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PAPER

BIOACTIVE AND PHARMACOKINETIC CHARACTERISTICS OF PRE-MATURED BLACK RASPBERRY, *RUBUS OCCIDENTALIS*

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ABSTRACT

Black raspberry (BR, *Rubus occidentalis*) is a berry originating from North America. It has a high anthocyanin and flavonoid content that seems to be dependent on ripening. Therefore, the bioactive and pharmacokinetic effects of BR were evaluated using pre-matured BR(PBR) collected from May to June. The total polyphenol, flavonoid and vitamin C content in PBR decreased while anthocyanin increased from 0.13 to 10.70 mg/g at 35 days after fruit set. The antioxidant activities due to DPPH and ABTS determination decreased as BRs were matured. Moreover, it is clear that the content of many phenolic compounds including gallic acid, caffeic acid, p-coumaric acid, ferulic acid, rutin, myricetin, luteolin and kaempferol was diminished during maturing. Both ferulic acid and rutin of BR generated the highest decrease as compared to other phenolic compounds. The pharmacokinetic T_{max} and C_{max} of PBR were 0.6 h and 0.264 μg/mL, respectively. Based on these results, PBR can generate qualified functionality as food and/or as a raw medicinal material.

Keywords: black raspberry, anthocyanin, flavonoid, polyphenol, pharmacokinetic

1. INTRODUCTION

Due to concerns for wellbeing and lifestyles of health and sustainability (LOHAS) resulting from a sharply increasing income, the consumption for functional foods is also growing very fast. Many studies have been conducted to develop functional food products containing natural, bioactive compounds to meet consumer demand. Berries – such as blueberry, strawberry, and black raspberry – are used as common functional food additives to meet demand from health-conscious consumers. Black raspberry (BR, *Rubus occidentalis*), which originated from North America, is one of the main functional food additives due to its pharmacological effects that were written about in *Donguibogam*, an ancient Korean medicinal textbook written by J. Hur (CHO *et al*., 2005). Most of the BR cultivated in Gochang, Korea belongs to *Rubus occidentalis*. However, the BR written of in *Donguibogam*, Korea is *Rubus coreanus* Miquel. BR is normally cultivated in soil with pH 6.0–7.2, and it shows medium water usage (OZGEN *et al*., 2008). Since BR is generally cultivated in moderate to low incident light on coarsely textured soil with average to low levels of organic matter and inorganic nitrogen, BRs form in June in Korea (BAJCZ, 2014). A morphological study indicates BR is a multi-stemmed shrub that forms broad colonies. BR contains thorns with three (ternate) or five palmately arranged leaflets (LEE *et al*., 2014). BR seems to generate a bright red flower in May, and it tends to breed a floral leaf that is shorter than its sepal. It has semi-spherical shape with naps. BR berry will turn to wine in color when the berry has matured. Normally, edible BR berries are harvested at the early stage of summer. However, some pre-matured BR berries are also gathered as medicines to improve sexual dysfunction of males, as mentioned in *Donguibogam* (ZHAO *et al*., 2011).

Approximately 1.7% of anthocyanin in BR tends to be responsible for most BR functions. The main anthocyanins of BR are cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sambubioside, and cyanidin 3-xylosylrutinoside (TIAN *et al*., 2006; TULIO *et al*., 2008; LEE *et al*., 2013). TORRE and BARRITT (1977) have indicated that both cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside of BR anthocyanin seem to contribute to the red or black color and antioxidant effect. In addition, as mentioned above, J. Hur during the Joseon Dynasty indicated that BR (*Rubus coreanus* Miquel) has anti-biotic, anti-cancer, and anaphylaxis effects (BAEK *et al*., 2005). Such effects have been confirmed by many current scientists (BAEK *et al*., 2005; LIU *et al*., 2005; DUNCAN *et al*., 2009; JEONG *et al*., 2010; LEE *et al*., 2011). A study conducted by CHO *et al*. (2005) showed that BR can increase antioxidant activities, and it was confirmed through ABTS, DPPH and TBARS when 1 g BR is added to 100 mL of 10 or 100% ethanol. The study also showed that protocatecuic acid is the most abundant phenolic compound in BR when analyzed *via* HPLC (CHO *et al*., 2005). CHAE *et al*. (2014) and BOBINAITE *et al*. (2012) demonstrated that BR contains a high amount of ellagic acid. They also reported that ellagic acid of BR possesses strong antioxidant effects (BOBINAITE *et al*., 2012; CHAE *et al*., 2014). Moreover, since ellagic acid is not easily metabolized by microflora and/or broken down by stomach acids, it is commonly selected as standard index material for berries.

To increase the use of BR as a functional food additive in Korea, BR was collected and its individual phenolic content was determined in this study. Based on the phenolic content of BR, its antioxidant effects were examined for BR depending on the stage of maturation. In addition, to evaluate the pre-matured BR (PBR, <28 days after fruit set) value and its quality as a functional food additive, the ellagic acid content, which is the standard material index for berries, was evaluated to determine the pharmacokinetic index of PBR.

2. MATERIALS AND METHODS

2.1. Sample preparation

According to research conducted by CHUNG *et al*. (2008), BR planting soil in Gochang, Korea mostly consisted of silt loam (64%), loam (35%) and clay (1%). BRs were cultivated in an open field with soil at pH 5.3~6.7 and harvested from 4-year-old BR plants. All BRs were sampled from May to June of 2014 with an interval of 3-4 days (Fig. 1). The average temperature and humidity of Gochang, Korea during harvest, was 17.4~21.7°C and 72.2~81.0 %, respectively (KMA, 2017). All BRs collected were similar to the Shuttleworth, which was confirmed by the random amplified polymorphic DNA (RAPD).
After harvest of the BR, they were dried at 60°C for 36 h and then powered for storage. 10 g of BR powder were sampled and mixed with 90 mL double distilled water (DDW). The mixture was distilled twice at 80°C for 2 h. All BR extracts were filtered, freeze dried, and stored at -70°C until use.



Figure 1. Pre-matured black raspberry (PBR, *Rubus occidentalis*) and matured black raspberry fruits harvested from May to June with an interval of 2-3 days.

2.2. Determination of the total phenolics, flavonoids, anthocyanins, and ascorbic acid content

The total phenolics of BR were measured using a modified Folin-ciocalteu colorimetric method (SINGLETON and ROSSI, 1965). 20 μL of filtered BR (1 mg/mL) was mixed with water and Folin-ciocalteu reagent. After 3 min of incubation at room temperature, 300 μL of 20% Na_2CO_3 were added. The mixture was then incubated in the dark for 30 min at

40°C. The absorbance was measured at a wavelength of 725 nm (Beckman Du 730, Beckman Coulter Inc., Fullerton, CA, USA) and expressed as its gallic acid equivalent.
The flavonoid content was determined using the method described by MEDA *et al*. (2005) with slight modifications. Briefly, 0.25 mL of BR (1 mg/mL) were mixed with 1 mL DDW, 0.075 mL of 5% $NaNO_2$, 0.075 mL of 10% $AlCl_3$, and 0.5 mL of 1 M NaOH. The final volume was then adjusted to 2.5 mL with DDW. The absorbance of each sample was then measured at a wavelength of 410 nm (Beckman Du 730, Beckman Coulter Inc., Fullerton, CA, USA). Quercetin was used as a standard. All readings were expressed as micrograms of quercetin equivalent.
Monomeric anthocyanin was evaluated using the modified pH differential method (CHAOVANALIKIT and WROLSTAD, 2004). Optimum dilution for BR filtrate was defined using 0.025 M of potassium chloride buffer. Individual BR was diluted with either potassium chloride buffer or sodium acetate buffer to reach an optimum state. After 15 min of incubation, the absorbance of each diluted BR was measured at a wavelength of 700 nm. The amount of anthocyanin was calculated as follows:

$$\text{Anthocyanin (mg/g)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

A: absorbance of diluted BR = $(A_{\lambda vis\text{-}max} - A_{700nm})$ pH1.0 – $(A_{\lambda vis\text{-}max} - A_{700nm})$ pH 4.5;
MW: expressed as cyanidin 3-glucoside (450);
DF: dilution factor;
ε: molar absorptivity (26,900).
The ascorbic acid content was determined using the modified method described by KAMPFERNKEL *et al*. (1995). Briefly, 0.8 mL of 10 % (w/v) trichloroacetic acid were added to 200 μL of BR filtrate (1 mg/mL). Each mixture was then pre-cooled and centrifuged at 3,000 rpm (4°C) for 5 min. Then, 1.5 mL water and 0.2 mL 0.2 N Folin-ciocalteu were added to 0.5 mL of BR supernatant. After 10 min of incubation at room temperature, the absorbance was recorded at a wavelength of 765 nm. L-ascorbic acid was used as a standard.

2.3. DPPH and ABTS radical scavenging activities

The 1,1-dipenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity of each extract sample was determined using the method described by BRAND-WILLIAMS *et al*. (1995). Briefly, freeze-dried BRs were diluted with methanol to obtain five different concentrations. Each diluted BR (10 μL from 1 mg/mL of BR) was mixed with 0.2 mM of DPPH in dimethyl sulfoxide. After 30 min of incubation at room temperature in the dark, the absorbance was measured at a wavelength of 517 nm using a spectrophotometer. The inhibition percentage was calculated from the equation below:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{Control absorbance} - \text{BR absorbance})/\text{Control absorbance}] \times 100$$

The 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activities of the BR extract were determined using the method described by RE *et al*. (1999). 2.45 mM of potassium persulfate was added to 7 mM of ABTS and the mixture was kept in the dark at room temperature for 12-16 h. The ABTS radical cation solution was diluted with phosphate buffer saline to obtain an absorbance value of less than 0.70 at a wavelength of 734 nm before the analysis. After adding 2.0 mL of diluted ABTS radical cation solution to 20 μL of BR sample (1 mg/mL), the reaction mixture was incubated in a cuvette at 30°C for 6 min. Trolox, an analog of vitamin E antioxidant, was used as control.

The ABTS radical scavenging capacity of the BR was calculated using the following equation:

$$\text{ABTS free radical scavenging activity (\%)} = [(\text{Control absorbance} - \text{BR absorbance})/\text{Control absorbance}] \times 100$$

All DPPH and ABTS values calculated using Graphpad Prism 5.0 (Graphpad Prism Version 5.0, Graphpad Software Inc., San Diego, CA, USA), were generated as the 50% inhibition concentration (IC_{50}).

2.4. Liquid Chromatography analysis for the phenolic quantification and ellagic acid identification

Five g of BR were homogenized in 20 mL of 80 % acetone containing 0.2 % formic acid for 1 min. BR was then concentrated, mixed with 10 mL of acidified water, and passed through an activated C18 Sep-Pak cartridge (Waters Corp., Milford, MA). All adsorbed phenolics onto the C18 column were then recovered with 2 mL of acidified methanol containing 3% formic acid. Individually recovered BR in methanol was filtered, and 10 μL of BR methanol extract was used to analyze the phenolics using liquid chromatography (LC) (Acquity H-class, Waters) equipped with an autosampler/injector and photodiode Array (PDA) detector. A Shiseido Capcellpak C18 UG (5 um, 4.6 × 250 mm) was used for separation. Both mobile phase A (0.2 M ortho-phosphoric acid, pH1.57) and B (20% 50 mM ammonium dihydrogen phosphate, pH2.6 in 80% acetonitrile) were used for elution. The flow rate was 1.0 mL/min, and detection was performed at 280 nm (gallic acid), 320 nm (caffeic acid, p-coumaric acid, ferulic acid) and 360 nm (rutin, myricetin, luteolin, kaempferol). A gradient was employed as follows: 0-10 min, 80% A; 10-15 min, 70% A; 15-20 min, 60% A; 20-25 min, 10% A; 25-30 min, 10 % A; 30-40 min, 95% A. All phenolics were identified and quantified using external standards (gallic acid, caffeic acid, $_p$-coumaric acid, ferulic acid, rutin, myricetin, luteolin, and kaempferol). Data scanned at 280–360 nm were collected. All LC handling steps were followed as mentioned in a previous study (CHAE *et al*. 2014).

2.5. Animal model and blood sample preparation

Male Sprague Dawley rats (7 wks, 220 g) were obtained from KOSA Bio, Korea. A total of six rats were acclimated in an environmentally controlled breeding room at a temperature of 22±2°C and relative humidity of 50±10 % with a 12 h dark/light cycle for one week before being used for the experiments. They were provided *ad libitum* access to commercial chow and water. All rats were handled in accordance with the recommendation of the Regulations for the Administration of Affairs Concerning Experimental Animals (BBRI-IACUC-16001). PBR at a dose of 150 mg/kg was orally administered to each rat. A serial of blood samples was collected at 0, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post oral administration. All blood samples were immediately transferred to a BD vacutainer lithium heparin tube and centrifuged at 3,000 rpm for 10 min at 4°C. The pH of rat plasma (100 μL) was adjusted to pH 2.5 using 1 M potassium dihydrogen phosphate solution. Serial addition of 50% phosphoric acid and acetonitrile was then performed. The plasma samples were centrifuged at 12,000 rpm for 8 min under refrigerated conditions. The supernatant was taken, dried, and re-dissolved in 0.1 mL methanol. Each supernatant sample was then used for ellagic acid analysis under the LC/MS/MS conditions summarized in Tables 1 and 2.

Table 1. Liquid chromatography (LC)/MS/MS[1] analytical conditions used for analysis of ellagic acid in Sprague Dawley rat serum.

Parameter	Condition		
HPLC system	Agilent 1290 Infinity Binary HPLC system with 6420 triple quadrupole LC/MS system		
Column	Shiseido Capcellpak C18 UG (5 μm, 4.6×250 mm)		
Column Temperature	40°C		
Flow rate	1.0 mL/min		
Injection volume	10 μL		
Mobile phase	A : 0.1% Formic acid		
	B : Methanol		
Gradient step	**Time (min)**	**A (%)**	**B (%)**
	0	70	30
	7.0	50	50
	13.0	10	90
	15.0	70	30
	18.0	70	30

[1]Ionization Type: ESI (Electospray ionization) Negative; Gas temperature (°C): 320; Gas flow (L/min): 9; Nebulizer (psi): 15; Capillary (v): 4,000; Scan mode: Multiple reaction monitoring (MRM).

Table 2. Liquid chromatography (LC)/MS analytical conditions used for analysis of ellagic acid in pre-matured black raspberry (PBR, *Rubus occidentalis*) extract.

Compound	RT[1] (min)	MW[2]	Precursor Ion (MS m/z)	Product Ion (MS, m/z) Confirm ion			Frag (V)	Collision Energy (V)
Ellagic acid	12.1~12.3	302	30[M-H]$^-$	229	257	185	170	20/16/15

[1]RT: Retention time; [2]MW: Molecular Weight.

2.6. Pharmacokinetic analysis

5 μL of different concentrations (0.625, 1.25, 2.5, 5.0, and 10.0 μg/mL) of ellagic acid in plasma were used to obtain the ellagic acid calibration curve (final concentrations of 31.25, 62.50, 125, 250, 500 ng/mL). As a result of ellagic acid calibration (R^2 = 0.998), a linear regression equation was obtained. Each plasma sample was then injected to LC/MS/MS. The pharmacokinetic parameters of ellagic acid were calculated using WinNonlin software version 6.3 on non-compartmental analysis (Pharsight Cor., Mountain View, USA).

2.7. Statistical analysis

All experiments were carried out in triplicates or quadruplicates and expressed as mean ± standard deviation. Statistical analyses were performed using SPSS program (SPSS version 12.0, SPSS Chicago, IL, USA). Unpaired t-tests or one-way repeated measures ANOVA were performed when appropriate. If significant in the ANOVA test, differences in the

means were determined using Duncan's multiple range tests. Statistical significance was considered when the *p*-value was less than 0.05.

3. RESULTS

3.1. Quality properties and phenolic compound of *Rubus occidentalis*

The anthocyanin content in BR increased from 0.13 to 10.70 mg/g, although the total polyphenols, total flavonoids, and vitamin C content in BR decreased during maturing (Table 3). The DPPH and ABTS radical scavenging activities of BR collected on June 19th were 8.44 and 4.53 times higher, respectively, compared to those of BR collected on May 23. This indicates that antioxidant activities decreased while maturing. Gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, myricetin, luteolin, and kaempferol were detected in BR (Table 4). Both gallic acid and ferulic acid were found to be its major phenolics. Depending on the BR harvest day, the amount of eight different phenolics decreased ($P < 0.05$) when collected on June 19th, the last day of harvest. Five phenolics (caffeic acid, *p*-coumaric acid, rutin, myricetin, and kaempferol) had the maximum contents on May 26 or May 30. However, the luteolin content was the highest on June 9th. For this reason, luteolin generated the least disappearance, and only 42.5% of luteolin disappeared when BR was collected on June 19. However, only 1% rutin was left at the end of harvest. The rutin and ferulic acid contents at the end of the harvest were 1 and 3%, respectively. The content decreases for both were the highest compared to the other six phenolics.

3.2. Pharmacokinetic and kinetic analysis

PBR was orally administrated to male Sprague Dawley rats. The plasma concentration-time profile of ellagic acid in Sprague Dawley rats (n = 5) is shown in Fig. 2. Ellagic acid was confirmed and quantified when plasma was sampled at 0.25, 0.5, 1, 2, and 4 h after oral administration of PBR.

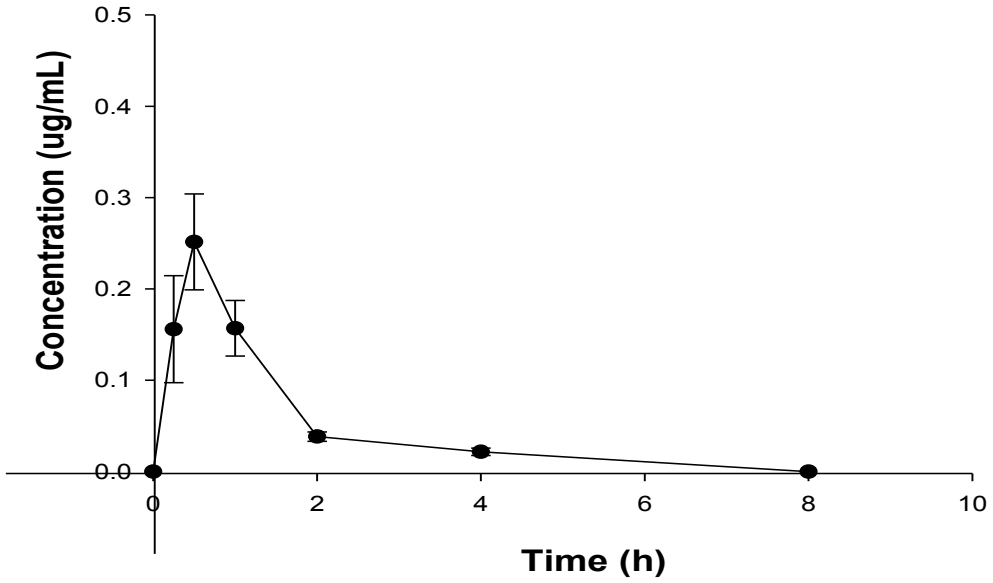


Figure 2. The mean plasma concentration-time profile of ellagic acid in rats after oral administration of pre-matured black raspberry (PBR, *Rubus occidentalis*) at 150 mg/kg (Mean±SD, n = 5).

Table 3. Chemical quality properties of pre-matured black raspberry (PBR, *Rubus occidentalis*)[1] harvested from late May to early June.

Days After Fruit Set (Date)	Total polyphenol (mg/g)	Total flavonoid (mg/g)	Anthocyanin (mg/g)	Ascorbic acid (mg/100g)	DPPH[2] IC_{50} (ug/mL)	ABTS[3] IC_{50} (ug/mL)
15 (May 23)	170.75±4.65^{a}	1.21±0.02^{a}	0.13±0.01^{e}	24.30±0.38^{a}	95.9±4.52^{e}	261.87±10.25^{d}
18 (May 28)	162.16±4.06^{a}	1.10±0.02^{b}	0.18±0.01^{e}	21.86±0.51^{b}	114.57±4.85^{de}	278.13±15.39^{d}
22 (May 30)	148.64±7.78^{b}	1.07±0.04^{b}	0.08±0.01^{e}	20.34±0.14^{c}	126.57±5.69^{de}	266.23±9.58^{d}
25 (June 2)	126.52±8.37^{c}	0.80±0.04^{c}	0.12±0.01^{e}	17.03±0.12^{d}	160.67±5.91^{de}	326.60±25.54^{d}
28 (June 5)	111.41±2.94^{d}	0.68±0.01^{d}	0.05±0.01^{e}	15.25±0.03^{e}	193.97±1.36^{d}	406.13±31.42^{d}
32 (June 9)	79.04±2.87^{e}	0.51±0.05^{f}	0.81±0.05^{d}	16.79±0.94^{f}	418.83±18.75^{c}	677.73±25.28^{c}
35 (June 13)	60.44±2.62^{f}	0.60±0.01^{e}	3.75±0.12^{c}	10.78±0.03^{f}	824.93±59.99^{a}	912.50±4.90^{b}
38 (June 16)	61.16±7.37^{f}	0.45±0.01^{f}	6.08±0.05^{b}	14.42±0.03^{g}	511.13±57.92^{b}	771.53±99.54^{bc}
41 (June 19)	45.15±0.71^{g}	0.52±0.02^{g}	10.70±0.1^{a}	10.62±0.03^{h}	809.77±99.82^{a}	1186.17±294.46^{a}

[1]Values are the Mean±SD (n = 3) based on dry basis.
[2]2,2-diphenyl-1-picrylhydrazyl.
[3]2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid.
[a-h]Values with different superscripts within the same columns are significantly different ($p < 0.05$).

Table 4. Phenolic compositions of pre-matured black raspberry (PBR, *Rubus occidentalis*)[1] harvested from late May to early June.

Days After Fruit Set (date)	Phenolic compound (µg/g)							
	Gallic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Rutin	Myricetin	Luteolin	Kaempfrol
15 (May23)	3321.72±1.25^{a}	381.71±52.17^{ab}	48.38±46.51^{ab}	1733.63±53.32^{a}	326.11±131.49^{a}	63.66±5.18^{ab}	2.52±0.13^{b}	11.40±0.31^{b}
18 (May26)	2638.79±6.67^{b}	390.88±72.29^{a}	52.40±22.98^{a}	1260.60±19.55^{b}	398.79±100.58^{a}	60.59±4.34^{b}	2.73±0.05^{b}	13.56±0.38^{a}
22 (May30)	3040.08±0.01^{c}	325.25±65.51^{ab}	31.54±13.53^{abc}	1241.73±15.96^{b}	324.48±126.41^{a}	70.12±5.70^{a}	2.33±0.23^{b}	11.30±2.26^{b}
25 (June2)	1826.81±0.14^{d}	330.65±53.15^{ab}	52.44±5.79^{a}	703.88±6.22^{c}	106.96±77.03^{b}	23.05±5.90^{d}	3.08±0.12^{b}	9.55±0.60^{cd}
28 (June5)	1225.55±2.72^{e}	310.03±33.78^{b}	23.17±14.66^{abc}	414.35±1.26^{d}	37.89±45.01^{b}	30.16±1.10^{c}	3.44±0.07^{b}	10.18±0.10^{bc}
32 (June9)	489.50±1.06^{f}	147.24±9.90^{c}	18.05±0.49^{abc}	119.31±5.28^{e}	38.89±14.71^{b}	12.63±2.46^{e}	6.35±3.39^{a}	8.24±0.17^{d}
35 (June13)	373.67±1.23^{g}	21.21±1.50^{d}	15.34±3.63^{bc}	77.24±0.32^{f}	46.04±53.14^{b}	13.19±4.02^{e}	3.35±0.15^{b}	4.93±0.43^{e}
38 (June16)	303.98±0.19^{h}	46.84±11.87^{d}	12.64±1.32^{bc}	53.15±3.08^{f}	73.47±63.69^{b}	8.79±0.24^{e}	3.22±0.03^{b}	4.47±0.10^{e}
41 (June19)	278.71±3.39^{i}	26.74±1.17^{d}	5.98±0.20^{c}	48.30±0.44^{f}	3.68±3.19^{b}	8.66±0.69^{e}	1.45±1.17^{b}	2.08±0.35^{f}

[1]Values are the Means ± SD (n = 3) based on dry basis.
[a-i]Values with different superscripts within the same columns are significantly different ($p < 0.05$).

Ellagic acid was below the detectable level at 8 h after oral administration of PBR. It had the maximum plasma concentration (250 ng/mL) at 0.5 h post oral administration of PBR. The plasma level of ellagic acid decreased as time went by, and its half life ($t_{1/2}$) was 1.018 h. The results of other pharmacokinetic parameters of ellagic acid are summarized in Table 5.

Table 5. Main pharmacokinetic parameters[1] of ellagic acid in rats plasma after oral administration of pre-matured black raspberry (PBR, *Rubus occidentalis*) at 150 mg/kg (Mean ± SD, n = 5).

	Unit	Mean[2]
AUC_{0-t}	ug h mL^{-1}	0.330±0.109
$AUC_{0-\infty}$	ug h mL^{-1}	0.363±0.118
$t_{1/2}$	h	1.018±0.195
T_{max}	H	0.600±0.223
C_{max}	ug mL^{-1}	0.264±0.109
MRT	h	1.494±0.309

[1]AUC_{0-t}: Area under the curve, $AUC_{0-\infty}$: Area under the curve, $t_{1/2}$: half life time, T_{max}: maximum time, C_{max}: maximum concentration, MRT: Mean residence time.
[2]Each value represents the Mean±SD.

4. DISCUSSION

BR was collected during its period of maturation at an interval of 2-3 days. The results for the amount of anthocyanin indicated that anthocyanin may not be a major component in BR contributing to its antioxidant effects before its complete maturation (Table 3). FLOEGEL *et al*. (2011) reported that high-pigmented and hydrophilic BR antioxidants seem to have both DPPH and ABTS scavenging activities, with higher activities toward ABTS than that for DPPH. In a study conducted by LEE *et al*. (2013), seven different anthocyanins were detectable in BR. Only cyanidin 3-glucoside, which has a lower amount compared to cyanidin 3-rutinoside and/or cyanidin 3-xylosylrutinoside, is found to have stronger antioxidant effect (DPPH scavenging activity) than ascorbic acid, but not Trolox (KÄHKÖNEN and HEINONEN, 2003). However, BORGES *et al*. (2010) suggested that the antioxidant activity of raspberries is influenced by ascorbic acid content rather than cyanidin 3-glucoside in this case, they showed 10.5 and 8.1 % antioxidant activity, respectively. In this study, PBR polyphenol and flavonoid contents were found to have significantly diminished with increase in maturation days. The study also indicated that the amount of ascorbic acid also showed a similar trend as evidenced by both polyphenol and flavonoid content. Therefore, in PBR, both DPPH and ABTS seem to be influenced by the amount of polyphenol, flavonoid and ascorbic acid rather than anthocyanin. This is similar to results obtained in a study conducted by OGAWA et al. (2008), which suggests that ascorbic acid and flavonols contribute to the antioxidant properties of berries.
In PBR, eight major phenolic compounds were detected. Each phenolic compound showed the least amount when BR was harvested on June 19th (Table 4). A study conducted by KANG *et al*. (2015), showed that whole black raspberry did not contain luteolin and myricetin but catechin, epicatechin, rutin, gallic acid and quercetin were detectable. Although our study did not trace any catechin, epicatechin and quercetin due to different analysis conditions, the amount of rutin was similar to that of fully matured black raspberry. In addition to that, many phenolic compounds of PBR were noted to decrease

during maturing periods. CHIRINOS *et al*. (2007) indicated that the contents of both phenolic compounds and flavan 3-ols during maturity stage of 10 mashua cultivars were different, and diminution of both phenolic compound and flavan 3-ols were attributed due to genotypic difference of mashua. Therefore, among BRs, the Shuttleworth at Gochang, Korea is a cultivar that may decrease some phenolic compounds in maturing stag, therefore, ascorbic acid and flavonols may be major factors that contributed to the DPPH and ABTS scavenging activities of BR at the early stage of the harvest.
To determine the LC protocol accuracy (recovery) and repeatability (precision) for ellagic acid determination in both PBR and rat plasma, ellagic acid was used. The recovery (103.01–104.23 %) and relative standard deviation (RSD, ≤ 3.35%) implied that the protocol had satisfactory accuracy and high recovery for ellagic acid. In addition, the RSD of intra- (1.92 %) and inter-day (0.61–2.14 %) differences indicated that the LC protocol was precise. Hence, the LC method seemed to be accurate and precise to quantify ellagic acid in PBR (32.13 ± 0.62 mg/g) (data are not shown). Based on the accuracy and repeatability of LC, ellagic acid plasma concentration-time profile in rats and six major pharmacokinetic parameters were analyzed. The pharmacokinetic results of PBR ellagic acid indicated that PBR ellagic acid in rats seemed to increase rapidly (Table 5), and the C_{max} reached 0.264 μg/mL. The half time of ellagic acid was found to be 1.018 h after oral administration. Similar results were reported in a pharmacokinetic study by LEI *et al*. (2003), in which the maximum concentration of pomegranate leaf ellagic acid in rat plasma is found to be 213 ng/mL after 0.55 h of oral administration. The pharmacokinetic characteristics revealed that ellagic acid seems to have poor absorption and rapid elimination after oral administration (SEERAM *et al*., 2004; BALA *et al*., 2006). The rapid elimination of ellagic acid has been confirmed *via* urine and feces analysis by SMART *et al*. (1986), with up to 70 % ellagic acid being detected in urine and feces.

5. CONCLUSIONS

This is the first study to characterize pre-matured black raspberry (PBR, *Rubus occidentalis*). PBRs are pre-matured black raspberry when BRs are collected from 15 to 28 days after fruit set. The results showed that PBR under 28 days after the fruit set seemed to produce a high content of phenolic compounds, flavonoids, and vitamin C, thereby possessing excellent antioxidant activities. Based on the PBR pharmacokinetic results and chemical analysis along with antioxidant results, PBR can be used as a functional food or medicinal material derived from the nature. Our data could be used us a baseline to develop nutraceuticals, natural colorants, and other related products.

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USE OF ELECTRONIC NOSE TO DISCRIMINATE MEATS FROM BULLS FED DIET WITH OR WITHOUT FLAXSEED INCLUSION AND SUBJECTED TO DIFFERENT AGING PERIODS

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ABSTRACT

A metal-oxide sensors array electronic nose (e-nose) was used to discriminate beef loins (*Longissimus thoracis*) obtained from Piemontese bulls fed without or with flaxseed and subjected to 3 different aging periods (2, 7, 10 days) at 4°C. At 7 days of aging, samples were also assessed for flavor intensity by panelists. A comparison between e-nose and panel assessments was performed subjecting a 7 days e-nose reading on cooked meat to partial least square regression for flavor prediction.

The e-nose could not discriminate populations in meat samples, however it could represent a valuable tool in supporting flavor scoring from sensory evaluation.

Keywords: aging, beef meat, electronic nose, flaxseed, sensory evaluation

1. INTRODUCTION

Red meat has been addressed has having high content of saturated fatty acids (SFA). Several studies have shown a positive relationship between dietary SFA and the onset and development of several widespread human pathologies, such as cardiovascular diseases and various forms of cancer (BOADA *et al*., 2016). In response to consumers demand, in recent years different feeding strategies aiming at reducing SFA and contemporarily at increasing polyunsaturated fatty acids (PUFA), particularly omega-3 fatty acids, in ruminant-derived food products have been developed (SHINGFIELD *et al*., 2013). Omega-3 PUFA bring numerous beneficial effects on human health as they favor normal embryogenesis and brain development, and protect against cancer, cardiovascular and neurodegenerative diseases (CALDER, 2013).

Flaxseed, one of the richest natural sources of α-linolenic acid (C18:3 n-3), has been shown to be an effective feed ingredient in increasing the content of omega-3 PUFA in beef (JUÁREZ *et al*., 2011). However, an increase of highly unsaturated fats may pose to alterations of meat flavor, mainly because of the derived greater susceptibility to oxidative breakdown (JUÁREZ *et al*., 2012). Moreover, when the proportion of C18:3 n-3 approaches 3% of muscle fatty acids, flavor liking scores assessed by human panelists can be significantly altered, even in case of only slight decreases of lipid oxidative stability (Wood *et al*., 2008).

Therefore, when applying feeding strategies to increase the omega-3 PUFA content of meat, the associated investigation of meat flavor is determinant. Flavor is a very complex attribute of meat palatability; it chemically acts on taste and smell receptors, and plays a key role in acceptability by consumers (KHAN *et al*., 2015). Meat flavor has traditionally been evaluated either by trained assessors or by head-space gas chromatography or mass spectrometry, these methods being time-consuming, labor-intensive and costly, particularly for routine quality control application. The development of objective automated non-destructive techniques that can easily and rapidly characterize meat flavor is an impelling need for the meat industry (NARSAIAH and JHA, 2012). Chemical sensor systems (i.e., electronic noses) are technologies for the at- or on-line discrimination of populations according to volatile compounds. These systems involve various types of electronic chemical gas sensors and with partial specificity which, combined to suitable statistical methods, allow for pattern recognition of simple or complex families of volatile chemical compounds (GHASEMI-VARNAMKHASTI *et al*., 2009). Over the last twenty years, several studies have been carried out using the electronic nose (e-nose), as a rapid and non-destructive method, to assess meat quality (GHASEMI-VARNAMKHASTI *et al*., 2009; Hong *et al*., 2012; LOUTFI *et al*., 2015). Some studies also showed the potential of e-nose to aid or replace olfactory sensory analysis of meat performed by trained panelists (MILDNER-SZKUDLARZ *et al*., 2007), but limited literature is currently available correlating e-nose response to flavor intensity assessed by sensory panels (LOUTFI *et al*., 2015).

The aims of this study were: (i) to evaluate whether the e-nose could be used to discriminate meat beef loins (*Longissimus thoracis* muscle; LM) samples obtained from bulls fed diets without or with flaxseed and aged for 2, 7 or 10 days, and (ii) to compare, at 7 days of aging, e-nose reading and sensory panel evaluation in the assessment of meat flavor intensity.

2. MATERIALS AND METHODS

2.1. Animals, dietary treatments and sampling procedures

Animal care and experimental procedures were carried out in compliance with European Union legislation on the protection of animals used for scientific purposes (EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION, 2010).
Eighteen male calves of the Piemontese breed (4.5 ± 0.59 months old; mean ± sd) were purchased from a local dealer and randomly allotted into two pens (9 animals/pen). Animals had free access to fresh water and were fed for 172 days the same base diet (adaptation period) consisting of a commercial concentrate for fattening cattle, ryegrass hay, corn meal, distillers dried grains, dried beet pulp and soybean meal. The adaptation period was followed by a treatment period of 135 days during which pens were randomly assigned to two treatment diets: control or flaxseed diet. The amount of ground flaxseed (dry matter (DM): 917 g/kg; ether extract: 360 g/kg DM; α-linolenic acid: 200 g/kg DM) in the flaxseed diet was set at 100 g/kg DM. All diets (Table 1) were formulated according to NRC (NRC, 2000) to fulfill the nutritional requirements of Piemontese young bulls.
At the end of the treatment period animals were slaughtered. A portion of the LM between the 8th and the 10th thoracic vertebra from the right side of the carcass was taken 24 h after slaughter and transferred under refrigerated conditions to the lab. Then, the LM was cut into three 2 cm thick steaks. Four equal subsamples were obtained from the first steak; each subsample was sealed in a commercial food grade polymer bag and kept for 2, 7 (two subsamples) or 10 days (d) in a controlled environment at 4°C, away from direct light, until e-nose headspace analysis. One subsample was aged for 7d at 4°C, then vacuum-packed and stored at -80°C until sensory evaluation by panelists.

2.2. Analysis of feed

AOAC International (2000; 2003) procedures were used to determine DM, ash, crude protein (CP), ether extract (EE) in flaxseed and diets. Feed chemical composition was expressed as g/kg DM.
Feed fatty acid (FA) composition was assessed as described by RENNA *et al.* (2014). Feed FA results are reported as g/kg of total detected FA.
The proximate and the main FA compositions of the diets are reported in Table 1.

2.3. E-nose procedure

A PEN 3 Portable Electronic Nose (Airsense Analytics GmbH, Schwerin, Germany) equipped with an array of 10 metal-oxide sensors (Table 2) and a pattern recognition software for data recording and processing (WinMuster, v. 1.6.2.13) was used.
The meat subsamples were subjected to e-nose reading either as raw (2, 7 and 10d) or cooked (7d). The meat was cooked in a flask in a water-bath at 70°C for 30 minutes. Before starting the e-nose assay a calibration procedure was carried out to account for variations in relative humidity of the air, temperature and possible drift of sensors over time. The air filtered through an active carbon filter was used as zero gas. At the end of the calibration procedure the sensors responses were recorded (G0). Then, about 50 grams of raw or cooked meat were cut into 2 cm^3 particles and put into a 250 mL flask equipped with teflon/silicon septum cup and let it stand for 30 minutes at 25°C to allow for a uniform distribution of gasses in the flask headspace before the e-nose analysis. Upon analysis, a needle connected to the e-nose was used to perforate the septum of the flask containing the meat sample and air of the headspace was absorbed into the air detection chamber

with a flow rate of 400 mL/min. Before each sample reading, the detection chamber was flushed for 330 s with reference air (air filtered through an active carbon filter) for sensors recovery. Then, upon flowing of headspace sample air the sensors' responses (G) were recorded once per second and for 60 s. The 60 s measurement interval was selected to allow sensors to reach a stable signal value. The sensor response to the substances in the headspace was defined by the conductance ratio G/G0. A G/G0 threshold value of 6 was set for the sensor number 2 in the array (W5S) through an automatic dilution system to protect the sensor array from overloading.

Table 1. Ingredients, proximate composition and main fatty acid profile of the experimental diets fed in the treatment period.

	Control	Flaxseed
Ingredients (g kg^{-1} DM)		
Concentrate A	333	258
Corn meal	254	294
Dried sugar beet pulp	160	102
Ryegrass hay	121	151
Barley meal	78	61
Ground flaxseed	0	100
Soybean meal	35	14
Concentrate B	22	23
Proximate composition (g kg^{-1} DM)		
DM (%)	86	87
CP	173	170
EE	38	75
Ash	66	60
Net Energy (MJ kg^{-1}DM)	7.92	8.19
Main fatty acid composition (g kg^{-1} of TFA)		
C16:0	173.20	109.20
C18:0	22.60	24.90
C18:1 n-9	260.65	240.95
C18:2 n-6	492.50	319.15
C18:3 n-3	31.15	292.80

Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract; TFA, total fatty acids.
Concentrate A: corn, wheat middlings, sunflower meal, roasted dehulled soybean meal, wheat bran, roasted soybean meal, vitamins and minerals; Concentrate B: corn germ meal, wheat middlings, wheat bran, corn, vitamins and minerals.

Table 2. Sensitivity and selectivity of the sensors in the portable electronic nose device (PEN 3 Portable Electronic Nose, Airsense Analytics GmbH, Schwerin, Germany).

Number in array	Sensor	General description	Reference
1	W1C aromatic	Aromatic compounds	Toluene, 10 ppm
2	W5S broad range	Broad range sensitivity react on nitrogen oxides and ozone, very sensitive with negative signal	NO_2, 1 ppm
3	W3C aromatic	Ammonia, used as sensor for aromatic compounds	Benzene, 10 ppm
4	W6S hydrogen	Mainly hydrogen, selectively (breath gases)	H_2, 100 ppb
5	W5C aromatic-aliphatic	Alkanes, aromatic compounds, less polar compounds	Propane, 1 ppm
6	W1S broad methane	Sensitive to methane (environment) ca. 10ppm, broad range, similar to W2S	CH_4, 100 ppm
7	W1W sulphur organic	Reacts on sulphur compounds (H_2S 0,1ppm) otherwise sensitive to many terpenes and sulphur organic compounds, which are important for smell (limonene, pyrazine)	H_2S, 1 ppm
8	W2S broad alcohol	Detects alcohol's, partially aromatic compounds, broad range	CO, 100 ppm
9	W2W sulphur-chlorine	Aromatic compounds, sulfur organic compounds	H_2S, 1 ppm
10	W3S methane-aliphatic	Reacts on high concentrations >100ppm sometimes very selective (methane)	CH_4, 100 ppm

2.4. Sensory evaluation

The steaks were placed in a refrigerator to thaw for 24 h at 4°C, then cooked without salt or spice addition in a double plate grill, preheated at 250°C, until the final internal temperature reached 70°C, which was monitored by individual thermocouples inserted into the geometric center of each steak (American Meat Science Association (AMSA), 1995). Upon reaching 70°C, the steaks were trimmed of external connective tissue, cut into 1.3 x 1.3 x 2 cm samples, wrapped in a foil pouch and labeled with three-digit random numbers. A sensory quantitative affective test based on intensity scale (MEILGAARD *et al*., 2006) was performed by 39 males and 24 females consumers, ranging in age from 21 to 60 years old. Panelists recruited for testing the samples, previously involved in surveys on beef preference/acceptance tests, were regular consumers of beef and had not diet restriction or allergies.
Samples from each treatment were randomly served one at a time to each panelist. Five sessions with approximately 12 panelists per session were carried out in individual booths in a sensory testing laboratory under artificial white lighting. Four samples (two per dietary treatment), each served 5 minutes apart, were offered to each consumer per session, for a total of 252 assessments over the five sessions. Panelists evaluated beef flavor intensity using an unstructured scale, consisting of a 15 cm long horizontal line, with anchor points labeled with the expression "extremely bland" (0 cm) and "extremely intense" (15 cm) (MEILGAARD *et al.,* 2006). The panelists expressed each evaluation by making a vertical line across the horizontal line at the point best reflecting their perception of the magnitude of flavor. The panelists were asked to rinse their mouth with still water served at room temperature during the one minute break imposed between consecutive samples.

2.5. Statistical analysis

All analyses were performed using SAS (Statistical Analytical System (SAS), 2003). Significance was declared at $P \leq 0.05$.
The e-nose measurement produced 60 readings for each sensor for a total of 600 readings for each sample. However, multiple readings from a sensor are correlated each other. Therefore, among the sixty available readings from each sensor only one (59^{th}) and in a plateau condition was considered for subsequent analysis, for a total of 10 values/sample.
The Mahalanobis Distance (MD) was used to calculate similarity between samples within classes (i.e. aging and diet).
The clustering of samples was investigated based on the sensors activation patterns. At first, variables (W1C, W5S, W3C, W6S, W5C, W1S, W1W, W2S, W2W, W3S) were subjected to the Stepdisc procedure and all of them were suited for entering the discriminant analysis. Then, the dataset was subjected to the Factor procedure for Principal Component Analysis (PCA) and the Candisc procedure for Canonical Discriminant Analysis (CDA).
Sensory evaluation data were subjected to the GLM procedure. The model included the diet as fixed effect whereas the panelist and diet x panelist interaction entered the model as random effects (NAES *et al.,* 2011).
The relationship between e-nose data, either on raw or cooked samples, and sensory scores assessed by panelists was analyzed by partial least square regression (PLS) for flavor prediction.

3. RESULTS AND DISCUSSION

3.1. E-nose analysis

When exposed to the headspace gas the sensor array produces a particular pattern in which each curve represents a different transient sensor response (Fig. 1). The x-axis represents the time of reading and the y-axis the sensors' ratio of conductance. For some sensors, the conductivity grows rapidly and then decreases to a stable condition whereas for some others the change in resistance, and therefore the G/G0 ratio is minimal or below the unity. Only one point toward the end of sample measurement was considered for each sensor and their mean responses within classification groups (i.e. diet and aging) are shown in Fig. 2. The polar plot suggests some sensors are more relevant than others in terms of signal response between control and flaxseed within the day of aging. Sensors W1C, W3C, W5C, W1W, W2W and W3S differentiate between diets at 7d of aging (P<0.05). Detailed information about sensors characteristics are not available in literature (SMYTH and COZZOLINO, 2013), as well as there are no reports that interrelate the response of a sensor to a particular chemical component. Nevertheless, association between sensors and groups or families of substances are outlined (Table 2) and from this we can address strongest reactive sensors at 7d (except broad range sensors: W1S, W5S) into three groups: sensors reactive to aromatic compounds (W1C, W3C and W5C; in which samples from flaxseed fed animals were in average 12% lower than control), sensors reactive to sulfur compounds (W1W, W2W; in which samples from flaxseed fed animals were in average 37% lower than control) and sensor W3S reactive to high concentration of methane in which samples from flaxseed fed animals were in average 10% higher than control.

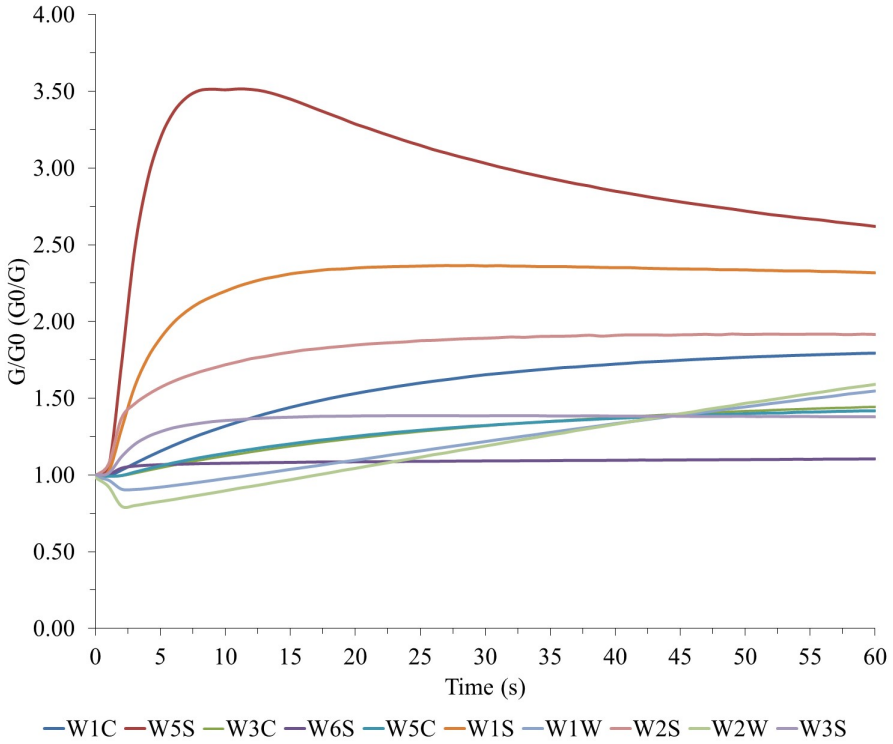


Figure 1. Example of electronic nose reading. The sensor gas response is expressed as G/G0 or G0/G (for sensors showing a negative behavior in presence of chemical compounds; W1C, W3C, W5C), where G and G0 represent the resistance of the sensor in sample gas and in zero gas air, respectively.

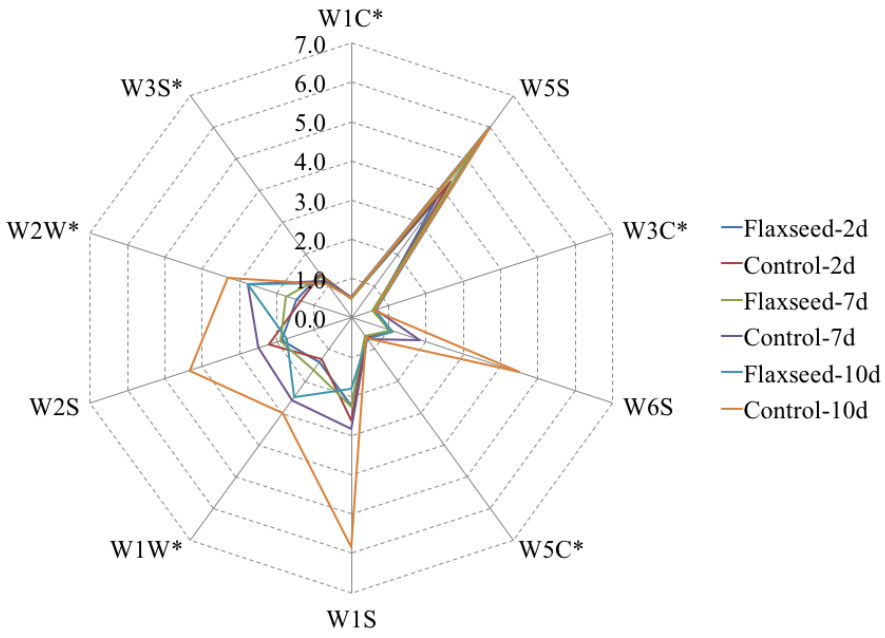


Figure 2. Polar plot of the average responses of sensors when exposed to raw meat samples at different aging (2, 7 or 10 days) and from animals fed control or flaxseed diet (n = 9). The gas response is expressed as the G/G0 ratio, where G and G0 represent the resistance of the sensor in sample gas and in zero gas air, respectively. Values with superscript (*) and within the 7 days aging differentiate for $P<0.05$

The MD classifies the observation into the nearest population by calculating the distance between the unit vector and the centroid for population. The MD takes into account the correlation of the data within the cluster, it is unit less and it measures how many deviations is the value from the cluster centroid. The MD enlarged with the increase of aging (Table 3).

Table 3. Mahalanobis distance of sensors (between class means).

	Control			Flaxseed		
Day	**2**	**7**	**10**	**2**	**7**	**10**
2	0	9.176	14.909	0	4.914	12.117
7	9.176	0	7.344	4.914	0	8.714
10	14.909	7.344	0	12.117	8.714	0

As previously reported (HONG *et al.*, 2012) also in the present work the e-nose seems capable of suggesting divergences between samples kept for different length of time at 4°C. The increments of the distances were different among samples from animals being fed different diets. The distances in flaxseed were 54% and 81% compared with control, respectively at 7d and 10d. Even though less pronounced (HONG *et al.*, 2012), we could speculate that in our condition the large MD in the control group might suggest an early

start of the aging related modification leading to different responses of the e-nose sensor array. Nevertheless, after an initial latency time, at 10d the MD of flaxseed seemed similar to the control.
Correlation patterns with sensors were obtained with PCA to help in the discrimination process among meat samples. The stepdisc procedure suggested all sensors could be included in the discriminant analysis (SAS, 2003) and a 0.66 value for the Kaiser measure of sampling adequacy exceeded the threshold value of 0.60 (STEVENS, 2009), therefore supporting the data set as suitable for the PCA analysis (CERNY and KAISER, 1977).
The PCA is a variable reduction method yielding linear combination of original variables (principal components, PC). The maximum number of PC equals the number of considered variable (i.e. number of sensors). The latent constructs were obtained with the PRIN method of the proc Factor procedure, with Varimax rotation, and retained in accordance to the eigenvalue-one criterion (STEVENS, 2009). Then, variables loading vectors and PC scores were obtained.
Following a Varimax orthogonal rotation, three factors were extracted explaining 95.2% of the total variability of data (Table 4). By giving a magnitude of at least 0.4 as indicator of a salient variable-factor relationship, the sensors W5S, W1W, W2W and W3S loaded on PC1 (32.89%), sensors W1C, W3C and W5C loaded on PC2 (31.39%) whereas sensors W6S, W1S and W2S loaded on PC3 (30.95%). The orthogonal factor rotation simplifies the interpretation of extracted factors and from that we could suggest PC1 as related mainly to the proteolysis activity, PC2 mainly addressing processes leading to aromatic compounds formation, whereas PC3 included the broad range sensors and a sensor reactive to hydrogen. To identify pattern of correlation among sensors responses, score coefficients for each variable were obtained and principal component scores for each sample were calculated. Fig. 3 shows a two-dimensional plot of the analysis score of meat samples with PC1 and PC2. The control at 7d and 10d and the flaxseed at 10d tended to cluster in the positive quarter for the considered PCs.

Table 4. Loading vectors of sensors on Varimax rotated extracted PC and proportion of explained variance.

	PC1	PC2	PC3
W1C	0.02	0.96*	0.03
W5S	0.90*	-0.28	0.08
W3C	0.11	0.99*	-0.04
W6S	0.32	0.02	0.94*
W5C	0.17	0.97*	-0.08
W1S	0.16	-0.07	0.98*
W1W	0.84*	0.31	0.36
W2S	0.22	-0.06	0.97*
W2W	0.87*	0.29	0.36
W3S	-0.90*	-0.19	-0.20
Proportion	32.89	31.39	30.95

PC = Principal Component, *Variables loaded on extracted components (i.e. loading vectors higher than 0.40).

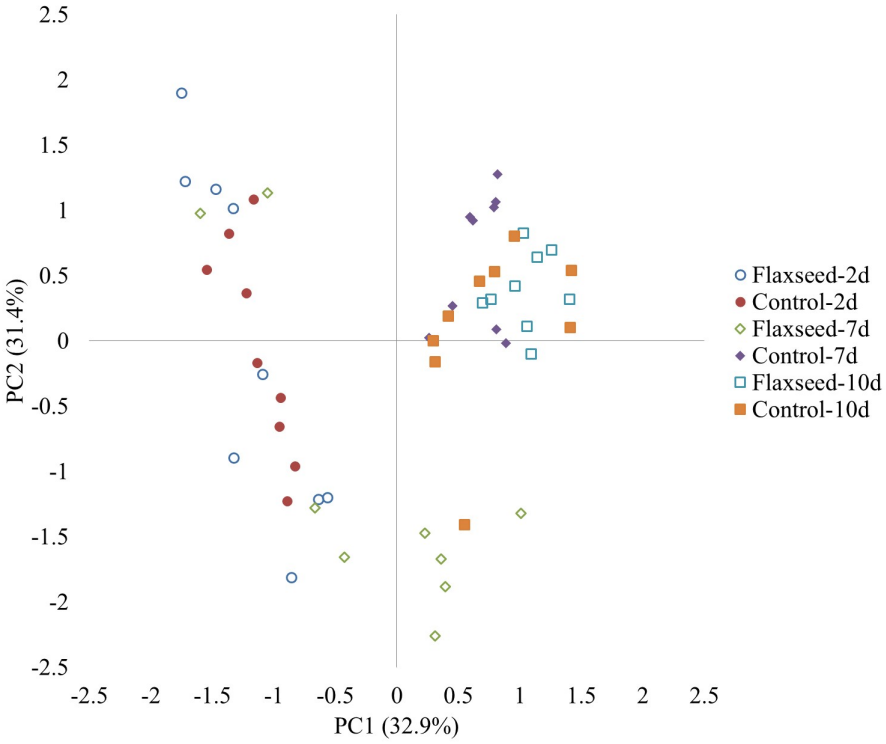


Figure 3. Score plot of principal component analysis (PC) of raw meat samples at different aging (2, 7 or 10 days).

As in PCA, also the CDA performs a dimension-reduction through linear combination of quantitative variables and helps to discriminate differences among classes. When performing the CDA the R squares indicated all sensors showed a significant (P<0.05) difference between the classification groups for all canonical variables (Can). The raw canonical coefficients of the first canonical variable showed that classes differ on the linear combination of the sensors selective for aromatic compounds (Table 5). The plotting of the first two canonical variables (Fig. 4) revealed Can1 has more discriminatory power and it is capable of discriminating meat samples into four groups: flaxseed at 2d, control at 2d and flaxseed at 7d, control at 7d and flaxseed at 10d, control at 10d. However, the better result in samples discrimination compared to PCA was inherent to the algorithm used for group separation since the CDA is a supervised learning method relying on group labels.

3.2. Sensory analysis

Sensory analysis was performed after 7d of aging when usually beef is offered for sale as retail cuts. The amount of fat in meat was similar between samples coming from differently fed animals (5.2 *vs.* 4.6 g kg^{-1}, respectively for control or flaxseed; data not shown). The flavor intensity was higher (P<0.05) in meat samples from flaxseed-fed animal compared with meat samples from control-fed animals (7.84 and 6.74, respectively). Feeding flaxseed doubled the intramuscular content of total n-3 PUFA (from 21.1 to 46.7 g/kg of total detected FA – data not shown) and the proportion of C18:3 n-3 in LM from flaxseed-fed bulls reached 3.0% while in control-fed bulls remained lower than 0.9% (data not shown); such modifications of the fatty acid profile of meat might explain the higher flavor intensity scored by panelists.

Table 5. Raw canonical coefficients for canonical variables.

	Can1	Can2	Can3	Can4	Can5
W1C	64.874	75.721	24.026	-118.107	1.820
W5S	0.798	-0.783	1.410	-0.636	-1.294
W3C	-71.757	-81.159	-137.678	96.442	-125.821
W6S	-5.924	-0.211	2.979	5.177	-5.727
W5C	57.716	20.960	150.921	-7.927	115.196
W1S	3.565	-3.336	-7.029	-6.538	6.833
W1W	-4.616	-5.562	13.041	3.326	0.050
W2S	3.976	6.841	7.977	4.485	-3.202
W2W	7.278	6.173	-15.159	-4.777	-0.785
W3S	-21.962	13.150	7.314	2.614	-25.212
Proportion, %	85.65	6.04	4.49	2.32	1.50
R^2	0.98	0.75	0.69	0.53	0.42

Can = Canonical variable.

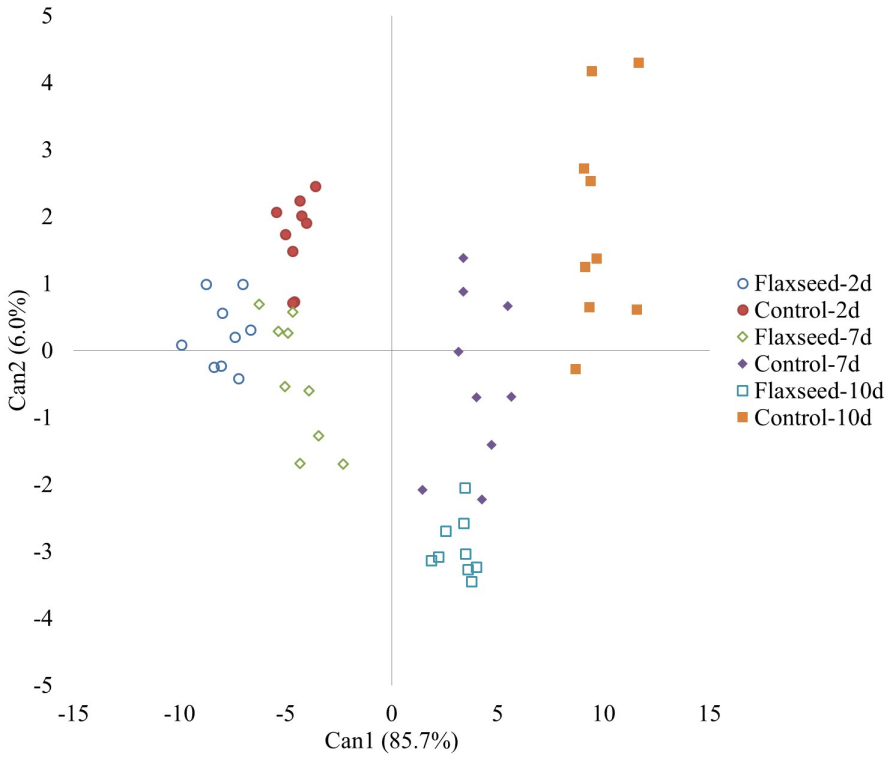


Figure 4. Score plot of canonical discriminant analysis (Can) of raw meat samples at different aging (2, 7 or 10 days).

Several studies have shown that animal diet can strongly influence the fatty acid composition of meat (PONNAMPALAM *et al.*, 2001; WOOD *et al.*, 2003; BAS *et al.*, 2007; VAHMANI *et al.*, 2015). The variation of fatty acid compositions has profound effects on meat quality, because fatty acid composition determines the firmness/oiliness of adipose

tissue and the oxidative stability of muscle, which in turn affects flavor and muscle color. High PUFA levels may produce alterations in meat flavor due to their susceptibility to oxidation and the production of unpleasant volatile components during cooking (WOOD *et al.*, 1999). Even if increases in overall liking scores were reported (VATANSEVER *et al.*, 2000), most studies have shown decreases in panelist preferences for meat from animals fed diets high in unsaturated fatty acids (CAMPO *et al.*, 2006), sometimes due to the related increase of oxidation products (YANG *et al.*, 2002).

3.3. Relationship between e-nose data and sensory scores

The 7d e-nose data on cooked meat were analyzed by PLS to investigate the relationship between sensors readings and flavor scores. Meat samples used in the two assays were cocked with different methods, water bath for e-nose and in a double plate grill for sensory evaluation. While the cocking methods could lead to different textural attributes of meat, the flavor is however not affected (CHOI *et al.*, 2016). The water-bath method selected for the e-nose assay was to minimize Maillard products and their reaction with volatile compounds (AASLYNG and MEINERT, 2017) and effects of high grilling temperatures on variability of volatile compounds and therefore on pattern observed during the e-nose assay. The e-nose sensor responses (predictor variable) were used to predict the flavor score (dependent variable) from sensory evaluation. Since the restricted number of samples, an independent data set for validation was not possible, therefore a one at a time cross-validation method was used to choose the number of extracted factors minimizing the predicted residual sum of squares (PRESS) and the van der Voet's test (VAN DER VOET, 1994) was used to select the fewest number of factors (i.e. with residual PRESS not statistically different than the minimum PRESS) (Table 6). The contribution of each sensor in fitting the PLS model was based on the Variable Importance for Projection (VIP) statistic of Wold (WOLD, 1994) with a minimum threshold value of 0.8. The VIP shows the contribution of sensors in fitting the PLS model for both sensors and flavor (Table 7). A small (in absolute value) coefficient of center and scaled parameter and a small VIP (i.e. <0.8) suggest low importance of the predictor in the PLS model. The parameter estimate in original scale represents the coefficients of each predictor in the PLS model. The predicted results by PLS *vs* observed results from sensory evaluation are shown in Fig. 5. The retained factors in PLS explained 99.6% and 82.5% of the variance of independent (sensors) and dependent (flavor) variables, suggesting in our condition the e-nose could represent a tool supporting the sensory evaluation by panelists.

4. CONCLUSIONS

The approach used in data evaluation could not clearly indicate the e-nose as capable of discriminating populations in meat samples from differently fed animals and with different days of aging. Within the sensor array used, sensors having major importance in discriminating power were the ones reacting to aromatic compounds, followed by sensors that could be related to proteolysis reactions. Differences among samples were observed at the 7d of aging. In our condition, when performing PLS regression the e-nose proved to be a valuable tool supporting the sensory evaluation. Additional efforts are needed to better understand the relationship between sensor activation and flavor intensity toward the identification of the substances acting in flavor intensity.

Table 6. Steps of the partial least square (PLS) method with cross-validation for the 7d cooked meat samples.

PLS Factors	Cross-validation	
	Root mean PRESS	Comparison Significance
0	1.059	0.034
1	1.035	0.047
2	0.997	0.072
3	0.869	0.095
4	0.912	0.079
5	0.911	0.04
6	0.956	0.036
7	0.792	0.101
8	0.931	0.011
9	0.659	0.064
10	0.613	1
Minimum root mean PRESS		0.613
Minimizing number of factors		10
Smallest number of factors with P>0.1		7

Retained factors	Percent Variation Accounted for			
	Independent variable		Dependent variables	
	Current	Total	Current	Total
1	32.338	32.338	48.745	48.745
2	35.152	67.490	7.229	55.974
3	26.017	93.507	2.970	58.944
4	3.608	97.115	11.002	69.946
5	1.896	99.011	2.042	71.987
6	0.514	99.525	4.166	76.153
7	0.066	99.591	6.364	82.519

Table 7. Variable importance for projection (VIP) and regression coefficients values for sensors in the prediction of flavor from e-nose reading on 7d cooked meat.

Variable	VIP	Centered and Scale Parameter estimates	Parameter estimates in original scale
Intercept	-	0	31.891
W1C	0.748	-0.247	-8.208
W5S	0.878	0.620	0.305
W3C	0.722	2.223	48.322
W6S	1.411	3.075	5.115
W5C	0.878	-3.922	-76.491
W1S	1.404	-1.693	-2.259
W1W	0.660	0.056	0.138
W2S	1.337	-1.624	-4.393
W2W	0.645	0.104	0.246
W3S	0.881	0.488	3.451

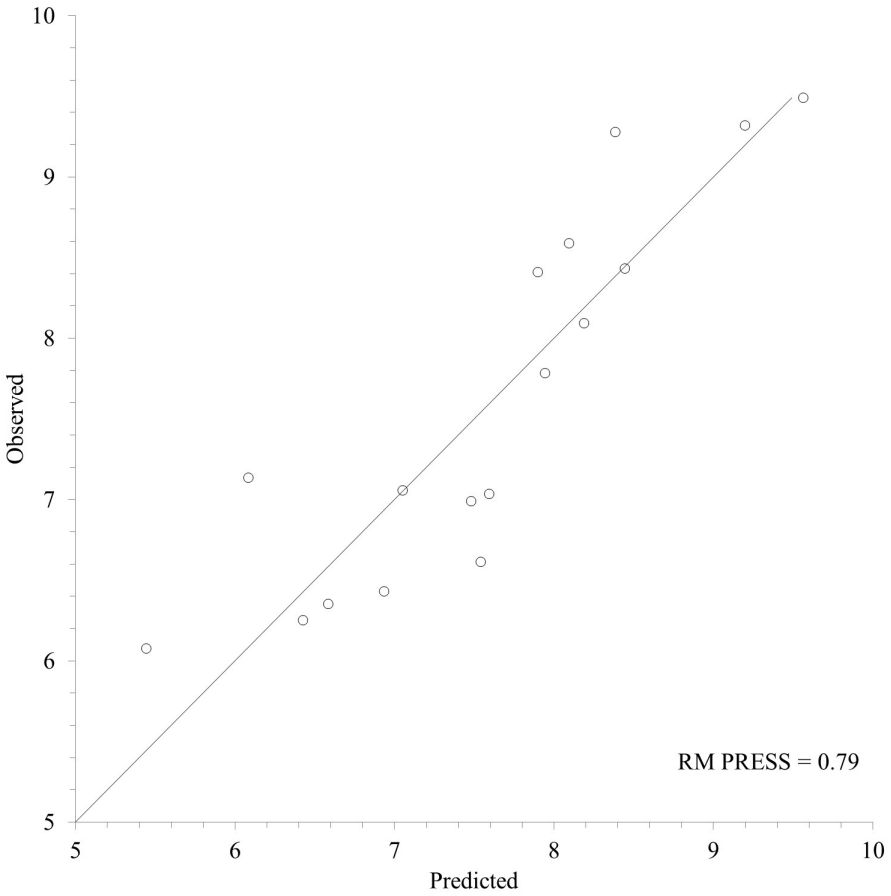


Figure 5. Observed and predicted flavor intensity scores (scale: 1 = poor to 15 = intense) evaluated by partial least squares regression with sensor responses as predictor matrix. RM PRESS = root mean square of predicted residual sum of squares.

