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ROSEMARY, THYME AND OREGANO ESSENTIAL OILS INFLUENCE ON PHYSICOCHEMICAL PROPERTIES AND MICROBIOLOGICAL STABILITY OF MINCED MEAT

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ABSTRACT

In this paper we have analysed the capacity of essential oils from aromatic plants in order to slow or stop down the physicochemical degradation and microbiological infestation of raw minced meat processes. Three essential oils: thyme, rosemary and oregano were chosen for the experimental study due to their acknowledged antiseptic effect and their flavour acceptance by consumers. The physicochemical and microbiological properties for a mixture of pork and beef meat were analysed throughout four days. Water content, total fat, total protein, easily hydrolysable nitrogen, pH and water activity were determined. The experimental measurements were performed considering the following concentrations: 0.5, 1.0 and 1.5% for the essential oil incorporated into meat. The evaluation of microbiological stability was determined as well. The results indicated that essential oils have a significant influence on the physicochemical properties correlated with microbiological stability when prolonging the shelf - life of minced meat.

Keywords: Essential oils, microbiological stability, minced meat, physicochemical analysis, shelf – life

INTRODUCTION

Alteration of meat consists in a set of processes that affect the substrate, leading to formation of low molecular weight compounds in conjunction with the development of microbial associations. The consequences are the modification on sensory properties, on nutritional quality and advanced alteration conducting to a negative impact of the product on consumer`s health. Different oils from aromatic plants can have preserving effect on meat. Antimicrobial effect of essential oils is acknowledged due to their hydrophobicity, being capable of damaging the cell wall of microorganisms, disturbing the metabolism of phospholipids from cytoplasmic membrane, damaging cell membrane proteins and increasing the permeability of cell wall, thus leading to loss of the liquid constituents of the cell (**Burt 2004; Bakkali *et al.*, 2008; Bassolé and Juliani 2012**). The antimicrobial activity of essential oils may be related to more than one component. It is considered that the strong character of antibacterial essential oils against food borne pathogens is due to their rich content in phenolic compounds such as: carvacrol, eugenol (2-methoxy-4- (2-propenyl) phenol) and thymol (**Faleiro *et al.*, 2003; Burt 2004; Erkan *et al.*, 2011a; Faleiro 2011; Proestos *et al.*, 2013a**). Phenolic compounds from plants contain the highest antioxidant potential, being able to decrease the local concentration of oxygen, form chelates with transition metals, inhibit the initialization of oxidative processes chain by free radicals. In addition they are capable for regenerating endogenous α-tocopherol in lipoprotein layer from tissues (**Kähkönen *et al.*, 1999; Frankel and Meyer 2000; Rocha-Guzmán *et al.*, 2007; Su *et al.*, 2014**). The preserving effect of oils from aromatic plants such as rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) was analysed in this paper. The synergic action of thymol and carvacrol in the essential oils of thyme and oregano inhibits the growth of several bacteria strains, e.g. *Escherichia coli, Bacillus cereus, Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas Aeruginosa* (**Helander *et al.*, 1998; Burt 2004; Cox and Markham 2007; Roldán *et al.*, 2010; Erkan *et al.*, 2011b; Bassolé and Juliani 2012; Erkan 2012**). Compounds such as carvacrol and thymol get the major antioxidant capacity from their essential oils (**Kulisic *et al.*, 2004; Erkan and Bilen 2010; Chatli and Joseph 2014**). Pork and beef meat added with 3% oregano essential oil showed lower levels of oxidation after 12 days of refrigerated storage (**Fasseas *et al.*, 2008**). Oregano essential oil (0.05%, 0.5% and 1%) could delay the growth of microorganisms and decrease the final counts of spoilage microorganisms (**Skandamis and Nychas 2001; Zhang *et al.*, 2010; Zhou *et al.*, 2010**). The effect of these oil compounds on food has become much more important since their antimutagenic, anti-inflammatory anti-carcinogenic, anti-haemolytic and immuno-stimulating effect was demonstrated (**Barnes *et al.*, 2005; Bakkali *et al.*, 2008; Chao *et al.*, 2008; Dung *et al.*, 2009; Ocaña-Fuentes 2012; Ozkan and Erdoğan 2011; Proestos *et al.*, 2013b; Ramchoun *et al.*, 2015; Sá *et al.*, 2014; Sivasothy *et al.*, 2012; Wojdyło *et al.*, 2007**).
The purpose of our study is to evaluate the possibility of extending shelf - life of minced meat with rosemary, thyme and oregano essential oils additions, under safety conditions by maintaining nutritional value and sensory properties without bringing any chemical or microbiological threat to consumer`s health.

MATERIAL AND METHODS

Samples

The matrix chosen was purchased from a local market from Romania, consisted in a minced meat (equal mixture of beef and pork), intended to immediate consumption and stored at 4°C. The whole amount was mixed and homogenized, and then the batch of minced meat was divided into ten portions. A portion was kept as a control sample and each of the other portions were further mixed with an appropriate volume of rosemary, thyme and oregano essential oil (final concentrations 0; 1 and 1.5% v/w). The samples were placed into 50 plastic containers, closed and stored at 4°C. All physicochemical and microbiological determinations were performed at equal time intervals over a period of 4 days, in compliance with the Romanian standards.

Reagents

All the reagents used were purchased from Sigma - Aldrich and they were of pro analysis purity: 2, 2'-diphenyl-1picrylhydrazyl (DPPH), ethanol, L(+)-ascorbic acid, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, quarter-strength Ringer's solution, peptone, meat extract, yeast extract, D(+) glucose, dipotassium hydrogen phosphat, sodium acetate trihydrate, triamonium citrate, magnesium sulphate heptahydrate, manganous sulphate tetrahydrate, malt extract, peptone, maltose, dextrin, glycerol, monopotassium phosphate, ammonium chloride, petroleum ether, H_2SO_4, NaOH, Tashiro indicator, MgO and paraffin oil.

Oils Analysis

Ten grams of rosemary, thyme and oregano essential oils were sterilized at 121°C for 15 min (Autoclave Raypa). The spectrophotometric assay was used for the analysis of the following properties of essential oils: antioxidant activity with 2, 2'-diphenyl-1picrylhydrazyl (DPPH) radical scavenging considering the method described by **Brand-Williams *et al.* (1995)** (UV VIS - NIR Spectrometer - Shimadzu, 3600, Japan) and total polyphenol content with Folin-Ciocalteu method (spectrophotometer Shimadzu UV-2550, Japan) by **Fujita *et al.* (2012)**.

Microbiological Analysis

Samples (10g) of minced meat were aseptically weighed, added to sterile quarter-strength Ringer's solution (90 ml) and homogenized in a stomacher (Lab Blender - BagMixer 400 P, INTERSCIENCE, France) for 60 s at room temperature. Decimal dilutions in quarter-strength Ringer's solution were prepared and duplicate 1 ml samples of appropriate dilutions were poured or spread on the following media made from basic ingredients considering the method described by **Tofan *et al.* (2002)**: MRS for lactic acid bacteria incubated at 35°C for 72 h and MMA for yeasts and moulds incubated at 30°C for 72 h. The number of colony forming units was obtained by reading at Colony Star (Funke Gerber, Germany) and the data (growth counts) were transformed to $\log_{10}$ values.

pH and Water Activity

The pH value was recorded by a pH meter (HQ11d pH by HACH), the glass electrode being immersed in the homogenate of minced meat at the end of microbiological analysis, according to SR ISO 2917:2007. The water activity (a_w) value was determined by a water activity meter (AquaLab LITE by Decagon, USA).

Physicochemical Analysis

The water content was determined by using gravimetric method according to SR ISO 1442:2010 and Soxhlet method was applied to establish the total fat content (Soxhlet Extractors - SER 148, VELP SCIENTIFICA, Italy), considering **SR ISO 1443:2008**. We determined total proteins by using Kjeldahl analysis method **(SR ISO 937:2007)**, with the following steps: digestion (mineralization) of organic substances in acidic medium (Kjeldahl Digestion Units - DK 6 VELP Scientifica, Italy), distillation and capturing nitrogen in a solution of 0.1N H_2SO_4 (Kjeldahl Distillation Units - UDK 129, VELP Scientifica, Italy), titration of 0.1N H_2SO_4 excess with 0.05N NaOH solution in the presence of Tashiro indicator. The determination of easily hydrolysable nitrogen (according to **SR 9065-7:2007**) was performed based on the following operations: distillation in aqueous solution (in the presence of MgO and paraffin oil) distillate capture in measured volume of 0.1N H_2SO_4 solution and final titration of the excess acid with 0.1N NaOH solution.

RESULTS AND DISCUSSION

The properties of essential oils

The DPPH method was used to evaluate the antioxidant capacity of basil, rosemary and thyme essential oils in comparison with known synthetic antioxidant L(+)-ascorbic acid. The disappearance of the DPPH radical based on the absorbance at 518 nm wavelength can be monitored by decreasing of optical density. Based on their antioxidant capacity, spice essential oils can be sorted in descending order: thyme (*Thymus vulgaris* L.) > oregano (*Origanum vulgare* L.) > rosemary (*Rosmarinus officinalis* L.). The DPPH radical scavenging activities of all the essential oils (oregano, rosemary and thyme) increased with the increasing of concentration.

Antioxidant capacities in concentrations series of oregano, rosemary and thyme essential oil and ascorbic acid were used to calculate the effective relative concentration EC_{50}. The concentration of essential oils that caused 50% neutralization of DPPH radicals (EC_{50} values), were calculated from the plot of inhibition percentage against concentration. A higher DPPH radical scavenging activity was associated with a lower EC_{50} value. EC_{50} values of scavenging DPPH radicals for rosemary, oregano and thyme essential oils were, 9.423 ± 0.06 µg/mL, 7.258 ± 0.02 µg/mL and 3.067 ± 0.09 µg/mL, respectively, while ascorbic acid registered an EC_{50} of 161.578 ± 0.03 µg/mL.

The results are consistent with previous reported data on antioxidant activity of essential oils of oregano (**Lagouri *et al.* 1993; Özcan 1999; Proestos *et al.* 2013b**), rosemary (**Hussain *et al.* 2010; Kadri *et al.* 2011**) and thyme (**Kähkönen *et al.* 1999; Juki and Milo 2005**) respectively. EC_{50} values generally vary considerably among studies, fact that can be explained by different chemical compositions of essential oils of oregano, rosemary and thyme, due to different environmental and genetic factors, different chemotypes and the nutritional status of the plants.

Thyme essential oil analyses by gas chromatography coupled with mass spectrometry (GC-MS) have revealed a significant quantitative differences between the oils (due to genotopic and environmental differences, namely, climate, location, temperature, fertility, diseases and pest exposure) within species (**Wojdyło *et al.* 2007**) for seven compounds: the alcohols α-terpineol and linalool, the two isomeric phenols carvacrol and thymol, their precursor α-terpinene, *p*-cymene and α-pinene. The concentration of these components varied greatly among the oils examined, in particularly that of carvacrol (from 2.8 to 20.6%), *p*-cymene (from 4.1 to 27.6%) and α-pinene (from 0.8 to 25.2%). Cosentino and others (1999) observed that the sum of the two phenols and their precursors constituted the bulk of each essential oil are: 53.6%, 74.9%, 86.9% and 77.3% of the total oil, respectively. Strong antioxidant activity is attributed to carvacrol and thymol (**Sacchetti *et al.* 2005**). Oregano oil has a high content of phenolic monoterpenes (35.0% thymol and carvacrol 32.0%) and monoterpenic hydrocarbons (10.5 % γ-terpinene, 9.1 % *p*-cymene, 3.6% α-terpinene) with a strong antioxidant (**Kulisic *et al.* 2004; Rocha-Guzmán *et al.* 2007**) and antimicrobial character (**Burt 2004**), more than 48 compounds being chromatographically identified (**Liolios *et al.* 2009**). Rosemary contains a number of phytochemicals, including rosmarinic acid, camphor, caffeic acid, ursolic acid and betulinic acid (**Keokamnerd *et al.* 2008**), the high levels of phenolic compounds leading to its great antioxidant activity. The antioxidant activity of rosemary extract and its constituents, carnosic acid, carnosol and rosmarinic acid is stronger than α-tocopherol, butylated hydroxyltoluene (BHT) or butylated hydroxylanisole (BHA) (**Özcan 1999; Almeida-Doria and Regitano- D'arce 2000**).

Total phenol content was calculated from the standard curve of gallic acid ($y=0.27x-0.022$, $r^2=0.9929$) and expressed as µg/mL samples and its values are: 33.12 µg/mL for rosemary oil, *27.58* µg/mL for thyme oil and *23.78* µg/mL for oregano oil.

The essential oils influence on microbiological stability

Plant extracts, essential oils and edible film coating treatments are proven to extend the shelf life of seafood by the use of natural sources. The potential effects of these treatments are delayed lipid oxidation, inhibited microbial growth and enhanced sensorial properties. Due to their antimicrobial and antioxidant properties, plant extracts and essential oils are promising their use instead of synthetic chemicals (**Aymerich *et al.* 2008; Karre *et al.* 2013; Erkan *et al.* 2015**). Contaminating bacteria are those specific to meat microbiota, such as Gram positive bacteria (*Micrococcus, Corynebacterium*) and Gram negative (*Acinetobacter*, *Flavobacterium*) bacteria prevail (**Banu *et al.* 2006**).

Lactic bacteria are the most resistant Gram-positive bacteria, they can be prevalent in the whole microbiota, without being affected by storage conditions (microaerophilic conditions). They are resistant to salt (4-7%) and sodium nitrite, being capable of growing at low temperatures in vacuum sealed packaging. Although lactic acid bacteria of *Lactobacillus*, *Streptococcus* and *Leuconostoc* species, are competitive with other spoilage bacteria which they inhibit by antagonism, still their presence is undesirable because they cause meat colour change (turning green) and their presence in high concentrations indicates failure to comply with hygiene conditions, before and after processing (**Banu *et al.* 2006**).

Gram positive bacteria are more sensitive to antimicrobial compounds from essential oils than Gram-negative bacteria. Fungi appear to be more sensitive than Gram negative bacteria, although there are very sensitive Gram-negative bacteria (**Aymerich *et al.* 2008; Nicolau 2006**).

The contribution of lactic acid bacteria to final flora dependents on types and levels of essential oil (Figure 1). It is necessary to mention that the rate of growth and final counts were affected by the addition of essential oil (Figures 1 and 2). The control samples showed the highest total viable counts of lactic acid bacteria and yeast and mould counts respectively, as compared to others containing rosemary, oregano and thyme essential oils with different concentrations. The relatively high initial counts of control samples may be attributed to the grinding process that introduced the microorganisms inside the meat and leads to the increase of total viable counts of meat. Lactic acid bacteria, yeasts and mould counts were gradually increased during cold storage in all samples with different ratios depending on the concentration of oil. Similar studies on the antimicrobial activity of essential oils were performed by **Salem *et al.* (2010)** and **Erkan and Bilen (2010).**

Hać-Szymańczuk *et al.* (2011), have suggested that rosemary preparations did not exhibit either antibacterial properties against aerobic mesophilic or psychrophilic bacteria. The essential rosemary oil was observed to inhibit the growth of coliform bacteria and enterococci, whereas the dried spice examined was found to increase the counts of aerobic mesophilic bacteria, coliforms and enterococci.

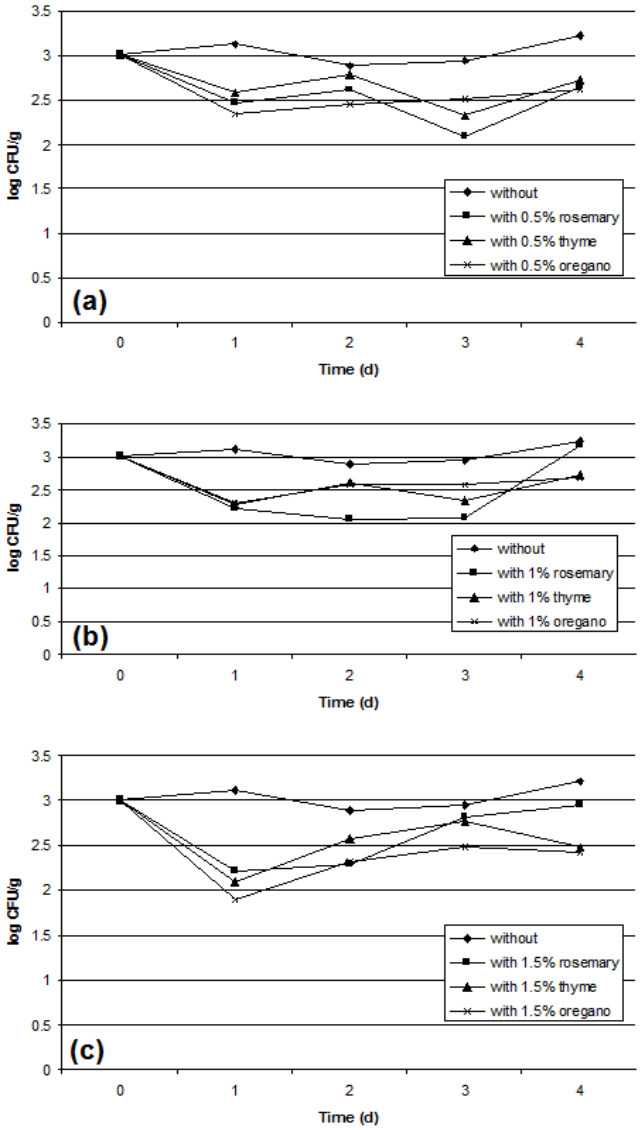

Figure 1 Changes on total viable count of lactic acid bacteria in minced meat without or with rosemary, thyme and oregano essential oils addition (concentration of: **(a)** 0.5%, **(b)** 1% and **(c)** 1.5%)

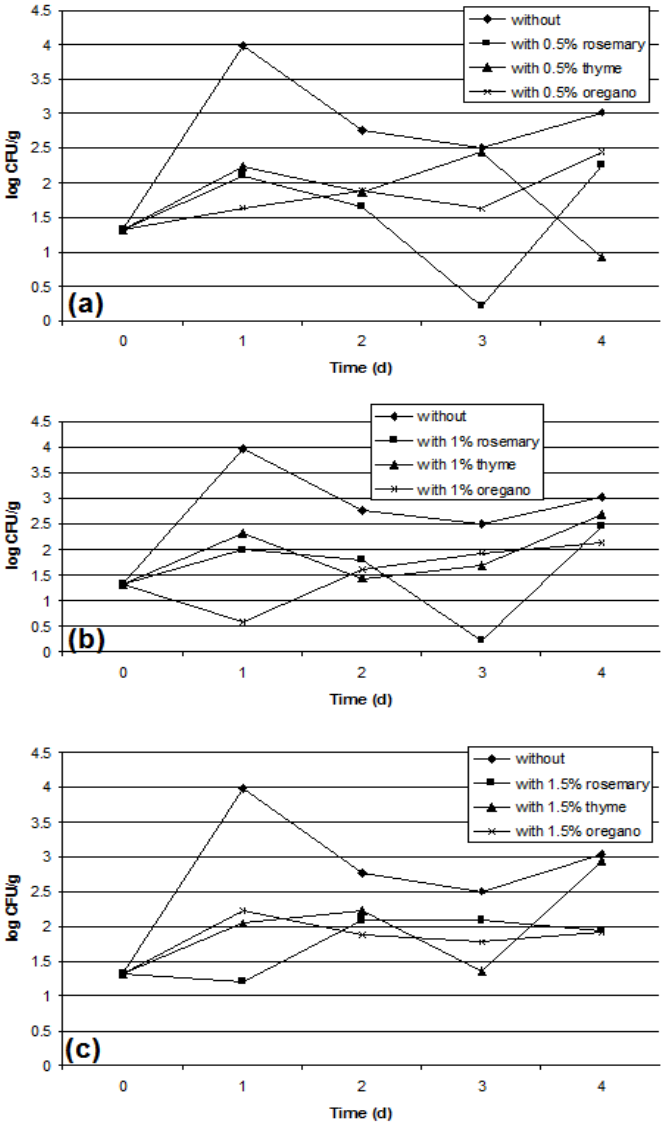

Figure 2 - Changes of yeast and mold counts in minced meat without or with rosemary, thyme and oregano essential oils addition (concentration of: **(a)** 0.5%, **(b)** 1% and **(c)** 1.5%)

Water Activity

The water activity value of minced meat ranged between 0.83 - 0.92, during storage for 5 days at 4°C, Figure 3. The addition of essential oils increased the water activity of minced meat because the water vapour transfer generally occurs through the hydrophilic portion of the film; thus, water vapour permeability depends on the hydrophilic-hydrophobic ratio of the film components from the surface of meat. Water vapour permeability increases with polarity, unsaturation and branching degree of the lipids, depending also on water absorption properties of the polar part of the film and due to the formation of chemical compounds of degradation by lipid oxidation and protein hydrolysis during storage.

