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FERMENTED TOMATO JUICE (*LYCOPERSICON ESCULENTUM MILL.*) PRODUCED VIA LACTIC ACID BACTERIA DURING COLD STORAGE

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ABSTRACT

This study was performed to evaluate the effect of using *Lactobacillus plantarum* ssp. *plantarum* EMCC 1027 and *Lactobacillus delbrueckii* ssp. *bulgaricus* EMCC 1102, used singly or together (1:1), (approximately 5-6×10^9 colony forming units (CFUs)/ml) to produce fermented tomato juice. Tomato juice inoculated with *L. plantarum*, *L. bulgaricus* or their mixture exhibited significant decreases in pH (3.1, 3.8 and 3.5, respectively) after 3 days. Reducing sugar levels were found to be 1.9, 2.5, and 2.8 g/100 ml, respectively. The viscosity increased with time, was greater for the juice incubated with *L. bulgaricus*, and increased by 72% by the end of storage. The ΔE values did not change significantly during storage; only the redness of the juice increased in the pasteurized sample. Compared to all examined samples, juice inoculated with *L. plantarum* exhibited higher scores. Thus, probiotication of juice could serve as a method to prepare healthy beverages for lactose-allergic consumers.

1. Introduction

Tomato (*Lycopersicon esculentum Mill*.) is a major vegetable that is widely consumed around the world. The health benefits of tomatoes are attributed to their abundant antioxidant components (Willcox et al., 2003);(Toor and Savage, 2005), such as lycopene and pro-vitamin A (Mayeaux et al., 2006), as well as ascorbic acid (Hanson et al., 2004), vitamin E (Lee et al., 2000), and other flavonoids (Dewanto et al., 2002). Tomatoes are consumed mainly as fresh or processed products. Tomatoes are processed for wide ranges of products, such as canned and sun-dried tomatoes, ketchup, pastes, purees, salads, sauces, soups, and juice products, and they provide significant sources of vitamins and minerals to the consumers (TSEN et al., 2008). Tomatoproducts play an important role in supplying lycopene to consumers; more than 80% of dietary lycopene consumed in the United States comes from a wide variety of tomato products (Willcox et al., 2003). Among tomato products, tomato juices are recognized as healthy beverages (Yoon et al., 2006). Tomato juices, as well as tomato paste and sauces, are the main contributors of dietary lycopene, accounting for 25% of the total daily lycopene intake in the Canadian population (Rao et al., 1998). There are extensive efforts to enhance the functionality of tomato juice products, with trials introducing value-added components or health-improving compounds to tomato juice products (Anese et al., 1999). One recent effort in improving the functionality of tomato juice products is the use of lactic acid bacteria (LAB) to ferment tomato juice as probiotics (Di Cagno et al., 2009). Although products made via probiotic bacteria are usually presented as fermented dairy products, fruit or vegetable juices can also serve as

excellent media for harvesting probiotics (Wang et al., 2009). Probiotic microorganisms are defined as live microorganisms that confer a health benefit to the host when administered in adequate amounts (Corrêa et al., 2008). Microorganisms from several selected bacterial genera, such as *Lactobacillus*, are now applied as probiotics; *L. plantarum* can produce pectolytic enzymes such as polygalacturonase, pectinlyase and pectinesterase (Fersht, 1985) that can degrade ester bonds and decrease the pectin content. Through the fermentation process, high- and low-esterified pectins from vegetable mash are depolymerized to increase the yield to a greater range, and the β-carotene content of vegetable juice also increases (Demir, 2000).

The positive properties of fermented vegetable juices can be attained by selecting the proper *Lactobacillus* strains for the lactic acid fermentation of individual raw materials. The criteria for a strain's suitability are as follows: the rate and total production of acids, the change in pH, the decrease in nitrate concentration and the production of biogenic amines (Karovičová et al., 1999).

In a study of lactic acid fermentative processes via selected probiotic bacterial species from the epiphytic microbiota of vegetables such as carrots and red beets, all examined strains were found to be able to rapidly utilize vegetables for cell synthesis and lactic acid production (Buruleanu et al., 2009). These strains produce a greater amount of lactic acid and reduce the pH of fermented juices from an initial value of 6.4 to below 4.4 after 48 h of fermentation. The lactic acid cultures in fermented juices gradually decrease the viability of the juice during cold storage.

One study (Nosrati et al., 2014) investigated LAB, including *Lactobacillus casei* and *Lactobacillus plantarum*, for the production of fermentative functional drinks from vegetable juice. The results indicated that vegetable juice that lacked any nutrient supplementation could be considered a proper matrix for the growth of LAB and for functional beverage production. However, there is no research on the fermentation of tomato juice with *Lactobacillus plantarum* ssp. *plantarum* (EMCC 1027), *L. delbrueckii* ssp. *bulgaricus* (EMCC 1102), and their mixed culture (1:1).

The aim of this study was to produce probiotic tomato juice via selected LAB as a healthy beverage for vegetarians and lactose-allergic consumers as an alternative to fermented dairy products. In addition, the changes in the physicochemical properties of fermented tomato juice during cold storage were examined.

2. Materials and methods

2.1. Materials

2.1.1. Source of fruit and vegetable media

Fresh raw tomatoes (*Lycopersicon esculentum* Mill.) were purchased from a local market in Zagazig city, El-Sharqia governorate, Egypt.

2.1.2. Preparation of tomato juice

The tomatoes were washed with tap water to remove soil and other impurities, dried at room temperature prior to use, and then peeled by blanching in water at 85°C for 1 minute for easy peeling, tenderization of the tissues, and inactivation of pectinase and peroxidase enzymes. The tomatoes were placed in cold water for 1 minute to cool the fruits, and a blender (Moulinex blender-LM241, France) was used to extract the juice. Approximately 840 g of tomato juice was obtained after blanching 1000 g of fresh tomato fruits. The extracted juice was filtered through a four-ply cheese cloth to separate the tomato juice and the cake containing peel and seed. The filtered juice was heated to 85°C for 10 minutes and cooled to 37°C.

2.1.3. Source of experimental starter cultures

Lactobacillus plantarum ssp. plantarum (EMCC 1027) and *L. delbrueckii ssp. bulgaricus* (EMCC 1102) were obtained from the Egyptian Microbial Culture Collection of

Cairo MIRCEN (EMCC), Faculty of Agriculture, Ain Shams University, Egypt.

2.1.4. Chemicals and Reagents

Sodium hydroxide, Rochelle salt (sodium potassium tartrate tetrahydrate), phenol, sodium bisulfite, phenolphthalein, 2,6-dichlorophenol indophenol, methyl alcohol, and 3,5-dinitro-2-hydroxybenzoic acid (3,5-dinitrosalicylic acid) were purchased from El-Gomhoria Company for Chemicals, Zagazig, Egypt. *Lactobacillus* Man, Rogosa and Sharpe (MRS) Broth and *Lactobacillus* MRS Agar were purchased from the Sigma-Aldrich Company (Cairo, Egypt).

2.1.5. Preparation of experimental fermented tomato juice

Forty-eight hours prior to the start of each experiment, cultures were revived by a series of two inoculations into 10 ml of MRS broth and incubation at 37°C for 24 h.

The used cultures were grown separately at 37°C for 24 h in MRS broth (Difco Laboratories, Detroit, MI, USA) to attain approximately 10^6 (CFUs)/ml as inoculate before inoculation into tomato juice at 0.5% (v/v). Cells were counted by plating serial dilutions of bacterial suspensions on MRS agar plates, incubating at 37°C, and counting the colonies after 48 h. Inoculation doses with *L. plantarum* and *L. bulgaricus* were chosen in the range of 10^5-10^7 CFU/ml, and lacto-fermented juices were processed.

2.2. Methods

2.2.1. Determination of pH-value

The pH was measured in all samples with a glass electrode and a digital pH meter (Model Mettler Toledo, Switzerland) (Horwitz, 2000).

2.2.2. Determination of titratable acidity

The acidity of samples was determined according to the general titration method based on lactic acid percentage (Horwitz, 2000)

2.2.3. Determination of total soluble solids (TSS)

The total soluble solids (TSS) and the refractive index were assayed using the refractometry method with a refractometer (ABBE DR-A1, Atogo, Tokyo, Japan) and corrected to the equivalent reading at 20°C (Horwitz, 2000).

2.2.4. Determination of reducing sugars content

Reducing sugars contents were assayed according to Miller (Miller, 1959) colormetrically. The colour intensities were measured in a UV spectrophotometer (Jenway-UV–VIS Spectrophotometer) at 575 nm.

2.2.5. Determination of vitamin (C) content

The vitamin C (ascorbic acid) content was determined using the 2,6-dichlorophenol indophenol reagent (Fluka, Deisehofen, Germany) according to the method described by AOAC (Horwitz, 2000).

2*.2.6. Determination of viscosity*

Each sample was measured at room temperature using a Brookfield digital viscometer (NDJ-85, Niryn Intelligent Company Limited, Shanghai). A suitable spindle (spindle 2) and rotational speed (60 rpm) were selected for this study (Sun et al., 2006).

2.2.7. Determination of Colour

Tomato juice colour was measured using a HunterLab system (HunterLab ColorFlex EZ, USA). The colour parameter (L*) indicates the degree of lightness to darkness, (a*) indicates the degree of redness to greenness, and (b*) indicates the degree of yellowness to blueness (Hunter, 1958).

2.2.8. Brix-acid ratio

The sweetness index (SI) was determined using the procedures outlined in a previous study (Agbemavor et al., 2014) and expressed according to the following equation:

SI = Soluble solids content (SSC) / Total titratable acidity (TTA).

2.2.9. Determination of the reducing sugars content

Reducing sugar contents were assayed colorimetrically according to Miller (Miller, 1959). The colour intensities were measured in a UV spectrophotometer (Jenway-UV–VIS Spectrophotometer) at 575 nm.

2.2.10. Microbiological analysis

Viable cell counts were determined by serial dilutions and the standard plate method after incubation. Dilutions of 10^{-7} and 10^{-8} CFU/ml were prepared from fermented samples and plated in double plates. Then, sterilized MRS agar (Merck, Germany) medium was poured on top (standard plate count method). The plates were incubated at 30°C for 48 h. Plates containing 30-300 colonies were counted and recorded as CFU per ml of solution (Vinderola et al., 2000). The viability of the lactic acid cultures was determined during the cold storage period using the mentioned method and was expressed as CFU/ml (Horwitz et al., 1970).

2.2.11. Sensory Evaluation

Sensory evaluation was performed as described in another study (Min et al., 2003). Ten panellists were selected (Staff of Food Science Department, Faculty of Agriculture, Zagazig University, Egypt) without selection for age or sex. Ten panellists were asked to choose their preference on a 9-point Hedonic scale with a degree of liking: 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, and 9=like extremely. In each session, five different samples were given to the panellists to rate the colour, flavour, texture, and overall acceptability of the samples.

2.2.12. Statistical analysis

All fermentation experiments were performed in triplicate, the results were determined in duplicate, and the average values were expressed as the mean ± standard deviation (S.D.).

3.Results and discussions

The changes in pH during tomato juice fermentation with *L. plantarum*, *L. bulgaricus*, and the mixed culture (1:1) are given in Table (1). Tomato juice containing *L. plantarum* showed a more rapid drop in pH (3.1) than *L. bulgaricus* juice (pH=3.8) and mixed culture juice (1:1, pH=3.5) after 3 days of cold storage, while at 12 days, the pH values were 2.9, 3.5, and 3.1, respectively. Previous reports studied the formulation and evaluation of yogurt and Rayeb (traditional Egyptian natural fermented milk) mixes with red and yellow carrot juices (El-Abasy et al., 2012). The pH fell considerably from 6.40 to 4.40 (control), from 6.36 to 4.10 for the yoghurt red carrot juice (YRC mix) and from 6.04 to 4.20 for the yoghurt-yellow carrot juice (YY mix); such decreases in pH were suspected to be caused by the formation of *lactic acid bacteria* throughout fermentation (El-Abasy et al., 2012). The researchers studied the suitability of milk/carrot juice drink stored at 4±2°C for up to 20 days for the production of non-fermented probiotic drinks using the survival counts of *Lactobacillus acidophilus* LA5, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 (Daneshi et al., 2012). The pH changes from *L. acidophilus* LA5, *L. plantarum* and *B. lactis* BB12 during the storage period were low and varied according to strain from 6.43 to 6.66 and 0.13% to 0.15% (according to lactic acid), respectively, and a comparison of the means showed no significant differences between the samples. The pH of carrot juice before the addition to milk was approximately 5.7.

As shown in Table (1), *L. plantarum* juice had more acid (1.60%) after 3 days of refrigerated storage than the other examined juice samples (1.30%) (Daneshi et al., 2012). One study (Shah et al., 1995) reported that the acid production ability of the LAB, especially

post-incubation (post-acidification), affected the cell viability of the probiotic bacteria including *L. plantarum* and *L. bulgaricus* (Shah et al., 1995). As a result, the plurality of changes observed in the microbial population, pH, acidity, sugar consumption, and lactic acid metabolism occurred between 30 h to 48 h of fermentation. Further extension of the fermentation process (from 48 h to 72 h) did not result in significant changes, and similar results were reported by others (Mousavi et al., 2011; Yoon et al., 2005).

The results for sugar consumption (Table 1) showed that the amount of reducing sugars for all samples dropped after 3 days due to bacterial growth and organic acid production. *L. bulgaricus* consumed higher levels of reducing sugars than the mixed culture (1:1) and *L. plantarum* culture, with values of 2.11, 2.45, and 2.51 g/100 ml after 3 days, and 1.45, 1.97, and 1.77 g/100 ml after 12 days of cold storage, respectively. These results are in agreement with a previous study (Tsen et al., 2003). The researchers investigated lactic acid production by *L. acidophilus* in medium based on mashed banana and used k-carrageenan gum to enhance fermentation. The selected strain metabolized low molecular weight sugars, i.e., fructose and glucose, as a carbon source for acid production. The researchers reported that the number of carbon sources with high molecular weight, i.e., fructo-oligo-saccharides, did not change during fermentation.

The concentration of reducing sugars was lower in fermented tomato juice than in fresh tomato juice due to the varied carbohydrate utilization by *Lactobacillus spp*. This process enhanced the viable count and increased the acidity when the fermentation time increased from 24 h to 72 h. An earlier study with fruit juices indicated the growth of *L. plantarum*, which resulted in a viable count of 8.0×10^8 CFU/ml after 72 h of fermentation (Mousavi et al., 2011). Similar results were reported in another study (Hou et al., 2000), with carbohydrate fermentation that varied with the type of substrate consumption and fermentation time; glucose was reported to be the primary energy source for *Lactobacillus* spp. Therefore, glucose has been introduced as the most important carbohydrate source for probiotic lactobacilli (Wang et al., 2003).

The results in Table 1 show a decrease in TSS % for probiotic tomato juice, especially *L. plantarum* (T2), *L. bulgaricus* (T3), and the mixed culture (T4), with values of 4.9, 5.2, and 5.3 and 4.2, 4.7, and 4.7 after 3 and 12 days of cold storage, respectively. This result is due to the cell synthesis and growth rate of the starter cultures.

The (T1) sample showed approximately stable values of 5.5 and 5.2, while the values for fresh juice (control) decreased from 5.3 to 4.7 after 3 and 12 days, respectively.

Tomato juice viscosity is influenced by its quantitative and qualitative composition and therefore, will depend on its treatment during processing. Table 1 shows the viscosity of tomato juice; the viscosity clearly increased in all samples. The sample (T3) had the highest viscosity of 41.3 (cps) and 47.8 (cps) after 3 and 12 days, respectively, of cold storage. Samples with the (T4) had values of 36.8 (cps) and 42.3 (cps), and the (T2) values were 30.3 (cps) and 35.6 (cps) after 3 and 12 days, respectively, of cold storage.

The increase in the viscosity of the fresh tomato juice (control) upon storage might be due to the increase in the microbial load, particularly moulds and yeasts, which lead to the spoilage of juice. Similarly, another study (in't Veld, 1996) reported that spoilage of food may result in physical changes, such as an increase in viscosity, gelation, and sedimentation or colour changes.

Table (1) shows that the vitamin C content decreased rapidly in samples (T2) to 22.4 and 17.3 mg/100 ml juice after 3 and 12 days, respectively, of cold storage. Samples with the (T4) had values of vitamin C 24.5 and 19.7 mg/100 ml juice, whereas (T3) had values of 25.7 and 21.2 mg/100 ml juice after 3 and 12 days, respectively, of cold storage. The decreasing content of vitamin C during storage

may be due to the metabolism, cell synthesis, and growth of LAB.

Fresh tomato juice (control) had values of 23.1 and 17.9 mg/100 ml juice after 3 and 12 days, respectively, while (T1) showed a slight decrease in the values to 27.3 and 23.3 mg/100 ml juice, respectively, at the end of storage.

Table (1) shows the decreasing Brix-acid ratios during the fermentation process and cold storage.

The sample (T2) had values of 3.06 and 2.47 after 3 and 12 days, respectively, of cold storage. Samples with the (T4) had values of 3.78 and 3.13, respectively, while (T3) had values of 4.00 and 3.35, respectively.

The fresh tomato juice (control) values were 7.57 and 5.80, while (T1) values were nearly stable at 13.01 and 7.76 during cold storage.

The balance between sweetness and acidity is a basic precept in man's judgement of the quality of many fruits. It was reported that berry soluble solids (expressed as degrees Balling) influenced the palatability of Perlette table grapes (Nelson et al., 1963). Coombe, Dundon, & Short, 1980 recommended the adoption of the Balling acid ratio as an index of palatability for grapes. Moreover, the ratio is often a better indicator of acceptability than either sugar or acid alone.

The tomato juice turbidity showed that sedimentation led to clarification after 12 days of cold storage, possibly due to acidification of the medium by LAB metabolites (organic acids, i.e., lactic acid). These results agree with an earlier study (Reiter et al., 2003) of carrot juice cloud stability on a pilot-plant scale using decanter technology. The production steps investigated were the model of acidification and different enzymatic mash treatments. The strongest effect on cloud stability was acidification. Cloud sedimentation could be enhanced by acidifying the coarse mash, resulting in cloud stability and acidification following juice extraction.

One study (Schultz et al., 2014) examined the effects of acidity on cloud stability in pasteurized carrot juice over the pH range of 3.5-6.2. The cloud sedimentation and particle diameter were measured at each pH to quantify the juice cloud stability and clarification during 3 days of storage. Acidification below pH 4.9 resulted in increased particle size and an unstable cloud, leading to juice clarification.

The effect of cold storage on the cell viability of the two lactic acid cultures in fermented tomato juice is presented in Fig. 1. The viable cell counts of *L. plantarum* and *L. bulgaricus* were greater than 1.0×10^6 CFU/ml even after 12 days of cold storage at 4±1°C. The viable cell counts of *L. plantarum* decreased gradually during cold storage and remained at 1.5×10^6 CFU/ml after 12 days of cold storage. The viable cell counts of *L. bulgaricus* decreased slightly during cold storage, but the cell viability remained at a considerably high level ($>10^6$ CFU/ml) after 12 days of cold storage (4±1°C). It is important to have a significant number of viable LAB present in probiotic products for maximum health benefits (Mousavi et al., 2011; Shah, 2001).

Several factors affect the cell viability of lactic acid cultures in probiotic food products. Probiotic cultures are commonly used in the dairy industry, and some products produced during lactic acid fermentation, such as lactic acid, diacetyl, and acetaldehyde, could be associated with the loss of viability of the added probiotic bacteria (Post, 1996). Probiotic lactic acid starters have been suggested to produce bacteriocin against probiotic bacteria and vice versa (Kumar et al., 2015; Prado et al., 2008).

In general, cell viability depends on the strains used, the interactions between species present, the culture conditions, the oxygen content, the final acidity of the product, and the concentrations of lactic acid and acetic acid. The main factors in the loss of viability of probiotic organisms are the decrease in the pH of the medium and the accumulation of organic acid as a result of growth and fermentation (Saarela et al., 2002).

Fig. 2 shows that colour values (L*, a*, and b*) decreased during the storage period. The L* values in samples with the mixed culture (1:1), *L. plantarum*, and *L. bulgaricus* at 3 days of storage were 23.47, 23.39, and 23.60, respectively. At 12 days, they were 22.55, 22.18, and 22.95.

The a* values in samples with the mixed culture (1:1), *L. plantarum*, and *L. bulgaricus* at 3 days of storage were 25.42, 22.45, and 23.69, while those at 12 days, were 23.65, 22.27, and 23.52, respectively.

The b* values in samples with the mixed culture (1:1), *L. plantarum*, and *L. bulgaricus* at 3 days of storage were 11.78, 11.01, and 11.16, while those at 12 days, were 11.13, 10.67, and 11.05, respectively.

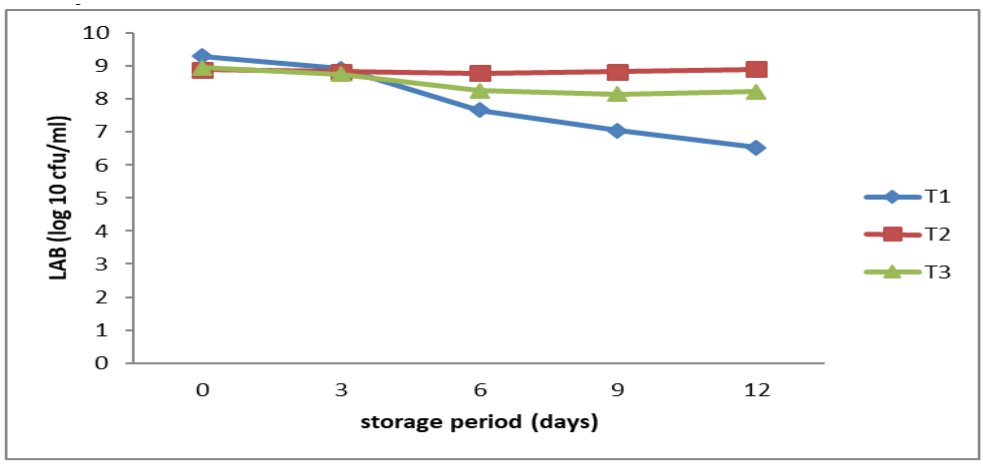
During storage, it was obvious that small decreases in colour attributes could be partly caused by the metabolism of bacteria or by the fermentation conditions, such as the temperature, pH, and acidity of the medium (Panda and Ray, 2007).

These results agree with authors (Kun et al., 2008) who previously determined that storage caused a decrease in β-carotene; it is thought that oxygen in the headspace causes the degradation of β-carotene. During cold storage, a decrease in colour attributes was observed until the final day and was possibly due to the total count of LAB or the pH and acidity conditions of the medium.

Figs. 2 and 3 show that the colour intensity (C), hue angle (h) and total colour difference (ΔE) values were highest for the sample with the mixed culture (55.2), followed by *L. plantarum* (53.0), and *L. bulgaricus* (53.9), on the ninth day of storage. The C values were highest for the sample with the mixed culture (28.0), followed by *L. plantarum* (25.0), and *L. bulgaricus* (26.1).

These results refer to decreasing values compared to the first day of cold storage, possibly due to bacterial metabolism.

Table 2 shows that fermented tomato juice with the *L. plantarum* culture was a favourable product compared to the *L. bulgaricus* culture product. The mixed culture (1:1) was the second most favourable product based on colour, flavour, texture, and overall acceptability. Generally, the panellists did not like the flavour of the products, possibly due to the higher acidity and contents of LAB by-products, such as diacetyl, acetaldehyde and lactic acid, which could negatively impact the flavour of the products.



*T1: tomato juice pasteurized at 85°C for 10 min., T2: tomato juice containing *L. plantarum*, T3: tomato juice containing *L. bulgaricus*; T4: tomato juice containing *L. plantarum* + *L. bulgaricus* (1:1).

Figure 1. Effect of cold storage (4±1^{0}C) on the viability of lactic acid bacteria cultures in fermented tomato juice

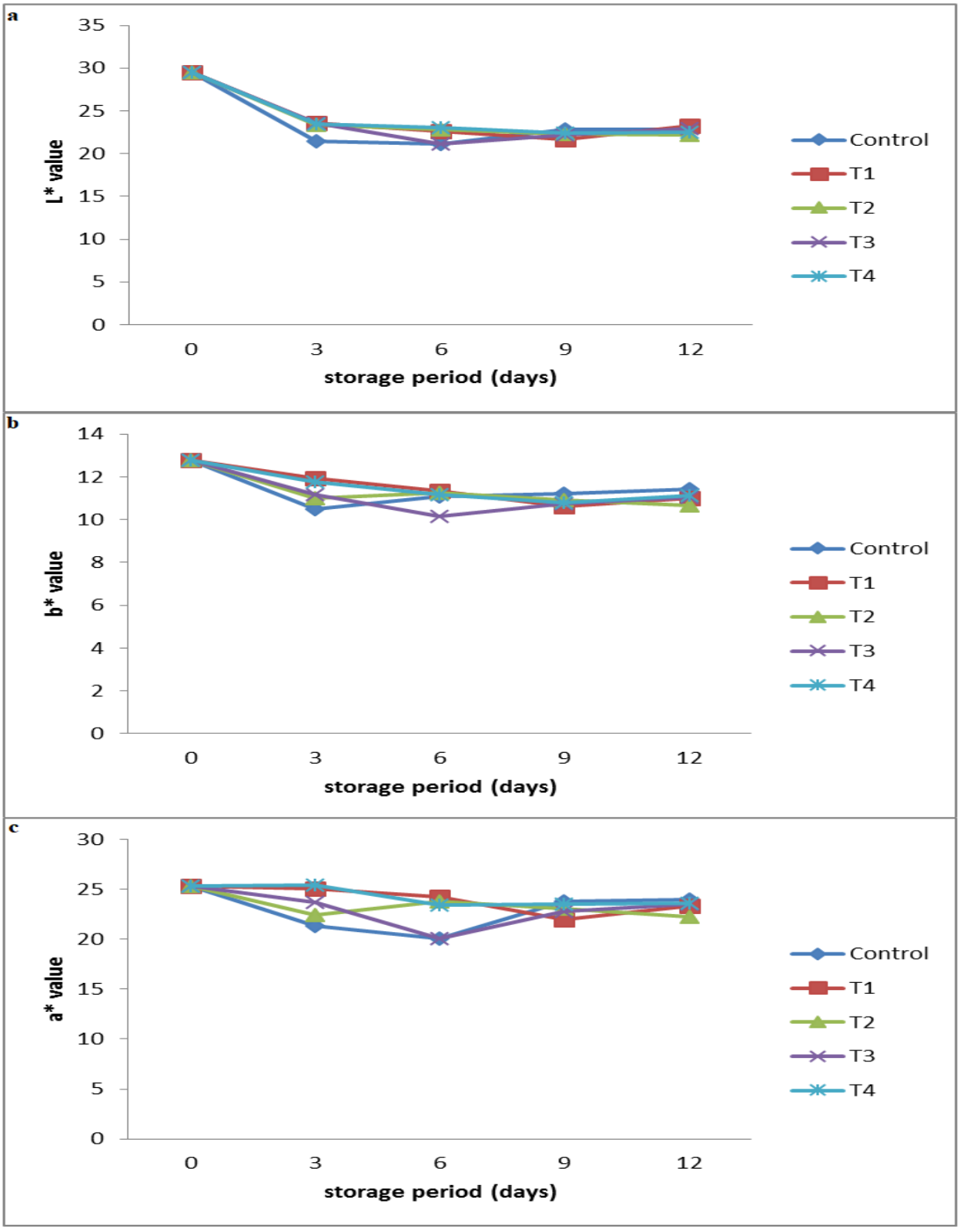
Table 1. Physicochemical properties of probiotic tomato juice during cold storage (4±1ºC)

parameter	Storage (days)	Control	T1	T2	T3	T4
pH	**0**	5.70 ± 0.0047	5.74± 0.0329	5.71 ±0.0081	5.75±0.0294	5.74±0.0339
	3	5.33±0.0205	5.73±0.0402	3.98±0.0294	4.25±0.0244	4.14±0.0374
	6	4.95±0.0326	5.64±0.0368	3.96±0.0163	4.15±0.0286	4.10±0.0294
	9	4.64±0.0169	5.53±0.0339	3.94±0.0169	4.15±0.0326	4.13±0.0329
	12	4.45±0.0286	5.33±0.0309	3.90±0.0326	4.13±0.0402	4.24±0.0262
Acidity (%)	**0**	0.15±0.0057	0.15±0.0094	0.15±0.0029	0.15±0.0024	0.15±0.0020
	3	0.34±0.0339	0.15±0.0188	0.67±0.0012	0.64±0.0053	0.65±0.0029
	6	0.44±0.0329	0.16±0.0032	0.67±0.0009	0.65±0.0061	0.65±0.0026
	9	0.48±0.0309	0.16±0.0036	0.68±0.0037	0.66±0.0014	0.65±0.0030
	12	0.54±0.0374	0.18±0.0032	0.69±0.0028	0.66±0.0024	0.68±0.0035
Reducing sugars content (g/100 ml)	**0**	2.95±0.0203	2.95±0.0206	2.97±0.0181	2.95±0.0186	2.97±0.0197
	3	2.77±0.0029	2.95±0.0185	2.51±0.0216	2.11±0.0020	2.45±0.0028
	6	2.35±0.0026	2.91±0.0075	2.33±0.0205	1.95±0.0030	2.33±0.0024
	9	2.23±0.0030	2.88±0.0032	1.98±0.0035	1.73±0.0016	2.15±0.0033
	12	2.01±0.0028	2.76±0.0094	1.77±0.0003	1.45±0.0016	1.97±0.0029
Total soluble solids (ºBrix)	**0**	7.80±0.0262	7.80±0.0205	7.80±0.0286	7.80±0.0169	7.80±0.0294
	3	7.70±0.0188	7.80±0.0216	7.60±0.0368	7.50±0.0235	7.60±0.0205
	6	7.60±0.0235	7.80±0.0309	7.40±0.0163	7.30±0.0262	7.20±0.0216
	9	7.60±0.0216	7.70±0.0294	7.20±0.0262	7.10±0.0355	7.00±0.0262
	12	7.50±0.0094	7.70±0.0216	7.10±0.0081	6.90±0.0249	6.90±0.0163
Vitamin (C) content (mg/100 ml)	**0**	5.6±0.0047	5.6±0.0377	5.6±0.0329	5.6±0.0282	5.6±0.0235
	3	5.2±0.0188	5.1±0.0141	4.2±0.0081	3.7±0.0047	3.8±0.0402
	6	4.8±0.0047	5.1±0.0094	3.9±0.0262	3.2±0.0124	3.4±0.0169
	9	4.3±0.0169	4.9±0.0124	3.8±0.0355	2.9±0.0402	3.2±0.0081
	12	3.9±0.0309	4.5±0.0262	3.5±0.0216	2.5±0.0355	3.0±0.0309
Brix/acid ratio	**0**	52.00±0.0040	52.00±0.0035	52.00±0.0030	52.00±0.0026	52.00±0.0021
	3	23.33±0.0028	52.00±0.0016	11.34±0.0033	11.71±0.0029	11.69±0.0024
	6	16.88±0.0026	48.75±0.0012	11.04±0.0016	11.23±0.0020	11.07±0.0004
	9	15.83±0.0012	48.12±0.0008	10.58±0.0037	10.75±0.0032	10.76±0.0030
	12	14.70±0.0088	42.77±0.0022	10.28±0.0023	10.45±0.0020	10.14±0.0018
Viscosity (cps)	**0**	5.02±0.0124	5.03±0.0141	5.03±0.0188	5.03±0.0235	5.05±0.0262
	3	7.06±0.0294	6.05±0.0235	8.04±0.0205	12.13±0.0262	8.07±0.0555
	6	9.15±0.0286	8.04±0.0141	11.12±0.0205	15.14±0.0339	9.14±0.0141
	9	13.17±0.0163	8.03±0.0124	12.15±0.0374	16.15±0.0339	12.18±0.0141
	12	15.16±0.0188	9.10±0.0612	14.16±0.0124	18.12±0.0852	14.14±0.0205

*Control: fresh tomato juice, T1: tomato juice pasteurized at 85ºC for 10 min., T2: tomato juice containing *L. plantarum*, T3: tomato juice containing *L. bulgaricus*; T4: tomato juice containing *L. plantarum* + *L. bulgaricus* (1:1).

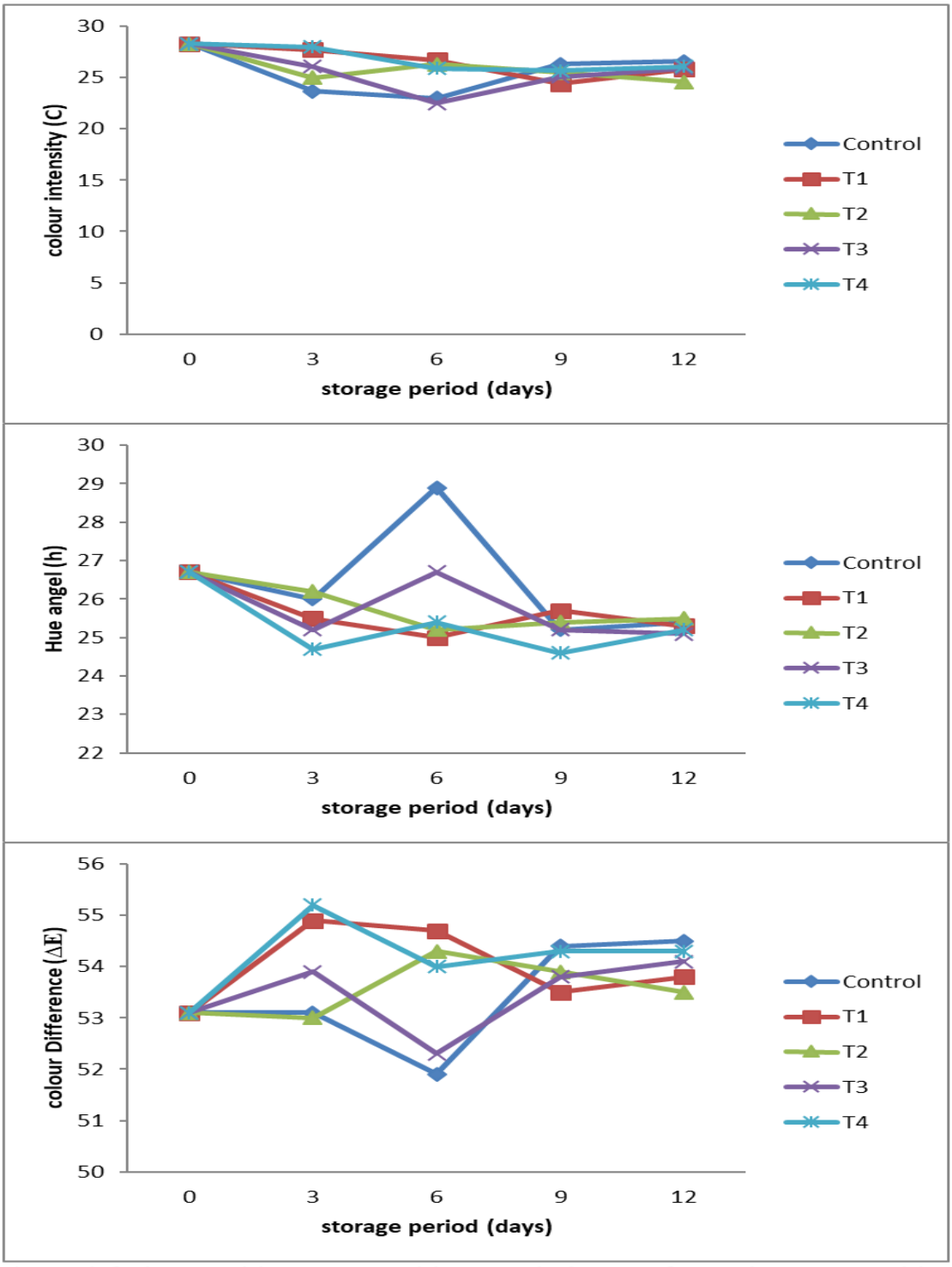
Table 2. Effect of cold storage (4±1°C) on sensory evaluation values in probiotic tomato juice

Sensory evaluation						
days	**Parameter**	**Control (9)**	**T1 (9)**	**T2 (9)**	**T3 (9)**	**T4 (9)**
0	Color	8.8±0.4898	8.7±0.4000	8.9±0.4582	8.8±0.3000	8.7±0.5000
	Flavor	8.8±0.4000	8.0±0.0000	8.9±0.4582	7.8± 0.4582	7.5±0.4898
	Texture	8.9±0.6324	8.9±0.4898	8.7±0.5000	8.9±0.4898	8.7±0.4582
	Overall acceptability	8.6±0.4582	8.5±0.3000	8.7±0.4000	8.5±0.3000	8.0±0.0000
3	Color	7.7±0.4582	8.1±0.3000	8.2±0.4000	8.3±0.4582	8.4±0.4898
	Flavor	7.00±0.0000	8.5±0.5000	8.4±0.4898	7.4±0.4898	7.3±0.4582
	Texture	7.4±0.4898	7.2±0.4000	7.00±0.0000	7.1±0.3000	7.3±0.6403
	Overall acceptability	7.3±0.6403	8.3±0.4582	7.3±0.4582	7.7± 0.4582	7.4±0.6633
6	Color	6.7±0.4582	7.00±0.0000	8.3±0.4582	6.5±0.5000	8.5±0.5000
	Flavor	6.3±0.4582	8.4±0.4898	6.5±0.5000	7.2±0.4000	7.1±0.3000
	Texture	7.6±0.4898	7.4±0.6633	7.4±0.4898	7.3±0.6403	7.00±0.0000
	Overall acceptability	6.6±0.4898	7.3±0.4582	7.2±0.4000	6.5±0.5000	7.6±0.4898
9	Color	7.7±0.6633	7.8± 0.4582	8.5±0.5000	7.2±0.4000	7.3±0.6403
	Flavor	7.7±0.3000	8.2±0.4000	6.5±0.5000	7.00±0.0000	7.3±0.4582
	Texture	7.5±0.5000	7.1±0.3000	7.3±0.4582	6.7±0.4582	6.7±0.4582
	Overall acceptability	6.3±0.4582	8.4±0.4898	7.4±0.4898	7.2±0.4000	6.6±0.4898
12	Color	6.2±0.4000	7.3±0.6403	8.5±0.5000	6.7±0.4582	7.1±0.3000
	Flavor	6.3±0.6403	7.6±0.4898	7.00±0.0000	6.6±0.4898	7.3±0.4582
	Texture	6.8±0.8717	7.1±0.3000	7.2±0.4000	6.8±0.4898	6.7±0.4898
	Overall acceptability	4.8±0.8000	4.7± 0.4582	4.9±0.4582	4.7±0.4582	4.4±0.4000



*Control: fresh tomato juice, T1: tomato juice pasteurized at 85°C for 10 min., T2: tomato juice containing *L. plantarum*, T3: tomato juice containing *L. bulgaricus*; T4: tomato juice containing *L. plantarum* + *L. bulgaricus* (1:1).

Figure 2. The effect of cold storage (4±1^{0}C) on color attributes in fermented tomato juice



*Control: fresh tomato juice, T1: tomato juice pasteurized at 85°C for 10 min., T2: tomato juice containing *L. plantarum*, T3: tomato juice containing *L. bulgaricus*; T4: tomato juice containing *L. plantarum* + *L. bulgaricus* (1:1).

Figure 3. Effect of cold storage (4±1^{0}C) on color intensity (C), (h) and (ΔE) in fermented tomato juice

4. Conclusions

This research demonstrates that tomato juices can be fermented with selected *Lactobacillus* species, such as *L. plantarum*, *L. bulgaricus*, as the probiotic microorganisms, either alone or as a mixed culture (1:1). Therefore, this research suggests that tomato juice fermented with lactobacilli can be developed as a potential probiotic product and may benefit consumers searching for an alternative beverage to replace fermented dairy products for lactose-allergic people and vegetarians.

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PRELIMINARY BIOCHEMICAL INVESTIGATION OF BIOACTIVE COMPOUNDS AND ANTIBIOFILM ACTIVITY OF *OCIMUM GRATISSIMUM* AGAINST FOOD-BORNE MICROORGANISMS

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ABSTRACT

The present study was aimed to investigate the antibiofilm activity and bioactive compounds in *Ocimum gratissimum* on bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum biofilm inhibitory concentration (MBIC) was carried out on the aqueous, ethanol and methanol extracts. The bioactive components of plant extracts were investigated using Gas Chromatography-Mass Spectrometry (GC-MS). *S. aureus* was more susceptible to the extracts than *P. aeruginosa*. The biofilm of *S. aureus* showed more susceptibility to the extracts of *Ocimum gratissimum* compared to that of *P. aeruginosa*. The ethanol extract exhibited greater activities against all test organisms than the methanol and aqueous extracts with MIC and MBC values <1 mg/ml; and MBIC values > 1 mg/ml with a biofilm inhibition of 61.70% and 43.14% for *S. aureus* and *P. aeruginosa*, respectively. None of the extracts was able to disrupt the growth of preformed biofilm by at least 50%. The ethanol and aqueous extracts inhibited biofilm formation in *S. aureus* but not *P. aeruginosa*. The methanol and aqueous extracts enhanced biofilm growth in *P. aeruginosa* while ethanol extract showed negligible activity against biofilm of *P. aeruginosa*. GC-MS analysis of ethanol extract of *Ocimum gratissimum* revealed the presence of fourteen compounds with oleic acid (21.11%) as the dominant compound, followed by n-Hexadecanoic acid (20.13%), squalene (16.82%) and z-2-Octadecen-1-ol acetate (11.53%). The antibiofilm activity of the plant could be used for the extension of the shelf-life in processed foods and against microorganisms both in locally made foods.

1. Introduction

Biofilms are multicellular clusters, permanently attached to each other in form of layers which is enclosed in a self-generated extracellular matrix of macro-molecules (polysaccharides, proteins and nucleic acids) which gives it an altered phenotype in terms of growth rate and gene expression as compared with planktonic bacteria (Costerton et al., 1999). Donlan, (2002) reported that biofilms are able to attach to surfaces and are often stable in the air-liquid interface. Biofilms can also grow on natural surfaces such as teeth,

inner lining of the heart valves, lungs, intestinal tract, middle ear, prosthetic joints and coronary angioplasty (Hoiby et al., 2010). The resistance that biofilms imparts on microorganisms has become a major issue due to their ability to withstand severe environmental conditions thereby causing loses in food industries and series of hospital cases.

Biofilms are characterized by increased resistance to antimicrobial agents like antibiotics in comparison to mobile cells (Costerton et al., 1999; Mah and O'Toole, 2001). The complete removal of biofilm forming organisms from food processing environments requires much effort because these bacteria attach to food contact surfaces and can strive even after cleaning and disinfection (Yang et al., 2012; Giaouris et al., 2013). The health of consumers may also be jeopardized due to contamination of food products owing to poor hygienic practices.

Staphylococcus aureus and *Pseudomonas aeruginosa* have the ability to form biofilms and are well known causes of food borne infections. The toxins produced by the Gram-positive *Staphylococcus aureus* is one of the main causes of food poisoning (Le Loir et al., 2003). Medicinal plants are made of bioactive compounds that possess antimicrobial properties making them an alternative to antibiotics (Essawi and Srour, 2000).

Ocimum gratissimum, a medicinal plant of the Lamiaceae family is common to Asia, Africa, and Central and South America. Commonly known as scent leaf due to its characteristic pleasant aroma; it is often used to improve the flavor of food. The antimicrobial activities possessed by the plant is attributed to its high alkaloid and polyphenol content (Cowan et al., 1999). Essential oils such as eugenol, linalol, methyl cinnamate, camphor and thymol has been isolated in *Ocimum gratissimum;* and are also regarded as the major bioactive compounds responsible for the antimicrobial properties and numerous medical uses (Matasyoh et al., 2007; Moghaddam, 2011; Mith et al., 2016). Compounds isolated from these plants could be used in the production of pharmaceuticals. This study tends to investigate the effect of *Ocimum gratissimum* extracts on the biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Collection and identification of plant material

The plant material *Ocimium gratissimum* (scent leaf) was purchased from Ubaani Main Market in Umuahia Local Government Area of Abia state, South-East, Nigeria. The identification of the plant material was done by Mr. Ibe Ndukwe of the Department of Forestry, Michael Okpara University of Agriculture, Umudike, Abia state, Nigeria. Voucher specimen of the plant MOUAU/1601771 was deposited in the herbarium of the department.

2.2. Preparation of plant materials

The plant material obtained was initially air dried at room temperature (25-28°C) for 14 days. The air dried plant material was pulverized to fine powder with an electric blender (SONIK R, JAPAN).

2.3. Preparation of extracts

The extracts of *Ocimium gratissimum* were prepared using the method as described by Remington (2000). The aqueous extract was prepared by adding 50 g of the powdered plant material was soaked in 150 ml of water and incubated for 3 h with shaking at intervals. It was then filtered using Whatman No. 1 filter paper in a Buchner funnel. The process above was repeated twice on the residue using 100 ml and 50 ml of water for the second and third extraction, respectively.

The ethanol and methanol extract prepared as discussed above were transferred separately into a conical flask. The filtrates obtained were concentrated using an oven at 50°C, and then

transferred to a flask and then evaporated at 45ºC for 24 h in a vacuum oven. The mass of the powdered extract was obtained and the percentage yield determined. The extracts were stored in sealed vials until further analysis.

The aqueous extract obtained was transferred into small conical flasks, sealed with parafilm and frozen at -4°C before drying. The extract was concentrated to dryness in a hot air oven (SELLECTA CE 0505) at 45°C with constant monitoring to prevent thawing of extracts. The concentrated extract was double sealed with parafilm and stored in a desiccator. The mass of the extract was obtained and the percentage yield was determined. The extract was stored in a caped plastic container at 4ºC until further analysis.

2.4. Preparation of cultures

Bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from the Diagnostic Centre of National Veterinary Research Institute Vom, Plateau State, Nigeria. The strains are *S. aureus* ATCC 12540, ATCC 12600, ATCC 12660, ATCC 12732 and *P. aeruginosa* ATCC 10200, ATCC 10325, ATCC 10145, ATCC 10528. Glycerol stock cultures of each organism were prepared and kept at -4°C prior to use. The strains were revived into sterile Tryptone Soy Agar (Oxoid, UK) and incubated at 37°C for 18 h. The identity of the organisms was confirmed using selective media. The organisms were then inoculated into sterile Tryptone Soy Broth (TSB) and incubated at 37ºC overnight. The culture was then standardized to a concentration of 1.0 x 106 Cfu/ml. This was done by diluting with TSB to obtain an absorbance (OD590 nm) of 0.02 (Sandasi et al., 2008).

2.5. Biofilm formation and quantification

The wells of a sterile 96 well flat bottomed microtitre plate were filled with 230µl TSB and thereafter, 200 µl of overnight bacterial culture standardized to a concentration of 1.0 x 106 Cfu/ml was poured into each well and incubated at 37°C for 24 h. The negative control wells contained TSB only. After incubation, the content of the plates was poured off and the wells washed three times with 300 µl of sterile distilled water. The remaining attached cells were fixed with 250 µl of methanol per well and emptied after 15 min and allowed to dry at a temperature of 28 ± 2°C for 3 h. The plates were stained with 250 µl of 0.1% (w/v) crystal violet stain for 5 min. Excess stain was rinsed by placing the microtitre plate under running water and air dried. The dye bound to adherent cells was re-solubilized with 250 µl of 90% ethanol per well. Absorbance was measured at 570 nm (Stepanovic et al., 2007).

2.6. Antimicrobial Activity

2.6.1. Determination of minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration of *Ocimium gratissimum* was determined using the tetrazolium microplate assay as described by Eloff (1998) with slight modifications. This assay was performed using the round-bottomed polystyrene 96-well clear microtitre plate with standard plate layout as proposed by Cos et al., (2006). The extracts were dissolved in Dimethyl Sulfoxide (DMSO) and an identical two-fold serial dilution was made to form 0.03125 – 4.0 mg/ml. 100 µl of the standard culture (1.0 x 106 cfu/ml) was then added to all the wells. The plates were sterile sealed with sealing tape and incubated at 37oC for 24 h. The MIC of the plant extracts was detected after addition of 50 µl of 0.2 mg/ml of 2-4-Iodophenyl-3-4-nitrophenyl-5-phenyl- 2-tetrazoliumchloride (INT) in all the wells and incubated for further 30 min at 37°C. Bacterial growths was determined by observing the colour change of INT in the microplate wells. The concentration at which there was no visually detectable bacterial growth was taken as the MIC.

2.6.2. Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) were determined according to the method described by Qandashtani et al. (2017). A hundred (100) microliter of culture medium from the microtitre plate wells that showed no changes in colour was re-inoculated on Mueller Hinton agar (Oxoid, UK) agar plates. After 24 h of incubation at 37°C, MBC was determined as the lowest concentration that showed no visually detectable bacterial growth. Ciprofloxacin (V. S. International pvt Ltd. INDIA) and Tryptone Soy Broth (TSB) (Oxoid, UK) were used as the positive and negative controls respectively.

2.6.3. Determination of minimum biofilm inhibitory concentration (MBIC)

The MBICs of *Ocimium gratissimum* was determined according to Cernohorská and Votava (2008) with slight modifications. The experiments were done in 96-wells polystyrene microtitre plates with round bottoms (Sigma Aldrich, Costa, USA). About 75 µL of an overnight standard culture of 1.0 x 106 cfu/ml was added to the wells of microtiter plate and the plate incubated for 24 h at 37°C. The wells were washed three times with phosphate buffered saline (PBS, pH 7.2) under aseptic conditions to remove unattached bacteria and dried in an inverted position at a temperature of 60°C for 1 h. Volumes of 100 µL of appropriate two-fold dilutions of the respective plant extracts were transferred into the dried wells with established biofilms. The microtitre plate was incubated for 18–20 h at 37ºC. Following incubation, 50 µl of 0.2 mg/ml of INT (2-4-Iodophenyl-3-4-nitrophenyl-5-phenyl- 2H-tetrazoliumchloride) sample was added in all the wells and incubated for further 30 min at 37°C and the MBIC determined, which corresponds to the lowest concentration of the extracts which inhibits growth of biofilm cells as indicated by the first clear well. The positive and negative controls were ciprofloxacin (V. S. International pvt Ltd. INDIA) and Tryptone Soy Broth (TSB) (Oxoid, UK).