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PAPER

EFFECTS OF TEMPERATURE ON BIOFILM FORMATION AND QUORUM SENSING OF *AEROMONAS HYDROPHILA*

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ABSTRACT

Aeromonas hydrophila is an emerging foodborne pathogen that causes infections more frequently in summer than in winter. This study evaluated the effects of temperature (4-37°C) on the biofilm formation and quorum sensing abilities of *A. hydrophila* on microtiter plates, stainless steel (SS), and crab surfaces. The incubation of the bacterium in Luria-Bertani broth at temperatures of 20-25°C significantly ($P < 0.05$) enhanced the biofilm formation and intra-species quorum sensing ability (via C4-AHL and C6-AHL). Field-emission electron microscopy revealed that the bacterium colonized the surface of crab and formed biofilms at 25°C. Thus, the present study demonstrates that temperature control in food processing environments may reduce *A. hydrophila* biofilm formation. Therefore, the study has significant applications in food processing plants.

Keywords: *Aeromonas hydrophila*, temperature, biofilm, quorum sensing, crab surface

1. INTRODUCTION

Aeromonas hydrophila has recently received much attention as an emerging opportunistic and foodborne pathogen and a causative agent of various human infections such as gastrointestinal tract infections, wound and soft tissue infections, and blood-borne dyscrasias (DASKALOV, 2006). The incidence of these infections is higher during summer, owing to elevated temperatures (JANDA AND ABBOTT, 2010). The importance of *A. hydrophila* in food safety (DASKALOV, 2006), fish diseases, and human infections (JANDA AND ABBOTT, 2010) as well as its role in quorum sensing and biofilm formation (CHOPRA *et al*., 2009) have been studied. The bacterium has been isolated from various fresh and estuarine water samples and animals living in these waters, including fish, crab, shrimp, and other mollusks (OTTAVIANI *et al*., 2011; DENG *et al*., 2014).

Microbial biofilms are sessile microbial communities that are attached to either biotic or abiotic surfaces. Biofilm formation is a common phenomenon in nature; for instance, biofilms are formed on foods and food-contact surfaces as well as in waste treatment plants. The effect of temperature on the production of biofilms and virulence factors has been studied using various microorganisms, including *Enterococcus* spp., *Salmonella* spp., and *Listeria monocytogenes* (DI BONAVENTURA *et al*., 2008; JAHAN AND HOLLEY, 2014). Although *Salmonella* is commonly associated with human and animals, it can also be found in the environment (CHRONICLE, 1997; GIAOURIS *et al*., 2005). As these are environmental microorganisms, such as *A. hydrophila*, temperature is expected to modulate their survival and ability to form biofilms.

Quorum sensing is a density-dependent process by which microorganisms coordinate and control intraspecies and interspecies communication (FUQUA *et al*., 1994). Several authors have reviewed the importance of quorum sensing in relation to food microbiology (SKANDAMIS AND NYCHAS, 2012; MIZAN *et al*., 2015). *A. hydrophila* carries quorum-sensing genes, including *ahyI* (encoding N-3-butanoyl-DL-homoserine lactone [C4-AHL]) and *ahyR* (encoding N-3-hexanoyl homoserine lactone synthase [C6-AHL]) (SWIFT *et al*., 1997). In addition, the bacterium produces autoinducer 2 (AI-2) for inter-species communication (KOZLOVA *et al*., 2008). Intra-species quorum sensing in *A. hydrophila* (via C4-AHL and C6-AHL) has been reported to control biofilm formation, motility, and virulence factors (KHAJANCHI *et al*., 2009).

Temperature is known to play an important role in governing the rate of microbial activity as well as for the propagation of biofilms and settlement of organisms in aquatic systems (RAO, 2010). Biofilm formation is influenced by several factors, available nutrients, and organic matter. MELO AND BOTT (1997) reported that the development and maturation of biofilm is dependent on temperature, nutrient availability, and water flow rate. As *A. hydrophila* is common in aquatic environments, it may face adverse environmental conditions such as fluctuations in temperature, humidity, and pH (DASKALOV, 2006). Survey studies have shown that aeromonads are found in high numbers in late summer/early autumn (temperatures are around 20–25°C) but are rarely detected during the cold months (GAVRIEL *et al*., 1998). The present study focused on the effects of temperatures on biofilm formation and quorum sensing by *A. hydrophila* on the surface of microtiter plates, stainless steel (SS), and crab shells. In the food industry, biofilm formation is a major concern as an important source of food contamination. Therefore, the results of this study will provide potential approaches to avoid contamination, as they reveal the temperatures favorable for *A. hydrophila* biofilm formation and quorum sensing.

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture media, and growth conditions

In the present study, the following strains were used: A. hydrophila KCTC 11533 (isolated from surface water) and KCCM 32586 (a clinical isolate), Vibrio harveyi strain BB120 and BB170, and *Chromobacterium violaceum* CV026. The bioreporter strain CV026 was provided by the Animal, Plant, and Fisheries Quarantine and Inspection Agency, Korea. Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) was used for the bacterial cultivation and violacein production assay. Prior to each experiment, frozen culture aliquots (100 μL) were thawed and inoculated into 5 mL of LB broth. The cultures were incubated at 30°C and 220 rpm for 24 h. These starter cultures were subsequently inoculated into fresh LB broth and cultured to a final optical density of 1.0 at 600 nm (OD600), followed by their dilution (1:50) for biofilm formation experiments and quorum sensing assay.

2.2. Quantitative biofilm formation assay in microtiter plates

The quantitative biofilm assay was performed as previously described (O'TOOLE, 2011; MIZAN *et al*., 2016). A. hydrophila was cultured in LB broth for 24 h without shaking, followed by its dilution at 1:50 in LB broth. A total of 100 μL aliquots were placed in the wells of 96-well polystyrene microtiter plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the microtiter plates were incubated at 4, 10, 15, 20, 25, 30, 35, and 37°C for 72 h without shaking. Biofilm formation was normalized to planktonic growth and determined using the following equation (1), according to TEH *et al*. (2010):

$$\mathrm{BFI} = \frac{(\mathrm{AB} - \mathrm{CW})}{(\mathrm{GB} - \mathrm{GW})} \qquad (1)$$

Where BFI is the biofilm formation index, AB is OD_{595} of the crystal violet (CV)-stained attached microorganisms, CW is OD_{595} of the stained blank wells containing sterile (microorganism-free) medium only, GB is OD_{600} of the cells in suspended culture, and GW is OD_{600} of the blank well. Biofilm production was classified into three categories according to MARTINEZ-MEDINA *et al*. (2009): weak ($0.1 > \mathrm{BFI} \leq 0.5$), moderate ($0.5 > \mathrm{BFI} \leq 1$), and strong ($\mathrm{BFI} > 1$).

2.3. Determination of biofilm formation on SS surfaces

Austenitic SS coupons (type 302, 2 × 2 × 0.1 cm; Chung-Ang Scientific Inc., Seoul, Korea) were processed as described by SHEN *et al*. (2012). Thereafter, *A. hydrophila* cell suspensions were diluted at 1:50 and inoculated into 7 mL of fresh LB in 50 mL Falcon tubes containing a completely submerged SS coupon. The tubes were incubated without shaking at 4, 10, 15, 20, 25, 30, 35, and 37°C for 24 h to allow biofilm formation on SS coupons. Following incubation, each SS coupon was transferred into a small Petri dish (55 × 12 mm) containing 1 mL of 0.1% peptone water (PW; Oxoid, Hampshire, UK) and agitated by rotation in clockwise and anticlockwise direction using sterile forceps. Agitation was always performed by the same person; thus, it was assumed that the same amount of pressure was applied to all coupons (JAHID AND HA, 2014). The cells were separated, vortexed, and diluted in PW for enumeration. Cell number was quantified using Bacto R2A agar (Difco, USA) following incubation for 24 h.

2.4. Preparation of inocula for crab surfaces

Cultures grown in LB broth were centrifuged (10,000 ×*g* for 10 min at 4°C) and the pellets were washed twice with Dulbecco's phosphate-buffered saline (DPBS). The pellets were re-suspended in a suitable amount of DPBS to obtain an absorbance of 1.0 at 600 nm wavelengths. To determine the cell density, serial dilutions were performed and plated on *Aeromonas* selective medium (Oxoid, Hampshire, UK). These inocula were used to inoculate the crab surfaces.

2.5. Biofilm formation on crab surfaces and detachment of cell population

Crabs (*Corystes cassivelaunus*) used in this study were purchased from a local grocery store in Anseong, Korea. A delimited area (cm^2) of crab shell was dissected and processed immediately, as described by JAHID *et al*. (2015). The shells were incubated at different temperatures (4, 10, 15, 20, 25, 30, 35, and 37°C) without shaking. The detachment of microbial populations from shell surfaces was performed as described by JAHID *et al*. (2014), with minor modifications. Briefly, the crab surfaces were washed twice with PBS to free planktonic cells and placed in 10 mL of PW in a sterile stomacher bag (Nasco Whirl-Pak, USA). These were processed using a stomacher (BagMixer, Interscience, Saint Nom, France) at maximum speed for 2 min to free the biofilm-forming microbes from the crab shells. *A. hydrophila* was enumerated after serial dilution and spread plating on *Aeromonas* selective medium containing ampicillin. The plates were incubated at 30°C for 48 h. Colonies were counted and the results for biofilm production were expressed as colony-forming unit (CFU)/cm^2 for biofilm populations. For each of three independent experiments, two plates per dilution were assessed to obtain the final data.

2.6. Quantification of violacein production

To quantify violacein production, the procedures described by KIM *et al*. (2013) and JAHID *et al*. (2015) were followed. *A. hydrophila* was cultivated in LB broth at different temperatures for 24 h and the supernatant was collected by centrifugation at 15,000 ×*g* for 15 min, followed by its filter sterilization using 0.22 μm filters (Millipore Corporation, Billerica, MA, USA). LB agar was prepared, cooled, and poured using the open side of a 1 mL pipette tip to make a well. A loop full of *C. violaceum* CV026 overnight culture was spread on the wall of the well and treated with 100 μL of supernatant from each condition at 28°C for 24 h in an upside up of petri dish. Next, whole CV026 cells grown on the plate were collected and solubilized with 250 μL of dimethyl sulfoxide (DMSO; Sigma Aldrich). The mixture was vortexed to ensure the release of violacein pigment. After centrifugation at 15,000 ×*g* for 15 min, the absorbance of 200 μL of colored DMSO from CV026 cells was measured at 585 nm wavelength using a microplate reader (Spectra Max 190; Molecular Devices).

2.7. Autoinducer-2 (AI-2) bioassay

The secretion of AI-2 by *A. hydrophila* during its incubation with crab surfaces at different temperatures (4-37°C) was assessed with minor modifications in previously described procedures (SONI *et al*., 2008). *A. hydrophila* was inoculated on crab surfaces in cyanobacteria BG-11 freshwater solution (Sigma Aldrich, Inc., St. Louis, MO, USA) and incubated at different temperatures without shaking. The cultures were centrifuged at 15,000 ×*g* for 10 min. The supernatant from the cell-free culture was passed through 0.2 μm Tuffryn syringe filters and stored at -20°C. The cell-free supernatants were tested for

the presence of AIs that induce luminescence of *V. harveyi* reporter strain BB170. This strain carries sensor 2, but not sensor 1, and is capable of sensing AI-2 but not AI-1. *V. harveyi* strain BB170 was grown overnight at 30°C with aeration in the autoinducer bioassay (AB) broth and diluted to 1:1,000 in AB medium (BASSLER *et al*., 1993). Next, 4.5 mL of diluted *V. harveyi* strain BB170 and 500 μL of the cell-free supernatant from each sample (*A. hydrophila* supernatant incubated with crab at different temperatures) was added to 50 mL Falcon tubes and shaken for 16 h at 220 rpm to allow the reporter strain to produce luminescence. A total of 100 μL samples were transferred to white microtiter plates and the luminescence was measured using a computer-controlled microplate luminometer (GloMax® 96 Microplate Luminometer for Luminescence, Promega, Madison, WI, USA). *V. harveyi* strain BB120 that produces AI-1 and AI-2 was used as a positive control. Control *V. harveyi* strains were grown overnight at 30°C with shaking at 220 rpm in LB broth and 1 mL of cell-free supernatant from each culture was prepared as described above.

2.8. Field-emission scanning electron microscopy

A. hydrophila biofilm formation was observed on crab surfaces at 4, 25, and 37°C by field-emission scanning electron microscopy (FESEM). The inoculation and incubation procedures were the same as those described above. FESEM samples were processed according to the previously described procedures (MIZAN *et al*., 2016). The dehydrated samples were sputter-coated with platinum and visualized with an FESEM microscope (Sigma, Carl Zeiss, Germany) at an accelerated voltage of 5 kV and a working distance of 5 mm. Digitized images of biofilms were collected for further analysis.

2.9. Statistical analysis

Biofilm formation and quorum sensing of *A. hydrophila* at different temperatures (4–37°C) were analyzed by analysis of variance (ANOVA) using SAS software (Version 9.2; SAS Institute Inc., Cary, NC, USA) for a completely randomized design to determine the significance of differences due to temperature variation. The mean separation was evaluated with Duncan's multiple-range test when the effect was significant ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Impact of temperature on biofilm formation

Temperature is one of the major factors that affect bacterial growth. Most of the clinically important pathogens are mesophiles that grow well at optimum temperatures between 25°C and 40°C (MURRAY *et al*., 2003). The optimum growth temperature is 20°C for some *Aeromonas* species and 37°C for others (EWING *et al*., 1961). MAALEJ *et al*. (2004) reported a decline in *A. hydrophila* population to a level below the detection level at 23°C and 5°C. The optimum temperature for *A. hydrophila* infection in goldfish (*Carassius auratus*) is 17–25°C (RAHMAN *et al*., 2001). The results from the examination of the biofilm formation on microtiter plates at different temperatures are shown in Table 1. A significant increase in the biofilm production was observed at 20–25°C for *A. hydrophila* strains 11533 and 32586. Biofilm formation declined at temperatures over 25°C (i.e., 30-37°C) or below 20°C. RACHID *et al.* (2000) reported the formation of dense biofilms with an increase in temperature.

Studies have shown that *A. hydrophila* may attach and produce biofilm on to SS surfaces (LYNCH *et al*., 2002), glass (WHITELEY *et al*., 1997), and vegetables (JAHID *et al*., 2014) in laboratory settings. LYNCH *et al*. (2002) reported that *A. hydrophila* produces a thin biofilm that may cover 40-50% of SS surface. Biofilm formation by *A. hydrophila* strains 11533 and 32586 on SS coupons is presented in Table 1. The trend observed was similar to that reported with microtiter plates. A significant increase ($P < 0.05$) in biofilm formation was observed at 20-25°C, indicative of the optimum temperature range for biofilm formation. Similar trend was observed for both *A. hydrophila* strains, although they had different origins (i.e., clinical versus environmental).

Biofilms on fish surfaces and bacteria from marine water source may contaminate seafood-processing facilities. *Vibrio*, *Aeromonas*, *Listeria*, and *Salmonella* isolated from seafood are known to cause foodborne illness and form biofilms (TAKAHASHI *et al*., 2009; ABEROUM and JOOYANDEH, 2010; NORHANA *et al*., 2010; JAHID *et al*., 2015; MIZAN *et al*., 2017). The ability of *A. hydrophila* to produce biofilms on crab surfaces at 4, 10, 15, 20, 25, 30, 35, and 37°C is shown in Table 1. Biofilm formation was significantly lower ($P < 0.05$) at 4, 10, and 15°C than at 20-37°C. A significant increase in the biofilm formation was observed for both strains at 20-30°C, while the biofilm growth gradually reduced at 37°C. No significant difference ($P > 0.05$) was observed in the biofilm formation between the two *A. hydrophila* strains.

Table 1. Viable counts of biofilm cells of *Aeromonas hydrophila* in Luria-Bertani (LB) medium with different temperature from 4°C to 37°C.

Support	Microtiter plates		Stainless steel surfaces		Crab surfaces	
Temperature (°C)	11533 (BFI±SEM**)	32586 (BFI±SEM)	11533 (log CFU/cm^2 ±SEM)	32586 (log CFU/cm^2 ±SEM)	11533 (log CFU/cm^2 ±SEM)	32586 (log CFU/cm^2 ±SEM)
4	0.29±0.02de	0.22±0.03d	4.56±0.26c	3.62±0.28d	2.05±0.10h	1.66±0.17f
10	0.38±0.03cd	0.47±0.01bc	4.89±0.15bc	5.14±0.22bc	2.76±0.09g	2.81±0.07e
15	0.79±0.02ab	0.63±0.04ab	5.26±0.11b	5.50±0.32b	4.74±0.26f	4.45±0.15d
20	0.91±0.08a	0.72±0.03a	6.23±0.07a	6.22±0.24a	5.98±0.03ab	4.77±0.14d
25	1.06±0.04a	0.70±0.07a	6.35±0.18a	6.72±0.24a	6.08±0.11a	5.55±0.09ab
30	0.34±0.05de	0.45±0.06bc	5.46±0.25b	5.29±0.12b	5.88±0.07abc	5.67±0.17a
35	0.24±0.04e	0.36±0.02cd	5.16±0.08bc	5.34±0.08b	5.43±0.24cd	4.87±0.09c
37	0.16±0.02e	0.38±0.06cd	2.65±0.26e	3.26±0.27de	4.96±0.30de	4.73±0.23d

**The values are mean ± SEM (Standard Error Mean) of 3 independent experiments. The values with same letters within a column were not significant ($p < 0.05$) according to Duncan's multiple-range test.

3.2. Violacein production

The modulation in the quorum sensing ability of *A. hydrophila* at different temperatures has been previously investigated (MEDINA-MARTINEZ *et al*., 2006). The biosensor *C. violaceum* strain CV026 (a *cvil* transposon mutant) with quorum sensing regulation defect fails to produce violet color in the absence of AHL, which controls violacein production. However, CV026 produces color if AHL is externally supplied. The mutant CV026 produces color when grown in the presence of *A. hydrophila*, as violacein is produced in response to AHL secreted by *A. hydrophila*. The intensity of the color is proportional to AHL concentration. Violacein production was significantly lower ($P < 0.05$) at 4-15°C than at 20-25°C. AHL produced by strain 11533 was significantly higher ($P < 0.05$) than that

produced by strain 32586 (Fig. 1). JAHID *et al*. (2015) stated that the production of AHL by *A. hydrophila* is strain-dependent. PONCE-ROSSI *et al*. (2016) reported *A. hydrophila* ATCC7966 to be negative for AHL production, while *A. hydrophila* Embrapa 029 was shown to produce AHL.

Both *A. hydrophila* strains produced the highest AHL levels at 25°C (Fig. 1). MEDINA-MARTINEZ *et al*. (2006) noted that the optimum temperature for AHL production was 22°C, and no AHL production was observed at 37°C. At 12°C, however, C4-HSL production was detected after 70 h of incubation.

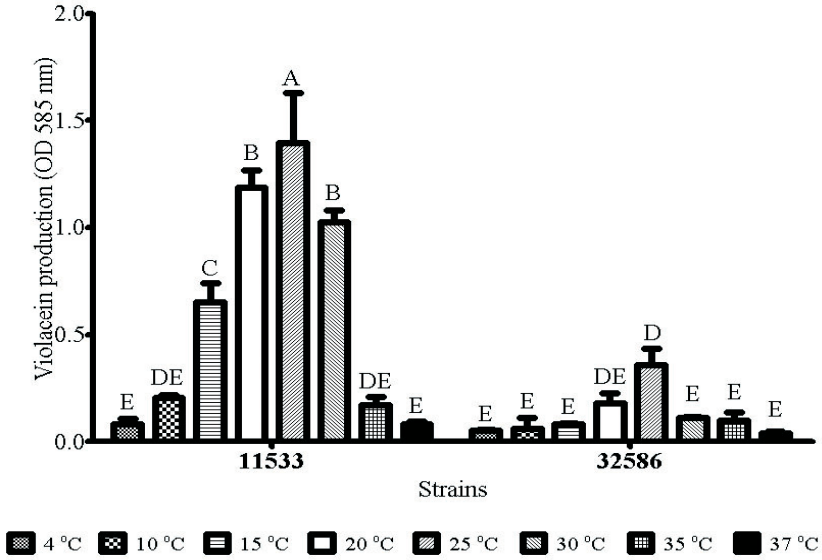


Figure 1. Violacein production in *A. hydrophila* at different temperatures. Values shown are the mean ± SEM of three independent experiments. Within each treatment, the values marked with the same letter are not significantly different according to Duncan's multiple-range test ($P < 0.05$).

3.3. AI-2 production

Although AI-2 production and biofilm formation may be associated with each other (MIZAN *et al*., 2016), no strong relationship was observed between AI-2 levels and biofilm formation, as the procedure for AI-2 measurement was too sensitive and variable to compare independent experiments (Fig. 2). Temperature modulates cell density, and quorum sensing is dependent on cell density. AI-2 production by *A. hydrophila* was analyzed in crab fresh water samples incubated at different temperatures. As shown in Fig. 2, AI-2 production by *A. hydrophila* strains was origin-dependent; more strains are necessary to justify the results. The environmental strain (KCTC 11533) produced lower levels of AI-2 than the clinical strain (KCCM 32586). A significant ($P < 0.05$) increase in AI-2 production was observed in both *A. hydrophila* strains at 20–30°C (Fig. 2). Further studies on the role of food composition and environment in the production of AI-2 and C4-HSL may increase our understanding of the synthesis and accumulation of these signal molecules in food products.

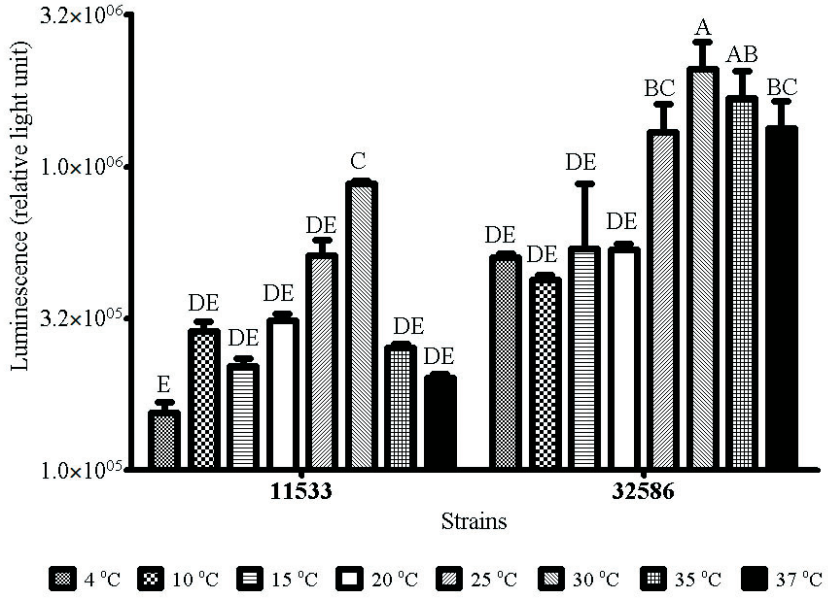


Figure 2. AI-2 production in *A. hydrophila* at different temperatures. Values shown are the mean ± SEM of three independent experiments. Within each treatment, the values marked with the same letter are not significantly different according to Duncan's multiple-range test ($P < 0.05$).

3.4. Analysis of crab samples with FESEM

ABEROUM AND JOOYANDEH (2010) reported that processing facilities of seafood products may act as a source of contamination and that *A. hydrophila* is commonly isolated from unprocessed and processed seafood products. TSAI AND CHEN (1996) observed that *A. hydrophila* contaminated 50% of oysters purchased from native marts of Taiwan. Biofilm formation may differ, owing to differences in the growth surface and temperature (NOORI *et al*., 2016). JAHAN AND HOLLEY (2014) observed dense biofilm formation (*Enterococcus* spp.) at temperatures lower than the optimum growth temperature. NOORI *et al*. (2016) reported that higher temperatures induced extensive biofilm formation, whereas lower temperatures resulted in the attachment of the bacterial cells (*V. parahaemolyticus*) as monolayers on crab surface. MIZAN *et al*. (2015) observed *A. hydrophila* cell attachment on crab shell. Biofilm formation on crab samples incubated at 4, 25, and 37°C (according to biofilm formation strength) is presented in Fig. 3. *A. hydrophila* failed to form biofilms on crab surfaces at 4°C; the bacterium attached on the crab surface (Fig. 3A). In contrast, *A. hydrophila* formed a strong three-dimensional structure at 25°C, (Fig. 3B). At 37°C, the bacterium formed biofilms on the crab surface (Fig. 3C). PONCE-ROSSI *et al*. (2016) evaluated biofilm formation in two strains of *A. hydrophila* at the same temperature but different incubation times. These authors found that the biofilm formation was maximum at 48 h and decreased at 72 h. ALMEIDA *et al*. (2017) studied *Salmonella* and observed that maximum biofilm formation may occur after 36 h incubation in contrast to other times evaluated.

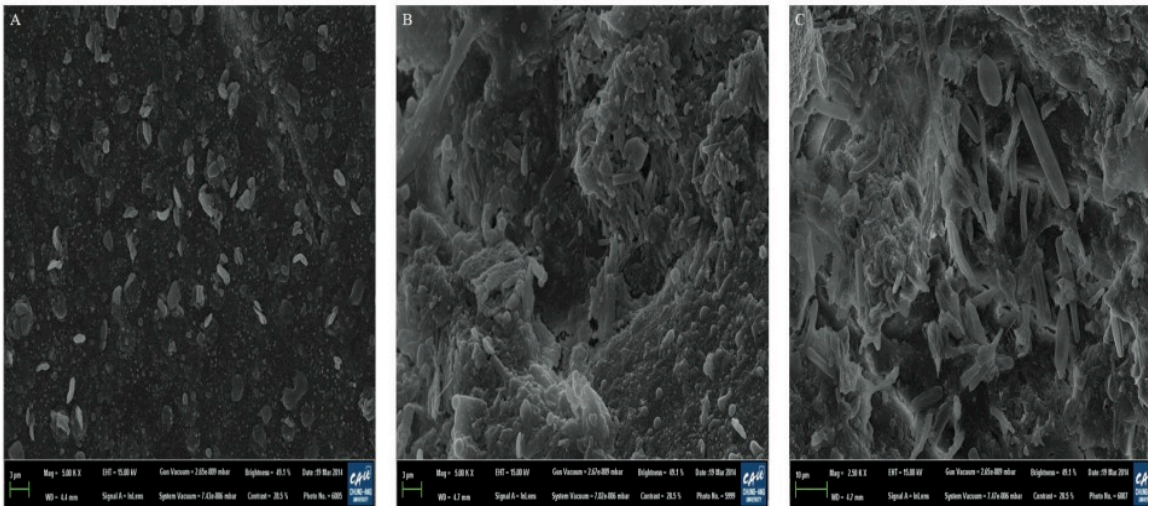


Figure 3. FESEM images of *A. hydrophila* biofilm formation on crab surfaces at different temperatures. The image shown is a representative result for strain KCTC 11533. (A) 4°C, (B) 25°C and (C) 37°C.

4. CONCLUSIONS

The two *A. hydrophila* strains, one originally isolated from surface water (strain KCTC 11532) and the other from clinical sample (strain KCCM 32586), showed significant variations in the tested phenotypes (i.e., biofilm formation and quorum sensing), indicating strain-specific regulation. Strain KCTC 11533 was found to produce high concentrations of AHL and lower concentrations of AI-2. Strain KCCM 32586, on the other hand, showed high AI-2 production and lower AHL activity. Therefore, the phenotypic properties differed between the two strains. Such studies will elucidate the effect of quorum sensing on the regulation of virulence factors produced by opportunistic pathogens such as *A. hydrophila* on microtiter plates, SS, and crab surfaces. However, the experimental scope of the study is limited to only one strain of environmental and clinical origin. Further studies are warranted to extend the application of this study in food quality and safety regulations.

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PAPER

EFFECT OF ULTRASOUND AND CHEMICAL COMPOUNDS ON MICROBIAL CONTAMINATION, PHYSICOCHEMICAL PARAMETERS AND BIOACTIVE COMPOUNDS OF CHERRY TOMATOES

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ABSTRACT

In this study, different sanitization methods were evaluated to understand their effects on the microbiological and physicochemical characteristics of cherry tomatoes stored at 7°C. Microbiological analysis showed that treatment with silver nanoparticles at 6 mg/L in combination with ultrasound treatment demonstrated a significant reduction in the assessed microorganism content of the cherry tomatoes. There were no changes in titratable acidity, pH, soluble solids, instrumental color, firmness, lycopene or beta carotene content after treatment followed by 10 d at 7°C. It was observed that cherry tomatoes treated with ultrasound had lower firmness values. Treatment with sodium dichloroisocyanurate, detergent-based surfactants, lactic acid, ultrasound treatment combined with detergent and ultrasound treatment combined with silver nanoparticles all led to increased ascorbic acid content during storage. An application of the silver nanoparticles promoted the best results in terms of microbial count reduction without significantly affecting the quality characteristics of the cherry tomatoes.

Keywords: food quality, organic acids, sanitizers, silver nanoparticles, vegetables

1. INTRODUCTION

A tomato variety with great popularity worldwide is the cherry tomato (*Lycopersicon esculentum* var cerasiforme) (WANG *et al*., 2008; ZHAO *et al*., 2010). The cultivation of cherry tomatoes is gaining ground due to the interest in incorporating the plant into modern cuisine (LENUCCI *et al*., 2006) as it is tasty and sweet and can be consumed either as a fruit or as an appetizer. The reduced size and great versatility of their applications have made cherry tomatoes very popular in diverse culinary preparations. The use of cherry tomatoes as adornments, appetizers and the main feature of several dishes has led to increased consumption of this vegetable. This vegetable is present in diets because it has many antioxidants, such as carotenoids, ascorbic acid and phenolic compounds that can promote beneficial effects (GUERREIRO *et al*., 2016).

The microbiological quality of fruits and minimally processed vegetables is directly related to the presence of spoilage microorganisms, which cause changes in sensory characteristics. Additionally, the pathogenic micro-organisms in a given population can cause damage to consumer health (RICO *et al*. 2007; LETHO *et al*., 2011). Fruits and vegetables often possess naturally high amounts of bacteria, yeast and mold (ROMEO *et al*., 2010). Microorganism contaminants come from various sources such as irrigation water, manure, cultivation, harvesting, postharvest processing and distribution handling (TORNUK *et al*., 2011; GUO *et al*., 2016).

Washing with sanitizing solutions is considered to reduce the number of spoilage and pathogenic microorganisms, thus contributing to product safety (ALLENDE *et al*., 2008; RAHMAN *et al*., 2011; SÃO JOSÉ & VANETTI, 2012). Among the sanitizers used in the food industry, especially to wash fresh produce, chlorine and chlorinated compounds are widely used (ALVARO *et al*., 2009). The ease of use, low cost, high antimicrobial activity and complete dissolution in water make chlorinated agents a common choice for disinfectants in the fruit and vegetable industry (ALLENDE *et al*., 2008; SANCHEZ *et al*. 2015; YAMANER *et al*., 2016).

New sanitization treatments have also been evaluated. The choice of sanitizer is important for hygienic and sanitary requirements and to maintain and, where possible, improve the sensory and nutritional characteristics of fruit and vegetables.

Organic acids are generally recognized as safe (GRAS) and can inactivate pathogens involved in food contamination (AKBAS & OLMEZ, 2007; TIRAWAT *et al*., 2016). Lactic acid is an organic acid that may be applied to fresh produce (SAGONG *et al*., 2011). Ultrasound is a technology recommended for use in the food industry for different applications. Among these uses is the removal of particles adhering to surfaces and the inactivation of microorganisms (GOLMOHAMADI *et al*., 2013; BEVILACQUA *et al*., 2014; SÃO JOSÉ *et al*., 2014). This inactivation is the result of a process called cavitation, which consists of the formation, growth and collapse of bubbles that generate mechanical energy where chemicals are located. When ultrasound treatment is used in conjunction with chemical agents, the intense pressure gradient allows for penetration of these agents into the cell membrane of microorganisms (PIYASENA *et al*., 2003; SÃO JOSÉ *et al*., 2014).

The use of detergents / surfactants in sanitizing solutions or washing water reduces the surface tension of the solution and thus improves contact with bacterial cells, causing a greater inactivation / removal of bacteria from vegetable surfaces (KESKINEN & ANNOUS, 2011). The combination of detergents with physical sanitizing agents may enhance their action (SAGONG *et al*., 2013).

Silver has been extensively evaluated as a therapeutic agent and for its use in water disinfection processes. Moreover, the interest in the use of silver has increased, especially as a possible replacement for chlorine compounds. Other prospective silver applications are related to post-harvest processing of fruits and vegetables (GOPAL *et al*., 2010).

It is known that fruits and vegetables undergo a series of changes after harvesting due to the environment, nutrient supplies and crop injury from processing. Sanitization treatments aim to eliminate spoilage and pathogenic microorganisms to provide consumers with a safe product with a longer shelf life. Sanitization should prevent undesired enzymatic reactions as well as microbial growth and multiplication to preserve the nutritional and sensorial aspects of the fruits and vegetables.
Growing consumer interest in food has led to looking beyond the basic functions of food for energy and nutrient supply and into purchasing products that have properties that promote health and prevent diseases. The quality of these food products is strongly related to their preparation, handling and storage. Some studies have shown that these operations have important effects on the content of bioactive compounds (SORIA & VILLAMIEL, 2010; RAWSON *et al*., 2011a; RAWSON *et al*., 2011b; PLAZA *et al*., 2011; ZINOVIADOU *et al*., 2015). In this context, this study was done to evaluate the effects of different methods of sanitization on the microbiological and physicochemical properties of cherry tomatoes stored at 7°C.

2. MATERIALS AND METHODS

2.1. Cherry tomato samples

Cherry tomatoes (*Lycopersicon esculentum* var. cerasiforme) were acquired from local retailers and from a single producer to avoid variation. Tomatoes were stored under refrigeration at 7°C for a maximum of 24 h before processing, and damaged or rotten tomatoes were discarded. Tomatoes were washed in water to remove dirt adhered to the surface and were then drained for 30 min in a laminar flow hood.

2.2. Preparation of sanitizers

The effects of the following sanitization methods were evaluated: sodium dichloroisocyanurate (Nippon®, Indaiatuba, São Paulo, Brazil) at a concentration of 200 mg/L (SD), 1 % lactic acid (95 %; Vetec®, São Paulo, Brazil) (LA), a solution of silver nanoparticles at 6 mg/L (NP), and detergent Nitrol WV 2640 (Nippon®, Indaiatuba, São Paulo, Brazil) (DET) with or without ultrasound treatment (US) at a low frequency of 40 kHz, 300 W, with a tank size of 6"L x 5.5"W x 4"D (Ultrasonic Cleaner Branson® 1510, St. Louis, USA). A 1 % solution of lactic acid (v/v) was prepared. The distilled water used to prepare the solution was previously sterilized and kept under refrigeration. The colloidal dispersions of silver nanoparticles were obtained according to the methodology developed by FERNANDES (2010).
To prepare the sanitizing solutions that were applied in conjunction with ultrasound, we used water at 5°C. Previously, it was found that the ultrasonic sanitization time implied a 2 degree increase in water temperature. The next sanitization step consisted of immersing the cherry tomatoes in the sanitizing solution for 5 min at a temperature of 7±1°C. A group of tomatoes exposed to treatment with sterile distilled water and a group of tomatoes exposed to no sanitization treatment were used as controls.
For each treatment, 2 kg of cherry tomatoes were sanitized in containers made of polyethylene terephthalate that had previously undergone exposure to ultraviolet light (254 nm) for 30 min in a laminar flow hood. Cherry tomatoes were distributed with 250 g in each container and stored at 7°C for 10 d and evaluated at days 1 and 10 for microbiological qualities and on days 1, 2, 4, 6, 8 and 10 for physicochemical and nutritional qualities.

2.3. Microbiological analysis

The procedures used in this step were performed according to the methods of the American Public Health Association (APHA) as described in the Compendium of Methods for the Microbiological Examination of Foods (DOWNES & ITO, 2001).
After each sanitization treatment, 25 g of cherry tomatoes were homogenized separately in 0.1% peptone water at a dilution of 10^{-1}. Homogenization was conducted in a stomacher (Seward Medical Co., London, United Kingdom) for 2 min at the normal speed. Appropriate dilutions were prepared, and aliquots of these dilutions were transferred to growth media specific for the detection of each microbial group. Plating rate experiments were performed in duplicate, and the results were expressed in colony-forming units per gram (CFU/g). To determine the number of aerobic mesophiles, inoculation was performed in standard plate count agar (PCA) (Difco®), followed by incubation for 48 h at 35±1°C. Mold and yeast aliquots were inoculated on potato dextrose agar (PDA) (Oxoid®) at a pH of 3.5 and incubated at 25±2°C for 5 to 7 d. Coliform counts were carried out at 35°C using the Petrifilm technique (3M®) according to the recommendations of the Association of Official Analytical Chemists (AOAC, 2005). Plates were incubated at 35°C for 48 h, enumerating *E. coli* as blue colonies entrapped with gas, and other coliform colonies as those colored red and associated with gas. Standard plate counts were used for aerobic psychrotrophics on Plate Count Agar (Himedia®), with plates incubated at 7°C±1°C for 7-10 d. Lactic acid bacteria counts were performed using the method of plating in depth using 1 mL of the sample and a spilling-out quantity of MRS agar (Himedia®) in Petri dishes followed by incubation at 30°C for 5 d.

2.4. Physicochemical analysis

Tomato samples were evaluated for their physical and chemical characteristics. These analyses were intended to identify possible changes in the quality of the cherry tomatoes after sanitization treatments compared to non-sanitized samples (controls).
The determination of titratable acidity (TA) was performed using titration according to the technique described by AOAC (2005). The titration was performed with 0.1 mol/L NaOH, and the results were expressed as % citric acid.
For pH determination, 10 g of tomato samples were randomly collected and homogenized together with 100 mL of distilled water (IAL, 2004). Readings were taken using a Digimed DM 20 pH meter (São Paulo, Brazil).
Total Soluble Solids (TSS) content was determined by refractometry using a portable digital ABBE refractometer at 25°C. Readings were taken using three drops of pulp juice made with 10 g of tomato. The results were expressed as % (IAL, 2004).
Color change was measured objectively by colorimetry using a Minolta colorimeter Color Reader CR 10 CIELAB system, calibrated with a direct reflectance reading of L* coordinates (lightness), a* (relative green to red) and b* (relative yellow to blue). For each sample, the average of three measurements in five random cherry tomato areas was used for each repetition of treatments in each evaluation day. The parameters L*, a* and b* were determined using the Hue angle (H°) and chroma through the following equations:

$$^{o}H = \tan^{-1} \cdot (b^*/a^*) \qquad \text{Chroma} = [(a^*)^2 + (b^*)^2]^{1/2}$$

The firmness of the cherry tomatoes was determined using an Instron Test Apparatus (Series 3367) with a 3-mm diameter probe, a speed of 5 mm/s and a penetration distance of 19 cm before contact with the sample. Five cherry tomatoes were analyzed for each treatment, with two measurements taken per tomato. The results of the firmness analysis were expressed as the maximum force (N).

2.5. Extraction and analysis of ascorbic acid

Ascorbic acid (AA) extraction conditions were optimized by CAMPOS et al., (2009). A 5-g sample of the cherry tomatoes and 15 mL of extraction solution (3 % metaphosphoric acid, 8 % acetic acid, 0.3 mol/L sulfuric acid and 1 x 10^{-6} mol/L EDTA) were mixed using microtrituration for 5 min, centrifuged at 1789 *g* for 30 min, and the supernatant was adjusted up to 25 mL with ultrapure water. AA was determined according to CAMPOS *et al*., (2009) using a high-performance liquid chromatograph (Shimadzu) equipped with a LiChrospher 100 RP-18, 250 x 4 mm, 5 μm chromatographic column. The flow of the mobile phase (1 μmol/L NaH_2PO4, 1 μmol/L EDTA, pH 3.0) was 1.0 mL/min, and the run time was 5.0 min. Elution was detected using a photodiode array detector (Shimadzu SOD-M10 AVP) with the wavelength set to 245 nm. AA identification was done by comparing the retention times obtained for the standard and for samples analyzed under the same conditions. Furthermore, the authors compared the absorption spectra of the standard and the peaks of interest in the samples using a diode array detector. The standard used was L-ascorbate (Vetec, Brazil). Quantification of the compound in the samples was taken from the analytical regression curve equation of ascorbic acid (y = 3,870,141.085x + 883,647.051; R^2=0.997), and results were expressed in mg of fresh matter.

2.6. Extraction and analysis of carotenoids

Carotenoid extraction was performed according to RODRIGUEZ *et al*. (1976) with modifications. Approximately 7 g of tomato were triturated in 60 mL of acetone (divided into three volumes of 20 mL), vacuum filtered on a Buchner funnel and transferred to 50 mL of cold petroleum ether. Extracts obtained from the samples were then concentrated in a rotary evaporator at a temperature between 35 and 37°C. Then, carotenoids were dissolved in 25 mL of petroleum ether and stored in amber glass vials in a freezer (at approximately -5°C) for chromatographic analysis.

Carotenoids were determined according PINHEIRO-SANT'ANA *et al*. (1998) using a high-performance liquid chromatograph (Shimadzu) equipped with a Phenomenex C18 RP-18 chromatographic column, 4.6 x 250 mm, 5 μm. The mobile phase was methanol:ethyl acetate:acetonitrile (50:40:10). Flow of the mobile phase was 2.0 mL/min, and running time was 10 min. Elution was detected using a photodiode array detector (Shimadzu SOD-M10 AVP) with the wavelength set to 450 nm. Identification of carotenoids was done by comparing retention times obtained for standards and samples analyzed under the same conditions. Furthermore, the authors compared the absorption spectra of the standard and the peaks of interest in the samples, using a diode array detector.

The standards used were β-carotene (Sigma Aldrich, USA) and lycopene (Sigma Aldrich, USA). Quantification of the compounds in samples was taken from calibration curves and regression equations for lycopene (y= 6.844.138,9670x – 13.153,9821 R^2 = 0.9996) and β-carotene (y= 7.994.514x+ 1.277,372 7; R^2= 0.999). The results were expressed in μg/100 g of fresh matter.

2.7. Statistical analysis

A completely randomized design was used, with eight treatment groups and a control group (no sanitizing) with tests run in triplicate. To evaluate the efficiency of sanitization treatments on microbial load at the beginning and end of the storage period, Student's t test was used to compare each treatment. To compare the treatments with each other within each period, the results of the counts (log CFU/g) were analyzed with an analysis of variance (ANOVA), with means compared statistically using a Duncan's test with a 5 % significance threshold.
A completely randomized design was used for physicalchemical and nutritional quality with split plots, where treatments were in the plots and time in the sub-plots, with three replicates. The results of the parameters were analyzed using ANOVA, and the means were compared statistically using Duncan's test for qualitative variables (treatment) and regression analysis was performed for quantitative variables (storage time). The significance level used was 5 %.
All statistical analyzes were performed using the Statistical Analysis System (SAS Institute, North Carolina, USA), version 9.1, licensed for use by the Federal University of Viçosa, Minas Gerais, Brazil.

3. RESULTS AND DISCUSSION

3.1. Microbiological analyses

After sanitization treatments, the mesophilic aerobic bacteria count was reduced between 0.27 and 2.33 log CFU/g compared to cherry tomatoes that were not sanitized (Fig. 1).

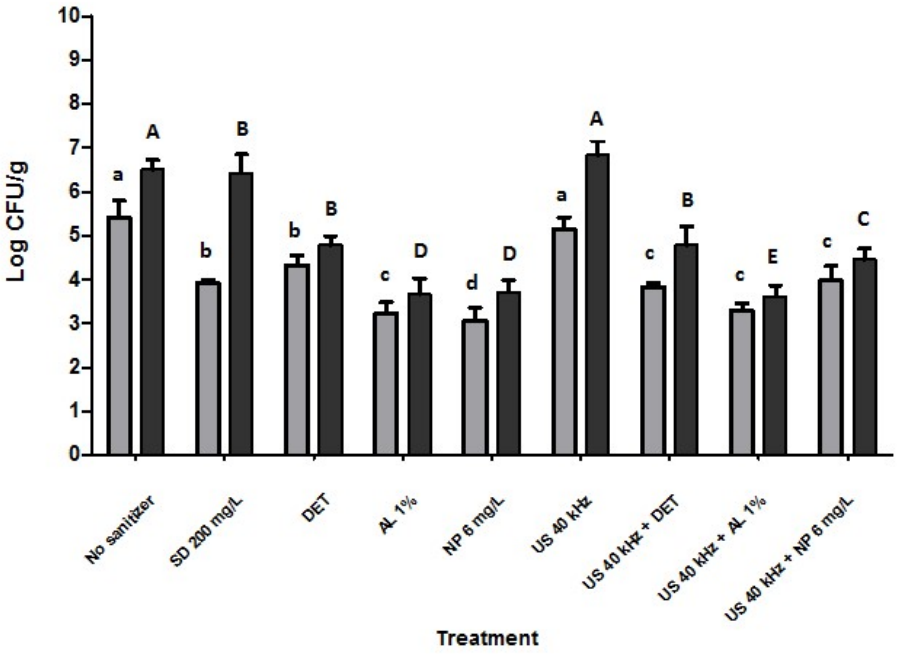


Figure 1. Mesophilic aerobic bacteria count and standard deviation after sanitization treatments (▭) and the tenth day (■) of storage at 7°C. Treatments indicated with the same lowercase letter did not differ ($p > 0.05$) between one another after sanitization treatments. Treatments indicated with the same capital letter did not differ ($p > 0.05$) between one another on the tenth day.

On the tenth day of storage at 7°C, counts were 6.83 log CFU/g of aerobic mesophilic bacteria on tomatoes sanitized by ultrasound at 40 kHz. Treatment with ultrasound did not achieve reduction of 1.0 log CFU/g, a result that was similar to that observed by SÃO JOSÉ & VANETTI (2012) using ultrasound with a frequency of 45 kHz for 10 min. Susana-Rivera, Venturini, Oria & Blanco (2011) observed a reduction of 1.0 log CFU/g of aerobic mesophilic bacteria by applying ultrasound to decontaminate fresh truffles (*Tuber aetivum*). The limitations for the reduction of microorganisms from the vegetable surface can be related to the presence of a hydrophobic cuticle composed of several layers and cutin wax molecules covering the skin of the fruits and vegetables (VELÁZQUEZ *et al*., 2009).

The combination of chemical agents with ultrasound promoted reductions between 1.73 and 2.11 log CFU/g. Immediately after sanitization, treatment with detergent promoted a reduction similar to that of sodium dichloroisocyanurate treatment ($p>0.05$). SÃO JOSÉ *et al*. (2014) and ROSÁRIO *et al*. (2017) mentioned that ultrasound has a potential to be used to inactivate bacteria but must be applied in combination with other sanitizing agents. Treatments with 1 % lactic acid, silver nanoparticles and both of these sanitizing agents combined with ultrasound demonstrated a superior and significant reduction in aerobic mesophilic bacteria counts when compared to the application of sodium dichloroisocyanurate ($p<0.05$). However, when lactic acid 1 % was combined with ultrasound treatment, there was no significant difference ($p>0,05$), indicating that in this combination of treatments, a greater reduction in bacteria was not found. When detergent was combined with ultrasound, we found a significant reduction in bacteria when compared to the reduction promoted by the application of only detergent solution ($p<0.05$). Lactic acid and 1 % silver nanoparticles at 6 mg/L reduced contamination with aerobic mesophilic bacteria by 2.18 and 2.33 log CFU/g, respectively. GOPAL *et al*. (2010) investigated the use of silver (as silver nitrate) and electrochemically generated silver in minimally processed lettuce and found that sanitization with silver was more effective than chlorinated solutions for different micro-organisms, such as mold, yeast, *Enterobactereacea* and *Pseudomonas*.

At the end of the storage period, the counts of mesophilic aerobic bacteria in the cherry tomatoes treated with lactic acid, detergent, silver nanoparticles and lactic acid + ultrasound and silver nanoparticles + ultrasound were significantly lower than in the sodium dichloroisocyanurate treatment group ($p<0.05$).

Fruits and vegetables can also be contaminated by mold and yeast. After harvest, fungal contamination may deteriorate plant material and produce toxic metabolites. Some molds and yeasts can also produce mycotoxins as they grow on these products and others, which present pathogenic risks to consumer health (TOURNAS, 2005). Untreated cherry tomatoes showed 4.70 and 6.13 log CFU/g of mold and yeast after sanitization and at the end of the storage period, respectively (Fig. 2). Treatments promoted reductions between 0.37 and 1.58 log CFU/g. At the initial time of storage, the greatest reductions observed in mold and yeast counts were 1.58 and 1.26 log CFU/g, as registered in samples of cherry tomatoes treated with silver nanoparticles and ultrasound combined with 1 % lactic acid, respectively. A smaller reduction was observed and recorded for mold and yeast counts after treatment with ultrasound applied by itself.

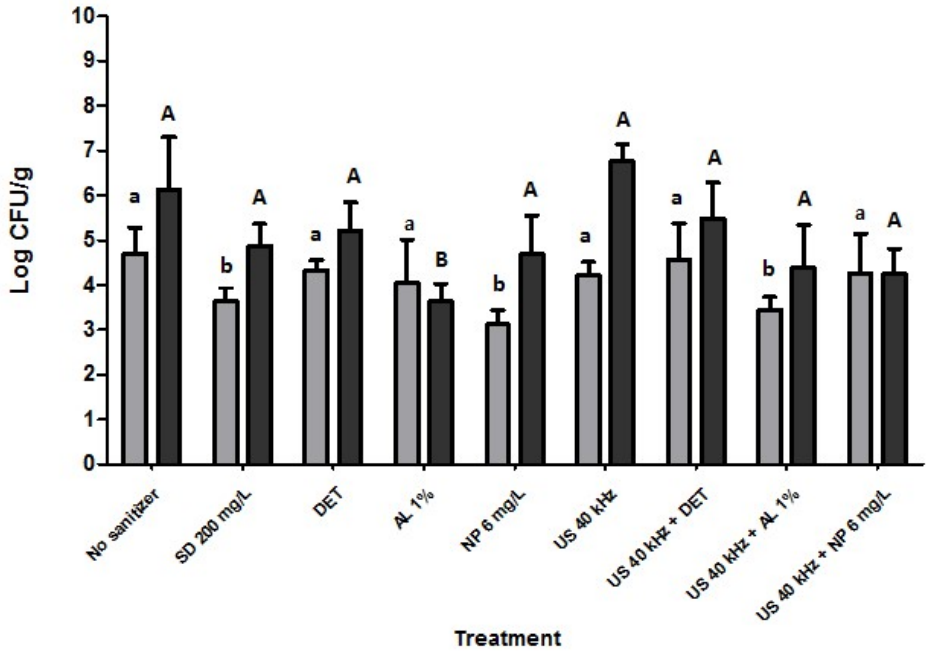


Figure 2. Mold and yeast counts and standard deviation after sanitization treatments ( ) and the tenth day ( ) of storage at 7°C. Treatments indicated with the same lowercase letter did not differ ($p > 0.05$) between one another after sanitization treatments. Treatments indicated with the same capital letter did not differ ($p > 0.05$) between one another on the tenth day.

Of the treatments proposed in this work, only sanitization with silver nanoparticles and ultrasound combined with lactic acid promoted reductions statistically equal to sodium dichloroisocyanurate ($p>0.05$). This result indicates that these treatments had similar effects in reducing mold and yeast counts. A loss of efficiency in the reduction of the mold and yeast counts was observed when detergent was applied to the silver nanoparticles in combination with the ultrasound. In evaluating the effect of ultrasound on the structure of the nanoparticles (data not shown) it was observed that the action of the ultrasound may have contributed to the removal of the surfactant present in the structure of the silver nanoparticle in order to facilitate the approximation of the silver particles. This aggregation of the particles possibly culminated in an increase in diameter and consequently a lower antimicrobial effect.

For coliforms at 35°C, non-sanitized samples showed 5.03 log CFU/g. Among the treatments that stood out, 1 % lactic acid, 6 mg/L silver nanoparticles and ultrasound combined with these two sanitizers promoted, respectively, reductions of 2.06, 2.34, 2.86 and 2.59 log CFU/g after the sanitization processes (Fig. 3). Oliveira *et al*. (2011) detected coliform bacteria in most samples of minimally processed vegetables, with populations of 3 log MNP/g. The presence of these microorganisms can also contribute to reducing the useful life of products (BERBARI *et al*., 2001). Brazilian law RDC No. 12 of January 2001 (BRAZIL, 2001) provides a value of 10^2 MNP/g for testing sanitized vegetable samples. HUANG and CHEN (2011) studied different sanitizing treatments in spinach and noted that 1 % lactic acid reduced *E. coli* O157:H7 to 1.9 log CFU/g.

The application of an isolated form of ultrasound was inefficient for inactivating coliform, yielding a reduction of 0.27 log CFU/g. Detergent alone promoted a coliform reduction of 0.64 log CFU/g. However, a combination of detergent with ultrasound increased inactivation, resulting in a reduction of 1.53 log CFU/g, and a synergistic effect could be

observed. The intense pressure generated by ultrasound can contribute to the penetration of chemical oxidants through the cell membrane, and the cavitation process assists in the breakdown of microorganisms that culminates in increased efficiency of the sanitizer (GOGATE and KABADI, 2009). It was observed that, at the end of the storage period, the cherry tomatoes treated with lactic acid, ultrasound and ultrasound combined with silver nanoparticles presented lower counts than the tomatoes treated with sodium dichloroisocyanurate.

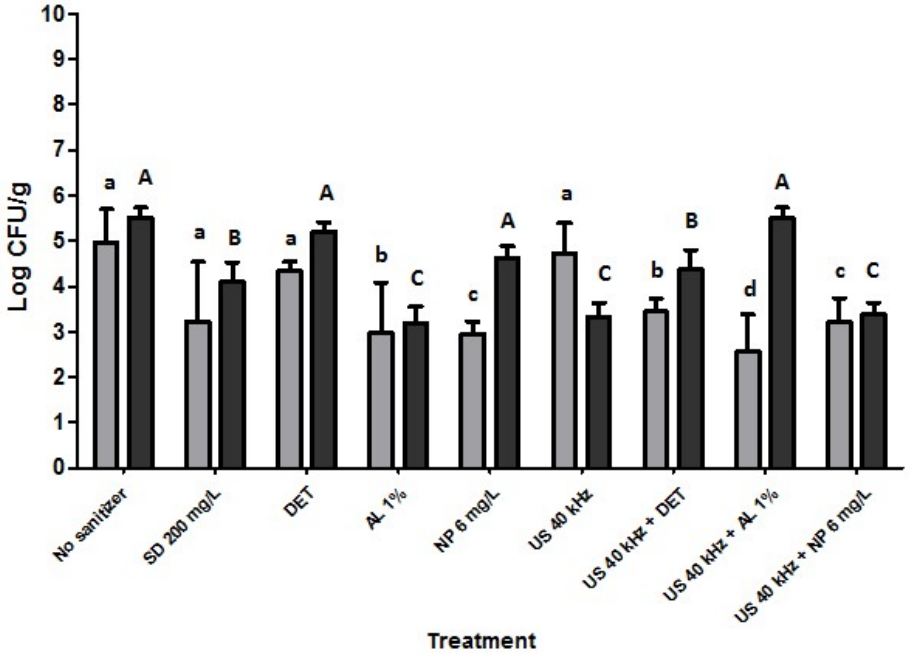


Figure 3. Coliform count and standard deviation after sanitization treatments (▭) and the tenth day (■) of storage at 7°C. Treatments indicated with the same lowercase letter did not differ ($p > 0.05$) between one another after sanitization treatments. Treatments indicated with the same capital letter did not differ ($p > 0.05$) between one another on tenth day.

Treatments promoted a reduction of between 0.41 and 2.64 log CFU/g of lactic acid bacteria (Fig. 4).

The counts obtained after treatment with 1 % lactic acid, ultrasound and ultrasound combined with silver nanoparticles did not differ statistically compared to the non-sanitized samples ($p>0.05$). Higher inactivation was achieved by treatment with sodium dichloroisocyanurate 200 mg/L, ultrasound combined with 1 % lactic acid and silver nanoparticles, which caused reductions of 2.02, 2.14 and 2.64 log CFU/g, respectively. As these treatments showed statistically similar reductions ($p>0.05$) in lactic acid bacteria count, this may suggest the possible replacement of sodium dichloroisocyanurate treatment with ultrasound combined with lactic acid or silver nanoparticles. Only treatment with silver nanoparticles was efficient in the microbiological control at the end of the storage period.

Psychotropic count after sanitization was between 3.90 and 6.29 log CFU/g, and at the end of storage, sanitized cherry tomatoes achieved scores of approximately 5 log CFU/g (Fig. 5).

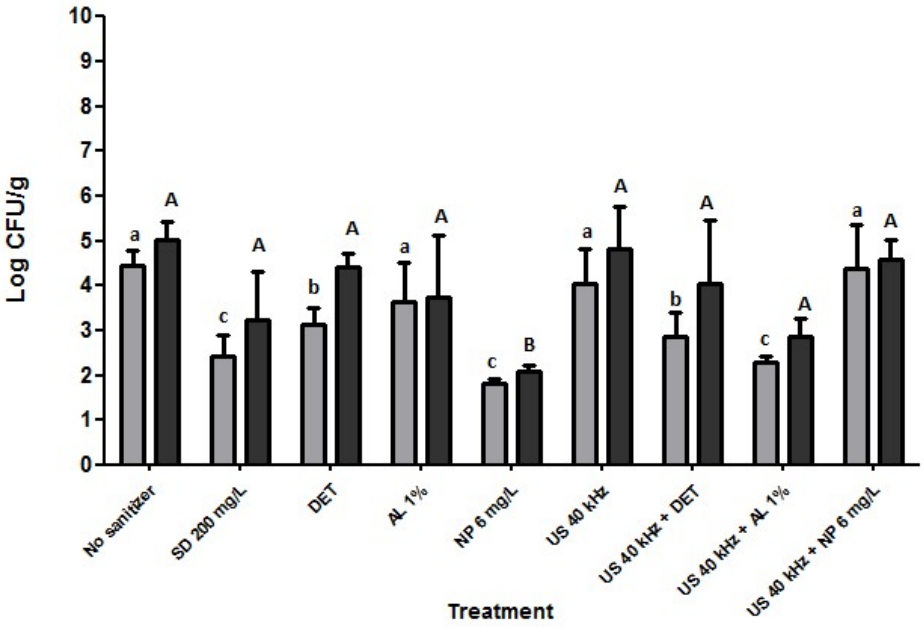


Figure 4. Lactic acid bacteria counts and standard deviations after sanitization treatments (▭) and the tenth day (■) of storage at 7°C. Treatments indicated with the same lowercase letter did not differ (p>0.05) between one another after sanitization treatments. Treatments indicated with the same capital letter did not differ (p > 0.05) between one another on the tenth day.

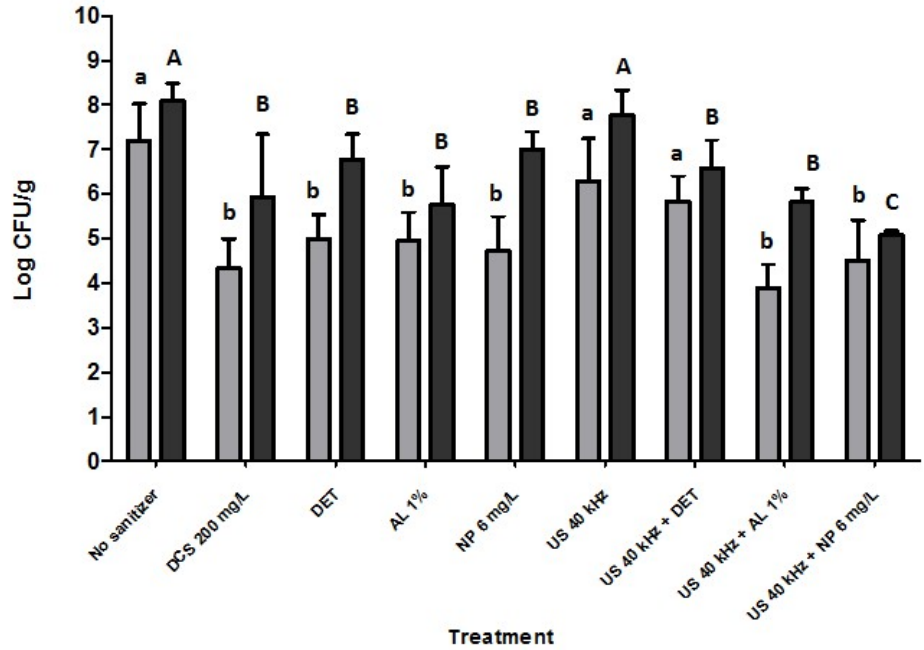


Figure 5. Psychrotrophic aerobic counts and standard deviations after sanitization treatments (▭) and the tenth day (■) of storage at 7°C. Treatments indicated with the same lowercase letter did not differ (p > 0.05) between one another after sanitization treatments. Treatments indicated with the same capital letter did not differ (p > 0.05) between one another on the tenth day.

OLIVEIRA *et al*. (2011) evaluated the contaminant microbiota of minimally processed vegetables and observed large populations of psychrotrophic aerobic bacteria in the samples, suggesting a short shelf life of the products. These counts can be related to the

storage condition of the product that favored this group of bacteria. Ultrasound, and ultrasound combined with detergent did not lead to significant reductions in bacterial counts ($p>0.05$). It is worth mentioning that the ultrasound treatment combined with silver nanoparticles promoted a significant reduction of the psychrotrophic aerobic count after the sanitization process and maintained the lower count at the end of the storage period.

3.2. Physicalchemical and bioactive compound analysis

Measurements of pH showed no significant differences ($p> 0.05$) resulting from sanitization treatments, time or interaction between time and treatments. Quality monitoring of this parameter after sanitization and during the storage period was important because sanitizers may influence the pH values of the vegetables, causing changes that may accelerate the deterioration of the product (RICO *et al*., 2007; SÃO JOSÉ and VANETTI, 2015).

Values of titratable acidity (TA), total soluble solids (TSS), and the ratio of TSS/TA had a significant association with storage time. TA values varied significantly with time, which is best represented by linear regression ($y= 0,01965x^2 + 0,57070$, $R^2 = 0,87$). For TSS, linear and quadratic models were not significant. For TSS/TA, linear regression ($y= -0,41861x^2 + 12,42302$, $R^2 = 0,99$) best represented the data. ADEKUNTE *et al*. (2010) evaluated the effect of ultrasound on tomato juice in different intensities and time and found no change in pH, TA or TSS.

Table 1 shows pH, TA, TSS values and the TSS/TA ratio for the different treatments. TA values ranged from 0.60 to 0.68 % citric acid. This parameter is involved in the assessment of food preservation, since, in most cases, the decomposition of food alters the concentration of hydrogen ions (IAL, 2004) in addition to influencing the sensory characteristics of food. This parameter can vary depending on the degree of maturation and growth conditions (CHITARRA and CHITARRA, 2005). Cao *et al*. (2010) treated strawberries with ultrasound at different frequencies and found that TA content decreased with storage time and that fruit treated with ultrasound at 40 kHz tended to have higher values of TA compared to the control fruit.

Average values of TSS for cherry tomatoes corroborated the mean value observed by PINHO (2011), who recorded 6.1°Brix in cherry tomatoes grown in conventional systems. TSS is a refractive index that indicates the ratio (%) of dissolved solids in solution. It is the sum of sugars (sucrose and hexose), acids (citrate and malate) and other components (phenols, amino acids, soluble pectins, ascorbic acid and mineral salts) present in minor proportions in tomato fruit pulp (BECKLES, 2012). According to the ABHorticultura (2011), the tomato has between 9 and 12 %, which features the highest concentration of soluble solids, especially sugars. CAO *et al*. (2010) evaluated the effect of ultrasound on TSS values and found a decrease in all treatments after strawberry harvest. These authors observed that treatment with 40 kHz ultrasound significantly inhibited the decline of TSS after six days of storage.

In this study, the TSS/TA ratio of cherry tomatoes tended to be lower after treatment with ultrasound combined with chemical sanitizers. This relationship is one of the indices most often used to determine the maturation and palatability of fruit and corresponds to fruit sugar and acid content and is thus an appropriate parameter to measure flavor perception by the consumer (SUAREZ *et al*., 2008; BECKLES, 2012). JAVANMARDI and KUBOTA (2006) found that the average TSS of tomatoes stored at an ambient temperature and at a low temperature ranged from 5.0 to 5.1 %.

In this study, treatment with sanitizers, storage time and the interaction between storage time and treatments were not found to significantly affect color parameters (Table 1). The color of the food is one of the more attractive attributes for consumers and may vary

between species and even between cultivars. The color of a food product is an important freshness-related attribute for consumers when evaluating quality (SÃO JOSÉ & VANETTI, 2015). GANI *et al*. (2016) observed that ultrasound treatment applied in 10, 20, 30 and 40 min lengths resulted in better retention of color during storage of strawberry samples. MUZAFFAR *et al*. (2016) observed that ultrasound treatment between 30 and 40 min showed better retention of color of cherries (*Prunus avium*) during the storage period at 4°C.

ADEKUNTE *et al*. (2010) observed a decrease in the values of L* and color parameters a* and b* in tomato juice treated with ultrasound under different conditions. They suggested that the color changes observed may have been caused by cavitation, which involves different physical, chemical and biological reactions.

In this study, because it involved the sanitization of intact cherry tomatoes, cavitation may have had less of an effect on color. The color of the tomatoes is mainly related to the presence of carotenoid pigments such as lycopene and is influenced by processing (LIANFU and ZELONG, 2008). SAGONG *et al*. (2011) evaluated the effects of 1 % lactic acid combined with ultrasound on other vegetables, including fresh lettuce, and observed no significant change in L*, a* or b* after 7 days of storage at 4°C.

Firmness values did not change significantly ($p>0.05$) due to the treatment, time or interaction between treatment and time (Table 2). The lack of significant changes in the firmness of the cherry tomatoes after the different sanitization treatments during the storage period indicates a preservation of the tomato structure. Firmness is a critical attribute of quality in the acceptability of fruit and vegetables by the consumer (CAO *et al*, 2010) and may decrease due to loss of turgor cells due to water loss during storage (AKBAS and ÖLMEZ, 2007).

Despite the changes in firmness between the different treatments not being statistically significant, it was observed that firmness values were lower after treatment with ultrasound compared to after the application of chemical agents. CAO *et al*. (2010) observed that the application of 40 kHz ultrasound markedly inhibited the softening of strawberries, which maintained high levels of firmness during storage through inactivation of polygalacturonase and pectin methyl esterase enzymes. YANG *et al*. (2011) evaluated the effects of ultrasound both as an individual treatment and combined with salicylic acid for 10 min on peaches and observed no change in firmness after 6 days of storage at 20°C. GANI *et al*. (2016) observed that samples treated for 20 and 30 min with ultrasound showed a maximum retention of firmness and the decrease observed at 40 and 60 min could be attributed to the prolonged exposure resulting in cell injury and loss of water.