Phenolic compounds containing alcohol groups in there chemical structure seems to be a good barrier as compared to aldehyde compounds (e.g., cinnamaldehyde, citral) as hydroxyl group has less affinity for water than for carbonyl groups. All the values obtained for minced meat with rosemary, thyme and oregano essential oil additions (concentration of: 0.5%, 1% and 1.5%) stored at 4°C for 4 days were between 0.88 and 0.97 (**Kingchaiyaphum and Rachtanapun 2012; El Adab *et al*. 2015**).

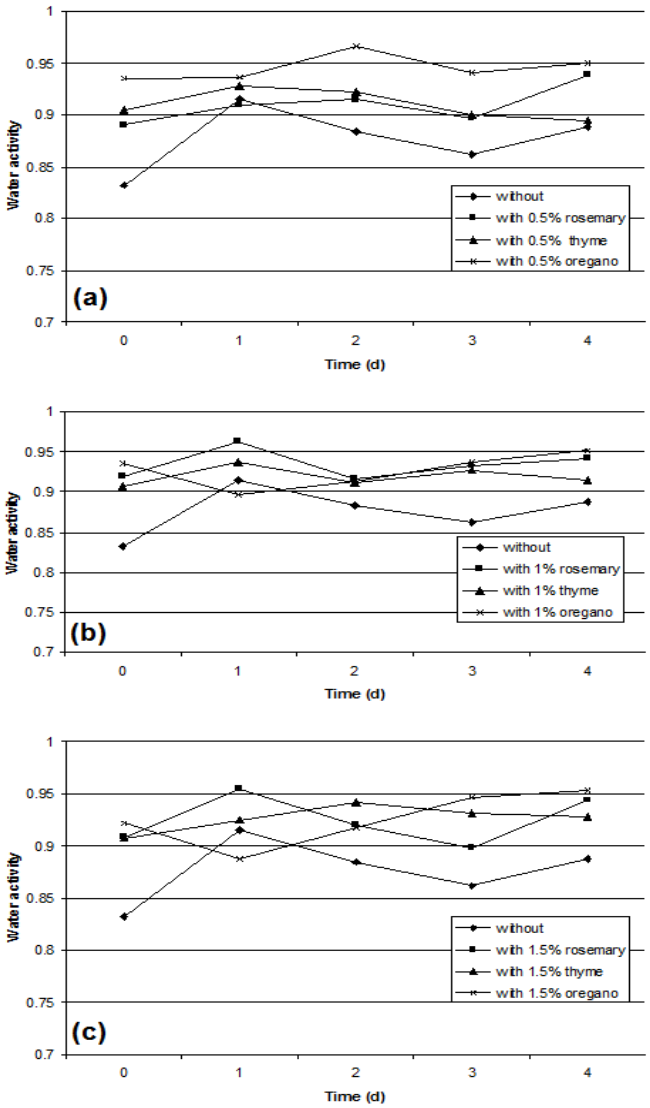

Figure 3 Water activity (a_w) in minced meat without or with rosemary, thyme and oregano essential oils (concentration of: **(a)** 0.5%, **(b)** 1% and **(c)** 1.5%) stored at 4°C for 4 days

pH and physicochemical properties of minced meat

Figure 4 illustrate the maximum range in which the values for the five groups of parameters studied differ in terms of their stability. If the values of moisture, total protein and total fat do not vary significantly in the case of easily hydrolysable nitrogen and pH, an increase in the minimum, maximum and average values can be notice.

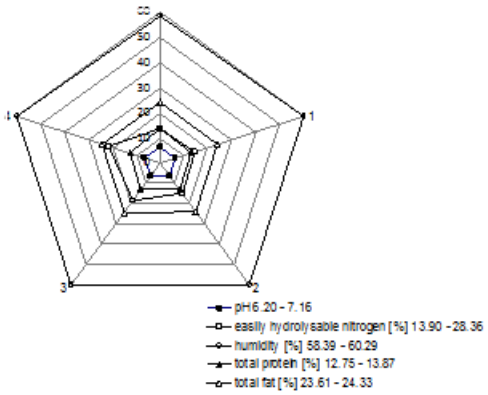

Figure 4 Graphical representation of descriptive statistics for the values of the following parameters: pH, easily hydrolysable nitrogen, humidity, total protein, total fat, on days 0-4, regardless of the essential oil used

This observation was supported by the data resulted from statistical processing: standard deviation values for moisture, total protein and total fat do not exhibit clear upward trend, while the standard deviation for the easily hydrolysable nitrogen shows a clear upward trend of about 8 times between day 1 and day 4, and about 7 times for the pH between the same days as in Table 1.

Table 1 Variation of standard deviation values for the following parameters: pH, easily hydrolysable nitrogen, humidity, total protein, total fat, on days 0-4, regardless of the essential oil used

Day	Parameters Analysis				
	pH	Easily hydrolysable nitrogen	Humidity	Total protein	Total fat
0	0.00	0.00	0.00	0.00	0.00
1	0.03	0.43	0.30	0.08	0.31
2	0.03	0.93	0.31	0.02	0.14
3	0.14	2.62	0.24	0.06	0.12
4	0.20	3.26	0.10	0.06	0.10

This fact suggests that there are differences in growth between the analysed samples. It is considered that some independent factors are causing these differences to a greater or lesser extent. In our case the growth it influenced by the amount and type of oil used. Similar results have been reported by **Erkan and Bilen (2010)** for bay leaf, thyme, rosemary, black seed, sage, grape seed, flaxseed and lemon essential oil on chub mackerel.

Figures 5 and 6 shows a similar situation both in the evolution of easily hydrolysable nitrogen parameter and in that of pH parameter. Thus, for both, the factor time (days) was the strongest determinant of the parameters evolution and significant differences between measurements every day were recorded. The analysis of individual differences between days regarding the percentage of easily hydrolysable nitrogen by slope of growth revealed that there are significant differences between days 2 - 3, and 3 - 4, suggesting an acceleration from day 2 in increasing the amount of easily hydrolysable nitrogen in the sample. The average difference between the percentages of easily hydrolysable nitrogen was 3.24% on days 2 and 3 and 2.70% between days 3 - 4. For pH, significant difference between two consecutive days were registered only on days 3 - 4, with a mean difference of 0.30 pH value units. Both in the case of easily hydrolysable nitrogen and in that of pH, the amount of oil used was significant to determine differences in the evolution of parameters.

The type of oil used did not influence significantly the evolution in time of easily hydrolysable nitrogen and pH indicators.

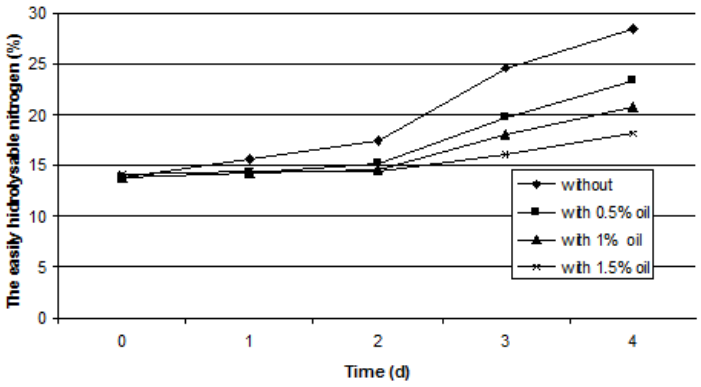

Figure 5 Variation of easily hydrolysable nitrogen percentage on days 0-4, for 0.5%, 1.0% and 1.5% concentration of oil added

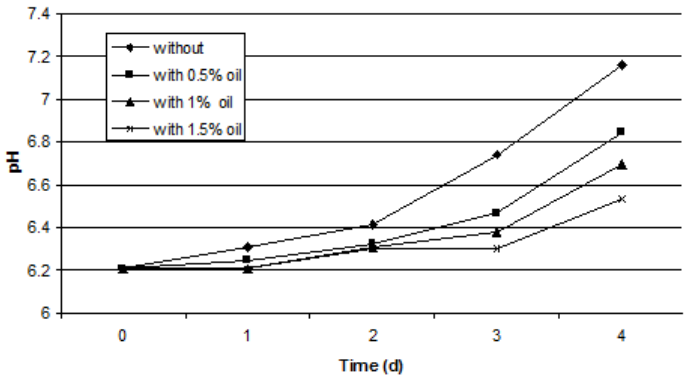

Figure 6 pH variation, on days 0-4, 0.5%, 1.0% and 1.5% concentrations of oil

Figure 7 illustrate the evolution of easily hydrolysable nitrogen percentage on 0-4 days, for the control sample and for each type of oil and the amount of oil incorporated. The lines representing the highest oil concentration used are located at the bottom of the graph, and thyme oil of 1.5% concentration corresponds to the lowest percentage of final easily hydrolysable nitrogen, the slope that increases the percentage of easily hydrolysable nitrogen in days 2-4 being the lowest. Figure 7 confirms the statistical results presented in Table 1, according to which the growth rate of easily hydrolysable nitrogen in all samples is accelerating from day 2, except for that of thyme oil in concentration of 1.5% which seems to adjourn the process to the 3rd day.

The pH evolution from Figure 8 confirms the results mentioned above, higher concentrations of the oil resulting in a less steep increase slope of pH value. Also, it can be seen that all three oils at maximum concentration of 1.5% incorporated adjourn the acceleration from day 2 to 3rd day that it was found to control sample. The differences between day 2 and day 4 remain considerable. Recent studies demonstrated that evaluation of essential oils in different products with refrigeration and frozen methods (**Erkan 2012; Erkan *et al.* 2011a**) or with modified atmosphere packaging (**Erkan *et al.* 2011b**) can be applied as effective conservation methods in order to extend the shelf-life of processed food without the use of chemical preservatives.

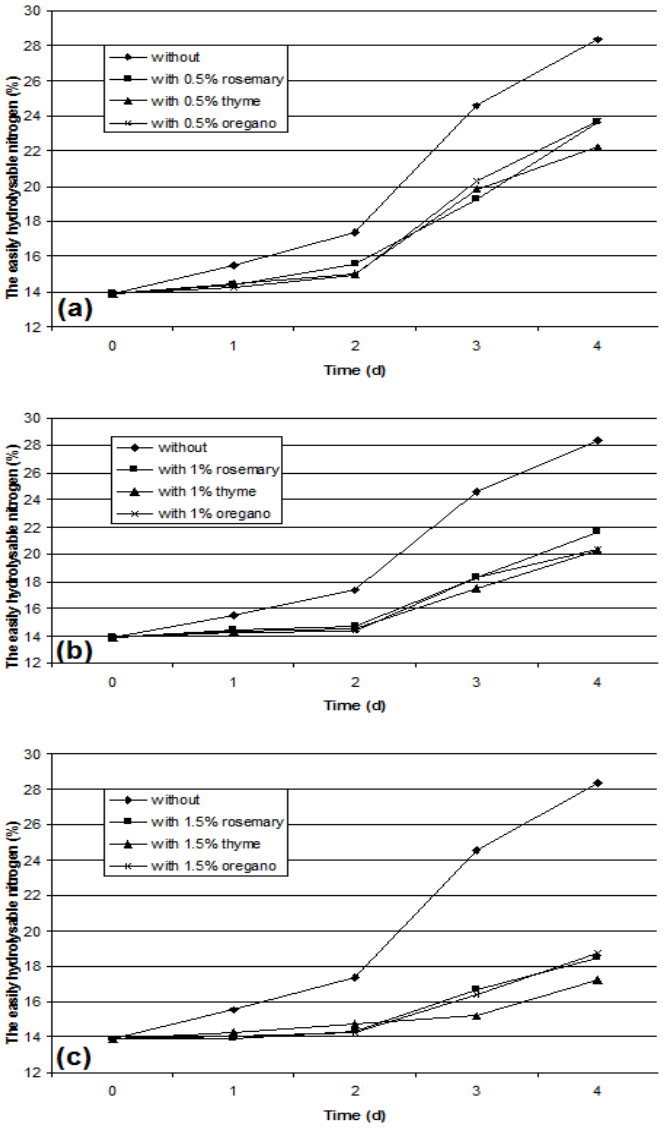

Figure 7 The evolution of the easily hydrolysable nitrogen percentage on days 0-4, depending on the type and amount of oil used (concentration of: **(a)** 0.5%, **(b)** 1% and **(c)** 1.5%)

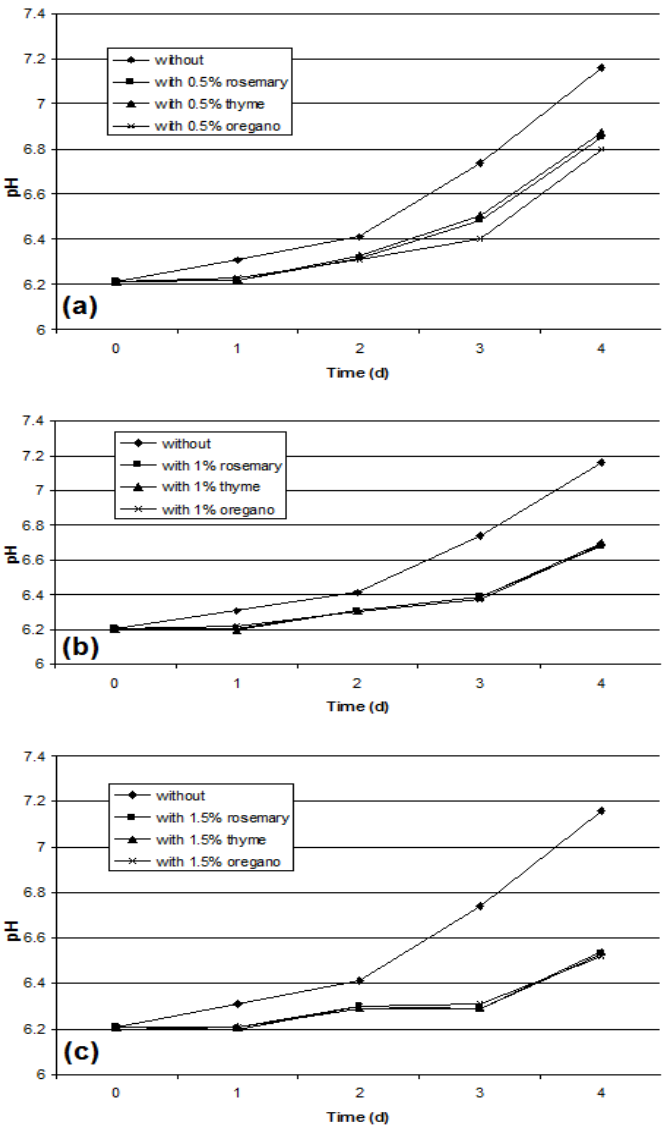

Figure 8 - Evolution of pH on days 0-4 depending on the type and amount of oil used (concentration of: **(a)** 0.5%, **(b)** 1% and **(c)** 1.5%)

CONCLUSION

In this paper was analysed the influence of three different oils rosemary, thyme and oregano on the physicochemical and microbiological properties of minced meat. The oils analysis were performed by applying DPPH method, also the microbiological activity was evaluated. From the analysis of experimental data and their statistical processing it can be concluded that the incorporation of essential oils in minced meat has differentiated effects depending on the parameter analysed. Sensory properties, with major impact on the purchasing decision, were maintained in the range of acceptability for the samples containing essential oils. Unlike these, the control sample at the end of the test, showed changes in odour and colour being unfit for consumption. Parameters such as humidity, total protein, total fat, are not affected by the type or content of essential oil. Indicators regarding the degree of meat degradation, easily hydrolysable nitrogen and pH showed increased values from day to day, but lower ones for the samples added with essential oils and much higher for the control sample. When increasing the concentration of essential oil added, the increase is balanced. No significant differences were found in the efficiency between the types of essential oil used.

It should be noted that for the previously named parameters, the rate of increase is not uniform throughout the testing period, an increasing in easily hydrolysable nitrogen accumulation and sample alkalization after the third day were observed.

In conclusion, the use of essential oils could improve both microbial stability and sensory quality. Thus, this study suggests the possibility of using thymus, oregano and rosemary oils as natural antioxidant in food industry, where they may be considered natural preservatives to replace synthetic preservatives of which consumers are increasingly distrustful. The pleasant taste, smell and low toxicity indicate their use as additives to prevent bacterial contamination.

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EVALUATION THE RISK OF TOXIC COMPOUNDS FORMATION IN GRAPE AND FRUIT BRANDIES

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ABSTRACT

Study to determine the quantities of accumulating harmful to consumer health compounds in grape and plum brandies was conducted. Gas chromatographic assay of the volatile components in grape brandies founded that the amounts of methanol (compound with the highest index of danger for health) ranging from 12.44±0.03 – 54.40±0.10 mg/100ml a.a., which values are several times lower than the limits legalized in the EU Regulation - to 1000 mg/100ml a.a. The results for the second component which also represents a health hazard to the consumer (acetaldehyde) varied from 2.08±0.10 to 4.17±0.04 mg/100ml a.a. This concentration is low and comparable with the data obtained by other researchers which established it as harmless to the consumer health. Studies on the plum brandies founded higher values of methanol – 416.00±0.12 – 458.02±0.10 mg/100ml a.a., which is understandable in view of the higher pectin levels in the plum fruit, which are precursor for the formation of methanol. The obtained results for plum brandies were within the maximum permissible levels described in Regulation №110/2008 of the European Union. The analyzes on grape and plum brandies indicated that they were safe for consumption.

Keywords: Plum brandies, grape brandies, methanol, acetaldehyde, higher alcohols

INTRODUCTION

Brandy is a traditional alcoholic drink in Bulgaria and other countries in Balkan region with a relatively high index of consumption. The lack of overall knowledge on the mechanisms of alcoholic fermentation and technological parameters in the production of brandies, leading in many cases to obtain of final product with low quality and great uncertainty with regard to health safety.

The fermentation process is usually realized in wooden, metal or plastic containers for food purposes. This microbiological process is usually performed without the addition of cultivated yeasts, and rely of spontaneous fermentation induced by wild yeasts that are normal present microflora on the fruit parts. In many cases the distillation process is carried out in copper stills into an open fire. It is claimed that this type of distillation does not allow the controlling constant temperature, which negatively affects the proper realization of the process (**Coldea *et al*., 2011; Tesevic *et al*., 2009; Kostik *et al*., 2014; Pomahaci *et al*., 1892**).

Storage and aging of this type alcoholic beverages are usually realized in oak barrels for a period at least 2-3 months, which ensures the formation of a specific aroma and bouquet and golden yellow color (**Stanimirovic and Stanimirovic, 1982**). For the flavor and aroma of the fruit and grape brandies essential importance have many volatile compounds formed during the fermentation by metabolic activity of the yeasts cultures (*Sacharomyces cerevisiae*) and during storage and aging of the beverages. Some of these compounds - acetaldehyde, methanol and some higher alcohols can have adverse toxicological effects on the human body after consumption of beverages containing them in high concentrations (**Kostik *et al*., 2014; Coldea *et al*., 2013; Obosamiro, 2013**). Because of this fact they are subject to regulation by the European and local legislations.

Methanol is a compound with very high degree of health hazard to the consumer. It is a naturally occurring component in brandies, especially these produced from rotten fruits with a high concentration of pectin (**Coldea *et al*., 2011**). The mechanism of methanol formation is connected with the action of the enzyme pectin-methyl-esterase, which contain in greater concentration in the stone fruits. Under the action of this enzyme occurs demethylation of pectin and release methanol together with pectinic acid and pectol (**Coldea *et al*., 2011; Lukic *et al*., 2011**). In general, the mechanism for action of pectolytic enzymes follows the scheme: initial decomposition of insoluble (protopectin) to soluble pectin under the action of the enzyme protopectinase; subsequent degradation of the resulting soluble pectin to the polygalacturonic acid and methanol under the action of the enzyme pectinesterase; subsequent decomposition of polygalacturonic acid to galacturonic acid by the action of polygalactouronase (**Marinov, 2005**). The consumption of beverages with concentrations of methanol exceeding permissible levels can lead to permanent blindness, surrogate toxicity **(Paine and Davan, 2001**), and after consumption of larger quantities - to the death of the consumer, has already been reported (**Lachenmeier *et al*., 2007**). The toxic mechanism in the ingestion of large amounts of methanol relates to the formation of formaldehyde and formic acid (cell poisons) in the body of the consumer (**Skrydlewska, 2003**).

Kostik *et al*. (2014) studied grape brandies obtained after distillation of grape juice, after distillation of grape mash and after distillation of wine. They founded a wide variation of the methanol content in the three test variants. The concentration of methanol in their study ranges from 3.5 to 883 mg/100ml a.a.