2.6.4. Inhibition of cell attachment to polyvinyl chloride

The *Ocimium gratissimum* extracts were tested for potential anti-adhesion properties at a concentration of 1 mg/ml. Two hundred (200) microlitres of the extracts were added to the 96-well microtitre plates, and equal volumes of TSB and ciprofloxacin (MIC value) were added as negative and positive controls respectively. 200 µl of standardized culture (1.0 × 106 CFU m/l) was then pipetted into the wells to yield a final volume of 400 µl in each well. The cultures were added into the wells in triplicate. The plates were sterile sealed with sealing tape and incubated at 37°C for 8 h without shaking to allow cell attachment and biofilm development. After incubation, the modified crystal violet assay according to Djordjevic et al. (2002) was performed to assess biofilm biomass. The incubated plates were washed three times with sterile distilled water to remove loosely attached cells. The plates were air-dried and then oven-dried at 60°C for 45 min. After drying, the wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unabsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 125 µl of ethanol to destain the wells. One hundred (100) microlitres of the destaining solution was then transferred to a cuvette, and the absorbance determined at 590nm using a spectrophotometer (SPECTRUMLAB S23A) and the results expressed as percentage inhibition (Sandasi et al., 2008)

$$\text{Inhibition (\%)} = \frac{\text{ODcontrol}_{590} - \text{OD sample}_{590}}{\text{OD control}_{590}}$$

Where: OD is the optical density at 590 nm

2.6.5. Inhibition of biofilm growth and development (preformed biofilm)

The biofilm was allowed to form for 4 h before the addition of plant extracts at a concentration of 1 mg/ml in the wells. The formation of biofilm was then achieved by aliquoting 100 µl of a standardized (1·0 × 106 CFU m/l) culture of the test organisms into a 96-well microtitre plate. The plates were incubated at 37°C for 4 h to allow cell attachment. Following the 4 h incubation, 100 µl of each plant extract was added to yield a final concentration of 1 mg m/l in the wells, and equal volumes of TSB and ciprofloxacin were added as negative and positive controls, respectively. The plate was further incubated for 24 h before the crystal violet assay is performed (Sandasi et al., 2008).

2.7. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC-MS analysis of *Ocimum gratissimum* ethanol extract was performed according to the methods described by Achi and Ohaeri (2015) on a GC-MS (Model: QP2010 PLUS Shimadzu, Japan) equipped with AOC-20i auto-sampler; VF-5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 µm film thickness. The column temperature was programmed at 80°C for 1 min, and was gradually increased to 200°C for 4 min and held for 10 min at 280°C. Helium gas (99.99 % purity) was used as a carrier gas at a constant flow rate of 1.58 ml/min. 4.0 µl of sample was injected in the split mode at a ratio of 1:10 and the sample injector temperature was maintained at 250º C. The electron ionization mass spectrometry was at 70 eV with a mass scan range of 40-600u. The total running time of GC-MS was 25 min and the relative percentage of each extract constituents were expressed as a percentage with peak area normalizations. The identification of the compounds was performed by comparing their mass spectra with that of the National Institute of Standard and Technology (NIST) 2008 libraries.

2.8. Statistical analysis

The data of each experiment are presented as mean values ± standard deviation of three determinations for antimicrobial and antibiofilm activity. One-way analysis of variance (ANOVA) was carried out using IBM SPSS Statistics version 21. Values of $p < 0.05$ were considered to be statistically significant.

3. Results and Discussion

3.1. Yields of plant extracts after extraction with different solvents

Table 1. Percentage yield of *Ocimum gratissimum* with various solvents

Ocimum gratissimum		
Solvent	**Actual Yield (g)**	**Percentage Yield**
Water	17.63	35.26
Ethanol	4.04	8.08
Methanol	13.88	27.76

Molecular weight of Water = 18.01528 g/mol; Ethanol = 46.07 g/mol and Methanol = 32.04 g/mol

Extraction of the plant material was performed using water, ethanol and methanol as described earlier. Quantitatively, the best solvent was water which yielded 35.26%. This was followed by methanol (27.76%) and ethanol (8.08%) as shown in Table 1.

3.2. Biofilm formation and quantification

Each of the four Bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were screened for biofilm formation. The results of the screening for biofilm formation and quantification are shown in table 2. *P. aeruginosa* ATCC 10325 produced the highest biofilm 0.83 ± 0.006 Biofilm Formation Index (BFI) and was quantified as strong biofilm producer. *S. aureus* ATCC 12540 produced the highest biofilm 0.70 ± 0.003 and was also quantified as strong biofilm producer.

Table 2. Biofilm formation and quantification of the test organisms

Name of organism	Biofilm (OD_{570})	Biofilm producing ability
Control (TSB)	0.14 ± 0.006	Nil
S. aureus ATCC 12540	0.70 ± 0.003^{b}	Strong producer
S. aureus ATCC 12600	0.15 ± 0.009^{g}	Poor producer
S. aureus ATCC 12660	0.27 ± 0.003^{f}	Poor producer
S. aureus ATCC 12732	0.42 ± 0.012^{d}	Moderate producer
P. aeruginosa ATCC 10200	0.39 ± 0.003^{e}	Moderate producer
P. aeruginosa ATCC 10325	0.83 ± 0.006^{a}	Strong producer
P. aeruginosa ATCC 10145	0.41 ± 0.003^{d}	Moderate producer
P. aeruginosa ATCC 10528	0.62 ± 0.007^{c}	Moderate producer

Each value is mean ± SD (n=3). $^{a, b, c, d, e}$ *$p < 0.05$ in comparison with Control (TSB).*

3.3. Antimicrobial screening of the plant extracts

3.3.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The ethanol, methanol and water extracts of *Ocimum gratissimum* was assessed on growth inhibitory ability against planktonic cells of *S. aureus* and *P. aeruginosa*. Inhibition of bacterial growth is observed when the solution in the well remained clear after incubation. The type of solvent used for the extraction greatly influenced the antimicrobial activities of the extracts, with the ethanol extracts showing greater activities against all test organisms than the methanol and aqueous extract (Table 3). *S. aureus* was more susceptible to the extracts than *P. aeruginosa* as evidenced by its lower MBC values compared to that of *P. aeruginosa* with MIC and MBC values <1 mg/ml. Also, biofilms of *S. aureus* showed more susceptibility to the extracts of *Ocimum gratissimum* compared to biofilms of *Pseudomonas aeruginosa* with MBIC values > 1 mg/ml. The positive control, ciprofloxacin belonging to the β-lactam antibiotics which is inhibitor of cell wall synthesis was found to possess high inhibitory activity against the two organisms under investigation.

Table 3. Antimicrobial activity of *Ocimum gratissimum* against *S. aureus* and *P. aeruginosa*

	MIC (mg/L)		MBC (mg/L)		MBIC (mg/L)	
	P. aeruginosa	***S. aureus***	***P. aeruginosa***	***S. aureus***	***P. aeruginosa***	***S. aureus***
Control	0.06 ± 0.10	0.05 ± 0.03	0.10 ± 0.04	0.05±0.08	0.13 ± 0.02	0.13±0.09
Water	0.63 ± 0.07^{a}	0.50 ± 0.07^{c}	0.75 ± 0.02^{a}	0.63 ± 0.11^{b}	1.50 ± 0.05^{b}	1.13 ± 0.07^{b}
Ethanol	0.63 ± 0.09^{a}	0.16 ± 0.06^{a}	0.75 ± 0.02^{a}	0.16 ± 0.06^{a}	1.13 ± 0.03^{a}	1.00 ± 0.12^{a}
Methanol	0.75 ± 0.05^{b}	0.38 ± 0.03^{b}	1.00 ± 0.05^{c}	0.75 ± 0.10^{c}	1.50 ± 0.01^{b}	1.13 ± 0.07^{b}

Each value is mean ± SD (n=3). $^{a, b, c}$ *$p < 0.05$ in comparison with Control (Ciprofloxacin).*

3.4. Antibiofilm activity of plant extracts

3.4.1. Inhibition of cell attachment

The extracts of the *Ocimum gratissimum* was assessed for the inhibition against cell attachment to PVC (Table 4). However, none of the extracts showed complete inhibition of cell attachment including the positive control ciprofloxacin. Inhibition was expressed as percentage values with the most active extract showing the highest percentage inhibition and vice-versa. The ethanol extract of *Ocimum gratissimum* showed the highest inhibition of 61.70% and 43.14% for the *S. aureus* and *P.*

aeruginosa, respectively. *P. aeruginosa* was more resistant than *S. aureus* as evidenced by its lower percentage inhibition values compared to that of *S. aureus.*

Table 4. % Inhibition of cell attachment of *S. aureus* and *P. aeruginosa* after exposure to plant extracts at a concentration of 1 mg/ml

Extracts	***S. aureus* Absorbance (OD_{590})**	**% Inhibition of cell attachment**	***P. aeruginosa* Absorbance (OD_{590})**	**% Inhibition of cell attachment**
Ciprofloxacin	0.10 ± 0.021	78.72	0.17 ± 0.038	70.59
O. gratissimium ethanol	0.18 ± 0.027	61.70[a]	0.30 ± 0.019	43.14[a]
O. gratissimium methanol	0.21 ± 0.019	55.32[b]	0.34 ± 0.050	33.33[b]
O. gratissimium aqueous	0.20 ± 0.015	57.45[b]	0.29 ± 0.042	41.18[a]

Each value is mean ± SD (n=3). [a, b] $p < 0.05$ in comparison with Control (Ciprofloxacin)

Table 5. % Inhibition of preformed biofilm of *S. aureus* and *P. aeruginosa* after exposure to plant extracts at a concentration of 1 mg/ml.

Extracts	***S. aureus* Absorbance**	**% Inhibition of preformed biofilm**	***P. aeruginosa* Absorbance**	**% Inhibition of preformed biofilm**
Ciprofloxacin	0.31 ± 0.033	55.71	0.49 ± 0.015	40.24
O. gratissimium ethanol	0.55 ± 0.019	21.43[a]	0.77 ± 0.050	6.10[a]
O. gratissimium methanol	0.65 ± 0.060	7.14[b]	0.85 ± 0.033	-3.67[b]
O. gratissimium water	0.54 ± 0.014	21.43[a]	0.84 ± 0.051	-2.44[b]

Each value is mean ± SD (n=3). [a, b] $p < 0.05$ in comparison with Control (Ciprofloxacin)

3.4.2. Inhibition of growth of a preformed biofilm

The ability of the extracts to inhibit growth of a preformed biofilm was accessed and none of the extracts was able to inhibit growth of a preformed biofilm by at least 50% (Table 5). The ethanol and aqueous extracts of the plant had the same percentage inhibition of 21.43% against *S. aureus* but not *P. aeruginosa*. The methanol and aqueous extracts of the plant enhanced biofilm growth in *P. aeruginosa* as evidenced by the increase in biomass compared to the control biofilm. *O. gratissimium* ethanol extract showed negligible activity against biofilm of *P. aeruginosa*.

3.5. GC-MS analysis of ethanol extract of *Ocimum gratissimum* leaves

The chromatogram result of GC-MS analysis of the ethanol extract of the *Ocimum gratissimum* (Fig.1) showed a total of fourteen compounds with their molecular weight identified. This includes four prominent peaks with retention time range of 18.558 - 27.883. The largest peak with retention time of 21.242 and peak area of 21.11% denotes Oleic acid. Other peaks with retention time of 18.558 (peak area 20.13%), 27.883 (peak area 16.82%) and 21.683 (peak area 11.53%) denotes the presence of n-Hexadecanoic, Squalene and z-2- Octadecen-1-ol acetate respectively. Other less prominent peaks at other retention times are shown in Table 6.

Table 6. Identified phytochemical compounds in *Ocimium gratissimum* with their retention time, molecular weight and composition.

NO	RT	Name of compound	Molecular formular	MW	Nature of Compound	Peak area %	Activity
1	4.958	Benzene, 1 methyl-2-(1-methylethyl)	$C_{10}H_{14}$	134	Monoterpene	0.93	Antioxidant
2	9.167	Benzenemethanol, 4-(1-methylethyl)	$C_{10}H_{14}O$	150	Alcohol	3.78	Antimicrobial
3	11.125	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl	$C_{15}H_{22}$	202	Sesquiterpene	0.39	Antimicrobial, anti-inflammatory
4	11.650	2, 6- Octadien-1-ol, 2, 7-dimethyl-	$C_{10}H_{18}O$	154	Alcohol	0.95	Anti-inflammatory, Analgesic activity
5	12.91	Benzene, (2-nitroethenyl)	$C_8H_7NO_2$	149	Nitrogen compound	1.24	No activity
6	14.533	1H-3a, 7-methanoazulene, octahydro-1, 4, 9, 9- tetramethyl	$C_{15}H_{26}$	206	Sesquiterpene	1.59	No activity reported
7	15.417	1-Octadecyne	$C_{18}H_{34}$	250	Alkyne	2.66	Catalytic polymerization
8	18.558	Palmitic acid (n-Hexadecanoic acid)	$C_{16}H_{32}O_2$	256	Fatty acid	20.13	Antibacterial
9	20.183	11- Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	Ester	6.35	Antifungal, anti-cancer
10	20.458	Phytol	$C_{20}H_{40}O$	296	Diterpene	4.11	Anti-proliferative, antioxidant, anticancer
11	21.242	Oleic acid (Cis-9-octadecenoic acid)	$C_{18}H_{34}O_2$	282	Fatty acid	21.11	Antimicrobial
12	21.683	Z-2-Octadecen-1-ol acetate	$C_{20}H_{38}O_2$	310	Ester	11.53	Antimicrobial
13	25.133	2-Isopropenyl-5-methylhex-4-enal	$C_{10}H_{16}O$	152	Aldehyde	8.43	Antifungal activity
14	27.883	Squalene	$C_{30}H_{50}$	410	Terpene	16.82	Antioxidant, cytotoxic, antitumor

RT = Retention time, MW = Molecular weight

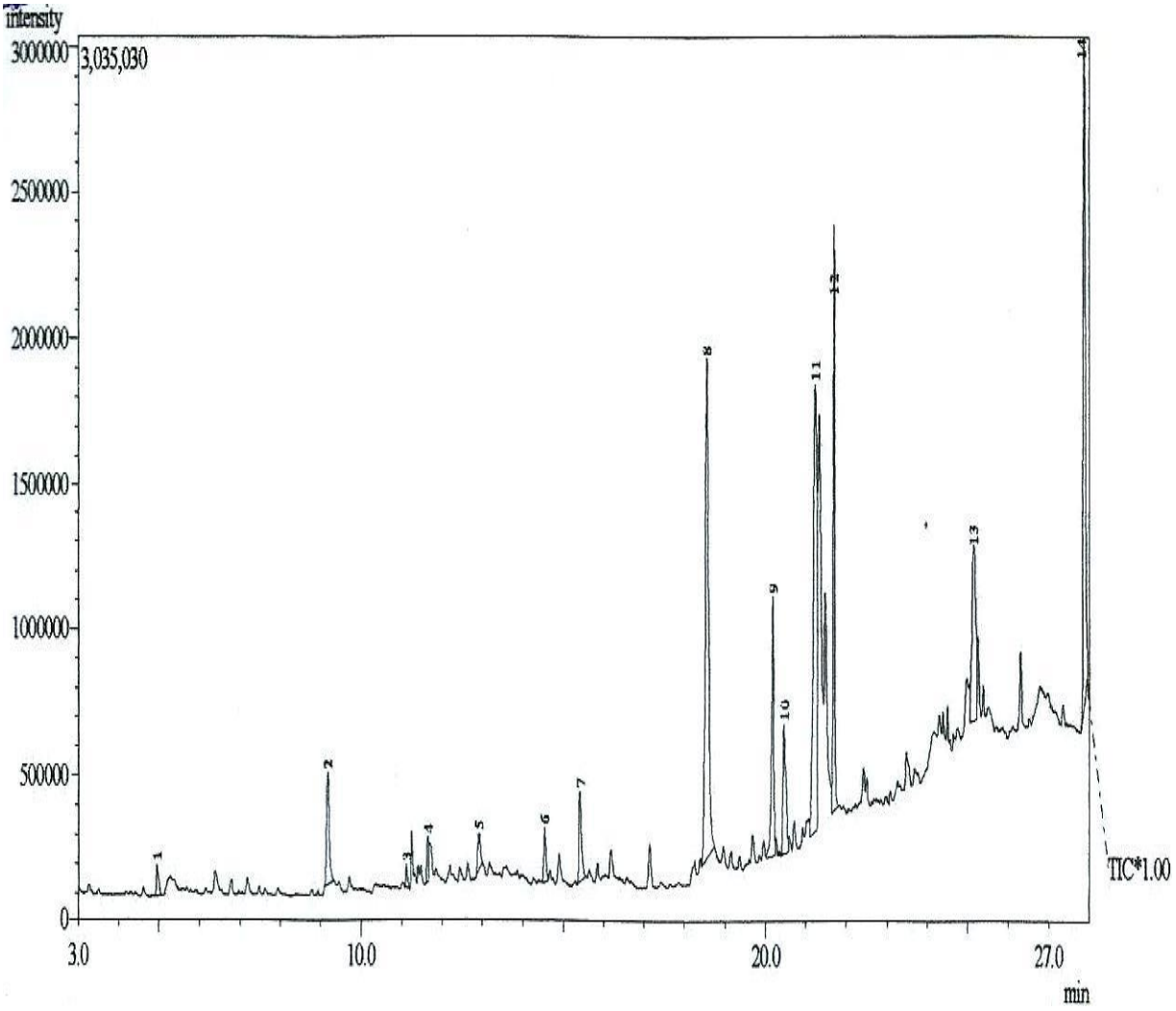

Figure 1. Chromatogram of *Ocimum gratissimum*

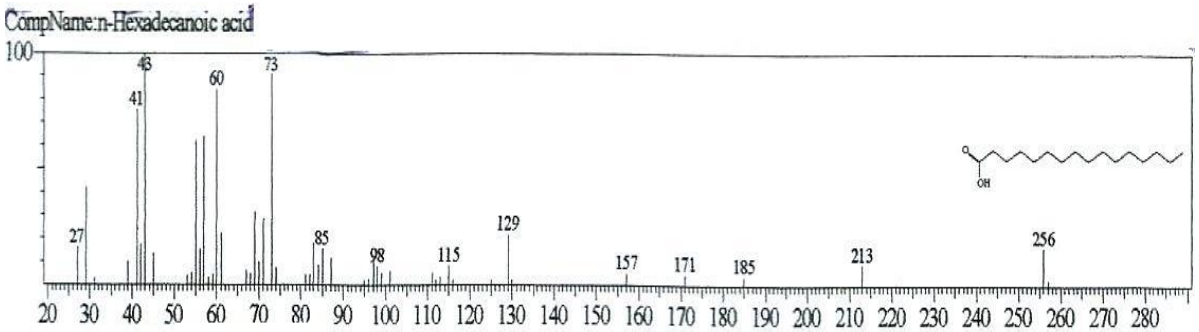

Figure 2. Mass spectrum of n-Hexadecanoic acid

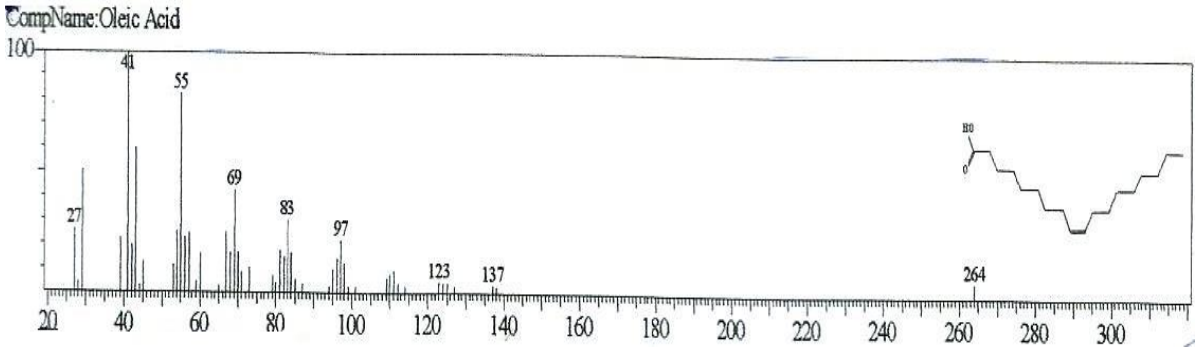

Figure 3. Mass spectrum of Oleic acid

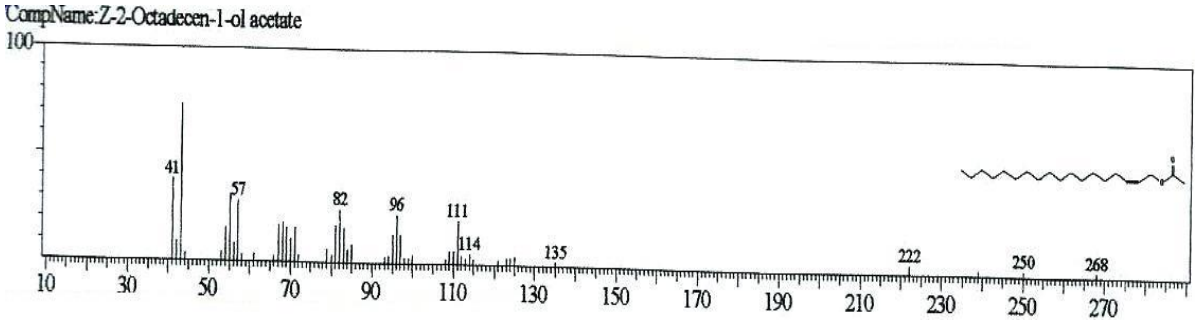

Figure 4. Mass spectrum of Z-2-Octadecen-1-ol acetate

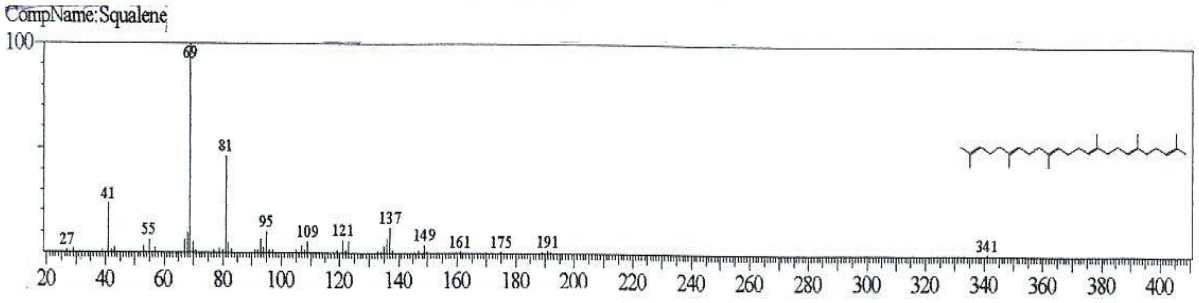

Figure 5. Mass spectrum of squalen

Ocimum gratissimum exhibited strong antimicrobial properties when extracted with ethanol. The difference in solubility which is as a result of differences in polarity among various solvent could be responsible for the variation in degree of activity. Bamidele et al. (2017) had earlier suggested in their study that the increase in activity of plant extracts could be as a result of the solvent used for extraction. Hence polar solvents tend to extract more polar compound in plants such as polyphenols and alkaloids. In this study, water showed higher yield of extracts compared to ethanol and methanol. This could be attributed to the bipolar nature of water which was able to extract more of non-polar constituents. Results obtained in this study revealed that the ethanol extract exhibited more antibiofilm activity due to the complete extraction of the bioactive components by the solvent. The bioactive compounds isolated from *Ocimum gratissimum* having high molecular weight were more of essential oils which is in line with the report of Matasyoh et al. (2007), Moghaddam et al. (2011) and Mith et al. (2016). The difference in composition of bioactive compounds and antimicrobial properties of the ethanol extract could be as a result of the extraction method.

The phenolic components are highly responsible for the antibacterial properties of essential oils (Cosentino et al., 1999). This is in agreement to the high phenolic content of *Ocimum gratissimum* reported by Igbinosa et al. (2013). Due to the hydrophobic nature of essential oils, they are able to disrupt structures because of separated lipid membrane of the bacterial cell which makes them more permeable (Sikkema et al., 1994). This makes the cell membrane prone to leakage of ions and other cell contents (Gustafson et al., 1998; Cox et al., 2000; Carson et al., 2002). In this study, phenolic compounds such as eugenol and thymol were not isolated in contrast to those by Matasyoh et al. (2007), Moghaddam (2011) and Dorman and Deans (2000).

Oleic acid is a naturally occurring monounsaturated fatty omega-9 fatty acid found in animals and vegetables oils; and is a major constituent of olive oil which is also present in fruits (Waterman and Lockwood, 2007). Oleic acid, practically regarded as non-toxic to humans has reported to be tolerated at an oral dose above 2.2 LB by humans above 70kg body (Gosselin et al., 1976).

The National Centre for Biotechnology Information database [http://pubchem.ncbi.nlm.nih.gov/compound/4456] highlighted that oleic acid is commercially used as a food addictive, an emulsifying agent in pharmaceuticals and as an antimicrobial agent in cosmetics. Oleic acid has been reported to show inhibitory and bactericidal properties against the Gram-positive bacteria Staphilococus aureus (Dilika et al. 2000; Desbois and Lawlor, 2013) which is in consonance with this study.

Palmitic acid is a saturated long-chain fatty acid naturally found in palm fruit. It is a colorless liquid with a density of 0.8527g/cu at 62°C and is soluble in organic compounds (Lide, 2005). A report by National Centre for Biotechnology Information: Palmitic acid [http://pubchem.ncbi.nlm.nih.gov/compound/ 985] shows low solubility in water with about 0.04 mg/L being sparingly insoluble at 25°C. This is in line with the low antimicrobial property observed by the aqueous extract. *Ocimum gratissimum* has an aroma threshold of 10ppm (Burdock, 2005) which makes it a good flavoring agent FAO/WHO (2014) Food additives evaluation: Palmitic acid (http://apps.who.int/food-additives contaminants-jecfa- database/chemical.aspx?chemID=652).

Palmitic acid can also find its use in the manufacture of laundry and skin care product where it exhibits its antimicrobial properties Cartron et al., 2014).

Squalene is an isoprenoid compound similar to beta-carotene with six unconjugated double bonds. Known to ancient fishermen as “miraculous oil” which is used to cure a wide range of ailments (Popa et al., 2015). It is a commercially obtained from shark liver oil and industrially used as a bactericide in the manufacture of pharmaceuticals (O’Niel, 2013), foods, cosmetics, health supplements and components of vaccines used to enhance the immune response WHO (2006) Squalene-based adjuvants in vaccines [http://www.who.int/vaccine_safety/commitee /topics/adjuvants/questions_and_answers/en].

In humans, squalene is a precursor in the biosynthesis of cholesterol and other steroids. About 60% of dietary squalene is absorbed and transported with very low density lipoproteins which is ubiquitously distributed in the human skin protecting the skin surface from lipid peroxidation due to exposure to UV rays (Kelly, 1999). Squalene demonstrates low toxicity to laboratory animals (Lewis, 2004). A dose dependent increase in cellular and immune function has been reported in squalene supplemented mice by Kelly (1999).

The diterpene Z-2-Octadecen-1-ol acetate has been isolated as one of the bioactive constituents of essential oil in several plants (Chalannavar et al., 2013; Nattudurai et al., 2014). Though much studies are yet to be carried out on its antimicrobial properties in the plant extract. The observed spectrum

antibacterial activity by the extracts of this plant could probably be attributed to the dominant presence of squalene, a terpenoid. These terpenoids have the potential to inhibit the growth of microbes and against biofilms through the detachment of planktonic cells from the biofilms which affects the integrity of the membranes (Perumal and Mahmud, 2013).

While screening the extracts for antimicrobial activity against planktonic bacteria, *Staphylococcus aureus* was found to be more susceptible to the extracts compared to *Pseudomonas aeruginosa* (Table 2). This was in line with the report of Fennel et al. (2004) that gram positive bacteria are susceptible to plant extracts than gram negative bacteria. This could be due to the fact that the cell walls of Gram-positive bacteria are more porous than Gram-negative bacteria (Nazzaro et al., 2013). The biofilms of *S. aureus* also showed more susceptibility to the extracts of *Ocimum gratissimum* compared to biofilms of *Pseudomonas aeruginosa*. Essential oils appear to be more active against gram positive than gram negative bacteria. (Burt, 2004; Yap et al., 2014). It has been assumed that the presence of lipopolysaccharide that surrounds the bacterial peptidoglycan inhibits the diffusion of hydrophobic essential oil into the cytoplasm (Vaara, 1992). However, studies on essential oils by Busatta et al. (2008); Prabuseenivasan et al. (2006); Wilkinson et al. (2003) came up with a different opinion that gram positive are less susceptible. (Busatta et al., 2008; Prabuseenivasan et al., 2006; Wilkinson et al., 2003). Another study by Ouattara et al. (1997) came to conclusion that no obvious difference between gram-positive and gram negative organisms after 24hrs. The highest antibiofilm activity was demonstrated by the positive control ciprofloxacin a standard antibiotic. This is probably due to fact that it exists in its pure form and has been proven to be effective against microorganisms (Prescott et al., 2002).

Essential oils are made of certain characteristics such as low pH, low water activity and low oxygen tension which enhances their role in food preservation. Antibacterial activity of essential oils depends on the availability of oxygen. When there is depleted oxygen, generation of energy via anaerobic metabolism makes cells more exposed to the toxic action of essential oils (Paster et al., 1990). Essential oils confer pleasant flavours on food when used as antibacterials (Burt, 2004). The distinctive flavour of *Ocimum gratissimum* porpularly known as scent leaf enhances the flavour of foods (Oriakhi et al., 2013). This flavor imparted on food could be of a great advantage when used as a food preservative.

4. Conclusions

Volatile hydrocarbons, terpenes and essential oils were isolated from the extract of *Ocimum gratissimum*. Among the isolated compounds, it was observed that more of the bioactive components were essential oils. Antimicrobial analysis showed that gram-positive *Staphylococcus aureus* are generally more sensitive to the plant extracts than gram-negative *Pseudomonas aeruginosa*. Antibiofilm potential and biosafety of bioactive compounds from *Ocimum gratissimum* reveals a prospective active principle that could be of use against biofilm associated menace in food, medicines and also as preservatives in cosmetics. The antibiofilm activity of the plant could be applied both in food processing industries and locally processed foods against food microorganisms and for the extension of the shelf-life of products.

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EFFECT OF FAR INFRARED PRE-PROCESSING ON MICROBIOLOGICAL, PHYSICAL AND CHEMICAL PROPERTIES OF PEANUTS

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ABSTRACT

In this study, we investigated the use of far infrared (FIR) heating for the surface decontamination of raw peanuts. We evaluated the reduction of natural microflora in terms of total mesophilic aerobic bacteria (TMAB) count which is an indicator of food hygiene. Depending on the treatment conditions, we obtained microbiologically sterile peanuts. We also analyzed the effects on the quality of various parameters (moisture content, colour, texture, peroxide value) of the FIR process. FIR heating not only significantly reduced the number of microorganisms, but also substantially protected the quality of the attributes. Our experimental study results suggest that continuous-system far infrared surface pasteurization equipment could be used commercially to reduce the microbial load of peanut samples immediately after harvest and to minimize the risk of post-harvest microbial growth during storage.

1.Introduction

Peanuts (*Arachis hypogaea*) are used worldwide as an economical food source as they contain high-quality proteins and unsaturated fatty acids while also being rich in minerals. They can be consumed whole, roasted or boiled and as ingredients in a variety products (Friedman, 1996; Duranti, 1997; Hammond et al., 1997; Atif et al., 2000; He et al., 2005; Kornsteiner et al., 2006; Sousa et al., 2011). However, peanuts are a food stuff that is very susceptible to aflatoxin contamination.

Aflatoxigenic fungi survive in a wide range of environments and their colonisation in pre-harvested peanuts often results in aflatoxin accumulations in the post-harvest period under conditions that favour contamination (Wu et al. 2016). Therefore, the elimination of this fungus before toxins are produced must be the primary objective, rather than the removal of toxins after production. While many strategies have been suggested for suppressing fungal growth, these often require sophisticated equipment and expensive chemicals or may cause unacceptable detrimental effects to nutritional quality (Basaran et al., 2008) As such, there is increased research interest in discovering practical, inexpensive, timesaving and effective treatment methods that reduce surface fungal contaminations while preserving food quality.

Several researchers have used infrared (IR) radiation in the decontamination and disinfection of food and food-contact surfaces and in the inactivation of bacteria, spores, yeast and mould in both liquid and solid foods (Rosenthal et al. 1996; Hamanaka et al. 2000; Hebbar et al. 2003; Jun & Irudayaraj 2004; Huang 2004; Tanaka et al. 2007; Krishnamurthy et al. 2008; Huang & Sites 2008; Staack et al. 2008). Due to its advantages of being a controlled, rapid and targeted heating application, IR technology shows great

potential in a variety of applications (Afzal et al. 1999).

In recent years, the application of IR heating for pathogen inactivation has gained much attention. The studies mentioned above show that infrared heating provides a significant reduction in the microbial load of food materials as well as significantly protecting the quality parameters. Therefore, our first objective was to investigate the detrimental effect of far infrared (FIR) treatment on the natural microbial load and, secondly, to investigate the changes in the physical and chemical quality characteristics of peanuts during FIR processing.

2. Materials and methods

2.1. Materials

2.1.1.Samples

We obtained unshelled peanuts with reddish-brown skin from a peanut storage and processing factory in Osmaniye, Turkey (Virginia type, NC 7 cultivar) and kept them in cold storage at 4 °C until analysis.

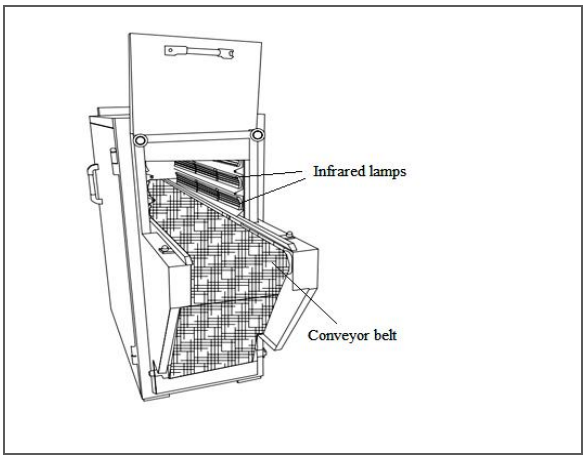


Figure 1. Schematic diagram of FIR heating equipment.

2.2. FIR heating process

We used custom-designed FIR heating equipment (Figure 1.) to determine the effect of infrared rays on the microbial load and the quality characteristics of the peanut samples. For a detailed description of the FIR system, please refer to Erdoğdu and Ekiz (2011). We spread the peanuts (15 ± 1g; 15–20 kernels) in a single layer on a wire mesh, which we then fed into the FIR tunnel oven at setting temperatures ranging between 150 °C to 400 °C and for different holding times.

2.3.Determination of microbial load

We determined the total mesophilic aerobic bacteria (TMAB) contents of the peanut kernels to investigate the influence of FIR heating on reductions in the natural microbial load. Following the FIR heating, we transferred the peanut samples from the wire mesh to sterile Petri dishes. We aseptically weighed 10 g of peanuts and placed them a 250-mL volumetric flask containing 90 mL of sterile peptone water (0.1% w/V). We then vortexed the flask (IKA, Germany) at 1400 rpm for 1 min. After performing serial dilutions in 0.1 mL steps, we spread the peanuts onto sterile plate count agar (PCA) to determine the TMAB count then, incubated Petri dishes at 37 °C for a period of 24–48 hours in an incubator (Velp, Italy). We then counted the colonies and determined the number of microorganisms in the samples.

2.4. Moisture content determination

We determined the moisture content of peanut samples by the oven drying method (AOAC, 2000), which involves measuring the amount of water removed from the sample after heating at 105 °C.

2.5.Colour measurements

Colour of the raw and FIR-treated peanut samples was measured using a Colour Quest XE colorimeter (Hunter Lab, U.S.A.) and determined their lightness (L*), redness (a*), yellowness (b*) and chroma (C) parameters. To do so, we filled Hunter Lab cuvettes with randomly selected samples and took colour readings from the middle area of both sides of the cuvette. We then averaged the total of ten readings for each temperature–time combination in the FIR heating procedure (Eser, 2012).

2.6. Texture analysis

We performed texture measurements at ambient temperature using a Texture Analyser (TA-XT2i, Stable Micro Systems Ltd., Surrey, UK) and used a twenty-five-mm diameter cylinder probe for the compression tests. We compressed each peanut kernel to 5 mm with the probe touching the sample at a test speed of 5 mm/s and performed twenty replications for each FIR-treated sample. To evaluate the textural properties of the peanut samples, we considered the total work done (total area under the force–deformation curve during compression) (Eser, 2012).

2.7. Peanut oil analysis

We fed peanut samples into the FIR tunnel, which we had set at 300 °C, and kept them there for 60 s, 120 s, 180 s and 240 s. Then we pressed the peanut samples and obtained peanut oil using a cold press machine (Koçmaksan KMS 10, İzmir, TURKEY). After centrifugation of the peanut oil, we determined its peroxide value, and fatty acids–methyl esters (FAME) compound. We determined the peroxide value using the standard American Oil Chemists' Society (AOCS, 2002) method. We express the values as milliequivalents of peroxide per kilogram of the sample, which oxidiseszes KI. We determined the fatty acid composition by the method outlined by Hammond (1991).

2.8. Statistical analysis

To determine the effect of FIR heating on peanut texture, we conducted an independent statistical analysis for each FIR setting temperature using IBM SPSS Statistics 20 software and used the Tukey test to adjust for multiple comparisons.

3.Results and discussions

3.1. Effect of FIR radiation on microbial load

We determined the total plate count, which is an indicator of the overall food hygiene. Figure 2 shows the reductions in the TMAB populations in the peanut samples during FIR heating. The allowable TMAB count in legume products is 10^4 CFU/g (Kayode et al. 2011). We observed a reduction in bacteria population of the peanut samples to a specified level of 1×10^4 CFU/g upon FIR temperatures of 150 °C to 400° C for holding times of 240 s, 150 s, 60 s, 50 s, 45 s and 25 s and sterilisation (total elimination) obtained more than 4 log reductions in terms of TMAB counts at all temperatures. Combined with the results of our previous work on the TMY (Total Mold and Yeast) population (Eser and Ekiz, 2016), the present data show that infrared heating is very effective method to reduce natural microflora of peanut. In parallel with these findings and using the same FIR heating equipment, Erdoğdu and Ekiz (2011, 2013) observed TMAB counts in cumin seeds to be reduced by approximately 2 log units after 2.5 min. processing at 300° C. Additionally, they found TMAB counts in black pepper samples to be reduced by about 7 log units after 4 min at 350 °C. So, we can see that a similar reduction in the TMAB count is achieved for both peanuts and spices.

The natural microbial load reduction indicates the potential of the FIR process to efficiently decontaminate all types of nuts. In further studies, the decontamination efficiency of FIR heating should be evaluated with respect to the reduction in moulds such as *Aspergillus flavus* and *A. parasiticus*, which are of key concern in the peanut industry.

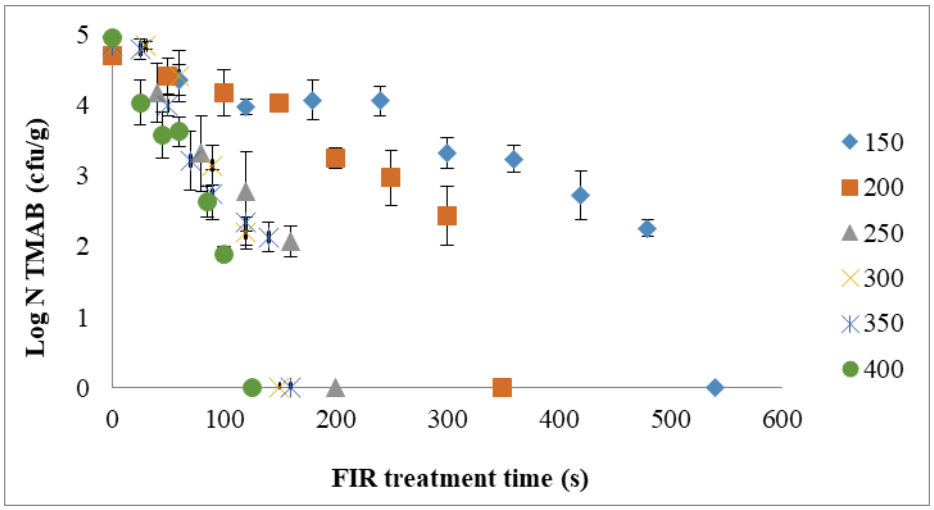


Figure 2. Effect of FIR treatment on total mezophylic aerobic bacteria counts of peanut kernels.

3.2. Changes in quality parameters

The moisture removed from the kernels was increased significantly with increasing time and temperature. Figure 3 shows the estimated moisture content for the selected FIR treatment conditions.

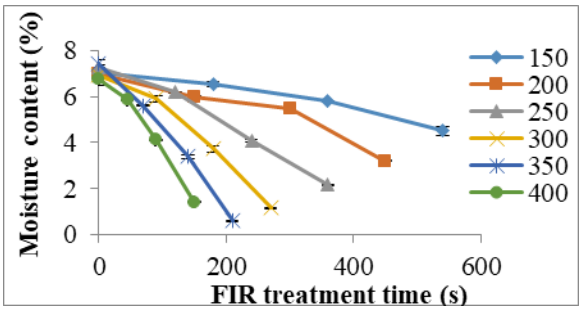


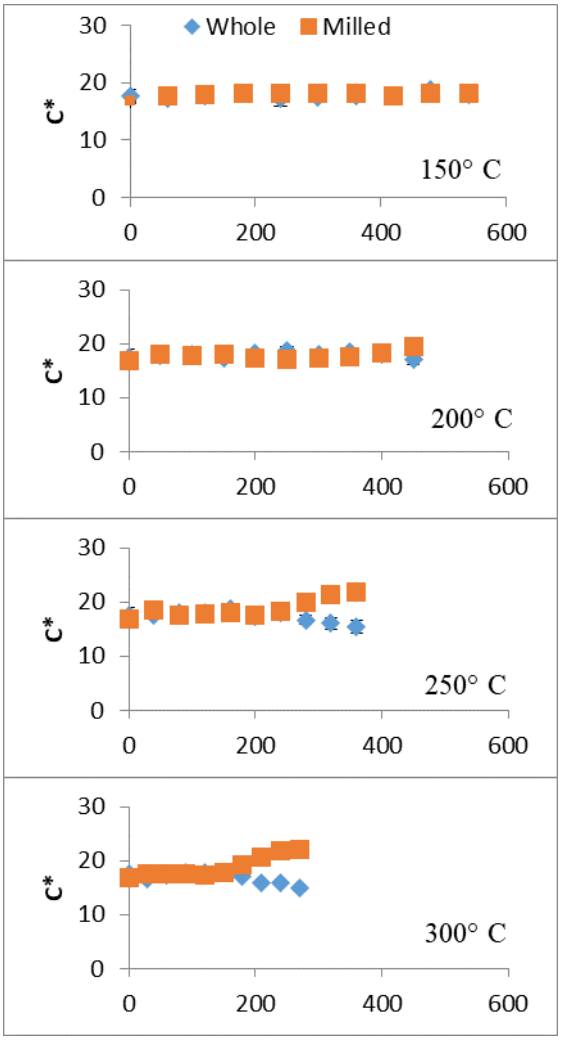
Figure 3. Effect of FIR treatment on moisture content of peanut kernels.

Fungi can grow exponentially in storage due to the combined heat and high humidity. Initial tolerable levels of aflatoxins become intolerable after improper drying and or improper post-harvest storage. To minimise the risk of post-harvest mould growth, each producer's goal should be to dry crops as soon as possible to obtain microbiologically safe products. IR heating is an efficacious drying method compared to conventional heating processes due to its highly efficient surface heating.

Next, we measured the L* (lightness), a* (redness) and b* (yellowness) parameters of the peanut samples. The initial L*, a* and b* colour parameters of whole peanut samples were 54.79, 9.12 and 14.47, respectively. There was no significant change in any of the colour parameters for short processing durations. However, longer treatment times and higher temperatures had a remarkable effect on the colour properties. Chroma is a measure of saturation or purity, and a sample with a high chroma is more vivid than one with a low chroma value. We determined C* (chroma) from the a and b coordinates, calculated using the square root of the sum of $(a^*)^2$ and $(b^*)^2$. We measured chroma in both the whole and milled samples (the milling process was performed after FIR treatment with a miller (IKA Werke M 20 Universal Mill, Germany). Figure 4 shows the beginning of colour changes during FIR heating. Detectable colour changes in the C* value occurred after FIR treatment for 540 s, 400 s, 240 s, 150 s, 120 s and 85 s at 150 °C, 200 °C, 250 °C, 300 °C, 350 °C and 400° C, respectively. This finding clearly shows that when there is a total elimination of the bacteria count of the samples, the peanut skin colour is not yet affected by the FIR

heating. Similarly, the colour parameters were not affected by infrared processing in previous studies conducted by Erdoğdu and Ekiz (2011, 2013) in cumin and black pepper, Huang (2004) in frankfurters, Bingöl et al. (2011) in almonds or Eliasson et al. (2014) in oregano.

Textural properties vary widely with the moisture content of food materials as low moisture content can cause brittleness, crispiness and harder surfaces. During FIR heating, we found the moisture content of peanut samples to be significantly reduced.



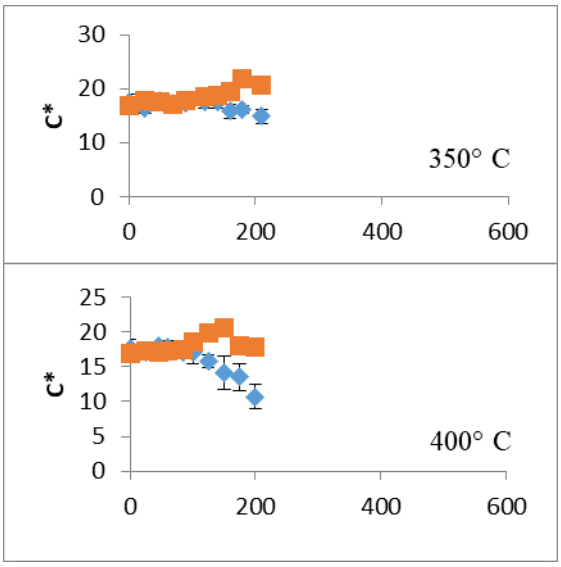


Figure 4. Effect of FIR treatment on C* value of whole and milled peanut samples.

This reduction is closely associated with the textural properties of peanuts, so we analysed the changes in total consumed work. We found the initial work for the compression to be 210 Nmm. We observed decreases with increased processing time, which indicates that the structure had become more brittle. Table 1 shows our statistical analysis of the texture of the FIR-treated peanuts. The different letters in the same columns indicate significant ($P < 0.05$) differences. Although the IR heating temperature and times did not affect the texture of the samples, we observed the untreated samples to differ from the treated ones at all temperatures.

Texture is one of the key quality attributes for evaluations of product quality and acceptability and plays a significant role in nut processing. Hence, to obtain products with desirable textural properties, it is essential to optimise the conditions for all processes (surface decontamination, drying, roasting). Based on our results, it is possible and practical to estimate the preferred physical and microbiological quality characteristics of peanut kernels by

controlling the surface temperature. The decreased hardness with increased processing time during pulsed infrared roasting of groundnuts was observed by Kumar et al. (2009), who found the compressive strength to be reduced from approximately 80 N to 17 N. Saklar et al. (1999) observed a significant decrease in the first fracture point during the roasting of hazelnuts. Although the effects of these roasting processes on textural properties were marginal, this marginal change resulted in nuts with better texture. Our purpose in this study was not to roast peanuts. As such, our results are not comparable with those mentioned above. Our primary aim was microbial decontamination without compromising the physical quality of peanuts.

We consider FIR heating to be a preservation method for peanuts that are stored for different uses such as peanut oil, peanut butter, peanut brittle and so on. Therefore, we examined the effect of FIR heating on the quality of peanut oil. The peroxide value (PV) provides a measure of the oxidative deterioration of oil or fat, as well as their storage stability. Here, the PV increased from 2.25± 0.62 meq/kg to 4.96 ±0.26 meq/kg after 240 s at 300 °C (Table 3). The maximum limit for the PV of cold pressed peanut oil is specified as 15 meq/kg in the Turkish Food Codex, Vegetable Oil Legislation: 2012/29. Our results were considerably lower than the given value.

Microwave roasting of peanuts has been found to increase the PV from 1.03 meq/kg to 3.08 meq/kg with increases in processing duration (Megahed, 2001). Makeri et al. (2011) investigated the effect of roasting temperature on PV and studied the conditions that increase the PV by 100–200%.

In addition to PV, we determined the fatty acid composition of peanut samples. As shown in Table 2, the major fatty acid components include oleic acid (57.75%), linoleic acid (22.58%) and palmitic acid (8.41%). Peanut oil also contains stearic acid, behenic acid, arachidic acid, lignoseric acid and eicosenoic acid. We found FIR heating to have no effect on the fatty acid composition of peanut oil.

Table 1. Effect of FIR treatment on textural properties of peanut samples.

FIR setting temperature (°C)	FIR heating duration (s)	Compression work (Nmm)
150	0	$206,5^{ab}$
	60	$222,4^{b}$
	120	$181,8^{ab}$
	180	$186,6^{ab}$
	240	$183,4^{ab}$
	300	$154,4^{ab}$
	360	$179,6^{ab}$
	420	$160,2^{ab}$
	480	$179,8^{ab}$
	540	$149,2^{a}$
200	0	$188,4^{ab}$
	50	$190,8^{b}$
	100	$171,6^{ab}$
	150	$160,1^{ab}$
	200	$157,4^{ab}$
	250	$179,2^{ab}$

	300	155,3[ab]
	350	132,3[b]
250	0	194,3[a]
	40	203,2[a]
	80	165,7[a]
	120	167,2[a]
	160	164,8[a]
	200	157,6[a]
300	0	187,1[c]
	30	210,2[ab]
	60	167,4[b]
	90	175,7[ab]
	120	175,0[ab]
	150	154,9[a]
350	0	210,3[a]
	25	178,5[a]
	50	163,1[a]
	70	182,8[a]
	90	179,3[a]
	120	158,2[a]
400	0	251,7[c]
	25	205,9[bc]
	40	196,1[abc]
	60	182,4[ab]
	85	173,5[ab]
	100	139,8[a]

Table 2. Effect of FIR treatment on fatty acid composition and peroxide value of peanut samples.

