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ANALYSIS OF PHENOLIC COMPOUNDS, PHYTOSTEROLS, LIGNANS AND STILBENOIDS IN GARLIC AND GINGER OIL BY GAS CHROMATOGRAPHY

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A B S T R A C T

The phenolic compounds, phytosterols, lignans, stilbenoids alongside the physicochemical, proximate and fatty acid composition of garlic and ginger obtained from a local farm in Nigeria were determined. We set out to confirm the presence of bioactive substances in these samples as found in our locality with the aim of dispelling or confirming previously stated bioactive substances obtained from same samples but from different regions of the world. Gas chromatography analysis revealed that the major phenolic compound in the samples was caffeic acid. The result of the analysis of phytosterol in the samples showed sistosterol as the major phytosterol in them. Analysis of the lignans showed that (9E, 12E, 15E)- 9,12,15- octadecatrien-1-ol was the major lignan, occurring up to 46% in the samples. Determination of the stilbenes showed that resveratrol was the major stilbenes present in them while unsaturated fatty acids were the major fatty acids present in the samples. These findings showed that the samples contained similar bioactives compounds alongside some additional bioactive compounds that were not detected in the previous studies. These findings further highlight high proportion of bioactive compounds in these samples especially lignans and stilbenes which may be of value in many biological processes and medical conditions such as in reducing the risk of cancer in humans.

Keywords: Lignan, stilbene, garlic, ginger.

INTRODUCTION

Characterization of oils and fats involve determination of the bulk physiochemical and bioactive compounds often present in them. Also, it has been found that the unsaponifiable matter in oils have important bioactive, nutritional and characteristic compositional properties that affect the quality of individual oils (Mitel *et al.* 2009). Among phytochemicals possessing these properties is phenolic compounds (Jahangir *et al*. 2009). Phenolic compounds commonly referred to as polyphenols are present in all plant but in different concentrations. It is a generic term that refers to a large number of compounds which are characterised by having at least one aromatic ring with one or more hydroxyl group(s) attached (Maria *et al.* 2011). It has been reported that intake of these phenolic compounds help to reduce the risk of coronary disease and cancer (Maria *et al*. 2011). Sterols are a group of naturally occurring substances derived from hydroxylated polycyclic isopentenoids. They occur as a mixture of different compounds although their structures are closely related and varied depending on the extent of modifications of the ring system and side chain variations. Sterols are known to have a wide range of biological activities and physical properties such as: inhibition of cholesterol absorption (Abidi, 2001), lowering of plasma cholesterol, acting as useful emulsifiers for cosmetic manufacturers, supplying the majority of steroidal intermediates and precursors for the production of hormone pharmaceuticals (Abidi, 2001).

Stilbenes are phenolic-based compounds, of which the most widely recognized is resveratrol (3,4′,5-trihydroxystilbene) (Aggarwal *et al.* 2004). Stilbenoids are chemical compounds, belonging to the family of phenylpropanoids including resveratrol, pterostilbene and piceatannol which can be found in grape skins and

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seeds, wine, nuts, peanuts (Norton, 2011). Resveratrol has attracted immense attention because of its biological properties including its anticancer effects (Aggarwal *et al.* 2004). Stilbenes especially resveratrol functions as a chemopreventive agent and has been shown to inhibit ribonucleotide reductase and certain other cellular events associated with initiation, promotion, and progression of carcinogenesis (Jang *et al*. 1997). Lignans are a group of chemical compounds found in plants. They are polyphenolic substance derived from phenylalanine via dimerization of substituted cinnamic alcohols (Milder *et al*. 2005). Lignans have multiple physiological functions in the body such as decreasing blood lipids (Hirata *et al*. 1996) and arachidonic acid levels (Ghafoorunissa, 2004). They also act as antioxidants thereby providing anti-inflammatory functions (Hsu *et al.* 2001).

Garlic (*Allium Sativum)* is cultivated worldwide and the potential medical properties of garlic have been recognised for thousands of years (Beato *et al.* 2011). Garlic compounds were reported to have tremendous antioxidant property which exerts actions by scavenging reactive oxygen species (ROS), enhancing cellular antioxidant enzymes and increasing glutathione in the cells (Borek (1981). Garlic health properties depend on the bioactive compounds of garlic especially organosulphur compounds (which are responsible for the pungent flavour of garlic), (Beato *et al*. 2011) and phenolic compounds which have interesting pharmacological properties (Lanzottti (2006). Garlic is claimed to help prevent heart diseases including: atherosclerosis, high cholesterol, high blood pressure and cancer (Block (2010). Animal studies and some early research studies in humans have suggested possible cardiovascular health benefits of garlic. A Czech study found garlic supplementation reduced accumulation of cholesterol on the vascular walls of animals (Durak *et al*. 2002).

Ginger is known botanically as *Zingiber Officinale*. It is used as a spice because it has a distinctive flavour and aroma, thus they are used to season foods. The characteristic odour and flavour of ginger is caused by a mixture of Zingerone, shogaols and gingerols (Abitogun & Badejo (2010). Phytochemical studies showed that the plant is rich in a large number of substances including zingiberene bisabolene (Jolad *et al*. 2005). Ginger is one of the most commonly used herbal supplements. Traditionally, ginger has been used to treat a wide range of ailments such as stomach aches, abdominal spasm, nausea, vomiting as well as arthritis.

Although, previous studies (Beato *et al.* 2011; Gorinstein *et al*. 2008; Drozd *et al.* 2011) have identified various bioactive compounds present in garlic and ginger, majority of the studies were done outside the locality of the present study. The composition of seeds or fruits may vary with environmental and climatic factors. Hence, we set out to establish whether our local garlic and ginger contain the same or additional bioactive substances as shown by previous authors with the aim of further highlighting their economic, medicinal and nutritional values.

MATERIALS AND METHODS

Garlic and ginger roots were obtained from local farms around Ado- Ekiti, Ekiti State, Nigeria. All solvents and reagents used in this work were of analytical grade. The roots were washed several times with distilled water, cut into smaller pieces and air dried. The roots were milled using electric grinder.

Extraction of the oils: The powders were extracted with petroleum ether in a soxhlet apparatus for 6hrs. The extracted oil was concentrated using rotary evaporator.

Physicochemical analysis: The acid value, saponification value and iodine value were determined by standard methods as described by Association of Official Analytical Chemists (AOAC (1990). The refractive index was determined with calibrated Abbey refractometer and viscosity was also determined by a calibrated viscometer (Stress Tech Rheological). The metal compositions of the samples were determined using an Atomic Absorption spectrophotometer S series 711430VI.26 following the manufacturer's specifications.

Proximate Analysis: Standard methods of AOAC (1990) were used to determine the moisture, ash, crude fat, crude fibre, crude protein and carbohydrate contents of each sample.

Fatty Acid Methyl Ester Analysis: 50mg of the extracted fat content of the sample was saponified for five minutes at 95^0C with 3.4ml of 0.5M KOH in dry methanol. The mixture was neutralised by using 0.7M HCl. 3ml of 14% boron triflouride in methanol was added. The mixture was heated for 5 min at 90^oC to achieve complete methylation process. The Fatty Acid Methyl Esters (FAME) was extracted thrice from the mixture with redistilled n- hexane. The content was

concentrated to1ml for gas chromatography analysis and 1µl was injected into the injection port of GC.

Sterol Analysis: Aliquots of the extracted oil were added to the screw- capped test tubes. The sample was saponified at 95^{0}C for 30 minutes, by using 3ml of 10% KOH in ethanol to which 0.2ml of benzene had been added to ensure miscibility. Deionised water (3 ml) was added and 2ml of hexane was used in extracting the non-saponifiable materials. Three extractions each with 2ml of hexane were carried out for 1 hour, 30 minutes and 30 minutes respectively to achieve complete extraction of the sterols. The hexane mixture was concentrated to 1ml in the vial for gas chromatography analysis and 1µl was injected into the injection port of GC. The GC used for both FAME and sterol analysis and the conditions of the instrument are: HP 6890 Powered with HP chemstation Rev. A 09.01 [1206] software. Injection temperature- split injection, Carrier gas – nitrogen, Split ratio - 20:1, inlet temperature 250^{0}C , Column type - HP INNOWax, Oven program - initial temperature @ 60^{0}C first ramping @ 10^{0}C/ min to 20 min, second ramping @ 15^{0}C/ min for 4 min.

Determination of phenolic acids: 50mg of the sample was extracted with 5ml of 1M NaOH for 16 hours on a shaker at ambient temperature as described by Kelley *et al*. (1994) and Provan *et al*. (1994). After extraction, the sample was centrifuged, rinsed with water, centrifuged again and the supernatants were combined and placed in a disposable glass test tube and heated at 90^{0}C for 2h to release the conjugated phenolic compounds. The heated extract was cooled, titrated with 4M HCl to pH < 2.0, diluted to 10ml, with deionised water, and then centrifuged to remove the precipitate. The supernatant was kept for subsequent purification and analyse using gas chromatography. The GC used for the analysis is HP 1 Column, column length – 30m, injection temperature – 250^{0}C, Detector – FID, Carrier – nitrogen, initial temp – 60^{0}C for 5 min, first rate – 15^{0}C/min for 15 min, second rate - 10^{0}C/min for 4 min.

Determination of stilbene: The samples were extracted with petroleum ether for 1hr in a soxhlet extractor at 40^{0}C. The oils were concentrated with rotary evaporator. 1mg of the extracted oil was derivatized with 100µl of a mixture of 3.5:1:0.5 (v/v/v) bis-(trimethylsilyl) trifluoroacetamide dimethyl formamide- methanol in a 2ml GC vial according to Rimando and Cody (2005). The vial was capped and heated at 70^{0}C for 1 hr. After, the vial was cooled to room temperature. 2µl was injected and analysed by GC- MS. The GC used for the analysis is HP 6890 Powered with HP chemstation Rev. A 09.01 [1206] software. Injection temperature- split injection Carrier gas – hydrogen, Split ratio 20:1, inlet temperature 250^{0}C , column type DB-5MS, oven program - initial temperature @ 40^{0}C for 5 min, first ramping @ 10^{0}C/ min to 200^{0}C, second ramping @ 8^{0}C/min to 300^{0}C.

Determination of lignan: The modified method of James et al, 2006 was used for the analysis of lignan. The samples were extracted as described above. The total extracts were concentrated with a rotary evaporator. 1mg each of the oil was then purified in 5ml acetone for the GC- MS analysis. The oil was concentrated to 1ml in the gas chromatographic vial for the analysis. The GC used for the analysis is HP 6890 Powered with HP chemstation Rev. A 09.01 [1206] software. Injection temperature- split injection, Carrier gas – nitrogen, Split ratio 20:1, inlet temperature 250^{0}C , column type HP 5, oven program- initial temperature @ 120^{0}C ramping @ 10^{0}C/ min to 20 min, second ramping @ 8^{0}C/min to 300^{0}C.

RESULTS AND DISCUSSION

Table 1 presents the result of the proximate analysis of garlic and ginger flour. The result shows that both samples are rich in carbohydrate with garlic having relatively higher protein value than ginger and these are in agreement with work reported by other authors like: Okolo *et al*. (2012) and Otunola *et al.*(2012) but contrary to that by Nwinuka *et al.* (2005) where a high protein value for ginger (17.35%) and low value for garlic (8.58%) was reported. The discrepancy in these studies may be due to environmental conditions and methods of storage of samples. The result of the physicochemical characteristics (Table 2) showed that the saponification values of garlic and ginger oil is 162.12 and 169.70 mg/KOH/g respectively. The saponification values obtained in this work are lower than 192mgKOH/g reported by Gafar *et al.* (2012) for garlic oil and 213.18mgKOH/g reported for ginger by Abitogun and Badejo (2010). The differences in these results may be due to changes in climatic conditions. The observed high saponification values in this study showed that these oils could be used in soap making as earlier noted by other authors (Wara *et al*. 2011).

