Man, Rogosa and Sharpe (MRS) (Oxoid) and Nutrient Broth (NB) (Oxoid), respectively, incubated at 30 °C for 24 h. Yeasts were reactivated in Yeast Peptone Dextrose (YPD) medium (Oxoid) incubated at 30 °C for 24 h.

2.4. Antibacterial activity determination

A cell density of approximately 10^7 CFU/ml of each strain was reached in the optimal growth substrate (BHI, MRS, NA or YPD) and EOs of *O. majorana* and *R. officinalis* were tested applying the paper disc diffusion method of Kelmanson *et al.* (2000) (Kelmanson *et al.*, 2000), with the modification reported by Cruciata *et al.* (2018). Briefly, an agar base support (2% [wt/vol] water agar) was overlaid with 7 ml of the optimal soft agar (0.7% [wt/vol]) medium, as indicated by the respective culture collection for each strain, previously inoculated with approximately 10^7 CFU/ml of a given test organism. Sterile filter paper discs (Whatman no. 1) of a 6-mm diameter were placed on the surface of the double agar layer and soaked with 10 µl of each EOs.

Additionally, a mixture of oregan and rosemary EOs at ratio 1:1 was tested. Sterile water was used as negative control, while streptomycin (10% w/v) and cycloeximide (0.01% w/v) represented the positive control for bacteria and yeast, respectively. The inhibitory activity was evaluated after incubation under proper growth conditions as described above. The diameters of the inhibitory halos around the paper discs were

measured. The experiments were performed in triplicate.

2.5. Minimum inhibitory concentration

The strains showing the highest sensitivity (i.e. showing the highest diameter of the inhibition halo) to the screening assay were used for MIC calculation. The MIC was defined as the lowest concentration of EOs inhibiting visible growth after 24 h of incubation as an expression of EO antimicrobial performances. In particular, the EOs were, serially, 2-fold diluted in the optimal growth substrate and the agar disc diffusion method was employed to determine the sensitivity to each dilution of the EOs (NCCLS, 2002) (Militello *et al.*, 2011). The culture medium without bacteria/yeast was used as negative control.

3. Results and discussions

3.1. Chemical composition of *Origanum majorana* EOs

A total of 29 constituents (Table 1) were identified in marjoram EOs and the main components were thymol acetate (21.77%), 4-carvomenthenol (17.82%) and γ-terpinene (15.81%). The oil average yield of the collected rosemary and origanum was determined to be 2% and 1,5 %, respectively.

Table 1. Chemical composition of *O. majorana* and *R. officinalis* EOs. Results indicate mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant and common peaks to all samples) x l00.

Chemical compound	*Origanum majorana*		*Rosmarinus officinalis*	
	Retention time (min)	**%**	**Retention time (min)**	**%**
Monoterpene hydrocarbons				
b-phellandrene	14.124	2.44	-	-
Camphene	-	-	11.229	2.78
Carene	13.71	5.97	-	-
Isosylvestrene	19.335	2.48	-	-
Limonene	-	-	14.127	2.02
Myrcene	12.885	1.39	12.889	1.16
p-cimene	13.991	2.81	13.994	1.85
Sabinene	12.19	2.54	-	-
α-phellandrene	13.278	1.09	-	-
α-pinene	10.703	0.54	10.708	8.8
α-terpinene	16.123	1.91	-	-
α-thujene	10.498	0.72	-	-
γ-terpinene	15.163	16.54	-	-
Oxygenated monoterpenes				
1.8 cineol (eucayptol)	-	-	14.225	30.82
4-carvomenthenol	18.93	18.65	18.93	0.77
Borneol	-	-	18.604	5.92
Bornyl Acetate	-	-	22.059	0.81
Carvacrol	20.594	0.31	-	-
d-camphor	-	-	17.901	12.73
p-menth-2-en-1-ol	17.219	0.41	-	-
Sabinene Hydrate	15.487	0.3	-	-

Thymol	20.857	1.75	-	-
Thymol Acetate	22.246	22.77	-	-
α-terpineol	19.335	2.37	-	-
Diterpenes				
Cembrene	38.38	3.48	-	-
Sesquiterpenes				
Cadinene	-	-	24.356	0.37
Caryophyllene	25.613	5.68	25.615	14.71
Guaiene	-	-	27.492	0.74
Valencene	-	-	26.102	0.43
α-calacorene	-	-	28.639	0.39
α-copaene	28.154	0.26	-	-
α-cubebene	-	-	24.473	1.61
α-humulene	26.469	0.6	26.469	1.8
β-bisabolene	27.78	3.77	27.776	0.51
β-cadinene	27.026	0.25	27.026	1.73
β-cubebene	27.153	1.71	27.945	1.71
Others				
Bicyclogermanene	27.537	3.75	-	-
Unknow	16.555	0.72	-	-
Unknow	21.198	0.83	-	-
Unknow	23.44	0.33	-	-
Unknow	-	-	28.157	4.38
Unknow	-	-	29.613	1.62
α-ionone	-	-	19.335	2.34

The last component has been reported as a precursor of carvacrol (Tongnuanchan and Benjakul, 2014) and it is one of the major chemical compound of EOs extracted from different *Origanum* species and subspecies, including *O. vulgare* (Sezik *et al.*, 1993), *Origanum scabrum* (Demetzos *et al.*, 2001) and *O. majorana* (Jan *et al.*, 2018). Similarly, 4-carvomenthenol (also known as 4-terpineol) was found as one of the major components of *O. majorana* (Baj *et al.*, 2018; Busatta *et al.*, 2017).

As reported in the literature, a number of oregano species are categorized by the presence of two main chemotypes, thymol and carvacrol. Another intermediate type would contain high content of two monoterpene hydrocarbons, γ-terpinene or p-cymene. Nevertheless, some species were found to contain high levels of linalool and other monoterpenes and sesquiterpenes (Baj *et al.*, 2018). Moreover, marjoram main EOs also include the bicyclic monoterpene cis-sabinene hydrate and sabinene (Busatta *et al.*, 2017), while the phenolic monoterpene carvacrol, arising from the "cymyl" pathway, is not a typical feature of this spice, as confirmed by our data (carvacrol 0.30%). Similarly, thymol and thymol acetate are not generally present at high yield in marjoram EOs (Baj *et al.*, 2018; Busatta *et al.*, 2017; Jan *et al.*, 2018). According to the literature, *O. majorana* from Pantelleria Island EOs contain oxygenated monoterpenes (44.61%),

monoterpene hydrocarbons (34.95%), sesquiterpenes (11.75%), diterpens (3.48%) and other compounds (5.46%). Surprisingly, despite literature data, *O. majorana* EOs from Pantelleria showed a very low percentage of sabinene (2.42%) and sabinene hydrate (0.29%).

These results indicated that *O. majorana* from Pantelleria is a thymol/terpinene-4-ol rich chemotype (Sellami *et al.*, 2009). Thus, *O. majorana* is chemotypically different from the same species growing in other parts of Sicily (Tuttolomondo *et al.*, 2013) and in other countries (Figuérédo *et al.*, 2006; Sellami *et al.*, 2009), which are characterized from different dominant constituents. Recently, EOs chemotypes have been defined on the basis of a single prominent monoterpene compound (La Pergola *et al.*, 2017). However, they are often hard to compare when considering the whole monoterpene pattern, because of differences in composition according to the harvesting period, taxonomic classification, variety, age and part of the plant analyzed as well as geographical origin. In particular, the prevalent production of thymol and carvacrol is thought to depend on some external factors, such as climatic conditions, harvesting time, soil and/or the amount of water to which the plant is exposed (Kimura *et al.*, 2006). The variation in the EOs composition of *O. majorana* from different origins could be attributed to both interactions between genetic (biotic) and environmental (abiotic) factors (Sellami *et al.*, 2009).

3.2. Chemical composition of *Rosmarinus officinalis* EOs

According to the GC-MS data, 23 constituents (Table 1) were identified in *R. officinalis* EOs. The essential oil was mainly composed of oxygenated monoterpenes (51.05%) and sesquiterpens (24%). Within oxygenated monoterpenes 1,8 cineol, also known as eucalyptol (30.82%), and d-camphor (12.73%) were the major chemicals detected, while among sesquiterpens, caryophyllene (14.71%) was the most abundant compound. Among minor components, only α-pinene exceeded 6 % in *R. officinalis* EOs, followed by borneol. High yields of 1,8-cineole, caryophyllene and camphor have been reported for different rosemary samples from Pantelleria (TP), classified as cineoliferum (high content in 1,8 cineol) chemotype (Napoli *et al.*, 2010). 1,8 cineol and α-pinene have been reported as the major constituents of *R. officinalis* cultivars growing in different areas of Uruguay, southern Brazil (Dellacassa *et al.*, 1999), and, along with camphor, in southern Spain (Tomei *et al.*, 1995).

Boutekedjiret et al. (1998) investigated the essential oil from flowering aerial parts of *R. officinalis* collected in Algeria. More than 90% of the components were identified, with 1,8-cineole (52.4%) and camphor (12.6%) being the major components. Moreover, the chemical composition of *R. officinalis* EOs was similar to those found in the oils from Iran, India, Tunusia and Turkey, characterized by a high amount of camphor, eucalyptol, α-pinene, ß-pinene and borneol (Gurbuz *et al.*, 2016). According to these authors, content, flavour notes and quality of rosemary EOs were influenced by the geographical location of the plants (Viuda-Martos *et al.*, 2007) and by the harvest time.

In particular, the concentration of 1,8-cineole was quite similar throughout the year, while the lowest concentrations of camphor and maximum concentrations of α-pinene were observed in winter (Boutekedjiret *et al.*, 1998). Interestingly, high yield of caryophyllene have not been previously reported for the EOs of *R. officinalis* collected in other regions (Napoli *et al.*, 2010).

3.3. Comparison of the chemical composition of EOs

Comparing the chemical profile of the two EOs, we observed that eight compounds were common to both EOs, but differed in percentage. Interestingly, rosemary EOs was

found to contain high levels of α-pinene (8.80 %) and caryophyllene (14.71 %), while the same components were present at 0.51 % and 5.42 % in marjoram EOs. On the contrary, *O. majorana* and *R. officinalis* EOs contained 4-carvomenthenol at 17.82% and 0.77%, respectively. This characteristic distribution of the constituents of the two EOs constituted the rationale for our hypothesis of a combined antibacterial action. The amount of β-bisabolene, p-cimene, β-cubebene, mircene and α-humulene was in the range of 0.51-3.60 % in both EOs. Many of this EOs constituents have been considered by the FDA as Generally Recognized as Safe (GRAS) substances and are registered by the European Commission as food flavorings (Tongnuanchan and Benjakul, 2014).

3.4. Antimicrobial activity of EOs

There is a growing interest in assessing the antimicrobial effects of plant secondary metabolites against a range of foodborne pathogens, in order to counteract bacterial resistance to antibiotics (Bajpai *et al.*, 2009). The application of natural products is of paramount importance for infection control and/or food preservation and to ensure consumers a safe, healthy, and nutritive food supply. Globally, all strains tested in this study (Table 2) showed sensitivity to both marjoram and rosemary EOs. Among Gram positive bacteria, *S. haemolyticus* ICE 182 and L. monocytogenes DHPS 179 were the most sensitive strains to *O. majorana* EOs, while *S. epidermidis* ICE 244 and *L. monocytogenes* DHPS 22 BO showed the most sensitivity to R. officinalis EOs. The high sensibility of *Staphylococcus* and *Listeria* species to EOs extracted from aromatic herbs is well known (Cao *et al.*, 2009). Coagulase negative staphylococci used in the present study, such as *S. epidermidis*, *S. haemolyticus* and *Staphylococcus warneri* can be involved in nosocomial infections(Vuong and Otto, 2002), while *L. monocytogenes* is responsible for human disease deriving from food poisoning (Swaminathan and Gerner-Smidt, 2007). Regarding Gram negative bacteria, *H. paralvei* 4G 53 and *A. guilloue* ICE24 growth was inhibited greatly by marjoram and rosemary EOs, respectively. *H. paralvei* belongs to the *Enterobacteriacee* family and is responsible for intestinal diseases, while *Serratia* and *Acinetobacter* species determine bloodstream infections (Wisplinghoff *et al.*, 2004). Generally, EOs have been reported as slightly more active against Gram-positive than Gram-negative bacteria because of the complexity of their double-layer cell membrane. However, our results showed an antimicrobial action of *O. majorana* EOs against several Gram negative strains. Thus, our results support its use to counteract food poisoning caused by *E. ludwigii* and other species of clinical relevance tested in this study, such as the multidrug-resistant bacteria *S. maltophilia* or *S. aureus* (Kot *et al.*, 2018).

The most sensitive strains were selected to calculate the minimum inhibitory concentration (MIC). MIC value for O. majorana EOs was 1.25 µl/ml for *L. monocytogenes* DHPS 179 and 0.62 µl/ml for *S. haemolyticus* ICE 182, *B. cereus* ICE 170 and *H. paralvei* 4G 53. Regarding R. officinalis EOs, MIC value was 1.25 µl/ml for both *L. monocytogenes* DHPS 22 BO and *B. cereus* ICE 170. On the basis of these results, both the EOs presented a broad spectrum of antimicrobial activity. However, a sharper drop in the microbial growth was observed using *O. majorana* EOs, with the exception of *L. monocytogenes* DHPS 5 BO. The difference in the inhibitory effects of EOs extracted from marjoram and rosemary against different microorganisms has been related to their particular component profile (Tongnuanchan and Benjakul, 2014).

Therefore, the major effect of *O. majorana* EOs compared to *R. officinalis* from Pantelleria Island could be mainly due to the stronger antimicrobial activity presented by its preponderant constituents (Djeussi *et al.*, 2013). Among the main

constituents of *R. officinalis*, camphor has oxygen functions in its structure and these functions are known to increase the antimicrobial properties of terpenoids (Tongnuanchan and Benjakul, 2014).

A recent study, also referred to caryophyllene anti-oxidant, anti-inflammatory, anti-cancerous and local anesthetic effects (Klauke *et al.*, 2014). On the other hand, 4-terpineol, abundant in *O. majorana*, has been reported as an important antifungal and antibacterial agent (Djeussi *et al.*, 2013; Pezzani *et al.*, 2017) while thymol and carvacrol are the most active constituents against multiple foodborne pathogens (La Pergola *et al.*, 2017). However, the acetylated form seemed to have low toxicity and enhanced biological effects. In particular, thymol and its synthetic derivative thymol acetate have shown antielmintic effect and antinociceptive activity (Angeles-Lopez *et al.*, 2010). The strong antimicrobial activity of marjoram species from Mexico have been linked to the high thymol and phenolic monoterpene content (Ortega-Nieblas et al., 2011). Nevertheless, specific effects of thymol acetate on the microbial vitality/activity have not been reported yet. This characteristic chemical composition of Pantelleria *O. majorana* species may be responsible for its wide antimicrobial spectrum and support the use of this spices as food natural additive.

Table 2. Inhibitory activity of *O. majorana* (*O.m.)* and *R. officinalis (R.o.)* EOs. [a] -, no inhibition; ±, low inhibition (< 9 mm diameter); +, clear inhibition (9 – 12 mm diameter); ++, strong inhibition (> 13 mm diameter). Results indicate the mean value of three independent assays.

Strains	**Inhibition[a]**			**Source of isolation**
	O.m.	***R.o.***	***O.m.+R.o.***	
Bacteria				
Acinetobacter guillouiae ICE 24	++	++	++	Ice cubes
Bacillus cereus ICE 170	++	++	+	Ice cubes
Enterobacter ludwigii 4G 145	++	+	++	Ready to eat salad
Hafnia alvei 4G 44	++	+	++	Ready to eat salad
Hafnia paralvei 4G 53	++	+	++	Ready to eat salad
L. monocytogenes DHPS 1 BO	++	++	+	Chopped meat
L. monocytogenes DHPS 11 BO	++	++	++	Meat factory
L. monocytogenes DHPS 12 BO	++	+	+	Ripened salami
L. monocytogenes DHPS 129	++	+	++	Human stool
L. monocytogenes DHPS 13 BO	++	+	++	Gorgonzola cheese
L. monocytogenes DHPS 131	++	++	++	Human stool
L. monocytogenes DHPS 133	+	+	-	Human stool
L. monocytogenes DHPS 179	++	+	++	Salmon
L. monocytogenes DHPS 180	+	+	++	Ricotta cheese
L. monocytogenes DHPS 182	++	++	++	Ricotta cheese
L. monocytogenes DHPS 184	++	++	-	Rice salad
L. monocytogenes DHPS 185	+	+	++	Beef
L. monocytogenes DHPS 186	++	++	++	Mozzarella salad
L. monocytogenes DHPS 187	++	+	++	Roasted chicken
L. monocytogenes DHPS 188	++	+	++	Green salad

L. monocytogenes DHPS 2 BO	++	+	++	Fresh salami
L. monocytogenes DHPS 20 BO	++	++	++	Gorgonzola cheese
L. monocytogenes DHPS 22 BO	++	++	++	Taleggio cheese
L. monocytogenes DHPS 24 BO	++	++	++	Taleggio cheese
L. monocytogenes DHPS 3 BO	+	+	+	Fresh salami
L. monocytogenes DHPS 4 BO	++	+	+	Ripened salami
L. monocytogenes DHPS 5 BO	+	++	+	Ripened salami
L. monocytogenes DHPS 6 BO	++	++	+	Ripened salami
L. monocytogenes DHPS 7 BO	++	++	++	Ripened salami
Lactobacillus sakei LMG 2313	++	+	+	Unknown
Listeria innocua 4202	++	+	+	Unknown
Listeria monocytogenes ATCC 19114	++	+	++	Animal tissue
Raoultella ornithinolytica 4G 594	++	+	++	Ready to eat salad
Serratia grimesii 4G 954	++	++	++	Ready to eat salad
Staphylococcus epidermidis ICE 244	++	++	+	Ice cubes
Staphylococcus haemolyticus ICE 182	++	+	++	Ice cubes
Staphylococcus warneri ICE 20	++	+	++	Ice cubes
Stenotrophomonas maltophilia ICE 272	++	+	+	Ice cubes
Yeasts				
Auerobasidium pullulans AD201	++	+	++	Wheat kernels
Candida intermedia 4G137	++	+	++	Ready to eat salad
Candida intermedia 4G307	++	+	++	Ready to eat salad
Candida intermedia ICE86	++	+	+	Ice cubes
Candida parapsilosis ICE214	++	+	++	Ice cubes
Candida zailades 4G362	++	+	++	Ready to eat salad
Criptococcus curvatus ICE84	++	++	++	Ice cubes
Pichia fermentans 4G140	++	+	+	Ready to eat salad
Rhododotorula glutinis AD64	++	+	++	Wheat kernels
Rhodotorula mucilaginosa ICE29	++	++	++	Ice cubes
Saccaromyces cerevisiae GR1	++	++	++	Grape

3.5. Antifungal activity of EOs

The development of natural protective agents against pathogenic fungi and yeasts causing food spoilage is currently in the focus of many research groups. Therefore, in the present investigation the antifungal activities of *O. majorana* and *R. officinalis* EOs was examined. Interestingly, both EOs showed marked antifungal activities against eleven yeasts, that appeared to be spices-dependent. All yeast growth was inhibited more markedly by marjoram than rosemary EOs. Both EOs showed antifungal action against pathogenic yeasts belonging to different genera including *Candida*, *Aureobasidium* and *Rhodotorula*.

Among them, *C. parapsilosis* has been commonly associated with blood, wound and tissue infections (Palmeira-de-Oliveira *et al.*, 2009) while *A. pullulans* and *R. glutinis*, despite their importance in biotechnology, have emerged as opportunistic human pathogens (Najafzadeh *et al.*, 2014; Nunes *et al.*, 2013). *Pichia fermentans*, a spoilage yeasts belonging to the *Saccharomycetaceae* family, has been frequently isolated from orange juice and fermented foods (Qvirist *et al.*, 2016). On the other hand the oleaginous yeast *C. curvatus* and

S. cerevisiae are acquiring growing importance in food industry (Liu *et al.*, 2017). To our knowledge, the effects of marjoram and rosemary EOs on the mentioned species have not been investigated elsewhere, with the exception of *Candida*, *S. cerevisiae* and *Rhodotorula glutinis* (Kunicka-Styczyńska, 2011; Palmeira-de-Oliveira et al., 2009; Tripathy, *et al.*, 2017). In addition, the EOs obtained from O. majorana have shown antifungal activity against *Aspergillus flavus* and *A. parasiticus* (Palmeira-de-Oliveira *et al.*, 2009; Tripathy *et al.*, 2017).

Surprisingly, we observed that *C. curvatus* ICE84 and *S. cerevisiae* GR1 were the most sensitive strains to marjoram EOs (MIC 1.25 μl/ml) and rosemary EOs (MIC 1.25 μl/ml), respectively. As stated in the literature, α-terpinene and other constituents of aromatic plant EOs affect ergosterol biosynthesis and sterol uptake, influencing yeast physiology (Parveen *et al.*, 2004).

3.6. Antimicrobial activity of EOs mixture

The combined use of the EOs from *O. majorana* and *R. officinalis* was tested to inhibit the survival of all bacteria and yeasts strains reported above. Our results showed that the application of the EOs alone or in mixture (1:1) caused the inhibition of the growth of all tested strains, with the exception of *L. monocytogenes* DHPS 133 and DHPS 184. Only for these two strains an antagonistic effect of the combined application of EOs may be supposed.

Conversely, an enhanced antimicrobial effect was observed *vs L. monocytogenes* DHPS 180 and DHPS 185, suggesting an interactions between the components of the two EOs. The combined EOs reduced the diameter of the inhibition halos of all other strains. These results might be due to the use of sub-inhibitory amount of each EOs in the mixture. Moreover, it would seem reasonable that the combination of EOs possessing compounds with similar structures may show additive rather than synergistic effect. The occurrence of additive interactions of these EOs could be related to their similar composition possessing phenolics (carvacrol and thymol) as main compounds, suggesting a similar mechanism of action (De Azeredo *et al.*, 2011). On the other hand, the increased antimicrobial activity caused by the mixture of these EOs could be partially explained considering the different compounds found for each EOs individually. Additive effects of mixture of EOs extracted from aromatic plant have been reported. However, increasing evidences indicate that the inherent activity of EOs may not depend exclusively on the ratio in which the main active components are present, but also interactions between these and minor constituents of the EOs. As an example, among hydrocarbons, p-cymene probably enables easier entrance of carvacrol into the cell membrane where it exerts its action. Moreover, the lipophilic properties and the characteristic functional groups of each component may influence the biochemical properties of the mixture (Hyldgaard et al., 2012).

In our study, *O. majorana* and *R. officinalis* EOs combined at sub-inhibitory concentrations were effective in inhibiting the growth and survival of pathogenic and spoilage microorganisms, although the underlying mode of action has to be better explored.

4. Conclusions

Experimental data indicated that *O. majorana* and *R. officinalis* EOs are effective against Gram positive and Gram negative bacteria and yeasts. The inhibitory activities of marjoram EOs were stronger than rosemary EOs. These properties could be partly due to the presence of some classes of compounds, such as monoterpene hydrocarbons and oxides, characteristics of the spices of Pantelleria Island. The fact that both *O. majorana* and *R. officinalis* EOs, alone or in combination, exhibited antimicrobial activities against the microorganisms studied supports their application in food industry.

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PNEUMATIC CONVEYING OF SUGAR: EFFECT ON THE RHEOLOGY OF COOKIE DOUGH

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ABSTRACT
This study evaluated the impact of the pneumatic conveying system on the white sugar particle size and its effects on the rheological behavior of cookie dough. Sugar samples, blended with soft wheat flour (1:2.4 w/w), were analyzed for rheological properties. These blends were used to develop cookie doughs, which were submitted to consistency, stability, and hardness analyses. The pneumatic conveying system reduced the white sugar particles sizes. Wheat flours blended with sugar after pneumatic conveying (APC) showed lower water absorption and higher starch retrogradation rates than the samples with sugar before pneumatic conveying (BPC). For the cookie dough, although the APC sugar resulted in a harder texture, the condition of sugar did not affect the resistance and stability. The particle size of white sugar affected water absorption and starch retrogradation rates of wheat flour and hardness of cookie dough.

1. Introduction

Sugar and wheat flour are two of the main ingredients used in cookie dough formulations, and, consequently, affect its quality (Manohar and Rao 1997). Starch and proteins present in wheat flour absorb water, forming the gluten, which increases the viscosity of dough (Dhaka and Khatkar, 2013). Sugar affects not only dough viscosity, but also increases starch gelatinization temperature and delays or inhibits gluten development (Pareyt and Delcour, 2008). In the food industry, pneumatic conveying is the best way to move ingredients; however, movement and handling of sugar can increase the proportion of finer particles, due to the breaking of crystals to a greater or lesser extent (Manley 2000).

White sugar particle size has an effect on various rheological characteristics of cookie dough, such as consistency, toughness and apparent viscosity (Manohar and Rao 1997). According to Cauvain and Young (2006), the main influence of white sugar particle size is on the dissolution rate of its crystals in water. Thus, it is of extreme importance to understand the influence of sugar particle size on the rheology of cookie dough to anticipate its behavior during the cookie production stages, as well as its impact on the appearance and texture of final product (Manohar and Rao

2002). The objective of this study was to investigate the impact of the pneumatic conveying system on the white sugar particle size and its consequent effects on the rheological behavior of cookie dough.

2. Materials and methods

2.1. Materials

White granulated sugar produced in 2012/2013 harvest was obtained from three different suppliers in the state of São Paulo, Brazil (designated Suppliers A, B, and C). These samples were separated into two groups: sugars A, B, and C collected before pneumatic conveying (BPC) and sugars A, B, and C collected after pneumatic conveying (APC).

Soft wheat flour was purchased from one supplier in the state of Rio Grande do Sul, Brazil and other ingredients for cookie dough production, such as fat, emulsifier, flavoring, acidifying, baking powder and salt were purchased from suppliers in the state of Santa Catarina, Brazil.

2.2. Methods

2.2.1. Pneumatic conveying system

The pneumatic conveying system utilized to move and fraction white sugar used a positive dilute phase pneumatic conveying, according to the classification suggested by Klinzing et al. (2010) and Mills (2004). This system included three air blowers, operating at a pressure range of 0.40 to 0.67 bar and pressure limit at 0.80 bar. The airflow generated by blowers was 16.5A m3/min, with an estimated airspeed of 22.0 m/s and conveying flow speed at approximately 11.0 m/s.

2.2.2.Determination of particle size distribution of white sugar

Particle size distribution of BPC and APC white sugar samples was determined with five replications for each sample, following the GS2/9-37 method of International Commission for Uniform Methods of Sugar Analysis (ICUMSA 2007). The average of particle size (millimeters) was calculated by the Rens method (ICUMSA 2007).

2.2.3. Flour rheological analysis

Rheological properties of wheat flour (utilized as the control) and the blends (wheat flour + white sugar, 1:2.4 w/w) were analyzed using Mixolab (Chopin, France), according to methods 173 (ICC 2011) and 54-60.01 (AACC 2000). Equipment settings followed recommendations of International Association for Cereal Science and Technology (ICC 2011), as seen in Table 1.

Table 1. Configuration of Mixolab equipment for rheological analysis

Settings	Values
Mix speed	80 rpm
Temperature 1st Phase (C1)	30°C
Temperature 2nd Phase (C2)	30 - 60°C
Temperature 3rd Phase (C3)	60 - 90°C
Heating rate	4°C/min
Temperature 4th Phase (C4)	90°C
Time 4th Phase (C4)	15 min
Temperature 5th Phase (C5)	90 - 50°C
Cooling rate	4°C/min

C1 to C5 correspond to each peak of a typical curve generated by the equipment

Analyses included water absorption (%), development and consistency (C1), protein reduction (C2), starch gelatinization (C3), amylase activity (C4), and starch retrogradation (C5). Codes C1 to C5 corresponded to each peak of a typical curve generated by the equipment and the unit used was the newton-meter (Nm) (Banu et al. 2011; Jia et al. 2011; Mironeasa et al. 2012).

2.2.4. Cookie dough production

The cookie dough was prepared using the "cream method" described by Moretto and Fett (1999) and Manley (2000). First, the fat, emulsifier, flavoring, acidifying, baking powder, salt and water were homogenized and then wheat flour, sugar and starch were incorporated. The percentage of ingredients is confidential information of industry.

2.2.5. Cookie dough consistency and stability

Analyses of consistency and stability of cookie doughs utilized the MIXOLAB equipment (Chopin, France), according to the method applied to the industrial process (Chopin 2009). For this purpose, 100 g of cookie dough was submitted to mechanical work at 100 rpm, for 10 min at 30°C. The torque generated in C1 (Nm) calculated cookie dough consistency and its stability was determined by the time of permanence in this consistency. The test settings are the same as those described in Table 1 and results were obtained by the average of three replicates.

2.2.6. Cookie dough texture

The TAXT plus texture analyzer (Stable Micro Systems, UK) measured the cookie dough hardness (expressed in Newton) using a 6 mm (P/6) cylindrical probe and an accessory (A/DP). Six replicates with 110 g of dough was evaluated and the test conditions were pre-test speed was 2.0 mm/s, test speed 3.0 mm/s, post-test speed 10.0 mm/s and distance was 20 mm.

2.2.7. Statistical Analysis

Results expressed the mean ± standard deviation. Statistical analysis of variance and Tukey test ($p < 0.05$) were performed using Statistica software (version 7.0).

3.Results and discussions

Pneumatic conveying had an impact on the distribution of white sugar particle size, reducing significantly the size of all samples, around 23% (Table 2). Although white sugar particle size from supplier A was smaller ($p<0.05$) when compared to suppliers B and C in the BPC condition, after pneumatic conveying, no differences were observed for the three suppliers samples. These results are important for the food industry, which can use raw materials from different suppliers, but need to maintain the same characteristics in the final product.

Bell (2015) relates that in a pneumatic conveying system, the reduction of particle size is typically caused by high-velocity impacts or attrition between particles or between particles and system walls. Reduction on particle sizes increases the dissolution rate of white sugar in water, accelerating the mixing process or dissolution of the ingredients (Oliveira et al. 2007). For cookie production, considering the relationship between the sugar particle sizes and cookie dough baking process, any interference that can cause variations in the sugar particle size will reflect on the dough rheological behavior.

Rheological characteristics of wheat flour blended with sugar samples (Table 3) showed a reduction in water absorption and stability when compared to the control (wheat flour). This result suggests that this reduction is associated with competition between sugar and wheat flour by the available water.

Wheat flour had lower protein reduction (C2) than the blends, indicating an increase in consistency of dough due to the presence of sugar. Starch gelatinization (C3), α-amylase

activity (C4), and starch retrogradation (C5) of the blends were also higher than the control, showing that white sugar reduced dough viscosity and α-amylase activity, but increased starch retrogradation.

Table 2. Distribution of white sugar particle size from A, B, and C suppliers, before pneumatic conveying (BPC) and after pneumatic conveying (APC)[1]

Condition of sugar	**White sugar particle size (mm)**		
	A supplier	**B supplier**	**C supplier**
BPC	0.57 ± 0.01^{bB}	0.61 ± 0.02^{aA}	0.62 ± 0.02^{aA}
APC	0.46 ± 0.04^{cC}	0.47 ± 0.02^{cC}	0.45 ± 0.05^{cC}

[1]Mean values ± standard deviation of five replicates. Different lowercase letters in the same line and different uppercase letters in the same column are significantly different by Tukey test ($p < 0.05$)

Table 3. Analyses of wheat flour blended with white sugar from A, B, and C suppliers before pneumatic conveying (BPC) and after pneumatic conveying (APC)[1]

Analysis	**Wheat flour**	**Wheat Flour + White sugar**					
		BPC			**APC**		
		Supplier A	**Supplier B**	**Supplier C**	**Supplier A**	**Supplier B**	**Supplier C**
WA (%)	61.70±0.00	29.50±0.00	29.50±0.01	30.0±0.00	27.40±0.00	27.40±0.00	27.40±0.00
C1(Nm)	1.12±0.01	1.11±0.01	1.20±0.06	1.04±0.08	1.15±0.06	1.17±0.08	1.10±0.02
Stability[2]	10.52±0.23	0.99±0.15	0.95±0.07	1.12±0.20	2.93±0.58	1.64±0.19	1.92±0.32
C2 (Nm)	0.47±0.02	0.74±0.02	0.78±0.03	0.71±0.05	0.71±0.41	0.88±0.07	0.88±0.02
C3 (Nm)	1.69±0.03	0.98±0.01	0.99±0.02	0.97±0.03	0.86±0.53	1.1±0.04	1.11±0.01
C4 (Nm)	1.42±0.04	0.21±0.01	0.19±0.01	0.19±0.00	0.54±0.54	0.21±0.01	0.21±0.01
C5 (Nm)	2.06±0.03	1.81±0.04	1.87±0.12	1.83±0.01	1.84±0.08	1.46±0.08	1.60±0.04

[1]Mean ± standard deviation of three replicates. [2]Values expressed in minutes. WA: water absorption; C1: development and consistency; C2: protein reduction; C3: starch gelatinization; C4: α-amylase activity; C5: starch retrogradation

Table 4. Rheological evaluation of wheat flour blended with white sugar based only on sugar condition: before pneumatic conveying (BPC) and after pneumatic conveying (APC)

Analysis	Wheat Flour	Wheat Flour + sugar (BPC)[1]	Wheat Flour + sugar (APC)[1]
Water absorption (%)	61.70±0.01[a]	29.50±0.28[b]	27.40±0.00[c]
C1 (Nm)	1.12±0.01[a]	1.12±0.09[a]	1.13±0.06[a]
Stability (min)	10.52±0.23[a]	1.02±0.15[b]	3.27±3.89[b]
C2 (Nm)	0.46±0.02[b]	0.74±0.04[a]	0.82±0.22[a]
C3 (Nm)	1.69±0.03[a]	0.98±0.02[b]	1.02±0.29[b]
C4 (Nm)	1.41±0.04[a]	0.19±0.01[b]	0.32±0.31[b]
C5 (Nm)	2.05±0.03[a]	1.83±0.07[b]	1.63±0.18[c]

[1]Mean ± standard deviation of three suppliers. C1: development and consistency; C2: protein reduction; C3: starch gelatinization; C4: α-amylase activity; C5: starch retrogradation. Different letters in the same line are significantly different by Tukey test ($p < 0.05$).

Table 5. Effect of white sugar before pneumatic conveying (BPC) and after pneumatic conveying (APC) on the consistency, stability, and hardness of cookie doughs

Sugar Sample		Consistency (Nm)1	Stability (min)1	Hardness (Nm)2	Hardness Average
BPC	**Supplier A**	1.70±0.05	9.73±0.06	1.50±0.14	1.38 ± 0.16[b]
	Supplier B	1.72±0.04	9.69±0.03	1.26±0.18	
	Supplier C	1.57±0.07	9.74±0.02	1.38±0.18	
APC	**Supplier A**	1.71±0.04	9.71±0.12	1.87±0.18	1.59 ± 0.28[a]
	Supplier B	1.71±0.19	9.69±0.07	1.51±0.13	
	Supplier C	1.67±0.17	9.63±0.03	1.40±0.28	

[1]Mean ± standard deviation of three replicates. [2]Mean ± standard deviation of six replicates. Different letters in the same column are significantly different by Tukey test ($p < 0.05$)

The conveying system was the only factor that affected rheological properties of blends ($p<0.05$). Thus, the Tukey test was applied to compare the averages of suppliers' results and to evaluate the influence of sugar condition (BPC and APC) on the rheological behavior of wheat flour and white sugar blends (Table 4). White sugar promoted a drastic reduction in

water absorption by wheat flour and the blend containing sugar APC showed the lowest rate of water absorption ($p<0.05$). This fact suggests that APC sugar competes for water more effectively than wheat flour, due to the fact that dissolution rate of smaller particles is faster (Cauvain and Young 2006; Oliveira et al. 2007). Results of development and consistency (C1) were the same for the samples and control, indicating that the sugar particle sizes did not affect gluten behavior and dough development. Mixtures containing BPC and APC sugars also had the same stability, but with the shorter time than the control. This fact may be related to the competition between sugar and wheat flour for water molecules.

The BPC and APC sugar blends showed no differences for C2, C3, and C4 parameters, suggesting that sugar particle size did not influence protein weakening, starch gelatinization and α-amylase activity of blends. On the other hand, the addition of these sugars affected the rheological behavior of wheat flour, reducing its starch gelatinization and α-amylase activity. An increase in the degree of starch gelatinization promotes a decrease in viscosity of blends (Pareyt et al. 2009). According to Champ (1992), much of wheat flour starch is encapsulated by a protein matrix that restricts the α-amylase activity. However, when flour interacts with water and is subjected to mechanical work and heating, this enzymes act on starches, and reduce the viscosity of the mixture.

Analysis of starch retrogradation (C5) differed for blends and control ($p<0.05$), with the highest value for control (2.05 Nm) and the lowest for APC sugar blend (1.63 Nm). In this case, not only the presence of white sugar but also the size of its particles influenced the consistency of mixture during the cooling process.

The BPC or APC white sugar condition did not affect consistency and stability of cookie dough; however, APC white sugar resulted in a harder texture of cookie dough (Table 5). The greater availability of this sugar to interact with other cookie dough ingredients (due to its small particle size) may be responsible for this fact, contributing to the structure of dough and, consequently, making it harder. For supplier factor, cookie dough made with white sugar from A supplier was significantly harder than those from B and C suppliers. This result can be explained by smaller particle size of white sugar from supplier A before submitting to the pneumatic conveying system, as seen in Table 2.

Cookie dough rheological analysis showed that sugar particle size did not influence their consistency and stability. These results are in accordance with those found for wheat flour blended with white sugar, showed in Table 4, which demonstrated that only addition of white sugar interfered in dough development ($p<0.05$).

4. Conclusions

White sugar submitted to the pneumatic conveying system had a reduction in particle size. The small particle size of sugar reduced water absorption and increased starch retrogradation rate of blends and resulted in a harder texture of cookie dough. Finally, although pneumatic conveying system promotes a reduction in the particle size of sugar, industries may continue to use this mechanism to transport sugar during the cookie manufacturing process, and further research on the effect of sugar particle size on cookie hardness is suggested.

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THE EFFECT OF BALANGU SHIRAZI (*LALLEMANTIA ROYLEANA*) GUM ON THE QUALITY OF GLUTEN-FREE PAN BREAD CONTAINING PRE-GELATINIZED SIMPLE CORN FLOUR WITH MICROWAVE

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ABSTRACT

In this study, pre-gelatinized simple corn flour with microwave and three levels of Balangu Shirazi gum were used in producing gluten free bread. The substructure of two types of flour was evaluated using the electronic microscope. The properties of bread samples including moisture, specific volume, porosity, crust color, texture and overall acceptance were studied. The results of electronic microscope showed that the granular structure of corn flour was coherent. However, the granular structure of pre-gelatinized corn flour had more structural expansion and swell because of demolished heat. In addition, samples containing pre-gelatinized corn flour and 2% Balangu Shirazi gum had the highest humidity in the first (22.5%) and third (19.4%) day. The highest porosity (23.3% and 23.5%) and specific volume (4.9 and 5.1 cm^3/g) and the minimal texture firmness were observed within 1 day after manufacturing (4.8 & 4.9 Newton) in samples containing pre-gelatinized corn flour and two levels of Balangu Shirazi gum (1% and 2%). Moreover, the results showed that increased L* colorful component (52% increase) caused by using the pre-gelatinized corn flour and raising the consumption level of gum. The presence of gum didn't have a significant impact on two colorful components such as a* and b*. The use of pre-gelatinized corn flour resulted in decreasing b* colorful component. Also, sensory evaluations gave the highest score of overall acceptance to samples containing pre-gelatinized corn flour and two levels of Balangu Shirazi gum (1% and 2%).

1.Introduction

Celiac disease is an autoimmune digestive disorder that created by the consumption of wheat gliadin, rye prolamin (secalin), barley (hordein) and oat (avenin). If a patient has a gluten-free diet continuously, an appropriate outcome is achieved through mucus and clinical improvement (Hamaker Bruce, 2008).

Gluten is an essential protein in forming the structure of flour and makes the elastic characteristics of bread dough. It has a role in the appearance and internal texture of many bakery products (Lopez *et al*., 2004). Removing this

protein from wheat flour or using gluten-free flours like corn, rice, millet, sorghum and so on in formulation of bread, cake, biscuits, muffin and other bakery products makes an important appearance and technological problems and results in decreased product marketing (Gallagher *et al*., 2004). In the segmentation of starch types, which include varieties of cereals, roots and tubers and the waxy, corn and wheat are in one group, namely, starch from cereals. The study of granular characteristics such as granule type and diameter, morphology, gelatinization temperature, amylose content, and baking characteristics indicate a high similarity between corn starch and wheat starch (Swinkels, 1985).

In the field of gluten-free products, food industry researchers commonly advice producers to use types of additives in order to solve this problem and follow the gluten's properties. On the other hand, promoting consumers' awareness about damages caused by chemical additives and trend toward natural and local additives to make nutritive and community health have been increased.

Balangu Shirazi (*Lallemantia royleana*) is one of these additives that extracted from local seeds. Benth plant *Lellemantia royleana (Benth. In Walla)* which is known as Balangu Shirazi in the Persian language belongs to dicotyledon class, sympetalous subclass, lamiaceae order, mint family and lallemantia genus. Mint family has a good distribution in different areas of Iran with 46 genera and 410 species and subspecies (Naghibi *et al*., 2005). Dark and oval seeds of this plant have completely two distinct dorsal and inner surfaces which covered by many tiny hollows. The dorsal surface is convex and has four or five subtle vertical lines. The inner surface has a distinguished vertical prominence in the middle. The narrower part of the inner surface is dent and has a small round and white buttony prominence (the junction of seed and the axis of an ovary) exactly at the top of the prominent midline and terminal edge (Razavi *et al*., 2008). Balangu's seed is a rich source of fiber, oil and protein has a healthy, nutritive and pharmacological effects (Naghibi *et al*., 2005). It creates a sticky and cloudy liquid in the water which has no taste (Razavi *et al*., 2008). It's applied to various traditional products like ice-cream, bread in Iran and Turkey and a kind of juice (chia) (Razavi and Karazhiyan, 2009). In addition, aromatic seeds of this plant are traditionally used in the treatment of failures such as gum bleeding, coughs caused by a cold and kidney problems and also applied as a pain relief, sedative, stimulant, and diuretic drug due to having mucilage.

Despite the use of additives as a gluten replacement, gluten-free products or those containing weak flour have stiff or dry texture. Also, the duration of their maintenance is short and become stale very soon. These products have no taste and scent and have a wrinkled and colorless crust. They're not acceptable for consumer (Sahraiyan *et al*., 2013). Different methods are recommended to decrease staling and improve the texture of these types of products like using flour and pre-gelatinized starch. Pre-gelatinized starch is one type of modified starches which sometimes called instant starch. To produce instant starch, first, natural starch is gelatinized and then changed to dry powder. An important difference between thermally modified starches and chemical modified ones is that increased viscosity would be made more quickly in gelatinized type. It means that they rapidly absorb water like a sponge (Anastasiades *et al*., 2002).

One of the thermal methods is to use microwave's waves to produce modified starches. These waves are a part of an electromagnetic spectrum that has 300-300000 MHz frequency. They're placed between dielectric and infrared waves in an electromagnetic spectrum (Zhongdong *et al*., 2005). However, microwave's waves have been applied for household uses more than half of the century, industrial uses of them have been limited to the recent years only (Vadivambal and Jayas, 2007). In a conventional thermal method, the heat is transferred from the warm point to cool spot which leads to increase temperature

and warm up substances gradually. In the thermal method with microwave, severe vibration oscillations were generated in bipolar molecules and ions. Interaction of these molecules with each other leads to increase foodstuff temperature very rapidly. On the other hand, warming up the foodstuff is accelerated quickly. So, thermal processing in the microwave is an effective factor to reduce waste of energy and time because of warming up the inner parts of foodstuff (Bilbao-Sainz *et al*., 2007; Zylema *et al*., 1985).

Zylema *et al*. (1985) compared the granular structure of the starch system in the water during thermal processing in the microwave and showed that there is no difference between the size of swell in samples heated by microwave and those heated by the conventional method under the condition the heat is equal. But, the time and energy were saved. In the case of microwave application, the amylose content, particle size diameter, pasting temperature and gelatinization temperature increase and parameters such as pasting viscosity, and solubility of all the heat-treated starches are reduced. Of course, it should be noted that due to the application of both types of microwave heating and conventional heating, changes in the structure of starch granules, loss of birefringence, rupture of granules and leakage of the constituent of starch occur (Uthumporn *et al*., 2016). Ndife *et al*. (1998) studied how to be gelatinized different starches' granule during heating in the microwave. They presented a quantitative model to designate the relation between gelatinization of granules and existing water content. Moreover, relatively wide researches have been performed about physicochemical properties and molecules and modification of different starches using the thermal processing with microwave (Anderson and Guraya, 2006; Horchani *et al*., 2010). Various studies have shown that pregelatin corn flour has a good ability to swell in cold water, which makes it easier for consumers to pre-gelatinized corn flour powder to be bake at a lower temperature (Joshi *et al*., 2014; Jiamjariyatam *et al*., 2015). So in this study, given the society need to gluten-free products with desirable sensory characteristics and texture, corn flour (pre-gelatinized flour with microwave or simple flour) and local gum of Iran (Balangu Shirazi) were used to produce gluten free bread and gluten free samples compared to the bread containing wheat flour.