Other studies evaluating the influence of chemical sanitizers on the firmness of fruits and vegetables (AKBAS and ÖLMEZ, 2007, SAGONG *et al*., 2011; ALEXANDRE *et al*., 2012) have similarly observed maintenance of firmness during storage.

Fruits and vegetables lose firmness and freshness characteristics when they are kept in refrigerated storage, even for short periods. In this work, maintaining firmness may have been favored by the intact sanitization of cherry tomatoes. This is an important finding for the processing of this product. An ideal sanitization treatment should be simple and easy to apply without causing physical damage and maintain the sensory characteristics of the food (SUSANA-RIVERA *ET AL*., 2011).

In addition to the quality features already discussed, the assessment of losses in carotenoid and ascorbic acid contents after sanitization treatments is also important. The content of lycopene and β-carotene in sanitized cherry tomatoes did not change significantly due to sanitization treatments, time, or the interaction of treatment and time (Table 3).

Table 1. Mean values* and standard deviations of pH, TA, TSS, ratio TSS/TA and color parameters of cherry tomatoes under different sanitization treatments stored for 10 d at 7°C.

Treatment	pH	TA	TSS	TSS/TA	*L**	*a**	*b**	Hue	Croma
No sanitizer	4.30±0.33	0.64±0.23	6.59±1.57	10.30±1.36	29.19±1.04	19.41±2.66	27.80±1.66	55.17±4.00	33.98±2.07
SD 200 mg L^{-1}	4.27±0.29	0.60±0.22	6.35±1.11	10.58±1.37	29.29±1.01	17.93±2.71	27.40±1.15	56.95±3.94	32.82±1.85
DET	4.30 ±0.37	0.63±0.13	6.22±1.33	9.87±1.44	29.78±1.19	16.78±2.80	26.94±3.94	57.95±5.20	31.88±3.76
1 % AL	4.29 ±0.35	0.62±0.17	6.05±1.04	9.75±1.95	29.80±1.12	18.10±3.69	27.38±2.02	56.78±5.16	32.95±2.95
NP 6 mg L^{-1}	4.31 ±0.34	0.63±0.18	5.83±0.84	9.25±1.64	29.59±1.10	17.71± 3.15	27.11±1.94	57.01±4.75	32.49±2.56
US 40 kHz	4.30 ±0.35	0.66±0.18	6.23±1.11	9.43±1.12	28.99±0.76	17.12±2.93	27.07±1.22	57.89±4.10	32.10±2.15
US 40 kHz+DET	4.33 ± 0.32	0.68 ±0.20	5.77±0.89	8.48±2.98	28.97±1.08	16.50±3.09	25.42±0.72	57.27 ±4.74	30.41±1.95
US 40 kHz+1 % AL	4.26±0.33	0.60±0.17	6.46±1.24	10.67±2.95	28.95±0.73	17.77±5.73	26.45±1.53	56.82±6.86	32.13±4.25
US 40 kHz+NP 6 mg L^{-1}	4.31 ±0.35	0.62±0.21	5.66±0.79	9.13±3.11	29.10±0.86	16.90±3.09	25.93±1.12	57.13±4.54	31.05±2.14

*Interaction (treatment × storage time) not significant ($p > 0.05$). Effect of the treatments were not significant ($p > 0.05$).

Table 2. Mean values* and standard deviations of firmness of cherry tomatoes under different sanitization treatments stored for 10 d at 7°C.

Treatment	Firmness (N)
No sanitizer	2.63±0.43
SD 200 mg L^{-1}	2.32±0.72
DET	2.33±0.60
1 % AL	2.21±0.58
NP 6 mg L^{-1}	2.56±0.75
US 40 kHz	2.07±0.75
US 40 kHz + DET	1.70±0.66
US 40 kHz + 1 % AL	1.75±0.55
US 40 kHz + NP 6 mg L^{-1}	1.72±0.63

*Interaction (treatment × storage time) not significant ($p > 0.05$). Effect of the treatments were not significant ($p > 0.05$).

Table 3. Mean* values and standard deviations of lycopene and β-carotene contents of cherry tomatoes subjected to different treatments sanitization stored for 10 d at 7°C.

Treatment	Lycopene (*μ*g/100 g FM)	β-carotene (*μ*g/100 g FM)
No sanitizer	771.82±180.47	646.43±102.72
SD 200 mg L^{-1}	769.24±221.42	704.94±122.04
DET	615.26±186.47	634.61±118.59
1 % AL	737.31± 216.39	635.95±99.93
NP 6 mg L^{-1}	682.81±207.61	643.81±119.75
US 40 kHz	797.44±196.56	636.47±120.09
US 40 kHz + DET	653.77±233.90	651.64±132.59
US 40 kHz + 1 % AL	730.44±205.15	630.11±182.98
US 40 kHz + NP 6 mg L^{-1}	831.54±207.54	675.78± 92.56

*Interaction (treatment × storage time) not significant ($p > 0.05$). Effect of the treatments were not significant ($p > 0.05$). FM = Fresh matter

Lycopene degradation can occur due to processing and storage conditions, which cause isomerization and oxidation of this compound. Environmental factors such as oxygen, light and temperature have an important role in this process (DEMIRAY *et al*., 2013). Free radicals, mechanical energy and hot spots (regions of high temperature and pressure) generated by ultrasonic cavitation can contribute to reducing the contents of nutrients in treated cherry tomatoes. However, this change was not observed, indicating that high retention values were obtained and that the applied sanitizing treatments seemed to have preserved the contents of lycopene and β-carotene.

Studies with other foods that evaluated other bioactive compounds (ALEXANDRE *et al*.., 2012, TIWARI *et al*., 2010) showed that ultrasound allows for greater retention of anthocyanin content, with the authors suggesting that degradation may occur due to oxidation reactions promoted by the interaction of free radicals formed during sonication. ANESE *et al*. (2013) observed that the total concentration of lycopene from tomato pulp was unaffected by processing with ultrasound, and total contents ranged between

5.10±0.80 and 6.60±1.10 mg/g of dry mass in untreated and ultrasound-treated tomato samples, respectively. Mild heat treatment in tomatoes during food preparation, such as heating, boiling and cooking does not significantly alter carotenoid levels in tomatoes or other vegetables (ANESE *et al*., 2013).

Storage at low temperatures inhibits lycopene formation (JAVANMARDI and KUBOTA, 2006); thus, the maintenance of the values of lycopene, as observed in this study, may also be associated with the storage conditions used here (7°C). It is important to emphasize the maintenance of lycopene and β-carotene content after sanitization using different treatments, because it is known that these carotenoids are directly related to the color displayed by tomatoes. This confirms the results obtained in the analysis of color parameters, which showed no significant changes due to sanitization treatments during storage. Although several studies (SASS-KISS *et al*., 2005; KOTÍKOVÁ *et al*., 2011; ILAHY *et al*., 2011) on tomatoes have indicated a higher proportion of lycopene to β-carotene, this study indicated similar concentrations of these two carotenoids. This result may be related to the differences between varieties and/or geographical locations and climates that can promote lycopene production to a greater or lesser extent than the other carotenoids (KOTÍKOVÁ *et al*., 2011).

Regarding ascorbic acid contents, the interaction between treatment and storage time was significant ($p<0.05$) (Table 4). With regards to this interaction, control treatments, ultrasound and silver nanoparticles showed no significant change over time. The ultrasound treatment combined with 1 % lactic acid exhibited non-significant regression coefficients, which suggests the unsuitability of linear and quadratic regression models to predict the behavior of ascorbic acid contents with respect to time. For the other treatments, regression equations were significant. Although vitamin C is the least stable of all vitamins and is easily destroyed during processing and storage, the present study indicated either maintenance or increased ascorbic acid content for some sanitizing treatments. CRUZ *et al*. (2008) additionally found that the rate of destruction is increased by the action of metals, particularly copper and iron, enzymes, oxygen availability, prolonged heating in the presence of oxygen and exposure to light. GANI *et al*. (2016) observed a decrease in vitamin C content in strawberries when ultrasound was applied for 10, 20, and 30 min.

Table 4. Regression equation models for ascorbic acid content in cherry tomatoes submitted to different sanitization treatments and stored.

Treatment	Regression model	R^2	p > F
No sanitizer	$\bar{Y}$=16.80	-	-
SD 200 mg L^{-1}	0.3674x+12.8796	0.42	0.0191
DET	0.8653x+12.2803	0.81	0.0024
1 % AL	0.5169x+14.2298	0.88	0.0017
NP 6 mg L^{-1}	$\bar{Y}$=15.18	-	-
US 40 kHz	$\bar{Y}$=15.84	-	-
US 40 kHz + DET	0.4562x+12.6830	0.46	0.0155
US 40 kHz + 1 % AL	Y=21.54	-	-
US 40 kHz + NP 6 mg L^{-1}	0.4955x+12.9953	0.75	0.0031

Y= mg of ascorbic acid. Function of storage time (x), determination coefficients (R^2) and probability levels (p).

During cavitation, free radicals may be produced, and these compounds may react with food. This interaction may be beneficial or not, depending on the process and the food matrix (SORIA and VILLAMIEL, 2010). Rawson *et al*. (2011a) studied the effect of ultrasound on carrot disks and observed retention of vitamin C content results similar to those observed in the present study, which found that ascorbic acid content was preserved in samples that were not sanitized, treated with ultrasound at 40 kHz and treated with silver nanoparticles at 6 mg/L during the storage period. For the other treatments, ascorbic acid contents increased, which may be related to the maturation of the fruit during storage. YAHIA *et al*. (2001) stated that ascorbic acid is subjected to oxidation and reduction reactions during tomato ripening. Oxidation products are free acid radicals that can be processed again to form ascorbic acid, which allows for the increase of the substance as the fruit ripens. ERCAN and SOYSAL (2011) observed no significant difference ($p > 0.05$) in the content of ascorbic acid of tomato extracts after treatment with ultrasound. CAO *et al*. (2010) found higher levels of vitamin C in strawberries treated with ultrasound at 40 and 59 kHz compared to controls. The differences observed in research reported earlier might be due to difference in food samples and treatment parameters.

4. CONCLUSIONS

An application of silver nanoparticles promoted the best results in terms of microbial count reduction. In the case of the mesophilic aerobic bacteria, the application of the silver nanoparticles contributed to the control after the sanitization process and until the end of the storage period. For mold and yeast, silver nanoparticle treatment and a combination of ultrasound and lactic acid were also effective. For coliforms, ultrasound associated with nanoparticles promoted the best effect. For control of lactic bacteria and psychrotrophic aerobic counts, silver nanoparticle treatment was more efficient.

All of the sanitizing treatments maintained pH values, TA, TSS, color parameters, firmness, and lycopene and beta-carotene contents throughout the storage period at 7°C. Ascorbic acid content ranged between treatments and during storage and showed an increase for several treatments, including sodium dichloroisocyanurate, detergent-based surfactants, lactic acid, ultrasound combined with detergent and ultrasound combined with silver nanoparticles. These results indicate that some proposed treatments have the potential for use in sanitizing cherry tomatoes and showed greater effects on reducing microbiological contamination without affecting other quality characteristics of the vegetable. It is worth noting that the proposed treatments presented similar or superior results than those promoted by sodium dichloroisocyanurate. This indicates a possibility of replacement. However, when choosing a sanitization method, it is necessary to evaluate not only the efficiency but also the cost benefit of each one.

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PAPER

EFFECT OF INFRARED THERMAL PRE-TREATMENT OF SESAME SEEDS (*SESAMUM INDICUM* L.) ON OIL YIELD AND QUALITY

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ABSTRACT

The aim of the study was to determine the effect of thermal infrared treatment of Indian sesame seeds (*Sesamum indicum* L.) on the yield and quality of extracted oil. The infrared radiation treatment of the seeds was applied for 30 s, 60 s, 90 s, 120 s, and 150 s at a temperature of 180ºC. The mean moisture and fat content in the seeds was 7,46 and 45,61%, respectively. The extracted oil was assessed in terms of the hydrolysis degree (AV), primary oxidation degree (PV), oxidative stability in the Rancimat test, and content of carotenoid and chlorophyll pigments.
The study has shown that the infrared thermal treatment of sesame seeds contributed to an increase in the oil extraction efficiency, compared to the control sample. The highest increase in the oil yield was found in the case of seeds heated for 120 s. Simultaneously, there was a reduction of the quality of these oils accompanied by increased oxidative stability. The induction time in oils extracted from infrared-treated seeds increased gradually together with the increase in the length of the heating process. The longest induction time (9,53 h) was noted in sesame oil obtained from seeds heated for 150 s.

Keywords: antioxidant additives, acid value, peroxide value, thermal infrared treatment, oxidative stability.

1. INTRODUCTION

Freshly pressed and non-refined vegetable oil is characterised by a delicate flavour and aroma typical for the seeds used in the process. It should not have an unpleasant bitter taste, as this may imply that the oil is not fresh. Such oils are biologically active, because they are pressed at low temperatures. Hence, they are also referred to as 'virgin', which means that they have the same chemical composition after extrusion as that in the seeds of plants from which they originate. These oils are not supplemented with pigments, antioxidants, and preservatives and are not devoid of phospholipids or tocopherols (vitamin E) as is the case of the common refined edible oils (PALA, 2001).
Cold-pressed oils contain mainly highly active antioxidants (tocopherols, polyphenols, carotenoids, feldspar), polyunsaturated fatty acids from the n-3 and n-6 groups, and bioactive sterols (ROSZKOWSKA *et al*., 2014).
The method of oil extraction with the use of a screw press is quite common. The method has been widely popularised as a simple and ecologically friendly technology that does not require excessive energy costs and investment expenditures. Unfortunately, there are some limitations to the method, e.g. low yields of the process (considerable oil content left in the pomace) and difficulties in achieving a stable quality of the product. Investigations have evidenced that the process of fragmentation or heating the seeds prior to oil extraction increases the efficiency of the pressing process (GUPTA, 2011).
A very important step in the oil extraction process is the hydrothermal pre-treatment of oil-bearing plants. In the oil industry, pre-treatment (T 30 - 40°C) is applied to achieve a homogenous temperature and humidity of the seed mass and to ensure a proper fragmentation process. In turn, the basic conditioning (roasting) of the seed pulp (T 80 - 100°C) is applied to facilitate fat extraction and to increase the oil yield (GUPTA, 2011).
Currently, investigations are being carried out to assess the potential of other alternative seed pre-treatment methods that are more time and energy efficient. An example of such methods is the infrared thermal treatment technique, which provides a higher temperature (over 160°C) within a shorter time than the conventional convection heating (AZADMARD-DAMIRCHI *et al.,* 2011).
Infrared radiation penetrates and heats the product inside, thereby leading to partial evaporation of water from the seed interior accompanied by an increase in intracellular pressure. The disintegration of the cellular structure expands the surface area of the oil release thus contributing to enhancement of extraction yields (AZADMARD-DAMIRCHI *et al*., 2011, YANG *et al.,* 2013).
The infrared thermal treatment of seeds applied prior to the pressing process not only increases the yield but also shortens the heating time, which is accompanied by reduced energy consumption (AZADMARD-DAMIRCHI *et al.*, 2011).
As shown by literature data, the pressing method and seed pre-treatment exert a significant effect on the quality, nutritional value, and oxidative stability of extracted oil (NIU *et al.*, 2013, YANG *et al.*, 2013).
The range of qualitative changes in such oils depends both on the pre-treatment and extraction parameters and on the species of pressed seeds. Therefore, the aim of the study was to determine the effect of infrared thermal treatment of Indian sesame seeds (*Sesamum indicum* L.) on the yield and quality of extracted oil.

2. MATERIALS AND METHODS

The research was conducted on Indian sesame seeds (*Sesamum indicum* L.) harvested in 2016 and oil extracted from the seeds. To balance the humidity of the seeds, the material was stored in a glass jar without a lid at room temperature (20±2˚C) for 14 days.
The infrared thermal treatment of the seeds was applied for 30, 60, 90, 120, and 150 s at a temperature of 180ºC. The choice of the temperature range was based on analysis of previous investigations conducted by ANDREJKO *et al.* (2011).
The seeds were heated with the use of an original laboratory device for thermal treatment of bulk plant materials. The device is equipped with two heating heads with four 400 W ECS-1 radiators manufactured by ELCER. These temperature radiators supplied by electricity (230 V) have a small fraction of visible radiation (dark radiators) in the spectrum and heat all plane points uniformly (plane radiators). The average temperature of the filament is approx. 500°C and the wavelength is λ = 2,5-3,0 μm. The belt conveyor carrying the research material is powered by a DC motor equipped with a voltage regulator facilitating smooth adjustment of the belt travel speed in the range from $5 \cdot 10^{-3}$ m·s^{-1} to $7 \cdot 10^{-2}$ m·s^{-1} (the time that the seeds stay in the heating zone ranges from 15 to 200 s, respectively).
After cooling, the seeds were pressed in a DUO screw press manufactured by Farmet (Czech Republic) with a capacity of 18-25 kg·h^{-1}, motor power 2,2 kW, and a screw speed of 1500 r·min^{-1}. A 0,4 kg seed batch was pressed with the use of a 10-mm diameter nozzle. Before the process, the screw press was heated to a temperature of 50°C, which was measured with an Ama-digit thermometer. After the pressing process, the oil was left for natural sedimentation for 5 days in refrigeration conditions (T 10±1ºC). Next, the oil was analysed.
The oil extraction efficiency was calculated based on the weight of extracted oil, the weight of the seed sample, and the percentage content of oil determined in the seeds. The extraction efficiency "W" was calculated with formula (1) (ROTKIEWICZ *et al.*, 2002):

$$W = \frac{m_{ol}}{Z_{ol}} \cdot 100 \ [\%]$$

Where: m_{ol} - mass of extruded oil, kg, Z_{ol} - mass of oil masa contained in seeds during pressing, kg.
The seed moisture was determined with the oven-drying method using a Radwag max 50/1/WH moisture analyser at a temperature of 120°C in accordance with standard PN-EN ISO 665:2004.
The fat content in the seeds and pomace was determined with the Soxtec 8000 device following the Soxhlet method based on application ASN 310 and in accordance with standard PN-EN ISO 659:201020.
The acid value (AV) defines the amount of free fatty acids in cold-pressed oils. The test was carried out with the AOCS Official Method Cd 3d-63 (Official Method, 2000).
The peroxide value (PV) defines the amount of primary oxidation products in cold-pressed oils (MALHEIRO *et al.*, 2013). The test was carried out with the AOCS Official Method 965.33 (OFFICIAL METHOD, 1999).
The oxidative stability was determined with the Rancimat accelerated oxidation test, which measures the induction time by detection of volatile acids formed during oil oxidation (MATHÄUS, 1996). The test was carried out with the AOCS method (OFFICIAL METHOD, 1989) with the use of the 893 Professional Biodiesel Rancimat device manufactured by Metrohm.
Oil samples (2,50 ± 0,01g) were accurately weighed, placed in a measurement vessel, and subjected to air flow of 20 l/h at a temperature of 120˚C. The results were expressed as the

induction time, which was automatically determined from the curve inflection point with the use of StabNet1.0 software provided by the company.
The overall colour was determined with a spectrophotometric method in accordance with standard PN-A-86934:1995. Measurements of the absorbance of the oil samples were performed after dilution at two wavelengths in the visible range: λ=442nm for the carotenoid pigment group and λ=668nm for the chlorophyll pigment group. The absorbance values were pooled and expressed as an integer
The determination was carried out in triplicate. The arithmetic mean of the repetitions was taken as the result. The statistical analysis of the results was carried out in the Statistica 10 package. The analysis of variance ANOVA was employed to determine the significance of the differences between the values. Inference was carried out at a significance level of 0,05. The mean confidence intervals were analysed in detail with Tukey's test.

3. RESULTS AND DISCUSSION

3.1. Seed moisture and fat content

The initial mean moisture and fat levels in the sesame seeds were 7,46 and 45,61%, respectively (Table 1). The seed moisture content was higher than the value reported by GHARBY *et al*. (2017) (6%), whereas the fat content was lower than that determined by NZIKOU *et al.* (2009) (54%).

3.2. Chemical determinations of sesame oil

Selected chemical determinations that are indicators of the quality of the extracted sesame oil assessed immediately after pressing are shown in Table 1. The acid value (AV) was 1,52 mg KOH/g and the peroxide value (PV) was 0,58 meq O_2/kg. This indicates that the analysed oil fulfilled the quality requirements for cold pressed oils specified in the Codex Alimentarius in terms of the acid value and peroxide value (AV ≤ 4 mg KOH/g, PV ≤ 10 meq O_2/kg) (2009). The acid value of the analysed oil was lower than that reported by RAO *et al.* (1955), i.e. 2,5 mg KOH/g. Similarly, the peroxide value determined in this study was significantly different from the results described by YOSHIDA AND TAKAGI (1997) (1,42 meq O_2/kg), OGBONNA AND UKAAN (2013) (3,95 meq O_2/kg), and GHARBY *et al.* (2017) (2,7 meq O_2/kg) for cold-pressed sesame oil. The differences between the results of this study and those reported by other authors may be associated with the quality of the seeds used. The raw material, e.g. the degree of damage, has a significant effect on the yield and quality of pressed oils (KRYGIER *et al*. 2000).

Table 1. Mean values of moisture and fat content in sesame seeds as well as the acid value (AV), peroxide value (PV), and induction time in the extracted oil.

Sesame seeds		Sesame oil	
Moisture (± SD) (%)	7,46±0,05	AV (± SD) (mg KOH/g)	1,52±0,02
Fat content (± SD) (%)	45,61±0,63	PV (± SD) (meq O_2/kg)	0,58±0,02
		Induction time (± SD) (h)	3,91±0,05

Oxidation is primarily responsible for deterioration of the quality of fats, disagreeable odour and flavour of fat products, and reduction of their nutritional value. Therefore,

oxidative stability is one of the most important indicators of the quality of oils, especially those extracted at low temperatures, which contain natural antioxidants (e.g. tocopherols, carotenoids, sterols, phospholipids, and phenolic compounds). Concurrently, they contain undesirable compounds with prooxidant activity (e.g. metals, chlorophylls), which are removed in the refining process (KOSKI *et al*., 2002; ZINE *et al*., 2013).
The Rancimat test revealed (Tab. 1) that the induction time of the analysed oil was 3,91 h, and this value differed from results reported by other authors. In their investigations, KAMAL-ELDIN AND APPELQVIST (1995) as well as GHARBY *et al*. (2017) demonstrated higher induction times of sesame oil, i.e. 6,1 and 28,5h, respectively. These differences may be associated with the different sesame species and Rancimat test parameters (T 100°C) used in the investigations.

3.3. Effect of seed thermal treatment on oil extraction efficiency and oil content in pomace

The thermal treatment contributed to an increase in the oil yield (Table 2) and exerted an effect on the traits of the sesame oil (Figs. 1-4).
The extraction efficiency of the sesame seed oil gradually increased together with the seed heating time until it reached 120 s. The highest extraction yield (56,25%) was achieved after the 120 s thermal treatment. In comparison with the control sample, the increase in the extraction efficiency was 12,68% ($p \leq 0,05$). The longer heating time (150 s) also contributed to an increase in the oil yield, in comparison with the control sample; however, the difference over two-fold lower (5,24%) than in the case of seeds treated thermally for 90 and 120 s (Table 2).

Table 2. Mean extraction efficiency of the sesame oil relative to the fat content in the pomace.

Heating time of seeds in T 180°C (s)	The fat content in the pomace (± SD) (%)	The oil yield (± SD) (%)
0	$26,31^{e}$ ±0,21	$43,57^{a}$ ±0,04
30	$25,73^{e}$ ±0,18	$45,12^{b}$ ±0,04
60	$24,65^{d}$ ±0,23	$47,31^{c}$ ±0,04
90	$22,05^{b}$ ±0,37	$55,6^{e}$ ±0,04
120	$21,25^{a}$ ±0,24	$56,25^{f}$ ±0,03
150	$23,77^{c}$ ±0,23	$48,81^{d}$ ±0,04

Mean values in the column denoted by different letters differ significantly statistically at $p \leq 0,05$.

The highest fat content, i.e. 26,31%, was determined in the pomace from the thermally non-treated seeds (Table 2). At the longer 120 s thermal treatment of the seeds, the pomace fat content declined from the value of 25,73% noted in the case of seeds heated for 30 s to 21,25% obtained at the heat treatment lasting 120 s. At the 150 s heating time, the fat content in the pomace increased to 23,77% (Table 2). Analogous changes in "Bakara" cultivar rapeseed were reported in the investigations conducted by RĘKAS *et al*. (2015). The oil yield from these seeds was in the range from 53,9% in non-heated samples to 65,3% in the case of seeds heated with microwaves for 3 minutes. The oil extraction efficiency in seeds heated for 7 minutes was reduced to 59,8%. The pomace fat content was 25,5% in non-heated seeds, 20,2% in the case of seeds heated for 3 minutes, and 22,2% in seeds heated for 7 minutes.

The higher fat content in pomace and the lower differences in the oil pressing yields in the case of the 150 s heating time, compared to the control sample, can be attributed to the insufficient moisture level caused by the excessive time of the thermal treatment. Similar conclusions were formulated in the study conducted by KACHEL-JAKUBOWSKA (2008).

3.4. Effect of seed thermal treatment on the physicochemical traits of the extracted oils

To assess the effect of the infrared seed treatment applied prior to the pressing process on the physicochemical traits of the oils, the basic quality parameters were evaluated (Figs. 1-4).
The acid value of the analysed oils ranged from 1,52 mg KOH/g in the oil extracted from the non-heated seeds to 2,02 mg KOH/g in the oil from seeds treated with heat for 150 s (Fig. 1).

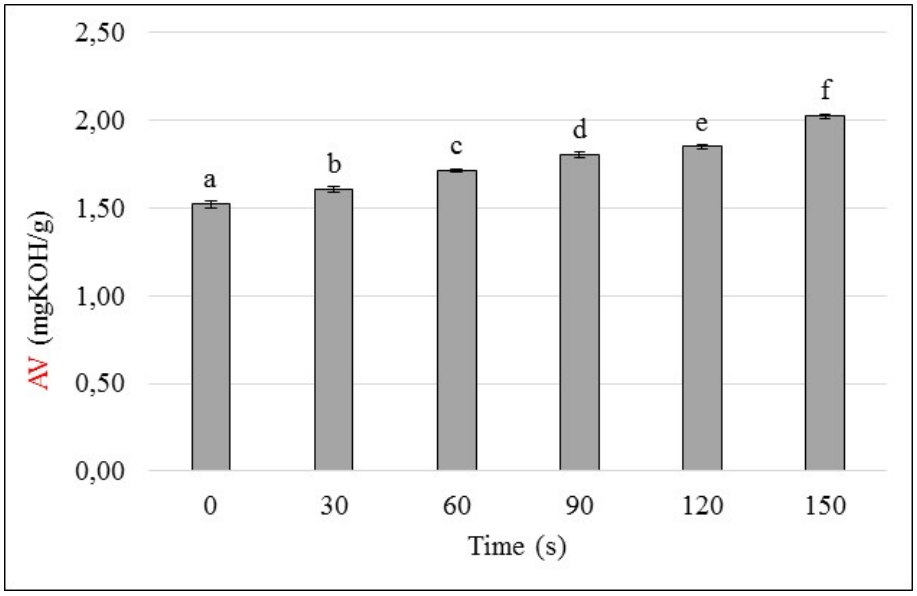


Figure 1. Acid values of oils pressed from sesame seeds relative to the time of seed thermal pre-treatment "t"; different letters above the bars indicate statistically significant differences.

The highest increase in the hydrolysis degree was observed in the oil extracted from seeds treated with 150 s heating.
Furthermore, the oils were characterised by a low peroxide value (Fig. 2), which determines the primary products of oil oxidation.
The peroxide value ranged from 0,58 meq O_2/kg in the non-heated seeds to 1,75 meq O_2/kg in the oil from seeds treated thermally for 150 s. All the analysed oils met the requirements for the acid value and peroxide value in cold-pressed oils specified in the CODEX ALIMENTARIUS (2009). A similar effect of seed thermal treatment on changes in AV and PV of extracted oils was observed by other authors. In investigations of sesame oil from seeds heated at 160°C for 10, 20, and 25 minutes, YOSHIDA AND TAKAGI (1997) demonstrated a PV range from 1,42 (non-heated seeds) to 5,38 meq O_2/kg (seeds heated for 25 minutes). KRALJIĆ *et al*. (2013) evidenced that PV of oil from different rapeseed species heated at a temperature of 80°C for 30 minutes ranged from 1,6 (non-heated seeds) to 2,6 mg KOH/g (heated seeds -Toccata cultivar), from 1,5 (non-heated seeds) to 2,3 mg KOH/g (heated seeds - Oase cultivar), and from 1,3 (non-heated seeds) to 2,3 mg KOH/g (heated seeds - Remy cultivar). In turn, the AV of these oils was in the range from 0,2 meq

O_2/kg (non-heated seeds) to 0,25 meq O_2/kg (heated seeds -Toccata cultivar), from 0,29 (non-heated seeds) to 0,36 meq O_2/kg (heated seeds - Oase cultivar), and from 0,43 (non-heated seeds) to 0,44 meq O_2/kg (heated seeds - Remy cultivar). In investigations of oil from sesame seeds heated with microwaves at a temperature of 100°C for 15 minutes, ABOU-GHARBIA *et al.* (2000) showed a PV range from 0,46 (non-heated seeds) to 0,98 mg KOH/g (heated seeds).
The analysis of the oxidative stability of the extracted oils (Fig. 3) proved that the parameters of the seed thermal treatment applied before the extraction process extended the oil induction time.

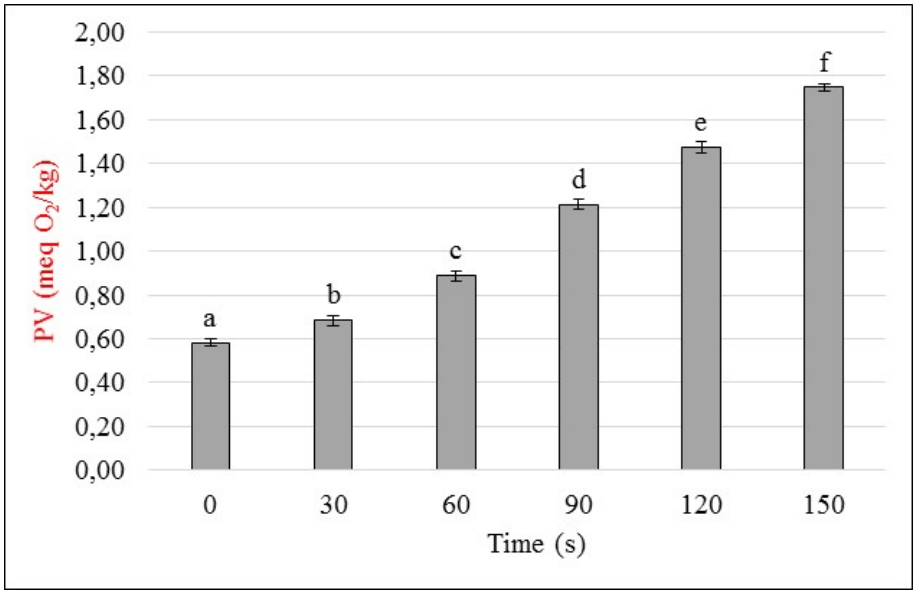


Figure 2. Peroxide values of oils pressed from sesame seeds relative to the time of seed thermal pre-treatment "t"; different letters above the bars indicate statistically significant differences.

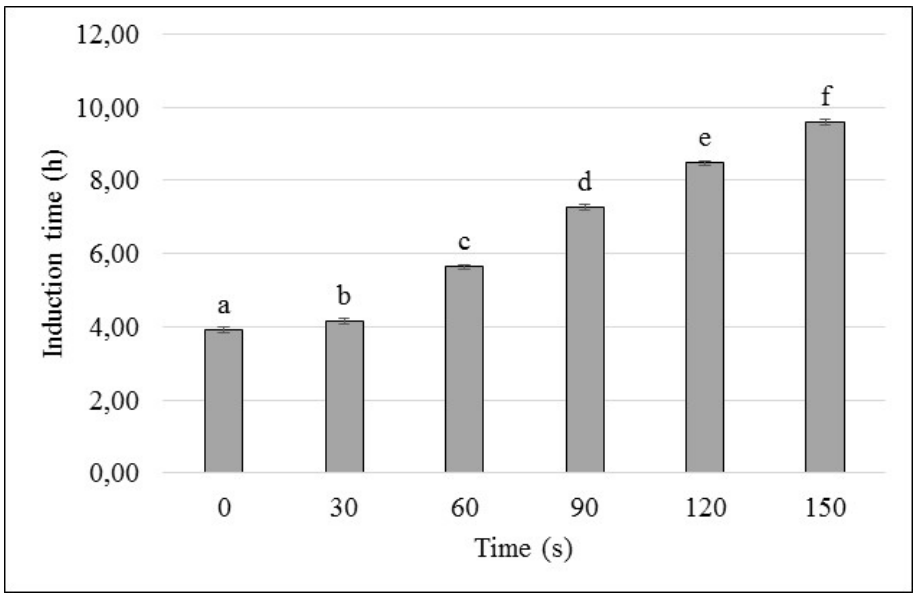


Figure 3. Induction time of oils pressed from sesame seeds relative to the time of seed thermal pre-treatment "t"; different letters above the bars indicate statistically significant differences.

The value of this parameter increased with the longer seed heating time and ranged from 3,91 h for the oil from the non-heated seeds to 9,6 h for the oil extracted from seeds heated for 150 s. The 150 s thermal pre-treatment of the seeds contributed to an almost three-fold increase in the oil induction time, i.e. by 5,69 h. The literature provides reports of the increase in the oxidative stability of oil extracted from thermally treated seeds. In their analyses of oil extracted from sesame seeds heated with microwaves at 100°C for 15 minutes, ABOU-GHARBIA *et al.* (2000) noted an induction time in the range from 9,49 (oil from non-heated seeds) to 18,68 h (oil from heated seeds).
Although there was a statistically significant increase in the LN value as a result of heating, this value was low (1,8 meqO_2/kg) even for oil extracted from seeds heated for 150 s. This did not influence the oxidative stability of the analysed oils. Similarly, FLACZYK *et al*. (2004) did not find a direct relationship between the content peroxides in oils and their oxidative stability. The higher content of natural antioxidants in the oils obtained from the heated seeds may have been a determinant of the long induction time in the analysed oil.

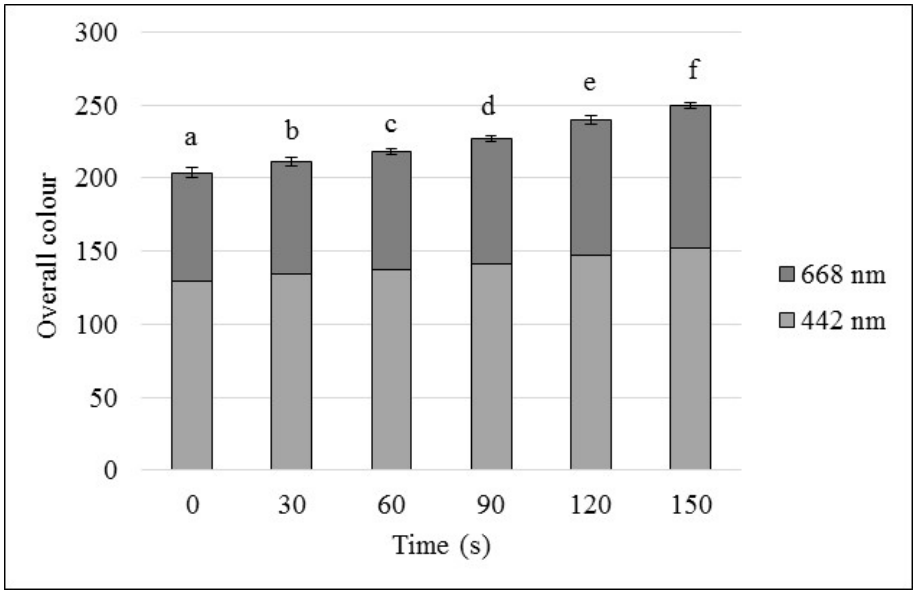


Figure 4. Colour of oils extracted from sesame seeds (1000 x (A442nm + A668nm)) relative to the time of thermal pre-treatment of the seeds "t"; different letters above the bars indicate statistically significant differences.

The analysis of the colour of the sesame oil (Fig. 4) revealed that the oil from the non-heated seeds was the lightest and clearest (overall colour 203). It contained the lowest content of carotenoid and chlorophyll pigments in comparison with the oils extracted from the heated seeds. The thermal treatment of the seeds caused significant darkening of the extracted oil and an increase in absorbance at the wavelengths of 442 and 668 nm. The colour of the oil from the heated seeds was in the range from 211 after the 30 s thermal treatment to 249,67 after heating the seeds for 150 s. Therefore, the oil extracted from seeds receiving the longest thermal pre-treatment was the darkest and the least clear. The same effect of thermal seed treatment on changes in the colour of extracted oils was reported by GHARBIA *et al.* (2000). In investigations of oil extracted from sesame seeds heated with microwaves at 100°C for 15 minutes, the authors demonstrated a three-fold higher content of total carotenoid and chlorophyll pigments in oil from heated seeds than that in oil

extracted from non-heated seeds. The darker colour of the oils extracted from pre-heated seeds can be explained by the presence of products of Maillard reaction and phospholipid and chlorophyll degradation products derived from the thermal seed treatment (AZADMARD-DAMIRCHI *et al.*, 2011; LAMORSKA AND TYS, 2011).

4. CONCLUSIONS

Based on the research results, the following conclusions were formulated:
Infrared thermal treatment of sesame seeds exerts a significant effect on the oil extraction efficiency. The highest increase in the oil yield was noted in the case of seeds heated for 120 s. The longer heating time of 150 s reduced the efficiency of the extraction process.
Infrared thermal treatment of sesame seeds causes a significant increase in the oxidative stability of oil. The longest induction time in the Rancimat test (9,53 h) was recorded for the oil extracted from seeds heated for 150 s.
Thermal seed pre-treatment contributes to an increase in the acid value and the peroxide value, a higher absorbance value at wavelengths of 442 and 668 nm, and a darker overall colour of oil. All the oils analysed in the study fulfil the quality standards in terms of the acid value (AV ≤ 4 mg KOH/g) and the peroxide value (PV ≤ 15 meq O_2/kg).

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PAPER

COMPARATIVE 1D- AND 2D-ELECTROPHORETIC PROTEIN PROFILES OF ANCESTRAL AND MODERN BUCKWHEAT SEEDS GROWN IN THE ITALIAN ALPINE REGION

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ABSTRACT

Buckwheat is an old crop whose seeds are under-utilized. The protein composition of these seeds, however, makes them suitable as much needed ingredients for the production of gluten-free products. Several buckwheat species and local cultivars are known worldwide. In this work, 1D and 2D electrophoresis were used to characterize and compare the seed protein profiles of two buckwheat species (*Fagopyrum esculentum* and *Fagopyrum tataricum*). The two analyzed cultivars of *F. esculentum* represent authentic landraces of an Italian Alpine valley, named Valtellina. The protein profiles of *F. tataricum* and the two *F. esculentum* cultivars did not show major differences. However, narrow but significant differences were present between these two landraces, allowing their discrimination at protein level. This work represents a molecular-based approach to the designation of origin and authenticity of local buckwheat varieties and their tracing in flours for human food.

Keywords: buckwheat, electrophoresis, *Fagopyrum* spp., historical landraces, proteome, traditional cultivation

1. INTRODUCTION

Buckwheat is a pseudo-cereal seed of the class Dicotyledoneae, genus Fagopyrum, and smartweed family. It originated from and was domesticated in Eastern Himalaya regions and can be cultivated in flat and mountainous regions, as long as the climate is cold. Buckwheat came to Europe in the late Middle Age (OHNISHI, 1993) and, early traces of its cultivation in Italy were found in property documents of a family in Teglio, Valtellina, dating back to the middle of the sixteenth century (FERRANTI *et al*., 2002).
After a long history of growth and food use and a remarkable decline in the last decades, there has been a renewed interest in the crop. Among the reasons for this recent reappraisal are the growing worldwide need for sustainable nutrient sources (DURANTI and SCARAFONI, 2015) and the claimed health benefits of various buckwheat components (LI *et al*., 2008; IZYDORCZYK *et al*., 2014; ZHOU *et al*., 2015), as it occurs for many other seeds (SCARAFONI *et al*., 2007).
Buckwheat seeds (*Fagopyrum* spp.) display a high fiber content, ranging from 12 to 18% with very low lipid levels (about 3%) and relatively low carbohydrate levels, around 50% (EGGUM *et al*., 1980). The presence of biologically-relevant compounds, such as flavonoids, flavones, phytosterols, thiamin-binding proteins (LI and ZHANG, 2001) and rutin, with antioxidant properties (KREFT *et al*., 2002), all add nutritional value to this seed. The protein content is similar to or greater than that of wheat, from 12% d.w. upward. The peculiar composition of the protein fraction makes these seeds and their proteins suitable as main ingredients for many food applications, including foods for coeliac patients.
Phylogenetic relationships and polymorphisms of buckwheat have been studied at both genetic and protein levels (LI *et al*., 2008; OHNISHI and MATSUOKA, 1996; YASUI and OHNISHI, 1998; DU *et al*., 2004; ZELLER *et al*., 2004; ROUT and CHRUNGOO; 2007).
Wide margins for implementing tailored and finalized applications exist, because not all molecular and compositional features of these seeds have been thoroughly investigated for their optimal exploitation.
Modern analytical approaches allow the use of molecular-based strategies for the comparison, selection and improvement of crops and these activities are becoming crucial for mankind in the near future. Electrophoretic techniques applied to the protein fraction may represent a complementary and effective approach to the genetic/genomic analysis of plants (GORINSTEIN *et al*., 2005; CAPRARO *et al*., 2008). In this work, we applied a fast and reliable methodology based on electrophoretic analyses of seed proteins to identify candidate quality marker to be used to test and guarantee the designation of origin and authenticity of local buckwheat varieties. We focused our attention on two remnant cultivars of *Fagopyrum esculentum*, L.; one which is locally named 'Nustran' and the other called 'Furest' or 'Francese' or else 'Curunin' which has recently been identified as an authentic Valtellina landrace by genetic analyses (BARCACCIA *et al*., 2016). In this work we will refer to this latter cultivar as 'Furest'. It is still cultivated in Teglio (Valtellina), an Italian Central Alpine valley village. The first landrace, 'Nustran', is an original Teglio ecotype, whereas 'Furest' has been cultivated in Teglio only since the beginning of the twentieth century. In appearance, the two cultivar seeds show only slight morphological differences (BARCACCIA *et al*., 2016). In addition, a modern variety of *Fagopyrum tataricum* has also been included in our comparative work.

2. MATERIALS AND METHODS

2.1. Materials

Buckwheat seeds were kindly supplied by Raetia Biodiversità Alpine, Teglio, Valtellina, Italy. *Fagopyrum esculentum*, L. was of the varieties Nustran and Furest; *Fagopyrum tataricum*was of variety n'Zibaria.

2.2. Methods

2.2.1 Protein extraction

Dry seed kernels were manually ground to a meal in a mortar. The protein fractions of the resulting flours were extracted under stirring at room temperature for two hours following two procedures: a non-denaturing one, consisting of a solution containing 50 mMTris-HCl and 0.5 M NaCl at pH 7.5, and a denaturing one (redry solution), consisting of a solution containing 8 M urea, 2 M thiourea, 20 mg/mL3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate(CHAPS) and 65 mM 1,4-dithiothreitol (DTT) at pH 8.5. The ratios, 1/20 (w/v) in non-denaturing conditions and 1/40 (w/v) in the denaturing buffer, were used. The slurries were centrifuged at 12,000*g* at room temperature for 20 min, and the extracted proteins were conserved at -20°C until used. Two experimental replicas for each condition were carried out.

2.2.2 Electrophoretic techniques

Isoelectric focusing (IEF) was performed on 7 cm pH 3–10 linear IPG strips (Amersham Biosciences) as described by CAPRARO *et al*. (2008).
SDS-PAGE for either 1D and 2D analyses was carried out according to LAEMMLI (1970) on 12% polyacrylamide gel using a mini-Protean II cell (Bio-Rad). The gels were stained with Coomassie blue. Protein extracts were analyzed in triplicate.

3. RESULTS AND DISCUSSION

Buckwheat seed proteins include either albumins and globulins, typical of the legume grains, and prolamins at high and low molecular weight (HMW and LMW) (NAIŁĘCZ *et al*., 2009), typical of cereals seeds. For this reason, the performances of two extraction protocols were assessed. The extraction of proteins under non-denaturing conditions using a saline alkaline buffer (Fig. 1, lanes marked with ND, namely T^{ND}, N^{ND} and F^{ND}) resulted in the prevalent solubilization of buckwheat globulins, as shown by 1D SDS-PAGE analysis. The presence of two main bands at about 40 and 25 kDa (L_A and L_B, respectively in Fig. 1) is typical of the reduced 13S globulin family of most species (CASEY *et al*., 1985) and the greater $M_r$minor band components around 60 kDa likely correspond to the 7S globulin subunits (V in Fig. 1). Under these conditions, a lower number of high M_r bands with respect to the samples extracted under denaturing conditions was visible. According to NAIŁĘCZ *et al*. (2009), these new bands likely correspond to the prolamin family, which are insoluble, unless denatured and reduced.

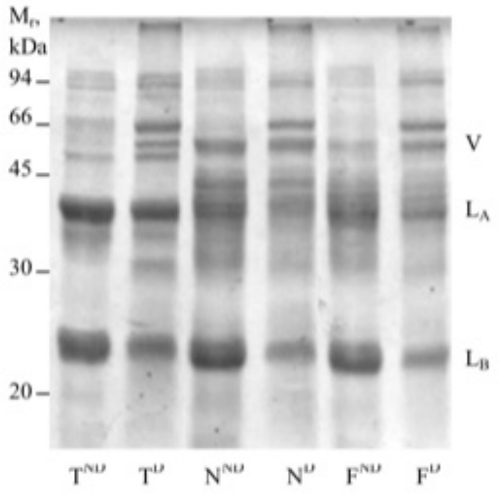


Figure 1. SDS-PAGE under reducing conditions of proteins from seeds of *Fagopyrum* species, namely *tataricum* (T), and *esculentum* landraces, 'Nustran' (N) and 'Furest' (F) extracted under non-denaturing (ND) and denaturing extraction conditions (D). L_A and L_B stand for 13S globulin acidic and basic subunits, respectively; V stands for 7S globulin family. See text for further details.

In the 1D separation of Fig. 1, a clearly distinct pattern of *F. tataricum* protein profile from those of the two *F. esculentum* cultivars was visible in the range 40-70 kDa. In particular, in samples T^D, three distinct bands were visible in the range 50 - 66 kDa while only two polypetides were detectable in the other two samples.
Overall, the 1D protein profiles of *F. esculentum* 'Nustran' and 'Forest' were more similar to each other than that of *F. tataricum*.
The greater resolution of 2D electrophoretic analysis was used to get a more detailed comparison of the three protein patterns (Fig. 2). Based on the results described above, the 2D IEF/SDS-PAGE analyses were carried out under denaturing and reducing extraction conditions to get the most complete picture of the respective proteomes. Indeed, the 2D maps of the analyzed *Fagopyrum* spp. allowed the identification of some of the seed's main protein components. The spot groups marked with L_A and L_B display intensities and positions which definitely identifies these spots as acidic and basic subunits of the 13S globulin, respectively. The 'train spot' group is typical of the high M_r 7S globulin chains with their peculiar pI isoforms (RADOVIĆ *et al*., 1996; MAGNI *et al*., 2007). In this latter case, however, closely migrating HMW prolamins with variable pI and M_r around 50 kDa were likely present too, as detailed by NAIŁĘCZ *et al*. (2009). Prolamins of intermediate and low M_r, though barely recognizable, seemingly spread in the map at more acidic pIs and with greater migration coefficients, according to NAIŁĘCZ *et al*. (2009).
The 2D electrophoretic maps confirm and detail the 1D profiles by showing a greater similarity between the two *F. esculentum* cultivars and their difference with *F. tataricum* spot pattern. Indeed, the region of the 13S globulin acidic subunits appears quite different and some major spots, which are circled in the panel T of Fig. 2, contributed to significantly diversify the map of *F. tataricum* with respect to those of *F. esculentum*. However, differences between the two *F. esculentum* landraces were also detectable. Quantitative differences among common spots, could be noted too. Most of these differences were found in the low M_r, neutral pH region of the 2D maps. The observed intervarietal differences cannot be attributed to climatic, pedological or edaphological causes, since the seeds arise from same cultivation area and grower. Likely, these

differences represent authentic biodiversity source. In an extension of this work, it would be interesting to identify those differing spots, which are unlikely related to classical storage proteins because of their low M_r and clear-cut shape of the spots, and to associate them with peculiar phenotypic or nutritional features of the two cultivars.

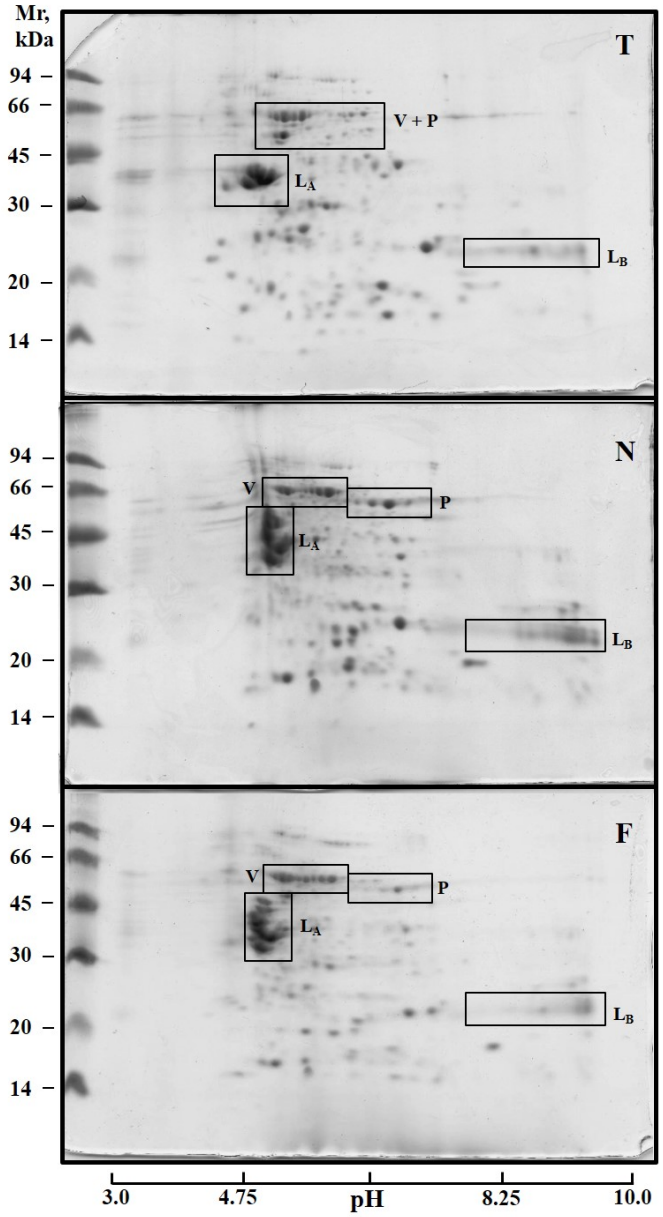


Figure 2. Two-dimension electrophoretic maps of proteins extracted from seeds of *Fagopyrum* species, namely *tataricum* (T) and *esculentum* landraces: 'Nustran' (N) and 'Furest' (F), all extracted under denaturing conditions (see details under Methods). V: 7S globulin chains; P: prolamins chains; L_A: 13S globulin acidic subunits; L_B: 13S globulin basic subunits.

4. CONCLUSIONS

In the perspective of future uses of landraces and their derivatives in foods suitable for people with coeliac disease or in nutraceutical formulations, the development of an agile

methodology to identify landraces components is needed. This work represents a first step in this direction, making available specific 2D electrophoretic maps for given landraces and thus helping in their identification and tracing in flours for human food. It is worthy of note that the two cultivar seeds are very similar in appearance, with 'Furest' being smaller in size and of lighter grey color (BARCACCIA *et al*., 2016). These minimal differences make the need for molecular fingerprinting more compelling.
This work, by revealing even minor interspecific and intervarietal differences in the protein expression patterns of buckwheat seeds, may open the gateway to the identification of hidden useful properties, such as resistance to adverse environmental conditions and seed quality characteristics, and to the valorization of these crop populations.

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PAPER

THE EFFECT OF TEMPERATURE AND METHOD OF DRYING ON ISOT (URFA PEPPER) AND ITS VITAMIN C DEGRADATION KINETICS

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ABSTRACT

This study investigated drying and vitamin C degradation kinetics in isot (red peppers) at different temperatures (55, 65 and 75°C) and conditions (vacuum and hot air drying). The drying temperature and method had a significant effect on the loss of vitamin C. Vacuum dried samples at 55°C retained the highest quantity of vitamin C, while the samples dried at 75°C in a cabinet dryer lost the highest amount of vitamin C. The results showed that vitamin C is especially sensitive to the presence of oxygen and higher temperatures. The Weibull model was found to provide the mathematical equation best describing the ascorbic acid degradation kinetics in red peppers, while the Page model best reflected the drying kinetics.

Keywords: drying, ascorbic acid, isot, degradation kinetics, mathematical modeling

1. INTRODUCTION

Red pepper (*Capsicum annuum* L.) is native to American tropical zones and largely grown in relatively moderate regions as well. Red pepper is widely consumed as a fresh vegetable crop or is processed into various food products like roasted, dehydrated, flaked or powdered pepper. One of the oldest and most common processing methods is drying, and it is utilized to enhance the shelf life of food crops. Lowering water activity during drying prevents microbial and biochemical decay of food (KAMILOGLU *et al*., 2014). Dried red peppers are used in the production of ready to eat soups, ketchup, snacks, potato chips, dressings and sauces.

Peppers are not just used as a colorant, or flavoring, and/or as a source of pungency, but pepper is a good source of vitamin C, antioxidants and bioactive compounds. Among the antioxidant phytochemicals, polyphenols deserve a special mention due to their free radical scavenging properties. The red pepper, especially, has a significantly higher total phenolic content than green pepper. Red pepper contains a higher level of β-carotene (5.4 μg/g), capsanthin (8.0 μg/g), quercetin (34.0 μg/g) and luteolin (11.0 μg/g) (NADEEM *et al*., 2011).

However, the drying process may negatively affect food quality parameters such as nutritional value, bioactive-compound content, color and texture (FAZAELI *et al*., 2011) Non-enzymatic browning reactions, loss of pungency and degradation of bioactive compounds are the major changes occurring in red peppers during drying.

Red peppers are also a good source of lycopene, β-cryptoxanthin, fiber and an array of vitamins such as A, K and C. Vitamin C is one of the numerous bio-functional compounds found in red peppers. Several studies reported on the ascorbic acid content of red peppers (LEE *et al*., 1995; KUMAR and SAPE, 2009). Even though there are large differences in varieties KUMAR and SAPE, 2009), red peppers are generally recognized as a good source of vitamin C, and its vitamin content can be as high as 186 mg/100 g of fresh weight. Vitamin C has been shown to be associated with alleviation of cardiovascular disease and high blood pressure, with increased immune function, increased iron absorption and the promotion of high iron stores. Retention of vitamin C in dried foods indicates that processing conditions were not harsh and thus the other micro-nutrients present in the food matrix were most likely retained (HIWILEPO-VAN HAL *et al*., 2012). As vitamin C is a hydrophilic, heat-sensitive vitamin that is especially prone to both chemical and enzymatic oxidation, its concentration in food systems can be considered as a quality factor in plant-based foods. The use of dried food crops is mainly to enhance the quality, flavor and acceptability of the prepared dishes. To minimize the loss or destruction of a food component such as vitamin C or its color during processing, kinetic models that describe destruction rates and their dependence on such factors as temperature, moisture content and water activity must be determined.

The study of drying behaviour of different materials has been a subject of interest for various researchers on both theoretical and application grounds for the past 16 years. Recently, there have been many studies on the drying behaviour of various vegetables and fruits, such as mushrooms and pollen (MIDILLI *et al*., 1999), potato (GOGUS and MASKAN, 1998), onion (SARSAVADIA *et al*., 1999), green beans and pumpkin (YALDIZ and ERTEKIN, 2001), grapes (YALDIZ *et al*., 2001), pistachios (MIDILLI, 2001) and peppers (DI SCALA and CRAPISTE, 2008; VERAS *et al*., 2012; DARVISHI *et al*., 2014).

Mathematical equations and kinetic models are required for the design of optimal procedures for food-processing steps such as drying and storage (KAYMAK-ERTEKIN and GEDIK, 2005). This study was carried out to report on the degradation kinetics of ascorbic acid and the drying kinetics of red peppers processed under different drying conditions at various temperatures. The loss of nutritional quality during food processing

has drawn more and more attention in recent years as nutrient deterioration can be the limiting factor determining the consumer demand, especially for vegetable-fruit-based foodstuffs. A review of the degradation kinetics of vitamins in fruits, vegetables and cereals during thermal processing was published by VILLOTA and HAWKES (1986). Thus, food processors are concerned in protecting nutrient quality and developing the technological capability to predict nutrient losses during handling and processing. This requires the identification and understanding of the processing parameters responsible for nutrient degradation.
Considering the rare reports available on ascorbic acid degradation kinetics in dried fruit or vegetables, and the growing interest in many bio-functional compounds including ascorbic acid in recent years, it is important to study the degradation kinetics of these compounds. The degradation kinetics of vitamin C gives a better insight into corresponding food processing conditions, and thus, helps to adapt the best processing methods to preserve the nutritional content of the products. This study was designed to determine the optimum drying conditions for preserving the micro-nutrients of Urfa pepper (İsot) that has a large trading potential in Urfa province. Therefore, we studied the drying of red peppers using different drying techniques at different temperatures and established the vitamin C degradation equations.

2. MATERIALS AND METHODS

2.1. Materials

Red pepper (*Capsicum annuum* L.) cultivated in Şanlıurfa Province was purchased from local markets and used for dried red pepper production. Peppers were sliced by hand before the drying processes. The samples obtained either during or at the end of the drying process were stored in a freezer (-20°C) until the analyses were carried out.

2.2. Chemicals

Meta-phosphoric acid (HPO_3) was obtained from Merck (Germany), ascorbic acid standard with 99% purity and methanol of HPLC grade were both obtained from Sigma-Aldrich Company (Germany).

2.3. Drying equipment

The drying processes were carried out in vacuum and cabinet (hot air) dryers. A vacuum dryer (WiseVen, WOV-70, Witeg, Germany) was used to dehydrate red peppers under vacuum conditions. The dryer has three metal shelves. Split red peppers were laid on each shelf in a density of 1600 g/m^2. Red peppers were dried under the conditions of -0.1 MPa atmospheric pressure and at temperature levels of 55, 65 and 75°C.
A cabinet dryer (elektro-mag, M7040-R, Turkey) was used for drying red pepper using hot air with a velocity of (1.2 m/s). The dryer has three grill shelves, a fan and ventilation hole. Split red peppers were laid on each shelf in density of 1600 g/m^2 and dried at three different temperatures (55, 65 and 75°C). The temperature values were determined according to the results of preliminary experiments. All drying processes were carried out in triplicate.

2.4. Calculations

The drying rate (DR) of peppers was calculated using Eq. 1, where M_t and M_{t+dt} are the moisture content (kg of water per kg of dry solid) at t and t +dt, where t is the drying time in minutes.

$$DR = \frac{M_{t+dt} - M_t}{dt} \quad (1)$$

For drying model selection, drying curves were fitted to 5 well known drying models, which are given in Table. 1. The moisture ratio (MR) of pepper during the drying experiments was calculated using the following equation:

$$MR = \frac{M_t - M_e}{M_o - M_e} \quad (2)$$

where M_t, M_o and M_e are the moisture content at any drying time (in minutes), and the initial and equilibrium moisture content (%, d.b.), respectively. The values of M_e are relatively small compared to those of M_t or M_o, hence the error involved in the following simplification is negligible (AGHBASHLO *et al*., 2008) and accordingly we can write:

$$MR = \frac{M_t}{M_o} \quad (3)$$

Table 1. Models used to fit the drying data.

Model	Equation	Reference	Eq. No.
Page	$MR = \exp(-kt^N)$	Diamonte and Munro (1993)	(4)
Modified Page	$MR = \exp[(-(kt)^N)]$	White *et al.* (1981)	(5)
Newton	$MR = \exp(-kt)$	Henderson (1974)	(6)
Henderson and Pabis	$MR = a\exp(-kt)$	Zhang and Litchfield (1991)	(7)
Wang and Singh	$MR = 1 + at + bt^2$	Wang and Singh (1978)	(8)

For the effect of drying temperature on the rate constant (k) for Eq. 4, Page's model was used with Arrhenius's equation (Eq. 9):

$$\ln(k) = \ln(k_o) - \frac{E_a}{RT} \quad (4)$$

Where, T, E_a, R and k_o are the drying temperature in K, the activation energy in kJ mol^{-1}, the ideal gas constant of 8.314×10^{-3} mol^{-1} K^{-1} and the pre-exponential factor, respectively. All calculations were done in triplicate.

2.5. Sample Preparation for HPLC analysis

Firstly, dried and frozen red pepper samples were blended (Yazıcılar, G1, Turkey) to make fine powder. Approximately 2 g of dried red pepper was weighed into a centrifuge tube

(50 mL) and it was combined with 50 ml of 3% metaphosphoric acid and shaken for 5 minutes and then centrifuged at 4000 rpm for 10 minutes. The supernatant was then transferred to a 100 mL volumetric flask with 3% metaphosphoric acid. Each sample was filtered into vials before injection into an HPLC Column.

2.6. Ascorbic Acid Analysis by HPLC

The detection and quantifying of L-ascorbic acid levels in the samples was carried out using HPLC equipped with a UV-DAD detector (Shimadzu), according to PUWASTEIN *et al.* (2011) and STEFANELLI *et al.* (2014). The HPLC equipped with an LC-20AD pump, autosampler, and an ODS C18 column (250 mm×4.6 mm×5 μm) was used, and the UV-DAD detector was set to a wavelength of 254 nm. The isocratic mobile phase was methanol: water (5:95, v/v) at a pH of 3 fixed by H_3PO_4, and the flow rate was 1ml/min with an injection volume of 20 μl. Various concentrations of standard ascorbic acid was used to obtain a calibration curve, and the peak areas were used to calculate the ascorbic acid content. Results were expressed as mg/100g of dry matter. All HPLC measurements were carried out in triplicate.

2.7. Statistical analyses

One-way analysis of variance (ANOVA) was carried out with SPSS 16.0 to determine the main influence of vacuum and hot air drying techniques on the drying and ascorbic acid degradation parameters. The Duncan multiple range test was employed to compare the differences among groups. The non-linear regression analysis was done using a Sigma plot (version 10) software package. The correlation coefficient (R^2) was one of the main criteria for selecting the best model. In addition to the coefficient of correlation, the goodness of fit was determined by root mean square error (RMSE) values, residual-predicted plot and experimental-predicted plot. For a quality fit, the R^2 values should be close to 1 and the RMSE values should be lower.

3. RESULTS AND DISCUSSION

3.1. Change of moisture ratio and drying rate during hot air and vacuum drying

Drying of the red peppers by both hot air and vacuum dryers started with an initial moisture content of around 91.26% (w.b.) and continued until a final moisture content of 12.50% (w.b.) was reached. The variations of moisture ratio with time at 55, 65 and 75°C for both hot air and vacuum dryers are given in Fig. 1. As expected, an increase in drying temperature resulted in a decrease in the drying time for both hot air and vacuum drying of red peppers at different temperatures (55, 65 and 75°C). The times to reach 12.50% (w.b.) moisture content from the initial moisture content of red peppers at 55, 65 and 75°C were found to be 960, 742 and 594 minutes for hot air drying, and 247, 231 and 195 minutes for vacuum drying, respectively.

Based on the final moisture content (12.50% w.b.), drying time decreased from 960 minutes to 247 minutes at 55°C when comparing hot air drying to vacuum drying. Similarly at 65°C, drying time decreased from 742 minutes to 231 minutes, and at 75°C drying time decreased from 594 minutes to 195 minutes. The vacuum drying was able to reduce the drying time of peppers by 74.1% at 55°C, 69.3% at 65°C and 67.0% at 75°C, compared with hot air drying (Fig. 1). These results are in good agreement with previous observations for

mint leaves (GIRI and PRASAD, 2007) and for mushrooms (THERDTHAI and ZHOU, 2009).

The changes in the drying rates (DR, % d.b min^{-1}) versus both drying time (minimum) and moisture content (%, d.b) for both hot air and vacuum dryers are shown in Fig. 2. It is apparent that the drying rate decreased with drying time for both hot air and vacuum drying. The drying rate also decreased continuously with decreasing moisture content or for increasing drying times. These findings are in the agreement with previous studies (TOGRUL and PEHLIVAN, 2002; YALDIZ and ERTEKIN, 2001; YALDIZ *et al*., 2001). There is no constant-rate drying period in these drying rate curves, and all the drying operations are seen to occur over the falling rate period.