Table 1. Result of proximate analysis of ginger & garlic flour.

Parameters	Garlic	Ginger
Moisture content(%)	5.02	10.50
Crude ash (%)	4.37	3.50
Fat content(%)	0.52	4.82
Crude fiber(%)	2.22	3.56
Crude protein (%)	15.35	7.50
Carbohydrate (%)	72.52	70.12

Table 2. Physicochemical properties of garlic and ginger oil.

Parameters	Garlic	Ginger
Acid value (mgKOH/g)	4.11	4.53
Iodine value (gI_2/100g sample)	134.0	96.0
Saponification value (mgKOH/g)	162.1	169.7
pH value	5.96	6.58
Viscosity	84.0	83.9
Refractive index	1.56	1.51

The acid values obtained in this study, is in agreement with other previous report (Gafar *et al.* 2012) and the low acid value is an indication of the good storage ability of the oils. The elemental composition of the oils showed that both samples contain high percentage of phosphorous: 3.80 and 4.16% for garlic and ginger respectively. No trace of chromium was detected in them and this makes them safe for consumption.

The result of the fatty acid composition is presented in Figure 1. The result showed that both samples contain high percentage of unsaturated fatty acid with their being present in relatively higher proportion in garlic oil than in ginger oil further highlighting their nutritional and health benefits respectively. This finding is in agreement with the report by Abitogun and Badejo (2010).

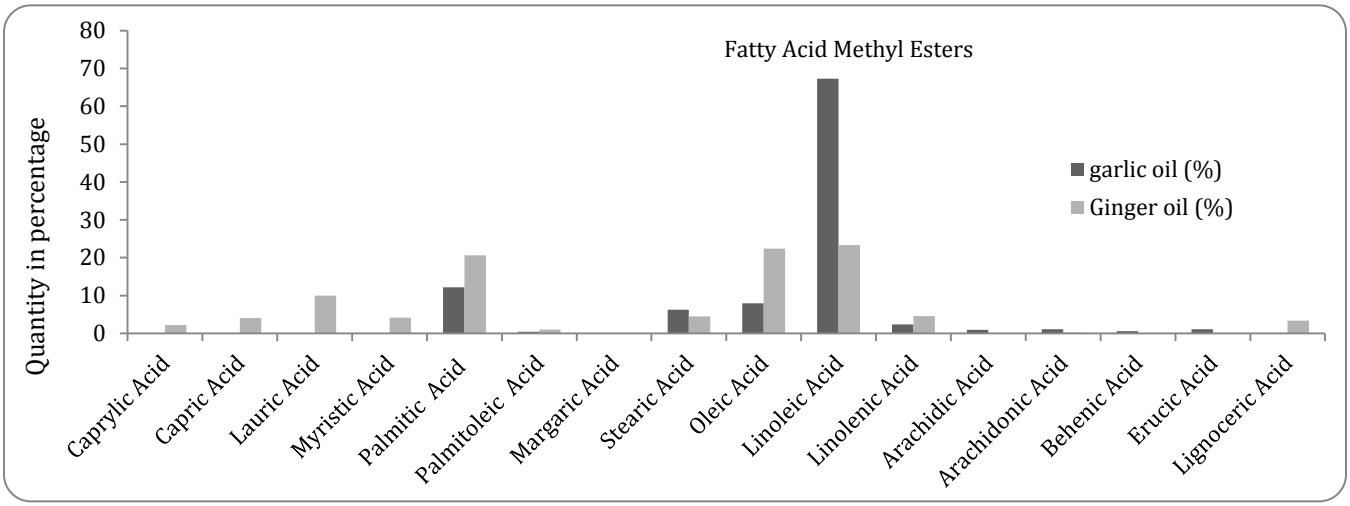


Figure 1. Percentage composition of fatty acid in garlic and ginger oil.

The high percentage of linoleic and oleic acid in the samples confirmed that the oil is liquid than solid. Therefore, the oil cannot easily congeal at ordinary temperature. It also implies that regular consumption of these sample oils is safe and can prevent the risk of heart problems because it offers better protection against increased blood pressure. (Abitogun and Badejo(2010).

The result of the phenolic compounds present in the samples showed that both garlic and ginger contained high percentage of caffeic acid; 46% and 45% for garlic and ginger respectively as shown in table 3 and figure 2. This is followed by 27% of ferulic acid in garlic. Percentages of protocatechuic acid, p – coumaric , p – hydroxybenzoic, piperic, chlorogenic, ellagic, rosmarinio were found to be approximately 3%, 3%, 5%, 5%, 4%, 3%, 1% in garlic oil.

Table 3. Phenolic compounds in garlic and ginger oil

Phenolic compounds	Garlic (%)	Ginger (%)
Protocatechuic acid	2.66	2.63
P – coumaric	3.46	3.80
Vanillic acid	0.15	7.25
O – coumaric	0.53	0.60
P – hydroxybenzoic acid	5.25	6.26
Gallic acid	0.36	4.32
Caffeic acid	46.13	45.77
Ferulic acid	27.08	3.39
Syringic acid	0.75	0.95
Piperic acid	4.48	5.41
Sinapinic acid	0.64	7.66
Chlorogenic acid	3.83	23.24
Ellagic acid	2.88	3.22
Rosmarinio acid	1.27	2.78

Ginger oil contain 23% chlorogenic acid, 7% vanillic acid, 6% p – hydroxybenzoic acid, 4 % gallic acid, 8% sinapinic acid, 3% ellagic acid, 3% protocatechuic acid, 4% p – coumaric acid, 3% ferulic acid, 5% piperic acid, 3% rosmarinio acid. This is in agreement with the result of Gorinstein *et al*. 2008 for phenolic acids in garlic. Beato *et al.* and Drozd *et al.* did not detect any protocatechuic acid in the garlic samples they studied (Beato *et al.* 2011; Drozd *et al.* 2011). The discrepancy between these studies and the present study may be due to differences in environmental conditions and methods of analysis.

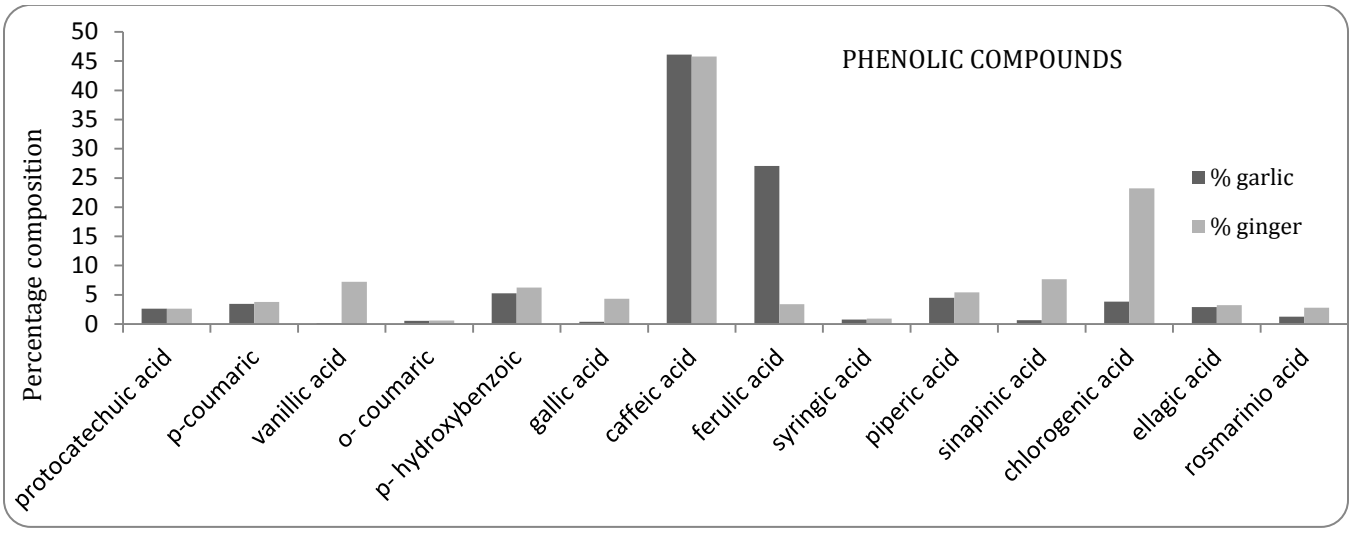


Figure 2. Percentage composition of phenolic compounds in garlic and ginger oil.

The result of the sterols (Figure 3) showed that both samples contain very high percentage of sitosterol; 86% and 87% for garlic and ginger respectively while campesterol, stigmasterol, and 5- avenasterol are present in approximately 7%, 2%, 5% and 6%, 3%, 4% for garlic and ginger oil respectively. Saturated oils like Cholesterol and cholestanol were found in very minute quantity in both samples. Sterols are known to inhibit oxidative deterioration of oils; serving as potential antipolymerization agents for frying oils (Abidi (2011). The unsaturated analogoues of phytosterols and their esters have been suggested as effective cholesterol – lowering agent by decreasing low density lipoprotein (LDL) cholesterol, mostly through interfering with the intestinal absorption of cholesterol thereby offering cardiologic health benefit (Law (2000).

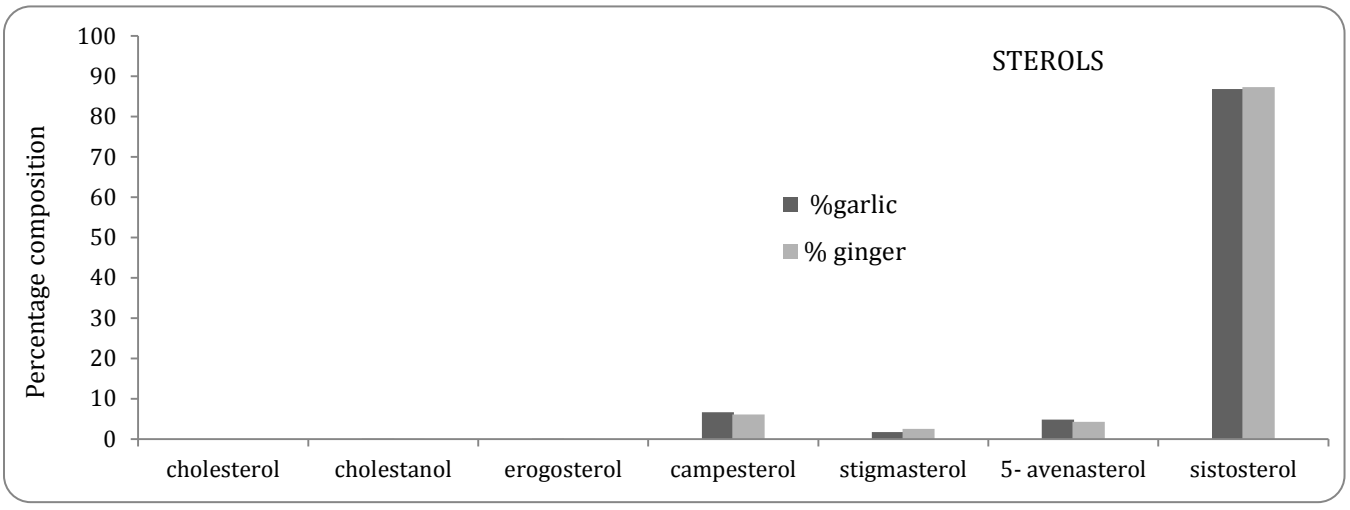
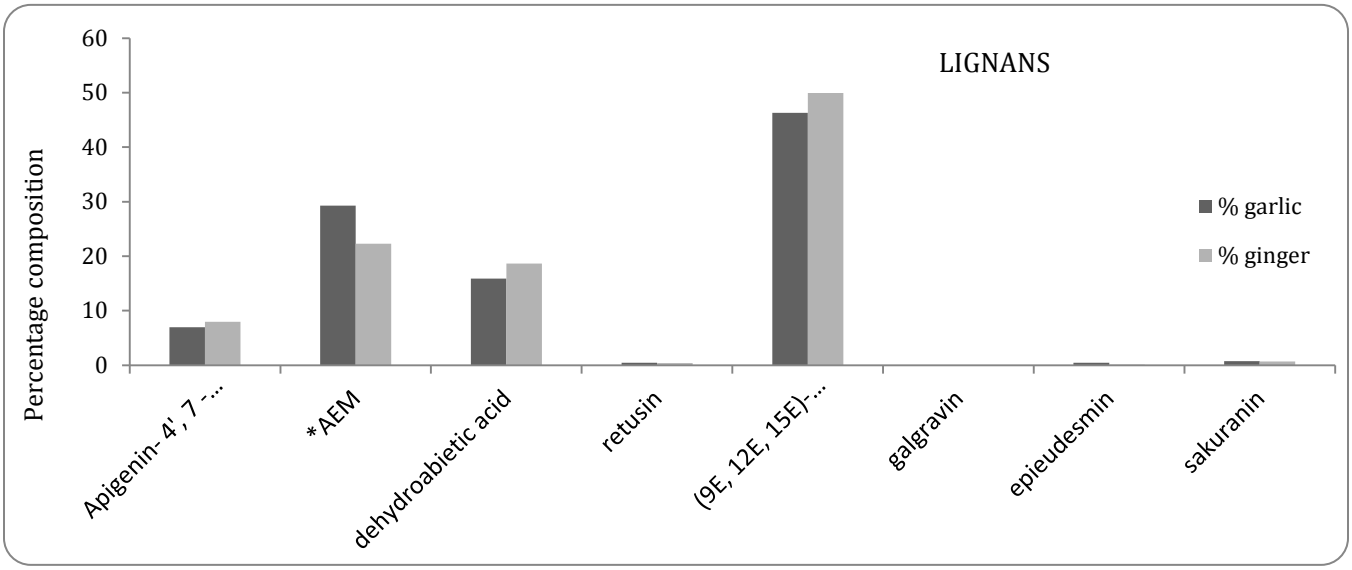


