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CONTENT


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ELECTROCHEMICAL BIOSENSOR FOR FOOD BORNE PATHOGENS: AN OVERVIEW

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ABSTRACT

Food safety is very significant for community fitness issue, as at present food borne diseases widespread and increasing public health issue all over the world. The fast and specific detection of food borne pathogens needed to control and avoid human food borne infections. Biosensors are fast and low price method of food borne pathogen detection. It uses the distinctive properties of biological and physical materials to identify a target molecule and effective transduction of an electronic signal. Many biosensors have been discovered, viz., electrochemical biosensor, optical biosensor and mass based biosensor. In this study, we review electrochemical biosensors for detection of food born pathogen. Electrochemical biosensors have many advantages over other biosensor such as the possibility to operate in disorganized media, sensitivity of instrument, and small size. Electrochemical biosensor are of different kinds like potentiometric, amperometric, potentiometric, impedimetric, or conductometric based upon different transducing elements used in it. From last few decade nanotechnology has arisen as a favorable field for solving food safety problems in terms of detecting contaminants. The nanomaterials used into electrical sensors to make them appropriate to reach over low detection limit, high sensitivity, and multi detection abilities.

1. Introduction

Food-borne pathogens are very diverse in nature and keep producing major public health problems all over the world. Therefore, food safety is very important issue for consumers and food industry. The globalization of food causes to changes in the dietary behaviors and changes in the food production, consumption and distribution because of these changes in food habits new safety issue arrived (Sankarankutty, 2014). Foodborne diseases produced by pathogens result in recurring intestinal swelling, chronic kidney infections, mental retardation, joints problems, impaired vision, and even death (Hoffmann *et al.*, 2015). Therefore, the food safety is very important and World Health Organization has promoted food safety as follows: “from farm to plate make food safe” on World Health Day, 2015 (WHO, 2015). Traditional methods for the recognition of toxins are sensitive and inexpensive, but they need many days to produce results. Conventional methods for recognition of pathogen detection are needs more manpower, low sensitivity and specificity, and need trained users (Fournier *et al.*, 2013). On the other side biosensors gives results very quickly with high sensitivity (Yang *et al.*, 2008). They can be used in different areas like in food industry for detection of pathogen and additives, environmental monitoring, clinical diagnoses and biodefense due to their high sensitivity, selectivity and fast response (Thakur and Ragavan, 2013). Biosensors are an aid to the food industry as they are intelligent

combination of biological component and technical component to find physical and chemical changes and to transmit it in to form of data. The implementation of nanotechnology increases the importance of biosensor in the field of pathogen detection. The sensitivity of the biosensor increased by using of nanomaterials such as magnetic Nano-particles (MNPs), carbon nanotubes (CNTs), Nano rods (NRs), and quantum dots (QDs). This review describes application of electrochemical biosensor for detection of food borne pathogens.

2. Electrochemical biosensors

The electrochemical biosensor is more popular than other biosensors due to its advantages such as low cost, high sensitivity and selectivity and small size for the detection of food-borne pathogens (Palchetti and Mascini 2008). This biosensor uses electrochemical transduction that typically measure conductivity or impedance changes, sensing methodology for electrochemical biosensor. Electrochemical biosensors uses chemical reaction comprising immobilized biomolecules and target analyte which effect measured electrical properties of solution such as an electric current or potential by producing or consuming ions (Zhang *et al.*, 2008). Block diagram for electrochemical biosensor given in figure 1. Accordingly, which type of transducer used electrochemical biosensor can categorized in amperometric biosensor, potentiometric biosensor and impedimetric biosensor.

The detection methods usually depend on the electrochemical properties of a particular electrode surface. Electrochemical methods include a reference, counter, and a working electrode. Reference electrode is make by silver chloride and put at a distance from the reaction-site to maintain a stable potential while sensing element work like a transducing element (Yunus, 2018). A counter electrode made contact in between electrolytic solution and electrode surface to supply current to the working electrode.

Milk used in this study obtained from Ege University Menemen Research and Application Farms. Beneo (Mannheim, Germany) Nutriz, rice bran formula obtained from Artisan Gida San. For the preparation of rice milk, 13.6 g of rice bran diluted in 100 mL of water. MYE 96-98 starter culture for yoghurt production containing *S. thermophilus* and *L. bulgaricus* was obtained from Maysa Gida San. In addition to the yoghurt culture, *Lactobacillus gasseri* ATCC 4963 and *Bifidobacterium longum* DSM Lafti B22 strains were used. Filling and packaging were done with packages obtained from Ege University Faculty of Agriculture Menemen Farms and Ege University Faculty of Agriculture Department of Dairy Technology.

2.1. Amperometric biosensors

Amperometric biosensors are universal way for finding the food pathogens. Transducer of amperometric biosensor measure the amount of electric current produced at constant potential between working and reference electrode (Sharma *et al.*, 2013). The equipment for amperometric biosensor contains three electrodes, a voltage source and a device for measuring current (Arora *et al.*, 2018). Equipment for amperometric biosensor displayed in figure 2. The amperometric biosensors make usage of Clarks oxygen electrode, which determines the quantity of oxygen (present in the analyte) reduced. It usually depends on an enzyme system that catalytically converts analyte into product that can oxidized at working electrode. Commonly Horseradish peroxidase (HRP) and alkaline phosphatase enzymes are used (Zourob *et al.*, 2008). Various types of amperometric biosensor are present for detection of food pathogen; examples are DNA based, immunosensor, and microbial metabolism based biosensor. A list of amperometric biosensors for detection of food pathogen presented in table 1.

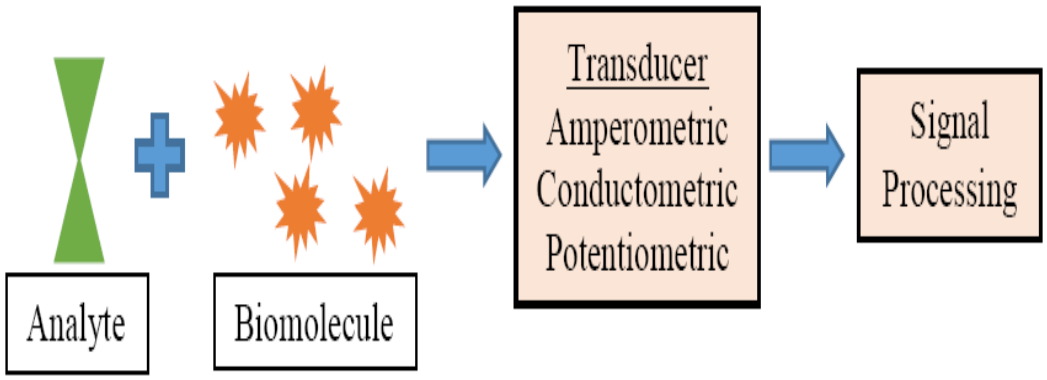

Figure 1. Electrochemical Biosensor

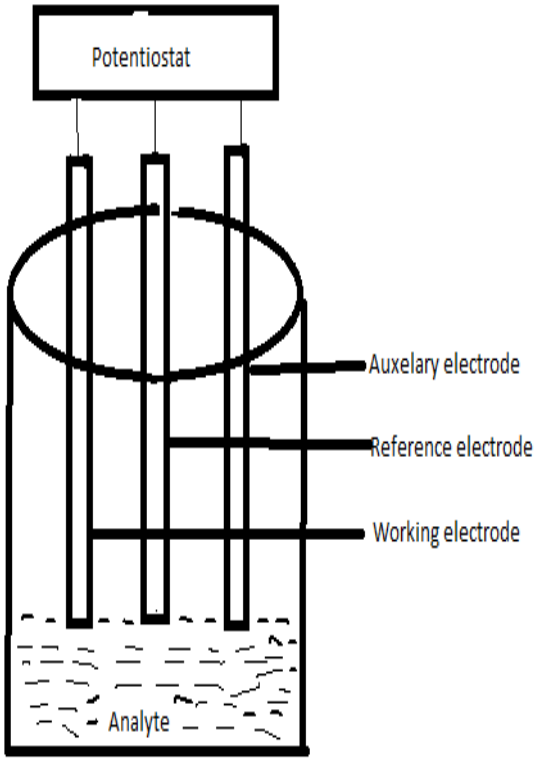

Figure 2. Amperometric Biosensor

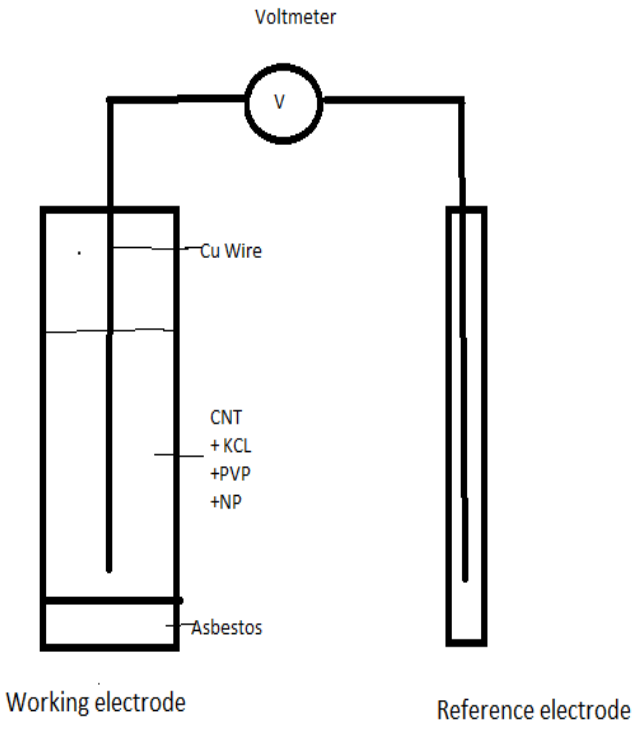

Figure 3. Potentiometric biosensor

Table 1. Amperometric biosensor for detection of food pathogens

Food sample	Pathogen	Bioreceptor	Electrode	LOD	Reference
Food sample	*Salmonella pullorum*	Antibody	Screen printed electrode (SPE) modified GNP	89 CFU/mL	Fei *et al*., 2016
PBS	*Streptococcus agalactiae*	Antibody	Screen-printed carbon electrodes	10 CFU/mL	Vásquez *et al*., 2016
Skim & whole milk	*Salmonella typhimurium*	Polyclonal antibody	Gold electrode	10 CFU/mL	Alexander *et al*., 2018
Milk	*L. monocytogenes*	HRP-labeled antibody	Novel multiwalled carbon nanotube	1.07×10^2 CFU/mL	Lu *et al*., 2016
Food sample	*E. coli* O157:H7 *S. aureus*	Antibody	Gold electrode	1×10^{-12} mol/L	Fernandes *et al*., 2014
Food sample	*E. coli*	Biotinyl Antibody	Saturated calomel electrode	$3x10^1$ – $3.2x10^6$ CFU/mL	Li *et al*., 2013
Blue-berry	*L. monocytogen*	Antibody	Gold nanoparticle modified screen printed carbon electrode	2 log CFU/g	Davis *et al*., 2013
Milk	*Staphylococcus aureus*	Antibody	DropSens screen-printed gold electrodes	1 CFU/mL	De Avila *et al*., 2012
Milk	*E. coli*	Antibody	Photo-lithographic gold	100 cells/mL	Laczka *et al*., 2011
Food sample	*S. aureus* nuc gene	Antibody	Gold electrode	3.23×10^{-14} mol/L	Sun *et al*., 2015

2.1.1. Amperometric DNA based biosensors

A DNA biosensor is device in which oligonucleotide incorporate with a known sequence of bases, either combined within or closely connected with the electrode (Zourob *et al.*, 2008). There are various kind of electrochemical DNA sensors developed for detection of the bacterial nucleic acid. A DNA based amperometric nanoparticle biosensor was developed by Fernandes *et al.*, (2014) for detection *of E. coli* O157:H7 and the nuc gene of *S. aureus.* This DNA sensor provide very low detection limit for both pathogen that is 1×10^{-12} mol/L. This DNA based amperometric

biosensor gives high specificity and selectivity in detection of target DNA. Another use of the electrochemical DNA biosensor includes detection of *S. aureus* gene sequence in the concentration range of 1.0×10^{-13}-1.0×10^{-6} mol/L with LOD of 3.23×10^{-14} mol/L (Sun *et al.*, 2015).

2.1.2. Amperometric immunosensors

In immunosensors microorganism detected by using antibodies that are immobilized on electrode surface or magnetic beads. This is one of the successful techniques for pathogen detection; various researchers reported immunosensors for detection of food pathogen. Laczka *et al.* (2011) reported immunosensor for the recognition of *E. coli* in a microfluidic system joined with immunomagnetic beads with LOD of 100 CFU/mL in milk. Lu *et al.*, (2016) established an amperometric immunosensor with LOD of 1.07×10^{2} CFU/mL by immobilization of HRP antibody against *Listeria monocytogenes* onto the surface carbon nanotube fibers.

2.1.3. Amperometric microbial metabolism based biosensor

Metabolic metabolism based biosensor uses specific marker enzyme for detection purpose (Arora *et al.*, 2011). Various researchers reported microbial metabolism biosensors for detection of pathogens in food. These biosensors commonly used for analysis of water samples to recognize coliform by their metabolic product released enzyme β-D-glucuronide, lucuronoso-hydrolase (GUS) and β-d-galactosidase (Arora *et al.*, 2018). Neufeld and coworkers (2003) developed an amperometric microbial metabolism based biosensors for quantification of coliform *E. coli* K-12 using bacteriophage immobilized on screen-printed carbon electrodes with the sensitivity of 1 CFU/100 mL of sample. Togo *et al*. (2007) developed a bacteria-based biosensors for GUS detection by immobilization of *Moraxella* species. Metabolic product of GUS enzyme of *E. coli* to *p*-nitrophenol (PNP) and D-glucuronic acid by *Moraxella* shows the occurrence of *E. coli*.

2.2. Potentiometric biosensors

Potentiometric biosensors uses the ion selective electrodes to find the potential of a solution based on particular relation with ions in the solution. These biosensors measure the electrical potential between working and reference electrode. The potential of reference electrode not change during the entire period of measurement while working electrode undertakes some change in its potential even for minor changes in concentration of analyte (Ahmed *et al.*, 2014). Equipment for potentiometric biosensor shown in figure 3. This technique is not very common for detection of food pathogen. A list of potentiometric biosensor given in table 2.

2.3. Conductometric biosensors

Conductometric biosensors use transducers, which measure deviation of the ionic strength of a solution that changes flow of current (Karunakaran *et al.* 2015). Conductometric biosensors have no need of reference electrode so it have miniaturization possibilities and low cost. These biosensors have advantages of low cost, small size (Salek-Maghsoudi *et al* 2018) and real-time monitoring (Bettazzi *et al.* 2017) but disadvantage of low sensitivity. Hnaiein *et al.* (2008) reported conductometric biosensor for finding of *Escherichia coli* with LOD of one CFU/mL. Tahir and Alocilja (2004) developed a conductometric biosensor for the detection of *E. coli* O157:H7 and *Salmonella* with an LOD of 81 CFU/mL. Pal *et al.* (2008) also reported a conductometric biosensor for *B. cereus* in different foods.

2.4. Impedimetric biosensors

Impedimetric biosensors developed by addition of impedance with biological recognition element (Yang and Bashir, 2008). This method is one of the oldest methods for detection of microorganism. G.N. Stewart in 1899 developed first impedimetric biosensors for detection of microorganism. Impedimetric transduction to find various kinds of foodborne pathogens. These biosensors have advantages of unobstructed measurement of the molecule of

interest, with no need for the enzymatic analyte and ability of multiple detection (Ahmed *et al.*, 2014). However, they have disadvantages of reproducibility and problems with nonspecific binding. Various researchers reported impedimetric biosensors for detection of food born pathogen. A list of impedimetric biosensors used for food pathogen are presented in table 3.

3. Commercially available electro-chemical biosensor for finding pathogen in foods

Although there are very large no of publication on biosensor for detection of pathogen but very few are commercially available. Table 4 shows list of commercially available electrochemical biosensor for detection of food pathogens. There are some limitations such as low lifespan of biological component, mass production and not easy to use. However, by application of nanotechnology in biosensor these problems can be solved in near future, as biosensors have unique ability in terms of sensitivity, specificity and quick response. There are costs and technical issue that can slow the commercialization of new systems.

Table 2. Potentiometric biosensor for detection of food pathogens

Sample	Pathogen	Bioreceptor	Electrode	LOD	Reference
Pig skin	*Staphylococcus aureus*	Aptamer	Single-walled carbon nanotubes	$8x10^2$ CFU/mL	Zelada *et al*., 2010
Milk, Fruit juice	*E. coli*	Aptamer	Single-walled carbon nanotubes	26 CFU/mL in juice and 6 CFU/mL in milk.	Zelada *et al*., 2010
Food sample	*Staphylococcus aureus*	DNA Aptamers	Carbon nanotube aptamer based electrode	Single CFU/mL	Hernandez *et al*., 2014
Food sample	*Staphylococcus aureus*	DNA Aptamers	Carbon nanotube aptamer based electrode	Single CFU/mL	Cao *et al*., 2009
Food sample	Sulfate-reducing bacteria	None	Glassy carbon electrode	$2x10^{-2}$ to $3x10^7$ CFU/mL	Wan *et al*., 2010
Lettuce, carrots	*E. coli*	Antibody	LAPS	10 cells/mL	Ercole *et al*., 2003

Table 3. Impedimetric biosensor for detection of food pathogens

Food sample	Pathogen	Bioreceptor	Electrode	LOD	Reference
Milk	*Listeria innocua*	Bacteriophage endolycin	Gold screen printed electrode (SPE)	10^5 CFU/mL	Tolba *et al*., 2012
Whole milk	*E. coli* O157:H7	Antibody	Alumina	83.7 CFU/mL	Joung *et al*., 2013
Ground beef, cucumber	*E. coli* O157:H7	Antibody	Gold Nano particle	1.5×10^4 and 1.5×10^3 CFU/mL	Wang *et al*., 2013
Fat free milk	*S. typhimurium*	Antibody	GNP and poly (amidoamine)-multi walled carbon nanotubes	10^3 CFU/mL	Dong *et al*., 2013
Milk	Gram +ve bacteria, selectively *L. monocytogen*	Antimicrobial peptide	Interdigitated gold microelectrodes	10^3 CFU/mL	Etayash *et al*., 2014
-	*Listeria monocytogenes*	DNA Aptamer	Platinum interdigitated array microelectrodes	5.39 ± 0.21 CFU/mL	Sidhu *et al*., 2016
ground beef, chicken	*E. coli* O157:H7, *Salmonella typhimurium*	Antibody	Screen- printed interdigitated microelectrode	2.05×10^3 CFU/g and 1.04×10^3 CFU/mL	Xu *et al*., 2016
PBS	*E. coli* O157:H7	Antibody	-	100 CFU/mL	Wan *et al*., 2016

Table 4. Commercially available biosensor for detection of food pathogens

Name of device	Type of Biosensor	Manufacturer	Target compound	Sample
Midas Pro	Amperometric	Biosensori SpA, Milan, Italy	Food pathogen	Food sample
Bactometer	Impedimetric	Bactomatic Inc., Princeton, USA	Food pathogen	Food sample
Bactometer	Impedimetric	Biomerieux, France	Food pathogen	Food sample
Bac Trac	Impedimetric	Sy-Lab, Austria	Food pathogen	Food sample
Malthus ATanalyzer	Impedimetric	Malthus Instruments	Food pathogen	Food sample
Malthus 2000	Potentiometric, conductometric, field Effect	Malthus Inc., Stoke-on-Trent, UK	Food pathogen	Food sample

Analyte 2000TM	Electrochemical	Research International Ltd.	*E. coli* O157:H7	Hamburger
Malthus systems	Electrochemical	Malthus Instruments Ltd.	*E. coli* O157:H7, Fungi, Yeast	Shell fish
RABIT	Electrochemical	Don Whitley Scientific Ltd.	Food pathogens	Vegetables
BioflashTM system	Electrochemical	Innovative Biosensors Inc.	*E. coli* O157:H7	Lettuce
Biosensor	Electrochemical	Michigan State University's, USA	*E. coli* O157:H7, *Salmonella*	Meat
Biosensor	Electrochemical	Massachusetts Institute of Technology. USA	*E. coli* O157:H7	Lettuce (Canary)
Biosensor	Electrochemical	Georgia Research Tech Institute, USA	*Salmonella* and *Campylobacter*	Pork industry

4. Nanotechnology in electrochemical biosensors

Application of nanotechnology in biosensor give advantage of increased sensitivity and selectivity, quick response and minimum cost of production. Four types of nanomaterial are very popular in electrochemical biosensor: these are GNP, graphene, carbon nanotube and photonic crystals. Many researcher developed electrochemical biosensor incorporated by nanomaterial for detection of pathogen. Ma *et al.*, (2014) developed electrochemical biosensor with gold nanoparticles (AuNPs) for detection of *Salmonella typhimurium* in pork with a detection limit of 3 CFU/mL. Zelada *et al.*, (2012) made potentiometric biosensor with carbon nanotube for detection of *S. aureus* in pigskin. A list of electrochemical biosensor incorporated with nanomaterials given in table 5.

Table 5. Biosensor using nanotechnology for detection of food pathogens

Biosensor	Food sample	Pathogen	Nanomaterial	LOD	Reference
Potentiometric biosensor	Pig skin	*S. aureus*	Carbon nanotube	800 CFU/mL	Zelada *et al.*, 2010
Electrochemical biosensor	Pork	*Salmonella typhimurium*	AuNPs	3 CFU/mL	Ma *et al.*, 2014
Electrochemical biosensor	Milk and infant formula	*Bacillus cereus*	AuNPs	10 CFU/mL	Izadi *et al.,* 2016
Impedimetric biosensor	Spiked food	*Salmonella typhimurium*	Copolymer	-	Sheikhzadeh *et al.*, 2016
Electrochemical biosensor	Food sample	*Salmonella enteritis* and *Bacillus anthracis*	Au NPs and MNPs	0.5 ng/mL and 50 pg/mL	Zhang *et al.*, 2010
Electrochemical immunosensor	Food sample	*E. coli* O157:H7	Carbon screen printed	$3.47x10^3$ CFU/mL	Dou *et al.*, 2013

5. Conclusions

Biosensors are the tools for detecting pathogen of foods. Electrochemical biosensors have lots of advantage over other methods like small size, low cost, easy to handle, high sensitivity. In the last decade, various new technique discovered. The application of nanotechnology in biosensor make it great tool for finding impurities of foods. Although biosensors have benefits over old-style methods, yet there are several problems in its development such as on site monitoring. Until now, few biosensors are commercially available for this purpose. It is expected that in future electrochemical biosensors, information technology will be included to help food industries and customers. Application of real time monitoring, nanoparticle and nanotechnology in electrochemical biosensor will be a great tool for detection of germs and pathogens in the foods.

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EVALUATION OF ASCORBIC ACID CONTENT AND TOTAL ANTIOXIDANT STATUS OF FRESH-SQUEEZED ORANGE JUICES

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ABSTRACT

This study aimed to evaluate i) consumer behaviour regarding fruit juices and ii) vitamin C content and total antioxidant status (TAS) of fresh-squeezed orange juices. To determine consumption habits of consumers regarding fruit juices, a questionnaire was applied. Fresh-squeezed orange juices were supplied from the stands, cafes and patisseries/bakeries/pastry shops. Ready-made orange juices were purchased from the markets. The pH, ascorbic acid (mg/100 g) and TAS (mmol/L) of orange juices were analyzed. This study found that there was a tendency towards fresh-squeezed juice types especially orange juice and the main motivational factors were naturalness/freshness, taste perceptions, vitamin C content and positive health effects. It was shown that ascorbic acid contents (42.4±6.71 vs. 17.0±11.09 mg) and TAS values (3.3±0.35 vs.1.2±0.90 mmol/L) of fresh-squeezed orange juices were higher compared to ready-made orange juices and vary depending on the place of purchase($p<0.05$). As a result of keeping the juices although they were freshly squeezed, their ascorbic acid contents decreased. Also, there was a positive correlation between ascorbic acid and TAS values in all orange juices ($r:0.902$, $p<0.05$). Supporting the perception of consumers, ascorbic acid content and antioxidant capacities of fresh-squeezed orange juices were higher compared to ready-made juices, and the fruit juices must be consumed without being kept even if they are freshly squeezed.

1.Introduction

Citrus, a genus belonging to the Rutaceae family, is one of the most demanded agricultural products in the world due to its rich bioactive content, and health benefits. Vitamin C, one of the component in citrus, is among the strongest antioxidant foods due to polyphenols, flavonoids and carotenoid compounds (Abudayeh *et al.,* 2019). Vitamin C, in particular, has been found to correlate with more than 65% of antioxidant activity in many fruits and fruit beverages (Mditshwa *et al.,* 2017).

Vitamin C is considered to be the most important water-soluble antioxidant and shows anti-atherogenic, anti-inflammatory, antimicrobial, anticancer, anti-epileptic and immune-enhancing effect in metabolism by directly cleaning the superoxide radical, single oxygen, hydrogen peroxide and hydroxyl radical (Abudayeh *et al.,* 2019; Kaur & Kapoor, 2001; Klimczak *et al.,* 2007). Due to such potential health benefits, the interest in consuming foods containing vitamin C is increasing day by day. Orange and orange juice is among the most important sources of vitamin C in the diet due to high consumption (Klimczak *et al.,* 2007). The vitamin C content of orange juice varies between 15-45 mg/100 mL and a glass of orange juice

(200 mL) can meet about 30-80% of the recommended daily intake of vitamin C (Klimczak *et al.,* 2007).

The vitamin C content of orange juice is affected by many factors. The type, variety, harvest time and post-harvest conditions of the orange used may affect the Vitamin C content of the final product (Mditshwa *et al.,* 2017). Post-harvest conditions include storage conditions and post-harvest stress, such as physiological disorders and mechanical damage. Conditions such as temperature, relative humidity, packaging material, and access to light and oxygen in the storage process of the orange used in the production of orange juice and orange juice offered for consumption affect the content of vitamin C (Klimczak *et al.,* 2007; Mditshwa *et al.,* 2017). In a study, an increase in anthocyanin, flavanone, hydroxycinnamic acid content and antioxidant capacity and decrease in vitamin C were observed in oranges stored at 6 °C for 65 days (Rapisarda *et al.,* 2008).

Nowadays, when grocery shopping, consumers take cognizance of the health benefits of the products besides their taste and content (Bech-Larsen & Grunert, 2003). So, they may tend to prefer fresh-squeezed fruit juices and consider them healthier than packaged ones. A study has shown that the most important factors for consumers when purchasing fruit juice are vitamin, mineral and antioxidant content, respectively (Oral *et al.,* 2016). It is extremely important to know the vitamin C and antioxidant levels of products that reach the end consumer, as orange juice is an important source of dietary vitamin C. Moreover, consumers' juice consumption has been increasing in recent years, and it is important to investigate the juice consumption trends and motivational factors in this regard.

This research was carried out to evaluate consumer behaviour regarding fruit juices and at the same time vitamin C content and total antioxidant status (TAS) of fresh-squeezed fruit juices.

2. Materials and methods

This study was conducted in two stages. While the tendencies of consumers towards fruit juices are determined in the first stage, some orange juice types (freshly squeezed and store-bought) were analysed in the second stage.

2.1. Determination of consumer behaviour regarding fruit juices

This stage of the study was conducted on a total of 246 adults (18-64 years), 128 of which were male and 118 were female. To determine individuals' consumption habits of fruit juices, a questionnaire was applied with a face-to-face interview technique. Thus, the types of fruit juice consumers often consume, the most frequently consumed fruit juices according to their fruit/aroma, and the reasons for consumption of fruit juice were determined.

2.2. Fruit juice analysis

2.2.1. Sample selection and supply

In line with the data obtained in the first stage of the study, it was determined that consumers mostly prefer fresh-squeezed orange juice. Therefore, fresh-squeezed orange juice was chosen as a basic sample in the second stage and further analysis of fresh-squeezed and store-bought orange juices were performed (Figure 1).

2.2.2. Fresh-squeezed orange juices

In Turkey, fresh squeezed juices are offered for sale mostly in municipal or private stands. Fruit juice is typically filled in transparent pet bottles immediately after the fruits are washed and squeezed using a manual extractor. In cases where freshly squeezed fruit juices are not purchased by consumers immediately, they are offered for sale in bottles kept in AHT coolers in ice.

In this study, fresh-squeezed orange juices in plastic bottles were purchased from the stands, cafes and patisseries/bakeries/pastry shops located in the 3 large central districts in Ankara province, Turkey by researchers and were analysed after brought to the laboratory by ensuring the cold chain. Fresh-squeezed orange

juices supplied from all points were distinguished as "freshly-squeezed/not kept" and "previously squeezed/kept".

2.2.3. Store-bought orange juices

Packaged orange juices in Tetra Pak were purchased from the big market chains in the districts where fresh-squeezed juices were supplied, and the samples brought to the laboratory for analysis were prepared for analysis in a dark environment isolated from UVs.

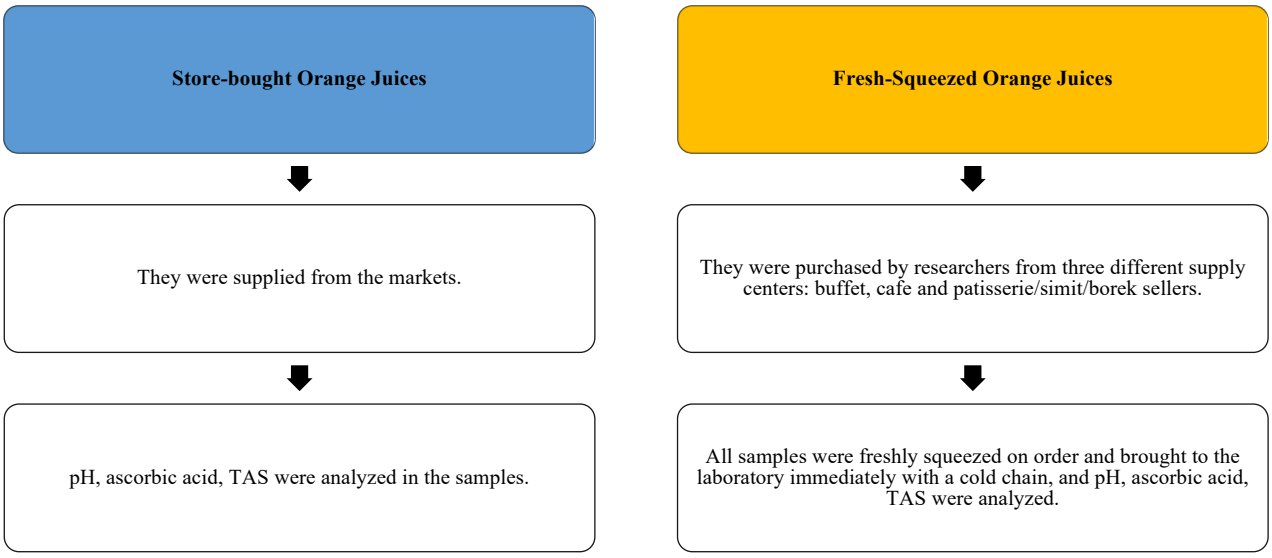

Figure 1. The flow of orange juices analysis

2.3. Analysis of samples

2.3.1. Determination of pH

Commercial pH meter (SelectaTM) was used for the pH determination of samples. The pH meter was calibrated before starting the analysis. For this, the pH meter was adjusted with buffer solutions of pH 4, 7 and 10 at 21 0C. Then, the samples were mixed until they were homogenized and the pH values were measured by taking them into a clean beaker.

2.3.2. Ascorbic acid analysis

In this study, the titrimetric method and Tillman reagent (2,6-dichlorophenolindophenol) were used for the determination of ascorbic acid in orange juices. The principle of this method is based on the principle that ascorbic acid is a strong reducing agent that bleaches the 2,6-dichlorophenolindophenol dye. In the last phase of the titration, the pink colour of the acid medium in 2,6-dichlorophenolindophenol, which can no longer react, appears due to the complete depletion of ascorbic acid in the medium.

All chemicals used in the analysis were provided before the study and all solutions were prepared fresh. Metaphosphoric acid solution (3%) was used as an extraction solution. Standard ascorbic acid solution (0.02%) was dissolved in 3% metaphosphoric acid solution and its volume was increased to 250 mL and titrated with 2,6-dichlorophenolindophenol and the amount of ascorbic acid in the samples was calculated as follows:

Ascorbic acid mg/100 g = [(V.f)÷m]x100 (1)

V: Amount of 2,6-dichlorophenolindophenol solution spent in titration (mL)
f: factor of 2,6-dichlorophenolindophenol solution
m: the amount of sample taken for titration

2.3.3. Determination of Total Antioxidant Status

All samples were centrifuged at 3000 rpm for 3 minutes at +4 °C and their supernatant fractions were separated. TAS levels were measured using commercially available kits (Relassay, Turkey). The novel automated method is based on the bleaching of characteristic colour of a more stable ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol Trolox equivalent/L. All experiments and analyses were carried out in duplicate.

2.4. Statistical Analysis

The data were analysed using SPSS 22.0. Percentage (%) and arithmetic mean±standard deviation ($\bar{x}$±SD) values were given as descriptive statistics for variables. "Mann-Whitney U Test" or "Kruskal-Wallis Test" was used to compare pH, ascorbic acid (mg/100 g) and TAS (mmol/L) values in orange juices according to the type, place provided and holding state. "Spearman Correlation Test" was used to determine the relationships between the variables. Level of significance was determined as α=0.05 in all analyses.

3.Results and discussions

Orange juices have been accepted as a part of healthy nutrition with their vitamin and bioactive compounds, and therefore antioxidant content for many years. Orange juices have types such as concentrated, frozen and pasteurized (Lee & Coates, 1999). In recent years, unpasteurized-hand and industrial fresh squeezed types have increased in line with the increasing health awareness of consumers all over the world (Gul *et al.,* 2011). In this context, this study was conducted to evaluate consumer trends regarding freshly squeezed orange juices and vitamin C and antioxidant contents which serve as hypothesis for these trends.

3.1. Determination of Consumer Behaviour Regarding Fruit Juices

87.0% of individuals consume fruit juices at least once a week. The factors that individuals pay attention to when purchasing fruit juices include production and expiration date (86.5%), contents (76.4%), price (59.3%) and nutritional value (45.1%), respectively (Data not shown in the table).

Figure 2 shows the types of fruit juice most frequently consumed by individuals. Accordingly, the most frequently consumed fruit juices are freshly squeezed juices (84.9%), 100% fruit juices (60.9%), nectars (39.0%) and flavoured juices (30.9%), respectively (Figure 2).

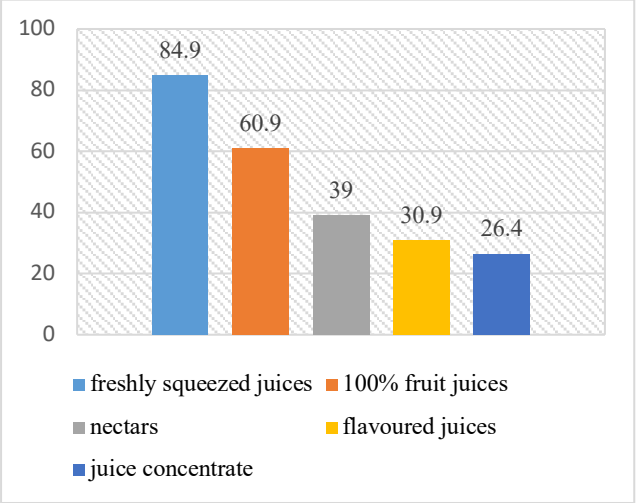

Figure 2. Fruit juices most frequently consumed by individuals

Figure 3 shows the most frequently consumed juice types according to fruit/flavour. Accordingly, the most frequently consumed fruit juices according to their fruit/flavour are orange juice (77.6%), cherry juice (58.9%), mixed juice (54.0%), peach juice (51.2%), pomegranate juice (36.1%), apple juice (32.5%), pineapple juice (24.3%) and mango juice (19.9%), respectively (Figure 3).

Individuals stated that they purchase juices mostly from the grocery stores (51.6%), while 36.6% stated that they prepare juices at home through their own means. The places where

individuals consume fruit juices most frequently are houses (84.5%), cafes (48.3%) and bakeries (24.8%).

74.7% stated that they consume fruit juices since they like the taste, 58.9% stated that they consume fruit juices since juices are healthy, 53.6% stated that they consume fruit juices since juices are rich in vitamin content, and 17.4% stated that they consume fruit juices since juices are affordable. 65.4 percent stated that they believe that freshly squeezed fruit juices are healthier because of being fresh (69.4%) and natural (77.6%), not containing added sugar (49.4%), and richness in vitamin C (44.7%) (Figure 4).

This study found that the majority of individuals purchase/consume fruit juices, and there is a tendency towards freshly squeezed juice types (Figure 3 and Figure 4). It was determined that naturalness/freshness, taste perceptions, vitamin C content and the thought that they have positive effects on health are the main motivational factors in fruit juice consumption (Figure 4).

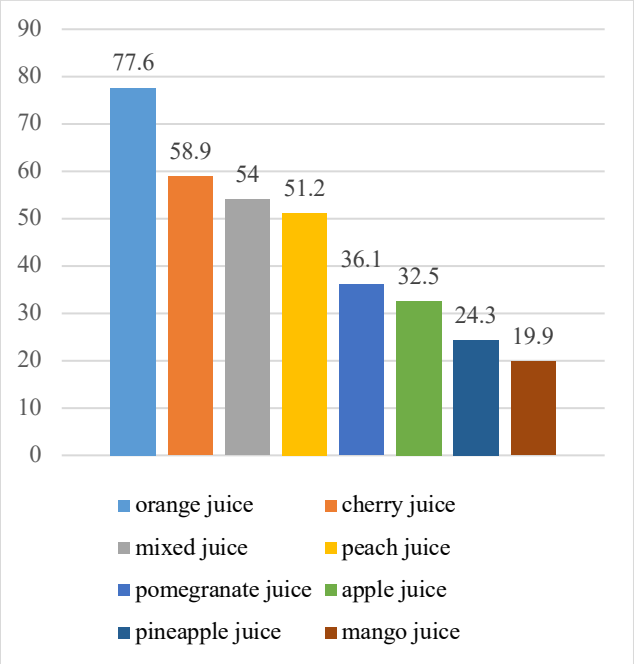

Figure 3. The most frequently consumed fruit juices according to their fruit/flavour

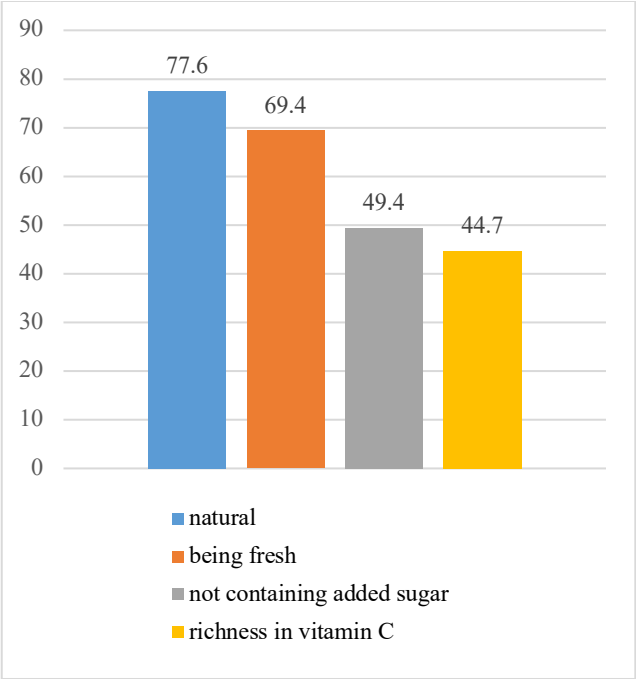

Figure 4. Reasons for consuming freshly squeezed fruit juices

Freshly squeezed unpasteurized fruit juices are often preferred by consumers because of their taste and nutritional value, and although they are typically costlier than pasteurized fruit juices, they are effectively marketed in many countries (Bagci & Temiz, 2011). It is reported in sensorial analyses that the most preferred juice is hand-squeezed orange juice compared to other juice extraction technologies (Baldwin *et al.,* 2012). Supporting the results of this (present) study, another study conducted in Turkey found that the opinion that the juice is healthy is widespread and especially vitamin content makes juices healthy (Bagci & Temiz, 2011). The aforementioned study reported that the opinion that fruit juices squeezed at home are healthier than packaged juices is very common (Oral *et al.,* 2016.). Moreover, another study determined that as the socioeconomic and cultural levels of adolescents increase, the consumption frequency of freshly squeezed fruit juice increases (22%), but the frequency of ready-made fruit juice consumption decreases (7%) (Gürel & Hisar, 2018). Another study reported that moderate consumption of 100% orange and grapefruit juices contributed to

nutrition because they contain vitamin C and other important nutrients such as potassium, folate, magnesium, and vitamin A, but it is not associated with anthropometric measurements in either children or adults (Rampersaud & Valim, 2017). Similarly, consumption of fruit juice and citrus fruits was found to be an important predictor of plasma vitamin C concentration in smokers and individuals with high body mass index (BMI) (Langlois *et al.,* 2016).

3.2. Fruit Juices Analysis

The pH, ascorbic acid (mg/100 g) and TAS (mmol/L) values of orange juice types are shown in Table 1. Accordingly, this study determined that ascorbic acid contents of freshly squeezed orange juices were higher (2.5 times) compared to ready orange juices (42.4±6.71 vs. 17.0±11.09 mg) regardless of pH levels ($p<0.05$). Similar to the findings of this study, Turkomp-Turkish National Food Composition Database reports that the average amount of L-ascorbic acid in orange juices are 46.8 mg/100 g (Turkomp Database, 2020). It is reported that there are many pre- and post-harvest factors affecting the content of vitamin C in foods as ascorbic acid and dehydroascorbic acid (Lee & Kader, 2000). Genotypic differences, climatic conditions, cultivation and processing techniques are among the effective factors (Lee & Kader, 2000). Furthermore, supporting the findings of this study, pasteurization/thermal processing in production of fruit juices is reported to cause significant losses in vitamin C. In the literature, it is suggested that fruit juices with no thermal processing have higher vitamin C content, but the shelf life of such juices is shorter and vitamin C losses may occur (Martí *et al.,* 2009).

When freshly squeezed orange juices were evaluated according to the place of purchase, the pH values of orange juices purchased from stands, cafes and patisseries/bakeries/pastry shops were statistically different ($p<0.05$). Ascorbic acid contents (39.8±8.78 mg/100 g) and TAS values (3.1±0.39 mmol/L) of freshly squeezed orange juices purchased from cafes were lower compared to those purchased from other places ($p<0.05$). It was determined that the pH (3.32±0.27 vs. 3.63±0.19), ascorbic acid (40.3±8.83 vs. 43.3±5.42 mg/100 g) and TAS values (3.2±0.45 vs. 3.3±0.29 mmol/L) of freshly squeezed orange juices kept in bottle for a while were lower compared to those instantly consumed ($p <0.05$) (Table 1).

In the present study, it was determined that the place of purchase was also important in terms of ascorbic acid content in freshly squeezed orange juices, but as a result of keeping the juices although they were freshly squeezed, their ascorbic acid contents decrease and pH levels change (Table 1). Storage conditions (temperature, pH, oxygen density of the environment, metal content, exposure to UV beam, etc.) are also important determinants of vitamin C (Martí *et al.,* 2009). A study evaluated the losses in vitamin C after storage of some juice types in plastic bottles at room temperature (29±1°C) or refrigerator temperature (4±1°C) for 4 weeks (Ajibola *et al.,* 2009). It was found that losses of vitamin C vary according to fruit type and storage method, but citrus juices such as orange, lemon and lime experience similar losses due to oxidation in vitamin C regardless of pH factor (Ajibola *et al.,* 2009). Another study found that there may be losses in vitamin C in fruits exposed to light by 10% when stored for 6 days at 5°C (Gil *et al.,* 2006). Moreover, it was reported that 0.34 mg/100 mL of vitamin C is lost per month, which was initially 32.8-40.6 mg/100 mL, even after freezing the freshly squeezed unpasteurized orange juices sold in polyethylene bottles (Lee & Coates, 1999). In this study, it is thought that keeping the orange juices especially in translucent containers, even if they are freshly squeezed, is the reason for the decrease in ascorbic acid content. Therefore, if consumers are to consume freshly squeezed juices in terms of vitamin C, it is believed that these juices must be consumed without being kept.

Table 1. pH, ascorbic acid (mg/100 g) and TAS (mmol/L) values of orange juices

	pH	Ascorbic Acid (mg/100 g)	TAS (mmol/L)
Type of fruit juice			
Freshly squeezed (n=45)	3.53±0.26	42.4±6.71	3.3±0.35
Ready-Made (n=7)	3.37±0.24	17.0±11.09	1.2±0.90
	p>0.05	**p<0.05**	**p<0.05**
Freshly squeezed fruit juices			
Place of Purchase			
Stands (n=13)	3.32±0.27	43.4±4.07	3.4±0.16
Café (n=18)	3.68±0.23	39.8±8.78	3.1±0.39
Patisseries/Bakeries/Pastry Shops (n=14)	3.54±0.15	44.8±4.45	3.3±0.38
	p<0.05	**p<0.05**	**p<0.05**
Holding state			
Freshly squeezed (n=31)	3.63±0.19	43.3±5.42	3.3±0.29
Previously squeezed/Kept (n=14)	3.32±0.27	40.3±8.83	3.2±0.45
	p<0.05	**p<0.05**	**p<0.05**

TAS: total antioxidant status

This study no statistically significant correlation was found between pH values and ascorbic acid (r: 0.078, p>0.05) and TAS (r: 0.031, p>0.05) values in all orange juices (p>0.05). In fact, ascorbic acid is very stable between pH 2 and 4. However; as pH level exceeds pK1 value 4.04, ascorbic acid loses its stability between pH 4 and 6 at the maximum level and irreversibly and swiftly gets hydrolysed to 2,3-diketo-L-gulonic acid, which has no vitamin activity (Gregory III, 1996). In this study, it is thought that the pH values of orange juices being acidic may be the reason for not detecting this relationship and therefore, it is not related to antioxidant capacity.

Moreover, there was a positive correlation (r:0.902, p<0.05) between ascorbic acid (mg/100 g) and TAS values (mmol/L) in all orange juices. A correlation (p<0.05) was found between ascorbic acid (mg/100 g) and TAS values (mmol/L) in both freshly squeezed (r:0.856, p<0.05) and ready-made orange juices (r:0.955, p<0.05). There is a positive correlation (p<0.05) between ascorbic acid (mg/100 g) and TAS values (mmol/L) in freshly squeezed orange juices purchased from stands (r:0.951, p<0.05), cafés (r:0.904, p<0.05) and patisseries/bakeries/pastry shops (r:0.697, p<0.05). In freshly squeezed orange juices; TAS values (mmol/L) are positively correlated (p<0.05) with ascorbic acid (mg/100 g) in both freshly squeezed juices (r:0.777, p<0.05) and kept juices (r:0.986, p<0.05) (Data not shown in the table).

In this study, similar to the vitamin C results, antioxidant capacities of freshly squeezed orange juices were found to be higher compared to ready orange juices (3.3±0.35 vs.1.2±0.90 mmol/L) and vary depending on the place of purchase (p<0.05) (Table 1). The antioxidant capacities of the freshly squeezed but kept orange juices were also found to be lower (p<0.05) (Table 1). Citrus fruits and juices contain many phytochemical/bioactive compounds that contribute to antioxidant capacity in addition to vitamin C. In a study evaluating the total antioxidant potential of beverages, it was determined that red grapes juice> mango juice> guava juice> cocktail juice> pineapple juice> orange juice> cherry juice> apple juice have antioxidant potentials, respectively (Ramadan-Hassanien, 2008). However, it is reported that fruit juice production processes have important effects on antioxidant potential, and contrary to manual squeezing, industrial production techniques can cause significant losses in bioactive compounds such as caffeic acid derivatives, vicenin 2 (apigenin 6,8-di-C-glucoside), and narirutin (5,7,4'-trihydroxyflavanone-7-rutinoside).