2. Materials and methods

2.1. Materials

Corn flour with 9.3% moisture, 9.4% protein, 3.3% fat and 2.6% ash was provided by Golha manufactory (Tehran-Iran). For this purpose, needed flour was prepared to do an examination and maintained in the fridge at $4^{\circ C}$. Also, yeast (Saccharomyces cerevisiae) that was in the form of active dry yeast powder and packed as a vacuum, supplied by Razavi Yeast company (Mashhad, Iran). Other products like lecithin powder, albumen powder, Balangu Shirazi seed and sugar, salt and vegetable oil were supplied by Toosarjan company (Mashhad, Iran), Golpoodr Golestan company (Gorgan, Iran), local market and stores with confectionary raw materials, respectively. Other chemical substances were provided by Merck company (Germany).

2.2. Preparation of pre-gelatinized corn flour with microwave

Majzoobi and Farahanky method (2008) was used with changes to produce pre-gelatinized corn flour. In this method, 12g flour was poured within 20 ml water inside the glass container and mixed for five minutes at the temperature of $55^{\circ C}$ in the hot water (W350b model, Electronic Fater Company of Iran). Then, the obtained mixture was placed into the household microwave (LGMC-789Y, made in Korea) with the 360W power and 2450MHz frequency for five minutes until it dried. Finally, the produced sample was ground and sieved until a complete homogeneous powder obtained.

2.3. Preparation of images from substructure of corn flour (simple and pre-gelatinized) with the electronic microscope

Before using it with an electron microscope, they use a very thin layer of gold to create conductivity in the sample, so that the photos have sufficient resolution. The sputter-coated with gold, in addition to the injection of gold on the sample, also applies a vacuum. In the case of higher than normal moisture, the sputter-coated with gold does not function properly. For this reason, flour sample was dried during 24hrs using the freeze dryer, FD-10V model, made in Iran. A thin layer of samples was fixed on the special aluminum bases by liquid glue. Then, the gold metal was spread on the samples by sputter-coated with gold. In this study, the device was a product of Bal-tec Company from Swiss. Samples that were completely covered by gold, transferred to the electron microscope with a maximum voltage of 30KW. Also, the magnification was 1500. In this study, scanning electron microscope (SEM) made by Philipps Company from Netherland, XL30 model, was used (Ahlborn *et al*., 2005).

2.4. Producing dough and pan bread

To produce bread dough, the below formula was used based on 100g corn flour: flour (100g), water (100g or equal to the weight of corn flour), salt (1.5g), sugar (1g), albumin (0.5g), lecithin powder (0.75g), Saccharomyces cerevisiae yeast (2g), and different levels of Balangu Shirazi gum (zero, one and two g). Samples were produced with two types of corn flour such as simple and pre-gelatinized. Table 1 shows the treatments in this study. To produce bread, first, all of the solid substances blended with each other and needed water added to it. The dough was mixed with 150 revolutions per minute during 10 mins and 1% oil added to the formulation in the sixth minute. The preliminary fermentation was performed in the fermentation container with 80% relatively moisture and 30°C temperature during 30 mins. Doughs were divided into 50g pieces and placed in the template with 4 cm diameter and height. Final fermentation was carried out during 40 mins, 30°C temperature and 80% relative moisture. After steaming for 10s, baking was done in the rotary oven (Zuccihelliforni, Italy) for 20 mins with 200°C temperature. After cooling, each sample was packed in polyethylene bags for the next examination and maintained at the ambient temperature (25°C).

Table 1. Treatments containing two types of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum

	Treatments
1	Corn flour
2	Corn flour + 1% Balangu gum
3	Corn flour + 2% Balangu gum
4	Pre-gelatinization corn flour (PG-Corn)
5	Pre-gelatinization corn flour (PG-Corn) + 1% Balangu gum
6	Pre-gelatinization corn flour (PG-Corn) + 2% Balangu gum

2.5. Moisture

To do this examination, AACC 2000 standard with the number of 16-44 was used. For this purpose, samples were placed in the oven (Jeto tech, OF-O2G model, South Korea) during 2hrs interval with 100°C-105°C heat after baking.

2.6. Porosity

Image processing technique was used in order to evaluate the porosity amount of bread during 2hrs interval. For this purpose, a slice of bread with the dimension of 2×2 cm was provided using the 120W electric saw knife (41600 model). It was shot using the scanner (HP Scanjet G3010 model) and 300-pixel resolution. The produced picture was analyzed by Image J software (National Institutes Health, USA). Gray level images were produced with activating of a part bit of the software. To change gray level images to binary ones, binary Images section of software got activated. These images are a collection of bright and dark spots. Calculating the ratio of bright to dark points is

estimated as an index for porosity amount of samples. It is obviously understood that the more proportion the more porosity of the achieved bread might exist (Naji-Tabasi and Mohebbi, 2015). With activating analysis part of the software, the ratio was calculated and porosity percent of samples was measured (Barcenas and Rosell, 2006; Sabanis *et al*., 2008).

2.7. Specific volume

Rapeseed replacement method was used according to AACC 2000 standard, 10-72 number, to measure specific volume. For this purpose, a piece of bread with the dimension of 2×2 cm was produced from the geometric center of it during 2hrs interval after cooking and its specific volume was determined.

2.8. Texture

To perform a penetration test, texture analyzer QTS, CNS Farnell, UK model, was used during 2 and 72hrs after baking. The main purpose of this test is to determine required force for a distinguished compression. The amount of resistance is considered as an index of sample staling. AACC 2000 standard, 74-90 number, a method was used to do this test. For this purpose, a 25 mm sample was separated from a central part of the bread. The pressure test was done at the speed of 10 mm per minute using the related probe with 21 mm diameter. During the first compression, the obtained maximum power was considered as a bread firmness and resistance per Newton.

2.9. Crust color evaluation

The analysis of crust color was performed by assigning three indicators of L*, a* and b*. L* indicator shows the sample brightness and its domain is variable from zero (pure black) to 100 (pure white). a* indicator shows that how the sample color is close to green and red colors. Its domain is variable from -120 (pure green) to +120 (pure red). b* indicator shows that how the sample color is close to blue and yellow colors. Its domain is variable from -120 (pure blue) to +120 (pure yellow). To measure these indicators, first, a slice of bread crust was provided in a dimension of 2×2 cm using the 120W electric saw knife, 41600 model. It was shot by the scanner (HP Scanjet G3010) with 300-pixel resolution. Then, the pictures were analyzed by Image J software. With activating LAB space in the plugins part, the above indicators were calculated (Sun, 2008).

2.10. Sensory test

To do this, 10 referees were collected among the faculty members of Razavi Khorasan research and education center of agriculture and natural resources (Mashhad-Iran) based on Gacula and Singh method (1984) and triangle test. In this method, two identical and similar samples (control) are compared with one of the different treatments. After selecting a different sample and expressing the cause of the difference (better or worse), this sample is compared with the two samples, using the five-point hedonic method to this sample and each sensory characteristics in comparison with the points given to the sample. Then, sensory properties of bread were assessed in terms of shape (asymmetric shape, rupture or destruction of a part of bread and the existence of any hollow or internal space), the properties of upper surface (such as burn, unusual color, wrinkle and abnormal surface), the properties of bottom surface (like burn, wrinkle and abnormal surface), porosity (abnormal pore and more compression), texture firmness and softness (being dough or abnormal softness, being firmness and fragility and crispiness), chew ability (being dryness and firmness, being dough in the mouth and sticking to the teeth) and smell and taste (spicy taste, raw and rancidity smell and/or natural scent). These properties aren't effective equally. So, 4, 2, 1, 2, 2, 3 and 3 coefficient ranking was given to each property after studying references. Finally, total score (quality number of bread) was calculated using the below formula with having this information. The evaluation of properties was from very bad (1) to very good (5). Overall acceptance (quality

number of bread) was calculated by Equation (1):

Equation (1) $$Q = \frac{\sum(P \times G)}{\sum P}$$

Q = Total acceptance (quality number of bread)
P = Coefficient of properties ranking
G = Coefficient of properties assessment

2.11. Statistical analysis

All samples were evaluated in three batches. In order comparison of the type of simple and microwave heated corn flour, as well as to determine the effects of Balangu Shirazi (*Lallemantia Royleana*) gum and to assess significant differences among treatments, a complete randomized design of triplicate analyses of six samples was performed using the Statistical Package for the Social Science (SPSS) 14.0 software (SPSS Inc., Chicago, IL, USA). The significant differences between the mean values of in the bread sample analyses were determined by using analysis of variance (ANOVA) independent t-test and Duncan's new multiple range tests were used to study the statistical differences of the means with 95% confidence.

3. Results and discussions

3.1. The obtained images from the electronic microscope

Figure 1 (A and B) shows the microscopic images of corn flour and pre-gelatinized corn flour with microwave, respectively. As the images show, granular structure of simple corn flour (unheated) is coherent and has a steadier surface while the structure of pre-gelatinized corn flour is destroyed due to microwave's heat. These granules have a more structural expansion and swell and, figure 1 (B) shows an uneven surface. In this regard, Majzoobi and Farahanky (2008) reported similar results and represented that starches which are influenced by thermal processes (electrical oven or microwave), their granular structure is destroyed whereby starches' molecules easily interact with the water. Also, Majzoobi and Farahanky (2010) reported that natural starch has a coherent granular structure and spherical shape and in an ambient temperature it's not able to absorb water and increase viscosity based on their own results of the paper. Further studies with electron microscopy on pre-gelatinization buckwheat flour with thermal processes (roasting, steaming, extrusion, boiling and microwave) (Sun *et al*., 2018), as well as corn flour and rice flour pre-gelatinised by microwave (Uthumporn *et al*., 2016), altered natural structure of starch granules was very distinct.

3.2. Moisture

The results of produced samples' moisture have shown in table 2 during 2 and 72hrs interval after baking. As results show, the moisture of samples containing pre-gelatinized corn flour is more than those which have simple corn flour. In addition, the results show that the increase of Balangu Shirazi gum formulation can cause an increase in produced samples' moisture. This samples' moisture was similar in both 2 and 72hrs interval after baking. The sample which contains pre-gelatinized corn flour and 2% Balangu Shirazi gum has the most amount of moisture and has a similarity with a moisture of control sample (containing corn flour). It is likely that the higher amount of moisture in samples with pre-gelatinized corn flour is because of increasing water absorption than samples containing simple corn flour. This increase can be caused by the connection between water's molecules and starch chains (Tester and Karkalas, 1996).

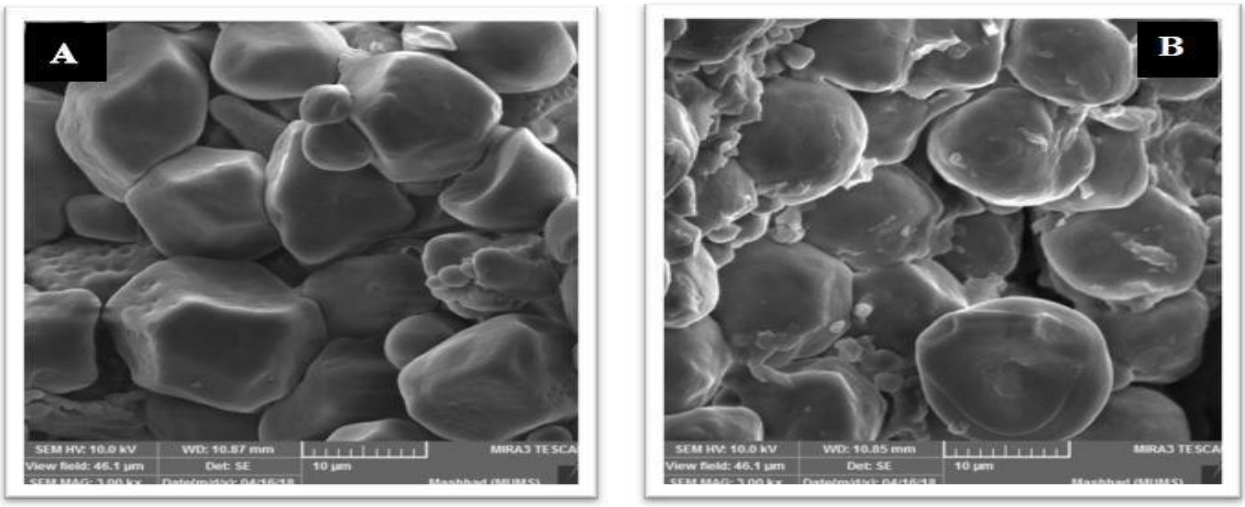

Figure 1. Microscopic images from corn flour (A) and pre-gelatinized corn flour with microwave (B)

On the other hand, heating up corn flour with microwave along with vibrational motions of water molecules can lead to more penetration of water into the starch's chains (Palav and Seetharaman, 2007). Moreover, Majzoobi and Farahanky (2008) presented that starches which are influenced by thermal processes, their granular structure is destroyed and starches' molecules interact with the water easily and result in increased absorption of water of dough and increased the moisture of final product. In a way of increasing amount of moisture with an increased amount of gum in a formulation of bread, Sahraiyan *et al*. (2013) presented that increased water maintenance capacity in gums is due to hydrophilic properties and existence of hydroxyl groups in gums' structure. In addition, McCarthy *et al*. (2005) reported that gums interact with the water because of a hydrophilic essence and decrease water release and strengthen the presence of water into the system by which water absorption is increased and the moisture of final product is maintained after the baking process.

Table 2. The effect of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum on the moisture of gluten-free bread during 1- and 3-day interval after baking

	Treatment	Moisture	
		First day	Third day
1	Corn flour	14.8 ± 0.2^{f}	9.7 ± 0.3^{e}
2	Corn flour+1% Balangu gum	19.7 ± 0.0^{d}	14.8 ± 0.4^{c}
3	Corn flour+2% Balangu gum	21.4 ± 0.5^{b}	17.9 ± 0.5^{b}
4	Pre-gelatinization corn flour	17.4 ± 0.5^{e}	13.4 ± 0.3^{d}
5	Pre-gelatinization corn flour+1% Balangu gum	20.6 ± 0.3^{c}	15.1 ± 0.3^{c}
6	Pre-gelatinization corn flour+2% Balangu gum	22.5 ± 0.2^{a}	19.4 ± 0.5^{a}

Similar words in each column don't have a significant difference statistically in P<0.05 level.

In a review by Kang *et al*. (2018) which was used in combination with modified starches and gums for the production of bread, it was found that the interaction of water with starch was higher than that of water with flour, due to the migration of moisture inside bread and the achievement of equilibrium during storage can affect bread's longer shelf life.

3.3. Porosity

Figure 2 shows the results of produced samples' porosity. According to the results, samples containing pre-gelatinized corn flour and 1% and 2% Balangu Shirazi gum have most porosity among produced samples. The porosity amount of these two samples doesn't have a significant difference ($P<0.05$). But the porosity in a sample containing corn flour and 2% gum is more than 1%. It shows that gelatinization of flour or starch in a formulation of gluten free bread is associated with a decreased gum. It is more likely that increased dough viscosity can cause decrease of consumable gum in case of using the gelatinized flour. Ziobro *et al*. (2012) found similar results based on a study of the effect of modified starches on gluten-free bread and dough properties and said that use of modified starch could cause an increased amount of porosity than simple starch in gluten-free products formulation by decreasing size, increasing gas cells and their homologous distribution in product texture. In addition, corn starch contains about 25% amylose and 75% amylopectin, because amylopectin, due to its lateral branches, can improve the volume, increase the smoothness of gaseous cells and porosity, and ultimately create a coherent structure in the final product (Lopez *et al*., 2004; Rathnatake *et al*., 2018).

Another important parameter of the kernel of bakery products is porosity. It totally implies the pore structure in the kernel of this category of foodstuffs and is an effective factor in qualitative properties of products kernel in baking industries (Armero and Collar, 1996). The porosity of texture kernel is impressed by the number of hollows in the kernel of texture and their distribution. The porosity amount of final product will be more if the number of hollows and gas cells are more and their distribution is more homolog. In a study conducted by Ozkocozge *et al*. (2009), it was presented if gums are used in desirable levels in formulation of foodstuffs so that they don't disturb the distribution of existing air bubbles in the sample, they strengthen the gas cells by decreased connection of gas cells due to making a thick layer on the surface of cells. So, each cell remains separately and its size is smaller. This issue has an impressive effect on increased porosity. In this study, it seems that the best result has been obtained for porosity using the gelatinized corn flour and 1% Balangu Shirazi gum. It means that the produced product has a desirable number of gas cell (by maintenance the gas cells in the dough and during the baking process) and homologous distribution of these cells in gluten-free bread.

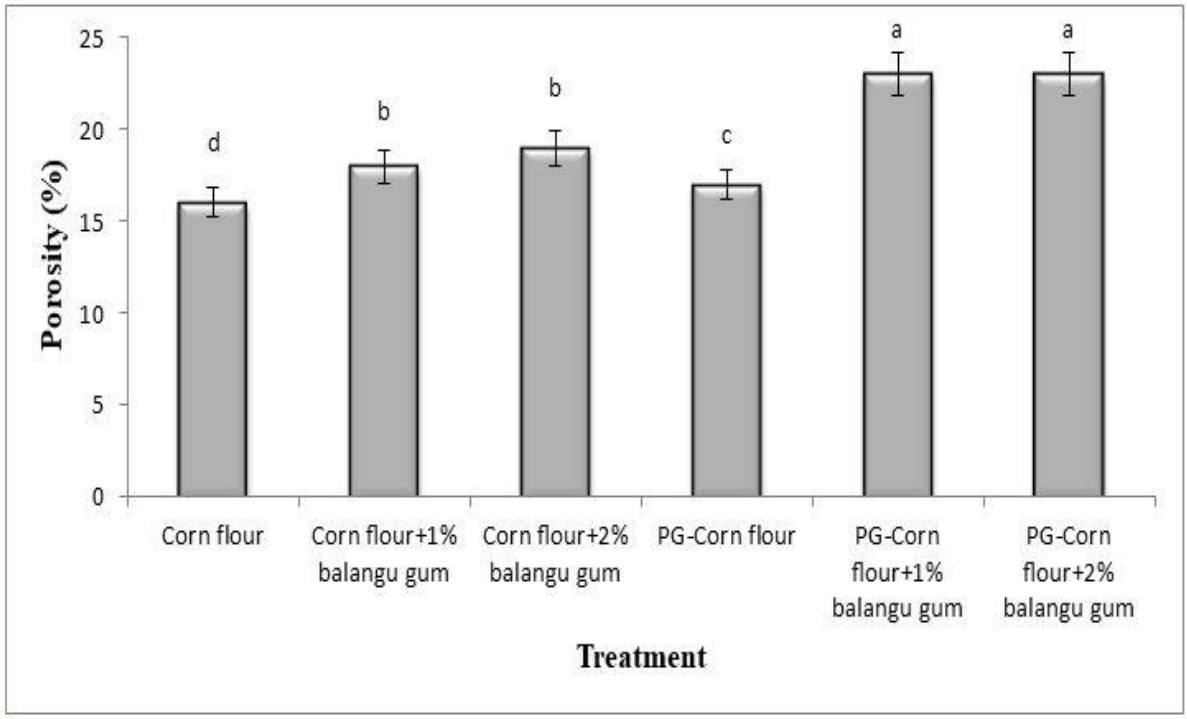

Figure 2. The effect of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum on the porosity of gluten-free bread

Similar words in each column don't have a significant difference statistically in P<0.05 level.

3.4. Specific volume

Table 3 shows the results of the specific volume of produced samples. Based on results, samples containing pre-gelatinized corn flour with 1% and 2% Balangu Shirazi gum have the most amount of specific volume among produced samples. As results show, samples containing pre-gelatinized corn flour have more specific volume. Also, the results demonstrate that there isn't a significant difference in specific volume of produced samples with adding 1% or 2% Balangu Shirazi gum to a formulation of gluten-free bread (P<0.0.5).

The specific volume of gluten-free bread or breads containing weak flour depends on several factors. One of these factors is the activity of bakery yeast and production of a desirable number of gas cells by the yeast. The second factor is the maintenance of gas cells in bread dough and prevention from their exit before baking process. Dough viscosity in a cold water can be an important factor in this regard. The third factor is to maintain the gas cells during the baking process. The wall of existing gas cells in gluten-free bread dough is strengthened during this process. Increased temperature causes the expansion of gas cells. If the wall doesn't have a good resistance, cells are ruptured and several gas cells join each other and the final product doesn't have a good volume (Sahraiyan *et al*., 2013). Therefore, given the reports by Sahraiyan *et al*. (2013), it seems that heated corn flour in the microwave (gelatinized corn flour) has a more viscosity of cold water and could maintain the number of gas cells with increasing viscosity. In this regard, Majzoobi and Farahanky (2010) reported that natural starch is not able to absorb water and increase viscosity because of coherent granular structure in ambient temperature while in thermal processes, the viscosity is made in the cold water due to gelatinizing starch. In addition, Majzoobi and Farahanky (2008) presented that starches which are influenced by thermal processes, their

granular structure is destroyed and starch's molecules interact with the water easily and cause the increase of viscosity in an ambient temperature. Balangu Shirazi gum can strengthen the wall of gas bubbles and prevent from their rupture during the baking process. In this study, it's observed that there's not a significant difference between samples containing 1% and 2% gum ($P<0.05$). It shows that produced samples need to most volume to achieve 1% gum. Even it's possible the volume of sample is decreased with increasing gum more than 2%. Because the wall of gas cells becomes thick and strong and prevents from the expansion caused by increased temperature during the baking process. In this regard, Shittu and Abulud (2009) reported similar results and represented that the exact consumable level of gum is essential in bakery products because if the right concentration is chosen given the formulation, the gas maintenance ability is increased by raising the stability and permeability of the wall of gas cells. If the use of this gum is more than required need, it acts reversely. Reducing volumes as a result of excessive gum consumption is due to the increased consistency and increased elasticity, whereby gaseous cells can not be well bonded, grow and affect the increase of the specific volume of the product (Yaseen *et al.*, 2010; Bourekoua *et al.*, 2016).

Table 3. The effect of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum on specific volume and stiffness of gluten-free bread texture during 1- and 3-day interval after baking

	Treatment	Specific volume (Cm3/g)	Firmness (N)	
			First day	Third day
1	Corn flour	1.9 ± 0.2 ^{c}	11.9 ± 0.4 ^{a}	22.5 ± 0.2 ^{a}
2	Corn flour+1% Balangu gum	3.1 ± 0.4 ^{b}	7.1 ± 0.5 ^{c}	10.7 ± 0.4 ^{c}
3	Corn flour+2% Balangu gum	3.2 ± 0.2 ^{b}	8.0 ± 0.3 ^{bc}	10.5 ± 0.7 ^{c}
4	Pre-gelatinization corn flour	2.1 ± 0.2 ^{c}	9.2 ± 0.4 ^{b}	18.2 ± 0.4 ^{b}
5	Pre-gelatinization corn flour+1% Balangu gum	5.1 ± 0.5 ^{a}	4.9 ± 0.2 ^{d}	8.6 ± 0.4 ^{d}
6	Pre-gelatinization corn flour+2% Balangu gum	4.9 ± 0.4 ^{a}	4.8 ± 0.1 ^{d}	6.5 ± 0.9 ^{e}

Similar words in each column don't have a significant difference statistically in $P<0.05$ level.

3.5. Texture

The results of texture firmness have shown in table 3 during 2 and 72hrs interval after baking. Based on results, samples containing pre-gelatinized corn flour with 1% and 2% Balangu Shirazi gum have the minimum amount of texture firmness among produced samples during 2hrs interval after baking. Also, the results of texture firmness during 72hrs interval after baking show that sample containing pre-gelatinized corn flour and 2% Balangu Shirazi gum has a minimum amount of texture firmness. It's needed to be mentioned that the firmness of all produced samples was raised during 72hrs maintenance.

It seems that the reason of superiority of pre-gelatinized corn flour with the microwave than simple corn flour is that exertion of heat on corn flour and production of pre-gelatinized corn flour increases the expansion of molecular

structure of starch in the water. It leads to more penetrate water into the starch network and improve the interactions of starch with the water. It could be expected that the final product has a soft texture with increased interaction of starch with the water and the maintenance of product's moisture. In this regard, Jiang *et al*. (2011) reported similar results with studying the physicochemical properties of the starch gel of rice. The texture firmness of products of baking industries depends on factors like the maintenance of product's moisture during the baking process and some technologic properties such as volume and porosity immediately after baking. The maintenance of moisture prevents from firmness and frangibility and the texture of product becomes softer after baking. On the other hand, two impressive factors that influence texture softness and decreased compression are increased volume and porosity due to the presence of a numerous number of air bubbles in the product and equal distribution of them. So, it's expected that samples with most moisture, volume and porosity have a fewer firmness.

Bread production and storage, especially based on starch, has led to numerous structural changes that ultimately lead to product destruction. These changes are mainly due to the immigration of water from crumb to crust and hardening of starch granules. Additives such as gum can be used to reduce this undesirable phenomenon (Horstmann *et al*., 2017; Gao *et al*., 2018; Witczak *et al*., 2019). Many searches have been conducted about using the gum in gluten-free products. In studies conducted by Moreira *et al*. (2013), Crockett *et al*. (2011), Demirkesen *et al*. (2010) and Onyango *et al*. (2009), similar results have been reported based on the decreased firmness of texture of produced products with adding 1% and 2% gum to a formulation of gluten-free products. Staling and increased firmness of bread that are started immediately after baking and increased during maintenance is a complicated process.

Several factors like amylopectin retrogradation, polymers rearrangement in an amorphous area, decreased moisture and/or distribution of moisture between amorphous and crystal areas are involved in this process (Ahlborn *et al*., 2005). Any kind of additives that can prevent from these factors especially decreased moisture and its immigration from internal texture to the crust, decrease the texture firmness during a maintenance period. It seems that the presence of Balangu Shirazi gum can prevent from an abnormal increase of product with the maintenance of moisture in internal gluten-free bread texture. Ribotta *et al*. (2004) attributed the increased firmness to bread moisture migration from the crumb to crust and recrystallization of starch in the absence of gum in a formulation of bakery products.

3.6. Crust color evaluation

The results of continuous colorful components (L^* a^* b^*) have shown in table 4. The results of this section show that use of pre-gelatinized corn flour and increased level of consumption of Balangu Shirazi gum in a formulation of gluten-free bread cause the increase of L^* colorful component. The presence of Balangu Shirazi gum didn't have a significant effect on two a^* and b^* colorful components ($P<0.05$) while the use of pre-gelatinized corn flour results in the decrease of b^* colorful component. Purlis and Salvadori (2009) represented that crust surface changes are responsible for crust brightness and soft and regular surfaces have more ability to increase L^* colorful component of crust than wrinkled surfaces. Obtaining these results weren't unexpected because samples containing pre-gelatinized corn flour and Balangu Shirazi gum were effective in making an equal flat surface with the maintenance of moisture during baking process and prevention from rapid immigration from internal texture to the crust. Color as one of the fundamental parameters may be influenced by factors such as process conditions, formulation and even the presence of specific materials (Marti *et al*., 2016). Also, it should be mentioned that texture properties like volume, porosity and texture firmness can affect the surface of final product except for the

maintenance of moisture. If the number of gas hollows is few, the extent of hollows is larger and their distribution is unequal and the texture firmness was more, light reflection is fewer from the surface because of excessive compression of sample surface and existence of large hollows in the surface. So, the glitter of a sample or L^* colorful component is decreased.

Similar results were reported about increased glitter by adding garden cress gum to the bread containing wheat and rice flour in a Sahraiyan *et al*'s study (2013) and adding xanthan gum to gluten free bread in a Mohammadi *et al*'s study (2014). On the other hand, Phattanakulkaewmorie *et al*. (2011) said that the presence of pigments and fibers in the flour and interaction that happens among components in formulation during producing dough and baking product are effective factors on a^* and b^* colorful components. It seems that the exertion of heat on corn flour and production of pre-gelatinized corn flour causes a decrease of yellow pigments in the flour. Consequently, b^* colorful component in gluten-free samples producing from this type of flour has decreased.

Table 4. The effect of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum on the color of gluten-free bread

	Treatment	Crust color value		
		L^*	a^{*ns}	b^*
1	Corn flour	31.6 ± 0.7 ^{e}	11.2 ± 0.5	24.2 ± 0.9 ^{a}
2	Corn flour+1% Balangu gum	39.2 ± 0.4 ^{c}	11.7 ± 0.8	24.8 ± 0.3 ^{a}
3	Corn flour+2% Balangu gum	42.8 ± 1.5 ^{b}	11.2 ± 2.3	24.1 ± 0.7 ^{a}
4	Pre-gelatinization corn flour	36.4 ± 0.8 ^{d}	12.5 ± 1.2	22.5 ± 0.5 ^{b}
5	Pre-gelatinization corn flour+1% Balangu gum	42.2 ± 0.6 ^{b}	10.5 ± 0.7	22.3 ± 0.3 ^{b}
6	Pre-gelatinization corn flour+2% Balangu gum	48.2 ± 0.8 ^{a}	9.8 ±1.2	22.5 ± 0.2 ^{b}

Similar words in each column don't have a significant difference statistically in P<0.05 level.
ns: Produced samples don't have a significant difference statistically in P<0.05 level.*

3.7. Overall acceptance

The score of overall acceptance of gluten-free pan breads has shown in figure 3. Sensory evaluators gave the score of overall acceptance to the samples containing pre-gelatinized corn flour and 1% and 2% Balangu Shirazi gum. The score of overall acceptance of these two samples didn't have a significant difference (P<0.05). Given the studied properties, obtaining this result wasn't unexpected. Taste referees gave the most score of porosity, texture softness and firmness, chew ability and shape to the samples containing pre-gelatinized corn flour and 1% and 2% Balangu Shirazi gum that affect the score of overall acceptance. The score of the properties of the upper and bottom surface is influenced by the color of the product greatly. Sensory evaluators gave the most score to the two mentioned samples in terms of surface properties. Given the results of colorful component evaluation of the crust, obtaining this score was predictable because these two samples had a high glitter. Also, samples containing pre-gelatinized corn flour and 1% and 2% Balangu Shirazi gum had desirable smell

and taste. It's likely that improvement of texture (firmness and softness, porosity and chew ability) in these two samples and perception of better feeling during chewing cause desirable

smell and taste. In this regard, many searches have been performed to study the effective factors on a release of taste. Most researches believed that perception of taste severity and release of tasty substances depends on the type of final texture of a product (Koliandris *et al*., 2008). For instance, a perception of the taste has been reported better in the texture having desirable firmness, cohesion, and coherence. The cause of this issue was the different interaction between tasty substance and texture structure according to Boland *et al*'s study (2004). In researches on the production of gluten-free bread based on pea flour Mastromatteo *et al*. (2015) and small broken rice berry flour (Numfon, 2017), addition of gum increased the parameters such as porosity, specific volume, texture, crust color, chew ability, and sensory properties so that the most score of overall acceptance was attributed to gum samples.

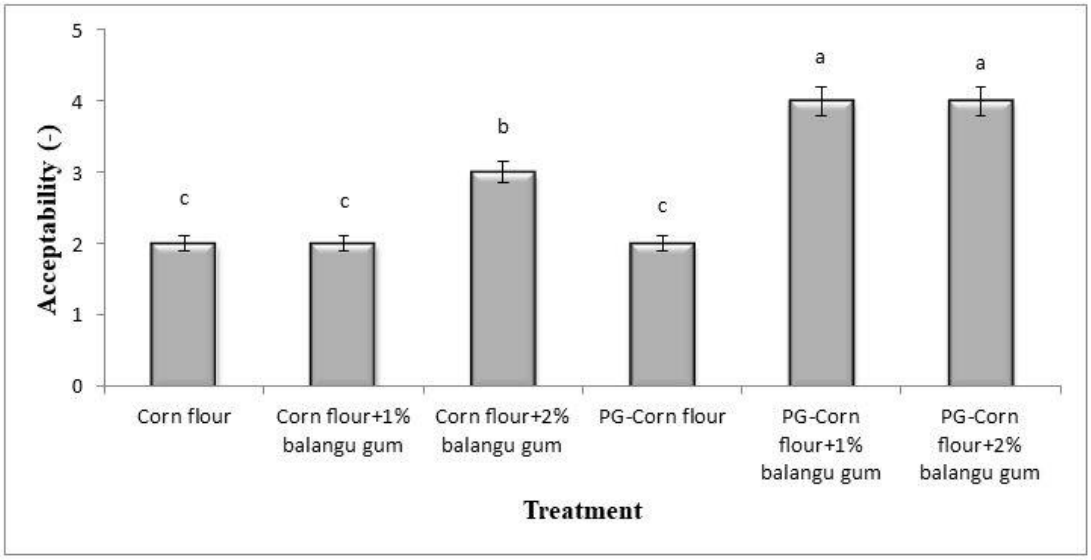

Figure 3. The effect of corn flour (simple and pre-gelatinized) and different levels of Balangu Shirazi gum on the overall acceptance score of gluten-free bread

Similar words in each column don't have a significant difference statistically in P<0.05 level.

4. Conclusions

Based on the results of this study, it could be said that pre-gelatinized corn flour is effective in increasing specific volume, porosity, and softness of gluten-free pan bread texture. In addition, high-quality gluten-free bread can be produced using the pre-gelatinized corn flour and 1% local gum of Balangu Shirazi which has a good marketing. Taste referees gave the highest score of overall acceptance to this sample and presented that sample containing pre-gelatinized corn flour and 1% local gum of Balangu Shirazi has a new and desirable smell and taste which is attractive for a consumer.

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VOLATILE COMPONENTS OF STRAWBERRY JAM

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ABSTRACT
Strawberry jams of such cultivars as "Ducat", "Honey" and "Polka" were studied to define the content of aromatic volatiles using the methods of highly efficient liquid chromatography. Volatiles contain a considerable amount of acids (65.6-76.8%), a small amount of furanone (8.3-14.6%) and that of aldehydes (3.4-10.8%). The share of esters in jams exceeds 0.7-3.1% of the total volatile amount. Typical compounds for strawberry jam flavor are hexanoic (caproic) acid, hexadecanoic acid, 2-ethyl hexanoic (capronic) acid, trans-cinnamic acid, linoleic acid, furil hydroxy methylketone, 2,5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane), furfural, 5-hydroxymethylfurfural, vanillin. As to aroma activity furanone derivatives dominate: 2.4-dioxy-2.5-dimethyl-3(2H)-furan-3-one, 2.5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane) та 2.5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol); they add sweet caramel scents to jams. For strawberry jams of cultivar "Polka", ethyl 2-methylbutanoate, decanal are active components which add fruity and grassy scents, strawberry jam made of cultivar "Ducat" – 2-decenal and γ- decalactone, 'Honey' - γ- decalactone (fruity, sweet) and linalool (sweet and floral scent).

1.Introduction

Garden strawberry is the most popular and valuable berry crop due to its high flavoring characteristics, fast and early ripening, not-demanding to growing conditions, high yielding capacity and high economic efficiency of growing. Strawberries are rich in sugars, organic acids, vitamins, phenol compounds, mineral substances, they have well-expressed typical flavor; they are in constant demand among consumers due to their high gustatory properties (Markovskyi *et al.*, 2008). Strawberries are consumed both as fresh and as juices, drinks, wines, puree, stewed fruits, jams (Amaro *et al.*, 2012).

Strawberry flavor is a combination of esters, aldehydes, ketones, ethyl, lactones, terpenic compounds, furanones (Larsen *et al.*, 1992; Larsen *et al.*, 1992, Forney *et al.*, 2000, Kafkas *et al.*, 2005). Esters are the main components (25-90% of the total amount), aldehydes and furanones – 50% (Larsen *et al.*, 1992; Larsen *et al.*, 1992). The latter add fruity and floral green and sweet or caramel flavors to strawberries (Pérez *et al*, 1996; Jetti, 2005; Kim *et al.*, 2013). Strawberry taste develops during ripening and it changes during storage (Zabetakis *et al.*, 1997; Forney, 2000) and processing for canned products (Lambert, *et al.* 1999).

One of the most popular processing products made of strawberry is jam which is due to high organoleptic properties, availability for consumers and long shelf-life of the product. To get strawberry jam with well-felt strawberry

flavor it is advisable to use the most fragrant berries (Lesschaeve *et al.*, 1991; Suutarinen *et al.*, 2002).

The main volatile compounds of strawberry jams belong to the classes of acids, alcohols and esters (Barron D. *et al.*, 1990; Kimura *et al.*, 1994.); they have natural origin and can be formed as a result of heat treatment (Sloan *et al.*, 1969). Thus, due to high temperatures of the treatment, changing sugar into caramel and Maiyar reaction, the product gets boiled, burned and caramel taste (Avasoo *et al.*, 2011), whereas green and fruity flavor, typical for fresh berries, becomes less expressed (Ozcan *et al.*, 2011). High furanol concentrations add typical caramel and sweet flavors to strawberry jams (Lesschaeve *et al.*, 1991; Pérez *et al.*, 1996).

However, not enough information is available in scientific literature concerning the content and composition of volatiles of jam made of strawberries of some pomological cultivars.

The purpose of our research was to identify the content and composition of volatiles of jam made of strawberry cultivars Ducat, Honey and Polka.

2. Materials and methods

2.1. Materials

The work was done in 2013-2014 with the berries of varieties "Ducat", "Honey", "Polka" in the laboratory of the department of the technology of storage and processing of fruits and vegetables at Uman national university of horticulture and at the experimental center of foodstuff quality control at the National institute of grape and wine "Magarach" (Ukraine).

2.2. Technological process

Strawberries were harvested at a technical stage of ripening, sorted by quality, cleaned and washed. Jams were made of the prepared berries according to current technological instruction (1992) adding pectin in concentration of 0.3%. Jam was boiled until the content of dry soluble substances reached 62%, packed in glass jars (250 cm^3). Jam was made of each pomological cultivar in five replications. The product was kept for six months at 20 °C.

2.3. Main instruments and equipment

Agilent Technologies 6890 chromatograph with mass-spectrometric detector 5973 and chromatographic capillary column DB-5 - internal diameter 0.25 mm and length 30 м – was used to identify volatiles of finished jam.

2.4. HPLC analysis on volatile components

A sample (0.75 g) was put in a 2-ml vial, and internal standard was added. Trydekan (50 mkg per sample) was used as an internal standard. To extract volatile substances, 0.5 ml of chloride methylene was added, the exposure time was 24 hours. The vial was carefully shaken several times. The extract received was taken with a micro-syringe, put into a 2-ml vial and digested in the flow of specifically clean nitrogen to the volume of 50 mkl. The concentrate was chromatographed.

A sample was injected into a chromatographic column in splitless mode, i.e., the flow was not divided; this allowed to eliminate losses and to increase the sensitivity of chromatography method considerably (10-20 times). The speed of the sample injection was 1.2 ml/min, and it took 0.2 min.

To identify the components, a library of mass spectrums NIST05 and WILEY 2007 with the total number of spectrums more than 470000 in combination with identification programs AMDIS і NIST was used.

The method of internal standard was used for quantitative calculations.

The calculation of the component content was made using the equation where:

$$C = K_1 \times K_2$$

C – volatile component content, mg/kg,

$$K_1 = \frac{П_1}{П_2}$$

П1 – peak area of the substance studied, П2 – peak area of the standard;

$$K_2 = \frac{50}{M}$$

50 – mass of the internal standard (mkg), introduced into a sample, M – a sample (g).

2.5. Statistical Analysis

Statistic analysis was made using StatSoft STATISTICA 6.1.478 Russian, Enterprise Single User (2007).

3.Results and discussions

38 components were identified in volatile concentration in strawberry jams made of the varieties studied: esters, aldehydes, ketones, furanones, acids, aroma compounds, lactones, terpenic compounds. The concentration of volatiles in strawberry jams was 12.1-33.54 mg/kg depending on the variety (Table 1). The most meaningful shares are: acids – 65.6-76.8%, furanes – 8.3-14.6% and aldehydes – 3.4-10.8% (Fig.1). The share of esters in jams exceeds 0.7-3.1% of the total volatile content. It is important to mention that in strawberry jams made of Polka cultivar the share of esters and aldehydes is much higher: 3.1 and 10.8%, that of furanes and acids, on the contrary, is the lowest – 8.3 and 65.6% which proves strong expression of scents typical for fresh strawberries.

Characteristic compounds for strawberry jam flavor made of the studied cultivars are hexanoic (caproic) acid (0.84-6.89 mg/kg), which is 6.9-22.9 % of the total volatile amount depending on their quantity for each cultivar, hexadecanoic acid (2.5-12.4%), 2- ethyl hexanoic (capronic) acid (3.1-10.7%), trans-cinnamic acid (17.5-25.3%), linoleic acid (0.3-7.2%), furil hydroxy methylketone (3.1-6.0%), 2.5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane) (7.4-13.7%), furfural (0.8-3.1%), 5-hydroxymethylfurfural (0.8-5.2%), vanillin (0.2-0.8%).

The availability of furfural (0.8-3.1%), 5-hydroxymethylfurfural (0.8-5.2%) 5-methylfurfural (0.7%) in strawberry jams indicates non-fermentative darkening during thermal treatment (Barren *et al.*, 1990; Kimura *et al.*, 1994).

High content of 2-heptenal – 0.9% of the total volatile content and hexanol (1.9%) were found in strawberry jam made of Polka cultivar, and as to cultivars "Ducat" and "Honey" – high content of 2-methylbutyric acid (6.3-6.6%) γ, and 2.5-dimethyl-3(2H)-furanone (0.4-1.1%). According to Schwab (2013) 2.5-dimethyl-3 (2H)-furanones is synthesized via sets of fermentative changes in fruits.

γ-decalactone – 0.55 and 1.73 mg/kg which is 1.8-5.2% – was found in strawberry jams (cultivars "Ducat" and "Honey"). This volatile compound adds "fruity", "sweet" and "peachy" scents (Ulrich *et al.*, 2007).

Small amounts of 2H -pyran-2.6(3H)-dion and 3.5-hydroxy-2-dimethyl-4H- pyran-4 – (0.09-0.66 mg/kg) which, depending on the cultivar, is 0.5-0.9% of the total volatile content in jams, were found; and according to (Barren *et al.*, 1990) they are the products of Maiyar reaction resulted from the reaction of glucose with glutamic acid, glycine, butylamine, lysine, hydroxypyroline and/or phenylalanine (amino acids).

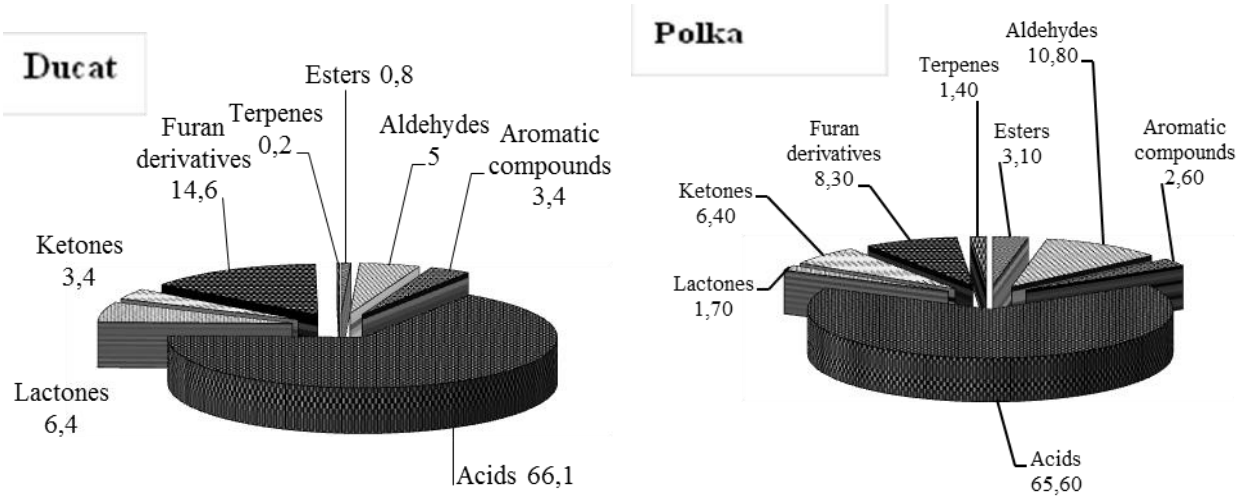
Terpenic compounds of strawberry jams are presented by small amounts of limonene (0.1 mg/kg, which is 0.8%) and α-terpineol (0.2-1.3%), they were found in fresh berries by Bianchi *et al.* (2014); these compounds add aromatic scent to fresh berries (Ulrich *et al.*, 2007, Bianchi *et al.*, 2014); oxyde bisabolol A (0.1-0.3%), trans - linalool oxyde (0.1-0.3%), cis - linalool oxyde (0.3%), however no data concerning their presence in fresh strawberries is available. Despite a large volatile amount, strawberry jam flavor is developed under the effect of their small amount.

To determine the share of each compound in the flavor, its activity is defined by dividing substance concentration on its threshold concentration (OAV = concentration (ppbv)/threshold value (ppbv) (Table 2) (Rothe *et al.*, 1963, Kim *et al.*, 2013). If the result of OAV exceeds 1, it proves the contribution of a component to the flavor.