FIR treatment time at 300° C					
Fatty acids	**0**	**60 s**	**120 s**	**180 s**	**240 s**
Palmitic	8,41	8,45	8,39	8,51	8,39
Stearic	3,60	3,66	3,74	3,68	3,72
Oleic	57,75	57,55	57,81	57,80	58,35
Linoleic	22,58	22,52	22,24	22,49	22,13
Arachidic	1,84	1,89	1,91	1,86	1,85
Eicosenoic	1,10	1,10	1,10	1,08	1,06
Behenic	3,27	3,36	3,36	3,20	3,15
Lignoceric	1,44	1,47	1,47	1,38	1,34
Peroxide value	2,25	3,02	3,76	4,64	4,97

Based on these results, we conclude that we achieved complete elimination of the natural microflora of the samples. In our investigation of the effects on quality parameters (moisture content, colour, texture, PV) of FIR processing, we found FIR heating to not only significantly reduce microorganisms, but also substantially protect the quality attributes. Therefore, IR is a promising technology for the surface pasteurisation of raw peanuts that does not compromise the raw peanut quality attributes. *In parallel with our study, many scientists* have reported the effects of infrared heating on the microbial load and quality characteristics of a variety of food samples. Maria et al. (2008) used IR heating to determine its effects on *Salmonella* Enteritidis in almonds and determined that the skin morphology, meat texture and

kernel colours of almonds were indistinguishable from those of untreated samples. Yang et al. (2010) reported a notable industrial intervention that used IR and sequential IR and hot air (SIRHA) in dry roasting almonds. They found SIRHA roasting to be the best roasting method for producing pasteurised roasted almonds, with the potential for reducing the roasting time compared to current hot air roasting methods. Bingöl et al. (2011) studied the reducing effect of infrared rays on the *Pediococcus* levels in raw almond samples contaminated with *Pediococcus* up to a load of 10^8 CFU/g. In that study, the authors achieved significant microbial reduction without affecting the colour parameters. Similarly, Erdoğdu and Ekiz (2011, 2013) investigated the effect of FIR rays on the TMAB and TMY loads in cumin and black pepper seeds. In both studies, the microbial loads of cumin and black pepper samples were reduced to the targeted levels within short treatment times without causing any changes in the volatile oil contents and colours of the samples. Additionally, Erdoğdu (2011) reported in her doctorate thesis the sterilisation of cumin samples in terms of TMY by the use of industrial-scale FIR heating equipment. Whereas the initial microbial load of cumin was $10^{3.6}$ cfu/g, after heating for 1.5 min. at 200 °C in this equipment, the cumin samples were determined to be free of mould and yeast. Eliasson et al. (2014) researched IR heating as a novel decontamination technology for oregano inoculated with *B. cereus* spores. When treated at 90 °C, the authors achieved a significant microbial reduction, with the colour only slightly affected by the IR treatment. Also, the key aroma compounds carvacrol and thymol indicated that the characteristic oregano aroma had been preserved after IR treatment.

4. Conclusions

In this study, we successfully used far infrared heating in the post-harvest decontamination of peanut surfaces. We completely inactivated the microorganisms (< 1 log cfu/mL) without causing changes in the quality characteristics of raw peanut samples. With the microbial elimination, we found no significant difference between untreated and FIR-treated samples with respect to colour, texture or PV. Therefore, we suggest that far infrared heating technology would be an effective method for rapidly achieving the desired moisture content, thereby reducing the microbial load and protecting the quality of the kernels during storage. In summary, the infrared processing of nuts is clearly beneficial for minimising post-harvest mould growth and the risk of toxin occurrence. However, in light of the limited number of studies on the infrared decontamination of aflatoxin-forming moulds, we plan to undertake further research to determine the detrimental effects, if any, of far infrared heating of target microorganisms in nuts.

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INOCULATION OF SELECTED LACTIC ACID BACTERIA IN RED WINES – DETERMINING THE RIGHT TIME

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ABSTRACT

Scientific research undertaken worldwide in wine biotechnology reveals that, along with natural factors, biological factors will determine the quality of wines. The main objective of the paper is to determine the right time for inoculating the selected lactic acid bacteria. In this paper, we tested the efficiency of the commercial preparation Inoflore R (containing the *Oenococcus oeni* species) to induce and perfect the malolactic fermentation of red wines produced in the Miniş-Măderat vineyard/Arad County/Romania. We experimented with 3 work alternatives: direct inoculation (without reactivation), wine reactivation, and must reactivation. Environment composition, the method of reactivation and the use of bacterial cultures, as well as the time of inoculation influence the duration of the malolactic fermentation and wine quality. The shortest duration of malolactic fermentation takes place in the case of must reactivation, and the longest is recorded for direct wine inoculation (without reactivation). In addition, musts, by their chemical composition, represent a good medium for the growth and development of lactic acid bacteria, and wines from these musts have a low content of undesirable substances, as confirmed by a moderately volatile acidity and a D-lactic acid content within reasonable limits. In conclusion, the results obtained in this paper certify the microbiological and technical-economic advantage of applying the protocol of must reactivation.

1. Introduction

Spontaneous malolactic fermentation is unpredictable and can be initiated only after long periods of delay. This "waste of time" may represent a considerable cost for producers. On the other hand, delays increase the risk of developing contaminating microorganisms that can generate abnormal tastes and smells. For this reason, it is absolutely vital to manage malolactic fermentation by using selected malolactic bacteria (Henick-Kling and Acree, 1998). If spontaneous malolactic fermentation involves lactic acid bacteria of the genera *Lactobacillus*, *Leuconostoc* *Pediococcus* and *Oenococcus*, the *Oenococcus oeni* species specializes in the initiation and development of directed malolactic fermentation (Davis *et al*., 1988).

Scientific research has proved and winemaking has confirmed the need for malolactic bacteria commercial preparations to obtain top quality red wines and to obtain white and rosé wines with complex sensory characteristics from crops with high acidity (Croitoru *et al*., 2003). The first selected malolactic bacteria cultures appeared in the early '90s and required an intense phase of reactivation and acclimatization in wine for it to "work". These malolactic bacteria

required a long and rigorous protocol for the preparation of the inoculum, as an obvious consequence of physiological difficulties that may be encountered in wine, due to their low adaptability to harsh environments, rich in alcohol (Fuster and Krieger, 2002). The success of bacterial inoculation into wine depends on the adaptability of the bacteria after the introduction into the fermentation medium, and on the moment of inoculation. The problem of environmental adaptability of bacteria appears to have been overcome today by applying direct inoculation bacterial preparations that do not require reactivation and which proved effective for most wines (Henick-Kling, 1995).

Inoculation with selected malolactic bacteria can be performed at different stages of alcoholic fermentation, i.e., before the onset of alcoholic fermentation (Croitoru, 2003), immediately after the onset of alcoholic fermentation (Renouf *et al*., 2008), during alcoholic fermentation (Rosi *et al*., 2003), in the final stage of alcoholic fermentation (Gindreau and Dumeau, 2005; Croitoru, 2009), or immediately after the completion of alcoholic fermentation (Alexandre *et al*., 2008).

Until now, more researches have been made which were aimed at determining the timing of the selected malolactic bacteria inoculation. Some authors (Beelman and Kunkee, 1985) insisted on the idea of co-inoculation consisting of the simultaneous administration of yeasts and selected malolactic bacteria into musts so that the two fermentation processes (alcoholic fermentation and malolactic fermentation) take place simultaneously. Most authors (Gindreau and Dumeau, 2005; Alexandre *et al*., 2008) are skeptical regarding the inoculation of musts, and suggest that the administration of selected malolactic bacteria be performed on wines and only after the full depletion of sugars. The same research direction is seen also with the authors (Rosi *et al*., 2003; Pillet *et al*., 2007a, 2007b), who have drawn attention to the risk of the occurrence of noticeable amounts of acetic acid and lactic acid in wine due to the metabolism of sugars in fermentation medium under the action of malolactic bacteria.

Still, even at small scale (pilot station), the available bacterial solution cannot be successfully used, but only after a reactivation phase (Krieger, 2002). Introduction of lactic bacteria directly in wines, induces cell death and reduces the efficiency of the process. Preparation leaven is an indispensable phase, because the anhydrous bacteria continue their growth in wine and to guarantee sowing success. The more efficient the use of this leaven is, the sooner is developed after the alcoholic fermentation, obtaining an energy economy, in so far as the wines keep a temperature between 16-20ºC (Valade and Laurent, 1995 apud. Gheorghiță *et al*., 2006).

Oenological equipment manufacturers, offers starter cultures of lactic bacteria in a wide range: liquid cultures, lyophilised cultures and frozen cultures.

2. Materials and methods

2.1. Materials

In this paper we experimentally evaluate the efficiency of the commercial concoction INOFLORE R/France (which contains the *Oenococcus oeni* species), for introduction and development of the malolactic fermentation in Oporto, Burgund, Cadarca, Pinot noir and Merlot wines, obtained from Minis-Maderat vineyard (Romania).

The commercial concoction INOFLORE R has biotechnological properties as follows:

- the bacterial population size $2x10^{11}$ UFC/g powder;
- minimal quality keep: 12 months at 4ºC, 18 months at -18ºC;
- optimal inoculation temperature 18-22ºC;
- pH tolerance 3.2 for an inferior pH;
- resistance to SO_2 : free < 10 mg/l, total < 60 mg/l;
- ethanol tolerance, maximum concentration 14% vol.

Figure 1 INOFLORE R commercial preparation used for managing malolactic fermentation (supplied by Enzymes&Derivates Romania)

2.2. Samples

The preparation of lactic bacteria leaven by reactivation in wine involves the following succession of technological operations:

- preparation of a 1 hl container ("pilot" container) to which 25 liters of wine and 25 liters of chlorine-free water will be added, resulting in a diluted suspension;
- administering a dose of 4 g/l of FERMOPLUS MALOLACTQUE, a nutrient needed for the growth and development of bacterial cells based on yeast cell walls;
- ensuring an optimal temperature of 22-24° C;
- inoculating the suspension with a dose of 1 g of selected malolactic bacteria (INOFLORE R), providing a density of $2x10^7$ cells/ml;
- maintaining an optimal temperature of 20-22° C for 24 hours in order to acclimate the selected malolactic bacteria to the restrictive environmental conditions; periodic homogenization is recommended at an interval of 3-4 hours;
- a 2-7 days temperature control is achieved at 20-22° C, allowing the metabolism of 2/3-3/4 of the initial malic acid content of the wine; the suspension in which the malic acid was metabolized is the final reactivated leaven capable of triggering and carrying out the malolactic fermentation under industrial conditions.

Further, the inoculation of the container with the reactivated leaven involves:

- administering a 30 g/hl dose of FERMOPLUS MALOLACTQUE in wine 24 hours before the reactivated leaven is added;
- mixing the final leaven with the wine in an "industrial" container with a capacity of 100 hl (1% inoculation percentage), and daily monitoring of the malolactic fermentation.

Were tested three working methods: direct inoculation (without reactivation), wine reactivation and must reactivation. In reactivation on wine, the inoculation of concoction INOFLORE R is made after the following scheme:

Wine (to inoculate): 25 l
Water: 25 l
Reagent: 4 g/l
Lactic acid bacteria: $2x10^7$ cells/ml

↓

Thermostating at 22ºC until the end of malolactic fermentation (from 2 to 7 days)

↓

+ wine (to inoculate): 50 l

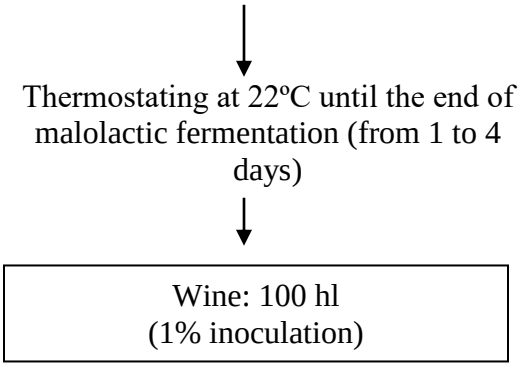

Figure 2. Inoculation of malolactic bacteria in wine – TWI Protocol (Gerbaux and Nault, 1994)

The preparation of leaven from lactic acid bacteria by reactivation in must involves the following succession of technological operations:

- preparation of a 20-liter "pilot" container to which 5 liters of unsulfured must, 5 liters of chlorine-free water, 2 g/l of autolyzed yeast (FERMOPLUS MALOLACTQUE) and 0.5 g of selected malolactic bacteria (INOFLORE R) will be added, providing a density of $2x10^8$ cells/ml;
- maintaining an optimal temperature at 25° C for 24 hours in order to acclimate the selected malolactic bacteria to environmental conditions;
- mixing the final leaven with the must in a "industrial" container with a capacity of 100 hl (0.1% inoculation percentage), and daily monitoring of the malolactic fermentation.

The protocol proposed by the Technical Wine Institute in France (TWI) determines the amount of inoculum for must at 1 $^0/_{00}$, similar to the one specific to active dry yeasts. The INOFLORE R concoction inoculation, in reactivation on must is made after the following scheme:

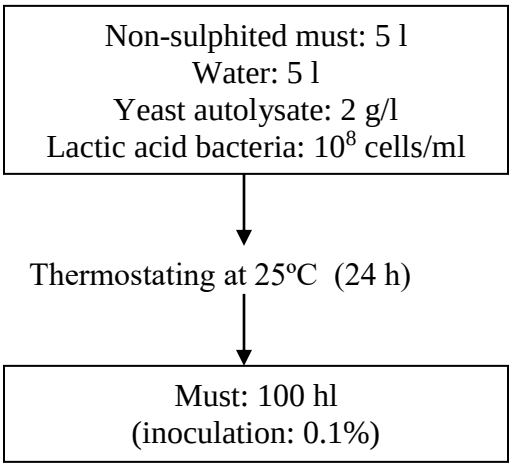

Figure 3. Inoculation of malolactic bacteria in must – TWI Protocol (Gerbaux and Nault, 1994)

3. Results and discussions

Before the malolactic initiation, the main physico-chemical properties of musts and wines were determined. The characteristics assessed in the musts were: sugars concentration, total acidity, pH, malic and lactic acids concentrations and total SO_2 (table 1). The characteristics assessed in the wines were: alcohol concentration, total and volatile acidity, pH, malic and lactic acids concentrations and total SO_2. The same physico-chemical properties were determined on wines after the malolactic fermentation.

The physico-chemical characteristics of the wines presented in table 2 (alcoholic concentration, total and volatile acidity, pH) do not satisfy the nutritional requisitions that must have an optimal culture environment for the growth and development of lactic bacteria. On the other hand, the musts, by their chemical composition, represent a propitious environment for the growth and development of the lactic bacteria. Still, the content in malic acid and total acidity is higher in musts, comparative to wines. Also, the content in lactic acid in musts is totally missing, while in wines, the content in L-lactic acid ranges between 0.2-0.4 g/l.

This is happens because malolactic fermentation starts at the end of the alcoholic fermentation before obtaining the wine.

Table 1. Physico-chemical characteristics of musts before the INOFLORE R concoction inoculation (reactivation on must method)

Types of must	Sugars g/l	Total acidity g/l	pH	Malic acid g/l	L-Lactic acid g/l	D-Lactic acid g/l	Total SO_2 mg/l
Oporto	217	3.33	3.40	1.9	traces	traces	28
Burgund	207	5.48	3.15	3.1	traces	traces	27
Pinot noir	205	4.45	3.28	2.0	traces	traces	28
Merlot	228	5.48	3.15	2.5	traces	traces	25
Cadarca	178	6.37	2.88	4.2	traces	traces	28

Table 2. Physico-chemical characteristics of the wines before the INOFLORE R concoction inoculation

Types of wines	Alcohol % vol	Total acidity g/l	Volatile acidity g/l	pH	Malic acid g/l	L-Lactic acid g/l	D-Lactic acid g/l	Total SO_2 mg/l
Oporto	12.5	3.21	0.28	3.4	1.5	0.34	0.1	28
Burgund	12.0	5.25	0.29	3.2	2.7	0.4	0.1	27
Pinot noir	11.9	4.40	0.29	3.3	1.5	0.28	0.1	25
Merlot	13.2	5.35	0.29	3.2	2.0	0.3	0.1	25
Cadarca	10.3	6.28	0.35	2.9	4.2	traces	0.2	28

Depending on the modality of work used to conduct malolactic fermentation (direct inoculation, reactivation on wine or reactivation on must), small differences on the values of the volatile acidity were found. The higher values of the volatile acidity were recorded in case of direct inoculation of the INOFLORE R concoction, nigh values by those recorded in case of wine reactivation version (volatile acidity 0.4-0.55 g/l). The lowest values of the volatile acidity are recorded in case of must reactivation of the INOFLORE R concoction (volatile acidity 0.32-0.47 g/l).

From figure 4, we see that the wines malolactic fermented by must reactivation method have the lower content in volatile acidity and lactic acid by malolactic fermented wines compared with reactivation version on wine, which is reflected by a more quality and better biological stability. In the version of must reactivation, the concentrations of lower alcohol wines can be explained by intense competition between lactic acid bacteria and yeasts during fermentation simultaneously (alcoholic and malolactic), the competition was ultimately won by lactic acid bacteria (Popescu-Mitroi *et al*., 2011).

From the data given in figure 4 it is noted that in the wine, after the conducted malolactic fermentation, L-lactic acid content ranges between 1.2-2.8 g/l and D-lactic acid content within 0.15-0.62 g/l. The results can be explained by the fact that the yeast and the lactic acid bacteria present in the spontaneous flora of wine degrade sugars to D-lactic acid, and L-malic acid is always degraded to L-lactic acid. In case of the reactivation of the malolactic bacteria preparation on must, D-lactic acid contents are higher than for the reactivation on wine, which is explained by the higher contents of sugars present in wine, releasing larger amounts of D-lactic acid. D-lactic acid is a normal constituent of wine, its presence is not always a sign of deterioration, as small amounts of D-lactic acid are produced in the

course of alcoholic fermentation by yeast action. A significant increase in the concentration of D-lactic acid during malolactic fermentation gives rise to suspicion on the defect of lactic souring of the wine. However, the results are normal and confirm that the wines have developed normally during malolactic fermentation.

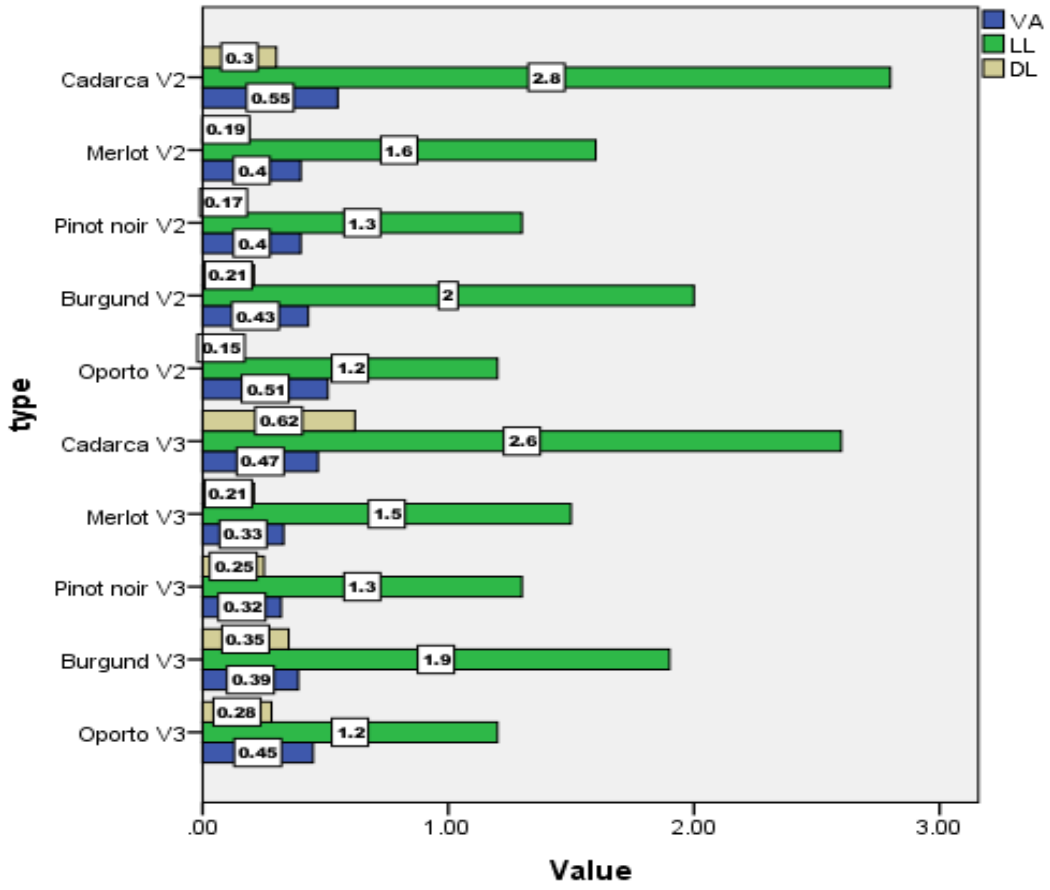

Figure 4. Physico-chemical characteristics of wines after the conducted malolactic fermentation
Legend: V_2 - wine reactivation version; V_3 - must reactivation version; VA - volatile acidity (g/l); LL - L- lactic acid (g/l); DL - D-lactic acid (g/l)

The centralized results in figure 5 show that simultaneously running alcoholic and malolactic fermentations (the must-reactivation variant) positively influences the physicochemical parameters of the wine at a pH of 3.3. Running simultaneous fermentations results in a better metabolism of the malic acid over time, with lower lactic acid and acetaldehyde contents. The other analyzed compounds (glucose, fructose) show no significant variations between the two variants. However, at pH values below 3.0, the physicochemical parameters of the wine obtained by simultaneously running alcoholic and malolactic fermentations (the must-reactivation variant) worsen due to the increase in volatile acidity (unpublished results).

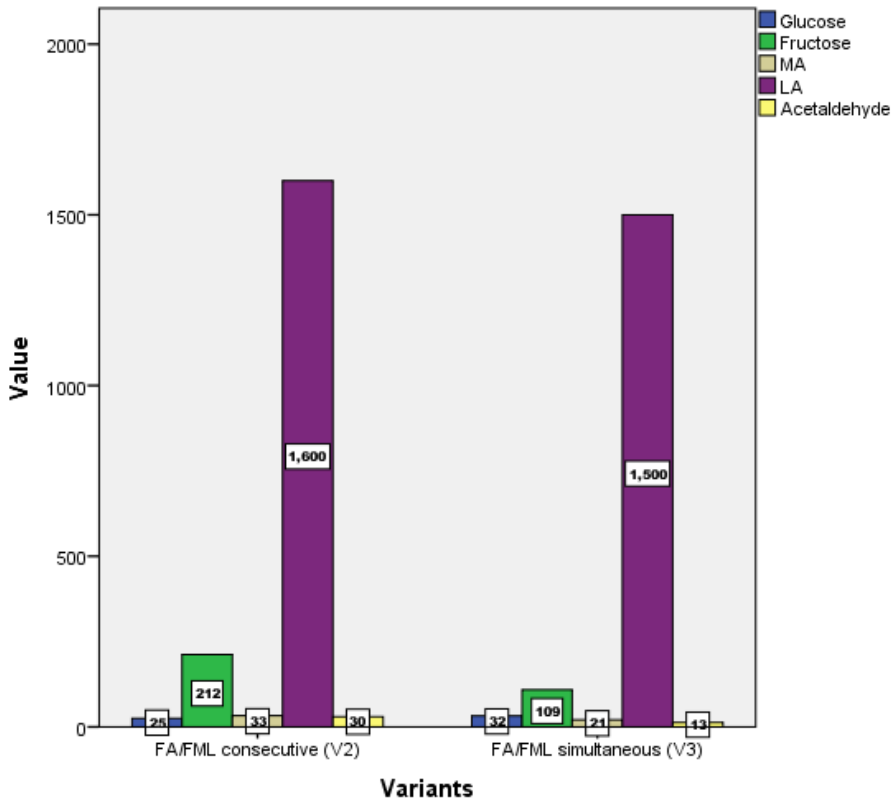

Figure 5. Values of several parameters in wine Merlot produced by consecutive and simultaneous alcoholic and malolactic fermentations

Legend: FA- alcoholic fermentation; FML- malolactic fermentation V_2 - wine reactivation version; V_3 - must reactivation version; MA- malic acid (mg/l); LA - lactic acid (mg/l)

Merlot wine (obtained from reactivation on must variant) and Merlot wine (obtained from reactivation on wine variant) have a very close sensorial profile but the experimented tasters can distinct them by comparison (figure 6). At degustation, at first sight, both wines are marked by vegetal flavors. Merlot wine (obtained from reactivation on must variant) presents "green pepper" and "pepper" vegetal flavor reference due metoxipirasynes. Merlot wine (obtained from reactivation on wine variant) has a rosemary, coffee and especially caramel flavor reference. The responsible molecules of the caramel flavor are phuraneol and homophuraneol. Still, by graphic representation of the olfactory profile for Merlot wine (obtained from reactivation on wine variant) and Merlot wine (obtained from reactivation on must variant), we realize that the flavors of these wines are quite close which creates the confusion premises of these wines, especially when are evaluated by non-experimented tasters.

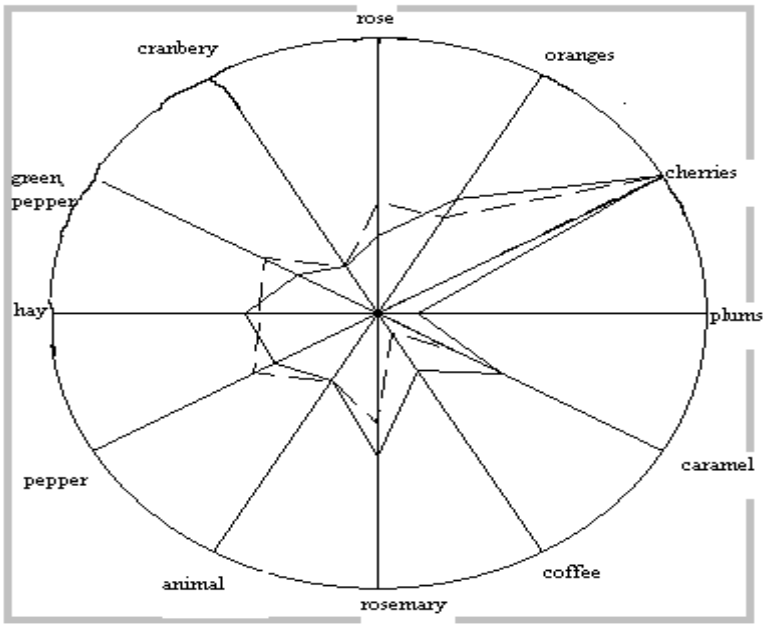

Figure 6 Graphic representation of the sensorial profile of wine Merlot (obtained from reactivation on must variant and reactivation on wine variant)
Legend: - - - Merlot obtained from V_3 (reactivation on must variant)
— Merlot obtained from V_2 (reactivation on wine variant)

The conducted malolactic fermentation is developing much faster, comparative with the spontaneous malolactic fermentation. Even within the conducted malolactic fermentation, there are differences dependent on the modality of work: direct inoculation (without reactivation), reactivation on wine and reactivation on must. The lower duration takes place in case of reactivation on must, and the higher duration is registered in case of direct inoculation (without reactivation). According to physico-chemical characteristics of wines, the duration of the conducted malolactic fermentation ranges between 6 and 19 days, being dependent on the reactivation version of the bacterial concoction, while in case of spontaneous malolactic fermentation (with inner flora), lasted 25-40 days (figure 7). It is worthy of note that the Cadarca wine does not succeed to malolacticly yeast in "spontaneous" conditions, not even in 120 days, because of a markedly low pH (2.9) for initiating the malolactic fermentation.

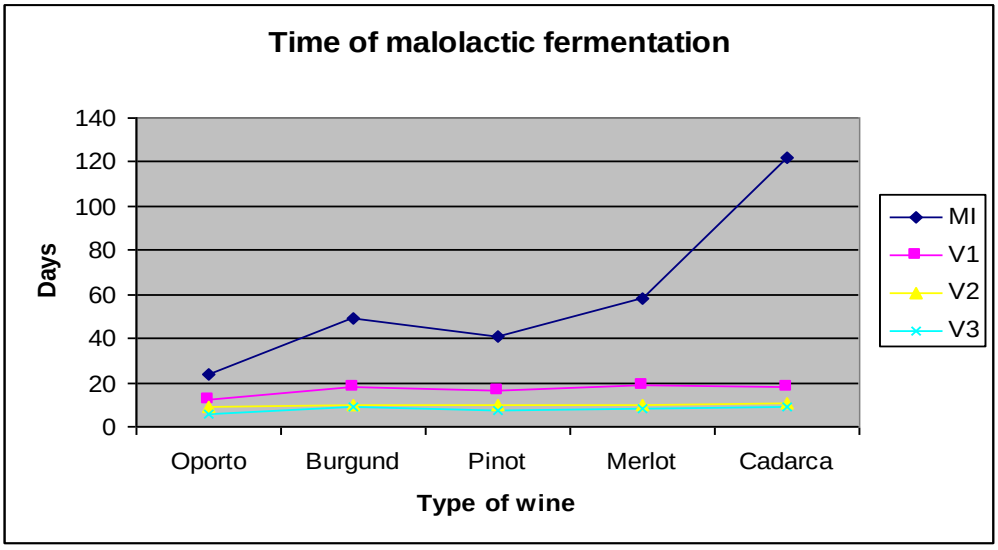

Figure 7 The influence of bacterial inoculation on the duration of achieving malolactic fermentation

Legend: MI – indigenous microflora; V_1 – direct inoculation version (without reactivation); V_2 – wine reactivation version; V_3 – must reactivation version

4. Conclusions

Previous studies conducted by the our team (PhD thesis Popescu, 2008) demonstrated that simultaneous alcoholic/malolactic fermentations do not have a negative impact on fermentation kinetics, nor on the final parameters of the wine. In this work we gave new insites on this metodology demonstrating the microbiological and technical advantage of applying a wine reactivation protocol.

Compared with wine inoculation, must inoculation has several advantages:

- faster onset of malolactic fermentation and reduction of winemaking, resulting in a lower cost to producers;
- achievement of malolactic fermentation even for "difficult" wines; there is the possibility of achieving malolactic fermentation for some "difficult" wines (with lower pH, high alcohol level, high level of sulphitation), as a result of an acclimatization of selected malolactic bacteria from the must phase;
- simplification of the reactivation protocol;
- an absolute adaptation of the lactic bacteria, seeing the lack of alcohol from the environment;
- standardization of the inoculation moment; low values of the volatile acidity and the assurance that undesirable compounds are not obtained.

However, the technique of co-inoculation should not fully substitute the sequential inoculation with selected yeasts and malolactic bacteria, and the decision to establish the time of inoculation of malolactic bacteria belongs to the oenologist, depending on the type of wine to be obtained.

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STUDY OF THE CHANGES ON LEAD AND CADMIUM LEVELS IN RICE UNDER THE INFLUENCE OF COOKING PROCESS

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ABSTRACT

Rice is considered as one of the major foods, it has a high share in the household baskets and is consumed daily by people. It can contain large amounts of heavy metals so it can be an important source of transmission of contamination of metal toxins to human. The main problem of heavy metals is that they are not metabolized in the body and thus are aggregated in body tissues. Heavy metals adversely affect nervous system, liver, kidney, skin, bones, and teeth. The method of cooking the rice, the amounts of water consumed for cooking, and its cooking time can be among the factors influencing the levels of metal toxins remained in the rice. This research studies the effect of the mentioned factors in order to achieve the best cooking conditions in terms of reducing the risk of contamination of the rice with metal toxins.

The samples of rice were purchased from wholesale stores across Mashhad City with a fully random design. All samples were soaked before cooking by water, after which their water was fully removed. Next, the rice samples were cooked under conventional method of draining (boiling in profuse water). Eventually, the level of cadmium and lead in the dry, soaked, drained, and cooked rice ready for consumption was measured and evaluated by atomic absorption device.

The comparison of the results indicated that the extent of the effect of the rice soaking stage has been more effective than other stages in reducing the level of cadmium and lead. Furthermore, the reduction in the level of lead and cadmium was greater in drained rice in comparison with brewed rice. Eventually, the greatest reduction in the level of lead and cadmium was obtained for "soaked, drained, brewed rice" as much as 61.3-87.8% for lead and 40-73.7% for cadmium. Also, the results revealed that across all stages, the extent of reduction in lead was greater than in cadmium. Indeed, it shows the higher resistance of cadmium in comparison with lead to reduction. Generally, the results indicated that all stages of soaking water, rice draining, rice brewing, as well as the amount of water and time required at each stage have influenced the extent of reduction in lead and cadmium present in rice.

1.Introduction

Although there is no clear definition of heavy metals, they are generally referred to metals whose specific weight is five times greater than water. They are a group of metal elements in the periodic table that show metallic properties. Toxic metals is another term referring to heavy metals. Although heavy metals are toxic, some of the toxic metals do not belong to the group of heavy metals. Iron, cobalt, copper, and manganese are a group of heavy metals that are required by the human body at trace values. Iron prevents from anemia and zinc is involved in more than 100 enzymatic activities in our body. Overabsorption of the mentioned metals is dangerous and toxic. If you pay attention to the nutritional value table present on the foods of newborn babies or some nutritional supplements, you will see the level of content zinc, iron, and copper. There are at least 20 heavy metals, but cadmium, lead, mercury, and arsenic are more important than the others (Rokni, 1378; AOAC, 2000; Houston and Kohler, 1970).

The main problem of heavy metals is that they are not metabolized in the body. Indeed, when introduced to the body, they are not discharged out of the body and thus are aggregated in body tissues. This causes incidence of diseases and various complications in the body. Heavy metals adversely affect nervous system, liver, kidney, skin, bones, and teeth. These compounds also enhance growth and development of viral, bacterial, and fungal infections and also replace other minerals and nutrients required by the body. The harmful effects of heavy metals include neurological disorders (Parkinson's, Alzheimer's, depression, and schizophrenia), different types of cancers, deficiency of nutrients, imbalance of hormones, obesity, abortion, respiratory and cardiac disorders, damage to kidneys, liver, and brain, allergy and asthma, infertility, fatigue and anemia, gene destruction, skin disorders, etc. Therefore, investigation of the level of heavy metals in food products is of crucial significance (Jarup, 2003; Laparra et al, 2005).

Even at trace levels, lead causes disorders in learning, hyperactivity and attention deficit in children, and decreased intelligence quotient. Memory loss, delayed reaction time, and declined learning capacity are among the long-term complications of lead-containing foods. Based on animal models, lead can have the potential to develop lung and stomach cancer in humans. Cadmium brings about adverse effects on kidneys and bone disorders such as osteoporosis and osteomalacia. In animal models, cadmium has developed cardiovascular disorders, yet to be confirmed in human beings. Further, cadmium has been categorized as a carcinogenic compound (woldermar and zdzislaw, 2005).

Soil, water, and polluted air are still among sources food contamination with heavy metals. Therefore, humans might be exposed to these metals through polluted air, soil, and food. All food products with an animal or plant origin have the potential to be contaminated by heavy metals. On the other hand, the way food products are consumed also influences the absorption of its toxins. For example, when foods are eaten completely, all of their toxins are probably absorbed by the body, while with regard to decoctions in which boiling happens, part of the toxins permeate into the water and are removed from the plant (woldermar and zdzislaw, 2005; Batista et al, 2012).

Following wheat, rice is regarded as the second most important grain in the world. As a plant product exposed to water, soil and air and due to the conditions of planting to harvest, rice can contain large amounts of heavy metals. Rice is considered as one of the major foods, it has a high share in the household baskets and is consumed daily by people. As a result, it can be an important source of transmission of contamination of metal toxins to human body and thus the consequences and toxicity of consuming these toxins in people. Therefore, paying attention to factors that bring about a reduction in the level of these toxins in ready-for-use rice is of crucial importance. The method of cooking the rice, the amounts of water consumed for cooking, and its cooking time can

be among the factors influencing the levels of metal toxins remained in the rice. Today considering the ever-increasing growth of population in Iran, domestic production of rice does not respond to the needs of people and thus considerable amounts of rice are imported from abroad (Raab, 2011; wikipedia, Lyon et al, 2000; Cheng et al, 2005; Ramesh, 2000).

This research studies the effect of the mentioned factors in order to achieve the best cooking conditions in terms of reducing the risk of contamination of the rice consumed by the household with metal toxins and thus reduction of adverse effects of these compounds on the health.

2.Materials and methods

2.1.Sampling

The samples of rice were purchased from wholesale stores across Mashhad City with a fully random design. These samples included two types of rice: Iranian and foreign, packaged in 1-kg packages. At the time of the test, four packages of each sample were mixed with each other. The treatments were chosen as follows:

2.2.Preparation of the samples

All samples were soaked before cooking by water, after which their water was fully removed. Next, the rice samples were cooked under conventional method of draining (boiling in profuse water). In this method, the rice is boiled in a dish containing profuse boiling water until its starch is fully gelatinized (fixed time for all samples). Thereafter, all of the water in the dish is removed, and until the rice is completely cooked, it will remain on heat in a closed dish (around 30 minutes).

The amount of water and time required for soaking the rice as well as the amount of water needed for boiling the rice were studied in two separate treatments. Eventually, the level of metal toxins in the dry, soaked, drained, and cooked rice ready for consumption was measured and evaluated.

2.3.The test method

The procedure is based on experimental observations and conclusion on the ground of laboratory findings obtained through device analysis. In the device analysis section, the level of cadmium and lead was measured by atomic absorption device with a graphite kiln (VARIAN GTA 120) according to AOAC (Chapter 9, p 19). All dishes and pipettes were soaked in nitric acid 10% (v/v) and then in HCl 10% (v/v) before the experiment for one night. They were then drained by two-time distilled water.

The stock standard solutions of lead and cadmium are created out of the relevant titrazoles (1000 mg/L) and then diluted for the working standards relevant to the concentration of the desired metal. All standards have an analytic grade (BDH, England). Next, the standards were sprayed into graphite kiln, followed by plotting the calibration curve.

As much as 10 g of fully dried sample is weighed by an analytical balance with an accuracy of 0.001 g inside a crucible, already reached a fixed weight. The sample was burnt inside the crucible and then transferred to an electric kiln at 450 °C for 8 hours until a white or light ash is obtained. Otherwise, 1-3 ml of distilled water is added to the ash, then dried at 100-450 °C, then retransferred to the kiln until becoming white. After being cooled, the obtained ash was placed on a water bath to which 5 mL HCl (6 M) is added. It is then heated until being dried. The residual is dissolved in 10 mL HNO_3 (0.1 M), then remains for 1-2 h. Thereafter, it is diluted by nitric acid (0.1 M) at 25 mL and its concentration is read by a device.

2.4.Statistical analysis

In order to analyze the data, MINITAB software was used. Data analysis was conducted based on ANOVA table.

3.Results and Discussion

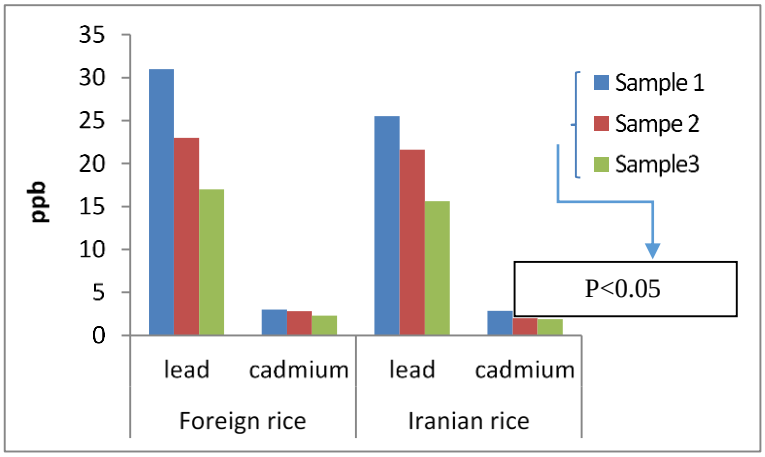
3.1.The effect of soaking stage

The level of lead and cadmium remained in foreign and Iranian rice after 30 minutes of soaking (in both water ratios of 2:1 and 4:1) was

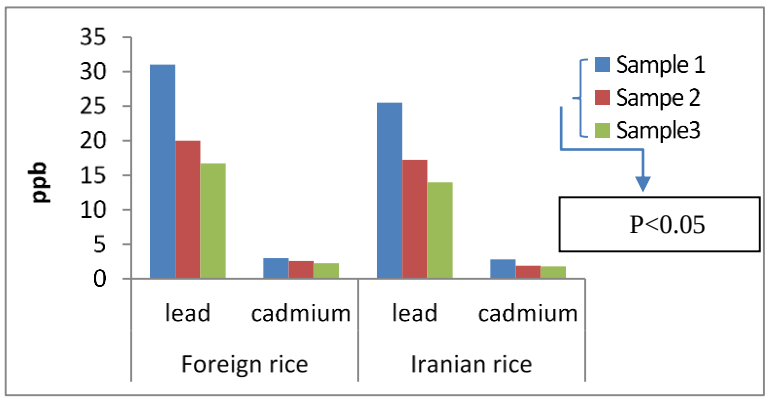
greater in comparison with 60 minutes of soaking (p value<0.05). The average level of lead remained in foreign and Iranian rice after 30 minutes of soaking with 2:1 water ratio was 84.7 and 74.2% and average level of cadmium remained was 70.2 and 93% respectively. Also, the average level of lead remained in foreign and Iranian rice after 60 minutes in the same water ratio was 58.8 and 64.5% and average level of cadmium remained was 66.7 and 86.7% respectively (p value<0.05).

This was also true for 4:1 water ratio, where the average level of lead remained in foreign and Iranian rice after 30 minutes was 61.2 and 54.8% and average level of cadmium remained was 66.7 and 76.7%, respectively. Also, the average level of lead remained in foreign and Iranian rice after 60 minutes in the same water ratio was 38.4 and 53.9% and average level of cadmium remained was 63.2 and 76.6%, respectively (p value<0.05) (Figure 1).

The results indicated that as the water used for soaking the rice increased (30 to 60 minutes), the level of lead and cadmium remained in rice (at both 2:1 and 4:1 water ratio) declined.



Sample1 : dry rice
Sample2 : 30 minutes soaking in the ratio 2: 1 water
Sample3 : 30 minutes soaking in the ratio 4: 1 water



Sample1 : dry rice
Sample2 : 60 minutes soaking in the ratio 2: 1 water
Sample3 : 60 minutes soaking in the ratio 4: 1 water

Figure 1. The effect of soaking time (at different amounts of water) on the level of cadmium and lead (p value<0.05)

3.2.The effect of the draining stage

The results showed that drain the rice to reduce the amount of lead and cadmium in both Iranian and foreign rice has a direct impact. This reduces was associated with the amount of water used to drain rice such that an increase in the amount of water ratio of 4: 1 to 6: 1, the amount of lead and cadmium reduced in rice.

3.2.1.In the rice drained by 4:1 water ratio

The average level of lead remained in (foreign and Iranian) rice after 30 minutes of soaking by 2:1 water ratio was 61.3 and 38.1% and the average level of cadmium remained was 80 and 66.7%, respectively. Also, the average level of lead remained after 60 minutes of soaking by the same water ratio was 44.2 and 33.7% and the average level of cadmium remained was 56.7 and 42.1%, respectively (p value<0.05).

This was also true for soaking water by rice with 4:1 water ratio, where the average level of lead remained in (foreign and Iranian) rice after 30 minutes of soaking by 4:1 water ratio was 47.1 and 36.9% and the average level of cadmium remained was 63.3 and 42.1%, respectively. Also, the average level of lead remained after 60 minutes of soaking by the same water ratio was 24.8 and 27.5% and the average level of cadmium remained was 40 and 36.1%, respectively (p value<0.05).

3.2.2.In the rice drained by 6:1 water ratio

The average level of lead remained in (foreign and Iranian) rice after 30 minutes of soaking by 2:1 water ratio was 45.2 and 22% and the average level of cadmium remained was 43.3 and 38.6%, respectively. Also, the average level of lead remained after 60 minutes of soaking by the same water ratio was 26.1 and 20.4% and the average level of cadmium remained was 53.3 and 35.1%, respectively (p value<0.05).

This was also true for soaking water by rice with 4:1 water ratio, where the average level of lead remained in (foreign and Iranian) rice after 30 minutes of soaking by 4:1 water ratio was 27.1 and 25.1% and the average level of cadmium remained was 56.7 and 49.1%, respectively. Also, the average level of lead remained after 60 minutes of soaking by the same water ratio was 20 and 18.8% and the average level of cadmium remained was 36.7 and 29.8%, respectively (p value<0.05).

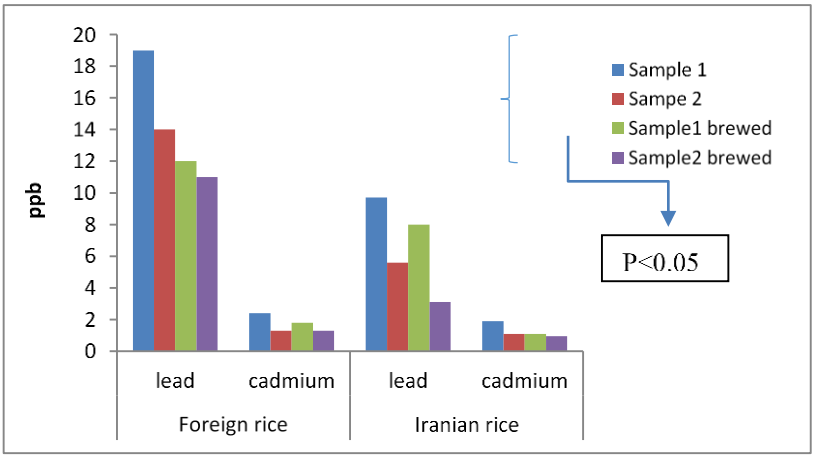
Finally, brewing of soak and drain rice also reduced the amount of lead and cadmium residues in rice (p value<0.05). This reduction has direct connection with the amount of water used to soak and drain the rice (Figure 2 and 3).

Few similar studies by other researchers on the rice is done. Daomukda et al. (Daomukda, 2011) investigated the effect of different cooking methods including brewing, draining, utilizing pressure cooker, and microwave on the physiochemical properties of rice. Their results indicated that in conventional methods, the level of protein and fat declines in comparison with other methods. On the other hand, a water-to-rice ratio of 2 was very influential in the tissue and softness of the rice (Mahadevamma and Tharanathan, 2007). Shaeibi et al. (Shoeibi, 2010) examined the effect of cooking process on the remaining of three types of toxins of pesticides. They observed that the amount of these toxins during the cooking processes has decreased by 35, 55, and 78%. Raab et al. (Raab, 2011) evaluated the effect of cooking methods on the amounts of arsenic present in rice. They concluded that the level of arsenic decreased in the rice cooking process such that its level is in cooked rice 35-40% less than in raw rice (Mondal et al, 2010).

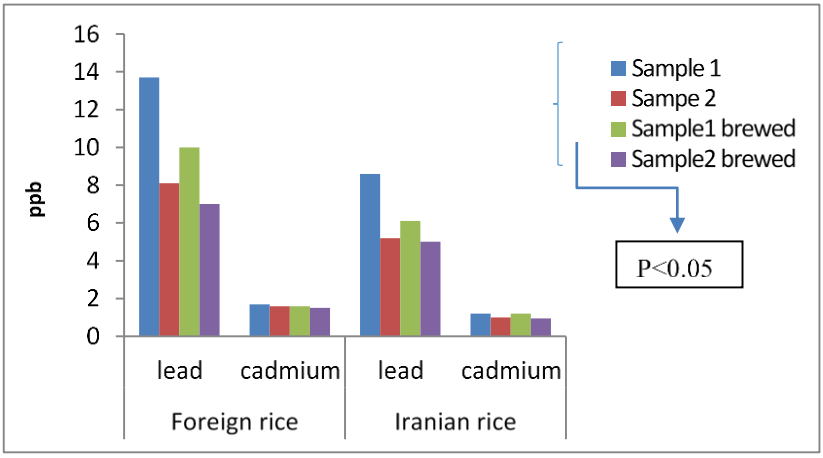
Generally the results of our study indicated that the extent of the effect of rice soaking stage on the extent of reduction in lead and cadmium was obtained to be 25.8-46.1 and 7-36.8%, respectively. In this case, the minimum reduction of the percentage of these toxins was obtained after 30 minutes of soaking by water 2:1, while the maximum reduction was gained after 60 minutes by water 4:1. Therefore, the amount of water used for soaking rice as well as

the time of soaking have been effective in reducing the level of lead and cadmium.

The extent of the effect of rice draining stage on the extent of reduction in lead and cadmium was obtained to be 38.7-81.2% and 20-70.2%, respectively. In this case, the minimum reduction of the percentage of these toxins was obtained draining by water 4:1, while the maximum reduction was gained by water 6:1. Therefore, the amount of water used for draining rice has been effective in reducing the level of lead and cadmium.



Sample1: drained by 4:1 water ratio (with sample soaked 30 minutes in the 2: 1 ratio of water)
Sample2: drained by 6:1 water ratio (with sample soaked 30 minutes in the 2: 1 ratio of water)
Sample1 brewed: drained by 4:1 water ratio (with sample soaked 30 minutes in the 2: 1 ratio of water) then brewed
Sample2 brewed : drained by 6:1 water ratio (with sample soaked 30 minutes in the 2: 1 ratio of water) then brewed



Sample1: drained by 4:1 water ratio (with sample soaked 60 minutes in the 2: 1 ratio of water)
Sample2: drained by 6:1 water ratio (with sample soaked 60 minutes in the 2: 1 ratio of water)
Sample1 brewed: drained by 4:1 water ratio (with sample soaked 60 minutes in the 2: 1 ratio of water) then brewed
Sample2 brewed : drained by 6:1 water ratio (with sample soaked 60 minutes in the 2: 1 ratio of water) then brewed

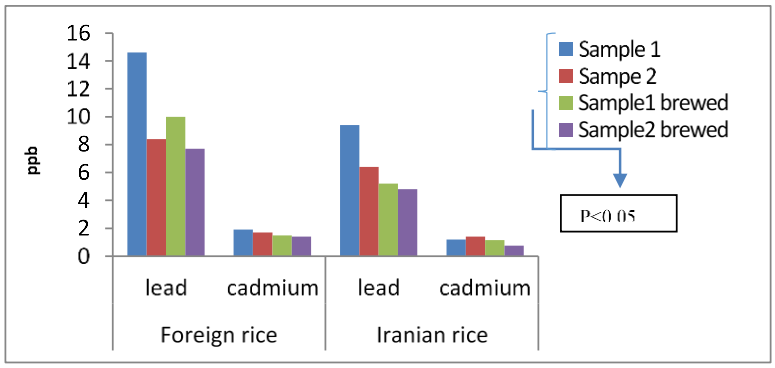
Figure 2. The effect of draining process by different amounts of water on the level of cadmium and lead (In the sample soaked by 2:1 water ratio), (p value<0.05)

The extent of the effect of rice brewing stage on the extent of reduction in lead and cadmium was obtained to be 61.3-87.8% and 40-73.7%, respectively. In this case, the minimum reduction of the percentage of these toxins was obtained draining by water 4:1, while the maximum reduction was gained by water 6:1. Therefore, brewing rice has also been effective in decreasing the level of cadmium and lead.