However, it was determined that such changes have no significant effect on the total antioxidant capacity of fruit juices, and pulp and L-ascorbic acid contribute to the antioxidant capacity of orange juices (77-96%) (Gil-Izquierdo *et al.,* 2002). Similarly, the present study also found a correlation between the vitamin C content and TAS values of orange juices. In a similar study, daily consumption of 500 mL of orange juice increased plasma C vitamin levels and decreases the concentration of 8-epi-PGF (2 alpha) and improves antioxidant status especially in smokers (Sánchez-Moreno *et al.,* 2003). Moreover, 8 fl. oz. doses of orange juice are reported to reduce lipid peroxidation, similar to 70 mg daily dose of vitamin C supplements, in healthy women (Johnston *et al.,* 2003). In terms of antioxidant activity, the bioavailability of bioactive compounds and antioxidant components in fruit juices is also an issue under discussion. A study found that the bioactive compounds of orange pulp and juice show high bioavailability, and especially the fruit itself contributes to antioxidant activity (De Ancos *et al.,* 2017). However, another study reports that the vitamin C bioavailability and antioxidant activity of freshly squeezed orange juices may vary depending on the orange type rather than the processing method (Mennah-Govela & Bornhorst, 2017). In this study, no characterization study was performed for bioactive compounds other than vitamin C, which may exhibit antioxidant activity, and it is thought that it will be beneficial in future studies.

4. Conclusions

In summary, this study determined that the majority of consumers have a tendency especially towards freshly squeezed fruit juice types, and the important motivation factors include nutritional value and health perception. Supporting the perception of consumers, ascorbic acid content and antioxidant capacities of freshly squeezed orange juices were determined to be higher compared to ready-made juices, and it was emphasized that the fruit juices must be consumed without being kept even if they are freshly squeezed.

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MICROENCAPSULATION OF *LACTOBACILLUS ACIDOPHILUS* 5 WITH ISOMALTO-OLIGOSACCHARIDE

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ABSTRACT

Co-extrusion microencapsulation of *Lactobacillus acidophilus* 5 (La-5) was performed using isomalto-oligosaccharide (IMO) as prebiotic, alginate as shell material and chitosan as the coating. The optimization of alginate (1.3% (w/v) to 1.7% (w/v)) and IMO concentration (1.0% (w/v) to 5.0% (w/v)) was evaluated based on bead size and microencapsulation efficiency of La-5. Subsequently, the chitosan-coated alginate with or without IMO were subjected to sequential digestion. It is found that 3.0% (w/v) IMO and 1.5% (w/v) alginate were the optimal concentration based on microencapsulation efficiency (MEE). The morphology of the beads containing IMO was found to be smooth and spherical, with diameter of 622.00 µm. The addition of IMO and chitosan are effective in protecting La-5 under gastric conditions but not effective in protecting the viability of La-5 under intestinal digestion.

1.Introduction

In recent years, research on probiotics as nutraceuticals and functional food has received increasing attention globally. Probiotics are bacteria defined as "live microorganisms in which when administered in adequate amounts confer a health benefit on the host" (Siang et al., 2019). Probiotics are used as food supplements as they can enhance the immune system and improve protection in terms of gastrointestinal health against pathogens (Chaikham et al., 2012). In addition, consumption of probiotics has been associated with several health benefits such as boosting immune function, maintenance of mucosal integrity, treat atopic and allergic diseases (Liserre et al., 2007).

Some probiotics might lose viability in the gastrointestinal tract since they are sensitive to low acidic conditions, presence of trypsin, pepsin, and bile salts in the stomach (Sahadeva et al., 2011). Hence, to have beneficial effects to the body, the probiotics must survive through these harsh conditions (Martín et al., 2015).

Prebiotics are non-digestible food components that are stimulating the growth of beneficial colonic bacteria of the host (Siang et al., 2019). Probiotics incorporated with prebiotic in functional foods are known as synbiotics (Roberfroid, 1998). With prebiotic consumption, it is reported that it can prevent colon cancer, lower cholesterol levels, and reduce diarrhea (Patel and Goval, 2012). Fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, and lactulose are common prebiotics incorporated into food.

Isomaltooligosaccharide (IMO) are one of the emerging prebiotics, naturally found in fermented food such as soy sauce, miso, or sake and honey (Gourineni et al., 2018). Health claims of IMO reported includes the activation of the immune system, improving liver and

kidneys function, enhancing the resistance to diseases as well as improving lipid metabolism (Li et al., 2009).

Microencapsulation is a process that encapsulates sample with an encapsulating matrix or membrane (Krasaekoopt et al., 2004). It protects probiotic bacteria against the harsh condition in the digestive tract (Etchepare et al., 2016). In microencapsulation, the semipermeable membrane surrounds the liquid core. This allows the excretion of secondary metabolites with penetration of oxygen supply to reach entrapped live probiotic bacteria.

Microencapsulation has been a prominent method for protecting probiotic from harsh conditions (Ozyurt and Ötles, 2014). Studies on the encapsulation method of probiotic includes extrusion, spray-drying, and emulsion techniques (Chew et al., 2019; Gandomi et al., 2016). Co-extrusion method can produce uniform and smaller size beads as compared to extrusion technique (Krasaekoopt et al., 2004). However, there are fewer reports on application of co-extrusion technique to encapsulate probiotic as compare to extrusion techniques (Silva et al., 2016; Olivares et al., 2017). In our previous work, co-extrusion microencapsulation of probiotic such as *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. lactis BB-12 (Lai et al., 2020; Ng et al., 2019; Siang et al., 2019, Yee et al., 2019; Yong et al., 2020).

This study aims to produce microencapsulated probiotic bacteria, *Lactobacillus acidophilus* 5, using the co-extrusion technique. The effectiveness of prebiotic and co-extrusion microencapsulation in protecting La-5 from the gastrointestinal condition were investigated.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Lactobacillus acidophilus La-5 was purchased from Bio-Life, Malaysia. All the chitosan and sodium alginate were of food-grade, while the chemicals and reagents were of analytical grade.

2.2. *Microencapsulation of LA-5 using co-extrusion technique*

Microencapsulation of La-5 was carried out using Büchi Encapsulator B-390 (Büchi, Switzerland) through the co-extrusion method as described by Ng et al. (2019) with modification. The core fluid (comprised of La-5 suspended in PBS with or without IMO) and shell fluid (sodium alginate solution) were added into two separate pressured bottles connecting to the Büchi Encapsulator B-390. During the microencapsulation process, core fluid and shell fluid were pumped simultaneously through the concentric nozzles with a diameter of 200 μm (inner nozzle) and 300 μm (shell nozzle) by the air pressure of 600 mbar to give a core-shell fluid stream. The vibration frequency of the nozzle was set at 300 Hz, with an amplitude of 3 and a voltage of 1.5 kV.

2.2.1. Optimization of Alginate

The optimization process was carried out using different concentrations of alginate from 1.3% (w/v) to 1.7% (w/v), with the concentration of calcium chloride fixed at 2.0% (w/v). The alginate beads were determined based on the microencapsulation efficiency of probiotics and bead size.

2.2.2. Optimization of Isomalto-oligosaccharide (IMO)

The optimization process was carried out using different concentration of IMO from 1.0% (w/v) to 5.0% (w/v), with a concentration of alginate fixed at 1.5% (w/v) (Siang et al., 2019). The optimum concentration of IMO was determined based on bead size and the viable cell counts in colony-forming unit per milliliter (CFU/mL) (Equation 1). Microencapsulation efficiency was calculated using Equation 2.

Colony forming unit (CFU/mL) = (Average number of colonies)/(Dilution factor × volume plated) (1)

Microencapsulation efficiency (%) = ($Log_{10}N$/ $Log_{10}N_0$) x 100 (2)

Where N represents the number of microencapsulated probiotics released from beads, and N_0 represents the number of probiotics in the initial microbial suspension.

2.2.3. Morphology and size of bead

The morphology and mean diameter of 10 randomly selected beads were determined and size measured using an optical microscope (Olympus, Japan), with x100 magnification, fitted with a micrometer scale (Lai et al., 2020).

2.3. *Sequential digestion of La-5*

Sequential digestion of La-5 was adapted from method reported by Yee et al. (2019) with slight modification. About 1 g of beads or 1 mL of free cells were added to 15 mL falcon tube (BD FalconTM, USA) containing 9 mL of sterile SGJ at pH 2.0. Simulated gastric juice (SGJ) consists of 3.5 mL of hydrochloric acid (Merck KGaA, Germany), 1 g of sodium chloride (R&M Chemicals, UK) in 500 mL distilled water, with pH adjusted to 2.0. It is then autoclaved at 21°C for 15 min, before adding 1.6 g of pepsin (Chemsoln, India).

The mixture was then incubated at 37°C and agitated gently at 150 rpm and for 1 hour and 2 hours in the incubator. After 1 hour and 2 hours exposures, SGJ was removed by centrifugation at 4200 rpm, 4°C for 10 min to test for the viability of La-5. After incubation in SGJ for 2 hours, the beads or free cells were transferred into 9 mL of SIJ. Simulated intestinal juice (SIJ) was prepared, according to Yong et al. (2020), with modification. SIJ consisted of 3.4 g potassium dihydrogen phosphate (Bendosen, Germany), 95 mL of sodium hydroxide (Merck KGaA, Germany) in total of 500 mL solution, adjusted to pH 7.5. It was then sterilized at 121°C for 15 min before adding 3 g of bile salt (Chemsoln, India)

The mixture was then incubated at 37°C for 1 hour and 2 hours with constant agitation at 150 rpm in an incubator. After incubation, the mixture was centrifuged at 4200 rpm, 4°C for 10 min, and the SIJ discarded. Before cell enumeration, the filtered beads were washed with sterile PBS to remove the excess SIJ solution. 9 mL of 4.0% (w/v) tri-sodium citrate (Merck KGaA, Germany) was added and followed by vortexing to release bacteria from retrieved beads. For cell enumeration, an aliquot of 0.1 mL of the mixture was spread on the MRS agar plate and incubate at 37°C for 24 hours. The viable cell counts for microcapsules and free cells were expressed as logarithm colony forming unit per gram (log CFU/g) and logarithm colony forming unit per milliliter (log CFU/mL), respectively. Survivability (%) of probiotic after exposure to SGJ and SIJ was calculated using equation 3.

Survivability (%) = $Log_{10}N_t$/ $Log_{10}N_0$ x 100 (3)

N_t is the number of viable cells in the free cell (CFU/mL) or beads (CFU/g) after exposure to SGJ or SIJ, and N_0 is the number of viable cells in the free cell (CFU/mL) or beads (CFU/g) at 0 hours.

2.4. *Statistical Analysis*

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analyzed using MINITAB 16 (Minitab Inc, Pennsylvania, USA). One-way analysis of variance (ANOVA) was carried out, with Tukey's HSD test to determine the significant difference set at $p \leq 0.05$.

3.Results and discussions

3.1. Optimization of concentration of alginate and IMO

The effect of alginate concentrations on size of bead produced and microencapsulation efficiency *Lactobacillus acidophilus* 5 (La-5)

were exhibited in Table 1. Chitosan and calcium chloride concentration were fixed at 0.4% (w/v) and 2.0% (w/v), respectively. It was found that there was no significant difference ($p>0.05$) on beads size among beads produced with different alginate concentration

From Table 1, it was observed that the microencapsulation efficiency increases when alginate concentration increased from 1.3% (w/v) to 1.5% (w/v), with the maximum microencapsulation efficiency (91.26%) at 1.5% (w/v). However, with further increase, the microencapsulation efficiency decreases slightly (87.90%) at alginate concentration 1.7% (w/v).

This is in agreement with Lotfipour et al. (2012), who reported that encapsulation of *Lactobacillus acidophilus* with alginate concentration 1.5% (w/v) to 2% (w/v) and calcium chloride concentration at 2.5% (w/v), have high microencapsulation efficiency at 98%.

Table 1. Effect of different concentration of alginate on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

Alginate (% (w/v))	Calcium chloride (% (w/v))	Diameter (μm)	Microencapsulation efficiency (%)
1.3	2.0	481.00 ± 88.6^{a}	83.39 ± 0.80^{c}
1.4	2.0	490.00 ± 94.4^{a}	84.70 ± 0.61^{c}
1.5	2.0	425.00 ± 70.4^{a}	91.26 ± 0.45^{a}
1.6	2.0	408.00 ± 141.8^{a}	89.58 ± 0.68^{ab}
1.7	2.0	411.00 ± 110.7^{a}	87.89 ± 0.67^{b}

$^{a-c}$Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

According to Mandal et al. (2010), increased alginate concentration could lead to higher encapsulation efficiency. The cross-linking of sodium alginate with calcium chloride produces a tight junction between the guluronic acid residues (Rajinikanth et al., 2003). The further increase of alginate concentration will then lead to increase in number of the cross-linking points formed, as there is greater availability of active calcium-binding sites in the polymeric chains (Mandal et al., 2010).

Table 2, on the other hand, shows the effects of different concentrations of isomalto-saccharide (IMO) on produced bead size and microencapsulation efficiency of La-5. The concentration of alginate was fixed at 1.5% (w/v).

Table 2. Effect of different concentration of isomalto-oligosaccharide (IMO) on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

IMO (% w/v)	Alginate (% w/v)	Diameter (μm)	Microencapsulation efficiency (%)
1.0	1.5	599.00 ± 87.1^{a}	87.93 ± 0.27^{b}
2.0	1.5	610.00 ± 103.7^{a}	84.75 ± 1.114^{b}
3.0	1.5	622.00 ± 87.4^{a}	94.42 ± 1.42^{a}
4.0	1.5	587.00 ± 123.3^{a}	91.84 ± 2.57^{a}
5.0	1.5	539.00 ± 111.5^{a}	86.71 ± 0.57^{b}

$^{a-c}$Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

From Table 2, it was found that there was no significant difference ($p>0.05$) among the beads size when produced with different IMO concentration. Studies by Haghshenas et al. (2015) and Ng et al. (2019) found that size of beads is not affected by increasing the prebiotic concentration. The bead produced in this study, which ranges from 539 to 622 μm, was in agreement with the work of Yee et al. (2019), who reported that the bead size of microencapsulated *Lactobacillus acidophilus* NCFM were in the range of 543 to 613 μm.

During microencapsulation, the addition of prebiotics will influence the size of bead, as observed in Table 2, as compared to Table 1. The diameter of beads without prebiotics was smaller than the beads with prebiotics for all encapsulated probiotics (Krasaekoopt and Watcharapoka, 2014).

From Table 2, it was also observed when the IMO concentration increases, the microencapsulation efficiency increased, with 3% (w/v) IMO showed the maximum microencapsulation efficiency (94.42%). La-5 produces oligo 1-6 glucosidase enzymes to hydrolyze IMO from the sucrase-isomaltase complex into D-glucose. IMO helps in stabilizing and increase probiotics resistance in gastrointestinal conditions.

3.2. Morphology, size and microencapsulation efficiency of bead

Figure 1 shows the size and shape of microcapsules measured with a scale micrometer, while bead size and microencapsulation efficiency of with and without IMO addition are shown in Table 3. From Figure 1, it was found that the bead produced was white and surrounded by a thin layer of the membrane. It was noted that the shape of the beads was generally spherical, with some oval shaped.

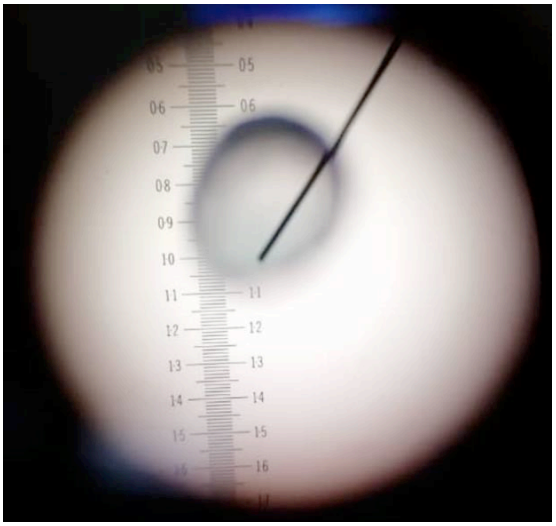

Figure 1. Shape and size of microcapsules measured with a stage micrometer (Ladd Research model 75545) using an optical microscope (Olympus model CX31)

Table 3. Effect of different concentration of isomalto-oligosaccharide (IMO) on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

Probiotic	Prebiotic	Diameter (μm)	Microencapsulation efficiency (%)
La-5	-	532.00 ± 0.07^{b}	95.32 ± 1.25^{a}
La-5	IMO	616.00 ± 0.09^{a}	92.51 ± 0.32^{b}

[a-b]Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

Bead produced using sodium alginate generally has a smooth surface (Solanki et al., 2013). This is important as bead with a broken surface (protrusion of cell) will lower the survivability of encapsulated cells because the chances of the free cell in the bead expose to the external environment and unable to protect encapsulated probiotic (Krasaekoopt and Watchapoka 2014).

The size of beads produced without prebiotic was smaller (532.00 μm) as compared to those with prebiotic (616.00 μm) (Table 3). Different concentrations and viscosity of alginate, calcium chloride solution, and the size of the nozzle could influence the difference in bead size (Solanki et al., 2013). High sodium alginate viscosity resulted in larger size of beads, as the higher coaxial air flow rate is needed to cut the droplet (Bhujbal et al., 2014). Also, increment in bead size can improve the stability of bead due to cross-linkage formation between divalent ions and alginate bead (Bhujbal et al., 2014).

Furthermore, the production of beads with micron-size create a smooth texture when it is added into food product (Fahimdanesh et al., 2012; Zanjani et al., 2012). Large beads size gives better protection on probiotics, but it will affect the sensory properties when it is used for consumption. While for the size of beads smaller than 100 μm, will prevent the coarse texture from being detected in mouth (Zanjani et al., 2017).

The beads sphericity may prevent the problem of cell overgrowth in encapsulated beads (McMaster et al., 2005). The range between 200 μm to 3000 μm of beads size can protect probiotic against harsh conditions (Heidebach et al., 2012). However, the study of showed that the co-extrusion technique is useful in producing beads which are smaller than the extrusion technique. Co-extrusion method produces bead size with smaller and more consistent in size, as compared to extrusion method (Krasaekoopt and Watchapoka 2014).

From Table 3, the microencapsulation efficiency of La-5 without IMO is higher (95.32%), as compared to the ones with IMO (92.51%). This is in agreement with the work of Ng et al. (2019) where the encapsulated beads without FOS has microencapsulation efficiency of 97%, as compared to beads with FOS (93%), as the prebiotic serves as food for probiotic to improve its growth.

3.3. Sequential digestion for free cell encapsulated La-5 with and without IMO

Sequential digestion is the continuous incubation of cell or beads in simulated gastric juice (SGJ) and subsequently incubate in simulated intestinal juice (SIJ) to mimic the human gastrointestinal condition (Minekus et al., 2014). 1 to 2 hours are required to digest the food in the stomach and followed by the partially digested food entering through the small intestine is 1.6 to 4.8 hours. However, the duration may be varied due to factors such as eating time, type of food intake, body type, and body size (Hellmig et al., 2006). Figure 2 shows the viability and survivability of free cells, encapsulated La-5 with and without IMO under sequential digestion.

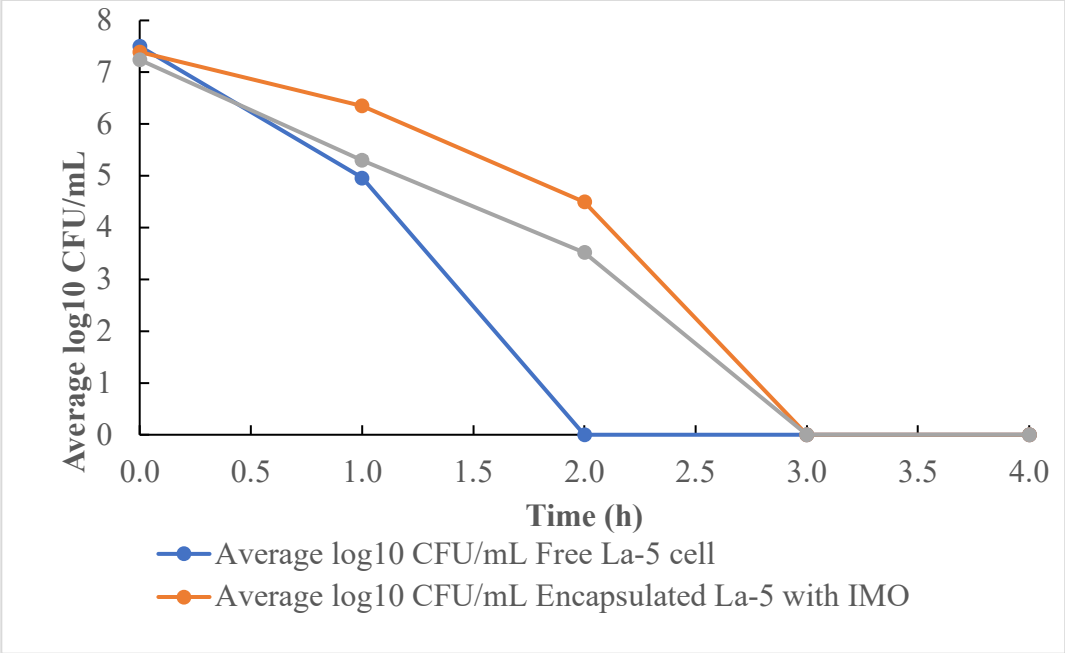

Figure 2. Average $\log_{10}$ CFU/mL and survivability of free cell encapsulated La-5 with and without IMO under sequential digestion.

From Figure 2, after exposure to acidic conditions, the viability of the La-5 free cell decreased notably compared to encapsulated La-5 with and without IMO. The viability of free La-5 cells dropped drastically by 33.83% from 100% (7.50 log CFU/mL) to 66.17% (4.96 log CFU/mL) after 1 hour of incubation in SGJ, followed by drastic reduction was below the detection limit (≤ 2 log10 CFU/mL) after exposure to 2 hours of simulated gastric juice (pH 1.2). This is because stomach probiotics in free form were easily damaged by stomach acid (Shi et al., 2013). Gebara et al. (2013) reported that La-5 cells reduced 3.54 log units after exposure to 5 hours of simulated gastric juice (pH 3.0) and simulated intestinal juice (pH 7.0).

The viable cell count of encapsulated La-5 without IMO showed a greater reduction, with viable cells count of encapsulated La-5 without IMO decreased significantly from 100% (7.24 log CFU/mL) to 73.25% (5.30 log CFU/mL) after 1 hour of exposure and to 48.63% (3.52 log CFU/mL) after 2 hours of exposure in SGJ.

On the other hand, the viable cells count of encapsulated La-5 with IMO reduced slightly from 100% (7.39 log CFU/mL) to 85.95% (6.35 log CFU/mL) after 1 hour of exposure in SGJ. However, after 2 hours of exposure in SGJ, the viable cell count dropped significantly to 60.92% (4.50 log CFU/mL).

Generally, encapsulated La-5 with IMO has best survivability, followed by encapsulated La-5 without IMO and lastly free La-5 cells. Alginate beads improve the physical and chemical characteristics of beads. The structure of the beads is stronger and denser, protecting probiotics and minimizing the exposure in gastrointestinal tract (Zhou et al., 1998; Krasaekoopt et al., 2004).

Chavarri et al. (2010) reported that alginate-chitosan were able to enhance microencapsulated *Lactobacillus gasseri* and *Bifidobacterium bifidum* maintained cell concentration at about 10^7 CFU/mL as compared to free cells, which decreased drastically in simulated gastric conditions after 2 hours of incubation. The electrostatic interactions between chitosan and alginate

happens when chitosan binds to alginate forms a strong membrane of the beads (Gaserod et al., 1998).

On the other hand, when subjected to SIJ, all 3 cells form (free cells, encapsulated La-5 with and without IMO) have 0 viability. Alginate is stable in low pH solution, which hydrochloric acid (HCl) presents in gastric juice but swelling in a weakly base condition (Annan et al., 2008). Krasaekoopt et al. (2004) also reported that encapsulated *Lactobacillus acidophilus* shows lower resistance in the presence of 0.6% (w/v) bile salt solution. Hence, further enhancement of encapsulation material is needed.

4. Conclusions

In this study, *Lactobacillus acidophilus* 5 (La-5) was microencapsulated with IMO as prebiotic. 1.5% (w/v) alginate and 3.0% (w/v) IMO was selected as optimized parameters, with 622.00 μm bead size and highest microencapsulation (94.42%). When subjected to SGJ, both IMO and chitosan have enhanced the survivability of La-5 in gastric conditions but not in intestinal conditions. Further studies are required to enhance the effect of IMO and chitosan in protecting La-5 from intestinal juice to confer health benefits for humans.

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TRADITIONAL FERMENTED WHEAT: NUTRITIONAL QUALITY AND SENSORY EVALUATION OF BREAD PRODUCED FROM COMPOSITE FERMENTED WHEAT FLOUR

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ABSTRACT

In this study, nutritional quality of traditional fermented wheat was determined, followed by assessment of organoleptic acceptance of the bread produced from composite traditional fermented wheat flour. The results showed that samples of traditional fermented wheat are more or less rich in proteins (4.59%), crude fibers (1%), fat (11.29%), polyphenols (10.48 mg AGE/g) and flavonoids (7.99 mg QE/g). They also show interesting antioxidant activity. Furthermore, the bread made with a mixture of 10% traditional fermented wheat flour was highly appreciated and had the best sensory qualities.

1.Introduction

Cereals are considered one of the most important sources of dietary protein, carbohydrates, vitamins, minerals and fiber for people all around the world (Kohajdovà and Karovicovà, 2007), however, Chavan *et al.* (1989); reported that the nutritional quality of cereal grains and the sensory properties of their products are inferior. In this regards, fermentation of cereals comes to extend shelf-life, improve palatability, digestibility, nutritive value (Shekib, 1994; Holzapfel, 2002) texture, taste and aroma (Deshpande, 2000; Kohajdovà and Karovicovà, 2007).

Several authors have demonstrated that the spontaneous fermentation of cereals can involve lactic acid bacteria, yeasts and molds (Kohajdovà and Karovicovà, 2007; Viéra-Dalodé *et al.,* 2007). Lactic acid fermentation processes are the oldest and most important economical forms of production and preservation of food for human consumption (Blandino *et al.,* 2003). This fermentation exerts chemical changes in food accelerated by enzymes of lactic acid bacteria. It is estimated that the largest spectrum of lactic acid fermented foods exists in Africa (Holzapfel, 2002). Moreover, spontaneous fermentation processes at the household or small industry level are often used to prepare fermented foods and beverages using maize, sorghum and millet as the main cereals in Africa (Blandino *et al.,* 2003). These spontaneous fermentation processes also take place in parts of Eastern Algeria to produce fermented wheat in order to prepare traditional foods.

To store cereals, several methods are employed by Algerian farmers but the traditional techniques are still used and give special products. Such techniques are based on the use of underground holes or silos built near the farm at generally high places. These stuffs are called 'Matmours'. This method gives to durum wheat brown color and very strong acid odor, following the natural fermentation due to

native micro-organisms, giving rise to the traditional fermented wheat locally named 'Mzeyet' and in other parts of Algeria it is called 'Elhammoum', which is used as the main ingredient in the preparation of a valued traditional Algerian dish (Couscous).

The aim of this paper is to examine the current knowledge on a traditional Algerian fermented food (fermented wheat, Mzeyet or Elhammoum), to provide more information regarding its biochemical and nutritional quality, as well as to evaluate its potential to be used as a supplement in bread technology.

2. Materials and methods

2.1. Sample collection

The different samples of traditional fermented wheat (TFW) were collected from two regions situated in Eastern Algeria namely Jijel and Mila. The eight samples were obtained after being subjected to traditional spontaneous fermentation in Matmours during approximately 10 months in rural areas.

2.2. Biochemical analysis

The traditional fermented wheat samples were subjected to biochemical evaluation; the pH and the greasy acidity were measured according to Multon (1982) and AFNOR (NF V 033-712 1982) procedure respectively. The protein contents (Kjeldhal method) and the starch content were determined according to Lecoq (1965), crude fiber content was measured employing AOAC procedure (1995) and fat content according to Serna-Saldivar (2012) procedure. Carbohydrates were determined by difference as described by Srivastava *et al.* (2002):

Carbohydrate (%) = 100 – [protein (%) + fat (%) + ash (%) + moisture (%)]. (1)

2.3. Antioxidant activity of methanolic wheat extract

Lyophilized traditional fermented wheat was homogenized with 70 % methanol during 48 h and the mixture was filtered using a filter paper. The filtrate was concentrated at the temperature of 45 °C (Bruneton, 1999).

2.3.1. Total polyphenolic and flavonoid contents

The total phenolic content (TPC) was investigated using the Folin-Ciocalteau assay (Othman *et al.*, 2007). Briefly: 0.2 ml of sample was mixed with 1.5 ml of Folin-Ciocalteau reagent. After 5 min, 1.5 ml of 7 % Na_2CO_3 solution was added and the mixture was incubated for 90 min, then the absorbance was measured at 750 nm. The total phenolic content was expressed as mg gallic acid equivalents per g of dry weight (mg GAE / g). To determine the total flavonoid content (TFC), aluminium chloride complex forming assay was used according to Djeridane *et al.* (2006). In this test, 1.5 ml of sample was added to 1.5 ml of aluminum chloride solution (2 %). The mixture was allowed to stand in darkness for 30 min. The absorbance of this reaction mixture was recorded at 430 nm and the results are expressed as mg quercetin equivalents per g of dry weight (mg QE/g).

2.3.2. DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

Antioxidant activity of the Traditional fermented wheat extracts (TFWE) was measured as scavenging free radical potential in ethanolic solution of DPPH, as described by Brand-Williams *et al.* (1995). 100 µl of TFWE were added to 3 ml of 0.025 g / l DPPH ethanolic solution freshly prepared. After incubation for 30 min at room temperature and in darkness, the absorbance was recorded at 517 nm and the antiradical activity was calculated as percentage of DPPH discoloration compared to the control using following formula:

Inhibition percentage = [(A-B) / A] x 100

Where (A) is the absorbance of pure DPPH in oxidized form and (B) is the absorbance of the sample.

2.4. Bread preparation and analysis

2.4.1. Flour blends and backing process

Three flour blends were prepared by mixing wheat flour with traditional fermented wheat flour (TFWF) in the proportions 90 / 10, 80 / 20 and 70 / 30 (w / w), while 100 % unfermented wheat flour (UFWF) was used as control. The four flour samples were stored at room temperature for bread production.

Bread was backed from the flour samples using the usual dough method in a commercial bakery, located in Jijel, Algeria. The formulations of the breads with TFWF were developed by modification of the traditional formulation, using different levels of TFWF addition (0 %, 10 %, 20 % and 30 %) in substitution for part of the UFWF (Table 1).

To obtain bread, the ingredients (UFWF, TFWF, fresh yeast, salt, flour improver and water) were mixed manually in laboratory then kneaded. After kneading, the dough was divided into 250 g dough pieces and these were balled and allowed to rise at room temperature for 20 min. The dough pieces were then shaped before being placed for 40 min in a fermentation chamber (humidity - temperature: 75 % - 35 °C). The baking takes place at 210 °C for 40 min in a plate oven equipped with a steam injection system. Once baked, breads were allowed to cool down for 30 min, and kept at room temperature for further assessment (Abdourahamane *et al.,* 1999).

Table 1. Bread making ingredients

Ingredients	Formulation			
	UFWF	10 % TFWF	20 % TFWF	30 % TFWF
UFWF	250 g	225 g	200 g	175 g
TFWF	00 g	25 g	50 g	75 g
Yeast	12.5 g	12.5 g	12.5 g	12.5 g
Salt	4.25 g	4.25 g	4.25 g	4.25 g
Flour improver	0.1 g	0.1 g	0.1 g	0.1 g
water	-	-	-	-

UFWF: unfermented wheat flour, TFWF: traditional fermented wheat flour, -: variable amounts

2.4.2. Gas retention and sensory evaluation of bread

The properties of gas retention during fermentation were evaluated by manually measuring the dough height during 60 min.

The bread samples were subjected to sensory evaluation about 1 h after baking by a jury composed of 6 tasters that were very familiar with bread. The sensory characteristics of the breads were evaluated according to the following criteria: loaf color, crust, crumb texture, aroma, taste and overall acceptability of the bread sample. The panelists rated their acceptability of the product on a 01-09 point hedonic scale (Ijah *et al.*, 2014).

2.5. Statistical analysis

All data were performed using SPSS software version 22.0 for windows. The data obtained from the analyses are expressed as the mean ± standard (SD). Statistical differences were analyzed by one way analysis of variance (ANOVA) at $p < 0.05$. Correlation analysis between some parameters was performed using Pearson correlation at $p < 0.05$.

3. Results and discussions

3.1. Biochemical analysis of traditional fermented wheat

The eight samples under study showed significant differences in pH values ($p < 0.001$***), these values varied from 3.94 to 6.89 with an average of 5.25. The most acidic sample was WM4 (3.94 ± 0.09), while, the least acidic one was WM5 (6.89 ± 0.17) (Table 2). Similar results were reported in an earlier study conducted by Gourchala *et al.* (2014) (5.63 ± 0.014), in which they compared naturally fermented wheat samples to unfermented ones. However, these values were higher than those found by Doukani *et al.* (2013), where a value of 4.45 was recorded as being the most acidic. The lowest pH value (3.94) makes the WM4 sample very acidic. This can be explained by a long fermentation time in Matmour, which leads to a large bacterial activity responsible for the accumulation of organic, lactic and other acids,

subsequently causing a decrease in pH (Kohajdovà and Karovicovà, 2007).

Table 2. Biochemical characteristics of traditional fermented wheat

Means ± SD	WM1	WM2	WJ1	WM3	WM4	WJ2	WM5	WM6
pH (***)	5.61 ± 0.30	5.75 ± 0.02	4.93 ± 0.09	4.57 ± 0.02	3.94 ± 0.09	4.04 ± 0.03	6.89 ± 0.17	6.33 ± 0.26
Greasy acidity (***)	10.86 ± 3.74	10.43 ± 3.21	8.39 ± 3.24	9.18 ± 0.62	7.44 ± 0.00	12.19 ± 1.00	2.19 ± 0.27	6.46 ± 0.69
Total sugars (%) (***)	80.52 ± 0.57	49.08 ± 0.29	79.13 ± 0.02	55.10 ± 0.72	79.81 ± 0.73	65.86 ± 0.67	87.70 ± 0.65	81.83 ± 0.65
Starch (%)	38.60 ± 0.07	13.20 ± 0.60	31.20 ± 0.07	10.00 ± 0.04	11.50 ± 0.12	13.70 ± 0.07	39.00 ± 1.00	56.00 ± 8.00
Crude fiber (%)	1.00 ± 0.00	2.01 ± 0.00	0.29 ± 0.00	0.26 ± 0.02	0.51 ± 0.02	0.29 ± 0.03	1.37 ± 0.20	2.30 ± 0.30
Total proteins (%)	0.17 ± 0.00	5.46 ± 0.00	5.84 ± 0.00	18.81 ± 0.00	1.96 ± 0.00	1.75 ± 0.00	1.42 ± 0.00	1.31 ± 0.00
Total fat content (%)	ND	ND	5.30 ± 0.00	ND	6.13 ± 0.00	ND	0.75 ± 0.00	1.30 ± 0.00
Total phenolic content (mg GAE/g) (***)	10.36 ±0.11	9.37 ±0.09	9.72 ± 0.10	9.81 ±0.14	10.61 ±0.08	10.79 ±0.16	11.21 ±0.12	11.97 ±0.03
Total flavonoid content (mg QE/g) (***)	8.36 ±1.13	8.25 ±0.28	9.11 ±0.41	9.22 ±0.17	9.24 ±0.07	8.51 ±0.42	3.77 ±0.00	7.51 ±0.00

WM1, WM2, WM3, WM4, WM5, WM6, WJ1, WJ2: traditional fermented wheat samples. ND: not determined. Values are mean ± SD, differences were evaluated by one-way analysis of variance (ANOVA) ($p < 0.05$).

In the same table, greasy acidity values range from 2.19 ± 0.27 to 12.19 ± 1 ($p < 0.001$***). The lowest value was found in sample WM5 and the highest one in sample WJ2. According to Doukani *et al.* (2013), the wheat fermented in a Matmour has a greasy acidity of about 1.51 %. The increase of greasy acidity level in the eight samples may be explained by inadequate grain storage conditions, which lead to hydrolysis of triglycerides by endogenous and exogenous lipases, and thereafter, the accumulation of free fatty acids (Feillet, 2000). Indeed, we noted a significant correlation between greasy acidity values, and the recorded pH values ($r = -0,611$**, $p = 0.002$**).

Sugar contents in the eight samples varied significantly ($p < 0.001$***). They ranged from 49.08 ± 0.29 % to 87.70 ± 0.65 %. We noticed that sample WM5 had the highest carbohydrate content (87.70 ± 0.65 %) followed by WM6, WM1, WM4 and WJ1. However, low values were registered in samples WM2, WM3 and WJ2 (49.08 ± 0.29 %, 55.10 ± 0.72 % and 65.86 ± 67 % respectively). The low content of sugar in TFW samples is due probably to excessive fermentation in underground silos. The same results were found in previous studies (Deshpande, 2000; Doukani *et al.*, 2013; Gourchala *et al.*, 2014). Wheat seeds contain amylolytic enzymes which release maltodextrins, maltose and glucose. These endogenous enzymatic activities play an important role in starch degradation, which is considered a source of fermentable sugars (Ganzle, 2014). The decrease in carbohydrate content may be due to their use as fermentable substrate by wheat microflora during fermentation. For this reason, fermented wheat is suitable for diabetics.

Starch represents a major part of carbohydrate in the mature wheat kernel (Matz, 1991; Bushuk and Rasper, 2012). As shown in Table 2; starch contents of TFW samples ranged from 10 ± 0.04 % to 56 ± 8 % (26.68 % in average). These values are low compared to

that found by Gourchala *et al.* (2014) who recorded a value of 48.17 %. According to Matz (1991), starch content in unfermented wheat varied from 60 to 68 %. During fermentation, the starch is the mostly degraded substrate (Bekhouche *et al.,* 2014), and this is due to high amylase activity (Ganzle, 2014), which explains the low starch content in TFW samples.

The protein content is an important criterion for assessing the quality of wheat. As shown in Table 2, values of total protein contents varied from 0.17 % to 18.81 %. WM1 protein content (0.17 %) seemed to be very far from the limit suggested by Matz (1991) and Feillet (2000), who required a protein content of 7 to 18 % and 10 to 15 % respectively. The low protein content in the seven samples can be linked to protein degradation during fermentative process, which favors the action of certain endogenous cereal proteases (Kamal Eldin, 2012). On the other hand, the production of free amino acids resulting from hydrolysis of cereal proteins during fermentation (Thiele *et al.,* 2004) improves cereal nutritional quality (Blandino *et al.,* 2003) by increasing the content of the essential amino acids such as lysine, methionine and tryptophan (Adams, 1990). Otherwise, the high level of protein content in WM3 sample (18,81 %) can be explained by bacterial synthesis of new protein products during spontaneous natural fermentation (Kohajdovà and Karovicovà, 2007).

The results revealed that total fat contents values varied between 0.75 % and 6.13 % with an average of 3.37 %. The values appeared to be higher than those found by Doukani *et al.* (2013) (1.08 %). In fact, Matz (1991) and Feillet (2000) suggested a fat content of 1.5 to 2 % and 2 to 3 % respectively. WM5 and WM6 fat contents (0.75 % and 1.3 %) are lower than the values pointed to above; this can be explained as hydrolysis of triglycerides and liberation of fat acids during storage.

The data given in Table 2 indicate that TFW samples have a crude fiber content ranging from 0.26 and 2.3 % with an average of 0.71 %, which is low compared to the limits given by Matz (1991) and Feillet (2000), which are respectively 1.5 to 2 % and 2 to 4 %. However, these results are in agreement with those obtained by Doukani *et al.* (2013), who noted a decrease in crude fiber content in fermented wheat grains contrary to the control sample. The decrease can be explained as cellulose hydrolysis due to yeast activity during grain fermentation (Jesperson, 2003).

3.2. Antioxidant activity of wheat extract

Phenolic compounds play a role in free radical scavenging capacities. They are one of the most effective antioxidative constituents that contribute to the antioxidant activity (Govindarajan *et al.*, 2007). According to Table 2, the TPC of the samples varied significantly ($p < 0.001$***), the highest TPC was obtained in wheat extract of sample WM6 (11.97 ± 0.03 mg EAG / g). These results are not in perfect agreement with those found by Gourchala *et al.* (2014). In fact, they quantified total polyphenols in Elhammoum 'durum wheat fermented product', and they noted a significant increase in the total polyphenols content in the fermented wheat (23,75 mg EAG / g) compared to the unfermented wheat sample (18,32 mg EAG / g). The same results were found by Zhang *et al.* (2012). On the other hand, our present results seem to be in agreement with those reported by El hag *et al.* (2002), who found a decrease in total polyphenols during fermentation but after shelling.

The same table shows total flavonoid contents in the eight samples of TFW ($p < 0.001$***). The sample coded WM1 has the higher value, but compared to previous studies, these values are very low. Zhang *et al.* (2012) and Sandhu *et al.* (2016); proved that fermentation using fungus species promotes the increase of total polyphenols and flavonoids contents, Dordevic *et al.* (2010), found that fermentation using lactic acid bacteria and

yeast can enhance polyphenol and flavonoid contents, which is not confirmed by our results.

Figure 1 shows the DPPH antioxidant activity of TFWE samples. The decrease in the absorbance of DPPH radicals at 517 nm induced by antioxidants determines its reduction capacity. During radical scavenging assay, DPPH radical without extracts of TFW was stable over the time. However, in the presence of several concentrations of TFWE, DPPH radical is reduced to non-radical DPPH-H. This reduction depends on the used concentrations. It was found that the DPPH scavenging effect of TFWE increased with the increase of their concentration.

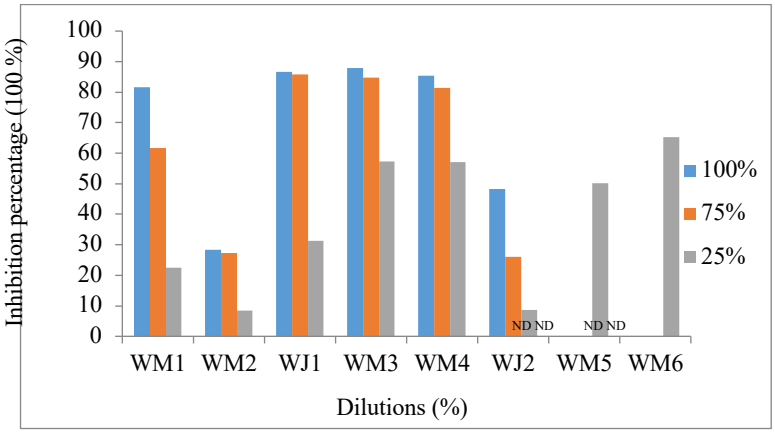

Figure 1. DPPH scavenging activity of different concentrations of TFW extracts
ND : not determined

According to Figure 1, the highest DPPH antioxidant capacity was attributed to WM3 fermented wheat sample (87.97 %) followed by WJ1 (86.55 %). At 75 % dilution, WJ1 also recorded the highest activity (85.77 %). The lowest activity was observed in WM2 sample at all dilutions. A positive correlation was also noted between flavonoid contents and antioxidant activity ($r = 0.856^*$, $p = 0.03^*$). DPPH antioxidant activity is effective in traditional fermented wheat, this can be explained by enhancement of antioxidants like polyphenols and flavonoids by fermentation. According to Zhang *et al.* (2012); DPPH antioxidant activity was more effective in wheat fermented using *Cordyceps militaris* than in unfermented wheat.

3.3. Bread analysis

The breads obtained are presented in Figure 2.

Table 3 shows measurement of dough height at 0, 20, 40 and 60 min. The dough height increases as time fermentation increases. At 20 min, the sample with (90:10 %) has the highest value while the bread obtained from (70:30 %) has the lowest value. The same results are noted at 40 and 60 min. It seems that the bread from (90:10 %) and (80:20 %) has better gas retention than that from unfermented wheat (100%) and from (70:30 %). The decrease in gas retention as fermented wheat flour proportions increase is probably due to a decrease in the elastic property of the flours, which depends on the gluten content. Such proteins could undergo an enzymatic degradation during the fermentation of the grains in the Matmour. The results obtained in our study are in agreement with those found by Okafor *et al.* (2012) for wheat mushroom powder bread, and Oloyede *et al.* (2013) for fermented unripe plantain flour, in regards to measurement of loaf volume of bread samples. Comparing the results for TFW dough gas retention to those of UFW dough, heighst values in TFW could be explained by the fact that the TFW flour is rich in simple products (such as fermentable sugars) used during

fermentative process of *Saccharomyces cerevisiae*.

According to Figure 3 which represents the mean sensory scores of experimental and control bread samples, after the bread from unfermented wheat, the bread from (90:10 %) was the most appreciated with a score of 7.03 ± 0.21, while the bread obtained from the (70:30 %) mixture was the least appreciated with a score of 4.83 ± 0.00. It is possible that the most brownish color of the crumb (Figure 2), which is dependent on percentage incorporation of TFWF, is the cause of the decrease in the acceptance of the products by consumers, who prefer bread with a lighter color (Gomes *et al.*, 2016), this result is confirmed by negative correlation between percentage incorporation of TFWF and sensory score of bread samples (r = - 0.991**, p < 0.001***). Oloyede *et al.* (2013); also reported low overall acceptability for bread produced from 30 % fermented unripe plaintain flour substitution. Although the bread from (90:10 %) had a score close to that of the bread from 100 % unfermented wheat, it is less acceptable than the latter. This result is similar to that found by Ameh *et al.* (2013), who noticed that the 100 % wheat bread was organoleptically more acceptable than the wheat bread supplemented with rice bran. So, breads of good sensory qualities could be produced from up to 10 % fermented wheat flour substitution in unfermented wheat flour. It is the same conclusion obtained by Olaoye *et al.* (2006) when they used soy flour substitution in wheat flour.