Figure 3. Percentage composition of sterols in garlic and ginger oil.

The result of the analysis of lignan is presented in figure 4. The result showed that garlic oil contains (9E, 12E, 15E)-9,12,15 - octadecatrien-1- ol, 2- allyl-5- ethoxy -4-mehtoxyphenol, dehydroabietic acid, and apigenin – 4',7 – dimethyl ether in percentages 46, 29, 15, 7 respectively while ginger oil contains 22% (2- allyl-5- ethoxy- 4-

methoxyphenol), 49% (9E,12E,15E)9,12,15 – octadecatrien – 1- ol), 8% (apigenin - 4',7- dimethyl ether) and 18% (dehydroabietic acid). The major lignan found in both samples is (9E, 12E, 15E) 9,12,15 – octadecatrien - 1- ol. Intake of lignans has been associated with reduction in the risk of breast cancer, lung cancer and postate cancer (Kanu *et al*. 2010). Lignans also help to reduce hair loss (Stephen (2006). The result of the stilbenoids (Figure 5) showed that 98% of the total stilbenes in both samples is resveratrol while piceatannol and pterostilbene accounted for the remaining 2%. Resveratrol has been found to be a promising agent in promoting cardioprotection against coronary heart disease. It has angiogenic, antihypercholesterolemic and antidiabetic effects (Bacciottinin *et al*. 2007) thus highlighting the health benefits of these plants.



*AEM - 2-allyl-5- ethoxy- 4- methoxyphenol

Figure 4. Percentage composition of lignans in garlic and ginger oil.

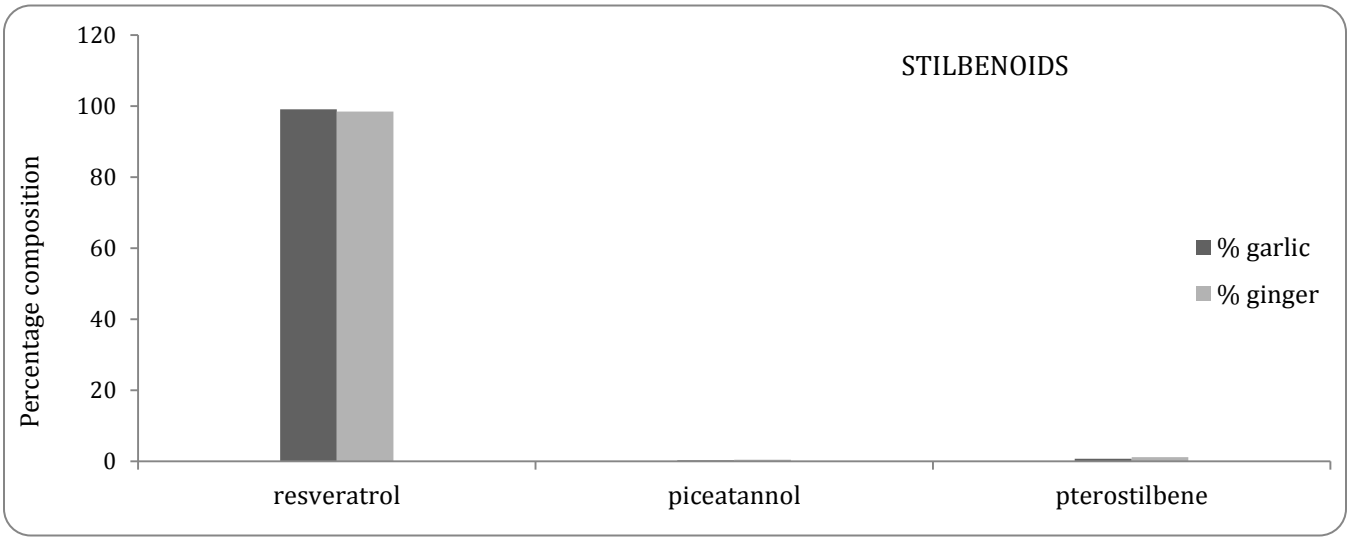


Figure 5. Percentage composition of stilbenoids in garlic and ginger oil.

CONCLUSION

This study highlights the presence of some previously identified composition of garlic and ginger alongside new compounds in them which were not detected hitherto by some previous authors and this shows that, environmental conditions may have effect on the chemical composition of seeds and fruits. Also, the additional bioactive compounds found in garlic and ginger used in this study showed that their oil will offer better protection against many human health problems such as increased

blood pressure, cancer, heart diseases, hyperlipidemia and those diseases linked with lipid peroxidation and body tissue destruction by free radicals. There is need for more local studies on these samples to further unmask their hidden potentials with the aim of highlighting their economic, nutritional and medicinal values.

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EFFECT OF DIFFERENT ATMOSPHERES ON QUALITY CHANGES OF KURDISTAN STRAWBERRY

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A B S T R A C T

The effects of two modified atmosphere packaging conditions (MAP) and a normal atmosphere on quality parameters of Kurdistan strawberry were investigated. For this purpose, tow containers made up of polypropylene were used to package the strawberry under two different initial headspace gas compositions (MAP1: O_2 6%; CO_2 7.2%; N_2 86.8% and MAP2: O_2 0.2%; CO_2 10%; N_2 89.8%), and control where the strawberry samples were stored without packaging in normal atmosphere. Physico-chemical and microbiological properties were monitored during a 7-day storage period at refrigerated temperature (5 °C). Results showed that two MAP packaging conditions significantly prevent product decay and improved the shelf life when compared with the unpackaged product. In this study, the best results were recorded with the MAP1, which assured a shelf life more than 7 days with flavour remaining. MAP, as a technique to extend shelf life, can be used to maintain the quality of Kurdistan strawberry.

Keywords: Kurdistan **s**trawberry, modified atmosphere, quality parameters.

INTRODUCTION

Perishability is an important concern in fruit and vegetable. MAP as "the packaging of a perishable product in an atmosphere which has been modified so that its composition is other than that of air (Hintlian & Hotchkiss, 1986)" has been studied extensively. Physiological changes have profound effects on shelf life and quality of fresh products after harvesting. Fruits and vegetables undergo perhaps the most complicated physiological changes after harvesting. These changes are related to environmental conditions such as gas atmosphere, humidity, temperature, and their physical condition. Changes in concentration of atmosphere gas may cause stressed metabolism in fruits or vegetables, thereby producing undesirable compounds, which affect their flavour. Therefore, MAP must be applied with extreme caution and requires a strict quality control.

After some of the more recent important works include experimenting on the extension of shelf life of cake with MAP (Seiler, 1965); working widely on MAP of different types of bakery products (Ooraikul, 1988); on fruits and vegetables (Kader, 1989); on fresh meats (Gill, 1995); and on meat and poultry products (Hotchkiss & Langton, 1995), MAP has now been commercially applied to practically all fresh produce and processed products (insert the most recent references on MAP for different cultivars of strawberries in other countries). It has been accepted as a notable element in hurdle technology (Leistner & Gorris, 1995).

Strawberry is an important fruit of the Kurdistan region in Iran, production of which is increasing in the last years. Fruit from this region has an excellent flavour but is very sensitive to spoilage, after harvesting, mostly due to high levels of moisture. Iran has produced 38500 tonnes of strawberry in 2007 (FAO, 2009). Kurdistan grows about 80% of the total strawberry production in the Iran. In Kurdistan, the predominant cultivar used in planting and processing is 'Kurdistan strawberry'. The overall production in Kurdistan accounts for 30,000 tonnes. Processed strawberries account for about 4.5 tonnes in Kurdistan.

The strawberry harvest begins in Kurdistan in May and peaks between May and June and continues until July. There is a lack of reports about studies on the quality of Kurdistan strawberry under different atmospheres.

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Therefore, the objective of this work was to study physico-chemical, microbiological, and sensory score of Kurdistan strawberry cultivar under different atmospheres during 7 days of cold storage. The temperature of 5ºC was used as the average temperature of storage for Kurdistan strawberry because it is the temperature usually applied to keep the fruit, when available for the end user.

MATERIALS AND METHODS

Origin of the strawberries: The study was carried out on strawberry cultivar of Kurdistan obtained directly from market. They were transported to the lab in transparent polystyrene containers with a capacity of 500 g and stored at 5ºC. The fruits were carefully selected to be uniform in appearance and free from physical damage and deterioration.

Packaging methods: Polypropylene (PP) packaging containers were used for packaging. In this study three different gas compositions (normal atmosphere; MAP1: O_2 6%; CO_2 7.2%; N_2 86.8% and MAP2: O_2 0.2%; CO_2 10%; N_2 89.8%) were applied. After setting up the experiment, fruits were stored in a cool storage at 5°C, for 7 days.

Quality parameters: Firmness of strawberry was determined with a penetrometer for fruit firmness testing (The Wagner FT 02 Penetrometer - Italy). The maximum force to penetrate the fruit sample was recorded as firmness. Each result was the mean of 10 determinations and was expressed in Newton (N).

After analysing the firmness , strawberries were cut into small pieces and homogenized in a grinder. Total soluble solids percent (%TSS) was measured in the juice of ground strawberries using an Atago RX-2500 digital refractometer (Atago Co. Ltd., Tokyo, Japan) at 20°C. A drop of the juice was placed on the lens and the reading was taken in degree Brix. Calibration was made with deionized water and the lens was carefully rinsed between samples. Total sugar as total reducing sugar was determined according to Iran's Standard based on Lane-Inon measurement (ISIRI, 2007).

The pH was recorded by pH meter (pH-526; WTW Measurement Systems, Wissenschaftlich-Technische Werkstätten GmbH, Wellhelm, Germany). In the case of titratable acidity, six grams of ground strawberry was suspended in 100 mL of distilled water and then filtered. The filtrate titrated against 0.1 N NaOH up to pH 8.3 and expressed as citric acid. Anthocyanin content of strawberries was determined using a spectrophotometric method. 2 g of ground strawberry sample mixed with 20 ml of acidified methanol (1% HCl) using a homogenizer and then centrifuged at 2000 g for 15 min. Anthocyanin content was estimated as pelargonidin 3-glucoside at 510 nm, using a molar absorptivity of 36000 $L.cm^{-1}.mol^{-1}$.