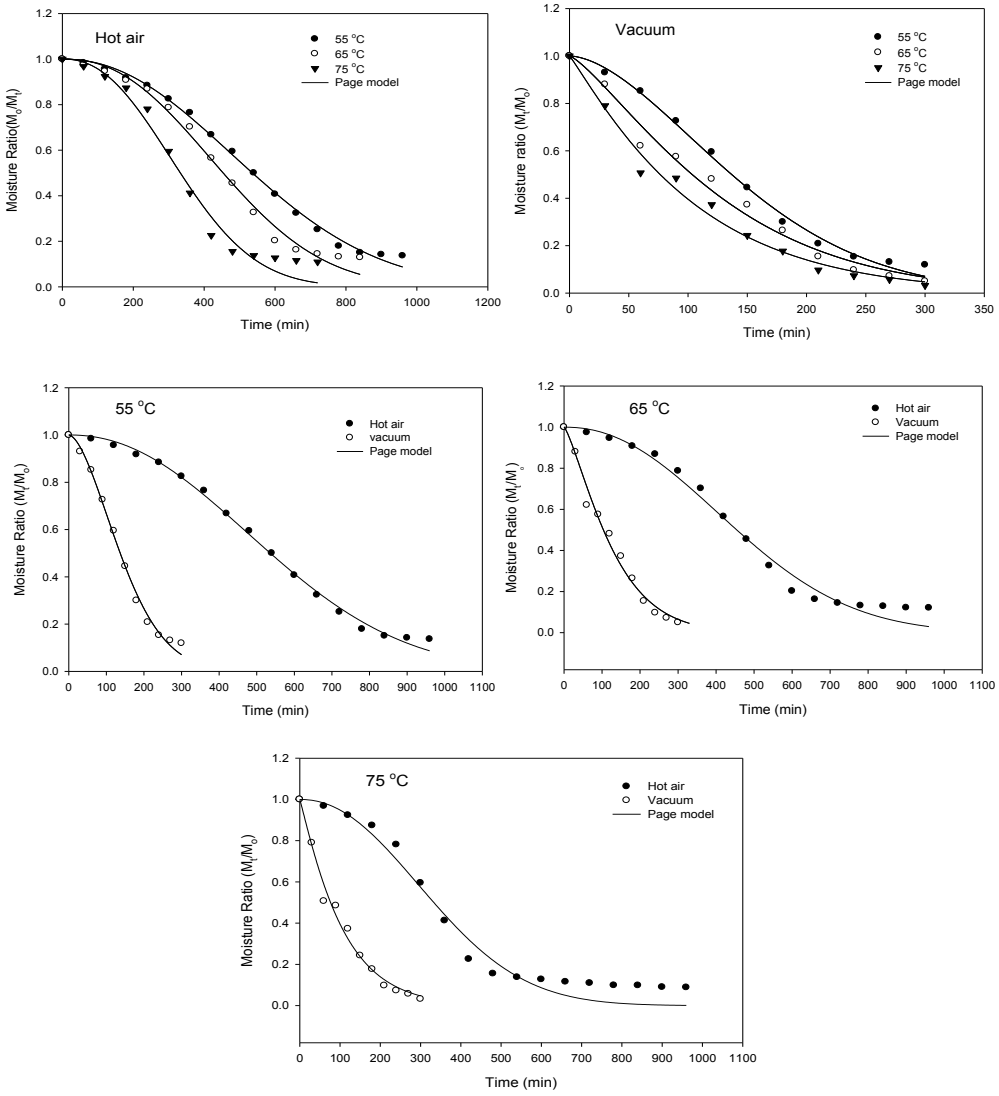
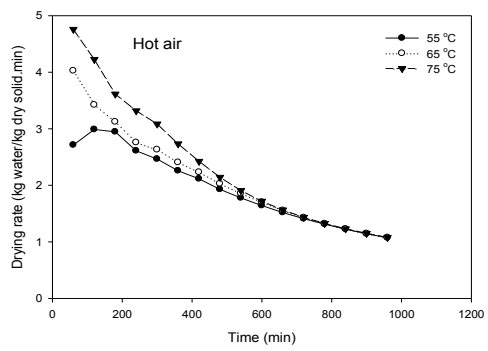
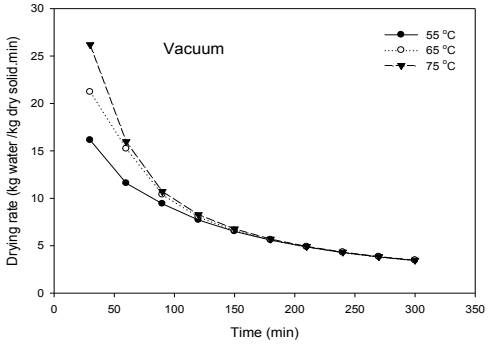
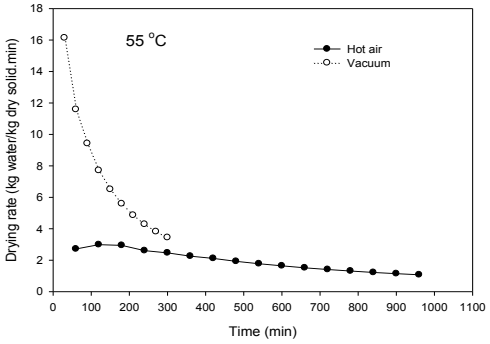
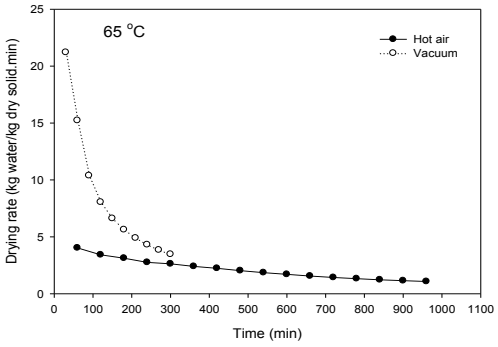


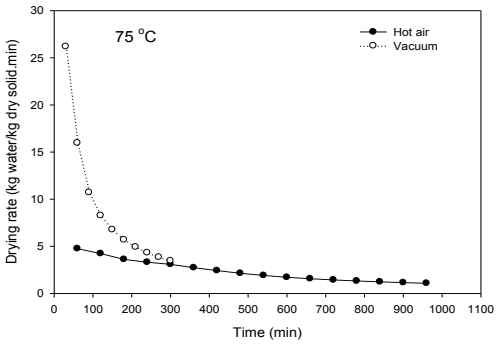
Figure 1. Fitting of the average experimental and simulated data of hot air and vacuum drying to the Page model at 55, 65 and 75°C temperatures.

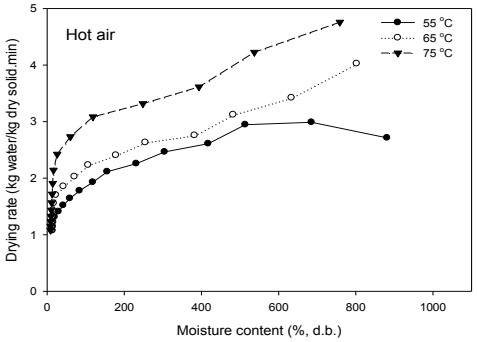

Hot air
55 °C
65 °C
75 °C
Drying rate (kg water/kg dry solid.min)
Time (min)

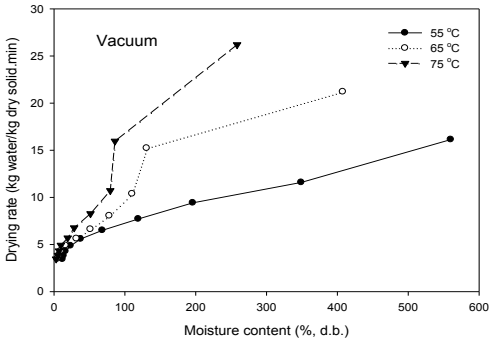

Vacuum
55 °C
65 °C
75 °C
Drying rate (kg water /kg dry solid.min)
Time (min)

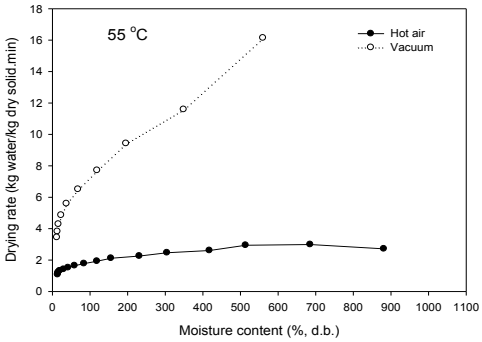

55 °C
Hot air
Vacuum
Drying rate (kg water/kg dry solid.min)
Time (min)


65 °C
Hot air
Vacuum
Drying rate (kg water/kg dry solid.min)
Time (min)


75 °C
Hot air
Vacuum
Drying rate (kg water/kg dry solid.min)
Time (min)


Hot air
55 °C
65 °C
75 °C
Drying rate (kg water/kg dry solid.min)
Moisture content (%, d.b.)


Vacuum
55 °C
65 °C
75 °C
Drying rate (kg water/kg dry solid.min)
Moisture content (%, d.b.)


55 °C
Hot air
Vacuum
Drying rate (kg water/kg dry solid.min)
Moisture content (%, d.b.)

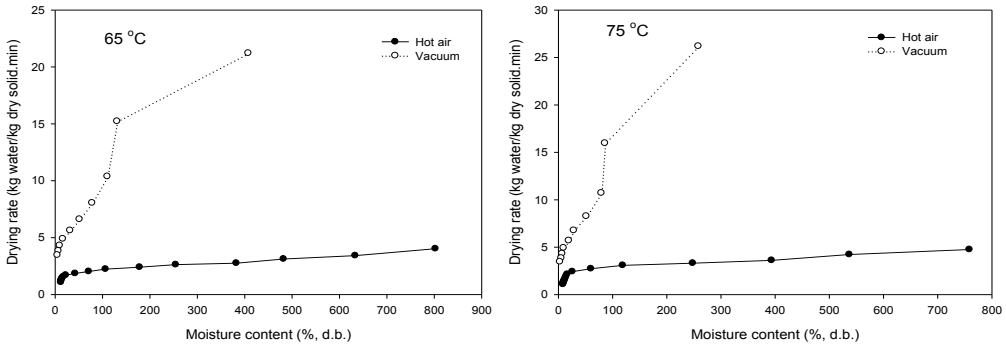


Figure 2. Drying rate versus time and moisture content at different temperatures for hot air and vacuum drying of peppers.

Increases in the drying temperature of red peppers also increased the drying rate and decreased the drying time (Fig. 2). The drying time is shorter when the temperature is higher, such as 75°C, and can be explained by the increase in the drying rate due to the increased heat transfer potential between the air and the peppers, therefore favoring the evaporation of water from the peppers. The effect of temperature on the drying rate has been also studied and reported on by some researchers (HENDERSON and PABIS, 1961; AKPINAR *et al*., 2003).

The vacuum drying was able to increase the drying rate of peppers by 76.6% at 55°C, 73.6% at 65°C and 70.2% at 75°C, compared to hot air drying (Fig. 2), respectively. Increases in the drying rates confirmed the decrease in drying times when the peppers were dried with a vacuum dryer. These results are in good agreement with the study by GIRI and PRASAD (2007) for mushroom vacuum drying with a drying time reduction range of 70 to 90%, and earlier observations (KAYMAK-ERTEKIN, 2002; AKPINAR *et al*., 2003).

3.2. The drying kinetics of peppers

3.2.1 Modeling of pepper moisture ratio as a function of drying time

Fig. 1 shows the change in moisture ratio of pepper samples with time at 55, 65 and 75°C for hot air and vacuum drying. It can be seen that the moisture ratio decreases continuously with drying time for both hot air and vacuum drying. The mass transfer within the samples was more rapid during higher temperatures because more heat generation within the sample created a large vapor pressure difference. The advantage of vacuum drying is to accelerate the drying process, to increase the mass transfer through an increased pressure gradient between the inner and outer layers, and to maintain the drying process at lower temperatures (PERE and RODIER, 2002).

The statistical and regression results from the 5 different models are given in Table 2. The R^2 and RMSE values for models were found to be in the range of 0.8596 to 0.9966 and 0.13 to 0.02, respectively.

Based on the criteria of the highest R^2 (0.9996 to 0.9697) and the lowest RMSE (0.02 to 0.06), Page's model was selected as the most suitable model to represent the hot air and vacuum drying behavior for red peppers. The model parameters were significant with a 95% confidence interval, and the predictive curves arising from the Page model (Fig. 1) adequately described the experimental data. The residual-predicted plot for the Page

model regressed on the data is displayed in Fig. 3 and the residual points seem to be randomly distributed, with most residuals lying within two standard deviations.

Table 2. Statistical parameters of the drying models for different conditions.

Process	Temp. (°C)	Model	a	b	k (min^{-1})	N	R^2	RMSE
Hot air	55	Page			$8.73x10^{-7}(\pm0.03)^{a,x}$	$2.16(\pm0.01)^{a,x}$	0.9966	0.02
		Modified Page			$1.57\ x10^{-3}$	2.16	0.9966	0.02
		Newton			$1.42\ x10^{-3}$		0.8596	0.12
		Hend.andPabis	1.17		$1.72\ x10^{-3}$		0.9056	0.10
		Wang and Sing	$-6.92x10^{-4}$	$-3.40x10^{-7}$			0.9691	0.05
	65	Page			$1.18x10^{-6}(\pm0.06)^{b,x}$	$2.15(\pm0.02)^{a,x}$	0.9841	0.04
		Modified Page			$1.86x10^{-3}$	2.15	0.9841	0.04
		Newton			$1.77x10^{-3}$		0.8616	0.13
		Hend.andPabis	1.18		$2.12\ x10^{-3}$		0.9033	0.11
		Wang and Sing	$-1.14x10^{-3}$	$1.00x10^{-7}$			0.9308	0.09
	75	Page			$2.76x10^{-6}(\pm0.03)^{c,x}$	$2.14(\pm0.03)^{a,x}$	0.9697	0.06
		Modified Page			$2.53\ x10^{-3}$	2.14	0.9697	0.06
		Newton			$2.49\ x10^{-3}$		0.8867	0.12
		Hend.andPabis	1.17		$2.90\ x10^{-3}$		0.9174	0.10
		Wang and Sing	$-1.96x10^{-3}$	$1.01x10^{-6}$			0.9229	0.10
Vacuum	55	Page			$7.31x10^{-4}(\pm0.02)^{a,y}$	$1.71(\pm0.02)^{a,y}$	0.9950	0.02
		Modified Page			$5.90\ x10^{-3}$	1.71	0.9950	0.02
		Newton			$5.77\ x10^{-3}$		0.9254	0.09
		Hend.andPabis	1.11		$6.49\ x10^{-3}$		0.9465	0.08
		Wang and Sing	$-4.05x10^{-3}$	$2.95\ x10^{-6}$			0.9722	0.05
	65	Page			$9.34\ x10^{-4}(\pm0.03)^{b,y}$	$1.28(\pm0.01)^{a,y}$	0.9858	0.04
		Modified Page			$7.25\ x10^{-3}$	1.28	0.9858	0.04
		Newton			$7.41\ x10^{-3}$		0.9692	0.06
		Hend.andPabis	1.05		$7.80\ x10^{-3}$		0.9734	0.05
		Wang and Sing	$-5.51x10^{-3}$	$7.64\ x10^{-6}$			0.9902	0.03
	75	Page			$1.57\ x10^{-3}(\pm0.08)^{c,y}$	$1.09(\pm0.02)^{a,y}$	0.9875	0.03
		Modified Page			$9.32\ x10^{-3}$	1.09	0.9875	0.03
		Newton			$9.45\ x10^{-3}$		0.9857	0.04
		Hend.andPabis	1.02		$9.59\ x10^{-3}$		0.9860	0.04
		Wang and Sing	$-7.04x10^{-3}$	$1.30x10^{-5}$			0.9839	0.04

Means in the same column with different superscript letters are significantly different, a to c (temperature), x to y (dryer), and $p \leq 0.05$. Values in parentheses are standard deviations.

Furthermore, the experimental-predicted plot was close to linear, indicating a good model fit to the Page and Weibull models in describing the drying characteristics of red peppers (Fig. 3).

From the Page model, N was found to be greater than 1.0 (the lowest value was 1.09), which means that the relationship between the moisture ratio and time was unlikely to be first order kinetics. Thus, Page's model offered improved predictability of drying kinetics

over other models. For Page's model, k for the pepper samples increased from 8.73×10^{-7} min^{-1} to 2.76×10^{-6} min^{-1} (a 68.37% increase) when drying temperature changed from 55°C to 75°C for hot air drying. Also, a 97.52% increase was obtained in the value of k for vacuum drying within the same temperature range. When comparing hot air and vacuum drying of peppers at 55°C temperature, the k value for the Page model increased from 8.73×10^{-7} min^{-1} to 7.31×10^{-4} min^{-1}, a 99.88% increase. Vacuum drying produced a similar percentage increase in the k value at 65°C and 75°C (99.87 and 99.82%). These results are supported with results in Section 3.1, which is related to the increased drying rate and decreased drying time with vacuum drying.

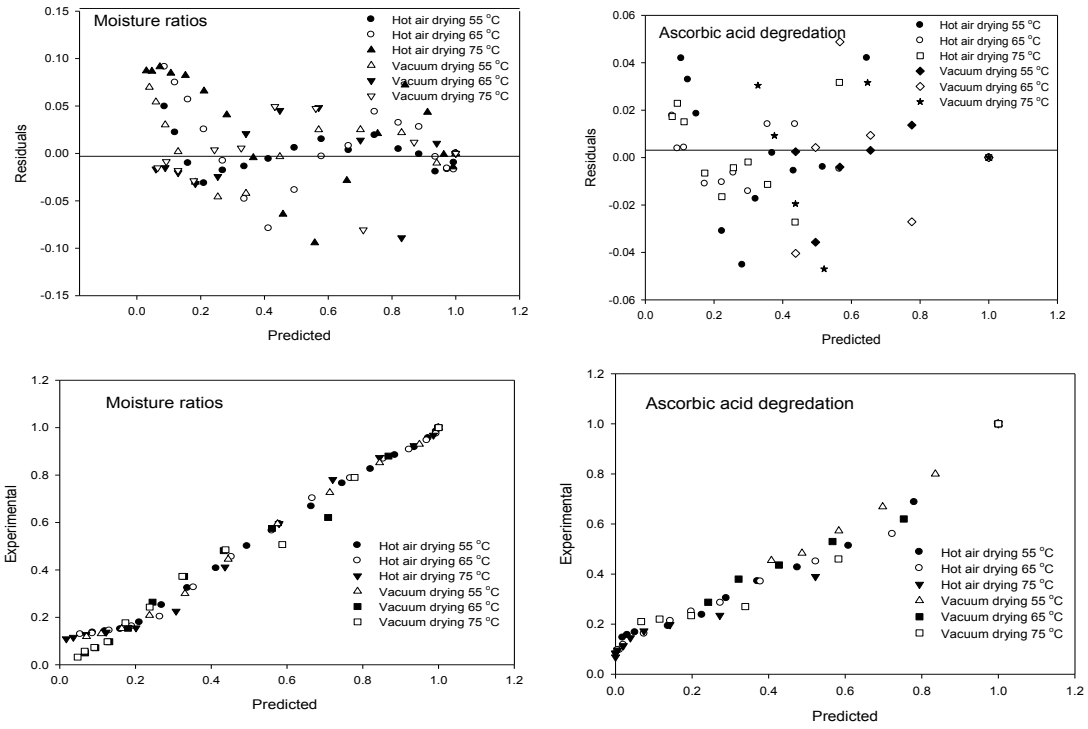


Figure 3. Residual-predicted and experimental-predicted plots for Page and Weibull models at different temperatures (55, 65 and 75°C) for hot air and vacuum drying

3.2.2 A general model to describe the moisture ratio (M_t/M_o) as a function of drying time and temperature

Arrhenius plots of the natural logarithm of the rate constant (k) versus the inverse of T (K) (Eq. 9) for both hot air and vacuum drying of peppers are superposed in Fig. 4. The activation energy, E_a, is related to the slope of this graph, and shows that the temperature dependence of the drying rate constant (k) was fitted to a linear model. The computed values of E_a were found to be 54.4 kJ mol^{-1} for hot air and 36.0 kJ mol^{-1} for vacuum drying of peppers, respectively. Compared to the hot air drying, the activation energy decreased when the vacuum drying was applied for drying red pepper. This corresponds to the increase of the drying rate and decrease of the drying time. A similar comparable result was obtained by THERDTHAI and ZHOU (2009) for mint leaves for both hot air and vacuum drying. Also, the activation energy is shown to be sensitive to the drying rate constant against temperature. The greater activation energy value, the higher sensitivity of

the *k* value of pepper to the temperature. In general, activation energy values for food and agricultural crops lie in the range of 12.7 to 110.0 kJ mol^{-1} (AGHBASHLO *et al*., 2008).

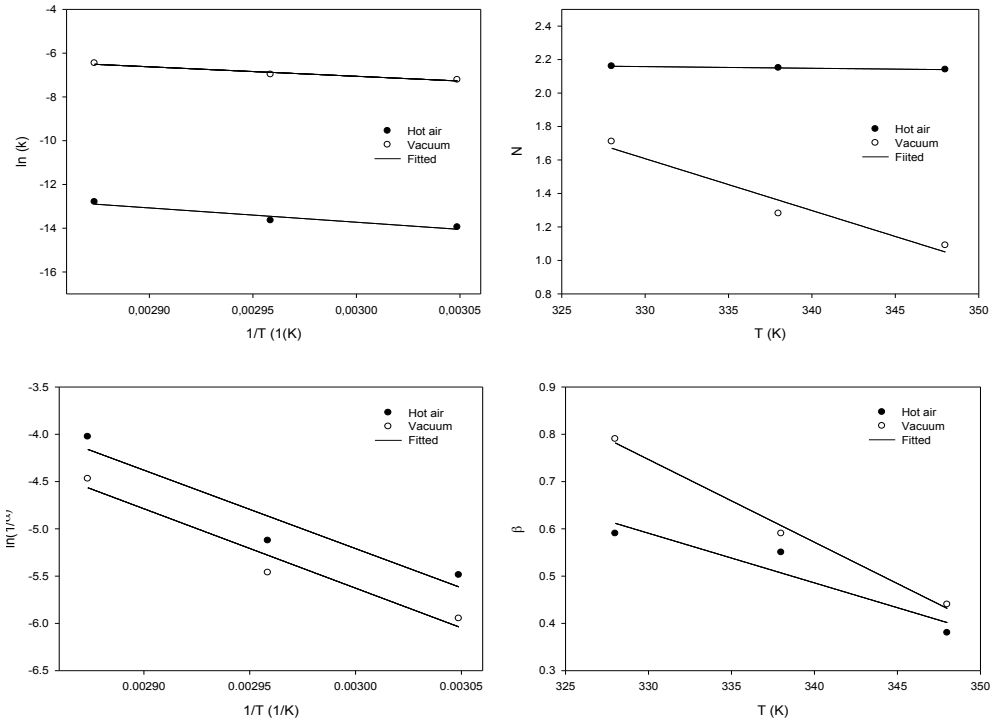


Figure 4. Arrhenius plot of the Page (drying rate constant, *k* and *N*) and Weibull (kinetic reaction rate constant for ascorbic acid, $1/\alpha$ and β) models for temperatures of 55, 65 and 75°C for hot air and vacuum drying of peppers.

The activation energy of pepper for the present study compares satisfactorily with similar data for red pepper reported in published literature by TURHAN *et al*. (1997), KAYMAK-ERTEKIN (2002) and DI SCALA and CRAPISTE (2008), at 28.4, 42.8 and 33.8 kJ mol^{-1}, respectively.

The parameter values of Page's model obtained for *k* and *N* by the least square fit of experimental points are given below for both hot air and vacuum drying:

Hot air drying $\ln(k) = 5.8966 - \frac{6540.9601}{T}$ (R^2=0.9211) (10)

$N = 2.488 - 0.001 * T$ (R2=0.9999) (11)

Vacuum drying $\ln(k) = 5.9359 - \frac{4331.2057}{T}$ (R2=0.9524) (12)

$N = 11.838 - 0.031 * T$ (R2=0.9524) (13)

Combining Page's model with Equations 10 to 13, the following general models can be derived to describe the drying kinetics of pepper as a function of time and temperature:

$$MR = \exp[-363.798 * \exp(-6540.9601\frac{1}{T}) * t^{2.488-0.0010*T}] \quad \text{(for hot air drying)} \quad (14)$$

$$MR = \exp[-378.380 * \exp(-4331.2057\frac{1}{T}) * t^{11.838-0.031*T}] \quad \text{(for vacuum drying)} \quad (15)$$

These expressions can be used to estimate the moisture ratio of red pepper and for temperature at any time with great accuracy during the hot air and vacuum drying processes. Similar expressions were also obtained by several researchers for different agricultural products (AKPINAR *et al*., 2003; KAYMAK-ERTEKIN, 2002; GOWEN *et al*., 2007; YILDIRIM *et al*., 2011; CHAYJAN *et al*., 2011).

3.3.1 Ascorbic acid degradation kinetics for pepper during drying

Ascorbic acid degradation was modeled using first order and Weibull models (Eq. 16 and 17). The Weibull model is flexible owing to the inclusion of a shape constant in addition to the rate constant and has been employed to describe microbial, enzymatic and chemical degradation kinetics (CUNHA *et al*., 1998; MANSO *et al*., 2001).

$$\frac{C_t}{C_o} = \exp(-k_1 * t) \quad (16)$$

$$\frac{C_t}{C_o} = \exp(-(\frac{t}{\alpha})^{\beta}) \quad (17)$$

where C_t is the ascorbic acid concentration at a time t, C_o is the initial ascorbic acid concentration, C_t/C_o is the ascorbic acid degradation ratio at any time, k_1 is the rate constant, β (dimensionless) is the shape constant (first order kinetics model is applicable when β=1) and α is the scale parameter in minutes.

In general, according to the literature, the average vitamin C content for red peppers is around 23.84-78.67 mg/100g (ANDREWS, 1995; TEODORO *et al*., 2013). The vitamin C content for the red peppers used in this study was 54.35 mg/100 g. Red pepper is known to be one of the richest sources of vitamin C compared with other fruits and vegetables (MARIN *et al*., 2004; SARKER and GOHDA, 2013), thus vitamin C was selected as the quality parameter for this study. A graphical representation of the experimental and predicted vitamin C degradation in the pepper samples during vacuum and hot air drying at 55, 65 and 75°C is shown in Fig. 5. As seen in the figure, at any time during drying, the loss of vitamin C increased with increasing temperature ($p<0.05$) in both drying conditions due to the heat-sensitive nature of vitamin C. The heated air inherently exposes the products to oxidation, reducing their ascorbic acid content (VEGA-GALVEZ *et al*., 2008).

The degradation kinetics of vitamin C was assessed with first order and Weibull models. The Weibull distribution function has an interesting potential for describing microbial, enzymatic and chemical degradation kinetics (CUNHA *et al*., 1998; OMS-OLIU *et al*., 2009). Table 3 shows the results of fitting vitamin C retention in red pepper to the first order model and Weibull model distribution. The Weibull model (Eq. 17) yielded a good fit to the vitamin C experimental data, and the Weibull distribution seemed to be suitable considering the high determination coefficients (R^2 = 0.9904 to 0.9975) and the low RMSEs (0.01 to 0.03). The values of kinetic constants (α) and (β) of the Weibull model were obtained by fitting Eq. 17 to the experimental data. The α and β values obtained from the Weibull model were directly affected by drying temperature. The β values for hot air drying were 0.59, 0.55 and 0.38 at 55, 65 and 75°C, respectively, and 0.79, 0.59 and 0.44 for vacuum drying at the same temperature levels. The constant β represents a behavior

index, and if $\beta < 1$ the reaction rate decreases with time and a degradation rate higher than the exponential is observed at the beginning of the process (CUNHA *et al*., 1998; MARFIL *et al*., 2008). Fig. 5 supports this idea as there was an initial high rate of vitamin C loss at relatively higher moisture contents, followed by a period of less rapid degradation as the moisture content decreased. Similar tendencies in vitamin C degradation during drying were also observed in other studies (DI SCALA and CRAPISTE 2008; ERENTURK *et al*., 2005). Therefore, retention of vitamin C is not only dependent on drying conditions but also on the sample moisture content. The α values were 241.83, 168.08 and 56.07 at 55, 65 and 75°C, respectively for hot air drying, and 383.05, 235.79 and 87.45 for vacuum drying at the same temperature levels (Table 3). Higher α values indicate lower degradation rates or, in other words, a longer time before nutrient collapse (MARFIL *et al*., 2008). The parameter α was dependent on both temperature and dryer type in this study. The degradation rate was less in vacuum drying and for lower drying temperatures. An oxygen-deficient medium and less drying time could be reasons for the lower degradation rate of the vitamin C in vacuum drying (YILMAZ *et al*., 2017).

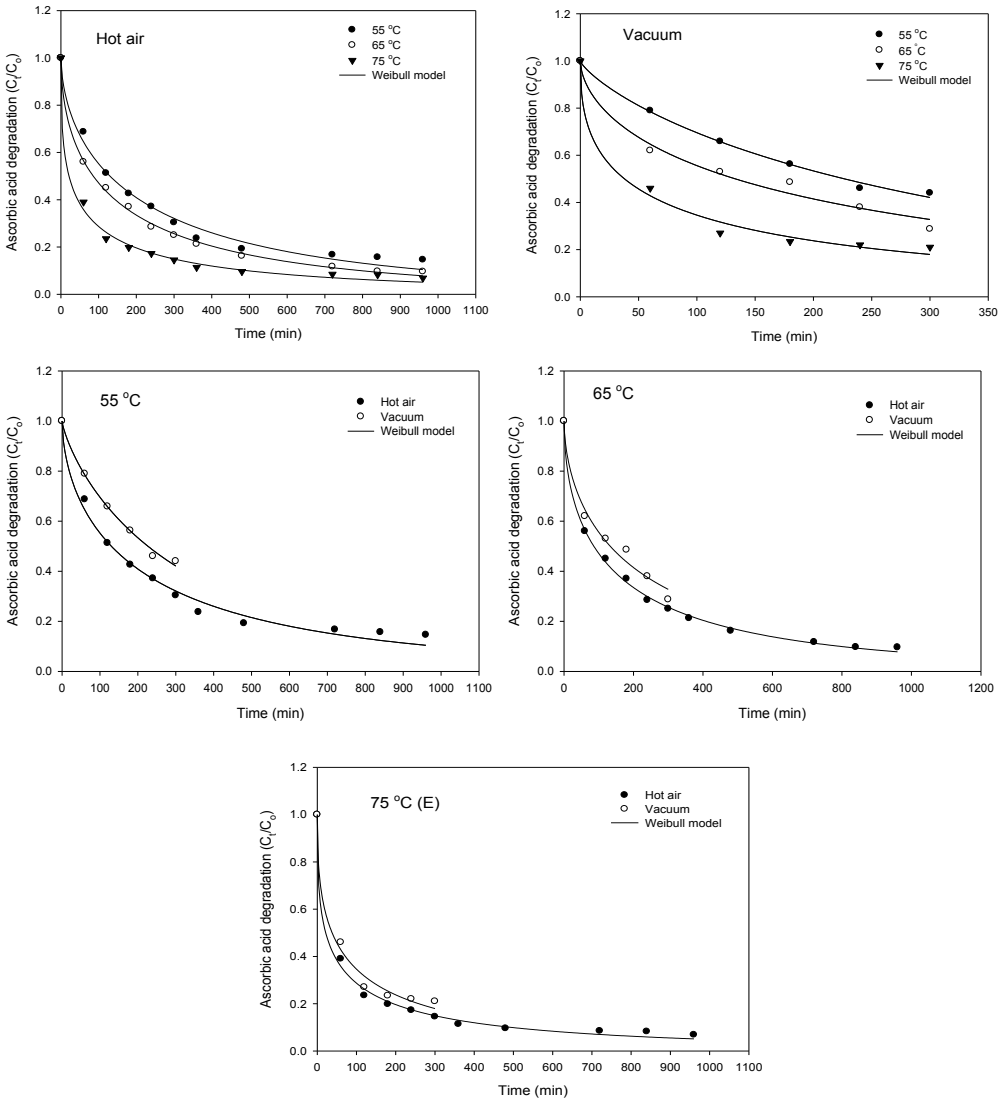


Figure 5. Ascorbic acid degradation curves for pepper samples using the Weibull model for hot air and vacuum drying at 55, 65 and 75°C.

The final vitamin C contents of the vacuum dried peppers were 45, 43 and 28% of the initial value at 55, 65 and 75°C respectively, as the drying operation was terminated at 12.50% moisture content (w.b.). Vitamin C retention for hot air drying was 20, 19 and 15% at 55, 65 and 75°C, respectively. As seen in these results, the degradation rate was higher for hot air drying and elevated temperatures. VEGA-GALVEZ *et al*. (2008) reported the ratio of final to initial vitamin C was in the range 0.18, 0.17, 0.15 and 0.16 for the Lamuyo variety red pepper dried at 50, 60, 70 and 80°C, respectively. SIGGE *et al*. (1999) reported 25 to 40% retention of vitamin C during dehydration of green peppers processed between 55 and 75°C. Indeed, drying at 55 and 65°C in this study produced similar vitamin C degradation. The results from DI SCALA and CRAPISTE (2008) also showed the same nutritional degradation level for red peppers from the ascorbic acid retention standpoint at 60 and 70°C. Vitamin C degradation rates during drying depended mostly on the combination of temperature and time parameters. In addition to that, air composition, sample shape and also food composition (a_w, enzymes, pH) are directly related to vitamin C retention (SANTOS and SILVA, 2008).

Table 3. First order and Weibull model parameters for drying pepper samples at different temperatures during hot air and vacuum drying.

Process	Temp. (°C)	Model	α	(1/α)	β	k_1	R^2	RMSE
Hot air	55	First order				$4.13x10^{-3}$	0.9024	0.08
		Weibull	$241.83(\pm1.35)^{c,x}$	$4.14x10^{3}(\pm0.04)^{a,y}$	$0.59(\pm0.01)^{c,x}$		0.9883	0.03
	65	First order				$5.39x10^{-3}$	0.9048	0.08
		Weibull	$168.08(\pm0.87)^{b,x}$	$5.95x10^{3}(\pm0.02)^{b,y}$	$0.55(\pm0.02)^{b,x}$		0.9975	0.01
	75	First order				$10.80x10^{-3}$	0.8926	0.08
		Weibull	$56.07(\pm2.22)^{a,x}$	$17.83x10^{3}(\pm0.05)^{c,y}$	$0.38(\pm0.01)^{a,x}$		0.9955	0.02
Vacuum	55	First order				$3.00x10^{-3}$	0.9790	0.03
		Weibull	$383.05(\pm3.84)^{c,y}$	$2.61x10^{-3}(\pm0.03)^{a,x}$	$0.79(\pm0.04)^{c,y}$		0.9972	0.01
	65	First order				$4.73x10^{-3}$	0.9226	0.06
		Weibull	$235.79(\pm2.45)^{b,y}$	$4.24x10^{-3}(\pm0.03)^{b,x}$	$0.59(\pm0.02)^{b,y}$		0.9940	0.02
	75	First order				$9.02x10^{-3}$	0.8897	0.09
		Weibull	$87.45(\pm3.42)^{a,y}$	$11.43x10^{3}(\pm0.05)^{c,x}$	$0.44(\pm0.01)^{a,y}$		0.9904	0.03

Means in the same column with different superscript letters are significantly different, a to c (temperature), x to y (dryer), and $p \leq 0.05$. Values in parentheses are standard deviations.

3.2.3 A general model to describe ascorbic acid degradation as a function of drying time and temperature

The temperature dependence of ascorbic acid degradation on the basis of Weibull's model can be described by an Arrhenius type equation where plots of $\ln(1/\alpha)$ Eq. (18) versus the reciprocal of drying temperature in absolute degrees resulted in straight lines (Fig. 4).

$$\ln(1/\alpha) = \ln(1/\alpha_o) - \frac{E_a}{RT} \qquad (18)$$

Where, T, E_a and R are the drying temperature in K, the activation energy in kJ mol^{-1} and the ideal gas constant of 8.314×10^{-3} mol^{-1} K^{-1}, respectively. The parameter values obtained by Weibull's model (α and β) by least square fit of the experimental points are given below for both hot air and vacuum drying:

Hot air drying $\ln(1/\alpha) = 19.68 - \frac{8298.00}{T}$ (R^2=0.9131) (19)

$\beta = 4.06 - 0.0105 * T$ (R^2=0.8867) (20)

Vacuum drying $\ln(1/\alpha) = 19.57 - \frac{8398.94}{T}$ (R^2=0.9555) (21)

$\beta = 6.52 - 0.0175 * T$ (R^2=0.9932) (22)

The high activation energies for both hot air (68.99 kJ mol^{-1}) and vacuum drying (69.83 kJ mol^{-1}) obtained in this study based on Weibull's model suggest that vitamin C is highly susceptible to temperature changes (OMS-OLIU *et al*., 2009; MARFIL *et al*., 2008; KARAASLAN *et al*., 2014). The degradation rates of ascorbic acid during vacuum drying was lower than that of hot air drying, but its activation energy was higher. These results indicate that vacuum drying was more stable than hot air drying.
From the regression of linear fit of Arrhenius curves for Weibull's model, the following general model can be derived to describe the ascorbic acid degradation kinetics of pepper during drying as a function of time and temperature:

Hot air: $\frac{C_t}{C_o} = \exp[-(3.54x10^8 * \exp(-8298\frac{1}{T}) * t)^{4.0557-0.0105*T}]$ (23)

Vacuum: $\frac{C_t}{C_o} = \exp[-(3.15x10^8 * \exp(-8398.9383\frac{1}{T}) * t)^{6.5217-0.0175*T}]$ (24)

With the models developed (Eqs. 23 to 24), the time and temperature dependent (instantaneous) ascorbic acid content can be estimated for both the dryer types. This study revealed the effects of dryer type, drying temperature, drying time and moisture removal on the vitamin C content of the red pepper. Also, our findings demonstrated that the Weibull distribution is likely to be a useful tool for describing vitamin C changes in red pepper under different drying conditions.

4. CONCLUSIONS

The drying rate of peppers increased under vacuum drying by 76.6% at 55°C, 73.6% at 65°C and 70.2% at 75°C, respectively. Vacuum drying could reduce the drying time of peppers by 74.1% at 55oC, 69.3% at 65°C and 67.0% at 75°C, compared to hot air drying. The drying time is shorter when the temperature is higher due to the increase of the drying rate, which means an enhanced heat transfer potential between the air and the peppers, therefore favoring the evaporation of water from the peppers. The Page model provided the best fit of five drying kinetic models with the highest R^2 values (0.9697 to 0.9996) and the lowest RMSE values (0.06 to 0.02). Weibull's model yielded a good fit with ascorbic acid degradation data, and the model distribution seemed to be suitable considering the high determination coefficients (R^2 = 0.9904 to 0.9975) and low RMSE values (0.01 to 0.03). Increases in drying temperature increased the percentage of the *k*

value in Page's model to 68.37% for hot air drying and 97.52% for vacuum drying. Vacuum drying increased the *k* value of the Page model with a 99.88% increase at 55°C, 99.87% at 65°C and 99.82% at 75°C. The activation energy of peppers based on drying kinetics decreased from 54.4 kJ mol^{-1} to 36.0 kJ mol^{-1} with vacuum drying. This confirms the increase in drying rate and decrease in drying time. On the other hand, high activation energies for both hot air (68.99 kJ mol^{-1}) and vacuum drying (69.83 kJ mol^{-1}), based on ascorbic acid degradation that were obtained in this study, suggest that vitamin C is highly susceptible to temperature changes. The degradation rates of ascorbic acid during vacuum drying were lower than that of hot air drying, while its activation energy was higher. These results indicate that vacuum drying was more stable than hot air drying. New general expressions obtained for both drying and ascorbic acid degradation kinetics can be used to estimate the moisture ratio and the ascorbic acid degradation ratio of red pepper at any time and temperature with a great accuracy during the hot air and vacuum drying processes. We concluded that vacuum drying can be more advantagoeus over hot air drying with respect to drying time and ascorbic acid degradation.

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ABBREVIATIONS

Co	Initial ascorbic acid concentration in mg $100g^{-1}$
Ct	Ascorbic acid concentration at any time in mg $100g^{-1}$
DAD	Diode Array Detector
d.b.	Dry base
DR	Drying rates
Ea	Activation energy in kJ mol^{-1}
HMF	Hydroxymethylfurfural
K	Drying temperature in K (Kelvin)
ko	Pre-exponential factor
k, N	Parameter values of Page's model
M.C.	Moisture content
MR	Moisture ratio
Mt	Moisture content at any time
Mo	Initial Moisture content
Me	Equilibrium Moisture content
R2	Coefficient of determination
RMSE	Root mean square error
R	Ideal gas constant: 8.314×10^{-3} mol^{-1} K^{-1}
w.b.	Wet base
α, β	Kinetic constants of the Weibull model

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PAPER

TRIMETHYLAMINE AS A FRESHNESS INDICATOR FOR SEAFOOD STORED IN ICE: ANALYSIS BY GC-FID OF FOUR SPECIES CAUGHT IN THE TYRRHENIAN SEA

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ABSTRACT

In seafood products, trimethylamine (TMA) is an indicator of the conservation status. It is almost absent in freshly caught samples, and its content increases during spoilage. In the present work, a new simple GC-FID method that uses a commercial capillary column, specifically designed, was applied. TMA was measured at increasing time intervals in four marine species caught in the Tyrrhenian Sea and stored in ice; 852 individuals were analyzed. An assessment of the maximum allowable time of storage in ice was made for each species. Existing guidelines for the level of trimethylamine are reviewed and discussed.

Keywords: Trimethylamine (TMA), seafood, freshness, shelf life, storage in ice, Gas Chromatography-Flame Ionization Detector (GC-FID)

1. INTRODUCTION

Fish, mollusks, crustaceans, and other marine species are among the most perishable food products (BOURIGUA *et al*., 2011; DIMOGIANOPOULOS and GRIGORAKIS, 2014; ŠTERNIŠA *et al*., 2016), and their spoilage leads to the formation of some substances that may cause intoxication when ingested (BIJI *et al*., 2016). Seafood spoilage leads also to the formation of low-molecular-weight volatile amines, which results in off-flavors. The main compound responsible for the typical smell of spoiled fish is trimethylamine (TMA). TMA is a good chemical marker of freshness (POPELKA *et al*., 2014): its concentration increases with spoilage by bacterial degradation of TMAO (trimethylamine N-oxide), an important osmoregulatory organic molecule that is commonly found in the muscle of marine fish (TREBERG and DRIEDZIC, 2002).
A bad conservation status of seafood represents a topic of safety concern. Ideally, seafood should be stored under conditions such that bacteria cannot grow at all: when preserved in ice, the product is safe only within a limited period. As such, an indicator of freshness may be very useful.
TMA is actually used as an indicator of seafood conservation status in ice (BOURIGUA *et al*., 2011; TONIOLO *et al*., 2014; BALIÑO-ZUAZO and BARRANCO, 2016). The various analytical techniques for the determination of TMA include colorimetric assays (PENA-PEREIRA *et al*., 2010), flow injection analyses (RUIZ-CAPILLAS and HORNER, 1999), biosensor analysis (BOURIGUA *et al*., 2011), capillary electrophoresis (TIMM and JØRGENSEN, 2002), and HPLC utilizing derivatization (BALIÑO-ZUAZO and BARRANCO, 2016). For gas chromatography, there are no simple instrumental methods available that allow a direct split/splitless injection into a capillary column. Packed columns were used for this purpose, especially in the past (VECIANA-NOGUES *et al*., 1996). Alternative methods use complex hyphenated techniques such as headspace-gas chromatography and solid phase microextraction-gas chromatography (KRZYMIEN and ELIAS, 1990; DEHAUT *et al*., 2016). In the present work, an innovative instrumental method was applied. Thanks to a capillary column specifically designed for the volatile amines and coated with a proprietary phase, simple instrumental analysis using gas chromatography with a flame ionization detector (GC-FID) was possible. This was done by injecting the liquid sample in split mode.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Trimethylamine hydrochloride (TMA·HCl) and *n*-propylamine hydrochloride (n-PA·HCl) were purchased from Sigma Aldrich® (St. Louis, MO, USA). Toluene, potassium hydroxide (KOH), and trichloroacetic acid (TCA) were from Carlo Erba Reagents® (Milan, Italy). Testmix CP0043 was from Varian® (Walnut Creek, CA, USA).
Standard amine solutions were prepared by dissolving TMA·HCl and n-PA·HCl in distilled water in order to obtain the desired concentrations expressed as free bases (TMA and n-PA).

2.2. Seafood samples

Red mullet (*Mullus barbatus*), European anchovy (*Engraulis encrasicolus*), and deep-water rose shrimp (*Parapenaeus longirostris*) were caught by professional fishermen during the months of June, July and September for the summer campaign and during the months of

February, March and April for the winter campaign. Atlantic mackerel (*Scomber scombrus*) was caught in the summer campaign only. For red mullet two different sizes were selected and analyzed in order to verify whether spoilage is influenced by this parameter. In some cases, the study had to take into account the amount of sample available at the moment. Fishing was done by trawling along the coast of the Tyrrhenian Sea, near Civitavecchia located in the region of Latium in Central Italy. The collected seafood samples were selected directly on board and separated by species and size, and then were placed in polystyrene boxes and covered with ice. The polystyrene boxes were stored in a refrigerated cell at 0-1°C until the day of the analyses that started on day 1 and continued on days 3, 6, 8, 10, and 13.
The size and number of the individuals collected are reported in Table 1.

Table 1. Size of the seafood species collected (minimum – maximum) and total number of individuals analysed.

	Weight (g)		Length (cm)		Number of individuals	
	Summer	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**
Red mullet, big size *(Mullus barbatus)*	52-276	23-99	16-27	13-20	17	42
Red mullet, small size *(Mullus barbatus)*	19-61	7-33	12-18	9-15	48	173
European anchovy *(Engraulis encrasicolus)*	10-18	9-23	12-14	12-16	120	138
Atlantic mackerel *(Scomber scombrus)*	51-135		19-26		25	
Deep-water rose shrimp *(Parapenaeus longirostris)*	8-25	6-24	10-16	10-14	109	180

2.3. Sample preparation

2.3.1 Filleting and homogenization

On the day of the analysis, a minimum of 2-4 and a maximum of 10-30 individuals of each species, based on the number available, were pooled. This was followed by gutting, skinning, and filleting. Subsequently, homogenization was carried out for 30 seconds at a low speed with a Waring blender (model 8010E, Waring® Products Division, New Hartford, CT, USA) and by using a previously cooled stainless-steel cup. The resulting homogenate was ready for TMA extraction.

2.3.2 TMA extraction

Analyses were performed in duplicate. Sample preparation followed the methods of PEREZ MARTIN *et al*. (1987) and VECIANA-NOGUES *et al*. (1996) with minor modifications.
Approximately 10 g of homogenized product was weighed into 250 mL plastic bottles. To the weighed sample, 40 mL of TCA 6% (w/w) solution was added, and then a second homogenization was carried out for 1 min at 11000 rpm with an Ultra Turrax homogenizer (Model T25B, IKA®, Staufen, Germany); the plastic bottle was kept immersed in ice to prevent any heating.

Subsequently, centrifugation was carried out for 10 min at 4°C (12000 rpm, 22214*g*) using a Beckman Coulter Avanti™ J25 ultracentrifuge. The supernatant was collected in a 100 mL volumetric flask by using a funnel with an inserted Whatman N° 2 filter paper 15 cm in diameter. The filter, residue pulp, and plastic bottle were washed with distilled water to ensure that all of the TMA extracted was quantitatively collected. Finally, the solution was brought to volume with distilled water. The solution (TCA extract) was transferred into 10 mL tubes and kept at -30°C until the day of gas chromatographic analysis.

2.3.3 Sample preparation for GC injection

On the day of the gas chromatographic analysis, the TCA extract from the previous step was left to thaw at room temperature. To 7 mL of the TCA extract in a glass tube, the internal standard (IS) n-PA·HCl was added. The added amount corresponded to a concentration of 19.04 mg/L of n-PA as free base. Subsequently, 2 mL of toluene and 10 mL of KOH 65% (w/v) solution were added, and the tube was shaken at 1500 rpm for 1 min by means of a vortex mixer (Heidolph®, Schwabach, Germany). One microliter of the toluenic upper layer was injected in GC-FID.

2.4. Instrumental analysis

The apparatus used was a 6890 Agilent gas chromatograph with a flame ionization detector (GC-FID), equipped with a CP-Volamine fused silica capillary column (60 m × 0.32mm I.D., 0.45 mm O.D., 5 μm film thickness, from Varian®). Helium as the carrier gas was used in constant flow mode at 0.9 mL/min.
The operating conditions were as follows. The initial oven temperature was 45°C, which was held for 10 min and then increased to 250°C at a rate of 20°C/min. This final temperature was maintained for 10 min. The FID temperature was 300°C, while the injector temperature was 270°C. Injections were made in split mode (5:1) with an injection volume of 1 μL. Fig. 1 shows two gas chromatograms for a shrimp sample and a standard solution of TMA.

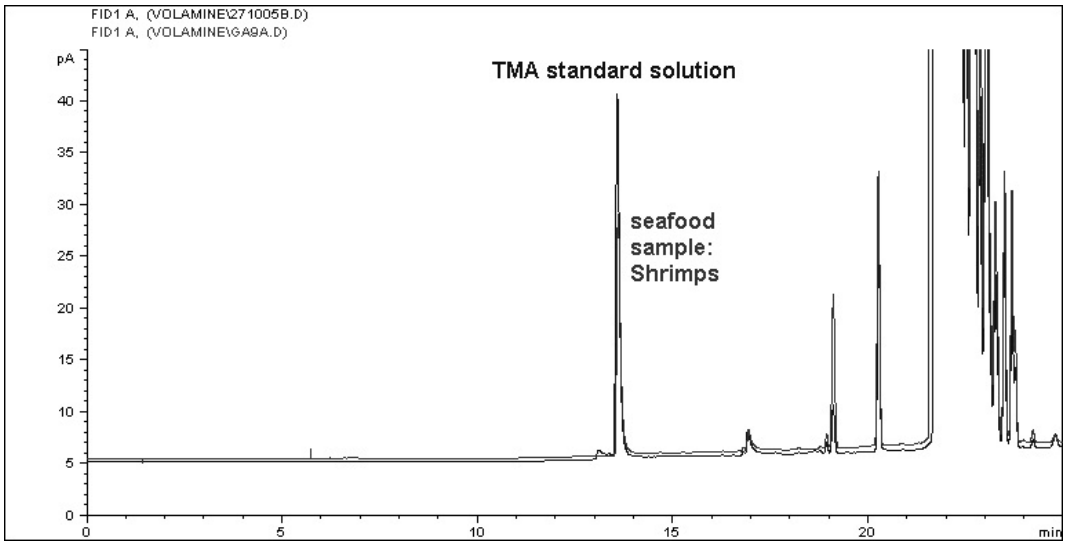


Figure 1. GC-FID chromatograms, peak of TMA. Standard solution of TMA and a shrimp sample. Chromatograms are overlaid.

2.5. Analytical quality control

Instrumental performance was investigated by using the Testmix CP0043 provided by Varian®. The composition of the Testmix was 0.1% of each component, including TMA, in isopropanol.
For the quantitative analysis of TMA, an appropriate calibration curve was constructed (Fig. 2).

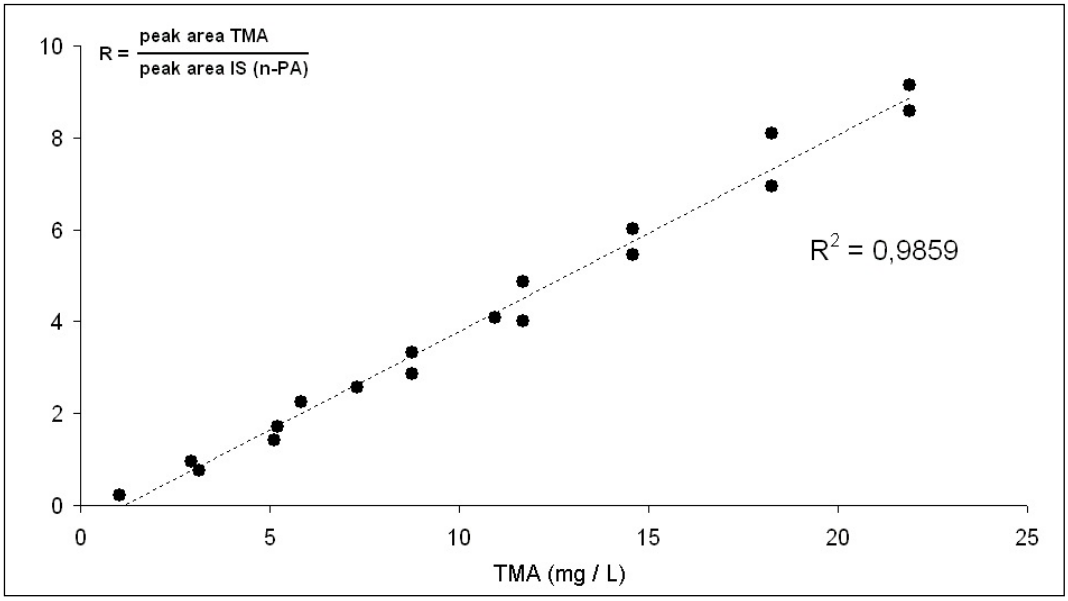


Figure 2. Calibration curve used to quantify TMA in the seafood samples.

Solutions of known composition for the points of the calibration curve were prepared in TCA by using pure TMA·HCl. Pure n-PA·HCl was used as IS. The real samples that were observed to have very high levels of TMA were again prepared and appropriately diluted before the GC injection to fall within the linear dynamic range of the calibration curve.
The limit of quantitation (LOQ) is the amount of TMA in the seafood sample that is easily quantifiable because of the good signal-to-noise ratio of the final chromatographic peak. The limit of detection (LOD) is the amount of TMA with a signal-to-noise ratio that is sufficient only for the detection of its presence without the possibility of a reliable integration. For the present method, we measured a LOQ of 3 mg of TMA per kilogram of seafood sample and a LOD of 2 mg/kg (corresponding to a LOQ of 0.07 mg N/100 g and to a LOD of 0.05 mg N/100 g when TMA is expressed in mg N/100 g of sample). Blanks and recovery measurements were carried out to ensure that no false positives nor false negatives were produced.
For the recoveries, the TCA extract (section 2.3.2) coming from a red mullet sample was spiked with TMA at three different concentration levels, as reported in Table 2. Recovery was 90.2±8.0%; this is similar to that reported by other researchers (PEREZ-MARTIN *et al*., 1987).

Table 2. TMA recovery measurements.

Sample		Measured concentration of TMA in the native TCA extract (mg/L)	Expected concentration of TMA in the spiked TCA extract (mg/L)	Measured concentration of TMA in the spiked TCA extract (mg/L)	Recovery (%)
Red mullet, big size winter campaign (T2)	No adding	2.11			
	Level 1, adding		3.20	2.70	84.4
	Level 2, adding		4.29	4.26	99.3
	Level 3, adding		5.37	4.66	86.8

After each addition of TMA, the extract was normally processed and injected in GC-FID.

3. RESULTS AND DISCUSSION

3.1. Guidelines for TMA concentration

TMA is considered a good indicator of fish spoilage when the product is preserved in ice; however, there is no regulation for TMA levels (MACÉ *et al*., 2012). The European Community Regulation cites that when the organoleptic examination reveals any doubt as to the freshness of the fishery products, samples may be taken and subjected to laboratory tests to determine the levels of TMA (Reg. CE No 854/2004); surprisingly, the Regulation itself does not provide any tolerance or reference value. There are, however, generally accepted values or values recommended by international organizations.
TMA is sometimes reported in milligrams of TMA per kilogram of fish; in other cases, it is reported in milligrams of TMA-N (milligrams of Nitrogen) per 100 g of fish, a situation that can be potentially confusing. For clarification, we report the equation for converting TMA into TMA-N and vice versa:

$$TMA\,(mg/kg) \times 0.0237288 \;=\; TMA-N\,(mg/100g) \qquad (1)$$

The Food and Agriculture Organization of the United Nations (FAO, 1988) reports that good-quality cold-water fish contains less than 63 mg/kg of TMA.
EL MARRAKCHI *et al*. (1990) investigated the freshness of marine fish (*Sardina pilchardus*) both by TMA determination and by sensory evaluations by two official veterinary inspectors. In their study the product was judged as fresh up to a TMA level of 50 mg/kg (1.19 mg/100g of TMA-N). This is in good agreement with what has been reported by FAO, and is the same as the maximum reference TMA content cited by the Italian Istituto Zooprofilattico Sperimentale (IZSUM, Ministry of Health) for the freshness of marine fish (HAOUET, 2001). VECIANA-NOGUES *et al*. (1996) reported that hake can be graded as excellent quality when its TMA level is lower than 42 mg/kg (1 mg/ 100 g of TMA-N).
We see that recommendations by national and international organizations, as well as experimental works of researchers, agree that the range 42-63 mg/kg of TMA (1-1.5 mg/100g of TMA-N) in marine fish is the limit below which the freshness status is at an optimum.

3.2. Deep-water rose shrimp

As Table 3 shows, shrimps exhibited a TMA content suggestive of a bad freshness state after three days of storage in ice (151.7 mg/kg in the summer campaign). We must emphasize that on day 3 the colour of the shrimps had in fact turned black from the original pink. It can be concluded that the shelf life of deep-water rose shrimp in ice is very limited when the product does not undergo to any conservative treatment, as is generally done (ZHANG *et al*., 2015). Another work (LAGHMARI and EL MARRAKCHI, 2005) confirms the short shelf life (3-5 days) of *P. longirostris* stored in ice.
As it can be seen for shrimps in Table 3 after many days of storage in ice the TMA content in some marine species could also stabilize or even decrease: this is a known phenomenon because the content of the precursor TMAO runs out, but that does not mean, of course, an improvement in the conservation status.

3.3. Red mullet

Red mullet (*M. barbatus*) stored in ice showed a status of good freshness until 8 days, when the TMA level remained constantly below 65 mg/kg for both sizes and campaigns studied (Table 3). It appears that even over 8 days the product in some cases is in an acceptable status of conservation. At long storage times in ice (i.e. 8 days and 10 days) the bigger size tends to release a lower quantity of TMA than the smaller size. This means that the larger size is more resistant to degradation than is the smaller one. It was already observed in other fish species a better resistance to degradation for bigger sizes (ORBAN *et al*., 2011).
A similar shelf life was obtained in a study in which sensory and microbiological analyses were carried out on red mullet (ÖZYURT *et al*., 2009).

3.4. Atlantic mackerel

Atlantic mackerel (*S. scombrus*) was caught in the summer campaign only. *S. scombrus* is a species of great commercial importance that is mainly distributed as a canned product. A bad conservation process of the product can lead to high levels of histamine in mackerel during storage, which may cause health problems for consumers (scombroid fish poisoning). This is due to the relatively high content of free histidine in this species, which during bad storage is converted to histamine (BENNOUR *et al*., 1991). Therefore an indicator of freshness is extremely useful.
From Table 3 it can be deduced that at 8 days in ice, the rejection status was reached being 170.2 mg/kg the TMA content; up to six days, the product can be considered in good state. A similar shelf life for mackerel stored in ice has been reported (BENNOUR *et al*., 1991). In the study by BENNOUR, the histamine concentration was also measured. It was concluded that even when mackerel is allowed to spoil in ice until it becomes unfit to eat (over eight days), the level of histamine does not rise much above 5 mg/100 g of flesh, the level established by the United States Food and Drug Administration as a guidance value (FDA, 2011).

3.5. European Anchovy

From the TMA content in Table 3 we can be deduce that the maximum allowable time of storage in ice for anchovies is 5-6 days in summer. In fact, the TMA concentration at day 6 is 68.1 mg/kg in the summer campaign.
This result is perfectly in agreement with a study on *E. encrasicolus* collected in the Mediterranean area during summer (PONS-SÁNCHEZ-CASCADO *et al*., 2006). Such

work performed microbiological and sensory assays on anchovies stored in ice. A team of eight panel members trained in fish freshness assessment developed the schemes proposed for raw anchovies, and the final judgement was that the limit of acceptability for anchovies is reached after 5 days of storage in ice. The mean size of the individuals was practically the same in the work of PONS-SÁNCHEZ-CASCADO *et al*. and in the present one.
The winter campaign seems to indicate a possible slightly longer time of storage in ice.

Table 3. Concentrations of TMA measured for different seafood species at different days of storage in ice.

Days of storage in ice	Species	TMA (mg/kg)		TMA-N (mg/100g)	
		Summer	Winter	Summer	Winter
1 day (T0)	Red mullet, big size	n.d.	n.d.	n.d.	n.d.
	Red mullet, small size	5.4±0.2	< 3	0.13±0.00	< 0.07
	European anchovy	28.8±2.6	16.2±0.3	0.68±0.06	0.38±0.01
	Atlantic mackerel	18.2±0.6		0.43±0.01	
	Deep-water rose shrimp	40.3±0.3	18.3±15.8	0.96±0.01	0.43±0.37
3 days (T1)	Red mullet, big size	11.2±0.2	< 3	0.27±0.00	< 0.07
	Red mullet, small size	7.8±0.1	5.2±0.4	0.18±0.00	0.12±0.01
	European anchovy	32.6±0.1	21.7±0.1	0.77±0.00	0.51±0.00
	Atlantic mackerel	48.1±1.6		1.14±0.04	
	Deep-water rose shrimp	151.7±2.3	76.8±4.8	3.60±0.06	1.82±0.11
6 days (T2)	Red mullet, big size	24.9±0.2	18.3±0.0	0.59±0.00	0.43±0.00
	Red mullet, small size	23.6±0.1	20.0±0.1	0.56±0.00	0.47±0.00
	European anchovy	68.1±0.7	49.6±0.0	1.62±0.02	1.18±0.00
	Atlantic mackerel	80.2±0.0		1.90±0.00	
	Deep-water rose shrimp	283.4±11.1	623.9±98.9	6.72±0.26	14.81±2.35
8 days (T3)	Red mullet, big size	33.8±1.8	24.5±6.8	0.80±0.04	0.58±0.16
	Red mullet, small size	46.6±0.0	64.8±1.1	1.10±0.00	1.54±0.03
	European anchovy	153.4±0.5	57.0±8.4	3.64±0.01	1.35±0.20
	Atlantic mackerel	170.2±0.3		4.04±0.01	
	Deep-water rose shrimp	548.4±8.6	1041.0±12.6	13.01±0.20	24.70±0.30
10 days (T4)	Red mullet, big size		98.4±0.8		2.33±0.02
	Red mullet, small size	46.3±0.1	142.5±14.0	1.10±0.00	3.38±0.33
	European anchovy	192.9±0.4	71.6±1.7	4.58±0.01	1.70±0.04
	Atlantic mackerel	187.3±0.6		4.44±0.02	
	Deep-water rose shrimp	638.6±1.1	679.2±40.3	15.15±0.03	16.12±0.96
13 days (T5)	Atlantic mackerel	261.6±2.2		6.21±0.05	
	Deep-water rose shrimp	1229.1±257.7	1089.0±87.2	29.16±6.11	25.84±2.07

Results are reported as mean±standard deviation (n = 2) and are expressed both in mg/kg of TMA and in mg/100g of TMA-N (see equation 1, section 3.1).
n.d. = not detected (below the LOD)

3.6. Comparison among species

Figures 3 and 4 plot the TMA content measured in all species as a function of the days of storage in ice. It is evident that shrimps constantly released a much higher amount of TMA when compared with fish species: this resulted in a much lower shelf life.

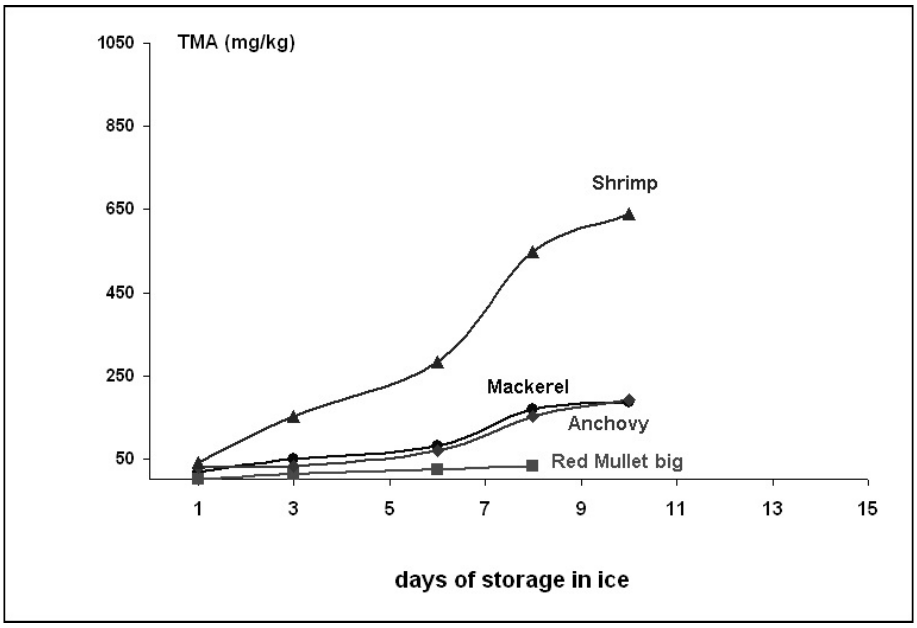


Figure 3. TMA content of the different species as a function of the days of storage in ice (summer campaign).

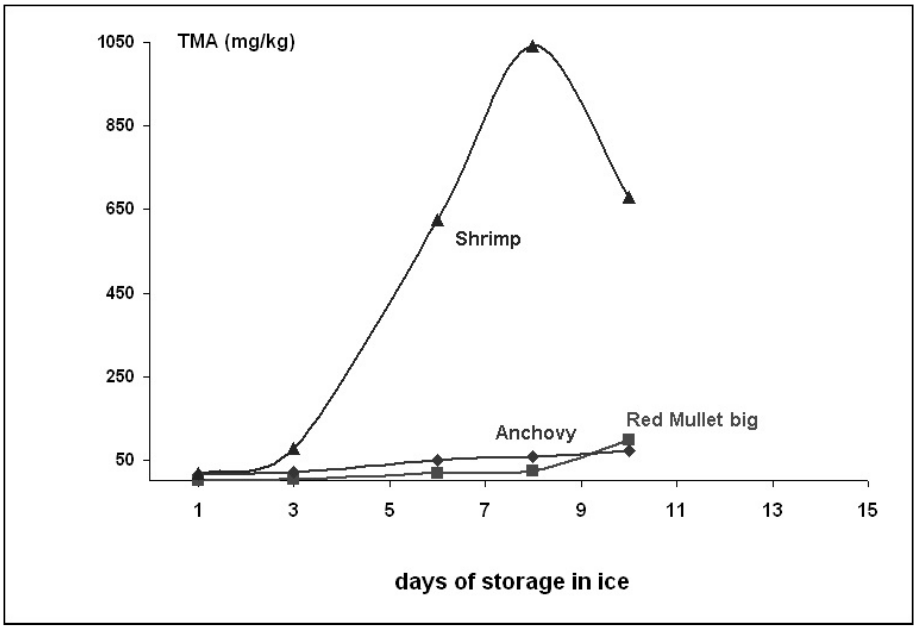


Figure 4. TMA content of the different species as a function of the days of storage in ice (winter campaign).

3.7. Method validation

In order to assess the reliability of the method presented here, a very different approach for evaluating the freshness status was applied. The species investigated for the TMA content (red mullet big size, red mullet small size, European anchovy, Atlantic mackerel) were also analyzed for their total volatile basic nitrogen (TVB-N) content, a widely accepted spoilage indicator that was established by the European Community (Comm. Reg. EC No 2074/2005). Measurements were carried out according to the EC official method (Decision 95/149/EC). They involved acid extraction followed by alkalinization, distillation, and titration.
Comm. Reg. EC No 2074/2005 stipulates that fish is unfit for human consumption when the TVB-N content is above 25-35 mg/100 g.
On the other hand, the value 1.5 mg/100g of TMA-N is the limit above which the fish begins to lose the optimum state of freshness (section 3.1).
We can easily see in Table 4 that the two indicators are in perfect agreement.