Because of the high index of danger from brandy consumption (produced at improper realized production technology), permissible concentrations of methanol are regulated by European legislation. The maximum permissible amount of methanol in grape and fruit brandies is assessed by the European Commission of 10 g/l of pure ethanol, which is equivalent to 1000 mg/100ml absolute alcohol (**EEC Council Regulation №110, 2008**).

Acetaldehyde is produced by the dehydrogenation of ethanol and may occur during fermentation, as a minor component (**Coldea *et al*., 2011**). Acetaldehyde is a highly toxic metabolite. It increased in the aging period of brandies, due to the chemical oxidation of ethanol and the subsequent oxidation of the resulting acetaldehyde to form acetic acid (**Silva and Malcata, 1998; Obosamiro, 2013**). Consumed in high concentrations it can result in various harmful effects on the body - dizziness, vomiting and nausea. European legislation does not provide permissible limits of acetaldehyde concentrations, which makes its quantification extremely important. **Apostoloupolou et al. (2005)** founded the range for the content of this component in brandies from 1.3 – 59.7 mg/100ml a.a.

Ethyl acetate is an ester of ethanol and acetic acid. Its high concentrations in alcoholic drinks represent an indicator for incorrectly performed alcoholic fermentation and prolonged storage period (**Tesevic *et al.*, 2009**). Established levels of this component in a variety of fruit brandies were in range – 7.66 – 692.1 mg/100ml a.a. (**Apostoloupolou *et al.*, 2005**).
The higher alcohols are final products of amino acid metabolism of the yeasts. Their quantities in alcoholic beverages are highly dependent of the concentrations of amino acids in the fruit raw material which are subject to a fermentation process. The main property of the higher alcohols is their strong influence on the aroma and taste of the drink (**Bonte, 1987**), giving the drink fruity notes.

The aim of this study was to identify and evaluate a quantitative accumulation of harmful for the health of the consumer compounds in grape and plum brandies.

MATERIAL AND METHODS

Origin of tested samples

Five samples for analysis were provided - three of them were grape brandies and two of them were plum brandies. All five grape and fruit brandies were produced in the town of Pleven, Bulgaria.

Analysis of the volatiles

To determine the major volatile compounds, brandies were directly injected into the gas chromatograph Varian Model 3900 with a capillary column VF-Wax MS (30 m, 0,25 mm ID, DF = 0,25 μm), equipped with a flame ionization detector (FID). The carrier gas was He. Hydrogen was supplied to the chromatograph via a hydrogen generator (model Parker Chroma Gas; Gas Generator 9200). The amount of injected material into the chromatograph was 2 μl. The injection was manual by microsyringe with calibrated scale (Hamilton Co.) with a maximum capacity of 10 μl.
The parameters of the gas chromatographic determination were: injector temperature – 210 °C, initial oven temperature - 40 °C / retention- 2 min, rise to 200 °C with 15 °C/min for 17,67 min. Total time of chromatography analysis - 19,67 min; temperature of the detector – 250 °C.
The major volatile compounds identified by GC-FID were: acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 2-butanol, 3-methyl-butanol, 1-pentanol, 1-hexanol, 1-heptanol. The compounds were identified by comparison of their retention times with those of authentic pure compounds. Solution of pure compounds with known concentrations was injected in an amount of 2 μl. The detected retention times of injected pure compounds are presented in Table 1.

Table 1 The retention times of pure compounds after injection of the standard solution

№	Volatile compound	Retention time (Rt)
1	acetaldehyde	2.312±0.011
2	ethyl acetate	3.600±0.013
3	methanol	3.709±0.009
4	1-propanol	5.001±0.010
5	2-methyl-1-propanol	5.171±0.012
6	1-butanol	5.793±0.011
7	2-methyl-1-butanol	6.433±0.008
8	2-butanol	7.011±0.010
9	3-methyl-butanol	7.416±0.011
10	1-pentanol	7.697±0.013
11	1-hexanol	8.352±0.008
12	1-heptanol	8.916±0.010
13	1-octanol	9.252±0.006

The quantitative evaluation of each of the tested compounds in brandies was carried out by the method of the internal standard. As an internal standard was used 1-octanol.

Chemicals and reagents

Chemically pure substances were used: acetaldehyde, ethyl acetate, methanol, 1-butanol, 2-methyl-1-butanol, 2-butanol, 3-methyl-butanol, 1-propanol, 2-methyl-1-propanol, 1-pentanol , 1-hexanol, 1-heptanol, 1-octanol (internal standard). All of them with a purity exceeding 99%. They were purchased from Fluka (Sigma Aldrich Co.).

Statistical analysis

The statistical analysis of the data is carried out by determining the standard deviation (SD), with triple repetition of the analyses. It is performed with the Excel 2007 software application of the Microsoft Office 2007 suite (Microsoft Corporation, USA).

RESULTS AND DISCUSSION

The ethanol content (vol. %) of the tested samples is presented in Table 2. In grape brandies it was in range of 40.20 vol. % to 45.80 vol. %; in plum brandies it was in range of 48.00 vol. % to 51.7 vol. %. These indicators shall be brought to standard alcoholic degrees regulated by the Bulgarian and European legislation.

Table 2 Identified concentrations of ethanol in grape and fruit brandies

Brandy samples	Content of ethanol, vol. %
Grape brandy 1	45.80
Grape brandy 2	45.70
Grape brandy 3	40.20
Plum brandy 1	51.70
Plum brandy 2	48.00

On the base of injection of standard solution consist pure substances were found the components of tested brandies by the established retention times. The chromatogram of standard solution is shown in Figure 1.

After injection of the three samples grape brandies, on the base of added internal standard to each, concentration of the searched components was identified. The data of the available identified main volatile components in grape brandies are presented in Table 3.

Table 3 Concentrations of identified by GC-FID compounds in grape brandies

Compounds	Concentrations of identified volatile compounds, mg/100ml a.a.		
	Grape brandy 1	*Grape brandy 2*	*Grape brandy 3*
acetaldehyde	2.08±0.10	5.34±0.12	4.17±0.04
ethil acetate	11.25±0.05	4.78±0.05	4.56±0.08
methanol	12.44±0.03	22.86±0.11	54.40±0.10
2-methyl-1-propanol	3.19±0.09	ND	4.80±0.12
1-butanol	21.76±0.10	7.17±0.05	5.93±0.13
2-methyl-1-butanol	ND	ND	1.79±0.07
2-butanol	260.75±0.15	149.23±0.12	59.79±0.02
1-hexanol	ND	ND	1.51±0.03
1-heptanol	0.841±0.12	ND	ND

** ND – Not detected*

The quantities of methanol in grape brandy samples varied from 12.44±0.03 mg/100ml a.a. to 54.40±0.04 mg/100ml a.a. The formation of methanol in grape brandy was less pronounced, because pectin concentrations in fruits of grape are lower, as compared for example with stone fruits. When the distillation was performed properly the levels of methanol resulted in minor concentrations which not affect human health after consuming of alcoholic beverages. The highest concentration of methanol in our study was found in grape brandy 3 – 54.40±0.10 mg/100ml a.a. Our results were in correlation with the data of **Kostik *et al.* (2004)**.
All established methanol concentrations in this study meet the requirements of Regulation №110 of the EU, 2008 which enshrines a maximum legal limit of methanol content - 1000 mg/100ml a.a. Our research established significantly lower methanol content than the maximum limit. This is clear indicator that the procedures for the production of grape brandies were conducted accurately and correctly.
Acetaldehyde is the second dangerous component, which in high concentrations can reflect negatively on the consumer health status. The amount of acetaldehyde in the studied grape samples ranged from 2.08±0.19 mg/100ml a.a. to 5.34±0.12 mg/100ml a.a. The obtained data were correlated with the results of **Apostoloupolou *et al.* (2005)**. The obtained results for the concentration of acetaldehyde were an indication of properly conducted fermentation process. The

formed acetaldehyde in grape brandies does not represent a danger for the consumer health.

Ethyl acetate has an important effect on the formation of taste. It gives fruity notes of brandies. However, this positive effect is achieved when concentrations of ethyl acetate in the product are low. The higher levels of this ester leads to the formation of unacceptable taste. In the studied grape brandies low amounts ethyl acetate were established. It varied from 4.56±0.08 mg/100ml a.a. to 11.25±0.05 mg/100ml a.a. The established content of this ester in Bulgarian grape brandies was in correlation with the data of **Apostolopolou *et al.* (2005)**.

Higher alcohols are also important for the formation and expression of the product taste characteristics. The highest concentration in the tested fraction of higher alcohols was established for 2-butanol. It varied between 59.79±0.02 mg/100ml a.a. and 260.75±0.15 mg/100ml a.a. The data were in correlation with studies of **Apostoloupolou *et al.* (2005)**. In the brandy sample 1 we established a slightly higher concentration of 2-butanol, which is likely due to the use of raw material with higher content of amino acids which are a precursor for the formation of higher alcohols.

The reported values for the presence of 1-butanol ranged from 5.93±0.13 – 21.76±0.10 mg/100ml a.a. and also correlated with the results of other researchers (**Apostoloupolou *et al.*, 2005; Claus and Bergland, 2005; Cortes *et al.*, 2010**).

The data for the second type of studied brandies which made from plums are presented in Table 4.

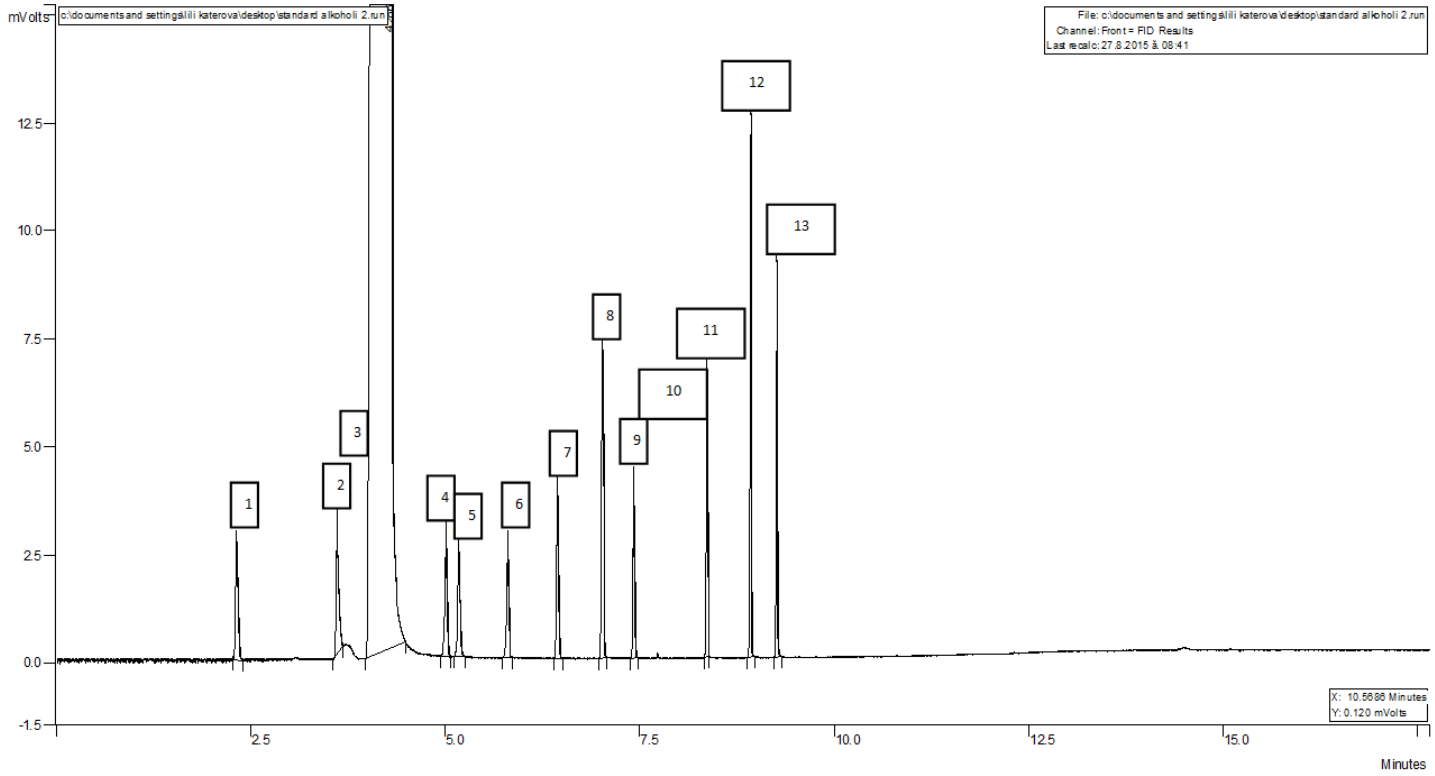

Figure 1 Chromatogram of identified pure compounds in standard solution (**1-** Acetaldehyde; **2-** Ethyl acetate; **3-** Methanol; **4-** 1-propanol; **5-** 2-methyl-1-propanol; **6-** 1-butanol; **7-** 2-methyl-1-butanol; **8-** 2-butanol; **9-** 3-methyl-butanol; **10-** 1-pentanol; **11-** 1-hexanol; **12-** 1-heptanol; **13-** 1-octanol)

Table 4 Concentrations of identified by GC-FID compounds in plum brandies

Compounds	**Concentrations of identified volatile compounds, mg/100ml a.a.**	
	Plum brandy 1	*Plum brandy 2*
acetaldehyde	2.36±0.05	1.51±0.12
ethil acetate	1.54±0.11	1.32±0.04
methanol	458.02±0.10	416.00±0.12
2-methyl-1-propanol	2.44±0.05	ND
1-butanol	3.89±0.12	2.11±0.02
2-butanol	76.63±0.13	2.61±0.08

** ND – Not detected*

The obtained results for the primary toxic compound - methanol in plum brandies showed a trend of higher quantities compared to grape brandies. The resulted concentrations of methanol in plum brandies varied from 416.00±0.12 to 458.02±0.10 mg/100ml a.a. This results are explained by the fact that the plums used as raw material for the production of brandy are a rich source of pectin, which is a precursor for the production of methanol in the final product. Despite having found a higher content of methanol in plum brandies than that of grape brandies, the concentration of this component is within the legal limit of 1000 mg/100ml a.a., approved by the European Union (**Reg. 110/2008)**. This makes the product safe for consumption with acceptable levels of methanol.

CONCLUSION

The conducted research on the assessment the risk of toxic compounds formation in fruit and grape brandies founded concentrations of harmful components that are within permitted concentration levels regulated in European legislation. Moreover, the obtained results correlated with the data of other researchers.

In plum brandies higher levels of methanol were obtained, which is understandable in view of the higher content of pectin in the fruit raw material used for brandy production, but these higher levels are within concentrations of legalized maximum limits of EU.

The obtained results of this research are evidence for properly perform production process of studied grape and fruit brandies, making them safe for consumption.

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ANTI-OXIDATIVE POTENTIAL OF HONEY AND ASCORBIC ACID IN YOGHURT FORTIFIED WITH OMEGA-3 FATTY ACIDS

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ABSTRACT

Processing of Nile perch (*Lates niloticus*), a commercial fish in Eastern Africa; results in omega-3 polyunsaturated fatty acids (PUFA) rich by-products. Oil derived from such by-products can be incorporated in commonly consumed foods; however, these fatty acids are highly susceptible to oxidation. Honey and ascorbic acid are natural anti-oxidants that could play a role in preventing lipid oxidation. In the current study, omega-3 rich oil was extracted from *L. niloticus* viscera and added to yoghurt samples. The aim of the study was to investigate the biochemical and anti-oxidative parameters in honey and lemon juice and use them as antioxidants in the fortified yoghurt samples. Stability of the fortified yoghurt was monitored over one month storage period. Ascorbic acid Equivalent Antioxidant Capacity (AEAC) of lemon juice and honey were 312 ± 2.34 and 197 ± 3.65 mg/L, respectively. The DPPH radical scavenging activity showed that honey (86.16± 1.43%) tended to be highly active in the reaction with DPPH compared to lemon juice (71.29± 3.52%).After four weeks of storage, the peroxide value (PV), anisidine value (AV) and (free fatty acid) (FFA) contents were within the acceptable range with the honey fortified sample being most stable. The ascorbic acid content was highest in lemon juice fortified samples (30 mg/100g) while in honey fortified samples were below 1mg/100g. The pH in all the samples decreased slightly over time. Honey and lemon juice are therefore good natural anti-oxidants and their anti-oxidative potential can be utilized in the prevention of lipid oxidation in omega-3 fortified yoghurts.

Keywords: Omega-3 fatty acids, honey, ascorbic acid, yoghurt, antioxidants

INTRODUCTION

Omega-3 fatty acids are examples of bioactive substances whose interest has increased over the years in scientific research due to their proven health benefits. The human body is not able to synthesize these fatty acids and hence must be provided through diet (**Amegovu *et al*., 2014**) or supplementation. For this reason, fortification of foods with omega-3 PUFAs has therefore been proposed as a practical approach towards increasing the consumption of these fatty acids **(Metcalf *et al*., 2003)**. However, the incorporation of these fatty acids in foods, the processing and handling is associated with nutritional challenges for their healthy delivery **(Huber *et al*., 2009)**. The extreme sensitivity of fish oils to oxidation can easily lead to the development of off-flavors and cause significant loss of product quality, stability, nutritional value, bio-availability and the overall acceptability of the food product **(Jellinek, 1971; Pak, 2005)**.The high rate of fish oil oxidation can be reduced by incorporation of synthetic or natural antioxidants **(Huber et al., 2009)**. The use of natural antioxidants is more preferred due to many health risks such as cancer associated with synthetic antioxidants **(Stip & Bels, 2009**). Honey and vitamin C derived from citrus fruits are natural antioxidants that could be used to delay or inhibit oxidation of Omega-3 PUFAs. Natural honey is associated with several biological properties ranging from anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-biotic and wound healing to immune-stimulatory properties **(Seema & Simon, 2013)**. Anti-oxidative potential of honey is attributed to the presence of high concentrations of important phenolic, flavonoid and carotenoids **(Alvarez-Suarez et al., 2010)**.The amount and type of these antioxidant compounds depends largely on the floral source/variety of honey, climatic conditions and processing **(Mohammed et al., 2010)**. Vitamin C is a natural antioxidant whose consumption is required for the prevention of scurvy and maintenance of healthy skin, gums and blood vessels. As an antioxidant, it has been reported to reduce the risk of arteriosclerosis, cardiovascular diseases and some forms of cancer **(Sarkar et al., 2009).**The aim of this study was to evaluate the potential of honey and ascorbic acid in inhibiting lipid oxidation in omega-3 fortified yoghurt. The effectiveness of the antioxidants in the preservation of the fortified yoghurt product over one month period was also evaluated.

MATERIALS AND METHODS

Materials

Nile perch viscera were purchased from a local Nile perch processing plant in Nairobi (W.E. Tiley ltd, Nairobi, Kenya). Fresh milk, skimmed milk, sugar, citrus fruits (lemon, tangerine and orange) and three honey samples (green forest, Amboseli and Baringo) were obtained from a local store (Tusky's Supermarket Ltd). Starter cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were purchased locally (Pradip enterprises ltd). All the solvents and chemicals used were of analytical grade.

Analysis of Honey

Proline content

The method described by the International Honey Commission **(IHC, 1999)** was used to determine the proline content. An aliquot (0.5mL≈ 0.05mg) of honey solution was transferred to a test tube. For blank test, 0.5 mL was transferred to a second tube and 0.5 mL of (0.032mg/mL) proline standard solution was dispensed into three other tubes. To each tube, 1 mL of formic acid and 1 mL of ninhydrin (Fisher Scientific, United Kingdom) solution was added. The tubes were capped carefully and shaken vigorously for 15 min. The tubes were then placed in a boiling water bath for 15 min and there after transferred to another water bath and incubated at 70°C for 10 min. 2-propanol water solution (5 mL) was added to each tube followed by immediate capping. The tubes were left to

cool for approximately 45 min and the absorbance values measured at 510 nm. Proline concentration in mg/kg of honey was calculated as follows:

Proline (mg/kg) = (Es/Ea)x(E_1/E_2)x80,

Where: **Es** is the absorbance of the sample solution; **Ea** is the absorbance of the proline standard solution (average of three readings); $E_1$is the mg of proline used for standard solution; $E_2$is the weight of honey in grams; **80** is the dilution factor. The mean of three readings was used.