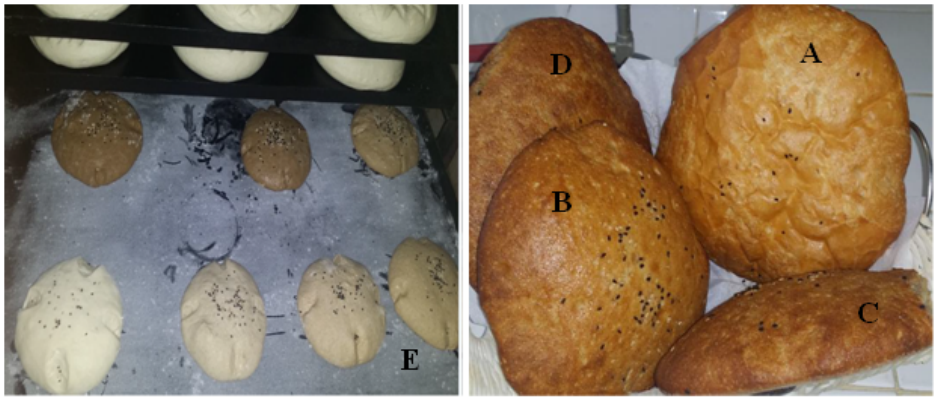

Figure 2. Aspect of produced bread
A: UFW (100 %), B: TFW (10 %), C: TFW (20 %), D: TFW (30 %) and E: aspect of bread during fermentation

Table 3. Physical properties of bread samples (dough height) (cm)

Fermentation time (min)	UFW (100%) (Cm) NS	UFW / TFW (90:10%) (Cm) NS	UFW / TFW (80:20%) (Cm) *	UFW / TFW (70:30%) (Cm) **
00	2 ± 0.00	2 ± 0.00	2 ± 0.00	2 ± 0.00
20	2.3 ± 0.00	3.15 ± 0.21	3.05 ±0.63	2 ± 0.00
40	2.6 ± 0.00	3.85 ± 1.06	3.5 ± 0.28	2.9 ± 0.14
60	3.2 ± 0.00	4.25 ± 1.06	3.95 ± 0.21	3.1 ± 0.14

UFW: unfermented wheat, TFW: traditional fermented wheat. Values are mean ± SD, differences were evaluated by one-way analysis of variance (ANOVA) ($p < 0.05$)

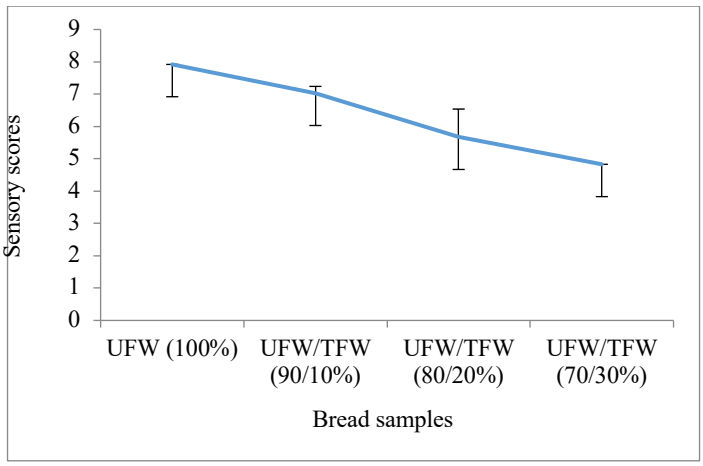

Figure 3. Sensory scores of bread samples
UFW: unfermented wheat, TFW: traditional fermented wheat

4. Conclusions

The fermentation of wheat in underground silos gives the grains special organoleptic characteristics and allows to obtain the Algerian traditional fermented product (Mzeyet or Elhammoum), which is much appreciated by Algerians and especially diabetics because it has a moderate carbohydrate content. Samples of traditional fermented wheat represent a satisfactory quality from a biochemical and nutritional point of view. The use of the flour obtained after milling the samples as supplementation in bread technology represents a very interesting task and makes it possible to obtain breads of good quality and organoleptic acceptability through the use of a proportion of incorporation at a ratio of 90:10 (unfermented wheat : traditional fermented wheat).

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CAN ESSENTIAL OILS STABILIZE FRYING OIL?! INSIGHTS TO THE EFFECT OF ESSENTIAL OILS FROM *FERULAGO ANGULATA*, *MENTHA PULEGIUM*, AND *CUMINUM CYMINUM* ON FRYING OIL DURING DEEP-FRYING OF POTATO SLICES

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ABSTRACT

The effect of essential oils (EOs) from *Ferulago angulata* (F), *Mentha pulegium* (M), and *Cuminum cyminum* (C) was considered on oxidative stability of frying oil during frying of potato slices. The EOs were applied in concentrations of 200 and 400 ppm and a mixture sample (140 ppm of each essential oil). Tertiary butyl hydroquinone (TBHQ) was used as a standard at 100 ppm. The efficacy of EOs was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, and parameters of free fatty acid content (FFA), peroxide value (PV), p-anisidine value (P-AnV), total polar content (TPC), and sensory evaluation during three cycles frying. The scavenging activity of TBHQ was higher followed by M-400, F-400, M-200, C-400, C-200, Mixture, and F-200, respectively. The obtained results from chemical parameters were in agreement with each other and except acidity, the content of PV, P-AnV, and TPC almost in all samples containing EOs were higher than the control during cycles of frying. Sensory evaluation data also showed the superiority of synthetic antioxidant followed by control and EOs. According to the results, EOs had a weak antioxidant effect on frying oil due to their volatility and sensitivity to high temperatures during frying.

1. Introduction

Deep-fat frying is an old and popular process that produces desirable color, flavor and crispy texture in fried foods. It includes immersing of food in hot oil at temperatures of 150-190℃. Simultaneous mass and heat transfer of oil, air and food during this process causes uthe nique quality of fried materials. In addition to desirable properties, deep-fat frying poses undesirable characteristics including off flavor, rancid odor and discoloration due to producing of volatile and nonvolatile components as a result of oxidation, hydrolysis and polymerization reactions that affect the ensory, functional and nutritional value of oils. Frying oil, time and temperature of frying, type of fryer and antioxidants affect these reactions (Choe and Min, 2007).

Today, due to increasing awareness of people and scientists from harms of synthetic additives including antioxidants, there was a

considerable tendency for substitution of these materials with natural ones.

Spices and herbs due to their flavor, perfume, and preservative properties have been used in different products and cosmetic and medical industries from ancient times (Bakkali *et al*., 2008). Essential oils are a mixture of volatile organic components that obtain from non-woody parts of the plant by hydro-distillation and steam (Batish *et al*., 2008). *Ferulago* is a member of the *Apiaceae* (*Umbelliferae*) family. It includes two subspecies: subsp. *Carduchorum* (Boiss and Hausskn) and subsp. *Angulata* (Schlecht). *F.angulata* (Schlecht) Boiss as title Chavir in Iran, grows in the east of Turkey, and north of Iran and Iraq. It has been used as an additive to animal oil for many years due to its preservative effect in Kermanshah province (Sadeghi *et al*., 2016 a). Antioxidant and antimicrobial effect of this plant has been proved in several studies (Taran *et al*., 2010; Sadeghi *et al*., 2016 a; Sadeghi *et al*., 2017). *Mentha* spices are in *Lamiaceae* family. *Mentha pulegium L.* (Pennyroyal) is one of these spices that are founded in damp and humid areas including north Africa, south Eroup and near east countries. In folk medicine, it is used as carminative, antispasmodic, diaphoretic, stimulant, sedative, antiseptic bronchitis, diuretic, and for skin diseases (Lawrence, 2007). The essential oil of this plant has antioxidant, antibacterial, antifungal and insecticidal activity (Teixeria *et al*., 2012; Zekri *et al*., 2013). *Cuminum cyminum L.* (Cumin) belongs to the *Apiaceae* family. It is cultivated in China, Iran, India, Japan, Morocco, Indonesia, South Russia, Turkey, and Algeria. Cumin seed is a popular spice used for the treatment of dyspepsia, epilepsy, diarrhea, jaundice, and toothache in folk medicine and as a flavoring agent. Antioxidant effect of cumin has proved in some studies (Bettaieb *et al*., 2010).

Although, the mentioned essential oils have shown antioxidant effect in various studies, the effect of them in high temperatures such as frying has not considered. Therefore, in this study, we considered the efficacy of Chavir, Cumin, and Pennyroyal essential oils on the stability of frying oil in the frying process of potato slices for a response to this question: if essential oils can stabilize frying oil?

2. Materials and methods

2.1. Materials

Frying oil without antioxidant including 50% palm-olein, 30% soybean and 20% sunflower oils were prepared from Agro-industry Complex & Vegetable Oil of Mahidasht, Kermanshah, Iran. *F.angulata and M.pulegium* were supplied from Dalahoo Mountains, Kermanshah, Iran, and Sari mountains, Mazandaran, Iran, respectively. *C.cyminum* seeds as dried were collected from the local market in Kerman province, Iran. The mentioned plants were identified by the Institute of Medicinal Plants, Tehran, Iran. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich. Other chemical materials with analytical grade were prepared from Merck. The potato was supplied from the local supermarket.

2.2. Essential oils extraction and identification

Essential oils (EOs) were extracted by Clevenger apparatus and were analyzed by GC-MS in prior studies (Sadeghi *et al*., 2016 a; Sadeghi *et al*., 2016 b; Sadeghi *et al*., 2013).

2.3. Sample preparation and frying process

Each of EOs was added to frying oil in concentrations of 200 and 400 ppm (F-200, F-400, M-200, M-400, C-200, and C-400 for *F.angulata*, *M.pulegume*, and *C.cyminum*, respectively). Also, a mixture of these EOs (140 ppm of each EOs) was used. TBHQ was applied as standard at 100 ppm and a control sample was prepared without antioxidant. Potatoes sliced in the dimension of 8 × 0.8 × 0.8cm. The slices washed with water and dehydrated by specific cloth before frying. Various treatments of EOs in frying oil in the volume of 1L were poured in the domestic fryer and after reaching of temperature to 180℃,

slices (200 gr) were added to oil and frying was carried out for 5 min in 3 batches with intervals of 1hr. Samples from frying oil and fried potatoes were taken after each time, cooled to room temperature and stored in -20℃ for analysis.

2.4. Chemical parameters

Official methods of AOCS were used for measurement of peroxide value (cd 8-53), free fatty acid content (cd 3a-63), P-anisidine value (cd 18-90), and polar compounds (cd 20-91) (AOCS, 1990).

2.5. DPPH radical scavenging assay

Efficacy of EOs for scavenging of DPPH free radical was assayed according to Gyamfi *et al*. (1999). Thus, a methanol solution of EOs at mentioned concentrations was prepared and 50 µl of them was added to 5 ml DPPH methanol solution (0.004%). After vertex (15s), solutions were incubated at 25℃ in dark for completion of reaction for 30 min. The absorbance of samples and control (DPPH methanolic solution) was read at 517 nm by UV-Visible spectrophotometer. Percent of the radical scavenging calculated as follow:

$$\% \text{ RSA} = 100 \times (A_C - A_S)/A_C \quad (1)$$

Where A_C and A_S are absorbance of the control and sample, respectively.

2.6. Sensory evaluation

Samples analyzed by 7 semi-trained panelists. Sensory evaluation was carried according to 9 points hedonic scale for flavor, aroma, color and total acceptability of fried potatoes with score 9 for extraordinary well and 1 for unacceptable (Sadeghi *et al*., 2017).

2.7. Statistical analysis

The data were analyzed by SAS software and Duncan's multiple range tests were applied for the assay of significant differences ($p<0.05$).

3. Results and discussions

3.1. Essential oils extraction and identification

The main components of *F.angulata, M.pulegium*, and *C.cyminum* essential oils are shown in Table 1 adapted from previous works. The antioxidant materials in plants are including various mixtures of polyphenolics, tocopherols, ascorbic acid or terpenoids (Grabmann, 2005). They inhibit oxidation with different mechanisms such as autoxidative chain reaction breaking (primary antioxidants) and chelating of prooxidant metals, decomposition of hydroperoxides, donating of hydrogen to primary antioxidants, quenching of singlet oxygen, or absorption of ultraviolet ray (secondary antioxidants). Terpenoids specifically monoterpenes and sesquiterpenes are predominant compounds of essential oils (Akoh and Min, 2008). Antioxidant properties of the mentioned EOs are attributed to the identified ingredients in their essential oils that mostly are monoterpenes and sesquiterpenes.

Table 1. The main components of essential oils from *Ferulago angulata*, *Mentha pulegium*, and *Cuminum cyminum*

F.angulata	Percentage	*M. pulegium*	Percentage	*C. cyminum*	Percentage
Cis-Ocimene	30.17	Pulegone	36.68	Cuminaldehyde	29.02
α-Pinene	15.4	Piperitenone	16.88	α-Terpinene-7-al	20.70
Trans-β- Ocimene	5.7	1,8 Cineole	14.58	γ-Terpinene	12.94
γ-Terpinene	5.57	α- terpineol	5.98	γ-Terpinene-7-al	8.9
Germacrene-D	5.03	Menthone	4.72	ρ-Cymene	8.55
Limonene	4.88	Cis- Salvene	3.56	β-Pinene	7.72
Bornyl Acetate	4.57	Piperitenone oxide	3.27	Cis-Dihydrocarvone	4.45

Myrcene	3.62	δ- terpineol	3.19	Myrcene	1.1
Camphene	2.41	Endo borneol	3.04		
Noe-Allo-Ocimene	1.87	β-Caryophyllene	1.79		
β-Phellandrene	1.84	Caryophyllene oxide	1.57		
α-Terpinolene	1.7	Carvacrol	1.34		
Bicyclogermacrene	1.29	Limonene	1.26		
δ-Cadinene	1.18				

3.2. Chemical parameters

3.2.1. Free fatty acid content (FFA)

When food is heated in frying oil; oxygen, water, and steam initiate some reactions in food and oil. Water and steam cause hydrolysis of oil and production of mono and diacylglycerols, FFAs, and glycerol. Thus, the content of free fatty acids increases during frying (Chang *et al*., 2004). FFAs were significantly increased after the first cycle of frying for all samples and gradually enhanced during the process (Figure 1). These outcomes were in accordance with other studies (Alizadeh *et al*., 2016; Naz *et al*., 2005; Sayyad, 2017). In end of 3 cycles frying, control sample had the highest FFA (0.16%) followed by Mixture (0.153%), F-200 (0.134%), M-400 (0.125%), M-200 (0.124%), C-400 (0.123%), C-200 (0.121%), F-400 (0.12%), and TBHQ (0.12%), respectively. According to obtained results, all of the antioxidant samples had lower acidity than control but there was a slight difference among them during cycles of frying (see supplementary, Table S1). Inanc and Maskan (2014) obtained an equivalent result for palm oil treated with Carvacrol during potato frying. Also, the concentration of essential oils had no effect on the decrease of FFAs. However, FFA content is not a very authoritative factor for evaluation of frying oil degradation, because it is hard to distinguish FFA produced by hydrolysis or oxidation (Ramadan *et al*., 2006).

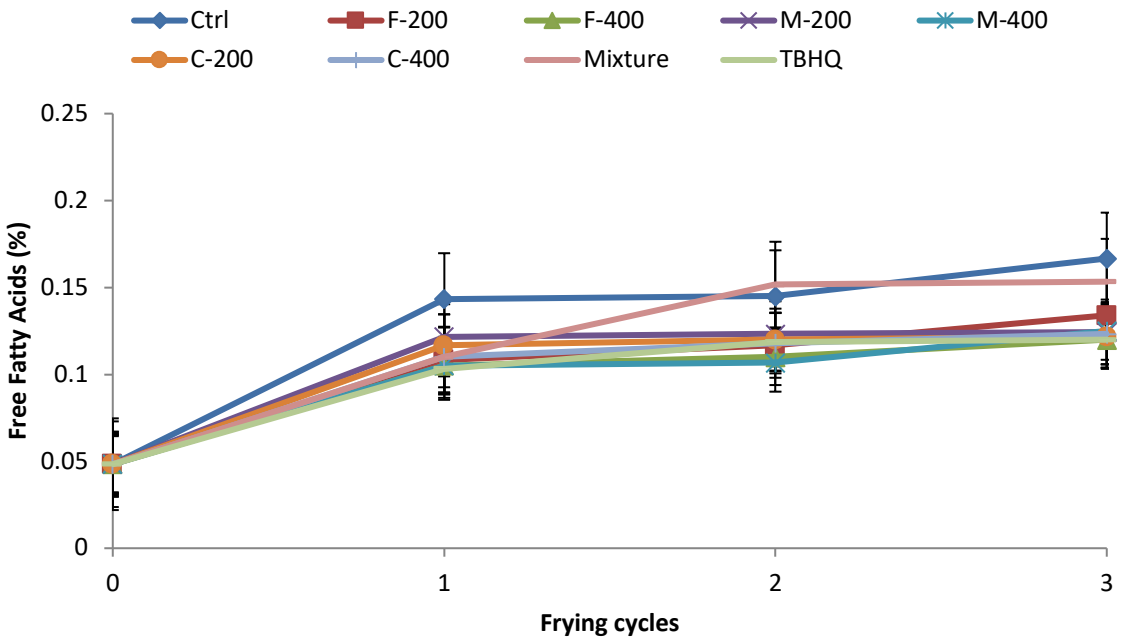

Figure 1. The effect of essential oils on free fatty acid content of frying oil during frying cycles

3.2.2. Peroxide value (PV)

Oil oxidation causes the formation of peroxides as primary products that are a good index of oil quality under normal condition. For control, PV increased with frying cycles (Figure 2). In samples of F-200 and F-400, PV during cycle 2 decreased and then increased at cycle 3. M-200, M-400, and C-200 showed enhancement in peroxide during frying. The content of peroxides of C-400, Mixture, and TBHQ rose to cycle 2 frying and then reduced at cycle 3. Acquired results showed all of the essential oils in a dose of 400 ppm had a lower PV than 200 ppm samples. DU and Li (2008) observed similar results with consideration antioxidant effect Cassia essential oil on deep frying of beef in soybean, peanut, rapeseed, sunflower, and palm oils. At the end of 3 cycles frying, M-400 had the lowest PV followed by C-400, TBHQ, Control, Mixture, F-400, F-200, C-200, and M-200, respectively. This show EOs exceptionally M-400 and C-400 had PV higher than control that is not in accordance to Bensmira *et al*. (2007) study on the effect of Lavender and Thyme essential oil in sunflower oil. They declared peroxide value of lower in oil with antioxidant rather than untreated samples. Moreover, TBHQ and control samples had no significant difference at the end of the process. Totally, there were significant differences between antioxidants and frying cycles (Table S1). Abatement in PV at cycles 2 and 3 frying is related to the decomposition of peroxides because these products are unstable and readily break at frying temperatures. Therefore PV cannot be a very safe indicator for prediction of frying oil quality.

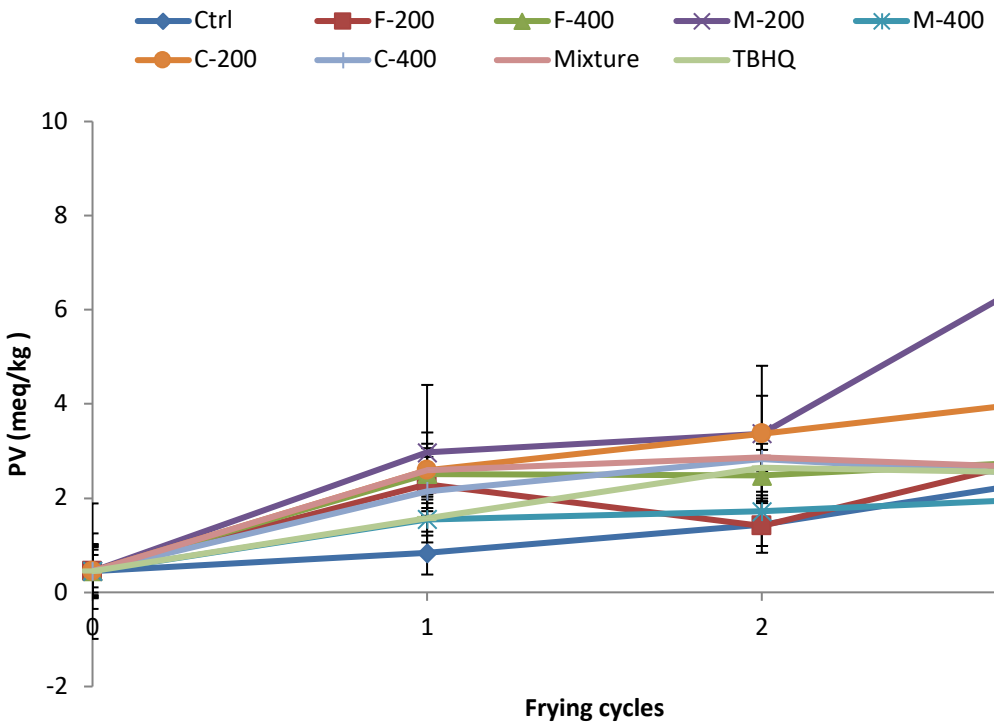

Figure 2. The effect of essential oils on peroxide value of frying oil during frying cycles

3.2.3. P-Anisidine value (P-AnV)

As previously mentioned; decomposition of peroxides at frying high temperatures leads to the formation of oxidation secondary products. P-AnV measures compounds such as α and β-alkenals and other components that react with P-anisidine reagent (Guillen and Cabo, 2002). The P-AnV content of samples is presented in Figure 3. In all samples, anisidine value increased with frying cycles that are in

agreement to results of Alizadeh *et al*. (2016), Sayyad (2017), and Naz *et al*. (2008). Also, enhancement of AnV during the first frying cycle was severer than the next cycles. Researchers declared that degradation of carbonyl components and their absorption to fried food can reduce the increment rate of them during deep frying (Aladedunye and Przybylski, 2009). Moreover, AnV with increasing dose of EOs decreased that is in accordance with PV results. The lowest value was related to TBHQ (5.41) followed by M-400 (6.58), F-400 (6.62), Ctrl (7.21), F-200 (7.23), Mixture (7.5), M-200 (8.37), C-400 (9.29), and C-200 (9.87), respectively in end of 3 cycles frying. These consequences are representative of this fact that essential oils except M-400 and F-400 had AnV higher than control and also TBHQ was the best antioxidant. This is in agreement to observations of Zhang *et al*. (2010) by evaluation oxidative stability sunflower oil treated with Carnosic acid in comparison to synthetic antioxidants BHA, BHT, and TBHQ. The mixture sample was better than M-200, C-400, and C-200, alone. Perhaps, this event is due to the synergistic effect of EOs. Similar results were obtained in PV content and prior study (Sadeghi *et al*., 2016 a).

The higher anisidine value in some of the EOs rather than control is due to decomposition and evaporation of them at frying high temperatures because EOs are volatile. As previously stated, terpenoids are the main ingredients of EOs. They generally are susceptible to rearrangement process at elevated heats. McGraw et al. (1999) graded thermal decomposition of terpenes to four various oxidative reactions including epoxidation, breaking of double bands, allylic oxidation to aldehydes, ketones, and alcohols, and dehydrogenation to aromatic products. The lower content of active components in the essential oils is another reason for their low activity. Inanc and Maskan (2013) with the investigation of the effect of seven EOs (Sage, Cinnamon, Turmeric, Rosemary, Thyme, Clove, and Oregano) and BHT on corn oil at 150℃ and 180℃ observed essential oils except oregano were not effective antioxidants for corn oil.

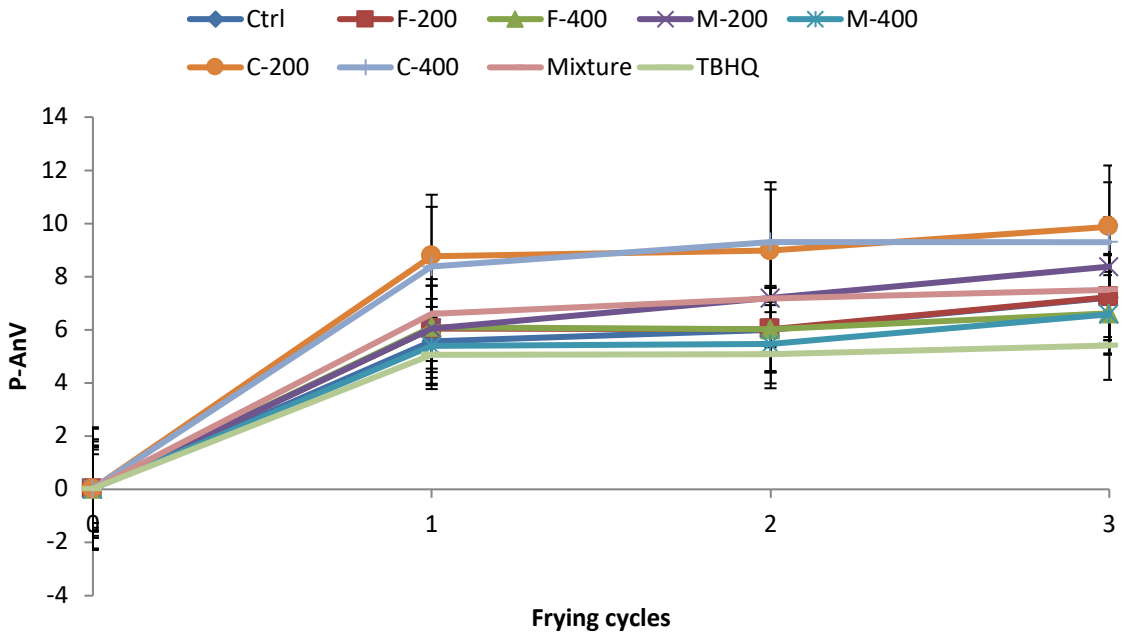

Figure 3. The effect of essential oils on P-Anisidine value of frying oil during frying cycles

3.2.4. Total polar compounds (TPC)

Polar compounds measurement is one of the best indicators for the evaluation of deterioration in frying oil that provide information about newly formed components with a higher polarity rather than triglycerides. The TPC is determined by solving of oil in fairly nonpolar solvents (such as benzene or toluene) and loading of it on silica gel column that absorbs polar materials. Then these compounds eluted directly from a column by diethyl ether or a mixture of methanol and chloroform or indirectly with solvent evaporation and weighting nonpolar fat and calculation of its weight difference with the primary sample. The content of the TPC is shown in Figure 4. It was 8.43% before frying. After the first cycle, the TPC was increased for all samples significantly that was in agreement with other parameters (see supplementary, Table S1). At the second cycle, the quantity of polar compounds in F-200, F-400, M-200, and M-400 samples slightly raised but for Control, C-200, C-400, mixture, and TBHQ slightly decreased. The small decrease in polar compounds perhaps is due to the exiting of volatile ones during frying. In the third cycle, this value enhanced for all samples except F-400. Similar results obtained by Farhoosh and Tavassoli-Kafrani (2010), and Tabee et al. (2009). Also, the TPC for all samples with a nominal difference was in the same range at 3 cycles frying process and ever there was no difference between TBHQ and control at cycle 3. Moreover, at the end of 3 frying cycles F-400 (10.11), C-200 (10.01), and mixture (10.12) had lower polar components content than the control (10.15). Nothing change in polar compounds maybe is due to this fact that sufficient time for increasing production of polar compounds had not given. In other words, probably during 3 cycles frying deterioration of oil does not happen significantly. Similarly, Casarotti and Jorge (2014) declared there was no significant difference among treats of Soybean oil, TBHQ, Rosemary extract and a mixture of TBHQ+ Rosemary extract during primary cycles of frying. Thus, frying time has a purposeful effect in the decomposition of frying oil and formation of polar mixtures (Corsini et al., 2009; Abdel-Razek et al., 2012).

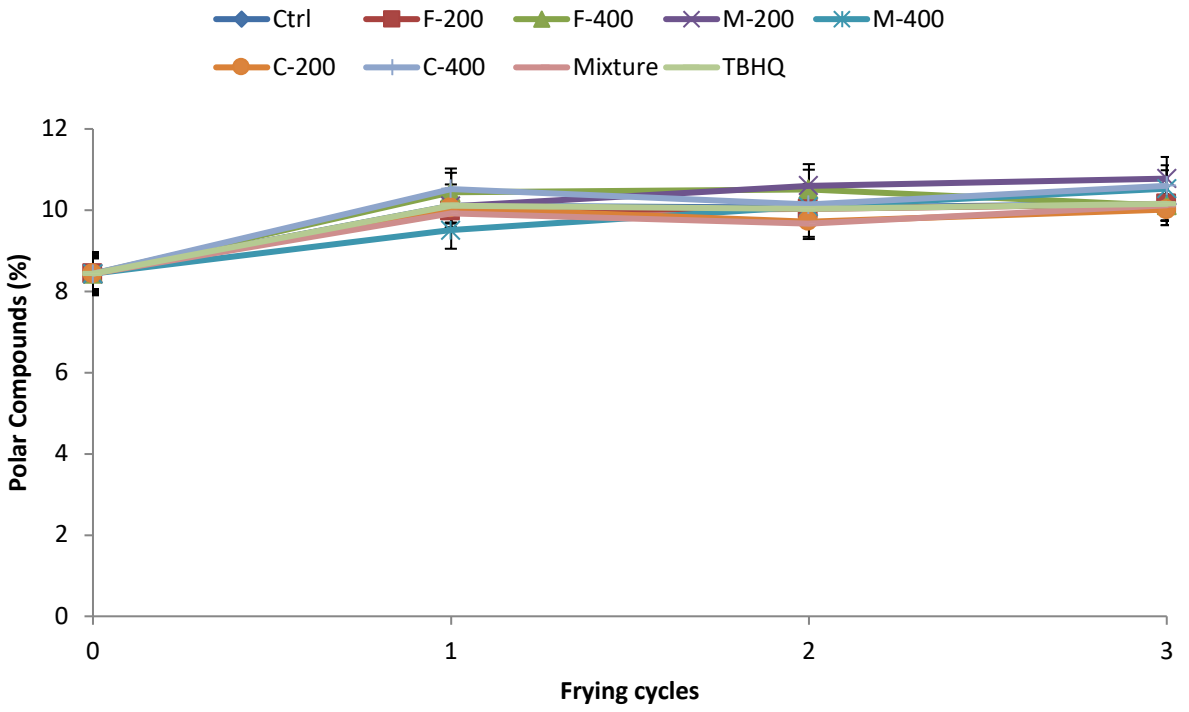

Figure 4. The effect of essential oils on content of polar compounds of frying oil during frying cycles

3.3. DPPH radical scavenging assay

This test evaluates the ability of essential oils for scavenging of radical DPPH by donating hydrogen to free radical and is a good indicator for investigation antioxidant activity (Chung et al., 2006). Antioxidant activity of EOs is presented in Table 2. According to the table, the highest activity was related to TBHQ followed by M-400, F-400, M-200, C-400, C-200, Mixture, and F200, respectively. Also, all EOs in concentration 400 ppm were more effective than concentration 200 ppm that was agreed with PV and P-AnV parameters but not for mixture sample. These observations showed that essential oils had low scavenging activity. Hosseini *et al*. (2012), Ghasemi Pirbalouti *et al*. (2016), Einafshar *et al*. (2012), and Bettaieb *et al*. (2010) acquired analogous results with studying of antioxidant activity *Ferulago*, *Mentha* and *Cuminum* essential oils. Lower scavenging activity in essential oils possibly is due to the lower content of active components in them (Inanc and Maskan, 2013).

Table 2. The DPPH radical scavenging activity of the essential oils from *Ferulago angulata*, *Mentha pulegium*, and *Cuminum cyminum* at different concentrations beside the synthetic antioxidant

Samples	F-200	F-400	M-200	M-400	C-200	C-400	Mixture	TBHQ
% RSA	1.54	2.12	2.11	2.46	1.93	2.06	1.69	8.54

RSA: Radical scavenging activity

3.4. Sensory evaluation

Results of the sensory evaluation are shown in Figure 5. The scores of aroma for TBHQ and control did not have a significant difference in all cycles of frying. Also, they had higher scores rather than other samples. Moreover, a significant difference was not observed among essential oil treats (see supplementary, Table S2). For flavor, the highest score was belonged to TBHQ, followed by control and essential oils. From the point of this characteristic, the difference among samples was meaningful. The obtained results for color were similar to aroma and flavor results and almost all samples had scores higher than 7. In fact, potatoes color for all treats was similar with well quality. Finally, the total acceptability of treats was too in agreement with stated properties. Concerning observed results for all investigated properties, the synthetic antioxidant had the highest score. Since it retards induction period of oxidation and prevents the formation of secondary products responsible for rancid aroma and flavor. After TBHQ, the control was the best treatment because the aroma and flavor of fried potatoes were changed by essential oils and this was effective on the acceptability of panelists. Furthermore, in no one of treats there were significant differences among cycles of frying perhaps because after 3 cycles process yet significant oxidation has not happened in the oil that was in agreement with chemical parameters (TPC & P-AnV, FFA) outcomes and issues of Alizadeh et al. (2016) and Lalas and Dourtoglou (2003) that notice significant difference among treatments after third and fourth cycles of frying, respectively.

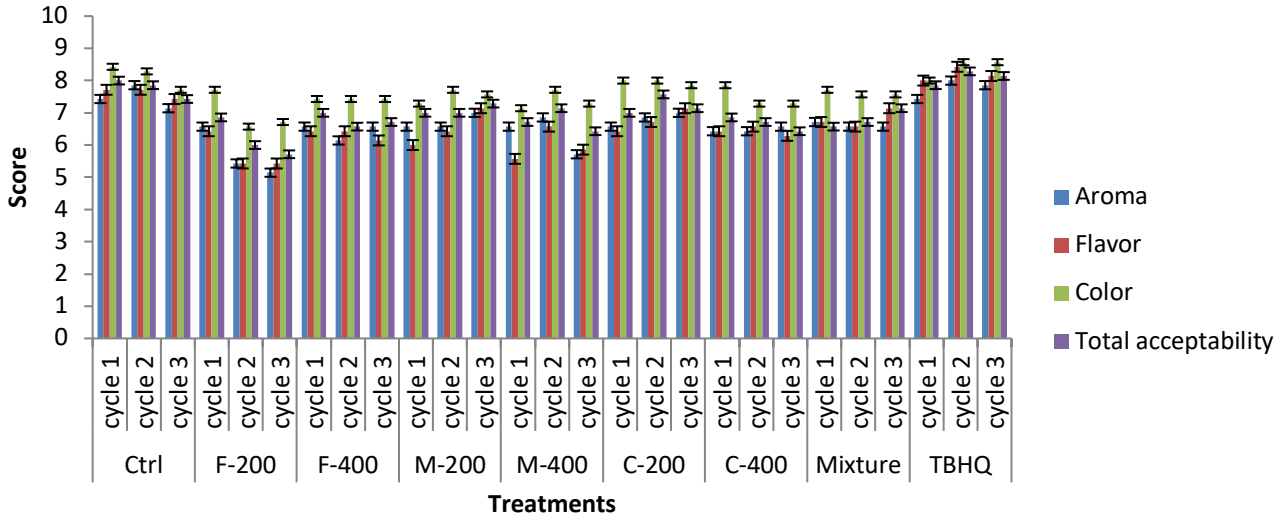

Figure 5. The effect of essential oils on sensory properties of the fried potatoes during frying cycles

4. Conclusions

Totally, we can say although M-400 was more effective than the other essential oils in PV and P-AnV and scavenging of DPPH, most of the EOs were not effective antioxidants during 3 cycles frying basically due to sensitivity natural components to high temperatures and volatility of them. Also, it is predicted that with increasing frying cycles, they cannot be effective antioxidants for the stability of frying oil in reason to the decomposition of these natural compounds. Thus, it is recommended EOs be used for storage stability of oils instead of frying processes.

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TEXTURAL AND SENSORY PROPERTIES OF FALSE ACACIA (*ROBINIA PSEUDOACACIA* L.) JELLIES WITH FUNCTIONAL COMPONENTS

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ABSTRACT

The aim of this study is to present the possibility of using *Robinia pseudoacacia* L. flowers in the composition of high-sugar pectin jelly. Basil seeds (*Ocimum basilicum*) are included in jelly's composition in order to improve its functional and sensory properties. The texture of the jellies was evaluated by the methods of penetration, durability and hardness. Relatively comparable indicators of the structural and mechanical properties of the gels were reported. No significant differences were noted in the maximum deformation at the point of destruction and the energy used to destroy the jellies. The basil seed-containing jelly showed higher gel strength, requiring greater both force (0.203 N) and energy (0.567 N.mm) for destruction. The sensory profiles clearly showed the higher panelist's preference of the seed-containing jelly, receiving 53% approval, with 14% for the plain jelly. The results obtained show good possibilities for the application of false acacia flowers in pectic jellies with the participation of basil seeds.

1. Introduction

The use of flowers from traditional and non-traditional plant species has been the subject of several studies (Oyeyemi *et al*., 2017; Navarro-Gonzalez *et al.,* 2015). Some authors emphasize the benefits of using plant flowers, both fresh and processed (Oyeyemi *et al.,* 2017; Stankov *et al.,* 2018). Their processing in the form of extracts, freeze-dried, frozen, as well as used in the production of jams, marmalades and jellies, causes their wide application during all seasons (Stankov *et al.,* 2018). In this case, food stabilization is achieved - besides the thermal treatment - by increasing the soluble solids content and the acidity. It could be achieved through the usage of different types of hydrocolloids (Genovese *et al.,* 2010) which, in addition to increasing the level of dry matter, also alter the texture of the model matrix. In another aspect, they improve the nutritional and biological value of the product (Kumar *et al.,* 2018). Such ingredients are textural moderators that alter the sensory perception of the product (Löfgren *et al.,* 2005). Most commonly, the production of jellies in industrial production uses highly esterified pectin (Endress *et al.,* 2005). There are studies about the effect of pectin concentration, pH, type, and concentration of cosolvent, on the viscoelastic properties of high methoxyl pectin gels (Löfgren *et al.,* 2005; Tsoga *et al.,* 2004). This may be attributed to the more complex structure and composition of jams, jellies, and marmalades, compared to

model pectin gels. Rheological properties are useful in determining ingredient functionality in the product development, quality control, and correlation of food texture to sensorial attributes (Dervisi *et al.,* 2001).

The use of various seeds, cereals, plant extracts, and other functional components in the composition of jellies and jams can have a beneficial effect on the quality of the product. Basil seeds offer great potential for use as an emulsifying, foaming, thickening, gelling, binding, fat replacing, binding, and stabilizing agent in the food industries (Naji-Tabasia *et al.,* 2017).

Despite extensive studies on the presence of substances with pronounced functional properties, the consumption of some plant species and individual parts of them is limited. This necessitates the search for alternative technological solutions for the production of the foods in which they are included, in the form of extracts, biologically active substances, structure-forming agents, and others.

The aim of this study was to analyze the textural and sensory indices of model false acacia pectin jelly with basil seeds.

2. Materials and methods

2.1. Materials

Materials: Acacia flowers (*Robinia pseudoacacia* L.) were collected in May 2018 from a ten-fifteen years old false acacia (black locust) tree, naturally growing in the foot of the Eastern Stara Planina Mountain, in Sliven, Bulgaria. Samples were identified and certified by an expert from the Agricultural University of Plovdiv, Bulgaria. High methylester (HM) pectin Ceampectin type MRS-4610 (CEAMSA, Porriño, Spain) was used as a gelling agent. Anhydrous citric acid Parafarm (Saporiti, Argentina) was used to regulate the pH.

Food grade sucrose, organic basil seeds (Basilikumsamen, Davert), and potable bottled water, each from the same batch, were bought in the local market.

2.1.1. Preparation of acacia syrup

The acacia flowers were stored at 5 °C for 24 hours. They were subjected to extraction with a boiling water-sugar solution (50%) for 30 min. Citric acid (1% by weight of the finished product) was added to the syrup. Total soluble solids content reached 70 °Brix; water activity - 0.67±0.01 (LabSwift-aw, Novasina, Switzerland); pH - 3.19±0.02. The prepared syrup was filtered and chilled stored in closed glass bottles.

2.1.2. Preparation of acacia jellies

Standard jellies (63 °Brix) were prepared by boiling the required amount of acacia syrup before the addition of pectin solution (5%). The desired pH-value (3.20±0.03) was adjusted by adding a 50% citric acid solution at the end of the cooking process. For the seed-containing jelly basil seeds (1% of the mass of finished product) were pre-hydrated in water at a ratio of 1:5 for 20 min; the prepared mucilage was mixed with the acacia syrup at 65 °C. The finished products were filled in cylindrical syringes with a volume of 60 cm^3, d^2 = 28.5 mm, and stored at 5 °C for 48h.

2.2. Method of analyses

2.2.1. Texture analysis

For the studies of instrumental texture parameters, a Stablemicrosystems TAXT2 texture analyzer was used in the laboratory of Food Research and Development Institute, Plovdiv with three different methods and probes on 2 slices of syringes. After the gel formation, the cylinder shape packs were immediately cut before measurement for slices of 10 mm height (6 slices of one syringe). The exact height was measured with the texture analyzer during the experiments. The methods used were determined: Penetration - (d1 = 5 mm; a1 [area] = 19.6 mm^2) to a relative deformation of 80%, a deformation speed of 1 mm/s and a unloading speed of 10 mm/s., and derived values for the consistency, Rupture stress, [kPa]; maximum deformation at break point, [mm], deformation energy, [kPa.mm], maximum unloading stickiness in pull out,

[kPa], sticky, area below unloading deformation axis, [kPa.mm]. Gel strength testing was performed by cone probe penetration (operating angle ∠45 °) to a relative deformation of 80%, a deformation speed of 1 mm/s and an unloading speed of 10 mm/s and results obtained for Rupture force [N], or as energy [N.mm], stiffness, the area below deformation axis, [N.mm]. Compression was performed with a cylindrical probe (D2 = 50 mm, A2 [area] = 1963.5 mm^2, d2 [probe diameter] = 28.5 mm, a2 [probe area] = 637.9 mm^2), obtained metrics: hardness, gel strength, such as rupture stress, [kPa] or rupture energy [kPa.mm] and maximum deformation at break point, plasticity [mm].

2.2.2. Sensory evaluation. Acceptance test.

A total of 100 untrained panelists (40 males and 60 females, 20 - 65 years old) participated in the study. They were students and staff of the University of Food Technologies (Plovdiv, Bulgaria), identified (in a survey previous to the test) as regular consumers were considered those who declared to consume these products at least 2 to 3 times per week. Samples were presented to the panelist coded and in random order. Plastic flat spoons were provided to the panelists, water was offered to cleanse their palates between sample tasting. Panelists were instructed to consider appearance, colour, flovour, taste, consistency, sweetness intensity, acidity intensity, succulence, aftertaste, and general perception of each sample on a 9 - point hedonic scale (1 - dislike extremely; 2 - dislike very much; 3 - dislike moderately; 4 - dislike slightly; 5 - neither like nor dislike; 6 - like slightly; 7 - like moderately; 8 - like very much; 9 - like extremely), (Stone *et al.*, 1993).

3.Results and discussions

3.1. Textural parameters

The textural parameters of the jellies obtained are crucial for their use in the food industry. In order to provide detailed information on the conduct of the jelly during processing and distribution, a number of studies have been carried out influence on its quality.
When penetrating the homogeneous the plain gel (jelly without basil seeds) showed, we get lower penetration values compared to the gel with pre-hydrated basil seed. There were no significant differences in the maximum deformation at the jelly breakpoints, as well as differences in the energy, required for gel breakage. The values of stickiness of the seed gel (jelly with added basil seeds) are significantly greater than those of the pure plain gel towards tension (stress) and breakout energy (Figure 1).

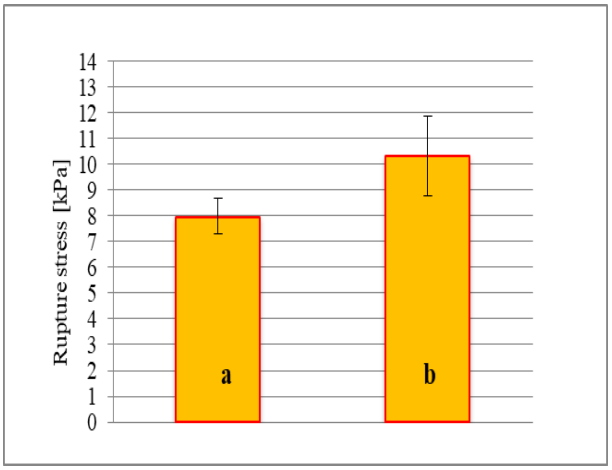

Figure 1. Consistency (stress) graphs of rheological analysis of jelly: (a) acacia jelly and (b) acacia jelly with basil seeds

As observed from the endurance tests, it was found that the seed gel was more durable than the pure plain HM-pectin jelly gel. The energy required to destroy the seed jelly (0.567 N.mm) with the seeds was significantly higher, indicating stronger gel due to the formation of a denser network microstructure. This assumption is supported by the recent findings (Samateh *et al.,* 2018), confirming the involvement of a nanoscale 3D network in the gelation process of the basil seed mucilage. By controlling the gel's microstructure, a wide variety of physical properties can be attained ranging from hard rubbery plastics to soft hydrogels.

Seed gel viscosity with seeds is significantly greater than that of the plain

(pure) gel in terms of strength and energy. It was not reported observed stress difference at the breakpoint during the compression, but in the deformation and the energy, the seed gel showed significantly higher values for the seed gel (Figure 2).

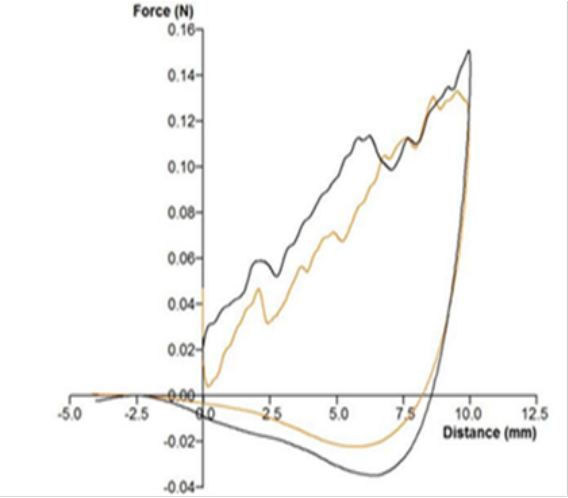

Figure 2. Graphs of rheological texture analysis of acacia jellies: red line – plain jelly; black line – jelly with acacia and added basil seeds.

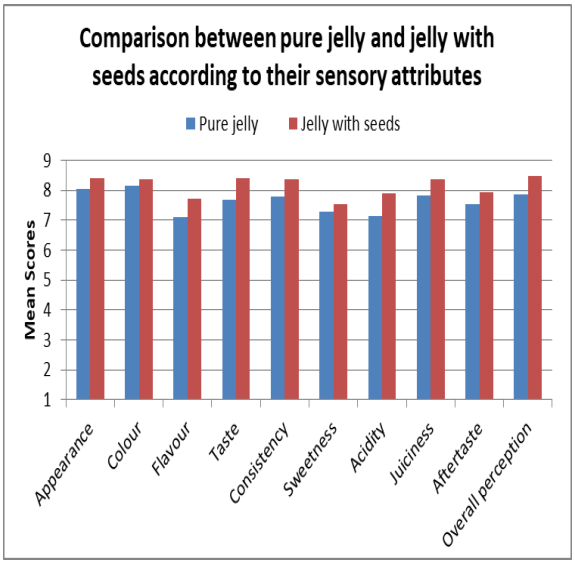

Figure 3. Comparison between pure jelly and jelly with seeds sensory evaluation scores of acacia jellies

The presence of basil seeds in the hydro molecular layer of the acacia gel changes its structural and mechanical performance. Because of the basil seeds applied, the energy used to destroy the gel (0.567 N.mm) was significantly increased compared to the gel without added basil seeds.

3.2. Sensory evaluation

Figure 3 presented the differences in consistency, juiciness, and overall perception between pure jelly and jelly with seeds.

The addition of raw materials in different quantity and quality, such as basil seeds, changed the textural and sensory characteristics of the food matrix (Table 1).

Table 1. Mean scores of the consistency, juiciness and overall perception ratings of pure jelly and jelly with seeds

Attribute	Acacia jelly	Jelly with basil seeds	Wilcoxon Signed Rank Test (two-tailed)	
			Z-values	p-values
Consistency	7.79	8.36	-5.13	2.76-07
Juiciness	7.84	8.38	-4.35	1.36E-05
Overall perception	7.86	8.47	-4.78	1.76E-06

Table 1 represents the mean scores for each combination between the jellies and these three attributes. The chosen level of significance is $\alpha = 0.05$.