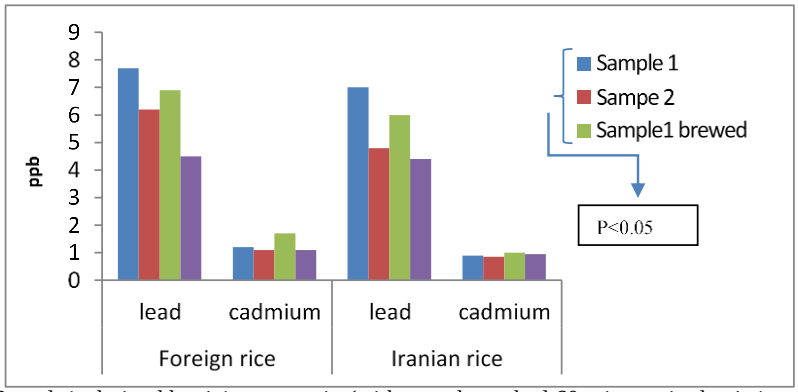
In general, the comparison of the results indicated that the extent of the effect of the rice soaking stage has been more effective than other stages in reducing the level of cadmium and lead (p value<0.05).

Furthermore, the reduction in the level of lead and cadmium was greater in drained rice in comparison with brewed rice (without draining) (p value<0.05). Eventually, the greatest reduction in the level of lead and cadmium was obtained for "soaked, drained, brewed rice" as much as 61.3-87.8 for lead and 40-73.7% for cadmium (p value<0.05). The conditions of work in this state involved 60 minutes soaking the rice at 4:1 water ratio, followed by boiling and draining of the rice at 6:1 water ratio, and eventually brewing the rice for 30 minutes.

On the other hand, the results revealed that across all stages, the extent of reduction in lead was greater than in cadmium. Indeed, it shows the higher resistance of cadmium in comparison with lead to reduction.



Sample1: drained by 4:1 water ratio (with sample soaked 30 minutes in the 4: 1 ratio of water)
Sample2: drained by 6:1 water ratio (with sample soaked 30 minutes in the 4: 1 ratio of water)
Sample1 brewed: drained by 4:1 water ratio (with sample soaked 30 minutes in the 4: 1 ratio of water) then brewed
Sample2 brewed : drained by 6:1 water ratio (with sample soaked 30 minutes in the 4: 1 ratio of water) then brewed



Sample1: drained by 4:1 water ratio (with sample soaked 60 minutes in the 4: 1 ratio of water)
Sample2: drained by 6:1 water ratio (with sample soaked 60 minutes in the 4: 1 ratio of water)
Sample1 brewed: drained by 4:1 water ratio (with sample soaked 60 minutes in the 4: 1 ratio of water) then brewed
Sample2 brewed : drained by 6:1 water ratio (with sample soaked 60 minutes in the 4: 1 ratio of water) then brewed

Figure 3. The effect of draining process by different amounts of water on the level of cadmium and lead (In the sample soaked by 4:1 water ratio), (p value<0.05)

4.Conclusions

Overall, the results indicated that all stages of soaking water, rice draining, rice brewing, as well as the amount of water and time required at each stage have influenced the extent of reduction in lead and cadmium present in rice.

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EXPLORING NUTRACEUTICAL POTENTIAL OF SELECTED VARIETIES OF *PIPER BETLE* L.

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ABSTRACT
Piper betle L. locally known as 'Paan', belongs to the family Piperaceae which is consumed all around the world. Present investigation deals with the evaluation of nutraceutical potential of betel leaf. The leaf of the plant is used traditionally and has immense antioxidant potential. For the present study hydro propanolic, acetonic, hydro acetonic and distilled water extracts of four varieties: meetha paan, bangla paan, paan (Assam), shillongi paan of betel leaves were used. For the present study the total phenolics, vitamin E and antioxidant activities viz. free radical scavenging activity (FRSA), total antioxidant capacity (TAC), hydrogen peroxide scavenging activity, nitric oxide radical scavenging activity and superoxide radical scavenging activity) was determined in the leaf extracts. The highest total phenolic compound and vitamin E content was observed to be 0.16 mg GAE/g and 3.69 mg/100gm and the lowest was observed to be 0.01 mg GAE/g and 3.20mg/100g. The antioxidant activity of all the extracts varied significantly ($p<0.05$). The present study will explore nutraceutical potential of P. betle L., will help build data base and promote utilization of betel leaf as natural herb.

1.Introduction

The theory "Let food be thy medicine, and medicine be thy food" stated by Hippocrates is a well established truth in today's world, which emphasise the relationship between food for health and their therapeutic benefits (Palthur et al., 2010). The concept of nutraceuticals is not entirely new, they are regarded as foods (or parts of a food) which provides medicinal benefits, and help prevent health problems (Sarin et al., 2012). Nutraceuticals are used as conventional foods or diet which represents the fastest growing aspect of food industry (DeFelice, 1995; Rishi, 2006). Nearly, 500 nutraceutical and functional food products are available in market world-wide with authenticated health benefits.

Traditional and non traditional neutraceuticals are available in today's market. Traditional nutraceuticals are naturally obtained and has various health benefits (Tank et al., 2010). Nature has been providing us medicinal and pharmacological agent for thousands of years; one such medicinal plant is betel leaf (*Piper betle* L.) that has great nutraceutical potential. It belongs to piperacae family which has a long standing history dating back 5500-7000BC (Arambewela, 2010). *Piper betle* L. locally known as *paan* in Hindi (India) has 100 varieties all over the world of which 40 varieties are found in India. It grows in deep, well drained, friable loamy and clayey soils which is rich in organic matter maintaining a pH of 7-7.5. These heart shaped leaves are aromatic in nature and due to the presence of essential oils it tastes's sweet to pungent (Pradhan et al., 2013).

The leaves of betel are nutritive and possess antioxidant activity (Choudhury and Kale, 2002), antimicrobial activity (Shitut et al., 1999), anthelmintic activity (Shilashar and Parasar, 1985), insecticidal activity (Arambewela, 2006), neuroprotective activity (Saravanan et al., 2003), antidiabetic (Murakami et al., 2000) and antitumor activity (Arambewela, 2006). Betel leaves contain variety of biologically functioning components like; hydroxychavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A and piperol B. The chief constituent of the leaf is a volatile oil known as betle oil. Leaves of *Piper betle* L. reported to produce an alkaloid: arakene, which has medicinal properties similar to cocaine (Kumari and Rao, 2015; Widawati and Riandi, 2015).

Free radical damages are related to various serious health issues like cancer, arthritis, atherosclerosis, alzheimer's disease, diabetes, asthma and other neurological disorders. These disorders can be prevented by antioxidants that scavenge free radicals and have been proven to be favourable for these diseases which can prevent cell damage. Antioxidant has the ability to scavenge free radicals, inhibit ion of lipid peroxidation, chelate metals and reducing capacity (Evans and Halliwell, 2001). Betel leaves contains a notable amount of antioxidants (hydroxychavicol, eugenol, ascorbic acid and β-carotene) that has the ability to fight against oxidative stress (Voon et al., 2014). The present investigation was designed to evaluate the antioxidant potential of different varieties of *Piper betle* L from different geographical locations and its extracts obtained using different solvents. This paper will help build database and promote utilization of betel leaf as a natural herb.

2.Materials and methods

2.1.Collection of plant material

Healthy and young betel leaves were collected from the local market of Assam and Meghalaya. The local names of different varieties taken for the study were: *meetha* paan, *bangla* paan, *paan* (Assam) and *shillongi* paan

2.1.1.Sample preparation

With the help of chilled mortar and pestle 10gm of fresh leaves were ground and dissolved in 100 mL of respective solvents i.e., distilled water, acetone, hydro acetone (50:50), 2 hydro propanol (50:50) (Himedia Laboratories, India). The crushed samples were incubated in shaker incubator (Remi Co., Model CIS 18 Plus, Mumbai, India) overnight at 28ºC. Centrifugation was done at 10,000 rpm for 10 minutes. The supernatant was collected in a pre-sterilised sample container. All the prepared samples were stored at -20ºC until further analysis.

2.2.2.Determination of total phenolics

The total phenolic content was estimated by the folin ciocalteau method with slight modification (John et al., 2014). Sample (200 µL) leaf extract was transferred into a test tube containing, 1mL of freshly diluted (10 fold) folin-ciocalateu reagent. . The mixture was kept undisturbed at room temperature. To the mixture, 3 mL of 7.5% (w/v) sodium carbonate was added and shaken manually. Incubation of the mixture was done at room temperature for 60 minutes. With the help of UV – visible

spectrophotometer (Shimadzu, UV-2600, Japan) the absorbance was noted at 756 nm.Acidified methanol was used as a blank. The calibration curve was plotted against gallic acid and expressed in terms of mg (GA) equivalents per g dry weight basis (mg GAE/g dw).

2.2.3.Determination of vitamin E content

Vitamin E content in the leaf was estimated as described by AOAC (2000). Different sample extracts (1.5 mL), standard and water was taken in different test tubes and were capped. To it 1.5 mL of ethanol was added to test and blank tubes. To the standard 1.5mL of water was added and all the tubes were centrifuged. To all the tubes xylene was added, mixed and centrifuged. The upper 1mL xylene layer was taken and 1mL of 2, 2-dipyridyl reagent was added and mixed. The extinction of test was read against the blank at 460 nm. Then, 0.33 mL of ferric chloride solution was added (including the blank) and mixed well. The absorbance was recorded at 520 nm after 15 minutes.

2.2.4.Determination of antioxidant activity

Free radical scavenging activity

The scavenging activity was determined by using DPPH radical on the basis of scavenging ability of the extracts of betel on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (Kekuda et al., 2010). Methanolic solution of DPPH (2 mL) was mixed with 2 mL of different concentrations of betel leaf extracts. The tubes were incubated at room temperature in dark for 30 minutes. The absorbance was recorded at 517 nm using spectrophotometer. T The scavenging activity other leaf extracts were calculated using formula

Scavenging activity (%) = [(A-B)] X 100

Where, A is absorbance of DPPH and B is absorbance of DPPH and extract combination.

2.2.5.Total antioxidant capacity (TAC)

Estimation of total antioxidant capacity was carried out by the phophomolybdenum method according to the procedure described by Peitro et al., (1999). To this 4.5 mL phosphomolybdenum reagent (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added and mixed properly. The capped tubes were incubated in boiling water bath at 95ºC for 90 minutes. The absorbance was measured at 695 nm in spectrophotometer using ascorbic acid (0.25 mg/mL) as standard. Total antioxidant content was calculated by using the formula

Total antioxidant acivity (%) = $[(A_s-A_c)/A_{aa}-A_{c)}]$ X 100

Where, A_c = control absorbance, A_s = sample absorbance and A_{aa} = ascorbic acid absorbance

2.2.6.Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity (%) was determined by the method of Zhao et al. (2006). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4, 50 mM). Extract (100 µL) was added to a hydrogen peroxide solution (0.6 mL, 40 mM). Decrease in absorbance was measured after 10 minutes of incubation at 230 nm.

The scavenging activity of the extract was calculated using the following formula

Scavenging activity (%) = [(A-B)/A] X 100

Where, A is absorbance of control and B is absorbance of sample.

2.2.7.Nitric oxide radical scavenging activity

Nitric oxide (NO) scavenging activity was determined by the method of Rintu et al. (2015) Sodium nitroprusside (1 mL of 10 mM) was mixed with 1mL sample of different concentrations of phosphate buffer (pH 7.4). The mixture was further incubated for about 150 min at 25ºC. Griess' reagent (1mL) was added to 1mL of the incubated solution. Absorbance was recorded at 546 nm and percent activity was calculated by using the following formula

Scavenging activity (%) = [(A-B)/A] X 100

Where, A is absorbance of control and B is the absorbance of sample.

2.2.8.Superoxide radical scavenging activity

The Superoxide (O_2^-) radical scavenging activity was measured by the method used by Dasgupta and De (2004) with some modifications. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). Nitro-Blue Tetrazolium (156 μM) 1 mL of strength, 1 mL Nicotinamide Adenine Dinucleotide - Hydrogen (468μM) and 3mL of sample were mixed. The reaction was started by adding 100 μL of phanazine methosulphate (PMS) (60 μM) and the mixture then incubated at 25ºC for 5 minutes, measured at 560nm. The percentage activity was calculated from the formula

Scavenging activity (%) = [(A-B)/A] X 100

Where, A is absorbance of control and B is absorbance of sample.

3.Results and discussion

3.1. Total phenolic compound

Plant polyphenols have gathered huge attention due to the antioxidant properties and effect in defending ultraviolet radiation or aggression by pathogens, parasites and predators. It has one or more aromatic rings with one or more aromatic groups (Dai and Mumper, 2010). For the antioxidant activity the major components of *Piper betle* L. are chavicol, chavibetol, allylprotocatechol and eugenol (Jaiswal et al., 2014). Total phenolic content (TPC) of four leaf varieties with different solvents; 2 propanol hydro assam (PHA), 2 propanol hydro shillongi (PHS), 2 propanol hydro bangla (PHB), 2 propanol hydro meetha (PHB); acetone assam (AA), acetone shillongi (AS), acetone bangla (AB), acetone meetha (AM); acetone hydro assam (AHA), acetone hydro shillongi, acetone hydro bangla (AHB), acetone hydro meetha (AHM); distilled water assam (DWA), distilled water shillongi (DWS), distilled water bangla (DWB), distilled water meetha (DWM) were evaluated. The TPC of betel leaf extracts varied significantly ($p<0.01$) amongst varieties and solvents. The observed descending order of total phenolic content of the four leaf variety can be written as PHM> DWM> AB> AHS> AS> PHB> DWA> AHB> AA> PHS> DWS> DWB> PHA> AHM> AM> AHA and the highest TPC value was observed to be 0.16 mg GAE/g in the 2 hydro propanolic extract (meetha) while the lowest value was 0.01 mg GAE/g in the hydro acetonic extract (Assam). Strong free radical activity is present in phenolic compounds (Proteggente et al., 2003). The presence of phenolic compound contributes for the antioxidant activity of the leaves and allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, heavy metal chelators and hydroxyl radical quenchers (Jha et al., 2011).The results are comparable as reported (Chakraborty and Shah, 2011) for *Piper betle* L. The variation in the phenolic content of different betel leaf extracts are depicted in Figure 1.

3.2.Vitamin E content

Vitamin E inhibits the activity of reactive oxygen species (ROS) that damage the cell membrane as it is a fat soluble antioxidant (Choe and Min, 2009). The highest vitamin E content was recorded to be 3.69 mg/100g in distilled water extract and the lowest was recorded to be 3.20 mg/100g in the 2 hydro propanolic extract. Yamauchi (1997) reported that the molecular mechanism of vitamin E is mediated by the antioxidant function or its membrane stabilizing effect. The vitamin E content showed similar trend in almost all solvents and varied significantly ($p<0.05$) amongst varieties. The decreasing order of the leaves of different varieties are DWM> AHM> DWB> AHB> AM> DWS> PHM> AHS> DWA> AHA> AB> AA> PHA> PHB> AS> PHS. The results were compared with the ethanolic extracts of *Piper betle* L. (Saravanan et al., 2003). The molecular mechanism of vitamin E is mediated by the antioxidant function or its membrane stabilizing effect. The variations in vitamin E content are depicted in the Figure 2.

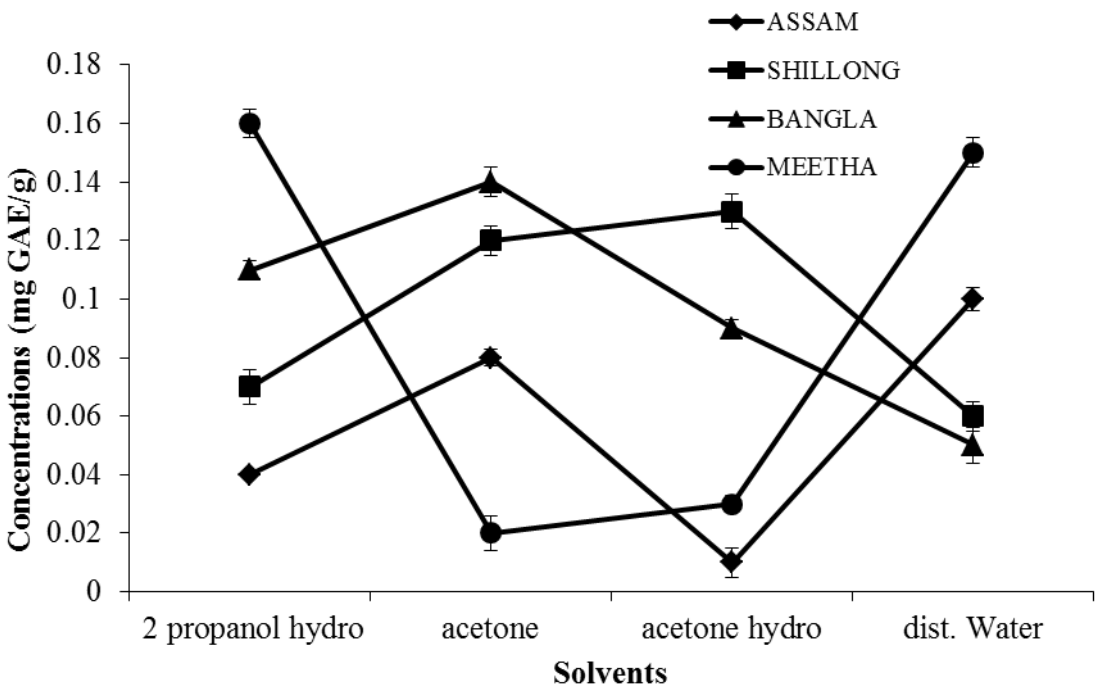

Figure 1. Total phenolic compound of different betel leaf extracts

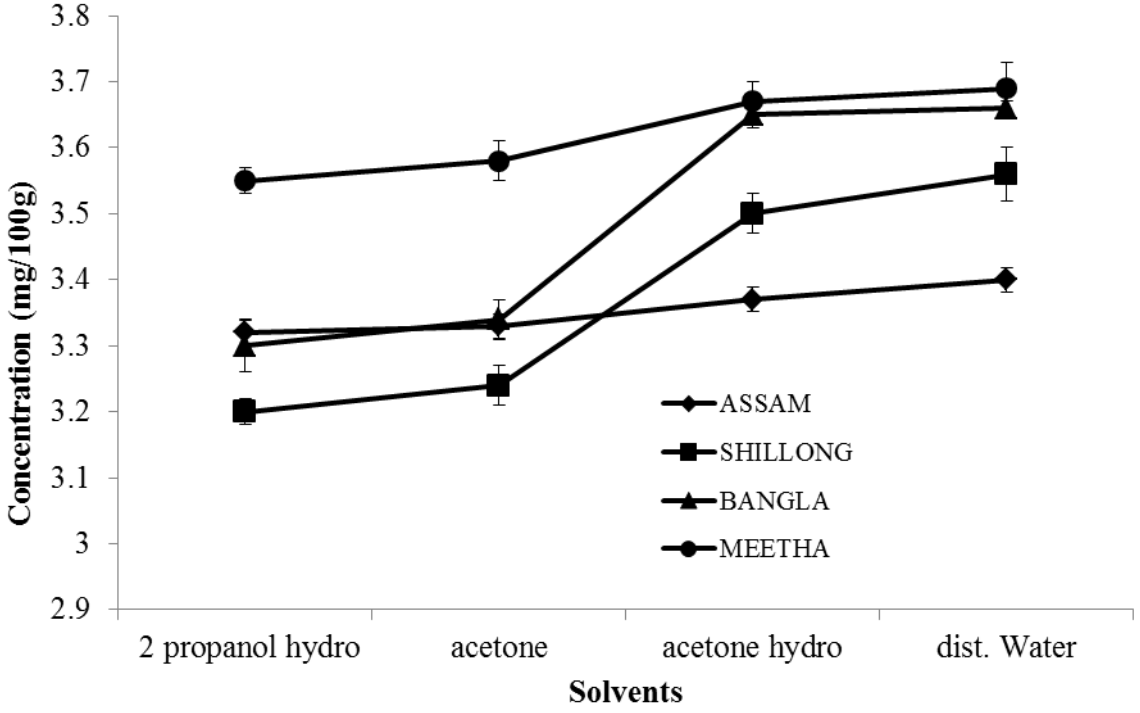

Figure 2. Vitamin E content of different betel leaf extracts

Table 1. Antioxidant activity of different varieties of betel leaf extract

Extracts	Free radical scavenging activity (% inhibition)	Total antioxidant capacity (% inhibition)	H_2O_2 scavenging activity (% inhibition)	Nitric oxide scavenging activity (% inhibition)	Superoxide radical scavenging activity (% inhibition)
PHA	69.45±0.01[a]	81.10±0.11[a]	88.07±0.04[a]	32.00±0.03[a]	73.40±0.05[a]
PHS	71.49±0.28[a]	77.50±0.06[a]	83.04±0.02[b]	20.04±0.20[a]	79.60±0.28[b]
PHB	74.05±0.07[a]	76.70±0.10 [a]	73.00±0.03[c]	13.03±0.40[a]	55.90±1.29[c]
PHM	80.99±0.03[b]	66.70±0.03[b]	55.01±0.12[d]	21.04±0.26[a]	75.80±0.20[b]
AA	60.20±0.14[c]	74.22±0.04[c]	62.01±0.23[e]	36.03±0.03[a]	71.40±0.03[a]
AS	77.42±0.03[b]	78.02±0.32[a]	66.05±0.05[e]	30.05±0.05[a]	59.30±0.30[c]
AB	78.10±0.05[b]	80.90±0.03[a]	78.02±0.02 [b]	14.06±0.23[b]	56.80±0.12[c]
AM	70.58±0.10[a]	87.07±0.22[d]	84.20±0.01[b]	17.06±0.20[b]	67.90±0.06[d]
AHA	73.63±0.01[a]	86.02±0.33[d]	89.45±0.02[a]	15.03±0.20[b]	77.90±0.08[b]
AHS	72.38±0.13[a]	75.34±0.06[c]	77.44±0.32[b]	23.02±0.04[a]	84.80±0.03[e]
AHB	66.87±0.13[d]	70.44±0.30[b]	72.34±1.12[c]	26.05±0.17[b]	62.00±0.05[f]
AHM	65.78±0.06[d]	72.28±0.20[b]	61.05±1.25[e]	41.03±0.03[c]	82.60±0.08[e]
DWA	56.07±0.04[e]	60.12±0.30[e]	57.03±0.11[d]	19.07±0.04[4]	80.00±0.03[e]
DWS	59.65±0.19[c]	67.00±0.44[b]	65.21±0.06[e]	18.08±0.30[b]	76.50±0.76[b]
DWB	64.00±0.13[d]	70.31±0.05[b]	63.00±0.03[e]	16.00±0.02[b]	89.20±0.46[g]
DWM	76.06±0.01[b]	65.00±0.04[b]	60.05±0.01[e]	22.03±0.55[a]	83.40±0.26[e]

PHA: 2 propanol hydro Assam, PHS: 2 propanol hydro Shillongi, PHB: 2 propanol hydro Bangla, PHM: 2 propanol hydro Meetha; AA: acetone Assam, AS: acetone Shillongi, AB: acetone Bangla, AM: acetone Meetha; AHA: acetone hydro Assam, AHS: acetone hydro Shillongi AHB: acetone hydro Bangla, AHM: acetone hydro Meetha; DWA: distilled water Assam, DWS: distilled water Shillongi, DWB: distilled water Bangla, DWM: distilled water Meetha

The value are presented as Mean ± SD

The value represented with different superscript ($p<0.05$) differ significantly

3.3.Antioxidant activity

Numerous diseases are associated with free radical damages in the body. For the present study antioxidant activity of different betel leaf extracts by Free radical scavenging activity, total antioxidant capacity, hydrogen peroxide scavenging activity, nitric oxide scavenging activity and superoxide radical scavenging activity. The variation in different varieties of betel leaf extracts are depicted in Table 1.

3.4.Free radical scavenging activity and total antioxidant capacity

Reactive oxygen species (ROS) are responsible for causing biological damage to the cells (Rathee et al., 2006). The free radical scavenging activity was found maximum in 2 hydro propanolic extract (80.99±0.03% inhibition) and lowest (56.07±0.04% inhibition) in distil water extract. Descending order of the DPPH scavenging activity of the extract can be arranged as follows- PHM> AB> AS> DWM> PHB> AHA> AHS> PHS> AM>PHA> AHB> AHM> DWB> AA> DWS> DWA. The results are comparable with that reported by Dasgupta and De (2004) for *Piper betle* L. Jaiswal et al. (2014) reported that antioxidant activity are high in the leaves because of the presence of phenolic compound hydroxyl-chavicol (4- allyl pyrocatechol) and is proved to be a better preservative. Total antioxidant capacity was highest (87.07±0.22%) in acetonic extract and the lowest (60.12±0.30%) was observed in the distilled water extract. The FRSA and antioxidant capacity rather varied significantly ($p<0.05$) amongst leaf varieties than amongst solvents used (Table 1).

3.5.Hydrogen peroxide scavenging activity

Hydrogen peroxide is hazardous and because of its ability to form radical emphasis should be given on the importance of its elimination. Hydrogen peroxide scavenging activity in different extracts of betel leaf in descending order can be arranged as- AHA> PHA> AM> PHS> AB> AHS> PHB> AHB > AS> DWS >DWB >AA> AHB > DWM > DWA > PHM. Maximum activity was found in the hydro acetonic extract (89.45±0.02% inhibition) and the lowest was found in 2 hydro propanolic extract (55.01±0.12% inhibition). It is already proven that dietary phenols plays an important role in protecting mammalian and bacterial cells from the oxidative stress caused by hydrogen peroxide (Nakayama, 1994). The observed scavenging of the hydrogen peroxide could be due to the presence of phenolic compounds. The results are comparable as reported (Tamuly et al., 2013) for *P. betleoides* and *P. wallichii*. The generation of low hydrogen peroxide in the organ system is important as it is an important ROS that also contributes to oxidative stress (Rathee at al., 2006).The H_2O_2 scavenging activity varied significantly ($p<0.05$) both amongst varieties and solvent extracts (Table 1).

3.6.Nitric oxide scavenging activity

Dasgupta and De (2004) reported that foods rich in antioxidant have the potential to prevent cardiovascular diseases and cancer. Nitric oxide (NO) is a potent pleiotropic mediator in physiological processes and a diffusible free radical too in the pathological conditions. Nitric oxide (NO) reacts with superoxide anion and forms 'peoxynitrite' (Besco et al., 2012). Nitric oxide activity was found in all the extracts. Rintu et al. (2015) evaluated 9 varieties of betel leaf for their regulatory effect on nitric oxide (NO) and observed that all varieties showed direct scavenging of NO and exhibited significant activity. Maximum inhibition was seen in hydro acetonic extract (41.03±0.03% inhibition) and the lowest was seen in 2 hydro propanolic extract (13.03±0.40% inhibition). The inhibitory activity of the leaf in decreasing order is as follows-AHM> AA> PHA> AS> AHB> AHB> AHS> DWM> PHM> PHS> DWA> DWS> AM> DWB> AHA> AB> PHB. The results were comparable as reported (Rintu et al., 2015) for nine varieties *Piper betle* L. of methanolic extract. There was no significant difference amongst propanolic and acetonic samples for NO scavenging activity. However, water extracts and hydroacetone extracts

showed significant ($p<0.05$) variation with all the solvents used.

3.7.Superoxide radical scavenging activity

Superoxide (O_2^-) may develop many health problems like radiation poisoning, hyperoxic injury, early aging through oxidative damage (Hayyan et al., 2016). Maximum superoxide radical scavenging activity was found in distilled water extract (89.20±0.46% inhibition) and the lowest was seen in 2 hydro propanolic extract (55.90±1.29). Descending order of the extracts are as follows- DWB> AHS> DWM> AHM> PHM> DWA> PHS> AHA> DWS> PHB> PHA> AA> AHB> AB> PHB. The results were compared with the investigation carried out by Rintu et al. (2015) for different extracts of *Piper betle* L. (water, methanol, ethyl acetate and ether). Choudhury and Kale (2002) reported that the activity of superoxide dismutase increased in different betel leaf extracts which increased the antioxidant level in Swiss albino mice in a dose dependent manner. The consumption of these leaves may play a vital role in preventing diseases like rheumatoid arthritis, cardiovascular disease and even dreaded cancer which are directly related to oxidative stress and reactive oxygen species. The O_2^- radical scavenging activity varied significantly ($p<0.05$) amongst all the varieties and solvent extracts.

4.Conclusions

The results revealed that the different variety of betel leaves have highly effective antioxidant activity. The phenolic and antioxidant activity of different leaf extracts are highly influenced by the extraction solvents. Addition of 50% water in propanol and acetone can increase the extraction process and extract more antioxidant compounds from the leaves. The leaves are good source of natural antioxidant for pharmaceutical industry and can be used to accomplish desirable therapeutic outcomes.

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CONSIDERATIONS ON PHYSICAL SEPARATION OF A NATURAL LIPID MIXTURE BY PRODUCTION OF LIPID FRACTIONS

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ABSTRACT
The impulse transfer or amount of movement is important in phenomena of separating semi-solid food materials by the occurrence of movement phenomena inside it. This paper proposes application of operation of physical separation and gravity on a semi-solid lipid model food, fluid, imposed conditions (homogenization, duration, temperature) in physical and mechanical forces. These forces act differentially on components in a fluid / solid mixture of densities and different particle sizes at which the flow is present.

1. Introduction

The idea of turning conducted under application of technologies "*green*", "*natural*", on physical principles, separator of the initial lipid mixture, animal and/or plant as by-products from processing various technologies concerned engineers food technologists (Jianu, 2010, Rinovetz *et al.*, 2009). Knowledge and application of environmentally friendly, non-polluting, environmentally friendly transport phenomena can be a viable technical and economic element in modifying, recovering, capitalizing, diversifying products with technological and nutritional benefits from products/by-products/fatty waste.

Hippolyte Mège Mouries, *(1869)*, is considered the *pioneer of physical (cold) separation of fats of animal origin*.

Also, by separating the beef tallow, it has obtained a liquid product (*oleo-margarine* or *tallow olein*).

After mixing this fraction with skimmed milk, a solid product called "*economic butter*" was produced, and later "*margarine MOURIES*" (O'Brien, 1998, Frank, 2006).

Separation on physical principles (hydrodynamic phenomenon) is characterized by the transfer of property, physical differences (mass, density, shape, characteristic size, viscosity, etc.) and specific response to physical stressors (time, temperature, action, magnitude of force), of molecular species that form the lipid mixture without a chemical response. This generates two separate: **1.** solid particles at the bottom of the separation vessel (***stearin*** (***S***)); **2.** upper, liquid phase (***olein*** (***O***)) (Figure 1) (Harris).

In the literature, the process is known as ***vinterisation***, evolving from the observation that refined cotton oil deposited during winter in reservoirs is physically separated into a solid

(stearin) and a clear liquid (olein) fraction. Finally, solid can be found in three states: **1.** Solid, in form of co-crystals; **2.** liquid; **3.** liquid, physically bound to surface of crystals. In practice, *physical separation of palm oil* is standard in comparing different separation operations, by carefully monitoring melting / solidification intervals of structural components in initial blend (Katsumata *et al.*, 1995, Tirtiaux, 1968, Seugé and Vinconneau, 1975). The wine-making operation follows the following phases: **1.** fatty melting of fat; **2.** nucleation (speed process is dependent on triglyceride composition, cooling rate); **3.** Growth of crystals (speed of process being temperature dependent, time (two, three days to obtain large crystals, optimum for separation) and presence/absence of agitation of mixture)); **4.** formation of solid/liquid separations; **5.** decantation (O'Brien, 1998, Frank, 2006). Advantages of applying physical separation of natural lipids are: **1.** obtaining products with new, technologically and/or biologically functional properties without occurrence of appropriate chemical changes; **2.** "*green*" process (non-polluting, no toxic effluents); **3.** absence of trans acids; 4. reversible process without molecular changes; **5.** economic process (Frank, 2006). The active role shows melting/solidification interval, duration and magnitude of action force, defining hydrodynamic, flowing, and final properties of resultant products (Kellens and Hendrix, 2000, DeMan *et al.*, 1991). It offers possibility of *advanced separation* by obtaining a wide range of by-products (salad oils or thermal treatments, "*hard*" or "*soft*" stearin as a ***substitutes of margarine***, cocoa butter, shorteners, frying fats) with multiple uses: pastry, chocolate, pasta, etc.) (Deffense, 1985). Data in literature show that palmitic acid tends to migrate into the stearin solid fraction and oleic acid in olein with properties similar to sunflower oil. By physical separation liquid oleins can be obtained at temperatures below 24°C and stearins with a melting range of 45-53°C. The compatibility of ***stearin*** (*pastry margarine*) with margarine, cocoa butter, makes this product a substitute/surrogate in the manufacture of spreads, pasta or mixed with oils as an alternative to hydrogenated products (Lin, 2002, Pantzaris, 1988, Siew, 2002, Deffense, 1993, Yella, 2010, Gibon and Tirtiaux, 2000, Deffense, 2008). It can be argued that careful identification and monitoring of melting / solidification range allows fixation of mono- and polyunsaturated organic acids in the liquid (olein) and unsaturated in the stearin fraction (Armaud *et al.*, 2004). Factors influencing hydrodynamics of separation are: **1.** flow dynamics (the velocities of elementary particles are maximal along the axis of flow and decreases towards the periphery of flow section, becoming null at the contact surface between fluid and solid contour due to adhesion of fluid molecules to solid surface); **2.** Temporospatial conditions (differential action of temperature and force). Assessment of flow behavior of raw material and solid/liquid fractions is a qualitative quantification of resulting products, whose physical characters are different from the original material (Gonzalez-Gutierrez and Scanlon, 2012). It can provide important information for: **1.** anticipating the structural behavior of lipids across the phenomenon of separation and/or processing; **2.** Developing predictions on separation dynamics; **3.** simulations for characterization of separations; **4.** dimming/designing appliances, machines, installations; **5.** optimizing operating parameters. (Groza *et al.*, 2009, Rivis *et al.*, 2008, Rivis *et al.*, 2013)).

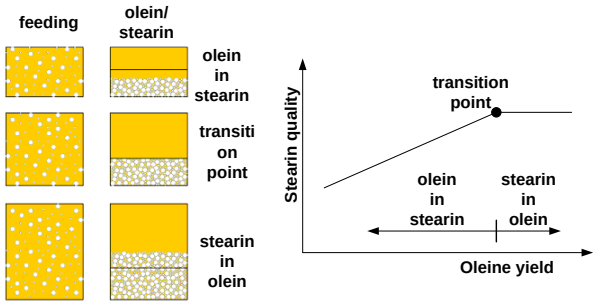


Figure 1. Mechanism for formation of separations (***oleins/stearins***) by physical principles

2. Materials and methods

2.1.Materials

Commercialized unhydrogenated palm oil (***UPO***), crystallization vessel, POL-EKO-NITECH incubator (temperature range 20-150^{o}C), analytical balance, Sigma-Aldrich analytical purity reagents.

2.2.Methods

Taking into consideration character of addressed problem, it was proposed to develop a block diagram of operations of physical separation in gravitational field of non-hydrogenated palm oil, following olein path (three fractionation stages (T1-3)) (Figure 2). Resulting fractions were characterized by comparison based on quality indicators [(iodine value (***IV***) (Wijs Method); density (d_{50}^{50}), picnometric method; melting/solidification point by capillary; dynamic viscosity (□)] (STAS 145/19-90, Popescu *et al.*, 1986, Enache *et al.*, 1997), to assess the effectiveness of separation operation. Unhydrogenated palm oil was deposited in a crystallisation vessel, which was melted at ***75°C*** (*specialty literature 65-80°C* (limit of oxidative degradation for solid fats) (DeMan *et al.*, 1991), ***duration 5h***, to "*destroy crystallisation memory*". As an advanced separation process, olein pathway was chosen, in which time (***12h***) was constant and the *variable temperature*: ***T1 - t^{o}C=35; T2 - t^{o}C=25 şi T3 - t^{o}C=15***. Resulting lipid fractions were collected in hermetically sealed plastic containers and stored under refrigeration conditions (4°C) for subsequent physico-chemical analysis.

3. Results and discussions

Because in literature formulations for solid/liquid interactions are vast, it has been attempted to question information on a limited segment. The hypotheses and conclusions can not cover the entire area of interest. However, this paper proposes to formulate new conclusions and assumptions based on conceptions, principles and hypotheses in literature. It can be argued that *fractional crystallization* and *mechanical separation* of a semi-solid (saturated/unsaturated) lipid mixture is a complex phenomenon, dependent on polymorphism, crystal-type size and newly formed supermolecular networks. Hypotheses formulated imply that separation of saturated/unsaturated phases occurs due to the *phase transition* to temperature variations in various crystallisation phases, which results in network changes. The role of non-lipidic elements (sometimes initiators of nucleation) is to modify nature of interactions between structural components as *plasticizing factors* and/or to "*brake*" redistribution of triglycerides in various phases of transition (refrigeration/separation, refrigeration/tempering, melting/refrigeration, etc.).

From a comparative evaluation of experimental data, taking into account the decisive natural influence of operating parameters, operating scheme was reflected in Figure 2. The raw material and obtained fractions (oleins/stearines (*substitutes*)) were physico-chemical evaluated Table 1) and graphically interpreted (Figures 3-5).

- Iodine value (Table 1, Figure 3) as an expression of degree of unsaturation shows an increase for liquid separations (oleins) and lower values for solid fractions (stearines). Differences occur as a result of the concentration of unsaturated components in oleic fraction compared to predominant saturated in solid fraction. Raw material has relatively high values being a mixture of triglycerides with different saturation degrees, but in which, however, predominantly unsaturated are present. Determined values are also caused by the differences in average molar mass of components in starting material. Solid separates have higher concentrations of saturated compounds with higher molar masses, while liquid separations are more concentrated in unsaturated compounds, so with lower molar masses.

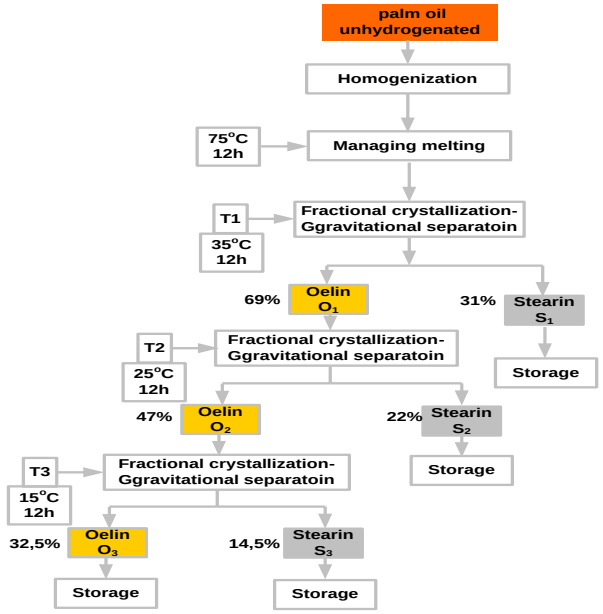


Figure 2. Operation chart proposed of gravity separation of unhydrogenated palm oil

● **Melting/solidification temperature** (Table 1, Figure 4a, b). Length of hydrocarbon chain, as well as degree of unsaturation, condition melting/solidification temperature of triglyceride mixture. Melting points vary depending on weight and nature of chemical entities present in the triglyceride structure (mono-and diglycerides have higher melting points than triglycerides with a similar composition in higher organic acids). Therefore, mixtures of triglycerides (fats) have a *melting range* and not defined melting points, depending on the nature of superior organic acids in their structure (simple triglycerides have defined melting points). Presence of complex fats (cholesterol, phospholipids, cephalins, etc.) constrains separation mode within operating parameters. From Figure 4a it is observed that separator liquid (oleins) exhibits decreasing solidification temperature values, and for solid separations, same phenomenon occurs in case of melting temperature. This indicator overlaps with degree of unsaturation. Data confirm that solidification temperature decreases in olein series (O1□O3), conditioned by reduction of hydrocarbon chain length under operating parameters. A sustained outbreak and increased unsaturation in this series. Higher values are encountered in the series of sterarines (S1□S3), increase due to presence of unsaturated hydrocarbons with longer lengths. An important role also belongs to the polymorphism phenomenon characteristic of lipids, especially the nature and weight of species of higher organic acids in triglyceride mixture, with influences on technological performances in various applications. In summary, melting temperature of triglyceride mixture (Up□35.7°C) is reduced compared to sum of oleins and/or stearins, a phenomenon that can be explained by distribution of mono- and diglycerides in mass of separates.

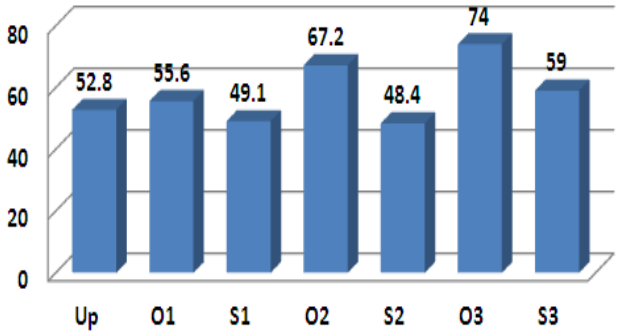


Figure 3. Comparison of the degree of unsaturation for liquid/solid separations

● **Density** (specific weight) (Table 1, Figure 5a). Analysis of density of solid/liquid lipid separations resulting from fractional crystallization and gravity field separation shows close results (0.906-0.8711 g/ml). There is some difference between samples, determined by mode and dynamics of flow phenomenon imposed by differentiated action of gravitational force. This finding further confirms that other operating parameters will be determinant in ensuring the efficiency of crystallisation and stratification.

● Viscosity (Table 1, Figure 5b). In Figure 5b, dynamic viscosity evolution (ç) is compared. Graphs confirm incidence of operating parameters in evolution of viscosity of separators, in sense of decreasing olein series and their increase for solid fractions (stearines).

Although, a number of studies have been carried out in this field, the nature of material (technological variant of production) and operator parameters remain responsible factors for technological and functional direction of resulting fractions.

Under similitude conditions, by extrapolation of laboratory data, material balance for a finished product - *olein* (***O***)/*stearin* (***S***) - was calculated for a quantity of 500 kg of raw material (Table 2).

Also, following values of weight of separates for each separation step resulted: **T1.** olein (O1) of 73% (361,715 kg) and 27% stearin (S1) (133,785 kg); **T2.** O2 (super olein) 58% (287.102 kg) and 15% S2 stearin (74.251 kg); **T3.** O3 of 49% (242.31 kg) and 9% stearin S3 (44,505 kg). Data in Table 2, reveals difference in distribution of two separate oleins/stearin, gravitationally, values confirmed by operating parameters (temperature, time, strength) supported by experimental data.

Stearin yield ($\boldsymbol{\eta_{ST}}$) was then determined (Table 3), based on the weight of solid resulting from each separation step of suspension (olein pathway ($\boldsymbol{m_{susp}}$)).

Obtained values show that combined action of force size, melting/solidification temperatures and time of action, with preliminary processing, directly influences flow phenomena, resulting in higher masses of oleins.

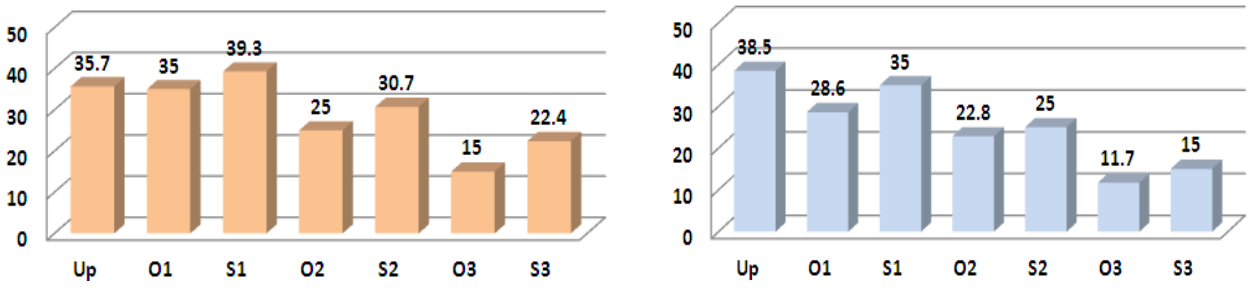
So, it can be said that the operating parameters accessed are the decisive factors in optimizing the process.

This confirms that efficiency of the process is dependent on:

1. conducting the crystallization process of suspension material (crystal size, distribution of crystals in suspension mass, olein mass retained on surface of crystals); **2.** mass of solid contained in mass of the suspension; **3.** choice of process parameters depending on the structure, the raw material characters and predicted formulation of finished product.

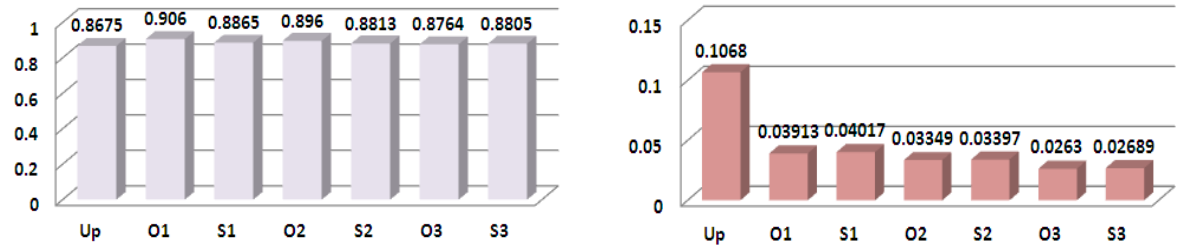
Overall, experimental obtained values and calculations made, allow some of following statements to be made regarding to the differential operation of operating parameters depending on nature of force acting on two separate ones.

To this is added lipid property of fractional crystallisation, depending on nature of triglycerides that induce melting-solidification range, as follows: **1.** lipid homogenate is a complex system with multiple interactions due to simultaneous presence of mono-, di-, triglycerides and presence free organic organic acids, which generate crystalline fractions; **2.** by the phenomena of polymorphism / polytropism, the presence of hydrogen bonds, the interactions of Van der Waals type, separation, is manifested with retention to phenomena of flow in gravitational field; **3.** the modest difference between quality indicators leads to the idea that proper choice of separation parameters is decisive in changing desired quality of product; **4.** achievement of higher yields and efficiency of separation by physical separation can only be achieved by thorough research, which subsequently allows the formulation of behavioral predictions that can be extrapolated on the basis of similarity on an industrial scale, observing laboratory constraints.



a **b**

Figure 4. Evolution of melting range (**a**)/solidification (**b**) for liquid/solid separations



a **b**

Figure 5. Evolution of density (d_{50}^{50}) (**a**) and viscosity (η) (**b**) for liquid/solid separations

Table 1. Comparison of physical-chemical indicators for raw material (non-hydrogenated palm oil) and liquid / solid separates

Operation/ Material	**Separate (symbol)**	**Quality indicators**					**Separation steps (T)**	**Separation parameters**	
		IV [g I_2/100 g]	**MP** [°C]	**SP** [°C]	d_{50}^{50} [g/ml]	**η** [Pas]		**Time [h]**	**Temperature [°C]**
Raw material	UPO	52.8	35.7	38.5[1)]	0.8675	0.1068			
Gravitatio-nal separation	O_1	55.6	35	28.6	0.906	0.03913	1	12	35
	S_1	49.1	39.3	35	0.8865	0.04017			
	O_2	67.2	25	22.8	0.8960	0.03349	2	12	25
	S_2	48.4	30.7	25	0.8813	0.03397			
	O_3	74	15	11.7	0.8764	0.02630	3	12	15
	S_3	59	22.4	15	0.8805	0.02689			

Table 2. Centralized balance material (kg) sheet for gravity separation

No.	**Incoming matter**					**Issued matter**				
	Operation / Material		**Symbol**	**Quantity**	**%**	**Operation / Material**		**Symbol**	**Quantity**	**%**
1	Reception	Palm oil unhydrogenated	UPO	500	100	Delivery	Lipid substitutes	-	499	99.8
		Total		**499.5**	**99.9**					
2	Homogenization	-	UPO	499		Material loss		P	1	0.2
3	Managing melting	-	UPO	498						
4	Crystallization/ Separation (**T1**)	Olein 1	O_1	341.9		**Total**			**500**	**100**
		Stearin 1	S_1	153.6						
5	Crystallization/ Separation (**T2**)	Olein 2	O_2	232.655						
		Stearin 2	S_2	108.912						
6	Crystallization/ Separation (**T3**)	Olein 3	O_3	160.718						
		Stearin 3	S_3	71.704						

Table 3. Presentation of solids yields (stearin), η_{ST}, fractionation steps

No.	Source	Symbol	Unit	Weight	η_{ST} [%] $\eta_{ST} = \frac{m_{ST}}{m_{susp}} \cdot 100$	Separation step
1	Olein	O_1	kg	341.9	30.6	1
2	Stearin	S_1	kg	153.6		
3	Olein	O_2	kg	232.655	31.85	2
4	Stearin	S_2	kg	108.912		
5	Olein	O_3	kg	160.718	30.81	3
6	Stearin	S_3	kg	71.704		

4. Conclusions

The literature study, supplemented by careful laboratory research, highlights the influence of operating parameters on lipid quality. Quality also manifested from perspective of "*history*" of raw material accessed, and especially in chosen case, of original destruction of "*memory*" of crystallization.

Data confirm responsibility of operating parameters (temperature, duration, strength), modest separation rates. So, knowledge and interpretation of quality indicators, along with separation parameters, are important in design and processing of lipid materials. This demonstrates that physical separation of a lipid system is a complex process in which simultaneous solid/liquid phenomena are manifested, which are conditioned by technical performances of accessed separation operations.

Selectivity of operation, absence of solvents and production of higher organic acids without appearance of trans-type, predicts that physical separation will be a dominant process in the 21st century compared to beginning of the modern oil and fat processing industry after ***Mège-Mouriès*** invented margarine.

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ACETYLCHOLINESTERASE INHIBITION AND ANTIOXIDANT EVALUATION OF POLYPHENOLIC FRACTIONS AND OIL FROM FOUR MELON SEEDS USED AS CONDIMENTS IN NIGERIA.