Total phenolic content

This was determined by a method described by **Singleton *et al*., (1999)**. Each honey sample (5 g) was diluted to 50 mL using distilled water. A 1 mL of this solution was mixed with 2.5 mL of 0.2 N Folin–Ciocalteu reagents (Sigma–Aldrich Chemie, Steinheim, Germany) for 5 min after which, 2 mL of 75g/l sodium carbonate (Na_2CO_3) was added. The reaction mixture was incubated for 2h at room temperature and the absorbance read at 760 nm against a methanol blank. Gallic acid (0–200 mg/l) was used as a standard to make a calibration curve. The mean of three readings was used and the total phenolic content expressed in mg of gallic acid equivalents (GAE)/100g of honey.

Carotenoids content

This was done according to the method described by **Ferreira *et al*. (2007)**. A sample of honey (100 mg) was vigorously shaken with 10 mLof acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper (Sigam Aldrich, USA). The absorbance of the filtrate was then measured at 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations:

Lycopene (mg/100 mL) = -0.0458 A_{663} + 0.372 A_{505}- 0.0806 A_{453};
β-carotene (mg/100 mL) = 0.216 A_{663}- 0.304 A_{505} + 0.452 A_{453}.

The results were expressed as mg of carotenoid/kg of honey

Color intensity (ABS_{450})

The mean absorbance of honey samples was determined according to the method described by **Beretta *et al. (*2005)**. Briefly, honey samples were diluted to 50% (w/v) with warm (45 – 50°C) distilled water, and the resulting solution filtered to remove large particles. The absorbance was measured at 450 and 720 nm and the difference in absorbance was expressed as milli absorbance *units* (mAU).

mAU of honey =(ABS_{700} –ABS_{450})

Determination of anti-oxidative properties of honey

DPPH radical scavenging activity

The method described by **Ferreira *et al*.(2007)** was used to determine the DPPH radical scavenging activity of the honey samples. The honey samples (12.5 µL - 100 µL/mL) were prepared in methanol. An aliquot (2 mL) of DPPH (Sigma - Aldrich, USA) solution (0.002 % in methanol) was added to 2 mL of the prepared samples. The samples were incubated at room temperature in the dark for 30 minutes and the optical density read at 517 nm. The absorbance of the DPPH control was also noted. The scavenging activity of the samples was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100.

Where: **A** is absorbance of DPPH and **B** is absorbance of DPPH and honey sample.

Ferric reducing power

This was done according to the method described by **Ferreira *et al.* (2007)**. Various concentrations of water honey solutions (2.5 mL) were mixed with 2.5 mL of 200mmol/L sodium phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. To the mixture, 2.5 mL of 10% trichloro acetic acid (w/v) (BDH Chemicals Ltd, Poole, - England) was added and the mixture centrifuged at 2000×g for 8 min. The upper layer (2 mL) was mixed with 5 mL of deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance read at 700 nm. Higher absorbance indicates higher reducing power **(Oyaizu, 1986)**.

Total Antioxidant content of honey

The antioxidant content was determined by measuring Ascorbic acid Equivalent Antioxidant Capacity (AEAC) values using the method of **Meda *et al*.(2005)**. Briefly, honey samples were dissolved in methanol to a final concentration of 0.03 g/mL. A 0.75 mL aliquot of the methanolic honey solution was then mixed with 1.50 mL of 0.02 mg/mL DPPH solution prepared in methanol. The mixture was then incubated at room temperature for 15 min, and the absorbance measured at 517 nm. The blank was composed of 0.75 mL of a methanolic honey solution mixed with 1.5 mL of methanol. Ascorbic acid standard solutions (2, 4, 6, 8 and 10µg/mL) prepared in distilled water were used to form a calibration curve. Measurements were performed in triplicate, and the mean value was expressed as mg of ascorbic acid equivalent antioxidant content per 100 g of honey.

Analysis of citrus fruit juice

Determination of total acidity and pH

Total acidity of the juices was determined by titration method according to **Rekha *et al*.(2012)**. A 10 % fruit juice was prepared and 10 mL titrated against standardized 0.1N NaOH (Sodium hydroxide) using Phenolphthalein as an indicator. The end-point was noted. Total acidity was calculated in terms of citric acid using formula:

Acidity (g/100 mL) = Normality of the juice x Equivalent weight of citric acid.
The pH of 10 % juice was determined using pH meter (Mettler Toledo, USA)

Estimation of Ascorbic acid content

Ascorbic acid content in fruit juices was estimated by titration method. Into a 100 mL volumetric flask, 50 mL of un-diluted fruit juice and 25 mL of 20 % meta-phosphoric acid was added as a stabilizing agent and distilled water added up to the 100 mL mark. A 10 mL volume of the solution was pipetted into a small flask and titrated using standard indophenols solution until a faint pink color persisted for 15 s. The mg of Ascorbic acid per mL of the sample was calculated as follows:

Vitamin C content (mg/100g) = (A-B) ×C ×100/10× 1/S ×100

Where: **A**= volume of the indophenols solution used to titrate the sample (mL)
B= volume of then indophenols solution used for the blank (mL)
C= mass in mg Ascorbic acid equivalent to 1.0mL of standard indophenols solution
S= volume of sample used

Determination of Antioxidant Activity of the Fruit Juices

DPPH free radical scavenging assay

This was determined following the method proposed by **Ferreira *et al*.(2007)** as described in the analysis of the honey samples.

Ferric reducing assay

This was determined following the method proposed by **Ferreira *et al*. (2007)** as described in the analysis of the honey samples.

Total antioxidant content of fruit juices

The antioxidant content of the fruit juices was determined by measuring Ascorbic acid Equivalent Antioxidant Capacity(AEAC) values using the method of **Meda *et al*.(2005)**. From the three citrus fruits evaluated, the fruit with the highest ascorbic acid content and hence antioxidant activity was selected for use in the preparation ofomega-3 fortified yoghurt. The recommended dietary intake (RDI) of ascorbic acid (60mg/day) was used to determine the volume of the juice to be added into the yoghurt, and taking care that the pH of the yoghurt does not to go below the recommended pH of 4.5.

Production of fortified yoghurt

Production of functional fortified yoghurt was done by a method developed in our laboratory. Two types of stirred fortified yoghurt samples were prepared. The first yoghurt sample was fortified with Omega-3 fatty acids and honey (YFH). Honey (95g) was incorporated into 1 litre of milk during the yoghurt mix formulation and the mixture homogenized to ensure a homogenous formulation. Skimmed milk powder containing Soy lecithin was added as a thickener and to solubilize the omega-3 rich oil in the yoghurt. The second yoghurt sample was fortified with Omega-3 fatty acids and 33.3 mL of lemon juice per 1litre of milk (YFL). The third yoghurt sample was a control, which contained only the oil sample without an antioxidant (YF). A fourth control comprised of plain natural yoghurt (PY).

Analysis of yoghurt after production and during storage

Determination of Quality parameters of the omega-3 fortified yoghurt was done by determining the PV, AV, Total oxidation (TOTOX) and FFA contents. pH and total acidity was determined at weekly intervals over one month storage period.

Statistical Analysis

The entire experiment was replicated three times and the means and standard deviations reported. The SPSS software (IBM SPSS statistic 19) was used to conduct analyses of variance (ANOVA) to determine the differences among treatment means in the various weeks and the *post hoc* Tukey's test was used. Correlation analyses were done using the SPSS software.

RESULTS AND DISCUSSION

Biochemical parameters of the honey samples

The Phenol content per 100 g of honey ranged from 58.56 mg to 71.56 mg GAE (Table 1). The commercial honey samples had higher phenolic content compared to the natural honey samples. The phenolic content of the samples is in agreement with phenolic content of honey reported in literature (Beretta et al., 2005; Gheldof et al., 2002). A general observation can be made that dark honeys (Amboseli and Green forest) were characterized by considerably higher phenolic content than the natural honey sample. This trend is similar to the relationship found in previous studies done on Burkina Faso and Italian honeys (Blasa et al., 2006; Meda et al., 2005). The natural honey sample had higher proline content of 2.41± 0.24 (mg/g), whereas the Amboseli and Green forest honey had less proline content. The proline levels obtained in all the samples exceeded the minimum limit, an indication that the honey samples were not adulterated (Table 1).Carotenoid such as β-carotene and lycopene were higher in the processed honey samples than in the natural honey. The color intensity (ABS_{450}) of the Natural honey (219 ± 23.43 mAU**)** was lower than that of the processed honey samples with Amboseli honey having highest ABS. This suggested a lower antioxidant activity in the natural honey compared to processed honey.

Table1 Biochemical and physical parameters of the honey samples

Sample	Phenol content (mg/100g)	Proline content (mg/g)	β-Carotene (mg/kg)	Lycopene(mg/kg)	Color intensity (mAU)
Baringo (Natural)	58.56± 1.78	2.41± 0.24	7.23 ± 0.92	5.31 ± 0.52	219± 23.43
Amboseli (Processed)	67.25± 0.89	1.62± 0.53	8.31 ± 1.02	6.71 ± 0.02	406± 12.34
Green forest (Processed)	71.56± 2.34	1.72± 1.02	8.92 ± 0.32	6.81 ± 0.01	394± 11.68

Values presented are mean ± SD of three determinations

Antioxidant activities of honey samples

The antioxidant activity of honey varied from 65.7 % to 86.2 % in the DPPH reaction system. The results of the DPPH radical scavenging activity showed that the processed honey tended to be highly active in the reaction with DPPH, while Natural honey had a lower radical scavenging activity. The Baringo sample had lower AEAC (280 ± 0.56) compared to the Amboseli (305 ± 1.23) and Green forest (312 ± 2.34) mg of AEAC/kg of honey (Table 2). Green forest honey had higher reducing activity (2.78 ± 1.69) compared to Amboseli (2.56 ± 1.23) and Baringo (0.98 ± 2.54).

Table 2 Antioxidant activity of honey samples

Sample	DPPH Scavenging activity (%)	Ferric Reducing power Abs at 700nm	Antioxidant content (mg of AEAC/kg of honey)
Baringo (Natural**)**	65.86± 2.94	0.98± 2.54	280± 0.56
Amboseli (Processed**)**	84.47± 2.58	2.56± 1.23	305± 1.23
Green forest (Processed)	86.16± 1.43	2.78± 1.69	312± 2.34

Values shown are means± SD of three replicate experiments

Correlations amongst biochemical parameters and antioxidant potentials

Several strong correlations were established amongst different biochemical and antioxidant parameters. A strong correlation was found between the color intensity of honey samples and antioxidant parameters (DPPH and AEAC) (Table 3). Strong correlations between the β-carotene, lycopene and AEAC suggest that these components contribute to anti-oxidative capacity of honey **(Ferreira, *et al.*, 2007)**. These findings suggest that honey color pigments such as β-carotene and lycopene may have a role in the observed antioxidant activities of honey samples. Similar to our findings, a strong correlation between the antioxidant capacity and ABS_{450} was reported by **Bertoncelj *et al.* (2007)** and **Beretta *et al.*(2005)** indicating that honey color intensity may be treated as a good initial indicator of its antioxidant capacity. A strong positive correlation was established between the antioxidant activity and total phenolic content (PC) (R^2=0.967 for PC/DPPH•, R^2=0.976 for PC/Ferric reducing assay, R^2=0.993 for PC/AEAC. This means that phenolic compounds are one of the main components responsible for the antioxidant activity of honeys. This correlation was in agreement with the findings of other authors such as the high correlation between radical scavenging activity and the total phenolic content at a level of p=0.5, **(Meda *et al.*, 2005)**. Overall, the strong positive correlations suggest that the honey samples had a strong antioxidant potential that could be utilized to prevent fish oil oxidation in the omega-3 fortified yoghurt.

Table 3 Correlation matrix showing the interrelation among biochemical parameters and antioxidant activity of honey

	Proline	βcarotene	Lycopene	ABS_{450}	DPPH	AEAC
Proline	1	-0.887	-0.985	-0.998*	-0.982	-0.947
β-carotene	-0.887	1	0.954	0.912	0.958	0.988
Lycopene	-0.985	0.954	1	0.993	1.000**	0.989
ABS_{450}	-0.998*	0.912	0.993	1	0.991	0.965
DPPH	-0.982	0.958	1.000**	0.991	1	0.991

*Correlation is significant at 0.05 levels (2-tailed)
**Correlation is significant at 0.01 levels (2-tailed)

Total acidity, pH and ascorbic acid content of Citrus fruits

Citrus fruits contain high levels of citric and ascorbic acid. In this study, ripe fruits were used since unripe ones contain lower pH than ripe fruits **(Rekha *et al.*, 2012)** which would further reduce the pH of the fortified yoghurt below unacceptable levels. The pH was lowest in lemon juice (2.9± 0.02) because lemon juice contains higher amounts of ascorbic acid in addition to citric acid. Orange and tangerine juice had a pH of 3.8 ± 0.01 and 4.1 ± 0.01, respectively. The total acidity was higher in lemon juice followed by orange juice and then Tangerine. pH and total acidity are important in determining the level of acidity of the juices which may affect the acidity of the yoghurt once added. The ascorbic acid content in the fruit juices ranged from 55.25 ± 0.34 to 62.82 ± 0.48mg/100 mL. The highest ascorbic acid content was observed in lemon juice followed by orange and tangerine (Table 4).

Table 4 Total acidity, pH, Ascorbic acid content, DPPH free radical scavenging activity, Ferric reducing activity and total antioxidant content (AEAC) of citrus fruit juices

Citrus fruit	pH	Total acidity (Citric acid g/100 ML)	Ascorbic acid(mg/100 mL)	DPPH (%)	Ferric reducing activity (Abs 700nm)	AEAC (mg/L)
Orange juice	3.8± 0.01	0.8± 0.01	55.25± 0.34	62.06± 2.56	0.98± 1.54	178± 2.49
Lemon juice	2.9± 0.02	3.7± 0.01	62.82± 0.48	71.29± 3.52	1.34± 0.65	197± 3.65
Tangerine juice	4.1± 0.01	0.6± 0.02	57.34± 1.54	59.02± 1.67	0.78± 1.92	169± 1.35

Values shown are means± SD of three replicate experiments

Radical scavenging and Ferric reducing Power

Radical scavenging activities of citrus juices investigated by DPPH radical scavenging assay showed that lemon juice had the strongest scavenging activity (71.29 ± 3.52 %) compared to orange (55.06 ± 2.56) and tangerine (51.02 ± 1.67) (Table 4). Antioxidant activity was found to be higher in lemon juice, which also had the highest level of ascorbic acid. This is in accordance with results obtained in previous studies by **(Gardner *et al*., 2000)**.Gardner *et al*, (2000) also showed that vitamin C is the main antioxidant in most of the citrus fruits. Ferric reducing assay showed that lemon juice had the strongest activity. This was in agreement with a study done by **Ali *et al.* (2011)**, which showed that citrus fruits had a high DPPH radical scavenging, and ferric reducing antioxidant potential. Although the antioxidant capacity evaluated by the DPPH method was higher than that evaluated by the Ferric reducing assay method, the correlations were good for both methods. This indicated that ascorbic acid contributed to the antioxidant activity of the citrus fruits. Other than the antioxidant potential of citrus fruits, a recent study by the World Health Organization showed convincing evidence of positive effects obtained from dietary intake of citrus fruits on cardiovascular disease **(WHO, 2003)**. They have also been shown to possess anti-inflammatory, antioxidant, antitumor and antifungal activities **(Ghafar *et al*., 2010)**.

Correlation matrix showing the relationship between Ascorbic acid content and Antioxidant activity of Citrus fruits

All the parameters evaluated to show the antioxidant potential of citrus fruit juices showed strong positive correlation (table 5). A strong positive correlation was evident between ascorbic acid content and total antioxidant activity (R^2 = 0.997). Citrus fruits are a good source of vitamin C and they also possess good antioxidant activity **(Berenguer *et al*., 2004)**. Lemon juice was the best in terms of ascorbic acid content and hence highest antioxidant capacity, it is available every season and very economical compared to the other studied fruits. Therefore, lemon juice was chosen for the fortification process of the yoghurt.

Table 5 Correlation matrix showing the relationship among Ascorbic acid content and antioxidant activity of Citrus fruits

	Ascorbic acid	DPPH	Ferric reducing activity	AEAC
Ascorbic acid	1	0.872	0.808	0.830
DPPH	0.872	1	0.993	0.997
Ferric reducing activity	0.808	0.993	1	0.999*
AEAC	0.830	0.997	0.999*	1

**Correlation is significant at 0.05 levels (2-tailed)*

Changes in Peroxide, Anisidine, Totox and FFA of the fish oil fortified yoghurt over four-week storage period

The PV, AV, and TOTOX values of the omega-3 fortified yoghurt increased slightly over time in all the samples with honey (YFH) having the least values followed by lemon juice and the highest values seen in the sample without antioxidant (YF). The FFA value in the lemon juice fortified sample (YFL) was higher than the sample with no antioxidant up to the third week. This is because FFA estimation is a titration method and thus the increased acidity in this sample gave the high FFA content. In the fourth week, a gradual increase in FFA in all the samples was observed overtime with the control (YF) having the highest FFA values (figure 1). When comparing samples with added antioxidants with those without anti-oxidants, it was found that the latter samples had lower values of these quality parameters. The antioxidants therefore, helped to reduce lipid per-oxidation of the fish oils. Honey was a better antioxidant compared to lemon juice. This is because honey has several compounds that play a critical role in antioxidant activity such as phenols, flavonoids, carotenoids**(Ferreira *et al*., 2007)** compared to lemon juice which has only ascorbic and citric acid as the major compounds enhancing its antioxidant activities. From this study, honey and vitamin C can be used as natural antioxidants in yoghurt fortified with omega-3 rich fish oil thereby extending its shelf life.

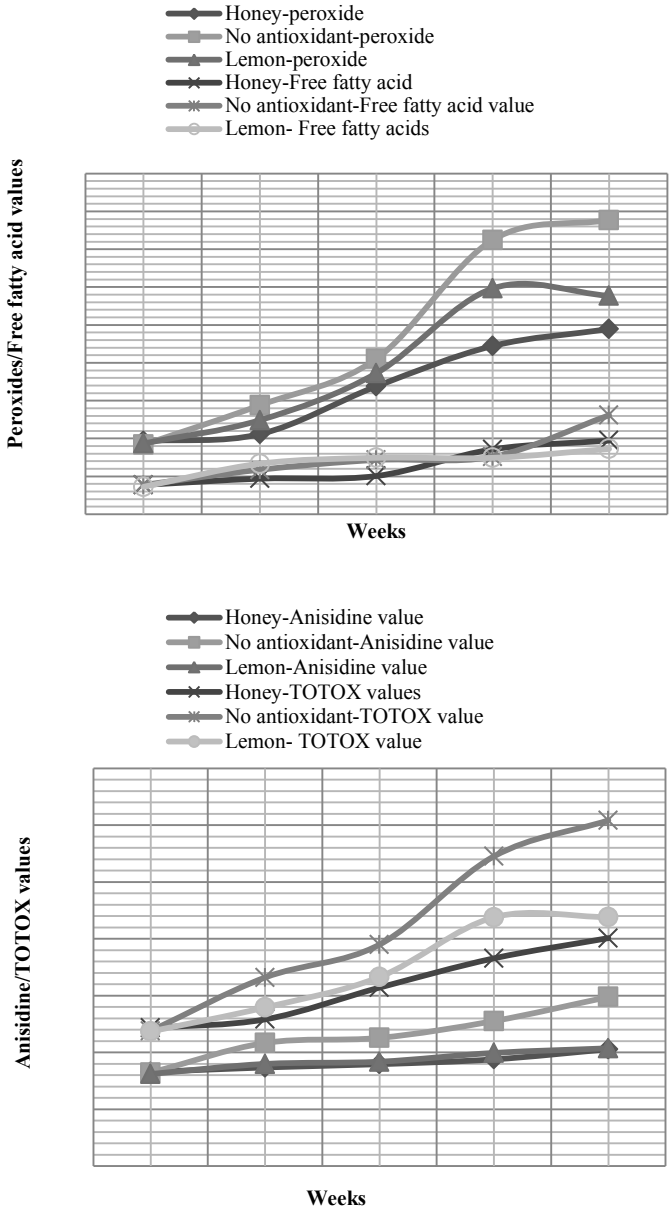

Figure 1 Changes in quality parameters of the fortified yoghurt over four-week storage period

Changes in pH, Total acidity over four week's storage period

Overall, the pH values in all the samples decreased slightly over time (figure 2). The total acidity increased slightly in all the samples with the highest % in the lemon juice fortified yoghurt (YFL). The lemon juice fortified yoghurt sample had the highest vitamin C content compared to all other samples. Generally, the pH values in all the samples decreased slightly over time. The sample with lemon juice as antioxidant had very low pH due to the presence of citric acid in addition to Ascorbic acid in the juice. The other three samples (PY, YF, and YFH) had close pH values with the plain natural yoghurt having the highest pH values. The total acidity increased slightly in all the samples with the highest percentage in the lemon juice fortified yoghurt. Starter cultures usually transform lactose in milk into lactic acid which is responsible for the initial acidification which coagulates the milk at pH 4.5 and also the post acidification during storage.