In order to choose the appropriate method for statistical analysis, the Shapiro-Wilk test for normal distribution was performed. It showed that the samples were not from normally distributed populations. Hence, the non-parametrical Wilcoxon Signed Rank Test was used in order to conduct the comparisons. Since the samples were big enough ($n = 100$) Z-values were used as an approximation to the W-values.

The last two columns of Table 1 represent the z and p-values of the test. All z-values are less than the negative two-tailed table value ($z_{(\alpha/2)}=\pm 1.96$) and p-values are less than the significance level $\alpha = 0.05$. Therefore there are significant differences between the ratings of the consistency, juiciness, and overall perception of the pure jelly and the jelly with

seeds and the ratings for the jelly with seeds score better.

The sensory evaluation revealed a positive influence of the added seeds on the colour, flavour, and consistency indicators (Figure 4).

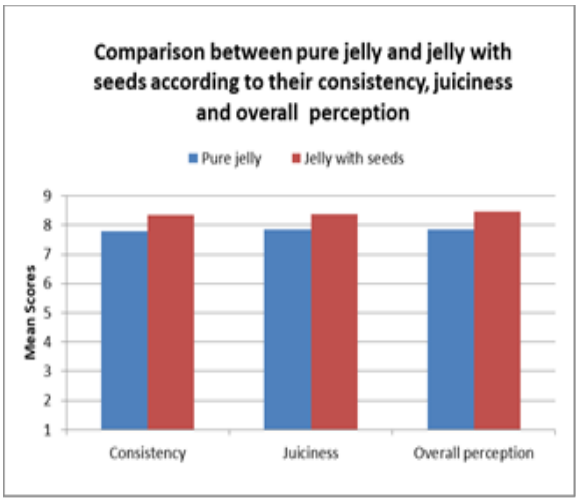

Figure 4. Comparison between pure jelly and jelly with seeds according to their consistency, juiciness, and overall perception.

When the textural and sensory characteristics of the two acacia gels were compared, it was determined that the pure gel had more pronounced juiciness.

Compared to the gel with the participation of basil seeds, confirming the experimentally established penetration hardness. It is observed that 47% of the panelists estimated the appearance of gel with the presence of basil seeds as "extremely liked", while only 24 % of the panelists assessed the pure gel as "extremely liked". The results showed that 56% of the panelists gave the highest mark for the colour of the gel with basil seeds, while only 37% of the panelists ranked the colour of the pure gel with the highest mark. Most of the evaluators (68%) assessed highly the gel with basil seeds, toward the remaining 22% of the panelists, who shared that they would prefer the natural, pure jelly without basil seeds.

4. Conclusions

The present study has shown good possibilities for the application of white false acacia in pectin jelly production. The light colour of the obtained jellies provided the opportunity to combine with other raw materials, such as seeds, nuts, and others, to improve the textural, sensory, and functional properties of the final product. It was proven the positive effect of basil seeds addition on the flavour, and overall evaluation of the jellies. The present study created opportunities for new researches with a view to the possibilities of combining different plant raw materials in composite solutions. Object of subsequent studies will be the determination of biologically active substances, chemical composition, and the possibilities of obtaining low sugar jelly with the participation of white false acacia (*Robinia pseudoacacia* L.).

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SEASONAL IMPACT ON THE RISK ASSESSMENT RELATED TO THE SPATIAL PREVALENCE OF ENTEROVIRUS IN OYSTERS FROM OUALIDIA LAGOON IN MOROCCO

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ABSTRACT
Enteroviruses are ubiquitous pathogens that are present worldwide and they are able to survive for long periods in a marine environment. They are transmitted through water and food contaminated with stools. The purpose of this study was to evaluate the prevalence of enteroviruses in oysters collected from Oualidia lagoon, using real-time RT-PCR. To achieve this objective, 624 oysters representing 52 batches were collected and tested for viral contamination using real-time polymerase chain reaction. The results show that 32.69% of the samples tested were positive for enteroviruses. The spatial distribution of the enteroviruses was statistically significant. Moreover, a prominent seasonal variation of enterovirus contamination was observed in this study. The consumption of oysters contaminated with this type of virus presents a major risk to human health, By causing serious illnesses such as gastroenteritis, hepatitis, and poliomyelitis, the presence of enteroviruses in oyster production areas represents a potential health risk.

1.Introduction

The surveillance systems implemented by the majority of the countries for assessment of the microbiological quality of shellfish and shellfish harvesting waters are mainly based on bacteriological parameters as indicators of fecal contamination (Anonymous, 2004, 2005). However, several studies have demonstrated the inadequacy of bacterial indicators for assessment of viral contamination in shellfish (Croci *et al*., 2000). In recent years, other putative indicators have been proposed, such as bacteriophages, in particular, the B40-8 phage of the Bacteroides fragilis (Havelaar, 1987). However, the use of these microorganisms as indicators of viral presence has been widely questioned (Xavier Abad *et al*., 1997).

Because shellfish are filter-feeding organisms, they can accumulate foodborne viruses, bacteria, and heavy metals in the digestive gland, the mucosa of the gills, and other tissues. As a consequence, they have frequently been implicated as major vectors in the transmission of many enteric diseases (Greening, 2006; Lees, 2000; Dong Joo Seo *et al*., 2014). Indeed, several viral outbreaks have been associated with the consumption of bivalve mollusks that had nonetheless met the bacteriological standards (Sugieda *et al*., 1996).

The frequency of foodborne viral infections associated with shellfish consumption is increasing worldwide. This is a public health issue that results in a lack of public confidence in shellfish and, therefore, in high economic losses for the seafood industry.

Human enteric viruses are present in large numbers in feces and sewage and they can be found in surface waters, groundwaters, and seawaters.

The viruses most often transmitted by contamination of the marine water are noroviruses (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), adenovirus (AdV), astrovirus (AV), rotavirus (RV), and enteroviruses (EV) (poliovirus, coxsackievirus, echovirus) (Griffen *et al*., 2003; Le Guyader *et al*., 2009). They are commonly transmitted by the fecal-oral route.

Enteroviruses are ubiquitous pathogens that are present worldwide and they are able to survive for long periods in the marine environment (OMS, 2013). Furthermore, the resistance of these viruses to conditions such as acidic pH and extreme temperatures facilitates their transmission (Bouseettine *et al*., 2020).

Enteroviruses are associated with a heterogeneous range of diseases. In addition to poliomyelitis, they can cause severe acute diseases such as hepatitis, gastroenteritis, meningitis, and non-specific febrile illness (Cristina and Costa-Mattioli, 2007; Gibson, 2014; Shulman *et al*., 2006).

Studies have shown the involvement of a panel of enteroviruses, such as enterovirus types 68-71, in acute flaccid paralysis (AFP) (Bahri *et al*., 2005; Delpeyroux *et al*., 2013; OMS, 2013).

Therefore, environmental monitoring can provide an additional tool to determine the different viruses present in a community (Pinto *et al*., 2007; Shulman *et al*., 2006) by examination of environmental samples, particularly from bivalve mollusks. Enteroviruses are one of the most frequently monitored viruses in environmental waterways and they are often used as a bio-indicator of viral contamination (Wurtzer *et al*., 2014).

In the past, the method for detecting enteroviruses has been based on virus infectivity of cell lines, but this method is expensive, time-consuming, and difficult to perform due to the absence of a susceptible cell line for each enteric virus.

Nucleic acid-based methods have proven to be highly specific and they can detect even very small amounts of viral particles in mussel homogenates (LeGuyader *et al*., 2001). However, successful application of molecular methods for enteric virus identification has been limited by the presence of RT-PCR inhibitors in shellfish.

Dissection of the digestive tract and diverticula (hepatopancreas) appears to reduce the presence of such inhibitors and it increases the sensitivity of molecular methods (Le Guyader *et al.*, 2006).

The purpose of this study was to evaluate the presence of enteroviruses in oysters collected for environmental monitoring and to analyze the seasonal variation of the contamination levels Additionally, our study aimed to illustrate the importance of including a routine virological analysis of shellfish in the monitoring of shellfish harvesting areas in Morocco.

2.Materials and Methods

2.1.Sample collection and processing

Oualidia lagoon (34°47'N - 6°13'W and 34°52'N - 6°14'W) is located on the Moroccan Atlantic coast in the province of Sidi Bennour, 168 km south of the city of Casablanca. It extends parallel to the coast for a distance of approximately 8 km and it is 0.5 km wide. Due to its location between two hills that constitute its watershed, Oualidia lagoon is a confluence of runoff water during rainfall in the region. On the other hand, fecal contamination (during the spreading of animal manure) and wastewater infiltration from septic tanks are the major mainland inputs to the lagoon (El Himer *et al*., 2013 ; Hassou *et al*., 2016). This lagoon is facing various environmental problems (liquid and agricultural practices) that will destroy the quality of the coast and threaten the collection

of various aquatic products for consumption (Hassou, 2014). Oyster (*Crassostrea gigas*) samples (n=12 individuals/sample) were collected bimonthly over a 13-month period from March 2018 to March 2019, amounting to a total of 52 samples. The sampling sites (S1 and S2) correspond to two oyster farms located in Oualidia lagoon (Figure 1).

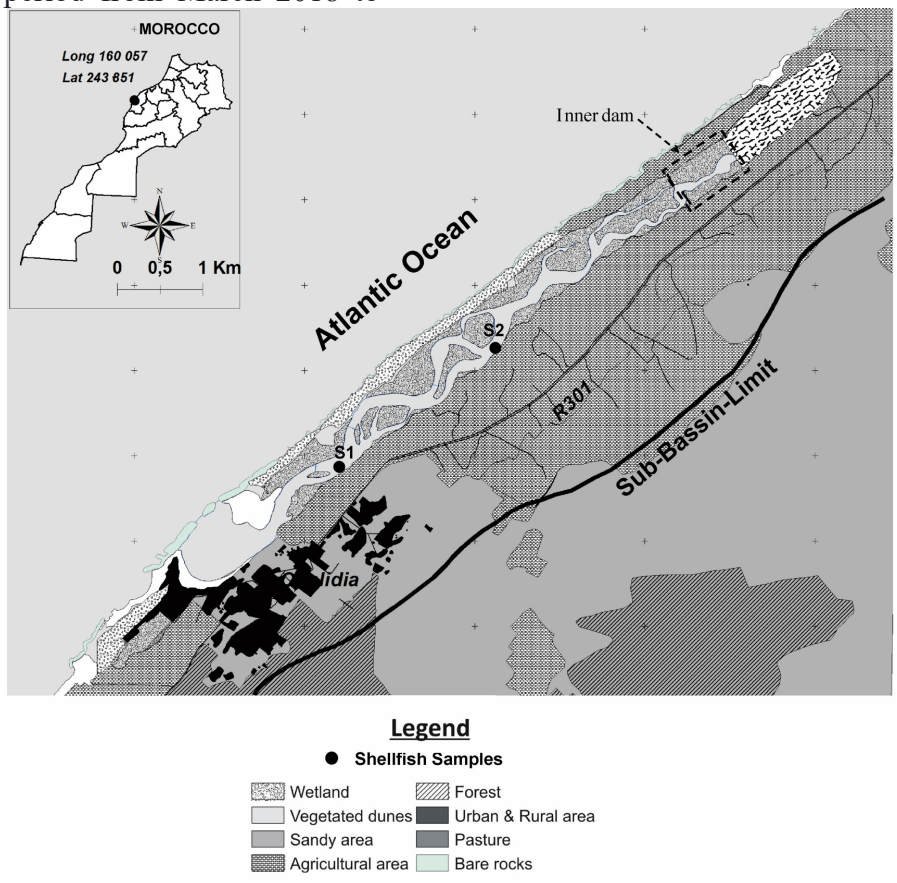

Figure 1. Shellfish collection sites

• S1, downstream of the lagoon (sandy sedimentary),

• S2 in the middle of the lagoon (muddy sedimentary),

Bivalve mollusks were transported to the laboratory under controlled temperature (+4 °C) within 24 hours of being harvested. They were processed immediately. The digestive tissues were dissected, finely chopped, and stored at -80 °C for further use. Aliquots of 2 grams were prepared for extraction of viral RNA. The pluviometry data for 2018-2019 were obtained from the local weather station. Based on the pluviometry data, the study period was divided into a dry period comprising June 2018 and July-August - September 2018 and a rainy period from October 2018 to May 2019.

2.2.Viral RNA extraction:

The tissues were homogenized with TRIzol reagent (1/1 w/v), and the homogenates were incubated at room temperature with mild agitation (200 rpm) for 20 minutes. After centrifugation at 3000 x g for 10 minutes, the supernatants were collected and mixed with 4 µl of the internal extraction control RNA. RNA was extracted from 500 µl of each supernatant using a PureLink™ Viral RNA/DNA Mini Kit (Invitrogen), according to the manufacturer's protocol. The RNA was recovered in DNase/RNase-free sterile water from the kit.

The RNA quality (ratio of absorbance at 260 and 280 nm) was assessed (30 µl of each eluate) using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA).

2.3.One-Step qRT-PCR

qRT-PCR assays for detection and quantification of enteroviruses were performed in a 7500 Fast Real-time PCR System (Applied Biosystems) using specific One-Step System kit Primerdesign™ Ltd enterovirus (5' non-coding region Genesig® Advanced Kit). Five microliters of RNA sample was amplified in a total volume of 20 µl with one-step reaction mix. Five microliters of each RNA sample was amplified in 20 µl of reaction mix that contained 1 X reaction mix, 10 µL of oasig® OneStep 2X RT-qPCR Master Mix, 1 µl of enterovirus primer/probe mix, 1 µl of internal extraction control primer/probe mix, and 3 µl of RNase/DNase-free water). The primers have previously been described by the manufacturer in the Genesig® Advanced Kit handbook. The amplification protocol was as follows: reverse transcription at 55 °C for 10 min was followed by 50 cycles of 2 min at 95 °C, 10 s at 95 °C, then 60 s at 60 °C for data collection, and then cooling at 40 °C for 30 seconds. Fluorescent signals were acquired during this step through the FAM and VIC channels. All of the samples were analyzed in duplicate. Two negative controls (only RNase/DNase-free sterile water) were included in each RT-PCR series. Five standards (from 2 copy numbers/µl to $2x10^4$ copy numbers/µl) were prepared by dilution of a positive control ($2x10^5$ copy numbers/µl). Sample purity (the absence of inhibitors that limit PCR efficiency) was tested using internal controls (IC) from the RT-PCR kits that were spiked in the samples. Briefly, 10 µl of the IC was added to each RNA sample and the Ct values were compared with the Ct value of the negative control (RNase/DNase-free sterile water) spiked with the IC. A threshold Ct value of 30 was used to determine sample purity.

The interpretation of the results was as follows:

Positive control: A positive result indicates that the primers and the probes for detecting the target enterovirus gene worked properly. In case of a negative result, the test results are invalid and must be repeated.

Negative control: A negative result indicates that the reagents had not become contaminated while setting up the run.

Internal RNA extraction control: The internal control is detected through the VIC channel and yielded a Cq value of 28 ± 3 depending on the level of sample dilution.

Sample results:

A positive quantitative result is thought to be the case when:

The negative control was verified as being negative,

The positive control was positive with a CT value between 16 and 23,

The internal control was positive with a CT value within the range of 28 ± 3

The sample could be amplified by 50 cycles of the OneStep RT-qPCR amplification protocol.

The positive control template is expected to amplify between CT 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

2.4.Statistical analysis

Spearman's rank correlation analysis was employed to correlate the results of positive samples pooled by month and pluviometry. These correlations were performed with the total accumulated rain of the previous month, assuming that each oyster harvesting area was mainly affected by the rains of the preceding month. All of the statistical analyses were performed using the statistical package SPSS Statistics 17.0. Statistical significance was determined by a P-value < 0.05.

3.Results and discussions:

Enteroviruses (EV) were detected by real-time RT-PCR in 36.54% of the oysters (Table 1). Based on the statistical analysis, the

detection rate of EV was significantly high in the oysters ($P < 0.05$).

Table 1. Prevalence of EV from bivalve molluscs collected in Oualidia Lagoon.

Stations / Month	S1		S2	
March 2018	+	+	-	+
April 2018	+	+	+	+
Mai 2018	-	-	-	-
June 2018	-	-	-	-
July 2018	-	-	-	-
August 2018	-	-	-	-
September 2018	-	-	-	-
October 2018	+	+	-	-
November 2018	+	+	+	+
December 2018	+	+	+	+
Junuary 2019	-	-	-	-
February 2019	+	+	-	-
March 2019	-	-	-	-
	12/26* (46.16%)		**7/26* (26.92%)**	
	19/52* (36.54%)			

*Significant difference of EV detection rate in S1 and S2 ($P < 0.05$).

Of the 26 batches of oysters collected at site 1, 46.16% were positive. Of the 26 batches of oysters from site 2, 26.92% were positive for these foodborne viruses. In terms of the seasonal variation, the detection rate of EV in oysters was 0.0% in the dry periods and 32.69% in the winter/rainy period (Table 2).

Table 2. Seasonal prevalence of EV detected in bivalve molluscs collected from Oualidia Lagoon.

Season	EV
Dry periode	0/52* (0.0)
Rainy periode	17/52* (32.69)

*Detection rates of EV were statistically significant in rainy season ($P < 0.05$).

These properties ensure that enteroviruses are very well-dispersed on the water surface or the wastewater from sewage treatment. Human enteroviruses are not inactivated in the water environment and will, therefore, often be caught and activated by the filter feeders of such mussels (Benabbes et al., 2013b). Most of the positive samples ranged from 4.31 to 2E+05 RNAc/g (Table 3).

Table 3. Monthly geometric mean quantification numbers of positive samples EV along the study period.

		CT value	Log Enteroviral Quantification	Enteroviral Quantification
S1	**November-18**	19	5,301	2,00E+05
	October-18	23	3,968	9,28E+03
	December-18	26	2,968	9,28E+02
	April-18	28	2,301	2,00E+02
	February-19	28	2,301	2,00E+02
	March-18	29	1,968	9,28E+01
S2	**November-18**	22	4,301	2,00E+04
	October-18	-	-	-
	December-18	30	1,634	4,31E+01
	April-18	32	0,968	9,28E+00
	February-19	-	-	-
	March-18	33	0,634	4,31E+00

EV yielded the highest average viral levels (2×10^5 RNAc/g DT) at site 1 in November. The results of the quantification show that the concentration of the enteroviruses had a spatio-temporal variation. These concentrations increased with the first heavy rains during October, November, and December, which caused septic tanks that had filled during the summer to overflow. The concentrations after the rains in March and April were lower due to the effect of the dilution caused by the rains of the winter months (Figure 2).

The results obtained for the two sampling sites were grouped to evaluate their seasonal prevalences. EV was detected in 6 out of 13 months in S1. EV was detected in 4 months, in March and April and between October and December in 2018, and in February of 2019. The average quantification level percentages of EV-positive samples over the study period are shown in Table. 3. Fisher's exact test showed highly significant differences in the virus prevalence between the dry period (May to September) and the rainy period (October to April) ($P < 0.0001$). A significant positive correlation between the number of positive samples and the average rainfall was observed.

Shellfish are known to accumulate human pathogens such as human enteric viruses, as demonstrated for rotavirus in mussels (Bosch *et al*., 1995) and enterovirus (McLeod *et al*., 2009), hepatitis A virus (Kingsley and Richards, 2003), and norovirus (Maalouf *et al*., 2010) in oysters. Shellfish contamination has been investigated in many countries (Benabbes *et al*., 2013; Coelho *et al*., 2003a,b; Croci *et al*., 2000; DePaola *et al*., 2010; Diez-Valcarce *et al*., 2012; Formiga-Cruz *et al*., 2002; Gallimore *et al*., 2005; Hansman *et al*., 2008; Kittigul *et al*., 2011; Mesquita *et al*., 2011; Namsai *et al*., 2011; Nishida *et al*., 2003; Romalde *et al*., 2002; Vilariño *et al*., 2009; Wang *et al*., 2008; Yilmaz *et al*., 2010). Although the elution concentrations and detection methods were different, the contamination level of foodborne viruses varied by country, the season, and the type of shellfish. In Morocco, the measurement of enterovirus levels in shellfish has been the subject of several studies in different lagoon regions or coastal areas and using different methods and techniques.

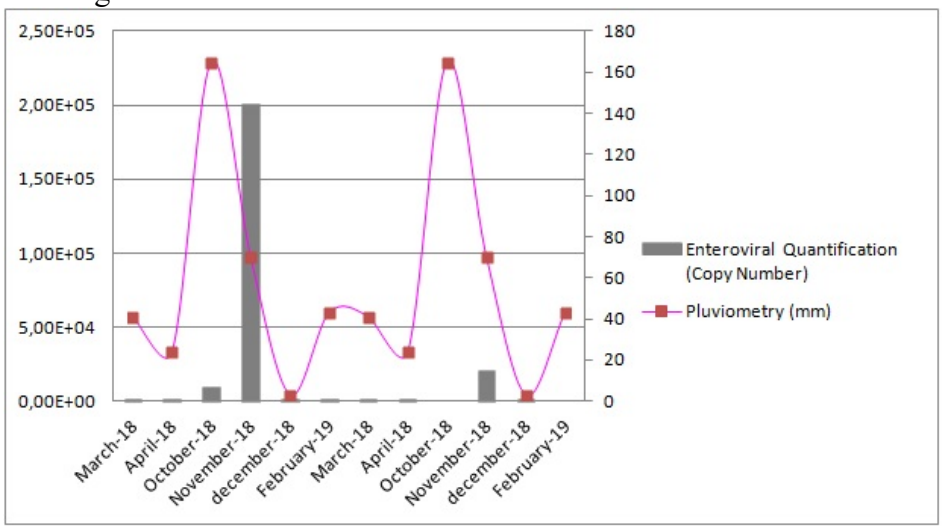

Figure 2. Spatio-temporal variation of enteroviruses concentration

As the technology for analytical methods develops, these viruses are being detected more and more. Indeed Karamoko *et al* in (2006) detected this virus in 10% of the samples tested from the Mohammedia area. Laila Benabbes *et al* in (2013b) found viral contamination of 36.3% of the samples collected from two shellfish production areas along the Moroccan Mediterranean coast, whereas in 2017 Lalla Meryem Idrissi Azzouzi

et al found that 75% of mussels were contaminated by enterovirus collected in three wild populations; Bouregreg estuary and Yaacoub Al Mansour In the present study, EV was detected significantly more frequently than NoV as found by (El Moqri *et al*., 2019). Moreover, the monthly prevalence was observed to be different, and many studies have reported a seasonal distribution of NoV in shellfish, water, and sewage samples, mainly in the cold months (Iwai *et al*., 2009; Le Guyader *et al*., 2000; Lowther *et al*., 2012b; Suffredini *et al*., 2012). Pluviometry is widely recognized to be one of the main environmental parameters that can influence viral detection in estuaries and shellfish due to the runoff or sewage treatment plant failures. Here, a correspondence between the number of detections and the pluviometry was statistically significant. Increases in pluviometry are more often associated with cold months; however, in the Oualidia region, heavy rainfalls typically occur in autumn and winter. The total rainfalls for the cold and the warm months during the study period were 237 mm and 64 mm, respectively. The relationship between the detection rate of EV and the sampling location showed that the prevalence of EV was significantly associated with the sampling site ($P < 0.05$). Several sites exhibited high EV contamination rates in shellfish. Indeed, Coelho *et al*. (2003b) found that oysters were readily contaminated in production areas that were close to human sewage (Rigotto *et al*., 2010). Thus, it is very important to control sewage or fecal contamination near shellfish production sites. In our study, monitoring of enterovirus detection in oysters was associated with seasonal variations. Indeed, a high EV contamination rate was found in winter. In several studies, seasonal variation of foodborne viruses has been explained by the fact that viruses are removed less effectively from shellfish in winter and that they can survive better in winter than in summer (Greening, 2006 ; Lees, 2000).

4.Conclusions

Our study showed that the prevalence of EV in oysters from Oualidia lagoon was quite high, and it was significantly associated with seasonal variation. Therefore, monitoring of foodborne viruses in oysters may contribute to the prevention of viral food poisoning and the promotion of public health. As a secondary conclusion, our study showed the importance of including routine virological analysis in the monitoring of shellfish harvesting areas in Morocco.

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CHARACTERIZATION OF ACID SOLUBLE COLLAGEN FROM THE SKIN OF SNAKESKIN GOURAMI (*TRICHOGASTER PECTORALIS*)

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ABSTRACT

The present study was aimed to isolate and characterized acid soluble collagen (ASC) from the skin of snakeskin gourami (*Trichogaster pectoralis*). ASC from gourami skin had a yield of 9.43% and 34.65%, based on wet and dry weight basis, respectively. The purity of ASC was superior with a distinct absorption peak at wavelength (WL) of 230.7 nm. Based on the electrophoretic pattern, gourami skin ASC was classified as type I collagen, as it comprised α1 and α2 as major components and higher molecular weight (MW) components γ, β were distinctly observed. ASC exhibited high T_{max} value of 33.43°C, which could correspond to its imino acids content of 188 residues/1000 residues. Fourier transform infrared (FTIR) spectrum and circular dichroism (CD) revealed that ASC extracted from gourami skin had greater structural integrity in its triple-helical form. Solubility of ASC was high at the pH range of 2-4 in which zeta potential exhibited highly positive charge. The highest solubility of ASC in the presence of NaCl was observed at 2% (w/v). Therefore, with all the characteristic features, ASC from snakeskin gourami skin can be a value-added product in the fish processing industry.

1. Introduction

Fish processing generates up to 70% of organic by-products, depending on species. Increasing interest has been paid from the fish processors to effectively utilize these by-products to produce value added products, especially collagen and gelatin (Kittiphattanabawon *et al*., 2019). Collagens with different properties have been extracted from bone, skin and scales of common carp (*Cyprinus carpio*) (Duan *et al*., 2009), skin of bigeye snapper (*Lutjanus lutjanus*) (Benjakul *et al*., 2010), scales of golden carp (*Probarbus jullieni*) (Ali *et al*., 2017) and the swim bladder of seabass (*Paralabrax* spp.) (Sinthusamran *et al*., 2013). Collagen is a major structural protein present in connective tissues (skin and bone) of animal. Collagen has wide range of applications ranging from cosmetic, biomedical, pharmaceutical, leather to film industries (Nalinanon *et al*., 2011; Ogawa *et al*., 2003). Fish skin being rich in collagenous protein, can serve as an excellent source for collagen production alternative to mammalian counter parts, which is mostly associated with several disadvantages such outbreak of mad cow disease and religions constraint, mainly

Islam and Judaism (Ali *et al*., 2018; Nalinanon *et al*., 2011).

Snakeskin gourami (*Trichogaster pectoralis*) is one of the common fish species of Thailand, habitat in shallow, sluggish or standing water with a lot of aquatic vegetation and in rice paddies and swamps. Due to its taste, it is highly valued for food and has been exported to other countries (Froese, 2014), and is one of the five most important aquacultured freshwater species in Thailand (Pongsri, 2005). Its flesh is appreciated for its good eating quality, and is eaten in the form of fried, grilled, fish soup or dried from (Froese, 2014). Due to its wide availability and acceptability, generation of its processing by-product, especially skin is in the significant amounts. This skin can be effectively utilized for the production of value-added products particularly collagen or gelatin which can be of highly significant as well as benefit the fish processers. Therefore, the objective of this study was to extract and characterize the acid soluble collagen (ASC) from the skin of snakeskin gourami in order to evaluate its possible use as an added value by-product for the fish processing industry.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Bovine hemoglobin, β-mercaptoethanol (β-ME), standard collagen type I were purchased from Sigma Chemical Co. (St Louis, MO, USA). Trichloroacetic acid, disodium hydrogen phosphate, sodium citrate, acetic acid, Folin-Ciocalteu's phenol reagent and tris(hydroxylmethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Coomassie Blue R-250 and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Sodium dodecyl sulfate (SDS) and bovine serum albumin were obtained from Fluka (Buchs, Switzerland).

2.2. Preparation of fish skins

Freshly available snakeskin gourami (*Trichogaster pectoralis*) was purchased from the local market situated in Samut Prakan province of Thailand. Fish were packed in a polyethylene bags, loaded in ice with a sample:ice ratio of 1:2 (w/w) and transported to the Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok within 1 h. The skins were manually removed with a filleting knife and washed with cold distilled water. Cleaned skins were cut into small pieces (0.5 × 0.5 cm^2) using scissors. The prepared skins were placed in a polyethylene bag and stored at −20 °C until used for collagen extraction.

2.3. Extraction of acid soluble collagens (ASC)

Acid soluble collagen (ASC) was extracted following the method of Kittiphattanabawon *et al*. (2019) with slight modification. All processes were carried out at 4 °C under continuous stirring condition. Non-collagenous proteins were removed by soaking the prepared skin in 0.1 M NaOH at a skin/ alkali solution ratio of 1:15 (w/v). The mixture was continuously stirred for 6 h and the alkali solution was changed for every 2 h. The treated skins were then washed in cold water to achieve neutral pH. Followed by defatting in 10% butyl alcohol at a sample:solvent ratio of 1:10 (w/v) for 18 h, the solvent was changed for every 6 h. The defatted skin was then washed with cold water. Collagen was extracted by soaking the pretreated skin in 0.5 M acetic acid at a solid:solvent ratio of 1:15 (w/v) for 48 h. After extraction, the mixture was filtered using two layered cheese cloth and the residue was re-extracted under the same condition. Both, the extracts were combined, and collagen was precipitated by adding NaCl at a final concentration of 2.6 M in presence of 0.05 M Tris-HCl (pH 7.5). The resultant precipitate was collected by centrifuging at 15,000 *g* for 1 h (Heraeus Primo, Thermo Scientific, Germany) and was dissolved in a minimum volume of 0.5 M acetic acid,

followed by dialyzing against 0.1 M acetic acid for 2 days. Thereafter, dialyzed against distilled water for 2 days by a changing the solution for every 12 h. The dialysate was freeze dried and was referred to as "ASC".

Hydroxyproline content of ASC was determined according to the method of Nalinanon *et al*. (2007). The yield of ASC was calculated based on the dry weight of prepared skin using the following equation:

Yield (%) = (Weight of ASC (g)/Dry weight of prepared skin (g)) x 100 (1)

2.4. Characterization of ASC

2.4.1. UV absorption

The samples were solubilized in 0.5 M acetic acid to obtain a final concentration of 2 g/L. UV absorption spectra of collagens were measured using a spectrophotometer (V-730, Jusco Co., Japan). Prior to measurement, the baseline was set with 0.5 M acetic acid. The spectra were obtained by scanning the wavelength in the range of 200–300 nm with a scan speed of 50 nm/min at room temperature.

2.4.2. Sodium dodecylsulfate-polyacerylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). The collagen samples were dissolved in 5% SDS and heated at 85 °C for 1 h. The mixtures were centrifuged at 4000 × g for 5 min to remove undissolved debris. The solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence or absence of 10% β-mercaptoethanol. Samples (15 µg protein) were loaded onto a polyacrylamide gel (4% stacking gel and 7.5% separating gel) and subjected to electrophoresis at a constant current of 15 mA per gel, using an electrophoresis apparatus (AE-6440, Atto Co., Tokyo, Japan). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid then destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Gels were imaged using a scanner (MFC-L2700DW, Brother, UK) and band intensities were quantified with the public domain digital analysis software, ImageJ (ImageJ 1.51t, National Institutes of Health, Bethesda, USA). High molecular weight marker (Sigma Chemical Co., USA) was used to estimate the molecular weight of proteins. Type I collagen from calf skin was used as a standard.

2.4.3. Amino acid composition

Collagens were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115°C for 24 h. The hydrolyzates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 mL was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

2.4.4. Differential scanning calorimetry (DSC)

DSC was conducted using a differential scanning colorimeter model DSC 7 (Perkin Elmer, Norwalk, CT, USA). The denaturation temperature (T_{max}) and enthalpy (ΔH) calibrations were determined using indium. ASC were rehydrated in a 0.05 M acetic acid solution with a sample:solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C. The sample (5-10 mg) was accurately weighed into aluminium pans and sealed. The sample was scanned at 1 °C/min over the range of 20-50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area of DSC thermogram.

2.4.5. Secondary structure

Fourier transform infrared (FTIR) spectra of collagen from the skin of snakeskin gourami was obtained using Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany). Collagens samples were placed onto

the crystal cell. The spectra were acquired over the range of 4000-800 cm^{-1} with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cell at 25°C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

Circular dichroism (CD) spectra of collagen was determined by dissolving the samples in 0.5 M acetic acid to obtain a concentration of 0.5 mg/mL The spectra of all the solutions were recorded from the wavelength of 300–190 nm (Jasco J-815, Japan Spectroscopic Co., Japan).

2.4.6. Solubility of ASC

2.4.6.1. Effect of NaCl on collagen solubility

The samples were dissolved in 0.5 M acetic acid at 4 °C for 24 h to produce a final concentration of 6 mg/mL. Next, 5 mL of prepared solutions were mixed with 5 mL of 0.5 M acetic acid containing various concentrations of NaCl (0%, 1%, 2%, 3%, 4%, 5% and 6% (w/v). The mixture was stirred continuously at 4 °C for 30 min, followed by centrifugation at 20,000 *g* at 4 °C for 30 min. Protein content in the supernatant was measured by the method of Lowry *et al*. (1951), using bovine serum albumin as a standard. Relative solubility was calculated as above.

2.4.6.2.Zeta potential

ASC were dissolved in 0.5 M acetic acid at a concentration of 0.5 mg/mL. The mixture was stirred at 4 °C for 12 h. The zeta potential was measured using a zeta potential analyzer (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). The zeta potential of ASC adjusted to different pH values with 1.0 M nitric acid or 1.0 M KOH using an Autotitrator (BI-ZTU, Brookhaven Instruments Co.) was determined. The isoelectric point was estimated from pH rendering zero zeta potential.

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed on the data and differences between means were evaluated using the Duncan's multiple range test. SPSS Statistic Program was used for data analysis.

3.Results and discussions

3.1. Yield of ASC

The yield of ASC extracted from the skin of snakeskin gourami was 9.43% on a wet weight basis and 34.65% on a dry weight basis, similar results were reported by Jongjareonrak *et al*. (2005). Kittiphattanabawon *et al*. (2005) reported that the yield of ASC from the skin of brownstripe red snapper was 9% (wet weight basis) whereas collagen from skin and bone of bigeye snapper yielded 9.38%. The hydroxyproline content of the ASC extracted from the skin of snakeskin gourami was 23.2±1.24 mg/mL which was about 3 times higher than that of its fresh skin (7.22±0.6 mg/mL). The skin raw material could not totally solubilize by 0.5 M acetic acid, indicating the present of high molecular crosslink components in the skin matrix. The differences in extraction yield might be attributed to the differences in fish species, tissue structure and composition, biological conditions, and preparative methods (Ali *et al*., 2018; Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2019; Sinthusamran *et al*., 2013).

3.2. UV absorption spectrum

Maximum UV absorption spectrum of ASC from snakeskin gourami was 230.7 ± 0.3 nm (Figure 1), which was similar to those of collagens from the skins of ornate threadfin bream (230 nm) (Nalinanon *et al*., 2011), channel catfish (232 nm) (Lui *et al*., 2007) and Rutilus Frisii Kutum (240 nm) (Naderi Gharagheshlagh *et al*., 2019). Generally, the absorption wavelength of protein is from tyrosine, tryptophan, and phenylalanine in the near UV region of 260-290 nm. Generally, the tyrosine and tryptophan residues are known to absorb UV light at 280 nm (Ali *et al*., 2017).

But the amount of tyrosine in ASC was 3 residues per 1000 residues. In addition, Edwards *et al*. (1997) found that ASC from *Oreochromis niloticus* skin showed a maximum absorption at 220 nm, which was related to the C=O, -COOH, -$CONH_2$ groups in polypeptides chains of collagen.

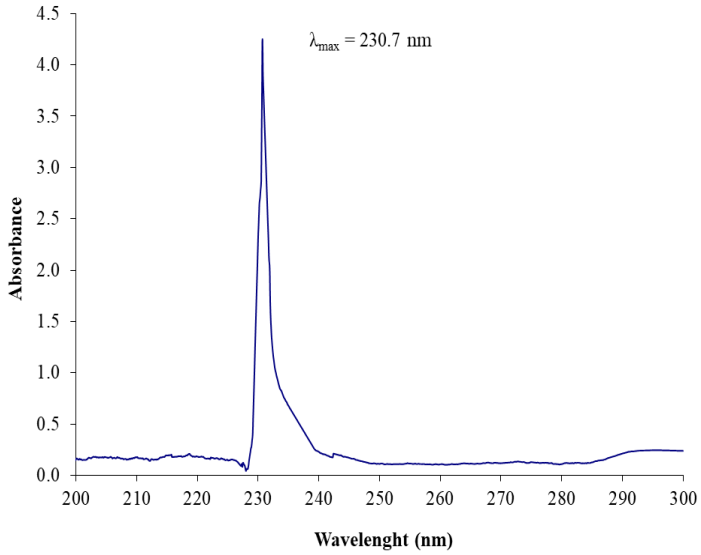


Figure 1. UV absorption spectrum of ASC from the skin of snakeskin gourami.

3.3. Protein patterns

Protein patterns of ASC and skin of snakeskin gourami are shown in Figure 2. The result revealed that both the samples contained α1-chain and α2-chain at a ratio of approximately 2:1, suggesting that type I collagen was the major protein component in the skin. It was noted that ASC mainly contained the highest band intensity of β-chains and γ-chain,which is consistent with findings reported for type I collagen from the skins of hake and trout (Montero *et al*., 1990), golden carp (Ali *et al*., 2018) and bigeye snapper (Kittiphattanabawon *et al*., 2005). Collagen from the swim bladder of seabass was also reported to be type I collagen (Sinthusamran, *et al*., 2013). The ratio between α1-chain and α2-chain of snakeskin gourami was 2.37, which is similar to that from skin of ornate threadfin bream (2.18) (Nalinanon *et al*., 2011). Band intensity ratios of ASC from snakeskin gourami skin (Figure 2A), Ornate threadfin bream, Deep-sea redfish, Black drum fish and calf skin (Table 1) show high population of cross-linked chains (γ and β). The results are in accordance with collagen from the skin of Ornate threadfin bream (2.18) and Deep-sea redfish (2.47) reported by Nalinanon *et al*. (2011) and Wang *et al*. (2007). ASC from the skin of snakeskin gourami found band intensity ratio of β/(α1 + α2) was 1.18 that was lower than in ASC from the skin of Ornate threadfin bream (1.39), and black drum fish (1.59) (Nalinanon *et al*., 2011; Ogawa *et al*., 2003). The different band intensity ratios of α1/α2, β/(α1 + α2), γ/(α1 + α2) and HMC/(α1 + α2) were specifically observed in collagen and could identify the proportion of monomer chain and cross-linked chain**.**

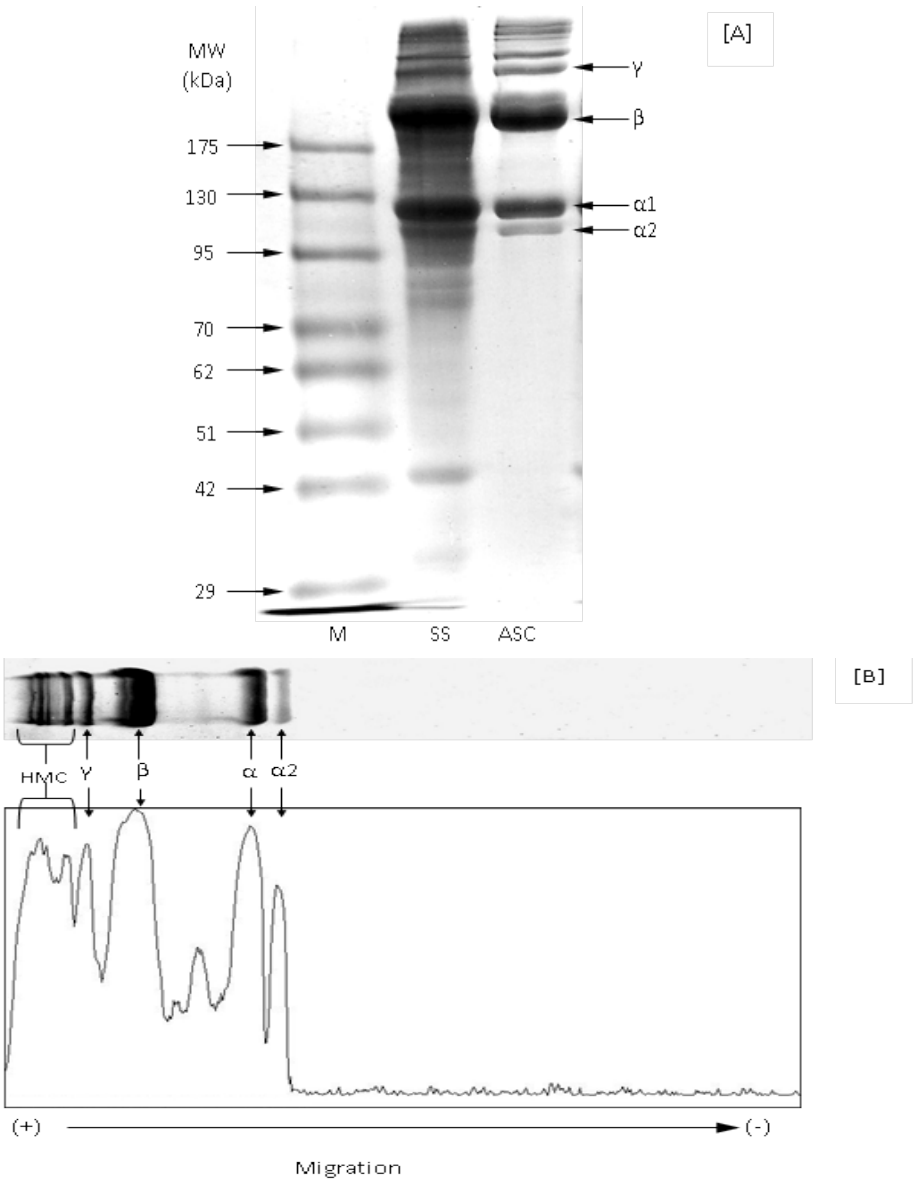


Figure 2. Protein patterns of the skin of snakeskin gourami (SS) and its acid soluble collagen (ASC) counterpart [A] and densitogram of ASC [B]. M denotes high-molecular weight protein markers. HMC is high molecular weight component.

Table 1. Band intensity ratios of collagens from the skin of snakeskin gourami (ASC) in comparison with ornate threadfin bream, deep-sea redfish, black drum, and calf skin.

Components	Band intensity ratio				
	ASC	Ornate threadfin bream[(a)]	Deep-sea redfish[(b)]	Black drum[(c)]	Calf [(c)]
α1/α2	2.37	2.18	2.47	ND	ND
β/(α1 + α2)	1.18	1.39	1.52	1.59	1.26

γ/(α1 + α2)	0.45	0.33	1.1	1.32	0.75
HMC/(α1+ α2)	1.15	0.62	ND	ND	ND

D = Not determined.

Sources :(a) Nalinanon *et al.* (2011); (b) Wang *et al.* (2007) and (c) Ogawa *et al.* (2003).

3.4. Amino acid composition

The amino acid composition of ASC was expressed as "residues per 1000 total amino acid residues" (Table 2). Type I collagen and ASC from the skin of snakeskin gourami were rich in glycine, alanine, proline, and hydroxyproline and had low or no residues of cysteine, tyrosine and hydroxylysine (Singh *et al.*, 2011). Glycine was the most abundant compound with 332 and 313 units of the total amino acids present in type I collagen and ASC, respectively. Generally, glycine in collagen represent almost one third of the total residues and occurs every third residue in collagen except for the first 14 amino acid residues from N-terminus and the first 10 residues from the C-terminus (Foegeding, 1996). Both collagens had proline (130 and 115 residues/1000 residues), alanine (108 and 124 residues/1000 residues) and hydroxyproline (95 and 73 residues/1000 residues). Alanine was the second most abundant amino acid in all collagens. All collagens contained no cysteine and negligible tryptophan. The imino acid (proline and hydroxyproline) content of type I collagen and ASC were 225 and 188 residues/1000 residues, respectively. ASC was relatively higher than those of ASCs from ballon fish skin (179 residues/1000 residues) (Huang *et al*., 2011), cod skin (179 residues/1000 residues), carp skin (179 residues/1000 residues) (Duan *et al*., 2009), swim bladders of seabass (128 residues/1000 residues) (Kaewdang *et al*., 2014) but was slightly lower than cobia skin (203 residues/1000 residues) (Zeng *et al*., 2012), brownbanded bamboo shark skin (204 residues/1000 residues) (Kittiphattanabawon *et al*., 2010), striped catfish skin (206 residues/1000 residues) (Singh *et al*., 2011). The imino acid content was used to determine the thermal stability of collagen and the formation of junction zones via hydrogen boding (Sinthusamran *et al*., 2013). The pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of polypeptide chains and help to strengthen the triple helix (Wang *et al*., 2007).

Table 2. Amino acid composition of acid soluble collagen (ASC) from the skin of snakeskin gourami and calf skin type I collagen (residues/1000 residues)

Amino acid	Type I collagen	ASC
Alanine	108	124
Arginine	49	55
Aspartic acid/asparagine	45	49
Cysteine	0	0
Glutamic acid/glutamine	73	75
Glycine	332	313
Histidine	5	6
Isoleucine	12	12
Leucine	24	27

Lysine	27	28
Hydroxylysine	7	6
Methionine	6	11
Phenylalanine	12	16
Hydroxyproline	95	73
Proline	130	115
Serine	34	33
Threonine	16	28
Tyrosine	3	5
Tryptophan	0	0
Valine	20	24
Total	**1000**	**1000**
Imino acids *	225	188
* Imino acids include proline and hydroxyproline.		

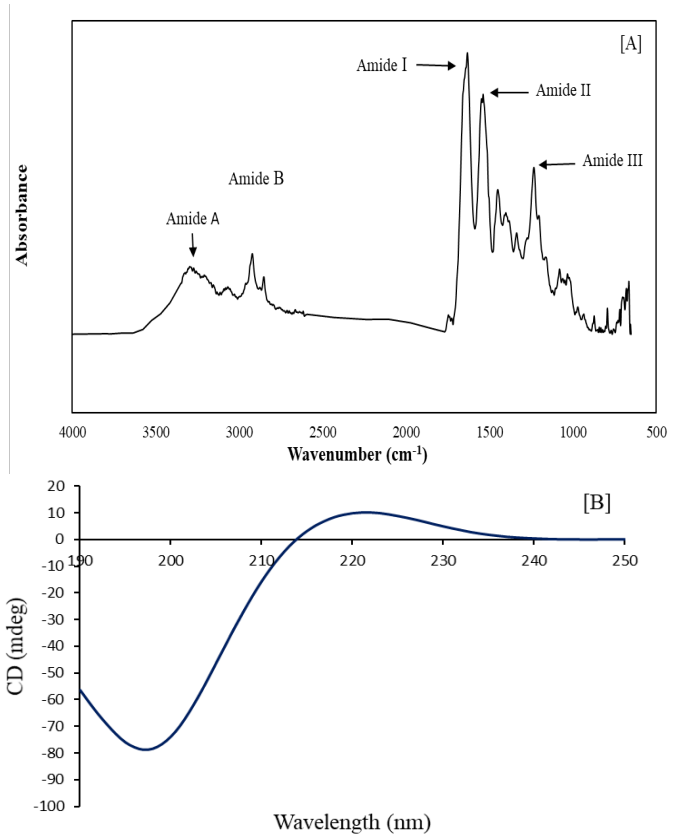


Figure 3. Fourier transform infrared (FTIR) [A] and circular dichroism (CD) [B] spectra of ASC from the skin of snakeskin gourami.