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ABSTRACT

Species of different melon seeds i.e. Citrullus lanatus (Thunb), Cucumeropsis mannii, Lagenaria siceraria (Mol.) Standl. and Citrullus colocynthis (L.) Schrad. are used mostly for culinary and health purposes in Nigeria, West Africa. Their health promoting potential may be connected to the antioxidant properties of their chemical constituents i.e. polyphenolic. Antioxidant evaluation was carried out on polyphenolic fractions and oils from the melon seeds' species using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging and 2, 2′-azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activities under in vitro conditions. Acetylcholinesterase inhibition effect of the polyphenolic fractions and oils were also investigated. The result of the antioxidant activity of the polyphenolic extracts and oils obtained by different in vitro methods varied remarkably on the basis of method used. Nevertheless, the oil from Citrullus colocynthis gave the best IC50 value of 3.88 µg/mL and the polyphenolic fraction from Cucumeropsis mannii gave the best result with IC50 values of 1.89 µg/mL and 22.49 µg/mL against DPPH and ABTS respectively. Polyphenolic fraction from seeds of Lagenaria siceraria and oil from seeds of Citrullus lanatus gave the best acetylcholinesterase inhibition activity with IC50 value of 47.40 µg/mL and 19.50 µg/mL. This study's results indicated that the different seeds of melon species are sources of natural antioxidants preventing oxidative damage and a good source of acetylcholinesterase inhibition preventing old-age related and neurodegenerative diseases i.e. Parkinson and Alzheimer's diseases..

1.Introduction

Three centuries ago, the great Philosopher Hippocrates gives credence to a principle which states "Let food be thy medicine and medicine be thy food" (Hasler, 1998). Besides, the nutritional value derived from food, they are needed for survival hence foods can be said to be functional. The term "functional food" should suggest that there is an extra importance and gain humans receive from food beyond basic nutritional requirements (Liang, 2005). The Institute of Medicine's Food and Nutrition Board (IOM/FNB, 1994) defined functional foods as "any food or food ingredient that provide a health benefit beyond the traditional nutrients it contains" (Meyer *et al.,* 1998). Seeds of different melon species are popular for culinary and medicinal purposes in West

Africa, their health promoting potential might be related to the pharmacological properties of their chemical constituents such as polyphenols, flavonoids, etc. Generally, plants contain natural products with remarkable role in traditional medicine. The consumption of naturally rich antioxidants medicinal plants and fruits have been identified with the management of various ailments and diseases (Johnson, 2001; Virgili *et al*., 2001).

Free radicals are harmful molecules which are responsible for serious damages that occur in the human body but antioxidants are plants' metabolites that not only protect the body against this but replenish this damages (Bello *et al.,* 2017). One of the main role of antioxidants is to help against oxidation, which is responsible for human cells' damage and leads to aging. This important function may ameliorate the immune role and reduce infection risk, heart failure and tumors. Antioxidants do exist has natural compounds in fruits and foods, vitamins and minerals. (American Dietetic Association, 2010). Stress which is caused by oxidative reaction is as a result of over production of free radicals and low defense from antioxidant i.e. imbalance between reactive oxygen species and antioxidants which give heed to chemical adjustment of biomolecules causing configurational and functional improvements (Hoake and Pastorino, 2002).

The primary physiological function of the enzyme acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) is the fast cessation of impulse transmission at cholinergic synapses by rapid hydrolysis of the cationic neurotransmitter acetylcholine (ACh) to yield acetic acid and choline (Barnard, 1974; Tõugu, V., 2001). Alzheimer disease (AD) and depression are quite related, people suffering from the latter, are prone to the former. The inhibition of acetylcholinesterase (AChE) is a major relieve to Alzheimer disease and its symptoms (Heinrich & Teoh, 2004). The main role of the biological enzyme is to speed up the hydrolysis of the neurotransmitter (acetylcholine) i.e. enhancing the amount of acetylcholine in the human cerebrum using acetylcholinesterase (AChE) inhibitors (Enz *et al.,* 1993). The inhibition of acetylcholinesterase (AChE) may be one of the major ways for the treatment of old age and neurodegenerative diseases i.e. Alzheimer's (AD) and Parkinson's disease (Brenner, 2000; Rahman and Choudhary, 2001).

To the best of our knowledge, the polyphenolic fractions and oils of seeds of *Citrullus lanatus, Cucumeropsis mannii, Lagenaria siceraria and Citrullus colocynthis* have not been investigated. The aim of this research is to evaluate the antioxidant and acetylcholinesterase inhibitory activity of oils and polyphenolic fractions from seeds of *Citrullus lanatus, Cucumeropsis mannii, Lagenaria siceraria and Citrullus colocynthis*.

2.Materials and Methods

2.1.Chemicals and equipment

The solvents employed in this work are of analytical grade (sigma Aldrich), solvents were redistilled before used where necessary. The reagents used for the assays (antioxidant and acetylcholinesterase) were bought from Santa Cruz Biotechnology, US.

2.2.Plant collection

The seeds of *Citrullus colocynthis* (Egusi melon), *Cucumeropsis mannii* (egusi-itoo), *Citrullus lanatus* (water-melon) and *Lagenaria siceraria* (Bottle gourd) were collected from Ilorin metropolis, Kwara, Nigeria, washed with water, air-dried, pulverized to fine powder and stored in air-tight bottles until solvent extraction.

2.3.Extraction

Twenty-five (250) gm of the air-dried, powdered seeds of melon species were

extracted individually using N-hexane at room temperature. The extracts were then concentrated using a rotary evaporator giving the crude N-hexane extracts. The residues were then air-dried and extracted using methanol. These were also concentrated to give crude methanol extracts.

2.4.Preparation of trans-esterified oil

To 2 gram of the crude N-hexane extract, 10 mL of 0.2 M methanolic HCl was added and refluxed for an hour. This was then poured into a separating funnel, 20mL of N-hexane was added and 20mL of water was used to wash the beaker into the separating funnel. The separating funnel was closed and shaked thoroughly. It was then allowed to separate into two layers (the N-hexane and water layers) and collected into different beaker. The N-hexane fraction collected was then poured back into the separating funnel and washed with another 20 mL of water twice. This procedure was repeated for the water fraction using N-hexane instead. Trans-esterified oil was obtained from the N-hexane fraction after filtration using Sodium sulphate and cotton wool.

2.5.Isolation of polyphenols

The methanolic extract of the melon seeds' species were dissolved in water, left for 1 hour and decanted. The water layer was poured into a separating funnel, then washed with petroleum ether several times until a clear upper layer of petroleum ether was gotten. Two layers were observed i.e. upper and lower layers. The latter which is the lower layer was then collected and treated with 1 litre of ethyl acetate containing 10 mL glacial acetic acid. Polyphenols extraction was then carried out for 36 hours at room temperature and the ethyl acetate layer was combined was and concentrated. The residue of the concentration was lyophilized and stored at -70 ^{0}C.

2.6.Antioxidant activity

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

The method employed was the one reported by Atolani *et al*., (2012) though with slight modifications (Atolani *et al*., 2012). Mean ± standard error of the mean of two independent experiments run in duplicate was used to present the results.

***2.6.2. Determination of 2, 2'-azino-bis-(3-ethyl) benzothiazoline-6-sulfonic acid (ABTS) radical cation scavenging activity*.**

The 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonate, ABTS radical cation decolourization assay based on the scavenging of $ABTS^{\cdot+}$ radicals by antioxidants component of the extracts was used. The assay follows the procedure of Atolani *et al.,* (2013), with slight modifications (Atolani *et al.,* 2013). All analysis was determined in duplicate.

2.7.Acetylcholinesterase (AChE) Inhibitory Activity

The AChE inhibitory assay analysis was carried out according to the method described by Lopez *et al*. (2002) with some modifications (Lopez *et al*. 2002) .

3.Results and discussions

3.1.Antioxidant activity

3.1.1.DPPH free-radical scavenging activity

Polyphenolic fractions and oil from seeds of melon species showed DPPH free-radical scavenging activity (Table 1, 2, 3 & 4). Oils and polyphenolic extracts from seeds of *Citrullus colocynthis* and *Cucumeropsis mannii* respectively showed lowest IC_{50} values, indicating remarkable DPPH free radical scavenging activity. The oil and polyphenolic extract from *Citrullus lanatus* has IC_{50} values of 117.5 and 100.9 µg/mL, respectively which showed the highest DPPH free-radical scavenging activity.

3.1.2 ABTS radical cation scavenging activity

All the oils and polyphenolic extracts from seeds of melon species showed ABTS radical cation scavenging activity except for oil of *Citrullus colocynthis* that hit a constraint (Table 1 & 2). Oils and polyphenolic extracts from seeds of *Lagenaria siceraria* and *Citrullus colocynthis* have IC_{50} values of 10.40 and 6.64 µg/mL respectively, this indicate high ABTS radical cation scavenging activity.

3.2.Acetylcholinesterase (AChE) Inhibitory Activity

All the oils and polyphenolic extracts from seeds of melon species showed AChE inhibitory activity (Table 1 & 2). Oil and polyphenolic extracts from seeds of *Citrullus lanatus* and *Lagenaria siceraria* respectively showed the lowest IC_{50} values, implying good AChE inhibition.

3.3.Analysis of Data

Analysis of data was achieved by using the GraphPad Prism, version 7 software (San Diego, USA) to determine the IC_{50} through a non-regression analysis. IC_{50} was taken as the concentration of sample that scavenged half of the radicals. Mean ± standard error of the mean was used to present the results.

3.4.Discussions

The IC_{50} values of DPPH and ABTS activities for *Citrullus lanatus*, *Cucumeropsis mannii*, *Lagenaria siceraria* and *Citrullus colocynthis* oils were found to be (µg/mL) 117.50 and 79.74, 4.17 and 24.53, 13.96 and 10.40, 3.88 respectively. From the comparative study of the results, the antioxidants activity of the oils is in the order of *Citrullus colocynthis* > *Cucumeropsis mannii* > *Lagenaria siceraria* > *Citrullus lanatus*. The IC_{50} values of DPPH and ABTS activities for *Citrullus lanatus*, *Cucumeropsis mannii*, *Lagenaria siceraria* and *Citrullus colocynthis* polyphenolic extracts were found to be (µg/mL) 100.90 and 100.20, 12.40 and 25.66, 1.89 and 22.49, 70.01 and 6.64, respectively.

Table 1.Antioxidant activity of oils from seeds of different melon species, as expressed (µg/mL) by inhibitory concentration (IC_{50}).

1.	***Plant Species***	***Inhibitory Concentration (IC_{50}) (µg/mL) ABTS radical cation***	***Inhibitory Concentration (IC_{50}) (µg/mL) DPPH free-radical***
2.	*Citrullus lanatus (oil)*	79.74 ± 30.50	117.50 ± 26.33
3.	*Cucumeropsis mannii (oil)*	24.53 ± 6.66	4.17 ± 3.12
4.	*Lagenaria siceraria (oil)*	10.40 ± 1.80	13.96 ± 4.49
5.	*Citrullus colocynthis (oil)*	NA	3.88 ± 4.26
6.	Ascorbic acid	376.50 ± 99.88	222.80 ± 129.20

Each value represents the mean ± SEM of two replicate experiments.

Table 2. Acetylcholinesterase activities of oils from seeds of different melon species, as expressed (µg/mL) by inhibitory concentration (IC_{50}).

	Melon Used	***Inhibitory Concentration (IC_{50}) (µg/mL) AChE***
1	*Citrullus lanatus (oil)*	19.50 ± 13.43
2	*Cucumeropsis mannii (oil)*	33.08 ± 14.61
3	*Lagenaria siceraria (oil)*	26.93 ± 8.55
4	*Citrullus colocynthis (oil)*	19.89 ± 17.52

Each value represents the mean ± SEM of two replicate experiments.

Table 3. Antioxidant activity of polyphenolic extracts (PPE) from seeds of different melon species, as expressed (µg/mL) by inhibitory concentration (IC_{50}).

	Melon Used	***Inhibitory Concentration (IC_{50}) (µg/mL) ABTS radical cation***	***Inhibitory Concentration (IC_{50}) (µg/mL) DPPH free-radical***
1	*Citrullus lanatus (PPE)*	100.20 ± 42.04	100.90 ± 24.21
2	*Cucumeropsis mannii (PPE)*	22.49 ± 6.10	1.89 ± 3.32
3	*Lagenaria siceraria (PPE)*	25.66 ± 6.98	12.40 ± 3.80
4	*Citrullus colocynthis (PPE)*	6.64 ± 2.19	70.01 ± 14. 57
5	*Ascorbic acid*	376.50 ± 99.88	222.80 ± 129.20

Each value represents the mean ± SEM of two replicate experiments.

Table 4. Acetylcholinesterase activities of polyphenolic extracts (PPE) from seeds of different melon species, as expressed (µg/mL) by inhibitory concentration (IC_{50}).

	Plant Specie	***Inhibitory Concentration (IC_{50}) (µg/mL) AChE***
1	*Citrullus lanatus (PPE)*	57.96 ± 13.96
2	*Cucumeropsis mannii(PPE)*	49.67 ± 8.11
3	*Lagenaria siceraria(PPE)*	47.40 ± 9.71
4	*Citrullus colocynthis(PPE)*	52.51 ± 11.35

*Each value represents the mean ± SEM of two replicate experiments***.**

From the comparative study of the results, the antioxidants activity of the polyphenols is in the order of *Cucumeropsis mannii > Citrullus colocynthis > Lagenaria siceraria > Citrullus lanatus*. The IC_{50} values of AChE inhibitory activity for *Citrullus lanatus*, *Cucumeropsis mannii*, *Lagenaria siceraria* and *Citrullus colocynthis* oils and polyphenolic extracts were found to be (µg/mL) 19.50 and 57.96, 33.08 and 47.40, 26.93 and 49.67, 19.89 and 52.51, respectively. From the comparative study of the results, the acetylcholinesterase inhibitory activity of *Citrullus lanatus > Citrullus colocynthis > Lagenaria siceraria > Cucumeropsis mannii* for oils and *Cucumeropsis mannii > Lagenaria siceraria > Citrullus colocynthis > Citrullus lanatus* for polyphenolic extracts. Hence, polyphenolic extract from *Cucumeropsis mannii* and the oil from *Citrullus colocynthis* gave the best antioxidant activity against DPPH and ABTS respectively. *Lagenaria siceraria* polyphenolic extract and *Citrullus lanatus* oil gave the best acetylcholinesterase inhibitory activity.

Citrullus colocynthis gave the best overall result and many studies have shown the biological advantages the seeds of this medicinal plant possess. Jayaraman and Christina, (2009) reported the presence of flavonoids in the seeds' extracts of *Citrullus colocynthis* through the phytochemical screening carried-out though other authors confirm the presence of flavonoids in the seeds also (Uma and Sekar, 2014; Jayaraman and Christina, 2009). Jayaraman and Christina, (2009) showed that extract of *C. colocynthis* has hepatoprotective effect and antioxidant nature which was indicated by employing DPPH ion radicals and reducing power method while the control (positive) employed was ascorbic acid. The antioxidant activity of the extract of *C. colocynthis* is dose dependent as revealed the study (Jayaraman and Christina, 2009). Gill *et al.*, (2011) studied the antioxidant nature of the seeds of *C. colocynthis* by DPPH and hydrogen peroxide free radical scavenging method spectrophotometrically. Gill *et al.*, 2011 reported the antioxidant activity of methanolic seed extract of *Citrullus colocynthis*, at the dose of 300 µg /mL it displayed inhibition of 79.4 and 72.4 % by 1, 1-diphenyl-2-picryl hydrazyl and hydrogen

peroxide method (Gill *et al.,* 2011). These findings justify the antioxidant nature of *C. colocynthis* showed in this work.

Presently, inhibitors of acetylcholinesterase (AChE) are the major approved medicines in the market against mild to moderate Alzheimer's disease. From ethnopharmacological point of view, there are plants used as memory enhancers, because of their apparent impact on brain activity, and therefore may contain compounds with activity against Alzheimer's disease. *Citrullus colocynthis* is being used in some parts of the world i.e. Anatolia, as a memory enhancer (Orhan and Aslan, 2009). The polyphenolics extracts from the seeds of *C. colocynthis* considerably inhibit the enzymatic activity of AChE with IC_{50} value of 52.51 µg/mL, though other melon seeds gave good results too. This gives credence to brain enhancing nature of the medicinal plant.

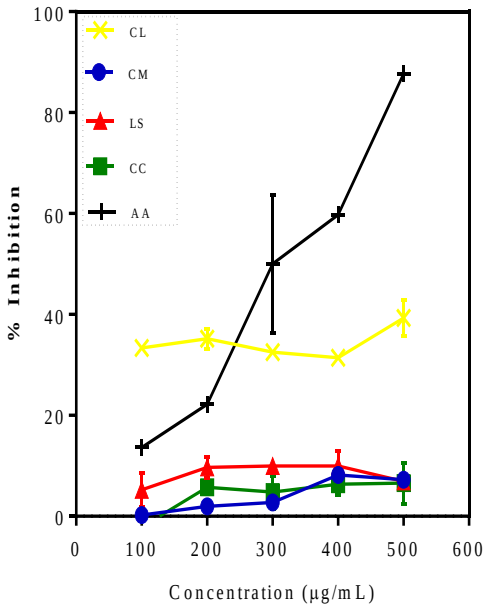

Figure 1. **DPPH** radical scavenging activity of oils from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

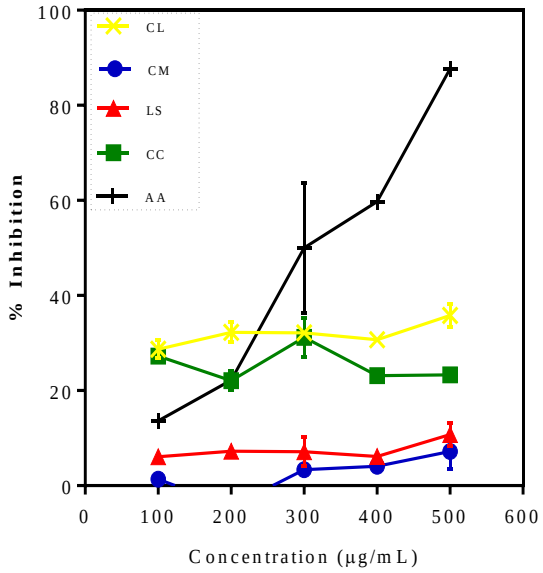

Figure 2.DPPH radical scavenging activity of polyphenolic extracts from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

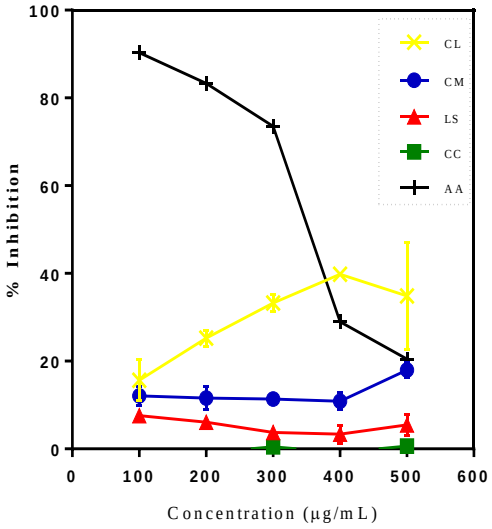

Figure 3. ABTS radical cation scavenging activity of oils from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

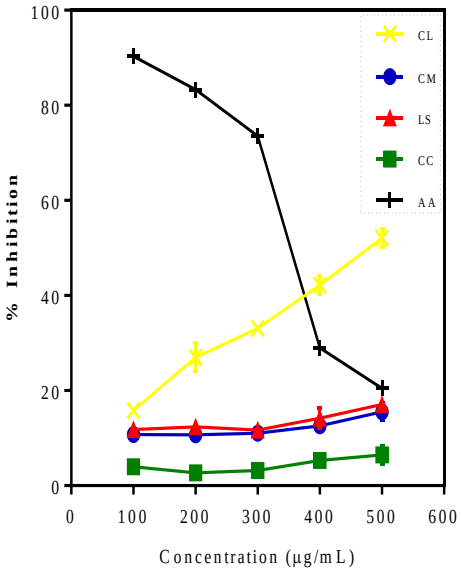

Figure 4. **ABTS** radical cation scavenging activity of polyphenolic extracts from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

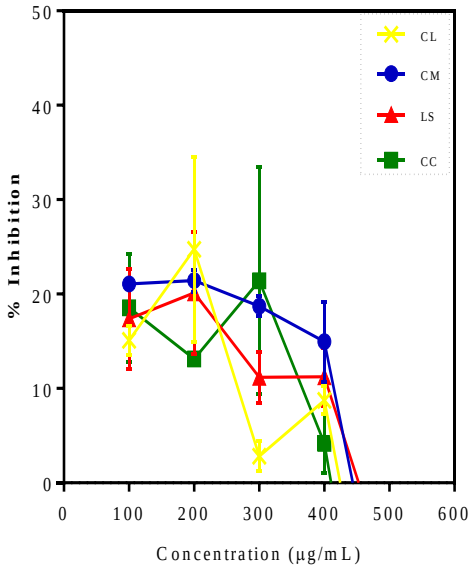

Figure 5. AChE radical cation scavenging activity of oils from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

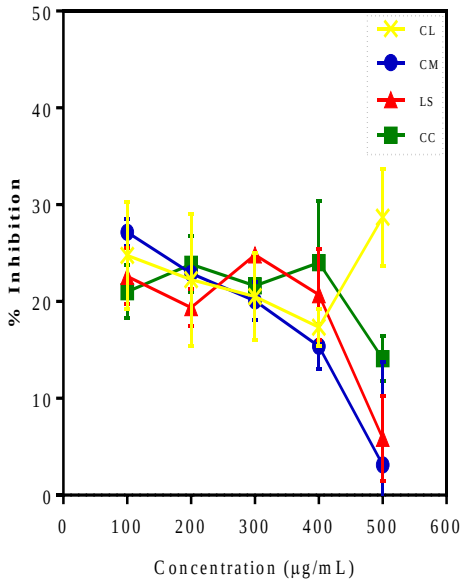

Figure 6. AChE radical cation scavenging activity of polyphenolic extracts from seeds of different melon species. Values are expressed as mean ± SEM. Values are mean of two experiments.

4.Conclusions

The results indicated that seeds from the different melon species are a good source of natural antioxidants with significant biological functions serving further as memory enhancer, fortifying material for maintaining shelf life. However, further studies should be carry out to develop a method for fractionation of polyphenols, identification and determination of the most active antioxidant compounds in this polyphenolic extract. Also, other biological activity should be carry out on the oils and polyphenols extracted from seeds of melon species in order to further ascertain their uses in medicine as antioxidant, anticancer, analgesic, anti-inflammatory, antidiabetic, antimicrobial, gastrointestinal agents, etc.

Table 5. Ethnomedicinal importance of the Melon Species Studied

	Names	Family	English Names	Vernacular Names	Traditional Uses	Pharmacological Uses	Phytochemicals	Isolated Compounds
1	*Citrullus lanatus (Thunb)*	Cucurbitaceae	watermelon	Bara *(Yoruba)* Elili *(Igbo)*	To maintain the healthy appearance and aid in its regeneration, in Central America and India the oil extracted from the seeds is applied to herpes lesions, venereal sores, stubborn leg ulcers and the face to treat acne vulgaris (Athar and Nasir, 2005).	Antimicrobial Activity (Oluba *et al*., 2011); Antioxidant Activity (Gill et al., 2011); Anti-Inflammatory Activity (Madhavi *et al.,* 2012); preventing stroke, cancer, heart attack and decreasing blood pressure (Azimabadi*.,* 1996; Yin-fang and Cheng-jun, 1999; Al-Jauziyah , 2003).	Alkaloids, flavanoids, tannins, aminoacids, carbohydrates, cardioglycosides, terpenoids	Lycopene, beta-carotene, xanthophylls, phenolics, vitamin C, 2-dodecyclobutanone, 2-tetracylcyclo butanone, glutelin, Glycoprotein-vicilin. lutein, beta-carotene, Cucurbitacin E (Ojieh *et al.,* 2008*;* Puerta and Cisneros*,* 2012*;* Sin *et al.,* 2006*;* Wani *et al.,* 2011*;* Yadav *et al.,* 2011*;* Olamide *et al.,* 2011)
2	*Cucumeropsis mannii Naudin.*	Cucurbitaceae	white-seed melon	òkôkòn *(Efik)* àhụ elu, aki *(Igbo)* agushi*(Hausa)* ẹgúsí-ìtóò, itóò, itórò *(Yoruba)*	juice of the fruit is used as healing ointment (Leung *et al.,* 1968).		amino acids	
3	*Lagenaria siceraria(Mol.) Standl.*	Cucurbitaceae	Bottle gourd	Egusi Igba (*Yoruba*) Lauki, Ghia (*Hindi*)	The fruits, leaves, stem, seeds and oil are traditionally used in the treatment of jaundice, diabetes, ulcer, piles, colitis, insanity, hypertension, congestive cardiac failure, and skin diseases (Minocha *et al.,* 2015). Its fruits are widely used in Ayurveda and other folk medicines traditionally, it is used for its cardioprotective,	Hepatoprotective, Immunomodulatory, Antihyperglycemic, Antihyperlipidemic, Analgesic and Anti-Inflammatory, Antibacterial and Diuretic properties (Kubde *et al.,* 2010), antiproliferative, immunosuppressive, antifertility (Shah *et al.,* 2010) antioxidant (Kubde et al., 2010) anticancer (Mazumdar, *et al.,*	Flavonoids, Triterpenes, Alkaloids,	avenasterol, codisterol, elesterol, isofucasterol, stigmasterol, sitosterol, campesterol, spinasterol (Rastogi *et al.,* 1990*;* Chopra *et al.,* 1992; Rahman *et al.,* 2003) Lagenin, cucurbitacins B, D, G, H and 22-deoxy cucurbitacin, fucosterol, campesterol aerpene byonolic acid (Shah *et al.,* 2010)

					cardiotonic, general tonic, diuretic, aphrodisiac, antidote to certain poisons, cooling effects and alternative purgative. It cures pain, ulcers and fever, and used for pectoral cough, asthma and other bronchial disorders, especially syrup prepared from the tender fruits	2011) analgesic (Shah et al., 2010).		
4	*Citrullus colocynthis (L.) Schrad*	*Cucurbitaceae*	bitter-apple, bitter-cucumber, vine-of-Sodom, wild gourd.	Egusi (Yoruba), Hagusi (Hausa)	Edible in daily meals	Antioxidant (Delazar et al. 2006; Kumar et al. 2008; Benariba et al. 2013), Anticancer, Analgesic, Anti-inflammatory (Rajamanickam *et al.,* 2010; Marzouk *et al.,* 2011), Antidiabetic, Antimicrobial, gastrointestinal (Gill *et al.,* 2001), reproductive (Pariza *et al.,* 2001) leprosy, asthma, bronchitis, jaundice, joint pain, cancer and mastitis (Chopra, 1958; Perveen *et al.*, 2007; Abo *et al.,* 2008; Asyaz *et al.*, 2010))	Flavonoids, Triterpene glycoside, alkaloids	isosaponarin, isovitexin and isoorientin 3'-*O*-methyl ether, 2-O-beta-D-glcoside and its aglycone, 2-O-beta-D-glcopyranosyl-cucurbitacin B, 2, 25-di-o-beta-D-glucopyranosyl, Colocynthosides A, and B, Curcurbitacins A, B, C, D, E, I, J, K, and L (Hussain *et al.,* 2014, Adam *et al.*, 2001; Yoshikawa *et al.,* 2007)

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SURVIVABILITY OF *LACTOBACILLUS ACIDOPHILUS, BACILLUS CLAUSII AND SACCHAROMYCES BOULARDII* ENCAPSULATED IN ALGINATE GEL MICROBEADS

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ABSTRACT

In this study, three probiotic species including *Lactobacillus acidophilus*, *Bacillus clausii* and *Saccharomyces boulardii* were double-layer coated by hydrogel encapsulation technique with sodium alginate and gelatin as first and second coating agents respectively. The free and encapsulated probiotics cells were subsequently inoculated in gastro-intestinal media for 120 min to examine the protective benefits of the layers. The results indicated that free bacteria were completely inactivated after 90 min incubation under low pH condition while those encapsulated by sodium alginate were less unaffected (in term of viable cell density). Double encapsulation resulted in a significant improvement in cell survival in which the viable cell density of the three probiotics remained stable for 120 min. Free yeast showed better acidic tolerance than bacteria with more than 3 log (cell density) survivals after 120 min prolonged under pH 2.0; viable cell density of encapsulated yeast, however, remained stable for the whole period. Tolerance of the three species under intestinal condition followed the similar pattern to that under gastric condition. SEM picture of microbeads encapsulated probiotics demonstrated that improvement in intolerance of probiotics to gastro-intestinal conditions was due to the protection of encapsulating molecules against external inhibition.

1.Introduction

Bioactive components in food can be divided into bioactive molecules and bioactive living cells (probiotics) (Vos et al., 2010). Maintaining the activity or integrity of probiotics throughout the human digestive system is one of the biggest challenges that has attracted considerable attention of many authors (Ding and Shah, 2007, Kim et al., 2008 and Sohail et al., 2013). Recently, encapsulation of probiotics in microgel has been considered as a method of choice due to its ability to control the release of probiotics at the correct time and proper place in the digestive system (Zhang et al., 2005; Sohail et al., 2011 & 2013). Microgel encapsulation based on gelation of sodium alginate with $CaCl_2$ through cross-linking of anionic chains of alginate and Ca^{2+} (Bhandari and Roos, 2012). However, alginate gel was often porous and probiotics therefore could release from the gel easily which made the control of target release become more difficult. Hence, several studies have employed double encapsulation to fix the porosity of the gel surface in an effort to control the release of probiotics (Wen-tao et al, 2005; Annan et al., 2008; Sohail et al., 2011 & 13). Gelatin which was reported to demonstrate excellent encapsulating properties (Saravanan & Rao, 2010; Teng et al, 2013) would be used in this

study to form the second layer to cover alginate gel bead.

In case of encapsulation of probiotics by gel entrapment, many encapsulation methods have been used such as: extrusion (Tan and Takeuchi, 2007), atomizing (Kwok et al, 1991) and aerosols (Sohail et al., 2013) which could produce particles with an average size of 5 mm, 5-15 μm and 40 μm respectively. It was also reported that protective improvement by gel entrapment was not affected by particle size (Sohail et al., 2013). However, extrusion often had one or more limitations such as scale-up restrictions and aerosols required special equipment (Sohail et al., 2011) which was very limited in most laboratories. In the present study, therefore, atomizing technique was employed due to its ability to scale up production and easy adoption of atomizing equipment in our laboratories.

Lactobacillus spp. and *Bifidobacterium spp* were reported as the most popular probiotics that have been used in many academic research and applications (Anal and Singh, 2007, Kim et al., 2008, Sohail et al., 2013). Previously, many probiotic strains have been used to investigate the effects of gel entrapment on resistance of probiotics to simulated gastric and intestinal fluids such as *Lactobacillus spp.* and *Bifidobacterium spp* (Kim et al., 2008). However, *Saccharomyces boulardii* which was the sole yeast that demonstrated probiotic properties (Czerucka et al, 2007) has not been investigated yet. In this study, gel entrapment of *Lactobacillus acidophilus*, *Bacillus clausii* and *Saccharomyces boulardii* was investigated to evaluate the resistance of encapsulated probiotics to simulated gastric and intestinal fluids.

2. Materials and methods

2.1. Culture preparation

B. clausii, *L. acidophilus* and *S. boulardii* were obtained from Food Microbiology laboratory at the University of Technology & Education. One colony from bacterial and yeast strains was inoculated in 100 mL of MRS (de Man, Rogosa, Sharpe) broth and in 100 mL of Hansen broth respectively. The inoculation was carried under aerobic condition at 37 ^{o}C for 24h (*B. clausii* and *S. boulardii*) and for 36 h (*L. acidophilus*) to achieve stationary phase. The cells were subsequently harvested by centrifuging the culture at 5000 rfc/g for 15 min at 5 ^{o}C and then washed with sterilized 0.85 % saline solution. The viable cell density in the harvesting phase was around 10^9 to 10^{10} CFU/mL. The microbial suspensions were kept in saline solution before use for encapsulation of probiotic cells.

2.2. Encapasulation of probiotics

Method for encapsulation of probiotics was conducted similarly to that of Kwok et al (1991) with some modifications.

The cells were firstly suspended in 5 mL of sterilized 0.1% peptone and then mixed with 45 mL of 2% (w/v) sodium alginate solution which had been sterilized at 121 °C for 15 min. Subsequently, the mixture was sprayed into a pan containing 500 mL of 1M $CaCl_2$ by the air-atomizing device. During this process, the $CaCl_2$ solution was constantly stirred by a magnetic stirrer. The pressure of the air-atomizer was 40 psi and distance between the orifice and the pan was 0.3 m. The resultant capsules were collected by centrifugation at 3000 rfc/g for 10 min at 5^{o}C by a centrifuge (Rotanta 460R, Hettich Zentrifugen, Tuttlingen, Germany). The capsules were then suspended in 0.3% (w/v) sodium alginate solution at room temperature for 4 min and immersed in 1% gelatin (Gelita Australia Pty.,Ltd., QLD, Australia) solution and shaken at 100 rpm for 30 min using an orbital agitator.

2.3. Determination of free and encapsulated cell numbers

2.3.1. Free cells

Free cell number was quantified by inoculating onto MRS agar plates for bacteria and Hansen agar plates for yeast. Dilutions of 10^1 up to 10^9 were made and 0.1 mL aliquots was plated on the agar. The inoculation was carried out at 37^{o}C for 36h.

2.3.2. Encapsulated cells

Determination of encapsulated cell numbers was carried out according to the method of Sohail et al. (2011). Encapsulated cells were firstly released from the gel matrix by adding alginate microbeads encapsulating probiotics (1 g) into test tubes containing 9 mL of 2% (w/v) sterile sodium citrate solution at pH 6.0. The test tubes were then gently shaken at room temperature for 15 min to obtain soluble solution by an orbital shaker. The following steps were similar to those in section 2.3.1.

2.4. Survivability of probiotics in simulated gastric and intestinal fluids

2.4.1. Survivability of probiotics in simulated gastric fluids (SGF)

Acid tolerance study of free and encapsulated probiotics was carried out according to the method of Ding and Shah (2007) with some modifications. MRS and Hansen broths were adjusted to pH 2.0 with 5.0 M HCl solution and sterilized at 121 °C for 15 min. One millimeter of aliquots (109 CFU/mL) of free B. clausii, L. acidophilus and S. boulardii were used as control samples. Test samples consisted of alginate micro beads (1 g) encapsulating probiotics produced by procedure in section 2.2. Test and control samples were added to test tubes containing 9 mL of modified MRS (for bacteria) and Hansen (for yeast) broths and incubated at 37oC for 30, 60, 90 and 120 min. After incubated for the specified time intervals, the test tubes were gently shaken and 1 mL aliquots of free and encapsulated cells were taken and the pH of each sample was adjusted 7.0 by adding 1M NaOH solution. Encapsulated cells were released from the beads by adding sterile sodium citrate solution. Media containing free cells or released cells was diluted with 0.1% peptone and inoculated onto MRS or Hansen agar plates for enumeration. All tests were repeated 3 times.

2.4.2. Survivability of probiotics in simulated intestinal fluids (SIF)

Survival of free and encapsulated probiotics in simulated intestinal fluids was carried out according to the method of Ding and Shah (2007) with some modifications. Bile salt (Himedia Laboratories Pvt. Ltd, Mumbai India) was added to the MRS and Hansen broths to achieve a concentration of 3% (w/v). The test and control samples were added to test tubes containing 9 mL of modified MRS and Hansen broths and then incubated at 37^{o}C for 30, 60, 90 and 120 min. After incubated for the specified time intervals, samples were collected and determination of cells density was carried out similarly to that in section 2.4.1.

2.5. Scanning electron microscopy (SEM) and Particle size distribution (PSD) of microbeads

The sample was placed on one surface of an adhesive tape and coated with gold by using an ion coater E-102 (Hitachi- Japan). The S-4800 model scanning electron microscope (Hitachi, Japan) was used to study the outer surface of the gel beads. The examination was operated at an accelerating voltage of 1 kV. The S-4800 software (Hitachi, Japan) was used to present the micrographs of the microbeads.

Suspensions of alginate gel microbeads in distilled water were mounted using a Model LA 920 laser diffraction particle analyzer (Horiba, Japan).

3.Results and discussions

3.1.Survivability of probiotics in simulated gastric fluids

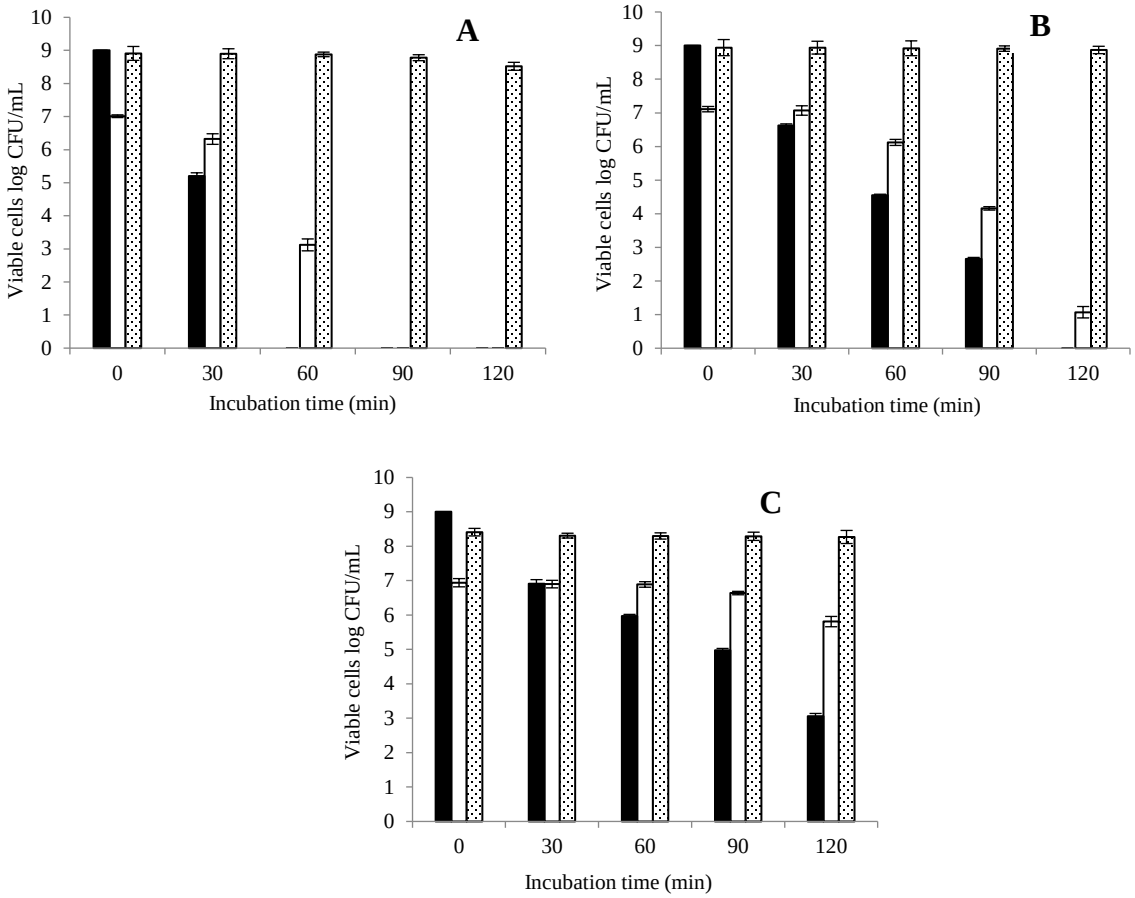

Figure 1. The viability of *L. acidophilus* (A), *B. clausii* (B) and *S. boulardii* (C) exposed to low pH environment: free cells (■), cells encapsulated in alginate microbeads (□) and gelatin-coated alginate microbeads (▒)

Fig. 1A, B and C demonstrates the viability of free and encapsulated *L. acidophilus*, *B. clausii*, and *S. boulardii* after 120 min exposure to low pH environment. As can be seen, the viability of free *L. acidophilus* and *B. clausii* decreased to undetectable levels after 60 and 90 min respectively whereas 3.06 (log CFU/mL) of free *S. boulardii* still be observed after 120 min. The survivability of probiotics was improved by alginate gel which extended the survival of *L. acidophilus* and *B. clausii* to 90 and 120 min respectively. Combination of gelatin and alginate greatly enhanced the survivability of probiotics at the high acid condition to the extent that only 0.30 (log CFU/mL) reduction (from 8.91 to 8.52) of *L. acidophilus* was observed within 120 min while survivability of *B. clausii* and *S. boulardii* remained statistically unchanged at 8.94 and 8.41 (log CFU/mL) respectively for 120 min ($P<0.05$). Kim et al. (2008) reported that free cells of *L. acidophilus* were completely inactivated after 60 min exposure at pH 1.2, while bacteria encapsulated in alginate beads remained survival above 10^4

CFU/mL after 180 min exposure at pH 1.5. Other studies have reported that survival of *Lactobacilli* in SGF was improved when the cells were immobilized in alginate beads (Ding and Shah, 2007; Mandal et al., 2006 & Mortazavian et al., 2008). A possible explanation is that alginate gel played a role as shelter to protect probiotics from pH stress due to its ability to isolate the cells from harsh environment (Sohail et al., 2013). Encapsulation by double layer technique (alginate coated by another surfactants) was reported to enhance survival of probiotics under low pH condition compared to single layer technique (Annan et al, 2008; Sohail et al., 2011). According to Annan et al (2008), if gelatin was used with a sufficient concentration, gelatin molecules could create cross-linking with Ca^{2+} of the alginate gel to form more stable gel that would prevent negative effects of low pH on gel structure.

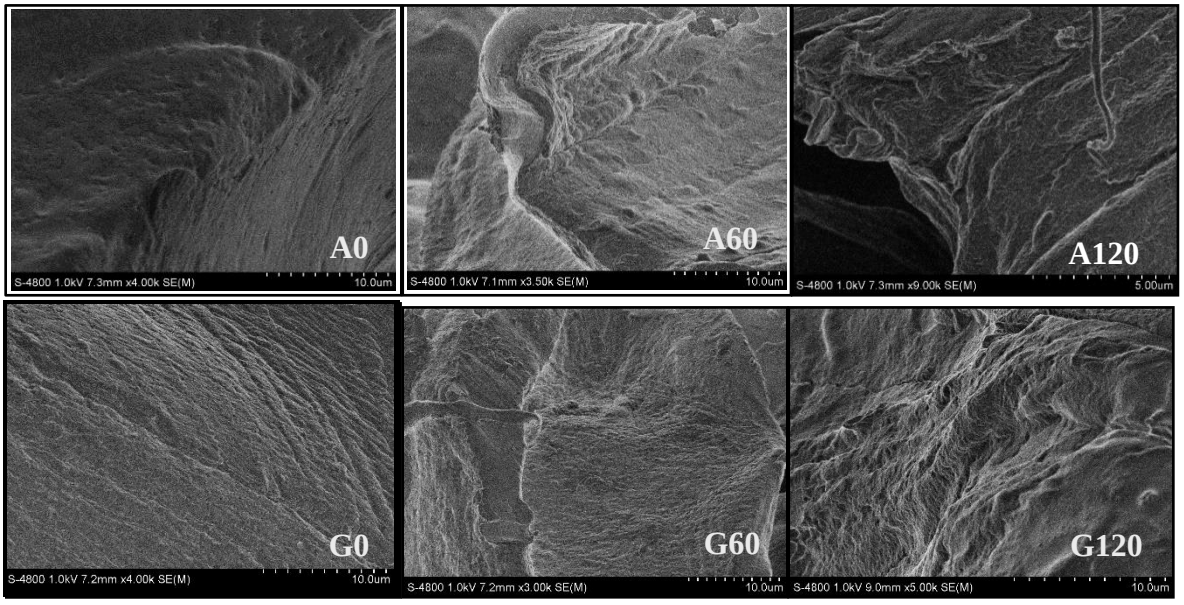

Figure 2. Sem pictures of microbeads from various samples: A0, A60, A120 (*L. acidophilus* encapsulated by alginate microbead after 0, 60 and 120 min immersed in low pH media respectively), G0, G30, G120 (*L. acidophilus* encapsulated by gelatin-coated microbead respectively)

For clearer understanding the encapsulation efficiency, Sem pictures of the microbeads encapsulated *L. acidophilus* at 0, 60 and 120 min exposure in simulated gastric medium were examined (Fig. 2). It can be clearly seen that the surface of microbeads (A0 and G0) before incubation was quite smooth and wrinkles were rarely discovered. However, microbeads of alginate (A60) demonstrated many cracks and holes after 60 min immersion while some cracks were occasionally observed on the surface morphology of gelatin-coated alginate microbeads. Similar observations were reported by Gouin (2004) and Hansen et al. (2002) when these authors reported that alginate gel structure was porous and not efficiency enough to trap microbial cells. As a result, microbial cells could easily release from the structure of alginate gel through

these fractures. In addition, gelatin (Saravanan & Rao, 2010) were reported to possess excellent coating property which could fix holes on the surface of the microbeads and that helped to prevent the cell from releasing out of the gel beads and consequently improve survival of the cells. When the incubation lasted for 120 min, gel structure of alginate (A120) was nearly destroyed with many cracks and big hole observed on the surface. Fractures on surface of gelatin-coated alginate microbeads were frequently seen but not holes. The surface morphology observed in Fig. 2 could be a clear explanation for improvement in survival of probiotics cells.

In the present study, free and encapsulated cells of yeast illustrated better pH resistance when compared to free and encapsulated bacteria respectively. It was reported that the viability of free *Lactobacillus* strains (*L. rhamnosus* and *L. acidophilus*) decreased more than 2 (log CFU/ml) (an approximately 99% decrease) after 60 min exposure in pH 2.0 at room temperature (Sohail et al., 2011) whereas free *Saccharomyces boulardii* still retained viability above 50% after 60 min exposure in pH 2.0 at 49^{o}C (Fietto et al, 2004). A possible explanation for this is difference in cellular composition of these strains. Yeast cell contains glucan and manan in the cell wall which possess excellent properties to resist changes of environment such as pH while bacterial cells do not have these constituents.

3.2.Survivability of probiotics in simulated intestinal fluids

Effect of simulated intestinal fluid on the survival of free and encapsulated *L. acidophilus*, *B. clausii* and *S. boulardii* respectively is shown in Fig. 3 A, B and C. The survival of the free cells of *L. acidophilus* decreased by 3.5 log (CFU/mL) within 60 min. The survival continued to drop to 4.0 log (CFU/mL) after 120 min exposure to SIF. By contrast, the survival of *L. acidophilus* encapsulated by alginate bead remained stable within the first 30 min, then decreased slightly to 6.43 log (CFU/mL) which showed a drop of only 0.58 log (CFU/mL) after 120 min. Combination of gelatin and alginate completely protected *L. acidophilus* from SIF and no significant reduction in survival of encapsulated *L. acidophilus* by double layer was observed. Similar observation was reported by Jasmina et al (2004) when the authors evaluated the survival of *Lactobacillus casei* encapsulation by soy protein coated – alginate in simulated intestinal condition.

B. clausii was found to be more resistant than *B. clausii* to SIF. The survival of the free cells of *B. clausii* decreased by 1.87 log (CFU/mL) within 60 min, then levelled off for the remaining time of the investigation while that of encapsulated cells (single and double layer) remained unchanged for 120 min. Hence, alginate beads resulted in improved protection in the present of SIF with or without combination with gelatin. *S. boulardii* was found to be best resistant to SIF. A decrease of 1.5 log (CFU/mL) of free yeast was observed within 120 min whereas no reduction in the survival of encapsulated yeast was found.

Previously, Sohail et al. (2011) reported a 1.70 log (CFU/mL) reduction of free *L. acidophilus* was observed after 2h exposure to SIF. Sohail et al. (2011) also emphasized that survivability of encapsulated bacteria is highly dependent on the strain encapsulated and this conclusion was supported by our results. In addition, in the present study, it was found that probiotic yeast showed a better resistant than bacteria to SIF which might be due to differences in cell-wall and cellular composition of these strains.

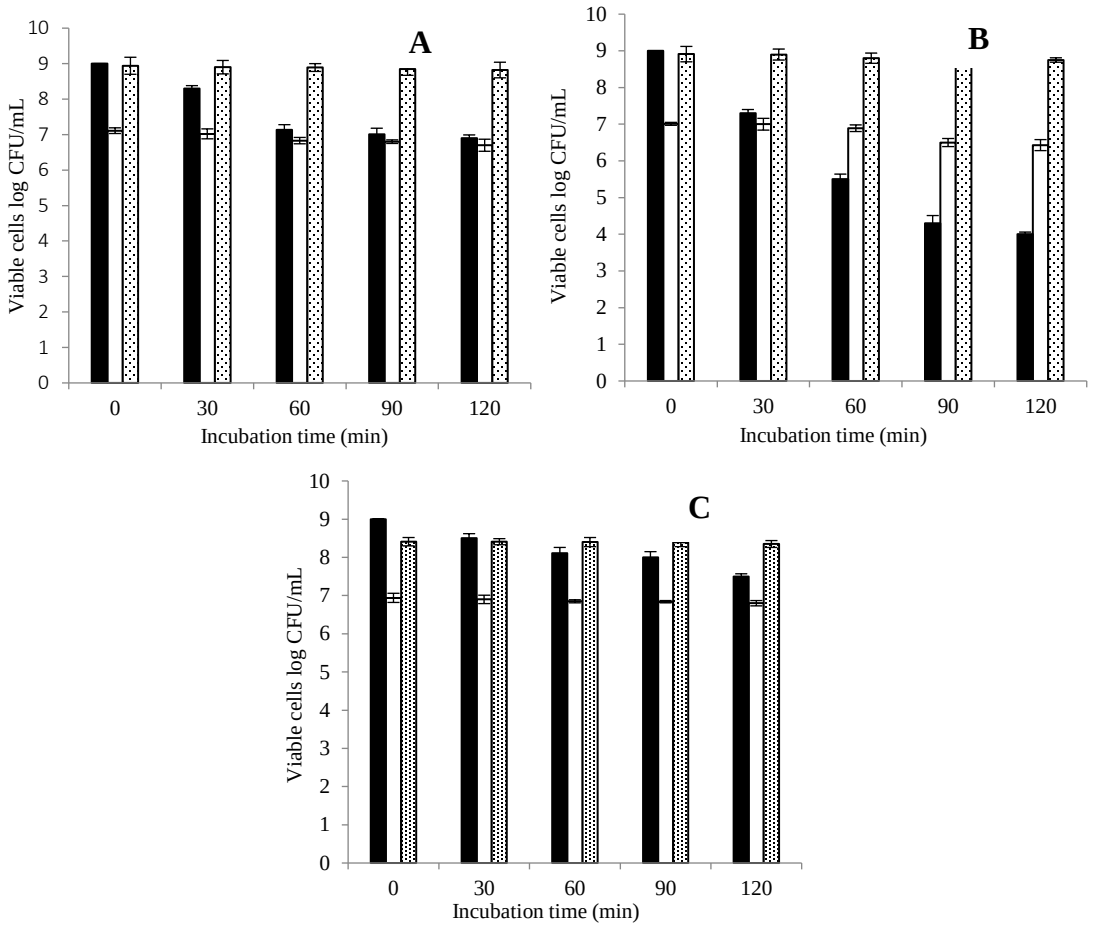

Figure 3. The viability of *L. acidophilus* (A), *B. clausii* (B) and *S. boulardii* (C) exposed to simulated intestinal fluid: free cells (■), cells encapsulated in alginate microbeads (□) and gelatin-coated alginate microbeads (▒).