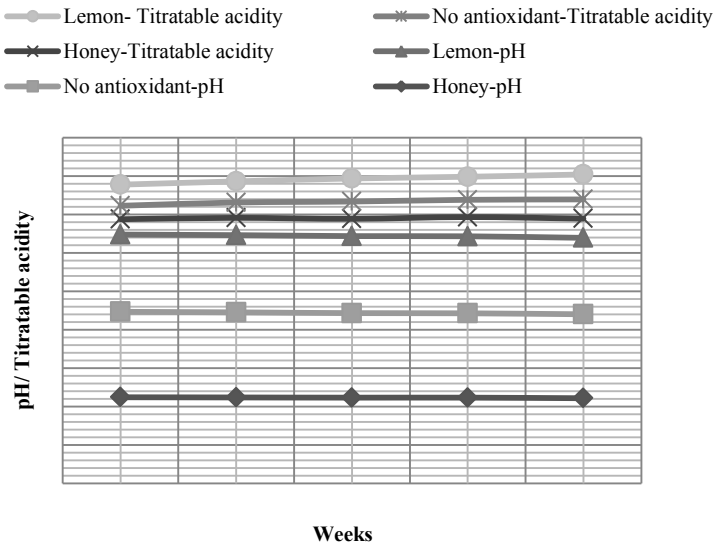

Figure 2 Changes in pH and titratable acidity of the different yoghurt samples over four weeks storage period

CONCLUSION

The greatest challenge in the addition of omega-3 rich oils to yoghurt as a source of n-3 FA is their extreme sensitivity to oxidation that leads to the development of off-flavors. The goal is to add sufficient levels of oil that can provide a significant contribution of omega-3 fatty acids into the daily diet while minimizing oxidation. This therefore calls for the need to add natural antioxidants and flavors to reduce oxidation and fishy flavor. Several natural antioxidants can be exploited to help delay oxidation of the fish oils in the fortified yoghurt. In our study honey and lemon juice were shown to have good anti-oxidative properties and this helped delay oxidation of the fish oils in the fortified yoghurt.

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QUALITY EVALUATION OF ZOBO (*HIBISCUS SABDARIFFA*) JUICE PRESERVED WITH MORINGA (*MORINGA OLEIFERA)* OR GINGER *(ZINGIBER OFFICINALE)* EXTRACTS AT DIFFERENT STORAGE CONDITIONS

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ABSTRACT

This study aimed to improve the storability of zobo juice naturally. Zobo juice infused with extracts of Moringa seeds or ginger at (0.5 and 1%), control (0% preservative) and, food vendor prepared (FVPZ) zobo were evaluated for 8 weeks. Samples were stored at ambient or refrigeration temperatures and physico-chemical, microbial load and sensory qualities of the juice were analyzed using standard methods. There were drops in pH values after pasteurization from (2.44- 2.75) to (2.31 - 2.58). Vitamin C increased with storage in preserved juice but reduced in control and FVPZ. There were significant ($p<0.05$) differences between samples in total titratable acidity. All of the samples had varying levels of microbial load. Microbial load of raw material ranged from (7.8×10^4 to TNTC), (3.0×10^3 to 3.0×10^4) and (5.6×10^4 to 8.0×10^4) CFU/g, while zobo juice on day 0 had counts ranging from (2.23×10^3 to TNTC); (3.0×10^2 to 8.0×10^4); (4.0×10^4 to 5.6×10^4), CFU/mL for total viable, staphylococcal and fungal count respectively. There was zero enterobacteriaceae count on day 0 but increased during storage. Moringa and ginger zobo juice overall had reduced microbial load during storage compared to zobo without preservative. On day 0 show that FVPZ was more liked: appearance (4.5), aroma (4.5), taste (4.4) and general acceptability (4.7) but scores degenerated during storage. At 8 weeks of storage, 0.5%GZ scored higher in all attributes. Refrigeration retarded microbial growth but did not influence sensory scores. Study concludes that incorporation of moringa or ginger extracts into zobo was effective in improving storability of juice but ginger preserved juice was preferred.

Keywords: Zobo juice, Roselle, ginger, Moringa seeds, drum stick, preservative

INTRODUCTION

Locally produced non-alcoholic drinks such as Kunun zaki and zobo are popular in Northern Nigeria (**Osuntogun, 2004**) and are gaining popularity in other parts of Nigeria because of the low price. Zobo is prepared from the dried calyces of the Roselle (*Hibiscus sabdariffa*) plant. Roselle is popularly acclaimed worldwide for its health claims (**Adegunloye *et al*., 1996; McKay *et al*., 2008**), uses in foods such as herbal teas (**Akanya *et al*., 1997**), jams (**Clydescale *et al*., 1979**), colourant (**Wrolstad, 2000**) and more. Although, zobo is a popular and cheap beverage, one of the major drawbacks is the quality of the raw materials (Roselle calyces, water and bottles) used in preparation and packaging of the juice. Furthermore, it has been documented that the drink deteriorates rapidly within 1-2 days (**Samy, 1980; Omemu *et al*., 2006**) if not refrigerated. This deterioration is most likely due to the activity of microorganisms (**Omemu *et al*., 2006**), making the drink microbiologically unfit, therefore unsafe for consumption.

Concerned consumers want foods and beverages that are wholesome and microbiologically safe. Preservation involves controlling the growth and metabolic activities of spoilage microorganisms in foods, in order to extend shelf life of preserved foods (**Jay *et al*., 2000**). Preservatives can be either chemical such as sodium benzoate or naturally occurring plant compound such as antioxidants. But the inclusion of chemical preservatives in foods is problematic because of toxicity effect on consumers (**Bedin *et al*., 1999; Adesokan *et al*., 2013**). Plant substances with aromatic, strong taste and antimicrobial properties are commonly used to enhance the taste of foods and inadvertently reduce microbial load (**Lanciotti *et al*., 2004**). *Moringa olifera* plant has been documented to have food and medicinal capabilities (**Lipipun *et al*., 2003; Ashok and Pari, 2003; Tahiliani and Kar, 2000**), and also antifungal activities (**Nwosu and Okafor, 1995**). Furthermore, chloroform extracted *M. olifera* seeds has been shown to have antifungal and anti-mould activity (**Caceres and Lopez, 1991; Bukar *et al*., 2011**). Spices such as cinnamon and ginger are also commonly employed in foods for their antimicrobial and preservative effect (**Bello and Adeleke, 2012**). Although a lot of work has been done on preservation of zobo with various spices (i.e. alligator pepper, ginger and garlic) and chemicals (**Nwachukwu *et al*., 2007; Ogiehor *et al*., 2008; Nwokocha *et al*., 2012; Braide *et al*., 2012**), there is no information on the effect of *Moringa olifera* seed extract on the shelf-life of zobo juice. Sensory evaluation is a scientific method that measures possible consumer perception of the product using the senses of sound, sight, smell, touch and taste (**Stone and Sidel, 2004**). Because of increased interest and potential in commercializing zobo juice, sensory analysis is also important in order to determine general acceptability or likeness of the product. Therefore, the objectives of this study were to improve the storability of zobo juice and investigate the effect of natural preservatives (moringa or ginger) extracts on the physico-chemical, microbiological load and general acceptability of the resulting zobo juice during storage.

MATERIALS AND METHODS

Dry calyces of *H. sabdariffa*, *M. oleifera* seeds and fresh ginger rhizomes were obtained from local Odo-Ori market in Iwo, Osun State, Nigeria. Dried Roselle calyces were manually cleaned to remove stones and other unwanted debris, washed thoroughly in sterile de-ionized water and dried in the cabinet dryer (Model F300, Chris Alex Engineering, Ibadan, Nigeria) at (50 ^{0}C) for 8 hours.

Preparation of *Moringa oleifera seeds* and ginger (*Zingiber officinale*) powder

Approximately 100 g of dried pods containing the seeds of *Moringa oleifera* were further dried at room temperature until the pods exposed the seeds. Also, about 100 g of fresh ginger rhizome was cleaned, chopped into small pieces and dried in a laboratory oven (Gen Lab, Widens, England) at 50 ^{0}C for 8 hours prior to the commencement of the study. After drying, both moringa seeds and ginger were ground (MasterChef blender MC-BL1544, China) into powder and stored in a clean air tight container in the Food Science Laboratory at room temperature (32±2 ^{0}C) until used.

Preparation of zobo juice

Zobo juice was prepared according to **Adesokan *et al.*, (2013)** with slight modification. Briefly, 300 g of cleaned calyces of *H. sabdariffa* was added to 1 L of hot boiling water, left to stand for 20 min and then filtered with white muslin cloth. The resulting zobo juice was separately blended with (0.5% and 1% w/v) of moringa or ginger powder. Then, 15g of sugar/L was added to each blend of zobo juice and the juice without any preservative served as control. About 250 ml of the filtrate was hot filled into sterile plastic bottles, and further pasteurized at 75 ^{0}C for 20 min. Overall; there were six sets of samples divided into 4 main groups thus:

a. Zobo juice without treatment (Control)
b. Zobo juice + Ginger (0.5% and 1%, w/v) inclusion
c. Zobo juice + Moringa (0.5% and 1%, w/v) inclusion
d. Food vendor prepared zobo

Food vendor prepared zobo juice was purchased same day this study's juice was prepared for comparison. Each sample set was further divided in two groups. One group was kept on the Laboratory shelf at room temperature (32±2 ^{0}C), while the other was kept in the refrigerator (10 ±2 ^{0}C).

PHYSICO-CHEMICAL ANALYSES OF ZOBO JUICE

Determination of pH

About 10 mL of each sample was dispensed into sterile beaker, diluted with 10 mL of distilled water and mixed thoroughly. Samples were allowed to equilibrate and pH reading in triplicates were recorded (Mettler Toledo AG 8603, Switzerland). Values were taken at day 0 and during storage (2, 4, 6 and 8) weeks.

Total Titratable Acidity

The total titratable acidity (TTA) (expressed as lactic acid) was determined on day 0, using Official method of analysis (AOAC, 1990). Briefly,
1 g of sample was diluted in 10 mL of distilled water and titrated against (0.025N) sodium hydroxide with phenolphthalein as an indicator. TTA was calculated as lactic acid and expressed as percentage.

Vitamin C determination

Ascorbic acid contained in the zobo juices was determined by titrimetric method using iodine according to AOAC, (1990). About 20 mL aliquot of the zobo juice was pipetted into a flask and 150 mL of distilled water plus 1mL of (0.5%) starch indicator solution was added. The samples were titrated with 0.005 mol L^{-1} iodine solution. The concentration of ascorbic acid in each sample was expressed as mg ascorbic acid/100 ml of sample.

Microbiological analysis

Microbial analysis was carried out on the raw materials (Ginger (*Zingiber officinale), Moringa oleifera, Hibiscus sabdariffa* and sugar) used in producing zobo juice. Microbial count was carried out using plate count agar (PCA) for total viable (TVC), Mannitol salt agar (MSA) for staphylococci count and Potato dextrose agar (PDA) for fungal count (Park scientific London, UK). One gram of each raw material was weighed separately into 9 mL of peptone water and further dilution up to 10^{-3} was made as described by **Ogbuile *et al.* (1998)**. Using the pour plate method, 1 mL of 10^{-3} dilution was aseptically transferred into the petri dishes in duplicates for each agar, incubated at 37 ^{0}C overnight and enumerated while PDA was incubated for 48 h. The isolates were characterized and identified by using Gram staining and biochemical tests. After zobo production, one bottle from each set of sample in each group was randomly selected on day of production and every 2 weeks for eight weeks of the study for microbial and sensory analyses. About 1 mL of zobo juice from each sample was pipetted into 9 mL of peptone water and further diluted up to 10^{-3}. Same plating method was used as previously described with the addition of Eosin-Methylene Blue agar (EMB) agar for enterobacteriaceae count.

Sensory analysis

Four sensory tests were performed (day 0, 2, 6 and 8 weeks of sample storage) at the Faculty of Agriculture, using ten untrained panelists cutting across students and staff at Bowen University. Different panelists were recruited every time to evaluate the various zobo juice samples. Refrigerated samples were taken out 3 h before the test in order to allow samples to normalize to room temperature before serving. A scoring test was used which was designed to determine which of the samples was most preferred. Water was provided to the panelist to cleanse their palate in between sample evaluation. Sensory attributes for scoring included appearance, aroma, taste (sweetness or tartness) and overall acceptability of the product. The samples were scored on a 5 point Hedonic scale where 1 = dislike extremely, 2 = dislike moderately, 3 = neither like nor dislike, 4 = like moderately and 5 = like very much (**Meilgaard *et al.*, 1991**). Sample code for sensory test and preparation ratios are presented in Table 1.

Table 1. Sensory codes and preparation ratios of preservative and zobo juice (w/v)

Sample code	Preservative-zobo juice ratio (w/v)	Sample name
BBA	0 g preservative: 1 L zobo	Control
BCA	Food vendor prepared zobo	FVPZ
CBA	5 g moringa: 1 L zobo	0.5%MZ
CDB	10 g moringa: 1 L zobo	1%MZ
ACC	5 g ginger: 1 L zobo	0.5%GZ
ABD	10 g ginger: 1 L zobo	1%GZ

Statistical Analysis

Data collected from sensory tests were analyzed using Statistical Package for the Social Sciences (SPSS) (2011). Analysis of Variance (ANOVA) was used to evaluate significant differences and separation of the mean values was carried out using Duncan Multiple Range Test at ($p<0.05$).

RESULTS

Physico-chemical analyse

pH

The pH values of the different zobo samples immediately after preparation was higher for all the samples (BFP), compared to after pasteurization (AP) and further increased during storage for both samples stored at room and refrigeration temperatures as shown in Figures 1a and1b. There were statistical significant differences ($p< 0.05$) among the samples. The average pH of the samples before pasteurization ranged from 2.44-2.75, with control as the lowest and food vendor prepared being the highest. After pasteurization pH dropped to 2.31-2.58 for control and FVPZ were lowest and highest. From week 4, there was an increase in pH FVPZ (4.12), control (4.07), while the increase in zobo with preservatives were not as high (Figure 1a). During storage there were similarities in pH trend for the samples stored at room and refrigeration temperatures. At room temperature, the pH of all the samples increased (Figure 1b).

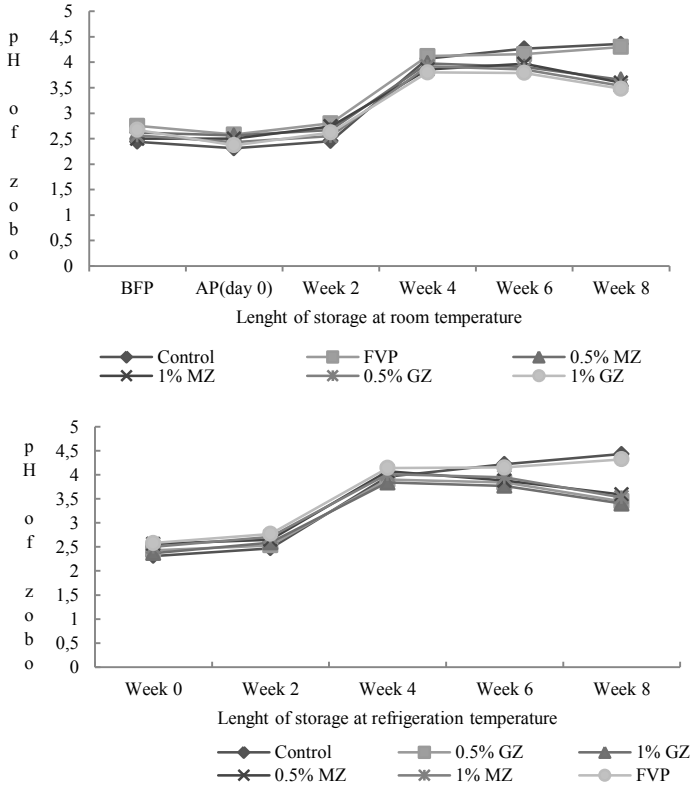


Figure 1 pH of different zobo juice before and after pasteurization and storage at room (A) and refrigeration (B) Temperatures

Total titratable acidity

Total titratable acidity was performed on all the zobo juice samples. The results obtained are reported in Table 2. In general, at week 0, the %Total titrable acidity ranged from .14 to .21% for food vendor prepared zobo and 1% GZ

respectively. There were significant ($p<0.05$) statistical differences between the samples.

Table 2 Percentage Total titratable (%TTA) analysis of zobo samples

Length of storage	Type of zobo					
	Control	FVPZ	0.5%MZ	1%MZ	0.5%GZ	1%GZ
Day 0 (% TTA)	.20^{b} ±0.02	.14^{d} ±0.00	.20^{b} ±1.05	.18^{c} ±0.02	.20^{b} ±0.03	.21^{a} ±0.06

Vitamin C

As reported in Table 3, for week 0, zobo juice with ginger had the highest ascorbic acid content. Juice with 1% ginger had (26.9 mg/100 ml) while 0.5% ginger had (26.7 mg/100 ml) and were significantly different from other samples. Throughout the period of storage at room (32±2 ^{0}C) and refrigerator (10±2 ^{0}C) temperatures ascorbic contents remained high and even increased for the zobo juices with preservatives while it was lower for control and FVPZ. Ginger preserved zobo consistently had higher ascorbic content than Moringa preserved zobo. Vitamin C content in
the preserved drinks during storage either increased or remained at the value in week 0.

Table 3 Mean[1,2] Vitamin C content of control, food vendor prepared and zobo with ginger and moringa during storage

Storage condition (T ^{0}C)	Treatment	Vitamin C content (mg/100ml) during storage in weeks				
		0	2	4	6	8
Room temperature (32±2 ^{0}C)	Control	24.7^{c} ±0.03	23.3^{c}±0.12	23.1^{c} ±0.02	22.5^{c} ±0.03	20.3^{c} ±0.05
	FVPZ*[1]	24.4^{d} ±0.15	23.3^{c} ±0.01	23.1^{c} ±0.03	21.3^{c} ±0.04	21.0^{c} ±0.01
	0.5%MZ**	25.5^{b}±0.04	25.5^{b} ±0.06	24.8^{b} ±0.03	25.3^{b} ±0.10	25.4^{b} ±0.06
	1%MZ	25.7^{b}±0.04	25.6^{b} ±0.04	24.6 ^{b}±0.16	25.3 ^{b} ±0.03	26.2^{b} ±0.21
	0.5%GZ*	26.7^{a}±0.10	26.7^{a} ±0.04	26.4^{a} ±0.07	27.5^{a} ±0.08	29.8^{a} ±0.03
	1%GZ	26.9^{a}±0.15	26.9^{a} ±0.03	26.5^{a} ±0.07	27.7^{a} ±0.03	29.7^{a} ±0.06
Refrigeration temperature (10±2 ^{0}C)	Control	24.7^{c} ±0.03	24.7^{c} ±0.03	23.1^{c} ±0.56	22.6^{c} ±0.01	20.3^{c} ±0.05
	FVPZ	24.4^{d} ±0.15	24.4^{c} ±0.06	23.1^{c} ±0.05	21.5^{c} ±0.05	21.0^{c} ±0.01
	0.5%MZ	25.5^{b}±0.04	25.6^{b} ±0.01	24.8^{b} ±0.01	25.6^{b} ±0.07	25.4^{b} ±0.06
	1%MZ	25.7^{b}±0.04	25.7^{b} ±0.01	24.7^{b} ±0.02	25.5^{b} ±0.08	26.2^{b} ±0.21
	0.5%GZ	26.7^{a}±0.10	26.8^{a} ±0.09	26.8^{a} ±0.04	27.7^{a} ±0.03	29.8^{a} ±0.03
	1%GZ	26.9^{a}±0.15	26.9^{a} ±0.03	26.3^{a} ±0.27	27.8^{a} ±0.01	29.7^{a} ±0.06

1 = mean of three replicates, 2 = means with same alphabets are not different ($p<0.05$) in each column, 3 = Control = zobo juice without ginger or moringa extract. * = Ginger zobo; **Moringa zobo; FVPZ*[1]= food vendor produced zobo.

MICROBIAL ANALYSIS

Microbial quality of raw material

In order to assess the microbial quality of the raw materials (Roselle calyces, ginger, moringa, and sugar) used in the preparation of zobo juice, were subjected to microbial analysis and the results are shown in Table 4. Overall there were growths in the different agars used, indicating that all the raw materials were contaminated. Ginger had the highest total viable count of too numerous to count (TNTC); sugar had highest staphylococcal count (3.0 x10^{4} CFU/g), and moringa had (8.0 x10^{4} CFU/g) and highest fungal count.