3.5. Thermal stability of ASC

The T_{max} and ΔH of ASC estimated from DSC thermogram were 33.43 °C and 1.15 J/g, respectively. Thermal stability of collagen was governed by pyrrolidine rings of proline and hydroxyproline and partially by hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul *et al*., 2010). However, the T_{max} value might also be determined by the conformation and amino acid sequence of collagen (Ali *et al*., 2018). T_{max} values of ASC from snakeskin gourami were higher than those previously reported for collagens from several fish species. T_{max} of ASC from the skin of snakeskin gourami was in accordance with those of cold-water fish such as cod (15 °C), Alaska pollack (16.8 °C), Japanese seabass (30 °C), skip jack tuna (29.7 °C) and ayu (29.7 °C) (Sinthusamran *et al*., 2013; Yu *et al*., 2014) or collagen from the skin of tropical fish such as brownstripe red snapper (31.5 °C), bigeye snapper (30.4 °C), black drum fish (34.2 °C), sheepshead seabream (34 °C) (Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2010; Ogawa et al., 2003). The differences in Tmax amongst collagens from different species were correlated with different imino acid contents, body temperature and environmental temperature (Duan *et al*., 2009; Kittiphattanabawon *et al*., 2005).

3.6. Secondary structure of ASC

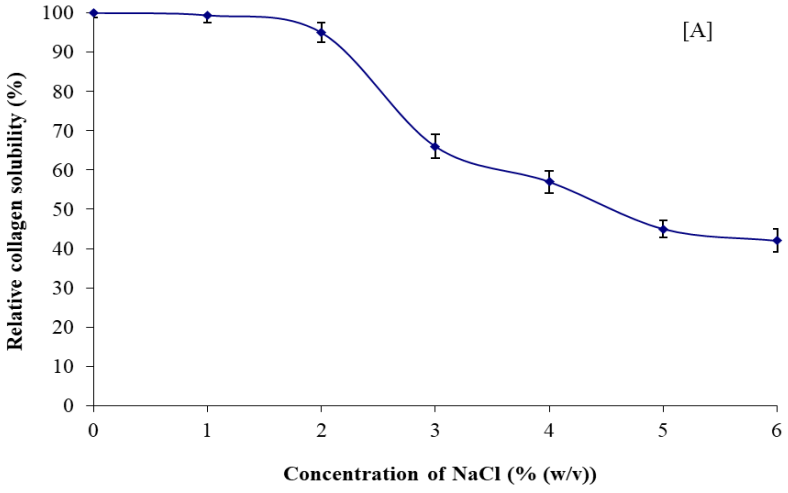
The FTIR spectrum of ASC from the skin of snakeskin gourami exhibited characteristic peaks of amide A and B, as well as amide I, II, III (Figure 3A). The major peaks found in ASC were similar to those of collagens isolated from other fish species (Kaewdang *et al*., 2014; Kittiphattanabawon *et al*., 2010; Li *et al*., 2020). The amide A was found at a wave numbers of 3288 cm^{-1} and associated with N-H stretching vibration, which occurs in the wave number range of 3400-3440 cm^{-1} (Purna Sai and Babu, 2001). Also, when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to a lower frequency (Ali *et al*., 2018). The amide B was found at a wave number of 3067 cm^{-1}, associated with the asymmetrical stretch of CH_2 (Muyonga *et al*., 2004). The wave number of amide I, amide II and amide III bands are directly associated with the configuration of collagen. The amide I band with the characteristic strong absorbance in the range of 1600-1700 cm^{-1} was mainly related to the C=O stretching vibration along the polypeptide backbone, and it could be a sensitive marker of peptide's secondary structure (Sinthusamran *et al*., 2013). The amide I band of ASC was found at the wavenumber of 1631 cm^{-1}. The amide II band of ASC was situated at a wave number of 1546 cm^{-1}, while the amide III band of ASC was located at wave number 1232 cm^{-1}. The amide II and amide III bands represent N-H bending vibration and C-H stretching, respectively (Naderi Gharagheshlagh *et al.*, 2019). As a consequence, ASC was still conserved in its secondary structure. The CD spectrum of ASC scanned in the range of 190-250 nm is shown in Figure 3B. The CD curves showed a rotatory maximum at 222 nm and minimum at 197 nm, and a consistent crossover point at about 214 nm, which is a typical characteristic of triple helical conformation of collagen (Ikoma *et al*., 2003). CD spectra represents backbone configuration of protein through absorption regions of peptide linkage (Ogawa *et al*., 2004). ASC exhibited distinct positive and negative absorbance for a native collagen, while denatured collagen has a more distorted spectrum, reflected by the disappearance of a positive peak at 221 nm as well as negative peak shifts to lower absorption than 198 nm as reported by Ali *et al*. (2017). The results confirmed that ASC had high structural integrity without denaturation.

3.7. Solubility of ASC

The effect of NaCl on collagen solubility is shown in Figure 4A. The high solubility of ASC in 0.5 M acetic acid was maintained in the presence of NaCl up to 1-2%. Solubility of ASC decreased gradually when the NaCl

concentration exceeded 2% and was also reduced when the NaCl concentration was 6%. This result is in accordance with the reports on the solubility of collagens from the skins of yellowfin tuna, dusky spinefoot, sea chub, eagle ray, red stingray, yantai stingray, brownstripe red snapper, bigeye snapper and striped catfish in acetic acid solution generally decreased with an increase in NaCl concentration (Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2005). This effect could be due to the "salting out" of collagen, which occurred at relatively high concentration of NaCl (Singh *et al*., 2011). According to Matmaroh *et al*. (2011), at low concentrations of NaCl, salt ions are bound weakly to the charged groups on the protein surfaces without affecting the hydration shell on those domains. These results support the use of collagen from fish scales and skin as an alternative source for use in food, pharmaceutical and nutraceutical industries.

The zeta potential is a key indicator of the stability of colloidal dispersions. ASC from snakeskin gourami at different pH levels is shown in Figure 4B. At pH 2-6, collagen sample were positively charged, and negatively charged between a pH of 7-10 with the net charge of zero at pH of 6.23. When pH values were above or below pI values of the proteins, the repulsion between the protein chains increased resulting in a high net charge and the solubility of protein molecules. When the net charge of a protein was zero, hydrophobic-hydrophobic interactions increased, resulting in protein precipitation and aggregation (Ali *et al*., 2018). Collagens extracted from various fish have been shown to exhibit different pI values. For example, the pI value of collagen from striped catfish skin was 4.27 (Singh *et al*., 2011), collagen from spotted golden goatfish scales had a pI of 4.96 (Matmaroh *et al*., 2011), and the pI of bamboo shark skin collagen was 6.12 (Kittiphattanabawon *et al*., 2010). The slight differences in pI between collagens from various fish species might be caused by the slight difference in their amino acid sequences and distribution of amino acid residues.



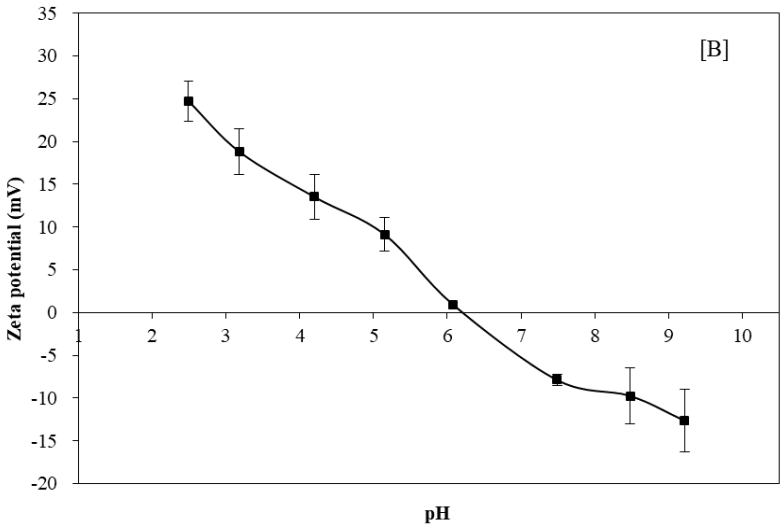


Figure 4. Solubility at different NaCl concentrations [A] and Zeta potential at different pHs [B] of ASC from the skin of snakeskin gourami.

4. Conclusions

Collagen from the skin of snakeskin gourami was simply extracted by using acetic acid. Based on SDS-PAGE and amino acid composition, ASC was characterized to be type I collagen. The structural integrity of ASC was well preserved as determined by FTIR and circular dichroism. The solubility of ASC was depended on pH and NaCl concentration. Since collagen has a wide range of commercial uses, and based on obtained results, ASC from skin of snakeskin gourami can be a promising means of alternative source to produce collagen mainly from the fish processing industries.

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BIOREFINERY FOR SEQUENTIAL EXTRACTION OF FUCOIDAN AND ALGINATE FROM BROWN ALGA *Sargassum cristaefolium*

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ABSTRACT

The critical step in the sequential extraction of fucoidan and alginate from brown algae is acid treatment, since it noticeably affects physicochemical properties of the both components. This study aimed to investigate the effects of acid treatment on the multiple responses of alginate and fucoidan yield from brown alga *Sargassum cristaefolium*. Box Behnken Design (BBD) from Response Surface Methodology (RSM) was established to understand the effects of temperature, time and pH in acid treatment on the fucoidan yield and multiple-response alginate as follows: yield, intrinsic viscosity, and molecular weight. The experimental results revealed that temperature, time and pH significantly affected fucoidan yield, alginate yield, intrinsic viscosity, and molecular weight of alginate. The optimum acid treatment was found at temperature 33.75 °C, time 58.22 min, and pH 3.07, resulting in fucoidan yield 1.22±0.068%, alginate yield 29.85±0.24%, intrinsic viscosity 409.72±8.23 ml/g and molecular weight 194.08±3.77 kDa with the desirability value 0.805.

1. Introduction

Brown algae is inarguably one of important sources for polysaccharides, e.g. fucoidan and alginate, with different physicochemical properties (Rioux *et al.,* 2007; Ale *et al.* 2011a; Torres *et al.*, 2007; Draget and Taylor, 2011). Alginate derived from brown algae is thermo-stable component, which is widely applied in a variety of industries as thickener, emulsifier, stabilizer, and gelling agent (Poncelet *et al.* 1999; Gomez *et al.* 2009; Rahelivao *et al.* 2013; Sellimi *et al*. 2015), while it is also massively used in food supplementation, pharmaceutical industry (producing slow-release characteristics), and antitumor (Sousa *et al*. 2007; Moebus *et al.* 2012; Jensen *et al.* 2012). Fucoidan isolated from brown seaweed is also reported capable of exerting beneficial health effects mainly on antitumor, anticancer, anti-inflammation, and immunomodulator (Asker *et al.,* 2007; Ye *et al.* 2008; Kim *et al*. 2010; Ale *et al.* 2011b; Costa *et al.,* (2011).

Although seaweed is industrially important, the processing has been currently hindered by some serious constraints, mainly related to low efficiencies and huge amount of waste. To deal with this, the use of integrated biorefinery for producing various products could gradually eliminate the mentioned constraints, while also rising economic benefits (Lorbeer *et al.* 2015). Such biorefinery processing is greatly possible for brown algae due to presence of fucoidan and alginate with existing or future potential applications (Jung *et al.* 2013; Ruiz *et al*. 2013). The parallel processing technology for producing fucoidan and alginate from brown algae could be a great opportunity in biorefinery industry (Sugiono and Ferdiansyah, 2019).

In general, acid treatment was applied to extract fucoidan and alginate from brown algae. It enables to induce destruction of cell walls, hydrogen bond cleavage, and solubilization of

extracted fucoidan (Kim *et al.* 2010; Ale *et al.* 2012; Ermakova *et al.* 2011; Sugiono *et al.* 2014), while the acid also simultaneously plays role in converting alginate-salts to alginate-acids, avoiding them from production of insoluble contaminants (Myklestad, 1968; Arvizu *et al.* 2007). During extraction of alginate, acid treatment serves to remove contaminants (fucoidan, laminaran and polyphenol) and produce alginate-acids which then increase their extractability using sodium carbonate (Hernandez-Carmona *et al.* 1999; Torres *et al.* 2007; Gomez *et al.* 2009; Sellimi *et al*. 2015; Rahelivao *et al.* 2013; Fertah *et al.* 2014; Sugiono *et al.* 2019a). Based on this mechanism, acid treatment becomes a basic principle for sequential biorefinery in extraction of fucoidan and pre-extraction of alginate.

Previous studies have reported the application of acid treatment for sequential extraction of fucoidan and alginate with regard to characterization of the components (Rioux *et al.* 2007), but their works were not exclusively directed to biorefinery processing. Therefore, the use of acid treatment with optimum levels allows us to perform biorefinery processing for extracting fucoidan and alginate from brown seaweed, resulting in high yield and quality. In low acid concentration, the yield was also poor; on the other hand, the excessive level of acid would degrade alginate structure, causing reduction of its viscosity. This present work aimed to determine optimum level of pH, temperature, and time in the acid treatment for isolating fucoidan and alginate from brown alga *Sargassum cristaefolium* with regard to biorefinery industry.

2. Materials and methods

2.1. Materials and reagents

Brown algae *Sargassum cristaefolium* was obtained from Poteran Island in Sumenep, Madura, and collected in Desember 2018. Chemicals (distilled water, HCl 37%, NaOH, ethanol 99.8%, Na_2CO_3) for extraction and analyses were analytical grade.

2.2. Sequential extraction of fucoidan and alginate

2.2.1. Pre-treatment of brown algae

Brown algae was washed using fresh water, dried, ground, and sieved at 60 mesh (Sugiono *et al.* 2014). The powder was then soaked in a solution containing ethanol: $CHCl_3$: distilled water (4:2:1), stirred overnight to remove phenol and protein. Last, the mixture was washed and dried at 45 °C for 6 h (Ale *et al.* 2012).

2.2.2. Fucoidan extraction

The pre-treated algae (7.5 g) was added with HCl (1:20, b/v; pH 1-5) and incubated in a shaking waterbath at 25 – 45 °C for 30 – 90 min. Subsequently, vacuum filtration was used to separate residue (A) from filtrate. The filtrate was mixed with ethanol 96% (1:2, v/v) and left overnight at room temperature until producing precipitate. Fucoidan was collected following centrifugation at 7000 rpm for 10 min, and dried using vacuum dryer at 45 °C for 18 h (Ale *et al.* 2012).

2.2.3. Alginate extraction

Residue A (collected from previous process) was added with Na_2CO_3 2.5% (1:20, b/v) and incubated in a shaking waterbath at 70 °C for 2 h, then followed by filtration to collect filtrate. The filtrate was centrifuged at 5000 rpm for 10 min, mixed with ethanol 96% (1:2, v/v) and filtered after incubation for 2 h. The alginate was washed twice using ethanol 70% and 96%, respectively, filtered and dried using vacuum dryer at 45 °C for 24 h. Ultimately, the dried alginate was ground and sieved at 60 mesh (Gomez *et al.* 2009).

2.3. Experimental design

Box-Behnken Design in Response Surface Methodology (RSM) was used, consisting of 3 variables, i.e. temperature (X_1: 25, 35, 45°C), time (X_2: 30, 60, 90 min), and pH (X_3: 1, 3, 5). The coded (±1 and 0) and actual of independent variables used in this experiment was presented in Table 1. A totally amounting of 15 experimental runs with three replicates in center

point (Table 2) (Montgomery, 2005). The center points were fixed according to preliminary study.

Regression analysis and model adjustment at the second order was carried out as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1} \sum\sum_{i<j} \beta_{ij} x_i x_j \quad (1)$$

where Y = response, β_0 = intercept coefficient, β_i, β_{ii}, β_{ij} = regression coefficient for linier, quadratic, and interaction, and x_i, x_j, = variables of pH and temperature, and time ($i \neq j$).

The data analysis was performed in Design-Expert version 7 software in order to find correlation coefficient (R) and determination coefficient (R^2), while the significance was set at P = 0.05. Accuracy between validation and estimated data by Design Expert was compared using paired sample t-test in Minitab 16 software.

Table 1. Coded and actual of independent variables

Independent variables	Symbols	Variables	
		Coded	Actual
Temperature (°C)	x_1	-1	25
		0	35
		+1	45
Time (min)	x_2	-1	30
		0	60
		+1	90
pH	x_3	-1	1
		0	3
		+1	5

Table 2. Box-Behnken Design from RSM and responses

No	Actual variables				Responses		
	Temperature (°C)	Time (min)	pH	Fucoidan yield (%)	Alginate yield (%)	Intrinsic viscosity (ml/g)	Molecular weight (kDa)
1	35	90	1	1.50	31.12	103.15	45.42
2	45	60	1	0.50	29.90	140.78	65.86
3	25	90	3	1.10	26.21	258.20	118.79
4	45	60	5	0.10	27.23	285.22	134.55
5	25	60	1	1.20	28.10	246.60	116.12
6	35	30	5	0.12	26.32	183.44	86.72
7	45	30	3	0.15	26.80	170.11	78.79
8	25	30	3	0.05	26.48	267.00	122.98
9	25	60	5	0.11	26.13	222.95	101.99
10	35	90	5	0.13	26.74	223.00	104.44
11	45	90	3	1.30	29.98	191.00	89.66
12	35	30	1	1.20	29.85	283.00	130.62
13	35	60	3	1.21	29.80	400.11	189.57
14	35	60	3	1.32	29.54	532.07	255.29
15	35	60	3	1.12	30.21	407.00	192.94
Pred.	33.75	58.22	3.07	0.71 ± 0.43^a	29.63 ± 0.72^b	448.8 ± 67.24^c	212.6 ± 32.26^d
Valid	33.75	58.22	3.07	1.22 ± 0.068^a	29.85 ± 0.24^b	409.72 ± 8.23^c	194.08 ± 3.77^d

2.4. Characterization

2.4.1. Yield

Yield was determined according to ratio of alginate or fucoidan weight over initial weight of brown algae, then multiplied by 100% (Torres *et al.* 2007).

2.4.2. Intrinsic viscosity

For alginate viscosity, viscometer capillary Ubbelohde (Canon, USA) with capillary diameter of 0.56 mm was employed at 25 °C. Alginate solution was made by dissolving 30 mg of alginate in 10 ml of aquabides, stirred for 5 h at room temperature (25 °C) and diluted at serial concentration of 0.05-0.3 g/dL (Chee *et al.* 2011). Relative viscosity η was determined according to ratio of flow time t over flow time for solvent t_0. Meanwhile, intrinsic viscosity [η] was calculated as follows:

Relative viscosity, $\eta=\frac{t}{t_0}$ (2)

Specific viscosity, $\eta_{sp}=\eta-1$ (3)

Reduction viscosity, $\frac{\eta_{sp}}{c}=\frac{\eta-1}{c}$ (4)

Intrinsic viscosity, $[\eta]=\lim_{c\to 0}\frac{\eta_{sp}}{c}$ (5)

2.4.3. Molecular weight

Molecular weight of alginate was determined according to relationship between averaged intrinsic viscosity and molecular weight. Calculation of molecular weight referred to Mark-Houwink, where k = 0.023 dL/g and a = 0.984 (Clementi *et al.* 1998). In this case, [η] represented intrinsic viscosity (dL/g), while M_w represented molecular weight (kDa).

$$[\eta]=kM_w^a \quad (6)$$

3. Results and discussions

3.1. Yield of fucoidan

The results showed that all studied variables showed linear relationship to the yield of fucoidan (Figure 1). The yield seemed to raise as increase in temperature, time, and acidity. The highest fucoidan extract (1.5%) was obtained at temperature 45 °C, time 90 min, and pH 1. This is in accordance with previous result reported by Ale *et al.* (2012), finding that a high temperature causes swelling of the algae cell wall due to thermal expansion, resulting in enhancement of fucoidan extractability (Sugiono *et al*. 2014). Meanwhile, the use of low pH and longer acid treatment enabled to soften cell wall of the algae, which increased solubility of fucoidan in HCl (Silva *et al*. 2015). Rodriquez *et al*. (2011) reported that the increase in temperature and time could improve extractability of fucoidan, then it gradually decreased after reaching the optimum level. Lorbeer *et al*. (2015) stated that fucoidan yield was higher when extracted under higher temperature and longer extraction time, while the low pH could destroy fucoidan structure.

3.2. Yield of alginate

In this present work, we found that all the variables demonstrated quadratic effects on the alginate yield (Figure 2), ranging from 26.21 – 31.12%. This was similarly reported by Silva *et al*. (2015) and Sugiono *et al*. (2019a). The yield showed an increase with the increased temperature, longer time, and reduced pH during pre-extraction with acid treatment. This is understandable since an increase in pH level and time could enhance conversion of Ca/H ions, thereby improving the solubility of alginate in Na_2CO_3. In addition, higher temperature and longer exposure to acid treatment noticeably contributed to formation of porous and softened structure in surface of the cell walls, which in turn also enabled to increase extractability of alginate (Sugiono *et al.* 2018b). Fertah *et al*. (2014) asserted that alginate extract was relatively increased with the increasing extraction temperature, after this condition it was continuously to decrease due to a degradation of alginate chain molecules. Nevertheless, pre-extraction of algae using acid treatment at pH 5 showed a contrary result. The exchange of Ca/H ions was logarithmically in proportional with acid concentration and pre-extraction time (Mykleasted *et al*. 1968; Lorbeer *et al*. 2015).

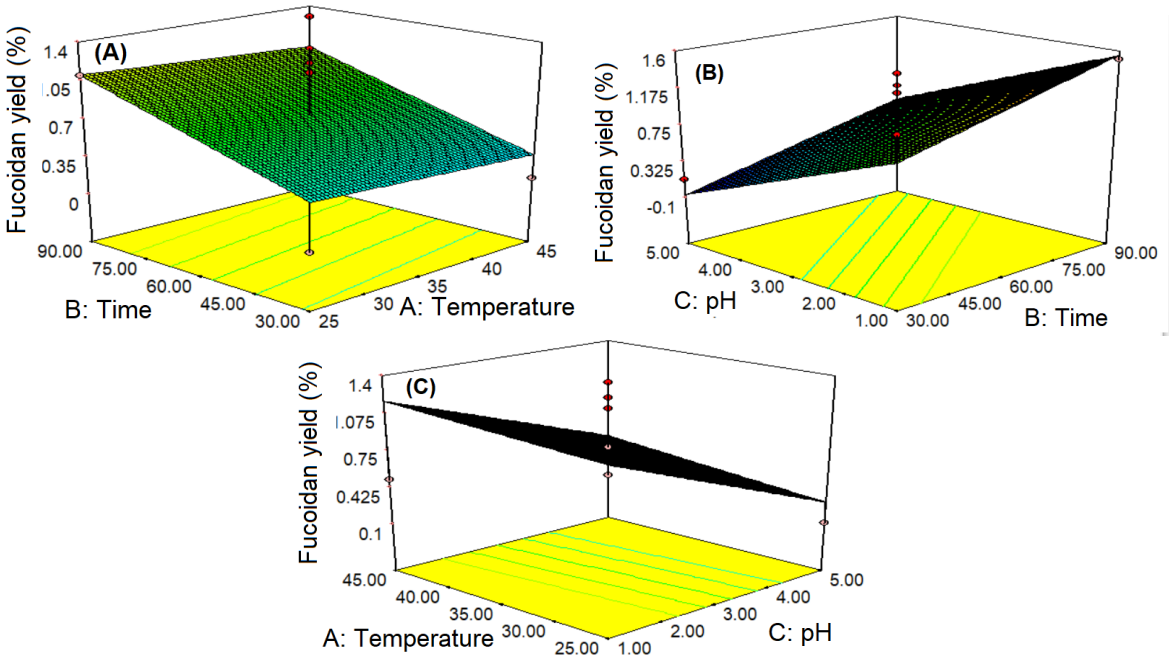

Figure 1. Response surface plots of fucoidan yields from brown algae *Sargassum cristaefolium* as a function of temperature and time (A), pH and time (B), temperature and pH (C).

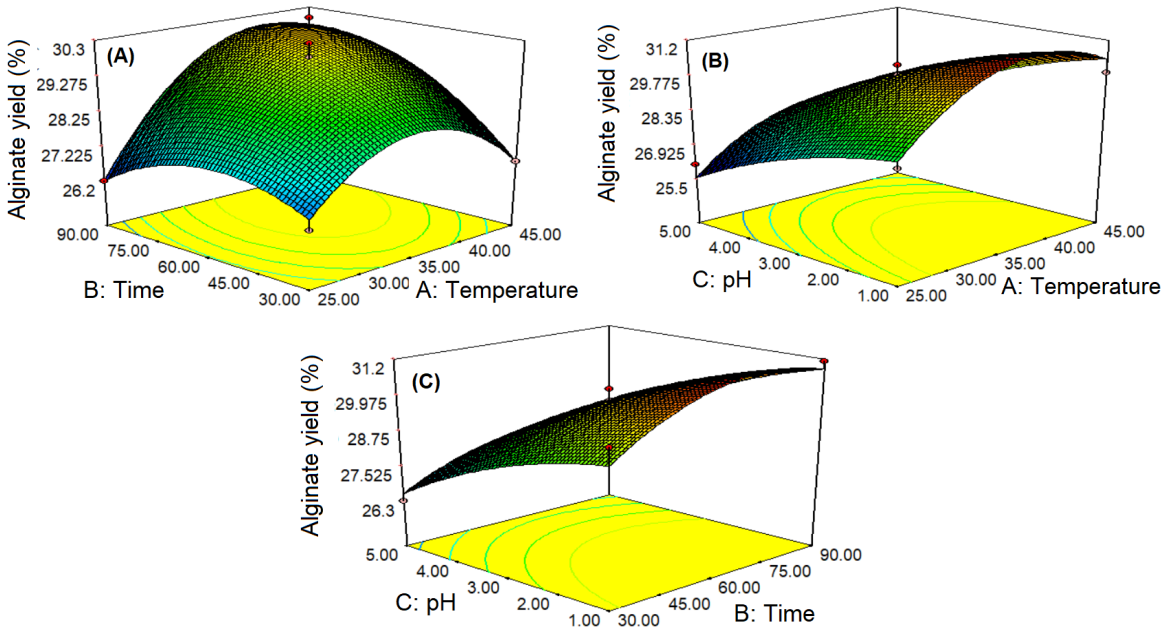

Figure 2. Response surface plots for alginate yield from brown alga *Sargassum cristaefolium* as a function of temperature and time (A), pH and temperature (B), pH and time (C).

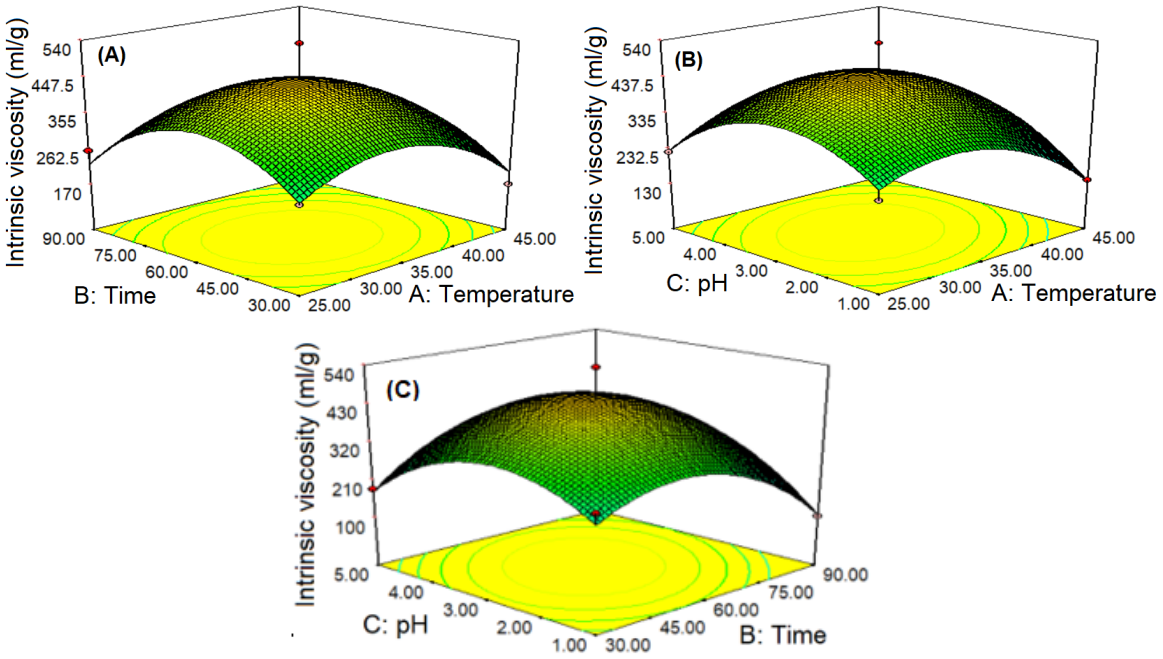

Figure 3. Response surface plots for alginate intrinsic viscosity from brown alga *Sargassum cristaefolium* as a function of temperature and time (A), pH and temperature (B), pH and time (C).

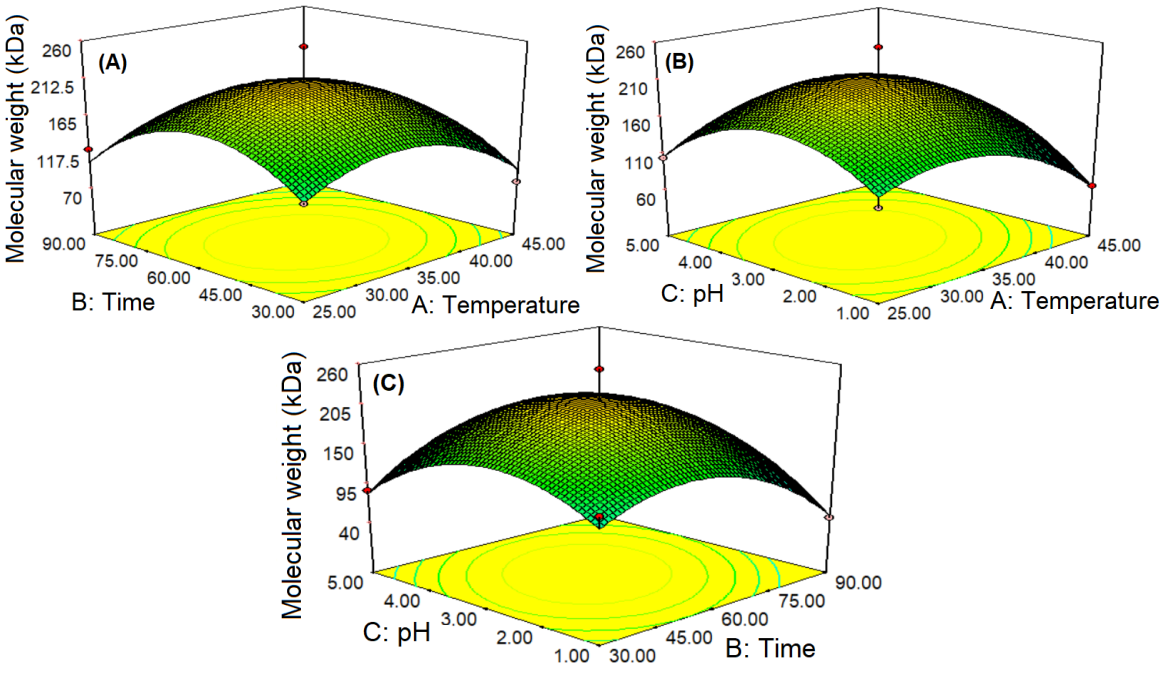

Figure 4. Response surface plots alginate molecular weight from brown alga *Sargassum cristaefolium* as a function of temperature and time (A), pH and temperature (B), pH and time (C).

3.3. Intrinsic viscosity of alginate

The results demonstrated that difference in temperature, time, and pH showed quadratic effect on intrinsic viscosity of alginate (Figure 3). An increase in temperature, time, and pH resulted in a growing viscosity, while it tended to be lower after reaching optimum condition. In this present work, intrinsic viscosity reached 103.15 ml/g to 446.39 ml/g, which was relatively similar to that reported by Torres *et al*. (2007), Fenoradosoa *et al*. (2010) and Rahelivao *et al*. (2013). The incrementation of alginate intrinsic viscosity was found at pH 3 and time 60 min; however, it showed a decrease at pH 1 and pH 5 in 30 – 90 min. The rising viscosity is associated with the increasing conversion of Ca/H ion exchange occurring at pH 3 within 30 – 90 min, thus improving the extractability of long-chain alginate.

Additionally, pre-extraction carried out at pH 5 seemed to be ineffective in reducing phenol compounds, in which their existence differently contributed to the increasing cleavage of main polymer chains of alginate during extraction in alkaline condition (Wedlock and Fasihuddin, 1990). Jayasankar (1996) reported that viscosity of alginate was higher after treated with acid compared to that without acid treatment. Meanwhile, extraction at pH 1 could induce degradation of alginate polymer chains (Haug *et al.* 1963; Smidsrod *et al*. 1969). Furthermore, the increase in temperature and time during acid treatment would induce cell wall to soften and swell, which remarkably enhanced the extractability of long-chain molecules of alginate (Sugiono and Ferdiansyah, 2018).

3.4. Molecular weight of alginate

Present work successfully found that concentration of alginate with high molecular weight tended to increase with a rising temperature, time and acidity (Figure 4). This is augmented by previous studies reported by Torres *et al*. (2007) and Lorbeer *et al*. (2015). It is noteworthy that pH levels in pre-extraction phase strongly caused positive effects on the incrementation of molecular weight, occurring up to pH 3 and time 60 min, although it was then declined at pH 1 and pH 5 within 30 – 90 min. At condition of pH 3, temperature 35 °C and time 60 min, the conversion of Ca/H ions was higher, thus escalating the solubility of alginate in Na_2CO_3. Myklestad (1968) found that Ca/H ion exchange occurred at a higher level with the increase in acidity and time during acid treatment. The higher temperature and longer period of acid treatment was responsible for degradation of cell wall, ultimately contributing to enhanced level of extracted alginate. Sugiono *et al.* (2018a) mentioned that the extraction of high molecular weight alginate could achieve more desirable results when carried out at high temperature, longer time, and low pH during pre-extraction acid treatment, after that, it tended to attenuate due to destruction of alginate polymer chains. At low pH, the polymer chain of alginate was destroyed because of β-elimination and hydrolytic reaction which was catalyzed by proton (Hernandez-Carmona *et al*. 1999; Silva *et al*. 2015). However, at pH 5, molecular weight of alginate was reduced due to oxidative depolymerization triggered by phenolic compounds through auto-oxidation process to release hydrogen peroxide; this free radical was capable of cleaving main chain of alginate molecules (Smidsrod *et al*. 1963). Wedlock and Fasihuddin (1990) also reported that acid treatment at pH close to 7 seemed to be less effective in phenolic compound removal; as commonly known, the component was not desired since it promoted degradation of alginate polymers in alkaline extraction stage. Furthermore, previous study found that molecular weight of alginate was higher at acid treatment of pH 3.5 compared to that extracted at pH 5 (Lorbeer *et al*. 2015).

3.5. Model accuracy

Box Behnken Design was used to evaluate the effects of temperature, time, and pH on yield (fucoidan and alginate), intrinsic viscosity, and molecular weight of alginate. The second order polynomial for biorefinery of sequential extraction was presented in Table 3.

The model accuracy on each response was evaluated using model significance, lack of fit, and correlation coefficient which are presented in Table 3. The acceptable model was achieved according to following criteria, i.e. significance of $P<0.05$, $R^2 \geq 0.8$ and lack of fit>0.1 (Montgomery, 2005). In this case, the second order polynomial for fucoidan yield and multiple response of alginate fitted entire criteria, suggesting that it is acceptable for predicting optimum response.

3.6. Optimization and verification

The optimum condition for extraction was achieved at temperature 33.75 °C, time 58.22 min, pH 3.07. In such condition, the response was predicted to reach fucoidan yield 0.71%, alginate yield 29.63%, intrinsic viscosity 448.8 ml/g, and molecular weight 212.6 kDa, with desirability value of 0.805 (Figure 5). Desirability ranges from 0 to 1, in which desirability close to 1.0 indicates that prediction of optimum condition generated by Design Expert possesses high validity (Sugiono *et al.* 2019b).

The predicted optimum condition was verified using 3 replicates, while the experimental value of response was described as follows: fucoidan yield 1.22±0.068%, alginate yield 29.85±0.24%, intrinsic viscosity 409.72±8.23 ml/g, and molecular weight 194.08±3.77 kDa. Based on paired sample t-test, the data obtained from prediction and validation did not differ significantly ($P>0.05$), suggesting that the experimental data showed a desirable suitability with optimum point as predicted by the model.

Table 3. Polynomial models, significance codes and fitting models

Coefficient	Fucoidan yield (%)	Alginate yield (%)	Intrinsic viscosity (ml/g)	Molecular weight (kDa)
Intercept				
β_0	+0.74	+29.85	+446.38	+211.46
Linear				
β_1	-0.051^{ns}	+0.87*	-25.96^{ns}	-12.33^{ns}
β_2	+0.31^{ns}	+0.57**	-16.03^{ns}	-7.61^{ns}
β_3	-0.49*	-1.57*	+17.64^{ns}	+8.34^{ns}
Quadratic				
β_{11}	-	+0.86^{ns}	-8.54^{ns}	+3.53^{ns}
β_{22}	-	-0.17^{ns}	+42.02^{ns}	+19.98^{ns}
β_{33}	-	-0.21^{ns}	+54.85^{ns}	+26.04^{ns}
Cross product				
β_{12}	-	-1.57^{ns}	-99.53**	-47.73*
β_{13}	-	-0.91*	-125.27*	-59.93*
β_{23}	-	-0.44***	-122.96**	-58.82*
Fitting model				
P value	0.0157*	0.0062**	0.0417*	0.0423*
Lack of Fit	0.0577^{ns}	0.1722^{ns}	0.7914^{ns}	0.7961^{ns}
R^2	0.9558	0.9575	0.9038	0.9032

Equation of the type $Y= \beta_{0+} \beta x_{1+} \beta x_2+ \beta x_3+ \beta x_1 x_2+ \beta x_1 x_3+ \beta x_2 x_3+ \beta x_1 x_1+ \beta x_2 x_2+ \beta x_3 x_3$

Significance codes: *** = $P<0.001$

** = $0.001<P<0.01$

* = $0.01<P<0.05$

^{ns} = $P>0.05$

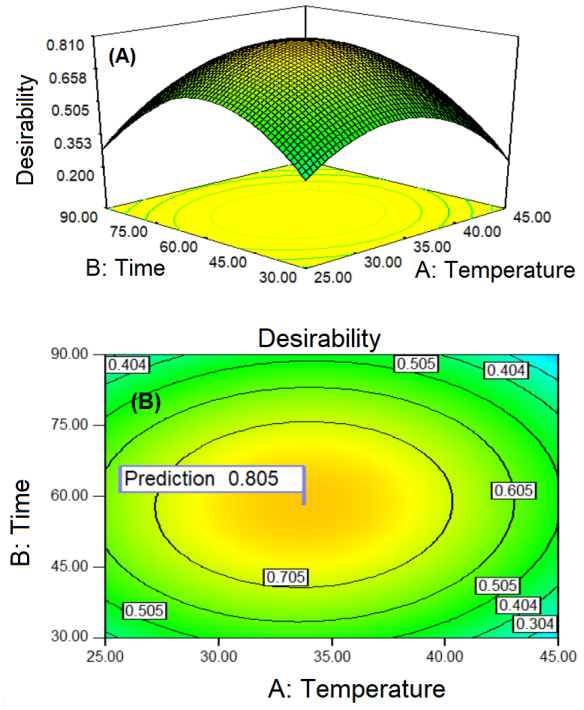

Figure 5. Response surface (A) and contour plot (B) of desirability for optimal points in biorefinery of fucoidan and alginate sequential extraction.

4. Conclusions

Biorefinery process for the sequential extraction of brown algae polysaccharides has been developed, this process can produce two products of fucoidan and alginate. The experimental results showed that all studied variables (temperature, time, pH) demonstrated linier effects on fucoidan yield, but displayed quadratic effects on alginate yield, intrinsic viscosity, and molecular weight. From the optimization, the best condition for acid treatment would be as follows: temperature 33.75 °C, time 58.22 min, pH 3.07. Such condition reached fucoidan yield 1.22±0.068%, alginate yield 29.85±0.24%, intrinsic viscosity 409.72±8.23 ml/g, and molecular weight 194.08±3.77 kDa.

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EFFECT OF POMEGRANATE (*PUNICA GRANATUM*) PEEL EXTRACT (PPE) IN INCREASING THE SHELF-LIFE OF HOME-MADE BUTTER

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ABSTRACT

Pomegranate (*Punica granatum*) fruit is known for its medicinal properties due to its various bio-active polyphenols and flavonoids which exhibit anti-microbial and anti-oxidant properties (Chaturvedula et. al, 2011). The extraction of these bio-active ingredients from fruit peels maximises fruit by-product utilisation and can be used as an alternative to chemical preservatives. These compounds of importance were extracted from powdered dried peels in ethanol using a water bath shaker to obtain pomegranate peel extract (PPE) with concentration of 125mg/ml. The preservative effect of PPE was studied on home-made butter for 15 days at 4°C using various concentrations like 1.25 mg/g, 2.5mg/g and 3.75 mg/g. The result showed the extended shelf life of PPE incorporated samples as compared to the control sample (R) which was found unacceptable on the ninth day and onwards. The rejection of control sample (R) was on the basis of sensory evaluation and increased number of microbial count, i.e., log 2.74 cfu/ml. The sample C (3.75 mg/g) showed better storage life of 15 days without any effect on its sensory attributes. PPE can be used as preservative in home-made butter as result indicates that the concentration of PPE inversely effects the microbial growth and hence there is a sharp decline in microbial count at high concentrations.

1. Introduction

With an environmental-friendly approach, there is an increased attention to devise and adopt suitable methods to utilize wastes as value-added products to reduce the problem of environmental pollution. Fruits and vegetable processing in India generate substantial quantities of waste. About 50% of the total fruit weigh corresponds to the peel, which is an important source of bioactive compounds such as phenolics, ellagitannins, flavonoids, etc. (Sreekumar et al., 2014 ; Ismail et al., 2012). These in turn have wide range of actions which includes anti-oxidants, antibacterial, antimutagenic, cardioprotective, antiviral and antifungal activities (Singh and Immanuel, 2014 ; Bhandari, 2012). Use of waste as a source of polyphenols and antioxidants may have considerable economic benefit to food processors, being cheap, efficient and environmentally sound.

It was reported that pomegranate peel contains antioxidant phenolic compounds like punicalagin, punicalin, ellagic acid, gallic acid, etc (Bharani and Namasivayam, 2016 ; Ibrahium, 2010 ; Murthy et al., 2002 ; Subashini, 2016). These compounds of importance can be extracted from the peels using organic solvents like ethanol which degrades cell wall, disrupts the cytoplasmic membrane, damage membrane proteins and interfere with membrane-integrated enzymes of cells and thus aids in food preservation due to its some preservative efficiency (Subashini, 2016 ; Tiwari et al., 2011).

Keeping in view the characteristics of pomegranate peel extract (PPE) experiments were planned and conducted to establish its use as a

natural food preservative. Home-made butter was selected because like all other dairy products, it is very perishable and prone to rancidity. The bio-active compounds in PPE are detrimental to psychrophilic microbes like *Pseudomonas flourescens* and coliforms like *E. coli* which cause deterioration in home-made butter (Kanatt et al., 2010). Synthetic antioxidants like BHT and BHA used to combat oxidative changes has decreased due to their suspected action as promoters of carcinogens, as well as for consumer rejection of synthetic food additives. Thus, the two basic reasons responsible for the spoilage home-made butter, microbial growth and lipid oxidation, made it ideal for incorporation of PPE in it.

2. Materials and methods

2.1. Materials

Pomegranate fruits were purchased from the fruit market of Sipri, Jhansi and the cream was extracted from Amul gold milk (milk fat-6% and SNF-9%) purchased from local market of Jhansi. Buttermilk from previous batch was used as a starter culture. Chemicals and apparatus were made available in food chemistry laboratory of Institute of food science and technology, Bundelkhand University, Jhansi.

2.2. Methods

2.2.1. Preparation of pomegranate peel extract (PPE)

Fruits were washed properly using distilled water, peeled and their edible portions were carefully separated. The fruit peels were dried in hot oven at 40°C for 48 hours, ground into a fine powder and passed through a 24-mesh sieve. 100g powdered sample was extracted with 800ml ethanol at room temperature for 24 hours in water bath shaker (Shan et al., 2009). The mixture was filtered through a Whatman filter paper No. 2 for removal of peel particles. The filtrate obtained as PPE having concentration of 125mg/g was stored in refrigerator at 4°C for further use (Murthy et al., 2002).

2.2.2. Preparation of home-made butter

Homemade butter samples were prepared by following the standard method using ripened cream (Deosarkar and Khedkar, 2016). Cream was collected in a clean and sanitized container and stored in refrigerated conditions at 4°C till use. It was then inoculated with buttermilk obtained from previously made butter and allowed to ripen overnight. The ripened cream was churned for the separation of butter from buttermilk at 9-11°C using chilled water. During churning the butter was emerged and was separated from butter milk using sieve. The butter granules were washed using chilled water (18-20°C) to remove adhering buttermilk. The butter granules were pressed by hand to convert into ball shape mass (De, 2013).

2.2.3. Sampling

The prepared PPE was added by thorough mixing, in each 50g sample of home-made butter taken with varying concentration. In this manner four samples were prepared (i) SAMPLE A-1.25mg/g, (ii) SAMPLE B-2.5mg/g, (iii) SAMPLE C-3.75mg/g and (iv) CONTROL (R)-sample with no PPE added. Sample R was kept with the PPE incorporated samples to examine its effect on butter.

All samples were stored in refrigerated conditions until the tests were conducted.

2.2.4. Physicochemical analysis

All the tests were conducted in triplicates.

The physicochemical analysis was conducted using the standard AOAC methods to ensure that the product developed has constituent fractions in accordance to the FSSAI standards with moisture not more than 16% and milk fat not less than 80%. pH was calculated as per the instructions mentioned in the testing kit of the pH meter.

2.2.5. Microbial analysis

The microbial tests were conducted for the enumerating the total bacterial count, yeast and mould, and coliforms using pour plate technique on the 0th day, 3rd day, 6th day, 9th day, 12th day, and15th day (Buch et al., 2014).

Standard Plate Count (SPC) method was used with Plate Count agar of CBH® JO 0479 to determine the population of viable bacteria in the milk product (butter) as per the recommended methods for testing dairy products in Microbiological Spoilage Of Dairy Products. The petri-plates with plate count agar were incubated for 48 hours at 35-37°C (Ledenbach and Marshall, 2009 ; Nwogu et al., 2012).

Potato Dextrose Agar (PDA) of CDH® JO 0013 was used as a culture media to enumerate the yeast and mould colonies of butter samples stored in a refrigerator (Ledenbach and Marshall, 2009). It was reported that butter is a good substrate for the growth of pathogenic fungus. Potato dextrose agar petri-plates were incubated for 120 hours in an incubator at 22-25°C (Ledenbach and Marshall, 2009 ; Nwogu et al., 2012).

E. coli count was determined using MacConkey agar using pour plate technique and incubated at 37°C for 24 hours (Ahmed et al., 2016).

The number of colonies present in the particular test sample were determined using the formula:

CFU/ml = CFU* dilution factor

Dilution factor =1/ diluent (Aneja, 2018)

2.2.6. Sensory evaluation

It was carried out on the basis of 9-point hedonic scale rating by fifteen partially trained panellist using score cards. The sensory evaluation was carried on as per the schedule of Analysis evaluation of storage study, that is, 0^{th} day, 3^{rd} day, 6^{th} day, 9^{th} day, 12^{th} day and 15^{th} day. The samples were coded in three figures including control sample.