Table 4. Maximum allowable time of storage in ice as indicated both by TVB-N and TMA-N content (mg/100g) for the summer campaign.

		Day 1	Day 3	Day 6	Day 8	Day 10
TVB-N	Red mullet, big size	13.66±0.29	15.50±0.15	15.78±0.17	17.44±0.27	not analyzed
	Red mullet, small size	12.11±0.52	14.86±0.04	16.40±1.22	18.74±0.17	19.83±0.56
	European anchovy	14.49±0.54	16.80±0.08	23.04±0.81	30.43±0.14	37.97±0.97
	Atlantic mackerel	19.30±0.27	26.54±0.23	25.72±1.24	35.81[a]	57.94±1.04
TMA-N	Red mullet, big size	n.d.	0.27±0.00	0.59±0.00	0.80±0.04	not analyzed
	Red mullet, small size	0.13±0.00	0.18±0.00	0.56±0.00	1.10±0.00	1.10±0.00
	European anchovy	0.68±0.06	0.77±0.00	1.62±0.02	3.64±0.01	4.58±0.01
	Atlantic mackerel	0.43±0.01	1.14±0.04	1.90±0.00	4.04±0.01	4.44±0.02

[a]single measure
Results are reported as mean±standard deviation (n = 2). n.d. = not detected
Analyses of TVB-N were performed on another aliquot of the same homogenate that was processed for TMA

From the TVB-N content we can conclude that red mullet is in a good conservation status up to 8 days (10 days for the small size), since the concentration remains always below 20 mg/100 g. An identical situation is indicated by TMA, which never exceeded 1.10 mg/100 g in red mullets.
For European anchovy and Atlantic mackerel, the TVB-N level is acceptable up to day 6 (≤25 mg/100 g), and it begins to exceed 30-35 mg/100 g on day 8, when degradation starts.
The same is valid for the TMA content, which shows that on day 8 degradation is starting (3.64 and 4.04 mg/100 g).
From the above it may be concluded that TMA, as measured in the present study using the developed GC-FID method, is a reliable freshness indicator.

4. CONCLUSIONS

A quick and easy gas chromatographic method for the analysis of TMA in seafood was developed and validated. A capillary column and a classical split injection were used so greatly simplifying the procedure. It was applied to different seafood species caught in the Tyrrhenian Sea that had been stored in ice. Thanks to its simplicity, the method appears very suitable for routine controls.
By comparison with fish species, crustaceans such as shrimps exhibited a much greater release of TMA, which resulted in a much shorter shelf life (1-2 days).
Fish species maintained a good freshness state for almost a week, up to 8-10 days for red mullet, with the bigger sizes being more resistant to degradation than the smaller ones.
In the present study, the existing guidelines for the TMA content are reviewed and discussed. Not always there is full clarity on this topic in the literature. The generally accepted criterion for an "optimum freshness state" is a TMA content below 42-63 mg/kg (1.0-1.5 mg/100g of TMA-N). Observations made in the present research fully confirm this limit, at least for the marine species here investigated.

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FERMENTATION CHARACTERISTICS OF CAMPBELL EARLY GRAPE WINE INOCULATED WITH INDIGENOUS KOREAN WINE YEASTS ENCAPSULATED IN CA-ALGINATE BEADS AFTER AIR-BLAST DRYING

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ABSTRACT

The aim of this study was to test the possibility of using yeast cells encapsulated in calcium alginate (Ca-alginate) beads as a starter for wine fermentation. Characteristics of Korean Campbell Early wines fermented by five free yeast cell types and those encapsulated in 2% Ca-alginate beads were compared using physicochemical analyses and sensory evaluation tests. The encapsulated yeast cells were shown to ferment Korean Campbell Early grapes with a similar efficiency as that exhibited by the five free yeast cell-types. After fermentation, the characteristics of free cells and encapsulated cells did not show significant differences in terms of content of reducing sugars, soluble solids, total acids, organic acids, and free sugars, as well as in terms of viable cell numbers and other physicochemical properties. The encapsulated cells did, however, produce more alcohol than the free cells. Encapsulation in 2% Ca-alginate beads was furthermore found to decrease the production of negative volatile compounds. The sensory evaluation of wines fermented by free cells compared with those fermented by Ca-alginate bead-encapsulated cells yielded similar scores for the following properties: color, taste, flavor, and overall preference. Overall, no significant differences were observed between the two grape wines, and yeast cells encapsulated in 2% Ca-alginate beads therefore showed high stability and served as an effective yeast starter for wine fermentation.

Keywords: Campbell Early grape, wine, calcium alginate bead, yeast cell immobilization, air-blast drying

1. INTRODUCTION

The Campbell Early grape (*Vitis labrusca* cultivar) is a major grape type used in winemaking and constitutes about 70% of the total grape production in Korea (SEO *et al*., 2007; HONG and PARK, 2013). Although the demand for domestic wine in Korea is increasing, the quality of Korean wines remains unreliable owing to high acidity and low sugar content in the wines as well as color weakness of the Campbell Early grape (KIM *et al*., 2017; LEE and KIM 2006; PARK *et al*., 2004). Owing to the short history of wine consumption and the wine industry in Korea, studies on these wines are in the preliminary stages (LEE *et al*., 2006; SEO and YOOK, 2007). Several researchers have recently attempted to address the problems associated with indigenous yeast and winemaking using Campbell Early grapes that have adapted to the Korean environment (HONG and PARK, 2013; LEE *et al*., 2004; PARK *et al*., 2004).

The wine fermentation process depends on the ability of yeast to convert grape sugars into alcohol and other compounds (PADILLA *et al*., 2016; ROMANO *et al*., 2003). Several studies have reported that indigenous yeasts can improve the sensory properties and the quality of local wines (CHAROENCHAI *et al*., 1997; ESTEVE-ZARZOSO *et al*., 1988; HONG and PARK, 2013; LEE *et al*., 2016). However, most Korean wines are fermented using an imported yeast starter (CHOI *et al*., 2011; KIM *et al*., 2007). The identification of a suitable Korean indigenous yeast in winemaking is an important goal for the local wine industry. Previously, *Saccharomyces cerevisiae* D8, M12, S13, *Hanseniaspora uvarum* S6 (previously SS6), and *Issatchenkia orientalis* KMBL5774 were isolated from Korean grapes to improve the wine quality and were found to enhance the local wine quality (HONG and PARK, 2013; KIM, 2006; SEO *et al*., 2007).

Techniques for drying microorganisms include freeze-drying, spray-drying, and fluidized bed-drying (BARBOSA *et al*., 2015; PODDAR *et al*., 2014). However, each of these techniques is associated with reduced microorganism viability. Recently, alternative drying processes have gained interest owing to their lower costs and faster processing times, as compared to freeze-drying (PODDAR *et al*., 2014; SANTIVARANGKNA *et al*., 2007). Immobilized yeast strains have been optimized based on properties such as survival rate, fermentation ability, viability, and ease of handling without the need for specialized laboratories (CAYLAK and SUKAN, 1998). A potential application of such immobilized yeasts is the use of Korean indigenous yeasts as starter cultures in the local wine industry. Different encapsulation techniques have been studied for the preservation and subsequent application of yeast cells (AKIN, 1987; CASSIDY *et al*., 1996; COLAGRANDE *et al*., 1994). Over the last three decades, cell immobilization specific to winemaking has been extensively studied owing to the technical and economic advantages such systems may offer over free cell systems (MARGARITIS *et al*., 1983; STEWART and RUSSELL, 1986; TSAKIRIS *et al*., 2004). The use of yeast immobilized in gel-forming materials such as calcium alginate (Ca-alginate), agar, carrageenan, cellulosic materials, and pectic acid in winemaking has been well documented (COLAGRANDE *et al*., 1994), and of the various reported methods, immobilization of microbial cells by entrapment in Ca-alginate gels is the most widely used approach. This is an attractive technique for various biotechnology, biomedicine, and food technology applications (DE VOS *et al*., 2009; KREGIEL *et al*., 2013; ROKSTAD *et al*., 2014). Ca-alginate encapsulation offers several advantages over the use of free cells: increased functional efficiency with high cell concentrations in the reactor, easy separation of the immobilized cells in the settling tank, short fermentation lag period, and increased stability of the fermentation system (BARDI *et al*., 1996). Several studies in yeast have reported improved survival rates after reduction of moisture content (BEKER and RAPOPORT, 1987; LIEVENSE *et al*., 1992; LIEVENSE *et al*., 1994), indicating that reduced moisture content enhances the preservation of yeast cells.

We previously showed that the immobilization of yeast cells via encapsulation in Ca-alginate beads yielded high survival rates and excellent storability (KIM *et al*., 2017). The aim of this study was thus to investigate the feasibility of using air-blast-dried Ca-alginate bead-encapsulated cells compared to the use of free yeast cells in wine fermentation.

2. MATERIALS AND METHODS

2.1. Strains and medium

Saccharomyces cerevisiae D8 (KACC 93245P), M12 (KACC 93246P), and S13 (KACC 93247P); *Hanseniaspora uvarum* S6 (previously, SS6 and KACC 93248P); and *Issatchenkia orientalis* KMBL5774 (*Pichia kudriavzevii* KMBL5774 and KACC 93124P) were obtained from the Food Microbial Biotechnology Laboratory, Department of Food Science and Biotechnology, Kyungpook National University (Daegu, South Korea). The yeasts were cultured aseptically in 100 mL YPD broth [2% yeast extract, 1% peptone, and 2% glucose (w/v); 2% (w/v) agar was added for solid medium] in a rotary shaker (JSSI-300C, JS Research Inc., Gongju, South Korea) at 30°C for 24 h. All yeast strains were stored in 15% glycerol at -80°C. Ca-alginate beads were prepared using a previously described method (KIM *et al*., 2017). In this study, 2% Ca-alginate bead-encapsulated cells were used and the encapsulated cells showed at least a 51% survival rate when stored at 4°C for 3 months (KIM *et al*., 2017).

2.2. Release and viability of encapsulated Ca-alginate beads

Beads containing yeast cells were rehydrated in 10 mL YPD broth and the release of the yeast cells from the 2% Ca-alginate beads was monitored for 48 h using an optical microscope (Nikon Eclipse, TE2000-U, Melville, NY, USA). After the yeast cells were released from the beads, a 1 mL sample of the resulting yeast culture was serially diluted in a 0.85% (w/v) NaCl solution.

2.3. Viable cell count

Samples were serially diluted with 0.85% NaCl using the appropriate dilution factors. Each sample was spread onto YPD agar medium plates. The plates were cultured at 30°C for 48 h, and the number of white colonies that formed on the YPD agar was counted to provide the number of colony forming units (CFU) (dilutions gave 30 to 300 CFU/mL).

2.4. Vinification processing

Campbell Early grapes (*Vitis labrusca* cultivar) were obtained from Sangju, Kyungpook province, South Korea in 2016. The vinification process was assessed using a general wine-making method (HONG and PARK, 2013). The grapes were washed, stemmed, crushed, and treated with potassium metabisulfite ($K_2S_2O_5$; 200 ppm) to inhibit harmful bacterial and yeast growth. The sugar content off the grape must was raised from 16.5° Brix (w/v; pH 3.2) to 24° Brix by the addition of sucrose. Following inoculation with the two types of yeast cells, namely a 24 h yeast pre-culture or cells immobilized in Ca-alginate beads [5% inoculum (w/v)], fermentation was carried out at 18°C for 14 days in glass bottles (5,000 mL volume) equipped with an airlock using 3,000 mL of grape must. Post fermentation,

free yeast cells, 2% Ca-alginate bead-encapsulated cells, and other lees were eliminated by centrifugation at 6,000 × *g* for 10 min.

2.5. Standard chemical analysis

The pH of the wine was measured using a pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). The soluble solid content (°Brix) was measured using a refractometer (RA250, ATAGO, Tokyo, Japan), the alcohol content was measured at 15°C using a vinometer, and the total acid content and reducing sugars were quantified using the AOAC method (CAPUTI, 1995). Total phenolic compounds (TPC) were assessed using the Folin-Ciocalteau phenol reagent method (SINGLETON and ROSSI, 1965) and the free sugar content was determined by high-performance liquid chromatography (HPLC) using a Sugar-Pak I column (⌀ 6.5 × 300 mm, Waters, Milford, MA) and a Ca-EDTA buffer (50 mg/L) at a flow rate of 0.5 mL/min. Organic acids were quantified by HPLC using a Shodex RSpak KC-811 column (⌀ 8 × 300 mm, Showa Denko KK, Kawasaki, Japan). The column was run with a mobile phase of 0.1% phosphoric acid at a flow rate of 1 mL/min at 40°C. Organic acids were detected using a refractive-index detector. Acetaldehyde, methanol, and various alcohols (fusel oil) were assessed using gas chromatography (6890N GC; Agilent, Santa Clara, CA, USA) and a flame ionization detector (FID). After distillation, samples were filtered through a membrane filter (Millex-HV, 0.45 μm, Millipore Co., Bedford, MA, USA) before injection. Separation was performed with an HP-FFAP column (⌀ 0.25 mm × 30 m, film thickness = 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) using helium as a carrier gas with a constant flow of 1 mL/min. The chromatographic oven temperature was initially kept at 60°C for 4 min, was then increased to 210°C, at 6°C/min, and was then maintained at 210°C for 2 min. The quantitative determination of volatile compounds was performed using the relative area calculated as the ratio. Samples were assessed in terms of Hunter's color values (L*, lightness; a*, red-green; and b*, yellow-blue) using a vertical-type spectrophotometer (CM-3600d, Konica Minolta, Inc., Tokyo, Japan). All measurements were replicated three times and average values (n = 3) were calculated.

2.6. Sensory evaluation

Sensory evaluation of wines was performed by a panel of twenty trained experts. The color, flavor, taste, and overall preference of the wines were evaluated on a scale of 1 to 5, where 5 was the best score. Overall preference according to the taste and flavor was evaluated using the mean value of a hedonic scale from 1 (very poor, dislike extremely) to 5 (excellent, like extremely) (PIGGOTT, 1988).

2.7. Statistical analysis

Data were expressed as mean±standard deviation (SD) of triplicate experiments. Statistical significance was determined by a Student's t-test for independent means, using Microsoft Excel (Microsoft, Redmond, WA, USA). A one-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine differences between means. The critical level for statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Characteristics of grapes fermented by free cells and Ca-alginate bead-encapsulated yeast cells

We previously investigated the effects of Ca-alginate beads and protective agents on the survival rates of five yeast strains using an air-blast drying method, which demonstrated that 2% Ca-alginate beads soaked in protective agents (10% skimmed milk and 10% solutions of various sugars) helped to protect free cells from the environment. Specifically, in this study, *S. cerevisiae* D8 cells with 10% sucrose, *S. cerevisiae* M12 cells with 10% raffinose, *S. cerevisiae* S13 cells with 10% trehalose, *H. uvarum* S6 cells with 10% trehalose, and *I. orientalis* KMBL5774 cells with 10% glucose, all encapsulated in Ca-alginate beads, exhibited the highest survival rates (90.67%, 87.73%, 92.05%, 90.81%, and 87.16% viability, respectively) after air-blast drying at 37°C for 5 h (KIM *et al*., 2017).
Changes in the pH of wine fermented by encapsulated cells compared to those of wines fermented by free yeast cells were shown in Fig. 1. pH has a marked effect on microorganisms: in a study by BAE (2002), it was suggested that a pH <3.2 results in sourness during wine fermentation, while a pH <4.0 is recommended for preventing contamination by other harmful bacteria. In this study, the pH of all samples ranged from 3.41 to 3.65 during fermentation. At the beginning of fermentation, the pH values of all samples were 3.59–3.63 and decreased slowly during fermentation to final values of 3.47–3.52. The changes in total acid content in encapsulated cell-fermented wine compared to free cell-fermented wine were also shown in Fig. 1, where it can be seen that the total acid content in all wines increased from 0.41–0.43 to 0.61–0.69 during fermentation. The total acid content of wines fermented using free cells was found to increase slightly after two days, while that of the wines fermented using 2% Ca-alginate bead-encapsulated cells was found to increase only slightly after three days.

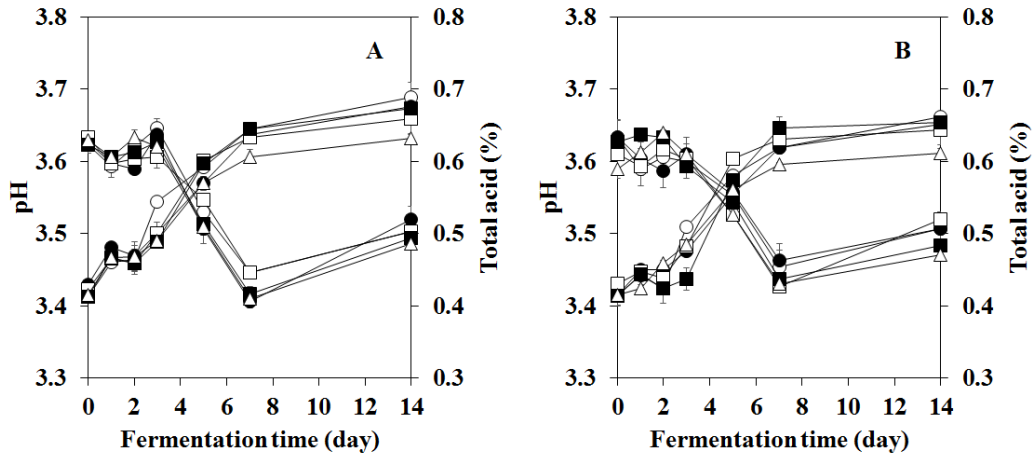


Figure 1. Changes in pH and total acid content during fermentation. (A) Free cells, and (B) cells encapsulated in 2% Ca-alginate beads. Open circles, *S. cerevisiae* D8; filled circles, *S. cerevisiae* M12; open squares, *S. cerevisiae* S13; filled squares, *H. uvarum* S6; open triangles, *I. orientalis* KMBL5774.

SEO and YOOK (2007) have reported that the total acid content during fifteen days of Campbell Early wine fermentation with a commercial wine dry yeast starter was 0.44–0.81. Similar to this, our study demonstrated low total acidity levels in wine fermented using

encapsulated cells for fourteen days. LEE and KIM (2006) reported that the Korean Campbell Early grape has a high level of acidity due to the presence of malic acid and tartaric acid, and investigated six different fermentation processes in terms of their capacity to reduce the levels of acidity. Based on the findings of the study, both carbonic maceration and precipitation methods were recommended for obtaining high quality wines.

The levels of soluble solids in the wines fermented using encapsulated cells were shown to decrease sharply after three days. In contrast, the soluble solid levels in the wines fermented by free cells decreased after two days (Fig. 2). We previously showed that the free and immobilized cell populations exhibited similar growth patterns; however, slight differences were observed at the early stage of the fermentation (KIM *et al*., 2017). It should be noted that, as the cell population in the beads increased, the diameter of the beads increased significantly due to the elastic properties of the alginate gel (VIVES *et al*., 1993). KIM *et al*., (2017) studied characteristics of red wine fermentation using Campbell Early and different sugars. The results revealed that wine fermentation with added glucose was faster, and it yielded a higher alcohol content, than that obtained with any other sugars. The wine fermented by the addition of sucrose and high fructose corn syrup showed results similar to that obtained by the addition of glucose. Similar results were obtained in our experiments; the soluble solid content of the two wines used in this study decreased upon the addition of sucrose, although the fermentation starting points differed. Due to conversion into alcohol, the reducing sugar content of all wine samples decreased sharply (from 25° Brix to 0.17° Brix) during fermentation. The reducing sugar content and the soluble solid content of samples fermented using 2% Ca-alginate bead-encapsulated cells decreased more slowly than that of samples fermented using free cells owing to pore size and different fermentation starting points (Figs. 2 and 3) (KIM *et al*., 2017).

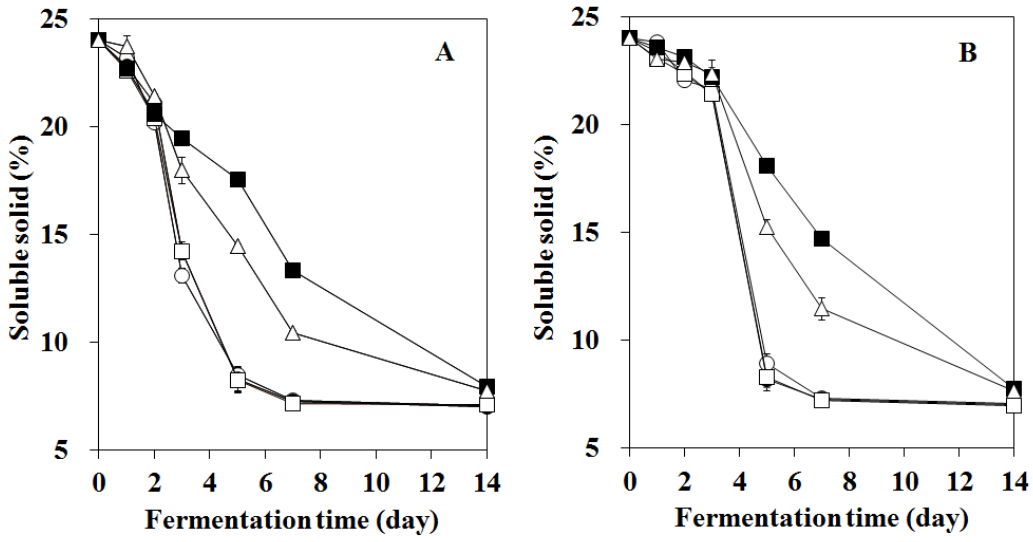


Figure 2. Changes in soluble solid contents during fermentation. (A) Free cells, and (B) cells encapsulated in 2% Ca-alginate beads. Open circles, *S. cerevisiae* D8; filled circles, *S. cerevisiae* M12; open squares, *S. cerevisiae* S13; filled squares, *H. uvarum* S6; open triangles, *I. orientalis* KMBL5774.

Most of the reducing sugar content and soluble solid content were consumed during the fermentation. Changes in alcohol content in the presence of bead-encapsulated cells compared to that in free cells during fermentation were shown in Fig. 3. The alcohol

content of wines fermented using free cells ranged from 9.20±0.06% to 12.97±0.09%, while that of wines fermented using encapsulated cells ranged from 9.75±0.04% to 13.43±0.24%. Although Ca-alginate bead-encapsulated cells fermented at a slightly slower rate than the free cells, their alcohol production was higher than that of free cells. ROUKAS *et al*., (1991) reported that free and immobilized *S. cerevisiae* cells produce the same maximum ethanol concentration under similar fermentation conditions. SINGH *et al*., (1998) also reported that the concentrations of ethanol produced in free cell and immobilized cell (Ca-alginate) batch fermentations were comparable. However, HOLCBERG and MARALITH (1981) reported that the rate of ethanol production by cells entrapped in agar, alginate, and polyacrylamide gels was higher than that of free cells. NORTON *et al*., (1995) reported that the resistance of yeast to ethanol was significantly higher in immobilized cells than in free cells. The findings reported here show that alcohol production using 2% Ca-alginate bead-encapsulated cells was higher than that using free cells. In both free cell and encapsulated cell systems, *H. uvarum* S6 and *I. orientalis* KMBL5774 exhibited slower alcohol production rates and lower maximal alcohol levels compared with *S. cerevisiae* D8, M12, and S13 (Fig. 3).

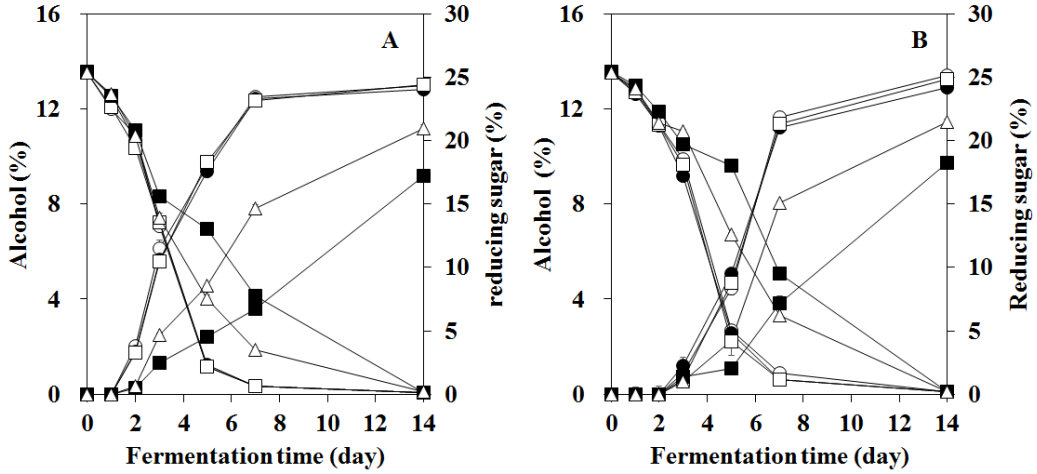


Figure 3. Changes in alcohol and reducing sugar content during fermentation. (A) Free cells, and (B) cells encapsulated in 2% Ca-alginate beads. Open circles, *S. cerevisiae* D8; filled circles, *S. cerevisiae* M12; open squares, *S. cerevisiae* S13; filled squares, *H. uvarum* S6; open triangles, *I. orientalis* KMBL5774.

A high cell density of a non-*Saccharomyces* (*H. uvarum*) yeast has been identified in *V. vinifera* grape must during the first 4-6 days of fermentation, until the ethanol content reached 4-7% (v/v). Accordingly, it has been suggested that the inoculation with pure cultures of *H. uvarum* cells may enhance ethanol production during fermentation (ROJAS *et al*., 2003). In agreement with these reports (MOREIRA *et al*., 2008; ROJAS *et al*., 2003), our findings revealed that *H. uvarum* S6 produced approximately 9.2±0.06 to 9.75±0.04% (v/v) alcohol. In a previous study, the effects of co-fermentation with various inoculation ratios (*S. cerevisiae* W-3: non-*S. cerevisiae; I. orientalis* KMBL5774) were investigated and led to the use of a mixed culture being recommended for better wine quality owing to the low alcohol production capacity of *I. orientalis* KMBL5774 (KIM *et al*., 2008). In our study, however, a low maximal alcohol content [11.20±0.06 to 11.47±0.29% (v/v)] was achieved by single fermentation and the use of Ca-alginate beads encapsulating a single strain (*I. orientalis* KMBL5774) was therefore studied.

The total phenolic compound levels in all the samples ranged from 0.13% to 0.15% (Fig. 4), while LEE *et al*., (2006) reported that the total content of phenolic compounds of Campbell Early wine in Korea was approximately 0.12%. The markedly higher total phenolic compound levels observed in the present study may be attributed to different conditions such as vintage, area, and climate (HUGLIN, 1978; HUGLIN and SCHNEIDER, 1998; SEGUIN, 1975; WINKLER *et al*., 1975). No major differences in total phenolic content were observed between free cell and encapsulated cell fermentations.

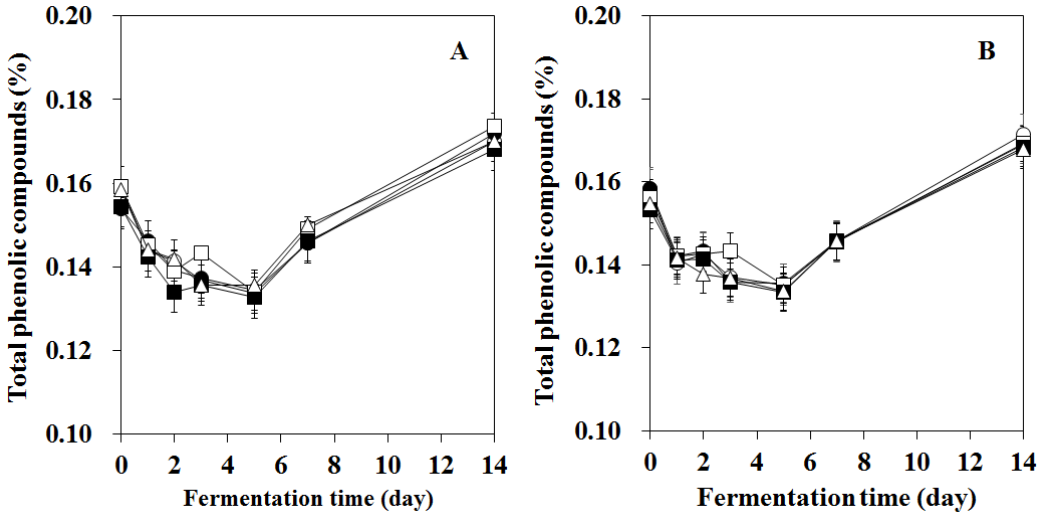


Figure 4. Changes in total phenolic compound contents during fermentation. (A) Free cells, and (B) cells encapsulated in 2% Ca-alginate beads. Open circles, *S. cerevisiae* D8; filled circles, *S. cerevisiae* M12; open squares, *S. cerevisiae* S13; filled squares, *H. uvarum* S6; open triangles, *I. orientalis* KMBL5774.

3.2. Yeast cell release from 2% Ca-alginate beads and viable cell count during fermentation

Encapsulated cells (initial cell concentration: 1.0±0.1 × 10^8 CFU/mL; stored at 4°C for 3 months) and cultured free cells (initial cell concentration: 1.0±0.2 × 10^8 CFU/mL) were inoculated into 5% (w/v) grape must. At the beginning of the fermentation, the free cells began to grow immediately, while the narrow and complex interior structure of the 2% Ca-alginate beads hindered the release of yeast cells from encapsulation following budding from 0 h (Figs. 5 and 6) (KIM *et al*., 2017). Accordingly, viable cell numbers in the 2% Ca-alginate bead samples increased more slowly than those in the free cell samples. As the cell population grows within the beads, the bead diameters increase significantly due to the elastic properties of the alginate gel (BABU *et al*., 1992; VIVES *et al*., 1993). This effect results in a delay in the rate at which the maximal cell population (10^8 CFU/mL) is reached (Fig. 6). KLINKENBERG *et al*. (2001) reported that the viable cell count (*Lactobacillus lactis* ssp. *lactis*) inside beads coated with alginate during milk fermentation remained significant at the beginning of fermentation, and that the alginate beads had a significant effect on the rate of cell release after 48 h of fermentation. In our study, however, the encapsulated cells were released slowly after 6 h and the release rate increased sharply after 48 h. In five to six days, the wines fermented by cells encapsulated in 2% Ca-alginate beads exhibited maximal populations similar to those exhibited by the free cell-fermented wines (Fig. 5).

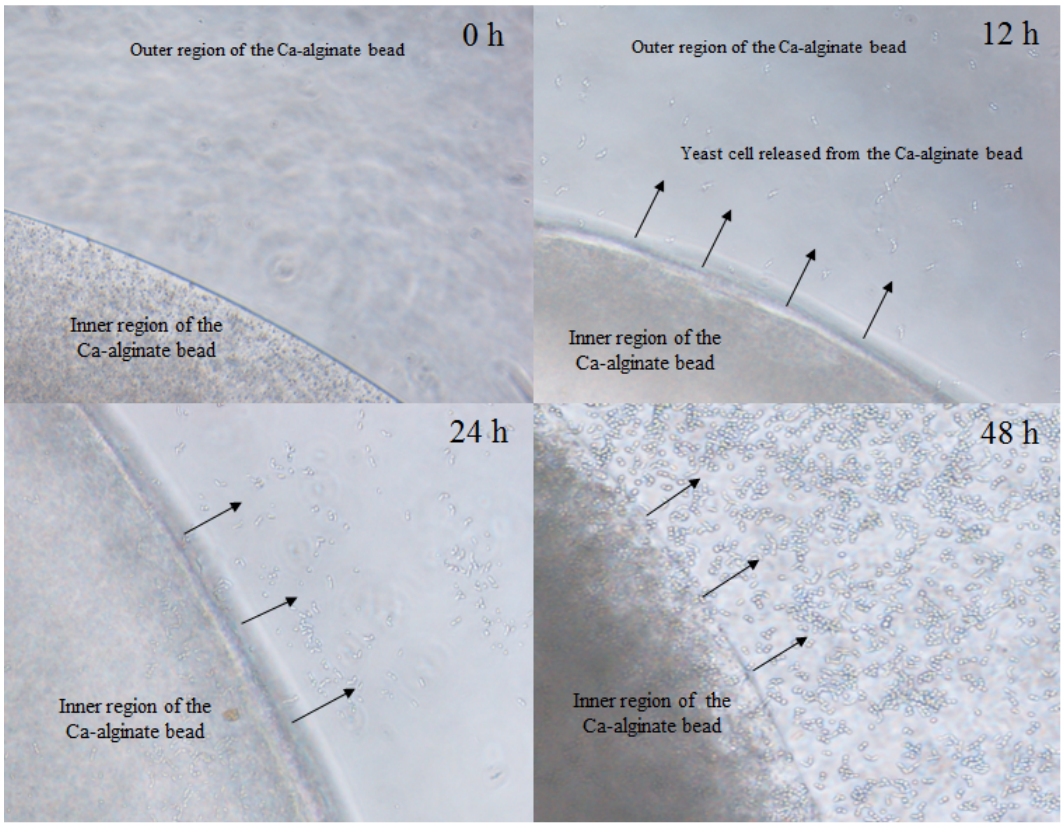


Figure 5. Optical microphotograph (×100 magnification) of yeast cells released from 2% Ca-alginate beads at different time intervals up to 48 h.

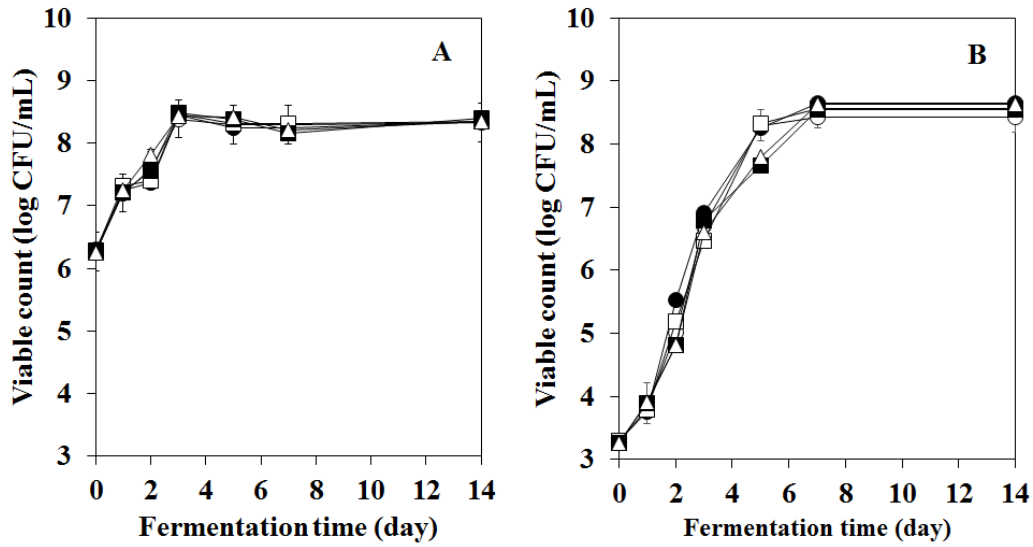


Figure 6. Changes in viable cell count during fermentation. (A) Free cells, and (B) cells encapsulated in 2% Ca-alginate beads. Open circles, *S. cerevisiae* D8; filled circles, *S. cerevisiae* M12; open squares, *S. cerevisiae* S13; filled squares, *H. uvarum* S6; open triangles, *I. orientalis* KMBL5774.

3.3. Physicochemical properties of wines fermented by free cells and encapsulated cells

After fermentation, sucrose, glucose, galactose, and fructose were identified as the sugar components in the wines and of these sugars, sucrose was found to be present at the highest concentration (1.32±0.01 g/L to 2.05±0.43 g/L) in both wine samples (Table 1). The two different fermentation processes were therefore found to yield similar sugar profiles. Free sugars are the major components of grape soluble solids and are related to the final alcohol content of wine (CONDE *et al*., 2007). Using HPLC, SHIRAISHI *et al*., (2010) showed that the major free sugars in grape wine (*Vitis* spp.) were glucose, fructose, and sucrose. KLIEWER (1966) have also reported the presence of glucose, fructose, galactose, sucrose, maltose, melibiose, raffinose, and stachyose in *Vitis* spp.

To determine the organic acid composition of the wines, the levels of malic acid, tartaric acid, citric acid, succinic acid, and acetic acid were assessed in the wines (Table 2). Malic acid and tartaric acid, which contribute 70–90% of the total acidity of grapes, significantly influence the sensory properties of wine (BEELMAN and GALLANDER, 1979; RUFFNER, 1982). Differences in organic acid content of both wines were negligible, nevertheless wines fermented using encapsulated cells exhibited slightly lower levels than those fermented using free cells. The wine fermented by *I. orientalis* KMBL5774, which has previously been shown to be able to rapidly degrade malic acid in medium where it represents the sole carbon and energy source (SEO *et al*., 2007), exhibited lower levels of malic acid than the other wines. As expected, the malic acid concentrations were lower in wines fermented with *I. orientalis* KMBL5774 than in wines fermented with other strains; however, other than malic acid, the levels of other organic acids did not differ markedly between free cell- and encapsulated cell-fermented wines. LEE and KIM (2006) studied the de-acidification of wine made from Campbell Early grapes, and recommended carbonic maceration and cold fermentation to decrease the organic acid content of Campbell Early wine. Several studies have shown that the process of malolactic fermentation results in the degradation of malic acid into lactic acid and carbon dioxide, the consequence of which is a reduction in total acidity (de-acidification) in the wine by strains of lactic acid bacteria (LAB) of the genera *Oenococcus*, *Leuconostoc*, *Lactobacillus,* and *Pediococcus* (BOULTON *et al*., 2013; VOLSCHENK *et al*., 1997; VILJAKAINEN and LAASO, 2000).

As shown in Table 3, the aldehyde content of wines fermented using free and encapsulated cells ranged from 44.88±0.49 to 65.28±4.32 mg/L and from 41.28±2.11 to 65.28±4.32 mg/L, respectively. Only small differences in aldehyde levels (lower in those fermented using encapsulated cells), if any, were observed between the free cell- and encapsulated cell-fermented wines. Acetaldehyde is considered to be a leakage product of alcohol fermentation by yeast, and *S. cerevisiae* and *Kloeckera apiculata* have been shown to produce 0.5–286 mg/L and 9.5–66 mg/L acetaldehyde (GEROYIANNAKI *et al*., 2007). GEROYIANNAKI *et al*., (2007) also reported that white and red grape pomace yielded 345 mg/L and 317 mg/L acetaldehyde, respectively. The lowest reported acetaldehyde level in wine was for cagaita wine produced using encapsulated cells (1.031 mg/L; *S. cerevisiae* UFLA CA11), whereas the free cell equivalent yielded 1.378 mg/L acetaldehyde (OLIVEIRA *et al*., 2011). All wines in this study (both free cell- and encapsulated cell-fermented) yielded acetaldehyde levels well below the official limit of 700 mg/L (KOREA, 2012). During wine production, methanol arises as a result of pectin methyl esterase activity during grape crushing (MASINO *et al*., 2008).

Table 1. Free sugar contents (g/L) in wines fermented using 2% Ca-alginate bead cells compared to those in wines fermented using free cells after fermentation.

Type	*S. cerevisiae* D8			*S. cerevisiae* M12			*S. cerevisiae* S13			*H. uvarum* S6			*I. orientalis* KMBL5774		
	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*- value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value
Sucrose	1.87±0.03	1.80±0.02	0.0420	1.71±0.01	1.72±0.05	0.6413	1.32±0.01	1.34±0.01	0.0512	1.45±0.05	1.32±0.06	0.0396*	2.05±0.13	1.96±0.07	0.3489
Glucose	1.11±0.08	1.09±0.04	0.7200	1.22±0.12	1.11±0.09	0.2321	1.07±0.08	1.01±0.10	0.4266	1.45±0.11	1.25±0.05	0.0477*	1.17±0.06	1.13±0.04	0.6547
Galactose	1.31±0.25*	1.10±0.08*	0.2459	1.22±0.07	1.10±0.08	0.1382	1.25±0.13	1.22±0.07	0.7296	1.29±0.06*	1.03±0.10*	0.0185*	1.32±0.07*	1.10±0.10*	0.0347*
Fructose	0.11±0.01	0.13±0.01	0.1340	0.13±0.01	0.13±0.03	0.8383	0.15±0.01	0.15±0.03	0.9068	0.11±0.00	0.12±0.00	0.0181*	0.10±0.01	0.10±0.02	0.8731
Total free sugars	4.40±0.26	4.12±0.11	0.1662	4.28±0.17	4.06±0.11	0.1382	3.79±0.13	3.72±0.18	0.5917	4.30±0.09	3.71±0.12	0.0026**	4.60±0.05	4.29±0.07	0.0021**

All data are expressed as mean±SD (n = 3).
*$p < 0.05$ and **$p < 0.01$ are considered to be statistically significant by student's *t*-test.

Table 2. Organic acid contents (g/L) in wines fermented by 2% Ca-alginate bead cells compared to those in wines fermented using free cells after fermentation.

Type	*S. cerevisiae* D8			*S. cerevisiae* M12			*S. cerevisiae* S13			*H. uvarum* S6			*I. orientalis* KMBL5774		
	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*- value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value
Lactic acid	0.78±0.02	0.77±0.05	0.7145	0.75±0.04	0.79±0.04	0.3038	0.74±0.05	0.85±0.03	0.0342*	0.82± 0.03	0.89±0.04	0.0598	0.82±0.02	0.94±0.05	0.0238*
Citric acid	0.14±0.06	0.20±0.02	0.2028	0.14±0.03	0.16±0.03	0.6491	0.21±0.04	0.16±0.03	0.1406	0.26±0.03	0.14±0.05*	0.0224*	0.21±0.01	0.16±0.04	0.1133
Tartaric acid	0.91±0.05	0.90±0.07	0.8561	0.83±0.02	0.86±0.03	0.1907	0.89±0.02	0.85±0.02	0.0920	0.95±0.07	0.93±0.09	0.8188	0.93±0.07*	0.92±0.10*	0.9261
Malic acid	1.72±0.01	1.70±0.01	0.0296*	1.89±0.04	1.71±0.06	0.0120*	1.63±0.01	1.65±0.03	0.4034	1.70±0.04	1.69±0.03	0.7625	1.33±0.03	1.03±0.06	0.0013**
Succinic acid	0.13±0.02	0.11±0.07	0.6800	0.12±0.03	0.11±0.01	0.4822	0.13±0.03	0.10±0.04	0.3218	0.09±0.06	0.09±0.04	0.9776	0.09±0.04	0.10±0.03	0.8686
Acetic acid	0.16±0.01	0.15±0.03	0.7050	0.11±0.01	0.13±0.04	0.4306	0.10±0.01	0.13±0.09	0.6319	0.16±0.01	0.12±0.03	0.1030	0.15±0.03	0.10±0.02	0.0714

All data are expressed as mean±SD (n = 3).
*$p < 0.05$ and **$p < 0.01$ are considered to be statistically significant by student's *t*-test.

Table 3. Aldehyde, methanol, and fusel oil contents (mg/L) in wines fermented by 2% Ca-alginate bead cells compared to those in wines fermented using free cells after fermentation.

Type	*S. cerevisiae* D8			*S. cerevisiae* M12			*S. cerevisiae* S13			*H. uvarum* S6			*I. orientalis* KMBL5774		
	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value
Aldehyde	64.17±2.13	64.27±3.32	0.9670	61.90±3.11	53.07±3.15	0.0259	65.28±4.32	54.66±3.45	0.0292*	44.88±0.49	41.28±2.11	0.0451*	52.01±1.26	47.56±1.65	0.0206*
Methanol	112.84±2.11	108.69±3.21	0.1346	110.26±3.22	110.28±2.01	0.9932	109.77±4.13	105.17±3.89	0.2329	105.39±1.25	106.30±1.02	0.3839	105.33±3.28	108.53±1.57	0.2021
Ethyl acetate	121.79±1.36	119.12±1.27	0.0678	126.2±2.14	115.74±2.35	0.0047**	125.33±1.46	113.35±1.43	0.0006$^{\#}$	158.06±3.52	148.61±3.29	0.0274*	132.19±1.14	130.09±3.21	0.3458
1-Propanol	191.67±5.21	155.83±2.74	0.0005$^{\#}$	172.78±3.22	106.20±1.25	0.0000$^{\#}$	155.05±2.14	97.98±1.98	0.0000$^{\#}$	158.25±1.58	121.10±2.04	0.0000$^{\#}$	139.07±2.95	117.35±3.05	0.0003$^{\#}$
Iso-butanol	361.79±2.56	389.12±1.07	0.0001$^{\#}$	346.22±3.14	345.74±2.65	0.8495	385.33±2.36	373.35±2.47	0.0037**	458.06±4.02	448.61±2.69	0.0277*	432.19±1.02	435.09±1.24	0.0352*
Iso-amyl alcohol	391.81±3.56	379.17±1.32	0.0045**	348.16±2.14	333.12±1.96	0.0009$^{\#}$	387.61±3.02	348.65±2.58	0.0001$^{\#}$	443.71±2.04	396.66±2.65	0.0000$^{\#}$	417.87±3.02	405.28±2.79	0.0061**

All the data are expressed as mean±SD (n = 3).
$^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#}p < 0.001$are considered to be statistically significant by student's *t*-test.

The methanol contents in all the wine samples in this study were found to range from 105.17±3.89 mg/L to 112.84±2.11 mg/L (Table 3), which are well below the standard maximal value for alcoholic beverages (1,000 mg/L), and were not affected by the Ca-alginate bead system. According to MATEOS *et al*., (2006), the major compounds that contribute to the overall volatile effects defining wine aroma are ethyl acetate and higher alcohols such as 1-propanol, isobutyl alcohol, and iso-amyl alcohol. As shown in Table 3, the fusel oil analysis conducted in this study revealed that iso-amyl alcohol, which contributes to wine quality (KOURKOUTAS *et al*., 2001) was present at 333.12±1.96 to 443.71±2.04 mg/L in the wines, and was the most prevalent fusel oil component. The ethyl acetate, 1-propanol, and iso-butanol concentrations ranged from 113.35±1.43 to 158.06±3.52 mg/L, from 97.98±1.98 to 191.67±5.21 mg/L, and from 346.22±1.96 to 443.71±2.04 mg/L, respectively. *H. uvarum* S6 cells (both free and encapsulated cells) yielded the highest levels of iso-butanol and iso-amyl alcohol during fermentation compared with the other yeast types. OLIVEIRA *et al*., (2011) have reported *Hanseniaspora* and *Issatchenkia* (non-*Saccharomyces*) as high ester producers. In this study, the wines fermented using encapsulated cells generally contained lower levels of fusel oil components than the wines fermented using the free cells.

In terms of Hunter's color values, there were no marked differences between the free cell and encapsulated cell fermented wines. The L* values ranged from 38.15±0.01 to 38.97±0.01, the a* values ranged from 7.24±0.04 to 7.66±0.16, and the b* values ranged from 0.00±0.02 to 0.20±0.02 (Table 4). There were no marked differences between the free cells and 2% Ca-alginate bead cells-fermented wines.

3.4. Sensory evaluation

The sensory characteristics of wines fermented using free and encapsulated cells were evaluated by a panel of twenty assessors. Preferences in terms of color, flavor, taste, and overall preference were determined on a scale of 1 (poorest) to 5 (best) (Fig. 7).

In terms of flavor and taste, the wines fermented with *H. uvarum S6* and *I. orientalis* KMBL5774 (both free and encapsulated cell fermentations) obtained the highest scores, while the wines fermented with *S. cerevisiae* D8, M12, and S13 obtained the lowest scores. In agreement with previous reports (MATEOS *et al*., 2006; TORRENS *et al*., 2008), differences in volatile compound levels between the wines seemed to correlate with the sensory evaluation. Among the wines in this study, high levels of acetaldehyde and low levels of ethyl acetate resulted in lower wine quality scores. When comparing wines fermented by free cells with those fermented by encapsulated cells, however, scores for color, taste, flavor, and overall preference did not differ, which indicates that encapsulated yeast cells represent suitable alternative starters for wine fermentation.

Table 4. Hunter's color values for wines fermented by 2% Ca-alginate bead cells compared to those for wines fermented using free cells after fermentation.

Type	*S. cerevisiae* D8			*S. cerevisiae* M12			*S. cerevisiae* S13			*H. uvarum* S6			*I. orientalis* KMBL5774		
	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*- value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value	Free cells	Bead cells	*p*-value
L*	38.97±0.01	38.97±0.02	1.0000	38.69±0.14	38.61±0.01	0.4276	38.44±0.03	38.44±0.01	1.0000	38.17±0.04	38.15±0.01	0.4481	38.28±0.02	38.28±0.01	1.0000
a*	7.66±0.16	7.58±0.03	0.4426	7.24±0.04	7.26±0.03	0.5265	7.46±0.13	7.32±0.03	0.1433	7.55±0.01	7.52±0.03	0.1757	7.47±0.04	7.47±0.03	1.0000
b*	0.01±0.02	0.00±0.02	0.5734	0.20±0.02	0.20±0.01	1.0000	0.13±0.02	0.14±0.02	0.5734	0.20±0.02	0.20±0.01	1.0000	0.05±0.21	0.07±0.12	0.8930
ΔE	38.92±0.01	39.72±0.03	0.0000$^{\#}$	38.64±0.01	39.12±0.13	0.0237*	38.46±0.02	39.15±0.04	0.0000$^{\#}$	38.19±0.02	38.91±0.03	0.0000$^{\#}$	38.30±0.01	39.00±0.02	0.0000$^{\#}$

All the data are expressed as mean±SD (n = 3).
$^{*}p < 0.05$ and $^{\#}p < 0.001$ are considered to be statistically significant by student's *t*-test.

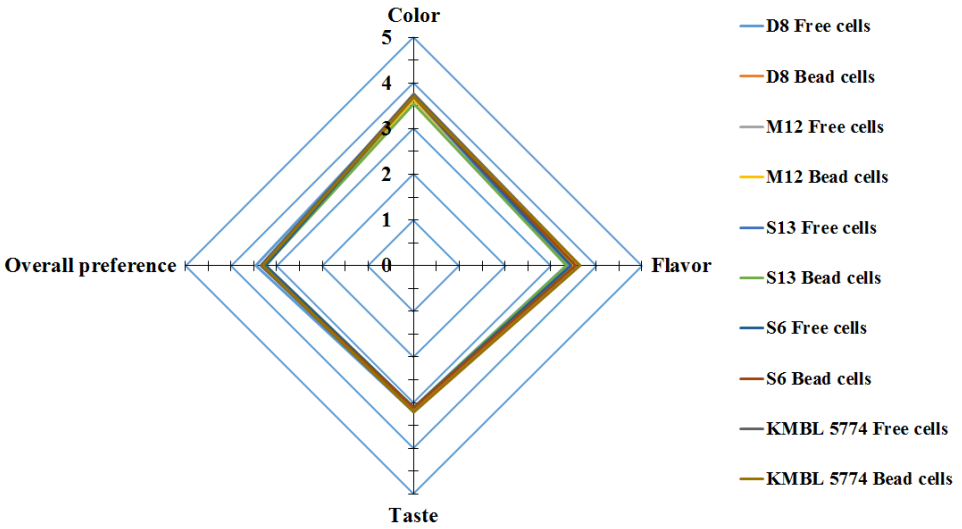


Figure 7. Radar plot of the sensory evaluation scores for wine fermented by 2% Ca-alginate bead cells compared to those for wines fermented by free cells after fermentation. The results reflects the means of scores from 20 semi-trained panelist.

4. CONCLUSIONS

In this study, fermentation characteristics of free yeast cell and yeast encapsulated in Ca-alginate beads (immobilized yeast) were compared. There was no significant difference between free yeast cell and immobilized yeast in terms of reducing sugars content, soluble solids, total acids, organic acids, free sugars, viable cell count and other physiochemical properties. However, immobilized yeast produced slightly higher alcohols and a lower total concentration of volatile acids (compound) than free yeast cell. These results suggest that yeast encapsulated in Ca-alginate beads have a potential to be used as alternative yeast for wine fermentation as it is cost effective compared to freeze-dried yeast.

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PAPER

DEVELOPMENT OF CHICKEN ROLL RECIPE USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The purpose of this study was to determine the optimum quantities of ingredients to yield a chicken roll product with desirable textural properties and coloring and a minimum cooking loss. Response surface methodology (RSM), a statistical technique, was applied for optimization. The optimum quantities for chicken roll production were found to be 8.66 g, 75.00 g and 53.83 g for wheat flour, distilled water, and minced chicken, respectively. The lowest cooking loss was achieved by a recipe with high wheat flour and distilled water content, whereas the highest cooking loss was observed in the recipe with the lowest wheat flour content and the highest minced chicken content.

Keywords: chicken, poultry, optimization, texture profile analysis, response surface methodology

1. INTRODUCTION

The consumption of poultry meat is gradually increasing around the world. Because of its high-quality protein and relatively low fat content, poultry meat, especially chicken meat, plays an important role in the human diet. In addition to this high nutritional value, the relatively low cost and great variety of chicken meat products make chicken meat a widely favored food (CHOULIARA *et al.*, 2008; MOK *et al.*, 2017).

In recent years, ready-to-eat (RTE) foods have become highly preferred food products. Some of the leading causes of the rising consumer demand for RTE foods include: changes in consumers' lifestyles, households in which both parents work long hours, the convenience of consumption, the minimal time required for RTE meal preparation, and the charm of flavorful products (BAE ET AL., 2010; JIANG AND XIONG, 2015). The consumption of RTE foods has risen almost 20% from 2007 to 2012; during these years, European consumers' demand for RTE meat and poultry products (e.g. meat balls, burger patties, sausages) has increased in accordance with this trend (FERREIRA *et al.*, 2016). In Australia, the consumption of RTE meat products has recently increased from 20% to 50% (JIANG and XIONG, 2015). According to a global report on the RTE food market, RTE food consumption is expected to rise 21.8% between 2018 and 2023 (Global Ready to Eat Food Market Report, 2017).

Hydrocolloids (e.g. pectin, xanthan, starch, guar gum, alginate) are frequently used in various food products as thickeners, gelling agents, emulsifiers, stabilizers, fat replacers, clarifying agents, flocculating agents, clouding agents, and whipping agents (LI and NIE, 2014; VIEBKE *et al.*, 2014). Guar gum, commonly used as a hydrocolloid, is a good stabilizer and water-binder, and it provides desirable structure and a slick fat-like mouthfeel for food products (ANDRÉS *et al.*, 2006). Starches occupy an important role in meat recipes (FENG *et al.*, 2013). Due to its unique white color, excellent mouthfeel properties, bland taste, and relatively small granules (2-7 μm), rice starch has become an alternative fat replacer that provides good textural properties for food products (PARK *et al.*, 2007; RESCONI *et al.*, 2015; TOMASCHUNAS *et al.*, 2013; WANI *et al.*, 2012). Because gum increases the viscosity of starch and affects gelatinization, the utilization of gum and starch in food systems has been researched in many studies (KIM and YOO, 2006; YOO *et al.*, 2005). The interactions between starches and gums enhance the rheological properties of starch, improve overall product quality, and reduce the cost of the products (KIM and YOO, 2006; SHI and BEMILLER, 2002). It was reported that guar gum and xanthan gum increased the viscosity of cationic tapioca starch suspensions (CHAISAWANG and SUPHANTHARIKA, 2005). FENG *et al.* (2013) studied the physicochemical properties, texture, and sensory evaluation of Chinese Cantonese-style sausage prepared from the Mesona Blumes gum-rice starch mixture; they reported that this gel could be used as a fat substitute in sausage (FENG *et al.*, 2013).

Alginate, a polysaccharide obtained from brown seaweed, is used as a thickening, film-forming, gel-producing, and emulsion-stabilizing agent in the food industry; this is due to its high water-solubility, high biodegradability, and low price, relative to natural casings (COMAPOSADA *et al.*, 2015; MARCOS *et al.*, 2016; NAKAUMA *et al.*, 2016). Mostly used in meat products, sodium alginate can be combined with calcium ions at room temperature to create a uniform, transparent, water-insoluble, and thermo-irreversible gel (BOLES and SHAND, 1998; COMAPOSADA *et al.*, 2015; LEON *et al.*, 2016). Calcium chloride solutions are most commonly used to provide the calcium ions necessary to achieve gelatinization (COMAPOSADA *et al.*, 2015; Hassan and RAMASWAMY, 2011). The US Food and Drug Administration applies the label ''Generally Recognized as Safe (GRAS)'' to citric acid, which is commonly used in meat and poultry processing for its antimicrobial and tenderizing effects (KHARE *et al.*, 2016). In some studies, combining

citric acid and lactic acid reduced the microbial load of chicken drum sticks, while negatively affecting the sensory parameters (ZHU *et al.*, 2016). Coating chicken fillets with carrageenan, cinnamon oil, and citric acid extends the shelf life of chicken meat, under chilled conditions (KHARE *et al.*, 2016).
In previous studies, chicken rolls were produced and analyzed with different formulations and methods (BRECLAW and DAMSON, 1970; DU *et al.*, 2003; FURUMOTO and STADELMAN, 1980; GILLETT and CARPENTER, 1992; WANG and CHEN, 1987; XIAO *et al.*, 2011; YIM *et al.*, 2015). However, to the best of our knowledge, there is no published study addressing the optimization of chicken roll formulation based on some preferred properties. The aim of this study was to determine the optimum quantities of ingredients for a chicken roll product of a desirable color, with textural properties such as low hardness and high resilience, and a minimum cooking loss.

2. MATERIALS AND METHODS

2.1. Experimental Design

RSM was employed to determine the optimum values of three independent variables and evaluate the combined effects of those parameters. As the purpose of this study was to evaluate the interaction effects between food hydrocolloids and other ingredients, the water used in the experiments needed to be free of salt or any other impurities. For example, common drinking water contains salts that can change the effects of calcium chloride and sodium alginate; thus, distilled water was used. The wheat flour (5-35 g), distilled water (35-75 g), and minced chicken (20-60 g) were the parameters and levels employed. The coded values and the original values of the independent parameters are shown in Table 1. For the purpose of evaluating the pure error and the curvature of the complete design, a three-level three-factor Box-Behnken Design (BBD) was implemented, which indicated the need to compose a total of 17 experiments with 5 replicates of the center point.

Table 1. Variables and their levels in response surface design.

Independent variables	Symbols	Coded levels		
		-1	0	1
Wheat flour (g)	A	5	20	35
Distilled water (g)	B	35	55	75
Minced chicken (g)	C	20	40	60

2.2. Sample preparation

Fresh skinless chicken breasts were obtained from Keskinoğlu Poultry and Hatchery Ind. Inc., Manisa, Turkey. Different amounts of wheat flour (5-35 g), distilled water (35-75 g), and minced chicken (20-60 g) were added to obtain chicken rolls according to the experimental design. The other ingredients, which are sodium alginate, rice starch, guar gum, citric acid, wheat flour, and calcium chloride, were obtained from Smart Chemistry Inc., İzmir, Turkey. Before preparing the chicken meat batter, breast meat was ground through the mincer. Sodium alginate (2.4 g), rice starch (1.7 g), guar gum (0.85 g), citric acid (0.2 g), and wheat flour were added to 60±5°C distilled water, and blended for 1 minute. The minced chicken was added to this mixture, which was then blended for 1

minute. The chicken meat batter was thinned to 2 mm and dipped in a 125 mL 7% (w/v) calcium chloride ($CaCl_2$) solution for 1 minute. Then the batter was molded into round shapes (each with an internal diameter of 10 cm), sealed into plastic bags, and cooked in a 90˚C water bath for 5 minutes (until the contents of the bags reached an internal temperature of 72˚C).

2.3. Color measurement

A Chroma Meter (Konika Minolta CR-5, Konika Minolta, INC., Japan) was used to measure the surface color of chicken rolls based on the Hunter Lab system. Hunter Lab values were expressed as L* (lightness), a* (redness), and b* (yellowness). Color measurements were taken from both sides of each chicken roll and the mean color values of three replicates were calculated.

2.4. Cooking loss determination

The chicken rolls were individually weighed before and after cooking. The cooking loss was calculated using the following formula, and expressed as a percentage.

$$\text{Cooking Loss } (\%) = [(Raw\ weight - Cooked\ weight)/Raw\ weight] \times 100$$

2.5. Texture Profile Analysis (TPA)

The TPA of the chicken rolls was performed using TA.XT Plus Texture Analyzer (Stable Micro Systems, UK). Four uniform test samples, each with a 25 mm diameter, were cut from each chicken roll for the TPA, and the test samples were compressed twice, to 50% of their original height, using a 36 mm cylindrical probe (P/36R) at a test speed of 10.02 cm/min. Force-time deformation curves were obtained, using a 50 kg load cell. The curves were calculated using Texture Exponent 2.0.6.0 software (Stable Micro Systems). Texture profile parameters were defined as below:
Hardness (N) = maximum peak force for the first compression cycle.
Adhesiveness (N.min) = negative force area for the first bite to pull away the compressing probe.
Springiness (ratio) = ability of the sample to recover its original shape between the end of the first bite and the start of the second bite. Cohesiveness (ratio) = ratio of the positive areas during the second compression area to the first compression area.
Gumminess (N) = hardness × cohesiveness.
Chewiness (N) = springiness × gumminess.
Resilience (ratio) = ratio of the area during the withdrawal of the first compression to the area of the first compression.

2.6. Statistical analysis

For optimization, the Design-Expert version 7.0.0 (State-Ease Inc., Minneapolis, MN, USA) was used to evaluate the experimental design, statistical analysis, and regression models. Linear, Quadratic, Cubic or 2FI models were obtained according to experimental data. Constant terms A, B, and C (linear coefficients for wheat flour, distilled water, and minced chicken, respectively), AB, AC and BC (interactive term coefficients), A^2, B^2, and C^2 (quadratic term coefficients) were the coefficients of the model. To evaluate the fitness of the model, correlation coefficient (R^2), adjusted determination coefficient (Adj-R^2) and adequate precisions were used. The model was determined to fit when its P value < 0.05,

lack of fit P value > 0.05, and Adeq. Precision > 4. For statistical significance, when a level was set at P < 0.05, an analysis of variance (ANOVA) was used to examine differences between means.

3. RESULTS AND DISCUSSION

3.1. Color measurement

L*, a*, and b* values of samples are shown in Table 2. L* values were between 68.140 and 76.540. The coefficients of the variables in the regression models and their significance are shown in Table 3.

Table 2. Design and results of Box-Behnken experiments (color values and cooking loss).

Runs	A	B	C	L*	a*	b*	Cooking loss (%)
1	0	0	0	72.839	-0.113	11.793	-0.50
2	0	1	-1	71.186	-0.824	9.724	-0.98
3	-1	1	0	75.268	-1.059	10.623	0.68
4	0	0	0	73.402	-0.334	11.738	-0.34
5	0	1	1	75.321	-0.018	11.871	2.12
6	1	0	1	70.067	0.474	12.479	2.27
7	0	0	0	73.433	0.013	12.385	3.76
8	1	-1	0	68.140	1.648	14.702	1.88
9	-1	0	1	76.540	0.373	11.067	8.82
10	0	-1	1	74.013	0.587	11.819	1.83
11	1	1	0	72.434	0.216	12.236	-1.41
12	0	-1	-1	69.260	0.593	13.353	2.63
13	-1	-1	0	75.288	-0.120	11.450	5.26
14	1	0	-1	68.195	0.558	13.003	2.50
15	-1	0	-1	72.358	-0.988	10.490	5.11
16	0	0	0	73.357	-0.194	12.120	2.05
17	0	0	0	72.897	-0.249	11.282	0.69

A: Wheat flour; B: Distilled water; C: Minced chicken.

Effects on the L* value of the chicken rolls that were statistically significant for the model ($p< 0.05$) included: the linear effects of wheat flour (A), distilled water (B), and minced chicken (C); the interaction effects of wheat flour and distilled water (AB), wheat flour and minced chicken (AC); and the quadratic effect of minced chicken (C^2) (Table 3). The L* value decreased when the amount of distilled water and wheat flour increased. However, it increased when the amount of minced chicken increased. Previous studies reported a high correlation between moisture and L* value for meat products (COSTA-CORREDOR *et al.*, 2009; GARCÍA-ESTEBAN *et al.*, 2003; SANABRIA *et al.*, 2004). DEVATKAL *et al.* (2011) determined that the addition of 10% sorghum flour into the chicken nugget formulation tended to lower the L* value of the products. Because meat muscles contain a high amount of water, increasing the meat content in the product increases the L* value (DEVATKAL *et al.*, 2011). In our study, increasing minced chicken content yielded greater lightness of the chicken rolls, but increasing the water content decreased the L* value of the samples. It is suggested that the cooking loss of the products increased in accordance with increased levels of water content. Thus, an increasing concentration of pigments (e.g., myoglobin) during the cooking process produces a darker coloration in the chicken rolls.

As we observed in our results, GARCÍA-ESTEBAN *et al.* (2003) also found that ham *Semimembranosus* (SM) muscle has a lower L* value, depending on dehydration (GARCÍA-ESTEBAN *et al.*, 2003).
The quadratic regression model for L* value is as follows:

$$L^* = +73.19 - 2.58A + 0.94B + 1.87C + 1.08AB - 0.58AC - 0.87C^2$$

a* values of the chicken rolls were between-1.059 and 1.648. The linear effects of wheat flour (A), distilled water (B), minced chicken (C), and the interaction effect of wheat flour - minced chicken (AC) were significant ($p < 0.05$). The 2Fl regression model for a* value results is shown below:

$$a^* = +0.033 + 0.59A - 0.55B + 0.26C - 0.36AC$$

a* values of the chicken rolls increased as distilled water content decreased, according to the regression model. As increased water content has a diluting effect on the myoglobin responsible for the color pigment of the meat, increasing the water content in the chicken roll resulted in a lower a* value. KHARE *et al.* (2015) also reported that decreasing the meat level in chicken noodles reduced the redness caused by the concentration of pigments (KHARE *et al.*, 2015).
The b* values of samples ranged from 9.724 to 14.702. Wheat flour (A) and distilled water (B) had a significant linear effect on b* value ($p < 0.05$). Thus, the decreased quantity of wheat flour yielded low b* values. The interaction effect of distilled water--minced chicken (BC) was statistically significant for the model ($p < 0.05$). It has been reported that the addition of 7.5% and 10.0% bean flour to beef sausages increases the yellowness of samples, depending on the dilution of the myoglobin in the meat and, to some extent, the color of the flour additives (DZUDIE *et al.*, 2002). The 2Fl regression model for b* value is as follows:

$$b^* = +11.89 + 1.10A - 0.86B + 0.92BC$$

Although the results of L* value are similar to some studies previously conducted on different poultry meat products, b* values were relatively higher than the values reported by previous studies (HEATON *et al.*, 2000; TANG and CRONIN, 2007; TANG *et al.*, 2005). Also, a* values were relatively lower than the values reported by TANG and CRONIN (2007) and TANG *et al.* (2005). Differences for a* and b* values could be related to the amounts of wheat flour and rice starch used in this study or to the different types of poultry meat used in previous studies.
Analysis of variance (ANOVA) of the regression models for all responses is shown in Table 4.