Overall, four genera of microorganisms were isolated and identified from the raw material. These were namely *Staphylococcus aureus* (Gram positive cocci, catalase positive and positive mannitol fermentation), *Bacillus subtilis* (Gram positive rods, catalase positive and starch hydrolysis positive) and *Saccharomyces cerevisiae* were from ginger, moringa and Roselle. Additionally, *Micrococcus spp.* (Gram positive cocci, catalase positive and negative growth on mannitol agar), was also isolated from sugar.

Table 4 Microbial load of raw materials used in the preparation of zobo juice

Raw material	**Microbial load (CFU/g*) of raw material**		
	Total Viable Count	**Staphylococcal spp.**	**Total Fungal Count**
Powdered ginger	TNTC**	1.0×10^{4}	NG[1]
Powdered *Moringa*	9.2×10^{4}	3.0×10^{3}	8.0×10^{4}
Sugar	9.2×10^{4}	3.0×10^{4}	7.7×10^{4}
Powdered *H. sabdariffa*	7.8×10^{4}	2.5×10^{4}	5.6×10^{4}

CFU/g* = Colony forming unit per gram of sample; TNTC** = Too numerous to count; NG[1] = No growth

Microbial quality of different zobo juice samples

Results of the microbial load of zobo juice with or without preservative and food vendor prepared are presented in Tables 3 - 6. Day 0 microbial load was performed after cooling but before pasteurization. The total viable count (TVC) on production day was too numerous to count for all the different zobo juice samples except 0.5%GZ (2.2 x 10^{3} CFU/mL) (Table 5). Continual increase in count ranging from (2.3 x 10^{5} CFU/mL to TNTC) was observed throughout the period of storage for control and food vendor prepared zobo (FVPZ) while in zobo with preservatives, TVC reduced ranging from (5.0×10^{2} to 2.0×10^{5} to 1.5×10^{3} to 8.5×10^{3}) for weeks 2, 4, 6 and 8 of storage. There were significant differences in counts between week 4 and weeks 6 and 8 of storage, indicated by the 2-3 log reduction. Refrigerated samples had slightly lower TVC during storage and it was observed that 1% ginger was slightly more effective in reducing total viable count. Staphylococcal counts are reported in Table 6. FVPZ had the highest count (8.0 x 10^{4} CFU/mL) on production day and throughout storage. Juice with preservative (0.5 -1%) inclusion did not have growth on day 0 except 1% moringa (3.0 x 10^{2} CFU/mL). The natural preservatives were able to keep staphylococcal growth in check during the eight weeks of storage while FVPZ and control zobo continued to show increased count. Refrigeration did not appear to reduce staphylococcal count until week 8 of storage indicated by (NG) in all the preserved zobo. There was no growth on EMB agar for day 0, probably because of the high temperature employed in preparing the zobo juice which may have resulted in microbial stress. Throughout storage period, only lactose negative enterobacteriaceae was observed and enumerated in all the juice samples. At week 2 of storage, 0.5% GZ had the highest count (3.0 x 10^{3}), but at week eight there was 0 count while the control (1.5 x 10^{2} to 1.4 x 10^{3}) and FVPZ (2.1 x 10^{3} to 1.4 x 10^{3}) CFU/mL were high for room and refrigerated samples (Table 7). It was also observed that storing the zobo samples at refrigeration temperature resulted in lower count, and that the ginger extract was more effective than moringa in reducing enterobacteriaceae count. Total fungal count was high on the production day with 0.5%GZ having the highest load (5.6 x 10^{4}) but subsequently reduction in count was observed at weeks 2, 4, 6 and 8 except the food vendor prepared zobo (Table 8).

Table 5 Total viable count (CFU/ mL) of control, food vendor prepared and treated zobo juice during storage

Samples	Total Viable count CFU/ mL during period of storage				
	Day 0	Week 2	Week 4	Week 6	Week 8
Rm Temp (32±2^{0}C)					
Control	TNTC[2]	TNTC	TNTC	TNTC	TNTC
FVPZ[1] juice	TNTC	TNTC	TNTC	TNTC	TNTC
0.5% MZ* juice	TNTC	1.2×10^3	2.0×10^5	1.5×10^3	1.5×10^3
1% MZ juice	TNTC	5.0×10^3	1.8×10^5	1.2×10^3	1.3×10^3
0.5% GZ** juice	2.2×10^3	1.1×10^3	1.3×10^5	1.5×10^3	9.4×10^2
1% GZ juice	TNTC	2.0×10^3	1.2×10^5	1.3×10^3	8.5×10^3
Chilling Temp (10±2^{0}C)					
Control	TNTC	TNTC	2.3×10^5	TNTC	TNTC
FVPZ juice	TNTC	TNTC	TNTC	TNTC	TNTC
0.5% MZ juice	TNTC	1.0×10^3	2.0×10^5	1.5×10^3	1.2×10^3
1% MZ juice	TNTC 2.2×10^3	5.0×10^2	1.5×10^3	1.2×10^3	1.2×10^3
0.5% GZ juice	TNTC	1.2×10^3	1.1×10^5	1.1×10^3	8.6×10^2
1% GZ juice		1.2×10^3	1.2×10^5	1.2×10^3	8.0×10^2

Means of two readings; *MZ = Moringa zobo; **GZ = ginger zobo; FVPZ[1] = Food vendor produced zobo; TNTC[2] = too numerous to coun

Table 6 Staphylococcal count (CFU/ mL) of control, food vendor prepared and treated zobo juice during storage

Samples	Staphylococcal count CFU/ mL during period of storage				
	Day 0	Week 2	Week 4	Week 6	Week 8
Rm Temp (32±2^{0}C)					
Control	4.5×10^4	1.6×10^2	1.6×10^2	3.5×10^2	9.5×10^2
FVPZ juice	8.0×10^4	TNTC	3.2×10^2	7.8×10^2	9.8×10^3
0.5% MZ juice	NG	3×10^1	1.9×10^2	1.5×10^2	3.5×10^3
1% MZ juice	3.0×10^2	1.1×10^2	NG	6.0×10^1	8.0×10^1
0.5% GZ juice	NG[a]	NG	1.2×10^2	NG	1.0×10^2
1% GZ juice	NG	NG	1.3×10^2	1×10^1	NG
Chilling Temp (10±2^{0}C)					
Control	4.5×10^4	1.0×10^2	2.2×10^2	1.8×10^2	8.1×10^2
FVPZ juice	8.0×10^4	1.5×10^2	8.0×10^2	1.2×10^3	1.0×10^3
0.5% MZ juice	NG	NG	1.4×10^2	1.5×10^2	NG
1% MZ juice	3.0×10^2	4.0×10^1	NG	1.4×10^2	NG
0.5% GZ juice	NG	NG	1.4×10^2	6×10^1	NG
1% GZ juice	NG	NG	9.0×10^1	6×10^1	NG

GZ = ginger zobo; MZ = moringa zobo; [1]FVPZ= food vendor produced zobo. TNTC = too numerous to count; NG[a] = no growth

Table 7 Enterobacteriaceae count (CFU/ mL) of control, food vendor prepared and treated zobo juice during storage

Samples	*Enterobacteriaceae* count CFU/ mL during period of storage				
	Day 0	Week 2	Week 4	Week 6	Week 8
Rm Temp (32±2^{0}C)					
Control	NG	1.0×10^3	3.6×10^2	1×10^3	1.5×10^2
FVPZ juice	NG	1.4×10^3	2.0×10^2	1.3×10^3	2.1×10^3
0.5% MZ juice	NG	1.0×10^3	9.6×10^2	2.5×10^2	8.0×10^2
1% MZ juice	NG	NG[a]	4.3×10^2	2.3×10^2	1.0×10^2
0.5% GZ juice	NG	3×10^3	1.8×10^3	8×10^1	NG
1% GZ juice	NG	NG[a]	2.0×10^2	NG	NG
Chilling Temp (10±2^{0}C)					
Control	NG	NG	6.0×10^1	8.0×10^2	1.4×10^3
FVPZ juice	NG	1.0×10^1	2.6×10^2	2.0×10^2	1.4×10^3
0.5% MZ juice	NG	NG[a]	1.1×10^2	1.5×10^2	NG
1% MZ juice	NG	4.0×10^1	8.0×10^1	1.4×10^2	NG
0.5% GZ juice	NG	NG[a]	1.1×10^2	1×10^1	NG
1% GZ juice	NG	NG[a]	6.0×10^1	NG	NG

Table 8 Fungal count (CFU/ mL) of control, food vendor prepared and treated zobo juice during storage

Samples	Total fungal count CFU/ mL during period of storage				
	Day 0	Week 2	Week 4	Week 6	Week 8
Rm Temp (32±2^{0}C)					
Control	4.0×10^4	7.0×10^2	7.1×10^2	2.4×10^3	2.4×10^3
FVPZ juice	4.0×10^4	7.6×10^2	TNTC	TNTC	TNTC
0.5% MZ juice	5.0×10^4	5.2×10^2	5.2×10^2	4.4×10^3	NG[a]
1% MZ juice	4.0×10^4	5.0×10^2	8.4×10^2	4.6×10^3	NG
0.5% GZ juice	5.6×10^4	4.0×10^2	6.4×10^2	NG	NG
1% GZ juice	5.5×10^4	3.6×10^2	5.5×10^2	NG	NG
Chilling Temp (10±2^{0}C)					
Control	4.0×10^4	3.0×10^2	1.9×10^3	2.4×10^3	2.5×10^3
FVPZ juice	4.0×10^4	7.0×10^2	TNTC	TNTC	TNTC
0.5% MZ juice	5.0×10^4	4.7×10^2	2.7×10^3	NG	3.4×10^2
1% MZ juice	4.0×10^4	NG	1.5×10^3	5.2×10^2	1.2×10^2
0.5% GZ juice	5.6×10^4	4.0×10^2	1.4×10^3	NG	NG
1% GZ juice	5.5×10^4	NG	7.6×10^3	NG	NG

SENSORY EVALUATION ANALYSIS

Sensory evaluation of the quality attributes of the various zobo samples were carried out on production day and every two weeks of storage for eight weeks except week four. Of the total number of panelists (40) who participated in sensory analysis, there were 24 (60%) females and 9 (22.5%) males, of which 13 (32.5%) were in age range of 18-19 and 19 (47.5%) were 20-30 years old.

Scores of appearance, aroma, taste and general acceptability of the juice on production day and during storage are presented in Figures 2- 5. For appearance, BCA (FVPZ) had higher degree of liking of (4.5) and the lowest was for 1%GZ (3.8) on day 0. However during storage (FVPZ) and control (BBA) deteriorated in appearance (1.5 - 2.2) but preserved zobo scored higher ranging from (4.4 - 4.7). Similar trend was observed for aroma FVPZ (4.5), control and (ABD) 1%GZ (3.9) were the highest and lowest respectively on day 0. During storage BCA and BBA were scored lower and preserved zobo higher (Figure 3). ACC was scored higher in all attributes except taste at refrigeration temperature 2 weeks after storage (Figure 3). In taste and general acceptability, BCA had (4.4) and (4.7) on day 0, compared to other zobo samples but lost likeability during storage (Figures 4 and 5). There were significant differences ($p<0.05$) between BCA (FVPZ) and BBA (control), ABD (1%GZ) CBA (0.5%MZ) and CDB (1%MZ) in taste and overall acceptability. At 6 and 8 weeks of storage zobo samples with ginger ACC and ABD had high general acceptability/likeability scores (4.6 and 4.5) while BCA and BBA (1.8 and 1.4) quality were scored very low as presented in Figure 5. Taste tests of BCA and BBA were not performed at weeks 6 and 8, because of the appearance and aroma of the juice and food safety concern.

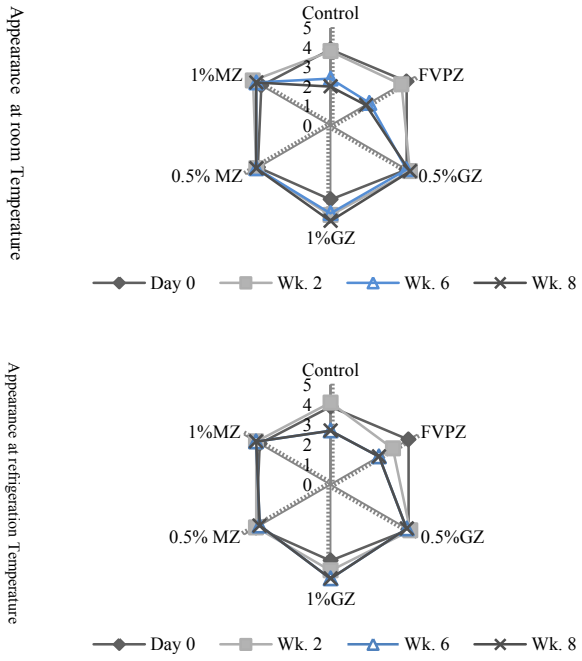
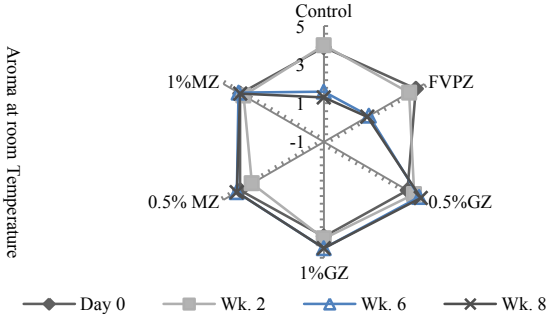


Figure 2 Appearance scores of zobo juice during storage at room (A) and refrigeration (B) temperatures.
BBA = control, BCA = FVPZ, CBA = 0.5%MZ, CDB = 1%MZ, ACC = 0.5%GZ, ABD = 1%GZ



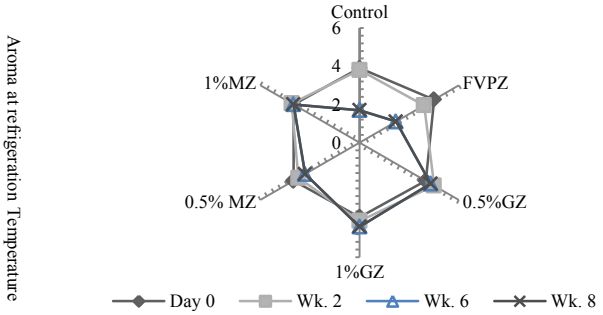


Figure 3 Aroma scores of zobo juice during storage at room (A) and refrigeration (B) temperatures.

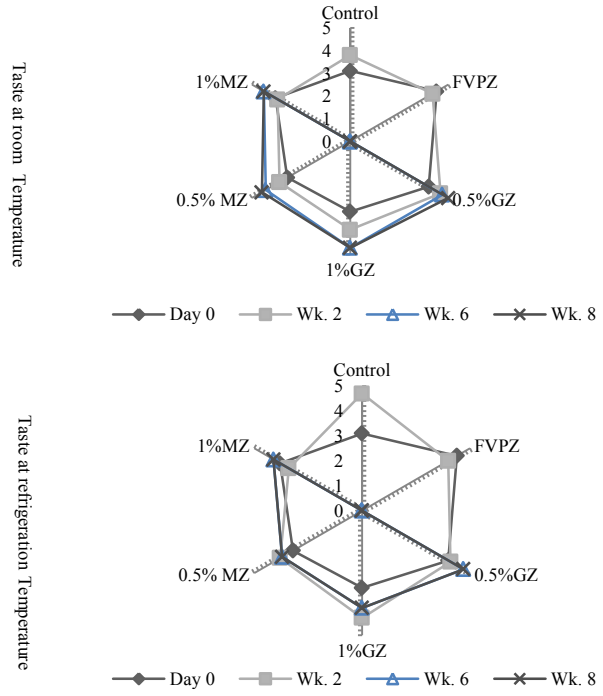


Figure 4. Taste scores of zobo juice during storage at room (A) and refrigeration (B) temperatures.

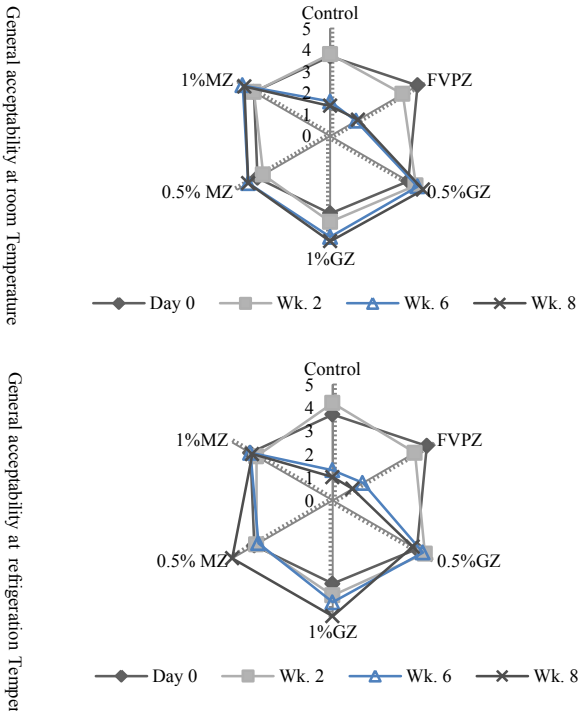


Figure 5 General acceptability scores of zobo juice during storage at room and refrigeration temperatures.

DISCUSSION

The pH results in this study is within range of other studies indicating that zobo drink is acidic in nature (**Doughari *et al.*, 2008; Omemu *et al.*, 2006; Ifie *et al.*, 2012**) but contradicts (6.63) and (7.05) values of **Adesokan *et al.*, (2013)**. Low pH value of 3.0 has been reported to be suitable for preservation of zobo juice (**Omemu *et al*., 2006**). Furthermore, according (**Leahu *et al.*, 2013**), orange juice has a pH of 3.30-3.82, pineapple juice 3.30-3.60 and grapefruit juice 2.90-3.25 and these fruit juices fall within the range of high acid foods. According to **Frazier and Westhoof (1988)**, zobo juice belongs to a class of foods referred to as high acid foods. High acid foods do not support survival of many pathogenic organisms but favours the proliferation of acidic organisms. The results of % TTA is low, low titratable acidity is also reported by **Bolade *et al.*, (2009)**, and it was attributed to low pH value and the status of zobo not being a product of fermentation. The vitamin C content in this study is within the range of (17.2 to 30.7 mg/100 ml) reported by **Bolade *et al.*, (2009)**. Furthermore, pH is the main factor affecting the stability of vitamin C, consequently high values of pH favour the oxidation processes of vitamin C (**Leahu et al., 2013**). Although *Hibiscus sabdariffa* has been documented to have antioxidants (**Oboh and Okhai, 2012**), the addition of moringa and ginger also contributed to the antioxidant content. Hence the preserved zobo drinks were able to withstand oxidation and prevent degradation of vitamin C during storage. The result also show that was no significant difference among the zobo drinks stored at room and refrigerator temperatures.

The total viable count for Roselle exceeds the allowable limit of < 10,000 CFU/g set by export markets (**FAO, 2004**). The high incidence of contamination could be due to poor harvesting and handling techniques, unclean containers, such as the bowls in which the raw materials were stored before sale, poor sanitary and largely unhygienic storage conditions and un-kept environment, where the raw materials are sold (**Ehiri *et al*., 2001**). Microorganisms isolated from the raw materials included *Bacillus subtilis, Staphylococcus aureus, Micrococcus* spp., and *Saccharomyces cerevisiae*. The majority of these microorganisms are normal plant microflora and inhabitant of the soil or they contaminate the material by human action. Other studies have also reported isolation of these microorganisms from the raw materials (**Nwafor and Ikenebomeh, 2009; Egbere *et al*., 2007**). Furthermore, some of the microorganisms isolated are persistent because they are able to withstand harsh environmental conditions and have ability to form spores (**Pelczar *et al*., 1993**). At the initial day of zobo preparation, total viable, staphylococcal and fungal counts were high. Although, according to **Frazier and Westhoff, (1988)**, the microbial loads for all the zobo juice samples were within the limits of (10^5 CFU/mL) for ready to consume food products. The presence of *Staphylococcus aureus* is indicative of poor personal hygiene conditions or excessive handling of the beverage (**Ajayi and Oluwoye, 2015**) and is of concern particularly because of multidrug resistant strains. It is noteworthy that enteric microorganisms were not recovered from the raw materials and on day 0 but were subsequently isolated from the juice during storage. Since common intestinal microorganisms particularly pathogens are readily inactivated by heat, it is possible that boiling may have inactivated the enteric microorganisms. Furthermore, temperatures above 60 ^{0}C for a few minutes have been documented to be sufficient to kill enteric microorganisms (**Backer, 2012**). Also, according to **Noor *et al.* (2013)**, increase in temperature cause stress to microorganisms by denaturing their proteins. During storage of zobo, only non-lactose fermenter enterobacteriaceae were isolated and enumerated and is in agreement with the report by **Omemu *et al.*, (2006)**, and could be from the water used in preparation. There were fluctuations in the microbial load of the juice during storage that may coincide with microbial growth phase and effect of the preservatives. For example, a decrease in count was observed during week 2 of storage, and then an increase in count was observed at week 4. Then reduction in counts was observed at weeks 6 and 8. Similar trends of increase in microbial counts observed in this study were also reported by **Omemu *et al*., (2006) and Ogiehor *et al.*, (2008)**. Reduction of microbial load was also reported by **Egbere *et al.* (2007) and Doughari *et al.* (2008)** using chemical preservatives.