2.2.7. Statistical analysis

All experiments were conducted in triplicates and the calculated mean was recorded for statistical data analysis through 'Analysis Of Variance- Two Way Classification' at 5% level of significance. MS Excel (Windows 10) was used for this analysis.

3. Results and discussion

3.1. Chemical composition

Proximate analysis conducted on the home-made butter in control sample R ensured the FSSAI standard. The fat and moisture content were 83% and 16% respectively which is in accordance to the FSSAI standards. Ash content was 1% and protein content was negligible (non-significant).

3.2. Microbial analysis

3.2.1. Effect of PPE on SPC

The graphical representation Figure 3.1 showed the effect of PPE on SPC of home-made butter. The control sample denoted as R represent a linear and continuous increase in CFU/ml. Lactic acid bacteria dominated made the sample highly unacceptable on the 9^{th} day onwards. In sample A (1.25mg/g), B (2.5mg/g) and C (3.75mg/g) the microbial growth showed a linear pattern but the number of microbial counts observed were less as compared to the sample (R). The graph also indicates the increased effect of concentrations of PPE on microbial growth i.e. as the concentration of PPE increases from sample A (1.25mg/g) to sample B (2.5mg/g) and sample C (3.75mg/g) the number of colonies appear to decrease from 3^{rd} day to 6^{th} day and from 6^{th} day to 12^{th} day. Sample C (3.75mg/g) showed the minimum colonies of standard plate count and hence the reduced sourness due to the inhibitory effect of PPE on lactic acid bacteria (Nikfallah et al., 2014).

3.2.2. Yeast and Mould Result

Figure 3.2 shows the effect of pomegranate peel extract (PPE) on yeast and mould in home-made butter samples during 15 days storage period at 4°C. The four lines in the figure 3.2, representing control sample R, sample A (1.25mg/g), sample B (2.5 mg/g) and sample C (3.75mg/g) showed an elevation from 0-100 colonies on the 0^{th} day of storage period. Few fungal colonies were reported on 0^{th} day samples without any spore formation. As the storage time increases from 3^{rd} to 15^{th} day the lines representing sample B (2.5 mg/g) and sample C (3.75mg/g) showed a very gradual increase from log 1.7781 to 2.3802 cfu/ml and 1.4771 to 2.2552

respectively, on the 15th day of storage. On the contrary, the lines representing control (R) and sample A (1.25mg/g) showed a sharp rise in the colonies to log 2.7242 cfu/ml and log 2.6020 cfu/ml respectively on the 15th day of storage.

Like SPC, yeast and mould colonies also indicate retarded growth due to effect of PPE but the ratio of decrease in colonies as compared to SPC was lower.

Table 3.1.SPC in cfu/ml.

DAYS ↓	**Microbial count (SPC) in cfu/ml.			
	CONTROL R (0 mg/g)	SAMPLE A (1.25 mg/g)	SAMPLE B (2.5 mg/g)	SAMPLE C (3.75 mg/g)
0th	2.3010	2.1461	1.8450	1.6020
3rd	2.4149	2.2552	1.9542	1.8450
6th	2.6020	2.5185	2.2041	2
9th	2.7403	2.6127	2.3010	2.079
12th	2.7853	2.6270	2.4149	2.2304
15th	2.8920	2.7160	2.4913	2.2787

*P<0.05 (Significant). **Above values are in log base 10.

Table 3.2.- Yeast & mould in cfu/ml.

DAYS ↓	**Microbial count (Yeast & Mold) in cfu/ml.			
	CONTROL R (0 mg/g)	SAMPLE A (1.25 mg/g)	SAMPLE B (2.5 mg/g)	SAMPLE C (3.75 mg/g)
0th	1.9030	1.6989	1.4771	1
3rd	2.0791	1.9030	1.7781	1.4771
6th	2.3222	2.2041	2.9542	1.7781
9th	2.5185	2.2787	2.0791	1.9542
12th	2.6127	2.5185	2.2552	2.1760
15th	2.7242	2.6020	2.3802	2.2552

*P<0.05 (Significant). **Above values are in log base 10.

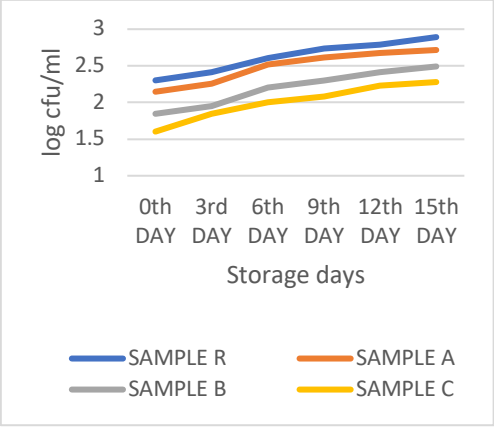

Figure 3.1.Effect of PPE on SPC

E. coli count remained absent in all the 4 samples, i.e., CONTROL and extract incorporated samples A (1.25mg/g), B (2.5mg/g) and C (3.75mg/g), on all days of test. The results, of investigation done to prove efficiency of pomegranate peel extract (PPE) to supress the growth of microbial colonies supported the work of Nikfallah et al., 2014, and Braga et al., 2004, who also stated in their work that PPE had antimicrobial activity against microorganisms like S. aureus, Klebsiella, L. acidophilus, L. subtilis and E. coli. The reduction in the number of colonies was due to the presence of polyphenols in PPE having antibacterial and antioxidant properties, caused the reduction in the number of microbial colonies (Bhandari, 2012 ; Bharani and Namasivayam, 2016 ; Subashini, 2016 ; Bopitiya and Madhujith , 2014).

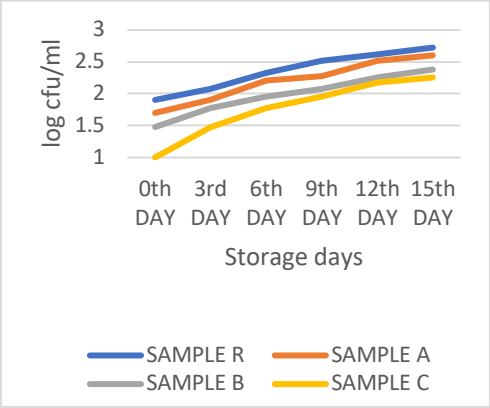

Figure 3.2.Effect of PPE on Yeast & Mould

3.3. Statistical analysis

These interprets that in both the cases there is a significant effect ($p < 0.05$) of varying concentration, i.e., 1.25mg/g, 2.5mg/g and 3.75mg/g of PPE incorporation in home-made butter samples upon microbial count and the days of storage.

Table 3.3. Statistical analysis of microbial enumeration

Analysis Of Variance (ANOVA) – 2 way without replication			
Microbial test	P-value	F value	F_{cri} value
SPC	$P_1<0.05$	12.7447	2.9091
	$P_2<0.05$	27.9878	3.2878
Yeast & Mold	$P_1<0.05$	19.1358	2.9012
	$P_2<0.05$	16.5667	3.2873

*5% level of significance

3.4. Sensory analysis

Figure 3.3 represents the overall acceptability (O.A.A.) of control sample (R), sample A (1.25mg/g), sample B (2.5 mg/g) and sample C (3.75mg/g), obtained by calculating the means of scores given by panellists upon various food attributes. Pomegranate peel extract (PPE) was found to be the most acceptable among extracts of other fruit peels (Murthy et al., 2002).

On the 0^{th} day of storage control sample (R) was found to be most acceptable.

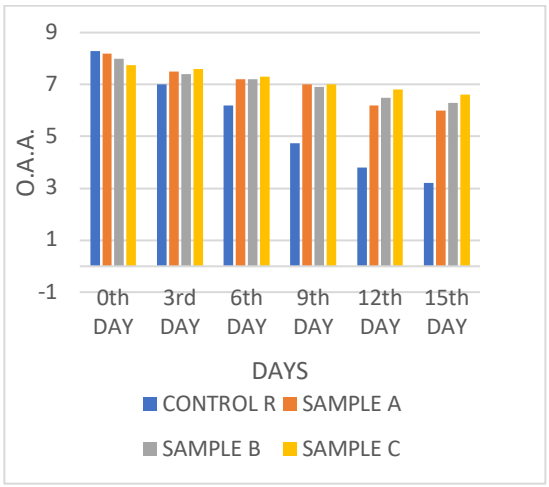

Figure 3.3.Sensory Analysis

The variation in the acceptability of the samples was attributed to the antioxidant activity of pomegranate peel extract (PPE) which supress lipid oxidation, thereby the extract incorporated samples do not turn sour and rancid. The antioxidant activity of PPE was capable of supressing oxidation in fats and oils (Bopitiya and Madhujith, 2014). The results also favour the work of Gandhi et al., 2013, which showed the capability of phenolics extracted from plant parts (using organic solvents like ethanol) in supressing oxidation in similar dairy products like ghee which is clarified butter fat.

4. Conclusions

The present work was conducted to establish the hypothesis that pomegranate peel extract (PPE) can be used as a preservative in home-made butter, to replace synthetic ones in the market. Results gathered after conducting the various tests demonstrated that the pomegranate peel extract (PPE) was capable of extending the shelf life of home-made butter by supressing the microbial growth. PPE incorporated samples had a pleasant taste which does not mask the sensory quality of home-made butter, thereby increasing its consumer acceptance.

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MINERALS ASSESSMENT IN WATER, SEDIMENT, AND FISH TISSUES OBTAINED FROM EARTHEN POND OF EKITI STATE UNIVERSITY, NIGERIA

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ABSTRACT

This study assesses the mineral levels in water, sediment, and fishes from Ekiti State University pond, Nigeria; and investigates the metal levels in locally made and imported feeds. Four fish samples (tilapia I and II; catfish I and II) from the pond were dissected to obtain livers, gills, and tissues. The water, sediments, feeds, and fish parts were analyzed using a Flame Photometer (Corning 400) and an Atomic Absorption Spectrophotometer (Buck Scientific 210). The levels of Na, K, Ca, Fe, Cu, Zn, and Mn in the pond water are 10.00, 8.89, 3.02, 6.67, 0.03, 0.25, and 0.18 mg/L, respectively. The concentrations of Na, K, Ca, Fe, Cu, Zn, and Mn in the sediment are 2.52, 4.21, 2.72, 29.78, 0.11, 0.95, and 0.42 mg/kg, respectively. The levels of metals in the feed samples (locally made and imported) range as follows: Na (47.56 – 76.06); K (181.06 – 190.05); Ca (95.43 – 244.53); Fe (2.38 – 4.16); Cu (0.17 each); Zn (1.03 – 1.31); Mn (0.59 – 0.83 mg/kg). The concentrations of metals in the liver, gill, and tissue range as follows: Na (4.90 – 54.56); K (5.02 – 69.07); Ca (21.42 – 522.57); Fe (0.72 – 9.54); Cu (0.06 – 0.44); Zn (0.24 – 1.17); Mn (0.02 – 0.52 mg/kg). However, Cd, Pb, Cr, Ni, and Co were not detected in all the samples. The results showed that the mineral levels in the fishes are within the permissible limits of the World Health Organization (WHO) and Standard Organization of Nigeria (SON).

1. Introduction

Heavy metals are toxic elements as a result of their high molecular weigths (Adewumi *et al.*, 2015). Heavy metals which are essential to living organisms only occur in trace amounts, their deficiencies and excesses in living system cannot be overemphasized (Szyczewski *et al.*, 2009). However, there is a close margin between the beneficial and toxic concentrations of some of the essential heavy metals (Tchounwou, 2008). Trace elements could be poisonous if higher concentrations are ingested into the body over a period of time. Heavy metals, such as Hg, Cd and Pb are very stable and not easily metabolized, they therefore bio-accumulate to attain toxic threshold. Most heavy metals do not have any nutritional role to perform in the body system, the more reason for their toxicity. However, essential metals like Fe, Zn, etc. can become toxic if their concentrations fall outside the permissible limits of international standards (Nabrzyski, 2006). Some metalloids such as arsenic are also in this category as they pose toxicity even at low level of exposure (Duffus, 2002).

Fishes totally depend on water for feeding, growth, salinity balance, reproduction, and metabolic activities (Bronmark and Hansson, 2005). Fish activities in aquatic system are

controlled by physicochemical parameters such as colour, odour, temperature, total dissolved solid (TDS), pH, temperature, electrical conductivity (EC), acidity, alkalinity, water hardness, etc. each parameter is governed by international standard (James, 2000). A fish pond could be a controlled pool, hand-dug lake, or tank (Burnett, 2008). Fish pond is not a flowing stream but a stagnant pool consisting of sand, decayed materials, and microorganisms. Vital nutrients get into the pond from water source, runoff, or feed supplements. Decomposition of animal and plant materials in the pond facilitates a balanced ecosystem. However, there could be metal toxicity in the pond if the concentration of metallic elements ingested by aquatic lives is higher than the efflux as a result of imbalanced metabolisms (Luoma and Rainbow, 2005).

Sediment is a major reservoir of metals, it has about 99% of the total metal contents that are present in the aquatic system (Demirak *et al.,* 2006; Aderinola *et al.,* 2009; Oztrurk *et al.,* 2009). Rashed (2001) reported that sediment attracts diverse pollutants such as pesticides and heavy metals, it also plays a major role in re-mobilizing contaminants in the aquatic systems under suitable conditions.

Pollutants, such as heavy metals are readily available in sediment and easily dispersed through ion exchange, absorption, and precipitation processes (Yuan *et al.,* 2004). Pollution in the environment has been a major concern in recent times (Zhang *et al.,* 2007). Heavy metals migrate into water bodies from different sources which could be natural or anthropogenic. They include discharged effluents from industries, domestic wastewater, application of pesticide and inorganic fertilizers on farmland, leaching from landfills, shipping, the use of fossil fuels, as well as weathering (Yilmaz, 2003; Marcovecchio, 2004; Nadafi and Saeed, 2006; Raja *et al.,* 2009; Yilmaz, 2009; Kamaruzzaman *et al.,* 2010). Heavy metals at varied concentrations are stored in different parts of fish in the aquatic systems (Dural *et al.,* 2006; Yilmaz *et al.,* 2007). Many parameters (biotic and abiotic) affect metal uptake and bioaccumulation in fish tissues: feeding system, age of fish, fish gender, body weight, pH, temperature, dissolved oxygen, as well as physiological conditions are few examples of such biotic and abiotic conditions (Fernandes *et al.,* 2007; Kamaruzzaman *et al.,* 2010). Fishes are important bio-indicators that are used to establish toxicity of heavy metals, vital information about the levels of heavy metals and their distribution in fish are necessary as they have direct effect on nature as a whole (Malik *et al.,* 2010).

This study is aimed at determining the physicochemical parameters of pond, and assessing the levels of some minerals in pond water and sediment, feed samples, and selected parts of African catfish (*Claria gariepinus*) and Tilapia (*Oreochromis niloticus*) that are being raised in Ekiti State University Farm, Ado-Ekiti, Nigeria.

2. Materials and methods

2.1. Description of the study area

Ekiti State University, Ado - Ekiti is located along Ado-Ifaki road, Ekiti State, Nigeria. Its geographical co-ordinates are 7.714103°N and 5.260058°E. It has an estimated population of 25000 students. The fish farm of Ekiti State University is of earthen type, located at the Faculty of Agricultural Sciences of the University. The geographical co-ordinates of the pond are 7.113200 °N; 5.2463760 °E.

2.2. Collection of samples

Water samples were collected into sample bottles and properly covered using stoppers before analysis. Samples that were meant for metal analysis were nitrified using 5 mL HNO_3. Sediment samples were collected from different points of the pond using a grab and then homogenised. Two pieces of catfish (I and II) and two pieces of tilapia (I and II) were collected from the pond. Two feed samples (locally made and imported) were collected from the farm and prepared for further analysis.

2.3. Analysis of pond water for physicochemical parameters

Water sample was analyzed for various physicochemical parameters such as pH, Total Solids (TS), Total Dissolved Solids (TDS), Total Suspended Solids (TSS), Electrical Conductivity (EC), Free CO_2, Total Hardness, Calcium Hardness, Alkalinity, Acidity, Chlorides, etc. according to standard analytical procedures as described by AOAC (2005). For Mineral analysis, 100 mL water sample, nitrified at the point of collection was transferred into a beaker, 2 mL of concentrated HNO_3 and 5 mL of concentrated HCl were added, and the sample was heated inside a fume cupboard until the volume was reduced to 15-20 mL. The sample was allowed to cool, and thereafter filtered into 100 mL volumetric flask, the filtrate was made up to the mark using distilled water and subsequently analyzed using Flame Photometer (Corning 400) and Atomic Absorption Spectrophotometer (Buck Scientific 210).

Fig. 1. Earthen fish pond of Ekiti State University farm

2.4. Analysis of sediment sample

Sediment sample was oven-dried at 105^oC, 2 g was weighed into a crucible followed by the addition of 10 mL of concentrated HNO_3. The mixture was heated up until near dryness, this process was repeated two more times. Distilled water was added to the residual material and the suspension was filtered into 100 mL standard volumetric flask using filter paper (Merck, 0.45 μm). The filtrate was made up to the mark using distilled water.

2.5. Analysis of fish sample

Fish sample was dissected into liver, gill, and muscle. The organs were analyzed according to the method described by AOAC (1990). For the liver, gill and muscle: 1 g of each was transferred into 250 mL beaker followed by the addition of concentrated HNO_3 (10 mL). The mixture was heated for 45 min and cooled, 5 mL of perchloric acid ($HClO_4$) was added to the mixture and heated until white fume was observed. 20 mL of distilled water was added, the mixture was further heated, cooled, and filtered into 100 mL volumetric flask according to Hseu (2004). The filtrate was then made up to the mark using distilled water and analyzed using AAS.

2.6. Analysis of feed sample

Feed sample (2 g) was weighed into a crucible and 20 mL of concentrated HNO_3 added, followed by 4 mL of $HClO_4$. The content was digested in a fume cupboard at 45^oC for 15 min. Immediately white fume was observed, the sample was cooled, filtered into 100 mL standard flask, made up to the mark with

distilled water, and subsequently analyzed using AAS.

3. Results and discussion

3.1. Physicochemical parameters of pond water sample

The physicochemical parameters of the earthen pond are presented in Table 1 and compared with the water quality standard of Standard Organization of Nigeria (SON, 2007) and World Health Organization (WHO, 2009). The colour of the pond water sample appeared brown. The pH value of the water was 6.51 which was within the standard permissible limits spelt out by WHO (2009) and SON (2007). This was also in line with the report of Boyd (1998) which inferred that the optimum pH for improved production of fish is 6.5 - 9.0. Values of pH outside the standard permissible limits could however cause destabilization of other physicochemical properties such as acidity, alkalinity, hardness, metal solubility, etc. The temperature of the water at the point of collection was 30.0°C. The temperature value fell within the values stipulated by WHO (2009). Ntengwe and Edema (2008) reported that optimum temperature range of 20 - 30°C is good for improved fish production. However, pond temperature depends on the intensity of sunlight or present weather condition as at a particular point in time. Conductivity is often used as an indicator of pollution status in pond water, this could be born out of persistent debris, feed formulation, run-off into ponds, metabolic wastes from fishes, as well as the activities of other living organisms in the pond (Solomon *et al.,* 2013). The value of electrical conductivity of the pond was 112.2 µS/cm, this value was within the maximum permissible limits stipulated by WHO (2009) and SON (2007). Also, WHO/FAO/IAEA acceptable limit for conductivity in aquaculture as reported by DWAF (1996) is 20 - 1500 µS/cm. The earthen pond under investigation is therefore suitable for fish production as far as electrical conductivity is concerned. The value of alkalinity of the sample was 131 mg/L which was in consonance with the permissible limits (<600 mg/L) stated by WHO (2009). Ehiagbonare and Ogunrinde (2010) reported alkalinity values of between 35 mg/L and 135 mg/L for fish pond water in Okada, Nigeria. Njoku *et al.* (2015) on the other hand, reported alkalinity values of 18 - 24 mg/L in earthen ponds within the Niger-Delta region of Nigeria; they suggested the optimum alkalinity for increased fish production to be 20 – 300 mg/L. James (2000), in his submission reported a suitable range of alkalinity for aquaculture to be 50 - 300 mg/L. Total hardness of water is a contribution of dissolved minerals, such as Ca and Mg compounds. Hardness of water determines the suitability of water for either domestic or industrial use as a result of the presence of bi-carbonates, sulphates, nitrates and chlorides (Solomon *et al.,* 2013). The total hardness value of the water samples was 360 mg/L. Sufficient level of hardness can bring down the level of ammonia and pH toxicity in water. Calcium hardness of the water sample was 92 mg/L and was within the limits set by WHO. The chloride content of the pond water was 123.2 mg/L and within the maximum permissible limits set by WHO (2009) and SON (2007). Although, chloride can be found in natural water, but high level of chloride is a pointer to pollution from either industrial or domestic wastes, seepage of saline water into fresh water system (Shyamala *et al.,* 2008). The values of total solids (TS), total dissolved solids (TDS), and total suspended solids (TSS) for the pond water were 4400, 3200, and 1200 mg/L, respectively. In production of fish, James (2000) opined that a maximum TDS value of 400 mg/L is adequate for the production of various species of fish. The pond water had high TS value which could be as a result of heavy downpour a day before sampling. Boyd (1998) suggested that, for total suspended solids, a range of 10 – 50 mg/L is good for optimum fish culture, although he also noted that the values could be higher in highly turbid fish ponds. Also, dissolved feeds that were not picked up by the fish could also contribute to high values of total suspended

solids in pond, which will invariably increase the level of turbidity.

Correlation analysis of the physicochemical parameters for the pond water was carried out using a statistical package (IBM SPSS V21) and the results are presented in Table 2.

A strong positive correlation was found between EC and TH ($r = 0.999$ at $p > 0.05$). This shows that calcium or magnesium salts are present as dissolved solids (Agarwal *et al.*, 2014), it also shows that increase in one of the parameters would lead to a decrease in the other. High positive correlations were observed between TS and TDS ($r = 1.000$ at $p > 0.01$); and TS and free CO_2 ($r = 0.999$ at $p > 0.05$), this implies that increase or decrease in the value of TS would lead to opposite effect in the other two parameters. TDS was also found to have high positive correlation with free CO_2 ($r = 1.000$ at $p > 0.01$). A positive correlation was observed between TH and MH ($r = 0.998$ at $p > 0.05$), this is because, in most cases, hardness is caused as a result of dissolution of only calcium and magnesium salts present in water body. Strong negative correlation values were observed between alkalinity and TH; and alkalinity and MH ($r = -0.999$ at $p > 0.05$; and $r = -1.000$ at $p > 0.01$), the negative correlation could be as a result of anthropogenic influence. Strong correlation between MH and Alkalinity showed that magnesium probably exists in the sample as magnesium carbonates and bicarbonate (Agarwal *et al.*, 2014).

Table 1. Physicochemical parameters of water sample from Ekiti State University farm earthen pond

Parameters	Pond water	WHO (2009)	SON (2007)
Colour	Brownish	-	-
Temperature (°C)	30	28-35	ambient
pH	6.51	6.5-8.5	6.5-8.5
Electrical conductivity (µS/cm)	112.2	300	-
TS (mg/L)	4400	-	-
TDS (mg/L)	3200	500	-
TSS (mg/L)	1200	-	10-50
Free CO_2 (mg/L)	15.53	-	-
Alkalinity (mg/L)	131	600	-
Acidity (mg/L)	126	-	-
Total hardness (mg/L)	360	600	-
Ca hardness (mg/L)	92	75-200	-
Mg hardness(mg/L)	268	-	-
Chlorides (mg/L)	123.2	200 -1000	200-600

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Table 2. Correlation coefficient for the physicochemical parameters of the pond water

	Temp (ºC)	**pH**	**EC (µS/cm)**	**TS (mg/L)**	**TDS (mg/L)**	**TSS (mg/L)**	**Free CO_2 (mg/L)**	**Alkalinity (mg/L)**	**Acidity (mg/L)**	**TH (mg/L)**	**CH (mg/L)**	**MH (mg/L)**	**Chloride (mg/L)**
Temp (ºC)	1.000												
pH	-.419	1.000											
EC (µS/cm	-.916	.021	1.000										
TS (mg/L)	-.990	.288	.964	1.000									
TDS (mg/L)	-.994	.317	.955	**1.000****	1.000								
TSS (mg/L)	-.971	.189	.986	.995	**1.000****	1.000							
Free CO_2 (mg/L)	-.995	.331	.950	**.999***	.995	.989	1.000						
Alkalinity (mg/L)	.875	.073	-.996	-.934	**.999***	-.966	-.917	1.000					
Acidity (mg/L)	.617	-.973	-.250	-.500	-.934	-.410	-.539	.159	1.000				
TH (mg/L)	-.900	-.019	**.999***	.952	-.500	.978	.937	**-.999***	-.211	1.000			
CH (mg/L)	.277	.756	-.639	-.410	.952	-.500	-.367	.708	-.585	-.669	1.000		
MH (mg/L)	-.874	-.075	.995	.933	-.410	.965	.916	**-1.000****	-.156	**.998***	-.710	1.000	
Chloride (mg/L)	.719	-.932	-.381	-.615	.933	-.532	-.650	.293	.990	-.344	-.468	-.291	1.000

*- Correlation is significant at 0.05 level; ****-Correlation is significant at 0.01 level. EC- Electrical Conductivity; TS- Total Solids; TDS- Total dissolved solids; TSS- Total Suspended Solids; TH- Total Hardness; CH- Calcium Hardness; MH- Magnesium Hardnes

3.2. Concentrations of minerals in pond water, sediments, and feed samples

The concentrations of minerals in pond water, sediments, and feeds (locally made and imported) are shown in Table 3. Minerals are essential supplements in human diet as they aid body processes such as rebuilding of tissues and maintaining ion gradients. The concentrations of potassium (K) in pond water, sediment, locally made feed, and imported feed were 8.89 mg/L, 4.21, 181.06, and 190.05 mg/kg, respectively. Mutlu and Uncumusaoğlum (2017) reported that one of the inorganic salts that contribute taste to water is K_2SO_4, and that K occurs in water mainly as K_2SO_4. The concentrations of sodium (Na) in water and sediment samples were 10.00 mg/L and 2.52 mg/kg, respectively. It was however observed that the concentration of Na in the water sample was within the standard limits stated by SON (2007) and WHO (2009). The concentration of Mn in the pond water (0.18 mg/L) was within the confines of permissible limits set by SON (2007). Mn in large concentrations can cause series of psychiatric disturbances, termed "Manganism" which have affected people working in production and processing of alloys derived from manganese (Nussey *et al.*, 2000). The concentrations of cadmium (Cd), lead (Pb), chromium (Cr), nickel (Ni), and cobalt (Co) in the pond water were below detection limits (BDL).

Table 3. Concentrations of minerals in pond water, sediment, and feed samples

Sample	Na	K	Ca	Fe	Cu	Zn	Mn	Cd	Pb	Cr	Ni	Co
Water (mg/L)	10.00	8.89	3.02	6.67	0.03	0.25	0.18	BDL	BDL	BDL	BDL	BDL
Sediment (mg/kg)	2.52	4.21	2.72	29.78	0.11	0.95	0.42	BDL	BDL	BDL	BDL	BDL
Locally made feed (mg/kg)	47.56	181.06	95.43	2.38	0.17	1.03	0.59	BDL	BDL	BDL	BDL	BDL
Imported feed (mg/kg)	76.02	190.05	244.53	4.16	0.17	1.31	0.83	BDL	BDL	BDL	BDL	BDL

BDL: below detection limit

The locally made and imported feeds were rich in sodium (Na) with values of 47.56 and 76.02 mg/kg, respectively. The concentrations of iron (Fe) in the locally made and imported feed samples were 2.38 and 4.16 mg/kg, respectively. The Fe content in the feeds is to improve the iron level of the fishes in order to meet up with the minimum iron requirement in fish that will benefit the consumers. However, the amount of minerals, most especially iron in a feed depends on the formulation of the feed. The concentrations of K, Ca, Cu, Zn, and Mn for locally made and foreign feeds were 181.06 and 190.05 mg/kg, 95.43 and 244.53 mg/kg, 0.17 mg/kg each, 103 and 1.31 mg/kg, and 0.59 and 0.83 mg/kg, respectively. The concentrations are within the permissible limits of WHO (2009) and SON (2007) standards.

3.3. Concentration of minerals in the fish samples

The concentrations of minerals in the various parts of tilapia and catfish samples are presented in Table 4. The concentrations of potassium (K) in the gill samples of tilapia I and tilapia II were 50.02 and 41.02 mg/kg; in the liver, 5.02 and 7.23 mg/kg; and in the muscle, 66.23 and 44.56 mg/kg. The potassium contents in the gills, liver and muscle of catfish I and catfish II were: 48.59 and 45.89; 9.10 and 9.56; and 73.47 and 69.07 mg/kg, respectively. The results showed that the highest concentration of K was observed in the muscle, and the lowest

concentration was observed in the liver. The highest concentration found in the muscle could be attributed to the fact that K is needed to: improve fish protein; build muscle; maintain normal body growth; control the electrical conductivity; and maintain the acid-base balance. The concentrations of manganese in the fish parts ranged from 0.02 – 0.51 mg/kg, with the gill having the highest concentration. The highest concentration in the gill could be attributed to the fact that, the gills, being the dominant site for contaminants and pollutants uptake due to the anatomical properties that maximize absorption efficiency of minerals from water. Cr, Cd, Pb, Ni, and Co were not detectable in any of the fish parts. Zinc (Zn) was detected in muscle, liver, and gills. The highest concentration (1.23 mg/kg) of Zn was observed in the gills of catfish I, while the minimum concentration (0.24 mg/kg) was observed in the liver of catfish II. In the case of copper (Cu), the results showed that the highest concentration (0.44 mg/kg) was observed in the liver of Tilapia I, while the minimum concentration (0.06 mg/kg) was observed in the gills of catfish II. The concentrations of Zn and Cu were however lower than those reported for a number of species of fishes such as *Tilapia nilotica* (Rashed, 2001); *Clarias gariepinus* and *Labeo umbratus* (Coetzee *et al.,* 2002); *Cyprinus carpio* (Zhang *et al.,* 2007); *Ctenopharyngodon idella* and *Labeo rohita* (Malik *et al.,* 2010). However, the results are in agreement with the reports of some authors whose observations demonstrated that heavy metal bioaccumulation in various tissues of fishes living in the same water body vary according to the species of fish (Canli and Atli, 2003; Mendil and Uluozlu, 2007; Uysal *et al.,* 2008). The concentrations of iron (Fe) in the body parts of tilapia I and tilapia II ranged from 1.07 – 9.54 mg/kg, where the gill (Tilapia II) had the highest concentration, and the minimum concentration (1.07 mg/kg) was observed in the muscle of Tilapia I. The trend of Fe concentration in the two tilapia fishes is as follows: tissue < liver < gills. The concentration of Fe in the body parts of catfish I and II ranged from 0.72 – 4.13 mg/kg. The highest concentration was observed in the gill of Catfish I, while the lowest concentration was observed in the muscle of catfish II. The trend of iron concentration in the two catfish samples is as follows: tissue < liver <gills. Similar reports were submitted on *Claria gariepinus* (Osman and Kloas, 2009) and *Oreochromis niloticus* (Saheed and Shaker, 2008). The levels of Fe in the fish samples are within the limits specified by FAO/WHO (1989). Among all the parts investigated for both catfish and tilapia samples, heavy metals tend to accumulate most in the gills, followed by livers and muscle, this is because fish gills are involved in gas exchange, regulation of ions, acid balance, and waste egestion (Shukla *et al.,* 2007). Fish gill is the major area used for the ingestion of dissolved heavy metals which could lead to lesion and damage of gills (Bols *et al.,* 2001).

Table 4. Concentrations of minerals in fish samples

Sample		Na (mg/kg)	K (mg/kg)	Ca (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)	Cd (mg/kg)	Pb (mg/kg)	Cr (mg/kg)	Ni (mg/kg)	Co (mg/kg)
Tilapia I	**Liver**	7.52	5.02	21.42	3.99	0.44	0.46	0.20	BDL	BDL	BDL	BDL	BDL
	Gill	50.93	50.02	428.53	8.57	0.37	1.17	0.35	BDL	BDL	BDL	BDL	BDL
	Tissue	46.25	66.23	200.51	1.07	0.10	0.73	0.14	BDL	BDL	BDL	BDL	BDL
Tilapia II	**Liver**	10.01	7.23	186.15	2.49	0.09	0.38	0.15	BDL	BDL	BDL	BDL	BDL
	Gill	54.56	41.02	522.57	9.54	0.16	1.33	0.51	BDL	BDL	BDL	BDL	BDL
	Tissue	44.58	44.56	281.53	1.08	0.22	0.81	0.17	BDL	BDL	BDL	BDL	BDL
Catfish I	**Liver**	14.32	9.10	44.56	3.37	0.07	0.57	0.03	BDL	BDL	BDL	BDL	BDL
	Gill	48.02	48.59	397.52	4.13	0.09	1.23	0.24	BDL	BDL	BDL	BDL	BDL
	Tissue	50.11	73.47	94.06	0.93	0.08	0.71	0.04	BDL	BDL	BDL	BDL	BDL
Catfish II	**Liver**	4.90	9.56	27.78	1.92	0.12	0.24	0.02	BDL	BDL	BDL	BDL	BDL
	Gill	32.16	45.89	360.58	2.35	0.06	0.91	0.45	BDL	BDL	BDL	BDL	BDL
	Tissue	35.24	69.07	59.87	0.72	0.07	0.55	0.07	BDL	BDL	BDL	BDL	BDL

3.4. Transfer Factor

Transfer factor is the ratio of concentration of one specific metal detected in the fish part to the concentration of the same metal in the water or sediment (Rashed, 2001). The transfer factors of fish parts with respect to the pond water and sediment were calculated as follows:

$$\text{Transfer factor } (TF) = \frac{M_{part}}{M_{water\ or\ sediment}} \quad (1)$$

Where M_{part} is concentration of metal in fish part; M_{water} or $M_{sediment}$ is the concentration of metal in water or in sediment. Tables 5 and 6 show the transfer factors between fish parts, pond water, and sediment using potassium (K) as a yardstick.

Table 5. Transfer factor of potassium (K) between fish parts and pond water

Fish body parts	M_{part}	M_{water}	Transfer factor
Tilapia I liver	5.02	8.89	0.56
Tilapia I gills	50.02	8.89	5.63
Tilapia I muscle	66.23	8.89	7.45
Tilapia II liver	7.23	8.89	0.81
Tilapia II gills	41.02	8.89	4.61
Tilapia II muscle	44.56	8.89	5.01
Catfish I liver	9.10	8.89	1.02
Catfish I gills	48.59	8.89	5.47
Catfish I muscle	73.47	8.89	8.26
Catfish II liver	9.56	8.89	1.06
Catfish II gills	45.89	8.89	5.16
Catfish II muscle	69.01	8.89	7.76

Table 6. Transfer factor of potassium (K) between fish parts and sediment sample

Fish body parts	M_{part}	$M_{sediment}$	Transfer factor
Tilapia I liver	5.02	4.21	1.19
Tilapia I gills	50.02	4.21	11.88
Tilapia I muscle	66.23	4.21	15.73
Tilapia II liver	7.23	4.21	1.72
Tilapia II gills	41.02	4.21	9.74
Tilapia II muscle	44.56	4.21	10.58
Catfish I liver	9.10	4.21	2.16
Catfish I gills	48.59	4.21	11.54
Catfish I muscle	73.47	4.21	15.45
Catfish II liver	9.56	4.21	1.80
Catfish II gills	45.89	4.21	10.90
Catfish II muscle	69.01	4.21	16.41

When transfer factor is less than 1, it means that bioaccumulation of metal (K) in the fish is not from the water or the sediment. When transfer factor is greater than 1, it means that bioaccumulation of metal in fish is from both the pond water and sediment. It can be observed from Table 5 that the transfer factors for Tilapia I and II in the liver were less than 1 which shows that bioaccumulation of K in the fish was not from the water or sediment. However, transfer factors for other Tilapia parts and all Catfish parts were greater than unity, which means that the minerals were transferred into the fish parts from either the pond water or the sediment.

4. Conclusions

This study showed that the feed samples contained the highest concentration of potassium since potassium is needed for building fish muscle and to maintain normal body growth, the more reason it is found in high concentration in the fish muscle. The levels of detectable (Na, K, Ca, Fe, Cu, Zn, and Mn) minerals were found to be within the confines of WHO and SON standards. However, Pb, Cd, Cr, Ni, and Co were not detectable in all the samples (fish, water, sediment, and feed) as the metal levels were below detection limit. In general, the fishes from the pond are free from contamination of heavy and toxic metals, hence are safe for human consumption.

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THE DEGREE OF RESIDUAL INVASION AFTER INFECTION WITH ANISAKIASIS FISH OF VARIOUS CULINARY PROCESSING

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ABSTRACT

The article presents the results of the study of the degree of residual invasion after infection with anisakiasis of herring *(Clupea harenqus* and *Alosa immaculate)* in various culinary processes and places of catch. During the experiment, the largest extent of the invasion was detected in marinated herring. The intensity of invasion with anisakis in marinated samples exceeded smoked products five times. A similar tendency of parasitic lesion was observed during autopsy. Most of the larvae were found in the abdominal cavity of pickled fish, and the least - in smoked fish. Smoked fish had the largest number of parasites in the wall of the abdominal cavity. In our opinion, the level of damage by fish parasites depends on a complex of external and internal factors. The type of culinary processing affects the intensity of the invasion. The intensity and localization of larvae of anisakis is significantly reduced after removal of the intestines from the fish.

1.Introduction

Fish and fish products with exceptionally high nutritional qualities are an important source of food are widely used in daily nutrition, diet and baby food. It is the source of the necessary protein, vitamins, trace elements and other substances necessary for the human body. According to international medical standards, a person must consume twenty kg of fish and fish products per year (Berezovskiy et al, 2013; Arcos, 2014).

In recent years, the culinary traditions of Japan, Korea, China and other countries of Southeast Asia have been widespread in Europe and in particular in Ukraine, where many dishes are cooked from raw or half-fat, crustaceans, squid and other mollusks. This represents a significant negligence in relation to human anthrop noses, in particular, invasive diseases, which fish carry as an additional or intermediate. Fish-invasive diseases, dangerous to humans, are found in marine and freshwater fish. The sea and the oceans produce on average more than two thirds of the total catch of fish (Gaponenko & Lysenko, 2009; Mikulich, 2013; Mok, 2015). In this case, sea fish is part of a trading network mainly in a chilled or frozen state.

In addition, there are different types of fish processing: marinades, cheeses, pickled, smoked and others. An increase in the supply of fish from abroad to the domestic market of Ukraine has increased the frequency of detection of helminthological pathogens previously diagnosed sporadically, in particular, opisthorchiasis, clonorhosis, metagoniosis, nanofeetosis, diophilobothiosis, coriandrosis, anisakidosis, and others (Berezovskiy et al, 2013; Arcos, 2014).

According to the literature (Gaevskaya, 2005; Mok, 2015), virtually all sea fish can be

infected with different types of worms, up to 30 species of which constitute a potential danger to humans or cause unwanted changes in fish, as in technological raw materials. Nematodes of the Anisakidae family are helminths dangerous to humans. Anisakis – pathogenic worms, the representatives of nematodes of the family *Anisakidae* (*Anisakis simplex, Pseudoterranova decipiens, Hysterothylacium aduncum, Contracaecum osculatum*). Localized in the body, on the surface or in the tissues of the internal organs, rarely in the muscles (often below the middle line of the body of fish), sea and passage Pacific fish (cod, mackerel, hake, flounder, knot, herring, pink salmon etc.) (Herrero et al, 2011; Buchmann & Mehrdana, 2016).

The aim of the study is to determine the degree of residual invasion in fish anisakiasis of various culinary processes With a sufficient and comprehensive study of the dynamics and degree of penetration of anisakis into popular types of fish products, it is possible to reduce the amount of low-quality products, break the life cycle of the parasite and reduce the level of infection with helminths population. Thus, our research will be able to participate in the improvement of the population and contribute to improving the sanitary safety of fish products.

2.Materials and Methods

To achieve this goal, the following tasks were set: to make a controlling selection of anisakiasis of fish products (freezing, salting, marinating, smoking Atlantic herring *(Clupea harenqus)* and Black Sea herring *(Alosa immaculata*) in the markets of Vinnitsa; carry out organoleptic and parasitological studies. The study was herring of freezing, salty, smoked, and the subject - the dynamics of lesion with anisakis.

25 samples were selected the 25 of fish products (freezing, salting, marinating, smoking Atlantic herring *(Clupea harenqus)* (the place of catch is the North Atlantic and the Baltic Sea) and Black Sea herring *(Alosa immaculate) (*the place of catch is the Black Sea). The selection was carried in the markets of Vinnitsa (Ukraine) out 2019 year by the work of the expansion network in accordance with the established rules and regulations.

In the course of research, such methods as organoleptic and parasitological are used. In the organoleptic assessment of fish, the appearance, the built in fish, the condition of the outer covers, mucus, eyes, gills (Berezovskiy et al, 2013; Gaevskaya, 2005).

The parasitological examination of fish reveals visible parasites, as well as parasites, muscles, under the skin or shines. Pay attention to the spots and inclusions, which differ in color or consistency from normal tissues, as well as in tumors, and form regions of meat of sparse consistency. To detect parasites in meat use a method of parallel cuts.

Detection of parasites or inclusion, like living parasites, is initially considered under magnifying glass or binocular. Then, if necessary, they are considered under the small and middle levels of the microscope. The vital activity of parasites is determined by the method of irritation.

The following parameters were determined to determine the dynamics of anisakis in different types of culinary processing: the number of damaged specimens, the severity of the invasion, the intensity of the invasion and the index of invasion. The number of specimens affected was determined by simple counting when the fish were opened and examined. Extensiveness of the invasion was determined by dividing the number of damaged specimens by the number of specimens (25 pieces) and multiplying by 100. The intensity of the invasion was determined by counting the parasites of one fish. The invasion index was determined by dividing the number of parasite larvae into the total number of parasites found in the sample (Bogatko et al, 2011).

The digital material was processed statistically. The resulting digital data was processed using the MS EXEL 98 and Windows program, statistically processed by Student. The results were considered statistically significant at $p < 0.1$, $p < 0.01$, $p < 0.001$. In the table material

of the work the following symbols are taken: * p <0.1, ** p <0.01, *** p <0.001.

3. Results and discussion

Experimentally it was established that fish of different culinary procedures had another infection with helmints (Table 1).

Table 1. Dynamics of anisakiasis of herrings for different types of culinary processing *(M ± m, n=25)*

Indicator	Kind of fish processing			
	freezing	salting	marinating	smoking
The number of specimens affected, thing	21.4 ± 0.16	20.9 ± 1.08**	23.7 ± 0.63	5.0 ± 0.09***
Extensiveness of the invasion, %	84.5 ± 3.25	83.6 ± 0.75	94.8 ± 2.87*	2.9 ± 0.13***
The intensity of the invasion, units	1-12	1-17	1-23	1-5
The invasion index, units	6.0 ± 0.34	8.3 ± 0.05***	10.3 ± 0.75***	1.8 ± 0.03***

From the digital material it is evident that the largest number of anisakis larvae was in pickled herring, and the smallest in smoked. The difference was 17.7 units (p <0.001). The experiment showed that the largest extent of anisakis invasion was marinated fish (the difference between frozen samples was 10.3% (p <0.1), and compared to smoked samples - 91.9% (p <0.001). *Anisakis* infestation intensity Herring of different types of culinary processing showed a similar tendency and most of the parasite larvae were found in marinated fish, which was confirmed by the calculation of the index of invasion: the index was greater by 4.3 units (p <0.001) compared with frozen fish, and in smoked fish - by 4.2 units (p <0.001).

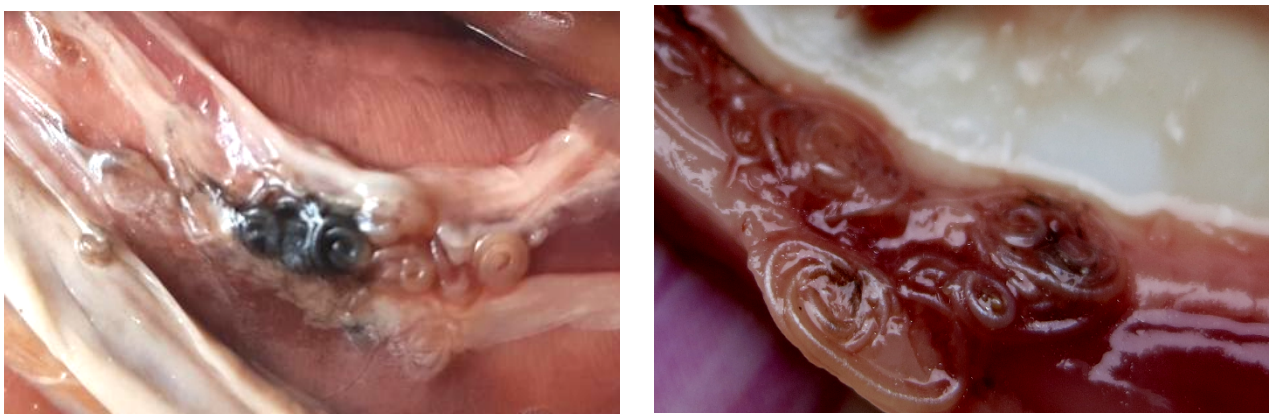
The study of the localization of helmints in fish of various types of culinary processing yielded similar results (Table 2).

Table 2. Placement of larvae of *A.simplex* in herring carcasses of different types of culinary processing *(M ± m, n=25)*

Indicator	Kind of fish processing			
	freezing	salting	marinating	smoking
Number of helmints in fish body, units	6.0 ± 0.34	8.3 ± 0.05***	10.3 ± 0.75***	1.8 ± 0.03***
Localization of parasites in the abdominal cavity, units	5.2 ± 0.05	6.6 ± 0.09***	9.2 ± 1.35**	0.2 ± 0.09***
% of the total number of larvae in one fish	86.67	79.51	89.32	11.11
Localization of parasites in the abdominal wall, units	0.7 ± 0.03	1.2 ± 0.01***	1.0 ± 0.83	0.8 ± 0.05*
% of the total number of larvae in one fish	11.67	14.46	9.71	44.44

Localization of parasites in the muscles of the back, units	0.3 ± 0.01	0.2 ± 0.08*	0.2 ± 0.03**	0.8 ± 0.08***
% of the total number of larvae in one fish	5.00	2.41	1.94	44.44

As the digital material shows, the largest number of larvae was found in pickled fish. At the autopsy it was found that marinated fish had 1.77 times more intruders in the abdominal cavity compared with frost fish, 1.39 times more than salty fish and more than 46 times more than smoked fish. At the same time, the difference was significant (p <0.1-0.001) (Fig. 1).



a **b**

Fig. 1 - Localization of parasite larvae in the abdominal cavity of saline (a) and marinated (b) herring

The largest larvae in the abdominal wall and the muscles of the back were smoked specimens (44.44%, p <0.1-0.001).

Experimental material indicates the connection of the type of culinary processing of fish with the degree of damage to its anisakis. Thus, the smallest extensiveness, intensity and index of damage were detected in smoked herring. In addition, she had the smallest larvae of the parasite in the abdominal cavity. Probably this is due to the fact that before the smoke the fish was subjected to the removal of the inwards and thorough cleansing.

The features of the invasion of herring anisakis from different places of capture were studied. At the same time, imported and domestic fish products were investigated. The research results are presented in table 3.