Table 3. The coefficients of the variables in the regression models and their significance.

	Constant	A	B	C	AB	AC	BC	A^2	B^2	C^2	Model
Hardness	89.05	17.02*	-16.33*	1.49	_	_	_	_	_	_	Linear
Adhesiveness	-9.97E-003	-2.39E-003*	-3.65E-003*	+1.35E-003	-1.65E-004	+3.42E-003*	-2.52E-003	_	_	_	2FI
Gumminess	69.68	12.40*	-13.65*	-0.21	_	_	_	_	_	_	Linear
Chewiness	59.79	9.57*	-13.04*	0.39	_	_	_	_	_	_	Linear
Resilience	0.38	-6.24E-003	-0.019*	-0.015*	_	_	_	_	_	_	Linear
L*	73.19	-2.58*	0.94*	1.87*	1.08*	-0.58*	-0.15	-0.53	0.13	-0.87*	Quadratic
a*	0.033	0.59*	-0.55*	0.26*	-0.12	-0.36*	0.20	_	_	_	2FI
b*	11.89	1.10*	-0.86*	0.083	-0.41	-0.27	0.92*	_	_	_	2FI
Cooking Loss	1.13	-1.83*	-1.40*	0.72	0.32	-0.98	0.97	1.87*	-1.40	1.67	Quadratic

*Significant at 5% level, A: Wheat flour; B: Distilled water; C: Minced chicken.

Table 4. ANOVA for examination of every regression model Adequac.

	Model					Lack of fit	
Responses	**F Value**	**P Value**	**R^2**	**Adj-R^2**	**Adeq. Precision**	**SS**	**P Value**
Hardness	23.51	<0.0001*	0.8443	0.8084	17.274	753.45	0.0735**
Adhesiveness	6.89	0.0041*	0.8051	0.6882	9.504	3.409E-005	0.5450**
Springiness	2.87	0.0771**	0.3985	0.2596	5.511	4.281E-003	0.9260**
Cohesiveness	3.36	0.0520**	0.4367	0.3067	5.834	4.169E-003	0.1790**
Gumminess	24.04	<0.0001*	0.8473	0.8120	17.486	432.80	0.1292**
Chewiness	17.34	<0.0001*	0.8001	0.7539	14.692	471.02	0.0980**
Resilience	4.92	0.0169*	0.5316	0.4235	7.626	2.698E-003	0.7154**
L*	50.07	<0.0001*	0.9847	0.9650	24.761	1.19	0.0854**
a*	15.77	0.0001*	0.9044	0.8471	14.717	0.61	0.0558**
b*	20.65	<0.0001*	0.9253	0.8805	16.417	0.91	0.5800**
Cooking Loss	4.00	0.0405*	0.8374	0.6282	8.227	4.46	0.7217**

Adj-R^2.adjusted determination coefficient; SS. Sum of square; *significant; ** not significant.

High F values (50.07, 15.77 and 20.65, for L*, a*, b*, respectively) and low P values (<0.0001, 0.0001, and <0.0001, for L*, a*, b*, respectively), indicate that the models were significant for L*, a* and b* values. The R^2 of predicted models for L*, a*, and b* values were 0.9847, 0.9044, and 0.9253, respectively. The Adj.R^2 value indicated a degree of linear fit between the predicted and experimental values, which were 0.9650 for L* value, 0.8471 for a* value, and 0.8805 for b* value. The P value of the lack-of-fit test was 0.0854 for L* value, 0.0558 for a* value and 0.5800 for b* value; these values indicate that the model fit the experimental data. Based on these results, the model of color parameters was adequate for predicting within the range of the variables employed.

3.2. Cooking loss determination

The cooking loss values of samples ranged from-1.41% to 8.82%, as shown in Table 2. The linear effects of wheat flour (A) and distilled water (B) and the quadratic effect of wheat flour (A^2) on the cooking loss of the chicken rolls were statistically significant for the model ($p< 0.05$) (Table 3). The negativity in cooking loss value can be explained by the interaction between the water in the water bath and in the chicken roll recipe during the cooking process.

Guar gum is widely used in the food industry for its thickening properties, which operate by its interaction with water and stabilizing effect on food matrices, and by its ability to favorably interact with gluten proteins and increase dough stability (LINLAUD *et al.*, 2009; SANDHU *et al.*, 2015). In our study, the thickening effect of guar gum on higher levels of water resulted in lower amounts of cooking loss. Therefore, increased quantities of distilled water produced low cooking loss values. WANG and CHEN (1987) also found that the cooking yields of poultry meats were higher when more water was added (WANG and CHEN, 1987).

The quadratic regression model for cooking loss results is shown below:

$$\text{Cooking loss} = +1.13 - 1.83A - 1.40B + 1.87A^2$$

The model was significant for cooking loss, with regard to F value (4.00) and P value (0.0405). The R^2 of the predicted model for cooking loss was 0.8374, indicating that more than 83% of the variability in cooking loss could be explained by the model. The regression model shows that cooking loss could be predicted within the range of the variables employed.

3.3. Texture Profile Analysis (TPA)

The results of the TPA are shown in Table 5. The hardness values of the chicken rolls were between 57.410 N and 123.069 N. Wheat flour (A) and distilled water (B) had a significant effect on the hardness of the samples ($p< 0.05$) (Table 3). The hardness of the chicken rolls rose when large amounts of flour were added to the formulation, but it fell when large amounts of water were added. This can be explained by the high water-and fat-absorption properties of flour and the softening effects of water. As we observed in our results, DEVATKAL *et al.* (2011) reported that the addition of 10% sorghum flour into chicken nuggets' formulation significantly increased the nuggets' hardness ($p<0.05$). By contrast, DZUDIE *et al.* (2002) found that the shear force and hardness of beef sausages decreased with the addition of common bean flour.

Table 5. Design and results of Box-Behnken experiments (TPA).

Runs	A	B	C	Hardness (N)	Adhesiveness (N.min)	Springiness	Cohesiveness	Gumminess (N)	Chewiness (N)	Resilience
1	0	0	0	82.077	-0.007	0.874	0.776	63.918	56.298	0.362
2	0	1	-1	72.848	-0.015	0.803	0.767	55.889	45.065	0.351
3	-1	1	0	67.193	-0.009	0.883	0.774	51.914	46.218	0.370
4	0	0	0	85.947	-0.013	0.889	0.808	69.472	58.818	0.372
5	0	1	1	78.032	-0.018	0.845	0.739	57.384	49.187	0.328
6	1	0	1	110.629	-0.007	0.859	0.762	84.605	73.447	0.361
7	0	0	0	81.591	-0.008	0.796	0.786	63.968	51.408	0.383
8	1	-1	0	123.069	-0.010	0.840	0.753	92.152	77.474	0.368
9	-1	0	1	78.479	-0.009	0.878	0.773	60.679	53.317	0.374
10	0	-1	1	103.109	-0.003	0.900	0.786	80.905	72.685	0.392
11	1	1	0	83.436	-0.015	0.814	0.768	63.492	51.823	0.375
12	0	-1	-1	119.455	-0.010	0.877	0.786	94.217	83.165	0.408
13	-1	-1	0	86.515	-0.005	0.895	0.818	70.623	63.327	0.410
14	1	0	-1	108.615	-0.018	0.860	0.818	88.664	76.981	0.409
15	-1	0	-1	57.410	-0.006	0.848	0.812	46.509	40.315	0.409
16	0	0	0	91.872	-0.008	0.832	0.798	72.836	61.076	0.394
17	0	0	0	83.586	-0.008	0.821	0.805	67.320	55.864	0.417

A: Wheat flour; B: Distilled water; C: Minced chicken.

This can be explained by the fact that the beef sausage formulation contained meat in large quantities and lacked any hydrocolloid that would have increased the hardness (DEVATKAL *et al.*, 2011).
The linear regression model for hardness is as follows:

$$\text{Hardness} = + 89.05 + 17.02A - 16.33B$$

Adhesiveness results of the samples ranged from-0.018 N.min to-0.003 N.min. The linear effects of wheat flour (A) and distilled water (B) and the interaction effect of of flour--minced chicken (AC) were statistically significant ($p < 0.05$) for the adhesiveness of chicken rolls (Table 3). VERMA *et al.* (2015) also reported that incorporating 8% pea hull flour into low-fat low-salt chicken nuggets resulted in a decrease in the adhesiveness value of the samples (VERMA *et al.*, 2015). The 2Fl regression model for adhesiveness is shown below:

$$\text{Adhesiveness} = -9.967E\text{-}003 - 2.393E\text{-}003A - 3.654E\text{-}003B + 3.420E\text{-}003AC$$

Springiness values of the chicken rolls were between 0.796 and 0.900. Cohesiveness results of the samples ranged from 0.739 to 0.818. The high P values for springiness and cohesiveness indicated that none of the regression models were a fit for these textural parameters.
Gumminess values of the chicken rolls ranged from 46.509 N to 94.217 N. The linear effects of wheat flour (A) and distilled water (B) were significant for the gumminess of the chicken rolls. These effects showed that decreasing the quantity of wheat flour or increasing the quantity of distilled water yielded less gummy chicken rolls. DEVATKAL *et al.*, (2011) found similar results with an addition of 10% sorghum flour, which enhanced the gumminess of the chicken nuggets (DEVATKAL *et al.*, 2011). Furthermore, it was reported that 10% sorghum flour or 10% finger millet flour significantly increased the gumminess of chicken patties ($p<0.05$)(DAS *et al.*, 2015). The linear regression model for gumminess is as follows:

$$\text{Gumminess} = +69.68 + 12.40A - 13.685B$$

Chewiness values of the samples ranged from 40.315 N to 83.165 N. As they were for gumminess, the linear effects of wheat flour (A) and distilled water (B) were statistically significant for the chewiness of chicken rolls. The chewiness value decreased as the quantity of distilled water increased, while chewiness increased as the quantity of wheat flour increased. It has been determined that incorporating 8% pea hull flour into low-fat low-salt chicken nuggets increases the chewiness of the samples. Adding 10% sorghum flour to the recipes for chicken nuggets and for chicken patties recipes resulted in significantly higher chewiness values ($p<0.05$)(DAS *et al.*, 2015; DEVATKAL *et al.*, 2011; VERMA *et al.*, 2015). The linear regression model for chewiness is shown below:

$$\text{Chewiness} = +59.79 + 9.57A - 13.04B$$

Resilience values of the chicken rolls were between 0.328 and 0.417. The distilled water (B) and the minced chicken (C) each had an individual linear effect that was statistically significant for the model of resilience. Thus, the resilience value of the chicken rolls increased when the amount of either the distilled water or the minced chicken was decreased. The linear regression model for resilience is as follows:

$$\text{Resilience} = +0.38 - 0.019B - 0.015C$$

While the values of hardness are similar to those of the turkey rolls reported by Tang *et al.*, 2005, the values of cohesiveness and gumminess are relatively higher than those of the same turkey rolls (TANG *et al.*, 2005). These differences with regard to cohesiveness and gumminess (between our chicken rolls and the previously studied turkey rolls) could be related to the cooking methods and food additives used in this study.

The P value, F value, R^2, Adj-R^2 and Adeq. Precision estimates of the adequacy of each model are shown in Table 4. The models are significant for hardness, adhesiveness, gumminess, chewiness, and resilience; this is reflected by high F values (23.51, 6.89, 24.04, 17.34 and, 4.92, respectively) and low P values (<0.0001, 0.0041, <0.0001, <0.0001, and 0.0169, respectively). As springiness and cohesiveness had low F values (2.87 and 3.36, respectively) and high P values (0.0771 and 0.0520, respectively), the models are not significant for springiness and cohesiveness. The R^2 of the predicted models are 0.8443 for hardness, 0.8051 for adhesiveness, 0.8473 for gumminess, 0.8001 for chewiness, and 0.5316 for resilience; however the R^2 for springiness and cohesiveness are relatively low (0.3985 and 0.4367, respectively). The Adj.R^2 values are 0.8084 for hardness, 0.6882 for adhesiveness, 0.8120 for gumminess, 0.7539 for chewiness and 0.4235 for resilience. The low Adj.R^2 values for springiness and cohesiveness (0.2596 and 0.3067, respectively) indicate quite a low degree of linear fit between the predicted and experimental values for these models. Based on these results, the models for hardness, adhesiveness, gumminess, chewiness, and resilience are adequate for predicting within the range of the variables employed, but the models for springiness and cohesiveness are not adequate for this purpose.

3.4. Optimization of quantities of ingredients and the validation of the model

To optimize textural properties, color, and cooking loss values, numerical optimizations were applied. The aim of this study was to provide the formulation of the chicken roll resulting in the highest values of L*, a*, springiness, resilience and the lowest values of b*, cooking loss, hardness, adhesiveness, gumminess, and chewiness. As optimization criterion, all relevant product characteristics of chicken rolls were selected on the basis of maximum yield and quality variables, as determined by previous studies (ANDRÈS *et al.*, 2006; ANDRÉS *et al.*, 2006; DU *et al.*, 2003; SOMBOONPANYAKUL *et al.*, 2007).The effects of wheat flour, distilled water, and minced chicken contents on L* value (Figs. 1a-c) and cooking loss (Figs. 1d-f) are shown in 3-D surface plots. The wheat flour of 8.66 g, the distilled water of 75.00 g, and the minced chicken of 53.83 g are identified as the predicted optimum quantities of ingredients for the chicken roll. Based on these optimized quantities, the predicted values are 76.153 for L*, -0.3576 for a*, 11.3484 for b*, 3.02492 for cooking loss, 60.8873 for hardness, -0.01428 for adhesiveness, 46.508 for gumminess, 39.7841 for chewiness and 0.3564 for resilience. The desirability value of these predicted values was 0.703.

To verify the model's adequacy, all experiments were performed with optimum formulations. The experimental values of each response were compared with the predicted values, and the calculated error rates are shown in Table 6. The error rates of all responses are lower than 10%. Thus, a high level of agreement was observed between the experimental values and the predicted values.

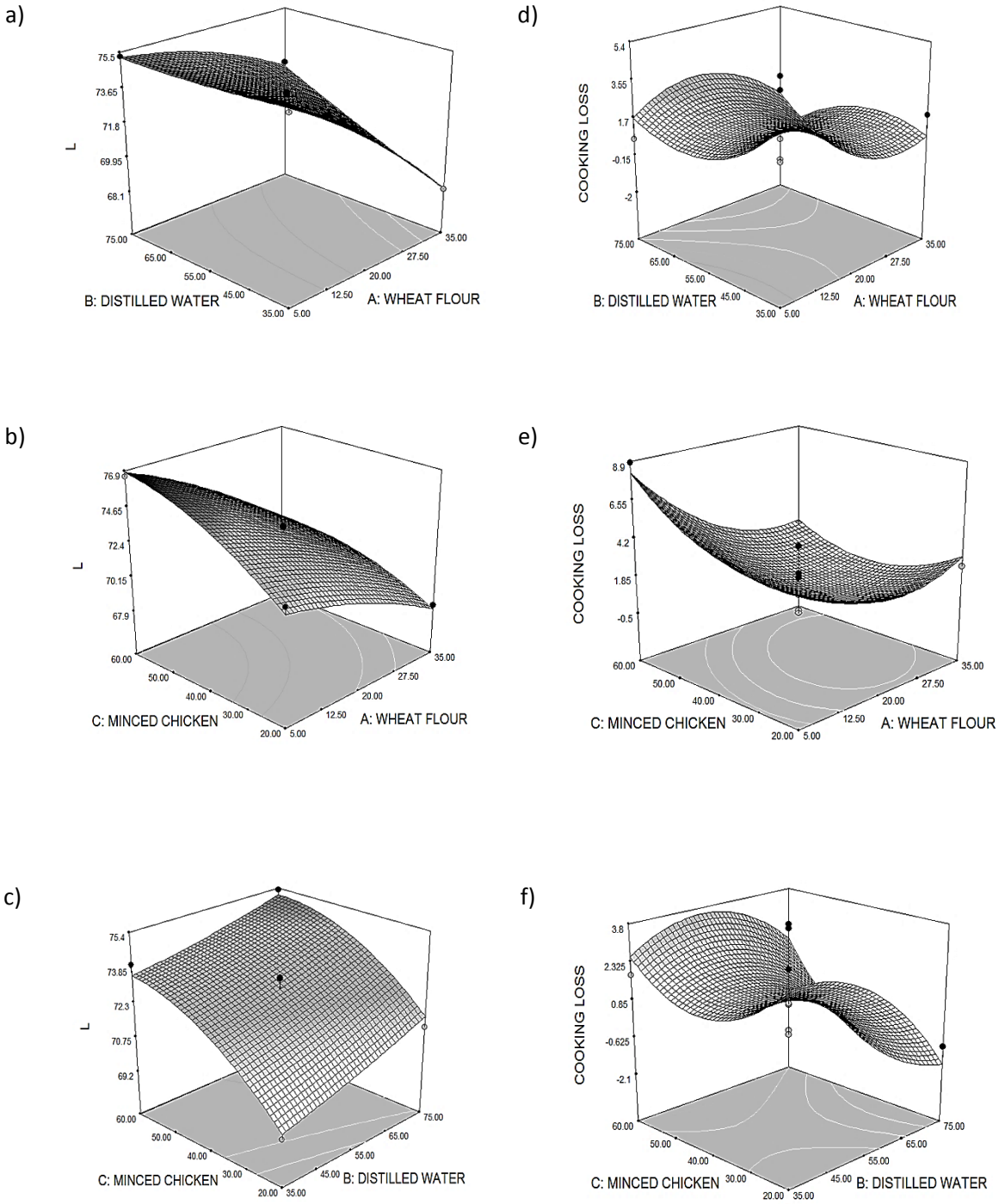


Figure 1. Response Surface Plots for L* Value (a,b,c) and Cooking Loss (d,e,f).

4. CONCLUSIONS

The interest in poultry and poultry products has grown over the last decade. To meet this increasing demand in today's living conditions, different fast food products need to be developed. A limited number of studies have been conducted on the optimization of new product development. In this study, the most suitable recipe for the chicken roll, a fast and healthy product with desirable textural and physical properties, has been determined. According to the results of this study, adding wheat flour to the chicken roll recipe increases the hardness and gumminess of the product. However, increasing the water content in the formulation decreases the hardness, gumminess and chewiness of the product. It was observed that the L* values of the chicken rolls made with a low wheat flour content and a high minced chicken content were relatively higher than those in the other experiments. As a result of this study, an alternative poultry product that is ready-to-eat and rich in desired textural and quality characteristics has been developed; future studies should aim to determine the product's sensorial and microbiological properties.

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PAPER

EFFECTS OF DIFFERENT PREBIOTICS ON VIABILITY UNDER *IN VITRO* GASTROINTESTINAL CONDITIONS AND SENSORY PROPERTIES OF FERMENTED MILK

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ABSTRACT

The effect of inulin, polydextrose and Hi-maize resistant starch on probiotic viability under simulated gastrointestinal conditions and sensory characteristics of ABT (*Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12, and *Streptococcus thermophilus*) fermented milk was investigated during 28 days. *B. animalis* presented higher survival rates under gastrointestinal stress than *L. acidophilus*. Although inulin addition enhanced viable counts of *L. acidophilus* more than those of other prebiotics during the gastric and enteric-1 phases, all samples showed similar *L. acidophilus* survival after the enteric-2 phase. The supplementation with Hi-maize indicated a protective effect on *B. animalis* tolerance to simulated gastrointestinal conditions on the 14th and 28th days. Inulin or Hi-maize did not affect the sensory properties of fermented milk whereas the product supplemented with polydextrose had the lowest scores specifically at the end of the storage period.

Keywords: *in vitro* gastrointestinal survival, fermented milk, prebiotic, probiotic

1. INTRODUCTION

Probiotics are live microorganisms, which when administered in adequate amount, confer a health effect on the host (FAO/WHO, 2002). To exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. Probiotic viability should be at a minimum level during the shelf life, which can range from 10^6 to 10^8 cfu/mL, and must survive through the gastrointestinal (GI) tract by tolerating acid, bile, and GI tract enzymes (pepsin, lipase, pancreatin) and then adhere and colonize the intestinal epithelium (CASAROTTI *et al.*, 2015).

Most studies on probiotics have focused on lactic acid bacteria, especially the genus *Lactobacillus* and *Bifidobacterium*. *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* are the frequently used probiotics in the production of fermented milks. Using different probiotic combinations (RANADHEERA *et al.*, 2014), microencapsulation (DE ARAUJO ETCHEPARE *et al.*, 2016), supplementation with prebiotics (OLIVEIRA *et al.*, 2009; NOBAKHTI *et al.*, 2009; CASAROTTI and PENNA, 2015), and the use of different matrices (CASAROTTI *et al.*, 2015) have been proposed to increase probiotic survival in the GI tract and in the product until the time of consumption. Among these options, the addition of prebiotics has been preferred in many studies to increase probiotic viability and their resistance to GI conditions.

Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (GIBSON *et al.*, 2004). Different prebiotics (e.g., inulin, Hi-maize resistant starch, lactulose, polydextrose, β-glucan, lactilol, and maltodextrin) have been used as supplements in the manufacture of fermented dairy products to improve the growth and activities of selected *Lactobacillus* and *Bifidobacterium* strains (OLIVEIRA *et al.*, 2009; NOBAKHTI *et al.*, 2009; HEYDARI *et al.*, 2011) in related studies.

Inulin, a compound extracted from the chicory root, is a fructan and cannot be digested by α-amylase or other hydrolases in the upper section of the intestinal tract (OLIVEIRA *et al.*, 2009; GONZÁLEZ-HERRERA *et al.*, 2015). Aside from its prebiotic property, inulin also presents some technical characteristics, such as being a fat replacer, sugar replacer, and emulsion and foam stabilizer (GONZÁLEZ-HERRERA *et al.*, 2015). Polydextrose is a low molecular weight randomly bonded polysaccharide of glucose with an energy contribution of 1 kcal/g (DO CARMO *et al.*, 2016). This low-calorie content of polydextrose is a result of its poor digestibility in the small intestine and incomplete fermentation in the large intestine (OLIVEIRA *et al.*, 2009). However, resistant starch is a small starch fraction that has the ability to resist digestion and can be fermented by the beneficial microbiota in the colon (ZAMAN and SARBINI, 2016). All inulin, polydextrose and Hi-maize resistant starch have been already reported (GONZÁLEZ-HERRERA *et al.*, 2015; ZAMAN and SARBINI, 2016; DE ARAUJO ETCHEPARE *et al.*, 2016) as prebiotics and have been showed to enhance the viability of *L. acidophilus* and *B. animalis* in fermented dairy products (OLIVEIRA *et al.*, 2009; NOBAKHTI *et al.*, 2009; BEDANI *et al.*, 2013; PADILHA *et al.*, 2016). However, there is no knowledge about the effect of these prebiotics on probiotic *in vitro* gastrointestinal tolerance in ABT-cultured (*Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium animalis*) fermented milk.

The aim of this study was to investigate the influence of the addition of the inulin, polydextrose and Hi-maize resistant starch on the viability of starter culture bacteria, probiotic survival under *in vitro* simulated gastrointestinal conditions, and sensory characteristics in ABT-fermented milk throughout 28 days of storage at 4°C.

2. MATERIALS AND METHODS

2.1. Cultures and ingredients

The ABT-10 culture (Chr. Hansen A/S, Hørsholm, Denmark), composed of *Streptococcus thermophilus*, *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12, skim milk powder (SMP) (Pinar Dairy Products, Izmir, Turkey), inulin (Fibruline® Instant, Cosucra, Warcoing, Belgium), polydextrose (Litesse® IP Powder, Danisco, USA), and resistant starch (Hi-maize, Ingredion, Hamburg, Germany), were used in this study.
The ABT-10 culture (pack size of 200U) was poured into 1 L sterilized reconstituted milk at 40°C and mixed thoroughly, and then 1 L of each milk base was inoculated with 1 mL of the culture. The reconstituted milk was prepared from skim milk powder and has 130g/L of total solids. This procedure gave initial counts after milk inoculation of approximately 7 log cfu g^{-1} for *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* Bb-12 and 8 log cfu g^{-1} for *S. thermophilus*.

2.2. Production of fermented milk

In the production of fermented milk, cow's milk containing 31 g/L fat and 29.2 g/L protein was supplied from Ege University, Agricultural Faculty (Izmir, Turkey). After standardizing it with skim milk powder to obtain 110 g/L of nonfat milk solids, the milk was divided into four lots. The control milk was not supplemented with prebiotics, whereas the other three groups were supplemented with 20 g/L inulin, polydextrose and resistant starch. After they were mixed properly, each milk base was heated to 90°C for 10 min by circulating it in a hot water bath and cooled to 42-43°C in an ice bath. At this point, they were inoculated with the ABT-10 culture. The mixtures were put into 100-mL plastic containers and incubated at 40°C until a pH of 4.75 was reached. After fermentation, the fermented milk samples were cooled and transferred to a refrigerator at 4°C, then stored at this temperature for 28 days during the analyses.

2.3. Determination of pH and microbiological analyses

The pH of the fermented milk was determined using a pH meter (model pH 211; Hanna Instruments, Woonsocket, RI).
The viability of bacteria in the ABT-10 culture was determined according to AKALIN and ÜNAL (2010). The counts of *S. thermophilus* were enumerated on M-17 agar (Merck, Darmstadt, Germany) after incubating the plates aerobically at 37°C for 48 h. *B. animalis* subsp. *lactis* Bb-12 was enumerated using MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paramomycin sulfate) agar. The inoculated plates were incubated anaerobically at 37°C for 72 h using an oxygen-free milieu and a CO_2 atmosphere in anaerobic jars (Merck, Darmstadt, Germany). The counts of *L. acidophilus* La-5 were enumerated on MRS-Sorbitol (Merck, Darmstadt, Germany) agar after incubating the plates anaerobically at 37°C for 48 h in anaerobic jars.

2.4. Survival of *L. acidophilus* and *B. animalis* subsp. *lactis* under simulated gastrointestinal conditions

The probiotic survival in the fermented milk samples subjected to gastric and enteric simulated conditions was evaluated after 1, 14 and 28 days of refrigerated storage according to the methods described by BEDANI *et al.* (2014) and CASAROTTI and PENNA (2015), but with some modifications. Each fermented milk sample was placed into

3 sterile flasks in order to perform the phases of simulated gastrointestinal conditions. 10 mL of sample, which was diluted in 0.5% NaCl, was used for the method. Prior to the gastric stage the sample is brought to pH 2.2-2.6 with 0.5 M HCl. Pepsin (from porcine gastric mucosa, Sigma-Aldrich) and lipase (Amano lipase G from *Penicillium camemberti*, Aldrich Chemical Company, St. Louis, MO, USA) solutions were added to the sample to reach a concentration of 3 g/L and 0.9 mg/L, respectively. The sample is placed for 2 h at 37°C in a shaking (150 rpm) waterbath (Mikrotest, MCS Series, Ankara, Turkey), leading to the simulated gastric phase. After then, the pH was adjusted to 4.3-5.2 with an alkaline solution (150 mL of 1 N NaOH and 14 g of PO4H2Na.2H2O and distilled water up to 1 L), which contained 10 g/L of bile (bovine bile, Sigma-Aldrich) and 1 g/L of pancreatin (from porcine pancreas, Sigma-Aldrich) in the final mixture. The sample was incubated again at 37°C in the water bath for 2 h for enteric phase 1. For the last stage, the pH level was adjusted to 7.0-7.3 using the same alkaline solution and the concentrations of bile and pancreatin were adjusted to 10 g/L and 1 g/L, respectively in the final mixture. The sample was then incubated at 37°C for the last 2 h for enteric phase 2. The viable counts of *L. acidophilus* and *B. animalis* were determined after each phase.

2.5. Sensory evaluation

A sensory evaluation of the samples was carried out according to the method modified from TURKISH YOGURT STANDARD (1989) and MARTÍN-DIANA *et al.* (2003). The panel group consisted of 8 experienced academicians from the Department of Dairy Technology (Ege University, Izmir, Turkey) who were familiar with attributes of fermented milk samples. Sensory evaluation consisting of appearance, aroma, taste, texture, and overall acceptability were based on 5-point hedonic scales (1: dislike extremely; 5: like extremely). Each sample was scored individually, and the samples were presented to the panelists inside individual plastic containers. Fermented milks, coded with 3 digits, were randomly presented to the panel group at each session. Panelists evaluated all of the samples after storage for 1, 14, and 28 d at 4°C.

2.6. Statistical analysis

The experiments, including fermented milk making, were performed in triplicate. Six values for each sample were averaged ($n = 6$). The results were analyzed using a one-way analysis of variance (ANOVA) and the general linear model (GLM) procedure of the SPSS software (version 11.05; SPSS Inc., Chicago, IL). The treatments were compared among each other in the same storage day, and the fermented milks of the same treatment were compared throughout the time in terms of *in vitro* simulated gastrointestinal tolerance. The means were compared using the Duncan multi-comparison test at the $p < 0.05$ level.

3. RESULTS AND DISCUSSION

3.1. pH values and microbiological characteristics

The pH values of fermented milk samples during refrigerated storage are shown in Fig. 1. The values for all fermented milk types ranged from 4.73 to 4.37 during storage. Although some fluctuations are observed, all products presented significant pH reduction ($p < 0.05$) at the end of the storage term when compared to the beginning.

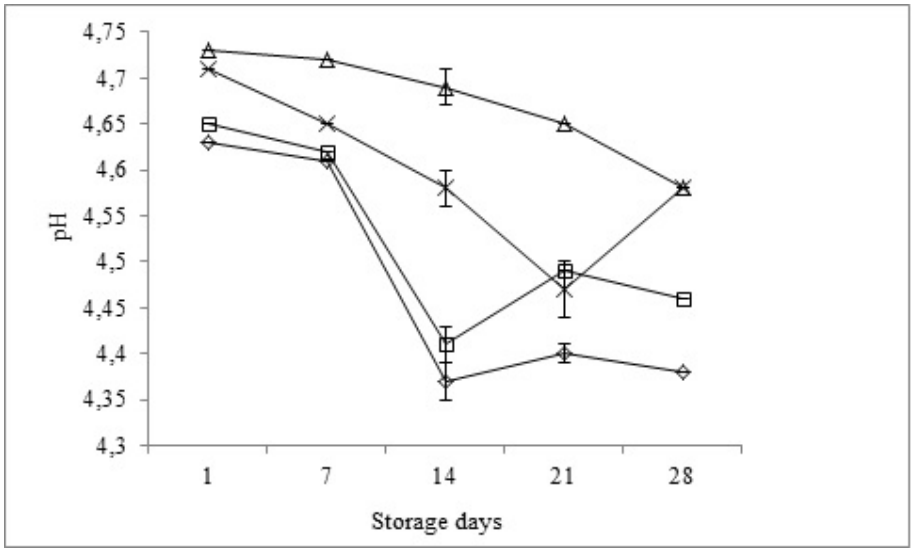


Figure 1. Changes in pH values in fermented milk control (FMC) without addition of prebiotic (◊), fermented milk with addition of 2% inulin (FMI) (□), fermented milk with addition of 2% polydextrose (FMP) (Δ), fermented milk with addition of 2% Hi-maize (FMH) (x).

The pH values of the control fermented milk were found to be lower than those of prebiotic added samples during 28 days, which can be attributed to the buffering capacity of ingredients used in the fortification of the fermented milk samples (HELLAND *et al.*, 2004). Similar results were obtained for the control product when compared to prebiotic added samples in other studies (NOBAKHTI *et al.*, 2009; BEDANI *et al.*, 2013). In contrast to our study, there were no significant differences between the pH values of the control products and prebiotic added products in some studies that could be attributed to the type of product or prebiotic used in these studies (OLIVEIRA *et al.*, 2009; HEYDARI *et al.*, 2011; SRISUVOR *et al.*, 2013).

The viability of *L. acidophilus*, *B. animalis* subsp. *lactis* and *S. thermophilus* during refrigerated storage lasting 28 days is presented in Table 1. The population of *S. thermophilus* remained above 8 log cfu/g throughout the storage period. However, the counts of probiotic bacteria (*L. acidophilus* and *B. animalis*) were maintained at the minimum effective dose for beneficial health effects, which has been suggested to be between 10^6-10^8 cfu/g, in all treatments during the storage time.

In general, the addition of inulin, polydextrose and Hi-maize provided a protective effect on the survival of probiotic bacteria by not allowing any decline in viability during the 28 days. Viable counts of *L. acidophilus* have been reported as lower in the Hi-maize added fermented milk drink than that of the control sample (NOBAKHTI *et al.*, 2009), which parallels our results. *L. acidophilus* has also been shown to not be stimulated by inulin in acidophilus-bifidus yoghurt by OZER *et al.* (2005) and in fermented soy product by BEDANI *et al.* (2013). Similar results were observed in some other studies (BURITI *et al.*, 2010; HEYDARI *et al.*, 2011). However, supplementation with polydextrose enhanced the survival rate of *L. acidophilus* more than supplementation with inulin throughout the 28 days of our study. ALLGEYER *et al.* (2010) obtained parallel results for yoghurt drinks containing both *L. acidophilus* La-5 and *B. animalis* Bb-12 during 30 days of storage.

The viable counts of *B. animalis* Bb-12 significantly decreased throughout the storage in all treatments except for the sample containing Hi-maize. The highest viability of Bb-12 was

detected in the control sample on 1st and 14th days, whereas fermented milk fortified with Hi-maize had the highest value at the end of the storage period ($p < 0.05$).

Table 1. Changes in the viable counts of *L. acidophilus*, *B. animalis*, and *S.thermophilus* during refrigerated storage of fermented milks (log cfu/mL).

	Storage days		
Products	**1**	**14**	**28**
	L. acidophilus		
FMC	7.45 ± 0.03^{Ab}	6.91 ± 0.03^{Ac}	7.51 ± 0.02^{Aa}
FMI	7.38 ± 0.02^{Ba}	6.94 ± 0.03^{Ab}	6.96 ± 0.17^{Cb}
FMP	7.42 ± 0.02^{Aa}	6.82 ± 0.08^{ABb}	7.36 ± 0.07^{Ba}
FMH	6.84 ± 0.05^{Cb}	6.77 ± 0.18^{Bb}	7.47 ± 0.07^{ABa}
	B. animalis		
FMC	7.95 ± 0.01^{Aa}	7.80 ± 0.03^{Ab}	7.63 ± 0.07^{Bc}
FMI	7.41 ± 0.03^{Db}	7.61 ± 0.05^{Ca}	7.22 ± 0.07^{Dc}
FMP	7.85 ± 0.05^{Ba}	7.70 ± 0.05^{Bb}	7.42 ± 0.01^{Cc}
FMH	7.50 ± 0.04^{Cc}	7.61 ± 0.04^{Cb}	7.74 ± 0.09^{Aa}
	S. thermophilus		
FMC	8.82 ± 0.09^{Aa}	8.31 ± 0.07^{Cb}	8.43 ± 0.14^{Ab}
FMI	8.30 ± 0.03^{Bb}	8.48 ± 0.07^{Ba}	7.99 ± 0.13^{Cc}
FMP	8.40 ± 0.09^{Bb}	8.74 ± 0.07^{Aa}	8.15 ± 0.16^{BCc}
FMH	8.10 ± 0.11^{Cb}	8.09 ± 0.09^{Db}	8.32 ± 0.11^{ABa}

Values are means of triplicates. FMC: fermented milk control without addition of prebiotic; FMI: fermented milk with addition of 2% inulin; FMP: fermented milk with addition of 2% polydextrose; FMH: fermented milk with addition of 2% Hi-maize

[a-c]Means ± standard deviations in the same row with different superscript lowercase letters are significantly different ($P < 0.05$).

[A-D]Means ± standard deviations in the same column with different superscript uppercase letters are significantly different ($P < 0.05$).

NOBAKHTI *et al.* (2009) reported that the addition of Hi-maize significantly increased the bacteria level of *B. animalis* Bb-12 in the fermented milk drink immediately after fermentation. However, there were no significant differences ($p > 0.05$) in *B. animalis* Bb-12 counts between ABY-type probiotic yoghurt samples supplemented with 1.5% inulin and 1.5% Hi-maize during 21 days of storage in another study (HEYDARI *et al.*, 2011).
Even though some fluctuations were observed in the population of *S. thermophilus*, in general, the counts significantly reduced at the end of the storage term when compared to the 1st day. Similar fluctuations in viable counts of this microorganism were also reported in other studies (AKALIN and ÜNAL, 2010; BEDANI *et al.*, 2013; CASAROTTI and PENNA, 2015). The viable counts were mostly lower in supplemented fermented milk samples compared with those of the control fermented milk during storage in our study; thus, it is obvious that the addition of the prebiotic did not improve the viability of *S. thermophilus*. In contrast, inulin addition improved the survival of *S. thermophilus* in fermented soy ABT milk during 28 days of storage. This difference can be related to the high ability of this bacterium to metabolize soy oligosaccharides (DONKOR *et al.*, 2007).

3.2. The survival of *L. acidophilus* and *B. animalis* subsp. *Lactis* under simulated gastrointestinal conditions

The survival of *L. acidophilus* and *B. animalis* exposed to *in vitro* simulated gastrointestinal conditions is shown in Figs. 2 and 3, respectively. In general, there was a significant reduction ($p < 0.05$) in the population of both La-5 and Bb-12 during the simulation of the *in vitro* GI stress, which was also observed in other studies (CASAROTTI and PENNA, 2015; CASAROTTI *et al.*, 2015). *B. animalis* Bb-12 presented higher survival rates during the *in vitro* assay than *L. acidophilus*, especially on the 1st and 14th days, this was also observed in many studies (CASAROTTI and PENNA, 2015; CASAROTTI *et al.*, 2015). The resistance of both probiotic bacteria to simulated GI conditions significantly decreased during storage time for all treatments (data not shown). This behavior can be related to the sensitivity of bacteria in that cells were more stressed and damaged by the cold storage at the end of the storage period compared to the beginning (WANG *et al.*, 2009). VINDEROLA *et al.* (2011) also reported a significant reduction in probiotic resistance to gastric stress in fermented milk throughout 20 days of refrigerated storage. It has been also reported that the bile resistance of probiotic bacteria can be poor when used in the presence of each other compared with monoculture. This might probably be related to the potential antagonism between each other in bile salt stress (RANADHEETA *et al.*, 2014). A competition between bacteria probably occurs so that each bacterium can use essential nutrients for its growth and survival (SRISUVOR *et al.*, 2013).

The counts of *L. acidophilus* decreased by 2-3 log cycles after 2 h of the gastric phase. This shows that *L. acidophilus* is highly susceptible to simulated gastric juice containing HCl and pepsin, because the highest reduction in survival was observed during the gastric phase during all storage days. It can be related to the acid tolerance of lactic acid bacteria, which varies by species and strains, as well as exogenous conditions, growth medium, and incubation parameters (MADUREIRA *et al.*, 2011). No recovery of viability of this microorganism was detected after the pH level was increased in the enteric phases of the assay.

Although there were significant differences among fermented milk samples for the gastric phase, the viability of *L. acidophilus* was generally similar after 6 h of assay on the 1st, 14th, and 28th days. On day 1, the supplementation with polydextrose and Hi-maize protected the *L. acidophilus* cells in the presence of low pH (2.2-2.6); however, fermented milk fortified with Hi-maize had the highest counts. There were no significant differences among all treatments when pH was increased to 4.3-5.2 ($p > 0.05$) at the beginning of the storage. However, fortification with inulin caused an increase in the survival of *L. acidophilus* during gastric and enteric phase 1 on the 14th and 28th days compared to the fortification with polydextrose and Hi-maize. The protective effect of inulin can be attributed to the resistance of inulin to hydrolysis by the GI tract enzymes and to its high degree of polymerization (DP) when compared to short chain fructooligosaccharides.

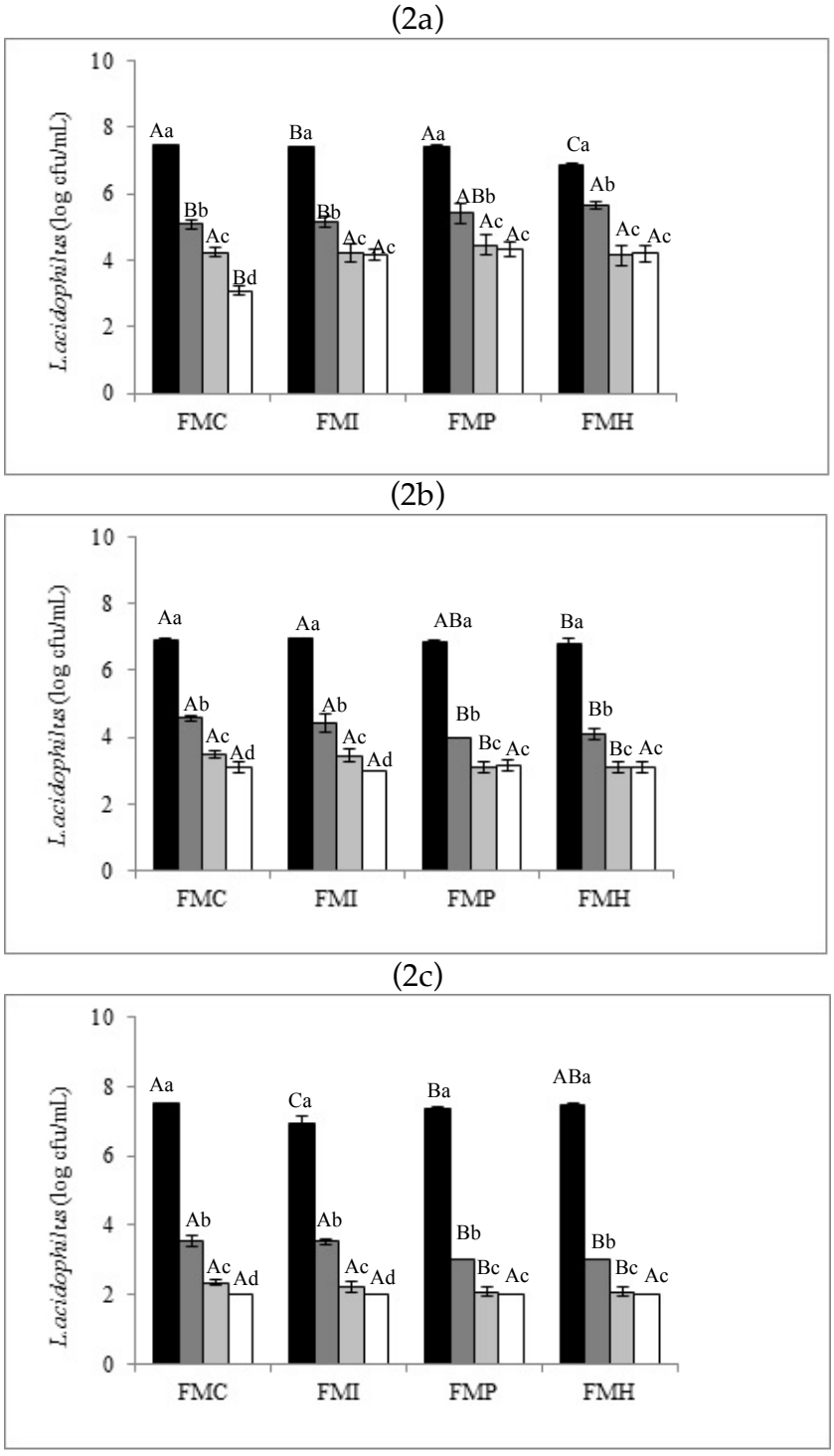


Figure 2. Survival of *L.acidophilus* La-5 (log cfu/mL) in fermented milk after 1, 14, and 28 days of storage (a, b, and c, respectively), before (black bar) and during exposure to *in vitro* simulated gastric conditions, for 2 h (dark gray bar) and enteric conditions, for 4 h (light gray bar) and 6 h (white bar). For the same storage period, $^{A-C}$Different superscript capital letters denote significant differences between formulations for the same sampling period of the *in vitro* assay ($p < 0.05$); $^{a-d}$Different superscript lowercase letters denote significant differences between different sampling periods of the *in vitro* assay for the same formulation ($p < 0.05$). FMC: fermented milk control, without addition of prebiotic; FMI: fermented milk with addition of 2% inulin; FMP: fermented milk with addition of 2% polydextrose; FMH: fermented milk with addition of 2% Hi-maize.

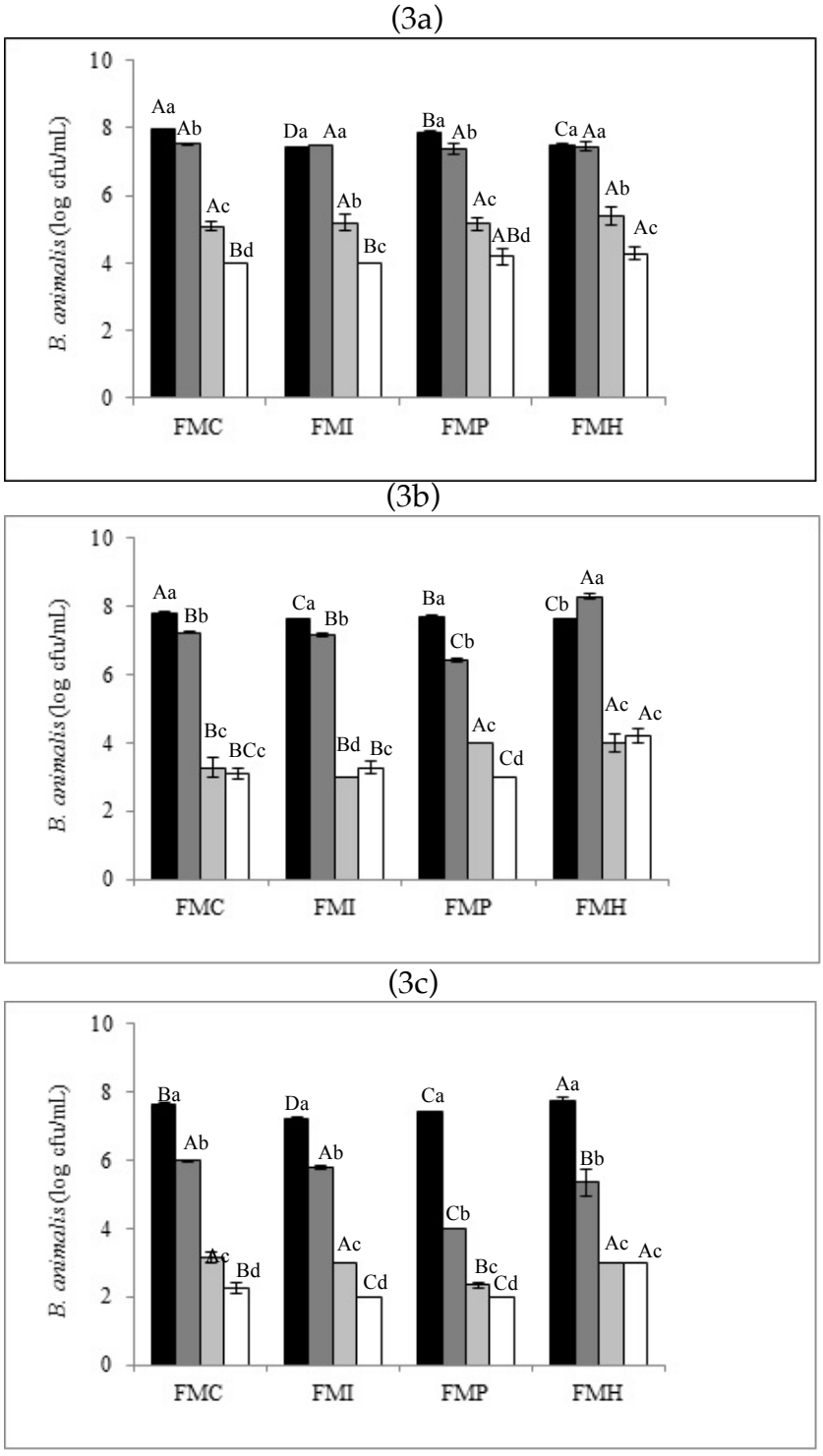


Figure 3. Survival of *B. animalis* Bb-12 (log cfu/mL) in fermented milk after 1, 14, and 28 days of storage (a, b, and c, respectively), before (black bar) and during exposure to *in vitro* simulated gastric conditions, for 2 h (dark gray bar) and enteric conditions, for 4 h (light gray bar) and 6 h (white bar). For the same storage period, $^{A-C}$Different superscript capital letters denote significant differences between formulations for the same sampling period of the *in vitro* assay ($p < 0.05$); $^{a-d}$Different superscript lowercase letters denote significant differences between different sampling periods of the *in vitro* assay for the same formulation ($p < 0.05$). FMC: fermented milk control, without addition of prebiotic; FMI: fermented milk with addition of 2% inulin; FMP: fermented milk with addition of 2% polydextrose; FMH: fermented milk with addition of 2% Hi-maize.

Inulin with high a DP has low solubility and an increased capacity to form a tridimensional network of microcrystals in the food matrix in which it is added (BURITI *et al.*, 2010). This structure containing small aggregates can act as a protective physical cover for bacterial cells against acid and bile (CASAROTTI *et al.*, 2015). On the other hand, HERNANDEZ-HERNANDEZ *et al.* (2012) reported that resistance to bile in *Lactobacillus* strains is dependent on carbon source. Hydrophobic index of bacteria, which is related to their adhesion capacity to intestinal cells, has been also reported to vary depending on the *Lactobacillus* strain by the same researchers. The addition of a mixture of inulin and fructooligosaccharide in the petit-suisse cheese containing the ABT-4 culture resulted in a protective effect for the probiotic survival during 6 h of *in vitro* simulated assay (PADILHA *et al.*, 2016). The authors emphasized that this protective effect of prebiotics might be specific for the food matrix. In this study, even though the prebiotics used had significantly different effects on the viability of *L. acidophilus* during *in vitro* simulated GI conditions among each other, they generally maintained the viable counts.

B. animalis Bb-12 was highly resistant to simulated gastric conditions in all fermented milks on the 1st and 14th days, whereas the viability decreased ($p < 0.05$) 1-2 log cycles after gastric phase at the end of the storage term. Although *B. animalis* showed higher survival rates in the presence of bile and pancreatin than *L. acidophilus*, significant reductions in the viability of *B. animalis* were observed during enteric phases of the assay.

The higher survivability of *B. animalis* Bb-12 compared to that of *L. acidophilus* during *in vitro* simulated GI conditions has also been reported by other authors (BEDANI *et al.*, 2013; 2014; CASAROTTI and PENNA, 2015). CRITTENDEN *et al.* (2001) demonstrated that *B. animalis* Bb-12 was both acid and protease tolerant among commercial strains and able to survive well in an *in vitro* model. The ability of *Bifidobacterium* strains to improve their own tolerance to gastrointestinal environment has been revealed, which is a considerable factor in the performance of strains in the GI tract. Bifidobacteria can adapt their enzymatic systems to the different barriers found along GI tract. They have the ability to increase the activity of the membrane-bound F_0F_1-ATPase enzyme, which pumps protons from cytoplasm to the extracellular environment. When the cells are previously exposed to acidic conditions, the F_0F_1-ATPase enzyme is overproduced, and better control of the intracellular pH is observed (SANCHEZ *et al.*, 2013). This can be the reason for the higher tolerance of *B. animalis* Bb-12 to simulated gastrointestinal conditions in this study. As some strains of bifidobacteria may have acid stress throughout the gastric conditions (Huang *et al.*, 2014), the intrinsic tolerance of the strains has a decisive influence. The high resistance of *B.animalis* subsp. *lactis* to both oxygen and gastrointestinal stress was also verified by other researchers (PERRIN *et al.*, 2000; ANDRIANTSOANIRINA *et al.*, 2013; AMBALAM *et al.*, 2014). Bile and bile components have been reported to affect the adherence of *Bifidobacteria* in the gastrointestinal tract (KOCIUBINSKI *et al.*, 2002). The improved tolerance of *B. animalis* Bb-12 in this study can also be attributed to having bile salt hydrolase activity, which is active during its transit through the gastrointestinal tract (PICARD *et al.*, 2005). However, BEGLEY *et al.* (2005) reported that the tolerance of Gram-positive probiotic bacteria to bile is a strain-dependent characteristic that should not be generalized in terms of species.

Supplementation with Hi-maize caused a significant increase in the survival of Bb-12 during the assay; however, the most effective improvement of Hi-maize on Bb-12 survival was observed in enteric phase 2 during all storage days. This probably can be caused by the slow degradation of resistant starch in the first part of the GI tract and so, it can reach the distal part of the colon and show a prebiotic effect (ZAMAN and SARBINI, 2016). The authors also reported that the amylose to amylopectin ratio is an important property to determine the resistance of a starch and its enzymatic digestibility. The related mechanism has been explained as the interaction of amylose molecules with amylopectin which can

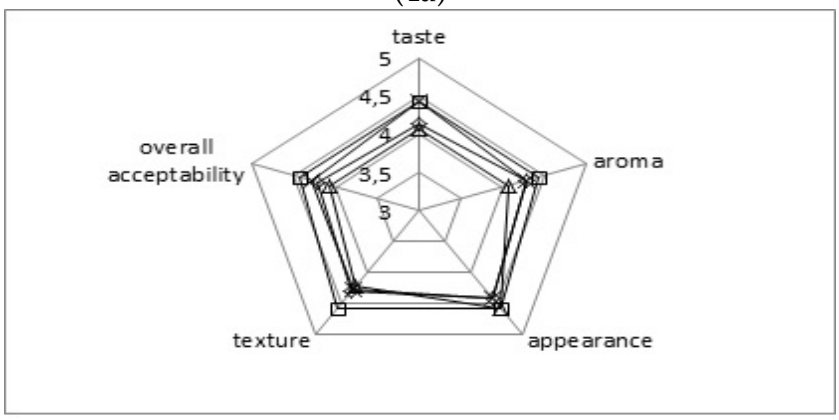
influence the accessibility of enzymes to hydrolyze starch molecules. In addition, NUGENT (2005) reported that Hi-maize, which belongs to Class 2 of resistant starches, comprises specially structured granules that prevent digestive enzymes from hydrolyzing them.

According to this study, supplementation with inulin may increase the viability of *L. acidophilus* La-5, whereas Hi-maize resistant starch can be preferred as a prebiotic ingredient to enhance the viable counts of *B. animalis* Bb-12 during the simulation of GI conditions. Therefore, choosing a suitable prebiotic for the manufacture of fermented dairy products can contribute to maintain the viability of probiotic bacteria in the gut. The *in vitro* analysis used in this study gives information about the survival rate of probiotic bacteria under gastrointestinal conditions but not about their probiotic activity. So, the probiotic activity of these bacteria should also been assessed by appropriate analyses. The species and strain specificity of probiotics to acid and bile tolerance should also not be forgotten (RANADHEERA *et al.*, 2014).

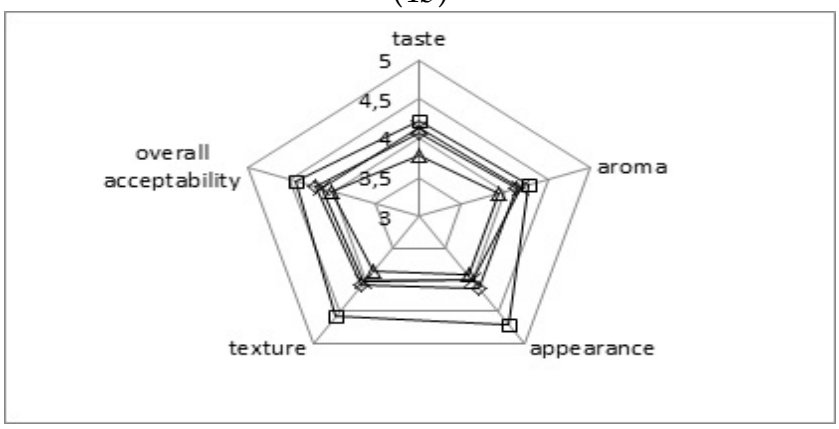
3.3. Sensory evaluation

The results of the sensory evaluation of the fermented milk samples are shown in Figs. 4a (d1), 4b (d14), and 4c (d28).

(4a)



(4b)



(4c)

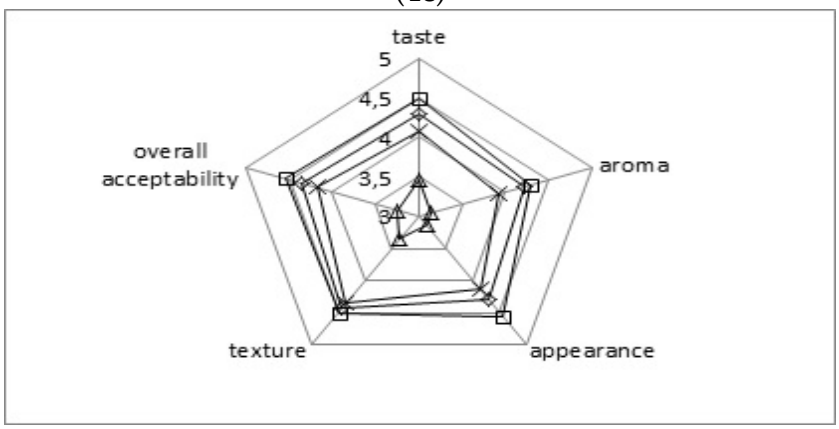


Figure 4. Sensory scores of (a) 1-d-old-fermented milks, (b) 14-d-old fermented milks, and (c) 28-d-old fermented milks; ◊ = fermented milk control without addition of prebiotic (FMC), □ = fermented milk with addition of 2% inulin (FMI), Δ = fermented milk with addition of 2% polydextrose (FMP), x = fermented milk with addition of 2% Hi-maize (FMH).

In general, the effect of storage time on the sensory characteristics of fermented milk samples was not found to be significant ($p > 0.05$). There were no significant differences ($p > 0.05$) among fermented milk samples in terms of taste, flavor, texture, and overall acceptability during the 14 days of storage. The addition of inulin or polydextrose at a ratio of 20 g/L also did not affect the flavor property of low-fat set yoghurts with probiotic-cultured banana purée (SRISUVOR *et al.*, 2013). The addition of inulin also did not negatively affect the sensory quality of sponge-cake products (ZBIKOWSKA *et al.*, 2017). Similarly, no significant inulin effect could be observed on the "firmness", measured as the force required to lift a spoonful of yoghurt in another study (GUGGISBERG *et al.*, 2009).

Fermented milk fortified with polydextrose had the lowest sensory scores among the samples at the end of the storage term ($p < 0.05$). The lowest appearance values were obtained again in the samples fortified with polydextrose throughout the storage period which can be attributed to the high solubility of polydextrose in water, thus resulting a non-viscous solution. Having a neutral taste without reflecting sweetness can also be a reason for indicating low scores (DO CARMO *et al.*, 2016). An artificially sweetened *misti dahi*, which is a popular fermented dairy product of eastern India, containing polydextrose was found to have lower flavor and lower overall acceptability scores when compared to the control sample and samples supplemented with other sweeteners (RAJU and PAL, 2011). ALLGEYER *et al.* (2010) also observed worse sensory attributes for a symbiotic yoghurt drink containing polydextrose compared with the control ($p < 0.05$).

The addition of inulin or Hi-maize did not negatively affect the sensory properties of the fermented milk samples throughout the 28 days. Similar results were also obtained in other studies (KIP *et al.*, 2006; RINALDONI *et al.*, 2012).

4. CONCLUSIONS

The importance of this study is that it provides knowledge about the efficacy of different prebiotics on the viability of probiotic bacteria in the fermented milk during *in vitro* simulated gastrointestinal conditions. According to the results obtained in the present

study, the supplementation with prebiotics showed a protective effect and did not allow a decline on the viability of *L. acidophilus* La-5 and *B. animalis* Bb-12 in ABT fermented milk, and the probiotics maintained the recommended level for the beneficial health properties, ranging from 6 to 8 log cfu/g during the 28 days of storage. *B. animalis* Bb-12 was more resistant to the simulated gastrointestinal conditions than *L. acidophilus* La-5 throughout the storage period. Although inulin added fermented milk had higher viable counts of *L. acidophilus* La-5 during the gastric and enteric-I phases, there were no significant differences among products at the end of the *in vitro* assay, except on the 1st day. However, it was obvious that the use of Hi-maize improved *B. animalis* Bb-12 resistance when subjected to simulated gastrointestinal conditions. The addition of inulin or Hi-maize to fermented milk had no influence on the sensory characteristics whereas the lowest scores were obtained for the sample supplemented with polydextrose especially on day 28. Therefore, the findings of this study suggest that an appropriate prebiotic can be suitable to ensure the viability of the probiotic strains during the manufacturing time and shelf-life of the product, and protect the probiotics during the passage through the gastrointestinal tract so that they can reach the colon. This can be a strategy for providing a broad range of health benefits of probiotic microorganisms to the host and for the development of future functional foods. In addition, further studies are necessary to follow which components of the prebiotics and/or technological processes might influence the probiotic tolerance to simulated gastrointestinal juices in fermented dairy products. The use of higher ratios of the prebiotics can be tested in the future studies and clinical trials should also be needed to support *in vitro* studies.

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PAPER

PRODUCTION OF INSTANT *NIGELLA SATIVA L.* BEVERAGE POWDER BY DRUM DRYING USING ARABIC GUM AS ADJUNCT

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ABSTRACT

This study determined the feasibility of drum drying process in producing drum dried *Nigella sativa L.* beverage powder using the response surface methodology to determine the effect and relationship between percentage of Arabic gum and drying temperature on the quality of the *Nigella sativa* powder produced. About 32.7 % of Arabic gum and 119.1°C of drying temperature were the optimum parameters for the production of *Nigella sativa* powder. Antioxidants and calcium content of *Nigella sativa* powder decreased significantly, while the iron, manganese, and sodium contents increased significantly after the drum drying process.

Keywords: Arabic gum, drum drying, *Nigella sativa* powder

1. INTRODUCTION

Nigella sativa L. (*N. sativa*), popularly known as black cumin or black seed, is a member of *Ranunculaceae* family. It is native to Southern Europe, North Africa, and Southwest Asia, but also widely cultivated in other countries such as Middle Eastern Mediterranean region, South Europe, India, Pakistan, Syria, Turkey and Saudi Arabia (KHARE, 2004). *N. sativa* seeds are extensively used for traditional medicinal purposes in treating various diseases such as skin diseases, jaundice, gastrointestinal problems, hypertension, and diabetes (FOROUZANFAR *et al*., 2014). Black seeds have also been used for thousands of years for culinary purposes as a spice or food (TIRUPPUR *et al*., 2010). Recently, *N. sativa* has gained the attention of researchers due to its health benefits, attributed to the presence of bioactive components. High phytochemical content in black seed makes it a healthy food product with nutraceutical and functional benefits for human consumption. Various studies have shown that black cumin seed provides anticancer, antidiabetic, antimicrobial, antiradical, anti-inflammatory, antihypertensive, hepatoprotective and renal protective properties (RAMADAN, 2007). Normally, *Nigella sativa* seeds are roasted to give a flavour similar to that of coffee. The roasting also contributes to a high percentage of water soluble extracts, making it a potential substitute to the coffee (KOMES *et al*., 2015). The significant health benefits of black seed and its unique flavour after roasting which is similar to coffee flavour, have increased the interest in developing it into a convenient substitute for coffee brew in the market. With the current consumer shift towards convenient healthy food products, such as easily rehydratable beverage powders, black seeds powder has the potential to open up a new possibility for the usage of black seeds as a healthy beverage in addition to the traditional medicinal and culinary uses. Drum drying method is widely applied in the food industry to convert liquid or paste from heat sensitive food products into a quickly rehydratable flake or powder form product. It is an inexpensive industrial practice for the production of foodstuffs including milk, coffee, fruit purees, baby foods, mashed potatoes, dry soup mixes, etc. The benefits of drum drying include; the ability to handle a wide range of products, economical operation, less labour intensive and high production yields (MUJUMDAR, 1995). However, drying can lead to a significant decrease in the phytochemical contents, thereby, reducing the phenolic contents, antioxidant activity, and flavonoids (RABABAH *et al*., 2015). To the best of our knowledge, a few research has been performed on drum drying *Nigella Sativa* seeds to produce instant beverage powder. The aim of this study was to determine the feasibility of drum drying process in producing *Nigella sativa L.* beverage powder, while using arabica gum as an adjunct. Arabic gum is popularly used as an encapsulating material by entrapping the core compound, helping to retain the volatile substances and provide protection from oxidation (RIGHETTO and NETTO, 2005). Encapsulation is often used in the food industry to isolate the active molecules such as aroma, vitamins, and antioxidants from the environment (TURCHIULI *et al*., 2014). Response surface methodology (RSM) was used to study the effect and relationship between percentage of Arabic gum and drying temperature on the quality of the black seed powder produced

2. MATERIALS AND METHODS

2.1. Materials

Roasted black seeds (*N. sativa*) were obtained from a local manufacturing industry in Penang, Malaysia. The seeds were packed in an airtight container. Arabic gum was supplied by Liangtraco & Sons Sdn. Bhd., Penang, Malaysia. The proximate composition

of *Nigella sativa* seeds and drum dried powder was analysed using standard methods prescribed by the Association of Analytical Chemists methods (AOAC, 2000); fat (AOAC method 960.39), moisture (AOAC method 950.46), ash (AOAC method 923.03), crude protein (AOAC method 960.52) and crude fiber (AOAC method 962.09).

2.2. Preparation of feed materials for drying

The roasted black seeds were mixed with distilled water at a ratio of 1:2 and blended using a high-speed blender for 1 min. The blended black seeds solution was then filtered with muslin cloth to remove insoluble particles. The feed material was prepared by mixing the specific amount of arabic gum into the filtered black seeds solution using a mixer (Pensonic Group, Malaysia). Arabic gum was added according to the experimental design in Table 1.

2.3. Experimental design

Response surface methodology (RSM) with statistical commercial package, Design Expert version 6.0 (Statease Inc., Minneapolis, USA) was used in this experiment to study the effects of percentage of arabic gum and temperature of the drum on physical properties (that is, yield, water activity, moisture content, colour, and solubility) of the powder. Face-centred central composite design with two factors and three levels were used as experimental design. The three coded levels used in this experiment were –1, 0 and +1, which corresponded to the low level, mid-level and high level of each independent variable, respectively. The independent variables and representative coded and uncoded levels are given in Table 1.