Table 1. Content of volatile components in strawberry jam, mg/kg

Volatiles	Variety		
	Polka	**Ducat**	**Honey**
Esters			
Methyl butanoate	0.14	0.03	-
Ethyl butanoate	0.06	0.15	0.09
Ethyl crotonate	0.02	-	-
Ethyl 2-methyl butanoate	0.02	-	-
Ethyl capronate	0.01	-	-
3,4-dihydropyran	0.13	0.10	0.12
Total esters	0.38	0.28	0.21
Aldehydes			
Benzaldehyde	0.04	0.10	0.14
Trans-2-heptenal	-	-	0.14
Hexanal	0.06	0.04	0.03
2-heptenal	0.11	-	-
Decanal	0.01	-	-
Furfural	0.38	0.38	0.23
5- hydroxymethylfurfural	0.63	0.66	0.24
Undecenal	0.05	-	-
2-decenal	-	0.07	-
Vanillin	0.03	0.20	0.23
5- methylfurfural	-	0.24	-
Total aldehydes	1.31	1.69	1.01
Aromatic compounds			
Hexanol	0.23	-	-
2H -pyran-2,6(3H)-dion	0.09	0.50	0.27
3,5-hydroxy-2-dimethyl-4H- pyran-4-on	-	0.66	-
Total aromatic compounds	0.32	1.16	0.27
Acids			
2-Methylbutyric acid	-	2.22	1.89
Octanoic acid	0.10	0.66	0.40
Nonanoic acid	-	0.09	-
Hexanoic (caproic) acid	0.84	4.85	6.89
Tetradecanoic acid	0.32	0.24	0.36
Palmitoleic acid	0.38	0.21	0.48
Hexadecanoic acid	1.50	0.85	1.37
2- ethyl hexanoic (capronic) acid	1.29	2.11	0.95
Trans-Cinnamic Acid	2.11	7.74	7.64
Dodecanoic acid	0.07	0.07	0.12
Pentadecanoic acid	0.21	0.10	0.26
Linoleic acid	0.87	0.85	0.10
Octadecanoic acid	0.24	0.11	0.21
Cis-Cinnamic acid	-	1.73	2.10
Oleic acid	-	0.33	0.38
Total acids	7.93	22.16	23.15

Lactones			
γ- Decalactone	-	1.73	0.55
Butyrolactone	0.12	0.38	-
γ-Caprolactone	0.08	-	-
δ- Caprolactone	-	0,04	-
Total lactones	0.20	2.15	0.55
Ketones			
2-acetylfuran	0.04	-	-
Furil hydroxy methylketone	0.73	1.13	0.95
Total ketones	0.77	1.13	0.95
Furan derivatives			
2,4-dioxy-2,5-dimethyl-3(2*H*)-furan-3-one	0.12	0.10	0.10
2,5-dimethyl-4-methoxy-3(2*H*)-furanone (mesifurane)	0.89	4.61	2.36
2,5-dimethyl-4-oxy-3(2*H*)-furanone	-	0.03	0.56
2,5-dimethyl-3(2*H*)-furanone	-	0.15	0.34
Total furan derivatives	1.01	4.89	3.36
Terpenes			
Linalool	-	-	0.08
α-Terpineol	0.03	-	0.40
Limonene	0,10	-	-
Oxyde bisabolol A	0.04	0.05	-
Trans - linalool oxyde	-	0.03	0.08
Cis - linalool oxyde	-	-	0.08
Total terpenes	0.17	0.08	0.64
Total amount	12.09	33.54	30.14
***LSD*$_{05}$**	*0.06*		



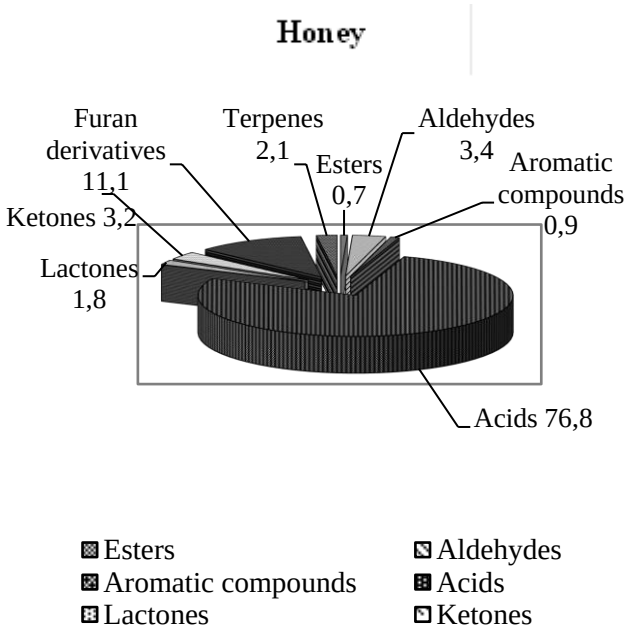


Figure 1. Volatile components of strawberry jams made of various cultivars, % of the total content

The calculation of volatile OAV of strawberry jams showed that furanone derivatives dominated: 2.4-dioxy-2.5-dimethyl-3(2H)-furan-3-one, 2.5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane) and 2.5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol); they add sweet, caramel scents to strawberry jams. Vanilla and hexanal are also active compounds, they are typical for vanilla and fresh grassy scents (Barren et al., 1990); 2-methylbutanoic acid, which adds sour-sweet taste, is active in strawberry jams made of "Ducat" and "Honey" cultivars.

For strawberry jams, ethyl 2-methylbutanoate, decanal along with furanes are active components which add fruity and grassy scents to strawberry jams made of cultivar "Polka". 2-decenal and γ- decalactone, 'Honey' – γ-decalactone (fruity, sweet) and linalool (sweet and floral scent) make an important contribution to the flavor of strawberry jam made of cultivar "Ducat".

Table 2. Activity of volatile components of strawberry jam flavor (OAV)

Volatiles	**Theshold, mg/kg**	**Activity of flavor volatile components (OAV)**		
		Polka	Ducat	Honey
Methyl butanoate	0.06	2.3	0.5	-
Ethyl butanoate	0.018	0.3	8.3	5.0
Ethyl crotonate	NA[1]	-	-	-
Ethyl 2-methylbutanoate	0.0001	200	-	-
Ethyl capronate	NA	-	-	-
Benzaldehyde	0.35	0.1	0.3	0.4
3,4-dihydropyran	NA	-	-	-
Trans-2-Heptenal	0.013	-	-	10.8
Hexanal	0.0045	13.3	8.9	6.7
2-Heptenal	NA	-	-	-

Decanal	0.0001	100	-	-
Furfural	3.0	0.13	0.13	0.08
Undecenal	0.005	10	-	-
2-decenal	0.0003	-	233	-
Vanillin	0.02	1.5	10	11.5
5- hydroxymethylfurfural	NA	-	-	-
5- methylfurfural	NA	-	-	-
Hexanol	2.5	0.09	-	-
2H -pyran-2,6(3H)-dion	NA	-	-	-
3,5-hydroxy-2-dimethyl-4H- pyran-4-on	NA	-	-	-
2-Methylbutanoic acid	0.25	-	8.9	7.6
Octanoic acid	0.910	0.1	0.7	0.4
Nonanoic acid	3	-	0,03	-
Hexanoic (caproic) acid	1.0	0.8	4.9	6.9
2- ethyl hexanoic (capronic) acid	NA	-	-	-
Trans-Cinnamic Acid	NA	-	-	-
Dodecanoic acid	10	-	-	-
Pentadecanoic acid	NA	-	-	-
Linoleic acid	NA	-	-	-
Octadecanoic acid	20	0.01	0.006	0.01
Cis-Cinnamic acid	NA	-	-	-
Oleic acid	NA	-	-	-
Tetradecanoic acid	10	0.03	0.02	0.04
Palmitoleic acid	NA	-	-	-
Hexadecanoic acid	NA	-	-	-
γ- Decalactone	0,01	-	173	55
Butyrolactone	NA	-	-	-
γ-Caprolactone	NA	-	-	-
δ- Caprolactone	NA	-	-	-
2-acetylfuran	10	0.004	-	-
Furil hydroxy methylketone	NA	-	-	-
2,4-dioxy-2,5-dimethyl-3(2*H*)-furan-3-one	0.00004[2]	3000	2500	2500
2,5-dimethyl-4-methoxy-3(2*H*)-furanone (mesifurane)	0.00003[2]	29667	153667	78667
2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol)	0.00004[2]	-	750	14000
2,5-dimethyl-3(2*H*)-furanone	NA	-	-	-
Linalool	0.006	-	-	13.3
α-Terpineol	0.330	0.09	-	1.2
Limonene	0.01	10	-	-
Oxyde bisabolol A	NA	-	-	-
Trans - linalool oxyde	NA	-	-	-
Cis - linalool oxyde	NA	-	-	-

[1]NA – not available. Threshold levels of compounds (in water) were obtained from the flavor base of Leffingwell & Associates.

[2]Siegmund B., Bagdonaite K., Leitner E. (2010)

Fruity and floral, green and sweet or caramel scents are emphasized in the aroma of fresh strawberries. Esters, ethyl acetate, butyl acetate, methyl butanoate, ethyl butanaote, ethylisovalerate, methyl hexanoate and ethyl hexanaote add fruity and floral scents to strawberry flavor. Hexanal, trans-2-hexenal, 2-hexenal, hexanol, cis-3-hexen-1-ol, hexyl acetate add green scents, and furaneols – sweet, caramel ones (Pérez *et al.*, 1996; Jetti, 2005; Kim *et al.*, 2013). Having analyzed volatile activity data of strawberry jams, it has been established that typical scents for them are sweet and caramel ones due to high furaneol activity, also there are vanilla, fruity and fresh grassy scents. The flavor of strawberry jams made of "Polka" cultivar is characterized with fruity and grassy scents, that of "Ducat" cultivar – fruity and sweet scents, and for "Honey" cultivar – sweet and floral scents.

4. Conclusions

The flavor of strawberry jams made of "Polka", "Ducat" and "Honey" cultivars consists of a complex mixture of compounds, the most active among them are furanone ones: 2.4-dioxy-2.5-dimethyl-3(2H)-furan-3-one, 2.5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane) and 2.5-dimethyl-4-hydroxy-3(2H) furanone(furaneol). Ethyl 2-methylbutanoate, hexanal, decanal, 2-decenal, vanillin and γ- decalactone make a great contribution to the flavor.

Sweet, caramel scents with vanilla and fresh grassy scents are very typical for strawberry jams: the flavor of strawberry jams made of "Polka" cultivar is characterized with fruity and grassy scents, that of "Ducat" cultivar – fruity and sweet scents, and for "Honey" cultivar – sweet and floral scents.

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DETERMINATION OF ESSENTIAL OIL COMPOSITION, PHENOLIC CONTENT, AND ANTIOXIDANT, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF MARIGOLD (*CALENDULA OFFICINALIS* L.) CULTIVATED IN ALGERIA

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ABSTRACT

Microwave-assisted hydrodistillation (MAHD) was used as an ecofriendly method to extract the essential oils from flowers and leaves of *Calendula officinalis* L. cultivated in Algeria. The results obtained were compared with the conventional extraction method, hydrodistillation (HD), and analyzed by gas chromatography-flame ionization detector (GC-FID) and GC-MS. For flowers oils, 33 compounds were identified with HD method *vs* 20 compounds with MAHD method. For leaves, 26 compounds were identified with HD method *vs* 19 compounds identified with MAHD method. It is interesting to note, furthermore, that the use of MAHD method during 90 min allowed us to obtain relatively similar yields than HD method during 180 min. The main abundant volatile constituent was *α*-cadinol with 31.9±0.71% for HD *vs* 39.7±0.26% for MAHD in leaves oils and 32.3±0.26% for HD *vs* 37.1±0.30% for MAHD in flowers oils. The oxygenated sesquiterpens was the most represented group of natural compounds contributing to the chemical composition in all oils. In the other hand, extraction of total phenolic compounds (TPC) and total flavonoids (TFC) was affected by the solvent type and, thus, 100% methanol was the better extraction solvent for both leaves and flowers. Highest levels were obtained from leaves. The highest antioxidant activity was recorded for leaves extract with 100% methanol. These values indicated a weak antioxidant activity compared to antioxidant standards. A correlation was established between the phenolic and flavonoids contents and the antioxidant activity of the crude extracts. A moderate to great antibacterial activity was observed against $Gram^+$ bacteria. Any antibacterial activity was detected against fungi strains and $Gram^-$ bacteria.

1.Introduction

Pot marigold (*Calendula officinalis* L.) is an annual or biennial plant belonging to the Asteraceae family (Rotblatt 2000).

Native from Europe, Southern Africa, Western Asia and USA, *Calendula officinalis* L. is cultivated in temperate regions all over the world for their ornamental and medicinal

purposes. The leaves are also used in paint coating , cosmetic and nylon industries (Muuse *et al.,* 1992). In folk therapy, this species have been considered as values remedies against gastrointestinal ulcers, dysmenorrhea, fevers and conjunctivitis diseases (Lim 2014). It is mostly known for their high wound-healing properties (Nicolaus *et al.,* 2017). In fact, it has been reported that the preparation of leaves applied in the form of compresses (Dei Cas *et al.,* 2015) , the oil and ointments from flowers (Jarić *et al.,* 2018) and the tinctures or infusions made with aerial parts were employed to relieve ,wounds , bruises, minor burns and skin damaged (Arora *et al.,* 2013). Furthermore, many pharmacological investigations have demonstrated the biological effects of *Calendula officinalis* L. including anti-microbial (Gazim *et al.,* 2008b) (Efstratiou *et al.,* 2012) (Chebouti *et al.,* 2014) (Shankar *et al.,* 2017), anti-inflammatory (Ukiya *et al.,* 2006, Amoian *et al.,* 2010), hypoglycemic (Yoshikawa *et al.,* 2001) antioxydant (Ćetković *et al.,* 2004) ,anti-leishmanial (Nikmehr *et al.,* 2014) and genotoxic effects (Bakkali *et al.,* 2005).

Over the past decades, the essentials oils from medicinal plants have been isolated mainly by using hydrodistillation, steam distillation, maceration or expression (Ferhat *et al.,* 2007). However, these conventional methods present some disadvantages such as the loss or the degradation of some thermo-labile components due to the long extraction time and elevated temperatures (Luque de Castro *et al.,* 1999). For these reasons, the various novel extraction techniques including hydrodiffusion , supercritical fluid extraction, ultrasound-assisted extraction and pressurized solvent extraction have been developed and investigated in order to decrease considerably the processing time, increase the extraction yield and enhance the quality of the extracts (Chan *et al.,* 2011) (J. Mason *et al.,* 2011). Among them, microwave assisted extraction in combination with hydrodistillation (MAHD) has been recognized as efficient alternative for isolation a complex mixture of bioactive compounds from plant sources (Farhat *et al.,* 2017; Thach *et al.,* 2013).

Concerning *Calendula officinalis* L., numerous authors have been reported that the chemical composition of essential oil have been conducted in which volatile constituents were extracted by hydrodistillation(HD) (Chalchat *et al.,* 1991), headspace-solid phase microextraction (HS-SPME) (Gazim *et al.,* 2008a) , and supercritical CO_2 (Crabas *et al.,* 2003). However, according to our knowledge, no data are available about the essential oils from *C. officinalis* L. cultivated in Algeria.

Driven by this goal, we reported in this present study, for the first time, the chemical composition of the essential oil of leaves and flowers from *Calendula officinalis* L. extracted by microwave-assisted hydrodistillation. This extraction method was compared to hydrodistillation (as the reference method), in terms of extraction yields, extraction time and aromatic composition of the essential oils. On another hand, we focused our study to evaluate the influence of the extraction solvent type (methanol, ethanol absolute and ethyl acetate) on the phenolic and flavonoid contents and on the antioxidant and antimicrobial activities.

2.Materials and methods

2.1.Reagents

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) ; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) , acide 2'2, azino-bis-(3-éthylebenzothiazoline)-6-sulfonique ($ABTS^{\cdot+}$), Folin-Ciocalteu reagent, aluminium trichloride ($AlCl_3$) , carbonate sodium (Na_2CO_3), Butylhydroxytoluene (BHT) , galic acid ,quercetine , persulfate ($K_2S_2O_8$) , extra pure sodium carbonate decahydrated (Na_2CO_3) were purchased from Sigma-Aldrich (Steinheim, Germany). Mueller-Hinton (MH) and Sabouraud dextrose agar (SDA) were obtained from Merck (Darmstadt, Germany).

All the solvents were of analytical grade. Alkane standards solutions (C_7-C_{27}) were obtained from Fluka-chemika (Buchs, Switzerland).

2.2.Vegetal material

The aerials parts of *Calendula officinalis* L. were cultivated and harvested from Birtouta area (36°38′59″ North, 2°59′56″ East) during mars 2017 at the flowering period. The authentication of the species was conducted in the department of Biology, University of Sciences and Technology Houari Boumediene, USTHB, Algiers.

2.3.Hydro-distillation apparatus

The vegetal material (400 g) was subjected to hydrodistillation with Clevenger- apparatus during180 min. The essential oils extracted from flowers and leaves were collected and stored in amber vials at 4° C prior to analysis. The extraction time was selected following the previous

2.4.Microwave-assisted hydrodistillation (MAHD) procedure

MAHD was performed at atmospheric pressure using a household microwave oven (MW8123ST, Samsung, United Kingdom) connected with Clevenger-apparatus with a maximum delivered power of 1550W in 100-W increments. The vegetal material (400 g) was immerged in a 2 L flask containing distillate water (1 L) and heating using a fixed power of 588W during 90 min. The flask was introduced in the oven cavity of microwave, and the Clevenger apparatus was used on the top, outside the oven, to collect the volatile extracts which were stored at 4° C until being analyzed. The experiments were conducted in triplicate under the same conditions and the mean value was reported.

2.5.General chromatographic conditions

The constituents of the oils were analyzed using Hewlett-Packard 6890 series Gas Chromatographic (GC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with flame ionization detector (FID) set at 280°C. The separation was achieved using HP-5MS apolar capillary column (30 m × 250 µm× 0.25 µm film thickness). This column consists of 95 % dimethylsilicone with 5 % phenyl groups. The column temperature was initially programmed at 60° C for 8 min and increased to 250° C at 2° C/min, then finally held isothermally for 20 min. The carrier gas was Helium at a flow rate of 0.5 $mL.min^{-1}$ in split mode with an injection volume of 1 µL.

Gas chromatography-Mass spectrometry (GC-MS) analyses were performed using Hewlett-Packard 6890series GC system coupled with a mass spectrophotometer MSD 5973C and equipped with the same apolar capillary column .Temperature programming is the same as that used in the analysis by GC-FID. Helium was carrier gas with a flow rate 1.5 mL/min. The injected volume was 0.2 µL and the split ratio was 1:50. Injector temperature was 250° C. The injected MS conditions were: MS source temperature: 230° C and MS quadrapole temperature: 150° C, the ionization mode used was electronic impact at 70 eV over a scan range of 29–550 atomic mass units.

In order to calculate the retention indices , the homologous *n*-alkanes C_7-C_{27} was injected in the same conditions as the essential oils in GC-FID and GC-MS. The identification of volatile components was established by comparing their GC Kovats retention indices (KI) with those available in the literature and by matching their recorder mass spectral fragmentations patterns with those stored in the Wiley 9 and NIST 7N mass spectral library and others published index data (Adams 2007). Relative percentage amounts of each component are based on the peak areas obtained with GC-FID.

2.6.Analyzes of phenolic compounds

2.6.1 Extraction of phenolic compounds

Powdered air-dried (40 g) flowers or leaves were macerated in solvents (200 mL) with different polarity: 100% methanol, absolute ethanol and 100% ethyl acetate at room temperature for 24 h. The suspension, thus obtained, was passed through the filter paper N°1 (Whatman Ltd., UK) and concentrated under the vacuum on a rotary evaporator (Laborita 4001) at 40° C. The final extracts were stored at +4° C until utilization.

2.6.2.Phenolic and flavonoid contents

The total phenolic contents of the extracts were evaluated using the Folin-Ciocalteu method (Singleton *et al.,* 1999). An aliquot (250 µL) of the extracts, previously dissolved in the ethanol was added at Folin-Ciocalteu solution (1250 µL). After 3 min of reaction, a solution of sodium carbonate (1000 µl) at a concentration of 75 $g.L^{-1}$ was added and the mixture was shaken a few seconds on the Vortex. The absorbance measurements were determined spectrophotometrically at 765 nm. Gallic acid was used as a standard. Total phenolic content was expressed as milligrams gallic acid equivalent (GAE) per gram extract of dry mass (mg $GAE.g^{-1}$ DM).

The trichloride aluminum method was used to quantify the total flavonoids contents of the samples(Menaceur *et al.,* 2013) . To do this, an aliquot (1000 µL) of the extract dissolved in the ethanol was mixed with of solution of trichloride aluminum $AlCl_3$ (1000 µL, 2% w/v).The absorbance was measured at 420 nm after 1 h of incubation at room temperature. The quercetin was considered as standard for the calibration curve. The flavonoids contents were expressed as milligrams of quercetin equivalent (QE) per gram extract of dry mass (mg $QE.g^{-1}$ DM). The experiments were carried out in triplicate.

2.7.Antioxidant activity

2.7.1.Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging effect

The DDPH free radical scavenging assay was used to evaluate the antioxidant activity of the obtained extracts according to the method previously described with minor modifications (Brand-Williams *et al.,* 1995). Briefly, an ethanolic solution (25µL) of each sample at different concentrations (100-200-400-600-800 and1000 $\mu g.mL^{-1}$) were added to 975 µL of DPPH-ethanol solution (60 µM). After 30 min of incubation in the dark at room temperature, the absorbance was recorded at a wave length of 517 nm by using a spectrophotometer OPTIZEN 3220 UV. Butyl hydroxytoluene (BHT) was used as a standard antioxidant.

2.7.2.ABTS free radical scavenging activity

The ABTS test was assessed using the method reported by Ling *et al* (Ling *et al.,* 2009) . The ABTS radical cation was produced by reacting 7 mM ABTS stock solution and 2.45 mM $K_2S_2O_8$. The obtained mixture was kept in the dark at room temperature during 12-16 h prior use. The ABTS solution was diluted with ethanol in order to have a maximum absorbance of 0.703± 0.025 at 734 nm. An aliquot of 20 µL of the samples at various concentrations was added at 980 µL of diluted ABTS solution. The decrease of absorbance measurement was monitored at 734 nm in the 6th min after adding the sample to the ABTS solution. The synthetic vitamin E (Trolox) was used as an antioxidant standard.

For both tests, the radical scavenging activity was calculated according to the following equation: % of radical scavenging activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorption values of the blank sample and Abs_{sample} is the absorbance of the tested sample. The results were expressed as IC_{50} corresponding to the efficient concentration of the sample required to inhibit 50% of the free radical. The

experiments were carried out in triplicate and the IC_{50} values were reported as means ±SD.

2.8.Determination of antimicrobial activity

The antibacterial activity of the crude extracts was evaluated following the paper disk diffusion method described by Bauer *et al* (Bauer *et al.,* 1966) with slight modifications. For the experiments, the samples were tested against three $Gram^+$ bacteria: *Bacillus subtilis* ATCC6633, *Listeria monocytogenese, Staphylococcus aureus* MRSA 639c and two $Gram^-$ bacteria: *Escherichia coli* ATCC43300 and *Pseudomonas aeruginosa* ATCC 9027. Two pathogenic fungal strains: *Aspergillus carbonarius* M333 and *Umbulopsis ramanniana* NRRL1829 were also investigated. The bacterial and fungal strains were cultured in Mueller-Hinton (MH) at 37° C and Sabouraud dextrose agar (SDA) at 30° C respectively. Amoxicillin (25 µg/disc) was used as the positive control while a disc impregnated with 25 µL of DMSO was considered as the negative control. The appropriate agar mediums (MH or SDA) poured into Petri dishes were seeded with the cultures of microbial inoculum (10^6 CFU/mL) using a sterile cotton swab. Afterwards, the DMSO-extract solutions (25 µL) were added on the filter discs which were placed in the surface of Petri dishes. After staying at 4° C in the refrigerator during 1 h for diffusion, the Petri dishes were incubated during 24h for the bacteria strains and 48h for the fungi strains. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones.

The extracts that showed antibacterial activity were screened for determination of minimum inhibitory concentration (MIC) by broth microdilution following National Committee for Clinical Laboratory Standards Guidelines with some modifications(Wikler 2009). A serial dilutions ranging between 7.8 to 500 $\mu g.mL^{-1}$ were carried out from an initial solution previously prepared in DMSO with concentration of 1000 $\mu g.mL^{-1}$. Then each solution (0.5 mL) was added to agar medium (5 mL) (MH or SDA). After that, the obtained mixture was poured into the Petri dishes which were inoculated with inocula strains of 10^6 $CFU.mL^{-1}$ and incubated immediately at 37° C during 24H for the bacteria strains and 48H for fungi strains. Amoxicillin and DMSO served as a positive and negative control respectively. The minimum inhibitory concentration (MIC) is defined as the lowest concentration which exhibited no growth. The assays were carried out in triplicate.

2.9.Statistical analysis

The experiments were done in triplicate. The data were subjected to statistical analysis using one-way analysis of variance (ANOVA) with XLSTAT software on Microsoft Excel 2007. The results were expressed as mean ± standard deviation (SD). The Student's t-test at $p<0.05$ were considered statistically significant. Correlation was calculated according Pearson's method.

3.Results and discussions

3.1. Quantitative and qualitative analyzes of essential oils

Essential oils were studied in leaves and flowers.

3.1.1.Effect of the extraction methods on the essential oils yields

The overall yields were 0.045 ± 0.004 % (w/w) for HD *vs* 0.044±0.002% (w/w) for MAHD and 0.025±0.003% (w/w) for HD *vs* 0.023±0.003% (w/w) for MAHD for the essential oils extracting from flowers and leaves respectively (Figure 1). The oils yields obtained in 90 min) using MAHD was relatively similar to that obtained after 180 min for HD method. Moreover, the extraction time required to reach an extraction temperature of 100° C to get the first essential oil droplet in only 5 min compared to 30 min for HD. It should be noted, furthermore, that the obtained

yields are lower than those reported in the survey literature (0.1-0.97%) (Chalchat *et al.*, 1991) (Gazim *et al.*, 2008a) (Khalid and El-Ghorab 2006).

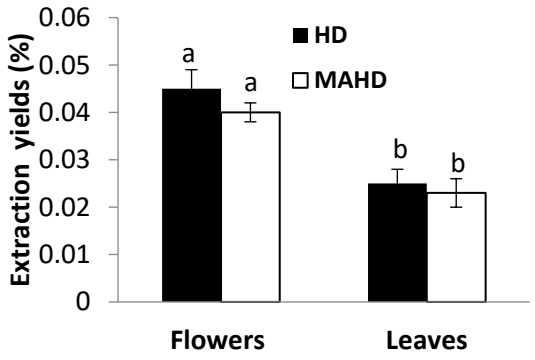

Figure 1. Extraction yields of essential oils from Calendula officinalis L. flowers and leaves with respect to extraction method.

Different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD. Error bars indicate standard deviation (SD).

3.1.2. Effect of the extraction methods on the chemical composition of the essential oils

The identified compound, their relative percentage, the experiments and literature retention indices are compiled in Table 1 and Table 2 for flowers and leaves essential oils respectively using HD and MAHD methods.

The analysis of the essential oils from flowers by GC and GC-MS allowed us to identify 33 compounds and 20 compounds representing 79.3% and 78.4% of the volatile oils constituents extracted by HD and MAHD respectively. The oils extracted were characterized in both methods by the predominance of two chemical families namely oxygenated sesquiterpens and sesquiterpens hydrocarbons. These compounds are responsible for the main biological activities of the vegetal matrice and their amounts depend closely to the used extraction method (Amorati *et al.*, 2013). The oil obtained by HD was little more concentrated in sesquiterpens hydrocarbons compounds compared with MAHD (22.6% HD *vs* 21.1% for MAHD). Oppositly , the oxygenated sesquiterpens compounds were more abundant in the oil extracted by MAHD (57.2%) than HD (53.7%). It was observed , in addition, that the monoterpens hydrocarbons compounds (such as *α*-pinene, *α*-phellandrene and *α*-terpinene) which represent 2.8% of the oils and the oxygenated monoterpens compounds (0.2%) such as (*α*-E)-ionone present with a relatively low percentage with HD method were absent or detected only at traces level using MAHD. The main abundant compounds were *α*-cadinol (37.1±0.26% for HD *vs* 32.3±0.3% for MAHD) followed by epi-*α*-cadinol (16±0.26% for HD *vs* 0% for MAHD) , epi-*α*- Muurolol (0% for HD *vs* 15.1±0.45% for MAHD) , *δ*-cadinene (13.1±0.52% for HD and 13.1±0.51% for MAHD), Torreyol (2.7±0.36% for HD *vs* 0.2±0.01% for MAHD) and Germacrene D (1.4±0.10% for HD *vs* 1.9±0.10% for MAHD).

Concerning the essential oils from leaves, 26 compounds were identified representing 80.1% of the oil extracted using HD while 19 compounds representing 88% were identified in oil extracted using MAHD. The major compounds identified and their respective percentage composition using both methods as follow as: *α*-cadinol (31.9±0.7% for HD *vs* 39.7±0.26% for MAHD), epi-*α*-cadinol (16.3±0.43% for HD *vs* 15.3±0.44% for MAHD), *δ*-cadinene (8.9±0.27% for HD *vs* 18±0.44% for MAHD), Torreyol (3.2±0.35% for HD *vs* 2.1±0.10% for MAHD) and *α*-Humulene (1.9±0.66%for HD *vs* 1.1±0.10% for MAHD). The established comparison between the chemical composition of the essential oils extracted by HD and MAHD methods showed a better abundance of oxygenated monoterpénes.

Table 1. Chemical composition of essential oils extracted by hydrodistillation (HD) and microwave-assisted hydrodistillation (MAHD) from flowers of *Calendula officinalis L.*

No	Compounds	KI[(a)]	KI [(b)]	HD (%)[(c)]	MAHD (%)[(c)]
1	*α*-Pinene	941	939	2±0.11	Tr
2	*α*-Phellandrene	1005	1002	0,1±0.01	-
3	*δ*-3-Carene	1013	1011	0,1±0.02	-
4	*α*-Terpinene	1018	1017	0,1±0.01	Tr
5	*p* –Cymene	1024	1024	0,1±0.01	-
6	*(Z-β)* Ocimene	1040	1037	0,1±0.01	Tr
7	*y* –terpinène	1062	1059	0,3±0.03	-
8	*Cis* Sabinene hydrate	1067	1070	Tr	-
9	*β*-bourbonene	1386	1388	0,1±0.02	0,1±0.01
10	*β* –Cubebene	1390	1388	0,1±0.01	-
11	*α*-gurgunene	1399	1409	0,3±0.02	0,4±0.03
12	*E*-Caryophyllene	1411	1419	1,1±0.10	-
13	*(E-α*) Ionone	1423	1430	0,2±0.01	-
14	*β* –copaene	1429	1432	-	0,1±0.03
15	*Cis* Cadina-1(6),4-diene	1464	1463	0,1±0.02	-
16	*Trans* Cadina-1(6),4-diene	1470	1476	0,2±0.01	0,2±0.05
17	*y* –Muurolene	1481	1469	0,8±0.02	-
18	Germacrene D	1484	1485	1,4±0.10	1,9±0.10
19	*β* –Selinene	1489	1490	0,2±0.02	-
20	*Trans*. Muurola-4(4),5- diene	1493	1493	0,2±0.03	0,3±0.03
21	*α*-Muurolene	1499	1500	2±0.50	-
22	cubebol epi	1502	1494	-	2,7±0.26
23	*y*-Cadinene	1506	1513	2±0.15	4±0.20
24	*δ*-Cadinene	1513	1518	13,1±0.52	13,1±0.51
25	*α*-Cadinene	1527	1530	0,5±0.02	0,8±0.02
26	*α*-calacorene	1545 [(d)]	1545	0,5±0.03	0,1±0.01
27	Murool-5-en-4-*β*-ol-cis	1552	1551	-	0,3±0.02
28	Murool- 5-en-4-*α*-ol-cis	1559	1561	-	0,5±0.07
29	Nerolidol	1560 [(e)]	1563	0,3±0.03	-
30	*β*-calacorne	1563	1565	-	0.1±0.01
31	Palustrol	1568	1568	0,3±0.01	0,3±0.03
32	caryophyllene oxide	1586	1583	-	Tr
33	Cubeban-11-ol	1596	1595	0,5±0.02	-
34	Guiol	1605	1600	0,2±0.02	-
35	*β*-Oplopenone	1609	1607	0,1±0.02	-
36	Cubenol (1,10-di-epi-)	1616	1619	0,4±0.05	0.8±0.02
37	Cubenol 1-epi-	1629	1628	0,9±0.05	-
38	epi *α* –Cadinol	1636	1640	16±0.26	-
39	*epi-α*-Muurolol	1644	1642	-	15,1±0.45
40	*α*-Muurolol (=Torreyol)	1646	1646	2,7±0.36	0,3±0.01
41	*α*-Cadinol	1655	1645	32,3±0.26	37.1 ±0.30
42	*Trans*- calamenen-10-ol	1672	1669	-	0,20±0.01
	Extraction time			180	90
	Yields			0.045±0.02	0.044±0.04
	Monoterpens hydrocarbons(%)			2.8	0
	Oxyenated monoterps (%)			0.2	0
	Oxygenated sesquiterpens (%)			53.7	57.3
	Sesquiterpens hydrocarbons (%)			22.6	21.1
	Total volatile compounds (%)			79.3	78.4

Note: [(a)]Experimental retention index relative to C_7–C_{27} *n*-alkanes on the HP5-MS (apolar capillary column), [(b)] literature retention index. [(c)] Percentage calculated by GC–FID on non-polar HP5—MS capillary column, [(d)] Gazim *et al. (*2008a), [(e)] Okoh *et al. (*2008), tr: traces (<0.1 %), - : absence of compound.

Compounds using HD (0.1%) than MAHD (0%) while a higher amounts of sesquiterpens hydrocarbons compounds were present in the essential oil extracted using MAHD compared to HD with a percentage of 27.4% and 24.6% respectively. Indeed, many volatile compounds including *α*-cadinene (0.9±0.01% for HD *vs* 1.3±0.25% for MAHD) and *α*-muurolene (3.4±0.40% for MAHD and 2.7±0.40% for HD) were present in low amounts with HD method or were absent such as *trans* cadina-1,4-diene (1±0.41% for MAHD *vs* 0% for HD) and *β*-copaene (0.2±0.02% for MAHD *vs* 0% for HD).

As the flowers oils, a greater proportion of the oxygenated sesquiterpens was observed in the leaves oil extracted by MAHD compared to HD (55.4% HD *vs* 60.6% for MAHD). This difference in the essential oil composition is probably due to the high absorption of microwave by these polar compounds in MAHD more than in HD extraction which favorise a more easily extraction of these compounds compared with others class of compounds such as monoterpens hydrocarbons that have lower dipolar moments (Ferhat *et al.,* 2006).

Furthermore, it is interesting to note that the quantities of the target compounds depend of the used extraction methods. According to the data values seen in the Table 2, the oxygenated sesquiterpens compounds of essential oil from leaves such as cubeban-11-ol and cubenol (1,10-di-epi) extracted by MAHD were present with a percentage of 1.2±0.66% and 0.6±0.020% respectively, or in the case of HD , these volatile compounds were present at 0.9±0.040% and 0.5±0.010% respectively. Considering another compounds such as Torreyol and β-oplopenone, the essential oil obtained by HD showed the presence of these compounds with a percentage of 3.2±0.35% and 0.1±0.01% respectively against 2.1±0.10% and 0% respectively in the MAHD extract. In the same way, the study of the chemical composition of essential oils from flowers (Table 1) reveals the presence of epi *α*-cadinol at 16±0.26% and 1-epi cubenol at 0.9±0.05% using MAHD whereas these volatile compounds were absent in the oil obtained by HD. Otherwise, *α*-muurolene and E-caryophyllene present at 2±0.50% and 1.1±0.10% respectively in the HD extracts were absent in the oils extracted by MAHD.

In addition, in both oils, some volatile constituents such as caryophyllene oxide and *α*-cubebene not detected using conventional extraction process were detected only at trace level by MAHD methods.

Regarding the aforementioned data, our finding was in agreement with those available in the literature. Indeed, the main component identified in this investigation in all oils (*α*-cadinol) was similar to that reported by Chalchat *et al* (Chalchat *et al.,* 1991) who studied *C.officinalis* L. from the French Central Massif. It has been reported also that the main components of essential oil from the Egyptian Pot Marigold cultivated under presowing low temperature were *α*-cadinol (up to 64.4%) following by *Δ*-cadinene, *δ*-cadenene and nerolidol (Khalid and El-Ghorab, 2006).

However, although the volatile compounds identified in this study were also mentioned by numerous authors, significant qualitative and quantitative differences were noted. This variation is probably due of the process factors, environmental conditions and/or genetic factors that affect the yield and quality of the essential oils (Duarte *et al.,* 2017). The essential oils from Brazilian Pot Marigold were dominated by sesquiterpens hydrocarbons (68.0 % of total area) and sesquiterpenols (27.0 % of total area) in which *δ*-cadinene (22.5%) and *α*-cadinol (20.4%) were the major constituents (Gazim *et al.,* 2008a). Flowers oils from Bucharest (Romania) obtained by steam distillation was

Table 2. Chemical composition of essential oils extracted by hydrodistillation (HD) and microwave-assisted hydrodistillation (MAHD) from leaves of *Calendula officinalis* L.

No	Compounds	KI[(a)]	KI[(b)]	HD (%)[(c)]	MAHD (%)[(c)]
1	Bornyl acetate	1285[(d)]	1288	0,1±0.01	-
2	*α*-cubebene	1388	1386	-	Tr
3	*β* –bourbounene	1390	1388	0,1±0.02	-
4	*α*-gurjunene	1408	1409	0,3±0.02	0,2±0.03
5	caryophyllene E	1418	1419	1±0.17	0,4±0.040
6	*β*-copaene	1430	1432	-	0,2±0.02
7	*β*-gurjunene	1434	1433	0,1±0.01	0,2±0.04
8	α-humulene	1452	1454	1,9±0.66	1,1±0.10
9	*Cis* cadina-1-(6),4-diene	1463	1469	-	0,4±0.02
10	*γ*-muurolene	1473	1479	1.8±0.20	Tr
11	*Trans.*cadina-1-(6),4-diene	1476	1471	-	1±0.49
12	*β*-selinene	1487	1490	0.4±0.01	-
13	*α*-muurolene	1500	1500	2,7±0.40	3,4±0.40
14	γ-cadinene	1513	1513	4,5±0.26	-
15	*δ*-cadinene	1523	1523	8,9±0.27	18±0.44
16	*Trans* cadina-1,4-diene	1534	1530	-	1±0.40
17	*α*-cadinene	1537	1538	0,9±0.01	1,3±0.25
18	*α*-calacorene	1543	1538	0,6±0.05	-
19	muurol-5-en-4-*β*-ol cis	1550	1551	0,3±0.02	-
20	muurol-5-en-4-*α*-ol cis	1561	1561	0,4±0.04	-
21	*β*-calacorene	1566	1565	0,6±0.02	-
22	Palustrol	1567	1568	0,3±0.02	-
23	cubeban-11-ol	1594	1595	0,9±0.40	1,2±0.66
24	*β*-oplopenone	1609	1607	-	0,5±0.01
25	cubenol(1,10-di-epi)	1616	1619	0,5±0.010	0,6±0.020
26	*α*-corocalene	1623	1623	0,6±0.10	-
27	cubenol 1-epi	1629	1628	1,2±0.10	1,2±0.20
28	epi *α*-cadinol	1641	1644	16,3±0.43	15,3±0.44
29	Torreyol	1646	1642	3,2±0.35	2,1±0.10
30	*α*-cadinol	1648	1654	31,9±0.26	39,7±0.71
31	calamenen-10-one-10-nor	1704	1704	0,3±0.01	-
32	Oplopenone	1740	1740	0,1±0.01	-
	Extraction time			180	90
	Yields			0.025±0.03	0.023±0.02
	Monoterpens hydrocarbons(%)			0	0
	Oxyenated monoterps (%)			0.1	0
	Oxygenated sesquiterpens (%)			55.4	60.6
	Sesquiterpens hydrocarbons (%)			24.6	27.4
	Total volatile compounds (%)			80.1	88

Note: [(a)]Experimental retention index relative to C_7–C_{27} n-alkanes on the HP5-MS (apolar capillary column), [(b)] literature retention index, [(c)]Percentage calculated by GC–FID on non-polar HP5-MS capillary column . [(d)] Gazim et al,. (2008a), tr: traces (<0.1 %), - : absence of compound.

characterized by a appreciable amounts of *α*-muurolene representing 41.5% of total area (Rădulescu *et al.*, 2000) of the plant, the essential oils of *Calendula officinalis* L. from South Africa were characterized by a greater proportion of *α*-cadinol, *α*-cadinene, *T*-muurolol, epi-bicyclosesquiphellandrene , limonene, 1.8 cineole and trans-*β*-ocimene belonging to class of monoterpens (Okoh *et al.*, 2007). The same author mentioned overwise, the effect of drying on the volatile composition of the oils: fresh flowers oil were dominated by *α*-thujene (26.9%), *T*-muurolol (24.9%) and δ-

cadinene (13.1%) as the main compounds. On the other hand, died leaves was dominated by 1,8-cinéole (29.4%), *α*-thujene (17.8%), *β*-pinene (6.9%) and *δ*-cadinene (9.0%) while the fresh leaves was found to be rich in T-muurolol (40.9%), *α*-thujene (19.2%) and *δ*-cadinene (11.4%) (Okoh *et al.*, 2008).

Many volatile constituents identified in this investigation were tested for their anticancer properties. Among of them , *α*-cadinol, showed selective toxicity against human colon adenocarcinoma cells (Sylvestre *et al.*, 2006) (He *et al.*, 1997). Otherwise, it was reported that the essential oils extracted from other medicinal plants containing *α*-pinene, *p*-cymene , *α*-copaene and *δ*-cadinene as volatile components have a potential antimicrobial and antifungal activities (Bel Hadj Salah-Fatnassi *et al.*, 2017).

3.2. Determination of phenolic content and evaluation of antioxidant and antibiological activities of samples extracts

3.2.1.Effect of solvent on total phenolic content (TPC) and total flavonoid content (TFC)

Three solvent systems were used (methanol, ethanol and ethyl acetate) for phenolic and flavonoid extractions from leaves or flowers of *Calendula officinalis* L. In the case of leaves, the extract obtained by 100 % methanol showed the highest total phenolic content, TPC (81.4 mg GAE.g^{-1} DM) and the highest total flavonoids content, TFC (24.11 mg QE.g^{-1}). Values obtained with 100 % ethanol were close but statistically different ($p<0.05$). TPC (46.16 mg GAE.g^{-1} DM) and TFC (19.75 mg QE.g^{-1}) obtained by extraction with 100 % ethyl acetate were lower.

In the case of flowers, the same extraction efficiency was recorded for the three solvents for both TPC and TFC but with low yields except for TPC extracted with ethyl acetate(49.6 *vs* 46.16 mg GAE.g^{-1} DM for leaves) (Figure2).

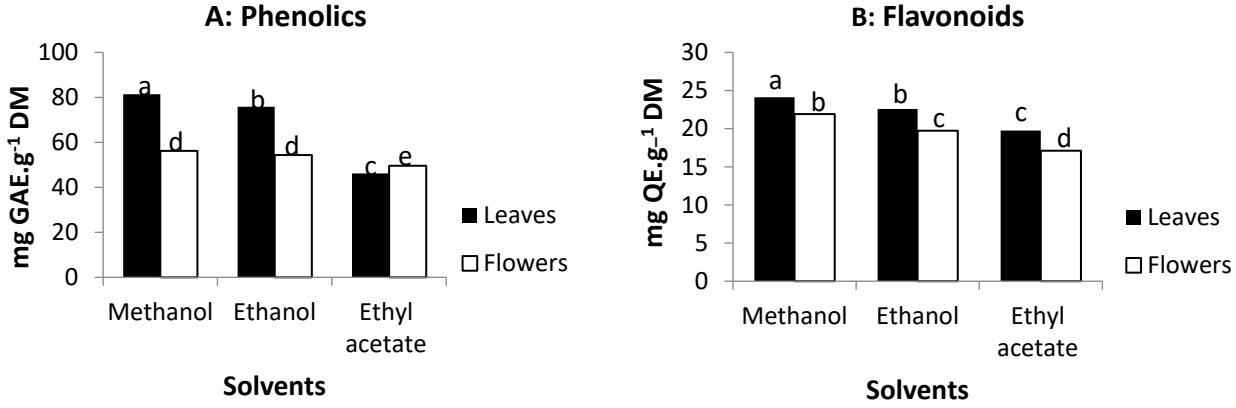

Figure 2. Total phenolics (A) and total flavonoids (B) contents of *Calendula officinalis* L. leaves and flowers according to extraction solvents.

For each graph, different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD. Error bars indicating standard errors (SD) are smaller than the symbol size.