Table 3. Comparative characteristics of invasion of anisakiasis of Atlantic herring *(Clupea harenqus)* and Black Sea herring *(Alosa immaculata)* depending on the place of catch and the method of preparation *(M ± m, n = 10)*

View and culinary processing of fish	Place of catch (importing country)	Extensiveness of the invasion, %	The intensity of the invasion, units	The invasion index, units
Atlantic herring *(Clupea harenqus)* (freezing)	North Atlantic (Norway)	93.5 ± 3.78***	5-19	11.5 ± 6.83**
Atlantic herring *(Clupea harenqus)* (freezing)	Baltic Sea (Latvia)	85.3 ± 11.35***	6-37	21.5 ± 9.75*

Black Sea herring *(Alosa immaculata)* (freezing)	Black Sea (Ukraine)	15.6 ± 5.33	1-10	5.7 ± 3.01
Atlantic herring *(Clupea harenqus)* (salting)	Baltic Sea (Latvia)	80.2 ± 7.25*	3-20	12.7 ± 7.85**
Black Sea herring *(Alosa immaculata)* **(**salting)	Black Sea (Ukraine)	70.3 ± 15.35	1-17	7.6 ± 3.33
Atlantic herring *(Clupea harenqus)* (marinating)	North Atlantic (Norway)	100	7-19	8.6 ± 5.67
Black Sea herring *(Alosa immaculata)* (marinating)	Black Sea (Ukraine)	5.3 ± 2.61***	3-15	7.3 ± 5.94
Atlantic herring *(Clupea harenqus)* (smoking)	North Atlantic (Norway)	8.3 ± 1.34*	0-9	4.3 ± 3.59
Atlantic herring *(Clupea harenqus)* (smoking)	Baltic Sea (Latvia)	9.1 ±3.25***	3-6	3.2 ± 1.26*
Black Sea herring *(Alosa immaculata)* (smoking)	Black Sea (Ukraine)	2.6 ±0.95	0-5	2.6 ± 0.87

Tabular materials suggest that imported products were more affected by larvae of *A. simplex* than domestic ones. Thus, in the analysis of fresh frozen herring imported from abroad, the difference in the degree of invasion was 69.7 - 77.9%, and the index of invasion - by 5.8 - 15.8%. A similar tendency of helmosis was found in fish of other types of cooking.

3.1.Discussions

Anisakiasis was first detected in the Netherlands in 1955 after eating slightly salted herring. Every year in many countries new cases are registered. According to studies of various scientists, the invasiveness of fish with anisakiasis reaches high rates: Atlantic herring (*Clupea)* is infected with larvae of anisakis by 41%, *Gadus* - by 25%, *Micromesistius* - by 41%, *Clupea harengus membras* - by 20%, *Theragra chalcogramma* - by 34%, *Scomberomorus niphonius* - 28 %, *Sprattus sprattus* - 16%, *Cololabis saira* - 28% etc (Berezovskiy et al, 2013; Carballeda-Sangiao, 2014).

Anisakis is a zoonotic helminthiasis characterized by a defeat of the gastrointestinal tract as a result of parasitism in the human body at the stages of the larvae of the worm family *Anisakidae*. The causative agents of human anisakiasis are the larval stages of the development of worms of the following groups: *Anisakis, Contracaecum, Pseudoterranova, Hysterothylacium*, belonging under the line *Ascaridida Skrjabin et Schikhobalova, 1940*, to the family *Anisakidae Skrjabin et Korokhin, 1945* (Gaevskaya, 2005; Faeste, 2014).

Additional mosquitoes of parasites have many species of sea fish, mollusks, large crustaceans, eating small crustaceans. The larvae of anisakis in the organism of intermediate hosts are localized in the body cavity, on the surface or within the various internal organs and muscle of the fish. They are inside semitransparent capsules - cysts or without them. The size of the cysts ranges from 1 to 7 mm on average. Anisakis larvae that do not form capsules, for example *P. dicipiens*, have a length of 1.5 to 6 cm (Gavryuschenko, 2016; Sondak & Gritsik, 2006).

Infection of end-owners occurs when they use infected intermediate hosts: fish, crustaceans and mollusks. If large intermediate hosts feed on small, invasive larvae of anisakis, then these larvae accumulate in the body of a larger, predatory fish.

Atlantic and Black Sea herring are the most popular among the domestic population. Atlantic herring inhabit the North Atlantic: from the Bay of Biscay to Iceland and southern Greenland and east to Spitsbergen and New Zemlya, including the Baltic Sea. In the western

part of the Atlantic Ocean, it is distributed from the southwestern shores of Greenland and Labrador to South Carolina, USA. This schooling bentopelagic oceanodrom fish keeps near the sea surface, winters and spawns in the near-bottom horizons (Herrero et al, 2011). It occurs at a positive temperature and high salinity and suffers considerable desalination. There are several separate subpopulations with different spawning periods. By the time of spawning, spring, summer, autumn and winter herring are distinguished. Spring and autumn herring are most numerous. The life cycle of herring of these populations occurs in the North Sea. Small, immature herring is distributed in three areas: the coastal waters of Norway and the Kola Peninsula, including fjords, bays, open waters of the Barents and White seas; the central and northeastern part of the Norwegian Sea; southeastern part of the Greenland Sea (Alekseenko, 2009; Daschner et al, 2012). The degree and dynamics of invasion by anisakis is determined by the place of catch. Thus, N.M. Bogatko, V. V. Vlasenko, O.Yu. Golub (Bogatko et al, 2011) reports that one hundred fish with a one-time spawning (for example, some salmon fish), after which they die, apparently, and anisakis larvae die. In fishes that spawn more than once, in particular herring ones, anisakis larvae do not perish and may eventually accumulate in the host organism. Changes in the hydrological conditions (when fish move from seawater to freshwater) and, above all, the salinity of the water do not have a detrimental effect on the larvae of the parasite.

For example, it indicates differences in the degree of invasion of herring from different places of catch and species specificity. For example, Dolinskaya herring (Southern Caspian, eastern coast) was infected with worms by 76.9%, Sarinskaya - by 33.3%, Eastern and Hasankulinskaya - by 50%, Krasnovodskaya - 76.5%, white-headed - by 93.3% Volgskaya - by 13.2 - 142%. The intensity of invasion of herring from different subpopulations also turned out to be different: there were up to 18 larvae in Dolinskaya herring, up to 4 larvae in Sarinskaya, 32 in eastern, 60 in Krasankovskaya, 51 in white-headed, Volgskaya, up to 21 larva (Alekseenko, 2009; Bogatko et al, 2011; Faeste, 2014).

Ye.L. Mikulich (Mikulich, 2013) reports that the main exporters of frozen fish to Belarus are: Russia, Norway, the Baltic countries, Denmark, Spain, the United Kingdom, Iceland, the USA, Canada, the countries of Latin America and Indochina. As a result of studies conducted on the internal organs of frozen fish, single larval stages of the *Anisakis simplex* were found, as well as *Nybelinia surminicola* larvae (the extensiveness of invasion was 70%, and the intensity of invasion was 2–6 parasites per fish). Scratches of the genus *Echinorhynchus* were found in gutted and decapitated codfish (the extensiveness of invasion was 50%, and the intensity was 2–4 parasites per fish).

Disinfection of seafood from larvae of anisakis can be freezing and heating. In the usual saline and acetic solutions used for cooking fish, lizards of anisakis can remain viable for many days or even months. Freezing of fish up to -18°C leads to the death of all larvae of the 14 days, at - 20°C they die within 4-5 days, at -30°C die for 10 minutes (Gaevskaya, 2005; Buchmann & Mehrdana, 2016; Baptista-Fernandes, 2017).

In Europe and the United States, sanitary rules regulate the freezing of fish, which can not be subjected to further heat treatment at -20°C for 5 days. Larvae can tolerate a temperature rise of up to 45°C, but at 60°C and above they die within 10 minutes. Thus, the thermal processing of fish in the range of temperatures 45-60°C does not guarantee its decontamination from the anisotropic larva (Mikulich, 2013; Nieuwenhuizen & Lopata, 2013).

In studying the localization of larvae of anisakis, it was established that the determining factor is not a kind of culinary processing, but a kind of processing of raw materials. Most of the invasions were found on the internal organs of fish. Therefore, in our opinion, removal of insects from the carcass of fish can reduce the level of invasion. The larvae may be twisted (shape of a spiral, wide ring) or elongated, in translucent capsules or without them. The size

of the cyst in the width - 3,5-5 mm, thickness 1,0-1,5 mm (*A. simplex*). The larvae obtained from cysts reach up to 4 cm in length, with a thickness of 0.4-0.9 mm. *Anisakis* genera are white to yellowish. Mature nematodes parasitize in the gastrointestinal channel of vertebrate animals living in an aqueous medium (Berezovskiy et al, 2013; Carballeda-Sangiao, 2014; Buchmann & Mehrdana, 2016).

The final hosts of these worms are marine mammals (cetaceans), predatory sea fish and violent birds that parasitize men and women with anisakis. The average length of a female is 60-65 mm, male - 50-55 mm. The fertilized eggs fall into the water, where the larva comes out of them, which is swallowed by the first intermediate hosts - crustaceans, often belonging to the family *Euphausiidae.* The relevance of the study of anisakiasis is obvious, since the danger of infection by these parasites is proven (Herrero et al, 2011). According to the results of our research, the largest number of larvae was found in the intestines of pickled fish, and the least - in cartridges of smoked fish. Ye.L.Mikulich. V.V. Sondak and et. there are cases of invasion not only in frozen fish, but even in fish canned fish from whole fish (Mikulich, 2013; Sondak & Gritsik, 2006; Buchmann & Mehrdana, 2016).

Samples of pickled fish were the most invasive. In our opinion, this is due to the selection of low-quality raw materials that have not been properly treated with cold. Another reason may be wrongly prepared marinade. Therefore, in the muscles of ready-to-eat pickled fish, the salt content was less than 14%. Such conditions, according to scientists, are favorable for the life of larvae. Therefore, pickled herring could have invasive larvae, dangerous for humans. It is dangerous that in most cases anisakiasis remains unnoticed. For example, in Japan 60% of lesions of worms were mistakenly diagnosed as appendicitis, cholecystitis, diverticulitis, tuberculosis peritonitis, and others (Daschner et al, 2012). According to S. A. Alekseyenko (Alekseenko, 2009), during the summer and autumn spawning of salmon in Khabarovsk there is an increase in the number of patients with acute abdominal pains. Helmints larvae were then detected in patients and fish products.

The total volume of herring samples of different types of culinary processing in Vinnitsa was somewhat higher than the data on the loss of imported fish, as shown in the article Ye.L.Mikulich according to their data, the degree of damage by parasites of fish was within 59%, the intensity of the invasion - up to 38, and the index of invasion - up to 12 (Mikulich, 2013). However, according to experimental data V. V. Grigoryeva (Gaponenko & Lysenko, 2009) the extensiveness of the invasion - the herring was 89%, the intensity of the invasion - 7-22 units. Apparently, discrepancies in the data are related not only to the type of culinary processing, but also to the observance of sanitary norms for the storage of fish raw materials and finished products. In our opinion, an important role in this is played by the place of catching fish.

For example, M. G. Gaponenko i S. Ye. Lisenko (Gaponenko & Lysenko, 2009) made the fish from the Azov-Black Sea basin anisakiasis mainly affect the abdominal cavity and internal organs (91.3%), less often the muscles of the abdominal wall (6.4%) and back muscles (2,3). %). The intensity of the invasion of the Black Sea herring was 1–12 individuals. In the case of imported fish, the abdominal cavity and internal organs (82.2%) are most often affected, less frequently the muscles of the abdominal wall (13.3%) and the back muscles (4.5%). The prevalence of herring invasion was highest at 59%, the intensity of Atlantic herring invasion was 1–38 individuals, and the invasion index was 12 larvae per fish carcass. Our studies have confirmed this tendency of contamination of herring with Atlantic and Black Sea anisakiasas. During our experiment, it was revealed that imported fresh-frozen fish from Norway and Latvia, regardless of the method of preparation, had higher levels of extensiveness, intensity and invasion than domestic fish.

4.Conclusions

The type of culinary processing of fish determines the degree of invasion of anisakis. The highest intensity, intensity and index of invasion were found in pickled fish. The analysis of carcasses showed that up to 89% of all larvae are localized in the abdominal cavity. Smoked fish had the slightest damage with anisakis. The removal of the intestines from the fish before smoking caused a decrease in the degree of invasion of the body by 78%. The practical significance of this study is to increase the efficiency of sanitary and epidemiological control of fish products, increase public awareness of the risk of anisakiasis and social responsibility for non-proliferation and detection of parasites.

Further research will be aimed at finding an optimal and quick method for the detection of living anisakis larvae and its use for the study of samples of fishery products of various types of culinary processing.

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EVALUATION OF ANTIBACTERIAL ACTIVITY, NUTRIENTS, AND TOTAL BACTERIAL COUNT OF MORINGA LEAF POWDER WITH VARIOUS DRYING METHODS

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ABSTRACT

Moringa leaves were widely used as raw material for food product. However, unsuitable drying method might cost the loss of antibacterial activity and nutrients content but still leave a high total bacterial count. The aim of this study was to determine the best drying method for moringa leaves, in terms of antibacterial activity, nutrients, and total bacterial count, in order to obtain valuable and safe product. Fresh leaves were dried by various drying methods, namely sun drying, room temperature (air) drying, oven drying, and freeze drying, and followed by grinding and sieving. Antibacterial actvity of moringa leaf powder was analysed by Kirby-Bauer method with agar disk diffusion, total bacterial count by Total Plate Count, bioactive compound by colorimetric method, β-caroten by Thin Layer Chromatography, and some minerals by Atomic Absorption Spectrophotometry. The results showed that moringa with all drying methods could not inhibit the growth of *Vibrio cholerae* and *Bacillus cereus*, but freeze-dried, oven-dried, and air-dried moringa leaf could inhibit the growth of *Escherichia coli*. Freeze-drying also showed the higher of bioactive compound than the other methods. Furthermore, total bacterial count of freeze-dried moringa leaf powder was $<2.50 \times 10^2$ cfu/g while that of other drying methods were significantly higher ($>10^6$ cfu/g). However, freeze-drying also caused greater decrease in mineral content compared to the other drying methods. In short, freeze drying could be the best choice to obtain safe and valuable moringa leaf powder but with compromised mineral content.

1.Introduction

Moringa (*Moringa oleifera*) is a tropical plant of Moringaceae family that all parts of the plant (especially the leaves) have pharmacological values for anti-diabetic, anti-inflammatory, anti-tumor, and anti-hypertension (Foline *et al*., 2011; Qureshi *et al.,* 2015). Besides, moringa contains a lot of macro and micro nutrients such as β-carotene, protein, sodium, phosphorus, iron, potassium, calcium, magnesium, vitamin C, vitamin B3, vitamin E, and vitamin A (Mulyaningsih *et al.*, 2018; El Sohaimy *et al.,* 2015). Moringa contains 7 times higher of vitamin C than oranges, 10 times higher of vitamin A than carrots, 17 times higher of calcium than milk, 9 times higher of protein than yoghurt, 15 times higher of potassium than bananas, and 25 times higher of iron than spinach (Rockwood *et al.,* 2013). Therefore, moringa is widely used as a basic ingredient for food products, supplements, and medicines. Even in some developing countries,

moringa is a primary source to cure malnutrition. In addition to nutrition, moringa leaves also contain various phytochemical compounds such as tannins, phenols, alkaloids, flavonoids, oxalates and saponins that can function as antibacterials against several Gram-positive pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus sp*., *Bacillus subtilis*), and Gram-negative *(Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*) (Ajayi *et al.,* 2015; Devendra *et al.,* 2011; Vieira *et al.,* 2010; Kalpana *et al.,* 2013).

Moringa leaves can be diversified into moringa tea, moringa milk, moringa biscuits, moringa cereals, moringa nuggets, etc. Generally, firstly, moringa leaves are made into powder by drying and grinding them. After that, they are added to the dough for further process to become food products. This drying process is also useful for extending the shelf life but it could decrease its nutritional and phytochemical content. Hussein *et al.* (2015) said that improper drying temperature can reduce the antibacterial activity in moringa leaf extract. In addition, it has also been reported that improper drying will leave a high number of bacterial total plates in food products. Adu-Gyamfi *et al.* (2014) reports that retail food products based on dried moringa leaves have a number of bacterial plate count of 5-8 x 10^{10} cfu/g, a million times higher than the limit, that is 10^4 cfu/g according to international standards and 10^2 cfu/g based on domestic standards. Such conditions will cause the quality decrease of food products in terms of nutrition and microbiological safety.

Optimization of drying techniques needs to be done to maintain the stability of mineral content and make safe foods based on microbiological criteria. J *et al*. (2015) stated that drying with various temperatures can affect phosphorus, potassium, calcium, magnesium, iron, and zinc found in moringa leaves. Foline *et al*. (2011) claimed that the total plate count (TPC) in moringa powder dried by sun drying technique was three times greater than air drying. Adu-Gyamfi *et al.* (2014) also said that the TPC of moringa leaves which were dried by air drying technique was higher than mechanical drying and solar drying. This drying optimization is useful for getting nutritious and safe food products.

The purpose of this study was to determine the appropriate drying method so that the antibacterial activity, bioactive compound, mineral content of moringa leaves would not be decreased and to evaluate the effect of drying techniques on microbiological safety through the determination of the total bacterial count (TBC). Hopefully, moringa leaves are processed with the right drying technique can be used as a basic ingredient in making various of food products quality in terms of nutrition and microbiological safety

2. Materials and methods

2.1.Sample preparation and collection

Moringa leaves were obtained from plantations located in South Tangerang, Banten. Moringa leaves were collected and washed in running water. Then, the leaves' surfaces were sterilized by using NaCl, ethanol, and distilled water (Mishra *et al.,* 2012). Next, moringa leaves were dried using various techniques. In sun drying, 300 g of moringa leaves were dried under the sun with 33^{0}C for 8 hours. Within air drying, moringa leaves were dried at room temperature (24^{0}C) for 5 x 24 hours. Using drying oven, moringa leaves were dried in the oven at a temperature of 45^{0}C for 24 hours. In freeze drying, moringa leaves were dried at -34° C for 6 hours 30 minutes. The dried leaves were then mashed with mortar. Then, they were sifted into a size of 0.5 mm - 1 mm.

2.2.Evaluation of Antibacterial Activity

The crude ethanolic extracts of moringa leaf powder were screened for their antimicrobial activity againts some Gram-positive and Gram-negative bacterial caused food-borne disease, namely *Escherichia coli* (ATCC 25922)*, Vibrio cholerae* (NCTC 11348)*,* and *Bacillus cereus (*ATCC 13061) by Kirby-Bauer method with agar disk diffusion.

The susceptibility of the bacteria were estimated by measuring the diameter of zone inhibition (Al_husnan *et al.,* 2016). Chloramphenicol was tested as a positive control.

2.3.Determination of moisture content, bioactive compound, β-carotene

The moisture of each sample of moringa leaf powder from those four drying techniques was measured using the AOAC method (1995) by placing the powder in air circulating oven with a temperature of 105^0C for 6 hours. β-carotene was analysed by adding chloroform into each sample of moringa leaf powder, then the filtrate was spotted into TLC layer and eluted by dicholoromethane : ethyl acetate as well as measured by TLC scanner at wavelength of 289 nm. Bioactive compounds (tannin and flavonoid) were also determined by AOAC method (1984).

2.4.Determination of Mineral Content

Determination of Ca, Mg and K were determined by percolation using ammonium acetate 1 M (pH 7) while Fe were determined by destructing the powder and drying with a gradual temperature from 100^0C until 200^0C. Then, they were measured using the Automatic Absorption Spectrophotometry (AAS) based on the AOAC method (2005).

2.5.Determination of Total Bacterial Count

Total Bacterial Count (TBC) was determined using the Total Plate Count (TPC) with spread plate technique. One g of moringa leaf powder was diluted serially in physiological NaCl solution 6 times. The last three dilutions were plated using the Nutrient Agar media (Merck) and incubated for 24 hours at 37°C.

The total bacteria was calculated and compared with Indonesian Standard (BPOM's Head Regulation Number 13 of 2019) concerning microbiological criteria in processed food.

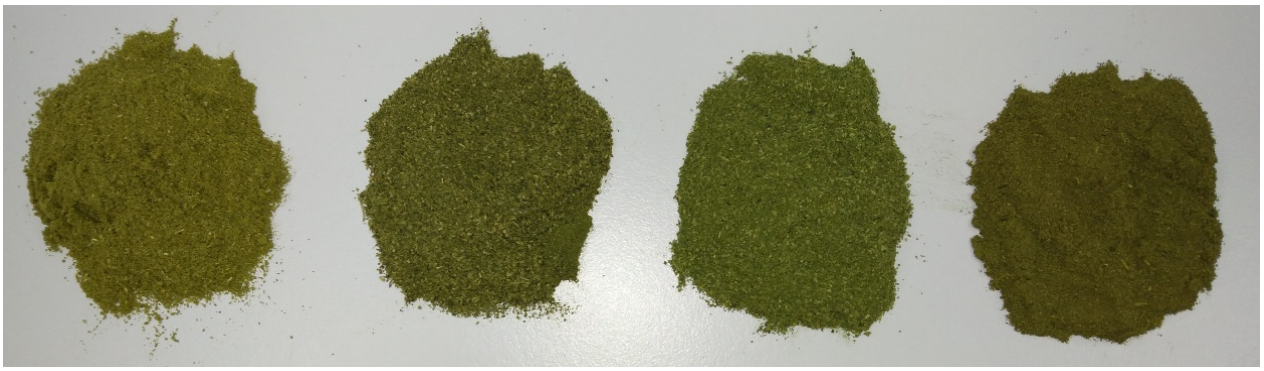
3.Results and discussions

3.1. Evaluation of Antibacterial Activity

Drying of foodstuffs aims to reduce moisture content so that it has longer shelf life (Garba *et al.,* 2019). Moringa leaves which dried with various techniques showing color differences (table 1 and figure 1). In spite of the difference between the four is not too significant, freeze-dried moringa powder was greener and brighter than sun, oven, air-drying techniques' results. These results were in line with Setyowati *et al.* (2017) which shows that the green colour of pandan leaves in freeze drying is brighter than oven drying. This is due to the low temperature which prohibit the release of magnesium ions in green pigments (chlorophyll) so that no brownish pheophytin compounds are formed. These pheoophytin compounds are formed when magnesium ions are released from chlorophyll and replaced with hydrogen ions due to an increase in temperature. Therefore, the higher the drying temperature of the leaves, the darker the colour will be.

Table 1. Colours character of moringa powder in various drying techniques

Drying Techniques	Colour
Freeze drying	Light green
Oven drying	Brownish green
Sun drying	Green
Air drying	Dark green



A B C D

Figure 1. Colours of moringa powder in various drying techniques: A. Freeze drying, B. Oven drying, C. Sun drying, D. air drying

The difference in the color characteristics of moringa leaves represents differences in quality of moringa, in term of antibacterial activity or phytochemical and nutrient content. Antibacterial activity of ethanolic extract of moringa leaf powder with various drying method are shown in table 2. Based on table 2, known that ethanolic extract of moringa leaf with all various drying method (freeze drying, sun drying, oven drying, and air drying) could not inhibit the growth of *Vibrio cholerae* and *Bacillus cereus.* However, three of drying method (freeze drying, sun drying, and air drying) could inhibit the growth of *Escherischia coli* with the diameter of zone inhibition is 2.75 mm, 4.5 mm, dan 1.25 mm, respectively. *V. cholerae, E. coli,* and *B. cereus* are bacteria that cause food-borne disease. *V. cholerae and E.* coli are Gram negative bacteria, while *B. cereus* is Gram positive bacteria. These three bacteria have different levels of sensitivity to antibacterial compounds (Valarmathy *et al.,* 2010; Vieira *et al.,* 2010; Moyo, 2012).

Table 2. Antibacterial activity of moringa leaf with various drying method to *E. coli*, *V. cholerae*, and *B. cereus*

Ethanolic extract of moringa leaf with various drying method	**Diameter zona hambat (mm)**		
	E. coli	***V. cholerae***	***B.cereus***
Freeze drying	2.75	-	-
Sun drying	-	-	-
Oven drying	4.5	-	-
Air drying	1.25	-	-
Fresh moringa leaf (control)	-	-	-

Legend: the sign (-) showed there is no inhibition activity

Table 3. Antibacterial activity of moringa leaf powder to *E. coli*

Type of extraction	**Diameter of zone inhibition (mm)**	**References**
Water	1(*)	Meshram *et al*., (2014)
Ethanolic	4(*)	Ojiako (2014)
Ethanolic	8(*)	Gebregiorgis Amabye *et al.,* (2016)

Legend : The sign of (*) is based on agar well diffusion technique

Some studies said that moringa leaves could not inhibit the growth of *V. cholerae* and *B. cereus* (Valarmathy *et al.,* 2010; Moyo, 2012), but could inhibit the growth of *E. coli* (Valarmathy *et al.,* 2010) even though the inhibitory value showed inconsistent results (Patel *et al.,* 2014; Ojiako, 2014; Gebregiorgis Amabye *et al.,* 2016) (table 3). These can be caused by several factors, namely: 1. Extract type, and 2. Antibacterial activity testing techniques. Altemimi *et al*., (2017) said that the type of solvent used to extract active compounds is very influential on the results obtained. According to him, methanol is the best solvent for extracting bioactive compounds of plant extract. The active compound extracted will certainly also affect its antibacterial activity. In addition, testing for antibacterial activity using agar well diffusion technique provide a high value of antibacterial activity compared to the method for agar disk diffusion. Valgas *et al.* (2007) said that the use of agar disk diffusion made bioactive compound which has many free hydroxyl group will be adsorbed to the surface of the disk and not diffuse into the agar, so that the antibacterial activity might be undetectable. In otherwise, agar well diffusion made that bioactive compound will diffuse directly into agar, so that the antibacterial activity might be higher than agar disk diffusion

3.2.Determination of moisture content, bioactive compound, β-carotene

Flavonoids can be antibacterial because they contain phenols which can denaturate lipoproteins in bacterial cell walls by forming protein-phenol complexes through hydrogen bonds (Rahmawati *et al.,* 2014; Sapara *et al.* 2016). Tannins are antibacterial by activating metabolic enzymes in bacteria and interfering with protein transport in the inner layer of cells (Sapara *et al.* 2016; Rahman *et al,* 2017). Based on inhibitory zone against *E. coli*, moringa leaves with oven drying have greater antibacterial activity, followed by freeze drying, and air drying. This can be related to the resistance of antibacterial compounds (tannins and flavonoids) to the drying temperature. Flavonoid and tannin levels will decrease along with increasing temperature (Obiang-Obounou *et al.*, 2013).

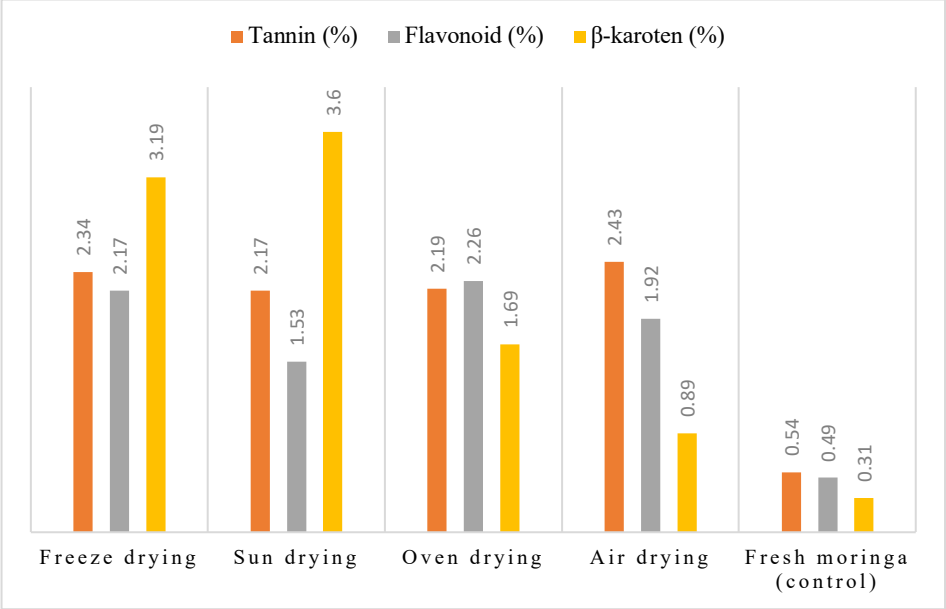


Figure 2. Tannin, flavonoid, and β-karoten of ethanolic extract of moringa leaf on various drying method

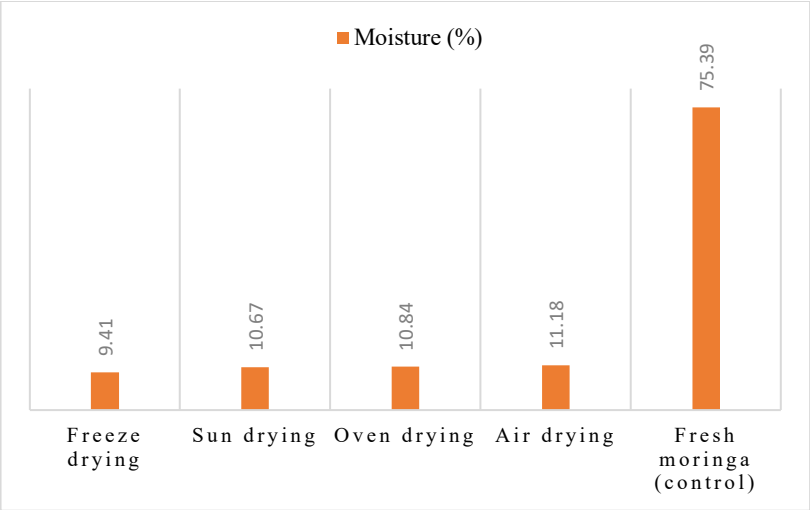


Figure 3. Moisture content (%) of ethanolic extract of moringa leaf on various drying method

Figure 2 showed that the tannin content of moringa leaves dried at air drying is greater than other drying methods, while the flavonoid levels in oven drying is greater than other drying methods. This showed inconsistent results, presumably non-uniform drying time contributes to the inconsistency of the results. However, total tannins and flavonoids in oven drying and freeze drying are greater than air drying. These results are consistent with their antibacterial activity (table 2). Fresh Moringa leaf extract does not show any antibacterial activity against the three bacteria and has the smallest level of antibacterial compounds. This can occur due to the presence of moisture content which gives relativity to leaf mass.

Figure 3 showed that the moisture content in moringa leaf powder with freeze dried is the lowest compared to other drying techniques so that the weight is lighter, although the difference between the four is not too significant. If used as raw material in food processing, then moringa leaf powder with freeze dried is needed in large quantities per mixture of the dough.

Although oven drying showed the best results on antibacterial activity, it turns out that β-carotene levels are not as good as the antibacterial activity. Figure 2 shows that freeze and sun drying techniques provide the best results on β-carotene levels. Mujumdar *et al.* (2016) said that freeze drying is one of the best way to remove water from a product while retaining its bioactive compound. β-carotene is a precursor of vitamin A that serves to sharpen vision at night. Fouad *et al.* (2019) said that β-carotene and flavonoids are natural antioxidants that are mostly obtained from moringa leaves which can be used as antibacterial and can overcome the problem of multidrug resistance.

3.3. Determination of Mineral Content

Reffer to the colour differences of moringa leaf powder, Wijaya *et al.* (2015) said that differences in mineral content in food contribute to differences in physical characteristics such as color, texture, taste, and stability. Minerals are essential constituents in food products even though the body needs a little of it.

Figure 4 showed the effect of drying techniques on the mineral content of moringa leaf powder. The mineral content of air-dried moringa shows the most stable number compared to sun-dried, oven-dried, and freeze-dried moringa. The content is almost equivalent to fresh moringa leaves; even the iron content has not decreased. It caused by the increasing temperatures which will make minerals become destructed. Oni *et al.* (2015) claimed that sun drying and oven drying decreases mineral

content of Mg, Zn, Mn, and Fe in edible botanicals because they are destructed by high temperatures. However, it is contradicted to Wijaya *et al.* (2015) who said that minerals cannot be structured due to exposure to heat, light, oxidizing, and extreme pH, but can be eliminated because of their solubility to water. In the same study, Oni *et al.* (2015) also said that the freeze-dried technique is an effective technique for preventing the decrease in mineral content. This is contrary to the results obtained in this study. The mineral content of the freeze dried technique actually decreases significantly, that is as much as 25% Ca, 35% K, 14.81% Mg, and 78% Fe. Referring to Wijaya's statement (2015), perhaps most of the mineral is dissolved with water so that it is eliminated along with the substrate of the water component when freeze drying is carried out. Freeze drying is a method of removing water by sublimating ice crystals from frozen material without passing the liquid phase. Water contained in foodstuffs is sublimated at -34°C for a certain time, then the remaining water is desorbed so that the material becomes completely dry (Gaidhani *et al.* 2015).

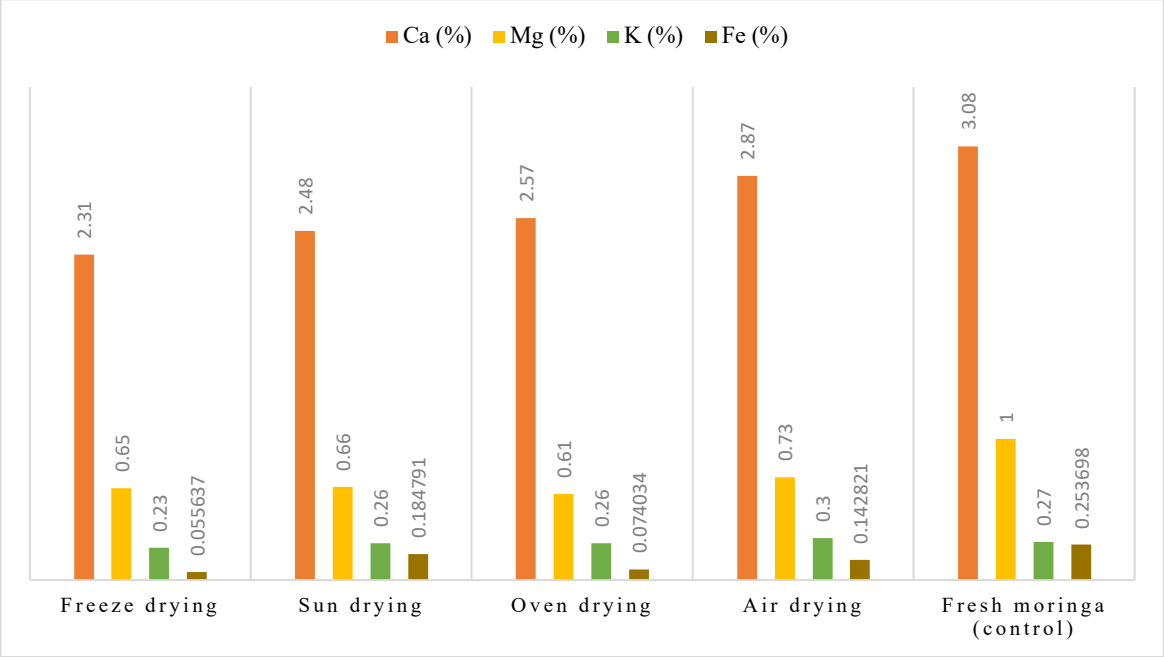


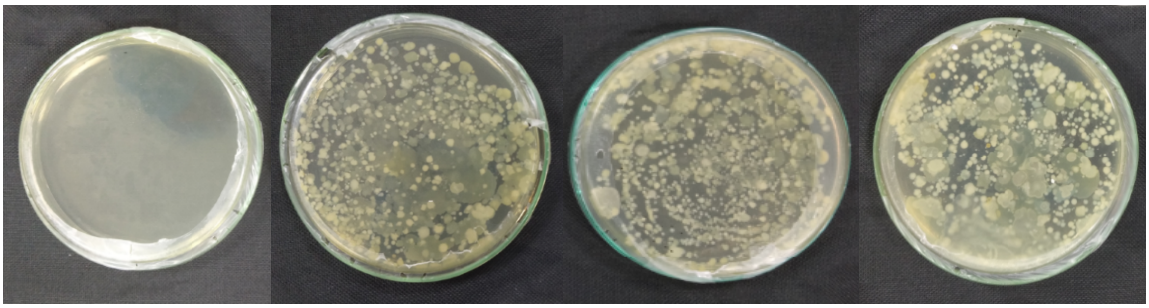
Figure 4. Mineral content of moringa leaf powder on various drying method

3.4. Determination of Total Bacterial Count

Drying technique can reduce moisture content which is useful for slowing decay by microbes and slowing down enzyme activity (Akani *et al.*, 2017; Garba *et al.,* 2019). However, dried-food can still contain high amounts of microbes. Adu-Gyamfi et al (2014) reported that retail food products based on dried moringa leaves have a total bacterial plate count of one million times higher than the international standard allowed. Table 4 and Figure 5 are the result of TBC determination on moringa leaf powder with various drying techniques.

Table 4. Total bacterial count on moringa leaves with various drying techniques

Drying Techniques	Total Bacterial Count (cfu/g)
Freeze drying	$< 2.5 \times 10^2$
Oven drying	4.08×10^6
Sun drying	1.95×10^7
Air drying	2.46×10^8



A B C D

Figure 5. Total Bacterial Count on moringa leaf powder with various drying techniques: A. Freeze drying, B. Sun drying, C. Oven drying, D. Air drying

TBC values on sun-dried, oven-dried, and air-dried showed above 10^6 cfu/g. Only freeze-dried moringa shows no bacterial growth (TBC value < 2.5×10^2 cfu/g). This can be caused by the moisture content of freeze-dried moringa leaf powder is lower than the results of other drying techniques. Therefore, it does not allow bacteria to grow in it. Sequentially, the drying technique of moringa leaf powder with TBC values can be ranked from the highest to the lowest bacterial growth, those are air-dried > sun-dried > oven-dried. This is consistent with the moisture content contained in the moringa leaf powder in each technique. According to BPOM's Head Regulation Number 13 of 2019 concerning the maximum limit of microbial contamination in processed food, the TBC value for herbal/spice powder should not be more than 10^4 cfu/g so that the only freeze-dried moringa leaves that meets the requirements of passing BPOM standards.

4. Conclusions

Drying techniques can affect the quality of food products in terms of antibacterial activity, nutrients, and total bacterial count. Based on the results of the study, the air-dried technique is the most effective technique for maintaining the stability of the mineral leaves of moringa leaf powder especially iron, but has a low antibacterial activity. However, from its microbiological safety, air drying still leaves high TBC value. The value is a thousand times higher than the BPOM standard. Moreover, the level of β-carotene has also decreased. In contrast, the freeze-dried technique is the most ineffective technique for maintaining the stability of the mineral, but it is best for microbiological terms. The TBC value is < 2.5×10^2 and it meets the BPOM standards. Freeze drying also has a better antibacterial activity especially to *E.coli* and has a higher β-carotene as well as bioactive compound than air-dried technique. Looking at the cost, air drying is recommended as long as the process should be sterile to avoid bacterial contamination. However, if consider the speed of time, freeze drying is recommended. Further research is needed to minimize the risk of nutrient loss in freeze drying.

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SURVEY OF FRAUD IN THE FOODS THAT WERE USED IN ITS PRODUCTION OF SAFFRON

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ABSTRACT

Food fraud is referred to as the intentional substitution addition tampering or misrepresentation of food. Food fraud is a broader term than the economically motivated adulteration. Different method are performed of fraud for instances include carcinogen colorants in foods, melamine in milk, species swapping of seafood, dilution of fruit juices, unauthorized repackaging**.**Although the vast majority of food fraud incidents do not pose a public health risk, some cases have resulted in actual or potential public health risks such as continuous consumption a substance that can be used as the artificial colors.It is not known conclusively how widespread food fraud is in Iran or worldwide therefore this deficit gave us the motivation for our review.In the total of 203 food samples tested in 72 (35.46) samples were detected food fraud therefore this survey showed that economically motivated food fraud and adulteration was an emerging and serious food safety problem in Iran.The results of the survey can be used not only to analyze food safety risks but also to prioritize target areas for food policy-making and enforcement of food safety regulation.

1.Introduction

1.1. Saffron

Saffron, the dark-red and dried stigma of Crocus sativus L,. very valuable for its Special aroma, color, taste and medicinal properties, belongs to the Iridaceae family and is native to Europe, Asia, and the Middle East such as Iran. At the moment considered the world's most expensive spice (Baghalian *et al*., **2010**; Fernández and Pandalai *et al*., **2004**). Nowadays, saffron are used almost in all cases for cooking purposes to give color and flavor to food products. With regards to the total amount of saffron production**,** Iran is the biggest producer country in the world, After the Iran located countries Greece, Morocco, India, Spain and Italy (Maggi *et al*., **2003**). Saffron has also been cultivated in Turkey, Azerbaijan, and China. But the saffron mine is Iran because now more than 90% of the total saffron produced in the world is produced in Iran (Ghorbani *et al*., **2008**).

The constituents of saffron that are considered pharmacologically active and main contains volatile agents (e.g,. safranal), bitter principles (e.g., picrocrocin), picrocrocin is the glycoside precursor of safranal (2,6,6-trimethyl-**1**,3-cyclohexadiene-**1**-

carboxaldehyde), that is in turn the most abundant of the volatile compounds responsible for the aroma of this spice and dye materials (e.g,. crocetin and its glycoside, crocin), crocins are crocetin esters with glucose, gentiobiose, neapolitanose or triglucose sugar moieties. These water-soluble carotenoids are responsible for saffron's yellowish color (Maggi *et al*., 2003; Anastasaki *et al*., 2010; Rios *et al*., 1996).

Saffron has shown antidepressant effects in several surveys (Schmidt *et al*., 2007; Abdullaev Espinosa-Aguirre *et al*., 2004; Hausenblas *et al*., 2015; Hausenblas *et al*., **2013**) and also anticonvulsant properties (Hosseinzadeh and Khosravan **2002**), anti-inflammatory properties, antinociceptive and (Hosseinzadeh and Younesi 2002) the anticancer properties (Zheng *et al*., **2011**; Bhandari **2015**) also proved. Another studies such as Hajime Fukui and et al reported that saffron odor may be effective in treating menstrual distress (Fukui *et al*., **2011**) and study of Soheila Pirdadeh Beiranvand and et al proved daily usage of Saffron is reduced of menstrual pain (Beiranvand *et al*., **2015**). In other study antioxidant activity (Serrano-Díaz *et al*., **2012**; Sánchez-Vioque *et al*., **2012**), antityrosinase (Li *et al*., **2004**), arterial pressure reducer (Fatehi *et al*., **2003**) of the saffron plant cited. In study of Concepción Pintado and et al (2011) mentioned compounds and probably their chemical relatives, are involved in the antibacterial activity of saffron, and that this effect can significantly reduce the risk of food contamination with Salmonella by this spice (Pintado *et al*., **2011**).

1.2.Food fraud

Food fraud is referred to as the intentional substitution addition tampering or misrepresentation of food. Food ingredients or food packaging for economic gain to the seller. Food fraud is a broader term than the economically motivated adulteration or EMA (Nenadis and Tsimidou **2016**; Wilson **2008**; Foster **2011**).

Examples of Food Fraud include:

- Horsemeat in ground beef
- Peanut Corporation selling known contaminated product
- Diluted or extra virgin olive oil
- Melamine in pet food and infant formula
- Over-icing with unsanitary water
- Unauthorized unsanitary repackaging (up-labeling or origin-laundering)
- Cargo Theft reintroduced into commerce (Spink *et al*., **2015**).

1.3.Food color

Throughout human history, food color has been a main feature of sensory quality assessment. With the emergence of processed food, food coloring has gained even more importance. According to consumer's belief, food coloring should be as natural as possible; however, there is actually no standard regarding the naturalness of food coloring. In addition to, word natural not surely mean edible. There are different methods for food coloring, include the addition of intensely colored food to coloration by synthetic dyes which means artificial colors. Using coloring food offers the most natural way to color food with food (Johnson **2014**). Legislation on the coloring and subsequent labeling of food is exist in Iran.

Many synthetic dyes have been banned because of their adverse effects on laboratory animals. Especially colors that as the fraud are used instead of saffron. It is of concern that Yellow **6** may be contaminated with significant levels of recognized carcinogens. Also, while rarely life-threatening, Yellow 6 causes mild to severe hypersensitivity reactions in a small percentage of the population and may cause hyperactivity in some children. Even if it does not cause cancer, Yellow 6 raises other, lesser concerns. Because it provides no health benefit whatsoever, Yellow 6 should be removed from the food supply (Kobylewski and Jacobson **2010**).

Considering that saffron is the most expensive aromatic compounds and food additives and also has lots of benefits caused

that many fraud to replace cheap materials used instead of saffron.

2.Materials and methods

2.1. Food sampling

Food samples collected include kebabs, rice and sweets that are used in the production of them saffron. The manufacture of sweets and restaurant centers of Qazvin province had a total includes 203 the center of preparation of food were sampled over one year. The samples were collected including 68 kebab, 93 rice and 42 sweets.

2.2. Analysis method

Fraud detection took place based on Iran National Standard 259-2 (IR-ISO NO 259-2).

2.3. Thin layer chromatography (TLC)

TLC is the easiest, economical and the most appropriate chromatographic technique for qualitative analysis of mixtures of analytes because of the possibility of obtaining better results in relatively short span of time (Kucharska and Grabka 2010). Reviewed various sample preparation techniques and chromatographic conditions for the analysis of food dyes in different food matrices by TLC (De Andrade *et al*., 2014).

3. Results and discussions

The tests results have are shown in under tables.

Table 1. Food fraud observed in the restaurants

Food	Color		Total	Percent food fraud
	Natural	Artificial		
kebab	37	31	68	45.58
rice	61	32	93	34.40

Table 2. Food fraud observed in manufacture of sweets

Food	Color		Total	Percent food fraud
	Natural	Artificial		
sweets	33	9	42	21.42

Table 3. Food fraud observed in the total food sampled

Total food fraud	Color		Total	Percent food fraud
	Natural	Artificial		
	96	72	203	35.46

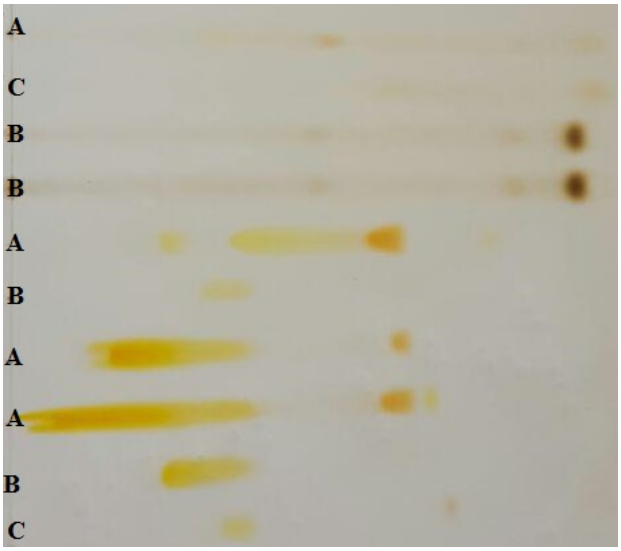

Figure 1. TLC paper: rice (A), kebab (B) and sweets (C)

Table 4. Artificial color observed in Samples of kebab

Color	Frequency	Percent
Tartrazine	15	48.38
Sunset Yellow	6	19.35
Tartrazine+ Sunset Yellow	10	32.25
Quinoline	0	0

Table 5. Artificial color observed in Samples of rice

Color	Frequency	Percent
Tartrazine	12	37.50
Sunset Yellow	8	25
Tartrazine+ Sunset Yellow	9	28.12
Quinoline	3	9.37

Table 6. Artificial color observed in Samples of sweets

Color	Frequency	Percent
Tartrazine	6	66.66
Sunset Yellow	0	0
Tartrazine+ Sunset Yellow	0	0
Quinoline	3	33.33

With human lack of awareness and carelessness, microbiological, chemical and physical hazards can by accidentally enter our foods and causing foodborne illnesses and deaths. While Casual food contamination of these hazards has always been a subject of food safety, a growing worry is in the introduction of hazards by deliberate human actions known as food fraud or economically motivated adulteration (FF/EMA) (Tähkäpää *et al*., 2014; Everstine *et al*., 2013). Recent sobering examples of FF/EMA that have drawn great attentions around the world include the 2008 incident of intentional tampering of infant milk formula with melamine in China and the 2013 horsemeat substitution scandal in Europe (Qiao *et al*., 2012; Bouzembrak and Marvin 2016). Therefore searching, communicating and managing risks from FF/EMA have become important tasks for food regulators and examiners in the interests of consumer protection and food safety (Zhang and Xue 2016) and also fraud detection in foods by reading the label on it is not possible (Charlebois *et al*., 2016).