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

		Coded values		
Independent Variables	**Symbol**	**–1**	**0**	**+1**
Concentration of Arabic gum (%)	X_1	15	25	35
Temperature (^oC)	X	90	120	150

2.4. Drying Operation

The feed materials prepared were dried using a double drum dryer (GF Dryer and Flaker, Model 215, Mathis Machine Corporation, USA) with drum rotation speed of 1 rpm and a 3 mm gap between drums. Drum temperatures were set at three levels: 90, 120, and 150°C. Dried flakes were collected, weighed and stored in a sealed aluminium layered plastic bag. The dried flakes were then ground into powder using a blender for further analysis.

2.5 Analysis of Physical Properties

2.5.1 Yield

The yield was calculated based on dry matter. Total weight of solid content used for the preparation of feed material and the weight of the powder obtained after drum drying were recorded. Total weight of solids content was referred to as the summation weight of roasted black seed and arabic gum. Product yield was calculated using Equation 1.

$$\text{Yield (\%)} = \frac{\text{Total weight of resulting powder}}{\text{Total weight of solids content}} \times 100 \qquad \text{Eq. 1}$$

2.5.2 Water activity

The water activity of the samples was measured directly by a water activity instrument (AquaLab, Model Series 3TE, Decagon Devices Inc., USA). Calibration of the instrument was done prior to measurement.

2.5.3 Moisture content

The moisture content of the samples was determined using oven method (AOAC, 1984). One to two grams of powder was weighed intoa moisture dish and dried in an oven (Memmert UM600, Schwabach, Germany) at 105°C for 24 h. The weight loss after drying in the oven was used to calculate the moisture content of the powder. The moisture content was expressed as the percentage of the original sample (Equation 2).

$$\text{Moisture content (\%)} = \frac{\text{Total weight loss}}{\text{Total weight of sample}} \times 100 \qquad \text{Eq. 2}$$

2.5.4 Colour measurement

The colour of the samples was measured using a spectrophotometer (Minolta Model Series CM-3500d, Minolta Corporation, Japan). Prior to the measurements, the spectrophotometer was calibrated using zero calibration box (CM-A100) and white calibration plate (CM-A120) provided by the manufacturer. The colour was expressed in terms of L* (lightness), a* (redness) and b* (yellowness) in CIE system.

2.5.5 Solubility

The solubility was determined using the method by CANO-CHAUCA *et al.*, (2005) with slight modifications, where 1 g of powder was mixed with 100 mL of distilled water and stirred for 10 min. Then, the solution was transferred to a centrifuge tube and centrifuged at 3,500 rpm for 10 min (Centrifuge Model 4000, Kubota Corporation, Japan). An aliquot of 15 mL of the supernatant was then transferred to pre-weighed crucibles and dried overnight in an oven (UM600, Memmert Corporation, Schwabach, Germany) at 105°C. The solubility was calculated by the weight difference after drying and expressed as a percentage of the original weight (Equation 3).

$$\text{Inhibition (\%)} = \frac{\text{Absblank weight loss}}{\text{Total weight of supernatant}} \times 100 \qquad \text{Eq. 3}$$

2.6. Analysis of antioxidant properties

2.6.1 Preparation of Sample Extract

A sample of 0.5 g was extracted with 20 mL of distilled water. Extraction was carried out at 80°C in a water bath (SW-23, Julabo, Seelbach, Germany) and shaken at 200 rpm for 120 min. After the extraction was complete, the suspension was centrifuged at 3500 rpm for 15 min by a centrifuge (Centrifuge Model 4000, Kubota Corporation, Tokyo, Japan). The

supernatant was collected. The extract was used for the determination of radical scavenging activity, total phenolic contents, and total flavonoid content.

2.6.2 DPPH free radical scavenging activity

Determination of DPPH free radical scavenging activity was based on the method described by VARASTEGANI *et al.*, 2015). An aliquot of 10 µL of sample extract was mixed with 1090 µL of distilled water. Then, 3.9 mL of 25 mM DPPH methanolic solution was added. The mixture was thoroughly done using vortex and stored under dark condition for 30 mins. Absorbance was measured at wavelength of 515.0 nm using a UV-V spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto Japan) against the blank. The blank was prepared by replacing the sample extract with distilled water. The free radical scavenging activity was expressed as a percentage of inhibition and was calculated from Equation 4.

$$\text{Inhibition } (\%) = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100 \qquad \text{Eq. 4}$$

where,
$\text{Abs}_{\text{blank}}$ = Absorbance of the blank
$\text{Abs}_{\text{sample}}$ = Absorbance of the sample

2.6.3 Total Phenolic Content (TPC)

Determination of total phenolic content was based on Folin-Ciocalteu (FC) method as described by SINGLETON and ROSSI, (1965) but with slight modification. An aliquot of 40 µL of sample extract was added with 3.12 mL of distilled water, followed by 0.2 mL of FC reagent. Then, 0.6 mL of 20% (w/v) sodium carbonate solution was added. The solution was mixed well using vortex, and incubated in 40°C water bath (SW-23, Julabo, Seelbach, Germany) for 30 mins. Finally, UV-Vspectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto Japan) was used to measure the absorbance of the samples at 765 nm. Standard solution of gallic acid with different concentrations was used to prepare a standard curve for TPC determination. TPC was calculated from the prepared gallic acid standard curve. The results were expressed as gallic acid equivalent (mg GAE/g).

2.6.4 Ferric Reducing Antioxidant Power (FRAP)

FRAP was assessed using a modified method proposed by BENZIE and STRAIN, (1996). This method is based on the reduction of Fe^{3+} ferric-TPTZ (tripyridyltrianzin) to a blue colour solution (Fe^{2+} ferric-TPTZ). FRAP reagent was prepared in the ratio of 10:1:1 by mixing 300 mM acetate buffer (pH 3.6), 20 mM ferric (III) chloride hexantydrate ($FeCl3{\cdot}6H2O$) and 2,4,6-tris (1-pyridyl)- 5-triazine (TPTZ) solution in 40 mM HCl. About 3.8 mL of the prepared FRAP reagent at 37°C was mixed with 200 µL of extract and vortexed. This mixture was then incubated for 30 min at 37°C in the dark, after which the absorbance was read at 593 nm (UVmini-1240, Shimadzu Corp, Japan) against a blank. A standard curve was made using the aqueous solution of ferrous sulphate heptahydrate ($FeSO4{\cdot}7H2O$) solution (200-800 µmol). The FRAP value was recorded in terms of ferrous equivalent (µmol/mg of sample).

2.6.5 Total Flavonoid Content (TFC)

Total flavonoid content was determined using a slightly modified colorimetric assay proposed by RAMAMOORTHY and BONO, (2007). A properly diluted 1.5 Ml extract was mixed with 1.5 mL of 2% aluminium chloride ($AlCl_3$) in methanol. After 10 min incubation at 37°C, absorbance of the mixture was read at 415 nm (UVmini-1240, Shimadzu Corp, Japan) against the blank. The blank was prepared by replacing the extract with distilled water in the mixture. A standard curve was prepared using (-)-Epicatechin (10–90 mg/L) to calculate the TFC. TFC was expressed on weight basis as mg (-)-Epicatechin equivalent (ECQ/g of sample).

2.7. Mineral content

Mineral content was determined based on the method described by SPEIGHT, (2015) although, with slight modification. Mineral contents (calcium, iron, manganese, and sodium) were analysed using atomic absorption spectroscopy (AAS). Atomic absorption spectrophotometer (AA-700, Shimadzu Corporation, Singapore) was used to analyse all the samples. Samples were digested for 1 h in microwave digester (MARS 6 240/50, CEM Corporation, USA) for extraction prior to mineral content determination. An aliquot of 10 mL of HNO_3 and 1 mL of 30% (v/v) H_2O_2 were added into 1 g of sample for digestion.

2.7.1 Calcium

A series of calcium standard solutions with concentrations of 0, 1, 2, 3, and 4 ppm was prepared with various amount of stock solution, 20 mL of lanthanum oxide (La_2O_3) and diluted up to 100 mL with deionised water. Sample was prepared by adding 20 mL of La_2O_3 into the digested sample and made up to 100 mL with deionised water. La_2O_3 solution was prepared by first adding 50 mL of deionised water into 58.64 g of La_2O_3 powder. Then, 250 mL was added into the mixture and the solution was made up to 1 L with deionised water in a volumetric flask.

2.7.2 Iron

A series of iron standard solutions with concentrations of 0, 1, 2, 3, and 4 ppm were prepared with respective amount of stock solution and diluted up to 100 mL with deionised water. The digested sample was diluted up to 100 mL with deionised water to obtain an analytical sample.

2.7.3 Manganese

A series of manganese standard solutions with concentrations of 0, 0.5, 1.0, 1.5, and 2.0 ppm were prepared from respective amount of stock solution and diluted up to 100 mL with deionised water. Analytical sample was prepared by diluting the digested sample up to 100 mL with deionised water.

2.7.4 Sodium

A series of sodium standard solutions with concentrations of 0, 0.5, 1.0, 1.5, and 2.0 ppm were prepared from respective amount of stock solution, 5 mL of 10% (w/v) potassium chloride and diluted up to 100 mL with deionised water. Analytical sample was prepared

by adding 5 mL of potassium chloride into the digested sample and made up to 100 mL with deionised water.

2.8. Sensory evaluation

Sensory evaluation of the beverage derived from the drum dried instant *Nigella sativa* powder was carried out with thirty panellists comprising students and staff at the School of Industrial Technology, University Sains Malaysia. Testing was conducted in the sensory laboratory. Panellists were required to evaluate the sensory attributes such as appearance (color), aroma, taste, texture (mouth feel) and overall acceptability of the reconstituted instant beverage derived from the spray dried product, using a 9-point hedonic scale with 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much, and 9=like extremely. The reconstituted instant black seed drink was served warm with the brewed roasted black seed drink as control. Every sample was uniquely coded, which made the evaluation single blinded.

2.9. Statistical analysis

The experimental data were reported as mean±standard deviation of triplicate measurements. Experimental data were analysed using IBM SPSS Statistics version 20 software (IBM Corporation, New York, USA). Statistical analysis was performed using Duncan's paired samples at the significance level of $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Proximate composition

The proximate compositions of the raw and roasted *Nigella sativa* seeds and drum dried powder are presented in Table 2. The moisture content was significantly lower in roasted black seed (1.31%) compared with other samples. The moisture content of spray-dried powder was lower than 5%, which is ideal for product shelf life. Protein content in raw black seed was significantly higher than in roasted and drum drying powder due to heat treatment in those samples.

Table 2. Proximate composition of *Nigella sativa* seeds and drum dried *Nigella sativa* powder.

	Ash (%)	Moisture %)	Fat (%)	Protein (%)	Fibre (%)	Carbohydrate (%)
Raw black seeds	2.86±0.07^{a}	1.80±0.03^{b}	38.74±0.32^{a}	2.97±0.002^{c}	9.64±0.18^{a}	43.99±0.56^{c}
Roasted black seed	2.01a±0.08^{b}	1.31±0.09^{c}	21.5±0.15^{b}	1.56±0.003^{a}	8.17±0.12^{b}	65.45±015^{b}
Drum Dried Powder	1.18± 0.05^{c}	2.89±0.18^{a}	2.58±0.02^{c}	0.61±0.005^{b}	2.12±0.04^{c}	90.53±0.18^{a}

Values are means of three replications±standard deviation, dry matter basis. Means in row not sharing the same letter are significantly different ($P < 0.05$).

Drum drying resulted in a significant decrease in fat content compared with the raw samples. This decrease in fat content could be advantageous to both the drying process and also the output powder in terms of extending shelf life (COSTA *et al.*, 2015). Drum

drying with Arabic gum as adjunct increased the amount of non-NS solids in the powder samples, which significantly lowered the fibre content of the resulting dried powder. The significantly elevated carbohydrate percentages found can be easily explained due to the addition of carrier agents, such as polysaccharides (Arabic gum). The resulting powder was also higher in carbohydrate percentage (COSTA*et al*., 2015).

3.2. Response surface analysis

The effect of concentration of arabic gum and drum temperature on the yield, water activity, moisture content, colour values, and solubility of the drum dried *N. sativa* beverage powder is shown in Table 3. The independent and dependent variables (responses) were fitted into respective model equation and goodness of fit was determined. Table 4 shows the significance of the independent variables on responses. The data indicated that percentage of arabic gum used and temperature were significant factors at 95% confidence level p- ≤ 0.05 for all the responses studied (that is, yield, water activity, moisture content, colour L, a*, and b* values, and solubility). There was no significant lack-of-fit (p > 0.05) in all dependent variables. This indicated that the independent and dependent variables are accurately fitted into the models. Coefficient of determination (R^2) determines the overall predictive capability of the model (BAŞ and BOYACI, 2007). All responses showed satisfactory level of R^2except colour values L and a*. This implied that the models used for colour values of L and a* did notproperly represent the model of the data. Therefore, they are used mainly for trend observation. The non-satisfactory level of R^2could be due to the large range of variations and noises.

Table 3. Central composite design and experimental results for the response variables.

Run	Independent variables		Response variables						
	X_1	X_2	Yield (%)	Water Activity, a_w	Moisture content (%)	Colour values			Solubility (%)
						L	*a**	*b**	
1	–1	–1	47.64	0.336	4.34	26.51	9.32	14.29	84.81
2	+1	+1	55.19	0.138	0.76	32.23	8.96	15.01	66.31
3	0	–1	56.25	0.326	4.10	27.56	8.68	14.80	80.82
4	0	0	49.89	0.179	1.26	28.01	8.8	14.15	77.78
5	–1	0	45.58	0.203	1.49	27.57	9.17	13.84	83.43
6	+1	0	55.49	0.178	1.14	30.08	8.79	14.40	73.60
7	0	+1	48.88	0.169	0.98	29.34	9.32	15.35	69.13
8	0	0	49.67	0.184	1.34	27.87	9.04	14.49	76.97
9	+1	–1	62.95	0.317	3.82	29.55	8.69	15.33	74.90
10	–1	+1	42.61	0.191	1.37	28.30	9.64	15.00	75.90
11	0	0	50.25	0.173	1.28	28.11	9.56	14.30	78.59
12	0	0	48.08	0.191	1.42	26.99	9.19	14.36	77.24
13	0	0	48.92	0.185	1.36	29.31	8.82	14.29	78.22

Table 4. Analysis of variance on the independent variables as model and interaction terms on the response variables.

Source	Response Variables						
	Yield (%)	Water Activity, a_w	Moisture Content (%)	Colour Values			Solubility (%)
				L	*a**	*b**	
Model (*p*-value)	< 0.0001	< 0.0001	< 0.0001	0.0005	0.0118	< 0.0001	< 0.0001
Lack-of-fit (*p*-value)	0.1850	0.5801	0.4718	0.6056	0.9578	0.5046	0.2925
R-squared (%)	96.21	99.38	99.80	77.79	58.85	96.09	98.14

3.3. Physical properties

3.3.1 Yield

As shown in Table 4, the percentage of arabic gum used and drum temperature were significant factors ($p \leq 0.05$) affecting the yield of *N. sativa* powder. The higher the percentage of arabic gum incorporated, the higher yield resulted (Fig. 1). Yield was calculated on dry matter basis. Higher percentage of arabic gum incorporated into the black seed had contributed to a higher total solid content. Thus, the percentage yield increased with an increase in the percentage of arabic gum. Meanwhile, the yield was lowered as the temperature increased (Fig. 1). Drum dryer is operated by heating the steam at the inner surface of the drum (TANG *et al.*, 2003). Therefore, the steam pressure used correlates with the temperature. Previous research also reported that the percentage yield of drum dried pitaya peel decreased with an increase in steam pressure (CHIA and CHONG, 2015b), which implied that the yield increased with the temperature.

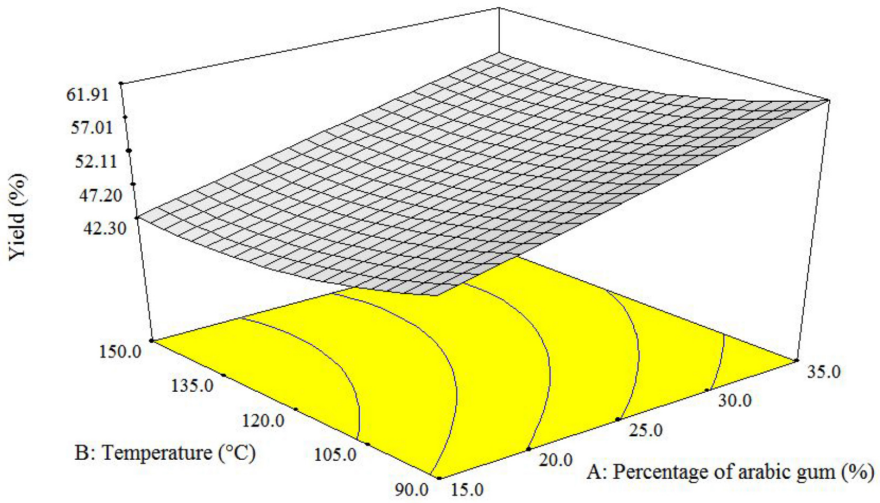


Figure 1. Response surface plot and contour plot for the interactive effects of percentage of arabic gum on the yield of drum dried N. sativa powder.

3.3.2 Water activity and moisture content

3.3.2 Water activity and moisture content

Water activity (aw) is defined as the ratio of vapour pressure of water in a food system to the vapour pressure of pure water at the same temperature (TROLLER, 1978). Water activity measures the availability of free water for any biochemical reactions. On the contrary, moisture content measures the water composition. The lower the water activity, the lesser free water is available for the biochemical reactions and hence, the longer the shelf life of black seed powder (QUE *et al.*, 2007). Figs. 2 and 3 demonstrated that water activity and moisture content decreased with the increase in drying temperature.

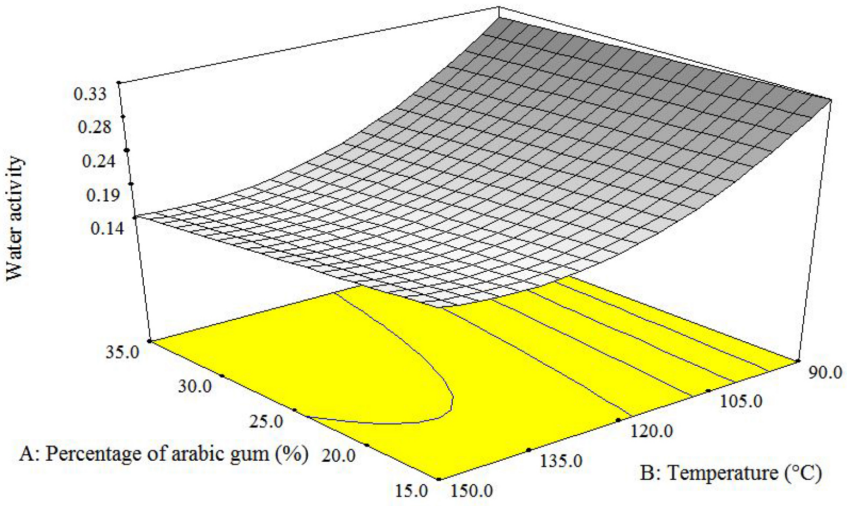


Figure 2. Response surface plot and contour plot for the interactive effects of percentage of arabic gum on the water activity of drum dried *N. sativa* powder.

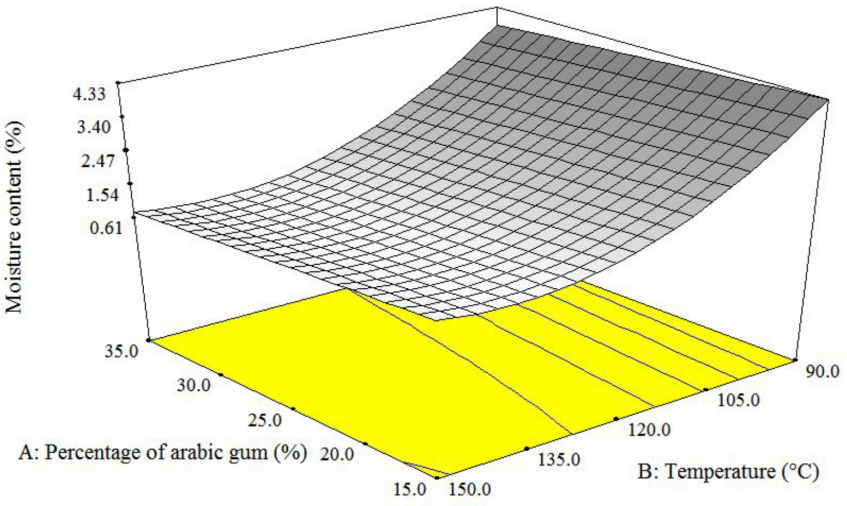


Figure 3. Response surface plot and contour plot for the interactive effects of concentration of arabic gum on the water activity of drum dried *N. sativa* powder.

The results were consistent with other findings in which the water activity and moisture content decreased with an increase in the temperature during the production of pregelatinized maize starch using the drum drying method (VALOUS *et al.*, 2002). The increase in the temperature increases the moisture transfer rate (JITTANIT *et al.*, 2011) and moisture removal rate (KAKADE *et al*., 2011), thus reducing the moisture content at a faster rate. Sorption isotherm explains the relationship between water activity and moisture content. High moisture content usually leads to high water activity, although the sorption isotherm is not in a linear regression (CHIA and CHONG, 2015a). Meanwhile, water activity decreased with the increase in the percentage of arabic gum. However, the decrease in the water activity was very small or negligible due to the effect of arabic gum percentage. These results were in agreement with the study by PUA *et al.* (2007), where they demonstrated that there was no significant contribution of percentage of arabic gum on the water activity of jackfruit powder.

3.3.3 Colour

In this study, results indicated that all the colour L, a*, and b* values were significantly affected by both variables at p≤ 0.05 (Table 3). These results were consistent with other research done. According to PUA *et al*., (2007), colour values of drum dried jackfruit powder were significantly affected by percentage of arabic gum used. The change in colour is probably due to colour nature of the arabic gum. Meanwhile, CAPARINO *et al*., (2012) reported that the change in colour of the drum dried mango flakes or powder was attributed to the high drying temperature. The change in the colour was due to Maillard reaction, which resulted in browning (CHIA and CHONG, 2015b). Colour L values indicate the darkness or whiteness while lower L value indicates darker black (TOPUZ *et al.*, 2009). The L value increased when the temperature increased. This indicated that the powder became browner or less black as the processing temperature increased. There is also a possibility that the Maillard reaction or the browning reaction incorporated brown colour in the drum dried product, leading to loss of the original dark black colour. Fig. 4 (a) and Fig. 4 (b) show that both the L and a* values increased with increase in drying temperatures.

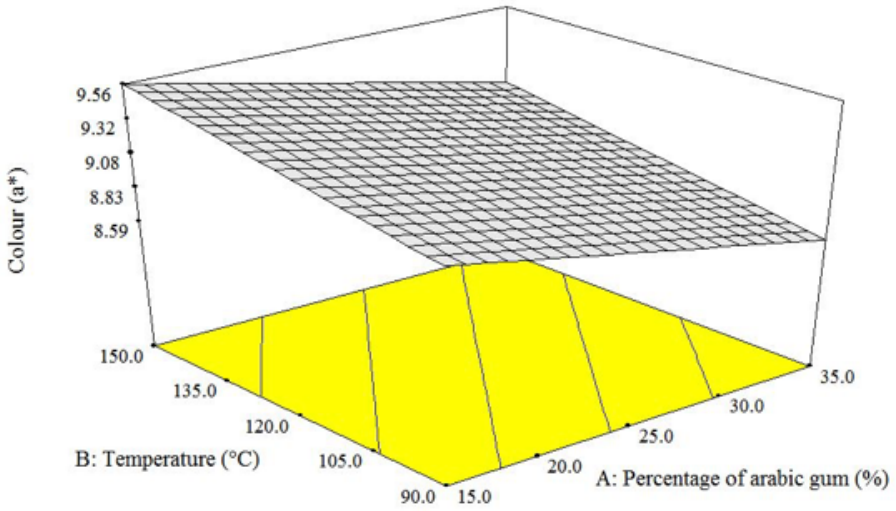


Figure 4A. Surface plot and contour plot for the interactive effects of percentage of arabic gum on the (*a**) colour values of drum dried *N. sativa* beverage powder.

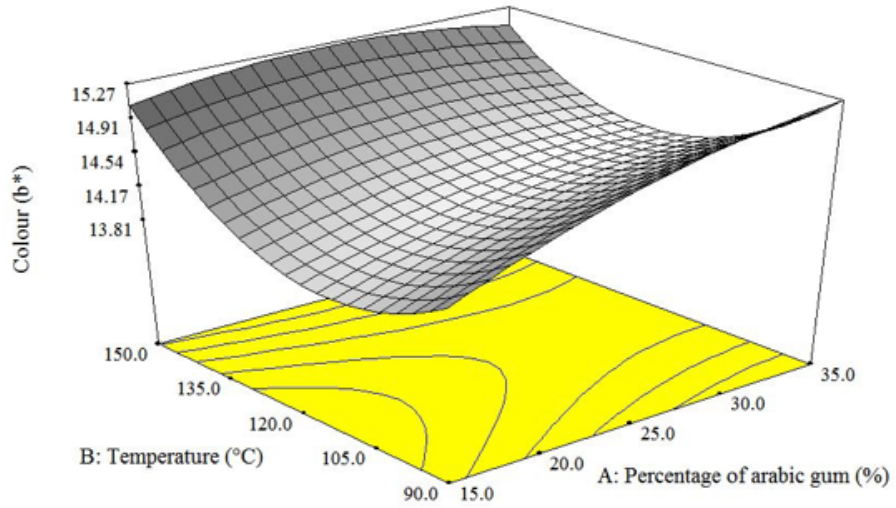


Figure 4B. Response surface plot and contour plot for the interactive effects of percentage of arabic gum on the (*b**) colour values of drum dried *N. sativa* beverage powder.

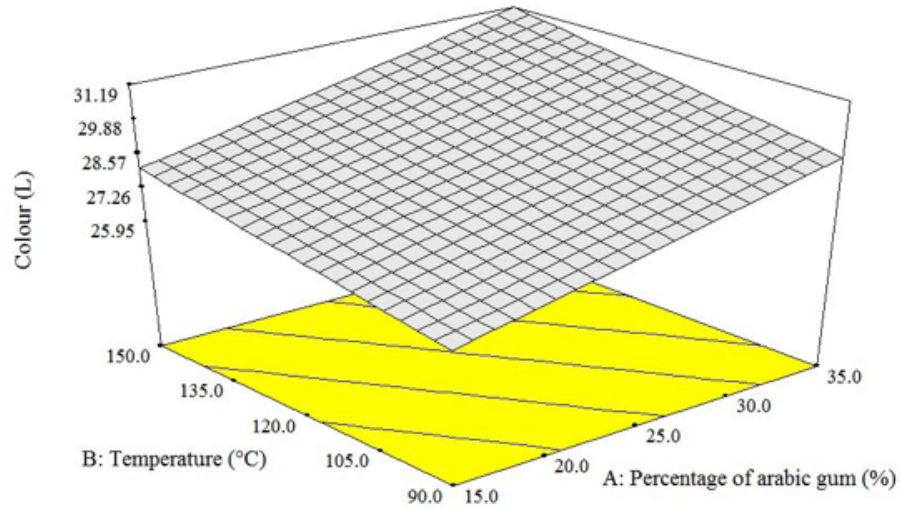


Figure 4C. Response surface plot and contour plot for the interactive effects of percentage of arabic gum on the (*L*) colour values of drum dried *N. sativa* beverage powder.

The trends showed that the L and a* values of drum dried powder significantly vary from its raw material when the temperature was higher. It can be speculated that such effect was due to the colour degradation caused by the high processing temperatures (ABONYI *et al.*, 2002).

3.3.4 Solubility

Solubility is achieved after the powder undergoes dissolution steps of sinkability, dispersibility and wettability (CHEN and PATEL, 2008). The solubility decreased as a function of both independent variables, such as percentage of arabic gum and drying

temperature (Fig. 5). CANO-CHAUCA *et al.* (2005) pointed out that solubility problems arise with high processing temperature and high concentration of solids. FERNANDES et *al*. (2013) reported that longer time is needed to dissolve solids if the production occurred at higher inlet temperature, while depending on the concentration of wall material (arabic gum). This correlates well with the solubility results achieved in this study. Higher temperature could result in further degradation of the structure. Collapsed structures have led to a more compact and rigid product with lower porosity (CAPARINO *et al.*, 2012). The water uptake ability is facilitated by highly porous structure (GOHEL *et al.*, 2004). Thus, solubility is higher in highly porous structures.

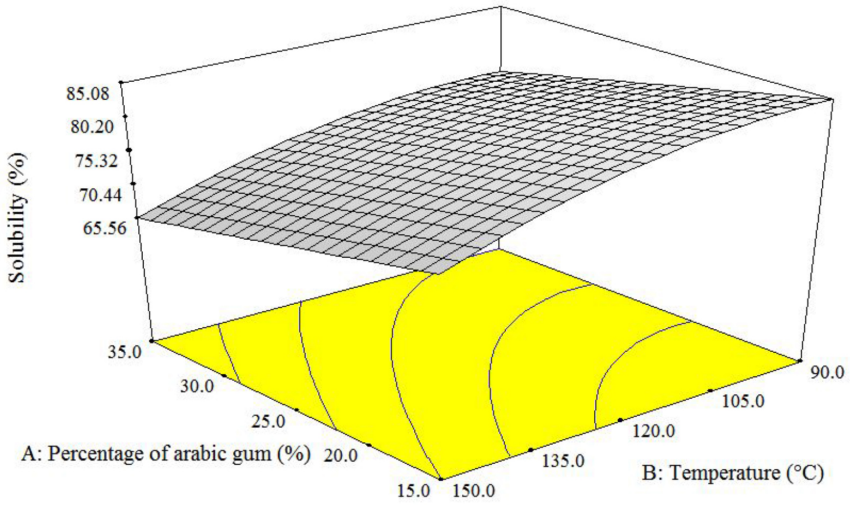


Figure 5. Response surface plot and contour plot for the interactive effects of percentage of arabic gum on the solubility of drum dried *N. sativa* powder.

3.3.5 Optimization

Optimization was carried out using response surface methodology to determine the optimum conditions for producing *N. sativa* powder with optimum physicochemical properties. This methodology utilizes graphical approach of superimposing the different response surfaces (Figs. 1-5) to generate an experimental region with desired values of the responses (ARTEAGA *et al.*, 1994). The suggested concentration of arabic gum and the temperature were 32.7% and 119.1°C respectively. Validation was done based on this optimal condition suggested, with modified drying temperature of 120°C due to the limitation of drum dryer. The results of the responses are recorded in Table 5.
Water activity of this drum dried N. powder fell under the same category as the whole milk powder. Water activity of less than 0.60 implies that the product is microbiologically stable (STEELE, 2004). The moisture content was within the range with reference to food products such as milk powder and instant coffee. Food standards by Food and Drug Administration (FDA) stated that the maximum desirable moisture content for milk powder is 5%, while the maximum desirable moisture content for instant coffee is 6% (F. D. A., 2013). However, Table 4 shows that the solubility of the powder produced at this optimum condition was not satisfactory, as no significant improvement was observed ($p >$

0.05) for the product to be reconstituted rapidly. Modifications on the processing methods or changing of the drying method should be made in order to achieve better solubility.

Table 5. Quality changes of roasted black seeds after drum drying.

Quality	Mean Value[A]±Standard Deviation		
	Raw Black seeds	Roasted Black Seeds	Drum Dried Powder[B]
Water activity, a_w	0.37±0.04[c]	0.31[a]±0.02	0.20[b]±0.03
Moisture Content (%)	1.53±0.05[c]	1.34[a]±0.06	1.26[a]±0.09
Colour:			
L	17.42±0.15[c]	20.23[a]±0.21	29.73[b]±0.16
*a**	1.73±0.12[c]	2.74[a]±0.67	8.77[b]±0.14
*b**	1.87±0.19[c]	2.15[a]±0.15	14.43[b]±0.22
Solubility (%)	68.53±0.75[c]	71.67[a]±0.76	75.38[a]±0.99

Different superscript letters in lower case are significantly different at $p< 0.05$ compared to roasted black seeds. [A]mean value±standard deviation (n = 3). [B]drum dried powder produced at optimal condition (percentage of arabic gum = 32.7%; drying temperature = 120°C).

3.4. Antioxidant properties

3.4.1 DPPH free radical scavenging effect

DPPH is a stable radical in which the maximum absorbance was observed at 515 nm. It steadily undergoes reduction by an antioxidant and is used to measure the antioxidant activity (ERKAN *et al*., 2008). The DPPH radical scavenging activity determines the inhibitory activity of antioxidant constituents. Inhibition activity is attributed to a series of antioxidant components such as polyphenols, carotenoids, ascorbic acids, and others (SHIN *et al*., 2015). Drying methods can affect the antioxidant activity significantly and the loss of 26.7% was observed with the roasted black seeds. HSU *et al*., (2003) reported that the highest radical scavenging activity was observed in freeze dried yam flour compared with drum dried and airdried yam flour. Table 6 showed that the reduction of inhibitory activity in drum dried sample was significant ($p < 0.05$) compared to the roasted black seed. CHIA and CHONG, (2015a) also reported significant loss of antioxidant activity in drum dried dragon fruit peel powder. Heat treatments can breakdown phytochemicals. Thus, the integrity of cell structure was affected, leading to various chemical reactions and migration of components (DAVEY *et al*., 2000). The reduction in antioxidant activity can be attributed to the degradation of phytochemicals during thermal processing (ZHANG and HAMAUZU, 2004).

3.4.2 Total Phenolic Content (TPC)

Phenolic compound constitute a large portion of plants antioxidants. The structure of phenolic compounds consists of one hydroxyl group substituted at different positions on the aromatic ring. Number and position of the hydroxyl groups affect the antioxidant as well as the identity of the main substituents. An example of the main substituent is carboxylic acid or linkage of the aromatic ring to another ring (RABABAH *et al*., 2015). Compared with the roasted black seeds, which are the raw material for drum dried black seeds powder, the level of TPC decreased significantly ($p \leq 0.05$) in the drum dried

seeds powder, the level of TPC decreased significantly ($p \leq 0.05$) in the drum dried powder (Table 6). Heating is capable of degrading phytochemicals. In addition, the inactivation of degradative enzymes such as polyphenol oxidases (which degrades phenolic compounds) occur during drying treatments leading to TPC loss. Intense heat treatment may be responsible for extensive loss of antioxidants, as most of these compounds are relatively unstable (LIM and MURTIJAYA, 2007). Extensive investigations were done previously on TPC reduction in relation to drying. However, no literature about drum dried *N. sativa* was recorded. Different heat treatments can lead to different degrees of TPC reduction. RABABAH *et al*., (2015) reported that the reduction of phenolic compounds in herbs was greater in oven drying method (40°C) than air drying method (24°C). In the present study, drum dried black seeds powder showed 31.4% of reduction in TPC. For drum dried products, CHIA and CHONG, (2015a) reported that 98.6% of TPC was retained in drum dried dragon fruit peel. In contrast, an almost complete loss of phenolic compounds such as tocopherols and tocotrienols was observed in drum dried oats. Other phenolic compounds such as avenanthramides and total cinnamic acids also showed a significant decrease in amount (BRYNGELSSON *et al*., 2002).

Table 6. Antioxidant properties of roasted black seeds and drum dried powder.

Antioxidant Properties	Mean Value[A]±Standard Deviation		
	Raw Black Seed	Roasted Black Seeds	Drum Dried Powder
TPC (mg/g)	17.83±0.30^{c}	34.69±3.03^{a}	24.74±0.54^{b}
TFC(mg/mL)	16.40±0.63^{c}	26.37±0.09^{a}	18.65±0.18^{b}
DPPH (%)	32.74±0.32^{c}	45.66±0.75^{a}	34.02±0.72^{b}
FRAP(mmol/mg)	0.70±0.002^{c}	0.87±0.003^{a}	0.75±0.005^{b}

Results display mean values±standard deviations (n = 6). [abc]Mean values within the same row that have different superscript letters indicate a significant difference ($P < 0.05$).

3.4.3 Total flavanoid content

The most abundant flavonoids found in Nigella sativa are epicatechin, (+) − catechin, quercetin, apigenin, amentoflavone and flavone (BOURGOU *et al.*, 2008). TPC and TFC are highly degradable due to heating and oxidation (FANG and BHANDARI, 2011; PATRAS *et al*., 2010). Results from this study showed that roasted black seeds were significantly higher in TPC, TFC, DPPH and FRAP compared to raw black seeds and the drum dried powder (Table 6). The low TFC, TPC and DPPH values in dried powder compared to roasted black seeds could be attributed to the presence of the carrier agent; Arabic gum. This has been proven in the past whereby increasing Arabic gum in samples of amla juice reduced the TPC, which in turn reduced the radical-scavenging capacity of the resulting powder (MISHRA *et al*., 2014). In spite of the decrease in antioxidant capacity, Arabic gum is known to be capable of preserving the antioxidative activity (COSTA*et al.,* 2015). This is evident from the fact that there was little difference in FRAP value, although significant. This could be due to the nature of Arabic gum, which has higher oxidative stability (COSTA *et al.,* 2015). Likewise, the heat in the drum drier could have contributed to the loss in the TPC. Heat treatments typically cause loss of naturally occurring polyphenol compounds, hence leading to reduced antioxidant capacity (DAGLIA *et al.,* 2000).

3.5. Minerals content

Table 7 shows the changes in various mineral contents after drum drying process. The results showed that calcium content decreased significantly ($p \leq 0.05$) after drum drying, whereas the iron, manganese and sodium content increased significantly ($p \leq 0.05$) after drying. However, calcium content was still the highest among the mineral content analysed, regardless of sample (either before or after drying). ÖZCAN *et al.,* (2005) reported that mineral content in basil were lost after drying. The decrease in mineral content and quality of dried product could be attributed to the high processing temperature used. The increase in concentration of mineral contents such as iron, manganese and sodium were most probably enhanced by the mineral contents found in the arabic gum itself.

Table 7. Minerals content of roasted black seeds and drum dried powder.

Minerals Content	Mean Value[A]±Standard Deviation		
	Raw Black Seed	Roasted Black Seeds	Drum Dried Powder
Calcium (ppm)	7.23±0.13[a]	6.63±0.27[b]	4.34±0.05[c]
Iron (ppm)	2.07±0.06[a]	0.72±0.01[c]	1.90±0.03[b]
Manganese (ppm)	0.82±0.02[a]	0.06±0.01 [b]	0.37±0.01[b]
Sodium (ppm)	6.32±0.14[a]	3.04±0.08[b]	5.54±0.10[b]

Results display mean values±standard deviations (n = 3). [abc]Mean values within the same row that have different superscript letters indicate a significant difference ($P < 0.05$).

3.6. Sensory characteristics

Results of sensory evaluation in terms of appearance, aroma, taste, texture and overall acceptability are presented in Table 8. The panellists judged each specific sensory parameter as acceptable, with a mean score equal to 6 (like slightly) or higher. The evaluation showed significant differences in taste and aroma among samples, with the beverage from roasted black seed powder scoring higher, compared to the beverage prepared from drum dried black seed powder.

Table 8. Mean of hedonic scales for panellist's acceptance of beverage prepared from drum dried black seed powder and from roasted black seed powder.

	Appearance	Aroma	Taste	Texture	Overall acceptability
Drum dried black seed	5.89±1.54[a]	5.89±1.08[b]	5.02±1.12[b]	6.12±0.87[a]	6.13±1.21[a]
Roasted black seed	6.14±1.03[a]	6.59±1.19[a]	6.32±1.75[a]	5.56±1.43[b]	6.10±1.37[a]

Results display mean values±standard deviations (n = 30). [abc]Mean for each characteristic followed by the same superscript letter within the same column are not significantly different at $P < 0.05$.

However, drum dried powder scored higher in texture than the roasted black seed powder. There was no significant difference in appearance and overall acceptability

between samples. Hedonic scales for the overall acceptability of both beverages from roasted black seed powder and drum dried black seed powder were higher than 6 (like slightly), indicating that both samples were at acceptable level.

4. CONCLUSIONS

RSM is useful in the optimisation of product formulation and processing conditions. The two independent variables, namely percentage of arabic gum and drying temperature significantly affected all the responses measured which include yield, water activity, moisture content, colour values L, a*, and b*, and solubility of the final product. Based on the optimization, the optimum percentage of arabic gum and the drying temperature are 32.7% and 119.1°C respectively. However, at these optimised conditions, the solubility of the powder was low, which could significantly affect the feasibility of using the method to produce instant N. sativa powder. The drum dried product showed significant reduction ($p \leq 0.05$) in total phenolic content as well as antioxidant activity as determined by the DPPH free radical scavenging activity. Significant loss ($p \leq 0.05$) was detected for calcium content in the drum dried product, but the iron, manganese and sodium contents increased significantly ($p < 0.05$) after drying. The overall sensory acceptability score was higher than 6 (like slightly), indicating the beverage was at an acceptable level.

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DEPURATION OF STRIPED VENUS CLAMS FOLLOWING ARTIFICIAL VIBRIONES CONTAMINATION

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ABSTRACT

Vibrio spp are widely distributed in the marine environments and are responsible for common illnesses in many countries. The main objective of this study was to investigate the depuration capacity of *Venus gallina* artificially contaminated with *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Contamination experiments were used to assess the accumulation capacity of clams in poorly, moderate and highly contaminated waters (*Vibrio spp*10^3/ml, 10^4/ml, 10^6/ml). All bivalves were exposed to contamination for 72 h and 8 experiments were performed. Accumulation capacity varied with respect to initial level. Comparing experiments dataset with two vibrios species, clams showed a different specific accumulation: in particular, molluscs evidenced a scarce accumulation capacity of *V.vulnificus*. Depuration trials were performed in close-circuit seawater-disinfection system that uses filtration, Ultra violet (UV) and ozone. Bivalves samples were collected every 12h until 3 days of depuration. Most of the depuration trials with *V.parahaemolyticus* showed a decrease in initial bacterial loads (4times lower values) after36-48 hours, but in subsequent periods, the trend remained stationary. In *V. vulnificus* tests, clams showed a scarce depuration capacity instead. Future studies are still required to assess the efficacy of the depuration process in reducing pathogenic Vibrio strain naturally accumulated in clams and to prevent significant economic losses to stakeholders due to long depuration periods.

Keywords: food safety, depuration, clams, *V. parahaemolyticus,V.vulnificus*

1. INTRODUCTION

The exploitation of bivalve molluscan shellfish is of great social and economic importance in the coastal ecosystems of southern Europe (BERTHOU *et al.,* 2005). In particular, the landing of *Venus gallina* play an important economic role in the central and northern Adriatic coasts of Italy, where it has considerably increased in the last decades with the introduction of the hydraulic dredges (MORELLO *et al.,* 2005; MOSCHINO and MARIN, 2006). The venerid bivalve, *Venus gallina,* is a mollusc distributed throughout the Mediterranean and Black Sea (MOSCHINO and MARIN, 2006). The interest towards this clam increased also in relation to its nutritional characteristics. In fact, it has interesting dietetic properties due to the low lipid and cholesterol contents, presence of phytosterols and the high percentages of healthy polyunsaturated fatty acids (ORBAN*et al.,* 2006). Since bivalves are filter-feeding organisms, they can accumulate pathogenic microorganisms (for example, bacteria, human viruses and microalgae), having a significant health risk if consumed raw or lightly cooked (COOK, 1991; LEES *et al.,* 2010). Generally, bivalves' bacterial composition is dominated by Gram-negative bacteria like Vibrionaceae and Enterobacteriaceae (CAO *et al.,* 2009). Among the indigenous microbiota of coastal environments, the family Vibrionaceae, particularly *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, is targeted as a causative agent of human disease due to the consumption of shellfish (BUTT *et al.,* 2004; NORMANNO *et al.,* 2006; RIPABELLI *et al.,* 1999). The occurrence of potentially pathogenic *Vibrio spp*. in coastal waters and shellfish of European countries has already been documented in Italy, Spain and France (BARBIERI *et al.,* 1999; HERVIO-HEATH *et al.,* 2002; MARTINEZ-URTAZA *et al.,* 2008, ROQUE *et al.,* 2009). The number of *Vibrio spp*. in shellfish varies widely and depends on the geographical area, environmental conditions and local parameters. Salinity and temperature are in fact important parameters in the dynamics of vibrios in marine systems (HSIEH *et al.,* 2008; BLACKWELL and OLIVER, 2008; DEPAOLA, 2003; PFEFFER *et al.,* 2003; RANDA *et al.,* 2004). Some studies have shown that *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* enter a viable but non-culturable state when water temperature average is below 15°C (COLWELL and GRIMES, 2000; ROSZAK and COLWELL, 1987). On the other hand, temperatures above 20°C favor the growth of Vibrio spp. in seawater (BLACKWELL and OLIVER, 2008; DEPAOLA *et al.*, 2003).

In order to reduce shellfish contamination and to minimize the inherent risks of shellfish consumption, legislation sets requirements for sample collection, wet storage, bivalve self-purification by depuration and/or relaying (tank construction and operation, packaging, labelling), shellfish processing, laboratory analytical methodologies and product distribution (Reg. N.853/2004/EC, Reg. N.854/2004/EC). The legislation employs a classification to the seafood harvesting areas according to bacterial indicators of sanitary quality (*E. coli*). This classification determines whether shellfish can be sent for direct consumption or must be treated prior to commercialisation (LEES, 2000). Furthermore, the current European legislation does not specify limits for *Vibrio spp*. (EC, 2001). Therefore, preventive measures should be implemented for *Vibrio spp*., taking into account that they are naturally found in seawater and normal constituents of mollusc flora (BARILE *et al.,* 2009) and some strains (such as *V. parahaemolyticus*) are the major cause of epidemics associated with the consumption of bivalves (MEAD *et al.,* 1999; WITTMANN and FLICK, 1995).

Relaying and depuration are common approaches to reducing bacterial loads in shellfish. In the relaying process, shellfish is transferred before harvest from polluted areas to an unpolluted waterway for natural biological purification. Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under

controlled conditions (LEES, 2000). Effectiveness of the depuration process depends on the diversity and physiology of shellfishes, initial loads of bacterial strains, environmental conditions (temperature, salinity, pH and so on.) and purification system (JOVEN *et al.*, 2011; SCHENEIDER *et al.*, 2009). Several studies have been carried out to evaluate the effect of depuration in physiological and microbiological aspects of some clams species, such as *Ruditapes decussatus*, *Venerupis senegalensis*, *Venus gallina* and *Mercenaria mercenaria* (EL-SCHENAWY, 2004; HOWARD *et al.*, 2003; MAFFEI *et al.*, 2009). In general, the depuration process is particularly efficient in the reduction of total viable counts and *E. coli* levels (ANACLETO *et al.*, 2013), but not with *Vibrio spp*. In fact, *Vibrio spp*. requires longer depuration periods than *E. coli* to become effective (COLAKOLU *et al.*, 2014; COZZI *et al.*, 2009; CROCI *et al.*, 2002; LOPEZ-JOVEN *et al.*, 2011). CROCI *et al.* (2002) indicated that 44 h of depuration process led to a decline in Vibrio by a factor of only 10, whereas, COZZI *et al.* (2009) describes that 72 h reduced Vibrio contamination to a level close or below the detection limit of the methods. Also, LOPEZ-JOVEN *et al.* (2011) reported that at least 10 days depuration at 20°C is effective in reducing Vibrio load.
In this context, the main objective of this study was to investigate the depuration capacity of *Venus gallina* artificially contaminated for three days with *Vibrio parahaemolyticus* and *Vibrio vulnificus* running tests.

2. MATERIALS AND METHODS

2.1. Collection of samples and treatments

Venus gallina specimens were taken at about 250-500 m from Molise coastline in classified areas. Clams were harvested with hydraulic dredges. The specimens (mean size: 25-32 mm) were taken to the laboratory in refrigerated containers (4°C) within 2 hours. Dead or damaged specimens were discarded and the remainder was divided into seven aliquots of 250 clams. Before the trial, specimens were acclimatised for 72h in aquariums with 100 litres of recirculated and artificial seawater (Instant Ocean Aquarium System salinity: 35‰). Temperature was fixed at 18°C.
After acclimatization, ten specimens for each aliquots underwent bacteriological analysis (*V. parahaemolyticus, V. vulnificus*) to evaluate the sanitary conditions and the initial load of each pathogen for determining the contamination levels.

2.2. Contamination experiment

Vibrio parahaemolyticus ATCC 17802 and *Vibrio vulnificus* ATCC 27562 were used for trials.
Bacterial contamination was conducted in 8 l (0.12 m^3) tanks filled with artificial seawater (Ocean Fish Marine Salts-Prodac). In this study, contamination experiments were conducted to assess the accumulation capacity of clams in poorly, moderate and highly contaminated waters (10^3/ml, 10^4/ml, 10^6/ml). Contamination levels were chosen from results of previous studies (BARILE *et al.* 2009; BARILE *et al.* unpublished). Details of each contamination trial are reported in Table 1.
To test the influence of temperature on *Vibrio vulnificus* accumulation in clams, two trials were conducted at different artificial conditions: 35‰salinity and 22°C temperature (test VV2), 30‰salinity and 25°C temperature (test VV3). Parameters were established considering results of previous studies (COLWELL and GRIMES, 2000; BLACKWELL and OLIVER, 2008).

All bivalves were exposed to contamination for 72 h. After contamination period, ten replicates for each test were analyzed to evaluate the variability of their accumulation capacity.

Table 1. Details of contamination trials.

Species	Bacterial load	Temperature	Salinity	Test
Vibrio parahaemolyticus	$2.30*10^3$	22°C	35‰	VP1
Vibrio parahaemolyticus	$3.75*10^4$	22°C	35‰	VP2
Vibrio parahaemolyticus	$9.37*10^4$	22°C	35‰	VP3
Vibrio parahaemolyticus	$1.12*10^6$	22°C	35‰	VP4
Vibrio vulnificus	$2.30*10^3$	22°C	35‰	VV1
Vibrio vulnificus	$7.5*10^4$	22°C	35‰	VV2
Vibrio vulnificus	$7.5*10^4$	25°C	30‰	VV3
Vibrio vulnificus	$1.12*10^6$	22°C	35‰	VV4

2.3. Depuration experiment

At the end of contamination, all clams were transferred to depuration system (Fig. 1), except for VV3 test. Specimens were placed in a single layer on a plastic grill to avoid contact with the bottom of the tank and thus, minimise recontamination.



Figure 1. Trial depuration system.

Depuration systems consisted of tanks fitted with a wool/Perlon prefilter and hyperactive carbon filter, an active biological filter using *Lithothamnium calcareum* algae and a UV sterilisation plant (Fig. 2), and filled with artificial seawater (Ocean Fish Marine Salts-Prodac). The pump takes water from the tank containing the biological filter (*L. calcareum*

algae) and pumps it into the UV sterilisation unit and then on, through the bubbler to reach the tank. It then crosses to the chamber fitted with the wool/Perlon and the carbon filters, through the serpentine cooling unit (in which ozonation also takes place) and back to the tank containing the biological filter. The water is then taken back up by the pump and recirculated. The water temperature is controlled by a thermostat (18°C).
The chemical, physical and microbiological parameters of water (temperature, salinity, dissolved oxygen, vibrios detection) and clams vitality were checked daily to control proper functioning of aquarium.
Bivalves samples were collected every 12h for 3 days. Samples were immediately processed using the methods reported below.

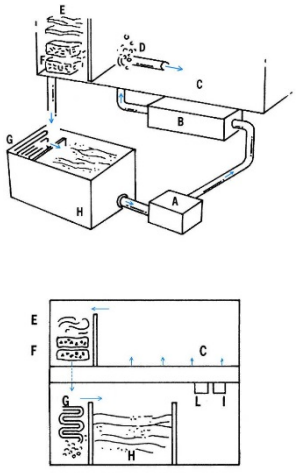


Figure 2. Depuration system:(A) Pump;B) UV sterilisation unit;C) Tank;D) Bubbler;E) Wool/Perlon filter;F) Carbon filter;G) Serpentine cooling unit;H) Biological filter;I) Ozonation air pump.

2.4. Enumeration of *Vibrio parahaemolyticus and Vibrio vulnificus*

For quantification of *Vibrio parahaemolyticus*, a MPN modified method was used. Clam meat was added to alkaline peptone water (APW). Serial ten-fold dilutions were prepared in APW, incubated at 37°C for 16-18h and subsequently plated on thiosulphate citrate bile salt sucrose agar (TCBS) and incubated at 37°C for 18-24h. Biochemical confirmation was performed with individual and miniaturised tests (API 20E: 4154106 for *V.parahaemolyticus*, 5346007 for *V. vulnificus*; API 20NE: 7077444 for *V. parahaemolyticus*, 7432055 for *V.vulnificus*). For the quantification of *Vibrio vulnificus*, analyses were conducted as described above, using the selective medium m-CPC (incubation at 40°C for 18-24h). Results are expressed in MPN/100g (Most probable Number).

3. RESULTS AND DISCUSSION

3.1. Clams mortality during trials

Mortality ranged from zero to five specimens during all trials. It should be stressed that, in this study, depuration experiments were carried out in a closed system, with a periodic control of water parameters. In addition, clams were placed in depuration systems at a

lower density than those commonly contained in bins of depuration centers. These excellent conditions could influence positively clams mortality rates.

3.2. Depuration system parameters

Chemical and physical parameters of water showed minimal variations (lower than 5%). No pathogen was detected in water samples.

3.3. Contamination experiment

Vibrio parahaemolyticus accumulation values were in one lower order of magnitude than water concentrations.
In fact, specimens of *Venus gallina* showed vibrios median values of 430MPN/100g, 9200 MPN/100g, 1100MPN/100g and15000MPN/100g respectively, at water levels of $2{,}3*10^{3}$/ml; $3{,}75*10^{4}$/ml; $9{,}37*10^{4}$/ml; and $1{,}12*10^{6}$/ml.
Considering experimental results, microbial loads changed markedly among different replicates in each trial (Table 2).

Table 2. *Vibrio parahaemolyticus* values after 72h contamination at controlled conditions: temperature 22°C and salinity 35%.

	TEST VP1 MPN/100 g	TEST VP2 MPN/100 g	TEST VP3 MPN/100 g	TEST VP4 MPN/100 g
Min	92	6.800	740	1.500
Max	930	17.000	2.200	360.000
average value	435	10.200	1.237	51.680
median value	430	9.200	1.100	15.000

Vibrio vulnificus accumulation values did not show a clear pattern regarding water concentrations (Table 3). This data was particularly evidenced in test VV4, where high contamination levels in water corresponded to a very low *Vibriovulnificus* loads in clams. Moreover, tests comparison between two vibrios species showed that shellfish accumulated fewer *Vibrio vulnificus* than *Vibrio parahaemolyticus*.

Table 3. *Vibrio vulnificus* values after 72h contamination at controlled conditions: temperature 22°C and salinity 35%.

	TEST VV1 MPN/100 g	TEST VV2 MPN/100 g	TEST VV4 MPN/100 g
Min	36	74	74
Max	230	15000	430
average value	125	4110	222
median value	92	1215	230

Besides, bivalve's inter- and intra-specie variability is well known in studies on microbiological contamination. In fact, the amount of water filtered is between twenty and one hundred liters per day, independent of the environmental conditions (RICHARDS,

1988; ROBERTSON, 2007). Bivalve molluscs feeding physiology determine the accumulation of pathogenic microorganisms filtered from the overlaying water (BURKHARDT and CALCI, 2000; HO and TAM, 2000). These phenomena may also partially explain seasonal and geographical differences in microbial content of bivalves (HERNROTH *et al.,* 2002).
In contamination experiments with *Vibrio vulnificus* conducted under different environmental conditions (test VV2 and test VV3), values were not comparable (Table 4).
In particular, at salinity of 30‰ and temperature of 25°C, most of the recorded values ranged from<30 to 350MPN/100g: it may indicate a lower accumulation capacity of clams oralower capacity of pathogen to survive and multiply under these conditions. These facts supported reports from other studies. KASPAR and TAMPLIN (1993) described that the greatest accumulation of microorganisms in hard-shelled clams occurred during certain periods in the spring, at temperatures ranging from 11.5 to 21.5°C. BURKHARDT *et al.* (1992) showed that temperatures outside the range of 13-22°C and salinities greater than 25 ppt reduced the survival of *V. vulnificus* in seawater.
Moreover, OLIVEIRA *et al.* (2011) reported that annual variation in water temperature and salinity influence shellfish's physiological state and therefore, affects the capacity of siphoning and accumulate microbial species.

Table 4. *Vibrio vulnificus* values after 72h contamination levels under different conditions: salinity of 35‰ and temperatures of 22°C (test VV2), salinity of 30‰ and temperatures of 25°C (test VV3).

	TEST VV2	**TEST VV3**
	MPN/100 g	**MPN/100 g**
min	74	36
max	15000	9200
average value	4110	1437
median value	1215	92

At the end, contamination experiment conducted in this study evidenced a variability infiltration capacity; the presence of such inhomogeneous results provide useful suggestions for planning future tests in terms of the number of organisms to be tested and number of organisms and replicas to be examined at different times. The remark of this variability, although, it led to greater difficulty in data processing, was a crucial and essential factor in the discussion of depuration tests.

3.4. Depuration experiment

In depuration trials, VP1-VP3 (Fig. 3), bacterial load showed a decrease after 48 hours with values four times lower than the initial contamination levels. Instead, in tests VP2 and VP4, after 36 hours of depuration, recorded values were four times lower than those at the start. Observed decreases remained fairly constant in the subsequent hours of depuration during all tests. This finding suggests that the initial phase of pathogen's elimination was followed by a "plateau" phase.
Concerning *V.vulnificus* levels during the first depuration trial (VV1), a not clear trend was found over time (Fig. 4). After 48 hours of depuration, lower values were recorded with respect to the initial load, and at the end of experiment (after 72 hours of depuration), values were in one lower order of magnitude with respect to the initial load.

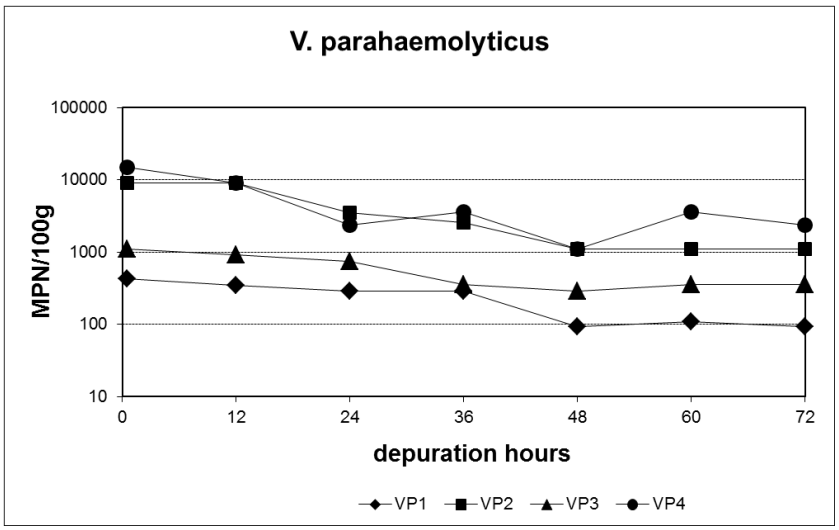


Figure 3. Temporal changes in *Vibrio parahaemolyticus* levels during depuration tests.

In tests VV2 and VV3 (Fig. 4), with low initial values (92 and 230MPN/100g), depuration levels lower than 30MPN/100g occurred after 36 hours. In test 2, this trend has remained constant, while in test 3, higher values (92MPN/100g) were detected after 48 hours. In all depuration treatments with *V. vulnificus*, recorded values were within the range defined by the initial contamination levels, then the apparent decrease may be associated with the variability in contaminated organisms and not to an effective depuration treatment.

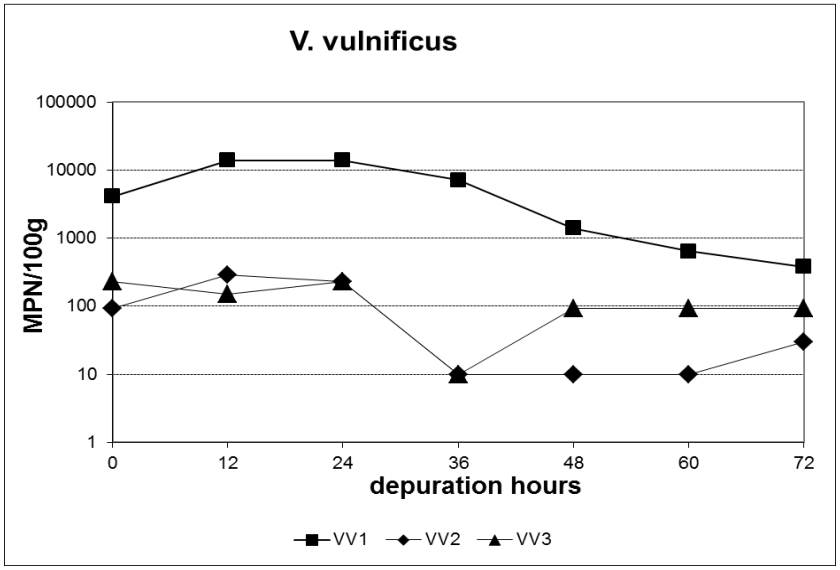


Figure 4. Temporal changes in *Vibrio vulnificus* levels during depuration tests.

In this study, although, it is known that the initial contamination levels affect the depuration efficiency and higher contaminated shellfish require longer purification periods, comparing trials with medium-high initial loads (10^3 to 10^5MPN/100g) showed similar trends. This evidence could be explained by a different response to purification processes by vibrios species. On the other hand, the presence of different pathogens could

affect clams filtration capacity differently. Such observations are in fact recorded for other bivalves: *Crassostrea virginica* oysters showed longer depuration times for *Vibrio spp*. than *E. coli* and *Salmonella tallahassee* (MURPHREE and TAMPLIN, 1991). Results of this research are in agreement with a previous study on depuration of *Venus gallina* by *E. coli*, *Salmonella tiphymurium*and *Vibrio parahaemolyticus* (BARILE *et al.*, 2009).
In addition, in regard to purification methods, previous studies reported that *V. parahaemolyticus* is sensitive to ultraviolet irradiation and chlorine dioxide (HAMAMOTO *et al.*, 2007, WANG *et al.*, 2010). REN and SU (2006) examined the effects of electrolyzed oxidizing (EO) water depuration in reducing *V. parahaemolyticus* and *V. vulnificus* in laboratory-contaminated oysters and found that both species could only be reduced by approximately 1.0-log unit after 8h at room temperature. Considering that adopted purification system was fitted with a UV sterilisation and ozonation plant, the data obtained confirmed those reported in literature. Concerning depuration experiments involving *V. vulnificus*, clams showed a scarce depuration capacity, although, further studies would be needed to give more strength to this hypothesis.
Clams purification capacity may have been influencedby temperature and salinityconditions of depuration processes (18°Cand 35‰).Some studies indicated the effects of water temperature on depuration of *Vibrio spp.* in other molluscan species, especially oysters. Limited reductions in *V. parahaemolyticus* (1.2 log MPN/g) and *V. vulnificus* (2.0 log MPN/g) were observed in oysters after depuration with a UV sterilizer at 22°C for 48 h (CHAE *et al.*, 2009). On the contrary, TAMPLIN and CAPERS (1992) reported that levels of *V. vulnificus* accumulated naturally in Gulf oysters increased by 5 log MPN/g after depuration in UV-sterilized water at 23°C for 48 h.
In this study, at temperatures of 18°C, reductions in one order of magnitude were recorded in only two tests with *V. Parahaemolyticus*. Specific studies are required to determine the optimal conditions for shellfish microbial depuration.

4. CONCLUSIONS

Shellfish production is done globally and their nutritional and economic value is well-known. However, filter-feeding bivalves is an efficient transmitter of seafood-born disease. In fact, the emergence of *Vibrio spp* as human pathogen is of particular concern to shellfish producers. Very few data are available on the number of pathogenic Vibrios and more information is needed to improve the quantitative risk assessment concerning the presence of Vibrios in shellfish (CANTET *et al.*, 2013; WHO, 2011). Bacterial indicators used for shellfish health evaluation were reported as inadequate predictors of the presence of autochthonous bacterial human enteric viruses. Over a long period of time, the high-risk nature of this product and underestimation factors have been well documented in many investigative reports and international agencies.
This study emphasizes a limited capacity of *Venus gallina* to release vibrios, in fact, clams with *V. Parahaemolyticus* showed a depuration capacity only in initial phases, while clams with *V. vulnificus* showed a scarce depuration capacity. Bivalves quality is reduced with time due to lack of feed in the depuration process. Stakeholders experience significant economic losses when depuration periods above 48h are implemented. More sensitive and reliable depuration procedures must be developed and a better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed.

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PAPER

JUICES OF PRICKLY PEAR FRUITS (*OPUNTIA* SPP.) AS FUNCTIONAL FOODS

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ABSTRACT

The prickly pear (*Opuntia* spp.) usually is consumed as fresh fruit. In this study of prickly pear juice *in vitro* we characterized and quantified secondary metabolites including antioxidant capacity of ten *Opuntia* spp. variants. Gallic acid was abundant in most variants. Catechin and epicatechin isomers, and procyanidins B1 and B2 were present in most variants. Ascorbic acid content was higher than 84 mg. Betacyanins stand out in red-colored juices, betaxanthins in the yellow ones; this caused lack of relationship between antioxidant capacity and total phenolic content. The soluble fiber content, sugars, betalains and ascorbic acid position this juice as a functional food.

Keywords: ABTS, antioxidant, betalains, FRAP, juice, prickly pears

1. INTRODUCTION

Normal metabolic processes produce free radicals, which cause oxidative damage. The human body possesses endogenous antioxidant mechanisms using substances that significantly delay or prevent oxidation. These substances include the cellular enzymes superoxide dismutase, glutathione peroxidase and catalase (WANG and QUINN, 2000). There are also non-enzymatic defense substances against oxidation stress, including vitamin E, an effective antioxidant of polyunsaturated membrane lipids, and vitamin C which, as a reducing agent or electron donor, reacts rapidly with the $HO^{\cdot}$ and the superoxide anion and also prevents the oxidation of membrane lipids (WANG and QUINN, 2000).

When endogenous antioxidant mechanisms are insufficient to offset the imbalance resulting from oxidative stress, physiological and biochemical changes take place such as protein glycosylation, lipid peroxidation, and glucose auto-oxidation (OPERA, 2004). Diseases associated with oxidative stress include Type-2 diabetes mellitus (DM2), hypertension, renal and hepatic impairment, cancer, and neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Huntington (JELLINGER, 2003; D'AMICO *et al*., 2013).

The intake of natural or synthetic antioxidants can reinforce the antioxidant capacity of the organism (HIDALGO *et al*., 2006). Recent research has shown that certain compounds present in plants, such as terpenes, flavonoids, betalains and anthocyanins, possess antioxidant properties that are more powerful than those of vitamins (HARASYM and OLEDZKI, 2014).