The general results suggest that the values obtained were affected by the type and polarity of extracting solvents. Indeed, this parameter plays a key role in the efficiency of the process because its influence the solubility of the target compounds and the penetrability into the matrix (Rostagno and Prado, 2013). As corroborate by numerous

authors, in our case , methanol was the best extracting solvent due to its higher polarity and good solubility for phenolic component from plants followed by ethanol and ethyl acetate (Belwal *et al.*, 2016; Roby *et al.*, 2013).

3.2.2. Evaluation of antioxidant activity of samples extracts

Various techniques are available to screen the antioxidant activity of vegetal matrices. However, the use of only one method was not efficient to identify all possible mechanisms characterizing an antioxidant. Thus, two complementary in vitro assays namely the DPPH and ABTS free radicals scavenging were selected to evaluate the potential antioxidant activity of methanol , ethanol and ethyl acetate extracts from flowers and leaves of *Calendula officinalis* L.

The obtained results showed that all the investigated samples extracts exhibits the ability to scavenge the DPPH free radical. Indeed, the IC_{50} which corresponding of the required concentration of an extract to inhibit the free radical by 50% present the values ranging between 149.10±1.36 µg.mL^{-1} and 312.86±1.40 µg.mL^{-1} *(*Table 3*)*. The highest DPPH-activity was observed for methanol extract with IC_{50} values of 149.10±1.36 µg.mL^{-1} and 175.21±1.57 µg.mL^{-1} respectively for leaves and flowers and the lowest one for acetate ethyl extract with IC_{50} of 285.52±1.14 µg.mL^{-1} and 312.86±1.40 µg.mL^{-1} respectively for leaves and flowers. Globally, the leaves showed a better antioxidant activity compared with flowers. Regarding the ABTS test, the same results were obtained with the highest IC_{50} values of 146.29±1.17 µg.mL^{-1} for leaves methanol extract and 168.44±1.76 µg.mL^{-1} for flowers methanol extract. Oppositely, the lowest IC_{50} values were 280.91±1.1µg.mL^{-1} and 307.22±1.06 µg.mL^{-1} for ethyl acetate extracts from leaves and flowers respectively. It should be noted, furthermore, that all the tested extracts exhibits a lowest antioxidant activity compared to the positive control BHT(IC_{50}=28.12±0.14 µg.mL^{-1}) and Trolox (IC_{50}=10.14±0.11µg.mL^{-1}) respectively for DPPH and ABTS tests.

Table 3. Effect of phenolic compound extracts of *Calendula officinalis* L. on antioxydant activities.

Samples extracts	IC_{50} DPPH	IC_{50} ABTS
Leaves extracts		
Methanol	149.10±1.36^{a}	146.29±1.17^{b}
Ethanol	187.94±1.23^{c}	184.83±1.62^{d}
Ethyl acetate	285.52±1.14^{e}	280.91±1.1^{f}
Flowers extracts		
Methanol	175.21±1.57^{g}	168.44±1.76^{h}
Ethanol	196.48±1.22^{i}	192.12±1.26^{j}
Ethyl acetate	312,86±1,40^{k}	307,22±1,06^{l}
Standards		
BHT	28.12±0.13	--
Trolox	--	10.41±0.11

-- not applied

Different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD.

It should be noted , that the differences noted between the antioxidant activities potential of these crude extracts maybe attributed at the quality and quantity of the phenolics compounds present in the extracts (Mokrani and Madani, 2016; Decker 1997)

3.2.3. Comparison between DPPH and ABTS methods

The DPPH and ABTS scavenging assays resulted in close values obtained through the two methods for the same organ and the same solvent but those obtained by DPPH method were slightly highest indicating a lower inhibition capacity compared to ABTS. Our observations are in line with those indicated by some authors which report that ABTS inhibition for samples from vegetables, fruits and beverages is higher than DPPH inhibition (Ghouila *et al.*, 2016; Floegel *et al.*, 2011). This is explained by the fact that the DPPH method is characterized by a lower sensitivity. The reaction of DPPH radicals with most active molecules is slower than ABTS radicals (Binsan *et al.*, 2008; Martysiak-Żurowska and Wenta, 2012) By mixing all data obtained for both organs and the three solvents, it resulted a

very high correlation (r=0.9997) between the two methods for determining the antioxidant activity (DPPH and ABTS) (Figure 3). These results suggest that the two methods led to similar antioxidant activity of *Calendula officinalis* extracts. Vamanu and Nita (2013) have reported the same observation for mushroom extract but with a lower correlation.

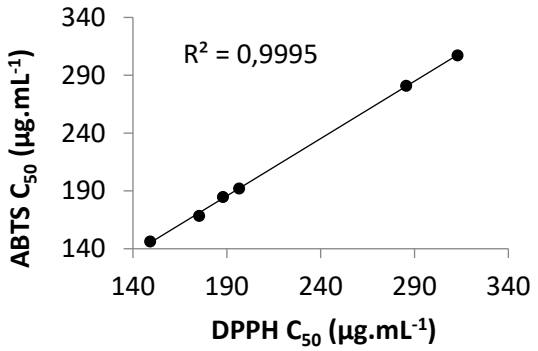

Figure 3. Correlation and regression of DPPH radical scavenging activity versus ABTS radical scavenging activity.
All values are issued from all phenolic compounds extracts (organ and solvent).

3.2.4. Relation between phenolic contents and antioxidant activity

A strong relationship between antioxidant capacity (DPPH and ABTS) and total phenolic or flavonoid contents was found (Table 4). This indicates that quantities of antioxidant molecules which were present in the extracts increased linearly with increasing concentrations. These results indicate also that flavonoids were the major contributors to the antioxidant properties of the studied plant. Correlation between phenolic compounds content and antioxidant activity has been reported by several authors and for many species. (Dudonné *et al.,* 2009; Li *et al.,* 2009; Piluzza and Bullitta 2011)

Table 4. Correlation of total phenolic and flavonoid contents with antioxydant activities (DPPH or ABTS).

	TPC Leaves	TPC Flowers	TFC Leaves	TFC Flowers
DPPH	0.9912	0.9925	0.9973	0.9471
ABTS	0.9909	0.9522	0.9943	0.9522

Values represent coefficient correlation (r) calculated according Pearson's method.

3.2.4. Antibiological activity

The prepared extracts from flowers and leaves of *Calendula officinalis* L. were screened against the selected Gram$^+$ bacteria, Gram$^-$ bacteria and fungi strains using the disc diffusion assay (Table 5). It has been observed that the effectiveness of the samples extracts depends of the tested microorganisms, the type of solvents used and the plant organ. Our finding showed that no antibacterial activity was detected against Gram$^-$ bacteria and fungi strains. The resistance of Gram$^-$ bacteria to inhibitory effect of the tested extracts could be attributed to lipopolysaccharides in their outer membrane, which make them inherently resistant to external agents, such as hydrophilic dyes, antibiotics and detergents (Hayouni *et al.*, 2007).

Otherwise, the tested extracts were globally most efficient against Gram$^+$ bacteria with a diameter of the growth inhibition zone ranging for 8.03±0.15 mm to 5.33±0.41 mm corresponding at moderate activities.

Methanol flowers extracts were efficient while tested against *Bacillus subtilus* ATCC6633 and *Listeria monocytogenes* with a diameter of the growth inhibition zone of 12.37±0.23 mm and 8.13±0.15 mm respectively . The same observation was done for the leaves extracts with an inhibition zone diameter of 10.9±0.78 mm and 12.16±0.12 mm against the same bacteria. However, any antibacterial activity was observed against *Staphylococcus aureus* 693c for both matrices. Ethanol extract from flowers present a moderate antibacterial activity with an inhibition zone diameter of 11.2±0.26 mm and 10.8±0.60 mm while no activity was observed against *Listeria monocytogenes*. In the same

way, tested against the same bacteria, ethanol leaves extracts didn't show any inhibition zone.

Oppositely, a moderate antibacterial activity was recorded against *Bacillus subtilus* ATCC6633 and *Staphylococcus aureus* 693c with an inhibition zone diameter of 10.9±0.78 mm and 12.16±0.12 mm respectively. Concerning ethyl acetate flowers extract, the antibacterial activity was observed only against *Bacillus subtilus* ATCC6633 with an inhibition zone diameter of 10.7±0.35 mm. As for the leaves extracts, an antibacterial activity was recorded against all the tested bacteria with a growth inhibition zone diameter of 8.1 ±0.36 mm, 10.1±0.20 mm and 8.03±0.15 mm for *Bacillus subtilus* ATCC6633, *Listeria monocytogenes* and *Staphylococcus aureus 693c* respectively. It should be noted furthermore, that among all the extracts, leaves methanol extract present the highest growth inhibition zone diameter with 25.33±0.41mm against *Bacillus subtilus* ATCC6633 corresponding at a great antibacterial activity. The amoxicillin exerted the strongest inhibitory effect against the tested microorganisms compared to all extracts. The Gram$^-$ bacteria and the fungi strains were not susceptible to amoxicillin at a concentration of 25 µg per disc.

Table 5. Effect of phenolic compound extracts of *Calendula officinalis* L. on growth inhibition zone diameter sizes (mm).

	Leaves extracts (25 µL)			Flowers extracts (25 µL)			Amoxicillin
	Methanol	Ethanol	Ethyl acetate	Methanol	Ethanol	Ethyl acetate	
Gram$^+$							
B. subtilis	25.33±0.41^a	10.9±0.78^b	8.1±0.36^c	12,37±0.23^d	11.2±0.26^e	10.7±0.35^e	45.13±0.21^f
L. monocytogenese	11.16±0.35^a	--	10.1±0.20^b	8.13±0.15^c	--	--	28.43±0.40^d
S. aureus	--	12.16±0.12^a	8.03±0.15^b	--	10.8±0.60^c	--	34.33±0.49^d
Gram$^-$							
E. coli	--	--	--	--	--	--	--
P. aeroginosa	--	--	--	--	--	--	--
Fungi							
A. *carbonarius*	--	--	--	--	--	--	--
U. *ramaniana*	--	--	--	--	--	--	--

-- no effect

For each line, different alphabetical letters indicate statistically significant differences between values ($p<0.05$, Student's t-test). All values are mean±SD.

The minimum inhibitory concentration (MIC) was estimated for the extract which showed the interesting antibacterial activity against the Gram$^+$ bacteria. A strong antibacterial activity was presented by a low value of MIC (Coulidiati *et al.*, 2009). Globally, perusals of table 6 showed that the IMC values varied from 7.80 to125 µg.mL^{-1}. The lowest minimal inhibitory concentration corresponding to strongest antimicrobial activity was observed for methanol extract from leaves against the standard strain *Bacillus subtilus* ATCC6633 with IMC of 7.8µg.mL^{-1}. The ethanol extract from flowers and leaves observed a relatively appreciable effectiveness against *Staphylococcus aureus* 693c with IMC values of 31.25µg.mL^{-1} and 15.62 µg.mL^{-1} respectively. Also, the leaves methanol extract present a moderate antibacterial activity against *Listeria monocytogenes* with IMC of 15.62µg.mL^{-1}. For each bacteria strain used, in both vegetal matrice, the lowest antibacterial activity was noted or was not detected for ethyl acetate extract.

This observation could be explained by the quantity of the polyphenols and flavonoids present in the extracts which influence the effectiveness of the extracts against microorganisms (Rodríguez-Vaquero *et al.*, 2013). In fact, the finding literature noted that

the bioactive compounds such as phenolic compounds, flavonoids, tannins and alkaloids are one of the most important antimicrobial agent present in the plant (Levy, 1994). Some of them act by altering the biochemical systems of microorganisms , binging their protein molecules or causing inflammation of the cells in order to inhibits their life process. (Garrod 1995)

Table 6. Results of minimum inhibitory concentration (MIC) in µg.mL^{-1} of crude extracts against microorganisms (Gram^{+} bacteria)

Microorganisms	**Flowers extracts**			**Leaves extracts**			**Amox.**
	Methanol	Ethanol	Ethyl acetate	Methanol	Ethanol	Ethyl acetate	
B. subtilis	62.5	62.5	125	7.8	62.5	125	7.8
L. monocytogenese	125	--	--	15.62	--	125	15.62
S. aureus	--	31.25	--	--	15.62	62.5	15.62

-- Not detected

4. Conclusions

The present study was aimed to investigate, for the first time, the effects of the use of MAHD extraction method on the chemical composition of the essential oils from leaves and flowers of *Calendula officinalis* L. cultivated in Algeria. This modern and green method allow us to obtain relatively similar extraction yields compared with conventional method (HD) while reducing the extraction time and saving substantial energy. In all extracted oils, the oxygenated sesquiterpens was the dominant family and the α-cadinol the main compound, present with different relatives amounts depending on the isolation methods. It showed that the phenolic and flavonoids contents are affected by the solvent type. Thus, in general, methanol was the better extraction solvent for both leaves and flowers of *Calendula officinalis*. On another hand, the potential antioxidant of methanol, ethanol and ethyl acetate extracts from leaves and flowers was conducted by capturing free DPPH and ABTS radicals in comparison with adequate positive controls BHT and Trolox respectively. Our finding observed the influence of the polarity of solvents on the biological activities of the tested extracts. In fact, methanol extract presented the highest antioxidant activity, for both matrices, while the ethyl acetate extract observed the lowest one. These general observations could be correlated with the total amount of phenolics and flavonoids present in the extract.

The antibacterial activity of the extracts against pathogenic microorganisms was evaluated using the disc diffusion and broth microdilution methods. The comparison was done with a standard antibiotic (Amoxicillin). A moderate to great antibacterial activity was observed against Gram^{+} bacteria. The efficiency of this antibacterial effect depends on extraction solvent and plant organ. Any antifungal and antibacterial activity against Gram^{-} bacteria was detected.

5. References

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ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *CITRUS LEMON* PEELS ENCAPSULATED IN PVA

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ABSTRACT

In this study, waste lemon peels were converted into a dietary supplement. Lemon peels were used because of the abundance of phytochemicals present in it and also they are easily available throughout the year. To improve the therapeutic efficacy, we used Polyvinyl Alcohol (PVA) as a nanocarrier of lemon peel methanolic extract. The lemon peel extract was encapsulated in PVA by the solvent evaporation method, to improve the solubility and stability of the compounds in the extract. Characterization of the prepared lime peel nanoformulation (LP-NF) was done by Scanning Electron Microscope, Zeta potential and Fourier Transform Infrared techniques. The antioxidant assays like DPPH(2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay and hydrogen peroxide assay showed a high scavenging activity when compared with commercial supplement with the IC50 value of 24 ± 0.05 and 26.07 ± 0.11 respectively. The Gram-negative bacteria, *E. coli* showed a zone of inhibition of 18 mm indicating the antibacterial property of LP-NF. The percentage release of the nanoformulation from sodium alginate beads was calculated and it showed the release of nanoparticle up to 83% after 7 hours in PBS at pH 7.4.

1.Introduction

Fruits and vegetable processing industries produce a large amount of wastes every year. The byproducts of fruits such as peels and pulp of many fruits are sources of sugars, minerals, organic acids, dietary fibres and phenolics (Sand et al., 2006).Pulp and seed contribute to the bulk of the fruit weight, comprising about 46% and 44%, while peel constitutes about 10%. Phytochemicals are non-dietary plant compounds such as carotenoids, flavonoids, isoflavonoids and phenolic acids. These phytochemicals play an important role in protecting cells against oxidative stress caused by harmful free radicals, which cause damage of biomolecules, such as DNA, lipids and proteins (Rui H et al., 2004). Citrus fruits also contain an impressive list of other essential nutrients, including both glycaemic and non-glycaemic carbohydrate (sugars and fibre), potassium, folate, calcium, thiamin, niacin, vitamin C and vitamin B_6, phosphorus, magnesium, copper, riboflavin, pantothenic acid and a variety of phytochemicals(Prasad et al., 2010). Bioactive compounds such as antioxidants like flavonoids, phenolic compounds and ascorbic acid are necessary for human nourishment. Flavanones, flavones and flavonols are three sorts of flavonoids that are abundantly present in Citrus fruit. The important types of flavonoids present in citrus fruits are naringin, narirutin and hesperidin. Essential flavonoid components like flavanones, flavanone glycosides and poly methoxylated flavones are novel to citrus fruits and are comparatively uncommon in other plants (Grohmann K et al., 2001). The peels of citrus fruits especially citrus

lemon are a great source of organic acids, polyphenolic compounds and dietary fibres.These compounds have a wide range of actions such as antioxidants, antibacterial, antiviral and cardio preventive activities. Lemon peels have shown to have protective effects against mouth, lung, skin, breast and colon cancer in many animal studies (Sheila et al., 2001). Utilisation of Citrus by-products as a source of polyphenols and antioxidants might have substantial financial profit to food processors and therefore an economical, efficient, and environmentally safe application of these wastes is required (Balasundram N et al., 2006).Studies show that the different bioactive compounds present in these peels are effective towards various bacteria (Keles et al., 2001).

Antioxidants are molecules that destroy free radical reactions and inhibit cellular damage. Overproduction of free radicals in the human body can cause a disparity that may lead to oxidative damage to large biomolecules such as lipids, DNA, and proteins. This damage causes pathogenesis of several human diseases like Cardiovascular Disease, certain types of cancers, and ageing (Gershoff, 1993; Harats*et al.,* 1998; Jacques *et al.,* 1997). Phenolic compounds are secondary metabolites of plants, which have antioxidant activities (Suryaprakash et al., 2000).Antioxidant activity depends on the extract concentration and increasing concentrations of extract correspond to increase in antioxidant activity. Grape seed extracts are known to have natural antioxidants, such as tocopherol and ascorbic acid and it was found that there was a difference in activity, depending on the assay. The superoxide anion scavenging activity was determined to be dependent on flavanol concentration (Yamaguchi et al. 1999).Diseases like cancer, diabetes, high blood pressure are more likely related to dietary habits. Functional foods recently have earned a lot of importance because they tend to reduce the occurrence of these diet-related diseases. Studies suggest that consuming foods like fruits and vegetables prevent our cells from oxidative damage, and are free scanvengers, help in preventing stress-induced diseases such as cardiac disorders, inflammatory and neurodegenerative diseases (Kaur C et al., 2001, Prakash etal.,2007). Antioxidants are used as food additives as they have the tendency to protect the food from spoilage (Soma Singh et al., 2014).

It was reveaed that the diets high in fruits and vegetables may decrease the risk of chronic diseases, such as cardiovascular disease and cancer, and phytochemicals including phenolics, flavonoids and carotenoids from fruits and vegetables play a key role in reducing such chronic disease risk (Rui H et al., 2004).Phytochemicals from pomegranate (Punicagranatum L) are known to stop cancer cell proliferation and cell apoptosis through the activation of cellular transcription factors and signaling proteins (Sand et al., 2006).Phytochemicals are known to have multiple antimicrobial mechanisms which include damaging the microbial cell wall, cytoplasmic membrane, etc. Reactive oxygen species (ROS) accumulation, Phosphatidylserineexternalization, DNA fragmentation are the few of the mechanisms of a photochemical induced death of cells. Exploiting the ability of phytochemical ought to encourage the advancement of better antimicrobial procedures which could effectively control the human infectious diseases (Omojate G et al., 2014).Extraction of natural antioxidants from orange, lemon and pomegranate fruit peels is done by using methanol as the solvent. Each extract was then utilised in paneer to determine the shelf life and antioxidant activity of value added paneer (Soma Singh et al., 2014).Antibacterial activity of citrus peel extract is due to the presence of essential components including flavonoids, limonoids, essential oils, alkaloids, and lacrone hypericin are effective against a wide range of bacteria. Other potent compounds like alcohols, terpenes and esters add to the antibacterial impacts of essential oils (Keles O. et al., 2001).Recently there has been an increase in concern about the development of antimicrobial resistance of pathogenic bacteria. Citrus peel, a natural substance is

said to have antimicrobial activities. The citrus peel contains various types of essential oils that repress the growth or kill pathogenic bacteria. Citrus peels were assessed for their ability to inhibit the growth of the pathogen like *E.coli* by well diffusion assay on MacConkey agar and a zone of 11mm was obtained indicating that citrus derived essential compounds have potential applications as inhibitory agents against *E.coli* (Ramakrishna N et al., 2008). The factors like quality of the original plant, the geographic origin, climatic condition, harvesting date and storage affect the quality of natural extracts and their antioxidant properties (Cuvelier et al., 1996).The temperature during drying and extraction affects the compound stability due to chemical and enzymatic degradation (Ibaanez et al., 1999).Temperature and light are the major factors affecting phytochemical compounds. These factors affect different compounds like flavonoids, carotenoids and terpenoids to different extents. High-temperature exposure to these phytochemicals can cause a reduction in free- radical scavenging activity (Larrauri et al., 1998).For the isolation of antioxidants from plants, solvent extraction is more frequently used. The yield of extraction and antioxidant activity of extracts are highly dependent on the polarity of solvents used, because different antioxidant potential of compounds react with different polarity of solvents (Duh P et al., 1995).For the extraction of orange peels, different solvents were used, the maximum total phenolic content was accomplished with methanol, whereas 50% acetone extracted more specifically the leucoanthocyanins. Lower IC50 values for the DPPH radical (amount of antioxidant needed for causing a reduction of 50% in the absorbance of DPPH) were observed for butanol extracts, followed by those in ethyl acetate (Julkunen-Tiitoa et al., 1985).For measuring the antioxidant activity of a particular substance, single assay can reflect the scavenging sources and antioxidants present in a system (Prior R et al., 2005).Generally important natural materials have been extracted with organic solvents and nonetheless, some of them are toxic, and the extraction conditions are often severe. Thus, a food grade ethanol rather than methanol is broadly utilizedin the extraction of phenolic compounds from different citrus peels (Li B at al., 2006).Antioxidant activity of a plant extract can be measured with different tests with different mechanisms. Chemical methods are based on the ability of extracts to scavenge different free radicals. UV-absorption and chelation ability are responsible for the antioxidant activity in oily systems. Tests measuring the scavenging activity with different challenges, for example, superoxide radical (O_2), hydroxyl (OH), nitric oxide (NO), alkyl peroxy radicals, ABTS+ (radical cation of 2, 20 – azinobis, 3 ethylbenzothiozoline-6-sulphonate), (2, 2-diphenyl-1-picrylhydrazyl) (DPPH) have been developed (Butler J et al., 1993).

In spite of the fact that, plants have huge potential as therapeutic compounds, its effectiveness and oral bioavailability is constrained by poor solubility and poor formulation characteristics because of high lipophilicity.Techniques used for preparing nanoparticles from biodegradable polymers are emulsion solvent evaporation, nanoprecipitation, salting out procedure, and a combined method. Nano-encapsulation of drugs/ plant extracts in biodegradable polymers like PVA (Poly Vinyl Alcohol), PCL (Polycaprolactone) has got consideration as a conceivable drug carrier system due to its faster mobility, high drug loading capacity and the possibility of controlled drug release to the specific target site. Moreover, these biodegradable polymers are approved by US Food and Drug Administration (FDA) and its final degradation products such as lactic and glycolic acids are perfect and safe, as they are either discharged by the kidneys or enter the Krebs' cycle to be in the end of the process eliminated as carbon dioxide and water (Leo E et al., 2004).Medicinal plants work at a very high dose, and these limitations can be addressed by formulating a suitable dosage form, that could offer better therapeutic

effectiveness at low doses. Drugs from plant origin require an approach which can avoid administration of high dose and also increase patient compliance. One such attempt is nanoparticle formulation. Several studies are reported for the incorporation of different phytoconstituents in the form of nanoparticles (Trickler W et al., 2008). Recently phytochemicals have been used greatly as a nutraceutical in pharmaceutical and food formulations. Even though lemon peels have great ability as a therapeutic compound, its effectiveness is poor because of its poor solubility and oral bioavailability. This issue can be fixed by converting the lemon peel extracts to lemon peel nanoparticle. Nanoparticles can be formed or prepared by various types of methods. Preparing nanoparticles from biodegradable polymers can be prepared using different methods like emulsion solvent evaporation, salting out technique, and nanoprecipitation. Nanotechnology has become an important part of the food industry. This technique has the ability to change sensory characteristics, change the nutritional functionality of the food product, change colour, flavour and also can enhance the shelf life of the food product (Ganesan S et al., 2014).

Nanoencapsulation uses the emulsion solvent evaporation method to capture or entrap essential compounds into a carrier, for transporting it to the target site and for releasing the compounds in a sustained manner (Chiu et al., 2007). Plant origin drugs require such an approach, which avoids administration of high dose and also increases the patient compliance.

Encapsulation of essential compounds of citrus species, into carriers or matrices helps in the protection, transport and release of the compounds in a controlled manner. Likewise, encapsulation could be utilised to increase the shelf life of materials for controlled delivery of essential compounds when ingested in the digestive system over a scope of physiological conditions (Gharsallaoui A et al., 2007).

Nanoparticle antioxidants are another form of therapies which are used for the prevention and treatment of diseases occurring due to oxidative stress (Chelarama et at., 2014). Nanoparticle antioxidants due to its size have effective and sustained interactions with biomolecules and work strongly against free radical-induced cell damage. These nanoparticles have shown a high-performance therapeutic activity in constricting oxidative stress with potential applications in treating and preventing neurodegenerative conditions (Wang J et al., 2009).Despite being a powerful bioactive agent and natural antioxidant, few fruit peels like pomegranate are practically water-insoluble. A solution to this issue would be the development of formulations of pomegranate peel nanoparticles to enhance its stability. The higher water-solubility could be ascribed to a larger surface area in contact with the solvent. The nanoformulation of the pomegranate had an antimicrobial effect stronger than the pure extract (Anand P et al., 2007).Polymers from normal sources have been utilised broadly in the pharmaceutical and nourishment industry. Among these polymers, polysaccharides have been broadly used in view of their biocompatibility, biodegradability, and low harmfulness). Alginate, a water-soluble, the natural polysaccharide consist of linear polysaccharide comprising of β-D-mannuronic acid and α-L glucuronic acid deposits combined together in blocks which are regularly utilised because of its mucoadhesive properties and its capacity to form matrix systems and can be gelled through ionic or covalent cross-linking. The alginate-based hydrogel systems are extremely effective in capturing and controlled delivery of various essential components like drugs, proteins, enzymes and cells (Poncelet R et al., 1992). The alginate matrices basically encapsulate nanoparticles and as the alginate loaded nanoparticles pass through stomach fluids at different pH, where there is a sustained release of the nanoparticles (Kawabata et al., 2010).Encapsulation of the drug loaded polymer nanoparticles into alginate- matrices provide protection and stability to drug amid

its transit along the gastrointestinal tract, in this manner increasing the amount of drug accessible to apply its pharmacological effect. Besides, the presence of alginate in the developed hydrogel matrices permits sustained release of the drug as the particles go down the gastrointestinal tract (George M et al., 2007).The aim of this study is to produce a high dietary nano supplement from waste lemon peels and determining its antioxidant, antimicrobial properties and incorporation of this nano supplement into alginate beads and checking the*in vitro* release activity.

2.Materials and methods

2.1. Collection of material

The lemon peels were collected from the local juice shop. The lemon peels were washed well-using tap water. The peels were cut into small pieces, then it was kept under the sun for drying over a period of 3-5 days. The dried samples were powdered using a mixiegrinder. The powder of the peels was stored separately in airtight bottles (Asia et al., 2015).

2.2. Preparation of extracts

2.2.1.Soxhlet extraction

The 25g powdered sample was extracted using 500ml of methanol at room temperature by Soxhlet extraction apparatus for 6 hours. The mixture was filtered through a Whatman filter paper and evaporated under reduced pressure at 60°C by a rotary evaporator. The extracts were placed in dark bottles and stored in the refrigerator at 4°C for further use (Hegazy et al., 2012).

2.3. Preliminary Phytochemical Analysis

The powered lemon peels were subjected to the following preliminary phytochemical screening tests(Kaur et al., 2013).

- Test for Saponins: 2ml of the extract added with 6ml of distilled water.
- Test for Phytosterols: 4-5 drops of extracts, added with 1ml Chloroform and few drops of concentrated sulphuric acid.
- Test for Flavanoids: 2 to 4 drops of ferric chloride added with 0.5 -1ml of the extracts.
- Test for Phenols: 1ml of the extract added with 5ml of Folin's reagent and 4ml of sodium carbonate.
- Test for Steroids: 0.5 ml of extract added with 3ml of chloroform and 2ml of concentrated sulphuric acid.
- Test for Tannins: 1ml of extract added with few drops of 1% ferric chloride.
- Test for Terpenoids: 5ml of extract added with 2ml of chloroform and 3ml of sulphuric acid.
- Test for Cardiac glycosides: 5ml of extract added with 2ml of glacial acetic acid with 1 drop of ferric chloride added with concentrated sulphuric acid.
- Test for Amino acids: 1ml of extract added with few drops of ninhydrin.

2.4. Determination of total phenolics content

Total phenolic content (TPC) was measured using gallic acid for the calibration curve. Results are presented as Gallic Acid Equivalents (GAE). The total phenol content was determined according to Folin-Ciocalteu's reagent method. 0.5ml of extract and 0.1 ml of 0.5 N FolinCiocalteu's reagent was mixed and the mixture was incubated at room temperature for 15 min. Then 2.5 ml of 20% sodium carbonate solution was added and further incubated for 30 min, at room temperature and the absorbance was measured at 765 nm. Gallic acid was used as a positive control. Total phenol values are expressed in terms of gallic acid equivalent for lemon peel (mg of gallic acid/g of extracted compound) (Kamtekar et al., 2014).

2.5.Preparation of lemon peels nanoformulation (LPNF) of methanolic extract

LPNF were prepared by solvent evaporation technique (Shreedhara, C.S et al., 2017).1:3 ratio of Lemon peel extract to Polyvinyl Alcohol (PVA) was used in this method. Briefly, 30 mg of PVA was dissolved in 10 mL milliQ water. 10 mg of

the lemon peel methanolic extract was dispersed in 10 mL acetone. The dispersed lemon peel methanolic extract in acetone was added drop wise to the PVA solution on a magnetic stirrer. The resulting mixture was kept on the magnetic stirrer for the organic solvent to evaporate. The suspension formed was homogenised using high-speed homogenizer at different time intervals and then sonicated using different sonicator parameters. Centrifugation was done at 2300 rpm for 30 minutes at room temperature. The supernatant was separated by centrifugation at 18000 rpm for 30 min in a cooling ultracentrifuge at 4°C. LPNF appeared as a sediment, which was separated and re-suspended in milliQ water. and freeze-drying was done by adding 2% mannitol. Nanoparticles thus formed were evaluated for yield, particle size and zeta potential.

2.5.Characterization Studies:

2.5.1.Determination of Yield (%):

The nanoparticles were weighed and the practical yield was calculated using the following equation:

Yield %= (Weight of nanoparticles obtained / Weight of extract and polymer used) ×100

2.5.2.Shape and surface morphology:

Shape and surface morphology of the nanoformulation was measured byHigh Resolution Field Emission Electron Microscope at an acceleration voltage of 5-20 KV.

2.5.3.Zeta potential:

Zeta potential was measured using Horiba Nanoparticle analyser. It is used for evaluating the surface zeta potential of the nanoparticles. The Zetasizer Nano ZS is a high performance two angle particles and molecular size analyser for the enhanced detection of aggregates and measurement of small or dilute samples, and samples at very low or high concentration using dynamic light scattering.

2.5.6.FT-IR Spectroscopy:

FTIR analysis of the nanoformulation was done using FTIR spectrophotometer by Agilent Technologies- Cary 600 series. The spectrum was recorded in the region of 4000 to400 cm-1. The FTIR spectra was compared with the FTIR spectra of the extract and PVA.

2.6.In vitro antioxidant properties

2.6.1.Determination of DPPH (1-1-diphenyl 2- picrylhydrazyl) radical scavenging activity

DPPH radical scavenging assay was performed according to the method of Rekha S et al., (2013) and absorbance was measured at 517 nm using a spectrophotometer. Percentage inhibition and IC50 values were calculated with respect to control. Ascorbic acid was used as the standard. 0.1 mM of DPPH solution was prepared in methanol and 1.0 ml of this solution was added to 1.0 ml different concentrations (10-50 µg/ml) of nanoformulation. After incubation for 30 minutes, the absorbance was measured at 517 nm against blank. Ascorbic acid was used as the reference standard. Radical scavenging activity was expressed as the percentage of inhibition and was calculated using the following formula:

$$\%\text{Inhibition} = [(A_o - A_t)/ A_o] \times 100$$

Here, A_o was the absorbance of the blank (without nanoformulation) and A_t was the absorbance of nanoformulation. All the tests were performed in triplicate.

2.6.2.Scavenging Hydrogen Peroxide:

The ability of the nanoformulation to scavenge hydrogen peroxide was determined according to the method of Bhakya S et al., (2016). A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer at pH 7.4 and concentration was determined spectrophotometrically at 230 nm. Nanoformulation at different concentrations (10-50 µg/ml) in distilled water was added to 0.6ml of hydrogen peroxide solution and the absorbance of the mixture was read at 230 nm after 20 minutes against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging activity of hydrogen peroxide of nanoformulation and

the control is calculated using the following equation:

$$\%Inhibition = [(Ao - At)/ Ao] \times 100$$

Here, Ao was the absorbance of the blank (without nanoformulation) and At was the absorbance of nanoformulation. All the tests were performed in triplicate.

2.7.Antibacterial Activity:

LPNF was tested against *Escherichia col*i for antibacterial activity by agar well diffusion method. and this was performed by determining the zone of inhibition. Pure cultures were subcultured into a nutrient broth and incubated at 37^{o} C for 24- 48 hours. The test organism was spread uniformly on the individual plates using spread plate technique. Three wells of 5 mm diameter were made on the agar plate. Using sterile micropipette tips, 0.1 mL (100 µL) of the sample solution was pipetted into each of the wells in all the plates. After incubation at 37^{0}C, the diameters of zone of inhibition were measured after 24hours (Kokila T et al., 2015).

2.8.Preparation of Sodium Alginate Beads:

Sodium alginate solution at 3 wt% was freshly prepared. The alginate powder was dissolved in double-distilled water while mixing with a magnetic stirrer. Lemon peel nanoformulation (LP-NF) was added to the alginate solution. The alginate solution containing LP-NF was dropped using a syringe through a 20-gauge needle into 0.5M $CaCl_2$ solution. Ionically cross-linked alginate beads were formed and cured in the $CaCl_2$ solution for 30 min at room temperature. The alginate beads were then collected by filtration and gently washed twice with deionized water (Kim et al., 2005).

2.9.In-vitro drug release study

The release of LP-NF from the sodium alginate beads was performed in 0.1M PBS. pH 2.1 (gastric pH) and pH 7.4(intestinal pH) of PBS were considered for this study.The first 2 hours were maintained at pH 2.1 and the subsequent hours were maintained at pH 7.4 at 37◦C. The prepared alginate beads in PBS were kept on a shaker. After anhour of incubation, aliquots were removed from the buffer solution,and wasanalysed by UV-Vis spectrophotometry at λ_{max} of 570nm.The percentage of LP-NF released was calculated from the standard graph of LP-NF in PBS at different concentrations such as 0, 5, 10, 15, 20, and 25 µg/ml (Guzman-Villanueva et al., 2013).

3.Results and discussion

Bioactive compounds are the main constituents in most fruits and vegetables and these are reported to contain antioxidant and free radical scavenging activities. Phenolics present in these bioactive compounds act as free radical scavengers which exhibit antioxidant activity by inhibiting lipid peroxidation and by preventing the oxidation of hydroperoxides. Flavonoids are one of the largest groups among the natural phenolics and are said to possess antioxidant properties acting as effective scavengers of harmful free radicals. Characterization of the prepared nanoformulation was done by scanning electron microscopy (SEM), Fourier transform Infrared Spectroscopy (FTIR) and Zeta potential.

3.1.Phytochemical analysis:

Different phytochemical tests were carried out. Figure 1 shows the result for phytochemical analysis followed by Table 1 which lists the presence or absence of the various phytochemicals in our lemon peel extracts.

Table 1. Phytochemical analysis of lemon peel extract in methanol. (+) indicating the presence of phytochemical and (-) indicating absence of the phytochemical.

Phytochemicals	**Methanol**
Saponins	-
Phytosterol	+
Flavanoids	+

Phenols	+
Steroids	+
Tannins	+
Terpenoids	-
Cardiac Glycosides	-
Amino acids	-

Figure 1. Phytochemical Test Results for Lemon peels

3.2.Total phenolic content:

In the present study, a high phenolic content of 0.923 GAE was observed in the methanolic extract of *Citrus limon* peels.This high content of phenol contributes to its potential antioxidant property and curative ability by adsorbing and neutralizing free radicals. The results are in agreement with the studies of Samidha Kamtekar, S et al., 2014 where, higher phenolic content (0.97 ± 0.11 GAE) was obtained in methanolic extracts of *Citrus limon* peels.

3.3.Yield%:

Different batches of LP-NF were prepared and these were subjected to different sonication and homogenization variables. Yield percentage was calculated and it was found to be highest in LP-NF3 (11.5%).

Table 2. Batches of Lime Nanoformulations subjected to different sonication and homogenization parameters.

Batches	**LP-ME (mg)**	**PVA (mg)**	**Sonication (a/t/p)**	**Homogenization (Seconds)**	**Yield (%)**
LP-NF1	10	30	60/10/5	10	7.2
LP-NF2	10	30	50/10/5	10	8.3
LP-NF3	10	30	60/10/5	20	11.5
LP-NF4	10	20	60/10/5	10	8.5
LP-NF5	10	50	50/10/5	20	7.8

3.4. Scanning Electron Microscopy:

The scanning electron microscopy photographs showed the formulation of nanoparticles. The structure of the nanoparticles plays an important role in determining their adhesion, interaction and

absorption with the body cells. It was shown that the LP-NF displays a particle.

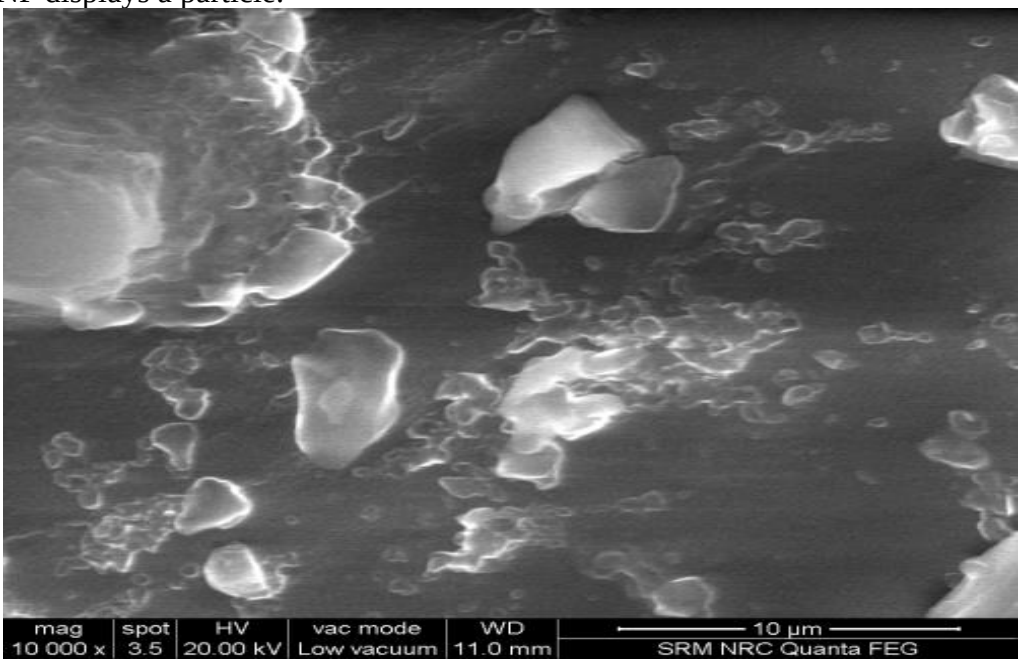

Figure 3.SEM image of LP-NF

3.5.FTIR Characterization:

The intermolecular interaction between PVA, Lemon peel methanolic extract and LP-NF was determined by FTIR spectroscopy are shown (Fig.7 and 8). The characteristic spectra of the PVA polymer showed in the region 2918 cm^{-1} and 929 cm^{-1} due to –CH stretchingvibrations, carbonyl –C=O stretching at 1735.53 cm^{-1} and –OH stretching at 3419.29 cm^{-1}. The lemon peel extract spectra showed characteristic bands in the region 3418.14 cm^{-1}due to –OH stretching vibrations and also bands in the region 1732.65 cm^{-1} and 1076.39 cm^{-1}due to C=O stretching vibrations. For LP-NF, the spectra showed that the OH stretching band (3200–3600 cm^{-1}) is slightly shifted to a lower wave length. The IR band of at 1076.39 cm^{-1} can be attributed to the –C–O– stretching vibrations of carboxylic acid, ester, and ether groups of the proteins present in the extract (LP-ME) and this peak shifted to 1095.56 $cm^{-1}$for LP-NF. The spectra obtained for PVA nanoparticles showed characteristic bands that were consistent with the studies of (Sowmya et al 2017). The slight shift in the spectra of OH-stretching band (3200–3600 cm^{-1}) for lemon peel loaded PVA nanoparticle may be due to increase in terms of energy absorption. These observations suggest that lemon peel methanolic extract is associated with the PVA polymer by hydrogen bonds. Also, the band corresponding for C=O stretching (1700–1800 cm^{-1}) was broader, indicating that the extract is associated with the PVA polymer by interactions between the carbonyl and the carboxyl groups of the flavonoids in the extract and the polymer

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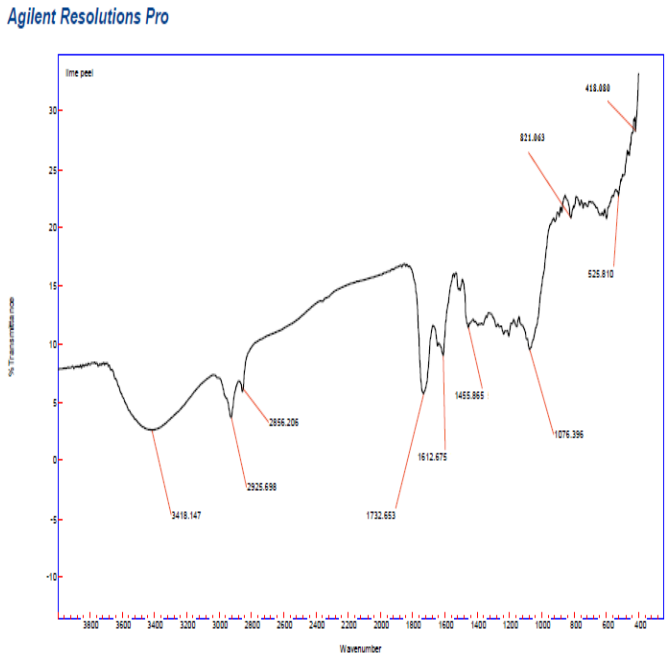


Figure 4.FTIR spectra of Lemon Peel Methanolic Extracts

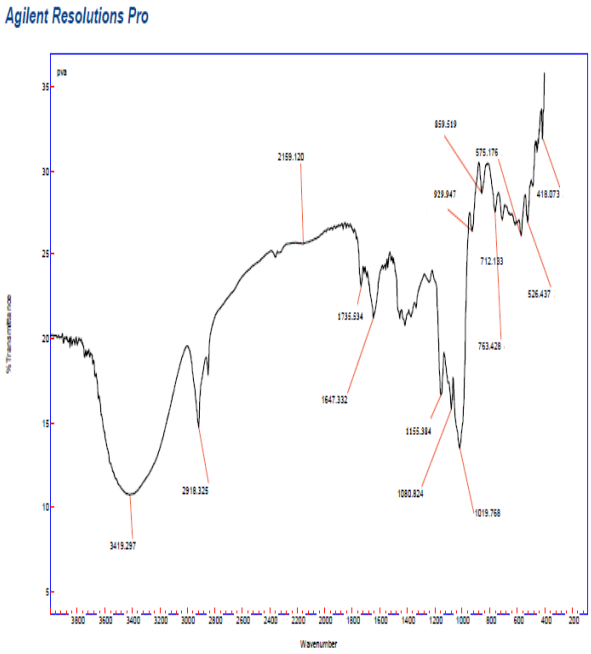


Figure 5.FTIR spectra of PVA

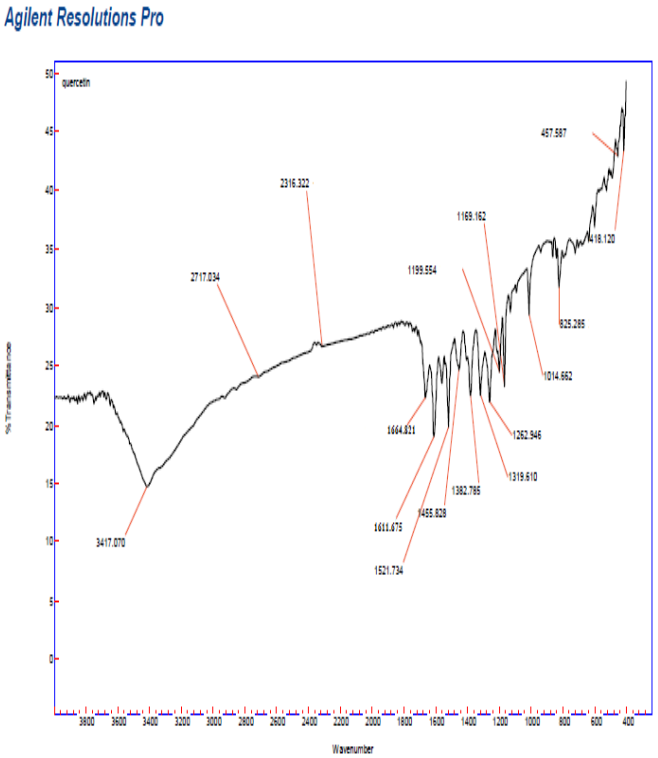


Figure 6. FTIR Spectra of Quercetin

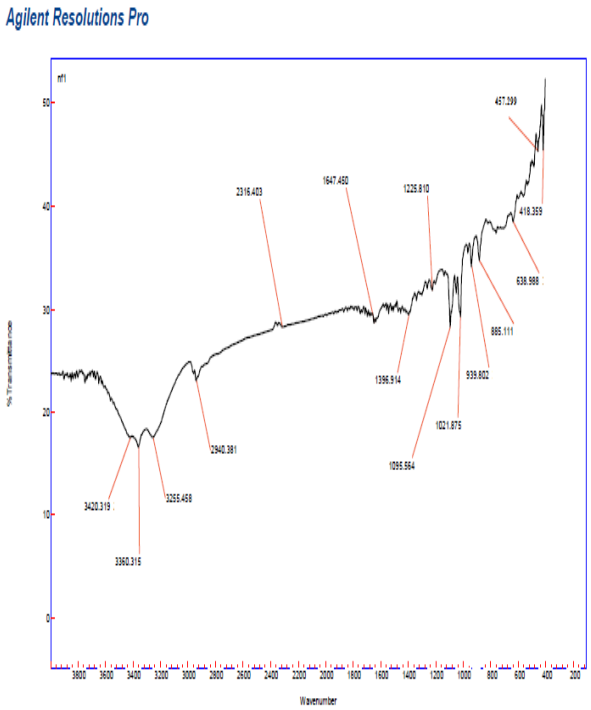


Figure 7. FTIR Spectra of Nanoformulation

3.6.ZETA POTENTIAL:

Zeta potential of the LP-NF was found to be -28.3 mV (Fig.8). Nanoparticles with zeta potential values greater than +25 mV or less than -25 mV are said to have high degrees of stability. As the observed zeta potential of the prepared nanoparticles of lemon peel is in the range of stability, the nanoparticles are said to be stable. Similar result was reported with a charge of -23 mV of the lemon nanoparticle was obtained (George M et al., 2007).