At week 8 there was a decrease in microbial load in all of the samples except the control sample and food vendor prepared zobo juice at both ambient and refrigeration temperatures. Decrease in microbial load observed in the samples that contained 0.5 and 1% ginger and moringa may be related to the antimicrobial effects and phytochemicals shogaol and zingerol present in ginger (**Kolapo *et al*., 2007**). Also, **Singh *et al.* (2009)** reported the presence of gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin glycosides, phenols, sterols, flavanol glycosides from the aqueous extracts of leaves, fruits and seeds of *M. oliefera* which may be responsible for the antimicrobial effects in zobo juice. Furthermore, *Moringa olifera* seeds contain isothiocynates which are documented to have antimicrobial properties (**Padla *et al.*, 2012**). Similar findings of microbial load reduction have been documented for related food items such as palm wine (**Ogiehor *et al*., 1998**) and African oil bean seed (**Ogbulie *et al*., 1993**). Microbial counts of the different types of zobo juice were further reduced during storage when combined with refrigeration. For example, at week 8 of storage, juice with preservatives had reduced TVC (8.0×10^2 to 1.2×10^3 CFU/mL); staphylococcal (NG); enterobacteriaceae (NG); and fungal counts ranged from (NG to 3.4×10^2). Reduction in microbial count during refrigeration was also reported by (**Fasoyiro *et al.*, 2005; Ogiehor *et al.*, 2008**). The decrease in microbial load of samples stored at refrigeration temperature may be the synergistic effects of boiling, addition of ginger or moringa, pasteurization and low temperature on the associated microorganisms in the juice. This helped to minimize the microbial activities by creating barriers which the microorganisms present were unable to overcome (**Ogiehor *et al.*, 2008**). Refrigeration in combination with preservative agents helps to stabilize the microbial, nutritive quality (**Ogiehor *et al*., 2008**) and extend the shelf life of the zobo juice. Similar findings have been reported for related indigenous and novel food items such as palm wine, apku and zobo (**Ogiehor *et al*., 1998; Ogiehor *et al*., 2004; Ogiehor, *et al*., 2008**). The shelf life of zobo juice is less than three days without any form of preservatives (**Samy, 1980**). The utilization of moringa as a preservative in zobo juice is a novel idea that can be further explored. Albeit the control sample and food vendor prepared zobo juice were also subjected to pasteurization, the microbial loads were higher due to the absence of preservatives in the samples at both storage conditions as recorded in Tables 3-6.

At the onset of the study food vendor prepared zobo was preferred in all attributes but as storage continued, deterioration in sensory quality was observed as the food vendor prepared zobo and control were slimy and have strong fermentative (sour and alcoholic) aroma. Refrigeration did not impact the sensory attributes scores in this study, although it has been documented that low temperature can be used to reduce microbial activity in foods (**Ogiehor, 2007; Ray and Bhunia, 2014**). An increase in volatile components when samples are heated has been reported by **Atkins and Locke, (2002)**, resulting in more intense odour (**Voirol and Daget, 1989**) and influencing sensory attributes. **Delwiche (2004)**, reported that food sample might contain volatile compounds that are below threshold levels at lower temperatures, which might explains why zobo samples stored at refrigerated temperature scored lower than room temperature in aroma and taste during weeks 6 and 8. Inclusion of natural preservatives (moringa or ginger) contributed to maintaining the sensory attributes such as appearance, aroma, taste and acceptability of the juice.

CONCLUSION

Some physico-chemical qualities of zobo juice stored at room or refrigerated temperatures for eight weeks were analyzed. During storage, pH of all the juice samples increased, but control and food vendor prepared juice increased more than the preserved juice and also there were significant losses in vitamin C content. However, the juice samples with preservatives were able to keep pH in check, thereby limiting loss of vitamin C. Microbial analysis report of the raw materials used in producing zobo show contamination with a variety of microorganisms such as *Staphylococcus, Bacillus subtilis, Micrococcus* and *Saccharomyces cerevisiae.* Addition of moringa or ginger extracts (0.5 and 1%) to zobo juice alone and in combination with refrigeration proffered resistance to the growth of microorganism, thereby reducing the total viable, enterobacteriaceae, staphylococcal, and fungal counts. Lower microbial counts extended the shelf life of the juice to 8 weeks. Ginger extract showed more effectiveness against microorganisms in zobo than moringa extract. From sensory qualities (appearance, aroma, taste and overall acceptability), panelists scored ginger spiced (0.5-1%) zobo higher during storage period in the attributes analyzed and is able to maintain the sensory qualities better than moringa zobo, zobo without preservatives and food vendor prepared zobo.

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QUALITY OF CHOK ANAN MANGO AS AFFECTED BY TAPIOCA-SAGO STARCH COATING SOLUTIONS STORED AT ROOM TEMPERATURE

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ABSTRACT

Chok Anan mango, a climacteric fruit, is easily susceptible to quality deterioration during storage. However, no research has been conducted related to the application of tapioca and sago starch coatings on Chok Anan mangoes. Therefore, the aim of this research was to study the effect of tapioca-sago starch coating towards the quality of Chok Anan mango through storage. Hard matures Chok Anan mangoes (stage 2) were coated with different ratios of tapioca (T) and sago starch (S), i.e. 25%T: 75%S, 50%T: 50%S and 75%T: 25%S. Uncoated mangoes were served as the control. Coated and uncoated Chok Anan mangoes were packed in corrugated box to ripen at ambient temperature (27±2˚C) for 13 days. Starch analysis demonstrated that sago contained higher amylose (28.15%) than tapioca starch and thus a higher percentage of sago starch in the coating solution (25%T: 75%S) contributed to higher coating's viscosity (75.90 cP). Analysis of coated mangoes exhibited significant better fruit quality as compared to the uncoated samples. Application of starch coating solutions with high percentage of sago starch (50%T: 50%S and 25%T: 75%S) were effectively delayed the increased of disease incidence, moisture loss and total soluble solids content as well as delayed the decreased of titratable acidity content during storage. Nevertheless, towards the final day of storage (day 13), no significant difference was shown in the peel color index, firmness and ascorbic acid content of Chok Anan mangoes between all coatings formulations applied.

Keywords: Chok Anan mango, quality, sago, tapioca

INTRODUCTION

Chok Anan mango is one of the mango varieties that gained high demand in Malaysia. However, mango, being a climacteric fruit, is highly perishable and easily susceptible to physiological deterioration after harvest **(Baldwin *et al.*, 1999)**. Normally, the shelf life of mangoes has been just around 4 to 8 days at ambient temperature storage (27±2˚C) **(Jha *et al.*, 2010; Habib *et al.*, 2009)**. This short shelf life gives rise to extreme difficulties, especially for long distance and export markets. Hence, further investigation is needed to encounter this issue.

One of the effective approaches to extend the shelf life of mango is through the application of edible coatings. Several researches have proved that edible coatings helped to reduce the post-harvest losses by slowing down the mango ripening, hence lead to better preservation of colour, firmness, titratable acidity, total soluble solids and total ascorbic acid contents **(Gracia *et al.*, 1998; Dang *et al.*, 2008; Kittur *et al.*, 2014).** Among all the coatings' component choices (polysaccharides, proteins and lipids), starch, which is under the groups of polysaccharides, is the most selective components used in fruits coating **(Bibi and Baloch, 2012; Kittur *et al*, 2001)**. This is due to its odorless, tasteless, colorless, impermeable to oxygen, biodegradable and safe to be consumed characteristics **(Pareta and Edirisinghe, 2006)**.

Nevertheless, due to the shortage and increasing prices of other starches (wheat, corn and soybeans), tapioca starch is viewed as an alternative source by food companies **(FAO, 2004)** in fruits' coating **(Chiumarelli *et al.*, 2011; Castricin *et al.*, 2012)**. Tapioca starch is known to have high viscosity **(Moorthy, 2004)** and clear appearance **(William, 2009).** However, currently, in Malaysia the total plantation area of tapioca starch has dropped steadily from 20913 ha in 2000 **(Tan and Idris, 2000)** to 2596 ha in 2011 **(Jabatan Pertanian Malaysia, 2013)**. Thus, starch from sago palm has gained more attention recently as this underutilized palm has been an extremely sustainable plant with the ability to thrive in most soil conditions **(Singhal *et al*, 2008)**. Despite of having almost the same characteristic with tapioca starch, sago starch is easy to gelatinize, high in viscosity and undergo low syneresis **(Takahashi, 1986)**. Moreover, in 2013, sago has been declared as the "Golden Crop in the 21st Century" **(Mahmud, 2013)**. Several researchers have studied the performance of sago starch in food systems and edible film makings **(Karim *et al.*, 2000; Javanmard *et al.*, 2012)**. However, studies on the performance of coated fruit by using sago starch are still scarce. Owing to the possible potential of sago starch being used as coating ingredients either exclusively or partially with other types of starch, and the lack of published work regarding sago in coating solutions, hence, the effect of different combination of of tapioca-sago starch coating solutions on post-harvest quality of Chok Anan mangoes stored at ambient temperature for 13 days storage were studied.

MATERIAL AND METHODS

Starch Samples

Tapioca starch (granule size ranged from 9.1- 20µm) and sago starch (granule size ranged from 13.9 - 37.6µm) were purchased from Eugene Chemicals Sdn. Bhd, Malaysia.

Total Amylose Content

Total amylose content in starch was determined quantitatively based on **Hoover and Ratnayake (2001)** method. An amount of 5.0ml of the sample (dissolved starch or amylose standard solution) prepared in were mixed with 5ml of I_2-KI solution. Then, the volume was adjusted to 20ml with distilled water, mixed vigorously and left at room temperature for 15 minutes to allow the development of a blue colour of amylose-iodine complex. Then, the absorbance of the sample was measured using a spectrophotometer (UV Vis-1800, Shimadzu, Japan) at 600nm against distilled water as a reference.

Preparation of Coating Solutions

The coating solutions were prepared according to **Gracia *et al.* (2010)** method with slight modification. An amount of 3% starch powder with different ratios of tapioca (T) to sago (S) starches i.e. F1 (50%T:50%S), F2 (75%T:25%S) and F3 (25%T:75%S) were dissolved in distilled water. The starch mixtures were stirred and heated until 90±2°C and maintained for 3 minutes at this temperature to

accomplish starch gelatinization. Then, 1% (w/v) of palm oil together with 0.5% (w/v) of glycerin were added and stirred continuously for 3 minutes, followed by the addition of 0.5% (w/v) potassium sorbate for 5 minutes. After that, the coating solutions were homogenized for 10 min and left it to cool down to room temperature.

Viscosity of Coating Solutions

The viscosity of tapioca-sago starch coating was measured using a Brookfield digital Viscometer model DV-E (Brookfield engineering labs. INC., Milddleboro, USA) with spindle number 2 at 100 rpm. Triplicate readings were taken by immersing the spindle into the coating solutions for 3 minutes to obtain thermal equilibrium between solutions and spindle with continued shearing **(Al-Hassan and Norizah, 2011)**. The readings were expressed in centipoises (cP). The viscosity measurement was conducted when the coating solutions reached room temperature (27±2°C).

Plant Materials

Hard mature Chok Anan mangoes (13 weeks after flowering) were obtained from Sui Yuan orchard, Kuala Bikam, Perak. Uniform size (250-300g), free from blemish and decay of Chok Anan mangoes were chosen. All the chosen mangoes were at index maturity stage 2.

Coated Chok Anan Mangoes

Chok Anan mangoes were washed, sanitized using 200ppm sodium hypochlorite solution for 2 minutes, rinsed and dried prior to coating. Then, Chok Anan mangoes were dipped in the coating solutions for 30 seconds and drip off for 2 min. After that, the coated Chok Anan mangoes were air-dried for 30 minutes at ambient temperature, packed in the corrugated box and stored at ambient temperature (27±2°C) for 13 days. Uncoated Chok Anan mangoes were served as the control. All the assessments on coated and uncoated Chok Anan mangoes were carried out for every 2 days of storage interval started on day 1.

Observation of the Disease Incidence

Chok Anan mangoes were considered infected when a visible lesion was observed. Visible microbial attacked on the mango was characterized as brown spots and a softening of the injured zone. The degree of disease incidence were assessed according to the percentage of disease area affected per fruit (**Khaliq, *et al.* (2015)**. The percentage score was then related to a 5-point scale, where 0 = 0% area affected, 1 = 1-5% area affected, 2 = 6-15% area affected (mild), 3 = 16-30% area affected (moderate) and 4 = 31-100% area affected (severe).

Determination of Physiological Weight Loss

The weight loss percentage of Chok Anan mangoes were calculated as the fresh weight change at each sampling time divided by the initial weight of the mangoes **(Mohamed, *et al.*, 2015)**.

Measurement of the Peel Color Indexes

The peel color of Chok Anan mangoes measurements i.e. lightness (L*), redness (a*), yellowness (b*), chroma (C*) and hue angle (h°) were determined using a Chroma meter (CR 400, Konica Minolta, Japan). Color was measured at each of the two equatorial opposite sides: exposed area and shaded area. Calibration was carried out prior to the determination process **(Mohamed, *et al.*, 2015)**.

Determination of Fruit Firmness

The firmness of Chok Anan mangoes was measured using a texture analyzer (TA. XTPlus, Stable Micro System Ltd, UK) by measuring the maximum penetration force required. The P2N probe was used to penetrate into Chok Anan mangoes at a rate of $5mms^{-1}$. The downward distance was set at 10 mm and automatic return **(Rojas-Grau, *et al.*, 2009).** Three noses of Chok Anan mangoes for each treatment were taken at three different areas (upper, middle and lower parts) for both sides. The firmness was reported as peak force and expressed in newton per gram of sample.

Determination of Total Soluble Solids (TSS) Content

TSS was determined according to the AOAC standard method where the analyses were performed with a Milwaukee MA871 digital refractometer with automatic temperature compensation. Edible flesh of Chok Anan mangoes was cut into small cube and the juice was extracted by hand, pressed the flesh in a piece of cheesecloth. Two drops of the mango juice were dropped on the prism of the refractometer. Readings were expressed in ºBrix.

Determination of Titratable Acidity (TA) Content

TA was measured by titration method with 0.1N NaOH and expressed in percent of citric acid/100ml of juice. Approximately 10ml of the Chok Anan mangoes were homogenized using blender with 40ml distilled water and then filtered through cotton wool. Three drops of 0.5% phenolphthalein were dropped inside the 5 ml of diluted juice solution and titrated using 0.1N NaOH to pH 8.2.

Determination of Total Ascorbic Acid(TAA)

The determination of the TAA concentration in Chok Anan mangoes was carried out according to the method reported by **Khan and Singh (2009)**. Approximately 5g of Chok Anan mango was homogenized in a glass mortar with 25ml of 6% metaphosphoric acid containing 0.18g of EDTA. The homogenate was centrifuged at 3000rpm for 15 minutes. The supernatant (500 µL), 3% of metaphosphoric acid (100 µL), 1400µL distilled water and diluted 200µL Folin reagent (Folin: distilled water, 1:5 v/v) were mixed and vortex for 2 minutes. Disposable cuvette (2 ml) was used to record the absorbance of the mixed sample after 10 minutes at 760nm wavelength using a UV-VIS spectrophotometer. Ascorbic acid concentration was quantified using a standard curve of L-ascorbic acid and was expressed as mg 100^{-1} fresh weight.

Statistical Analysis

A complete randomized design was used and all data were analyzed by using Statistical Analysis System (SAS 9.3, version 16, 2012). The experimental data for coated Chok Anan mangoes were subjected to two-way analysis of variance (ANOVA) (coating solutions treatment x storage time). LSD was calculated following a significant ($p \leq 0.05$) F-test. Data were stated as mean ± standard error of means.

RESULTS AND DISCUSSION

Starch Amylose Content

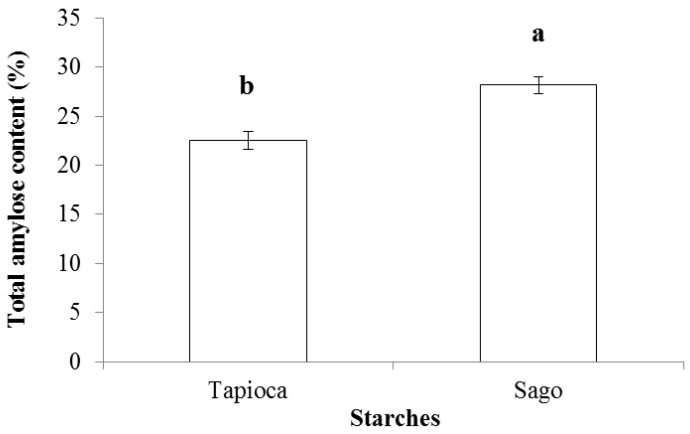


Figure 1 Total amylose content of starches using iodine-binding method. Vertical bars represent standard deviation (n=3)

It is evident that sago starch contained higher amylose content (28.15%) as compared to tapioca starch (22.55%) (Figure 1). As referred to **Al-Hassan and Norziah (2012)**, amylose content in sago starch was found to be in the range of 32-34%. The differences in amylose content in sago starch might probably due to the different parts of the harvested trunk **(Singhal *et al.*, 2008)**. Whereas, the amylose content of tapioca starch was slightly higher than the established value (17%) but it is well agreed with the published values of 21.5%-22.5% as reported by several researchers **(Chen *et al*., 2009; Sobolewska-Zielinska and Fortuna, 2010)**.

Coating Solutions Viscosity

Result showed that, viscosity was significantly higher ($p \leq 0.05$) in 25%T: 75%S (75.90 cP) starch coating solutions followed by 50%T: 50%S (75.33 cP) and 75%T: 25%S (73.85 cP) starch coating solutions measured at ambient temperature (27±2°C) (Table 1).

Table 1 Viscosity of tapioca-sago starch coating solutions measured at 27 ± 2°C

Coating Solutions	Viscosity (cP)
25%T: 75%S	75.93±0.10[a]
50%T: 50%S	75.33±0.11[b]
75%T: 25%S	73.85±0.12[c]
Least significant difference of means at p ≤ 0.05 level	0.32***

Mean ± standard deviation with different letters showed significant difference (p ≤ 0.05) by the least significant difference (LSD) with n=3

The result showed that the viscosity of tapioca-sago starch coating solutions increased with the increased of sago starch which might attribute by the higher percentage of amylose contained in its starch composition (Figure 1). This was explained by **Xie, *et al.* (2009)** that high amylose content contributed to high viscosity was due to the long linear amylose chains being more easily susceptible to entanglement compared with the inflexibility of the short branched chain in amylopectin.

Disease Incidence

No significant interaction (p>0.05) between the storage time and coating formulation was recorded for the disease incidence of Chok Anan mangoes. However, an increment (p≤0.05) for disease incidence of Chok Anan mangoes was shown after seven days of storage (Table 2). This might be associated with the increment of fruit ripening **(Khaliq *et al.*, 2015)**. Meanwhile, regardless of the storage time, disease incidence of Chok Anan mangoes coated with 25%T: 75%S starch solution was significantly lowered as compared to 75%T: 25%S starch solution and control. The mangoes coated with 25%T: 75%S starch solution exhibited the lowest scale (0.29) for the disease incidence parameter (Table 2). This formulation exhibited an approximately 77.52% inhibition of pathogen development as compared to the control. This might be due to the higher percentage of amylase content in sago starch (Figure 1) which promoted strong cohesive coating properties that can reduce coating porosity **(Wittaya, 2012)**. Thus, preventing the pathogen from entering through the skin surface to fruit tissue, **(Yaman & Bayındırlı, 2001)** and thereby reduced the disease incidence occurred on mangoes. On the other hand, disease incidence in mangoes coated with 75%T: 25%S (0.90) starch solution was comparable to control (1.29) and 50%T: 50%S starch solution (0.57) (Table 2).

Table 2 Effect of various tapioca-sago starch coating formulations and storage time of the disease incidence of coated Chok Anan mangoes stored at ambient temperature (27±2°C).