4. Conclusions

Survival of three probiotics strains including *Lactobacillus acidophilus*, *Bacillus clausii* and *Saccharomyces boulardii* was significantly enhanced by hydrogel encapsulation. Double layer encapsulation of alginate gel and gelatin showed better protection to probiotics than single layer encapsulation. Further studies should be the use of encapsulated probiotics for *in vivo* test as a next step for their potential application.

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APPLICATION OF PULSED ELECTRIC FIELD FOR INTENSIFICATION OF ESSENTIAL OIL EXTRACTION

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ABSTRACT
Chemical pretreatment processes using enzymes are generally employed to improve the release of essential oil from plants by hydro-distillation. This paper is aimed to investigate the application of high voltage electric pulses as a pretreatment step to increase the amount of essential oil extracted from Marrubium vulgare and Artemisia herba alba, which are local plants from Mascara, Algeria. Obtained results have shown that for both plants (i) the electrical conductivity of the plant sample increases due to the enhancement of the mass transfer (ii) the extraction process is significantly improved because the amount of the treated essential oil has increased up to three times.

1. Introduction

Food processing at industrial scale is nowadays considered as one of the most important issues of the world. The use of medicinal plants for healing different human affections, dates back to the ancient times – the prehistoric ones – when man living in the middle of nature, fighting through various ways to ensure his existence, has noticed that some plants are good to eat, or heal diseases and some others are toxic. The Global Health Organization recently announced that 75 – 80 % of the world's population treats themselves using natural remedies (Mocanuet, 2009).

In Africa, traditional medicine is used for the treatment of many diseases and infections. Plants readily synthesize substances for defense against the attack of insects, herbivores and microorganisms (Marjorie, 1999; Mayunzu, 2011).

Essential oils of plants are widely used in many applications such as in aromatherapy, pharmacy, perfumery, cosmetics and in food conservation, due to their wide spectrum of interesting biological activities (Paster, 1990; Nielsen, 2000). Currently, nearly more than 3000 essential oils are known, about 300 of them are employed in many industry fields (Bakkali, 2008; Tajkarimi, 2010).

Several plants contain essential oil but only in small amounts except the so-called "aromatic" plants. However, their extraction by hydro-distillation remains excessively expensive and requires a long time processing.

Marrubium vulgare, an official medicinal plant, is widely used as an expectorant and it is said to be the most popular of herbal cough

remedies. Other effects have been reported including hypoglycemic and hypolipidemic effects, analgesic and antioxidant activity (Javidnia, 2007). In Algeria, it is currently used in folk medicine to cure several diseases of the digestive tract, such as diarrhoea, as well as diabetes, rheumatism, cold and respiratory pains (Belhattab, 2006). Artemisia genus belongs to the Asteraceae family with more than 350 different species that are mainly found in arid and semiarid areas of Europe, America, North Africa and Asia (Nikolova, 2010). These plants have been the subject of several phytochemical studies because of their valuable essential oils (Da Silva, 2004).

The low extraction yield of the essential oil is mainly related to the low oil content in the blossoms and to the fact that the plants are tender and not durable. Various pretreatment methods are nowadays applied for disintegrating the tissue structure to improve the essential oil release and its separation by steam water. The enzymes have been used but they require specific conditions like an adjusted pH as well as significant processing times.

The traditional techniques used for the solvent extraction of natural products are associated with poor extraction efficiency (Vilkhu, 2008; Shirsah, 2012; Prokopov, 2017).

In this paper, the Pulsed Electric Field (PEF) pretreatment has been applied to induce permeabilization by electroporation of the biological membranes. The PEF pretreatment is employed in various processes in food technology such as drying, oil extraction and juice production. It has been shown that extraction yield from PEF-treated products such as apple, carrot or beet is much improved.

The PEF action mechanism is based on the electroporation of cell membranes by high-level electrical field pulses enhancing the mass transfer of the intracellular content. Typical processing parameters are electric field strength of 1-5 kV/cm and specific energy input of 5-20 kJ/kg.

The aim of this study is to investigate the effect of PEF pretreatment on Marrubium vulgare and Artemisia plants to enhance the extraction process, with special regard to yield increase and reduction of processing time.

Moreover, inactivation of microorganisms and acceleration of the drying process using PEF technique were demonstrated (He, 2016; Raluca, 2010).

2. Materials and methods

The plant samples shown in figure 1, were collected in March (2017) in Mascara, located in the North-West of Algeria (35°26' N.; 02°11' E.) with Mediterranean climate and mean annual precipitations of about 450 mm (Benarba, 2014).

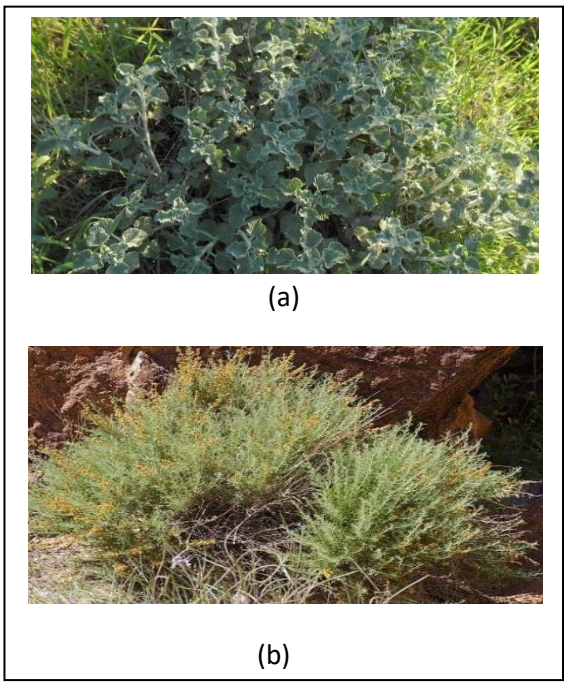


Figure 1. Photography of the plants used in the experiments.
a) Marrubium; b) Artemisia

2.1. Description of the extraction process

The diversity and the complexity of essential oils make the choice of the best extraction process difficult. The selected method should not induce biochemical reactions, thermal degradation, oxidation, reduction, hydrolysis, pH change or loss of volatile compounds (Fernandez, 2007). We opted in this work for the hydro-distillation process which is considered as the best extraction method both in laboratories

and in industry (Gourine, 2009; Rose, 1996). The advantages of this method are: high extraction yield, highly concentrated oil and direct contact "plant material-water" (Richard, 1992).

For each extraction experiment, we introduced 20 g of the plant leaves mixed with 250 ml of water in presence of pumice stones into a one-liter flask topped with a distillation head provided with a thermometer and connected to a refrigerant.

The mixture “leaves-water” is then heated up to boiling for a well-defined period of time by gradually adding water through the dropping funnel in order to keep constant the amount of water in the flask. When a distillate composed of oil and water is obtained, the two immiscible phases are then separated by decantation using an organic solvent (diethyl ether in our case).

The recovered essential oil is then mixed with diethyl ether (organic phase) and followed by a drying step by using anhydrous sodium sulfate to remove any existing water. The essential oil is then recovered after filtration and evaporation steps of the solvent using a rotary evaporator (Hahnvapor, HS-2005V-N).

2.2. Evaluation of the extraction yield

The extraction yield (Y) is defined as the ratio between mass m of the extracted oil and total mass M of the treated plant material, as follows:

$$Y(\%) = \frac{m}{M} \cdot 100 \quad (1)$$

For all the experimental work described in this paper, the experiments were repeated twice and the average value was considered for plotting.

2.3. PEF experimental setup

The leaves of the plants were dried for a period of 48h and then cut manually using scissors into small pieces of about 1-2 cm. For all experiments, a plant paw sample of mass 20 g was mixed with the same amount of distilled water to obtain a wet sample which is then placed in a polycarbonate cylindrical chamber comprising two parallel stainless steel planer disk electrodes, separated by a gap of 2.5 cm that represent the sample thickness.

The PEF experimental setup used in the present work is composed of a Direct Current high voltage (DC-HV) supply (Spellman 40 kV, 9 mA), a set of capacitors of total capacitance 2 µF, a spark gap switch and a treatment chamber (Figure 2). The DC-HV supply charges the capacitor sup to the spark gap's breakdown, causing an abrupt voltage applied to the treatment chamber where the sample is disposed (Bermaki, 2017).

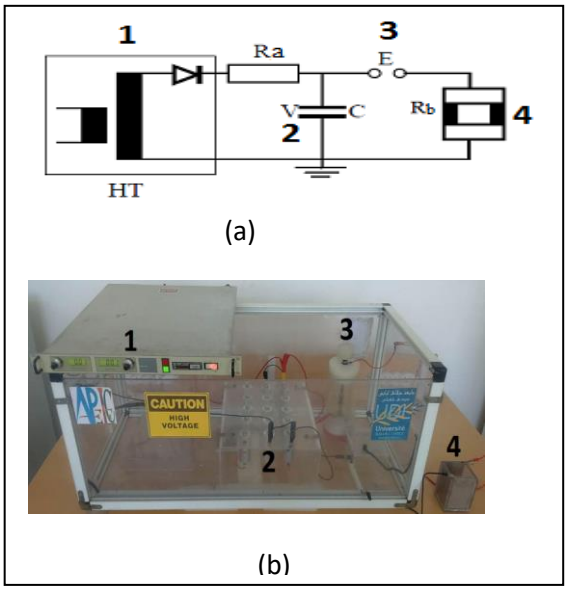


Figure 2. The pulse generator.
a) Descriptive schematic of the setup;
b) The photography of the setup
1- HV DC power supply, 2-Set of capacitors,
3- Spark gap switch, 4-Treatment chamber

2.4. Measurement of the electrical conductivity

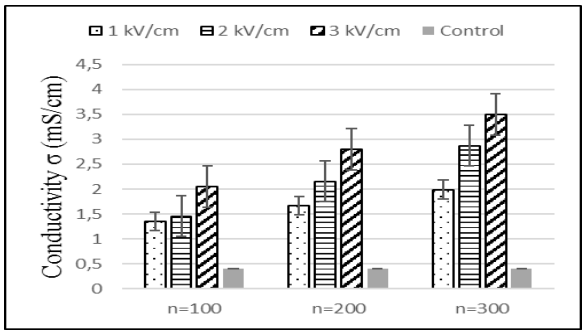
After PEF treatment, a determined amount of the distilled water of volume cm3 is taken from the treated sample and put in a glass tube for measuring the conductivity with a conductivity meter of liquid solution (Multiline P4 WTW).

3. Results and discussion

3.1. Electrical conductivity σ (mS/cm)

Obtained results plotted in figure 3, representing the variation of conductivity σ of

both MARRUBIUM and ARTEMISIA as functions of electric field E and pulse number n, show that indeed there is electroporation (also called electro-permeabilization) happening in the vegetable tissue. Once the cell membrane is electroporated, the solution conductivity is increased due to the mass transfer from intracellular to extracellular medium; the increase rate is greater for higher values of both E and n because of the high PEF energy injected to the sample. The application of high intensity electric field pulses to the biological tissue induces structural changes in the cell membrane through creation of pores.



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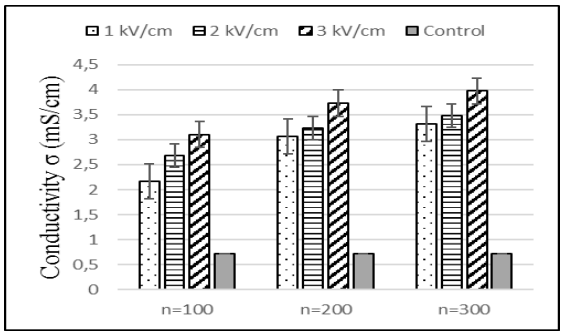


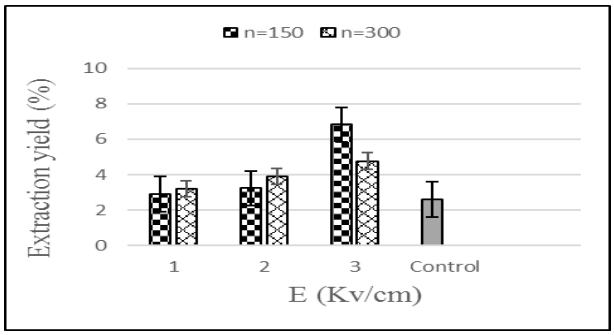
(b)

Figure 3. Effect of the PEF treatment on the electrical conductivity:
(a) Marrubium; (b) Artemisia

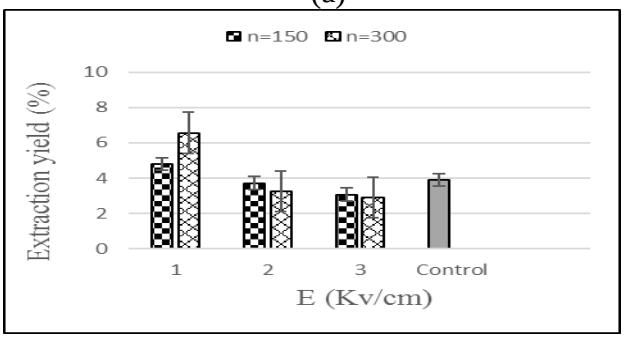
The cell membrane permeability caused by electroporation makes possible the movement of ions across the membrane. Moreover, a pronounced augmentation of the conductivity is caused by the increase of energy supplied to the cell following the growth of the electric field E and the number of pulses n.

3.2. Effect of the PEF treatment on the extraction yield

Wet samples of Marrubium and Artemisia of mass m= 20 g were put in the treatment chamber and PEF treated for different values of electric field E (0.8 kV/cm; 1.6 kV/cm; 2.5 kV/cm) and pulse number n (150; 300). This experimental work was carried out for two different values of the distillation process duration Δt (30 min and 60 min). The obtained results of extraction yield for both Marrubium and Artemisia represented in figure 4 and figure 5 respectively, indicate that for the same distillation time duration a significant enhancement of the extraction yield was observed compared to the untreated sample.

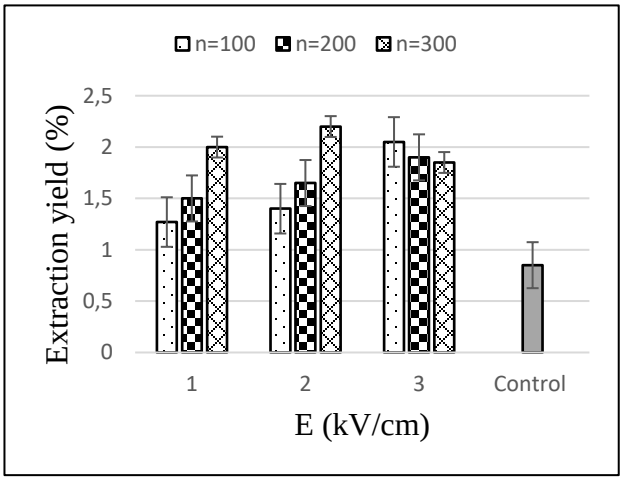


(a)



(b)

Figure 4. Effect of PEF treatment on the extraction efficiency of Marrubium for different values of the electric field and the pulse number: a) Δt = 30 min; b) Δt = 60 min



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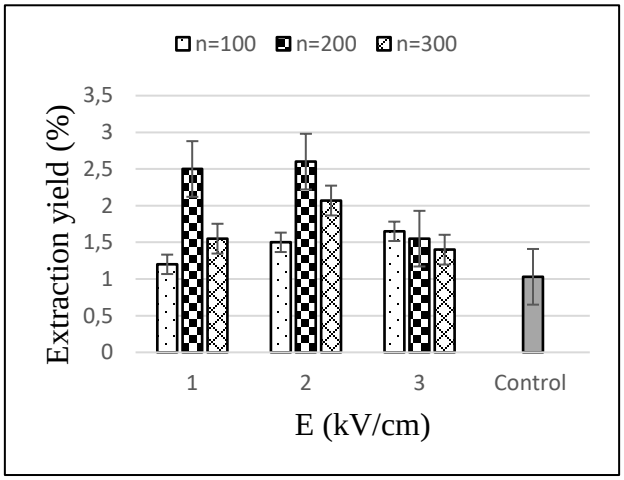


(b)

Figure 5. Effect of PEF treatment on the extraction efficiency of Artemisia for different values of the electric field and the pulse number: a) Δt = 30 min; b) Δt = 60 min

Furthermore, we notice that the PEF treatment accelerates the distillation process which is an important feature in case of industrial scale extraction. According to the plotted results in Figs. 4 and 5, a distillation period Δt = 30 min is sufficient to even cause more extraction than Δt = 60 min.

The maximum extraction increase rate for both plants estimated by:

$\Delta e = (Y_{max}-Y_0)/Y_0$

Where

Y_{max}: maximal extraction yield of the treated sample

Y_0: extraction yield of the untreated sample

is reported in Table 1.

Marrubium:

For Δt = 30 min (E=3 kV/cm) :Δe=((6.82-2.6)/2.6)*100=162.3 %

For Δt = 60 min (E=1 kV/cm) :Δe=((6.55-3.91)/3.91)*100=67.5 %

Artemisia:

For Δt = 30 min (E = 2 kV/cm): Δe = ((2.2–0.85) / 0.85) * 100 = 158.82%

For Δt = 60 min (E = 2 kV/cm): Δe = ((2.6–1.03) / 1.03) * 100 = 152.42 %

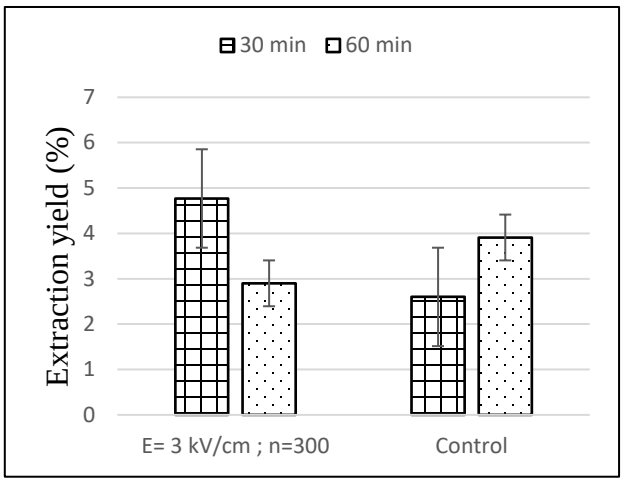
Table 1. The maximum extraction increase rate for both plants

	Marrubium	Artemisia
Δt = 30 min	162.3 %	158.82%
Δt = 60 min	67.5 %	152.42 %

Moreover, we notice that extraction yield increases with the electric field for a distillation time period of 30 min and vice versa for 60 min. The potential difference between the inside and outside of the cell membrane became larger with higher electrostatic force, resulting in the disintegration of organelles and cellular structures (Dobreva, 2010; Harrisonet, 1997), and thus the release of volatile substances is improved. But the too high electric field intensity will induce negative influence; a similar tendency has been reported previously (Lin, 2011).

When an intense electric field is applied to the sample (E = 3 kV/cm), the cell membrane is completely permeabilized and therefore almost all of the intracellular matter is extracted after only 30 min of distillation. Thus, for longer distillation process duration, the surplus of energy will cause the decrease of the extraction yield. This is further confirmed by the results shown in figure 6 representing the evolution of the extraction yield as a function of the distillation time for E= 3 kV/cm and n=300.The PEF pretreatment which is optimal for Δt= 30 min accelerates significantly the extraction process; more oil was extracted with the PEF-

treated sample after 30 min in comparison with the untreated sample after 90 min.



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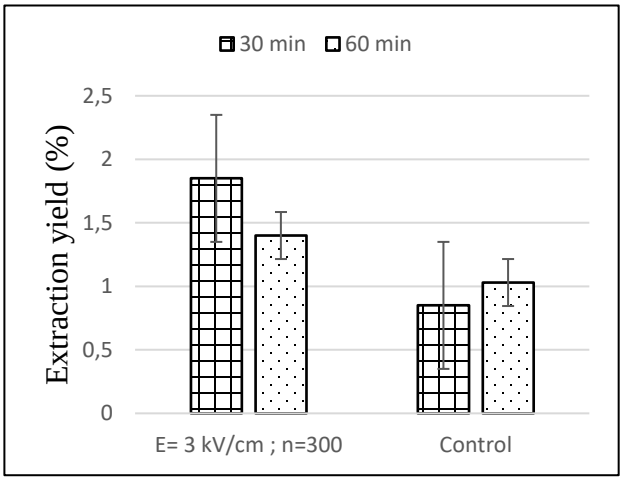

(b)

Figure 6. Variation of the extraction yield as function of the distillation process duration (E=3 kV/cm, n=300):
a) Marrubium ; b) Artemisia

4. Conclusions

We have shown in this paper that the PEF treatment would significantly improve the extraction rate of essential oil up to a ratio of 3 times. From the research that has been undertaken, it is possible to conclude that:
The Marrubium and Artemisia are susceptible to the PEF pretreatment.

The PEF indeed causes the electroporation of the cell membranes as shown by the variation of the electrical conductivity.

The extraction process is significantly improved after PEF treatment, the oil mass obtained with the treated samples increases up to three times.

The extraction process is much more accelerated after PEF treatment.

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EFFECT OF SOME PRO AND PREBIOTICS ON FATTY ACID AND ORGANIC ACID COMPOSITION OF SYMBIOTIC GOAT CHEESE

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ABSTRACT

The effects of probiotic cultures including *Enterococcus faecium*, *Bifidobacterium bifidum*, *Lactobacillus paracasei* subsp. *paracasei* and prebiotics including inulin, oligofructose and their blends on fatty acid and organic acid compositions of goat-milk white cheese were examined throughout 90-day storage period. Goat-milk cheeses were produced in an industrial plant following the standard production procedure for white cheeses, with some modifications. The highest value of saturated and unsaturated fatty acids in experimental cheeses were found be palmitic acid (C16) and oleic acid (C18:1) respectively. The most abundant organic acid among all experimental samples was lactic acid whereas the lowest one was acetic acid. The results indicated that the probiotic culture type and the percentage of prebiotic added to goat milk effected fatty and organic acid contents of goat-milk white cheeses at different levels.

1. Introduction

Symbiotics have been defined as a mixture of probiotics and prebiotics that positively affect the host. In addition to the direct introduction of beneficial bacteria to the gut, the number of beneficial *Bifidobacterium* and *Lactobacillus* species increase in the intestinal microbiota with the use of prebiotics. Prebiotics are selectively fermented ingredients that allow specific changes, both in the composition and in the activity within the gastrointestinal tract, that confer beneficial effects. These products are effective in the treatment of gastrointestinal disorders, respiratory infections and allergic symptoms. They also reduce blood cholesterol and improve immunity. Goats are an important component of the livestock industry having the adaptability to cope with different climates, which make them suitable for farmers who adopt innovative production methods. The contribution of goats in supplying milk and milk products is high, and they have a significant role in rural economy and health (Zenebe et al., 2014). Goats produce only approximately 2% of the world's total annual milk supply. Goat milk differs from cow milk in its higher digestibility, alkalinity, buffering capacity and certain nutritional and therapeutic properties. The composition of goat milk is similar to cow milk, although it has a smaller fat globule size, and a white color. Goat-milk fat contains more than 20 volatile branched-chain fatty acids including 4-methyl octanoic acid and 4-ethyl octanoic acid, which are related to a goat-type flavor. Goat milk is also poor in casein; casein micelles contain more calcium, inorganic phosphorus and non-centrifugal caseins. They are less solvated, less heat stable and lose beta casein more quickly than cow-milk casein micelles. Considering the survival and viability of probiotic cultures in

fermented dairy products, such as cheese, yoghurt and fermented milks, they are promising food delivery systems for these bacteria. It can also be said that cheese has been a better carrier of probiotic bacteria than other fermented milk for these microorganisms in the gastrointestinal tract. The characteristics of goat milk may be an advantage for adult humans suffering from gastrointestinal disturbances and ulcers. Goat milk is also important for the prevention of cardiovascular disease, cancer, allergies and microorganisms and is used for stimulation of immunity. Goat milk is recommended for infants and convalescent people (Zenebe et al., 2014).

In Turkey White cheese is the most popular cheese variety that has been produced for many years in various regions of Turkey. White cheese is a semi-hard cheese with medium moisture that is produced from raw or pasteurized cow, sheep or goat milk, or their blends. It is obtained after milk coagulation using rennet or proper coagulant enzymes, which are sometimes inoculated with selected lactic acid bacteria. White cheese is commonly marketed after 3 months of ripening at 4 to 5°C. This product has the most commercial value because its production technology is simple. The yield is high and consumers readily prefer it. Currently there is a lack of studies emphasizing the incorporation of probiotic culture and prebiotics into White cheese produced from goat milk, and on the influence of probiotics and prebiotics on some quality characteristics of the cheese during ripening. As a result, the main purpose of this study was to assess some physicochemical properties of symbiotic goat cheese supplemented with *Lactobacillus acidophilus, Bifidobacterium longum* and *Enterococcus faecium* as probiotics, and inulin, oligofructose and its blends as prebiotic substances during 90 days of storage.

2. Material and methods

2.1. Material

Cheese Manufacture

Cheeses were manufactured by the local dairy industry (Semsi Egi Dairy Company, Izmir, Turkey). For each cheese sample, raw milk was pumped into cheese vats at 25°C. The raw milk material was divided into four groups, then 3% inulin, 3% oligofructose, 1.5% inulin or 1.5% oligofructose (Beneo Orafti-Belgium) were added to the groups and heated at 65 ± 2°C for 15 minutes. After cooling to 35°C, 0.02% calcium chloride was added. Then the four different raw milk materials were sub-divided into four more groups and inoculated with four different commercial starter mixtures. The average microorganism counts and pH of cultures used in symbiotic goat cheese production are given in Table 1.

Table 1. The average microorganism counts and pH of cultures used in symbiotic goat cheese production

Culture	**pH**	**cfu/g**
Cheese culture	4.65	2.00×10^{11}
E. faecium	4.67	3.98×10^{9}
Lb. paracasei	4.80	1.72×10^{10}
B. longum	4.88	4.00×10^{10}

The commercial mesophilic starter mixture was containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (Maysa Gıda San. Tic. A. S., Istanbul, Turkey). The adjunct probiotic cultures used in the production were *Enterococcus faecium* NRRL B-2355 (ATCCC407), *Lactobacillus paracasei* subsp. *paracasei* NRRL B-4560 (ATCC 25598) and *Bifidobacterium longum* (Ezal®, Texel-France) respectively. Thirteen different samples including the control cheese were formed as seen in Table 2. The milk and starter adjunct-culture mixture were incubated for one hour and then 40 ml/100 L of commercial rennet (Maysa Gıda San. Tic. A. S., Istanbul, Turkey) was added to the milk. Following that, the milk

was allowed to set for approximately 60 minutes. The cutting process was started when the curd reached the proper consistency. The curd was then cut into 1 cm^3 cubes. The coagulant was placed in the cheese mold and drained. Then the cheese samples were shaped and soaked in brine (16% NaCl for about 4 hours at 18 to 20°C). The fresh cheeses were placed in cheese vats where they were left to ripen until the pH value reached between 4.85 to 4.90. Then, the cheese samples were ripened at 4 ± 1°C for 3 months. Analyses were carried out on all samples every 30 days starting from the first day.

Table 2. Experimental design of symbiotic goat cheese production and addition percentages to cheese milk

Cheese Sample	Culture				Prebiotics	
	Cheese culture0 .5%	*E. faecium* 0.25%	*Lb. paracasei* 2.5%	*B. longum* 1.5%	Inulin %	Oligofructose %
Control	✓					
EF-C	✓	✓				
EF-I	✓	✓			3.0	
EF-O	✓	✓				3.0
EF-IO	✓	✓			1.5	1.5
LP-C	✓		✓			
LP-I	✓		✓		3.0	
LP-O	✓		✓			3.0
LP-IO	✓		✓		1.5	1.5
BL-C	✓			✓		
BL-I	✓			✓	3.0	
BL-O	✓			✓		3.0
BL-IO	✓			✓	1.5	1.5

Thirteen different cheeses from each production were sampled at each ripening time interval. All samples were analyzed in triplicate.

2.2. Methods

Organic acid analysis

The organic acids of white cheeses were quantified by carrying out high-performance liquid chromatography (Agilent HPLC LC 1100 series-Germany) according to the methods described by Buffa et al. (2001) and Akalin et al. (2002).

Determination of free fatty acid composition

Lipids were extracted with purified kieselguhr and diethyl ether as described by Renner (1993). Fatty acid methyl esters were prepared according to AOAC (1997).

The instrumentation used for the analyses was a Hewlett Packard GC model 6890 equipped with Supelco SP-2380 fused silica capillary column (60 m x 0.25 mm ID, 0.2 µm film thickness; Supelco Inc. Bellafonte, USA) and a flame ionization detector. The temperature of the GC oven was programmed to go from 100 to 220°C at the rate of 4°C/min. The injector and detector temperatures were 300°C. Nitrogen was used as the carrier gas, and the flow rate was 1 ml/min.

Statistical analysis

Results were assessed using analysis of variance (ANOVA) and the SPSS 15.0 for Windows software package.

3.Results and discussions

3.1. Fatty acids

3.1.1. Evaluation of the Results of the Saturated Fatty Acid Analysis of Cheeses

Free fatty acids that have a role in the formation of the taste and aroma of the products are released as a result of lipolysis (Urbach, 1993). The released fatty acids function as precursors to other aromatic compounds (such as ketones and esters) (Urbach, 1997; Najera et al. 1994).

The changes in saturated fatty acid composition of experimental cheeses during storage, and in terms of sample type, are shown in Table 3.

Table 3. Saturated fatty acid composition of cheese samples.
Please refer to Table 2 for abbreviations.

Parameter	Day	Control	EF-C	EF-I	EF-O	EF-IO	LP-C
C4:0	**1**	1.69±0.02	1.81±0.03	1.73±0.08	1.58±0.09	1.55±0.04	1.84±0.12
	30	1.57±0.02	1.59±0.08	1.72±0.09	1.62±0.34	1.59±0.07	1.78±0.26
	60	1.67±0.09	1.66±0.26	1.53±0.41	1.76±0.07	1.52±0.33	1.82±0.27
	90	1.72±0.30	1.69±0.25	1.62±0.07	1.68±0.08	1.69±0.41	1.69±0.09
C6:0	**1**	2.02±0.59	1.96±0.26	1.96±0.23	2.09±0.09	2.14±0.71	2.40±0.44
	30	1.86±0.23	1.81±0.41	1.94±0.09	1.99±0.03	1.82±0.32	1.97±0.54
	60	1.92±0.47	1.80±0.51	1.83±0.62	1.79±0.03	1.86±0.21	1.90±0.14
	90	1.66±0.55	1.99±0.48	1.95±0.61	1.96±0.19	1.97±0.22	1.95±0.08
C8:0	**1**	2.26±0.25	2.47±0.31	2.10±0.19	2.33±0.09	1.61±0.24	2.09±0.08
	30	2.14±0.26	2.10±0.35	2.22±0.44	2.41±0.08	1.96±0.07	2.11±0.11
	60	1.97±0.19	1.98±0.06	2.20±0.09	2.19±0.54	2.14±0.69	2.23±0.37
	90	2.21±0.43	2.37±0.21	2.32±0.35	2.32±0.09	2.32±0.05	2.30±0.71
C10:0	**1**	7.92±1.26	9.21±1.31	9.74±1.47	9.30±0.09	9.99±0.76	8.97±0.82
	30	8.42±1.25	9.16±0.69	8.52±1.41	8.72±1.30	7.92±0.94	2.14±0.08
	60	9.02±0.67	8.89±0.88	8.16±0.79	7.83±0.56	7.81±0.58	7.76±0.08
	90	9.14±1.22	8.03±1.06	7.95±1.40	7.93±1.74	7.96±1.31	7.90±0.09
C11:0	**1**	0.26±0.08	0.26±0.16	0.38±0.18	0.35±0.21	0.55±0.24	0.32±0.19
	30	0.19±0.09	0.22±0.12	0.32±0.09	0.29±0.12	0.48±0.16	0.31±0.14
	60	0.20±0.09	0.19±0.07	0.21±0.03	0.18±0.11	0.17±0.12	0.18±0.08
	90	0.10±0.05	0.18±0.08	0.17±0.07	0.17±0.06	0.17±0.08	0.17±0.09
C12:0	**1**	3.30±0.19	3.20±0.23	2.93±0.17	2.94±0.18	2.88±0.24	2.90±0.12
	30	3.24±0.15	3.46±0.16	3.14±0.19	3.04±0.21	2.97±0.34	3.31±0.41
	60	3.36±0.34	3.14±0.32	3.09±0.31	3.14±0.36	3.04±0.09	2.98±0.07
	90	3.20±0.08	3.28±0.12	3.24±0.19	3.23±0.22	3.24±0.08	3.22±0.34
C13:0	**1**	0.06±0.03	0.03±0.01	0.03±0.02	0.04±0.03	0.04±0.01	0.03±0.02
	30	0.05±0.02	0.05±0.01	0.04±0.03	0.05±0.04	0.03±0.02	0.06±0.04
	60	0.06±0.03	0.04±0.01	0.04±0.02	0.05±0.02	0.05±0.04	0.06±0.03
	90	0.03±0.01	0.05±0.02	0.05±0.01	0.05±0.03	0.02±0.03	0.05±0.03
C14:0	**1**	9.93±1.23	10.10±3.45	11.08±2.59	9.55±3.59	12.60±1.98	11.76±4.05
	30	9.54±0.98	9.72±4.01	9.86±3.05	9.68±2.08	9.76±2.22	9.88±2.14
	60	9.86±3.15	9.73±3.65	9.62±2.98	9.59±3.09	9.81±4.05	9.17±3.26
	90	9.71±3.58	9.90±2.64	9.83±2.96	9.76±1.58	9.83±2.05	9.72±1.88
C16:0	**1**	30.93±2.36	29.56±3.05	30.15±3.33	31.16±4.16	31.07±2.58	27.43±3.15

	30	29.06±0.16	28.07±1.25	32.01±2.58	28.98±1.96	27.97±3.08	34.97±3.39
	60	25.70±1.77	28.81±1.59	33.17±1.42	31.77±2.05	29.76±2.68	28.82±3.04
	90	29.16±1.98	27.73±2.15	27.73±2.15	27.43±3.08	27.74±3.44	27.16±2.51
C17:0	**1**	0.38±0.09	0.48±0.12	0.34±0.14	0.37±0.06	0.54±0.09	0.87±0.21
	30	0.52±0.32	0.48±0.15	0.44±0.28	0.57±0.19	0.32±0.20	0.39±0.25
	60	0.62±0.32	0.41±0.17	0.38±0.12	0.34±0.09	0.52±0.16	0.56±0.15
	90	0.52±0.22	0.72±0.18	0.62±0.23	0.58±0.18	0.37±0.11	0.57±0.09
C18:0	**1**	15.59±1.59	15.78±2.36	14.35±1.26	15.83±2.08	13.91±3.54	15.42±2.08
	30	17.83±3.72	18.54±2.64	17.68±3.06	19.07±3.74	19.26±4.21	18.46±3.59
	60	20.14±1.58	20.38±3.46	17.46±2.74	19.56±1.98	21.04±4.74	19.16±3.59
	90	14.05±3.21	16.37±2.58	16.25±1.59	16.51±2.87	18.31±3.55	15.80±3.29
C20:0	**1**	0.14±0.03	0.10±0.05	0.19±0.06	0.26±0.02	0.34±0.04	0.55±0.07
	30	0.32±0.13	0.21±0.12	0.19±0.09	0.31±0.08	0.15±0.07	0.16±0.06
	60	0.17±0.11	0.19±0.04	0.33±0.03	0.36±0.01	0.24±0.04	0.32±0.02
	90	0.02±0.01	0.03±0.01	0.06±0.01	0.04±0.00	0.03±0.01	0.04±0.01

Table 3. (continued). Saturated fatty acid composition of cheese samples. Please refer to Table 2 for abbreviations.

Parameter	**Day**	**LP-I**	**LP-O**	**LP-IO**	**BL-C**	**BL-I**	**BL-O**	**BL-IO**
C4:0	**1**	2.05±0.25	1.55±0.09	1.87±0.03	1.50±0.05	2.01±0.32	1.76±0.05	1.86±0.34
	30	1.87±0.52	1.39±0.61	1.92±0.21	1.61±0.08	1.79±0.06	1.81±0.19	1.88±0.08
	60	1.67±0.16	1.73±0.04	1.79±0.06	1.68±0.07	1.73±0.08	1.59±0.14	1.69±0.05
	90	1.72±0.32	1.59±0.07	2.08±0.61	1.76±0.08	1.81±0.26	1.81±0.07	1.90±0.70
C6:0	**1**	1.91±0.26	2.01±0.23	1.48±0.19	1.92±0.08	1.63±0.09	1.70±0.47	2.31±0.51
	30	1.86±0.07	1.76±0.09	1.89±0.08	1.94±0.21	1.90±0.43	1.97±0.06	2.09±0.51
	60	1.83±0.07	1.92±0.31	1.69±0.09	1.96±0.04	1.93±0.06	2.04±0.08	1.97±0.08
	90	1.97±0.09	2.02±0.51	2.19±0.03	1.99±0.02	2.00±0.04	2.02±0.11	2.09±0.42
C8:0	**1**	2.26±0.60	1.89±0.05	2.14±0.15	1.80±0.36	2.50±0.47	2.57±0.08	2.00±0.09
	30	2.34±0.24	2.12±0.29	1.94±0.55	2.14±0.71	2.13±0.08	2.37±0.13	2.43±0.27
	60	2.19±0.21	2.36±0.74	1.89±0.08	2.36±0.09	2.29±0.11	2.29±0.51	2.33±0.36
	90	2.31±0.58	2.10±0.04	2.41±0.09	2.32±0.09	2.32±0.07	2.33±0.14	2.40±0.13
C10:0	**1**	9.32±1.19	10.22±1.24	11.29±1.34	11.13±0.84	10.92±1.58	12.89±1.47	11.50±0.65
	30	2.39±0.16	2.16±0.24	2.24±0.07	2.07±0.19	2.24±1.25	2.17±1.33	2.46±0.51
	60	7.94±0.11	8.24±0.24	7.91±0.62	8.14±0.42	7.96±0.51	7.63±0.21	8.19±1.20
	90	7.9±0.07	8.1±1.48	8.00±1.55	7.92±0.62	7.82±0.95	7.87±0.08	8.04±0.71
C11:0	**1**	0.27±0.09	0.69±0.15	0.41±0.13	0.67±0.17	0.50±0.19	0.38±0.26	0.67±0.22
	30	0.29±0.17	0.76±0.08	0.34±0.09	0.59±0.07	0.33±0.19	0.46±0.13	0.76±0.14
	60	0.21±0.01	0.19±0.08	0.17±0.10	0.19±0.17	0.22±0.05	0.16±0.11	0.18±0.13
	90	0.17±0.12	0.13±0.04	0.18±0.01	0.17±0.08	0.17±0.08	0.18±0.08	0.17±0.09
C12:0	**1**	2.99±0.14	2.38±0.09	3.51±0.08	2.49±0.07	3.05±0.01	3.32±0.02	3.38±0.06
	30	3.20±0.19	3.16±0.27	3.08±0.25	3.46±0.18	3.43±0.09	3.26±0.08	3.11±0.42
	60	3.23±0.38	3.26±0.37	3.34±0.21	3.46±0.22	3.21±0.37	3.39±0.13	3.24±0.11
	90	3.23±0.43	3.54±0.07	3.36±0.03	3.25±0.35	3.19±0.47	3.17±0.08	3.25±0.09

C13:0	**1**	0.03±0.01	0.05±0.02	0.07±0.03	0.04±0.01	0.05±0.02	0.06±0.03	0.06±0.04
	30	0.05±0.03	0.06±0.02	0.04±0.03	0.03±0.01	0.04±0.03	0.05±0.04	0.05±0.04
	60	0.06±0.01	0.04±0.02	0.03±0.01	0.04±0.01	0.04±0.02	0.05±0.01	0.06±0.02
	90	0.05±0.04	0.04±0.01	0.06±0.02	0.05±0.02	0.05±0.01	0.05±0.02	0.05±0.03
C14:0	**1**	9.89±1.28	9.08±1.39	10.95±2.08	11.06±3.07	12.25±2.45	10.26±4.06	11.09±3.09
	30	9.92±2.69	10.03±2.86	9.47±3.07	10.34±1.35	10.23±0.69	9.62±2.54	9.97±2.36
	60	9.28±3.54	9.34±2.91	9.26±3.61	9.96±3.79	9.27±2.88	9.34±3.09	9.59±2.07
	90	9.72±3.64	8.89±4.02	9.99±2.79	9.77±3.04	9.62±3.65	9.54±4.52	9.74±3.26
C16:0	**1**	29.65±4.08	31.38±2.55	29.93±3.01	28.86±3.64	31.78±3.99	30.19±4.00	28.40±2.84
	30	34.09±2.25	29.15±1.48	35.18±1.98	33.33±1.53	30.69±0.99	31.98±1.97	30.95±2.56
	60	26.87±2.95	29.37±2.87	28.19±3.41	31.12±3.06	29.66±1.22	29.94±1.94	29.98±1.48
	90	27.33±1.93	28.38±4.05	28.58±3.16	27.34±2.66	26.96±2.04	26.77±1.71	27.15±2.22
C17:0	**1**	0.59±0.31	0.71±0.21	0.37±0.31	0.68±0.19	0.47±0.14	0.58±0.26	0.70±0.47
	30	0.41±0.22	0.56±0.19	0.48±0.15	0.39±0.20	0.44±0.19	0.52±0.24	0.49±0.27
	60	0.43±0.21	0.38±0.09	0.41±0.21	0.37±0.09	0.46±0.08	0.39±0.11	0.52±0.20
	90	0.71±0.14	0.49±0.01	0.58±0.08	0.56±0.02	0.53±0.27	0.54±0.17	0.59±0.18
C18:0	**1**	15.05±4.57	14.33±1.26	11.86±5.12	12.20±4.23	10.09±3.25	10.18±3.19	11.10±2.47
	30	17.83±2.88	19.63±4.76	18.96±3.69	19.73±2.41	18.81±3.05	18.73±2.99	19.21±3.36
	60	21.44±5.49	18.16±2.08	19.41±3.72	17.81±2.85	19.16±3.84	20.23±2.69	17.89±3.21
	90	17.43±4.05	16.48±1.78	16.08±2.95	16.11±3.07	16.86±4.09	17.73±2.64	16.10±3.54
C20:0	**1**	0.38±0.09	0.37±0.08	0.38±0.07	0.49±0.06	0.29±0.05	0.42±0.09	0.60±0.12
	30	0.23±0.04	0.32±0.07	0.46±0.08	0.34±0.09	0.27±0.04	0.19±0.06	0.21±0.05
	60	0.4±0.06	0.28±0.04	0.19±0.09	0.17±0.08	0.21±0.08	0.16±0.06	0.3±0.14
	90	0.08±0.01	0.07±0.04	0.07±0.03	0.10±0.06	0.31±0.08	0.63±0.01	0.65±0.01

The highest value of saturated fatty acid rates in the experimental cheeses were found in C_{16}, while the lowest was found in C_{13} (see Table 3). This situation continued throughout the whole storage period. Although there were very small differences, the saturated fatty acid rates in all samples were: $C_{16}>C_{18}>C_{14}>C_{10}>C_{12}>C_8>C_6>C_4>C_{11}>C_{17}>C_{20}>C_{13}$, respectively. The saturated fatty acid rates of experimental cheeses varied from 72.84% to 77.22% on the first day of the storage period while, with slight changes, they were found to be from 71.34% to 73.65% at the end of the storage period. Saturated fatty acid rates changed over a limited range. The short-chain fatty acid (C4 – C8) rates of cheeses varied from 5.30% (EF-IO) to 6.33% (LP-K) on the first day and from 5.59% (K) to 6.69% (LP-IO) on the ninetieth day, while the medium-chain fatty acids (C_{10} – C_{14}) varied from 21.47% (K) to 26.91% (BL-O) on the first day of storage and from 20.07% (LP-O) to 22.22% (K) on the ninetieth day of storage; the long-chain fatty acids (C_{15} – C_{20}) changed from 40.8% (BL-IO) to 47.67% (EF-O) on the first day of storage and from 43.57% (LP-K) to 46.45% (EF-IO) on the ninetieth day of storage. Using prebiotics had no effect on the fatty acid ratios of samples $C_{8,}$ $C_{10,}$ C_{12}, C_{13}, C_{17} and C_{20} ($p>0.05$), while other saturated fatty acids were found to be affected ($p<0.05$). In the samples containing inulin, it was found that the cultures generally had an effect on C_{14} and C_{18} in the samples containing oligofructose, inulin and an inulin-oligofructose mixing/blend ($p<0.05$), whereas the cultures generally had an effect on C_{14}, C_{17} and C_{18} in the samples containing a mixture of inulin-oligofructose. The cultures had an effect on C_6 – C_8, and in the control group, the cultures had

an effect on C_{11}, C_{14}, C_{16}, C_{17}, C_{18} and C_{20} fatty acids ($p<0.05$), but they had no effect on other fatty acids ($p>0.05$). Comparing the results on the first and ninetieth day, no significant differences were found between short-, medium-, and long-chain fatty acids in symbiotic cheese samples. The long-chain fatty acid ratios of the control sample decreased to 43.75% from 47.04%, and in BL-K, BL-I and BL-O samples, to 21.16%, 20.85% and 20.81% from 25.39%, 26.77% and 26.91%, respectively. At the ninetieth day of storage, the long-chain fatty acids in BL-IO, with a 10% increase, increased from 41.37% to 45.67%, and the medium-chain fatty acid ratios in LP-IO, with a 22% increase, increased from 21.59% to 26.23%.

Georgala et al. (2005) reported that short- and medium-chain fatty acid levels of Feta cheese increased depending on the storage, and no significant changes were observed in long-chain fatty acids. Reggianito Argentino type cheeses were made by using a natural starter whey and different combinations of strains of selected *Lactobacillus helveticus* to determine the free fatty acid (FFA) contents at different ripening times. In the study, levels of individual FFAs from caproic (C6:0) to linoleic (C18:2) acids increased during ripening in all experimental cheeses. Palmitic (C16:0) and oleic (C18:1) acids were the most abundant FFAs throughout ripening in all cheeses (Perotti et al, 2005).

3.1.2. Evaluation of Unsaturated Fatty Acid Levels

It was found that the total unsaturated fatty acid ratios of samples changed from 22.78% (EF- I) to 27.16% (BL-K) on the first day of the storage. In a general evaluation, it was determined that the highest unsaturated fatty acid ratios was in $C_{18:1}$, and the lowest ratio was in $C_{18:3}$ (see Table 4).

Table 4. Unsaturated fatty acid composition of cheese sample
Please refer to Table 2 for abbreviations.

Parameter	Day	Control	EF-C	EF-I	EF-O	EF-IO	LP-C
C14:1	**1**	0.42± 0.21	0.45±0.13	0.51±0.14	0.74±0.09	0.41±0.24	0.67±0.30
	30	0.16±0.15	0.13±0.12	0.18±0.08	0.21±0.09	0.32±0.17	0.12±0.06
	60	0.24±0.08	0.19±0.11	0.23±0.13	0.18±0.07	0.16±0.09	0.11±0.04
	90	0.17±0.04	0.14±0.05	0.13±0.06	0.13±0.07	0.12±0.09	0.13±0.03
C15:1	**1**	0.01±0.00	0.02±0.00	0.01±0.01	0.03±0.00	0.04±0.01	0.01±0.01
	30	0.08±0.00	0.07±0.00	0.04±0.01	0.05±0.00	0.06±0.00	0.04±0.005
	60	0.03±0.00	0.07±0.00	0.08±0.01	0.04±0.00	0.05±0.00	0.05±0.006
	90	0.08±0.00	0.01±0.00	0.03±0.00	0.01±0.00	0.06±0.01	0.04±0.005
C16:1	**1**	0.56±0.08	0.40±0.06	0.51±0.05	0.38±0.04	0.64±0.02	0.52±0.03
	30	0.43±0.05	0.31±0.06	0.37±0.04	0.49±0.06	0.29±0.08	0.41±0.11
	60	0.37±0.08	0.41±0.08	0.51±0.06	0.33±0.07	0.27±0.08	0.53±0.04
	90	0.91±0.09	0.72±0.03	0.65±0.04	0.72±0.12	0.59±0.02	0.70±0.06
C17:1	**1**	0.77±0.12	0.78±0.14	0.56±0.16	0.65±0.17	0.51±0.06	0.89±0.12
	30	0.62±0.16	0.53±0.08	0.63±0.06	0.44±0.07	0.67±0.04	0.64±0.07
	60	0.73±0.07	0.71±0.11	0.80±0.21	0.67±0.15	0.59±0.16	0.66±0.18
	90	0.67±0.08	0.57±0.11	0.59±0.16	0.58±0.10	0.55±0.02	0.55±0.08
C18:1cis	**1**	20.11±3.45	19.76±2.58	19.83±4.15	18.50±1.26	18.29±1.58	20.07±2.35
	30	23.87±4.25	24.16±4.36	20.81±4.69	21.33±5.08	24.43±5.07	21.07±4.18

	60	22.63±4.19	20.16±3.66	19.24±4.78	18.76±5.25	19.87±4.12	22.33±3.89
	90	26.79±5.32	25.04±4.27	24.85±3.30	24.33±2.85	23.06±3.08	24.40±4.26
C18:2tr	**1**	0.56±0.08	0.60±0.11	0.59±0.07	0.70±0.06	0.60±0.21	0.40±0.33
	30	0.03±0.01	0.05±0.01	0.04±0.00	0.02±0.01	0.06±0.01	0.04±0.00
	60	0.05±0.01	0.07±0.01	0.08±0.00	0.07±0.00	0.06±0.00	0.03±0.00
	90	0.03±0.01	0.01±0.01	0.06±0.04	0.04±0.01	0.01±0.01	0.04±0.00
C18:2cis	**1**	2.04±0.24	2.13±0.88	2.01±0.65	2.37±1.23	1.84±1.49	2.10±1.11
	30	1.56±0.45	1.34±.29	1.27±0.41	1.35±0.08	1.07±0.62	1.44±0.55
	60	1.16±1.08	1.07±0.79	0.97±0.49	1.31±0.35	0.96±0.07	1.21±0.11
	90	0.37±0.34	0.49±0.16	0.51±0.24	0.57±0.16	0.65±0.21	0.63±0.31
C18:3	**1**	0.02±0.00	0.01±0.00	0.04±0.00	0.02±0.00	0.02±0.00	0.04±0.00
	30	0.02±0.00	0.03±0.00	0.05±0.01	0.04±0.00	0.07±0.00	0.05±0.00
	60	0.06±0.01	0.07±0.01	0.05±0.00	0.04±0.00	0.03±0.00	0.05±0.00
	90	0.07±0.01	0.03±0.00	0.02±0.01	0.03±0.00	0.03±0.00	0.03±0.00
C20:1	**1**	0.80±0.21	0.80±0.16	0.79±0.15	0.83±0.22	0.71±0.36	0.51±0.08
	30	0.08±0.04	0.07±0.05	0.10±0.06	0.21±0.04	0.09±0.08	0.19±0.02
	60	0.04±0.01	0.03±0.01	0.02±0.01	0.04±0.01	0.05±0.02	0.07±0.03
	90	0.07±0.03	0.09±0.00	0.03±0.01	0.07±0.03	0.08±0.01	0.05±0.00

Table 4. (continued). Unsaturated fatty acid composition of cheese sample
Please refer to Table 2 for abbreviations.