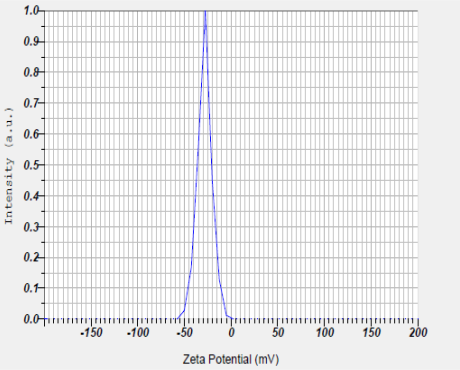
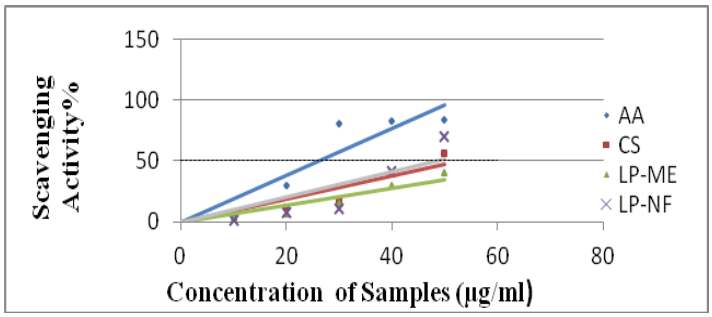


Figure 8. Zeta potential for LP-NF

3.7.Antioxidant Assays

3.7.1.DPPH Radical Scavenging Activity:

LP-NF has shown the reduction in IC_{50} values in all the tested models and the result shows higher scavenging activity than the LP-ME and CS. This observation indicates that the nanoformulation is therapeutically more effective. This method uses the principle of a stable free radical (DPPH) that accepts an electron or hydrogen radical to become an overall stable molecule. The reaction of DPPH is observed by measuring the absorbance of its radical at 517 nm. Lower the absorbance value, higher is the sample's scavenging activity. Upon reduction DPPH by an antioxidant, the absorbance at 517 nm disappears. The results revealed that LP-NF showed higher potency in scavenging the DPPH free radicals than pure extract of lemon peels and commercial supplement. Previous reports by Rekha S et al., 2013 also revealed enhanced scavenging activity in PVA encapsulated orange peel extracts.



AA- Ascorbic Acid
CS- Commercial Supplement
LP-ME- Lime peel methanolic extract
LP-NF- Lime peel Nanoformulation

Figure 9. 50% Scavenging activity of AA,CS, LP-ME and LP-NPsample

Table 3. Antioxidant activity for AA, CS, LP-ME and LPNF5 (IC_{50} µg/mL) with significance($p<0.05$)

S.No	Samples	IC_{50} Values
1.	AA	26.032 ± 0.21
2.	CS	52.524 ± 0.36
3.	LP- ME	72.421 ± 0.32
4.	LP-NF	48.823 ± 0.17

3.7.2. Hydrogen Peroxide Assay:

LP-NF showed reduction in IC_{50} values in all the tested models and the result shows higher scavenging activity than the CS. This observation indicates that the nano-formulation is therapeutically more effective.

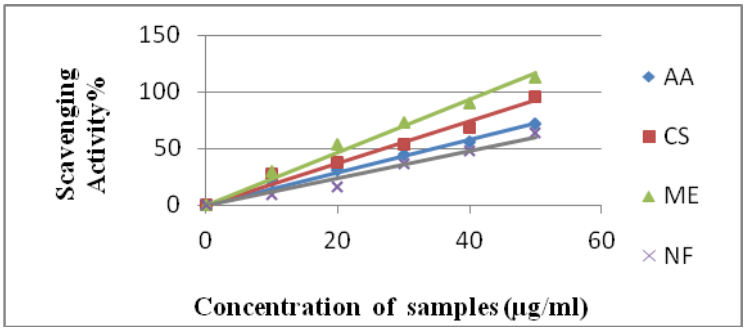


Figure 10. 50% Scavenging activity of AA, CS, LP-ME and LPNF

Table 4. Antioxidant activity for AA, CS, LP-ME and LPNF (IC_{50} µg/mL) with significance ($p<0.05$)

S.No	Samples	IC $_{50}$ Values
1.	AA	22.73 ±0.69
2.	CS	34.47 ±0.32
3.	LP- ME	41.52 ±0.11
4.	LP-NF	26.99 ±0.18

3.8. Antibacterial Activity:

The biologically synthesized Lemon Peel Nanoformulation showed antimicrobial activity against *E.coli* (Gram-negative bacteria). The zone of inhibition was measured and tabulated. The zone of inhibition for LP-NF against *E. coli* was determined to be 18mm. Kokila T et al., 2015 reported that *Citrus limon* peel showed zone of inhibition of 15mm against *E.coli*. This proves that antibacterial effect was found to be higher in nanoformulated extract.

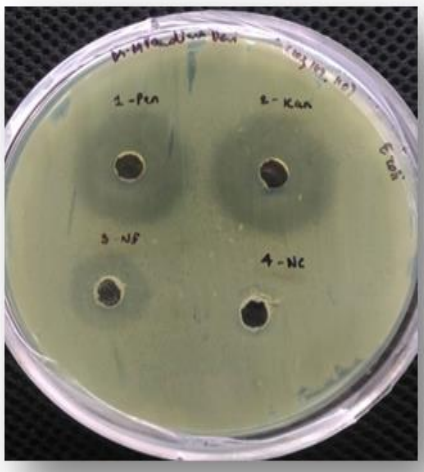


Figure 11. 50%Scavenging activity of AA, CS, LP-ME and LPNF

Table 5. Inhibitoryaction of LP-NF against *E.coli*.

S.No.	Drug	Zone of Inhibition
1.	Amikacin	25mm
2.	Kanamycin	28mm
3.	LP-NF	18mm
4.	Negative control	-

3.9. In vitro Percentage Release Study:

LP-NF percentage release from sodium alginate beads suspended in PBS buffer at pH 2.1 (gastric pH) and pH 7.4 (intestinal pH) is shown. The burst release of the nanoformulation was observed. It was found that 64% of the loaded drug was released in the first 7 h. Next, a sustained drug release phase was observed that continued upto 15 hours, when 83% of the nanoformulation was released. It was reported that at pH 7.4 and 70% of nanocurcumin from alginate beads was released (Guzman-Villanueva et al., 2013). The high release can be attributed to the presence of holes on the surface of the beads, which ease the diffusion of the release medium into thebeads loaded with LP-NF. Encapsulated bioactive compounds into nano delivery systems are being increasingly tested in food with the intention to improve the bioavailability of the hydrophobic phytocompounds. At present, biodegradable polymers are extensively used in drug delivery systems.

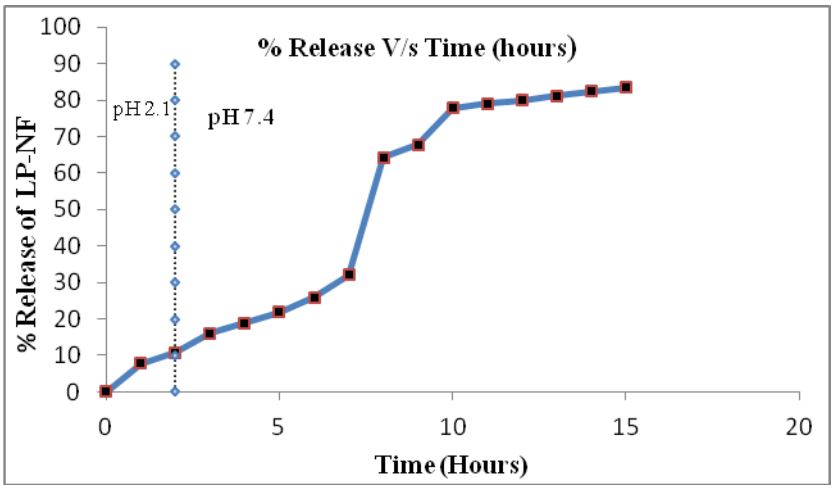


Figure 11.pH dependent release patterns of LPNF from Sodium alginate beads (pH 2.1 and 7.4) at 37°C

4.Conclusions

Lemons are a rich source of phytochemicals. Every year high amounts of lemon peels are wasted. In this project, the waste lemonpeels are converted into a valuable product. Since lemon peel extracts are poorly soluble and bioactive compounds of lemons have poor bioavailability they are loaded asnanocarriers. Biodegradable polymers are said to be a good nano carrier system for plant extracts. The antioxidant properties of lemon peel nanoformulation were determined by DPPH and hydrogen peroxide assays. The lemon peel nanoformulation showed a reduction in their IC_{50} values in both the assays.Well diffusion assay of LP-NF showed antibacterial activity against *E.coli*. In the in vitro release study of the nanoformulation, the percentage release of the nanoformulation from sodium alginate beads was calculated, and it showed a significant release after 7

hours in PBS at pH 7.4.The methodology employed used for preparation of plant extract nanoformulation is very simple, easy to perform, inexpensive, and eco-friendly.This suggests that the nanoparticles system can be applied to other medicinal plants that are known to be poorly soluble and have efficacy at higher dose.

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OPTIMISATION OF PECTIN EXTRACTION ASSISTED BY MICROWAVE FROM BANANA (*Musa sapientum* L.) FRUIT PEELS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT
In this study, microwave-assisted extraction (MAE) was used to extract pectin from waste banana (*Musa sapientum* L. CV. Kluai Namwa) fruit peels. Central composite design (CCD) was used to study and optimise the effects of processing parameters' variables (microwave irradiation, extraction time, and pH) on the pectin yield, degree of esterification (DE), and galacturonic acid content (GA) of pectins extracted from dried banana fruit peels with hydrochloric acid of 0.05 M. Extraction parameters applied in this study are microwave irradiation (300-600 watt), extraction time (5-15 min), and pH (1-3). The results showed that all the process variables had significant effect on the responses. The optimum conditions for the maximum pectin yield (13.47%), DE (92.45%), and GA (87.99%) were obtained at microwave irradiation of 580 watt, extraction time of 15.86 min, and pH of 1.71. Under the optimal conditions, close agreement between experimental and predicted values was obtained. From the results, second order polynomial model was developed and it adequately explained the data variation and significantly represented the actual relationship between independent variables and the response.

1. Introduction

Banana (*Musa sapientum* L. CV. Kluai Namwa) is one of the most important fruit crops that exists in large quantity in Thailand. Its fruits are used as snacks such as banana crisps or sweet banana crisps and their processed products include chips, puree, banana flour etc. that can be used to produce a variety of other products such as smoothies and nectars (Oliveira TÍ et al., 2016). Banana fruit peel is a by-product of the fruit industry and vendors; it is mostly used for fertilisation or as animal feed. However, proper utilisation of banana fruit peels can increase the economic value of the banana peels and reduce cost of waste disposal. Then, the use of banana fruit peels as a new raw material for the production of co-products in food industries such as pectin and other high value compounds such as cellulose nanofibres and phenolic compounds can be valued (Happi Emaga et al., 2008; Rebello et al., 2014). These are interesting propositions that not only can increase economic value, but also bring positive impact from the environmental perspective.

Pectin is a complex mixture of polysaccharides found in primary cell walls and middle lamella of plant tissues (Carpita and McCann, 2000). It consists of α-D-galacturonic

acid and some neutral sugars such as α-L-arabinose, α-D-galactose, and α-L-rhamnose (Basanta et al., 2012). The majority of pectin structure contains a backbone of α-(1, 4)-linked to α-D-galacturonic acid units that are partially esterified with methyl alcohol or mineral acid at the carboxylic acid (Xu et al., 2014). The degree of esterification (DE) is defined as the percentage of carboxylic acid units in pectin esterified with methyl alcohol. The DE of the pectin is an important factor for the definition of properties of pectin (Seixas et al., 2014). Depending on DE, pectin is divided into two major groups, namely high methoxyl pectin (HMP) with DE value higher than 50% and low methoxyl pectin (LMP) with DE value lower than 50% (Yapo et al., 2007). The HMP forms gels when heated in solutions with a low pH (2-3.5) and at high concentrations of sugar (55-75%), while the LMP can form gels in a wider pH range (2-6) without or with a little amount of sugar in the presence of divalent ions such as calcium (Wai et al., 2010). HMP and LMP have different physicochemical properties and thus different applications (Chan and Choo, 2013). In the food industry, pectin is widely used as food ingredient such as stabiliser, gelling agent, water binder, and thickener for the production of jellies, jams, fruit juices, and confectionery (Willats et al., 2006; Kratchanova et al., 2004). Among other uses, it has been used as a dietary fibre that has a positive effect on the digestive system and can reduce blood cholesterol. Moreover, LMP may be used as a gelling agent and texturising fat replacer to imitate the mouth-feel of lipids in low calorie food products for overweight and diabetic patients (May, 1990; Wai et al., 2010).

In the conventional extraction method, pectin is extracted using hot water (about 90 ^{o}C) acidified with mineral acids (Xu et al., 2014). However, this process is time-consuming, which normally takes at least three hours or even more than twenty hours to get sufficient amount of pectin and it leads to pectin degradation (Seixas et al., 2014; Liu et al., 2006). Therefore, there is a need to replace this extraction method with newer and better techniques to minimise the stated adverse effects. Microwave assisted extraction (MAE) is a non-conventional heating method that has been accepted as a powerful and potential alternative to conventional extraction methods, and it has many advantages such as higher extraction yield, shorter extraction time, less solvent extraction, and better quality products with lower capital cost (Maran et al., 2013b; Bagherian et al., 2011). Microwave is non-ionising radiation and it does not break down chemical bonds or cause molecular changes in the compounds by discharge of electrons. During extraction, microwave irradiation can directly extract plant constituents from sample solid matrixes and can induce the moisture inside the tissues to heat or evaporate, leading to tremendous increase in pressure on the cell walls. Finally, it breaks plant cell walls and releases the targeted compounds into the surrounding solvent (Dhobi et al., 2009). Over the last few years, MAE technique has been employed in pectin extraction from a variety of natural materials such as apple pomace, durian rind, pomelo peel, orange peel, and lime (Quoc et al., 2015; Fishman et al., 2006; Maran et al., 2013c)

From the extensive literature analysis, it has been found that many researchers have reported on pectin extraction from fruit peels. However, the optimal conditions for MAE technique of pectin extraction from banana fruit peel (*Musa sapientum* L. CV. Kluai Namwa) has not been reported yet. Hence, the purpose of this study is to develop a rapid MAE method for waste banana fruit peels as a new source of pectin and also to investigate and optimise the process parameters of MAE conditions such as microwave irradiation, pH, and extraction time for maximum extraction yield and quality of extracted pectin by using three factors, three-level central composite design. Response surface methodology (RSM) is designed to

optimise the pectin extraction process. The optimised conditions determined from MAE technique in this study should provide important reference data for subsequent studies.

2. Materials and methods

2.1. Materials

Banana fruit peels were collected from a local vendor in Ubon Ratchathani province. The banana fruit peels were washed, cut into smaller pieces, and treated according to the method reported by Tangwongchai et al. (2006) with slight modifications in which ethanol was used instead of blanching in boiling water and drying temperature was changed to 65 ^{o}C. Briefly, the sample was treated with equal amount of 95% ethanol (1:1 solvent to fresh weight of sample ratio) at 80 ^{o}C for 10 min. Then, the sample was washed with distilled water (3 times) and followed by drying of sample at 65 ^{o}C until weight of the sample was constant. The dried banana fruit peel (the moisture content of the sample was 3.88$\pm$0.04% in weight) was then ground into fine powder by a mechanical grinder and was passed through a pore size sieve (80 mesh) before kept in a dark airtight bottle at -20$\pm$2 ^{o}C until further experimental analysis.

2.2. Pectin extraction

Microwave assisted extraction of banana fruit peels was performed according to the method reported by Li et al. (2012) with slight modifications. The dried peel powder was poured into 0.05 M hydrochloric acid in a flask. The ratio of dried banana fruit peel to extracting solvent was 1:20 (w/v) and adjusted to the desired pH values (1, 2, and 3) and thereafter stirred. The solution was then extracted with three powers of 300, 450, and 600 watt for three times (5, 10, and 15 min). After extraction, the mixture was filtrated using filter paper and centrifuged (10000 g) for 30 min. Then, the supernatant was precipitated by adding 95% ethanol (the ratio of filtrate to ethanol was 1:2 (v/v)) and incubated at room temperature for 15 hours. After that, the coagulated pectin was filtered by using a Buchner funnel and was washed thrice by 95% ethanol and 95% acetone to remove the disaccharides and monosacharides (Minkov et al., 1996). In the next step, the wet pectin was dried at 60 ^{o}C in the hot air oven until a constant weight was obtained and was ground into powder. The pectin yield was calculated as follows:

$$\% \text{ Extraction yield} = \frac{\text{Weight of dried pectin (gram)}}{\text{Weight of dried powder sample (gram)}} \text{ x } 100$$

2.3. Determination of degree of esterification

The degree of esterification (DE) of the banana fruit peel pectin was determined by the titrimetric method (USP 26 NF21, 2003) with minor modifications. Briefly, the dried pectin sample (300 mg) was moistened with 2 mL of ethanol and dissolved in 100 mL of distilled water. The mixture was mixed until the sample was completely dissolved. Four drops of phenolphthalein indicator were added and the mixture was titrated with 0.5 M sodium hydroxide (V_1). Thereafter, 10 mL of 0.5 M sodium hydroxide was added and the mixture was stirred and kept for 15 min. Then, 10 mL of 0.5 M hydrochloric acid was added and the mixture was shaken until the pink colour disappeared. Finally, four drops of phenolphthalein indicator were added again and the mixture was titrated with 0.5 M sodium hydroxide (V_2) until a pale pink colour that persisted after vigorous shaking was obtained. The DE value (%) of the pectin sample was calculated as follows:

$$\% \text{ Degree of esterification (DE)} = \frac{\text{NaOH (V2)}}{\text{NaOH (V1)+NaOH (V2)}} \text{ x } 100$$

2.4. Determination of galacturonic acid

The galacturonic acid content was determined as described by Dedduang (2010) with some modifications. Briefly, dried pectin sample (0.1 g) was mixed and the volume was adjusted to 100 mL with 0.05 M of ethanol. The

mixture was stirred with a vortex and was incubated for 30 min. Then, 10 mL of pectin solution was adjusted to 100 mL with distilled water in a volumetric flask. After that, 2 mL of diluted pectin solution was transferred to an Erlenmeyer flask. Then, 1 mL of 0.1% of cabazole and 12 mL of sulfuric acid concentration were added and the mixture was stirred for 25 min at room temperature. Then, the sample was measured for absorbance at 525 nm. The galacturonic acid content was determined by comparison with a calibration curve obtained from standard galacturonic acid solutions (10-100 µg/mL).

2.5. Experimental design

A central composite design was used to the matrix of the experimental design to optimise and investigate the effect of independent variables (microwave radiation (X_1), extraction time (X_2), pH (X_3) on the three dependent variables; extraction yield, galacturonic acid content (GA), and degree of esterification (DE)). The independent variables and their coded and uncoded levels used in the central composite design are presented in Table 1.

Table 1. Independent variables and their levels used in the central composite design

Independent variables	**Code unit**	**Coded level**				
		α (-1.682)	**-1**	**0**	**1**	**α (+1.682)**
Microwave radiation (watt)	X_1	197.7	300	450	600	702.3
Extraction time (min)	X_2	1.59	5	10	15	18.41
pH	X_3	0.42	1	2	3	3.68

All computations and three-dimensional surface plots in this study were carried out using the Minitab 16.1.1.0 (Minitab Inc., State College, PA, USA) software.

2.6. Verification of model

The optimum extraction conditions for microwave-assisted extraction, which depends on microwave irradiation, extraction time, and pH, were obtained using RSM. The pectin yield, DE, and GA of banana fruit peels extracts were carried out under the optimum extraction conditions. The predicted and experimental values were compared to confirm the validity of the models by using two sample t-tests. The significantly different values at 95% confidence interval were analysed for each response.

3. Results and discussion

3.1. Statistical analysis

A central composite design or CCD is an experimental design to justify the relationship between independent and dependent variables. It is useful in response surface methodology for creating a second order or quadratic model for the dependent variable without using a complete three level factorial design (Choon-Hui et al., 2009). The experimental conditions of pectin extraction with hydrochloric acid and responses for the 20 pectin extraction treatments are shown in Table 2. A central composite design was used to investigate the effect of extraction time, microwave irradiation level, and pH on the pectin yield, DE, and GA of banana fruit peels. The results of the second order response surface models in the form of analysis of variance (ANOVA) for regression model are summarised

in Table 3. The results showed that the *p*-values of the developed model for pectin yield, DE, and GA were 0.013, 0.005, and 0.002, respectively, which indicated that the fitness of the model was highly significant ($p< 0.05$). The lack of fit tests showed insignificant results (*p*-value = 0.94, *p*-value = 0.074, and *p*-value = 0.19 for pectin yield, DE, and GA, respectively), which indicated that the models could be used to predict the responses. The values of R^2 for yield, DE, and GA were 0.81, 0.85, and 0.88, respectively. This indicated that the models explained 81%, 85%, and 88% of the total variation (Wai et al., 2010). The quadratic models for pectin yield, DE, and GA are shown in Table 4.

Table 2. Experimental conditions of pectin extraction with hydrochloric acid, and responses for the 20 pectin extraction treatments

	Independent variables			**Responses**		
Runs	Power	Time	pH	Yield	DE	GA
1	197.73 (-1.68)	10 (0)	2 (0)	6.98	70	50
2	600 (1)	15 (1)	1 (-1)	13.27	85	88
3	450 (0)	10 (0)	2 (0)	17.12	91	60
4	702.27 (1.68)	10 (0)	2 (0)	11.58	80	75
5	600 (1)	5 (-1)	3 (1)	9.38	61	27
6	300 (-1)	15 (1)	1 (-1)	9.93	69	71
7	450 (0)	18.41 (1.68)	2 (0)	13.44	80	80
8	450 (0)	10 (0)	2 (0)	13.17	85	56
9	450 (0)	10 (0)	3.68 (1.68)	9.49	75	32
10	450 (0)	10 (0)	2 (0)	15.72	84	58
11	600 (1)	15 (1)	3 (1)	10.39	96	58
12	450 (0)	10 (0)	0.32 (-1.68)	8.03	75	68
13	300 (-1)	15 (1)	3 (1)	15.68	80	43
14	450 (0)	1.59 (-1.68)	2 (0)	6.6	70	26
15	450 (0)	10 (0)	2 (0)	13.91	88	45
16	450 (0)	10 (0)	2 (0)	14	90	46
17	300 (-1)	5 (-1)	3 (1)	4.38	89	61
18	450 (0)	10 (0)	2 (0)	22.24	84	48
19	300 (-1)	5 (-1)	1 (-1)	5.82	72	48
20	600 (1)	5 (-1)	1 (-1)	12.45	70	30

Values in parentheses for independent variables are coded values according to the CCD design. Yield: pectin yield (%); DE: degree of esterification (%); GA: galacturonic acid content (%)

Table 3. Analysis of variance (ANOVA) for regression model of pectin yield, degree of esterification (DE) and galacturonic acid (GA)

Term	Pectin Yield					DE					GA				
	SS	DF	MS	F-value	p-value	SS	DF	MS	F-value	p-value	SS	DF	MS	F-value	p-value
Model	286.935	9	31.882	4.62	0.013	1352.98	9	150.331	6.09	0.005	5084	9	564.889	7.88	0.002
X_1-Microwave radiation	22.211	1	112.118	16.26	0.002	24.22	1	50.606	2.05	0.183	35.58	1	326.537	4.55	0.059
X_2-Irradiation time	60.496	1	58.54	8.49	0.015	208.24	1	29.45	1.19	0.3	2501.11	1	0.05	0	0.979
X_3-pH	0.049	1	45.58	6.61	0.028	65.77	1	184.169	7.47	0.021	862.71	1	151.652	2.12	0.177
X_1X_1	45.019	1	65.329	9.47	0.012	122.32	1	178.93	7.25	0.023	163.03	1	148.543	2.07	0.181
X_2X_2	39.043	1	50.26	7.29	0.022	146.61	1	178.93	7.25	0.023	0.01	1	0.317	0	0.948
X_3X_3	77.098	1	77.098	11.18	0.007	178.93	1	178.93	7.25	0.023	21.06	1	21.061	0.29	0.6
X_1X_2	23.052	1	23.052	3.34	0.097	492.82	1	492.823	19.98	0.001	882	1	882	12.3	0.006
X_1X_3	13.158	1	13.158	1.91	0.197	88.91	1	88.911	3.6	0.087	40.5	1	40.5	0.56	0.47
X_2X_3	6.808	1	6.808	0.99	0.344	25.17	1	25.17	1.02	0.336	578	1	578	8.06	0.018
Residual Error	68.959	10	6.896			246.71	10	24.671			717	10	71.7		
Lack-of-Fit	12.316	5	2.463	0.22	0.94	198.21	5	39.641	4.09	0.074	500.16	5	100.033	2.31	0.19
Pure Error	56.643	5	11.329			48.5	5	9.701			216.83	5	43.367		
Cor Total	355.894	19				1599.69	19				5801	19			
R-Squared			0.81					0.85					0.88		
Adj R-squared			0.63					0.71					0.77		

SS = sum of squares; DF: degree of freedom; MS: mean square; Yield: pectin yield; DE: degree of esterification; GA: galacturonic acid content

Table 4. Regression equations (for the coded variables) and statistical parameters of the models

Equations	*F*	R^2
$EY = -42.2412+0.1334X_1+2.5644X_2+11.3141X_3-0.0001X_1^2-0.0747X_2^2-2.3130X_3^3-0.0023X_1X_2-0.0085X_1X_3+0.1845X_2X_3$	4.62 (p=0.013)	0.81
$DE = 45.0156+0.0896X_1-1.8189X_2+22.7427X_3-0.0002X_1^2-0.1409X_2^2-3.5236X_3^2+0.0105X_1X_2-0.0222X_1X_3+0.3547X_2X_3$	6.09 (p=0.005)	0.85
$GA= 75.1882-0.2277X_1-0.0748X_2+20.6376X_3+0.0001X_1^2-0.0059X_2^2-1.2089X_3^2+0.0140X_1X_2-0.0150X_1X_3-1.7X_2X_3$	7.88 (p=0.002)	0.88

EY = extraction yield; DE: degree of esterification; GA: galacturonic acid content; X_1: microwave irradiation; X_2: time, X_3: pH

3.2. The effect of extraction condition on pectin yield of banana fruit peels

The pectin yield values of banana fruit peels ranged from 4.38% to 22.24% (w/w, based on dry weight of banana fruit peel) (Table 2), which depended on the extraction time, microwave irradiation, and pH (Figure 1(a-c)). The extraction time, microwave irradiation, and pH were important factors that affected on extraction efficiency in both linear and quadratic manners (Table 3). The results exhibited that the pectin yield increased with an increase in microwave irradiation from 400 to 600 watt (Figure 1(a, b)). The increase in microwave energy level can enhance the penetration of extracting agent into the pectin material matrix, deliver it to the materials efficiently through molecular interaction with the electromagnetic field, and offer a quick transfer of energy to the extraction solvent and matrix, allowing the components from the dissolution to be extracted (Gfrerer and Lankmayr, 2005; Yan et al., 2010). A longer extraction time shows a positive effect on the pectin yield (Figure 1(a, c)). It has been reported that a longer extraction time advocates the production of pectin yield (Li et al., 2012; Bagherian et al., 2011). This might be due to the time requirement for the exposure of the banana fruit peel pectin to the release medium where the solvent penetrated into the samples, dissolved the banana fruit peel pectin, and subsequently diffused out from the raw solid samples (Maran, 2014; Samavati, 2013). However, an excessively long extraction time exposure in the microwave field can cause degradation of the pectin (Maran et al., 2013c; Xianzhe et al., 2011). Therefore, the pectin yield decreased when the extraction time was extended beyond 15 min. Moreover, pH is one of the independent variables that influences the pectin yield and it is necessary to choose a suitable pH to get the highest extraction of banana fruit peel pectin. It was exhibited that the pectin yield increased with increasing pH values, before it began to decline (Figure 1(b, c)). A higher amount of pectin yield could be obtained at pH values of 1.5-2. The extraction solvent with high concentration of acidity had the ability to fuse with the insoluble pectin and favored the hydrolysis of the insoluble pectin into soluble pectin, therefore, it could increase the pectin recovery from plant samples (Maran and Prakash, 2015; Maran et al., 2013a; El-Nawawi and Shehata, 1988; Ma et al., 2013) and reached its maximum.

3.3. The effect of extraction condition on DE of banana fruit peel pectin

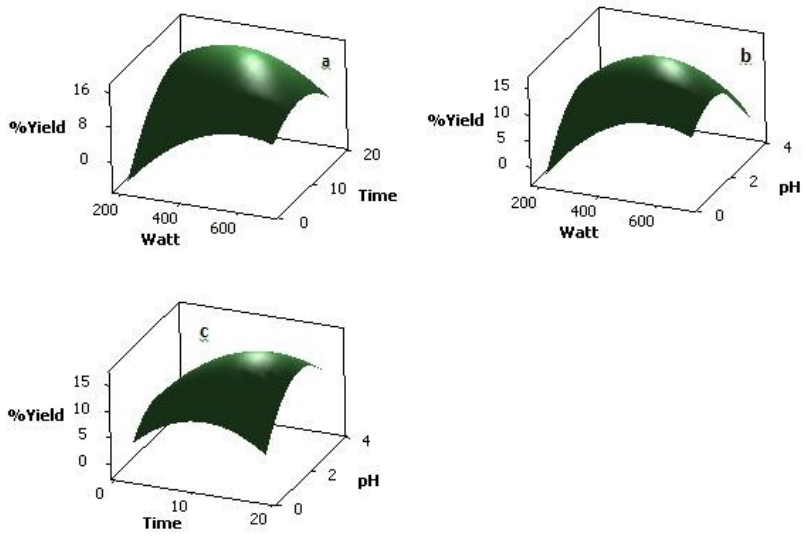

Figure 1. Response surface for effects of: (a) extraction time and microwave radiation level; (b) pH value and microwave radiation level; (c) pH value and extraction time on pectin yield from banana fruit peel.

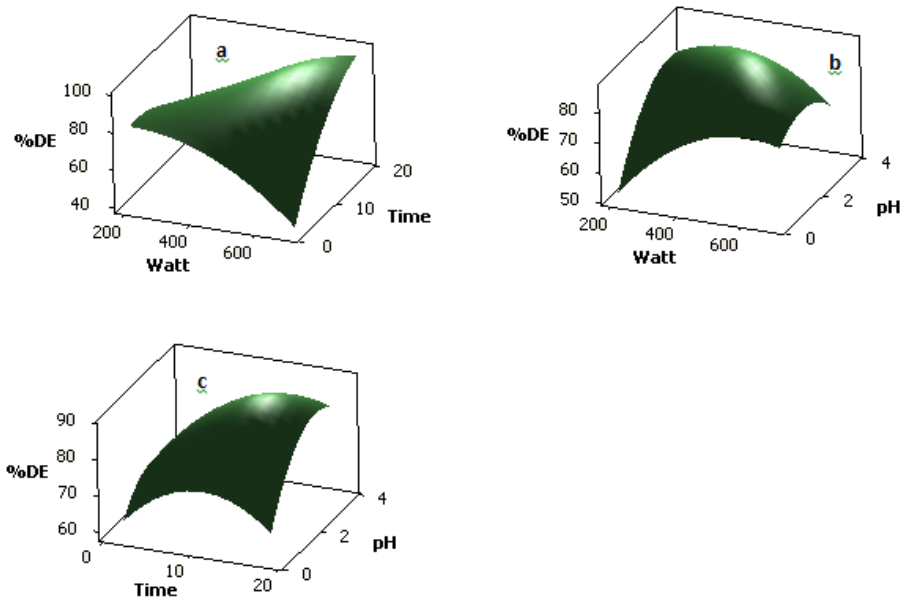

Figure 2. Response surface for effects of: (a) extraction time and microwave radiation level; (b) pH value and microwave radiation level; (c) pH value and extraction time on degree of esterification from banana fruit peel.

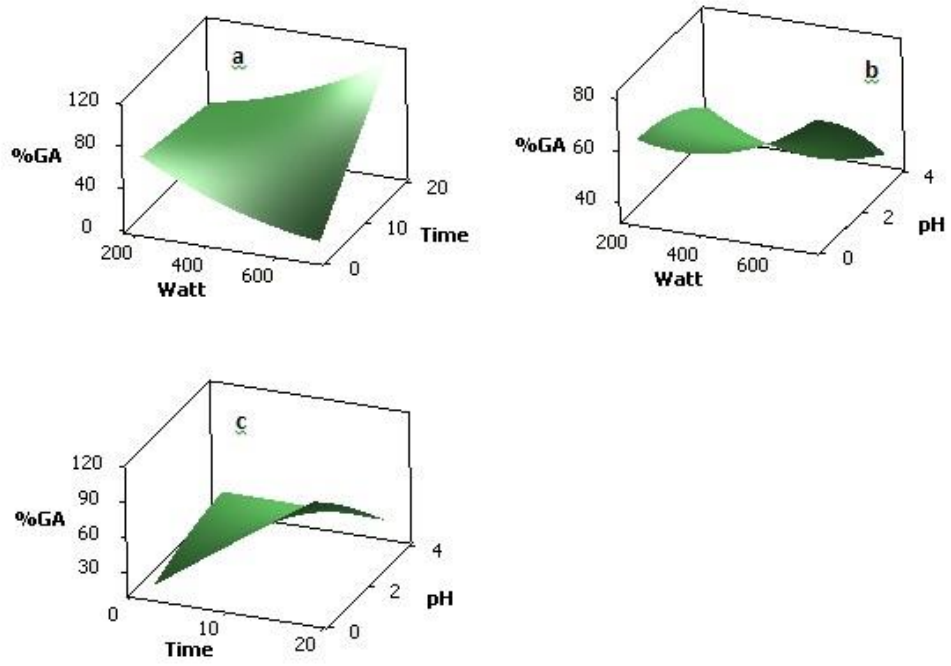

Figure 3. Response surface for effects of: (a) extraction time and microwave radiation level; (b) pH value and microwave radiation level; (c) pH value and extraction time on galacturonic acid from banana fruit peel.

The DE of banana fruit peel pectin ranged from 61% to 96% as presented in Table 2. Based on the results, the banana fruit peel pectin extracted exhibited DE values higher than 50% and thus can be classified as HMP. The effects of the microwave irradiation, extraction time, and pH on the DE of the extracted pectin are shown in Figure 2(a-c). The results exhibited that extraction time and microwave irradiation had insignificant effect on the DE, but pH had significantly ($p< 0.05$) affected the DE in linear and quadratic manners. The interaction between microwave irradiation and extraction time showed a statistically significant ($p< 0.05$) effect on the DE (Table 3). The DE of the banana fruit peel pectin increased with the increase in microwave irradiation and pH, before it began to decline. A higher amount of DE could be obtained at microwave irradiations of 450-550 watt, extraction time extended beyond 12 min, and pH between 2 and 3 as presented in Figure 2(a-c). The DE values from 61% to 96% were obtained, a range higher than that earlier reported by Oliveira TÍ et al. (2016) and Happi Emaga et al. (2008) when extracting pectin from banana fruit peel with citric acid and sulfuric acid, respectively. It indicated that highly methylated pectin was isolated from the cell wall when extracting pectin from banana fruit peels with hydrochloric acid as solvent extraction. In general, pectin obtained under very severe extraction conditions (high microwave irradiation, long extraction time, and low pH) have a high DE value because these severe conditions could increase DE of polygalacturonic chains (Wai et al., 2010).

3.4. The effect of extraction condition on GA of banana fruit peel pectin

The GA of banana fruit peel pectin ranged from 26% to 88% as presented in Table 2. The effects of the microwave irradiation, extraction

time, and pH on the GA of the extracted pectin are shown in Figure 3(a-c). The results exhibited that the extraction time, microwave irradiation, and pH had insignificant effect on the GA in linear and quadratic manners. The interaction between microwave irradiation and extraction time had significantly ($p< 0.05$) affected the GA and also the interaction between extraction time and pH (Table 3). The GA of the banana fruit peel pectin increased with the increase in microwave irradiation, before it began to decline. A higher amount of GA could be obtained at microwave irradiations of 500-600 watt and pH between 1 and 2 as presented in Figure 3(b). The GA increased with an increase in microwave irradiation of up to 500 watt and extraction time of up to 10 min as presented in Figure 3(a). The GA contents in the extracted pectin ranged from 26% to 88%, similar to that reported by Oliveira TÍ et al. (2016) but higher than that reported by Happi Emaga et al. (2008). In this study, it has been exhibited that the GA contents were enhanced by increasing microwave irradiation and extraction time and decreasing pH values up to an inflection point of maximum GA content (about 702 watt, 18.41 min, pH 1.95), beyond which the GA values began to decline. This trend (the increasing GA value to a maximum point and followed by decreasing GA value) can be explained by the combination of two phenomena occurring during pectin extraction with low pH values at high microwave irradiation, which can remove sugars as a product of pectin hydrolysis (it contributes to GA values) and can cause their degradation by low pH values and high microwave irradiation heating (Garna et al., 2004).

3.5. Optimisation of extraction parameters and validation of optimised condition

The objective of optimisation was to ascertain the MAE conditions that give the maximum values for each dependent variable. The desirability function approach was applied in the optimisation process. This individual optimisation technique evaluates a point that maximises the desirability function (Maran et al., 2013a) as presented in Table 5. The results of individual numerical optimisation exhibited that microwave irradiation of 457 watt, pH of 2.12, and 12.8 min of extraction time can result in an optimum pectin yield of 16.62%. The DE can result in maximum of 98.01% if the conditions of the MAE were set as pH of 1.95, extraction time of 18.41 min, and microwave irradiation of 702 watt. The optimum extraction condition of the MAE was pH of 1, extraction time of 14.66 min, and microwave irradiation of 450 watt, which can result in the maximum GA of 80%.

Table 5. Predicted and experimental response values at optimum conditions of each responses using the equation models under these conditions of MAE

Responses	Optimum MAE condition			Maximum value	
	Time (min)	pH	Irradiation (watt)	Predicted	Experimental
Extraction yield (%)	12.80	2.12	457.64	16.62	17.24± 0.40
Degree of esterification (%)	18.41	1.95	702.27	98.01	96.52 ± 1.87
Galacturonic acid content (%)	14.66	1	450	80	79.80± 1.14

* Mean ± standard deviation for 3 replication

Table 6. Multiple response optimisation of overall optimum conditions (580 W, 15.86 min, pH 1.71) using MAE

Responses	**Predicted value**	**Experimental value***
Extraction yield (%)	13.47	13.57 ± 0.76
Degree of esterification (%)	92.45	91.87 ± 3.23
Galacturonic acid content (%)	87.99	86.67 ± 2.31

* Mean ± standard deviation for 3 replication

A multiple numerical optimisation was carried out to find the optimum values of microwave irradiation, extraction time, and pH to the desired values for all responses. Several combinations of all the independent variables could give maximum pectin yield, DE, and GA. The multiple response optimisation using MAE showed that the overall optimum conditions were achieved at microwave irradiation of 580, extraction time of 15.86 min, and pH of 1.71 (Table 6). Under these optimum conditions, the predicted response maximum value of pectin yield was 13.47%, DE was 92.45%, and GA was 87.99% (Table 6). The suitability of the optimised conditions for predicting the optimum response values was tested experimentally using the selected optimal conditions. Triplicate experiments were carried out under the optimised conditions and the mean values obtained from real experiments demonstrated the validation of the optimised conditions.

The pectin yield under the optimum conditions was 13.47%, which was higher than the maximum yield of 2.18% obtained by Swamy and Muthukumarappan (2017) when extracting pectin from banana fruit peels by using continuous and intermittent microwave-assisted extractions. However, the conditions carried out by those researchers (Swamy and Muthukumarappan, 2017) were obtained with microwave irradiation of 900 watt, extraction time of 100 sec, and pH of 3.00 in the continuous method while in the intermittent process, they obtained the highest maximum yield of 2.58% at microwave irradiation of 900 watt, pulse ratio of 0.5, and pH of 3.00. On the contrary, the maximum pectin yield under the optimum conditions was 13.47%, which was lower than the maximum pectin yield of 21.7% reported by Happi Emaga et al. (2008) when extracting pectin from banana fruit peels with sulfuric acid by conventional solvent extraction method, and they obtained a high yield due to using harsher conditions (temperature 90 ^{o}C, pH 1.5, and time 240 min) than the optimum conditions of this study. Besides, the characteristics of the pectin yield of 21.7% reported by Happi Emaga et al. (2008b) had a degree of methylation and GA value of 49% and 40%, respectively, which were lower than those obtained in this study under the optimum conditions.

4. Conclusions

In this study, pectin was successfully extracted from banana fruit peel and MAE was optimised for the extraction of pectin. Three independent variables at three levels of central composite design were used to optimise the experimental conditions. Pectin was successfully extracted from banana fruit peels with hydrochloric acid under different conditions of extraction time, microwave irradiation, and pH. From the experimental results, microwave irradiation, extraction time, and pH were the most important variables that had affected on the responses. The optimal conditions were microwave

irradiation of 580 watt, extraction time of 15.86 min, and pH of 1.71, with maximum pectin yield of 13.47%, DE of 92.45%, and GA of 87.99%. Under the optimal conditions, the experimental values agreed with the predicted values. The optimised conditions determined from MAE technique in this study should provide important reference data for any subsequent study and the powdered pectin obtained can be produced with microwave-based extraction as a convenient pectin manufacturing process by the food industry.

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EFFECTS OF PROCESSING ON ESSENTIAL AND HEAVY METAL COMPOSITION OF POPULAR FISH SPECIES CONSUMED IN THE KARACHI COAST OF THE ARABIAN SEA

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ABSTRACT

This study analyzed three popular species of fish in raw and cooked (curried and fried) form, commonly consumed in Karachi coast and overall in Pakistan for their essential and heavy metal composition. The outcomes revealed that the content of toxic heavy metal (Hg) was observed in all raw samples particularly in raw Mulla fish *(Lethrinus nebulosus*) but heat treatment by frying and curry preparation of fish led to a decrease of Hg content in the muscles of all selected species especially in *L. nebulosus* after frying. While in *Acanthopagarus arabicus* Hg content was below detectable level. In this study the levels of Cd and Cr were observed at below detectable levels in all selected species except in *A. arabicus* where Cd tend to decrease after frying while the content of Cr slightly increase after both types of cooking. In the present study the essential metals namely Fe, Zn, Cu and Mn tend to increase in all fin fishes after cooking, especially in curried form. The above-mentioned result determined that these customary culinary practices of fish have an influence on their essential and heavy metal constituents. Furthermore, eating variety of fin fish species by applying different procedures of cooking is the finest attempt to attain better-quality of dietetic ways, minimizing mercury revelation and increasing chance to obtain vital elements.