Factors	Disease Incidence
Coating Formulations	
0% (Control)	1.29±0.29[a]
25%T: 75%S	0.29±0.21[c]
50%T:50%S	0.57±0.20[bc]
75%T: 25%S	0.90±0.10[b]
Storage Time (Days)	
1	0.00±0.00[d]
3	0.00±0.00[d]
5	0.08±0.08[d]
7	0.67±0.19[c]
9	1.08±0.23[bc]
11	1.42±0.29[b]
13	2.08±0.36[a]
Least significant difference of means at p ≤ 0.05 levels	
Coating Formulations (F)	0.37*
Storage Time (S)	0.49*
F × S	NS

Mean ± standard error of means followed by the different letters within the same factors denote a significant difference (p ≤ 0.05) by the least significant difference (LSD) with n=6. * = significant at p ≤ 0.05

Physiological weight loss

There was an interaction between the storage time and coating formulation (p≤0.05) for the physiological weight loss of Chok Anan mangoes. It is notable that the physiological weight loss of all coated and uncoated Chok Anan mangoes increased significantly (p≤0. 05) towards the 13 days of storage (Figure 2). This was further explained by **Khaliq *et al* (2015)** where the fruit weight loss during storage might be attributed by the loss of water during transpiration and respiration process on mangoes.

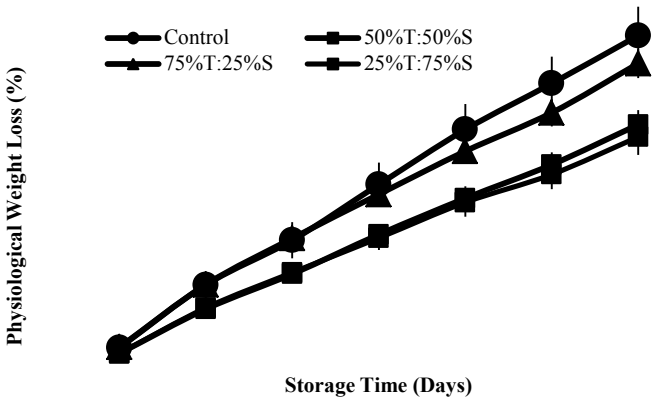
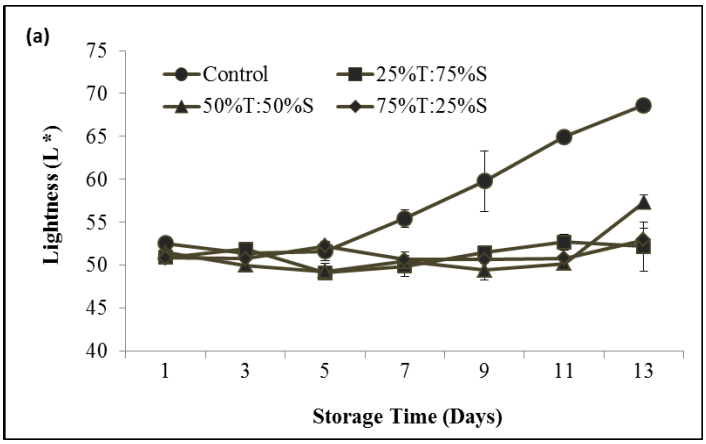


Figure 2 Physiological weight loss of Chok Anan mangoes as influenced by the storage time time and different formulations of tapioca-sago starch coating solutions towards 13 days of storage at ambient temperature (27±2°C). n=9 (3 fruits × 3 replications). Vertical bars represent the standard error of means and are invisible when the values are smaller than the symbol. LSD (P≤0.05): F=0.50, S = 0.49, F × S = NS.

Starting from day 5 until day 13 of storages, Chok Anan mangoes coated with 25%T: 75%S and 50%T: 50%S starch solutions showed the lowest percentage of fruit weight loss (p≤0. 05) compared to the control and mangoes coated with 75%T: 25%S starch solutions. However, Chok Anan mangoes coated with 75%T: 25%S starch solutions started to show a significant effect of physiological weight loss (p≤0. 05) with control after 11 days of storage, whereby the control sample obtained the highest percentage of fruit weight loss. This might be ascribed to the higher amylose content (28.15%) and viscosity of coating solutions (75.90 cP) which contained higher composition of sago starch. Higher amylose content in starch was responsible for the formation of coherent and effective strong coating **(Rindlav-westling *et al.*, 1998)**, while higher viscosity of solutions provided a great thickness of coatings as well as strong adhesion to the peel surface, hence lowered the water vapor permeability. This was similar to the research done by **Hernandezmunoz *et al.*, (2008)** who reported coating solution in 1.5% Chitosan resulted in greater viscosity and thickness of coating, further reducing moisture loss.

Peel Color Indexes

There was a significant interaction (p≤0.05) between the storage time and coating formulation for the pale color of Chok Anan mangoes stored at ambient temperature. Figure 5 showed that all the color indexes (except hue angle) of Chok Anan mangoes increased as storage time increased. A significant difference (p≤0. 05) was shown between coated and uncoated Chok Anan mangoes for all the peel color indexes. Starting from day 9 until day 13 storages, the L* of peel color value for uncoated mangoes increased significantly (p≤0. 05) in comparison with coated mangoes (Figure 3a). Besides, the peel color values of control, including redness (a*), yellowness (b*) and Chroma increased (p≤0. 05) with the decreased of hº after 9 days of storage as compared to coated samples.



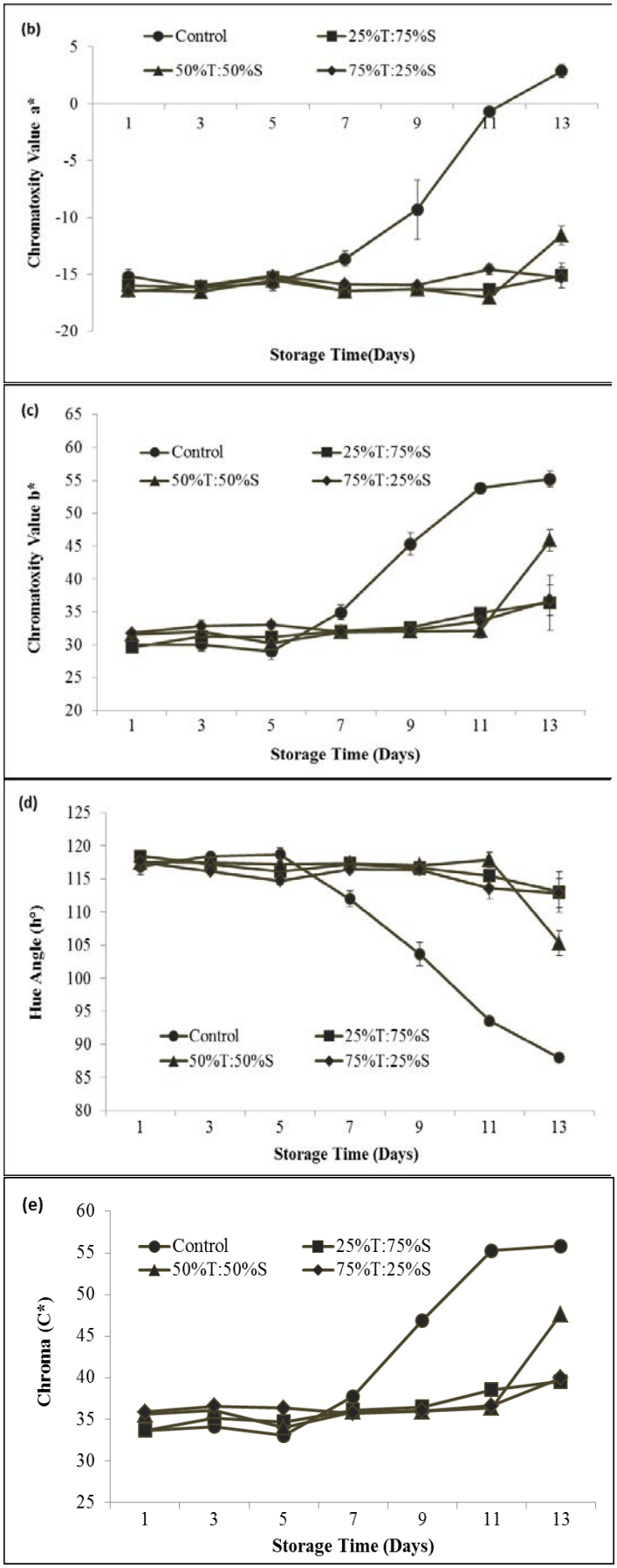


Figure 3 Color indexes of L* (a), a* (b) b* (c), hue (d) and Chroma (e) values of the peel of Chok Anan mangoes as influenced by the storage time and different formulation of tapioca-sago starch solutions stored at ambient temperature (27±2°C). n= 9 (3 fruits × 3 replications). Vertical bars represent the standard error of means and are invisible when the values are smaller than the symbol. LSD (P ≤ 0.05): L*, F = 1.31, S = 1.73, F × S = 3.45; a*, F = 3.80, S = 1.90, F × S = 7.27; b*, F = 2.03, S = 2.69, F × S = 5.38; b*, F = 2.03, S = 2.69, F × S = 5.38; C*, F = 1.80, S = 2.38, F × S = 4.76, h°, F = 2.12, S = 2.80, F × S = 5.60

The increased in L* value with the decreased of hº as storage time increased indicated that mangoes were lighter in color due to the development of yellowish colour in the peel mangoes. Whereas, the increased of Chroma value revealed that the peel mangoes shifted from a dull to a more vivid yellow/red colour **(Nunes *et al*., 2007)**. Similar result was presented by **Nunes *et al*. (2007)**, where the L* and Chroma values of 'Tommy Atkins' mangoes increased while hº decreased during storage. Surprisingly, all the coated mangoes maintained the peel color indexes throughout the 13 days of storage and no significant difference ($p>0.05$) was reported among coated samples. This indicated that coatings maintained the mango fruits natural color characteristic. This can be described as starch coatings provided a layer to fruit surface to reduce the gas permeability **(Pagella *et al.*, 2002)** and thus delayed the respiration rate of mangoes and prolonged the shelf life. With the delayed of the ripening process, conversion of organic acids to sugars were declined **(Wills *et al*., 2007a)**, and hence, the degradation of chlorophyll and synthesis of carotenoids was inhibited **(Carrilo-Lopez *et al.*, 2000)**.

Fruit Firmness

Significant interaction (storage time x coating formulation) were found in the firmness of Chok Anan mangoes stored at ambient temperature. It is clear from the results that the firmness of all coated and uncoated Chok Anan mangoes decreased as storage time prolonged (Figure 4). This was due to the substantial transformation and solubilisation of cell wall polymers such as cellulose, hemicellulose and pectin, which causes wall loosening and disintegration, resulting in fruits softening **(Zaharah & Singh, 2011)**. A significant difference ($p\leq 0.05$) was observed between coated and uncoated Chok Anan mangoes was reported after 9 days of storage. The starch coatings exerted a beneficial effect on mangoes' firmness in which, at the end of the storage periods (Days 13), all the coated samples were high in their firmness values as compared to control mangoes. However, between different formulations of coated mangoes, no significant difference ($p>0.05$) was observed (Figure 4). The application of mango coatings played a role in inhibiting the fruit softening during storage. This was due to the ability of the coating which functions to enclose the pores on the fruit surface **(Ali *et al*., 2011)** hence created a good barrier against gas transmission. With the lack of oxygen supply to the fruit, the respiration rate of fruits and the activity of enzyme hydrolysis were decreased; therefore, the softening of fruits can be delayed **(Dang *et al*., 2008).** In addition, the slower decrement of physiological weight loss in coated Chok Anan mangoes as discussed in section 3.4 also contributed to the delayed of fruit softening as water loss from was retarded and shriveling symptoms was reduced.

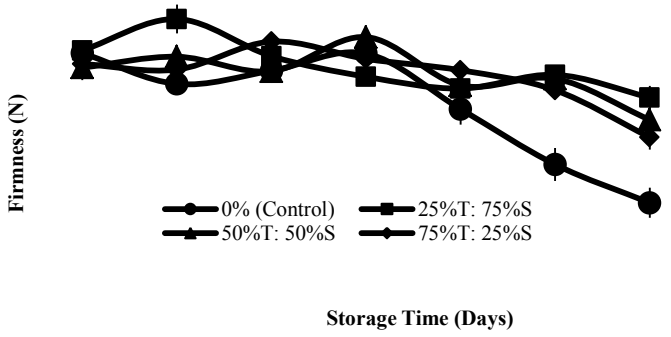


Figure 4 Fruit firmness of Chok Anan mangoes as influenced by the storage time and different formulation of tapioca-sago starch solutions stored at ambient temperature (27±2°C). n=9 (3 fruit × 3 replications). Vertical bars represent the standard error of means and are invisible when the values are smaller than the symbol. LSD (P ≤ 0.05): F = ns, S = ns, F × S = ns. ns = non-significant

Total Soluble Solids Content (TSS)

The TSS content of Chok Anan mangoes significantly interacted between the storage time and starch coating formulations. A significant increased ($p\leq 0.05$) was observed in control mangoes after five days of storage, whereas mangoes coated with 50%T: 50%S and 75%T: 25%S starch solutions, showed an increment after day seven. The most delayed increment in TSS value was mangoes coated with 25%T: 75%S starch solution. Chok Anan mangoes coated with this formulation only showed significant increase in TSS after 11 days of storage (Figure 5). The increased of TSS content with prolonged storage time indicated that the mangoes undergone ripening process in which pectin substances starch or other polysaccharide were converted into soluble sugar **(Eskin et al., 2013)**. Whereas, mango coated with higher percentage of sago

starch seemed to delay the transformation of polysaccharide into soluble sugar. This might be highly related to its higher viscosity property in higher percentage of sago starch (Table 1) which is believed to provide strong adhesion on the fruit surface and could reduce the porosity of the coating. This characteristic, thus, created a barrier that reduced the oxygen interchangeable on the fruit surface **(Khaliq *et al.*, 2015)** and hence inhibited the respiration rate as well as lowered the conversion of starch into sugar **(Ali *et al.*, 2011)**.

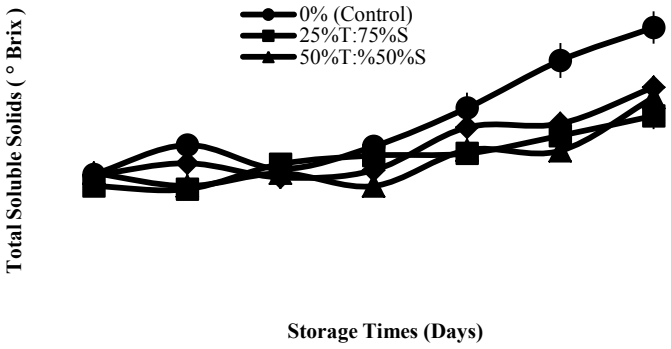


Figure 5 Total soluble solids (TSS) content of Chok Anan mangoes as influenced by the storage time and different formulation of tapioca-sago starch solutions stored at ambient temperature (27±2°C). n = 9 (3 fruits × 3 replications). Vertical bars represent the standard error of means and are invisible when the values are smaller than the symbol. LSD ($P \leq 0.05$): F = 0.45, S = 0.59, F × S = 1.18.

Titratable Acidity (TA)

It was notable that a significant interaction was shown between the storage time and coating formulation on the TA content of Chok Anan mangoes (Figure 6). The decreased of acidity content during storage might probably due to the conversion of starch into sugar and further utilizing the organic acid for respiratory metabolism **(Pauziah *et al*., 2014; Khaliq *et al*., 2015)**. Uncoated mangoes and coated with 75%T: 25%S starch solution showed significant decreased of TA ($p \leq 0.05$) after seven days of storage, while the other two samples (50%T: 50%S; 25%T: 75%S) decreased after nine days of storage. Whereas, Chok Anan mangoes coated with 25%T: 75%S starch solutions showed significantly higher TA as compared to sample coated with 75%T: 25%S and control after nine days of storage. Again, no differences ($p > 0.05$) in TA were observed for mangoes coated with more than or equal to 50% of sago starch (50%T: 50%S and 25%T: 75%S). This showed that starch coatings provided a protective oxygen barrier to the fruits, hence reduced the respiration rate and thus delayed the decrement of acidity content **(Ali *et al*., 2011)**

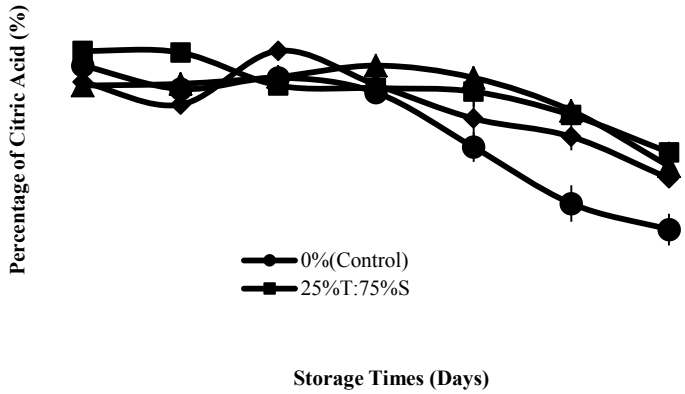


Figure 6 Titratable acidity (TA) content of Chok Anan mangoes as influenced by the storage time and different formulation of tapioca-sago starch solutions stored at ambient temperature (27±2°C). n = 9 (3 fruits × 3 replications). Vertical bars represent the standard error of means and are invisible when the values are smaller than the symbol. LSD ($P \leq 0.05$); F: 0.05, S: 0.07, F × S = 0.13.

Total Ascorbic Acid Content (TAA)

Both factors, i.e. storage time and coating formulation showed a significant interaction for the TAA content of Chok Anan mangoes at ambient temperature storage. As shown in Figure 7, the percentage of TAA content on coated and uncoated Chok Anan mangoes decreased gradually as storage time increased. The TAA content of uncoated Chok Anan mangoes started to decrease significantly after nine days of storage and showed the lowest ($p \leq 0.05$) TAA content as compared to coated Chok Anan mangoes. This might due to the presence of oxygen at surrounding during storage, which increased in respiration rate, thus, resulted in the release of water. This, thereby increased the degradation of ascorbic acid as ascorbic acid is readily oxidized in the presence of moisture **(Ottaway, 2010)**.

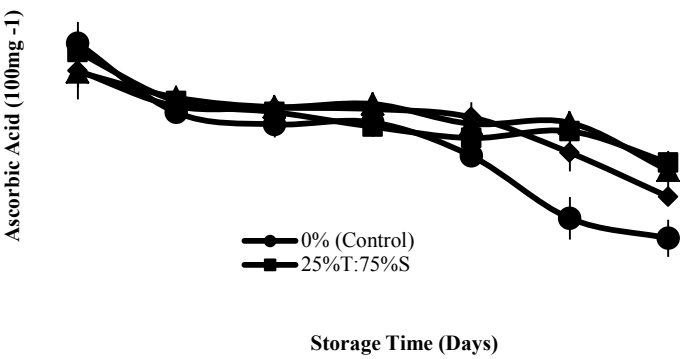


Figure 7 Total ascorbic acid (TAA) content of Chok Anan mangoes as influenced by the storage time and different formulation of tapioca-sago starch coating solutions stored at ambient temperature (27±2°C). n = 9 (3 fruits × 3 replications). Vertical bars represent the standard error of means. LSD ($P \leq 0.05$): F = 0.58, S = 0.77, F × S = 1.54.

However, sample coated with 25%T: 75%S starch solutions retained ($p > 0.05$) the TAA content throughout the storage periods. Surprisingly, no significant difference ($p > 0.05$) was observed between the coated Chok Anan mangoes until the last days of assessments (Days 13). This might attribute by the function of starch coating, which lowered the oxygen penetration into the fruit tissue, hence delayed the respiration rate. In this sense, the oxidations of ascorbic acid to dehydroascorbic acid were also decreased **(Yaman & Bayoındırl, 2002)**.

CONCLUSION

This study proved that all tapioca-sago coating formulations prolonged the storage life of Chok Anan mangoes by delaying the yellow peel color development and activity of fruit softening as well as slowing down the decrement of the ascorbic acid content as compared to uncoated sample throughout the 13 days of storage. On top of this, mangoes coated with 50%T: 50%S and 25%T: 75%S starch solutions were more effective in delaying the ripening process by inhibiting the pathogen development, retarding the fruit weight loss as well as slowing down the changes of soluble solids and acidity content at the end of storage (day 13). Hence, it can be concluded that mangoes coated with more than or equal to 50% of sago starch (50%T: 50%S and 25%T: 75%S) maintained the quality and prolonged the mango shelf life effectively.

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