The HPLC analysis was carried out to determine the vitamin C on a Shimadzu class LC VP HPLC system with class LC-VP software, a pump (LC-6AD), and a UV-VIS detector (SPD-10AV VP). The column used for measuring vitamin C was SGE (250 mm x 4.6 mm I.D., 5 µm). The mobile phase was water adjusted to pH 3 with phosphoric acid (vitamin C). Separation was carried out by isocratic elution with a flow rate of 0.4 ml min-1 and column temperature was ambient. The UV detector was set at 254 nm. Quantization was based on the peak area measurement.

Sample (10 g) was extracted in 10 ml water adjusted to pH 1.5 with 10 ml phosphoric acid-water (2%, v/v) for vitamin C. The extracts were filtered through filter paper. Then, 1.5 ml buffer (0.01 M KH_2PO_4, pH 8.0) was added to 1.5 ml sample extract. From this, 1.5 ml (organic acids) and 1 ml (vitamin C) of these mixtures were loaded on to C18 cartridges. After loading, 3 ml water adjusted to pH 1.5 with 2 ml phosphoric acid-water (2%, v/v) for vitamin C was passed through the cartridges. For HPLC, 20 µl of the eluents were injected.

For mould enumeration, strawberry samples of 10 g each were stomachered in a 1:10 dilution of sterile ringer (Seward Limited, London, UK). Ten-fold dilution series were made in as needed for pour plating. 1mL of the appropriate sample dilution was pour-plated on YGC agar (YGC, Merck, Germany) incubated at 30 ◦C for 5 days for moulds. Mould counts were expressed as cfu g^{-1}.

Sensory scores of strawberry samples were evaluated by ten trained staff members of the Kurdistan General Department of Standards and Industrial Research. The panelists were asked to evaluate the flavour. A twenty point scale was used where 20= excellent and 1= extremely poor. Accuracy and precision were statistically analyzed.

Data analysis: The results were analyzed using one-way analyses of variance (ANOVA) with the statistical software of SPSS (SPSS Inc., Chicago, IL, USA). Differences between means were studied with Duncan's test and differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Texture: Changes in texture over the storage time are showed in Figure. 1. As it is clear from the Figureure, the firmness of strawberries under MAP1, compared to MAP2, increased slightly. Strawberries with air packaging changed drastically in terms of texture after 5th day of storage. It has been reported that strawberries treated with CO_2-enriched atmospheres were firmer than air-stored fruit (Wszelaki & Mitcham, 2000; Van der Steen *et al.,* 2002; Pelayo *et al.,* 2003). This phenomenon has been usually linked to the accumulation of CO_2 in the packages. As a result of CO_2-enriched treatments, it was reported that cell-to-cell adhesion increased by 60% due to changes in the pH of the appoplast, with the subsequent precipitation of soluble pectins (Harker *et al.,* 2000).

Total sugar: The results from the sugar analyses are presented in Figure. 2. The initial total sugar concentration of strawberry samples was 5%. During the storage, sugar level in all the packaged samples increased slightly. This can be attributed to water loss of strawberry, thereby concentrating total sugar. More fluctuation in sugar level was observed in strawberry with normal atmosphere. At the end of storage time, there was no significant difference between strawberry of MAP1 and MAP2 ($p<0.05$) and sugar content was 5.94%.

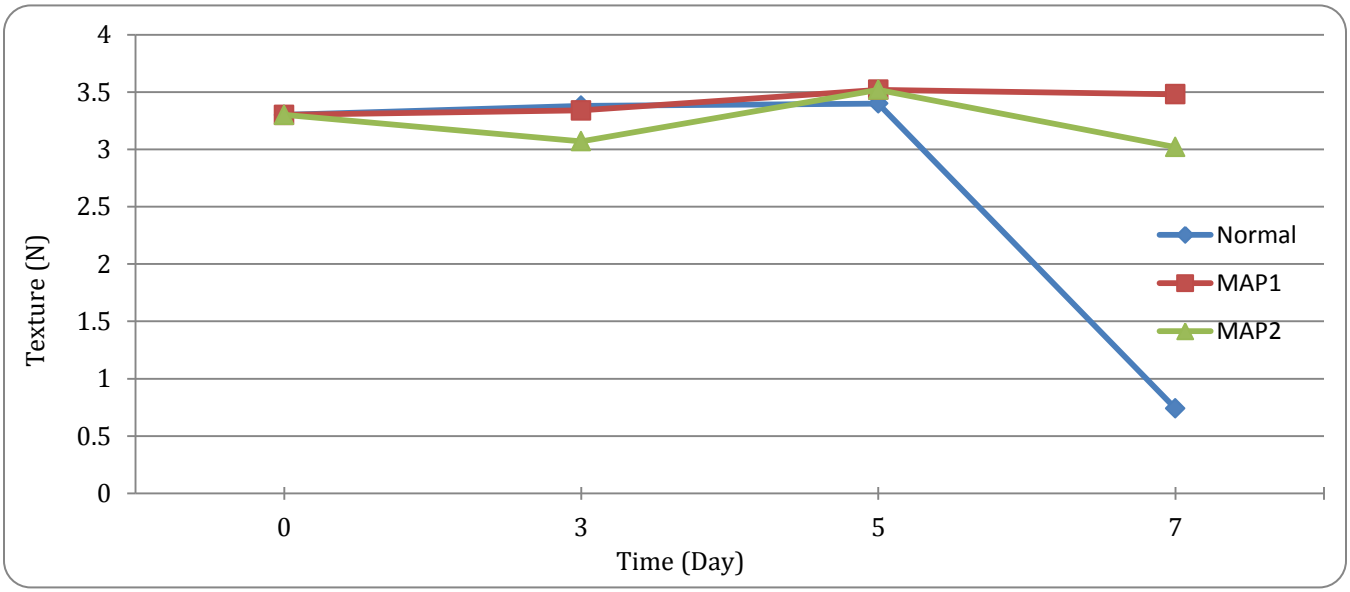

Figure 1. Changes of texture (N) of Kurdistan strawberry at 5°C under different atmospheres.

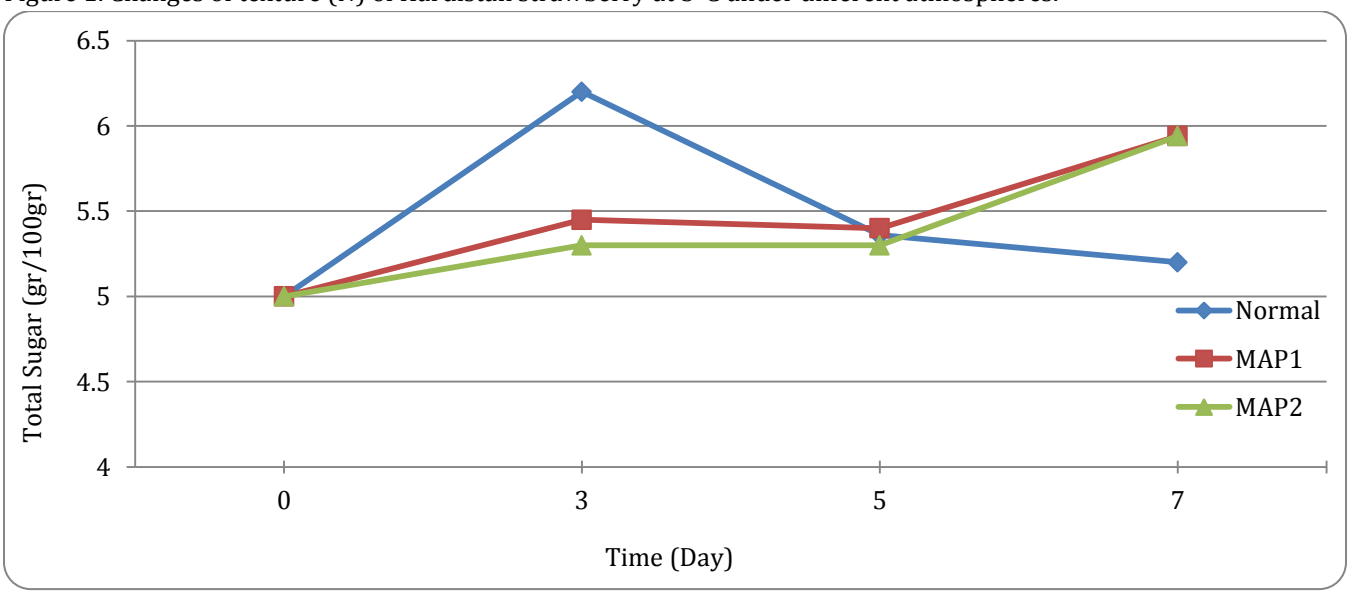

Figure 2. Changes of total sugar (gr/100gr) of Kurdistan strawberry at 5°C under different atmosphere.

TSS: TSS of strawberries was found to increase with storage time (Figure. 3). The observed increase in TSS content of the samples is an indication of high respiration rate and ripening (Pal and Roy, 1988). The changes were more considerable in untreated compared to the treated samples. The increase in total solids of samples of MAP was slower and more gradual than the other samples stored under normal condition. The total solid content of strawberries reached to 5.94, 5.94 and 5.2% at 7 d for MAP1, Map2 and normal atmosphere, respectively. This shows that the rate of senescence was the lowest in MAP1 and MAP2. Increase in TSS of modified atmosphere packed commodities over storage have been reported by Jafri *et al.* (2013) in mushrooms, Manurakchinakorn *et al.* (2010) in fresh-cut mangosteen, Diaz-Mula *et al.* (2011) in yellow and purple plums, and Magaraj *et al.* (2011) in pears.

pH and Titrtable Acidity: The total titratable acidity (TTA) calculated as citric acid, which is the dominant acid in strawberries, for the different atmospheres are displayed in Figure. 4. The acidity of strawberries under MAP1and MAP2 decreased slightly. Over the storage, no significant difference was observed between the acidity of MAP1 and MAP2. The variation in acidity of samples stored under air atmosphere is associated with growth of microorganisms and consequent production of organic acids (Heard, 2002).

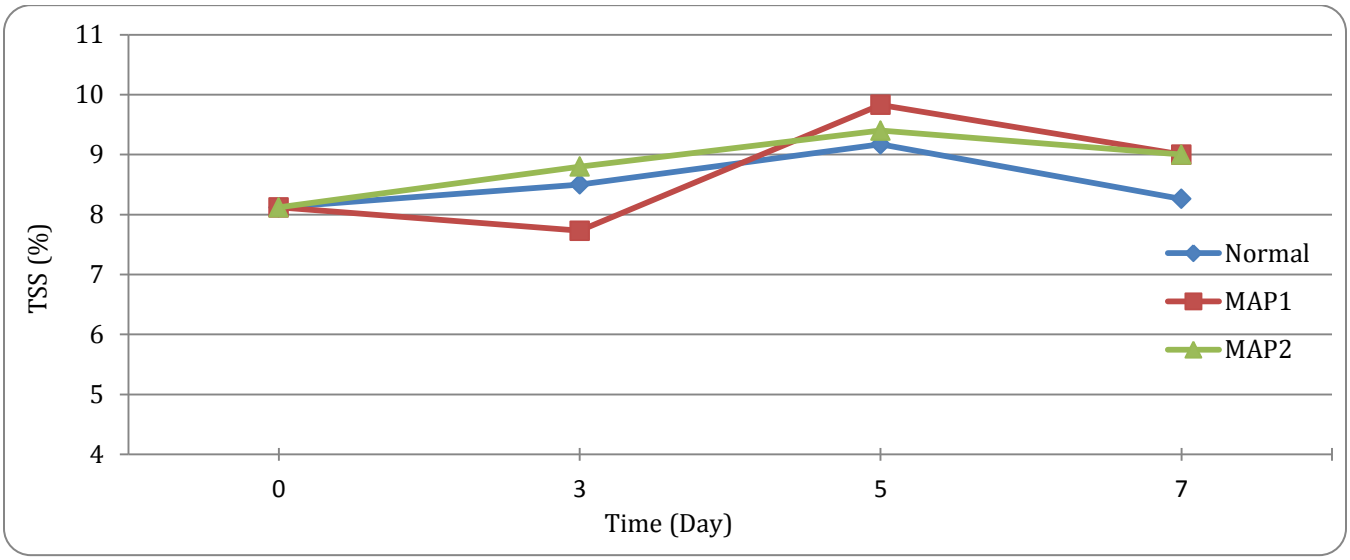

Figure 3. Changes of TSS (%) of Kurdistan strawberry at 5°C under different atmospheres.