1. Introduction

Toxic heavy metal pollution, which is obstinate and bio accumulative, progressively intimidates marine environment (Balkas et al., 1982; Bat et al., 2009; Bat, 2014). The metal pollutants in marine coastal system normally persist either in soluble or suspended form and finally tend to sink to the bottom or are taken up by the biota (Bat and Raffaelli 1998; Bat 2005; Khattak and Khattak, 2013; Bat and Özkan, 2015; Bat et al., 2015; Bat et al., 2017). Unfortunately, some heavy metals and their compounds are considered cancerous for humans and animals (Stanescu, 1998). Certain metals for instance Cd, Pb, and Hg are extremely lethal even at very slight amounts (Bat, 2014; Bat, 2017). For example, Cd and Hg are familiar to cause kidney diseases, high blood pressure, cancer, hepatic dysfunction and harm reproductive ability while Pb can cause renal malfunction, liver mutilation, reduced hearing or produce mental obstruction, whereas at high intensities in women can result in a reduced conception period (Iwegbue, 2011). Hg, lethal effects have been emphasized when combined poisoning reported after consuming large amount of fish by people (Renzoni et al., 1998; Chen et al., 2002). Cu, Fe, Mn, and Zn are

crucial and compulsory for regular body activity like the synthesis of metalloproteinase. Although lack of these metals might lead to illness, unnecessary consumptions of these metals could initiate prolonged inflammatory sickness and a possible factor of tumor (Naughton and Petroczi 2008). Heavy metals can be hazardous to consumer's health (Diaconescu et al., 2012; Bat, 2014; Bat, 2017; Bat and Arici, 2018). Usually heavy metals store in fleshy tissue of fish and so, the intensities determined in their tissues are able to reveal the former exposure (Ashraf, 2005).

Karachi, Pakistan is a coastal metropolitan and thus facing industrialization difficulty. Hence, adjacent coastal regions of the Arabian Sea are getting an immense amount of unregulated industrial manure discharges that eventually disturb aquatic life (Jaffer et al., 1995; Tariq et al., 1998). Earlier studies have evidenced enhanced levels of these metals in fish belonging to south west coast of Pakistan (Tariq et al., 1998).

Usually fish species are consumed in cooked form while most of research studies information made from uncooked/raw products (Domingo, 2011). For the meantime heavy metal evaluation in raw products does not present the accurate calculations of these metals intake via seafood ingestion and consequently, it is essential to establish the accumulations of heavy metals in raw and cooked fish (Kalogeropoulos, 2012). Some study proven that the heavy metals concentrations of fish can also be changed by processing and hence, it is likely to decrease the toxic heavy metal concentration in fillets by selecting a proper technique of cooking (Kalay et al., 1999; Ersoy et al., 2006; Diaconescu et al., 2013). Many investigations have recorded a remarkable decline of the heavy metals in fish after cooking, while specific works mentioned rise in the metal amount. The works that revealed a reduction of toxic metals usage cooking processes subjected to cooking terms, such as time, temperature, and cooking medium (Morshey et al., 2015). Metal concentrations in fish are highly dependent on fish size (Ahmed et al., 2016). According to Burger and Gochfeld (2011), the people must be well-informed prior to decide on which kind of fish is suitable to intake, how often and in what portion.

Though research studies are present on the heavy metal composition of raw fish, but studies to evaluate the essential and heavy metals composition of cooked fish in the Arabian Sea areas are rare. Furthermore, in Pakistan, almost none of the data exist on the impacts of traditionally processing on the essential and heavy metal concentrations in fish fillet. This study is consequently, an effort to evaluate the concentration of vital and toxic heavy metals in selected raw and traditionally proceed fish species usually consumed in Karachi.

2. Materials and methods

2.1. Materials

2.1.1. Collection of samples

Only the popular edible fish species of Karachi were included in this study. All fish were purchased from the local fish markets in Karachi city of Pakistan. Local, common, scientific names are presented in (Table 1). The selected culinary methods for selected fish species are common to most of the Pakistan and even in sub-continent.

Table 1. Common, Local and Scientific names of fish species included in the study

Common name	Local name	Scientific name
White Pomfret	Safeed Poplet	*Pampus argentus*
Arabian yellow-finned sea bream	Dhandya	*Acanthopagrus arabicus*
Spangled Emperor	Mulla	*Lethrinus nebulosus*

The fish were all captured from the coast of Karachi. On board they were put covered with ice and were later on shipped in a refrigerated truck to the central market. The selected fish species were purchased randomly during the period of August 2016 to February 2018. About 5 kg of each species of whole fish were captured, kept in icebox and immediately transferred to the PCSIR Laboratory of Karachi without delay,

where the identification and measurement of samples were taken.

For cooking purpose, vegetables like onion, tomato, garlic and mix spices, for coating of fish fillet, gram flour and sunflower cooking oil were purchased from local super market.

2.1.2. Sample preparation and cooking

Fish samples were washed with distilled water several times. The samples were cleaned as per usual cooking practices (scaling, beheading, gutting and removing the internal organs). Fillets were made for Yellow fin Bream, Mulla and Pomfret fishes. They were washed with water then fish fillet were divided into three groups, the first one is uncooked and the other two groups were cooked. Two different cooking methods were applied on fish species which are traditionally used on domestic and commercial level in local people. The frying of the marinated fish species was performed in a domestic non-stick pan (2-Litre capacity) at medium flame approximately for 15 minutes and fish curry was cooked with chopped vegetables and spices for 30 to 35 minutes in low to medium flame (Table 2).

Sunflower oil was used for frying and cooking in curry form. After cooking, samples cooled and kept below 4°C till analyzed in laboratory for essential and heavy metal concentration.

2.2. Methods

2.2.1. Heavy Metal Analysis

All chemicals used were of Analytical Regent (AR) Grade either of Merck or equivalent. Three fillet samples of each type raw or cooked fish selected at random and were dried in oven at 80°C for 3-4 hrs. The completely dried sample was homogenized with the help of pestle and mortar. For testing of Fe, Zn, Cu, Mn, Pb, Cr and Cd, 4-5 gm. of each sample in triplicate was weighed in a beaker and was soaked overnight in 5ml Conc. Nitric Acid. 10-15 ml deionized distilled water was added to the samples and the contents were heated at 60-80°C on a hotplate till samples were completely digested. Contents were filtered through Whatman 41in 25 ml volumetric flask and the volume was made with deionized distilled water. Sample blank was also prepared in the same manner, 5 ml Nitric acid was mixed with 10 – 15 ml deionized distilled water and heated on a hotplate for the same duration as for the samples. Samples were analyzed on Hitachi Z- 5000 Polarized Zeeman Flame/Graphite Furnace Atomic Absorption Spectrophotometer against standard curve. The instrument was handled as per manufacturer direction. Dilutions were made if needed to keep sample concentration between linear ranges of working curve.

For the analysis of Hg, 4-5 g of each sample in triplicate was reflux in 30 ml acid digestions mixture containing 1:1 nitric and sulfuric acid till contents are completely digested and all nitric acid is removed. Samples were diluted and solution was made up to 250 ml in volumetric flask with de-ionized distilled water. Sample blank was also made using similar method. Mercury was analyzed using same Hitachi Z 5000 Polarized Zeeman Flame/Graphite Furnace Atomic Absorption Spectrophotometer through cold vapor unit installed with the equipment against working curve. The instrument was handled as per manufacturer instructions. Dilutions were made if needed to keep sample concentration between linear ranges of working curve.

Metal contents were expressed as ppb for Hg, and ppm for Cu, Fe, Mn, Cd, Cr, Pb and Zn wet wt. of fresh fish.

2.2.2. Statistical analysis

The effect of different cooking methods on the proximate and heavy metal composition of selected fish was analyzed using standard deviation (SD).

The yearly quantity of fish consumed is 2 kg per person in 2006 (Food and Agriculture Organization of the United Nations 2009), which is same as 5.48 g/day for Pakistan. The EDI of metals was determined using the following equation (Bat and Arici, 2018).

$$EDI = C_{metal} \times W / b.w.$$

Where: C_{metal} is the concentration of metals in fish; W represents the per diem mean intake of fish; b.w. is the body weight.

Table 2. Ingredients and traditional methods of preparation of fish species commonly consumed in Karachi, Pakistan

Species	Cooking process	Sample size (g)	Oil used (ml)	Ingredients	Method of preparation	Cooking time (min)
Pampus argentus	Fried	100	200	*	***	10-15
	Curry	100	60	**	****	25-30
Lethrinus nebulosus	Fried	100	200	*	***	10-15
	Curry	100	60	**	****	25-30
Acanthopagrus arabicus	Fried	100	200	*	***	10-15
	Curry	100	60	**	****	25-30

* Spices containing mainly, salt, red chili powder, turmeric powder, fresh garlic paste and gram flour for coating.
** Spices containing yellow mustard seed, onion, tomatoes, yoghurt, salt, red chili powder, turmeric powder and fresh garlic paste.
*** Wash the fillet/shrimp with salt and vinegar mix water then clean tap water, fillets coated with spices mix gram flour batter leave for 15 minutes in refrigerator for batter grip of coated ingredients then fry in moderate hot sunflower oil till brown.
**** Wash the fillet with salt and vinegar mix water then clean tap water, roast/brown the grind vegetables, yoghurt and spices in hot sun flower oil then add fillet in gravy and cooked in low to moderate heat till done.

3. Results and discussion

3.1. Heavy Metals Content

The recovery of the spiked metals was close to 97- 99% for all tested metals by proposed method.The mean concentrations (±SD) of heavy metals in raw, fried and curried form of fish muscles are given in Tables 3-5.

In *P. argentus* Fe, Zn and Cu contents were increased conspicuously in fried forms as compared to their raw forms. While as compare to raw samples the amounts of Mn and Hg were decreased in fried fish. Mn value was slightly higher in curried form whereas Fe value was low. The Hg content was decreased in fried form as compare to its curried and raw. The Cd, Cr and Pb contents were below the detectable level in raw and cooked forms of white Pomfret fish (Table 3).

In *L. nebulosus* the amount of Fe was remarkably increased in curried form than fried form as compare to its raw form. The amount of Hg is very higher observed in raw form while after frying it was surprisingly decreased more than seven fold. The other heavy metal contents showed slightly increased after cooking. On the other hand Cd, Cr and Pb contents were below the detectable levels in raw and cooked forms (Table 4).

In *A. arabicus* Fe, Zn, Cu and Mn levels slightly increase in fried form while Cd amount decrease after frying and increase after cooked in curried form. The amount of Cr was slightly increased after both types of cooking as compare to raw form. The amounts of Hg and Pb were below the detectable levels in all raw and cooked samples of Bream fish (Table 5).

According to people well-being risk, the allowable weekly intakes were calculated by means of references for eatable tissues of fishes consumed by people. The EWI (Estimated Weekly Intake) and EDI (Estimated Daily Intake) levels showed in Tables 6-8, were estimated by assuming that a 70-kg person will consume 5.48 g fish per day which is even 38.36 g fish per week (see statistical analysis in Materials and Methods).

Table 3. Mean ± SD of heavy metal concentrations given mg/kg except Hg (µg/kg) wet wt. in Raw and cooked *Pampus argentus* (white Pomfret fish)

Form	Fe	Zn	Cu	Mn	Hg	Cd	Cr	Pb
Raw	86±1.1	36±0.42	2.2±0.21	14±0.96	11±0.29	Nd*	Nd*	Nd*
Fried	108±2.12	95±1.04	18±0.21	9.5±0.78	5.9±0.15	Nd*	Nd*	Nd*
Curried	72±1.14	49±0.49	3.7±0.22	17±0.29	7.9±0.26	Nd*	Nd*	Nd*

Nd*= non detectable (below the detectable level)

Table 4. Mean ± SD of heavy metal concentrations given mg/kg except Hg (µg/kg) wet wt. in Raw and cooked *Lethrinus nebulosus* (Mulla fish).

Form	Fe	Zn	Cu	Mn	Hg	Cd	Cr	Pb
Raw	34±0.5	16±0.21	1.4±0.17	0.7±0.15	144±1.21	Nd*	Nd*	Nd*
Fried	53±0.62	13±0.25	2.1±0.21	2.1±0.25	20±0.26	Nd*	Nd*	Nd*
Curried	74±1.36	19±0.26	1.7±0.23	3.9±0.29	47±0.98	Nd*	Nd*	Nd*

Nd*= non detectable (below the detectable level)

Table 5. Mean ± SD of heavy metal concentrations given mg/kg except Hg (µg/kg) wet wt. in Raw and cooked *Acanthopagrus arabicus* (Arabian yellow fin bream fish).

Form	Fe	Zn	Cu	Mn	Hg	Cd	Cr	Pb
Raw	16.3±0.15	3.79±0.006	1.53±0.03	0.29±0.04	Nd*	0.008±0.002	0.138±0.002	Nd*
Fried	16.71±0.02	4.41±0.006	2.68±0.05	0.59±0.02	Nd*	0.006±0.003	0.188±0.011	Nd*
Curried	19.98±0.24	1.03±0.06	1.32±0.03	0.7±0.01	Nd*	0.023±0.003	0.156±0.001	Nd*

Nd*= non detectable (below the detectable level)

Table 6. Estimated Weekly Intakes (EWI) and Estimated Daily Intakes (EDI) of heavy metals in edible tissues of *Pampus argentus* (white Pomfret fish) from the local fish markets in Karachi city of Pakistan.

Metals	PTWI[a]	PTWI[b]	PTDI[c]	EWI[d]			EDI[e]		
				Raw	Fried	Curried	Raw	Fried	Curried
Fe	5.6	392	56	3.29896	4.14288	2.76192	0.47128	0.59184	0.39456
Zn	7	490	70	1.38096	3.6442	1.87964	0.19728	0.5206	0.26852
Cu	3.5	245	35	0.084392	0.69048	0.141932	0.012056	0.09864	0.020276
Mn	2-5	140-350	20-50	0.53704	0.36442	0.65212	0.07672	0.05206	0.09316
Hg	0.005	0.35	0.05	0.0004	0.00023	0.0003	0.00006	0.00003	0.00004
Cd	0.007	0.49	0.07	Not detectable (below the detactable level)					
Cr	0.0233	1.631	0.233	Not detectable (below the detactable level)					
Pb	0.025	1.75	0.25	Not detectable (below the detactable level)					

[a]PTWI (Provisional Tolerable Weekly Intake) in mg/week/ kg body wt.
[b]PTWI for 70 kg adult person (mg/week/70 kg body wt.)
[c]PTDI (Permissible Tolerable Daily Intake) (mg/day/70 kg body wt.)
[d]EWI (Estimated Weekly Intake) (mg/week/ kg body wt.)
[e]EDI (Estimated Daily Intake) (mg/day/ kg body wt.)

Table 7. Estimated Weekly Intakes (EWI) and Estimated Daily Intakes (EDI) of heavy metals in edible tissues of *Lethrinus nebulosus* (Mulla fish) from the local fish markets in Karachi city of Pakistan.

Metals	PTWI[a]	PTWI[b]	PTDI[c]	EWI[d]			EDI[e]		
				Raw	Fried	Curried	Raw	Fried	Curried
Fe	5.6	392	56	1.30424	2.03308	2.83864	0.18632	0.29044	0.40552
Zn	7	490	70	0.61376	0.49868	0.72884	0.08768	0.07124	0.10412
Cu	3.5	245	35	0.053704	0.08056	0.065212	0.00767	0.01151	0.009316
Mn	2-5	140-350	20-50	0.026852	0.08056	0.149604	0.00384	0.01151	0.021372
Hg	0.005	0.35	0.05	0.0055	0.00076	0.00182	0.00078	0.00011	0.00026
Cd	0.007	0.49	0.07	Not detectable (below the detactable level)					
Cr	0.0233	1.631	0.233	Not detectable (below the detactable level)					
Pb	0.025	1.75	0.25	Not detectable (below the detactable level)					

[a]PTWI (Provisional Tolerable Weekly Intake) in mg/week/ kg body wt.
[b]PTWI for 70 kg adult person (mg/week/70 kg body wt.)
[c]PTDI (Permissible Tolerable Daily Intake) (mg/day/70 kg body wt.)
[d]EWI (Estimated Weekly Intake) (mg/week/ kg body wt.)
[e]EDI (Estimated Daily Intake) (mg/day/ kg body wt.)

Table 8. Estimated Weekly Intakes (EWI) and Estimated Daily Intakes (EDI) of heavy metals in edible tissues of *Acanthopagrus arabicus* (Arabian yellow fin bream fish) from the local fish markets in Karachi city of Pakistan.

Metals	PTWI[a]	PTWI[b]	PTDI[c]	EWI[d]			EDI[e]		
				Raw	Fried	Curried	Raw	Fried	Curried
Fe	5.6	392	56	0.625268	0.6409	0.76643	0.08932	0.0916	0.10949
Zn	7	490	70	0.14538	0.1692	0.03951	0.02077	0.0242	0.0056
Cu	3.5	245	35	0.05881	0.1028	0.0506	0.0084	0.0147	0.0072
Mn	2-5	140-350	20-50	0.0111	0.0226	0.0269	0.0016	0.0032	0.0038
Hg	0.005	0.35	0.05	Not detectable (below the detactable level)					
Cd	0.007	0.49	0.07	0.0003	0.0002	0.0009	0.00004	0.00003	0.0001
Cr	0.0233	1.631	0.233	0.0053	0.0072	0.0059	0.0008	0.0010	0.0009
Pb	0.025	1.75	0.25	Not detectable (below the detactable level)					

[a]PTWI (Provisional Tolerable Weekly Intake) in mg/week/ kg body wt.
[b]PTWI for 70 kg adult person (mg/week/70 kg body wt.)
[c]PTDI (Permissible Tolerable Daily Intake) (mg/day/70 kg body wt.)
[d]EWI (Estimated Weekly Intake) (mg/week/ kg body wt.)
[e]EDI (Estimated Daily Intake) (mg/day/ kg body wt.)

3.2. Discussions

According to recent reviews (Bat, 2014; Bat, 2017), results usually indicated that heavy metal absorptions remained minimal in the muscular tissues and maximal in the liver and gill. It has been revealed that destination tissues of toxic metals are metabolically active ones. Consequently, metal accumulation in destination tissues follow up greater amount compared to other tissues like the muscle, where metabolic activity is relatively weak (Kalay et al., 1999; Roesijadi and Robinson, 1994; Serra et al., 1993; Langston, 1990; Heath, 1987).

Overall the toxic heavy metal such as Pb was not observed or below detectable level in raw and processed muscles of selected species of fish, it indicates that our traditional culinary practice did not effect on it. Musaiger and D'Souza (2008) observed that low level content of Pb i.e. (≥ 0.02) µg/g in most of the cooked species of fish and shrimp of Arabian Gulf. In the present study the essential heavy metals such as Fe, Zn, Cu and Mn tend to increase in all fin

fishes after cooking, especially in curried form. It might be the reason of this increase is the usage of vegetables, yoghurt and citrus ingredients in traditional curry preparation (Musaiger 2006). The increases of Fe, Zn, Cu and Mn contents in fried form of finfish is may be the adding of gram flour batter coating on fillets. Gram flour is also rich in fibres, vitamin B-6, folate, thiamine, magnesium, phosphorus, potassium, manganese, iron, zinc and copper. It was also observed (Tawfik, 2013) the increase values of heavy metals after culinary practice, and was concluded that in the frying and marinated methods, the concentrations of metals increased. According to Bassey et al., (2014), the cooking methods produced remarkable raise in the concentrations of most metals compared to those of the uncooked samples. On the other hand, these culinary practices withal caused a decline in the amounts of metals in some fish species. This ways could be recognised to the interaction among the body size of the cooked fish, water loss, oil uptake and metal evaporation in the course of processing. However, according to Mitra et al. (2011), heat treatment by frying, boiling, steaming and curry preparation of fish lead to a reduction of the heavy metal amount in the muscle of all fish species. Devi and Sarojnalini (2012) observed the same as the decrease content of heavy metals in fry and curry form of Fish Amblypharyngodon mola.

In the present study the content of toxic heavy metal Hg was observed in all raw samples particularly in raw Mulla fish but heat treatment by frying and curry preparation of fish led to a decrease of Hg content in the muscles of all selected species especially in L. nebulosus after frying. However in A. arabicus, Hg content was below detectable level. According to Panichev and Panicheva (2016) cooking in sunflower oil might be the further example of thermal elimination of Hg from the fat fishes. They found 19.1% loss of Hg for rich in fat Yellowtail and only 5.9% for lean Cape hake fish.

In the present study the concentration of Cd and Cr was observed at below detectable levels in all selected species except in *A. arabicus* where Cd tends to decrease after frying. The conflicting result has been observed by Bassey et al. (2014) where Cd tend to increase in *Polydactylus quadratifilis* after frying and grilling. Musaiger and D'Souza (2008) reported that the remained steady value i.e. ≥ 0.02 µg/gm of (Cd) for all the methods of cooking. Decrease in the metal levels during fish processing may be associated to the discharge of these contaminants by the loss of water as free salts, maybe in relation to soluble amino acids and uncoagulated proteins (Bryan and Hummerstone 1971). In A. arabicus the content of Cr slightly increase after both types of cooking. The same observed by Tawfik (2013) in fin fish species *Oreochromis niloticus*, *Mugil cephalus*, *Sardinops saga* and fried shrimp *Penaeus monodon* which showed increase values of Cr after frying and marinated form. Ahmed et al. (2015) pointed out that the Cr is very important for human diet because of its key role in insulin function and lipid metabolism. With respect to Western Australian Food and Drug legislations, the recommended maximal permissible amount of Cr is 5.5 µg/g (Plaskett and Potter, 1979). According to Bassey et al. (2014) the increase of Cr in the course of frying could be due to moisture loss and uptake of Cr from the oil during frying.

In this study the species wise differences was not constant for all the detected heavy metals. The obtained variation of metal amounts in several species counts on feeding habits (Romeo et al., 1999) environmental requirements, metabolism (Canli and Furness, 1993), their habitats (Canli and Atli, 2003; Tuzen and Soylak, 2006) and age, size and length of the species (Linde et al., 1998). The practice of culinary therefore plays a key role in altering the absorptions of heavy metals.

The several ways of cooking have a significant effect on the nutrient and heavy metal composition of fish. The changes are subject to on culinary circumstances (time, temperature and medium of cooking). It is found that the traditionally fried *P. argenteus* and curried form of *L. nebulosus* as a routine portion of the diet

would be useful because of its rich content of Fe, Zn and Cu. In the present study the increase in essential heavy metal contents could be due to the ingredients used in traditionally cooking practices. Consequently, it is probable to decrease the heavy metals in fish muscles by selecting an appropriate process of cooking. Atta et al. (1997) found that the Cd, Cu, Pb and Zn levels in *Tilapia nolitica* decreased on steaming and baking. However the reduction in these metal levels on baking was much higher than on steaming (Atta et al., 1997). In this study our results make public that heat energy has the significant role in separation of heavy metals from the fish. Hence in conclusion it can be encouraged that polluted fish by toxic heavy metal (such as Hg in *L. nebulosus*) may be consumed after traditionally culinary practice of the study. Thus, it is likely to reduce the metal levels in fish by choosing a proper method of cooking. Consequently, such fish flesh should only be consumed after cooking (Atta et al., 1997).

The tolerable weekly intake of the metals as PTWI (Provisional Tolerable Weekly Intake), are established by the Food and Agriculture Organization/World Health Organization (FAO/WHO) Joint Expert Committee on Food Additives (JECFA). PTWI is the maximal level of a pollutant to which a person can be exposed per week over a lifespan with no health risk effects (National Academy of Science, 1989; WHO, 1996; Council of Europe, 2001; FAO/WHO, 2010; EFSA, 2010; EFSA, 2012). EWI levels of metals for a person (mg/70 kg body weight) consuming 38.36 g seafood/week were estimated using the mean ±SD metal levels (see Tables 6-8) for *P. argentus, L. nebulosus* and *A. arabicus*. Intake estimates were expressed as per unit body weight (mg/kg body wt. /weekly and daily). EDI values were calculated from EWI values.

4. Conclusions

In this study, the calculated EWIs and EDIs of the metals are under the permitted PTWIs and PTDIs and showed no hazard consequences to the consumers.

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EVALUATION OF THE THERAPEUTIC EFFECTS OF *MENTHA SPICATA* ESSENTIAL OIL AT THE LIVER LEVEL IN DEVELOPING WISTAR RATS CO-EXPOSED TO LEAD AND MANGANESE

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ABSTRACT

The objective of this study is to evaluate, on the one hand, the changes in hepatotoxicity induced by lead and manganese according to a biochemical and histological experimental approach in developing Wistar rats and, on the other hand, to test the effectiveness of mint essential oil (*Mentha spicata*) in restoring or not the harmful effects of the metals studied. by an intraperitoneal injection of 0,1 ml HEM/kg/day for a period of 21 days. The characterization of this essential oil by gas chromatography coupled with mass spectrometry indicates that the major components are: Carvone (42.2%), Menthone (20.89%), Piperitenone (17.41%) and Isomenthone (7.99%). The results of liver biochemical assays (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, albumin, total cholesterol, and triglyceride) show a correction of values following the administration of essential oil compared with those of co-exposed animals. Analysis of the antioxidant status in the hepatocyte indicates that this oil has restored the activity of the various antioxidant enzymes (superoxide dismutase (SOD: 0,87 U/mg), glutathione peroxidase (GPx: 38,62 U/mg), and catalase(CAT: 20,73 U/mg)). In fact, the histological study undertaken illustrated a clear improvement in liver tissue architecture in rats intoxicated and treated with oil, which justifies the great importance of *Mentha spicata* in traditional medicine and these therapeutic virtues.

1.Introduction

The heavy metals are a group of environmental chemicals which are both universal and non-biodegradable. Although the adverse effects resulting from exposure to them are well-known, their use and environmental concentration are increasing (Zhao et al, 2014). Numerous studies have revealed the toxicity of individual metals for living organisms (Basile et al, 2012). However, these metals do not only exist individually, but also combined in the environment (Smith et al , 2012)

In fact, lead is a non-essential element for the human body, due to its dispersal in the ambient air, as well as in many foods. Its toxicity is closely tied to its accumulation in certain tissues, and its interference with bio-elements, whose role is essential for a number of physiological processes. It has numerous adverse effects, including neurological,

behavioral, immunological, renal, hepatic, and in particular hematologic disorders (Annabi et al., 2007).

On the other hand, Manganese (Mn) is a natural trace element its toxicity is multifactorial, because Mn directly interrupts the activation of enzymes, prevents competitive mineral absorption, changing calcium homeostasis and lowering the level of antioxidants available in the body (Adli, 2017).

Due to the pervasiveness of both metals, Mn and Pb, reducing human exposure to toxic levels remains a global health challenge.

Moreover, aromatic plants produce active compounds (phytochemically having a pharmacological effect on living organisms) consisting in part of essential oils (Curutchet, 2014). Among these plants, *Mentha spicata* L., commonly called "naana" in Algerian medical systems, has many culinary and medicinal uses in the Maghreb. It is popularly consumed in the form of tea and added to several preparations as a flavor enhancer; dry or fresh spearmint leaf is added especially during tea brewing. Biliary disorders, menstrual pain, stomach aches, constipation, gingivitis, and waving are treated with decoction of spearmint leaves (Brahmi, 2012).

All these properties, typical of mint species, have been attributed to the combination of essential oil, essentially based on monoterpenoids (Brada, 2007), and polyphenolic derivatives (Dorman, 2003).

This study examined the impact of chronic co-exposure to lead acetate and manganese chloride at the hepatic level in Wistar rats during intrauterine life, lactation and after weaning, and then evaluated the effect of the essential oil of the plant *Mentha spicata* attenuated this hepatotoxicity in rats intoxicated by these two metals.

2. Materials and methods

2.1.Extraction and determination of the chemical composition of the essential oil by GC/MS

The leaves of spearmint (*Mentha spicata*) were harvested in Sidi Maàmar wilaya of Saida in the western Algerian highlands, then identified by taxonomic experts (Pr Hasnaoui department of biology, university Dr Moulay Tahar - saida). The sample was preserved, and the voucher specimen, coded P-200886, was deposited in the herbarium of the Biology Department of the Faculty of Sciences of the University of Saida, Algeria, for future reference.

The essential oil of mint (*Mentha spicata*) was extracted by hydrodistillation. The qualitative and quantitative analysis of this essential oil was then carried out by VARIAN CHROMPACK - CP 3900 gas chromatography by injecting 0.2 µl extract. The carrier gas used is helium (He) with a flow rate of 0.3 ml/min. The column used is a capillary column type VF5 (stationary phase nature: 5% phenyl-polysinoxane and 95% methyl), 30 m long and 0.25 mm inside diameter. The thickness of the stationary phase is 0.25 µm; the temperature of the initial injection column is programmed at 70ºC for 2.50 min, then rises in steps of 15ºC/min to 255ºC for 20 min; the detector used for this analysis is of the mass spectrometry type (Saturn 20200) with a temperature of 250ºC. The device is controlled by a menu computer, appropriate software for this type of analysis and a NIST database which allows the identification of the compounds.

2.2. Distribution of lots

The experiments were carried out on Wistar rats, weighing from 200 to 400 g. The rats are grouped in cages at a rate of 2 females and one male. They are placed in a ventilated animal house, at a temperature of 21 ± 1 ◦C with lighting artificiel which establishes a day/night cycle (day between 7 and 19 h). On the first

day of gestation, females are divided into two groups:

The intoxication of females begins on the first day of cohabitation with male rats which is represented by D0 and continues during the gestation period. Newborns are also exposed to Pb-Mn until weaning (21 days after birth). Progeny is subject to the same experimental condition.

the distilled water. Tested offspring are subject to the same conditions as their mother.

Group T-HEM: 24 hours after weaning, animals receiving distilled water are treated with HEM mint essential oil (0.1 ml/kg) with one intraperitoneal injection per day for 21 days (n = 07 male rats)

Group Pb-Mn-HEM: 24 hours after weaning, animals receiving Pb-Mn are treated with HEM mint essential oil (0.1 ml/kg) with one intraperitoneal injection per day for 21 days (n = 07 male rats) (Halder et al., 2011).

2.3. Biochemical tests

2.3.1. Determination of blood lead and manganese levels

After weaning and oil treatment, the animals are decapitated and the whole blood samples (100 µl) are recovered in a 5ml haemolysis tube containing a volume of 100µl of 0.1% newt. After vortex agitation for 30 seconds, 600µl of HNO_3 (1M) is added to deproteinize. Vortex the contents of the hemolysis tube for 10 minutes at room temperature. After centrifugation for 10 minutes at 3000 rpm, the samples are transferred into wells to determine blood lead and manganese levels using an atomic absorption spectrophotometer (SHIMA DZU AA6200).

2.3.2. Determination of liver parameters

The serum samples were used to measure the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) according to the methods of Reitman and Frankle (1957). Determination of the concentrations of albumin, cholesterol and triglycerides by following the methods of Thomas (1992) and Fossati et Prencipe (1982) respectively.

Group Pb-Mn: consisting of animals which receive orally lead acetate ($Pb(C_2H_3O_2)_2$) at 0.2% and manganese chloride tetrahydrate ($MnCl_24H_2O$) at 4.79 mg/ml in bidistilled water orally from the first day of gestation until weaning (n = 07 male rats) (Kahloula et al.,2009 ; Molina et al., 2011)

Group T: is the control batch (T) that receives

2.3.3. Measurement of antioxidant enzyme activity

The rat liver was weighed and homogenized in a buffer solution containing 0.32 M sucrose, 0.5 mM EDTA, 10mM Tris-HCl (pH 7.4) in ice (1mg tissue /4 ml buffer solution) using a glass/glass homogenizer. The fabrics were maintained at 4°C during all dissection and homogenization procedures. The homogenate was centrifuged at 1000xg for 15 minutes at 4°C. The supernatant thus obtained was centrifuged at 10000xg for 15 minutes at 4°C. The pellet constitutes the mitochondrial fraction and supernatant is re-centrifuged at 10,000g/30 minutes Both pellets thus obtained are solubilized in a buffer solution containing 0.32 M sucrose, 0.5 mM EDTA, 10mM Tris-HCl and 0.02% digitonin (pH 7.4), digitonin is added to release all imprisoned mitochondria in the synaptosomes and centrifuged a second time at 10000×g for 15mn at 4°C, the pellet thus obtained constitutes the fraction of the total mitochondria which will be solubilized in a solution containing sucrose (0.32 M at pH 7.4) (Rotruck ,1973).

Super oxidize dismutase (SOD) (EC 1.15.1.1) was analyzed on supernatant using the technique of Kakkar (1984); this method is based on inhibition of formation of adenine nicotinamide dinucleotide, phenazine methosulfate and formazan amino tetra zoliumblue. Activities and levels of antioxidants at the brain level such as catalase (CAT), glutathione peroxidase (GPx) were

analyzed by the (Sinha, 1972), (Rotruck ,1973) methods respectively.

2.4. Histological study

Samples of liver spleen were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Paraffin sections (5 µm thick) were prepared, routinely stained with hematoxylin and eosin (H&E) dyes (Suvarna et al., 2013), and then examined microscopically.

2.5. Expression and statistical analysis of results

The results are expressed as the mean (M) of the individual values, assigned from the standard error to the mean (SEM). The comparison of several means is carried out by an analysis of variance (one wayAnova) with the intoxication factor (Pb-Mn, T). Repeated-measure Anova were used for time factor analysis. A probability $p < 0.05$ is considered significative. Statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Performance and chemical composition of HEM

The hydro-distillation of the plant matter *Mentha spicata* allowed to obtain a yield of 0.83%. this disagrees with the work of **(**Priscilla et al.,2010) which reported a value of (0.23±0.04%) and with those of (Lucchesi et al.,2004), (0.60±0.06-%).This difference in yield can be attributed to several factors, mainly origin, species, harvest period, drying time and essential oil extraction technique (Karousou et al., 2005) ; moreover (Marotti et al.,1994) have shown that low yields can be associated with a series of factors such as genotype, plant development stages and environmental conditions that occur in semi-arid regions.

The analysis of the essential oil of *Mentha spicata* by gas chromatography identified 12 major compounds listed in Table 1 by order of elution. Twelve components representing the sum of the percentages of the components obtained were identified (98.51%), of which 96.71% are monoterpene ketones,1.75% are monoterpene hydrocarbons, 0.05% are oxides . The major components of this oil are: Carvone (42.2%), Menthone (20.89%), Piperitenone (17.41%) and Isomenthone(7.99%). The results obtained for the various works show that this oil is essentially made of Carvone (de Sousa Barros et al., 2015).

Table 1. Concentration in % and retention time of the different compounds obtained by gas chromatographic analysis of the essential oil of *M.spicata*.

Compounds	Retention time (min)	Concentration (%)
α-pinene	11.200	0.40
Sabinene	12.080	0.56
β-pinene	12.440	0.70
Octane-3-one	12.820	1.15
Paracymen	13.600	0.09
1.8 cineole	14.110	0.05
Menthone	18.213	20.89
Isomenthone	18.710	7.99
Pulegone	21.460	0.05
Carvone	21.480	42.2

Piperiton	22.010	7.02
Piperitenone	23.150	17.41
Total of identifiers		**98,51**

Table 2. Effect of the essential oil of the *M.spicata* plant on the various biochemical parameters in rats intoxicated by Pb-Mn compared to control rats.

Parameters	**Pb-Mn**	**Pb-Mn+HE**	**Control**	**HEM**
Lead levels (µg/dl)	34,1±0,81*	21,18± 4,01*	0,27±0,028	0,25±0,022
Manganese content (µg/dl)	2,38± 0,02**	1,21±0,1**	1,03±0,1	0,99±0,04
Glucose (g/l)	1,80±0,02*****	1,42±0,04*****	0,91±0,06	1,13±0,01
alanine aminotransferase ALT (IU/l)	42,10±0,87*****	26 ±0,21*****	21,08 ±0,8	20,57±0,3
aspartate aminotransferase AST (IU/l)	36,79 ±0,4*****	13,31 ±0,4*****	11,37 ±0,3	10,98±0,1
Alkaline phosphatase (U /l)	59,82 ±0,30*	46,66 ±1,9*	43,39 ±0,10	42,86±0,14
Albumin (g/dl)	3,05 ±0,2*	4,78 ±0,14*	5,03±0,04	5,15±0,07
total cholesterol (g/l)	2,66 ±0,02**	1,29 ±0,15**	1,09 ±0,03	1,05±0,02
Triglycerides (g /l)	0,89 ±0,02**	0,68 ±0,07**	0,63 ± 0,04	0,59±0,05

Values are reported as ± SEM on average (**: $p<0.01$,*: $p<0.05$).

3.2. Results of biochemical parameters

The results obtained from blood lead and manganese assays using atomic absorption spectrophotometry (AAS) are in the order of 2.38± 0.02 µg/dl for rats intoxicated by Pb-Mn (Table 02). These levels are significantly decreased ($p<0.01$) following administration of the plant extract (1.21±0.1) in the batch of previously intoxicated rats (Mn-HEM).

The level of Pb and Mn in the blood are effective biomarkers and representative of their exposure. Physiological blood lead is zero because lead is not necessary for the life of living things. After digestive absorption, lead passes into the blood where it is distributed in red blood cells, in a non-diffusible form before settling in tissues or being eliminated in urine. Blood is therefore the crossroads of all lead pathways in the body (Pezerat, 2006). The use of atomic spectrophotometry allowed us to record lead levels in the order of 34.1±0.81 µg/dl in rats exposed to Pb acetate. (Goullé et al .,2012) Recent experimental studies have determined lead concentrations in intoxicated rats to range from 31.8 to 58.7 µg/dl (Grizzo,2008).

The highest amount of Mn in the blood is contained in erythrocytes (about 66%), with a half-life of 37 days. (Lee et Kim, 2011). The Mn level found in our study is significantly elevated in intoxicated rats compared to control rats. however, studies have shown that during pregnancy, blood Mn concentrations increase over the three semesters and Mn penetrates the placenta by active transport (Krachler et al.,1999).the increase in Mn levels during pregnancy may also be related to the acceleration of erythropoiesis, intestinal absorption or the tissue that mobilizes Mn (Tholin et al., 1995).

Exposure to Pb-Mn resulted in hyperglycemia in poisoned rats compared to control rats. These results are consistent with those of Kasdallah et al. (2005) who confirm that exposure to Mn produces a stressful effect that results in hyperglycemia under the action

of stress hormones. Moreover, Kasdallah et al. (2005) ; Huang et al. (1989) report that chronic administration of lead and manganese during the development period leads to a dysfunction of energy metabolism this increase in blood glucose concentration is probably the result of glycogenolysis, neoglucogenesis which is confirmed by liver damage to cover the body's energy needs.

Analysis of liver function markers indicates that at serum levels ALT and AST activity are significantly higher in intoxicated rats (Pb-Mn) compared to control rats ($p<0.05$).treatment with *M.spicata* essential oil showed a significant decrease in transaminase (ALT, AST) activity compared to Pb-Mn intoxicated rats. These two enzymes are located in the cytosol and the increase in their serum concentration is due to their possible release from the cytoplasm following destruction of the plasma membrane and stress on the cell (Shyamala et al., 2003). Several studies have reported that lead induces high hepatotoxicity and causes changes in the architecture of the hepatocyte, leading to a sharp increase in transaminases (Dini et al., 1999). The work of (Fordahl et al.,2012) indicates that wide exposure to Mn has an effect on liver metabolites, mainly causing disturbances in lipid metabolism and thus excessive formation of oleic acid, hydroxy-butyric acid and ketone bodies, the latter being in surplus in the blood will be eliminated in the urine, adding that alterations in liver metabolites have been correlated with tissue increase in manganese. The liver participates in many phases of lipid metabolism, esterification and elimination of cholesterol, only free cholesterol determination and serum esterification has been widely used for the study of liver disease (Awde, 2014). However, the results presented in our study showed hypercholesterolemia and hypertriglyceridemia in poisoned rats compared to control rats. Our results are similar to the work of Hanan et al. (2012) ; Sharma et al. (2013) who found an increase in triglyceride and cholesterol levels in the blood after administration of lead acetate.

In addition, the relationship between lead acetate exposure and cholesterol levels suggests a possible alteration in lipid metabolism (Moussa et Bashandy, 2008). In contrast, Zwingmann et al. (2003) have shown that Mn can lead to biliary tract closure and inhibition of bile acid transfer to the gallbladder and its accumulation in the liver leading to hypercholesterolemia

The work of Fordahl et al. (2012) indicates that wide exposure to Mn has an effect on liver metabolites, mainly causing disturbances in lipid metabolism and thus excessive formation of oleic acid, hydroxybutyric acid and ketone bodies.

3.3. Activity of enzymes of oxidative status in the liver

Endogenous antioxidant enzymes are responsible for neutralizing the free radical and preventing its action responsible for tissue damage. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, which in turn causes a series of events deregulating cellular functions. Antioxidant enzymes, such as SOD, CAT and GPX form a team in solidarity with the defense against ROS (Bandyopadhyay et al., 1999).

After Pb-Mn exposure, SOD, GPx and CAT activity are significantly lower ($p<0.001$, $p<0.05$) respectively in intoxicated rats compared to hepatic control rats. After 21 days of treatment with HEM, a marked improvement in the activity of these enzymes was observed in treated rats compared to intoxicated rats (Table 03). It has been shown that the accumulation of lead in the various liver cell compartments produces oxidative damage by strengthening the peroxidation of membrane lipids and the oxidation of proteins, a deleterious process produced solely by free radicals (Villeda-Hernandez et al., 2001).

Several studies suggest that exposure to Mn induces a variety of cellular changes due to

increased oxidative stress, and inadequate energy systems metabolisms and antioxidants (Roth et Garrick, 2003). Studies have shown the link between oxidative stress and mitochondrial dysfunction due to exposure to this metal (Mn) (Milatovic et al., 2009).

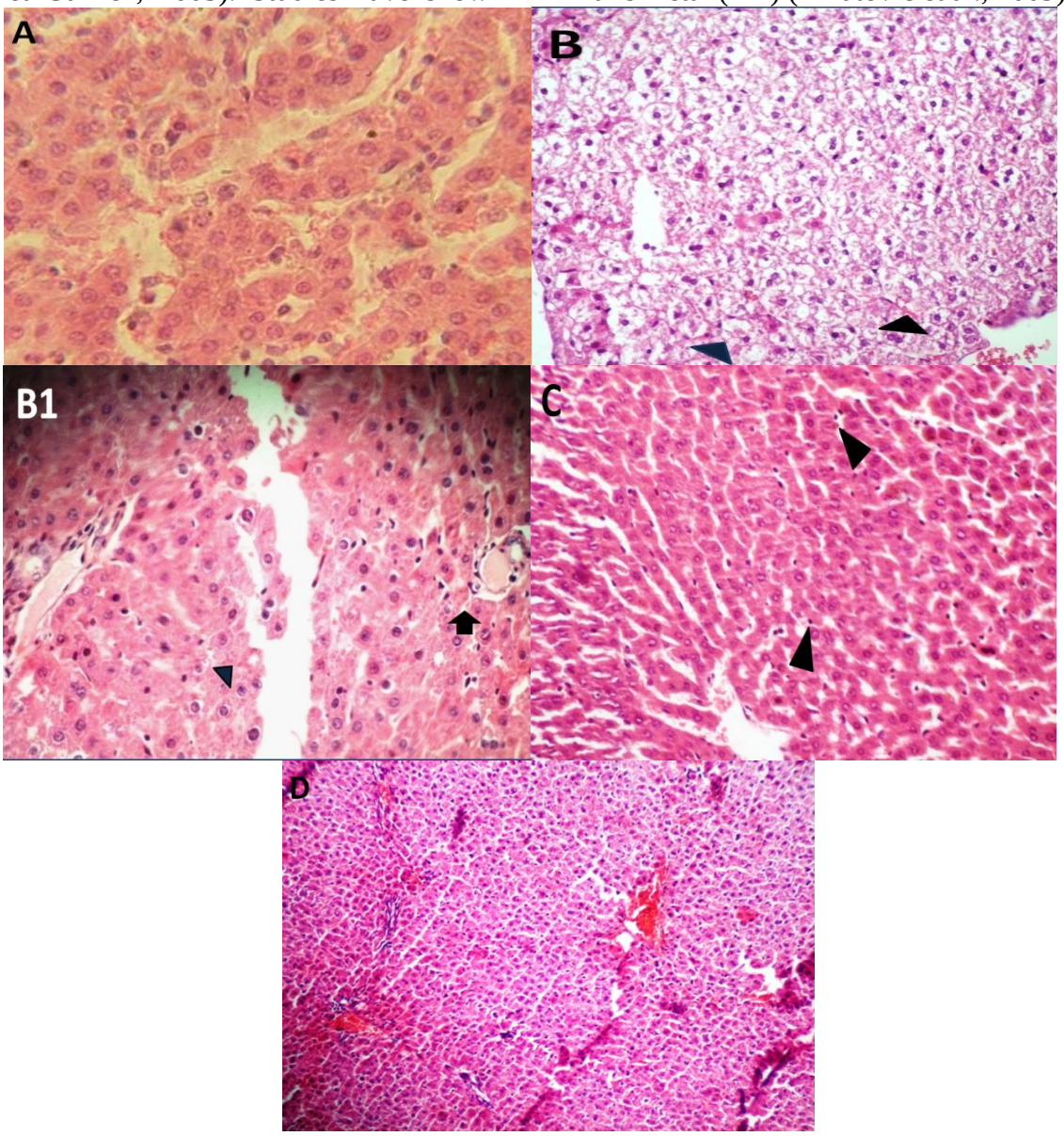

Figure 1: Optical microscopy of hepatic tissue stained with hematoxylin and eosin G :(x40). (A)G :(x100) in sections of the liver of control rats appeared with normal architecture (B, B1) Rats exposed to lead and manganese revealed a necrosis microfocus (blue arrow), portal inflammation (black arrow) (C) of rats intoxicated with Pb-Mn and treated with HEM illustrates moderate inflammation (black arrow). (D) normal liver sections of rats treated with HEM.