Parameter	**Day**	**LP-I**	**LP-O**	**LP-IO**	**BL-C**	**BL-I**	**BL-O**	**BL-IO**
C14:1	**1**	0.59±0.41	0.53±0.53	0.81±0.47	0.70±0.32	0.52±0.09	0.58±0.22	0.76±0.15
	30	0.20±0.08	0.17±0.04	0.33±0.09	0.26±0.11	0.40±0.34	0.22±0.05	0.19±0.06
	60	0.31±0.06	0.27±0.07	0.18±0.11	0.19±0.09	0.22±0.08	0.27±0.03	0.16±0.07
	90	0.13±0.07	0.11±0.04	0.17±0.02	0.39±0.01	0.19±0.06	0.21±0.04	0.35±0.17
C15:1	**1**	0.03±0.00	0.03±0.01	0.04±0.01	0.03±0.01	0.04±0.01	0.05±0.00	0.02±0.00
	30	0.02±0.01	0.03±0.004	0.05±0.01	0.06±0.00	0.07±0.00	0.04±0.01	0.03±0.00
	60	0.06±0.01	0.05±0.008	0.04±0.01	0.07±0.01	0.03±0.00	0.04±0.01	0.06±0.01
	90	0.00±0.00	0.05±0.003	0.01±0.00	0.03±0.00	0.01±0.00	0.03±0.00	0.00±0.00
C16:1	**1**	0.70±0.07	0.39±0.08	0.68±0.09	0.58±0.12	0.60±0.07	0.43±0.11	0.89±0.06
	30	0.37±0.09	0.28±0.05	0.38±0.06	0.51±0.04	0.47±0.08	0.33±0.07	0.37±0.06
	60	0.43±0.02	0.39±0.04	0.21±0.07	0.33±0.06	0.44±0.05	0.29±0.02	0.27±0.02
	90	0.71±0.04	0.68±0.05	0.58±0.08	0.73±0.07	0.72±0.13	0.54±0.04	0.55±0.50
C17:1	**1**	0.70±0.16	0.78±0.09	0.63±0.14	0.46±0.15	0.81±0.16	0.36±0.11	0.44±0.19
	30	0.73±0.14	0.52±0.18	0.78±0.22	0.43±0.21	0.37±0.14	0.41±0.08	0.39±0.09
	60	0.57±0.08	0.61±0.09	0.73±0.23	0.86±0.14	0.78±0.05	0.61±0.09	0.57±0.07
	90	0.57±0.09	0.54±0.06	0.60±0.04	0.58±0.11	0.58±0.06	0.57±0.08	0.58±0.07
C18:1cis	**1**	19.61±3.55	19.30±4.26	21.04±4.51	21.97±4.10	19.50±3.84	21.42±6.05	20.63±5.14
	30	23.19±4.93	23.26±3.21	22.38±22.58	21.17±4.16	23.06±3.88	23.14±4.02	22.21±3.66
	60	21.91±2.99	22.16±3.98	23.18±5.08	20.09±4.15	21.17±4.57	20.56±3.68	22.13±4.52
	90	23.70±2.65	23.78±4.98	24.26±4.06	24.49±3.88	22.99±3.46	25.40±2.86	24.48±3.44

C18:2tr	**1**	0.87±0.15	0.90±0.75	0.74±0.29	0.71±0.36	0.95±0.84	0.41±0.07	0.58±0.24
	30	0.05±0.002	0.07±0.00	0.04±0.01	0.03±0.00	0.07±0.01	0.05±0.00	0.06±0.00
	60	0.02±0.004	0.04±0.01	0.05±0.01	0.06±0.00	0.07±0.01	0.05±0.00	0.08±0.00
	90	0.04±0.002	0.03±0.01	0.07±0.00	0.04±0.02	0.05±0.01	0.07±0.03	0.04±0.03
C18:2cis	**1**	2.61±0.98	2.27±1.63	2.02±0.85	2.72±0.88	2.20±1.21	2.12±1.32	2.24±1.02
	30	1.56±0.71	1.83±0.27	1.34±0.26	1.76±0.38	1.63±0.41	1.72±0.71	1.66±0.18
	60	1.03±0.85	1.19±0.67	1.22±0.99	0.99±0.24	1.03±0.32	0.87±0.48	0.66±0.21
	90	0.58±0.22	0.62±0.14	0.13±0.08	0.56±0.19	0.61±0.28	1.09±0.32	0.46±0.30
C18:3	**1**	0.03±0.00	0.05±0.00	0.03±0.00	0.04±0.00	0.03±0.00	0.06±0.00	0.02±0.00
	30	0.03±0.00	0.06±0.00	0.04±0.00	0.03±0.00	0.04±0.00	0.05±0.00	0.06±0.00
	60	0.04±0.01	0.06±0.00	0.07±0.01	0.08±0.00	0.07±0.00	0.06±0.00	0.05±0.00
	90	0.04±0.01	0.02±0.01	0.04±0.01	0.07±0.00	0.08±0.00	0.06±0.00	0.03±0.00
C20:1	**1**	0.62±0.56	0.70±0.26	0.82±0.41	0.64±0.22	0.46±0.12	0.56±0.32	0.48±.0.40
	30	0.16±0.03	0.07±0.01	0.11±0.08	0.07±0.01	0.06±0.00	0.05±0.00	0.04±0.01
	60	0.08±0.04	0.06±0.01	0.04±0.01	0.07±0.02	0.05±0.00	0.04±0.00	0.08±0.01
	90	0.03±0.01	0.01±0.01	0.07±0.04	0.03±0.02	0.02±0.01	0.09±0.00	0.07±0.00

Fatty acid levels except for $C_{16:1}$ and $C_{18:2}$, were not affected by the type of prebiotics used ($p>0.05$). On the first day of storage, polyunsaturated fatty acid (PUFA) levels changed from 2.46% (EF-IO) to 3.92% (EF-O), and monounsaturated fatty acid (MUFA) levels changed from 20.32% to 23.69%. At the end of the storage period, monounsaturated fatty acid (MUFA) levels varied from 25.66% to 28.73%, and polyunsaturated fatty acid (PUFA) levels changed from 0.47% to 1.24%. Total unsaturated fatty acid rates varied from 26.42% to 29.43%. As can be seen, polyunsaturated fatty acid ratio of the samples increased significantly. As a result of the statistical analysis, it was found that the adjunct cultures and prebiotic types had an effect on the polyunsaturated fatty acids ($p<0.05$). During ripening, several physical, chemical and biochemical events take place together. In addition, the formation of the aromatic substances occurs during ripening. One of the most important factors affecting the formation of aromatic compounds is lipolysis. In lipolysis, fatty acids are decomposed and many compounds are formed as flavoring agents. Lipolysis occurs in polyunsaturated fatty acids. Starter cultures also have an impact on the lipolysis. Therefore, the significant impact of the adjunct cultures used in our study on the unsaturated fatty acids is an expected result. However, is also considered to be normal that the prebiotics that stimulate the starter cultures have a significant but indirect effect on lipolysis. There were no significant changes in the total saturated fatty acids ratio of the samples ($p>0.05$). In general, the highest ratio in unsaturated fatty acid composition of experimental samples was in $C_{18:1}$ and the lowest was in $C_{18:3}$. In all cheese samples, a decrease was observed in $C_{18:1}$ ratios on the ninetieth day of storage. MUFA and PUFA ratios were found to be quite close to each other in all cheese types. In another similar study by Zlatanos et al. (2002), it was reported that SFA, MUFA and PUFA ratios of Feta cheeses were 70.20%, 21%, and 4.7%, respectively. The results of this study are in line with the researchers' results.

3.2. Organic acid contents of symbiotic cheeses

Organic acids are formed by the degradation of proteins, fat, lactose and citrates during cheese ripening, and they have an important role, especially in the development of the cheese aroma. The organic acid results obtained from cheese samples are given in

Table 5. The highest level of organic acid among all experimental samples was lactic acid whereas the lowest level of organic acid was acetic acid. This trend continued until the citrate in milk was metabolized by lactic acid bacteria into flavor components such as acetic acid, acetaldehyde and diacetyl, until the end of the storage period. The oxalic acid and citric acid content of the cheeses on the first day of the production varied from 1018.83 to 2007.76 μg/g and from 8610.02 to 21197.17 μg/g. Different prebiotics and cultures used in production had no significant effects on the oxalic and citric acid content ($p>0.05$). Oxalic acid levels of the cheeses exhibited irregular increases and decreases during storage. At the end of the storage process, oxalic acid levels increased in EF-O, EF-IO, LP-K, BL-I and BL-IO samples, whereas they decreased in other cheese samples. It was found that the storage process, culture type used and prebiotic variety have significant effects on oxalic acid levels of the samples ($p<0.05$).

Table 5. Organic acid compound of cheese samples

Organic acids	Day	Control	EF-C	EF-I	EF-O	EF-IO
Oxalic acid	1	1775.38±125.43	1655.27±90.41	1183.1±79.81	2007.76±114.98	1410.72±86.21
	30	1320.06±90.47	1686.04±81.55	1023.18±74.69	1878.69±101.08	1235.28±44.17
	60	1803.32±116.17	1338.51±91.70	1806.71±124.52	1660.95±111.09	1859.35±155.81
	90	1233.46±80.26	1350.74±114.51	794.3±51.39	2742.01±140.73	2540.58±127.58
Citric acid	1	15075.56±321.47	16932.23±259.69	13416.17±211.87	12372.39±125.98	13970.01±200.64
	30	20798.52±359.29	13766.31±113.65	4276.49±114.62	5830.48±81.46	18602.65±300.17
	60	18784.21±288.64	8237.59±155.62	3410.02±66.43	4580.54±81.21	24874.73±260.11
	90	23323.84±278.25	12061.68±92.58	4828.78±59.21	1404.12±39.45	20311.78±187.29
Pyruvic acid	1	1882.38±121.20	570.22±66.98	484.65±48.51	566.26±68.21	1157.17±97.52
	30	4237.48±100.14	4170.16±118.54	2492.49±69.11	895.42±57.14	5956.48±129.84
	60	2788.82±144.32	3469.60±150.28	2317.43±110.54	4383.23±162.54	6924.03±176.84
	90	2269.06±136.54	689.79±69.87	2616.17±141.25	3352.66±165.32	6092.56±271.25
Succinic acid	1	488.54± 50.47	396.14± 44.65	119.76± 29.54	149.46± 21.36	290.7± 37.85
	30	-	-	-	-	-
	60	-	-	-	-	-
	90	-	-	-	-	-
Fumaric acid	1	1175.07±204.63	1551.39±144.25	1986.80±150.47	4190.54±259.47	1575.58±120.51
	30	4442.57±80.48	3942.03±97.21	4205.06±112.47	19117.05±326.78	9179.07±99.48
	60	14299.89±365.87	6018.65±125.31	6745.26±131.51	14446.93±251.72	14527.03±205.26
	90	9503.31±149.54	3142.31±68.97	8549.75±135.24	11776.04±150.80	13173.91±201.89
Lactic acid	1	172280.68±15610	118109.67±18654	178864.71±16895	267773.92±30258	147910.05±20487
	30	196153.93±17458	236020.84±21631	548759.07±49514	561020.11±50447	149895.47±21554
	60	305793.95±20145	106429.75±8796	987831.39±67845	478699.53±37896	234895.94±17489
	90	231354.89±9632	278576.84±12547	815426.93±36547	628065.01±15821	39275.64±2514
Formic acid	1	15464.22±1254.4	19660.85±1254	26250.39±968	16002.4±652	12576.04±654
	30	8597.01±110.47	5427.10±188.94	13464.02±451.29	16756.51±500	5328.92±193
	60	7213.47± 276.41	5150.71±199.47	4576.62±215.23	11096.57±740	6878.55±250
	90	2732.83±100.70	14389.66±841.39	8349.86±375.68	5390.46±201.35	9841.49±176.52
Acetic acid	1	32.98±3.02	63.77±4.59	24.00±1.15	27.44±1.06	50.39±2.01
	30	447.53±46.26	510.89±43.65	10.88±2.14	91.13±12.29	85.84±9.68

	60	367.99±40.26	237.14±20.57	24.07±6.71	126.69±11.94	65.53±5.22
	90	765.66±62.36	113.87±13.50	88.03±10.42	140.9±18.73	98.72±11.29
Propionic acid	**1**	29242.61±2514.3	22877.15±1874.4	27568.40±2005.4	16566.89±1254.8	16407.86±1102.8
	30	4295.06±195.35	13183.60±451.67	3515.59±142.25	8005.12±302.59	14743.43±1125.5
	60	3326.22±121.08	9032.28±200.47	7319.49±288.52	6825.29±300.49	16068.17±605.25
	90	10038.53±516.82	8100.08±321.19	7264.07±315.69	6494.91±302.51	9412.35±378.42

Table 5. (continued). Organic acid compound of cheese samples
Please refer to Table 2 for abbreviations.

Organic acids	**Day**	**LP-O**	**LP-IO**	**BL-C**	**BL-I**
Oxalic acid	**1**	1643.68±87.65	1217.19±90.53	1570.53±115.61	1043.00±80.24
	30	919.22±37.84	1214.32±88.24	1614.04±114.50	805.44±33.29
	60	1450.14±68.71	1064.35±34.22	1327.42±51.49	1271.95±65.48
	90	1096.75±53.79	1171.62±68.43	1287.22±74.59	1192.29±109.64
Citric acid	**1**	19289.22±177.52	19891.10±301.87	8610.02±341.84	11241.67±98.57
	30	21391.84±389.15	16243.84±248.41	21931.84±300.51	2373.17±49.51
	60	12476.97±119.48	17957.17±148.61	15947.57±132.51	15335.34±140.87
	90	12522.29±202.41	16175.04±289.41	9412.75±110.69	17591.26±236.95
Pyruvic acid	**1**	1642.03±125.32	1964.48±149.52	1193.15±171.25	1564.24±88.43
	30	1544.64±110.21	2373.17±121.26	4998.61±199.65	637.08±40.87
	60	3295.06±79.25	3179.46±100.36	343.65±68.30	1248.66±84.61
	90	4683.03±178.96	3950.35±154.21	2435.02±150.64	2244.92±90.57
Succinic acid	**1**	451.52±61.25	138.24±22.14	418.74±50.26	673.31±93.24
	30	-	-	-	-
	60	-	-	-	-
	90	-	-	-	-
Fumaric acid	**1**	4924.12±189.51	2354.17±155.26	1222.56±100.98	2359.80±125.47
	30	24234.38±301.56	7710.83±84.69	5367.84±77.48	14093.62±111.73
	60	9894.26±111.51	7355.65±154.20	2362.59±91.87	24612.82±501.47
	90	14571.08±221.89	13108.59±210.89	5539.09±99.58	20756.3±357.21
Lactic acid	**1**	442474.86±50417	402158.87±47956	192880.52±54186	329883±35622
	30	825931.44±66587	318097.5±25366	170197.36±12554	768770.32±50126
	60	488974.34±27841	573167.23±22145	158817.35±8963	812010.32±50418
	90	606433.39±14698	846367.42±29658	197474.95±8963	632760.72±21478
Formic acid	**1**	30827.10±1258	22552.26±784	24009.32±1925	8670.05±251.19
	30	8022.23±140	6626.63±258	11725.66±487	10254.03±415.20
	60	5698.10±174.86	9049.30±359	5793.05±454	13191.87±796.41
	90	8540.58±360.12	6706.25±295.45	3002.02±121	9495.18±330.57
Acetic acid	**1**	63.02±7.54	60.48±5.46	15.35±0.83	34.31±1.50
	30	28.03±3.78	23.29±3.21	481.18± 31.69	44.52±5.21
	60	37.97±4.06	22.45±3.73	285.66±26.70	41.43±9.05
	90	88.79±10.69	42.18±5.04	437.42±51.29	15.18±3.78
Propionic acid	**1**	12022.69±845.62	18398.09±759.84	26148.76±2154.20	13298.81±986.71

	30	6591.42±250.19	16305.16±512.25	9359.76±398.18	11467.30±402.29
	60	4593.32±199.81	700.15±286.79	4332.61±185.26	2960.58±115.14
	90	5149.15±220.19	9947.43±416.87	8487.56±385.26	6782.97±276.49

Irregular changes were also observed in the citric acid levels of the samples during the study (see Table 5). Comparing the first and ninetieth day of storage, it was found that there was an increase in citric acid levels of all BL types, except the BL-O, control and EF-IO samples, while citric acid levels showed a decrease in other sample types. According to the first day's results, using different prebiotics and cultures had no significant effect on the citric acid levels ($p>0.05$). At the end of the storage, evaluating the cheese groups between themselves, it was found that prebiotics had a significant effect, and using different cultures had a significant effect on citric acid levels ($p<0.05$). According to the first day's results, pyruvic acid levels of the samples varied between 468.48 μg/g and 1964.48 μg/g. Pyruvic acid levels of the cheeses containing inulin and oligofructose (EF-IO, LP-IO and BL-IO) were statistically different from the other cheeses ($p<0.05$). Also, it was determined that starter cultures had a significant effect on pyruvic acid levels of the samples ($p<0.05$). At the end, the pyruvic acid levels increased from 468.48 μg/g to 6092.56 μg/g in all samples. It was also found that the storage process had a significant effect on the pyruvic acid levels of the samples ($p<0.05$).

It was determined that the succinic acid levels of the cheese samples varied from 119.76 μg/g to 1058.01μg/g on the first day, after which succinic acid levels of all samples decreased over the first thirty days of storage. On the thirtieth, sixtieth and ninetieth days of storage, no succinic acid was found. Using different prebiotics had a significant effect on succinic acid levels ($p<0.05$). Fumaric acid levels of the samples varied from 1175.07 μg/g to 5488.44 μg/g on the first day of storage. In spite of irregular increases and decreases, fumaric acid levels on the ninetieth day of storage were found to be considerably higher and varied from 3142.31 μg/g to 30689.65 μg/g. In addition, evaluating each cheese group among themselves and between different prebiotic types, fumaric acid levels were significantly different.

Lactic acid had the highest organic acid level among the organic acids in the experimental cheeses. As is seen in Table 5, on the first day of storage, lactic acid levels varied from 118109.67 μg/g to 455180.07 μg/g. For samples containing inulin, using different cultures had no effect on the lactic acid levels ($p>0.05$), while using different cultures had a significant effect on the lactic acid production level of samples containing the oligofructose and inulin-oligofructose mixture ($p<0.05$). Furthermore, the lactic acid levels of the samples depended on the storage time and changed by the ninetieth day of storage. The increase in lactic acid levels were significant in all samples ($p<0.05$). Storage time had a significant effect on the lactic acid levels of all cheese samples ($p<0.05$). By the ninetieth day of storage, using different probiotics had a significant effect on the lactic acid levels of the samples ($p<0.05$).

Formic acid levels of the experimental cheese samples varied from 5124.32 μg/g to 32673.24 μg/g on the first day of storage. Using different types and amounts of prebiotics had a significant effect on the formic acid levels of the samples ($p<0.05$). At the end of the storage period, formic acid levels in all cheese samples decreased, except for the LP-K and BL-I samples. Formic acid levels showed irregular fluctuations during storage as in all other cheese samples. It was found that storage had a significant effect on formic acid levels of the samples ($p<0.05$).

Acetic acid can be produced from citrate, lactose and amino acids. It was found that acetic acid levels had the lowest levels in all cheese types (see Table 5). Acetic acid levels varied from15.35 μg/g to 81.65 μg/g on the first day of storage, while the levels varied from

1.518 μg/g to 765.66 μg/g by the end of the ripening of the cheese samples. Irregular changes were observed in acetic acid levels depending on the storage time. The storage process had a significant effect on the acetic acid levels of the samples ($p<0.05$). Among the cheese samples, it was found that using different adjunct cultures had a significant effect on the acetic acid levels of the samples ($p<0.05$). In the samples containing only oligofructose (EF-O, LP-O and BL-O) and only inulin (EF-I, LP-I and BL-I), it was found that different cultures had a significant effect on acetic acid levels.

Propionic acid levels of the cheese samples are given in Table 5. The lowest value was in BL-O with 7940.66 μg/g, whereas the highest level was in control group with 29242.61 μg/g. At the end of the storage period, propionic acid levels in all samples decreased. As a result of the statistical analysis, it was found that using different prebiotics had a significant effect on the propionic acid levels of the samples ($p<0.05$).

Abd-Elhamid (2012) observed the organic acid levels in Karish cheese made with free and encapsulated Bifidobacterium adolescents ATCC 15704. In that study, the initial acetic and lactic acids levels in Karish cheese with free and encapsulated *B. adolescents* ATCC varied from 0.63 g/kg to 3.94 g/kg, respectively, while the acetic and lactic acids levels at the end of the storage varied from 1.39 g/kg to 5.71 g/kg, respectively. This can be explained by the utilization of lactose by *Bifidobacterium* and other lactic acid bacteria, which can ferment the lactose, resulting in 3 moles of acetic acid and 2 moles of lactic acid per 2 moles of glucose.

Lactate and citrate are significant precursors for a serious of reactions leading to the production of lactic, acetic, citric, formic and pyruvic acids. Most of the lipase enzymes produce butyric acid during lipolysis (Murtaza et al., 2012). Temperature is also a major factor that affects bacterial growth in cheese during manufacture and ripening, and the increase in temperature increases the microbial activity and the rate of biochemical reactions and organic acid levels (Azarnia et al., 2006).

4. Conclusions

As a result of this research, adjunct probiotic culture and prebiotic substances added to goat milk in white cheese production had an effect on the chemical characteristics of the final product. The developments in technologies for the production of symbiotic cheeses as an innovative trend using adjunct probiotic culture and prebiotics (inulin, oligofructose) allows the production of cheese with good characteristics and satisfactory acceptance by consumers. It might also be an interesting and feasible opportunity for the dairy market to expand.

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EXTRACTION OPTIMIZATION AND CHARACTERIZATION OF ACID SOLUBLE COLLAGEN FROM MILKFISH SCALES (*Chanos chanos Forskal*)

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ABSTRACT

This study aims to determine the collagen from extraction optimization of milkfish scale collagen based on the yield and hydroxyproline content due to the treatment of CH_3COOH concentration and extraction time. [Method] This research used Response Surface Methodology (RSM) and Central Composite Design (CCD) with independent variable of extraction time(X1, hour) and concentration of acetic acid (X2, M), while dependent variables were yield (Y1,%) and hydroxyproline content (Y2,%). Design Expert software was used for this design. [Result] The result shows that the optimal condition of collagen extraction from milkfish scale is obtained by extraction time of 61.30 hours with CH_3COOH concentration of 0.66M, resulting in a yield of 0.737% ± 0.012 and hydroxyproline content of 7.223% ± 0.157, the difference in predictive value and Design Expert is less than 5%. [Conclusion] Result of FTIR, DSC and analysis amino acid show that milkfish scale collagen in this study resemble those of commercial collagen, thus it can be used as alternative source of collagen.

1.Introduction

Collagen is the most abundant protein produced by vertebrates and it is about 25% of the total vertebrates' protein (Ogawa et al., 2004). Collagen is commonly found in animals, it is on the skin, bones, tendons and other connective tissues. Generally, collagen sources are mostly obtained from cows and pigs (Minh Thuy et al., 2014). However, collagen from cows and pigs become particular concern to consumers due to the risk of dental and oral diseases, bovine sponge encephalopathy and avian influenza so that alternative sources are needed (Liu et al., 2015; Ogawa et al., 2004).

Milkfish scale processing can be an alternative for a safe source of collagen. The use of collagen from fish waste not only produces safe products but can also increase the income in fishing industry (Yung Huang, 2016). According to Hsiung Pan et al. (2010), collagen can be derived from the skin and scale of marine organisms. Fish skin is 6–10% of the fishtotal weight, while fish scale is only 3–4% of it. Therefore, the yield of collagen from fish skin is higher than the one from fish scale, but fish skin contains 3–6% fat and fish scale contains only 0.06% fat.

The waste from fish processing can reach 75% of the total weight of fish. Waste utilization is one of them that is used for animal feed, but many of them are polluting the environment because it is not utilized properly, so it is necessary to find the best utilization solution. One of them is to make it as a source of collagen (Woo et al., 2007). Several studies on fish scale collagen have been conducted. They are research by Duan et al. (2009) about collagen extraction of fish *Cyprinus carpio*, collagen extraction of *Labeo rohita* and *Catla catla* fish scale (Pati et al., 2010), fish scale

collagen from Japan and Vietnam oceans (Minh Thuy et al., 2014) and collagen of *Parupeneus heptacanthus* fish scale (Matmaroh et al., 2011).

This paper used Central Composite Design (CCD) with Response Surface Methodology (RSM). RSM is a mathematical and statistical model which was widely applied in food industry to evaluate the relationship between independent and dependent variables (Box and Wilson, 1951). Research on the optimization of collagen from fish scale is very limited. It was the reason of the researcher to conduct a study to determine the appropriate treatment to produce optimal collagen. This research aimed to determine the collagen from extraction optimization of milkfish scale collagen based on the yield and hydroxyproline content due to the treatment of CH_3COOH concentration and extraction time.

2. Materials and methods

2.1. Materials

Scale of milkfish with average body weight of 300-400 g was collected from Teaching Factory of Polytechnic Marine and Fisheries Sidoarjo. Milkfish scales were washed with cold water temperature ± 5 ° C and then stored in cold storage under the temperature of ± 18 °C before further process.

2.2. Extraction of acid soluble collagen

Fish scale was suspended in 0.1 M NaOH for 8 hours at a ratio of 1: 8 (w/v) to remove non-collagen proteins and pigments, the solution was changed every 4 h. Treated scales were washed with chilled water to achieve the neutral pH. Demineralization was carried out using 0.5 M EDTA-2Na solution (pH 7.5) for 24 h at the ratio of 1:8 (w/v), the solution was changed every 12 hours. Demineralized fish scale were extracted with acetic acid (0.2 M to 0.8 M) with extraction time for 24 – 96 hours at a ratio of 1: 6 (w/v), then the sample was filtered through cheese cloth. The collagen was precipitated with 0.9 mol/L NaCl for 24 hours, the precipitated was collected by centrifugation at 2000 rpm for 20 minutes. The pellet was dissolved with 0.5 M acetic acid and subsequently dialyzed in 0.1 M acetic acid, followed by distilled water. All processes were carried out at temperature of 4°C. Collagen was lyophilized using freeze dryer.

2.3. Experimental design

Response Surface Methodology (RSM) and Central Composite Design (CCD) were used for the extraction optimization of acid soluble collagen from milkfish scale consisting of independent and dependent variables. Independent variables were time of extraction (X1, hour) and concentration of acetic acid (X2, M), while the dependent variable were yield (Y1, %) and hydroxyproline (Y2, %). The design used was Design Expert 7.1.5 (State-Ease Inc., Minneapolis, USA) with an experimental design as displayed in Table 1.

The model was generated as a function of these variables in which the predicted response was first order polynomial and was represented as equation I.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta i X j + \sum_{j<i} \sum \beta i j X i X j \qquad (1)$$

Where β_0, β_i and β_{ij} are the regression coefficients for the intercept, linear and interaction coefficients, respectively, Y is the dependent variable or the response, x_i and x_j are the independent variables in coded units.

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

Variable	Treatment Code				
	-α	-1	0	1	+α
Extraction time (hour)	9.09	24	60	96	110.91
Acetic Acid (M)	0.08	0.2	0.5	0.8	0.92

2.4.Proximate analysis

Analysis of moisture, protein, ash and fat content were analyzed according to the AOAC method (2000).

2.5.Yield of collagen

Calculation of collagen yield was performed by comparing dry weight of milkfish scale collagen with weight of raw material. The extract yield was calculated using the following equation:

$$\text{Yield of collagen (\%)} = \frac{\text{Collagen dry weight}}{\text{Raw material weight}} \times 100\% \quad (2)$$

2.6.Hydroxyproline analysis

Five milligrams of collagen sample was hydrolyzed in 8 ml of 6 M HCL at 110°C for 22 h, the hydroxyproline content was determined according to the method of Woessner (1961) using $_L$-hydroxyproline as the standard.

2.7.Amino acid analysis

Amino acid composition of collagen sample was analyzed using the AOAC method (1995).

2.8.Analysis of Fourier Transform Infra Red Spectroscopy (FTIR)

Analysis of fourier transform infra red spectroscopy was performed using FTIR Spectrophotometer 8400S (Shimadzu, Japan) at the wavelength between 400 – 4000 cm^{-1}.

2.9.Analysis of Differential Scanning Calorimetry (DSC)

The flour sample was weighed for 0.0045 g in a special sample container (pan cell no. 201-53090), then the pan cell was closed to be airtight. The pan cell containing samples was analyzed by heating them using Differential Scanning Calorimeters (DSC 60 Shimadzu, Tokyo, Japan) from 30°C to 250°C with heating rate of 10°C/minute and the researcher flew nitrogen gas into the pan cell surrounding at the same time.

3.Results and discussions

3.1.Analysis of Central Composite Design experiment

Extraction optimization of milkfish scale collagen was aimed to explain the effect of extraction time with acetic acid concentration on collagen yield and hydroxyproline content. Program analysis of 13 treatments with design center was repeated five times to minimize the error rate. Design of the research using RSM-CCD method is displayed in Table 2.

3.2.Response analysis

The concentration between 0.2 to 0.8 M and extraction time of 24 to 96 h results in 0.54% to 0.74% yield and 3.6% to 7.6% hydroxyproline content. Analysis of variance (ANOVA) on the yield response and hydroxyproline content with quadratic model is displayed in Table 3.

The relationship between independent variables (extraction time and CH_3COOH concentration) and dependent variables (yield and hydroxyproline content) is presented in the following equation.

$$Y_1 = 0.10869 + 7.31318E\text{-}003\ X_1 + 1{,}27381\ X_2 - 1.71296E\text{-}003\ X_1 X_2 - 6.03781E\text{-}005\ X_1^2 - 0.83056\ X_2^2 \quad (3)$$

$$Y_2 = 1.25484 + 0.050994\ X_1 + 12.91867\ X_2 - 0.039352\ X_1 X_2 - 1.35995E\text{-}004\ X_1^2 - 8.25463 X_2^2 \quad (4)$$

Where: Y_1 = Yield; Y_2 = Concentration of acetic acid; X_1 = Extraction time; X_2 = Concentration of CH_3COOH

The analysis of variance shows that the quadratic model of the yield response is significant with P value 0.040, as well as the quadratic model of hydroxyproline content response is significant with P value 0.040. The lack of fit of the yield response is 0.134 (13.4%) while the one of the hydroxyproline content response is 0.077 (7.7%). The value

of the lack of fit is above 5% indicating that the experimental design model is significant (Chakraborty et al., 2017). Quadratic model of the yield response shows R^2 value of 0.755 and hydroxyproline content response shows R^2 value of 0.756. The model is quite appropriate because $R^2 \geq 0.75$. The model is considered appropriate if $R2 \geq 0.75$ (Le Man et al., 2010). Analysis of Variance (ANOVA) is displayed in Table 3.

3.3.Relationship between independent and dependent variables

The relationship effects between extraction time and CH_3COOH concentration to the yield and hydroxyproline content is illustrated in Figure 1. The effect of extraction time and CH_3COOH concentration on the yield could be seen in Figure 1a and The effect of extraction time and CH_3COOH concentration on the hydroxyproline content could be seen in Figure 1b.

Figure 1a illustrates the effect of collagen extraction time for 24 to 60 h causing the collagen yield to increase and decrease after extraction for 60 to 96 h, while the higher concentration of acetic acid causes the increase of collagen yield. Figure 1b illustrates that the extraction time between 24 to 60 h tends to increase hydroxyproline content and it slightly decreases after 60 h, but higher concentration of acetic acid results in slight increases the hydroxyproline content. It means that the yield and hydroxyproline content are affected by extraction time and concentration.

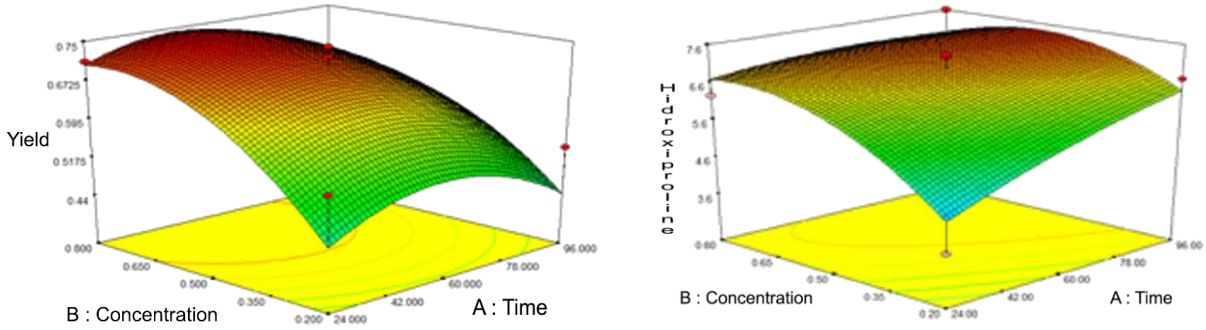

Figure 1. Response surface plot showing the effect of collagen extraction time and concentration of acetic acid on yield (a) and Hydroxiproline content (b)

Table 2. Central Composite Design of extraction optimization of milkfish scale collagen

Treatment	Code		Actual		Response	
	X1	X2	Concentration (M)	Time (Hour)	Yield (%)	Hidroxyproline Content (%)
1	-1	-1	0.2	24	0.56	3.67
2	-1	1	0.2	96	0.54	6.70
3	1	-1	0.8	24	0.71	6.27
4	1	1	0.8	96	0.62	7.60
5	0	-1.414	0.5	9.09	0.54	6.76
6	0	1.414	0.5	110.91	0.46	6.73
7	-1.414	0	0.08	60	0.29	4.90
8	1.414	0	0.92	60	0.72	6.33
9	0	0	0.5	60	0.72	6.33
10	0	0	0.5	60	0.74	7.26
11	0	0	0.5	60	0.74	7.33
12	0	0	0.5	60	0.6	7.33
13	0	0	0.5	60	0.74	6.93

The quadratic pattern shows that dependent variable values will keep increasing to the optimum point and decrease after reaching the optimum point in parabola. It indicates that the expected yield and hydroxyproline response reach maximum level. Higher concentration with a certain extraction time will accelerate the hydrolysis of collagen so that the yield and hydroxyproline will keep increasing to its optimum point. The result of the research by Wang et al. (2008) also showed optimization of collagen from carp fish scale with treatments of time and concentration resulted in quadratic patterns.

3.4.Optimization of extraction condition and validation of the model

Criteria of the response are the yield and hydroxyproline content reach as optimum point as possible. Determination of optimum point is based on Design Expert analysis giving extraction solution time of 61.30 h with CH_3COOH concentration of 0.66 M predicted to results in 0.740% yield and 7.206% hydroxyproline with 0.949 of desirability. Validation is performed to determine whether or not the optimum point comply with the optimization prediction of Design Expert program. The result is displayed in Table 4.

The difference of predictive value and result of the research is less than 5%. It indicates that the prediction is in accordance with the result of research or not significantly different. (Wu et al., 2006; Zou and Liu, 2014) stated that the model is considered appropriate if the difference of predictive value and result of the research is not more than 5%. Optimal collagen of the research is then compared with commercial collagen based on FTIR analysis, amino acid composition and thermal analysis.

Table 3. Analysis of variance (ANOVA) of the response surface regression model

Source	Sum of Squares	df	Mean Square	F Value	P-Value Prob>F
Yield					
Model	0.16	5	0.033	4.33	0.0407
X_1 - Time	6.449E-003	1	5.449E-003	0.86	0.3858
X_2 - Concentration	0.083	1	0.083	11.07	0.0126
X_1X_2	1.369E-003	1	1.39E-003	0.18	0.6828
X_1^2	0.043	1	0.043	5.65	0.0491
X_2^2	0.039	1	0.039	5.16	0.0574
Residue	0.053	7	7.538E-003		
Lack of Fit	0.038	3	0.013	3.39	0.1343
Pure Error	0.015	4	3.720E-003		
Cor Total	0.22	12			
Hydroxyproline					
Model	10.76	5	2.15	4.34	0.0407
X_1 - Time	2.33	1	2.33	4.70	0.0668
X_2 - Concentration	3.82	1	3.82	7.70	0.0275
X_1X_2	0.72	1	0.72	1.46	0.2667
X_1^2	0.22	1	0.22	0.44	0.5303
X_2^2	3.84	1	3.84	7.74	0.0272
Residue	3.47	7	0.50		
Lack of Fit	2.74	3	0.91	4.97	0.0777
Pure Error	0.73	4	0.18		
Cor Total	14.23	12			

Table 4. Data of optimum point validation

	Extraction time (Hours)	Concentration (M)	Response	
			Yield (%)	Hydroxyproline (%)
Prediction	61.30	0.66	0.740	7.206
Result of the research	61.30	0.66	0.737±0.012	7.223±0.157
Difference of predictive value and result of the research (%)	61.30	0.66	0.4	0.2

3.5.Proximate analysis of milkfish scale

Milkfish scale contains 53.87 ± 0.396% water, 14.37 ± 0.405% ash, 0.16 ± 0.032% fat and 31.34 ± 0.026% protein. Protein content in fish scale is similar to those of golden goatfish (Matmaroh et al., 2011), indicating that milkfish scale is potential to be a source of collagen. High ash content of the scale is due to calcium hydroxypthalite (Ca_5 $(PO_4)_3$ OH) contained in the outer and inner layer of the scale (Matmaroh et al., 2011).

3.6.FTIR analysis

Figure 2 is the result of FTIR analysis on milkfish scale collagen and commercial collagen. The regions of Amide A band (3300-3500 cm^{-1}) is related to N-H stretching vibrations. Amide 1 band (1600-1690 cm^{-1}) is related C=O stretching. Amide

II band (1480-1575 cm^{-1}) is related to NH bending and CN stretching. Amide III band (1229-1301 cm^{-1}) is related to NH bending and CN stretching (Muyongga et al., 2004; Kong and Yu, 2007). The presence of Amida A, I, II and III is detected on milkfish scale collagen and commercial collagen. The similar thing is also detected in collagen of perch fish skin which was produced in the area of Amida A, I, II and III (Muyongga et al., 2004).

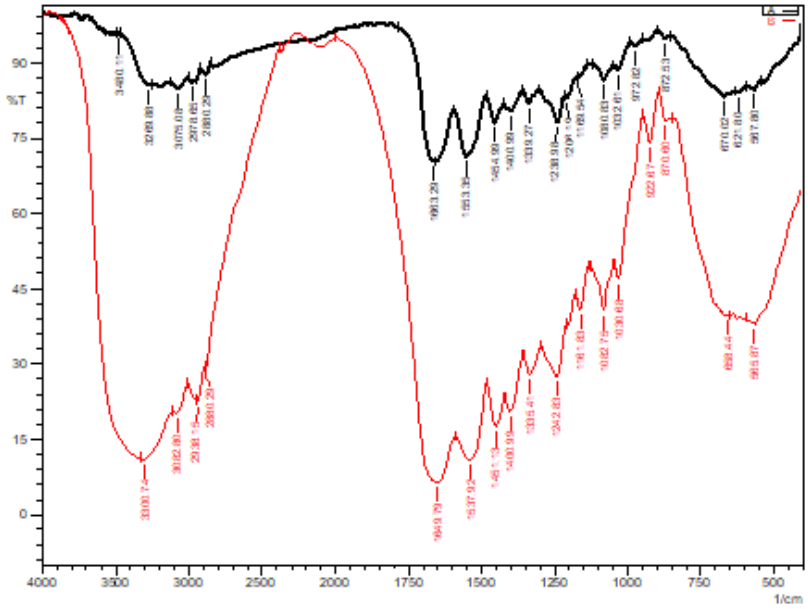

Figure 2. Infra red spectra of milkfish scale collagen and commercial collagen
A = Milkfish scale collagen, B = Commercial collagen

The group of Amida A in milkfish scale collagen and commercial collagen is detected at the wave numbers of 3480 cm^{-1} and 3300 cm^{-1}, while the group of Amida I is detected at the wave numbers of 1663 cm^{-1} and 1649 cm^{-1}. The value of Amida I in milkfish scale collagen is higher than that of commercial collagen. Nagarajan et al. (2012) explained that higher absorption apex compared with the one of Amida I group is found on gelatin. Yakimets et al. (2005) stated that the group of Amida I detected at the wave number of 1633 cm^{-1} is the structure characteristic called random coiled of gelatin.The structure is formed due to thermal depolymeration process that is the breaking up of organized structure of triple helix collagen due to the increase of temperature during the extraction process.

Amide II in milkfish scale collagen and commercial collagen is detected at the wave numbers of 1553 cm^{-1} and 1539 cm^{-1}, while Amide III is detected at the wave numbers of 1238 cm^{-1} and 1242 cm^{-1} showing intermolecular interactions on the collagen associated with CN stretching and NH bending (Kong and Yu, 2007). The triple helix structure of collagen can also be demonstrated by ratio intensity between the absorption apex of Amida III and the apex of area 1450 cm^{-1}. The result of this study indicates that the absorption apex of Amida III in milkfish scale collagen and commercial collagen is 1238 cm^{-1} and 1242 cm^{-1}, while the ratio value between apex area of Amida III and that of 1450 cm^{-1} are 1.17 and 1.16 respectively. Matmaroh et al. (2011) stated that a ratio value closing to 1.0

indicates that the collagen still has a triple helix structure.

Milkfish scale collagen and commercial collagen show the same secondary structure. The absorption areas of Amida I and III indicate that the triple helix structure of collagen is not changed or it is not undergoing a gelatinization. The band difference is believed due to differences in species and collagen molecular structures (Matmaroh et al., 2011).

3.7.Amino acid analysis

The amino acid composition of both milkfish scale collagen and commercial collagen is presented in Table 5. Amino acid is the major component of collagen that contributes in maintaining the stability of triple helix collagen structure (Ikoma et al., 2003). Furthermore, the structure of collagen integrity is affected by the amount of proline and hydroxyproline

The results of amino acid testing of milkfish scale collagen and commercial collagen show nearly similar values. Both collagen are rich of glysine at 24.09% and 24.38%, proline at 10.15% and 11.35% and hydroxyproline contents also high at 7.2% and 7.8%. Imino acid (proline and hydroxyproline) contents of milkfish scale collagen and commercial collagen are 17.35% and 19.15%, showing a similar tendency as other marine collagens. Amino acid in yellow fin tuna skin shows 25.1% glycine, 12.5% proline and 8.0% hydroxyproline (Woo et al., 2007). Hydroxyproline content on marine organisms tend to be lower than hydroxyproline content on mammals, marine organisms have 4–10% collagen while mammals might have more than 10% collagen (Khew and Tong, 2007).

3.8.Analysis of Differential Scanning Calorymetry (DSC)

Exothermic temperature of collagen can be measured by Differential Scanning Calorimetry. There are two exothermic apexes in the DSC analysis, the first apex indicates glass transition temperature where the hydrogen bond in collagen is interrupted resulting in the formation of an amorphous polymer called gelatin. The second exothermic apex shows the apex temperature of melting (T-max) (Wulandari et al., 2015). The result of DSC analysis on the milkfish scale collagen and commercial collagen in the form of thermogram curve is presented in Figure 3.

Milkfish scales collagen has an apex melting temperature (T-max) at the first apex of 101.12°C and at the second apex of 154.56°C. Commercial collagen has apex melting temperature (T-max) at the first apex of 166.81°C and at apex two of 180.35°C. Collagen of milkfish scale has lower thermal stability compared to commercial collagen and is also lower than cork bark fish collagen which peak melting temperature is 159.9°C (Wulandari et al., 2015) but it is higher than acid soluble collagen of Bambooshark fish skin which Tmax is 34.45°C (Kittiphattanabawon et al., 2010) and is also higher than collagen of golden goatfish scale which Tmax is 41.58°C (Matmaroh et al., 2011).

The difference in thermal stability is believed due to the difference in the amount of imino acid. The amount of imino acid in milkfish scale collagen is 17.35% and that in commercial collagen is 19.15%. Collagen thermostability is associated with imino acid content (proline and hydroxyproline). The collagen which has high imino acid content will be more resistant to heat (Bae et al., 2008; Kittiphattanabawon et al., 2005). Beside the difference in imino acid content, thermostability difference is might be due to

difference in the origin of raw materials and environmental temperature (Pati et al.,2010).

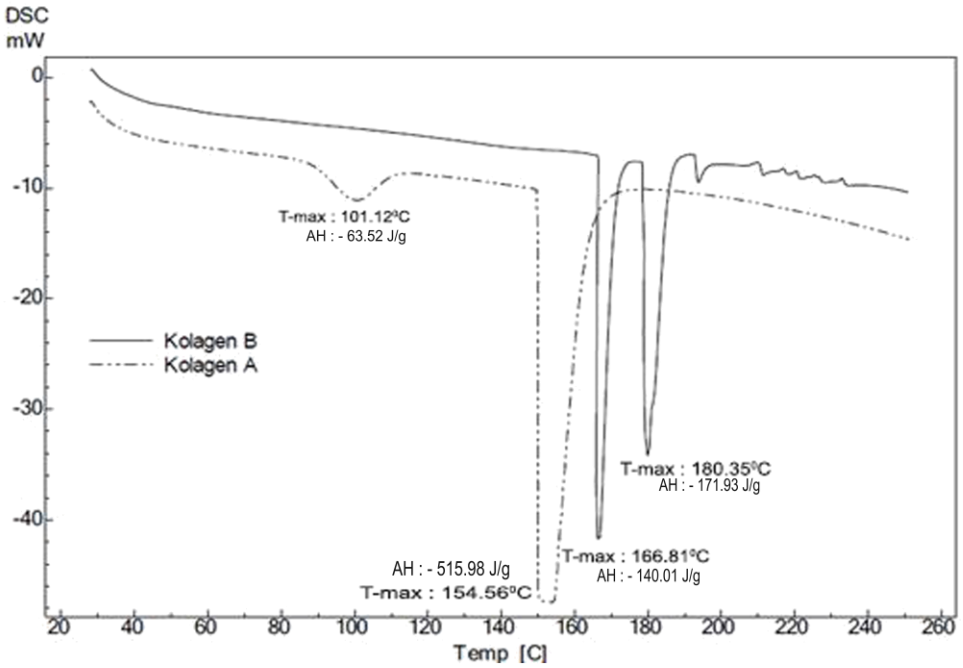

Figure 3. Thermogram of milkfish scale collagen
A = Milkfish scale collagen, B = Commercial collagen

Table 5. Amino acid composition of milkfish scale collagen and commercial collagen

Amino Acid	Milkfish Scale Collagen (%)	Commercial Collagen (%)
Glysine	24.09	24.38
Alanine	13.71	11.75
Valine	1.18	1.2
Leusine	1.87	1.78
Isoleucine	0.68	0.72
Serine	1.84	1.51
Threonine	3.41	3.45
Cysteine	0.58	0.43
Methionine	1.14	1.34
Aspartic Acid	4.95	4.75
Glutamate	11.49	11.68
Asparagin	0.1	0.9
Lisine	4.9	5,0
Hidroxicilline	0.05	0.04
Histidine	1,00	1,00
Phenylananine	1.73	1.33
Tyrosine	0.35	0.35
Tripthopane	0.0	0.0
Proline	10.15	11.35
Arginine	9.12	9,0
Hydroxyproline	7.2	7.8
Imino Acid	17.35	19.15

4. Conclusions

Methodology of response surface using Central Composite Design shows the effect of extraction time and acetic acid concentration on the yield and hydroxyproline content. Optimum result presents in the extraction time of 61.30 h with CH_3COOH concentration of 0.66 M. It results in the yield of 0.74% ± 0.012 and hydroxyproline content of 7.20659% ± 0.157, predictive value difference and Design Expert is less than 5%. Analysis results of FTIR, DSC and Amino acid show that collagen of milkfish scale has similarity with commercial collagen.

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EVALUATION OF MORPHOLOGIC METHOD FOR THE DETECTION OF NERVOUS TISSUE IN MINCED MEAT

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ABSTRACT

Producing meat products with ingredients which are not consistent with the label is considered fraud. Due to the high economic value of meat, the use of unauthorized tissue in meat products is possible. Aside from the adulteration aspect, it is important to note that some animal tissues like the brain and the spinal cord can bear infective agents which are transmissible to humans. Based on these observations, the aim of the present study was to apply morphological method for detection of nervous tissues in minced meat. Laboratory adulterated minced beef meat; each containing 0, 5, 10, 15 and 20% of beef brain was prepared. Then each sample was divided into three parts and four paraffin embedded blocks were prepared from each part. The sections were stained using sudan black and cresyl violet and also the immunohistochemical staining with fluorescent method were applied using anti-neurofilament 200 antibody for the determination of nervous tissue. Although the neuronal cell bodies and neuronal fibers were clearly detectable in Cresyl violet staining and sudan black staining, respectively, however, staining intensity did not show any difference according to different percentages of added brain. In contrary, immunohistochemical study revealed that neurofilament 200-immunolabeling was present in all percentages of added brain samples and the intensity of the labeling varying from weak to strong consisted by the increasing the amount of brain in samples.