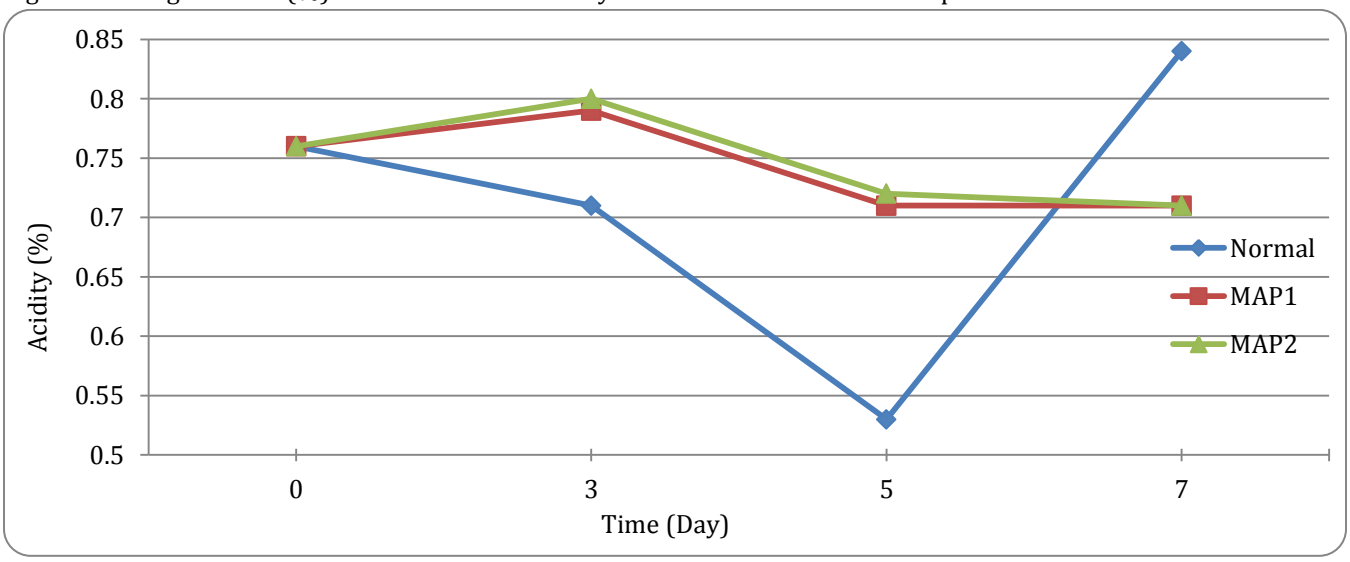

Figure 4. Changes of acidity (%) of Kurdistan strawberry at 5°C under different atmospheres.

During the storage, pH range was from 3.44 to 3.67. As shown in Figure. 5, pH increased in all samples. pH in MAP2 changed drastically with lesser change in MAP1. These results are in agreement with Siriphanich (1980), who reported that high-CO_2 treatments resulted in higher pH values than those of air control strawberries.

Vitamin C and anthocyanin: Vitamin C content of strawberry fruits was 32.76mg/100g. Over the storage time, the vitamin C level decreased in all samples under different atmospheres (Figure. 6). Vitamin C content in normal and MAP strawberry ranged from 32.76 to 1.42 mg Vit C 100 g^{-1}. Vitamin C, which is thermo-labile, is easily destroyed during processing and storage. At the end of storage period, levels of vitamin C in samples under normal atmosphere were significantly lower than in MAP samples ($P<0.05$). A possible explanation for these differences could be oxidation of vitamin C during storage. It has been reported that storage in CO_2-enriched atmospheres had beneficial effects on vitamin C retention in the first days of storage, but the levels decreased after 10 days of storage (Perez and Sanz, 2001). Our results showed that vitamin C content of strawberries stored under MAP was higher than that of air-stored samples at the end of the storage.

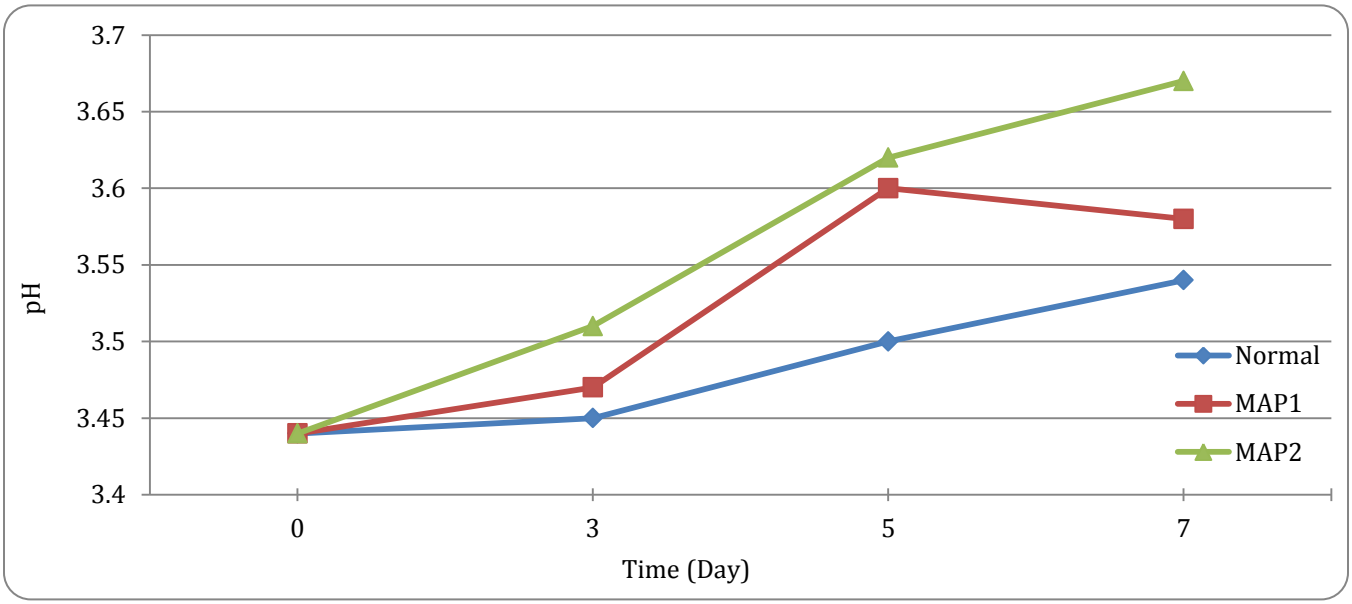

Figure 5. Changes of pH of Kurdistan strawberry at 5°C under different atmospheres.

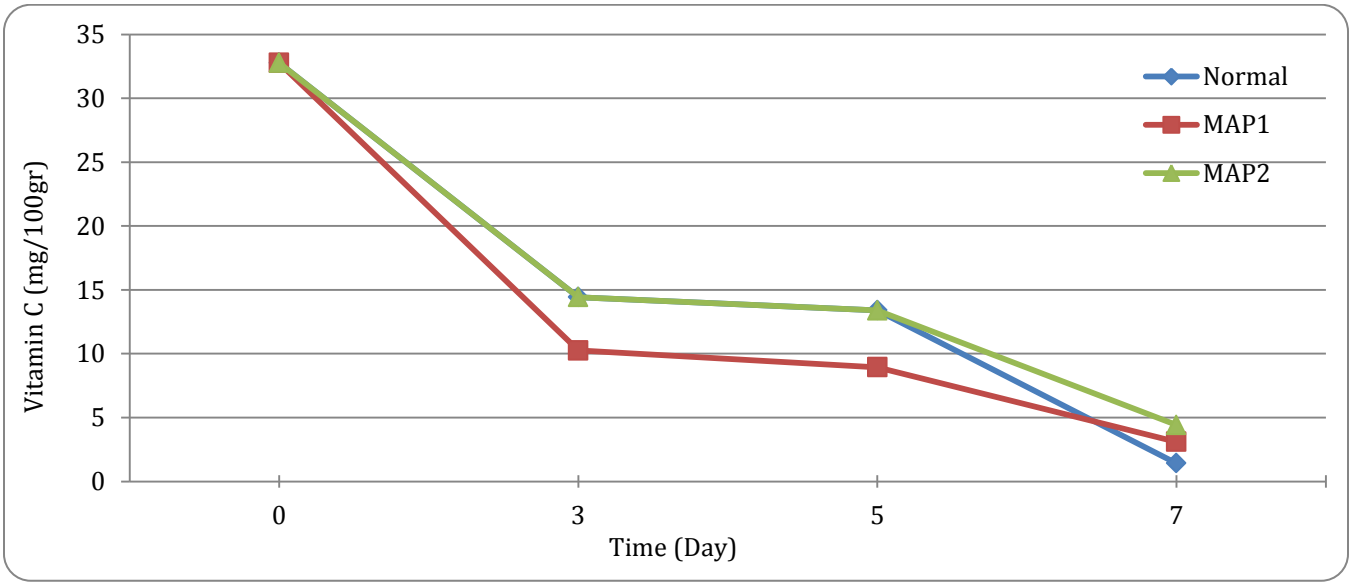

Figure 6. Changes of vitamin C (mg/100gr) of Kurdistan strawberry at 5°C under different atmospheres.

Several authors reported that phenolic contents, particularly anthocyanin, were lower in strawberries treated with atmosphere containing CO_2-enriched atmospheres when compared to air-stored fruit (Perez and Sanz, 2001; Zheng *et al.,* 2007). This phenomenon has been explained as a delay of the fruit ripening process caused by CO_2-enriched atmospheres (Wszelaki & Mitcham, 2000). Our results confirmed that high CO_2 level may reduce anthocyanin content (Figure. 7).

Mould spoilage: The rate of microbial growth is affected by the gas composition of atmosphere. This factor may be applied to a food product to delay or inhibit microbial development, thereby prolonging its shelf life. When applying MAP as a hurdle technique, one must consider the types of microorganisms which may cause spoilage and safety problems to the product.

The absence of oxygen would inhibit the growth of aerobic microorganisms. Obligatory aerobes such as moulds can be effectively controlled by the removal of oxygen. This was shown by Smith *et al.* (1986), who used an oxygen scavenger to control mould on baked products. An anaerobic atmosphere must be used with extreme caution because this atmosphere favors the growth and toxin production of *C. botulinum* (O'Conner-Shaw & Reyes, 2000). The results showed that decayed strawberry fruit increases with storage time but can be controlled by the use of a suitable atmosphere (Figure. 8).