Arrows indicate necrosis microfocus

Portal inflammation

Table 3. Liver antioxidant enzyme activity (SOD, GPx, CAT) in control rats, Pb-Mn and Pb-Mn-HEM.

Concentration in the liver	Pb-Mn	Pb-Mn-HEM	control	HEM
SOD(U/mg of protein)	0,22± 0,14***	0,87± 0,33***	1,03± 0,26***	1,10± 0,06
GPx(U/mg of protein)	25 ,31± 0,31*	38,62±0,08*	43,41± 0,12*	40,75± 0,62
CAT (U/mg of protein)	11,38± 0,15*	20,73± 0,97*	25,27±0,22*	26,08± 0,03

Values are reported as ± SEM on average (**: p<0.01,*: p<0.05).

3.4. The effect of lead and manganese on the structural architecture of the liver

Histological sections of the liver of rats intoxicated by lead and manganese show necrosis and the hepatic parenchyma site of swelling, ballooning of the hepatocytes and lipid vacuoles within the hepatocytes producing steatosis foci, a necrosis microfocus (Figure 01.B, B1) compared to the liver of the control rats. These results are similar with studies by Jarrar et al. (2012) suggesting that subchronic exposure to lead causes liver damage in rats. The appearance of inflammatory cells in liver tissue due to sub-chronic exposure to lead may suggest that lead may interact with proteins and enzymes in interstitial liver tissue interfering with the antioxidant defense mechanism and lead to reactive oxygen species (ROS) which in turn may mimic an inflammatory response (Johar et al ., 2004). In addition, the liver is the critical organ for regulating Mn homeostasis in the body. In the same way, Mn toxicity is rare and occurs mainly in the liver, probably because of the primary role this organ plays in eliminating Mn from the blood through biliary excretion (Crossgrove et Zheng, 2004), consumption of water contaminated with Mn causes liver overflow which can lead to liver damage (Peili et al., 2011).Indeed several studies have reported that Mn induces a histopathological alteration by a necrosis of the liver and appoptoseperiportal (Rahelié et al., 2006) which could compromise the detoxifying function of the liver to know how to eliminate manganese (Zhang et al., 2003). Mint is usually used in the treatment of biliary system disorders, liver problems, irritable bowel syndrome and inflammatory bowel disease (Bouchra et al.,2003). The effects of mint are related to its effect on bile flow and liver function (Taylor,1984). On the other hand it is observed that blood glucose levels are decreased in rats treated with *Mentha spicata* EHM compared to intoxicated rats. The administration of mint essential oil to rats exposed to Pb-Mn resulted in a decrease in blood glucose levels, it has been shown that mint extract performs hypoglycemic activity due to the presence of major components such as menthol and menthone (Khodadust et al., 2015) In addition, the results of Bayani et al. (2017) indicated that aqueous extract of *M. spicata* leaves possesses hypoglycemic, hypocholesterolemic and antioxidant properties in diabetic rats. In parallel, a significant decrease in transaminases (ALT,AST) was also observed following the administration of *M.spicata* HEM which indicates regulation of liver function biomarkers.Consistent with the results of this study, Rajesh et al. (2013) reported that elevated serum ALT biochemical markers in rats with hepatic lesions were decreased with the addition of ethanolic extract of mint leaves (*Mentha arvensis*) at 400 mg/kg . Barbalho et al. (2011) studied the effects of administration of mint juice twice daily for 30 days on certain biochemical parameters in humans, the results showed a 41.5% reduction in blood sugar, 58.5% in triacylglycerides, 66.9% in total cholesterol, 52.3% in LDL indices, 70% in AST levels, 74.5% in ALT levels.

Treatment with HEM in intoxicated rats showed an improvement in cholesterol values, triglycerides and oxidative status enzymes (SOD,CAT, GPx). These results are similar

with the work of Al-Fartosi et al. (2014) showed that phenolic compounds of *Mentha longifolia* and *Mentha spicata* leaf extract in diabetic rats significantly reduced serum cholesterol levels compared to untreated groups. The cholesterol-lowering effect of mint could be due to the menthol and menthone content which has the property of reducing the activity of a liver enzyme HMG CoA reductase (a primordial enzyme in the synthesis of cholesterol in the liver) and simultaneously reducing cholesterol levels. (Poltowicz et Wesyk, 2005). *Mentha arvensis* leaves have the highest phenol and flavonoid content which explains why it has the greatest antioxidant property and reduces cholesterol levels (Vishwakarma et al., 2014).

It has also been observed that the administration of HEM *M.spicata* protective action of liver tissue that could be explained by the chelating and antioxidant effect of phenolic components contained in HEM, which significantly reduces free radicals generated by intoxication that causes cell damage in liver tissue. (Muruganathan et Srinivasan, 2016) Our results are in harmony with Vinothkumar et al., (2013), which report that carvone reduces oxidative stress by improving enzymatic antioxidant activity in rats poisoned by 1,2-dimethylhydrazine. However, other authors have illustrated that treatment with carvone restored the activities of serum ALT and AST and ALP levels in rats treated with streptozotocin, indicating the maintenance of liver cell function and structure (Muruganathan et Srinivasan, 2016). Another study indicated that in vivo administration of peppermint alcohol extract reduced the adverse effects of CCl4 on liver function (Khodadust et al., 2015).

4. Conclusions

Exposure of developing wistar rats to Pb-Mn revealed hepatotoxic effects resulting in significant alteration of liver bio markers and the antiradical system represented by different enzymes. Treatment with *M. spicata* HEM clearly demonstrated effective hypoglycemia, hypolipidemia and remarkable liver protection in rats previously intoxicated with Pb-Mn.

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SENSORY ATTRIBUTES PROFILING OF DAMPIT ROBUSTA COFFEE LEAF TEA (*Coffea canephora*)

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ABSTRACT

Abundant leaf waste resulted from the maintenance of coffee plants can alternatively be made into coffee leaf tea. The results of this study indicate that old Robusta coffee leaf significantly have increasing total phenol, pH and color, but decreasing the level of caffeine. The fermentation process can significantly reduce total phenol, caffeine content, pH and color. Meanwhile, the brewing temperature only gives a real effect on the color of steeping tea. Based on the method of Rate-All-that-Apply (RATA), coffee leaf tea powder has aroma characteristics (green, wood, floral, earth and sweet), while the steeping tea has characteristics of having green aroma and flavor as well as bitter taste and astringent in mouth. The most dominant profile of aromatic compounds of coffee leaf tea with GCMS HS-SPME method is green which might be attributed by 2-heptanol (CAS), 2-hexen-1-ol, 1-furfuryl-2-formyl pyrrole, safranal, beta-cyclocitral, 4-heptanal,(Z)-(CAS), hexanal (CAS), nonanal, benzeneacetaldehyde, benzaldehyde, 2-heptanone (CAS).

1. Introduction

Coffee is one of the most widely consumed beverages worldwide, due to its distinctive flavor and aroma (Campanha et al., 2009). Brazil, Vietnam, Colombia and Indonesia are the main coffee producing countries because they are located in suitable areas to grow (WBCM and ICO, 2017). Coffee plants will be trimmed regularly because it can grow up to 1.5 - 4.6 meters. Pruning is needed to optimize fruit production and plant maintenance (Jonathan et al., 2009). However, pruning produces abundant waste in the form of coffee leaves that are not utilized in large quantities. Therefore, it is necessary to find out alternative use of coffee leaves.

One alternative use of coffee leaves is using them as a material for making tea. Coffee leaf tea is one of Indonesia's typical drinks, precisely derived from the realm of Minang in Padang, West Sumatra (Ratanamarno and Subskar, 2017). During the Dutch colonial period of 1847-1908, the population of West Sumatra Minangkabau experienced a system of colonial exploitation in the system of forced cultivation. Residents had to submit all the coffee beans cultivated to the Dutch, so people who wanted to enjoy coffee could only use the coffee leaves for beverages. Drinks from the coffee leaves are known as "Kopi Daawa Melayu or "Kopi Kawa" (Akira, 1977 in Booth et al., 1988).

Ratanamarno and Surbkar (2017) suggest that the differences of leaf age, the pre-treatment, and brewing time, affecting the differences on caffeine and catechin contents of Arabica leaf tea (*Coffea arabica*). Young and non-fermented coffee leaves tent to have hinger levels of caffeine and catechins than those of old leaves. They also suggest that the recommend brewing time is 5 minute, to minimize caffeine level. According to Yitayal and Achame (2015) revealed the effects of caffeine on a mixture of coffee leaves and spices that are processed differently by Roasted, Raw and Majengir. The caffeine content of the Roasted type was not significantly different from Majengir type in both solvents. Tea mixed coffee leaves and spices can be used as an alternative to coffee drinks that are low in caffeine in Ethiophia. In further research (Yitayal and Titalun, 2017) reported the nutritional value of coffee leaf tea in the form of proximate and mineral composition. Coffee leaf tea contains essential and vital nutrients for energy and health, so this drink can be used to meet the nutritional needs of the community.

It shows that coffee leaf tea can have health effects because they contain caffeine and polyphenols, but for some people caffeine can lead to increase heart rate, at risk of cholesterol buildup (Hoeger et al., 2001), stomach disorders, anxiety, diminished memory, and insomnia. The caffeine content also affects bitter flavor correlated with other bitter taste-forming compounds such as alcohol, caffeine, trigonelline and guinine, and correlated with chlorogenic acid, polyphenols, catechins and other compounds (Calvert et al., 2015).

This study aims to introduce the coffee leaf tea as a traditional drink from Sumatra Indonesia, focusing on profiling sensory attributes. This study also aims to determine the effect of leaf age, the process of manufacture, and temperature of serving on the physicochemical, sensory and aromatic compounds.

2. Materials and methods

2.1. Tea Samples and Physicochemical Analysis

Coffee leaves were harvested from Robusta coffee plants (*Coffea canephora*), growing under natural environment in Dampit, Malang, Indonesia. Young leaves were picked from bud to the 4th leaves, while old leaves were picked from the 5th to 8th leaves. The chemicals and reagents used consisted of folin-ciocalteu's phenol reagent and gallic acid for phenol total (Spectrophotometry), sugar total (refractometer), petroleum eter for fat content (soxhlet), indicator fenolpthhalein and NaOH for total acid (titration), and buffer pH 4 and pH 7 for pH measurement (pH meter).

2.2. Coffee Leaf Tea Processing

Non-fermentation method: young or old coffee leaves were washed and cut into small pieces, after that dried with an electric oven at the temperature of 70 ± 2 ºC for 4 hours. As for the method of fermentation: young or old coffee leaves were washed and cut into small pieces, then withered and rolled at room temperature of 23-27 ºC for 18-24 hours, followed by drying with an electric oven at the temperature of 70 ± 2 ºC for 4 hours. The 4 samples of dried coffee leaf tea were ground using a blender at 3000 rpm and 3 ± 1 minute duration, then they were divided into small plastic clips and given a three-digit random code of 2 grams per bag. Samples of dried coffee leaf tea were brewed with hot water at 90-96 ºC. Then each sample was presented in 3 different serving temperatures i.e. cold temperatures ranging from 8-12 ºC, normal temperature of 23-27 ºC, and warm temperature of 40-44 º C.

2.3. Phenol Total Analysis

Phenol total analysis followed Pal et al., (2012). The 1 ml sample was diluted with

distilled water at a ratio of 1:10. The mixture was placed on the test tube, and 1ml ethanol was added. 5 ml of distilled water and 0.5 ml of the Folin-Ciocalteu reagent (50%) were added to the test tube and mixed. After 5 minutes, 1 ml of Na_2CO_3 (10%) was added in the tube and mixed until homogeny. The mixture was then placed in a darkened room or covered with aluminum foil for 60 minutes. After that, the absorbance was measured with a wavelength of 725 nm. The standard curve was made in the same way by replacing the sample with an error acid made with some concentration. Total polyphenol content in tea functional drinks was expressed in mg/L.

2.4. Caffeine Analysis

Caffeine stock solution (1000 ppm) was prepared in distilled water. Different working solutions were prepared by serial dilution with the addition of 1.0 ml hydrochloric acid. The 0.25 g sample was weighed and diluted accurately in water and prepared with a net volume of 20 ml with distilled water. The 20 ml sample solution was pipetted in a 250 ml flask and 10 ml of 0.01 mol/l hydrochloric acid and 2 ml acetic acid solution were then added. The final volume was prepared with distilled water. The 50 ml solution was filtered and added to a 100 ml flask. 0.2 ml of 4.5 moles of sulfuric acid was added and again made to a clean and filtered volume. The absorption of work standards and samples was measured on the UV/Vis (Shimadzu) spectrophotometer. The level of caffeine from the sample was calculated through the regression equation of the best line according to the standard (Christian, 1994).

2.5. Volatile Compounds Evaluation

Analysis of volatile compounds in coffee leaf tea was done by GCMS (Gas Chromatography Mass Spectrometry) and SPME (Solid Phase Micro Extraction) 4000 variant of GCMS (Variant, Inc., USA) consisting of 3,800 GC with CP-8410 auto injector (Bruker, USA), and 4,000 Ion Traps MS detector with CP-1177 split/spitkess injectors, at a temperature of 300 °C and a 1.0 µL injection volume. Each splitless mode: Agilent focus liner with glass wool deactivation was done in triplicate Manually Chromatographic. Separation was done by using Zebron MultiResidue-1 columns (30 m × 0.25 mm x 0.25 µm; Phenomenex Inc., USA); internal diameter × 0.25 µm; film thickness) capillary column (Restek, Bellefonte, PA, USA) (Lee et al., 2013).

2.6. Sensory Analysis

Sensory analysis was used the RATA (Rate-All-that-Apply) method with two stages, namely to know the characteristics of powder sensory and coffee leaf tea steeping. The panelists were used untrained panelists of 110 people, who consisted of men and women with an age ranging from 18 to 45 years. The main instrument was used the RATA questionnaire with scores 1-3 to describe the sample of sensory characteristics, i.e. score 1 for low intensity attributes, score 2 for moderate intensity attributes, and score 3 for high intensity attributes. Sensory attributes that do not describe sensory characteristics need not be ticked (Ares et al., 2014).

2.7. Data Analysis

The sensory intensity attributes responding on leaf age factor, pre-treatment , and serving temperature were analyzed using GLM (General Linear Model). In addition, GLM was used to find out the results of chemical and physical tests on samples with different factors. The datas were analyzed using statistical analysis program MiniTab 17.

3.Results and discussions

3.1. Characteristics of Coffee Leaf Tea

Table 1 shows that the various leaf ages of the processing stage gives significant difference ($p < 0.05$) to water content, ash content, fat content, total sugar, total acid, reddish color intensity (a +) and yellowish (b +), but not

significant (p> 0.05) for brightness intensity (L *).

Young leaf tea in Table 1 tends to have higher water content than old leaf tea, because the older the age of the leaves the more decreasing the water content. Young leaves have a relatively soft texture, because they have more moisture content than old leaves (Osman et al., 2004). Fermentation of the old leaves causes a difference in water content which increases along with the longer fermentation time. This is due to the oxidative fermentation reaction of catechins with oxygen to produce water vapor and result in increased water content (Bradley and Vanderwam, 2001).

When the water content (H_2O) in the material increases, the ash content decreases. Thus, the ash content on old leaves decreases with the fermentation process. This is as a result of the abundance of water-soluble minerals and fats, so it will come together during the drying process. Decrease of the ash content can occur because of during the fermentation process there will be an increase in organic material due to the degradation of substrate by microbes. The less organic matterdegradation , the less decrease of ash content (Hatakka, 2001).

In old coffee leaf tea, the fat content tends to be higher in Table 1 because the moisture content of the leaves is lower. During the drying process there has been decomposition of the water molecule bonding component H_2O and increasing the sugar, fat, minerals and protein content, there by rising the ash content. In addition, the decrease in the fat content is caused by the degradation of lipase enzyme (Bhara, 2009).

Sucrose is a disaccharide found in plants, included in non-reducing sugars because the active groups are already bonded to each other. Sweet-tasting components such as sugar, aldehydes, sulphide and benzoate emerge during the fermentation. Reduced sugar content will increase due to sucrose hydrolysis to glucose by invertase enzyme. The decrease in total sugar in Table 1 and the drying process breaks down the water molecule bond component (H_2O) and increases the sugar content (Bhara, 2009).

Coffee leaf tea has caffeine content which will evaporate when drying or roasting, forming components such as acetone, furfural, ammonia, trimethylamin, formic acid and acetic acid (Khan and Mukhtar, 2007). The longer the fermentation, the more the acidity of coffee leaves as described in Table 1. This is because of the formation of aliphatic acids during fermentation. If the fermentation is extended, there will be aliphatic chemical composition changes to esters of carboxylic acids resulting in the taste of rottenness (Famwort, 2005).

Table 1. Physical characteristics of coffee leaf tea powder

Sample	Water Content (%)	Ash Content (%)	Fat Content (%)	Total Sugar (%)	Total Acid (%)	L*	a+	b+
MNF	10.57^{a}	5.01^{d}	1.23^{c}	0.61^{d}	2.11^{b}	41.90^{a}	2.73^{a}	9.77^{b}
MF	10.56^{a}	5.29^{c}	0.82^{d}	4.17^{b}	2.85^{a}	42.93^{a}	2.87^{a}	10.70^{a}
TNF	6.73^{b}	6.51^{a}	4.28^{a}	0.80^{c}	1.87^{c}	43.40^{a}	1.90^{ab}	11.00^{a}
TF	6.83^{b}	6.10^{b}	3.22^{b}	4.37^{a}	2.87^{a}	42.87^{a}	1.03^{b}	9.07^{b}

Note: (MNF) young leaf with a non fermented method; (MF) young leaf with a fermentation method; (TNF) old leaf with a non fermented method; (TF) old leaf with a fermented method; (^{a,b,c,d}) notation of real difference with Tukey statistics (p <0,05)

Table 2. Physicochemical characteristics of coffee leaf tea

Factor	Treatment	Phenol Total mgGAE/g	Caffeine (%)	pH	L*	a+	b+
Leaf Age	Young Leaf	67.76[b]	0.56[a]	5.66[b]	29.67[b]	2.82[b]	5.27[b]
	Old Leaf	78.77[a]	0.52[b]	5.77[a]	32.14[a]	3.42[a]	10.39[a]
Process	Non Fermented	75.23[a]	0.55[a]	5.73[a]	32.34[a]	4.53[a]	10.06[a]
	Fermented	71.31[b]	0.53[b]	5.69[b]	29.47[b]	1.70[b]	5.61[b]
Temperature of serving	Cold	73.11[a]	0.54[a]	5.69[a]	31.03[a]	3.19[b]	8.62[a]
	Room	73.96[a]	0.53[a]	5.72[a]	32.92[a]	4.32[a]	9.73[a]
	Warm	72,73[a]	0,53[a]	5,73[a]	28,77[b]	1,83[b]	5,14[b]

([a,b,c,d]) notation of significant difference with Turkey statistics (p <0,05)

Table 3. Volatile components of coffee leaf tea analyzed with GCMS HS-SPME

No	Compound	MNF	MF	TNF	TF	Aroma Description
1	(methyl benzoate) Benzoic acid, 2-(acetyloxy), methyl ester (CAS)	√			√	fragrant, fruity
2	2-furanmethanol (CAS)	√		√		caramellic, coffee, bready, sweet, burnt, brown
3	2-heptanol (CAS)	√	√		√	fresh, herbal, sweet, floral, green, fruity, green, earthy
4	Linalool	√	√	√	√	fruity, floral, rose-like
5	Phytol	√	√	√	√	floral, oily, balsamic, herbal, magnolia, orchid
6	2-Hexen-1-ol	√			√	fresh, fruity, green, grassy
7	1-Hexanol,2-ethyl- (CAS)	√	√	√	√	newly cut grass
8	1-Furfuryl-2-formyl pyrrole	√			√	roasted, chocolaty and green
9	(Safranal)1,3-Cyclohexadiene-1carboxaldehyde,2,6,6trimethyl(CAS)	√	√	√	√	herbal, woody, sweet, green floral, herbaceous, somewhat tobacco
10	Beta-Cyclocitral	√	√			minty, herbal, rose, green, fruity, tea
11	4-heptanal,(Z)-(CAS)	√		√		green, biscuit, cream, fat, fishy, rotten
12	2,4-heptadienal	√	√	√	√	fatty, nutty, hay, fishy
13	Butanal-2-methyl (CAS)	√		√	√	almond, cocoa, fermented, hazelnut
14	Hexanal (CAS)	√	√	√	√	green, fatty, leafy, fruity and woody
15	(Furfural) 2-furancarboxaldehyde (CAS)	√		√		almond-like
16	Eugenol	√	√	√	√	sweet, spicy, woody, phenolic, warm
17	Nonanal		√		√	grassy, green, tea, vegetable
18	Benzeneacetaldehyde	√	√	√	√	honey, floral, rose, chocolate, spicy
19	2-hexenal	√	√	√	√	green, banana, aldehydic, fatty, herbal, spicy, fresh
20	Pentanal	√		√		almond, bitter, malt, oil, pungent
21	Benzaldehyde		√	√	√	bitter almond, burnt sugar, cherry, green, roasted pepper
22	6-methyl-5-hepten-2-one	√		√		citrus, pepper, strawberry
23	1-pentanol (CAS)	√				mild, fusel-like, unpleasant
24	Benzeneethanol (CAS)	√	√	√	√	floral, rose, burn, almond
25	Pyridine	√	√	√	√	sour, putrid, fish-like
26	Napthalene	√	√	√	√	floral, fruity

27	(Methyl Isopalmitate) Pentadecanoic Acid,14methyl-, methyl ester	√			√	oily, waxy, fatty, oris
28	Methyl salicilate	√	√	√	√	sweet, spicy, minty
29	(guaiacol) Phenol,2-methoxy-(CAS)	√	√	√	√	phenolic, woody, smoky, spicy, eugenol-like
30	(BHT) Phenol,2,6-bis)1,1 dimethylethenyl)-4-Methyl	√		√		slightly, phenolic, off-odor, off-flavor
31	Alpha-ionone	√		√		woody, floral, nutty, berries
32	2-heptanone (CAS)	√				fruity, banana-like, nut, spicy, green, blue cheese
33	Beta-ionone	√		√	√	woody, floral (rose-like), berries, cherry
34	Acetyl pyrole	√	√		√	musty, nutty, walnut, bready, coumarinic, tea-like
35	(3,5-cocoa pyrazine) Pyrazine,2-ethyl-3,5dimethyl-(CAS)	√		√		nutty, roasted, coffee, cocoa, sweet, corn, caramelic

The fermented samples of chlorophyll content will be lost due to maillard reaction, i.e. non-enzymatic browning which turns the color of coffee leaves to brown resembling a coffee bean. In addition, phenolic acids will decrease as temperature increases. With the presence of heat and oxygen, the phenolic compounds can be oxidized because the activity of polyphenol oxidase enzymes to form reactive ortho quinone radicals and can react further into amino compounds form a brown product (Reblova, 2012).

3.2. Characteristics of Coffee Leaf Tea

Table 2 shows that coffee leaf tea with different leaf age and pre-treatment factors shows a significant effect ($p < 0.05$) on total phenol, caffeine, pH and color, but the presented temperatures only give a significant difference ($p < 0.05$) to color.

3.3. Total Phenolic Compound

According to the Table 2, coffee leaf tea from old leaves has a higher total phenol value compared to tea from young leaves. According to Farhoosh et al. (2007), the old leaves of Robusta coffee have higher phenol levels. The high total content of polyphenols is due to the relatively soft texture and high moisture content in young leaves. The long sedimentation time provides greater heat penetration so that the polyphenol oxidase enzyme is inactive faster and results in less polyphenol damage (Osman et al., 2004). Non-fermented coffee leaf tea has a higher total phenol value than tea with fermentation process. According to Pou (2016) during the fermentation process the enzymes present on the leaves will be contact with air and begin to oxidize. Phenolic acids will decrease due to rising temperatures, heat and oxygen, so that the phenol compounds are oxidized because the enzyme activity of polyphenol oxidase forms a reactive radical orthosemiquinone and can react further into amino compounds.

3.4. Caffeine

According to Table 2, the percentage of caffeine in young coffee leaves tea is higher than old coffee leaf tea. According to Zheng and Ashihara (2004), caffeine contents in young leaves are higher than old leaves and gradually replaced by theacrine, which is converted into liberine as the dominant purine alkaloid in young leaves. The older coffee leaves contain smaller caffeine biosynthesis (Keya et al., 2003). The non-fermentation process produces coffee leaf tea with a higher caffeine percentage than the coffee leaf tea with a fermentation process. According to Mandal (Mandal, 2010), during caffeine fermentation other components such as acetone, furfural, ammonia, thrimrthylamine, formic acid and acetic acid evaporate and form. Ratanamarno

and Surbkar (2017) suggest that the fermentation is one method to decrease caffeine in coffee. Increasing fermentation time, reducing caffeine levels in coffee. During the fermentation, caffeine is converted into uric acid, 7-methylxanthine and xanthine. (Todar, 2010).

3.5. pH

According to Table 2 in the leaf age factor, the pH of steeped old coffee leaf tea is higher than steeped young coffee leaf tea. The young leaves contain higher moisture content and softer texture than the old leaves, so that the timber will provide greater heat penetration and result in easier polyphenol damage (Osman et al., 2004). According to Fulder (2004), the processing step results in oxidizing polyphenol component to theaflavin. If there is further oxidation, it will turn into thearubigin. If more thearubigin is formed, then the pH will decrease.

3.6. Color

The boiling or brewing processes oxidize the flavonoid compounds into phenol compounds, which later become quinone due to oxidize of heat. Further oxidation of the quinone will produce thearubigin which causes darker color in boiled tea water (Shahidi and Neczk, 2005) According to Reblova (2012), tea production requires heating, as the increase in temperature can lead to oxidize and browning marked color change in tea drinks. Temperature changes trigger the reactions of amino acids such as alanine, leucine, isoleucine, valine and tannins in the tea, releasing carbon dioxide molecules that will form the aldehyde complex that affects the brewing color.

3.7. Correlation of Powders and Coffee Leaf Tea

In general, coffee is consumed by the community in the form of powder that is brewed using hot water. Meanwhile, in the processing and brewing of tea, the tea should be brewed with the possible optimum concision to maximally utilize the content in the tea powder. Tea brewed with different water temperatures and durations will create different tastes. Tea brewed over makes more caffeine extracted. Even though polyphenols such as tannins require longer time to be extracted to give color and flavor, because too long brewing will leave a bad aftertaste (McAlpine and Ward, 2016). The extraction time refers to the amount of tea leaf time in contact with water and thus, the brewing time may occur. If the time is optimal, some compounds in the tea leaf will reach the point of equilibrium, meaning that the concentration of the compound in the leaves will be equal to the concentration of the compound present in the brewing water (Gerbely, 2016).

3.8. Panelists' Response to Coffee Leaf Tea

According to panelist’s responses, coffee leaf tea has green smell as described in Figure 1. Green and similar terms such as grass have been used in many sensory analysis descriptions of various foods (Baldwin et al., 2004). Hexane is the compound most often associated with green characteristics such as cut grass (Buettner and Mestres, 2005) and herbs (Jordan et al., 2003). Alex Probyn in Grey (2013) mentions that coffee leaf tea has a very fresh taste, resembling fresh leaves like green tea, pungent and greenish.

Higher responses show that steeped old coffee leaf tea has a sweet flavored attribute than young coffee leaf tea, as described in Figure 2. According to Campbell et. al., (Campbell et al., 2006) carbohydrates in young leaves are still abundant in the form of starch, so the sweet taste is still low. Starch will be broken down into simple sugars such as glucose, fructose and sucrose during the aging process or maturation, so it becomes sweeter.

The bitter taste attribute is one significant difference, referring to Figure 2. The young leaves tea has a higher average intensity than old leaves. The higher bitter taste in younger leaves is thought to be due to the influence of caffeine content in younger leaves that are

higher than in the old leaves. Ratanamarno and Subskar (2017) suggest that caffeine and phenol form the immune systems in leaves , causing bitterness. The caffeine content on young leaves is hinger than the old leaves. (Izzreen and Fadzelly, 2013).

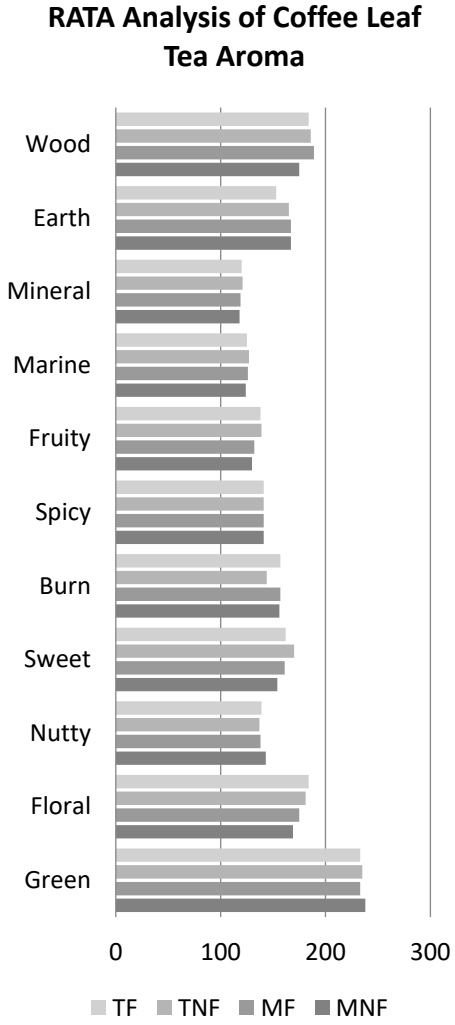

Figure 1. RATA analysis of coffee leaf tea aroma

Another distinct attribute is thesour taste . Young coffee leaf tea has a higher intensity than that in old coffee leaf tea, as described in Figure 2. The pH of young leaf tea is lower than that of old leaves. Lower pH indicates a more sour taste. According to Chan et. al. (2016), as the pH increases, the hydrogen ion concentration in the sample decreases, resulting in the release of hydrogen ions by phenolic compounds (antioxidants) in the sample. Furthermore, the decreasing pH decreases the antioxidant activity.

Earth and woody flavors in this study also have significantly different results, as described in Figure 2. The earth flavor in the tea is resulted from the 2-6-dimethylcyclohexanol and 2-isobutyl-3-methoxypirazin compounds (Lee and Chambers, 2006; Kumazawa and Masuda, 2002). Zecuppa (2017) explains that flavor wood resembles dry wood, wooden barrels, oak, dead wood, or cardboard. Woody flavors in coffee leaf tea can be caused by α-Ionone and β-Ionone (Lee and Chambers, 2006).

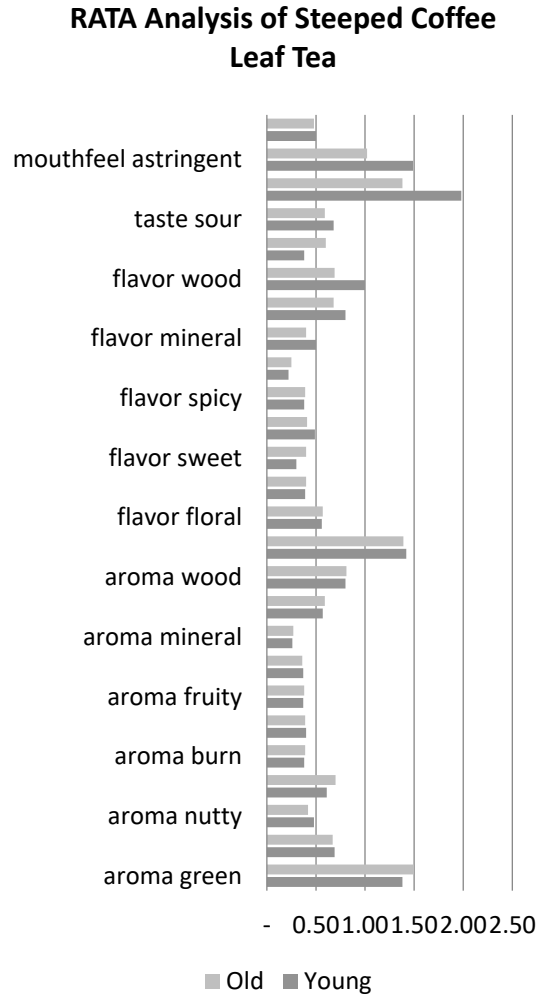

Figure 2. RATA analysis of steeped coffee leaf tea

3.9. Volatile Compound on Coffee Leaf Tea

The volatile compound is only about 0.01% of the total weight of the dry tea, but its contribution to overall tea quality is very important (Pripdeecech and Wongpomchai, 2013). In the coffee leaf tea analyzed by GCMS using SPME extraction, there were 71 volatile compounds in a non-fermented young leaf (MNF), 54 volatile compounds in fermented young leaf (MF), 62 volatile compounds in non-fermented old leaf (TNF), and 60 volatile compounds in the fermented of old leaf (TF). Large 35 volatile compounds presented in coffee leaf tea and aroma description can be seen in Table 3.

Volatile compounds of coffee leaf tea are dominated by green and floral fragrances that can be seen in Table 3. The predominant smell identified in coffee leaf tea is green in 11 out of 35 large compounds, such as 2-furanmethanol (CAS), 2-heptanol (CAS), 2-heptanol (CAS), safranal, beta-cyclocitral, hexanal (CAS), 4-heptanal(Z)- (CAS), benzeneacetaldehyde, benzaldehyde, nonanal, and alpha-ionone. The "green" sensory characteristics are commonly used in describing the characteristics of various fresh vegetables, raw fruits, food products, and gentle fragrances. The term green and similar terms such as grass have been used in many sensory analysis descriptions of various foods (Balwon et al., 2004). Waltner-Law *et. al.* (2002) grouped volatile compounds found in green tea into the categories of green flavors,

floral scents, baked and spicy scents, and off-flavor (stale, burning smoke).

3.10. Effect of Volatile Compounds on the Sensory Attributes of Coffee Leaf Tea

Sensory attributes of coffee leaf tea are described in Figure 1. Aroma attribute consists of green, floral, spicy, fruity, marine, nutty, sweet, burn, minerals, earth, and woody. Meanwhile, the sensory attributes of steeped leaf coffee tea are categorized into aroma, taste, flavor, and mouthfeel . The flavor and aroma attributes consist of green, floral, spicy, fruity, marine, nutty, sweet, fire, minerals, earth, and woody. The taste attribute is detected using papilla on the tongue. The taste attribute consists of 3 attributes i.e. sweet, sour, and bitter. The mouthfeel attribute consists of astringent and oily, as described in Figure 2.

The sensory attribute of powder and coffee leaf tea is the green aroma, as described in Figure 1 and 2. This is due to the presence of hexenal, benzaldehyde, 2-hexenal, 2-heptanone, alpha-ionone, and 2-hexen-1-ol. According to Kim *et al.*, (2016), the smell of leaves in the tea is due to the presence of volatile components such as hexanal, benzadehyde, 2-hexenal, and 2-heptanone. The high intensity aroma sensory attributes of other coffee leaf tea are wood, floral, earth and sweet.

The highest intensity sensory attribute is thebitter taste , illustrated in Figure 2. Alek Probyn in Gray (2013) argues that coffee leaf tea has a bitter but acceptable character. Coffee leaves contain caffeine of 21.9 g/kg per dry weight, epicatechin, mangiferin , isomangiferin n, and some other phenolic components. Among the four basic tastes, bitterness is the most complex and least understood (2005). Other sensory attributes that also have high intensity are green, flavor green, and astringent. Alex Probyn in Gray (2013) also mentions that coffee leaf tea has a very fresh taste, resembling fresh leaf like green tea, pungent and greenish. The different processing and serving temperatures do not have a significant effect to the sensory attribute.

4. Conclusions

The results conclude that the coffee leaf tea has high intensity of green, wood, floral, earth and sweet. The steeping tea has characteristics of bitter taste, strong aroma and flavor of fresh leaves (green). The age of coffee leaves gives a significant influence on the 6 sensory attributes, namely the sweet taste, sour taste, bitter taste, sweet flavor, wood flavor, and earth flavor. Processing treatment and serving temperature do not give a significant effect on any sensory attributes.

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EFFECT OF ORGANIC FERTILIZATION ON THE QUANTITY AND QUALITY OF MELON PRODUCTION

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ABSTRACT

Growing environmental impact of conventional farming practices, gradually impacts soil structure and biological balance. This has forced the development of alternative practices to reduce and mitigate these effects. Types of fertilizers influence the quantity and quality of production. Following this idea, in 2016, in a crop of melon in a polyethylene tunnel, Lignohumat organic fertilizer was used, a granular humic product, which was applied during vegetation, in three stages and three fertilization doses: 100; 150 and 200 g/ha. Melon plants (Charentais variety) fertilized with Lignohumat at different doses showed positive results compared to the unfertilized variant. Results showed that application of the 150 g/ha dose significantly improved ($P \leq 0.05$) the average number of fruits/plants, their average weight, fruit/plant production and the production/m^2 but also the quality of fruits: soluble dry matter – 8.55%, total dry matter – 12.12%, reducing sugars – 3.40%, vitamin C - 21.12 mg/100 g, carotene - 36.42 mg/100 g f.m and antioxidant activity of 175.92 µMTE/100 g (ABTS method) and 228.16 µMTE/100 g (DPPH method). Antioxidant activity was evaluated using 2.2-diphenyl-1-picrylhydrazyl (DPPH) 2.2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS). Significant positive correlations were identified for very importants parameters.

1.Introduction

High demand for agricultural products in order to obtain food and consumer goods in modern society has generated significant increases in agrarian activities over the past decades. Consequently, it has been necessary to implement methods that will, among other things, improve crop efficiency, mitigate the negative impact on soil, reduce the use of chemical fertilizers and increase crops economic return.

Organic fertilization involves use of organic fertilizers, decomposition of organic matter debris, use of domestic waste water, manure and microorganisms (fungi and bacteria) (Chirinos *et al.*, 2006). In recent years, lignocellulosic materials have been used as fiber raw materials in the production of biodegradable nutrient pots for vegetable seedlings in containers. Considering the high degradation potential of these materials, they can also be used as sources of biofertilizers (Nechita *et al.*, 2010).

All these are used to improve nutrient fixation in the rhizosphere, to produce growth stimulators for plants, to improve soil stability by ensuring effective biological control, and promote micorization, symbiosis and bioremediation in soils contaminated with toxic substances (Rivera-Cruz *et al*., 2008).

Bio-fertilizer application improves surface productivity in a relatively short time, reduces soil and water contamination, increases soil fertility, thus promoting antagonism and biological control of phytopathogenic organisms (Chirinos *et al.*, 2006). These issues provide real benefits for farmers as a result of lower fertilization costs and higher yields. Implementation of fertilization techniques requires feasibility studies, monitoring of environmental variables involved in metabolic processes, capital investment, time and trained personnel (Vanegas, 2003).

Biofertilizers have a significant effect on production due to nutrients that mobilize microorganisms that contribute to the availability of macro and micro elements and their forms (El-Sanafawi, 2006, Ahmed and Fraihat, 2011). Application of biofertilizers improves plant growth morphological elements, chlorophyll content and N, P, K elements, production elements (weight and number of fruit/plant) and quality (Dinu *et al.*, 2013). Therefore, the purpose of this study was to evaluate Lignohumat organic fertilizer based on humic acids on quantitative and qualitative elements of melon fruit and to establish an optimal dose for a polyethylene tunnel culture, thus reducing the negative impact on soil and environment. *Cucumis melo* L. culture was chosen to diversify the diet and because melon fruits are also a significant source of polyphenol antioxidant phytochemicals which provide health benefits, especially to the cardiovascular system, as largely shown for other foods.

2. Materials and methods

2.1. Plant material

Experience was placed in a polyethylene tunnel of the Faculty of Horticulture and Agriculture (University of Craiova), Romania. Biological material was represented by Charentais variety. The experience had four variants and was placed in randomized blocks in three rehearsals. Variant specifics were as follows: V_1 (Control) - unfertilized; V_2- fertilized with Lignohumat 100 g/ha; V_3- fertilized with Lignohumat 150 g/ha; V_4- fertilized with Lignohumat 200 g/ha. Culture was set up by planting on 03.05.2016 according to the following technological scheme: 50 + 110 (3) + 50 x 30 cm (3.2 pl/m^2).

Lignohumat organic fertilizer was applied foliarly at 10 days interval. Lignohumat is a highly effective humic product with chelating microelements with growth stimulating effect and plant protection against stress. Prior to the establishment of the crop, agrofond was given 30 t/ha of composted grape marc. Culture technology was that specific to yellow melons grown in polyethylene tunnels.

2.1.1.Samples preparation

A quantity of 100 g of each sample was broken up with a blender. 1 gram of homogenized sample (pulp) was mixed with 10 ml 80% methanol, intensely agitated for 10 minutes using a vortex. Extraction of phenolic compounds was carried out by keeping the mixture in an ultrasonic bath for 70 minutes. The mixture was filtered, and to the solid residue was added 5 ml of 80% methanol and the extraction procedure repeated. The two obtained extracts were combined and analyzed

using protocols specific work to determine the total polyphenol and antioxidant capacity.

2.2.Soluble dry matter (S.D.M) content was determined gravimetrically and the result was espressed in percentages.

2.3.**The soluble solids** content (SSC) **%** was determined using a digital refractometer (Kruss Optronic DR 301-95) from the juice pressed from the fruit the result was espressed in percentages.

2.4.The titratable acid content (acidity) was determined by titration with 0.1N sodium hydroxide (NaOH) and expressed as % citric acid.

2.5.**Carbohydrates** (**reducing sugars)** (%) were extracted in distilled water (1:50 w/V) and assayed colorimetric with 3.5 dinitrosalicylic acid using glucose as standard. Ascorbic acid was extracted in 3% metaphosphoric acid (1:50 w/V) and determined by using redox titration with 2,6-dichloroindophenol.

2.6.The determination of total carotenoids

The weighed samples, having been put separately in 95% in acetone (50 ml for each gram), were homogenized with Braun MR 404 Plus for one minute. The homogenate was filtered and was centrifuged using the Hettich Universal 320/320R centrifuge at 2500 rpm for ten minutes. The supernatant was separated and the absorbances were read at 400-700 nm on Cary 50 spectrophotometer. It was recorded that Chlorophyll a showed the maximum absorbance at 662 nm, chlorophyll b at 646 nm and total caroten at 470 nm. The value of these pigments was calculated using the method described by Dinu *et al*., (2013).

2.7.Total phenolic content

The amount of total phenolic compounds in the leaf and petiol of sweet potato extract was determined colorimetrically with Folin-Ciocalteu reagent by using the method described by Singleton and Rossi (1965) with some modifications. To 1 ml extracts (diluted 1:10 with ultrapure water), 1 ml bidistilled water (blank), 1 ml of each standard solution were introduced in laboratory flasks of 25 ml and added every 5 ml reactive Folin-Ciocalteu (diluted 1:10 with ultrapure water). After 2 min, 4 ml of sodium carbonate solution 7.5% was added and they were kept in the incubator during 2 h at the room's temperature.

The absorbance was measured at 765 nm by using a model evolution 600, double beam scanning UV-visible spectro-photometer, PC control with VISION pro software. A standard curve was prepared by using 50, 100, 150, 200 and 250 mg/L solutions of gallic acid in methanol and water (60:40, v/v). Gallic acid was used as the reference standard and the results (total phenolic content) were expressed as gallic acid equivalents (GAE) and the results were expressed as mg/g F.W.

2.8.Antioxidant activity

Extracts for the determination of antioxidant activity were prepared into 80% aqueous methanol (1:10 w/v) at 24°C for 16 h. The resulting slurries were centrifuged at 4000g for 5 min and the supernatants were collected. The total phenolics content was determined colorimetric by using the Folin-Ciocalteu reagent (Dinu *et al.,* 2016) and expressed as mg of gallic acid equivalents (GAE)/100g f.w. The capacity of extracts to reduce the radical 2,2-diphenyl-1-picrylhydrazyl was assessed colorimetric. The Trolox calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The results were expressed as μM Trolox equivalents (TE)/100g.

The ABTS radical cation scavenging activity of the methanolic extract was assessed colorimetric. The final results were expressed as μM Trolox equivalents (TE)/100g fw.

2.9.Statistical analisis.

The significance of differences between variants was statistically determined using variance analysis using ANOVA and

calculating the limit differences, LSD≤ 0.05%, (LSD = least significant difference). The correlation coefficients between quality parameters were performed by multiple correlation coefficientes.

3.Results and discussions

3.1. Production Determinations

Production determinations in the experimental variants concerned the average number of fruits/plants, average weight/fruit, production/plant and production/m^2.