In conclusion, the immunohistochemical technique with fluorescent method is an effective method for evaluations of additive brain tissue in minced meat with high sensitivity.

1. Introduction

Meat products may contain ingredients which are not consistent with the label (Ballin, 2010). Since only muscle tissue is considered as meat from the point of view of labeling (Gout et al., 2004), several reports are available for the detection of different animal tissues in the meat products, in which histological technique has been used (Tremlova & Starha 2003; Gout et al. 2004; Prayson et al. 2008a; Prayson et al. 2008b, Ghisleni et al., 2010; Botka-Petrak et al., 2011; Latorre et al., 2015; Sadeghinezhad et al., 2015).

Some additives in meat products are not only important in meat product quality but also for the food safety. The central nervous system

tissues (CNST) including brain and the spinal cord can bear infectious agents, such as prions can cause transmissible spongiform encephalopathies (Prusiner, 1998). There is increasing evidence that bovine spongiform encephalopathy (BSE) can be transmissible to humans as variant Creutzfeldt Jacob disease (Almond and Pattison, 1997; Ridley and Baker, 1999; Roma and Prayson, 2005). Therefore, various methods like direct tissue examination (Bauer et al., 1996) and cholesterol content analysis (Lucker et al., 1999; Schmidt et al., 1999) has been used for the detection of CNST in meat products. The standard histological method has been applied for examination of CNST in meat products with some contradictions in literature (Linke, 1959; Wnisch et al., 1999). However, immunohistochemical method (IHC) has been used for the detec¬tion of CNST in meat products due to high specificity (Wenisch et al., 1999; Tersteeg et al., 2002).

The current experimental study was designed to evaluate the neurohistological staining (sudan black and Cresyl violet) and immunohistochemical staining (using neurofilament antibody) for the determination of CNST in raw minced meat with known levels of added brain tissue.

2. Materials and methods

Preparation of meat samples- Samples of minced beef meat, each containing 0%, 5%, 10%, 15% and 20% beef brain were prepared. All the samples with different percentages were evaluated for flavour, tenderness, juiciness, and overall acceptability by 10 different people. Then, each sample was divided into three equal parts, four pieces of each part were collected, and fixed in 10% neutral-buffered formalin.

Histological study- The tissues were routinely processed for light microscopy and embedded in paraffin. Each paraffin-embedded block was cut into 7µm sections and mounted on polysined slides. Then, one slide from each block was taken and stained using Sudan black and Cresyl violet in order to detect the neurons and myelin sheats, respectively.

Immunohistochemical study- The mounted slides were dewaxed in xylene and then rehydrated through graded ethanol up to water. For antigen unmasking, sections were heated in sodium citrate buffer (pH 6.0) in a microwave (two cycles of 5 min at 800 W). To reduce the background, the tissues were incubated in PBS containing 20% normal goat serum for 1 h at room temperature (RT). The tissues were then incubated overnight at 4°C in a humid chamber, in a primary antisera (anti-NF200 rabbit polyclonal antibody, 1: 200 - N4142, Sigma-Aldrich) diluted in a suitable medium (1.8% NaCl in 0.01 M phosphate buffer containing 0.1% Na-azide). After washing in PBS (3×10 min), the tissues were incubated for 1 h at RT in a humid chamber in a secondary antibody (Goat anti-rabbit IgG FITC, 1:200 - Calbiochem-Novabiochem) diluted in PBS. The tissues were then washed in PBS (3×10 min) and mounted in buffered glycerol pH 8.6.

Light and fluorescence microscopy- The slides were observed under a microscope (Nikon E600, Japan) equipped with electronic eyepiece (E-eye, MB-2250, Germany) and the Axiovision software (Carl Zeiss, Oberkochen, Germany) for the detection of brain tissue. The appropriate filter cubes to distinguish fluorescein isothiocyanate (FITC) fluorescence aid to locate CNST by the presence of a fluorophore that labelled the antigen. The images were processed using Adobe Photoshop CS (Adobe system, San Jose, CA).

3. Results and discussions

The organoleptic evaluation showed that added CNST was not detectable in the prepared minced meat, also at high percentages.

The striated skeletal muscle fibers were detectable using Sudan black and Cresyl violet staining; in all the examined slides, the multiple nuclei were displaced to the periphery of the muscle fibers. The neuronal cell bodies were clearly detectable in Cresyl violet staining, while were less observable in Sudan black staining, due to the blue-black color of the myelin sheet, (Figure 1). However, staining

intensity did not show any difference according to different percentages of added brain.

Immunohistochemical study revealed that NF200-immunolabeling was present in all percentages of added brain samples (Figure 2). The intensity of the labeling varying from weak to strong consisted by the increasing the amount of brain in samples.

There are reports indicating that BSE can be acquired by humans with consuming contaminated beef which lead to the loss of confidence in consumption of meat and meat products (Wenisch et al., 1999). The bovine skull including brain, spinal cord, eyes and tonsils has been considered as specified risk materials and thus have been banned as raw materials in processed meat products by European Union (Tersteeg et al., 2002). Thus of particular concern, various methods have been used for the detection of the brain tissue in meat products (Gout et al., 2004).

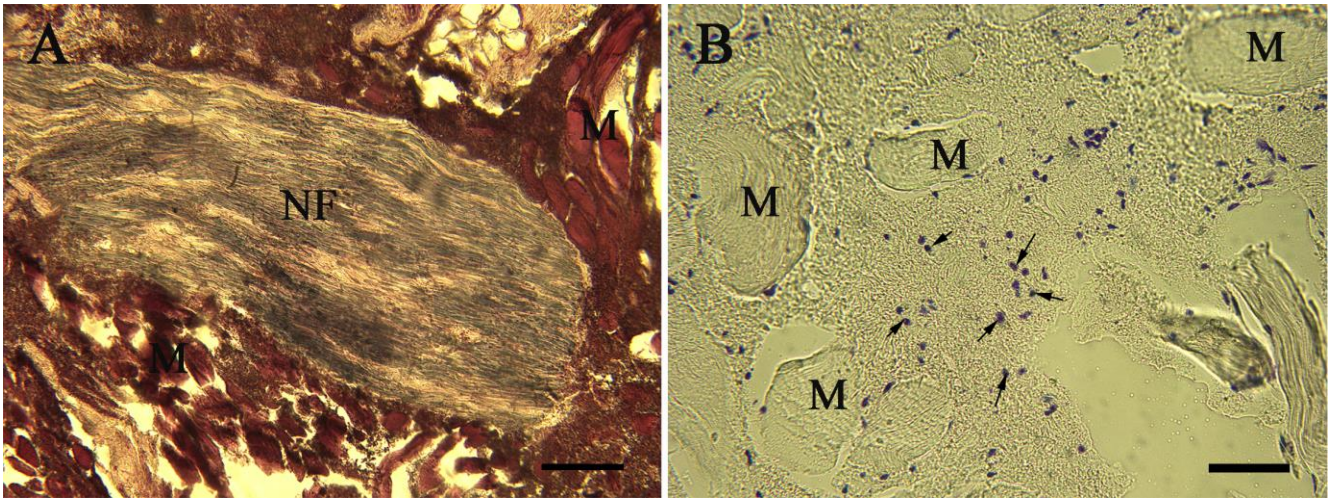

Figure 1. Photomicrograph of histological staining of additive nervous tissue in minced meat. A: The histological section shows the striated skeletal muscles (M). The Neuron fiber (NF) has been specified (Sudan black, scale bar 100 µm). B: The photomicrograph shows muscle fibers (M) and neuronal cell bodies (arrows) (Cresyl violet, scale bar 50 µm).

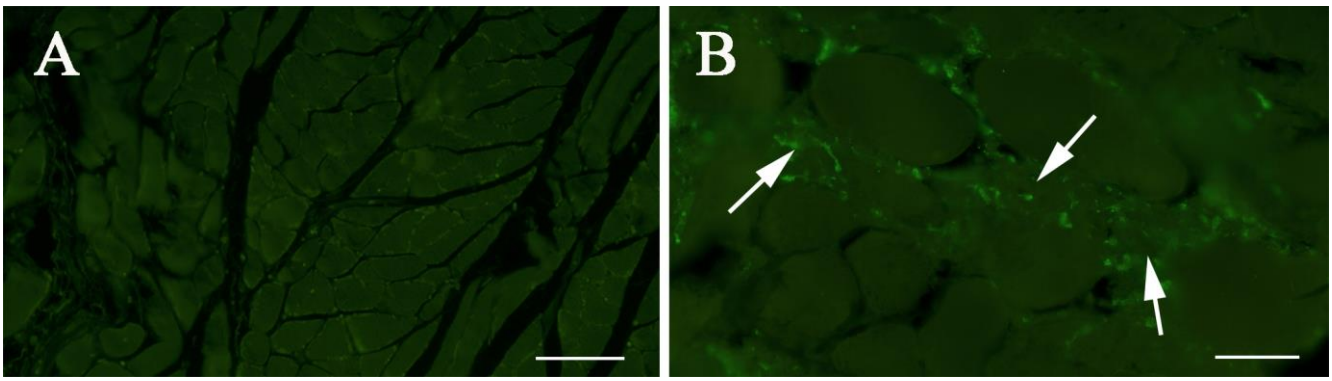

Figure 2. Photomicrograph of additive nervous tissue immunolabelling in minced meat with the antibody anti-NF200. A: In minced meat without nervous tissue (0 %) no reaction was seen (scale bar 100 µm). B: The neural fibers are easy recognizable (arrows) in minced meat with nervous tissue (scale bar 50 µm).

The histological technique used in this study despite revealed striated skeletal muscles and fragments of neuronal processes and the neuronal cells. Linke (1959) indicated the brain tissue in Frankfurter sausage using histological examination. In contrast, Wenisch et al. (1999), using H&E and also special stainings, stated that no neuronal cell body nor neuronal process were not detectable within cooked sausage due to homogenization in cutting mixer and heating pressure. In overall, this method cannot be recommended a reliable technique for the detection of CNST in minced meat because of destruction of the CNST in this product.

Immunohistochemical method (IHC) has mostly been used for the detec¬tion of CNST in meat products due to high specificity (Tersteeg et al., 2002). The neuron-specific enolase (NSE) immunoreaction was suggested as a reliable marker of CNST in cooked sausage as a consequence of the extraordinary resistance of the enzyme (Wenisch et al., 1999). However, Tersteeg et al. (2002) used four different antibodies including anti-neurofilament (NF), anti-myelin basic protein (MBP), anti-glial fibrillary acidic protein (GFAP) and NSE for detection of bovine (0%, 1%, 5%, 10%, and 20%) and porcine brain (5%) tissues in raw, pasteurized and sterilized meat products using DAB (3,3'-Diamino-Benzidine) staining. Overall, all the antibodies were useful for the raw meat product and the anti-MBP was suggested as a most useful antibody in detecting brain tissue of the heated meat products.

The problem with using IHC is the heating process or even certain meat manufacturing processes can cause conformational and chemical changes in the reactive side of the antigens (Tersteeg et al., 2002).

In minced meat despite the mechanical manipulation of the meat, the neuronal nuclei and processes are strongly labeled due to the lack of cooking in this type of product. Furthermore, florescent method for detection of the neuronal marker used in this study facilitate the detection of CNST and showed that staining intensity differ according to the different amount of added brain.

4. Conclusions

In conclusion, the immunohistochemical technique with fluorescent method is an effective method for evaluations of additive brain tissue in minced meat with high sensitivity. In addition, this method provide a facilitate detection of CNST in minced meat.

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COMPARATIVE STUDY ON POLYPHENOLS CONTENT AND ANTIOXIDANT EFFECT OF SOME GRAPE VARIETIES GROWN IN CENTRAL GREECE

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Total phenols.

ABSTRACT

Five local and six European grape varieties grown in Central Greece, have been studied for evaluating and comparing their polyphenols content and antioxidant properties. It has been established that total phenols (TP) of the local and European grape varieties move in broad ranges depending on the variety type, chemical soil properties, and with the ecological conditions. The content of TP in the grape varieties of the ethanol extracts ranged from 278.2 to 1813.9 mg GAE kg^{-1} FW, non-flavonoid phenols (NFP) ranged from 97.2 to 665.9 mg GAE kg^{-1}, flavonoid phenols (FP) from 170.7 to 1148 mg GAE kg^{-1} and total flavanols (F-3-ols) ranged from 43.3 to 183.7 mg CE kg^{-1}. The highest content of TP, NFP, FP and F-3-ols has been found in the red European grape variety Merlot, and the lowest in the white European variety Macabeo. The antiradical activity DPPH• ranged from 382 to 1569 μmol DPPH kg^{-1} FW, while the antioxidant activity FRAP ranged from 1768 to 6612 μmol FRAP kg^{-1} FW. A strong positive correlation is observed between the contents of total phenols of the grapes and the antioxidant and the antiradical activity (r^2 = 0.8927 and 0.8525 respectively).

1. Introduction

The reactive oxygen and nitrogen containing free radicals, which are formed in the human cells as strong oxidants can cause oxidative damage of the basic biological molecules (lipids, proteins). These changes of the biological molecules induce the occurrence of many diseases. (Aruoma *et al*.,2006; Pryor *et al*., 2006; Pham-Huy *et al*., 2008; Sen *et al*., 2010; Sies, 2015).

The epidemiological investigations demonstrate that the oxidative destruction of biomolecules can be reduced by using endogen and exogenous antioxidants such as vitamins C, E, caretenoids, polyphenols, etc. which are contained in fruits and vegetables (Pellegrini *et al*., 2003;2006; Scalbert *et al.*, 2005; Scalzo *et al*., 2005; Valko *et al*., 2006).

Fruit and vegetable rich food reduces the risk of degenerative diseases such as cardiovascular and cancer (Zhang *et al*., 2016). Greece is a country with rich tradition in grape and wine-making. The polyphenols contained in them are characterised by high antioxidant and

health effect (Gougoulias, 2009; Hosu *et al*., 2011; Gougoulias *et al*., 2015).

Phenolic compounds of grapes they contribute to grape and wine organoleptic characteristics. The biosynthesis of polyphenols in grapes is associated with many physiological and biochemical processes, depends from environmental factors and the climatic conditions of plants growth, from cultivation systems, fertilization, irrigation and from variety (Koundouras *et al*., 2009; Mulero *et al*., 2010; Xu *et al*., 2011).

The purpose of the present study is to investigate and compare the content of polyphenols and their antioxidant action of the grape on the basis of the local and European varieties, grown in central Greece.

2. Materials and methods

2.1. Experimental

The experiment was conducted in the non-irrigated vineyard Derkou in 2016 in the area of Flampouro Larissa, Central Greece (latitude 39°58′15″ N, longitude 22°15′10″ E, 510 m altitude). Eleven grapes varieties cultivated in the Farm on an area of 0.3 hectare (about 250m^2 per variety). The Five are varieties local (Roditis, Asyrtiko, Xinomavro, Limniona and Moschomavro) and the six are European varieties (Chardonnay, Macabeo, Merlot, Syrah, Muscat Hambourg and Cabernet Sauvignon). For all grape varieties the planting took place the 2003 with the vineyard desing is 1.5m x 1.5m (plant spacing x row spacing). The region of Flampouro is characterized by a continental climate with cold dry winters and hot summers. Average winter temperature 4.5^{0}C, average summer temperature 25.1 ^{0}C and average annual precipitation 690 mm. 250 kg commercial product Patentkali for fertilizing per hectare were added, corresponding to 75 kg K_2O, 25 kg Mgo and 105 kg SO_3. Also, 150 kg commercial product Yaraliva nitrabor per hectare were added, corresponding to 23 kg Nitrogen, 28.8 kg Calcium and 0.3 kg Boron. The grapes was collected in the phase of technological maturity from which 100 grape berries were randomly selected for chemical analysis.

2.2. Preparation of the ethanol extracts

Twenty g of the grapes samples were two rounds treated by 20 ml of 80% aqueous ethanol. Samples were incubated for 1-hour at stirring in the extractant at dark and room temperature. The extract was gathered after centrifugation and filtration. The pellet was re-treated with aqueous ethanol for 1-hour at stirring at ambient temperature. The extract was gathered after centrifugation/filtration. The collected extracts were brought to 50 ml with aqueous ethanol and used for chemical analysis (Yi *et al*., 1997).

2.3. Methods of analyses

Soil analysis. Soil was analyzed using the following methods which are referred by Page (1982). Organic matter was analyzed by chemical oxidation with 1 mol L^{-1} $K_2Cr_2O_7$ and titration of the remaining reagent with 0.5 mol L^{-1} FeSO4. Soil pH and Electrical conductivity, (EC), measured in the extract (1 part soil : 5 parts H_2O).

Inorganic nitrogen was extracted with 0.5 mol L^{-1} $CaCl_2$ and estimated by distillation in the presence of MgO and Devarda's alloy, respectively. Available P forms (Olsen P) was extracted with 0.5 mol L^{-1} $NaHCO_3$ and measured by spectroscopy. Exchangeable form of potassium was extracted with 1 mol L^{-1} CH_3COONH_4 and measured by flame Photometer (Essex, UK).

Available forms of Mn, Zn, and Cu were extracted with DTPA (diethylene triamine pentaacetic acid 0.005 mol L^{-1} + $CaCl_2$ 0.01 mol L^{-1} + triethanolamine 0.1 mol L^{-1}) and measured by atomic absorption. The samples were analyzed by Atomic Absorption (Spectroscopy Varian Spectra AA 10 plus, Victoria, Australia), with the use of flame and air-acetylene mixture (Varian, 1989).

The pH, the Brix degrees and the total acidity were measured in grape must. The Brix degrees by a Zeiss refract meter, while the total acidity by titration with 0.1N NaOH solution and expressed in g of tartaric acid L^{-1} grape must.

Determination of total polyphenolics (TP). Total polyphenolic content was determined with the Folin-Ciocalteu (F-C)

reagent according to the method by (Singleton and Rossi, 1965) using the microvariant proposed by (Badenschneider *et al*., 2015) and the results were expressed as gallic acid equivalent (GAE) in mg kg^{-1} fresh weight.

Nonflavonoid phenols (NFP). The content of NFP was determined with the F.-C. reagent after removing the flavonoid phenols (FP) with formaldehyde according to the method by (Kramling and Singleton, 1969) and was expressed as gallic acid equivalent (GAE) in mg kg^{-1} fresh weight.

Flavonoid phenols (FP). Flavanoid phenols were determined as a difference between the content of total phenols (TP) and nonflavonoid phenols (NFP). Their amount was evaluated as gallic acid equivalent in mg kg^{-1} fresh weight.

Total flavanols (F-3-ols). The amount of total flavanols (catechins and procyanidins) was determined using p-dimethylaminocinnamaldehyde (p-DMACA) reagent after the method by (Li *et al*., 1996) and was presented as catechin equivalent (CE), in mg kg^{-1} fresh weight.

The anthocyanins (AC) were determined according to the method of (Ribereau-Gayon and Stonestreet, 1965), slightly modified by (Burns *et al*., 2000) by following the change in color at different pH. They were presented as μmol malvidin-3-glicoside (m.-3-.gl) g^{-1} fresh weight.

DPPH• assay. The radical scavenging activities by antioxidants in the grape extracts were evaluated using the stable free radical 2,2′-diphenyl-1-pycrylhydrazyl radical (DPPH•), as a reagent, according to the method by (Brand-Williams *et al*., 1995) and the results were expressed as μmol DPPH• kg^{-1} fresh weight.

Determination of the inhibition coefficient (IC_{50}). The inhibition coefficient (IC_{50}), represents 50% reduction in the colour intensity of the DPPH radical by the total phenols in the studied extracts after plotting the dependence of the TP content on the bleaching of DPPH• solutions. The inhibition coefficient (IC_{50}) was calculated using the following equation:

$$\% \text{ inhibition} = [(E0 - Ex)/E0] \times 100$$

where E0, is the extinction of the radical solution before the reaction and Ex, after polyphenols addition (Yen and Duh, 1994).

Ferric reducing antioxidant power assay (FRAP). The ferric reducing antioxidant power of the grape extracts was evaluated according to the method by (Benzie and Strain, 1999) and the results were expressed as μmol FRAP reagent kg^{-1} fresh weight.

2.4. Statistical analysis

Data were analyzed using the MINITAB (Ryan *et al*., 2005) statistical package. The experiment had four replications. Analysis of variance was used to assess treatment effects. Mean separation was made using Tukey's test when significant differences (P=0.05) between treatments were found.

3. Results and discussions

Table 1 shows chemical properties of the grape must, on the stage of technical maturity. The soil of the experiment for depth 0-30 cm was Sandy Loam (SL), the physicochemical characteristics are presented in Table 2.

Table 1. Chemical properties of the grape must on the stage of technical maturity

Grape varieties	Color	Origin	pH	Brix degrees	Total acidity g tartaric acid L^{-1}
Roditis	white	Greece	3.89 ± 0.18	20 ± 0.9	4.87 ± 0.22
Asyrtiko	white	Greece	3.60 ± 0.17	18.2 ± 0.9	4.65 ± 0.23
Chardonnay	white	France	4.13 ± 0.18	23.7 ± 1.1	2.93 ± 0.16
Macabeo	white	Spain	4.26 ± 0.22	14 ± 0.8	3.08 ± 0.18
Merlot	red	France	4.17 ± 0.25	24 ± 1.1	5.25 ± 0.28
Xinomavro	red	Greece	3.96 ± 0.19	16.2 ± 0.9	5.25 ± 0.27
Syrah	red	France	4.38 ± 0.27	22.2 ± 1.1	3.00 ± 0.16

Limniona	red	Greece	4.03 ± 0.19	20.1 ± 1.2	3.30 ± 0.17
Muscat Hambourg	red	Europe	4.36 ± 0.20	18.5 ± 0.8	3.20 ± 0.16
Cabernet Sauvignon	red	Europe	3.74 ± 0.16	20.5 ± 1.1	4.50 ± 0.26
Moschomavro	red	Greece	3.88 ± 0.17	18.8 ± 0.9	5.10 ± 0.31

Data represent average and SE deviation. (n) = 4

Table 2. Soil chemical properties at the beginning of crops

Soil properties	**Vineyard**
	Soil depth 0-30 cm
Texture	Sandy loam
pH, (1:5)	7.30 ± 0.31
EC, (1:5), dS m^{-1}	0.13 ± 0.01
$CaCO_3$ (%)	0.67 ± 0.02
Organic matter (%)	1.02 ± 0.06
N-inorganic (mg kg^{-1})	46.0 ± 4.53
K-exchangeable (mg kg^{-1})	100.6 ± 5.10
Na-exchangeable (mg kg^{-1})	41.4 ± 1.17
CEC (cmol kg^{-1})	17.0 ± 0.62
P -Olsen (mg kg^{-1})	48.3 ± 3.84
Cu-DTPA (mg kg^{-1})	2.04 ± 0.14
Zn-DTPA (mg kg^{-1})	0.71 ± 0.02
Mn-DTPA (mg kg^{-1})	2.40 ± 0.18
Fe-DTPA (mg kg^{-1})	2.17 ± 0.19

CEC, cation exchange capacity; EC, electrical conductivity; Data represent average and SE deviation. (n)=4

The single extraction of the grapes with 80% ethanol results in the extraction of total phenols for the white grapes varieties which ranges from 278.2 to 474 mg TP (GAE) kg^{-1} FW, while in the grapes of the red varieties ranges from 582.4 and 1813.9 mg GAE kg^{-1} fresh mass (Table 3). Among the grapes studied, with the highest content total phenols is characterized the red variety Merlot with 1813.9 mg GAE kg^{-1} FW, while with the lowest content total phenols is characterized the white variety Macabeo with 278.2 mg GAE kg^{-1} FW.

Our results are in agreement with the data obtained by other authors who have established differences in phenols content in the red and white grape varieties (Cantos *et al*., 2002; Borbalan *et al*., 2003). Most probably, these variations in the polyphenols content, could be explained from the differences in the genetic characteristics of the various varieties. According to some authors, the environmental conditions have a strong effect on the polyphenols biosynthesis, and on their antioxidant capacity (Downey *et al*., 2006; Guidoni *et al*., 2008).

Non-flavonoid phenols (NFP) content in the grapes of white varieties ranges from 97.2 to 156.1 mg GAE kg^{-1} FW, while in the grapes of red varieties ranges from 134.4 to 665.9 mg GAE kg^{-1} FW. Among the grapes studied, with the highest content non-flavonoid phenols is characterized the red grape variety Merlot with 665.9 mg GAE kg^{-1} FW, while with the lowest content non-flavonoid phenols is characterized the white grape variety Asyrtiko with 97.2 mg GAE kg^{-1} FW (Table 3). The comparison of the content of non-flavonoid phenols with respect to the total phenols in the white grape varieties studied shows that they represent 27.5-38.6 % of total phenols, while in the red grape varieties they represent 23.1-39.5% of total phenols. The NFP fraction consists mainly of hydroxybenzoic, coumaric, cinnamic and other acids, and their derivatives (Lorrain *et al*., 2011).

The variation of phenols fractional composition within one and the same variety under the action of climatic factors is observed by many authors (Frankel and Meyer, 1998).

Flavonoid phenols (FP) content in the grapes of white varieties ranges from 170.7 to 337.4 mg GAE kg^{-1} FW, while in the grapes of red varieties ranges from 417.1 to 1148 mg GAE kg^{-1} FW. Among the grapes studied, with the highest content flavonoid phenols is characterized the red grape variety Merlot with 1148 mg GAE kg^{-1} FW, while with the lowest content flavonoid phenols is characterized the white grape variety Macabeo with 170.7 mg GAE kg^{-1} FW (Table 3). The comparison of the content of flavonoid phenols with respect to the total phenols in the white grape varieties studied shows that they represent 61.4-72.5 % of total phenols, while in the red grape varieties they represent 60.5-76.9% of total phenols. Red grapes varieties are characterized by higher content flavonoid phenols in comparison with white grapes varieties (Table 3).

Total flavanols (flavan-3-ols) content in the grapes of white varieties ranges from 43.3 to 117.6 mg CE kg^{-1} FW, while in the grapes of red varieties ranges from 62.8 to 183.7 mg CE kg^{-1} FW (Table 3). Among the grapes studied, with the highest content flavan-3-ols is characterized the red grape variety Merlot with 183.7 mg CE kg^{-1} FW, while with the lowest content flavan-3-ols is characterized the white grape variety Macabeo with 43.3 mg CE kg^{-1} FW. According to some authors, the flavan-3-ols fraction consists mainly of gallocatechin, procyanidin B1, procyanidin B2, procyanidin B4, procyanidin C1, catechin, and epigallocatechin (Cantos *et al*., 2002). According to these authors, flavan-3-ols are the most abundant phenolic compounds in the white grapes varieties, and there are present mainly in the skin of grapes.

Anthocyanins content (equivalent to malvidin-3-glicoside) in the red grapes varieties ranges from 85.6 to 260.1 μmol (m.-3-gl.) g^{-1} FW (Table 3). Among the red grapes varieties studied, with the highest content anthocyanins is characterized the red grape variety Merlot with 260.1 μmol (m.-3-gl.) g^{-1} FW, while with the lowest content anthocyanins is characterized the red grape variety Moschomavro with 85.6 μmol (m.-3-gl.) g^{-1} FW. Anthocyanins are the main phenolic compounds in red grapes (Cantos *et al*., 2002).

Table 3. Content of total phenols, non-flavonoid phenols, flavonoid phenols, total flavanols, and anthocyanins of the grapes

Grape varieties	**TP**	**NFP**	**FP**	**F-3-ols**	**Anthocyanins**
	mg (GAE) kg^{-1} FW			mg(CE) kg^{-1}FW	μmol (m.-3-gl.) g^{-1} FW
Roditis	474.0^{f}	136.6^{ef}	337.4^{e}	117.6^{b}	-
Asyrtiko	353.1^{g}	97.2^{f}	255.9^{f}	66.8^{d}	-
Chardonnay	474.0^{f}	156.1^{de}	317.9^{e}	89.8^{cd}	-
Macabeo	278.2^{h}	107.5^{ef}	170.7^{g}	43.3^{e}	-
Merlot	1813.9^{a}	665.9^{a}	1148^{a}	183.7^{a}	260.1^{a}
Xinomavro	662.0^{d}	197.8^{d}	$464,2^{cd}$	129.2^{b}	86.0^{d}
Syrah	896.1^{c}	353.7^{b}	542.4^{c}	103.1^{bc}	131.8^{b}
Limniona	689.9^{d}	272.8^{c}	417.1^{de}	104.2^{bc}	87.2^{d}
Muscat Hambourg	862.0^{c}	311.2^{bc}	550.8^{c}	77.1^{d}	100.6^{c}
Cabernet Sauvignon	1009.3^{b}	364.2^{b}	645.1^{b}	129.4^{b}	135.8^{b}
Moschomavro	582.4^{e}	134.4^{ef}	448.0^{d}	62.8^{d}	85.6^{d}

TP, total phenols; NFP, non-flavonoid phenols; FP, flavonoid phenols; F-3-ols, total flavanols; GAE, gallic acid equivalent; CE, catechin equivalent; m.-3-gl., malvidin-3-glicoside; For each chemical property, the same letter on the columns of the table do not differ significantly according to the Tukey's test (P=0.05).

The antioxidant activity (FRAP) in the grapes of white varieties studied ranges from 1768 to 2366 μmol FRAP kg^{-1} FW, while in the grapes of red varieties ranges from 2652 to 6612 μmol FRAP kg^{-1} FW (Table 4). Among the grapes studied, with the highest antioxidant activity is characterized the red grape variety Merlot with 6612 μmol FRAP kg^{-1} FW, while with the lowest antioxidant activity is characterized the white grape variety Macabeo with 1768 μmol FRAP kg^{-1} FW (Table 4). The red grapes varieties are characterized by higher antioxidant activity in comparison with white grapes varieties. There is linear correlation between the amount of total phenols and antioxidant activity of the grapes (r^2 =0.8927).

The antiradical activity (DPPH) in the grapes of white varieties studied ranges from 382 to 612 μmol DPPH kg^{-1} FW, while in the grapes of red varieties ranges from 673.1 to 1569 μmol DPPH kg^{-1} FW (Table 4). Among the grapes studied, with the highest antiradical activity is characterized the red grape variety Merlot with 1569 μmol DPPH kg^{-1} FW, while with the lowest antiradical activity is characterized the white grape variety Macabeo with 382 μmol DPPH kg^{-1} FW. The order of antiradical activity (DPPH) of the varieties studied are the following: Merlot > Cabernet Sauvignon > Syrah, Muscat Hambourg, Limniona > Xinomavro, Moschomavro > Roditis, Chardonnay > Asyrtiko and Macabeo (Table 4). The red grapes varieties are characterized by higher antiradical activity in comparison with white grapes varieties. There is linear correlation between the amount of total phenols and antiradical activity of the grapes (r^2 =0.8525).

Our results are in agreement with the data obtained by other authors who have established differences in antioxidant and antiradical activity in the red and white grape varieties (Borbalan *et al*., 2003). The comparative studies of the polyphenols complex of the local and European wine grape varieties from the region of the Flampouro Larissa, Greece confirm the studies of other authors for polyphenols rich in antioxidant and antiradical activity. Polyphenols show a stronger antioxidant effect than vitamins C, E and carotenoids, inhibiting the oxidation of the bad cholesterol and appearance of cardio-vascular and other diseases (Nuttall *et al*., 1999). Also, our results confirm the hypothesis that the polyphenols in the grape and wines from Central Greece due to the antiradical and antioxidant properties, contribute to the reducing many diseases.

The results expressed as IC_{50} are demonstrated in Table 4. The lower IC_{50} values (78 and 91 μg ml^{-1}), which indicated higher antioxidant potential, were observed for the samples from the varieties Limniona and Merlot respectively. The antiradical activity of the ethanol extracts of the investigated grapes from the various varieties determined as the inhibition coefficient (IC_{50}), correlates with TP (Table 4). The antioxidant activity of the extracts is not only due to the monomeric polyphenols contained in the solutions but also in a synergistic action of the polyphenols with other compounds.

Table 4. Antioxidant activity (FRAP) and Antiradical activity (DPPH) of ethanol extracts of the grapes

Grape varieties	**FRAP**	**DPPH**	**IC_{50}**
	μmol kg^{-1} FW		μg ml^{-1} extract
Roditis	2366^{ef}	612^{e}	145^{cd}
Asyrtiko	1966^{f}	460^{f}	200^{e}
Chardonnay	2298^{ef}	602^{e}	164^{d}
Macabeo	1768^{f}	382^{g}	208^{e}
Merlot	6612^{a}	1569^{a}	91^{a}
Xinomavro	2987^{d}	735.7^{d}	124^{b}
Syrah	3450^{c}	819.5^{c}	114^{b}
Limniona	3060^{d}	790.6^{c}	78^{a}

Muscat Hambourg	3403^{c}	810.2^{c}	128^{bc}
Cabernet Sauvignon	4459^{b}	1098^{b}	119^{b}
Moschomavro	2652^{e}	673.1^{de}	140^{c}

For each chemical property, the same letter on the columns of the table do not differ significantly according to the Tukey's test (P=0.05).

4. Conclusions

In the grapes cultivated in the same area and on the same soil in Central Greece, depending on the variety, some differences in the content of the total polyphenols, flavonoid phenols, non-flavonoid phenols, flavan-3-ols and of the antioxidant activity were observed. A strong positive correlation is observed (r^2) between the antioxidant and the antiradical activity of the grapes and of the contents of total phenols.

Our results confirm the hypothesis that the polyphenols in the grape and their products from Central Greece, due to the antioxidant and antiradical properties contribute to the reducing many diseases and are a source of bioactive components that could be included in functional foods composition.

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STUDY THE ANTIOXIDANT ACTIVITIES OF EDIBLE CHITOSAN AND WHEY PROTEIN ISOLATE FILMS CONTAINING *MENTHA PULEGIUMS* ESSENTIAL OIL

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ABSTRACT

Stability, low cost and film forming ability of chitosan which is enriched with presence of essential oil of medicinal plants make it good choice for production of edible film for food products. The present investigation was done to study the antioxidant activities of edible chitosan and whey protein isolate (WPI) films containing Mentha pulegiums essential oil. M. pulegiums plant was collected and its essential oil was extracted using the Clevenger. High molecular weight chitosan was used for film preparation. Whey protein powder and distilled water were used for preparation of the edible WPI film. M. pulegiums essential oil was added to films in concentrations of 0, 0.5, 1 and 2 percent. DPPH radical scavenging effects of films and also their inhibitory activities against oxidation of linoleic acid was studied. BHT had the highest antioxidant effects. We found that increase in the percent of essential oil caused increase in the antioxidant effects of films ($P < 0.05$). Treatment of the 2% M. pulegium chitosan and WPI-based films had the highest DPPH radical scavenging (42.90±4.19 and 40.31±3.62 percent) and also inhibitory effects on oxidation of linoleic acid (84.11±7.71 and 82.92±8.15 percent) ($P < 0.05$). Statistically significant difference was seen between the percent of M. pulegiums essential oil and its antioxidant effects ($P < 0.05$). Production of chitosan and WPI-based films containing the M. pulegiums essential oil at concentration of 2% is applicable approach to increase the shelf life of various types of food products.

1.Introduction

Packaging is a best method to decrease the risk of microbial and also chemical spoilage in food products. It is used for preparation of the optimal moisture and atmosphere of the food product and prevent from the entry of foreign materials and therefore increased the survival of food products in their best condition (Claudio, 2012; Vilarinho, Sanches-Silva, Vaz, & Farinha, 2016). However, according to the environmental problems caused by synthetic packaging material, the food industry has paid rising consideration to biopolymer and edible films (Claudio, 2012; Vilarinho et al., 2016).

Chitosan, deacetylated derivative of chitin, is the second most abundant polysaccharide after cellulose (Altiok, Altiok, & Tihminlioglu, 2010; Zimoch-Korzycka, Bobak, & Jarmoluk, 2016). Its low cost and also good film-forming ability have made it good-looking for active food packaging (Altiok et al., 2010; Zimoch-Korzycka et al., 2016). It is also a source of antioxidant and antimicrobial components which mainly can increase the quality of food

packaging (Altiok et al., 2010; Zimoch-Korzycka et al., 2016). To improve the shelf life of food products, chitosan-based films and packages have been enriched with natural antimicrobial and antioxidant components like plant extracts and essential oils as an alternative for synthetic compounds (Altiok et al., 2010; Zimoch-Korzycka et al., 2016).

Among essential oils, the preponderance of reports on effective antioxidant properties is directed toward extracts from plants in the Lamiaceae family and especially *Menta Pulegium* (*M. pulegium*) (Kamkar, Javan, Asadi, & Kamalinejad, 2010; Mikaili, Mojaverrostami, Moloudizargari, & Aghajanshakeri, 2013; Shahmohamadi, Sariri, Rasa, & Aghamali, 2014). *M. pulegium* L. or mint or oregano is a plant of Lamiaceae or Labiatae family which can be found throughout the world. *M. pulegium* is one of Menta species which is typically called Pennyroyal. It is a herb native to Europe, north Africa, minor Asia and the Middle-East especially in damp and wet places (Kamkar et al., 2010; Mikaili et al., 2013; Shahmohamadi et al., 2014). *M. pulegium* is traditionally used in the treatment of colds, sinusitis, cholera, food poisoning, bronchitis and tuberculosis (Kamkar et al., 2010; Mikaili et al., 2013; Shahmohamadi et al., 2014). Phenolic acids, flavonoids, terpenoids and volatile compounds have been identified as the major compounds found in different extracts of mint (Kamkar et al., 2010; Mikaili et al., 2013; Shahmohamadi et al., 2014). It is also full from antioxidant and antimicrobial components (Kamkar et al., 2010; Mikaili et al., 2013; Shahmohamadi et al., 2014). Its antimicrobial and antioxidant effects make it suitable for production of edible films and also packaging for food products.

In despite of the high antioxidant effects of chitosan and *M. pulegium* essential oil, there were no previously published data on their application as an edible film. Therefore, the present investigation was done in order to study the antioxidant activities of edible chitosan film containing *M. pulegiums* essential oil in Iran.

2.Materials and methods

2.1.Plants

Aerial parts of the *M. pulegium* plant was collected from the height of the Shahmirzad region in Semnan province, Iran. Plants were immediately transferred to the Food Hygiene and Quality Control Research Center of the Faculty of Veterinary Medicine, University of Tehran. Exact species of collected plants were identified by an expert person in the field of Medicinal Plants in the University of Tehran. The collected plant was dried at ambient conditions for 10 days. The dried plant was used to extract the essential oil.

2.2.Extraction of essential oil

Dried *M. pulegium* was milled using a crusher. The essential oil of *M. pulegium* was extracted by hydrodistillation in Clevenger apparatus for 4 h. The essential oil to dry weight ratio was 0.67% w/w. The extracted essential oil was stored in colored glass at 4° C.

2.3.Preparation of chitosan-based edible film

Chitosan-based film was prepared by dissolving high molecular weight chitosan (Fluka, Sigma-Aldrich, St. Louis MO, USA) in an aqueous solution (1% v/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50 ˚C) which typically required 3 h stirring. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper and followed by vacuum filtration to eliminate insolubles and remove any undissolved particles. Film preparation was done based on the molding method (Moradi, Tajik, Razavi Rohani, & Oromiehie, 2011). Treatments of the *M. pulegium* were added to the chitosan-based films in concentrations of 0, 0.5, 1 and 2 percent. Solution was then mixed using the homogenizer device in 13000 rpm for 1 min. Jars of glass with a surface of 8 cm^2 were used for this purpose. Glass jars were kept at a perfectly smooth surface at room temperature for 36 h. After complete drying, films were kept at a constant condition (50±2%

moisture and 25±2 ˚C) in desiccator containing a saturated solution of sodium bromide (Merck, Germany) for 48 h.

2.4.Preparation of whey protein isolate film

Whey Protein Isolate (WPI) and glycerol at 5 wt% each were dissolved in DD water (90 wt%) with continuous stirring to obtain a film-forming solution. The pH of the film forming solution was adjusted to 8.0 with 2 M NaOH. Then, the solution was heated to 90 ± 2 °C for 30 min in a water bath while being stirred continuously, and rapidly cooled in an ice bath for 10–15 min to avoid further denaturation. The solution was then filtered through two layers of muslin cloth to remove any coagulation. The filtered WPI solution was subjected to ultrasonication levels of 0%, 10%, 50% and 100% sonication (i.e. 0, 16, 80 and 160 μm amplitudes), denoted as A, B, C and D respectively. The film forming solutions of 10, 15 and 20 g were cast on sterile polystyrene petri dishes (Fisher brand, USA). These were dried at 35 ± 1 °C for 24 h in a hot air dryer having 24% relative humidity (RH), then kept in a 50% ± 2% RH chamber for at least 24 h before the films were peeled and tested at room temperature (24 ± 1 °C).

2.5.Antioxidant effects of films

The antioxidant activity of the film samples was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. Briefly, 3 mL of film extract solution were mixed with 1mL of 1 mM methanolic solution of DPPH (Merck, Darmstadt, Germany). The mixture was vortexed and incubated in the dark at ambient temperature for 30 min. When the DPPH solution was mixed with the sample mixture acting as a hydrogen atom donor, a stable non radical form of DPPH is obtained with simultaneous change of the violet color to pale yellow. The absorbance was then measured at 517 nm. The percentage of DPPH free radical quenching activity was determined using the following equation:

$$\text{Percent of the DPPH scavenging effect} = \frac{Abs\ of\ DPPH - Abs\ of\ extract}{Abs\ of\ DPPH} \times 100$$

Where Abs DPPH is the absorbance value at 517 nm of the methanolic solution of DPPH and Abs extract is the absorbance value at 517 nm for the sample extracts. DPPH radical scavenging of films was also compared with the BHT synthetic antioxidant.

2.6.β-carotene-linoleic acid test

Antioxidant activity of the film samples was determined using the β-carotene-linoleic acid test (Taga, Miller, & Pratt, 1984). Approximately 10 mg of β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in chloroform (10 mL). The carotene–chloroform solution, (0.2 mL) was pipetted into a boiling flask containing linoleic acid (20 mg, Sigma–Aldrich) and 200 mg Tween® 40 (Sigma–Aldrich). Chloroform was removed using a rotary evaporator (RE-52AA) at 40 °C for 5 min, and distilled water (50 mL) was added to the residue slowly with vigorous agitation, to form an emulsion. A portion of the emulsion (5 mL) was added to a tube containing the sample solution of the film containging various concentrations of *M. pulegium* (0.2 mL) and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C and the oxidation of the emulsion was monitored spectrophoto-metrically by measuring absorbance at 470 nm over a 60 min period. Control samples contained 200 μL of water instead. Butylated hydroxytoluene (BHT, Sigma–Aldrich), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation: AA = 100(DRC -DRS)/DRC, where AA = antioxidant activity; DRC = degradation rate of the control = [ln(a/b)/60]; DRS = degradation rate in presence of the sample = [ln(a/b)/60]; a

= absorbance at time 0; b = absorbance at 60 min.

2.7.Statistical analysis

All data were presented as mean ± standard deviation (SD). SPSS. Ver. 20 was used for statistical analysis. Comparison of means were studied using the one-way ANOVA and also post hoc turkey test. *P* value ≤0.05 was determined as significant level. All tests were done 3 times.

3.Results and discussions

3.1.Results

We found that all of the chitosan-based films containing the *M. pulegium* essential oil had a considerable antioxidant effects. Among chitosan-based films, treatment of the 2% *M. pulegium* had the highest DPPH radical scavenging effects (42.90±4.19 percent) (*P* <0.05). Among WPI-based films, treatment of the 2% *M. pulegium* had the highest DPPH radical scavenging effects (40.31±3.62 percent) (*P* <0.05). DPPH radical scavenging effects of the chitosan-based films were entirely higher than WPI-based film, while there were no statistically significant differences between them except for treatments of 0.5% *M. pulegium*, (Table 1).

Table 1. Percent of the DPPH radical scavenging of the chitosan and WPI-based films containing the *M. pulegium* essential oil.

Treatments		Percent of the DPPH radical scavenging
BHT		87.17±7.25[a*]
Chitosan-based film	0% *M. pulegium*	4.73±0.38[d]
	0.5% *M. pulegium*	36.77±3.41[b]
	1% *M. pulegium*	38.16±3.67[b]
	2% *M. pulegium*	42.90±4.19[b]
WPI-based film	0% *M. pulegium*	4.22±0.28[d]
	0.5% *M. pulegium*	31.16±2.73[c]
	1% *M. pulegium*	34.27±3.11[b]
	2% *M. pulegium*	40.31±3.62[b]

*Dissimilar letters in each column shows significant differences less than 0.05.

Table 2. Percent of the inhibition of the oxidation of linoleic acid of the chitosan and WPI-based films containing the *M. pulegium* essential oil.

Treatments		Percent of the inhibition of the oxidation of linoleic acid
BHT		87.50±6.33[a*]
Chitosan-based film	0% *M. pulegium*	38.23±3.08[c]
	0.5% *M. pulegium*	47.42±4.18[c]
	1% *M. pulegium*	81.43±6.94[b]
	2% *M. pulegium*	84.11±7.71[b]
WPI-based film	0% *M. pulegium*	36.67±3.07[c]
	0.5% *M. pulegium*	40.23±3.57[c]
	1% *M. pulegium*	79.24±7.45[b]
	2% *M. pulegium*	82.92±8.15[b]

*Dissimilar letters in each column shows significant differences less than 0.05.

Table 2 represents the percentage of inhibition of the oxidation of linoleic acid in various treatments of chitosan-based and WPI-based films containing various concentration of *M. pulegium*. We found that BHT had the highest antioxidant effects against oxidation of linoleic acid (87.50±6.33 percent). Among chitosan-based films, treatment of the 2% *M. pulegium* had the highest inhibitory effects on oxidation of linoleic acid (84.11±7.71 percent) (P <0.05). Among WPI-based films, treatment of the 2% *M. pulegium* had the highest inhibitory effects on oxidation of linoleic acid (82.92±8.15 percent) (P <0.05). Oxidation inhibitory effects of the linoleic acid of the chitosan-based films were entirely higher than WPI-based film, while there were no statistically significant differences between different treatments (P >0.05).

3.2.Discussions

The present study showed that chitosan and WPI-based edible films containing the *M. pulegium* essential oil had a considerable DPPH radical scavenging effects and also inhibitory effects on the oxidation of linoleic acid. Increase in the concentration of *M. pulegium* essential oil caused increase in the beneficial effects of films. However, their radical scavenging effects and also inhibitory effects on the oxidation of linoleic acid of chitosan-based films were lower than BHT in all treatments. Possible explanations for the high antioxidant effects of chitosan and WPI-based edible films containing the *M. pulegium* essential oil are maybe presence of antioxidant components such as phenolic and flavonoid materials in the essential oil of the *M. pulegium*. Phenolic and flavonoid compounds have been shown to be responsible for the antioxidant activity of plant materials (Adebayo, Dzoyem, Shai, & Eloff, 2015; Khodaie, Bamdad, Delazar, & Nazemiyeh, 2012; Mahboubi & Haghi, 2008).

Several investigations have been focused on the determination of the antioxidant effects of chitosan and WPI-based edible films (Bonilla, Atarés, Vargas, & Chiralt, 2012; Han & Krochta, 2007; López-Mata et al., 2015; Mehdizadeh, Tajik, Rohani, & Oromiehie, 2012; Min & Krochta, 2007; Oussalah, Caillet, Salmiéri, Saucier, & Lacroix, 2004; Wang et al., 2015).

López-Mata et al. (2015) reported a significant increase in the antioxidant capacity of the chitosan films containing cinnamon bark oil at 0.5% (6.01 times) and 1.0% (14.5 times) concentrations. They showed that increase in the concentration of the cinnamon bark oil caused increase in the antioxidant activities of chitosan films which was similar to our findings. Mehdizadeh et al. (2012) showed that DPPH radical scavenging activity of the starch-chitosan films significantly was increased ($P \leq$ 0.05) with increasing the concentration of *Thymus kotschyanus* which was also similar to our findings (Mehdizadeh et al., 2012). DPPH scavenging assay was used to indicate antioxidant activity of the film. This assay was based on the ability of DPPH, a stable free radical, to be quenched and thereby decolorize in the presence of antioxidants resulting in a reduction in absorbance values (Siripatrawan & Harte, 2010). In a study which was conducted by Wang et al. (2015), antioxidant activity and physicochemical properties of chitosan films incorporated with *Lycium barbarum* fruit extract for active food packaging was studied (Wang et al., 2015). They showed that DPPH radical scavenging effect of the chitosan films incorporated with *L. barbarum* fruit increased near ten-folds and reached up to 35.8%. Hafsa et al. (2016) indicated that chitosan films containing *Eucalyptus globulus* essential oil had considerable inhibitory effects on the DPPH free radicals (Hafsa et al., 2016). Similar findings were also reported by Genskowasky et al. (2015) and Bonilla et al. (2013) (Bonilla, Talón, Atarés, Vargas, & Chiralt, 2013; Genskowsky et al., 2015).

Oliveira et al. (2017) reported the considerable antioxidant and antimicrobial effects of the whey protein-based films incorporated with oregano essential oil (Oliveira, Bertan, De Rensis, Bilck, & Vianna,

2017). Min and Krochta (2007) showed that the WPI films reduced lipid oxidation in peanuts during early storage at 23 °C and throughout the storage at 50 °C (Min & Krochta, 2007). The peroxide values of WPI-coated peanuts at 35 °C for 70 days were >30 which showed its high antioxidant effects. Oussalah et al. (2004) reported the high antioxidant effects of the milk protein-based film containing essential oils for the preservation of whole beef muscle (Oussalah et al., 2004). They showed that the milk protein-based film cause reduction in the levels of thiobarbituric and increase in the levels of total phenolic components. They showed that milk protein-based film had a high antiradical effects.

4.Conclusions

In conclusion, using from chitosan and WPI-based films containing 2% *M. pulegium* essential oil had the best antioxidant effects on DPPH free radicals and also inhibitor effects of the oxidation of linoleic acid. Chitosan-based film had the higher antioxidant effects than WPI-based one. An antioxidant chitosan composite film incorporated with *M. pulegium* essential oil is promising and has decent potential to enhance the safety of foods and food products. Future research could be conducted to evaluate the sensory aspects of prepared films using these natural essential oil compounds, as well as to characterize their stability and other physico-mechanical properties. Moreover, the anti-microbial effect of chitosan and WPI-based films incorporated with *M. pulegium* essential oil should be determined on an entire model food.

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