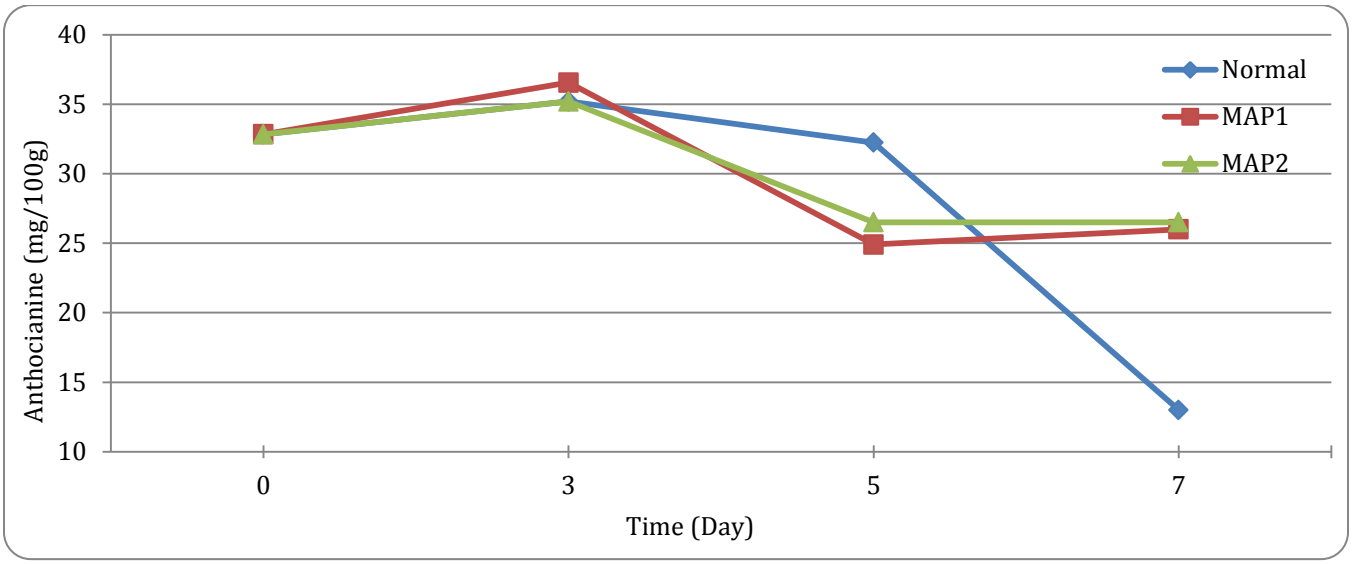

Figure 7. Changes of anthocyanin (mg/100gr) of Kurdistan strawberry at 5°C under different atmospheres.

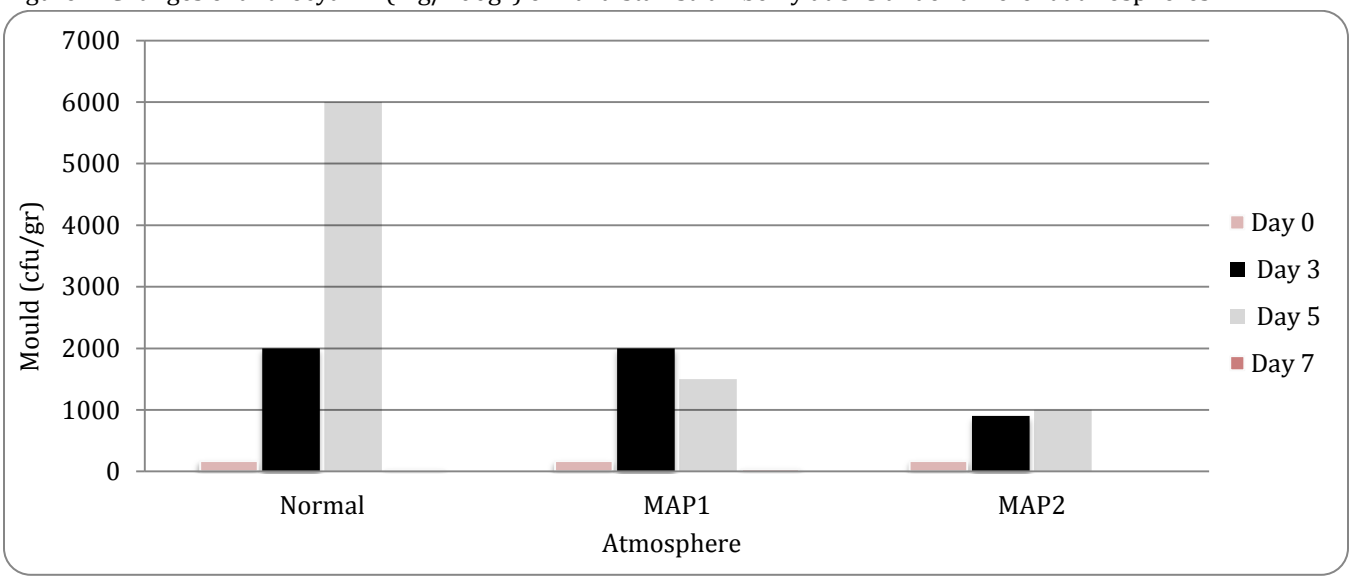

Figure 8. Changes of mould number (cfu/gr) of Kurdistan strawberry at 5°C under different atmospheres.

Thus, the number of moulds in air-stored strawberries was uncountable on 7th day of storage. As shown in Figure. 8, due to O_2 concentration, mould growth was significantly different in MAP1 and MAP2. On 7th day the number for MAP1 and MAP2 was lower than 30 cfu/gr.

Flavour analysis: The flavour analysis was performed to differentiate any flavour between the different packaging conditions during 7 days of storage (Figure. 9). There were significant differences in the flavour of strawberries kept in the different packaging conditions. None of the samples including air-stored and MAP1 showed significant changes after 5 days of storage. However, on 5th day the flavour of strawberries with MAP1 condition changed significantly. After 7 days of storage, the reduction of flavour was more noticeable in control samples when compared to strawberries in MAP1 and MAP2. This negative effect on flavour of strawberry could be stated as the most adverse effect of MAP. Similar trend has been reported by Allende *et al.* (2007). Acetaldehyde is a very volatile intermediate product of anaerobic respiration in fruit and vegetables. In the presence of sufficient O_2, most fruit and vegetables respire aerobically and at low O_2 concentrations, there is a chance of fermentation.However, when there is inadequate NADH to reduce all of the acetaldehyde to ethanol, result the accumulation of acetaldehyde. CO_2-enriched atmosphere has a role in the induction of anaerobic respiration and ethanol production (Kimmerer & Kozlowski, 1982). Kader (1986) stressed that the effects of both low O_2 and high CO_2 concentrations in the induction of anaerobic respiration are additive. Ethanol in the tissue stored under modified atmosphere at constant temperature suggests partial induction of anaerobic respiration when the O_2 concentration dropped below 10% and the CO_2 concentration rises above 5% (Kader, 1987).

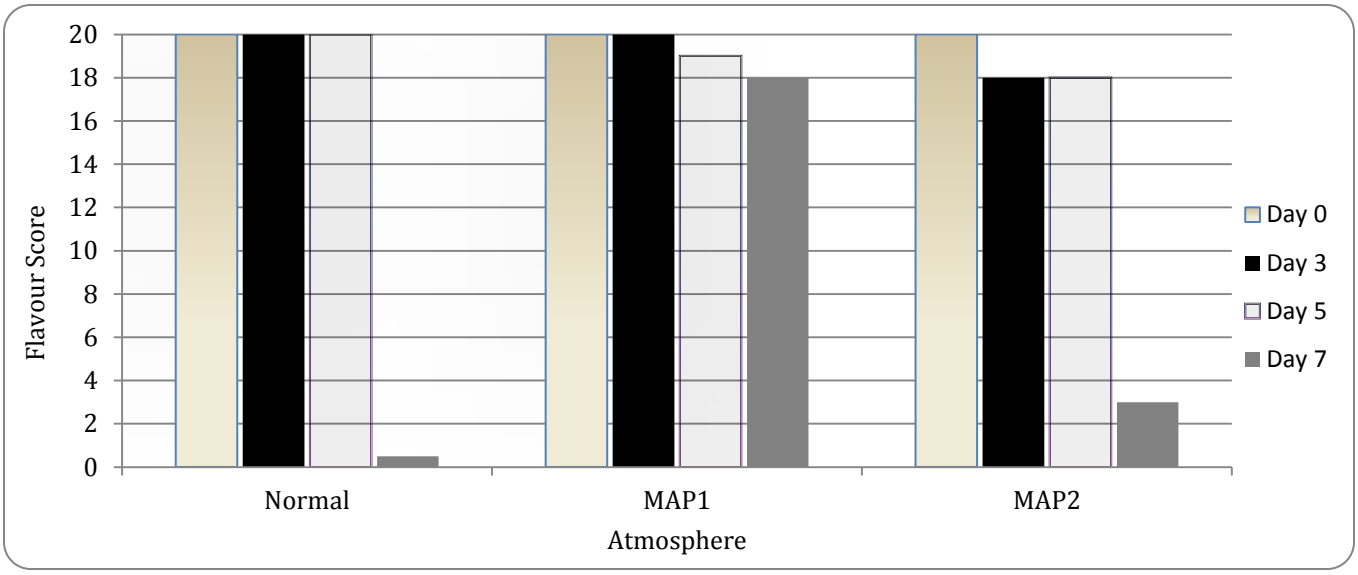

Figure 9. Changes of flavour score of Kurdistan strawberry at 5°C under different atmospheres.

Although high CO_2 atmospheres reduced decay, they increased production of fermentative metabolites that impart negative organoleptic properties to strawberries (Van der Steen et al., 2002; Pelayo *et al.,* 2003). Therefore, in this study, flavour scores of active MAP strawberries were significantly (Probability $P<0.05$) lower than in air-stored samples after 3rd day of storage.

CONCLUSIONS

The postharvest treatments (name the treatments) tested under the described conditions affected the physico-chemical properties of 'Kurdistan' strawberries. In general, the use of high CO_2 atmospheres significantly reduced anthocyanin content of strawberries after treatment. At the end of storage time, the content of vitamin C under normal atmosphere after 7th day was significantly lower than MAP samples ($p<0.05$). MAP conditions delayed the fungal growth when compared to air-stored samples (include the temperature = 25°C and RH = 35%). Flavour scores of MAP in Kurdistan strawberries were significantly lower than those of air-stored samples after 3 days of storage.

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QUALITY EVALUATION OF HONEY FROM THE DIFFERENT REGION OF AZERBAIJAN

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A B S T R A C T

The main objective of this study was to evaluate the quality of Azerbaijan honey, verifying their compliance with international standards. Honey samples used in the research represent main honey producing regions of Azerbaijan. The physico-chemical characteristics of 29 from the 53 honey samples (54 % of all samples) analyzed in this study completely agree with the European Commission and the Codex Alimentarius indicating adequate processing, good maturity and freshness. 15 samples (28 % of all samples) did not meet characteristics established in European and Codex standards relative to the diastase activity (min. 8 Schade units), although the other physico-chemical parameters were within the range of the allowable limits. In the 53 samples analyzed, the HMF content is quite elevated 4 samples (less than 8 % of all samples) exceed the limit of 40 mg/kg, and 34 samples (64 % of all samples) show values lower than 10 mg/kg, which are typical of fresh unheated honeys, according to the current quality criteria. 2 samples (4 % of all samples) show a fructose + glucose content lower than 60 g/100g, only both of the same samples exceed the value of 5 g/100g for sucrose. 7 samples (13% of all samples) show the protein values below 0.1%.

Keywords: Azerbaijani honey, chemical composition, quality of honey, water content, sugar content.

INTRODUCTION

Honey is a complex mixture produced by honeybees from the nectar and also exudates from plants and it is consumed as a sweetener. Honey is a supersaturated solution of sugars with fructose and glucose as main saccharides. Antimicrobial effects of honey against microorganism associated with disease or infection have been reported and honey biological activity has been attributed not only to the high sugar concentration but also to different compounds such as acids, phenolics, proteins, vitamins, minerals and carbohydrates (Beatriz A. Rodriguez, Sandra Mendoza et al., 2012).