Concerning the average fruit/plant number (Table 1), it varied from 5.4 to V_1 to 7.2 at V_3, the variant with significant results. Regarding the average weight/fruit this was higher in the fertilized variants compared to unfertilized variant, which shows that the fertilization dose directly influences production. Significant differences between variants are observed in production obtained per plant (4.7 kg per V_3 versus 2.7 kg at V_1) and production per m^2 - 15 kg/m^2 at V_3 versus 8.6 kg/m^2 at V_1 unfertilized. Results are similar to those obtained by Mohamed and Mohamed (2016) in an experiment with fertilizer variants in melon culture, but also those reported by Adam *et al.*, (2009) and Naidu *et al.*, (2014).

3.2. Biochemical determinations

Main biochemical components of melon fruit are shown in Table 2. High content of soluble solids (SSC) corresponds to a good accumulation in sugars, which is very important from a sensory point of view. It was different at each variant. Highest content, of 8.55% was recorded at V_3, followed by V_2 and V_4 and the lowest in V_1, non-fertilized variant. Dry substance (%) values recorded in this study confirm results of Ahmad and Al-Fraihat (2011). Total soluble dry matter had the highest value (12.12%) in the fertilized variant with 150 g/ha Lignohumat and the lowest in the unfertilized version (6.80%). V_2 and V_4 had values close to each other but greater than V_1 and much lower than V_3 (Table 2). The total dry substance values (%) obtained in the study at the University of Craiova, Romania, are superior to those obtained by Mohamed and Mohamed (2016) in a study with several variants of fertilization of melons, one of which was compost + humic acids + microorganisms.

Table 1. Elements of production at studied variants

Variant	Average fruit/plant (number)	Average weight / fruit (g)	Production/plant (kg)	Production./m^2 (kg)
V_1- control	5.4^{b}	471^{b}	2.7^{c}	8.6^{c}
V_2	6.0^{ab}	552^{ab}	3.5^{bc}	11.2^{b}
V_3	7.2^{a}	643^{ab}	4.7^{a}	15.0^{a}
V_4	6.4^{ab}	680^{a}	4.5^{ab}	14.4^{a}
LSD≤0.05	1.2	180.4	1.1	1.8

The different letters in the same row indicate significant differences P≤0.05 between the variants

Table 2. Biochemical determinations of melon fruits

Variant	Soluble solids (%)	Soluble dry matter (%)	Acidity (citric acid %)	Carbohydrates (%)	Vitamin C (mg/100g f.m.)
V_1- control	5.80^{c}	6.80^{d}	0.085^{d}	2.75^{b}	16.88^{c}
V_2	7.25^{b}	8.29^{c}	0.105^{c}	2.66^{b}	20.10^{b}
V_3	8.55^{a}	12.12^{a}	0.147^{a}	3.40^{a}	21.12^{a}
V_4	7.05^{b}	8.91^{b}	0.133^{b}	3.01^{a}	19.40^{b}
LSD≤0.05	0.944	0.514	0.008	0.451	0.887

The different letters in the same row indicate significant differences P≤0.05 between the variants

Acidity recorded low levels ranging from 0.085 to 0.147% citric acid in melon fruit on experimental variants. The highest content was recorded at variant fertilized with 150 g/ha Lignohumat (0.147%), still small quantities compared to other fruit or vegetable species. These values are also supported by Kolayli *et al.,* (2010) and Obando-Ulloa *et al.,* (2009). Carbohydrates, an important parameter for determining fruit quality, ranged from 2.66% to V_2 to 3.40% at V_3 with statistically significant differences for V_3 and V_4 variants that were fertilized with the highest doses of Lignohumat. Lingle and Dunlap (1987) stated that sugary melons are influenced by environmental factors. Thus, climatic factors, fertilization regime and nature of fertilizer influence the content in sugars, and implicitly the quality of the fruit. Vitamin C is a very important natural antioxidant. Its biological importance is based on the ability to participate in various enzymatic processes, hydroxylation, oxidation-reduction. Vegetable vitamin C content may vary depending on environmental factors and stress, such as: light intensity, temperature, humidity, pollution (Dinu *et al.,* 2016). The values in Table 2 for vitamin C content ranged between 16.88 mg/100g f.m. in unfertilized variant and 21.12 mg/100g f.m. in the fertilized variant with 150 g/ha of Lignohumat. The determined values are similar to those in literature. Thus, Laster and Hodges, (2008), in a study of three melon varieties, determined an ascorbic acid content of 34.7-44.7 mg/100 g. Obando-Ulloa *et al.,* (2009) reported an ascorbic acid content, determined at 29 melon isogenic lines from 6.0 to 19.7 mg/100g. Highest values of ascorbic acid were determined for melon varieties followed by grafted melons and the lowest values for hybrids. Laster and Hodges, (2008) stated that culture technology directly influences the accumulation of ascorbic acid in melon varieties.

Total polyphenols represent the largest class of secondary metabolites in plants. Phenolic content and composition of fruits and vegetables depend on genetics, environmental conditions, and storage conditions after harvest. Table 3 presents the values of total melanin fruit polyphenols for the experimental variants. It is noted that the highest content was recorded in variant 3 of 94.10 mg GAE/100 g f.m followed by V_4 with 40.65 mg GAE/100 g f.m. Variance analysis highlighted variant 3. The results of our study are superior to those obtained by Preciado-Rangel *et al*., (2015) in a melon culture treated with different types of organic fertilizers. They claim that low nitrogen, magnesium and phosphorus content in organic fertilizers could cause nutritional stress in melon plants during growth, thus favoring the increase in phenolic compounds.

Carotenoids are one of the main lipophilic constituents, which contribute to total antioxidant activity and to content of provitamin in potato but also in other vegetals (Lachman *et al.,* 2016). Variance analysis for total carotene content showed a significant difference ($P<0.05$) between studied variants. Results show a variation from 24.96 mg/100 g f.m to V_1 to 36.32 mg/100 g f.m to V_3. Highest value was at V_3, fertilized with 150 g/ha of Lignohumat, followed by V_4 and V_2. High values and differences between variants may be the result of different fertilizer doses and orange pulp color (Charentais variety) and are also supported by Henan *et al*., (2013).

Results of this study are similar to those obtained by Woblang *et al.,* (2010), which studied the influence of pre and post-harvest factors on β-carotene content and antioxidant capacity of melon. In the present study, values greater than 31 mg/100 g f.m are the differentiated result from fertilization dose. Menon and Ramana Rao, (2012) reported values of carotene content close to those of this study, their research being focused on melon plants nutrition at various stages of ripening.

Evaluation of antioxidant activity is a parameter for assessing nutritional quality. DPPH radicals are generally used as a screening method for evaluating a wide variety of compounds. Antioxidant activity of the

studied variants was determined by the ability of the extracts to reduce the ABTS radical cation. It had values between 103.30 μMTE/100g f.m to V_2 and 175.92 μMTE/100g f.m to V_3. DPPH method varied between 104.0 μMTE/100g f.m at unfertilized variant and 228.16 μMTE/100g f.m at variant fertilized with 150 g/ha of Lignohumat. From the results it can be said that the fertilizer dose of 150 g/ha has significantly influenced antioxidant activity of melons. Other doses did not influence antioxidant activity compared to non-fertilized variant.

Table 3. Biochemical determinations of melon fruit

Varianta	Total phenolics content* (mg GAE/100g f.m.)	Total carotene (mg/100 g f.m.)	Antioxidant activity	
			ABTS (μMTE**/100g f.m.)	DPPH (μMTE**/100g f.m.)
V1-Control	34.76^{c}	24.96^{c}	123.00^{b}	104.3^{b}
V2	36.90^{c}	31.28^{b}	103.32^{b}	112.0^{b}
V3	94.10^{a}	36,42^{a}	175.92^{a}	228.1^{a}
V4	40.65^{b}	31,52^{b}	134.09^{b}	107.5^{b}
LSD≤0.05	2,44	1,62	36,7	39,42

*gallic acid equivalents; ** equivalents Trolox; The different letters in the same row indicate significant differences P≤0.05 between the variants

Table 4. Correlations between the biochemical compounds analyzed in the melon variants fertilized with Lignohumat

Specification	S.S.M (^{0}Bx)	S.D.M (%)	Carbo-hydrates (%)	Total phenolics content mg (GAE/100g f.m	Total caroten (mg/100g f.m.)	Antioxidant activity ABTS (μMTE/100g f.m)	Antioxidant activity DPPH (μMTE/100g f.m)
Vitamin C (mg/100g f.m)	0.936***	0.856***	0.598*	0.682*	0.972***	0.471	0.679*
S.S.M (^{0}Bx)	-	0.960***	0.771**	0.853***	0.994***	0.680*	0.847***
S.D.M (%)	-	-	0.915***	0.946***	0.950***	0.857***	0.930***
Carbohydrates (%)	-	-	-	0.922***	0.767**	0.982***	0.885***
Total phenolics content (mg GAE/100g f.m)	-	-	-	-	0.812**	0.924***	0.996***
Total carotene (mg/100 g f.m.)	-	-	-	-	-	0,660*	0,799**

*r – P 5% = 0.58; **r – P 1% = 0.71; ***r – P 0,1% = 0.82

Variation of antioxidant potential depends on plant maturity stage or analyzed plant parts, genotype, culture method, environmental conditions (Soare *et al.,* 2016) but also of the fertilizers used, therefore, Preciado-Rangel *et al.,* (2015). recommend fertilization with organic nutrients applied foliar to melon culture to improve the nutritional qualities of production.

Antioxidant activity begins to increase with the onset of fruit maturation, reaches a peak in the ripening stage, after which it decreases at overmaturation (data not shown). Although all four variants of melon were grown under same

environmental conditions, study results show that 150 g/ha Lignohumat dose significantly influenced the production elements as well as biochemical characteristics of melon fruit, thus there were significant differences between unfertilized variant and V_3. The correlations between certain quality parameters of melon fruit obtained in the fertilized experience with different doses of Lignohumat are presented in the table 4. For the majority of the parameters, significant positive correlations were identified. Literature confirms that when fruits and vegetables with high carotene content are consumed, there is a strong antioxidant activity in the body because there is a strong correlation between these two elements.

Corrigan *et al.*, 2000 support the view that pumpkin fruits with a high sugar content and high total carotenoids are considered to be good quality fruits and can be eaten raw.

4. Conclusions

Our results have shown that the use of the Lignohumat natural fertilizer in three different doses at melon culture in polyethylene tunnel has led to the production of qualitative and quantitative yields superior to the unfertilized control. The average number of fruit/plant (7.2), average fruit weight (643 g) and average yield/m^2 (15 kg/m^2) were recorded at V_3 (150 g/ha). Also in this variant, the organoleptic properties of melon fruit recorded the highest values compared to the non fertilized control but also with the other two variants fertilized with different doses.

Significant positive correlations were registered between all parameters studied regardless of the fertilization dose. These results may recommend the use of Lignohumat 150 g/ha for the fertilization of some vegetable crops in the polyethylene tunnel, knowing that in the vegetable sector, due to the superintensive character, high doses of fertilizers can be used which can cause soil and groundwater pollution. Organic fertilization with Lignohumat is a viable alternative to a source of organic nutrients in order to obtain melon productions grown in the polyethylene tunnel with superior nutraceutical qualities.

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RESEARCH OF TECHNOLOGICAL PARAMETERS AND CRITERIA FOR EVALUATING DISTILLATE PRODUCTION FROM DRIED JERUSALEM ARTICHOKE

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ABSTRACT

In this paper, it is proposed to use dried Jerusalem artichoke as a raw material for distillate production. The purpose of the research was to develop a distillate technology from dried Jerusalem artichoke and to determine the criteria for assessing its quality. The work revealed patterns of changes in the concentrations of the main distillate volatile components, depending on the strength of the fermented wort and its composition. It was established that the increase in the strength of fermented wort by 2.0-2.5 %, leads to an increase in the yield of distillate on average by 3.0 % and enriching it with valuable volatile components. A new technical solution was proposed, based on the regulation of the strength of fermented wort from Jerusalem artichoke due to the addition of distillate. A high degree of correlation was established between the tasting evaluation of Jerusalem artichoke distillates and the concentration of 1-propanol, ethyl caproate, ethyl caprylate, the sum of enanthic ethers, the ratio of C_3 and C_4 alcohols, and was found the ratio of the amount of enanthic ethers to ethyl acetate. It is proposed to use the methanol concentration, the content of enanthic ethers, the total content of carbonyl compounds, the ratio of the sum of enanthic ethers to the concentration of ethyl acetate as criteria for assessing the quality of distillates from dried Jerusalem artichoke.

1. Introduction

The unflagging interest in the Jerusalem artichoke use as a raw material in the food industry in the Russian Federation and abroad has been associated with its unique biochemical composition for several decades. The main areas of researches in the field of application of this crop are developments aimed at producing inulin, a polymer of fructose, which is widely used in creating functional foods and dietary supplements (Bekers *et al.*, 2008; Lisovoy *et al.*, 2016; Barkhatova *et al.*, 2015), use of Jerusalem artichoke prepared in various ways as an ingredient of food products (Yakovleva and Arsen'eva, 2012; Shazzo *et al.*, 2013; Baranenko and Borisova, 2014) and pectin production (Toshkov *et al.*, 2015). Known the developments of Russian and foreign experts in obtaining ethyl alcohol by rectification of fermented wort from Jerusalem artichoke (Nakamura *et al.*, 1996; Ponomareva *et al.*, 2009; Pornthap Thanonkeo *et al.*, 2011).

This type of inulin-containing raw material can also be promising in the production of alcoholic drinks based on distillates. The climatic conditions of the Russian Federation make it possible to grow this crop in sufficient quantities for production. Interest in the use of Jerusalem artichoke in the wine industry is also due to its relatively low purchase price compared to fruit raw materials.

It is known, that the tubers of fresh Jerusalem artichoke are difficult to store as raw materials due to the characteristics of the covering tissues (Ilchenko and Patlasov, 2016). In order to increase its storage time, methods have been proposed earlier, involving

the use of a controlled gaseous medium or lower temperatures (Khripko and Kozhukhova, 2003). However, these methods require the use of specialized expensive equipment. Therefore, the organization of year-round production of distillate from fresh Jerusalem artichoke tubers is not economically feasible. In this regard, German specialists use Jerusalem artichoke tubers to produce distillates exclusively as seasonal raw materials (Dürr *et al.*, 2010).

As an alternative to fresh raw materials, was conducted research on the use of dried Jerusalem artichoke. The advantages of dried Jerusalem artichoke are possibility of year-round production, the high stability of its biochemical composition and microbiological purity, the partial depolymerization of the main carbohydrate components of the raw material and, consequently, an increase in their accessibility to enzymatic hydrolysis (Krikunova et al., 2016). The use of dried Jerusalem artichoke allows to simplify the technological process, eliminating the stage of washing and crushing.

Earlier, the main regularities of changes in the carbohydrate complex of dried Jerusalem artichoke in the preparation of the saccharified wort were identified and technological modes of preparation of raw materials for distillation were developed (Oganesyants et al., 2016). Was showed the advantages of using Fermiol alcohol yeast, as compared to the use of wine and brewing yeast, allowing to intensify the fermentation process, to get a wort with a maximum strength and a minimum content of acetaldehyde and methanol (Oganesyants et al., 2017).

The purpose of this work was to identify patterns of changes in the main volatile component concentrations of distillate from Jerusalem artichoke, depending on the strength of the fermented wort and its composition, to develop a new technological method, aimed at increasing the yield of distillate and improving its organoleptic characteristics.

2.Materials and methods

2.1. Materials

Dried Jerusalem artichoke from the tubers of the Skorospelka variety was obtained from Topinambur LLC (Tver Region, Russian Federation). The method of dried Jerusalem artichoke production involves washing, sorting, inspection, calibration, cleaning, additional cleaning, cutting, blanching, drying and subsequent grinding (Golubev and Volkova, 1995). The biochemical composition of dried Jerusalem artichoke we studied previously (Krikunova *et al.*, 2016).

Samples of fermented wort from dried Jerusalem artichoke, obtained by two-and one-stage methods (control); wort samples prepared for distillation (experimental); distillate fractions, selected by strength and organoleptic characteristics; samples of distillate were used in this study.

2.1.1. Obtaining a fermented wort

The control sample C 1 of fermented wort from dried Jerusalem artichoke was obtained according to the previously developed two-stage method, namely:

- *at the saccharified wort obtaining stage:* hydronic module 1÷4.5; enzymatic hydrolysis of raw materials polymers due to its own Jerusalem artichoke enzymes and microbial inulinases (3.0 units/g of inulin raw material) and proteases (0.01 units/g of protein raw material); the duration of hydrolysis is 3 hours at 50 °C.
- *at the saccharified wort fermentation stage:* the use of dry Fermiol alcoholic yeast with an application rate of 100 mg/100 g of wort; fermentation at a temperature of 28-30 °C for 48 hours.

A control sample of C 2 fermented wort from dried Jerusalem artichoke was obtained by a one-step method, including mixing the raw material with water at a water ratio of 1÷4.5, acidifying the wort with H_2SO_4 solution to pH 4.5; the use of microbial inulinase with a dosage of 4.0 units/g of inulin raw materials; the introduction of a fermentation activator; the use of Fermiol dry alcoholic yeast with an application rate of 100 mg/100 g of wort; fermentation at a temperature of 28-30 °C for 72 hours. At the same time, Vitamon Kombi (Erbsloeh, Germany), which is a mixture of pure ammonium phosphate and thiamine (vitamin B_1), was used as a fermentation activator.

2.1.2. Experimental samples preparation

The experimental samples of wort were prepared by introducing into the control samples a certain volume of distillate, obtained as a result of fractionated distillation of control samples previous batches. The amount of introduced distillate was determined on the basis of increasing the strength of the fermented wort by 1.0-4.0 % by volume. The increase in the strength of samples S 1.1 and S 2.1 was 0.9–1.1% vol.; samples S 1.2 and S 2.2 - 1.9-2.1% vol.; samples S 1.3 and S 2.3 - 2.9-3.1% vol.; samples S 1.4 and S 2.4 - 3.9-4.1% vol.

2.1.3. Obtaining distillates

Control and experimental samples of fermented wort were subjected to single fractionated distillation in a direct distillation unit with a strengthening column and a reflux condenser (Kothe Destillationstechnik, Germany). The temperature of the heating vapor was maintained from 102 °C (at the beginning of the distillation) to 105 °C (at the end of the distillation). The heating vapor pressure in the distillation process was 0.2-0.5 mPa. The selection of the head, average (heart) and tail fractions of the distillate was carried out according to organoleptic characteristics and strength.

2.2. Methods

The qualitative and quantitative composition of volatile components in the objects of research was determined by gas chromatography using a Thermo Trace GC Ultra gas chromatograph (Thermo, USA) with a flame ionization detector (detection limit not more than 3•10-12 g/s). Chromatography column HP FFAP: length 50 m, internal diameter 0.32 mm with a membrane thickness of the stationary phase of 0.5 μm (State Standart, 2016). In order to conduct a comparative analysis of the samples studied, the concentration of volatile components was expressed in mg/dm^3 of absolut alcohol (mg/dm^3 of a.a.). For processing the research results, a statistical method for processing experimental data was used, during which the average values of measured values from 3-5 replications, the standard deviation and the confidence interval were determined (Borovikov, 2003; Grachev and Plaksin, 2005). Data in tables and figures are presented as averages of 3-5 dimensions. Mathematical planning and processing of experimental data was carried out using the methods of mathematical statistics using Excell 2007. The results of the correlation analysis were evaluated using tabular data on the critical values of the Pearson's code. For p=0.05 with the number of degrees of freedom 18, r=0.44.

3. Results and discussions

At the first stage of researches, was made a comparative assessment of volatile components composition and concentration in the control samples of fermented wort from dried Jerusalem artichoke, depending on the method of preparing raw materials for distillation (Table 1).

Table 1. The Main Control Samples Volatile Components Content of Fermented Wort from Dried Jerusalem Artichoke

Volatile Components Content, mg/dm^3 of a.a.	**C 1 (7,31 % vol.)**	**C 2 (7,58 % vol.)**
Acetaldehyde	685	565
Ethyl acetate	114	126
Methanol	1181	971
Higher alcohols, including:	2254	2191
- 1-propanol	588	577
- isobutanol	536	497
- isoamylol	1130	1117
Enanthic ether	28	22
Phenylethyl alcohol	224	214
The sum of the components*	**4532**	**4146**

* In this table and in the subsequent when calculating the amount of volatile components, all identified impurities were taken into account, some of them are not included in the illustrative material.

It was established that the method of obtaining fermented wort from dried Jerusalem artichoke affected on the total content of volatile components and the concentration of individual substances. In sample C 2, there was a decrease in acetaldehyde and methanol compared to sample C 1 by 17.5 and 17.8%, respectively. The concentration of higher alcohols did not depend on the method of producing wort.

When comparing the data, presented in Table 1, and in a previously published paper (Krikunova *et al.*, 2017), significant differences were found in the control samples of fermented wort from dried Jerusalem artichoke and samples of fermented wort from fresh tubers. Concentrations of higher alcohols and methanol in fermented wort from fresh raw materials, compared with the wort from dried Jerusalem artichoke, exceeded their values by 1.4–1.9 and 4–5 times, respectively. Therefore, it can be concluded that in terms of methanol content, an indicator characterizing the safety of alcoholic drinks, dried Jerusalem artichoke has significant advantages over fresh raw materials. At the same time, the marked lower content of higher alcohols, components that form the basis of the aroma of distillate-based alcoholic drinks, could adversely affect on the intensity of the aromatic characteristics of the finished product, which should be considered when developing a new distillate technology from dried Jerusalem artichoke.

In order to increase the mass concentration of aroma-forming components and the possibility of changing their volatility in the distillation process, wort prototypes (experimental samples) were obtained. They were prepared from control wort samples by adding distillate as described above.

The conditions for the transition of volatile components to distillate depend on many factors, including their solubility in ethyl alcohol and aqueous-alcoholic solutions of various concentrations, on their mutual miscibility and the type of distillate plant (Prado-Ramirez *et al.*, 2005). Differences in the behavior of volatile components affect the organoleptic characteristics of individual fractions, selected during the distillation process and their yield (Claus and Berglund, 2005). On this basis, the addition of distillate to the fermented wort from dried Jerusalem artichoke could be a significant factor influencing the processes occurring during distillation. The content of the main volatile components of the experimental samples of wort is presented in Table 2.

Table 2. Volatile Composition of Experimental Wort Sampels

Volatile Components Content, mg/dm^3 of a.a.	**S 1.1**	**S 1.2**	**S 1.3**	**S 1.4**	**S 2.1**	**S 2.2**	**S 2.3**	**S 2.4**
Acetaldehyde	706	725	747	765	583	598	614	630
Ethyl acetate	122	129	137	144	134	141	148	156
Methanol	1346	1508	1668	1825	1118	1262	1404	1544
Higher alcohols, including:	2583	2906	3224	3536	2478	2752	3023	3291
- 1-propanol	650	711	771	830	639	698	757	814
- isobutanol	620	702	783	862	568	636	701	768
- isoamylol	1313	1493	1670	1844	1271	1418	1565	1709
Enanthic ether	34	40	46	51	29	35	42	48
Phenylethyl alcohol	223	223	222	222	214	213	213	212
The sum of the components	**5065**	**5589**	**6105**	**6612**	**4652**	**5073**	**5525**	**6171**

As can be seen from the obtained data, the increase in the strength of the fermented wort due to the introduction of a certain volume of distillate led to a change, as compared with the control, of both the total concentration and the content of individual volatile components. The total concentration of volatile components in the test samples increased on average by 10-30 %. At the same time, the mass concentration of acetaldehyde, a component that gives rigidity to taste and aroma of distillates, increased, depending on the amount of distillate

introduced, by 3.0 - 12.0 %. However, its relative content in the amount of volatile components decreased by 1.0-4.0%. The absolute content of higher alcohols in the test samples of the wort increased by 13-57 % compared with the control ones. The concentration of the components of enanthic ether, represented by ethyl caprate, ethyl caprylate, ethyl caproate, increased in the wort samples by 30-120 %. It is believed, that these volatile components give specific floral shades to the distillates aroma and harmonize the organoleptic characteristics of alcoholic drinks (Li *et al.*, 2012; Kostik and Memeti, 2013).

It was estimated the effectiveness of a new method of preparing of dried Jerusalem artichoke to distillation on the basis of experimental data by the yield of distillate (average fraction or heart) calculated on anhydrous alcohol when processing 10 kg of prepared wort. The initial data for the calculation of the fractions yield presented in Table 3. While the strength of fermented wort increased from 7.31% to 11.15% (sample 1) and from 7.58 % to 11.48 % (sample 2) revealed a clear tendency to increase the volume of all fractions. The head ranged from 6 to 50 %, heart - from 13 to 34 %, tail - from 5 to 30 %. At the same time, no regularities were revealed for changing the strength of individual fractions. The strength of heart (final distillate) averaged 84-85% vol., that is, it practically did not depend on the strength of the distilled wort.

Table 3. Baseline Data for Calculating the Yield of Distillate Fractions Depending on the Strength of the Fermented Wort

Indicator Name	**C 1**	**S 1.1**	**S 1.2**	**S 1.3**	**S 1.4**	**S 2**	**S 2.1**	**S 2.2**	**S 2.3**	**S 2.4**
Volume of a.a. from 10 kg of fermented wort. cm^3	730	830	925	1020	1115	760	860	955	1050	1150
Fraction volume. cm^3:										
- head	75	80	90	110	145	70	75	75	100	135
- heart	655	750	850	925	990	690	800	900	970	1030
- tail	850	900	950	1000	1210	900	950	950	1115	1200
Alcohol volume proportion in the fraction. %:										
- head	84.7	85.0	85.1	85.0	84.8	84.1	84.4	84.7	84.0	84.2
- heart	84.2	84.5	84.7	84.5	84.2	83.9	84.0	84.1	83.7	84.0
- tail	10.7	11.4	10.5	11.2	10.0	11.4	10.4	11.7	10.9	11.6
Losses of a.a.. %	3.3	3.0	3.1	3.2	3.3	2.6	2.9	2.4	3.1	2.8

The data, presented in Table 3 allowed us to calculate the yield of fractions by absolut alcohol (Figures 1, 2).

It was established that with an increase in the strength of the wort by 1-2%, the share of the head fraction decreased by 0.3-1.0%, and a further increase in the strength of the wort (by 3-4%) led to an increase in the yield of the head fraction by 0.3-2.3%, depending on the method of Jerusalem artichoke preparation for distillation. The yield of the tail fraction on the contrary was characterized by a tendency to decrease.

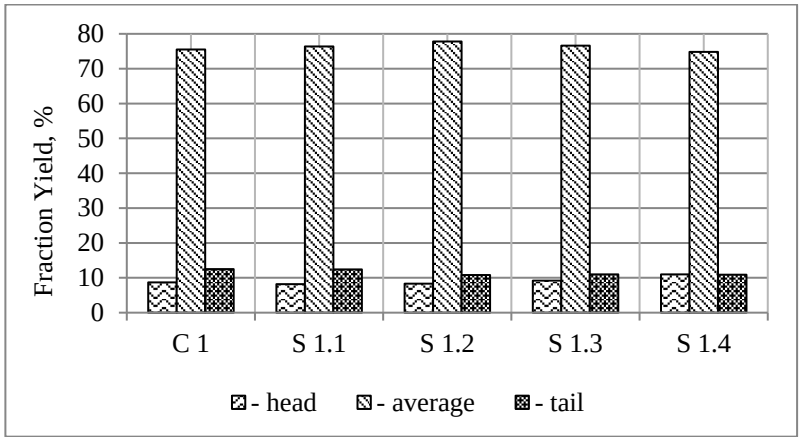

Figure 1. Fractions Yield by Absolut Alcohol during the Wort Samples Distillation, Obtained by the Two-Stage Method (Sample 1)

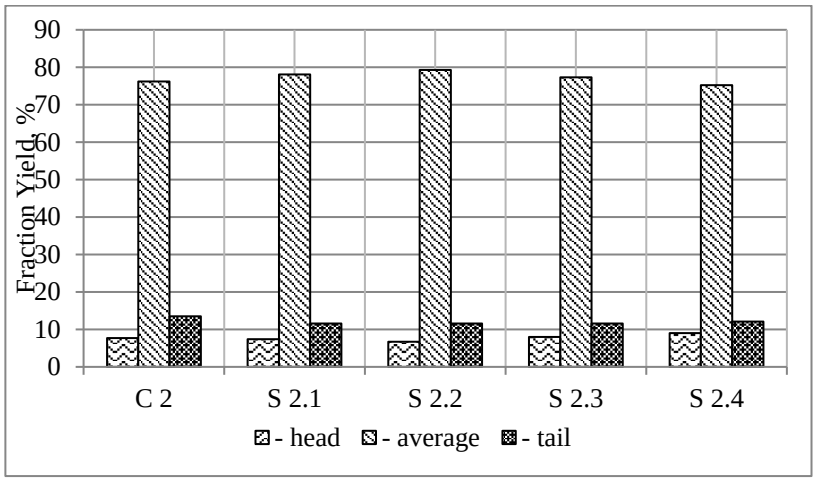

Figure 2. Fractions Yield by Absolut Alcohol during the Wort Samples Distillation, Obtained by the Single-Stage Method (Sample 2)

The data presented in Figures 1 and 2 showed that to achieve the maximum yield of the average fraction (distillate from dried Jerusalem artichoke), increasing the strength of the original wort by adding additional distillate volume, obtained from fractional distillation of previous batches of control samples should be 2-2.5 %. For example, the yield of distillate in test samples S 1.2 and S 2.2 was maximum compared with the control and increased by 2.8 and 3.1%, respectively.

Distillate-based alcoholic drinks are a special group of elite expensive products. In its production of these spirits, it is necessary to take into account not only the economic aspects, determined by the output of products from a unit of raw materials, but first and foremost consumer properties. In this case, the concept of consumer properties includes safety indicators and organoleptic characteristics of the product. The organoleptic characteristics of distilled drispirits, produced without exposure in contact with the wood of oak or other wood species are determined by the composition of the fragrance forming volatile components.

The study of content of volatile components in experimental samples of distillates have shown that the qualitative composition and quantitative content of volatile components in the distillate prototypes from dried Jerusalem artichoke differed significantly in dependence on the level of increase in the initial strength of the wort. These changes were reflected in the results of organoleptic analysis. As a rule, when distillates and distilled drinks sensory evaluation, tasters use a system of descriptive descriptors and an intensity scale. The choice of descriptors system is a fundamental element

in preparation for an organoleptic analysis and includes a descriptive characteristic of appearance (color, transparency, the presence of sediment), flavor, its shades and taste (Brochet, Dobourdieu, 2001).

To characterize a new type of product (distillates from dried Jerusalem artichoke), we used certain descriptors for flavor and taste evaluation.

The following descriptors were used to evaluate the flavor: intensity: bright, strong, moderate, weak; character: peculiar to initial raw materials, fruit, flower and honey, including undesirable - fusel, alcohol, musty, sharp; shades: enanthic, grassy, including undesirable - salty, boiled down, medicinal, oxidized.

The following descriptors were used to evaluate the taste: character: soft, refined, oily, including undesirable - sugary, pungent, sharp, rough; harmony: harmonious, inharmonious, disorganized; typicality: typical, atypical, with a foreign tint.

Color and transparency were estimated in the range of 1-2 points. The aroma and taste were estimated in the range of 1-3 points.

According to the results of the organoleptic analysis of distillates from dried Jerusalem artichoke, all samples (control and experimental) were colorless and transparent and received the highest rating for these.

indicators. The main differences between the samples were identified by the nature and intensity of aroma and taste (Table 4).

Table 4. Organoleptic Analysis of Dried Jerusalem Artichoke Distillates Samples

Sample	Colour (max 2 pts)	Clearness (max 2 pts)	Odor (max 3 pts)	Taste (max 3 pts)	Total (max 10 pts)
C1	2	2	2.6	2.5	9.1
S 1.1	2	2	2.7	2.6	9.3
S 1.2	2	2	2.9	2.8	9.7
S 1.3	2	2	2.9	2.8	9.7
S 1.4	2	2	2.7	2.7	9.4
C 2	2	2	2.7	2.5	9.2
S 2.1	2	2	2.9	2.7	9.6
S 2.2	2	2	3.0	2.8	9.8
S 2.3	2	2	3.0	2.8	9.8
S 2.4	2	2	2.8	2.8	9.6

The results processing of the organoleptic analysis using selected descriptors was carried out graphically, which is widely used in the statistical processing of the organoleptic evaluation of various types of food products. Figures 3 and 4 show the aromatic and taste profiles of distillates from dried Jerusalem artichoke (sample 1 - preparation of the wort in a two-stage process).In the study of the distillate aromatic profiles revealed significant differences between the samples according to the nature and intensity of the aroma.

Unlike the control sample (C 1), which had a weak, unexpressed flavor, the best prototypes (S 1.2 and S 1.3) were characterized by a pronounced aroma of raw materials with bright floral-honey and fruit shades. It was noted that the sample (S 1.4) with the maximum addition of distillate into the wort, was distinguished by a sharp aroma with a strong fuss shade.Analysis of the taste profiles of distillates from dried Jerusalem artichoke made it possible to establish that the control sample (C 1) had an inexpressive taste. The introduction of a certain volume of distillate into the wort (an increase in strength by an average of 2.0% vol.) resulted a product characterized by a pronounced, soft, harmonious, typical taste with oily tints. A further increase in the volume of distillate, introduced into the wort led to the appearance of coarse and sharp tones in the taste of the distillate (S 1.4).

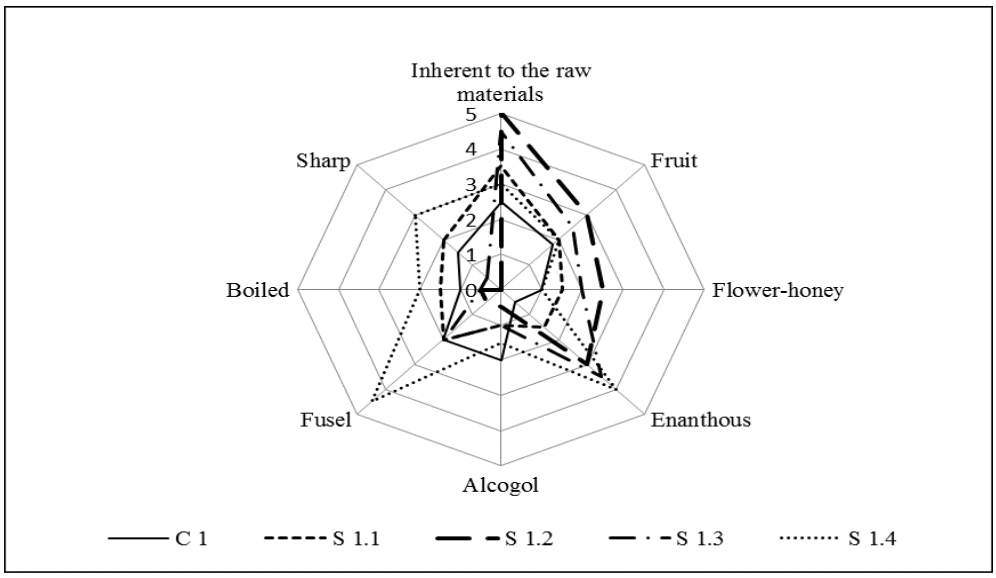

Figure 3. Distillate Aromatic Profiles from Dried Jerusalem Artichoke

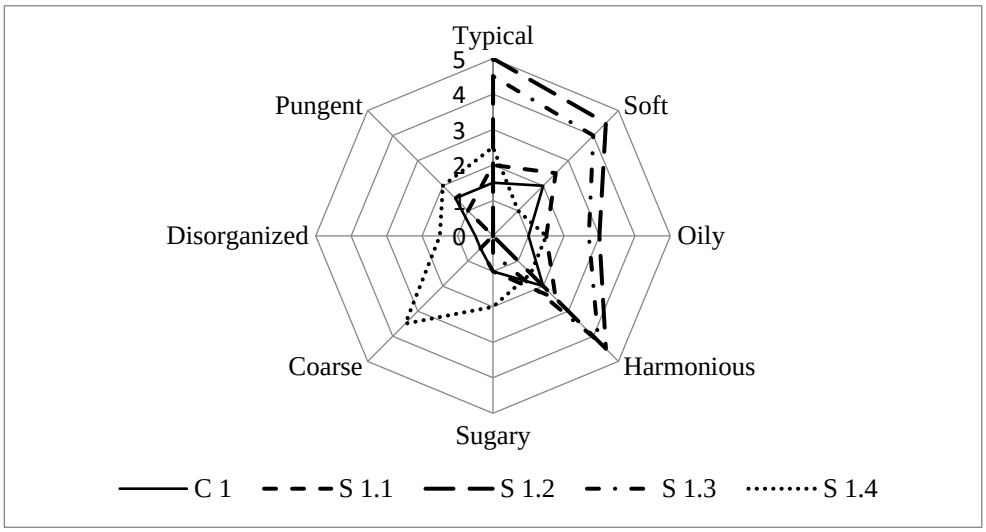

Figure 4. Distillate Taste Profiles from Dried Jerusalem Artichoke

For a deeper understanding of the volatile component influence degree on the taste and aromatic characteristics of distillates, we calculated the pair correlation coefficients between the concentration of individual volatile components, as well as the ratios of individual groups of components, and the tasting assessment (Table 5).

According to the data obtained, the composition of the volatile components of all distillate samples from dried Jerusalem artichoke was dominated by higher alcohols, which accounted for 62-69% of the total amount of volatile components. From aliphatic higher alcohols in the control and experiment samples of distillates 1-propanol, 2-propanol, isobutanol, 1-butanol, isoamylol, hexanol were identified. Despite the high content of this group of volatile components, no significant correlation was found between their total concentration and tasting assessment. Isobutanol and isoamylol, which are the main components of fusel oils, were contained in the studied samples in concentrations from 598 to 1100 mg/dm^3 a.a. and from 1300 to 2459 mg/dm^3 a.a, respectively, which significantly exceeded their threshold concentrations in aroma perception (Arrieta-Garay *et al*., 2014). However, it was found that the coefficients of

pair correlation of these components with the organoleptic evaluation of distillates had low values (0.115 and 0.087), respectively.

The detected concentrations of hexanol turned out to be significantly lower than the threshold ones, while the pair-correlation coefficient for this substance had a numerically significant negative value, well above the critical value. The concentration of 1-propanol with a pleasant, oily-floral aroma ranged from 532 to 821 mg/dm^3 a.a. The threshold perception of 1-propanol is at the level of 100-500 mg/dm^3. The high value of 1-propanol was llowed us to make a conclusion about its positive effect on the formation and character of the aroma of distillates from dried Jerusalem artichoke. Also, the data, presented in Table 5 showed that the ratio of C_3 and C_4 alcohols is significant for evaluation of character of flavor and taste.

Phenylethyl alcohol was found in insignificant amounts. Given that the threshold perception of it is in the range from 10 mg/dm^3 to 80 mg/dm^3, and the value of the r_{xy} for it was 0.011, it can be argued that this component does not affect on the flavor of the distillate from dried Jerusalem artichoke.

Table 5. The Relationship of the Volatile Components Qualitative and Quantitative Composition and the Tasting Evaluation of Jerusalem artichoke Distillates

Indicator	Volatile Components, mg/dm^3 of a.a.										r_{xy}
	C 1	S 1.1	S 1.2	S 1.3	S 1.4	C 2	S 2.1	S 2.2	S 2.3	S 2.4	
Ethanol , % v/v	84.2	84.5	84.7	84.5	84.2	83.9	84.0	84.1	83.7	84.0	
Methanol	1407	1496	1536	1587	1970	1050	1241	1302	1421	1802	-0.074
Acetaldehyde	214	202	186	187	204	143	130	120	118	115	-0.584
Isobutyraldehyde	2	2	1	1	2	1	0	0	1	1	-0.670
Acetone	7	8	5	5	6	6	5	4	4	6	-0.872
2-propanol	5	4	2	3	4	4	4	2	2	3	-0.941
1-propanol	544	659	796	821	705	532	609	725	751	740	0.728
Isobutanol	706	826	853	904	1100	598	710	765	832	904	0.115
1-butanol	24	19	13	15	25	23	18	10	13	19	-0.935
Isoamilol	1552	1800	1939	2058	2459	1300	1461	1611	1800	1987	0.087
Hexanol	16	14	8	10	13	14	10	5	7	10	-0.967
Phenylethyl alcohol	8	8	7	7	7	9	8	9	8	8	0.011
Isoamylacetate	5	6	8	10	9	7	9	11	12	7	0.862
Ethyl acetate	68	64	64	75	98	70	70	70	89	109	0.059
Ethylcaproate	13	14	15	15	12	14	14	15	15	14	0.807
Ethyl lactate	4	3	1	1	2	3	3	2	1	2	-0.801
Ethyl caprylate	11	12	16	15	14	13	14	17	17	15	0.952
Ethylcaprate	24	29	41	48	61	28	41	49	70	81	0.492
Aldehydes and ketones	223	212	192	193	212	150	135	124	123	122	-0.603
Higher alcohols	2847	3322	3611	3811	4306	2471	2812	3118	3405	3663	0.199
Ethers	125	128	145	164	196	135	151	164	204	228	0.401
Enanthic ethers sum	48	55	72	78	87	55	69	81	102	110	0.588
The ratio of alcohols C_5 to the sum of C_3, C_4	1.24	1.21	1.18	1.19	1.36	1.15	1.10	1.08	1.13	1.21	-0.604
The ratio of alcohols C_3 / C_4	0.77	0.80	0.93	0.91	0.64	0.89	0.86	0.95	0,90	0.82	0.717
Σ of enanthic ethers / ethyl acetate	0.71	0.86	1.12	1.04	0.89	0.79	0.99	1.16	1.14	1.00	0.966
Tasting evaluation, score	7.4	7.5	7.7	7.7	7.5	7.5	7.6	7.8	7.8	7.6	

The ethers in the studied samples were represented by ethyl acetate, isoamyl acetate, ethyl caproate, ethyl lactate, ethyl caprylate, ethyl caprate. Ethyl acetate, whose threshold concentration ranged from 50 to 100 mg/dm^3 was found in the largest quantities in studied distillates. It has been established that there is practically no correlation between the concentration of ethyl acetate and the tasting assessment (r=0.059). The mass concentration of enant ethers (ethylcaproate, ethylcaprylate, ethylcaprate) significantly exceeded their threshold concentrations. The values of r_{xy} for ethylcaproate and ethylcaprylate were close to 1.0, which indicates their significant role in formation the distillates aroma. A high positive correlation was noted between the tasting evaluation and the ratio of the sum enanthic ethers and ethyl acetate (r = 0.966).

The concentration of isoamylacetate, which in its pure form has a sharp, fruity smell like pears, in the studied samples ranged from 5 to 12 mg/dm^3 a.a. The correlation coefficient for isoamyl acetate was 0.862, which, taking into account the threshold concentration of this component (0.5-5.0 mg/dm^3), indicates its importance in the perception of the distillates aroma from Jerusalem artichoke.

Of the carbonyl compounds (aldehydes and ketones) in the studied samples, acetaldehyde was the main (in concentrations exceeding the threshold by 1.5–2 times). Isobutyraldehyde was presented in trace concentrations. All carbonyl compounds had high negative correlation coefficients with a tasting score.

The concentration of methanol in distillates from dried Jerusalem artichoke varied from 1050 to 1970 mg/dm^3 a.a. The dependence of the increase in methanol concentration on the level of increase in the strength of fermented wort was revealed. The absolute values of this indicator in samples of distillates from dried Jerusalem artichoke did not exceed the permissible maximum content of methanol in distillates (2 g/dm^3) established in the Russian Federation. According to the results of the correlation analysis, it was concluded that methanol in the indicated concentrations had no effect on the organoleptic characteristics of the distillates obtained (r=-0.074).

In general, the obtained results allowed us to single out a number of individual components and groups of compounds that, to one degree or another, influence the character of the aroma and the taste perception of distillates from dried Jerusalem artichoke. On the basis of the obtained results, we recommended the following criteria for assessing the quality of distillates from dried Jerusalem artichoke: the total concentration of methanol should not exceed 1.6 g/dm^3 a.a., enanthic ethers should be at least 70 mg/dm^3 a.a., the total content of carbonyl compounds should not exceed 200 mg/dm^3 a.a., the ratio of the sum of enanthic ethers to the concentration of ethyl acetate should be at least 1.1.

4. Conclusions

Revealed regularities of changes in the concentrations of the main volatile components of the distillate from dried Jerusalem artichoke depending on the strength of the fermented wort and its composition. It is shown that an increase in the strength of fermented wort is on average 2% vol. leads to an increase in the yield of distillate and its enrichment with valuable volatile components (higher alcohols and enanthic ethers).

A high degree of correlation was established between the tasting evaluation of distillates from dried Jerusalem artichoke and the concentration of individual volatile components, groups of compounds and their ratios: positive for 1-propanol, ethyl caproate, ethyl caprylate, amounts of enanthic ethers, ratios of C_3 and C_4 alcohols, ratio of the amount of enanthic ethers to ethyl acetate; negative for acetaldehyde, isobutyraldehyde, the sum of aldehydes and ketones, ethyl lactate, the ratio of alcohols C_5 to the sum of alcohols C_3, C_4.

A new distillate technology from dried Jerusalem artichoke has been developed, based on the regulation of strength and composition of fermented wort, which allows to increase the efficiency of the process.

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