Global trends in food and nutrition indicate a growing interest in the consumption of fruits and vegetables, given their nutritional value and benefits for the functions of the human body. These trends in eating patterns have led to a new area of research and development in nutrition related to the so-called "functional foods", defined as any food, either natural or processed, which in addition to its nutritional components contains substances that boost a person's health, physical ability and mental state (KONIGSBERG-FAINSTEIN, 2008). Functional compounds include exogenous antioxidants, which safely interact with free radicals and disrupt their chain reaction before they damage vital molecules (OROIAN and ESCRICHE, 2015).

The prickly pear, the fruit of cacti of the genus *Opuntia* is widely available throughout Mexico's South Highland. More than 189 species of wild prickly pear cacti are known, 83 of which are Mexican; of these, 29 are distributed in the north-central region of Mexico, in an area of approximately 300 000 km^2 that stretches across part of the states of Aguascalientes, Guanajuato, Hidalgo, Jalisco, Queretaro, San Luis Potosí, Zacatecas, and around México City. In this area, a number of variants with different degrees of humanization can be found, from the wild *O. streptacantha* and the cultivated *O. hyptiacantha*, *O. megacantha* and *O. albicarpa*, to *O. ficus-indica*, the species considered as the one with the highest degree of domestication (REYES-AGÜERO *et al*., 2005). Prickly pears are consumed mainly as fresh fruit and display marked differences in size, shape, color and flavor, as well as in seed quantity, size and hardness; prickly pear is also processed to produce jelly, jam and paste. Chemical compounds found in prickly pear include polyphenols and betalains (FIGUEROA-CARES *et al*., 2010; YEDDES *et al*., 2013). These antioxidant metabolites either prevent or control the excessive production of highly unstable free radicals and reactive molecules that have the ability to disrupt the functions of various biomolecules, i.e., oxidative stress (RODRÍGUEZ *et al*., 2001; SOOBRATTEE *et al*., 2005).

Evaluations of the prickly pear fruit indicate its potential to be considered as a functional food due to its content of ascorbic acid, phenols, carotenoids and betalains at levels that

exceed those in plums, nectarines or peaches (FERNÁNDEZ-LÓPEZ *et al.*, 2010). These phytochemicals may contribute to mitigation of the effects of prolonged hyperglycemia and reinforcement of the antioxidant system in normal glycemic patients. Antioxidants have been shown to increase the sensitivity of insulin receptors or may moderate the rise in blood glucose concentration after the ingestion of carbohydrates by inhibiting the action of digestive enzymes and glucose transporters SGLT-1 (BRYANS *et al*., 2007). In addition, these phytochemicals have been associated with anti-inflammatory, antioxidant, immunomodulatory and apoptotic properties (KAULMANN and BOHN, 2016). Phytochemicals, which locally reduce oxidative stress, are widely studied as cancer-protective agents (MOORE *et al.*, 2016). Indeed, animal assays indicate that supplementation with green and black tea (rich in polyphenolic compounds) led to a decrease in postprandial blood glucose levels in Sprague-Dawley rats (ZEYUAN *et al*., 1998). Furthermore, *in vivo* studies showed a drop in glycosylated hemoglobin (FUKINO *et al*., 2008) and increased insulin activity after consumption of tea extracts (RICHARDA and DOLANSKY, 2002).
Based on the above, the objective of this study was to supplement existing assessments of prickly pear juice as a functional food by identifying and quantifying antioxidant compounds in the juice of fruits of *Opuntia* and to investigate their antioxidant capacity *in vitro*.

2. MATERIALS AND METHODS

2.1. Selection of variants and sample preparation

Ten prickly pear variants, six of them cultivated, were evaluated as ripe fruits: Rojo Pelón (*Opuntia ficus-indica*), Blanca (*O. albicarpa*), Amarilla Monteza, Pico Chulo, Torreoja and Sangre de Toro (*O. megacantha*), and four wild variants: Cardona (*O. streptacantha*), Charola (*O. streptacantha* ssp. *aguirrana*), Tapona and Tapón Rojo (*O. robusta*). Fruits were collected in the municipality of Villa de Arriaga, state of San Luis Potosí, México. *Opuntia* variants were selected based on: (a) degree of humanization, (b) abundance and economic potential in the state of San Luis Potosí, and (c) fruit color. The skin of prickly pears was removed, then the juice was extracted from the pulp with a stainless-steel blender (International LI-12-106), and seeds were separated with an 8 mesh filter; the juice was stored in sterile containers at -20°C until use.

2.2. Total phenolic compounds

Total phenolic compounds in prickly pear juice was quantified using the Folin-Ciocalteu method modified by YEDDES *et al*. (2013), and expressed as gallic acid equivalents (mg GAE g^{-1}). To extract phenols, cool absolute ethanol was added to 0.15 g of lyophilized juice stored at -50°C (Frezer dryers IIshin, Corea), the mixture was sonicated for 10 min and then maintained under constant stirring for 2 h at 4°C. The solution was filtered through Whatman Grade 42 filter paper. Extracts were brought to 15 mL with ethanol and stored protected from light at -20°C. Total phenols were measured in triplicate; to this end, 437.5 μL of 1N Folin-Ciocalteu reagent (Sigma) were added to 35 μL of the ethanol extract and were left to react at room temperature for 3 min. Afterwards, 2187.5 μL of a 20% Na_2CO_3 solution were added and the volume was brought to 3500 μL. The mixture was left to stand at room temperature in the dark for 2 h for the development of color. Absorbance was read at 760 nm in a spectrophotometer (Agilent Technologies, Germany), using blank samples made of distilled water and the reagents used. The amount of phenolic

compounds was estimated by comparing the absorbance values of samples with those of the gallic acid standards.

2.3. Phenolic acids and flavan-3-ols

Phenolic compounds were extracted with the method used by RODARTE *et al.* (2007). Two grams of lyophilized prickly pear juice were mixed with 3 mL of acidified methanol (0.1% hydrochloric acid), and sonicated in a water bath for 10 min; then, the supernatant was collected and the previous procedure was repeated five times with the precipitate to obtain six extractions. Supernatants were collected and centrifuged for 10 min at 5000 rpm; the centrifuged fraction was concentrated under vacuum on a rotary evaporator at 30°C (Heidolph, Alemania) followed by reconstitution with 2 mL of methanol. All samples were filtered through 0.45 μm nylon filters.
Phenolic acids were identified and quantified in a liquid chromatograph (Spectra-Physics UV6000LP), with a LiChrospher® 100 RP-18 column (250 mm x 4.6 mm, 5 μm particle size). Formic acid (10% in water) (A) and acetonitrile/water/formic acid (45:45:10) (B) were used as mobile phase, with a flow rate of 1 mL/min. The identification was made by comparing the retention times and UV-Vis spectra obtained using a diode array detection system (Thermo Scientific, USA), with reference standards. Hydroxybenzoic acids were quantified at 280 nm; hydroxycinnamic acid esters, at 315 nm.
Flavan-3-ols were identified and quantified in a liquid chromatograph (Thermo Spectra Physic Series P100, USA), coupled to a fluorescence detector (Perkin Elmer Series 200ª, USA), with a LiChrospher® 100 RP-18 column (250 mm x 4.6 mm and 5 μm particle size). Acetonitrile (A) and acetic acid (B) were used as mobile phase, with a flow rate of 1.4 mL/min. Flavanols were identified using the wavelengths λexc = 280 nm and λem = 320 nm. In this study, procyanidins were quantified as catechins.

2.4. Identification and quantification of betalains

Betalains were measured using the method of CASTELLANOS-SANTIAGO and YAHIA (2008). To do this, 100 mg of lyophilized prickly pear juice were weighed and 10 mL of 80% methanol acidified with 0.5% HCl were added; this mixture was sonicated for 15 min and filtered through a 0.45 μm nylon filter (Agilent Technologies, Alemania). An electronic scan was run between 400 and 700 nm in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Alemania), which identified the absorption peaks of betalains at 547 nm and 490 nm. Absorbance units were converted to concentration units.

2.5. Ascorbic acid quantification

Ascorbic acid was quantified using the method of SDIRI *et al.* (2012). To this end, 2 mL of 4.5% meta-phosphoric acid were added to 0.1 g of lyophilized juice; this mixture was sonicated in a water bath for 2 min, then centrifuged for 10 min at 5000 rpm, and finally filtered through 0.45 μm nylon filters. Ascorbic acid was quantified in a HPLC chromatograph (Thermo Spectra Physic Series P100), coupled to a UV detector (Thermo Finnigan Spectra System UV2000), with a LiChrospher® 100 RP-18 column (250 mm x 4.6 mm and 5 μm particle size), and KH_2PO_4 (0.2 M at pH=2.3-2.4) was used as mobile phase, with a flow rate of 1.0 mL/min for 15 min at λ = 243 nm and an injection volume of 20 μL. This compound was estimated using the following calibration equation y = 76165x - 161251, r^2= 0.9993.

2.6. FRAP (ferric reducing antioxidant power) method

The FRAP assay assessed the capacity of juice samples to reduce the ferric ion (Fe^{+3}) in a complex with 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), to the ferrous ion (Fe^{+2}), in accordance with FIRUZI *et al.* (2005). The FRAP reagent was prepared daily by mixing 10 mL of 300 mM sodium acetate buffer solution (pH 3.6) with 1 mL of 20 mM ferric chloride hexahydrate and 1 mL of 10 mM TPTZ dissolved in 40 mM hydrochloric acid. Twenty five microliters of prickly pear juice diluted 1/20 with methanol were added to 96-well flat bottom microplates in triplicate, followed by the addition of 175 μL FRAP solution. A control treatment was prepared with 200 μL methanol; another control was prepared by mixing 25 μL methanol with 175 μL FRAP; for a third control, 25 μL ferric sulphate and 175 μL sodium acetate buffer were added; finally, 25 μL of juice sample were added to 175 μL sodium acetate buffer solution. Readings were recorded with a Multiskan Ascent reader (Thermo Electron Corporation 100-240 VAC Type: 354) at 595 nm. The first reading was taken at time 0, and the plate was incubated at 37°C immediately afterwards. The second reading was taken after 60 min.
The calibration curve was constructed with ferric sulphate heptahydrate (7.194 mM) dissolved in methanol at concentrations of 108 μM to 864 μM. The resulting calibration equation was y = 0.0011x - 0.069, r^2= 0.9971.
The FRAP value for the curve was calculated according to the following equation:

$$FRAP(M) = \left({}^{\Delta a_t FI}/_{\Delta a_t Fe^{2+}}\right) x10^{-5}$$

Where
$\Delta a_t FI$ = change in absorbance of the analyte after the time interval.
$\Delta a_t Fe^{2+}$= change in absorbance of iron sulfate at the same concentration and after the time interval.

The results of each sample are expressed as μM $FeSO_4$ eq.

2.7. Estimate of the trolox equivalent antioxidant capacity (TEAC) with the chemical mediator $ABTS^{\cdot+}$

This estimate was made in accordance with the methodology of NENADIS *et al.* (2004). The TEAC of samples was based on 2,2'-azino-bis(3-ethylenebenzothiazoline-6-sulfonic acid) (ABTS), which produces the radical $ABTS^{\cdot+}$ and is compared with an antioxidant (trolox). For each evaluation, the $ABTS^{\cdot+}$ solution was prepared by mixing 5 ml of 7 mM ABTS and 88 μL of 140 mM potassium persulphate; the mixture was stored in the dark covered with aluminum foil and left to stand for 12 h at room temperature to produce the radical; afterwards, 500 μL of the solution were mixed with 25 mL of ethanol and its absorbance was read in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Alemania) to confirm that it was between 0.7 and 1 at 734 nm. Samples and controls were measured in triplicate (20 μL sample plus 230 μL $ABTS^{\bullet+}$) and placed in a 96-well flat bottom plate; after adding the radical ion, the plate was covered with aluminum foil and after 6 min the reading was recorded at 734 nm in a Multiskan Ascent Reader (Thermo Electron Corporation 100-240 VAC Tip: 354).
The percent inhibition of the standard was obtained with the following equation:

$$\% \text{ inhibition} = \left[\frac{Abs\ control - Abs\ sample}{\underset{control}{Abs}}\right] * 100$$

The absorbance of the sample was subtracted from the absorbance of the control to obtain the true absorbance.
The calibration curve was constructed with 50 μM, 100 μM, 200 μM, 300 μM, 400 μM and 500 μM trolox standards, adding 20 μL of the standard solution and 230 μL $ABTS^{\cdot+}$; the resulting regression equation was y = 0.2263x + 7.5033; r^2= 0.9979. The results were expressed as μg·mol trolox equivalent (TE) per gram of juice (dry weight).

2.8. Experimental design and statistical analysis

The experiment was performed according to a completely randomized experimental design. Treatments were the juices from the 10 prickly pear variants, which were tested for content of phenolic compounds, betalains and ascorbic acid, as well as their antioxidant capacity through FRAP and ABTS. Three replicates were used for each of these measurements. The data were subjected to an analysis of variance and Tukey's multiple comparison test. A Pearson correlation was carried out between FRAP and ABTS variables (SAS, version 8.0; SAS Institute, Cary, North Carolina).

3. RESULTS AND DISCUSSION

The potential of these prickly pear juices as functional foods is high due to outstanding content of soluble fiber and the adequate content and proportion of glucose to fructose (ZENTENO-RAMÍREZ *et al*., 2015). In order to determine whether consumption of prickly pear juices can help prevent or cure diseases associated with the excess of free radicals it is essential to identify and quantify the compounds with antioxidant capacity contained in prickly pear juice. Phenols in fruits, flowers and vegetables have attracted the attention due to their antioxidant potential. It has been shown that various parts of *Opuntia* (pulp, fruit skin, seeds and cladodes) are rich in polyphenols (GALATI *et al*., 2003; VALENTE *et al*., 2010; TOUNSI-SAIDANI *et al*., 2011). In addition, various studies have demonstrated their antioxidant effects (DOK-GO *et al*., 2003; TESORIERE *et al*., 2004; SIRIWARDHANA and JEON, 2004; OSORIO-ESQUIVEL *et al*., 2011).

3.1. Total phenol content

Due to their ubiquitous presence in plant foods, phenolic compounds are normally included in the daily human diet. The daily intake ranges between 25 mg and 1 g, depending on the amount of fruits, vegetables, pulses, tea and spices consumed (HAGERMAN *et al*., 1998). Raw extracts of phenol-rich plant products are attracting interest in the food industry, since these slow down the oxidative degradation of lipids, and hence improve the quality and nutritional value of food; their antioxidant power protects against heart disease and cancer, in addition to other chronic degenerative diseases (KÄKHÖNEN *et al*., 1999). The protection against LDL oxidation is not due to a single compound, but results from the effect of several phenolic compounds (RICCHELLE *et al*., 2001).
The total content of polyphenols was estimated in the ethanol extracts of lyophilized juice samples (Table 1). The statistical differences between prickly pear variants seem to be unrelated to fruit color and degree of humanization, and it should be noted that the four

O. megacantha variants evaluated showed the highest total content of phenolic compounds. Among the variants evaluated by MABROUKI *et al.* (2015), the highest concentration was observed in the pulp of *O. streptacantha*, followed by *O. ficus-indica*, with 104.6 GAE per 100 g of juice. In general, it has been pointed out that the concentration of phenolic compounds in prickly pears range from 54 mg/100 g to 104 mg/100 g fresh weight (KATABI *el al*., 2013; FIGUEROA-CARES, *et al.*, 2010). Thus, the concentration of phenolic compounds in prickly pear juice is similar or higher than in pineapple, tomato, banana, mango and cucumber (1.7, 2.0, 2.3, 2.6, and 3.8, all in mg/g dry weight, respectively) (MUÑÓZ-JÁUREGUI and RAMOS-ESCUDERO, 2007).

3.2. Quantification of phenols by high performance liquid chromatography (HPLC)

Table 1 shows the concentration of phenolic acids in the studied prickly pear variants, which show significant differences ($P < 0.0001$). Gallic acid was recorded in all variants except Tapona, and was the main phenolic compound in most of them, with varying concentrations between 32.6 μg/g and 81.2 μg/g. Syringic acid was absent only in Torreoja and Cardona, and ellagic acid in Blanca, Sangre de Toro and Tapón Rojo. Protocatechic acid was recorded only in Pico Chulo (41.6 μg/g). Pico Chulo showed the four phenolic acids and recorded the highest total phenolic acid concentration (176 μg/g). By contrast, Blanca showed the lowest total phenolic acid content (79.4 μg/g).

Table 1. Average concentration (μg/g) of total phenols and phenolic acids in lyophilized juices of 10 prickly pear variants.

Variant*	Total phenols	Gallic acid	Syringic acid	Ellagic acid	Total phenolics acids
Rojo Pelón	1.92±0.11^{de}	32.6±0.6^{g}	29.2±0.9^{d}	25.0±0.9^{e}	86.9±2.3^{e}
Blanca	1.93±0.25^{de}	53.7±0.6^{e}	25.6±0.4^{e}	n.d.	79.4±0.8^{e}
Amarilla Monteza	3.81±0.75^{bc}	74.8±3.6^{bc}	13.6±0.3^{h}	33.5±0.1^{d}	122.0±3.8^{b}
Pico Chulo	2.81±0.39^{bcd}	63.6±0.6^{d}	20.0±2.7^{f}	50.5±2.3^{b}	176±7.9^{a}
Torreoja	3.90±0.06^{ab}	49.7±1.8^{e}	n.d.	41.9±1.9^{c}	91.6±3.6^{d}
Sangre de Toro	5.21±0.83^{a}	42.4±0.5^{f}	66.5±0.1^{a}	n.d.	109±1.2^{c}
Cardona	1.67±0.36^{de}	81.2±0.7^{a}	n.d.	26.7±0.4^{e}	108.0±1.0^{c}
Charola	1.69±0.48^{de}	78.3±1.0^{ab}	16.9±0.3^{g}	73.2±1.5^{a}	168±2.2^{a}
Tapona	2.52±0.42^{cde}	n.d.	45.3±1.1^{b}	68.3±4.1^{a}	114.0±5.2^{bc}
Tapón Rojo	1.45±0.14^{e}	71.5±0.1^{c}	38.4±0.3^{c}	n.d.	110.0±1.2^{c}
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Variants are sorted from highest to lowest degree of humanization.
n = 3. Treatments with different letters in the same column are statistically different (<0.05).
n.d.= not detected

Flavonoids are the dominant class of phenols in food, accounting for approximately two thirds of the phenols consumed in the human diet (LOTITO and FREI, 2006). Table 2 shows the concentrations of the flavan-3-ol derivatives found in the juice of all prickly pear variants studied, with significant differences ($P < 0.0001$) between them. These four derivatives were recorded in the juice of all variants; however, catechin was not found in Blanca, being the derivative found at the lowest concentration in all variants except Charola, where epicatechin attained the lowest concentration. The derivative registered at the highest concentration in these prickly pear juices was either epicatechin or procyanidin

B2, according to the variant. The highest epicatechin concentrations were found in Tapona juice, and the lowest in Cardona, with 90.8 μg/g and 17.2 μg/g, respectively. With regard to the total content of flavan-3-ol derivatives, the Tapona juice showed the highest concentration (223±6.09 μg/g), and also the highest levels of each individual derivative; in contrast, the Cardona juice showed the lowest concentration of these derivatives (73.7 μg/g).

Table 2. Average concentration (μg/g) of flavan-3-oles in lyophilized juices of 10 prickly pear variants.

Species	Variant*	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2	Total flavan-3-oles
O. ficus-indica	Rojo Pelón	13.30±0.63^{de}	19.26±1.68^{f}	16.71±0.13^{f}	28.36±0.38^{e}	77.6±1.30^{f}
O. albicarpa	Blanca	n.d.	60.94±1.26^{b}	32.50±1.49^{d}	38.76±0.43^{c}	132±3.18^{c}
O. megacantha	Amarilla Monteza	14.23±0.69^{cd}	37.4±0.16^{c}	21.03±0.74^{ef}	33.93±0.55^{d}	107±4.82^{d}
	Pico Chulo	14.87±0.6^{cd}	24.58±0.02^{e}	23.59±1.25^{e}	29.51±1.19^{de}	92.5±1.86^{e}
	Torreoja	19.61±0.94^{b}	32.10±0.39^{d}	25.40±0.87^{e}	20.37±0.40^{f}	97.5±1.80^{de}
	Sangre de Toro	19.61±1.60^{b}	61.93±0.77^{b}	40.67±1.40^{c}	51.62±1.31^{a}	174±1.88^{b}
O. streptacantha	Cardona	10.44±0.18^{e}	17.15±0.45^{f}	22.41±0.51^{e}	23.68±1.08^{f}	73.7±2.21^{f}
O. streptacantha ssp. *aguirrana*	Charola	27.25±0.95^{a}	17.97±0.98^{f}	47.06±2.19^{b}	44.45±0.28^{b}	137±4.40^{c}
O. robusta	Tapona	27.89±0.97^{a}	90.81±2.18^{a}	59.47±0.90^{a}	45.20±2.05^{b}	223±6.09^{a}
	Tapón Rojo	17.56±1.14^{bc}	19.16±0.30^{f}	24.67±1.56^{e}	23.63±1.81^{f}	85.1±4.21^{ef}
P value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Variants are sorted from highest to lowest degree of humanization.
n = 3. Treatments with different letters in the same column are statistically different (<0.05).
n.d.= not detected

3.3. Concentration and identification of betalains

According to STINTZING *et al*. (2005), prickly pear color is due to betalains, since these authors recorded indicaxanthin and betaxanthins (84 mg/kg and 100 mg/kg, respectively) in yellow-orange prickly pears, while red prickly pears contained betacyanins at concentrations of 400 mg/kg, as the chemicals responsible for this color. As shown in Table 3, the juice of red-colored prickly pears have a higher betacyanin content, while betaxanthins predominate in the yellow variants (Amarilla Monteza and Pico Chulo), a finding that is consistent with other studies (STINTZING *et al*., 2005; CHÁVEZ *et al*., 2009; YAHIA and MONDRAGÓN, 2011). The Tapona juice showed the highest content of betacyanins and betaxanthins, but its purple-red color derives from the prevalence of betacyanins. The intermediate values of both compounds in the juice of the red *O. megacantha* variants is worth noting, as well as the minimum content of them in Blanca juice; this finding coincides with the results of CASTELLANOS-SANTIAGO and YAHIA (2008) for the same species.

Table 3. Average content of betaxanthins and betacyanins (mg/g dry weight) in lyophilized juices of 10 prickly pear variants.

Species	Variant*	Betaxanthins	Betacyanins	Total betalalains
O. ficus-indica	Rojo Pelón	0.148±0.005^{f}	0.149±0.010^{g}	0.298^{g}
O. albicarpa	Blanca	0.018±0.003 ^{g}	0.021±0.004^{h}	0.044^{h}
O. megacantha	Amarilla Monteza	0.120±0.007 ^{f}	0.011±0.001^{h}	0.130^{h}
	Pico Chulo	0.085±0.017^{f}	0.019±0.004^{h}	0.105^{f}
	Torreoja	0.313±0.029^{e}	0.358±0.030^{f}	0.671^{ef}
	Sangre de Toro	0.810±0.007^{c}	1.580±0.030^{c}	2.390^{c}
O. streptacantha	Cardona	0.423±0.030^{d}	0.800±0.008^{d}	1.213^{d}
O. streptacantha ssp. *aguirrana*	Charola	0.290±0.007^{e}	0.660±0.020^{e}	0.945^{e}
O. robusta	Tapona	1.450±0.017^{a}	2.610±0.030^{a}	4.074^{a}
	Tapón Rojo	1.230±0.04^{b}	2.380±0.060^{b}	3.610^{b}
P value		<0.0001	<0.0001	<0.0001

*Variants are sorted from highest to lowest degree of humanization.
n = 3. Treatments with different letters in the same column are statistically different (<0.05).

3.4. Quantification of ascorbic acid by HPLC

Ascorbic acid is one of the most effective and abundant antioxidants in fruits and vegetables (LOGANAKI and MANIAN 2010), participating in various biological functions that include the synthesis of collagen, hormones and neurotransmitters. The increase in the consumption of ascorbic acid is associated with a lower risk of chronic diseases such as cancer, cardiovascular disease and cataracts. This may be due to its ability to eliminate free radicals in biological systems. This study only measured ascorbic acid content (Table 4) without performing the reduction of dehydroascorbic acid (DHAA), necessary to obtain the total vitamin C content (SDIRI *et al*., 2012).

Table 4. Average concentration of ascorbic acid (mg/g dry weight) in lyophilized juices of 10 prickly pear variants.

Species	Variant*	Ascorbic acid
O. ficus-indica	Rojo Pelón	1.328±0.003^{a}
O. albicarpa	Blanca	0.316±0.003^{d}
O. megacantha	Amarilla Monteza	0.327±0.016^{d}
	Pico Chulo	0.542±0.004^{c}
	Torreoja	0.327±0.004^{d}
	Sangre de Toro	0.652±0.041^{b}
O. streptacanta	Cardona	0.325±0.006^{d}
O. streptacanta ssp. *aguirrana*	Charola	0.191±0.000^{e}
O. robusta	Tapona	0.527±0.029^{c}
	Tapón Rojo	0.691±0.006^{b}
P value		<0.0001

n=3. Treatments with different letters in the same column are statistically different (<0.05).
*Variants are sorted from highest to lowest degree of humanization.

Ascorbic acid concentration showed significant differences between the prickly pear juices evaluated ($P < 0.0001$).
The highest ascorbic acid content was recorded in Rojo Pelón, followed by Sangre de Toro and Tapón Rojo; Charola was the variant with the lowest ascorbic acid concentration in juice. However, all concentrations measured were sufficient to meet easily the minimum daily intake (84 mg) of ascorbic acid in the human diet (SÁENZ *et al*., 2007).
Among the variants evaluated by YAHIA and MONDRAGÓN (2011), the highest ascorbic acid concentration was recorded in the juice of the Camuesa prickly pear (*O. robusta*), followed by Cardona (*O. streptacantha*), with 4.0 mg/100 g and 2.1 mg/100 g fresh weight, respectively, while the lowest concentration was observed in the juice of Naranjona (*O. megacantha*), ranging between 1.2 mg/100 g and 1.4 mg/100 g fresh weight; according to these authors, DHAA showed a pattern similar to that of ascorbic acid. In general, it has been pointed out that the concentration of ascorbic acid in prickly pear (*Opuntia* spp.) ranges from 12 mg/100 g to 81 mg/100 g fresh weight (FEUGANG *et al*., 2006). Thus, the concentration of ascorbic acid in prickly pear juice is similar to or higher than in grapes, apple and pear (0.5 mg/g, 0.3 mg/g and 0.2 mg/g edible dry weight, respectively), but lower than in guava and kiwi fruit (9.4 mg/g and 4.9 mg/g edible dry weight, respectively) (LOTITO and FREI, 2006). To note, the variants with the highest and lowest degree of humanization showed the highest concentrations of this antioxidant, suggesting that this process is unrelated to the concentration of this antioxidant.

3.5. Antioxidant capacity of prickly pear juice *in vitro*

The antioxidant capacity of prickly pear juice was estimated through ABTS and FRAP, since both are the assays most frequently used and they measure most antioxidants present. ABTS is typically used for mixtures or complex beverages, and measures mainly SET (single electron transfer) antioxidants, without excluding HAT (hydrogen atom transfer) antioxidants, in both water-soluble and fat-soluble media. In contrast, FRAP is applicable mostly to vegetables with SET and HAT antioxidants, mainly phenols and ascorbic acid (SURVESWARAN *et al.*, 2007; GÜLÇIN, 2012). These methods were considered as mutually complementary and were contrasted through the correlation between their respective results.
SURVESWARAN *et al*. (2007) point out that various herbs, fruits and vegetables show a direct relationship between antioxidant capacity and total phenolic content. In the prickly pear juices evaluated, this trend was not observed due to their contrasting differences in color, related to the presence of antioxidants such as ascorbic acid and betalains (Table 5). The data obtained with ABTS were normally distributed, but those with FRAP had to be log-transformed before being analyzed. The estimates of antioxidant capacity obtained with both methods (FRAP and ABTS) for total phenols, betalains and ascorbic acid in the juice of the 10 prickly pear variants were compared through a simple linear correlation analysis (Table 6).
All correlation values for each comparison had the same sign, which evidences a consistent general trend in the estimates obtained with both methods. The results show that the estimates of the reduction ability of betalains with FRAP and ABTS were positively and significantly correlated ($P < 0.0001$). On the other hand, the estimates for ascorbic acid and phenolic compounds were not significantly correlated. The significant correlation of the total antioxidant capacity between both methods is explained by the abundance of betalains and because both methods produced similar estimates of the antioxidant capacity for all other compounds tested.

Table 5. Antioxidant total capacity of juices of 10 prickly pear variants.

Species	Variant*	ABTS$^{\cdot+}$ TEAC (μM/g dry weight)	FRAP (μM eq. FeSO4/g dry weight)
O. ficus-indica	Rojo Pelón	43837±2601^{abc}	49102±4280^{cd}
O. albicarpa	Blanca	39570±8473 ^{c}	45202±4098^{cd}
O. megacantha	Amarilla Monteza	37504±6726^{c}	38490±2591^{d}
	Pico Chulo	38307±6833^{c}	39157±2583^{d}
	Torreoja	46264±8198^{abc}	42378±9272^{cd}
	Sangre de Toro	51422±400^{abc}	79066±7562^{b}
O. streptacantha	Cardona	45501±3565^{abc}	60141±4645^{bc}
O. streptacantha spp. aguirrana	Charola	40778±3741^{bc}	57543±4843^{cd}
O. robusta	Tapona	62117±10439^{a}	112651±15066^{a}
	Tapón Rojo	59968±12243^{ab}	118790±16262^{a}
P value		<0.0016	<0.0001

*Variants are sorted from highest to lowest degree of humanization.
n=3. Treatments with different letters in the same column are statistically different (<0.05).

Table 6. Correlation (r) between estimates of antioxidant capacity generated by ABTS and FRAP methods in the juices of 10 prickly pear variants.

Antioxidant	ABTS	FRAP
Total	0.7845 ***	0.9436 ***
Total phenolic compounds	0.0848	-0.11811
Phenolic acids	-0.2033	-0.0943
Flavan-3 ols	0.3943	0.4781
Betalains	0.7828***	0.9511***
Ascorbic acid	0.1829	0.1635

Significance *** $P < 0.0001$.

4. CONCLUSIONS

The higher content of betalains in the red prickly pear variants Tapona, Tapón Rojo and Sangre de Toro, and of ascorbic acid in Rojo Pelón, Tapón Rojo and Sangre de Toro, resulted in their total antioxidant capacity being higher than in the other color variants, and explains the lack of a significant correlation between the estimates of the antioxidant capacity of phenols. Of the variants evaluated, Pico Chulo was the richest in phenolic acids, and Tapona in flavan-3-ols. Unlike other table fruits, prickly pear is an important source of phenols, in addition to having the most common antioxidant phytochemicals, such as betalains and ascorbic acid.
Therefore, this study of prickly pear juice *in vitro* provides further support for recommending consumption of prickly pear juice as a functional food due to its antioxidant properties similar or superior to the juice of various marketed fruits. The study confirms the antioxidant capacity of the analyzed fruit, however before prickly pear fruit can be considered a potential functional food it is important to highlight the importance of running *in vivo* studies (animals and humans) in order to confirm

bioavailability of the compounds analyzed in the study and to find whether consumption of prickly pear fruit actually induces positive effect in promoting a healthy status.

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PAPER

EFFECT OF CHITOSAN COATING ENRICHED WITH CUMIN (*CUMINUM CYMINUM* L.) ESSENTIAL OIL ON THE QUALITY OF REFRIGERATED TURKEY BREAST MEAT

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ABSTRACT

A solution containing 2% chitosan, 1% cumin seeds' essential oils and 1% acetic acid was applied for the coating preparation. In all of the treatments the total viable counts and applied psychrophilic bacteria decreased significantly compared to the control through the storage time. The pH, total volatile basic nitrogen (TVB-N), peroxide value (PV) and sensory attributes in all the treatments were significantly detected lower than the same parameters of the controls. The results of our investigation revealed that chitosan+cumin and ascorbic acid retarded spoilage and oxidative changes in refrigerated turkey breast meat.

Keywords: antibacterial properties, lipid oxidation, meat products, natural antioxidant

1. INTRODUCTION

Products produced by poultry meats are considered as the food products with considerable growing interest in market in many parts of the world due to their low production cost compared to further meat products including beef, lamb and pork meats. They contain low fat, high nutritional value as well as distinct interesting taste and flavor. However, poultry meats due to their nature and composition, (i.e high moisture and protein contents) as well as high pH value (low acidity value), they have ideal and appropriate environments for pathogenic microorganisms' growth (LATOU *et al.,* 2014; VAITHIYANATHAN *et al.,* 2011). The approach to extend and increase the shelf life of raw poultry meats and its products may present a challenge for meat and poultry industries not only in Iran but also in further areas. Microbial growth as well as lipid oxidation reactions may lead to undesirable and unwanted organoleptic alterations through the storage period in meat products (OUATTARA *et al.,* 2000). One of the most commonly used methods to preserve food products safe and increase their shelf life is the addition of natural compounds with antibacterial and antioxidant properties in food products. Since synthetic antioxidants have been reported to behave as carcinogens and mutating agents, more attention has been directed to the employment of natural antioxidants (TAHERI *et al.,* 2012; WANNES *et al.,* 2010). Several studies have been performed in this area including application of essential oils obtained from plant sources (RAEISI *et al.,* 2015; CHEN *et al.,* 2014; TAHERI *et al.,* 2013, ALLAHGHADRI *et al.,* 2010), chitosan obtained from natural origins (OJAGH *et al.,* 2010; NO *et al.,* 2007), and organic acids of natural resources (SHALTOUT *et al.,* 2014; BIN JASASS, 2008) with the aim of retardation of microbial deteriorations and oxidation reactions (LATOU *et al.,* 2014).
Chitosan is categorized as a modified and natural carbohydrate polymer derived from deacetylation of chitin [poly-β-(1 → 4)-N-acetyl -D-glucosamine), and is a major component of the shells of crustaceans including crab, shrimp, and crawfish and is ranked second in the abundance among further natural biopolymers (after cellulose) (NOWZARI *et al.,* 2013; NO *et al.,* 2007). Due to the chitosan's intrinsic antimicrobial and anti-oxidative properties and having appropriate characteristics in film preparation, as well as its biocompatibility and biodegradability properties, chitosan has attracted much attention as a natural additive (preservative) in not only nutraceutical but also in pharmaceutical and cosmetic industries (YUAN *et al.,* 2016; KANATT *et al.,* 2013; FAN *et al.,* 2009). Several studies have been performed on the antibacterial, antioxidant, and potential health benefit properties of the essential oils extracted from spices and herbs particularly from *Apiacea* family (ALLAHGHADRI *et al.,* 2010; ZHANG *et al.,* 2009). One of these valuable plants is cumin (*Cuminum cyminum* L.), named "zira" growing in Middle East particularly in Iran. This plant species is also commonly grown in Cyprus, Lebanon, Morocco, Malta, Turkey, Spain, Russia and India as well as China (ERDENI *et al.,* 2013). Antioxidant and antibacterial activities are detected as one of the most considerable functional properties of cumin seeds. Cumin seeds may have considerable potential to be used as an antioxidant agent in food products; the achieved results of several performed studies have shown that γ-terpinene is a predominant detected compound with potential health benefits in cumin seeds (RAEISI *et al.,* 2015; CHEN *et al.,* 2014; IACOBELLIS *et al.,* 2005). Application of a biodegradable film or coating is considered as a novel approach to protect not only meat products but also further food products against deteriorating agents (NOWZARI *et al.,* 2013). A combination of chitosan and cumin essential oils may be formulated to prepare biodegradable films with considerable protective effects. The aim of the present study is to evaluate and assess the effect of chitosan coating enriched by cumin essential oil in combine of acetic acid to be used in enhancements of the shelf life and quality of turkey fillet stored at (4±1°C).

2. MATERIALS AND METHODS

2.1. Turkey breast preparation

Fresh turkey breast samples (18 kg) were purchased from a local market in Ahvaz (Khuzestan province, South Iran). The average weight of each breast piece was set on 3 kg. The turkey breast samples were placed and sealed in an ice box with ice (refrigeration temperature) and transferred to Food and Drug Administration Laboratory at Jundishapur University within 30 minutes. Turkey breast samples were filleted manually and carefully washed with cold water. The weight of each fillet was set on 110±5 g. The prepared fillets were used for the selected experiments.

2.2. Cumin seeds

Cumin seeds' essential oil (*Cuminum cyminum* L.) and the required chemicals and reagents for GC-MS analysis (gas chromatography - mass spectrometry) were provided from Barij Essence Company in Iran.

2.3. Preparation of the coating film and treatment of the fillets

A chitosan solution was prepared with 2% (W/V) chitosan (Sigma Chemical Co, medium molecular weight, viscosity 200-800 cP) in 1% v/v acetic acid. 20 g of chitosan solution was mixed well with 900 mL of distilled water and the obtained mixture was stirred for 10 min, afterward 10 mL of glacial acetic acid was added to the mixture and stirred at room temperature by achieving a smooth solution. The solution was reached and diluted up to 1000 mL by the addition of distilled water. Glycerol was added as a plasticizer to the achieved solution with the concentration of 0.75 mL. g^{-1} and was stirred for 10 min (NOWZARI *et al.*, 2013; OJAGH *et al.*, 2010). Thereafter the cumin essential oil (CEO), (mixed with Tween 80 (Aldrich Chemical Co., Steinheim, Germany), was added to the final prepared solution (BAZARGANI GLILANI *et al.*, 2015; OJAGH *et al.*, 2010) to comfort distribution and complete incorporation of cumin seeds' oil in the final solution. The final coating solution consisting of 2% chitosan, 1% acetic acid, 0.75 % glycerol and 0.2 % Twen 80 as well as cumin seeds' essential oil 1% was homogenized under aseptic conditions for 1 min. The fillet samples were divided in 3 separated groups. Samples of the first group were left untreated (Control, Sterile distilled water), and two other groups were treated by the following solution: chitosan 2% - CEO 1% and acetic acid 1%. Each sample was immersed for 30 min in the solution (YUAN *et al.*, 2016). Then the prepared meat samples were removed from the solution and allowed to drain for 30 min before packaging (coating) (KANATT *et al.*, 2013). After that, all of the fillet samples were individually packed in sterile LDPE containers; all of the containers were kept in a refrigerator at the temperature of 4±1°C. Fillet samples were experimented on days 0, 3, 6, 9, 12 and 15 after packaging and storage, and analyzed for chemical and microbial tests as well as sensory alterations analysis. Each experiment was done in triplicate.

2.4. Microbial analysis

10 g of each sample was mixed with 90 mL of sterile saline (0.85 % NaCl) solution in a sterile stomacher bag and was stomached continuously for 1 min. Other decimal dilutions were prepared from the achieved uniform solution or dilution of stomacher and cultivated in an appropriate microbial medium regarding the type of the microbial test. The Total viable count (TVC) was determined by pour plate medium approach with the use of plate

count agar (PCA) (Merck, Germany). The inoculated plates were incubated at 37 °C for 48 h for total viable count, and at 10 °C for 7 days for psychrophilic count. The achieved results were expressed as log 10cfu. g^{-1} of samples (NOWZARI *et al.*, 2013).

2.5. pH value determination

For determination of pH value, 5 g of turkey breast samples (of each treatment) were homogenized for 1 min with 45 mL of distilled water. The pH value was measured using a standardized portable pH meter (TOA, Kobe, Japan; TAHER*I et al*., 2013).

2.6. Determination of peroxide value (PV)

Peroxide value (PV) was determined in the lipid extract according to the method described previously by (AOAC, 2000). The achieved results were expressed as milli-equivalents peroxide value per each kg of lipid (meq O2/kg lipid).

2.7. Determination of Total Volatile Basic Nitrogen (TVB-N)

Total volatile basic-nitrogen (TVB-N) values were detected by the direct distillation approach according to (GOUDLAS and KONTOINAS, 2005) method. The micro diffusion method was determined by distillation after the addition of MgO to the homogenized turkey breast samples. The TVB-N value (mg nitrogen. 100 g^{-1} breast sample) was determined regarding the consumed volume of sulphuric acid reagent.

2.8. Sensory evaluation and analysis

Sensory analyses were performed by an instructed taste panel consisting of five experienced judges, according to the guidelines presented in Table 1 (OCTAVIAN and OCTAVIAN, 2010). Three different categories were ranked in the prepared questioners: 3 scores for excellent, 2 scores for acceptable, and 1 for unacceptable rates. The assessed and studied parameters in sensory assessment were detected as the following: appearance, odor, color, consistency and elasticity.

Table 1. Sensorial attributes and quality of refrigerated turkey breast.

Attribute	Excellent	Acceptable	Unacceptable
Appearance	Without slime present on surface	Slime present in some part of the surface	Slime present on the entire surface
Muscular elasticity	Fast return	Slow return	No return
Odor	Characteristic	Off odors (slight sulphurous or ammoniacal)	Foreign (rancid, acid, putrid)
Color	Pink	Dark pink	Pale pink

2.9. Statistical analysis

SPSS software version 22 was applied for data analysis in the present study. All of the selected experiments for each sample were performed in triplicate. Nonparametric statistics were used to analyze the achieved data. The analysis of Variance (ANOVA) test

was applied to detect the significant difference among the obtained data in the confidence level of 0.05. Duncan's multiple range tests were applied to compare the achieved means in the confidence level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Microbial analysis

It has been well known that through the refrigeration storage time of poultry meat, an extensive range of bacterial species may be characterized (VASILATOS and SAVVAIDIS, 2013). Changes in the value of total viable counts (TVC) in turkey breast fillet during the refrigerated storage are presented in (Fig. 1). The initial TVC (log10 cfu. g^{-1}) of the control, acetic acid and chitosan-cumin treatment were detected 4.77, 4.62, and 3.90 (log10 cfu. g^{-1}), respectively. According to the recommended and standard limits (7 log10 cfu. g^{-1}) for fresh meat (RAEISI *et al.*, 2015), the samples have shown acceptable quality. These achieved results were in solid agreement of those from BAZARGANI-GILANI et al. (2015) and LATOU et al. (2014) for fresh chicken meat, and RAEISI et al. (2015) in term of fish fillet. All of the studied samples expressed an increased TVC value with enhancements in storage time ($p<0.05$). In 6 days after storage, the obtained mean of TVC of control samples was detected 6.54, which was close to the maximum allowed limit of TVC (7 log10 cfu. g^{-1}) for raw meats, indicating the shelf life confined 5 - 6 days. This phenomenon (6 days shelf life) may be attributed to the longer acidic environment started after the bleeding of slaughtered animals (SHALTOUT *et al.*, 2014). For untreated samples (control), the TVC presented a value rather than the acceptable healthy limit in 9 days after storage time (8.76 log10 cfu. g^{-1}). In contrast, the TVC values for the treated samples with Acetic acid (1%) and Chitosan (2%) + Cumin (1%) was determined lower than the proposed standard value by the end of day 15^{th} of storage period. Reduction in microbial count by floating of the samples in the prepared solution mixed of chitosan, cumin and acetic acid, have been reported previously for chicken meat by (BIN JASASS, 2008), turkey breast by (VASILATOS and SAVVAIDIS, 2013) and shrimp by (YUAN *et al.*, 2016). In the present study, the Chitosan + Cumin 1% treatment was the most effective formulation against TVC.

Gram-negative psychotrophic bacterial species (PTC) are detected as the major group of microorganisms leading to spoilage of aerobically stored fresh meats at chilled temperatures (NOWZARI *et al.*, 2013; OJAGH *et al.*, 2010). In the current study, the initial PTC (day 0) in the control sample was detected 3.55 (log10 cfu. g^{-1}), it was determined 4.04 and 3.194 (log10 cfu. g^{-1}), in meat samples coated by AA and Ch + C, respectively. Furthermore, the growth pattern on TVC and PTC expressed an increasing rate through storage period (Fig. 2). For all of the treatments, storage time had significant ($p < 0.05$) effects on the PTC value (log10 cfu. g^{-1}), On day 6^{th} of storage time, the mean value of PTC of the control samples increased up to 7.57 and spoilage started to appear as a slight foul smell. A significant reduction ($p<0.05$) of PTC value was detected in the samples treated by AA compared to the control ones. The effectiveness of acetic acid against microorganisms may be attributed to a decrease in pH and metabolic inhibition by the undissociated acid molecules as detected and reported by BIN JASASS (2008). In control samples, the PTC was detected rather than the acceptable limit on day 6^{th} of storage time (7.57 log10 cfu. g^{-1}). In contrast, the TVC values for the treated samples with chitosan + cumin (5.78 log10 cfu. g^{-1}) remained lower than the proposed standard value by the end of day 15^{th} of storage period. The antimicrobial activity of cumin essential oil might be attributed to the phenolic compounds (BAZARGANI-GILANI *et al.*, 2015; RAEISI et al.,

2015; ALLAHGHADRI *et al.*, 2010). Chitosan has been reported to be effective as an antimicrobial agent (SHAHIDI *et al.*, 1999; FAN *et al.*, 2009; KANATT *et al.*, 2013). Chitosan may act on the cells of the microorganisms and pathogens, therefore by alterations in the permeability of the cytoplasmatic membranes, may lead to the leakage of intracellular electrolytes and protein compounds out, as a result, it may lead to the death of the cells (YUAN *et al.*, 2016; BAZARGANI-GILANI *et al.*, 2015). The mechanism of action of chitosan appears to be associated with the disruption of the lipopolysaccharide layer of the outer membrane of gram-negative bacteria (NOWZARI *et al.*, 2013; PEREDA *et al.*, 2011), as well as its function as a barrier against oxygen penetration (OJAGH *et al.*, 2010; JEON *et al.*, 2002).

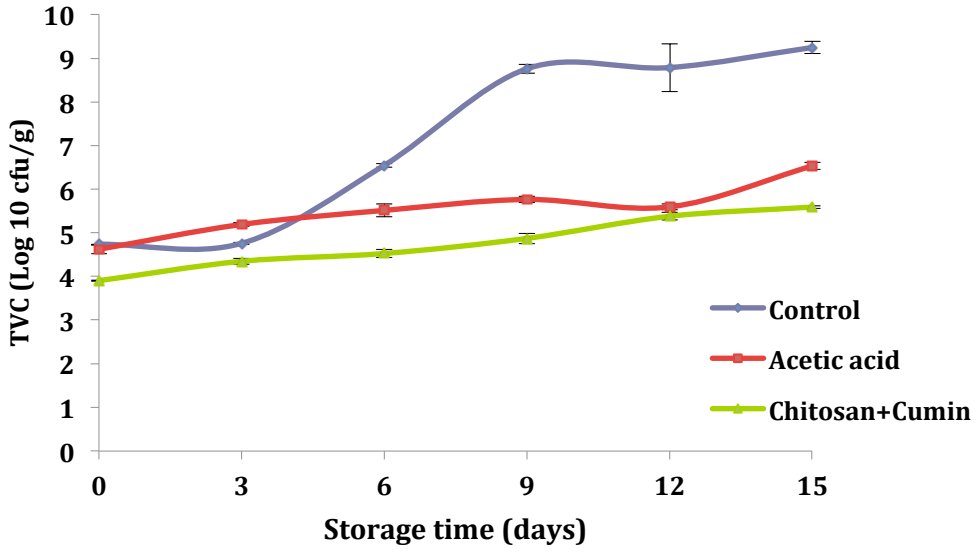


Figure 1. Changes in total viable count (TVC) of turkey breast samples during refrigerated storage.

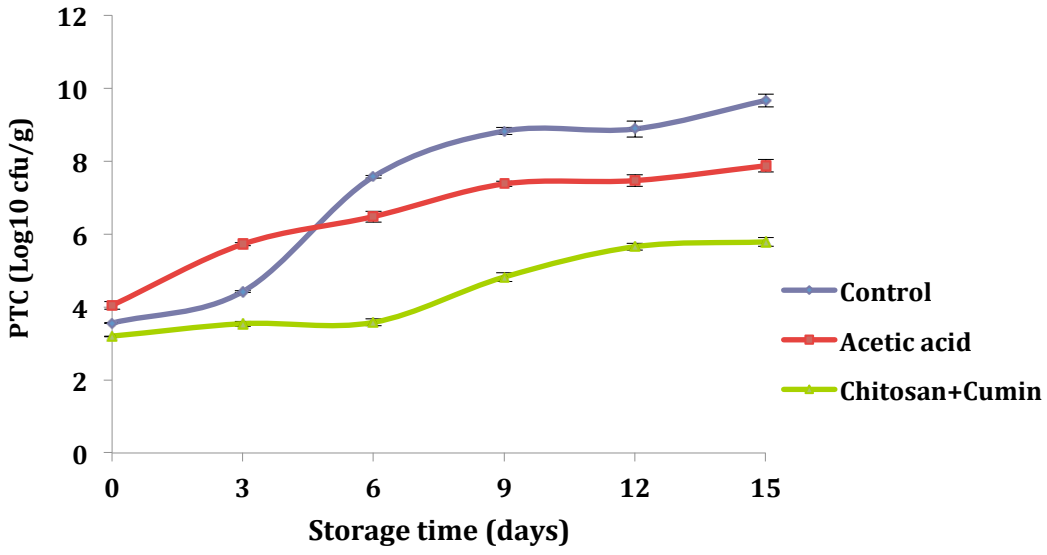


Figure 2. Changes in psychrotrophic counts (PTC) of turkey breast samples during refrigerated storage.

3.2. pH value

The pH and its alteration during storage time (0, 3, 6, 9, 12 and 15 storage days) for turkey breast in the control and two other treatments (AA and Ch+C) have been shown in (Fig. 3). An increase in pH from 5.86 to 7.07, 5.32 to 5.87, and 5.14 to 5.20 in the Control, samples treated by AA 1%, and Ch 2 % + C 1% was observed respectively, during 15 days storage time. For all of the treatments, storage time showed a significant ($p < 0.05$) effect on the pH values.. The initial pH value of the control sample was significantly ($p < 0.05$) higher than those from all of the treated samples. In all of the turkey breast samples, the values of pH showed a decrease by the day 6th of storage time, and then increased significantly. Decrease of pH may be attributed to increasing of solubility of CO_2 at storage time, affecting growth of aerobic microflora (TAHERI *et al.*, 2013), while an increase in pH of the control sample may be due to an increase in volatile compound contents (e.g. ammonia and trimethylamine), produced by either endogenous or microbial enzymes through storage time (FAN *et al.*, 2009; BAZARGANI–GILANI *et al.*, 2015). No significant difference was observed between the pH values of treatment groups (AA and Ch+C) through 15 days of storage time ($p>0.05$), with exceptions in samples stored for 3 and 15 days ($p< 0.05$). Samples treated by acetic acid, chitosan and cumin essential oils expressed a gradual increase throughout storage period, probably due to the presence of the acidified antimicrobial agents (BAZARGANI–GILANI *et al.*, 2015) and phenolic compounds (IBRAHIM and EL-SHERIF, 2008). The achieved results of the present study are in solid agreement of those for chicken breast (containing pomegranate juice and chitosan and essential oil) (BAZARGANI–GILANI *et al.*, 2015), beef meat (containing organic acid) (SHALTOUT *et al.*, 2014) and marinated chicken thigh (sodium lactate and lactic acid) (SMAOUI *et al.*, 2012).

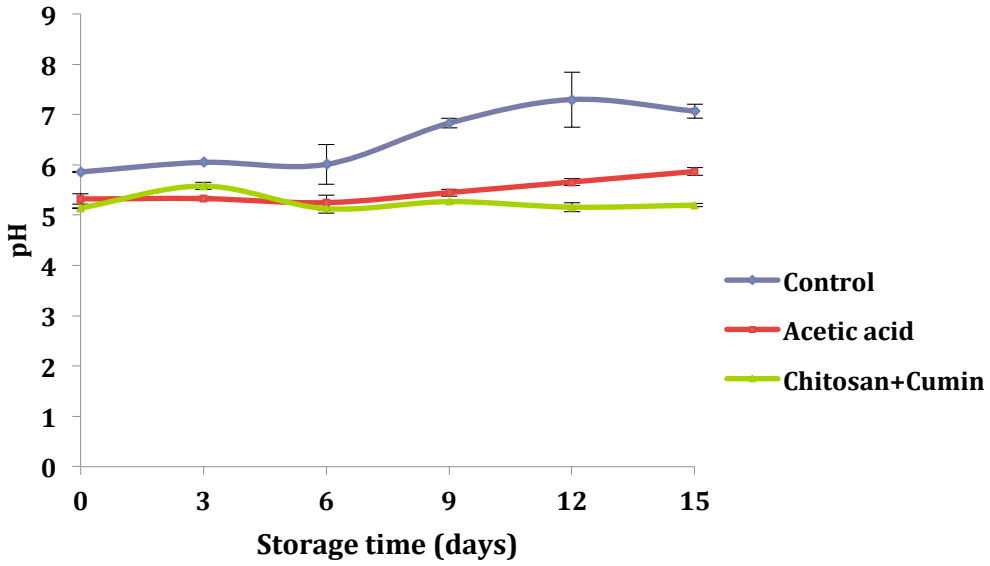


Figure 3. Changes in pH values of turkey breast samples during refrigerated storage.

3.3. Total volatile basic nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) mainly composed of ammonia and primary, secondary and tertiary amines, is extensively used as an indicator for meat deterioration determination. An increase in this parameter may be attributed to the activity of bacterial species and endogenous enzymes (FAN *et al.*, 2009; KYRANA *et al.*, 1997; NOWZARI *et al.*, 2013; DUAN *et al.*, 2010). The TVB-N values for turkey breast samples through storage period have been presented in (Fig.4) as well.

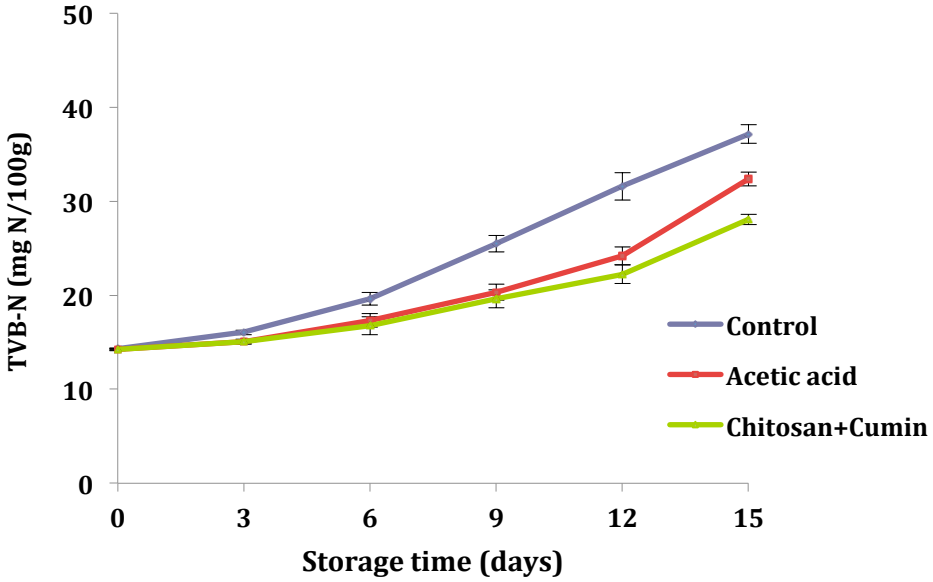


Figure 4. Changes in TVB-N values of turkey breast samples during refrigerated storage.

An increase in TVB-N value was observed from 14.30 to 37.16, 14.23 to 32.40, and 14.23 to 28.06 mg N. 100 g^{-1}, in Control, samples treated by AA 1%, and Ch+C 1%, respectively, through storage time for 15 days. According to (BALAMATSIA *et al.*, 2006), the value of 28-29 mg N. 100 g^{-1} is proposed as the highest acceptable level in poultry meat products. In the present study, the TVB-N values were detected higher than the acceptable limit by 12 and 15 days of storage time for the untreated samples (control) and the treated ones by acetic acid (1%), respectively. However, TVB-N values in treated samples by chitosan + cumin remained lower than the limit of acceptable index throughout the entire storage time. Higher microbial counts resulted in a significant increase in the basic nitrogen fraction for the untreated samples compared to chitosan treated samples (NOWZARI *et al.*, 2013; MOHAN *et al.*, 2012). The TVB-N level presented enhancements gradually along with the time of storage for the control and both of the treated samples ($p<0.05$). By 6 days after storage, the TVB-N value of the control samples increased significantly compared to the treated samples, ($p<0.05$). At the end of the storage time, the TVB-N value of the control sample was detected significantly rather than the studied treatments ($p<0.05$). These achieved results are in line with those of previous studies that reported, the TVB-N value of fish and chicken meats treated by acetic acid, chitosan, and cumin essential oil showed reductions significantly ($p<0.05$) (RAEISI *et al.*, 2015; SMAOUI *et al.*, 2012). An increase rate in TVB-N value of turkey breast treated by chitosan coating was also

inhibited compared to the control, that is in agreement with the achieved results of (Yuan *et al.*, 2016) and (OJAGH *et al.*, 2010). In addition, an increase in TVB-N value of turkey breast treated by chitosan + cumin coating was significantly lower than the samples treated by acetic acid by 15 days after storage period, presenting the synergism effect of chitosan coating on TVB-N value once used in combination with cumin seeds' essential oils.

3.4. Peroxide value (PV) Determination

Lipid oxidation value was detected regarding the PV formation (primary oxidation compounds). The peroxide value of the studied samples indicates the concentrations of peroxide and hydroperoxide compounds produced during early stage lipid oxidation process. The peroxide value is commonly determined for a sample, and a sharp increase indicates the end of the shelf-life for the studied samples (TAHERI *et al.*, 2013). Alterations in PV values of the control sample and both treatments (AA and Ch + C) during 15 days of storage time at the temperature of 4±1°C have been presented in (Fig. 5).

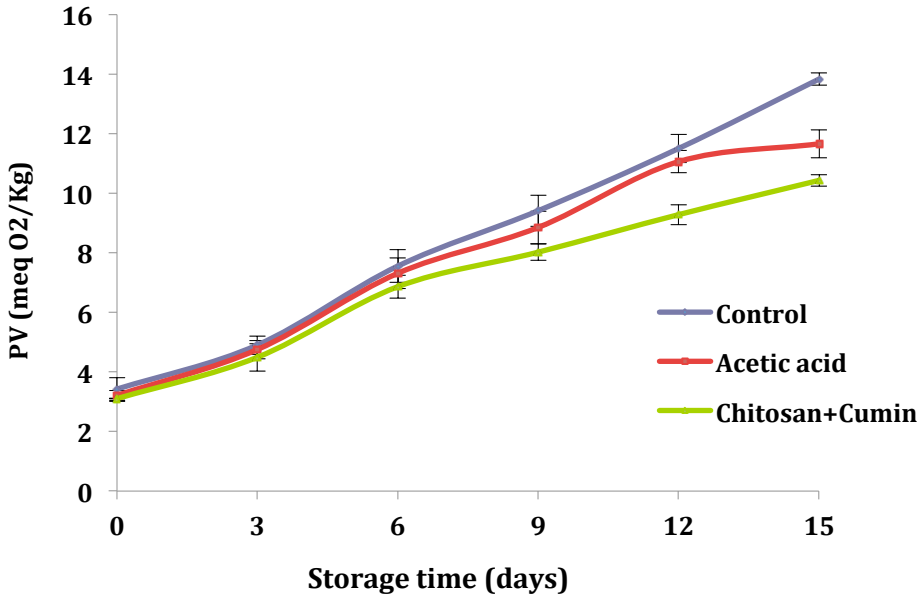


Figure 5. Changes in PV values of turkey breast samples during refrigerated storage.

Initial PV values of control, samples treated by acetic acid, and chitosan + cumin were determined as the following: 3.41, 3.21, and 2.10 meq O_2. Kg^{-1} followed by enhancements up to 13.83, 11.66, and 10.33, respectively. All of the samples expressed an increased PV value in turkey breast fillets once the frozen storage time increased ($p<0.05$). In control samples, PV values were detected higher than other samples in the current study during the storage time. The differences among detected peroxide values of the treated and untreated samples (control) may be associated with different bioactivity properties of the materials with natural origins. Plant phenols and flavonoids are known to inhibit lipid peroxidation by quenching lipid peroxy radicals and reduce and/or chelate iron ions in lipoxygenase enzymes, and as a result of it, may prevent the initiation of lipid peroxidation reactions (ALLAHGHADRI *et al.*, 2010). The antioxidant property of cumin

seeds' essential oil may be associated to phenolic and γ-terpinene compounds as well as monoterpenes (CHEN *et al.*, 2014; RAEISI *et al.*, 2015). According to the achieved results, it is concluded that cumin seeds' essential oils may have a significant effect on lipid oxidation retardation. Similar results were reported by further scientists (RAEISI *et al.*, 2015; ALLAHGHADRI *et al.*, 2010). The results of the present study indicate that chitosan coating is effective in retarding the production of PV in turkey breast fillets stored by refrigeration. JEON et al. (2002) demonstrated that chitosan may be considered as a potential natural antioxidant for stabilizing shelf life enhancements in food products containing lipid. These results are in solid agreement with those of (HU *et al.*, 2002), who reported that chitosan loaded by cinnamon essential oils was effective against lipid oxidation in pork meats stored at 4±1°C. The achieved results of the present research confirmed the obtained results by (BAZARGANI-GILANI *et al.*, 2015), who reported that chitosan coating was effective in retarding of lipid oxidation reactions in chicken breast fillets stored at 4±1°C. Moreover (NOWZARI *et al.*, 2013) reported, that chitosan coating is effective in retarding of POV in trout fillets stored at 4±1°C.

3.5. Sensory evaluation

The sensory qualities of turkey breast samples were assessed in terms of appearance, color, odor, meat consistency and elasticity, with the use of a three-point hedonic scale (1 representing unacceptable and 3 indicating excellent). The turkey breast samples were considered acceptable for customers by 2, suggested by (OCTAVIAN and OCTAVIAN, 2010). The obtained results of the sensory evaluation of turkey breast samples have been shown in (Table 2).

Table 2. Changes in sensory attributes score of turkey breast samples stored at 4±°C.

Sensory attributes	Treatment	Storage period (days)					
		0	3	6	9	12	15
Appearance	Control	3.0±0.00^{aA}	3.0±0.00^{aA}	2.6±0.54^{bAB}	1.2±0.44^{bB}	1.0±0.00^{cB}	1.0±0.00^{bB}
	AA	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	2.6±0.54^{aAB}	2.0±0.70^{bB}	1.8±0.44^{aB}
	Ch+C	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	2.8±0.44^{aAB}	2.4±0.54^{aB}	2.0±0.00^{aC}
Meat elasticity	Control	3.0±0.00^{aA}	2.8±0.44^{aA}	2.8±0.44^{aA}	1.8±0.44^{bB}	1.0±0.00^{cC}	1.0±0.00^{cC}
	AA	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	2.6±0.54^{aB}	1.8±0.83^{bC}	1.6±0.54^{bC}
	Ch+C	3.0±0.00^{aA}	3.0±0.00^{aA}	3±0.00^{aA}	2.8±0.44^{aAB}	2.4±0.54^{aB}	2.0±0.00^{aC}
Odor	Control	3.0±0.00^{aA}	2.6±0.54^{bB}	2.0±0.00^{bC}	1.6±0.54^{bD}	1.0±0.00^{cE}	1.0±0.00^{bE}
	AA	3.0±0.00^{aA}	3.0±0.00^{aA}	2.6±0.54^{abB}	2.2±0.44^{abC}	1.6±0.54^{bD}	1.6±0.54^{abD}
	Ch+C	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	2.8±0.44^{aAB}	2.4±0.54^{aB}	2.0±0.00^{aC}
Color	Control	3.0±0.00^{aA}	2.8±0.44^{aA}	2.8±0.44^{aA}	1.6±0.54^{cB}	1.0±0.00^{bC}	1.0±0.00^{bC}
	AA	3.0±0.00^{aA}	3.0±0.00^{aA}	2.8±0.44^{aAB}	2.4±0.54^{bBC}	2.2±0.44^{aC}	1.4±0.54^{bD}
	Ch+C	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	3.0±0.00^{aA}	2.2±0.44^{aB}	2.0±0.00^{aB}

Means in column with different small letters indicate significant differences ($p<0.05$) among treatments, and means in row with different capital letters indicate significant differences ($p<0.05$) as result of refrigerated storage time.

At the beginning of the evaluations, the appearance, odor, color, and consistency of breast fillets were fresh. As expected, progressive quality deteriorations of samples were observed, as a result of an increase in storage time ($p<0.05$). The sensory evaluation result is associated with the microbial and chemical properties. Due to a high lipid oxidation and microbial growth, the control samples (untreated) of turkey breast fillet showed deterioration, appearing as off-odor, and slimy as well as discoloration after 6 days of

storage period. In comparison, chitosan + cumin coated samples showed an acceptable sensory score up to 15 days of storage time. Thus, antioxidant and antimicrobial effects of chitosan + cumin coating may minimize the oxidative reactions, and as a result extending the products' shelf life while maintaining their quality as well (OJAGH *et al.*, 2010). The achieved results indicated that chitosan + cumin treatment led to the highest score among other treatments in all of the studied sensory properties through storage time. This phenomenon may be attributed to the unique flavor of cumin seeds' essential oil. Addition of cumin seeds' essential oil to chitosan coating enhanced the beneficial effects on color, odor, and overall acceptability of turkey breast fillets significantly ($p<0.05$) in the final days of storage periods. Considerable correlations among the microbial and chemical qualities as well as sensory attributes were found by (BAZARGANI-GILANI *et al.*, 2015; KANATT *et al.*, 2013; SHALTOUT *et al.*, 2014; VASILATOS and SAVVAIDIS, 2013; RAEISI *et al.*, 2015; OJAGH *et al.*, 2010) where were in solid agreement of the achieved results of the current study.

4. CONCLUSIONS

Fresh meats and their products are very susceptible to deterioration by microbial growth and oxidative reactions. The shelf life of refrigerated turkey meat is normally short due to the chemical and microbial activities in it, leading to quality loss and spoilage. The achieved results of microbial (TVC and PTC), physicochemical (pH, TVN-B and PV) and sensory evaluation analyses indicated that acetic acid and Ch + C (chitosan and cumin oil) coating on turkey breast fillets may lead to the maintenance of qualitative characteristics, improvement of microbial safety and extension of the shelf life of meat products through the chilled storage period. Chitosan + cumin treatment could maintain turkey breast fillet shelf life till the end of the storage period (day 15), while acetic acid treatment and the control sample had a shelf life just for 9 and 6 days, respectively. Therefore, natural preservatives such as chitosan + cumin oil combination may be used as a safe preservation approach to extend the shelf life of chilled turkey meat and its products.

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