The number of honey types that are being produced depends on the geographical region and climatic conditions (Kaspar Ruoff, 2006a). This variety of physical and chemical parameters of honey does not allow establishing standard criteria. Currently used standards (The "REVISED CODEX STANDARD FOR HONEY") is intended for voluntary application by commercial partners and not for application by Governments (Codex Alimentarius. 2011). According to these standards, quality of honey is characterized by parameters; sugar profile, moisture content, acidity, diastase number, the amount of HMF and protein content.

The amount of amino acids and proteins are relatively small, at the most 0.7 % thus having relatively small nutritive effects. However these components can be important for judging the honey quality. The honey proteins are mainly enzymes and other amino acids (Stefan Bogdanov, August 2009). In general, of the total honey protein, about 1/3 relates to pollen and has plant origin and the remaining 2/3 includes enzymes and proteins with insect origin (honey bee) (Hassan Nazarian, Razieh Taghavizad and Ahmad Majd, 2010). Of the 8 to 11 proteins found in various honeys, 4 are common to all, and appear to originate in the bee, rather than the nectar (J.W. White and Landis W. Doner, 1980).

For testing of thermal treatment of honey, hydroxymethylfurfural (HMF) content in honey is usually determined. HMF in honey is formed from carbohydrates, mainly from fructose, which is thermally more labile than sacchorose and glucose.

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Fructose disintegrates at approximately 60 ^{0}C (Belitz H.D., Grosch W., 1992).

The main objective of this study was to evaluate the quality of Azerbaijan honey, verifying their compliance with international standards. Honey samples for this research were bought from sales fair and exhibition of beekeeping products, organized by the Ministry of Agriculture of the Republic of Azerbaijan. During 2012 in Azerbaijan produced 110 tons of honey. The annual production of honey samples used in the study is the 17% of the total production (http://azinterkom.com).

MATERIALS AND METHODS

Honey samples: Honey samples used in the research represent main honey producing regions of Azerbaijan. Honey samples were bought from sales fair and exhibition of beekeeping products, organized by the Ministry of Agriculture of the Republic of Azerbaijan. Honey samples coming from the regions which are covered by Greater Caucasus (North), Lesser Caucasus (West) and Talysh Mountains (South) (figure 1.). In 2012 in Azerbaijan was produced 110 tons of honey. The annual production of honey samples used in the study is the 17% of the total production. These samples were stored at room temperature in a dark place before analysis.

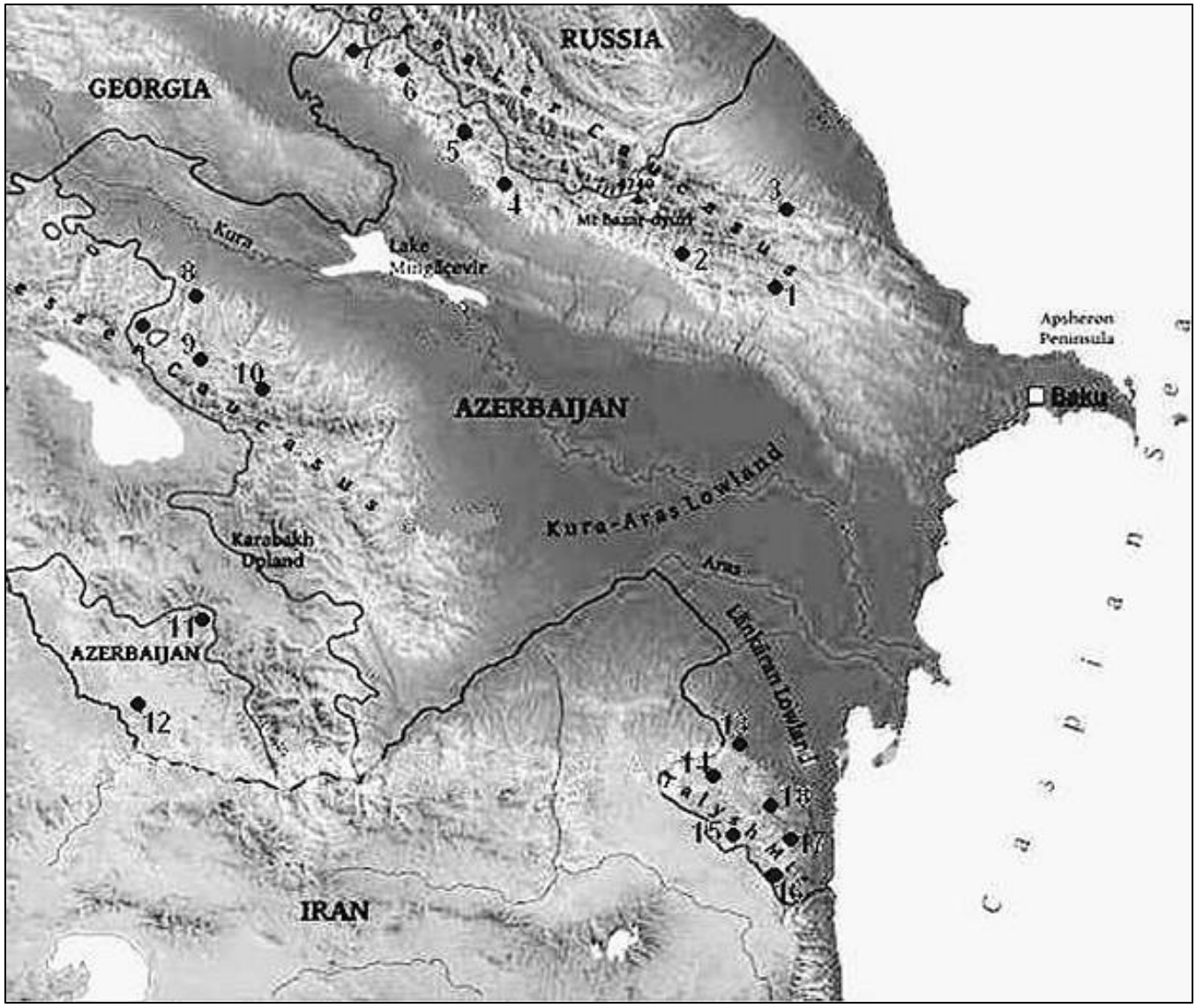

Figure 1. Location of honey bee sampling points in Azerbaijan. 1. Shamakhi, 2. Ismayilli, 3. Guba, 4. Sheki, 5. Gakh, 6. Zagatala, 7. Balaken, 8. Tovuz, 9, Gadabay, 10. Dashkasan, 11. Sharur, 12. Nakhchivan, 13. Jalilabad, 14. Yardymly, 15. Lerik, 16. Astara, 17. Lenkoran, 18. Masalli.

All chemicals (sodium hydroxide, sodium chloride, potassium iodide, iodine, bromocresol green, methyl red, sulfuric acid, hydrogen peroxide) analytical-reagent grade from Merck Chemicals. HPLC solvents were LC grade – LiChorsolv Acetonitrile and ultrapure water (Sartorius Stedim Biotech, arium 611DI, Gottingen, Germany. 18 MΩ/cm resistivity). Quality of honey was characterized by various chemical and physicochemical parameters.

Moisture content: For moisture content of natural honey max. value 20% was taken into account. Refractometric analysis method has been applied (Harmonised Methods of the İnternational Honey Commission, 2002a). (Refractometer – Bellingham Stanley, thermo controlled.)

Free acidity: The free acidity of honey is the content of all free acids, expressed in milliequivalents/kg honey, determined by following prosedure. The 10 g sample was dissolved in 75 ml of carbon dioxide - free water in 250 ml beaker. The pH measured and the solution titrated with 0.1N sodium hydroxide solution to pH 8.3 (Harmonised Methods of the İnternational Honey Commission, 2002b).

Sugar profile: HPLC method was used to determine the sugar profile of honey. After filtration of the solution, the sugar content were determined by HPLC (Agilent 1200) with RI – detection. Peaks were identified on the basis of their retention times. Quantitation was performed according to the external standard method on peak areas (Harmonised Methods of the İnternational Honey Commission, 2002c).

For the Agilent Zorbax carbohydrate column (4.6 mm diameter, 150mm length, 5µm particle size).

The following conditions have been used to give satisfactory separation.

Flow rate: 1.3 ml/min

Mobile phase: Acetonitrile / water (75/25 , v/v)

Column and detector temperature: 30 ^{0}C

Sample volume: 20 µl

Enzyme activites: With the appointment of Diastase points an idea about the freshness of honey was obtained. Enzyme activites in honey were principally measured to evaluate possible heat defects. Even if alpha – amylase and alpha – glucosidase are derived mostly from the bees , the different honey types however show considerable differences in enzyme activities (Persano Oddo L., Baldi E., Accorti M., 1990). However, as the enzyme activites in honey decrease during storage and heat treatment, indications to botanical origin can only be obtained from fresh honeys (Kaspar Ruoff, 2006b).

The traditional method for the measurement of diastase activity in honey is the Schade procedure. One unit of diastase activity (or more specifically, α – amylase) the Schade or Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end - point in one hour at 40 ºC. For determination of diastase activity, five grams of honey were dissolved in 15 ml water; and transferred to a 50 ml volumetric flask. According to the method, 9 different pre-defined volumes of this solution were mixed with 5 ml 0.25 % starch solution in a tube and incubated during 15 minutes and then, tubes were cooled. In each of tube were added 0.5ml KI + I solution. A standard solution of starch, capable of developing with iodine, a color in a defined rage of intensity. The diminution in the blue color is measured at intervals (V.I.Krishtafovich, I.F.Zhebeleva, 2001).

Hydroxymethylfurfural: Hydroxymethylfurfural (HMF) was determined in aclear, filtered, aqueus honey solution using reverse phase HPLC equipped with UV detection at 285 nm. The signal was compared with those from standards of known concentration. Five grams of honey samples were diluted up to 50 ml with distilled water, filtered on 0.45 µm filters and immediately injected in a HPLC equipped with a UV detector. The HPLC column was a C 18 -reversed phase material, 250_4 mm, fitted with a guard cartridge. The HPLC conditions were the following: mobile phase, 90% water and 10% acetonitrile; flow rate 1 ml/min; injection volume, 20 µl. The wavelength range was 220–660 nm and the chromatograms were monitored at 285 nm. HMF was identified by splitting the peak in honey with a standard HMF (Harmonised Methods of the İnternational Honey Commission, 2002d; M. Zappal, B. Fallico, E. Arena, A. Verzera, 2005a). HPLC separates HMF from other components and thus avoid interference in the determination (Wootton M., Ryall L. 1985). HPLC method seems to be the more appropriate for HMF determination in honey, because the presence of substances, probably derived by heat or storage damage, which interfere with the UV methods did not reveal (M. Zappal, B. Fallico, E. Arena, A. Verzera, 2005b).

Protein content: Protein content was determined with

"UDK-152 Automatic Distillation and Titration Unit" by Kjeldahl method. 1 q honey was taken for analyze, and was kept in sulfuric acid for 60 min. at 420 ^{o}C for digestion (AOAC, method 960.52, 104).

Statistical Analysis: Results represent the average of at least three replications for moisture content, free acidity, sugars, protein content, hydroxymethylfurfural (HMF) and enzyme activity. Statistical analysis was carried out by the use of Microsoft Excel Statistical Packages and GraphPad Instat program.

RESULTS AND DISCUSSION

Considering altogether the results of these physicochemical analyses, we can observe that, as far as the quality is concerned, only 54 % of the examined samples can be evaluated as wholly compliant, while about 45 % of them present some quality defect (Table 1).

Table 1. The results of physico - chemical analysis of Azerbaijani honey samples (n = 53).