Our survey showed 68 samples Kebab tested there was fraud in 31 (45.58%) samples and in 93 rice samples tested there was fraud in 32 (34.40%) samples and in 42 sweet samples tested there was fraud in 9 (21.42%) samples and In the total of **203** food samples tested in **72** (**35**.46) samples were detected food fraud. Other studies have confirmed the presence of fraud in foods like ours study. In study of Nunes and et al (2016) fraud consisted of injecting solutions of non-meat ingredients (NaCl, phosphates, carrageenan and maltodextrin) in bovine meat, aiming to increase its water holding capacity (Nunes *et al*., 2016).

In the study of Ghovvati and et al (2015) showed that none of the samples were contaminated with porcine residuals, but 40% of sausages samples and 30% of cold cut samples were contaminated with poultry residuals. Also the ground meat samples were not contaminated with poultry residuals (Ghovvati *et al*., 2009).

And also in study of Gheisari and et al (2008) showed 31.25% of whole samples of honey was tested is in a way of spurious (Gheisari1 and Hamidian Shirazi 2008).

4. Conclusions

Food fraud has always existed and still exists so the only way to control this issue is not careful supervision by government. The most appropriate strategy is to alert the conscience of the people who are involved in food production that in this case warning about the effects of food fraud is an efficient way because the many forgers are not aware about of the consequences of their work.

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STUDY OF BIOTECHNOLOGY RAISE ANTIOXIDANT PROPERTIES OF OLIVE OIL AND BLACK SEED OIL

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ABSTRACT

The aim of this study was to improve the functional and antioxidant properties of olive oil using 10%, 15%, 20% and 25% black oil as a substitute. The compositions of olive oil with black cumin seed oil were created to develop the formulation of the product with enhanced functional and antioxidant properties. Antioxidant properties of olive oil, black cumin seed oil and the obtained compositions of olive oil with black cumin oil were studied by chemical and photometric methods. The results showed that olive oil with the addition of 15 % - 20 % black cumin seed oil is optimal both in antioxidant activity and organoleptic properties. Therefore, this oil composition can be recommended as a functional edible oil.

1.Introduction

Olive oil from European olivium (Olea europaea L.) is one of the most important foods that is in increasing demand all over the world, which makes it possible to increase values and antioxidant properties (Makarova and Borodinova, 2011). As additives that increase antioxidant properties, olive oil can be used as enzymatic and lipolytic properties, from which derived medicinal preparations. Unlike other vegetable oils, black cumin oil (Nigella sativa L.) contains such essential unsaturated fatty acids as (linoleic -55-65%; oleic -15-18%; palmitic -10-12%; eicosenic - 4- 5%; stearic - 1-3%; alpha-linolenic - 1%), lipase, tocopherols, multivitamins, groups A, B, P, essential oils, enzymes and other components (http://www.gabris.ru/gabris /). health / cumin / oil), also has strong bactericidal and antiseptic properties (Elshafey et al., 2014).However, the compositions for comparison with the original oils and olive oil. It is known that black cumin oil has a bitter unusual taste. Therefore, studies of the organoleptic characteristics and antioxidant activity of various compositions with different mass fractions of oil in olive oil are relevant (Lemekhova et al., 2011).These studies showed an increase in the functional and antioxidant properties of olive oil using 10%, 15%, 20% and 25% black oil as a substitute (Khasanov et al. 2004).

2. Materials and methods

2.1. Materials

Sampling of oils for the study of organoleptic and antioxidant quality indicators was made in supermarkets in Astrakhan. Oil samples were taken from the fruit of extradition planted olives produced in Italy (Borgeos Olive Oil, Carapelli Olive Oil). Black cumin seed oils (chernushki) were purchased from the central market outlets (FitOil Black Cumin Seed Oil, NigOil Black Cumin Seed Oil). Samples of oils from black cumin seeds and olive fruits were

prepared for research: A - FitOil black cumin seed oil; B - black cumin seed oil NigOil; C - Borgeos olive oil; D - Carapelli olive oil.

2.1.1. The composition of olive oil

The composition of olive oil (Borgeos) with the addition of black seed oil (FitOil) to it in the following% ratios: E - 90:10 (%) (A: 10%); F - 85:15 (%) (A: 15%), G - 80:20 (%) (A: 20%) and H - 75:25 (%) (A: 25%).

2.2. The antioxidant properties of vegetable oils

The antioxidant properties of vegetable oils and their compositions were investigated by a chemical method described (Fabris, et al., 2013), which consists in the colorimetry of free stable radicals based on the reaction of an antioxidant sample (AH) with DPPH dissolved in ethanol (2,2-diphenyl -1-picrylhydrazyl (C18H12N5O6, M = 394.33). A DPPH test (DPPH) was performed by photometric method with 0.5 millimolar (mmol) DPPH alcohol solutions (Sigma-Aldrich) at a wavelength of 517 nm, measured on an AP- 101 (APEL, Japan). The working solutions of the samples were prepared on 96% ethyl m alcohol; measurements were carried out 60 minutes after the start of the reaction of DPPH with a sample of antioxidant (AH) (Kouakou et al., 2018.) The degree of radical seizure — P% and antioxidant activity — AA% were calculated. P% was calculated using the formula: P (%) = [(But - NaOH) / But] x100%, where Ho and Naooh are the height of the photometric signal in the absence and presence of antioxidant, respectively (Shpigun et al., 2010). AA in% was calculated using the formula: AA = [1 - (Ai-Aj) / Ac] x100%, where Ai is the absorption of the test solution with a DPPH reagent, Aj is the absorption of the test substance, Ac is the absorption of a DPPH solution (Fabris et al., 2013).In parallel, the potential antioxidant activity was determined by the method of cyclic voltammetry (Baizer and Lund, 1988). To remove cyclic voltammograms (CVA), a potentiostat "IPC-Pro", coupled with IBM, was used as a three-electrode, diaphragm-free cell (V = 2 ml). A platinum electrode (D = 1 mm2) was used as a working one, the reference electrode was Ag / AgCl in a saturated KCl solution with a waterproof diaphragm, the auxiliary electrode was platinum (S = 70 mm2) (Baizer and Lund, 1988).As a background electrolyte, n-Bu4NClO4 (C = 0.1 M) was used to increase the electrical conductivity of the analyzed solutions (Bordwell ang Cheng, 1989). Samples of oils were dissolved in methylene chloride, which was purified by the method. The method is based on the ability of organic components of oils with biological activity to be oxidized to a one-electron stage at a certain potential value under the conditions considered.

3. Results and discussions

The antioxidant activity of the studied samples of oils from the fruits of olives and black cumin seeds is presented in Table 1 and the graph in Figure 2. The highest degree of radical seizure of a stable free radical (DPPH) by the samples studied was found in oils of black cumin seeds of sample A and sample B, slightly lower than the antioxidant activity in olive oil sample C, then sample D.

Table1. Comparative studies of the antioxidant properties of various samples of olive oil and black cumin oil

Test substance	The degree of radical seizure,%	Antioxidant activity,%
Black Cumin Seed Oil FitOil - A	117,7	129,23
Black Cumin Seed Oil NigOil - B	96,5	91,53
Olive oil Borgeos - C	83,6	78,58
Olive oil Carapelli - D	64,6	61,89

The antioxidant activity index is distributed in this method in the same correspondence: the best sample is A, then the sample is B, then the sample is C, and the least antioxidant activity in olive oil of the sample is D. There is a correspondence between these two indicators, the higher the degree of radical seizure , the higher the degree of antioxidant activity. Olive oil samples also exhibited antioxidant

properties when interacting with a stable free radical, but to a lesser extent than black cumin seed oil samples (Fig. 2), which is consistent with literature data (Makarova and Borodinova, 2011).

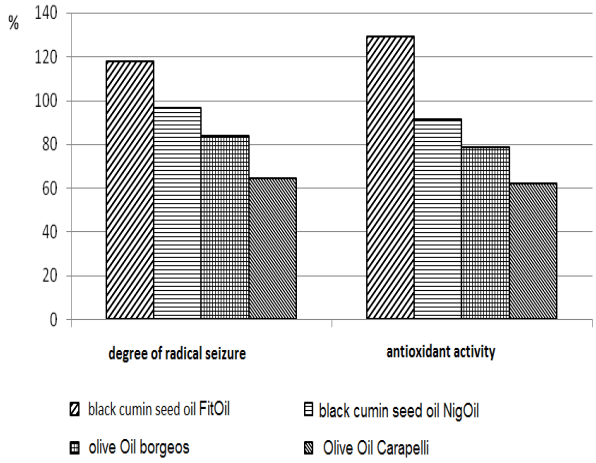

Figure 2. Comparative degree of antioxidant activity depending on the degree of radical seizure of various samples of olive oil and black cumin oil.

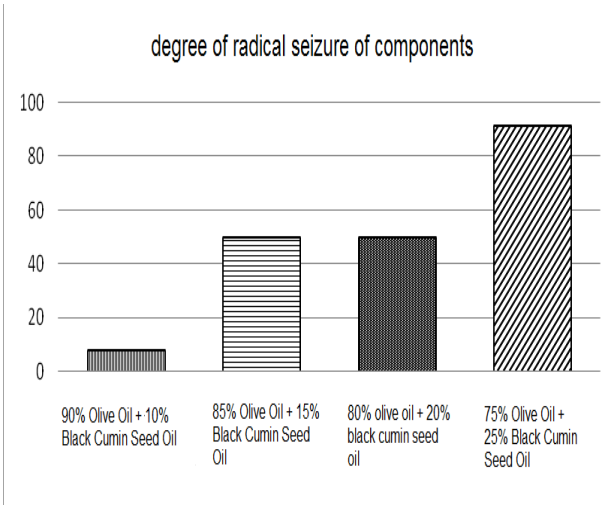

Figure 3. The change in the degree of radical seizure depending on the content of black cumin seed oil in olive oil

Black cumin seed oil was found to exhibit higher antioxidant activity than olive oil samples. Although samples of olive oil had a relatively high degree of radical seizure, i.e. antioxidant activity. Created compositions based on olive oil with the addition of black cumin seed oil in order to increase the degree of antioxidant activity of olive oils were investigated to change the antioxidant activity. Four different compositions of olive oil with the addition of black cumin seed oil: E - (A: 10%); F - (A: 15%); G - (A: 20%); H - (A: 25%) showed the following results of an increase in antioxidant activity (Fig. 3).

The presented results of changes in the degree of antioxidant activity of olive oil (AA) (radical free radical capture (DPPH) showed that the best results were achieved by adding 25% of black cumin seed oil to it, sample H - (A: 25%). When it is contained in olive oil 10% black cumin oil (sample E - (A: 10%), olive oil practically does not change its antioxidant properties. Composition of olive oil F (A: 15%); and G with 20% black cumin oil (A: 20%) have almost the same degree of antioxidant activity, and the ledge According to activity, only sample H (A: 25%) with a 25% black cumin oil content. It should be noted that the latter composition H (A: 25%) was not inferior in terms of the degree of antioxidant activity to sample B of pure black cumin oil, however, it had less pronounced peculiar smell and taste of black cumin oil.As can be seen in the diagram (Fig. 3), the higher the content of black cumin oil in the mixture, the higher the antioxidant properties of the composition, however, the organoleptic characteristics of the compositions may vary depending on the original ingredients, which is a reason to consider options for improving the taste and antioxidant properties of this product. combination with the developed dish.The oxidizing ability of the components of olive oil, black cumin oil or their compositions was analyzed by cyclic voltammetry (CVA) in the anodic region of potentials from 0.5 to 2.0 V. From literature sources (Sampiyev et al.,2014) it is known that in this range spatial-obstructed phenols (from 1.0 to 1.3 V), which have a high antioxidant activity (Sampiyev et al., 2014). At higher potentials, five-membered O-, N-, S-heterocyclic compounds (from 1.5 to 2.0 V) with high biological and physiological activity are oxidized. The CVA method, being a qualitative and quantitative analysis, which

shows a linear dependence of the peak of the anodic current on the concentration of the active components of the studied oil samples (A - H), showed (Fig. 4) that with increasing concentration of the substance (black cumin oil in olive oil) in the electrochemical cell oxidation peaks grow according to current.

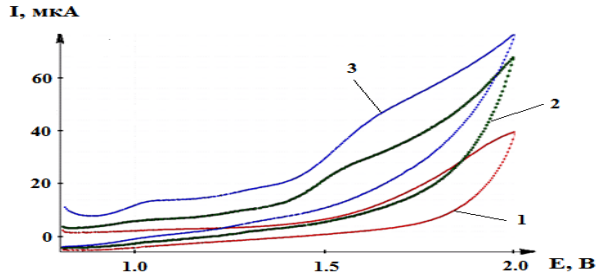

Figure 4. Cyclic voltammogram of oxidation: 1 - background electrolyte; 2 - components of black cumin oil (sample A) (V = 100 µl); 3 - components of black cumin oil (sample A) (V = 300 µl) (Pt-anode, Ag / AgCl, CH2Cl2, 0.1 M n-Bu4NClO4).

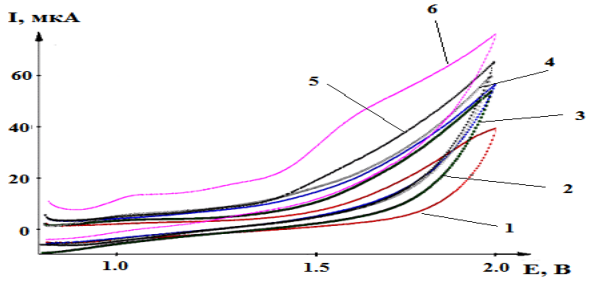

Figure 5. Cyclic voltammogram of oxidation (V = 300 ml): 1 - background electrolyte; 2 - components of olive oil (sample C); 3 - components of a mixture of olive oil (90%) and black cumin oil (10%) (sample E - A: 10%); 4 - components of a mixture of olive oil (80%) and black cumin oil (20%) (sample G (A: 20%); 5 - components of a mixture of olive oil (75%) and black cumin oil (25%) (sample H - A: 25%); 6 - A components of the oil black cumin oil (Pt-anode, Ag / AgCl, CH2Cl2, 0.1 M n-Bu4NClO4).

Based on the analysis of cyclic voltammograms of oxidation of components contained in samples of black cumin oil (A, B), olive (C, D) and their compositions (E, F, G, H), it was found that in black cumin oil (A, B) there is the highest concentration of substances similar to spatially obstructed phenols (Fig.5). Also in this sample A, the maximum content of heterocyclic oxygen -, nitrogen - or sulfur-containing compounds is observed, and their concentration is 4-5 times higher than compounds of the class of phenols (Kouakou et al., 2018).

Accordingly, in the sample of olive oil C, the content of these valuable components is minimal.

The results of the study of the compositions of olive oil and black cumin oil in various percentages allow us to conclude that increasing the mass fraction of black cumin oil in olive oil of samples E, F, G, H from 10% to 25% increases the biological value and antioxidant activity of the mixture oils. This is due to the increase in the concentration of biologically active components and substances with antioxidant activity in it, as evidenced by the data of CVA method (Fig. 5).

According to the results of studies of literature data in the issues of improving consumer properties of the organoleptic characteristics and quality of vegetable oil, according to the specifics of the human body, methods of blending two or three different types of oil have been proposed (Sikoev, 2009).

4. Conclusions

On the basis of the obtained data, it is clear that the highest index of antioxidant activity was found in sample A and B of black cumin seed oil, which is consistent with the authors' opinion on the high nutritional, perfume and therapeutic activity of chernushka oils (black cumin — Nigella sativa). Samples of olive oil (C, D) in this work had half the antioxidant activity (Muratova et al., 2016). It turned out that by adding black cumin seed oil to olive oil, it is possible to improve not only its organoleptic properties, but also antioxidant activity, which increases the possibility of creating new food products not only with improved taste data, but also with a targeted antioxidant effect (Makarova and Borodinova, 2011). The composition of olive oil with 15–20% content of black cumin seed oil (samples F, G) is optimal both for antioxidant activity

and organoleptic properties. Therefore, this composition of oils can be recommended as a functional edible oil, which increases their consumer qualities (Mizhueva and Balashov, 2010). Earlier, an organoleptic analysis was performed, where the indicators taken into account included taste, aroma and appearance. As a result, according to the criterion for assessing the organoleptic characteristics of research objects expressed in points and their weight coefficients and based on the results of tasting, it was determined that the composition containing 80% olive oil and 20% black cumin oil had the best organoleptic characteristics (Shahin and Dolganova, 2014).

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″GABRIS″ food, black cumin oil. http://www.gabris.ru/gabris/health/tmin/oil/

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CLASSIFICATION BY ARTIFICIAL NEURAL NETWORK FOR MUSHROOM COLOR CHANGING UNDER EFFECT UV-A IRRADIATION

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ABSTRACT

In this paper, the classification of the effect of irradiation with the number of different UV-A lamps (0.20, 40, 60 and lumens) on the variation of L*, a* and b* was investigated using input data L *, a* and b* with the artificial neural network. In this experiment, to the classification two networks of radial basic and multi-layered perceptron functionalities with the hyperbolic tangent activation function in one and two hidden layers were used. According to the results obtained, the best value for R and Percent Correct for the number of lamp 0 (Percent Correct = 100 - R^2= 0.652, lamp number 20 (Percent Correct = 100 - R^2= 9999), for lamp number 40 (Percent Correct = 100 - R^2= 0.652) and for lamp number 60 were also (Percent Correct = 100 – R^2 = 0.9994). Also, the highest numbers of correctly detected data for the number of lamp in the number of 20 were observed in the MLP network, and this network has been able to categorize 100% for the total number of 0, 20, 40, and 60 correctly. Generally, the MLP neural network is better than the RBF network, and the network with 4 and 8 neurons in the hidden layer is appropriate, and the network with 1 layer and 2 layers has acceptable accuracy for classification.

1. Introduction

Non-modern statistical methods for modeling difficult and nonlinear calculations are often unusable, especially if the relationship between output and measured characteristics of the model is not clear. One type of computation that is used to address the bugs of non-modern methods is intelligent predictive methods such as artificial neural networks. These types of systems have properties such as learning capability, generalizability, information dispersion, parallel processing, and robustness, use in pattern separation, grading, approximation of function, and correlation equation. Generally, wherever one needs to learn a linear or nonlinear mapping, it should be used. Artificial neural networks today play an important role in predicting process parameters as a powerful tool (Van Dam 2014; Johnsson *et al.,* 2018). The most important advantage of artificial neural networks is that they do not require a basic model for linking

incoming and outgoing data to predict different parameters to predict different parameters (Rahal *et al.,* 2018). The other advantages of the neural networks, as compared to other intelligent systems, are the ability to learn them on a very small scale from the surrounding area and the ability to generalize this learning. In general, this method tries to establish a linear or nonlinear model between independent and dependent variables based on the inherent relationships between the data. Different types of artificial networks have been introduced, which are mainly used in applications such as classification, clustering, pattern recognition, and modeling, approximation of functions, control, estimation and optimization (Hertz., 2018). Other uses of the artificial neural network include the use of these networks by researchers in agricultural sciences to simulate parameters such as evapotranspiration (Chowdhary *et al.,* 2010), evapotranspiration (Ashrafzadeh et al. 2018)، air temperature prediction (Rodrigues *et al.,* 2018), solar radiation(Işık and Inallı 2018), flood prediction, and soil water retaining capability (Lykhovyd., 2018). Neural network models, unlike mathematical models, without the need to extract the relationship between parameters, are able to identify the relationship between them, and in this regard are considered as a very powerful tool in modeling. In this method, the relationship between the parameters in the stage of network education is introduced and in the next stage, it is act similar to the human brain, and with training, the neural network will be able to predict the process and the problems associated with extracting the relationship between the parameters are eliminated. Therefore, neural network models are often used in cases where the relationship between parameters is unknown or very difficult (Ghasemi *et al.,* 2017). The most common neural networks, the type of multilayer perceptron that consists of the input layer, the hidden or middle layer, and the output layer (Alam *et al.,* 2018). Classification is defined as a method in which each sample is in a predetermined class and, using a series of initial information, the samples are assigned to certain categories. The classification of large databases makes it possible to use a large amount of information to be assigned to several smaller, more consistent categories, in various modeling processes(Gonçalves *et al.,* 2018). Fuzzy clustering and artificial neural network methods are among the algorithms used in the classification. Fuzzy clustering is a method that matches the fuzzy logic in which each dataset belongs to a group that is characterized by its membership grade (Moorthi *et al.,* 2018). With the advent of computers in recent years, it has been possible to carry out in-house procedures on a large volume of data and the use of classification methods has been expanded. Classes can be divided into generic and advanced divisions. General categorization methods include the maximum probability and the minimum distance. Also, advanced classification, artificial neural networks, decision tree, backup vector machine, and object-oriented classification can be mentioned (Das *et al.,* 2017). Barbero *et al*., (2006) examined the amount of ultraviolet radiation by two methods, one based on traditional statistical techniques and another artificial neural network, and acknowledged the high ability of artificial neural network (Barbero *et al.,* 2006). Huang *et al*., (2017) studied the capability of ethyl hexalyl salicylate to absorb ultraviolet radiation by artificial neural network and found that this material has high economic potential (Huang *et al.,* 2017). Luque *et al.,* (2006) Used spectroscopy, ultraviolet ray, and pattern recognition techniques for differentiating and classifying wines, and the speed of analysis and review was Impressive (Luque *et al*., 2006) . Rahman *et al.,* (2016) with the classification of fresh and non-fresh fish, based on the effect of ultraviolet radiation on the fish's eyes, obtained an appropriate assessment of fish's liveliness and shelf-life (Rahman *et al.,* 2016). Due to the fact that mushrooms are air-sensitive products and have many color changes, so it is necessary to use different methods to investigation the prediction and classification of these products

that the artificial neural network is one of the prediction methods. The aim of this paper is to classify the color change of the fungus in UV-A irradiation, whether the neural network has the proper ability to classify the amount of UV radiation. Also, the sensitivity coefficient L *, b* and a* is obtained using artificial neural network.

2. Materials and methods

2.1. Sample preparation

Samples from the mushroom Production Plant in Golestan province were freshly prepared and sent to the Laboratory of Bio-system Mechanics Department of the University of Agricultural Sciences and Natural Resources, and after washing with standard methods, the moisture content of mushroom was measured using oven (Figure 1).The moisture content of mushroom was 89.6 ± 1.5% wet basis. After measuring the moisture content, samples were cut into slices with a thickness of 5 mm and placed after encoding in the cellophane (Polyolefin film). The encoded samples were placed in three groups of 20, 40 and 60 lamps, to be irradiated, and all experiments were carried out with three replications. Also, for color variation to be checked, control treatment with no irradiation on it was selected.

Figure 1. The mushroom samples placing method in into plate

2.2. Irradiation of samples

After preparing, the samples were exposed to UV, A, 20, 40 and 60 for 10, 20 and 30 minutes. The irradiation interval was 0.5 cm from the specimens, and in Figures 2 and 3 were showed how the lamps are illuminated and the distance and placement of the sample.

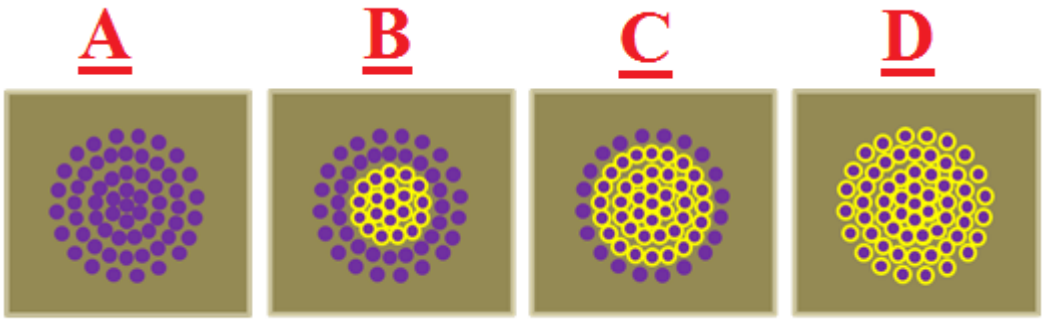

Figure 2. Arrange lamps method for irradiation – A: The total number lamps B: Twenty lamps C: Forty lamps D: Sixty lamps

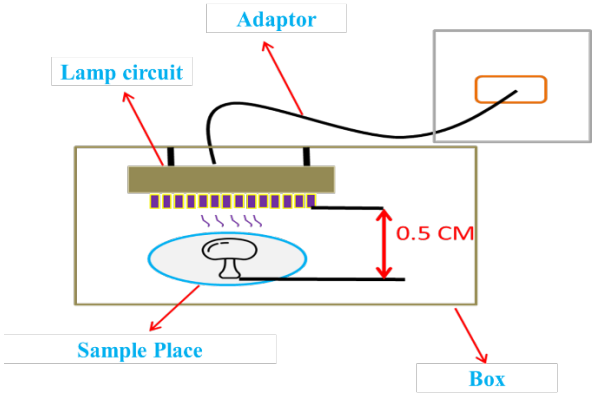

Figure 3. Sample place in box and irradiation UV-A

2.3. Imaging samples

All photos were taken before irradiation and then, after irradiation of each sample, photos were taken every one hour in 24 hours (in one day) for each specimen. Photography was done using the canon ixus 132hd camera and the camera was mounted Perpendicular to the sample product on the camera's location. The photography was taken in a closed box with the same exposure for all specimens to prevent different light exposure to the product.

2.4. Color analysis

In the analysis of color values, the values of L*, a* and b* were used and this is due to the independence of this analysis from the device and covers a wider range than RGB and CMYK. Image J software was used to analyze images and obtain color values. First, it was done to improve the images and remove unnecessary components in the image for all pre-processing images. In the process of image processing, the general purpose at this stage is to identify features of the image that can be used for their intended use. The images were converted from RGB to XYZ and then to L *, a* and b* using two steps. Using (Eq.1), which is the method used by San and colleagues, the images can be converted from the RGB color space to the XYZ color space. Also using (Eq. 2 to 4), the XYZ images can be converted to L *, a* and b* in the next step.

$$\begin{bmatrix}\hat{X}\\ \hat{Y}\\ \hat{Z}\end{bmatrix}=\begin{pmatrix}0412456 & 0.257580 & 0.180423\\ 0212671 & 0.715160 & 0.072169\\ 0.019334 & 0.119194 & 0.950227\end{pmatrix}\begin{bmatrix}\hat{R}\\ \hat{G}\\ \hat{B}\end{bmatrix} \quad \textbf{(1)}$$

$$\hat{L}=\begin{bmatrix}116\times\left(\frac{\hat{Y}}{Y'}\right)^{\frac{1}{3}}-16\\ 903.3\times\left(\frac{\hat{Y}}{Y'}\right) ELSE\end{bmatrix}(2)\hat{a}=500\times\left[\left(\frac{\hat{X}}{X'}\right)^{\frac{1}{3}}-\left(\frac{\hat{Y}}{Y'}\right)^{\frac{1}{3}}\right]$$

(2)

$$\hat{a}=500\times\left[\left(\frac{\hat{X}}{X'}\right)^{\frac{1}{3}}-\left(\frac{\hat{Y}}{Y'}\right)^{\frac{1}{3}}\right] \quad \textbf{(3)}$$

$$\hat{b}=200\times\left[\left(\frac{\hat{Z}}{Z'}\right)^{\frac{1}{3}}-\left(\frac{\hat{Y}}{Y'}\right)^{\frac{1}{3}}\right] \quad \textbf{(4)}$$

Where X ', Y' and Z 'are XYZ values for the 5 D 65standard.

$$\begin{bmatrix}\hat{X}\\ \hat{Y}\\ \hat{Z}\end{bmatrix}=\begin{pmatrix}95.047\\ 100\\ 108.883\end{pmatrix} \quad \textbf{(5)}$$

2.5. Artificial Neural Network Modelling

In this research, a multi-layered perceptron (MLP) and a radial base function (RBF) neural network were used to classify UV-A lamps. For these networks, 1 and 2 layers were hidden and 4 and 8 neurons were selected, the selected networks were separately trained and formed and analyzed using NeuroSolution6 software. The hyperbolic tangent activation function of (Table 1).Which is most used for processing, was selected and used in both of input and output. In this research, the Levenberg-Markott optimization method was used for network training. The values of L *, a* and b* were selected as inputs for the classification and number of bulbs (0.20, 40 and 60) as the output of the networks. 70% of the data for training and 15% for the test, and 15% for the evaluation of the network were used. In the formation of these networks, 5 repetitions were selected for simulating artificial neural network data to achieve the minimum error rate and maximum network stability, averaging 5,000 Epoch to simulate data by neural network. Error Estimating Algorithm in Networks the compilation was performed using an error-back propagation algorithm. For estimating the network, two factors of the coefficient of explanation (R^2) and root mean square error were used. The correlation coefficient determines the correlation between the output data of the neural network and the observed data and is calculated from (Eq.6), whose idea

value is equal to 1.The mean square error defines the difference between the predicted data and the actual data and its root is calculated from (Eq.9). The goal of a good network is to reduce this error to the lowest value, and its appropriate value is zero.

Table 1. Neural Network Relationships

Formula	Formula Number	Reference
$\text{Tanh} = \frac{e^{x}-e^{-x}}{e^{x}+e^{-x}}$	**(6)**	(Soleimanzadeh *et al.* 2015)(Kariman *et al.* 2019)
$R^2 = 1-\frac{\sum_{i=1}^{n}(P_i-O_i)^2}{(P_i-O)^2}$	**(7)**	(Azadbakht *et al.* 2016;Azadbakht *et al.* 2018a)
$r = \sqrt{1-\frac{\sum_{i=1}^{n}(P_i-O_i)^2}{(P_i-O)^2}}$	**(8)**	(Azadbakht *et al.*2019)
$RMSE^1 = \sqrt{\sum_{i=1}^{n}\frac{(P_i-O_i)^2}{n}}$	**(9)**	(Khoshnevisan, Sh. Rafiee, M. Omid 2013)(Azadbakht *et al.*2018b)
$MAE^2 = \frac{\sum_{i=1}^{n}\lvert P_i-O_i\rvert}{n}$	**(10)**	(Azadbakht *et al.* 2017)

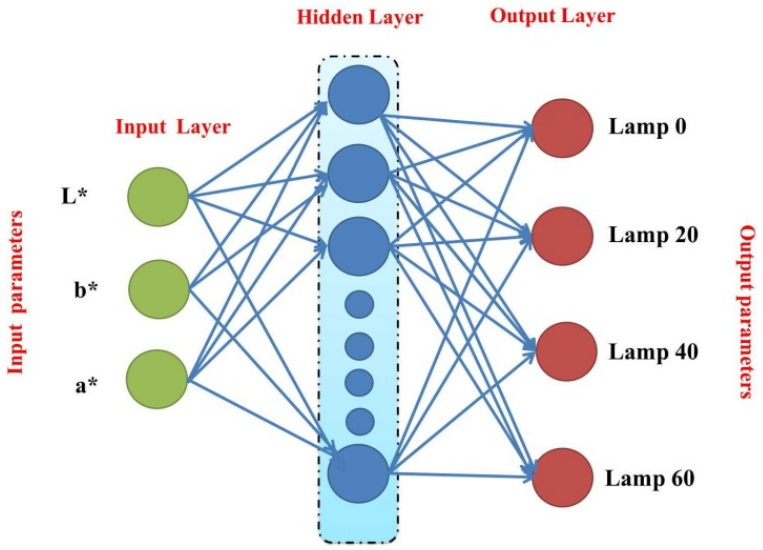

Figure 4. ANN schematic

[1] Root mean squared error
[2] Mean Absolute Error

3.Results and discussions

Table 2 shows the RMSE, MAE, R^2 and Percent Correct values. Accordingly, the best values for the MAE, R^2 and Percent Correct in the MLP network with 4 neurons with two layers were hidden. The best values for the number of lamps are 60 RMSE $_{Train}$=9.49×10^{-5}, MAE $_{Train}$=7×10^{5}, R^2 $_{Train}$=0.99999998, Percent Correct $_{Train}$=100 and for the number of bulbs, the best 40 values of the RMSE $_{Train}$=8.37×10^{-5}, MAE $_{Train}$=5.6×10^{-5}, R^2 $_{Train}$=0.99999998, Percent Correct $_{Train}$=100 For the number of lamps 20, the best values are RMSE $_{Train}$=6.32×10^{-5}, MAE $_{Train}$=4×10^{-5}, R^2 $_{Train}$=0.99999998, Percent Correct $_{Train}$=100 and finally for the number 0 bulbs, The best values were, RMSE $_{Train}$=2.24×10^{-5}, MAE $_{Train}$=1×10^{-5}, R^2 $_{Train}$=0.99999998, Percent Correct $_{Train}$=100. According to the results obtained, the best value for $R^2$and Percent Correct for the number of bulbs 0 (Percent Correct = 100 - R^2= 0.652, lamp 20 (Percent Correct = 100 - R^2= 9999), For lamp number 40 (Percent Correct = 100 – R^2 = 0.652) and for the number of bulbs, 60 were also (Percent Correct = 100 - R^2 = 0.9994). Also, the highest numbers of correctly detected data for the number of lamp in the number of 20 were observed in the MLP network, and this network has been able to categorize 100% for the total number of 0.20, 40, and 60 correctly. Also according to the results obtained when using the MLP network, the accuracy and predictability of the network were greater than the RBF network. Also, the closer RSME is to zero, the better the network performance, which is less than RBF in MLP networks, which means the accuracy of these networks are higher. According to the results, it can be stated that at a time when 4 neurons were used for the MLP network in hidden layers in a network with two hidden layers, they obtained better values than R^2, RMSE, and Percent Correct and when 8 neurons are used in hidden layers, a hidden double-layer network has been able to show better values and for the RBF network it's exactly the opposite of the MLP network. In general, it can be argued that the MLP network has been able to display better R^2, RMSE, and Percent Correct values for classification

Table 2. Error values in predicting experimental data using optimal artificial neural network

Network	Input layer	Lamp number	RMSE		MAE		R^2		Percent Correct	
			Train	Test	Train	Test	Train	Test	Train	Test
4 Neuron- MLP	1	60	0.024495	0.667083	0.0147	0.436	0.9986	0.386	100	33.333
		40	0.03	0.667832	0.0206	0.433	0.9988	0.2369	100	55.33
		20	0.03873	0.044721	0.0289	0.035	0.9992	0.999	100	100
		0	0	0.004472	0.0045	0.004	0.9996	1.000	100	100
	2	60	9.49E-05	0.447258	7E-05		0.99999998	0.99693	100	83.66
		40	8.37E-05	0.632487	6E-05	0.20003	0.99999998	-0.57344	100	0
		20	6.32E-05	0.447225	4E-05	0.40004	0.999999989	0.66666	100	100
		0	2.24E-05	0.4036	1E-05	0.20002	0.999999998	1	100	100
8 Neuron- MLP	1	60	0.004472	0.449555	2.2E-03	0.2256	0.9999578	0.615	100	100
		40	0.004583	0.449555	2.4E-03	0.2277	0.9999595	0.652	100	66.667
		20	0.000933	0.155563	7.0E-04	0.0738	0.9999981	0.935	100	100
		0	0.000616	0.000316	4.4E-04	0.0002	0.9999998	0.9036	100	50.33
	2	60	0.00945	0.443847	6.93E-03	0.210	0.999950	0.631	100	50
		40	0.009429	0.441588	7.04E-03	0.216	0.99989	0.89	100	50
		20	0.003606	0.094868	3.12E-03	0.051	0.99999	0.976	100	100
		0	0.002938	0.070711	2.30E-03	0.040	0.99998	0.991	100	100
4 Neuron RBF	1	60	0.016125	0.028284	0.01226	0.0222	0.99949	0.9994	100	100
		40	0.013038	0.024495	0.00970	0.0194	0.99967	0	100	50.33
		20	0.009487	0.022361	0.00766	0.0178	0.99983	0.9997	100	100

		0	0.008944	0.01	0.00646	0.0070	0.99975	0.9999	100	100
	2	**60**	0.5	1.28841	0.5	0.3999	0.0989799	0.400005	100	0.166
		40	0.5	1.28841	0.5	0.3999	0.0984165	0.400006	0	0.166
		20	0.5	1.32365	0.5	7.82E-12	0.0978989	2.35E-06	0	3.36
		0	0.5	1.296399	0.5	1.89E-12	0.0989369	1.08E-06	0	0989
8 Neuron- RBF	**1**	**60**	0.6245	0.465403	0.404	0.2482	-0.399	-0.3907	0	0
		40	0.474342	0.404846	0.325	0.2663	0.608	0.6830	100	100
		20	0.451664	0.484045	0.216	0.2731	0.567	0.5641	100	100
		0	0.337639	0.356371	0.211	0.2300	0.734	0.6679	0	0
	2	**60**	0.042426	0.595651	0.0224	0.3109	0.9971	0.2949	100	50
		40	0.022361	0.641716	0.0150	0.4134	0.9990	-0.2721	100	0
		20	0.014142	0.336749	0.0125	0.0449	0.9995	0.8912	100	100
		0	0.014142	0.144914	0.0101	0.0009	0.9992	0.9993	100	100

Mean square error (MSE) - Root mean square error (RMSE) - Mean absolute error (MAE) – R^2 : Coefficient of determination

In Table 3 the results of classification are shown using the neural network for the number of 60, 40, 20 and 0 lamps. According to the results, it can be stated that in the MLP network all data is correctly predicted.

Table 3. Classification values for RBF and MLP networks

Network	**Lamp number**	**Input layer**	**Lamp number**							
			0		**20**		**40**		**60**	
			Train	**Test**	**Train**	**Test**	**Train**	**Test**	**Train**	**Test**
4 Neuron- MLP	60	1	0	0	0	0	0	0	**60**	1
		2	0	0	0	0	0	1	8	0
	40	1	0	0	0	0	8	0	**40**	2
		2	0	0	0	0	5	0	0	0
	20	1	0	0	8	1	0	0	**20**	0
		2	0	0	5	2	0	1	0	0
	0	1	1	1	0	0	0	0	**0**	0
		2	4	1	0	0	0	0	0	0
8 Neuron- MLP	60	1	0	0	0	0	0	1	**60**	1
		2	0	0	0	0	0	0	6	1
	40	1	0	0	0	0	6	2	**40**	0
		2	0	0	0	0	6	0	0	1
	20	1	0	0	5	1	0	0	**20**	0
		2	0	0	8	1	0	0	0	0
	0	1	5	0	0	0	0	0	**0**	0
		2	2	2	0	0	0	0	0	0
4 Neuron- RBF	60	1	0	0	0	0	0	0	**60**	2
		2	5	0	7	0	5	1	5	2
	40	1	0	0	0	0	7	0	**40**	0
		2	0	0	0	0	0	1	0	1
	20	1	0	0	6	2	0	0	**20**	0

		2	0	0	0	0	0	0	0	0
	0	1	3	1	0	0	0	0	**0**	0
		2	0	0	0	0	0	0	0	0
8 Neuron- RBF	60	1	0	0	0	0	0	0	**60**	0
		2	0	0	0	0	0	1	6	1
	40	1	0	0	0	0	6	1	**40**	1
		2	0	0	0	0	7	0	0	1
	20	1	4	1	4	2	0	0	**20**	0
		2	0	0	6	1	0	0	0	0
	0	1	0	0	0	0	0	0	**0**	0
		2	3	1	0	0	0	0	0	0

It is possible to choose the best network using RMSE and R^2, and for the prediction percentage, no difference between the 4 and 8 neurons in the hidden layers of the 1 layer and 2. This demonstrates the very good ability of this network to predict and classify, and for the neural network RBF network trained with 4 neurons and hidden layers, and 8 neurons and two hidden layers, has been able to categorize 100% of the data. Table 4 shows the results of learning the neural network. According to the results shown in the Table, it can be stated that the fastest network is RBF network with 8 neurons and one hidden layer and for Cross Validation, the fastest network is RBF with 4 neurons and one hidden layer but according to the results, the R^2 and RMSE were not good enough. Also Azadbakht *et al*., (2020) reported that RBF neural network is faster than MLP neural networks.

Table 4. Some of the best MLP and RBF neural network topologies to predict test value

	Input layer	Cross Validation	Training	
4 Neuron- MLP	**1**	4	2	**RUN**
		4999	66	**EPOCH**
	2	4	1	**RUN**
		199	2020	**EPOCH**
8 Neuron- MLP	**1**	5	1	**RUN**
		48	2017	**EPOCH**
	2	3	1	**RUN**
		61	2022	**EPOCH**
4 Neuron- RBF	**1**	1	1	**RUN**
		15	2014	**EPOCH**
	2	1	1	**RUN**
		23	299	**EPOCH**
8 Neuron- RBF	**1**	5	1	**RUN**
		7	117	**EPOCH**
	2	1	1	**RUN**
		18	2018	**EPOCH**

The sensitivity coefficient for L *, a* are shown in (Figures 5 and 6), and the sensitivity coefficient b* for both the MLP and RBF with the number of neurons and various layers has been zero. For L *, the highest color-sensitivity coefficient was observed in the number of bulbs 20 and in the MLP network, and for the number of bulbs 0 and 60, the coefficient of sensitivity was lower than L *.In the number of lamps 20, 40 and 60, the highest sensitivity was found in the MLP network and the network with 4 neurons in the hidden layer and one hidden layer. For the a* value, a very low sensitivity coefficient for the number of bulbs has been obtained and the number of lamps 20 with both RBF and MLP networks in both Neuron 4 and 8 neurons, and 1 and 2 layers of the hidden layer were able to obtain a relatively high sensitivity coefficient and the sensitivity coefficient has decreased as the number of lamps has increased, and the sensitivity factor of 60 bulbs has been less than 40 lamps

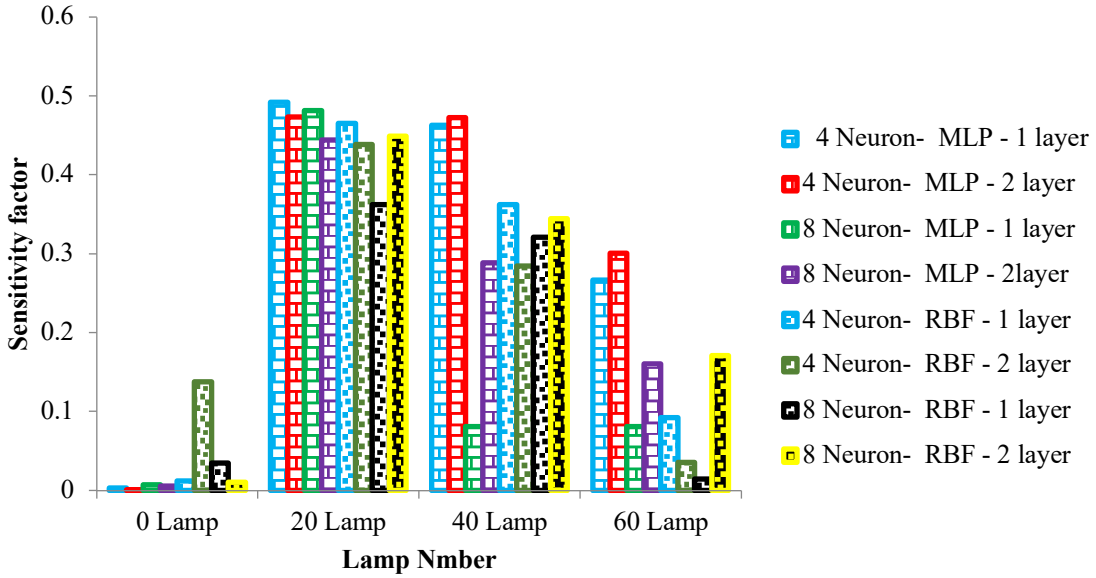

Figure 5. The sensitivity coefficient L * in number of different lamps

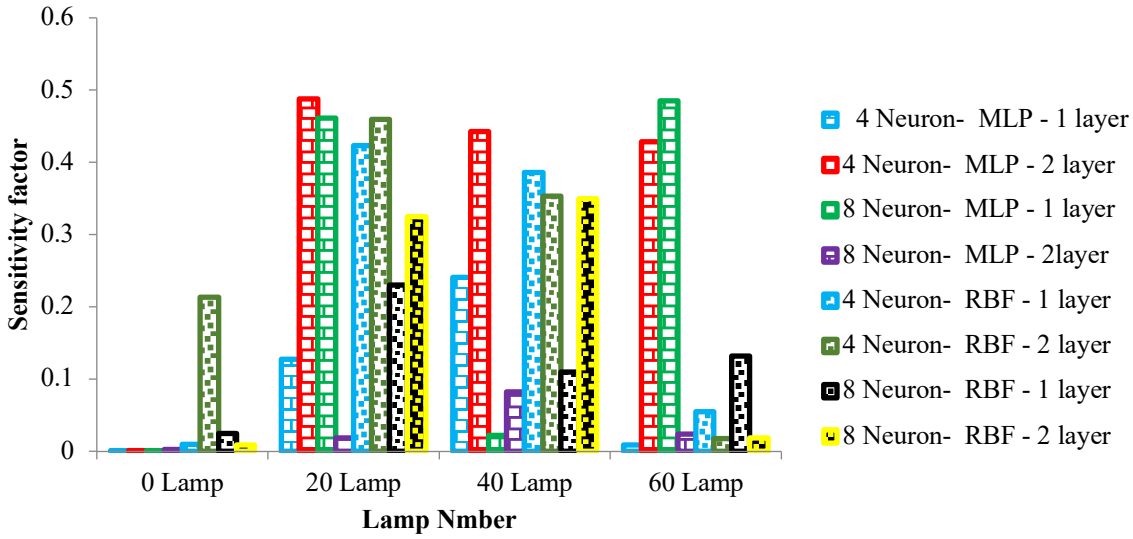

Figure 6. The sensitivity coefficient a* in number of different lamps

4. Conclusions

According to the results of the network, the MLP network classification to categorize all the data accurately (100%) for the training, and the network have an appropriate R-value, and all R^2 for networks with 4, 8, and 1 neuron And 2 hidden layers above 0.99, which indicates the high ability of this network. Also, the network with 2 hidden layers and 4 neurons in the hidden layer had the least amount of MSE, and for a network with 8 neurons, the MSE was less than the MLP network with 4 neurons and 1 hidden layer, as well as the RBF network. For the sensitivity coefficient, the results also showed that for L*, the number of lamps were 20, the highest was the sensitivity coefficient and was the lowest in 0 lamps. According to the results of the MLP network with 4 neurons in the hidden layer in the 1st and 2nd layer, the highest sensitivity coefficient was found for the number of 20, 40 and 60 lamps. The sensitivity coefficient of 0 bulbs with the RBF network had the highest sensitivity coefficient and in total it can be argued that the MLP neural network efficiency was better for the color change classification than the RBF network.

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