Parameter	Mean value	Min – Max values	Limits of EU standards	Samples exceeding limits of EU standards
Diastase	9.69	6.5 - 23.8	min. 8 Schade units	24 samples
HMF mg/kg	14.53	0.23 - 160.68	max.40	4 samples (34 samples show values lower than 10 mg/kg)
Water %	16.52	15.2 - 18.7	max. 20	not detected
Free Acidity mmol/kg	22.26	8.4 - 43.6	max. 50	not detected
Fructose %	39.83	29.21 - 44.02	not fixed limit	
Glucose %	32.09	23.68 - 37.86	not fixed limit	
Frcts+Glcs %	71.91	52.89 - 72.91	min. 60	2 samples
Sucrose %	4.87	ND - 28.58	max.5	2 samples
Glcs/Frcts	1.24	1.02 - 1.44	not fixed limit	
Glcs/Water	1.94	1.41 - 2.44	not fixed limit	
[a]Protein %	0.31	0.01 -0.67	min.0.1	7 samples

[a]Total protein of honey is between 0.1% to 0.65% (Hassan Nazarian, Razieh Taghavizad and Ahmad Majd. (2010). Origin of honey proteins and method for its quality control. Pakistan Journal of Botany, 42(5), 3221-3228.).

Water content: For the water content, most of the samples show quite low values. Moisture shows an average value 16.5 %, this variety depends on climatic factors, season of production and maturity of honey. 20 % of moisture is the maximum allowed to avoid fermentation. (Cantarelli M.A., Pellerano R.G., Marchevsky E.J., Camina J.M., 2008.).

Sugar profile: Sugar profile is quite in agreement with Bogdanov et al. (Bogdanov S. et al., 1999.) and with the international standards: only 2 samples show a fructose + glucose content lower than 60 g/100g, both of the same samples exceed the value of 5 g/100g for sucrose.

pH and free acidity values: pH values do not indicate a significant amount of honeydew. All free acidity values fall under the prescribed limit of 50 meq/ kg.

Enzyme (diastase activities): Enzyme (diastase activities) 23 samples have a diastase value lower than 8 Schade units. The EU standards establish a limit of not less than 8 Schade units for diastase. It is to be noted that the use of enzyme activities as indicators of honey freshness is often criticized, since the initial enzyme activity may be very different in the various honey types (White J.W. 1994.). The enzyme activities in honey depend on the intensity of the nectar flow and the amount of nectar processing by the honey bees. Therefore honey from very rich nectar sources e.g. often show low natural enzyme activities. When honey is adulterated by addition of inverted sucrose or hydrolyzed starch namely high fructose corn syrup (HFCS), then such dilution of honey leads to the reduction of diastase number (M.Voldrich, A.Rajchl, H.Cizková and P.Cuhra. 2009).

The measure of HMF content: It is used to evaluate honey freshness. The EU standards establish a limit of not more than 40 mg/kg for HMF and not less than 8 Schade units for diastase. Fresh honey does not contain hydroxyl-methyl-furfural. Thus HMF is not a useful criterion for the botanical classification of honey. However, before determining storage dependent measurands such as enzyme activity, one should ensure that honey are fresh and do not express any heat defects by checking the HMF content is below 15 mg/ kg.(Kaspar Ruoff. 2006c).

In the 53 samples analyzed, the HMF content is quite elevated (14.5 mg/kg on average) 4 samples exceed the limit of 40 mg/kg, and 34 samples show values lower than 10 mg/kg, which are typical of fresh unheated honeys, according to the current quality criterion. As far as enzyme activities are concerned, 23 samples have a diastase value lower than 8 Schade units.

Evaluation of the protein content: The honey proteins are mainly enzymes and other amino acids (Stefan Bogdanov, 2009). In general, of the total honey protein, has plant origin and proteins (enzyme) with insect origin (honey bee). Pollen is the major source of protein for honey bees.

During processing, sugar feed enriched with protein. Feeding honey bees sugar in syrup form is the most popular and probably most effective method in Azerbaijan too, and some beekeepers use this method for adulterating honey. In the experiments of Shenfelda (1955) protein content increased only to 0.08%, after the bee feeding syrup. But in blossom honey protein content is 0.2-0.4%. Blossom or nectar honey is derived from the nectarines of flowers and honeydew honey comes from the sugary excretion of some hemipterous insects on the host plant or from the exudates of the plants (Saxena S., Gautam S. and Sharma A., 2010). Nectar is secreted by glands at the base of the flowers, known as nectarines. Field bees collect nectar from blossom in the field. At this stage, the nectar has a high level of sucrose sugar with some laevulose and dextrose and high moisture content, with traces of other substances such as minerals, vitamins, pigments, aromatic substances, organic acids and nitrogen compounds (Goulburn, 2000). Nectar is primarily a carbohydrate source, but can contain some amino acids and lipids (Baker H.G., Baker I., 1975). The main source of the sugar in honey is nectar or honeydew. Pollen is the major source of protein for honeybees. Pollen is made up of various substances, including proteins, fats, lipids, carbohydrates, vitamins, minerals and many others. Honeybees rely on pollen as their source of protein, lipids, sterols, vitamins, minerals and certain carbohydrates (Todd F.E., Betherick O., 1942).

There are some protein compounds in honey in addition to sugars, lipids and mineral compounds. Relative quantity of proteins in honey compound is considered as a quality index. Determination of the quantity of plant origin (pollen) and animal origin (honey bee) of the proteins of honey is an important. (Hassan Nazarian, Razieh Taghavizad and Ahmad Majd, 2010).

First are reactions allowing protein or peptide quantification as the Kjeldahl method (IDF standard, 1964) based on the determination of the nitrogen content after a sample mineralization step. Nowadays, the evaluation of the protein content is mainly based on the Kjeldahl method.

The protein content of 53 honeys considered quite high, with an average of 0.31% and only 8 samples (15%) show values lower than 0.1% (figure. 2).

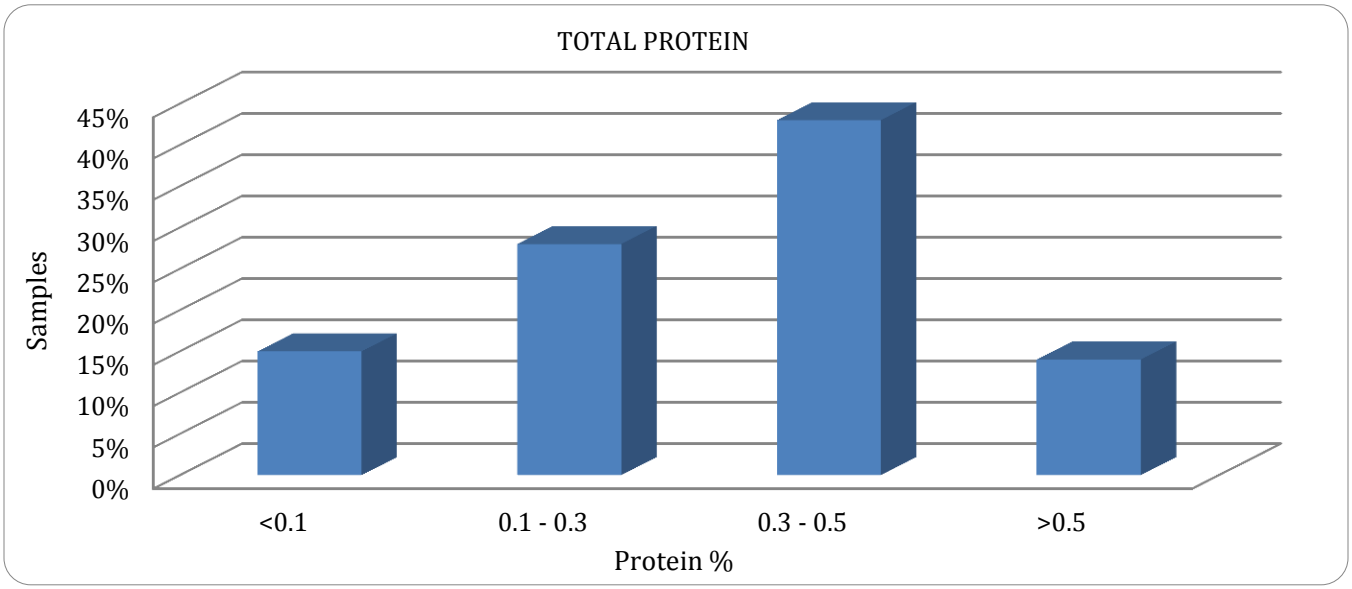

Figure 2. Distribution of the 53 honey samples according to the total protein content.

By comparing the freshness indicators, 9 unheated (HMF lower then 10mg/kg) samples are judged not to correspond to fresh honey, 6 of unheated non-fresh samples show protein values lower than 0.1 %. This can be accounted for diastase by inadequate processing or storage conditions, more feeding sugar to a colony, but partly for it could also be due to the climate of the production area. The physico-chemical characteristics of 28 from the 53 honey samples analyzed in this study completely agree with the European Commission and the Codex Alimentarius indicating adequate processing, good maturity and freshness. 15 samples did not agree with characteristics established in European and Codex standards relative to the diastase activity, although the other physico-chemical parameters were within the range of the allowable limits (figure 3).

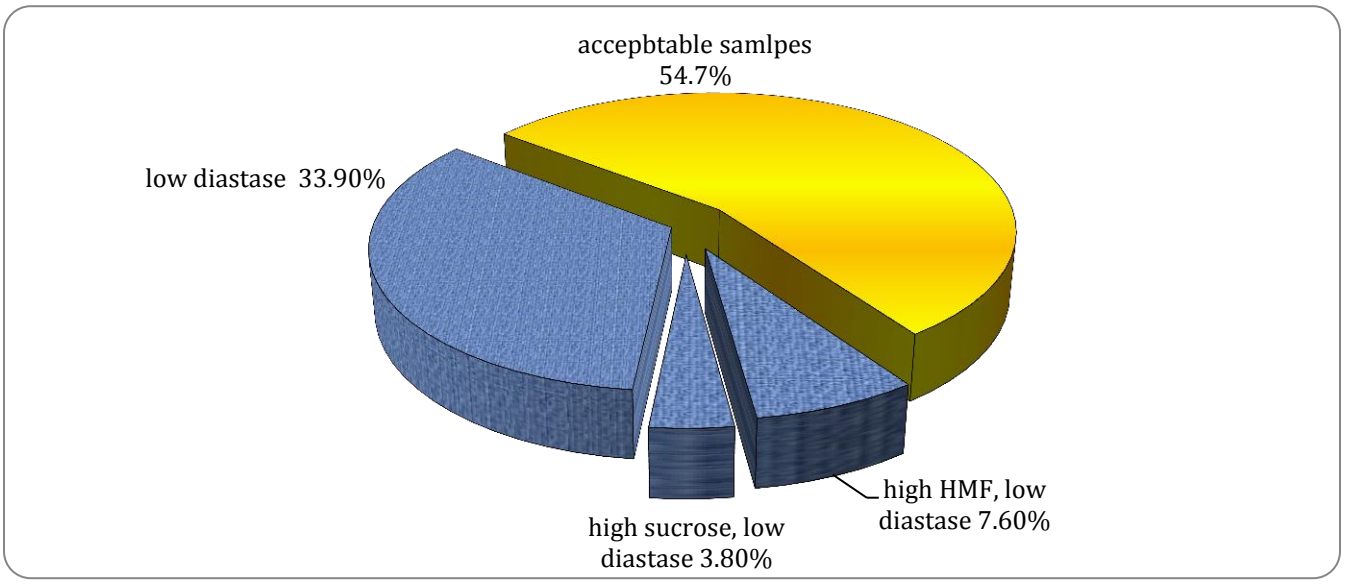

Figure 3. Defects found in the honey samples.

Table 2 is designed to study the influence of geographical conditions on the parameters of quality. Table was used to study the physical and chemical quality of honey purchased in three different regions of Azerbaijan. Means of physical and chemical results were found in samples of honey that are acceptable quality on the basis of EU standards shown Table 2.

Table 2. Physico - chemical quality of honey samples from different regions of Azerbaijan

Parameter	Regions			Total mean value	Limits of EU standards
	North	West	South		
Diastase	12.9	11	9.7	11.2	min. 8
HMF mg/kg	4.2	8.2	9.8	7.4	max.40
Water %	16.2	16.3	17.0	16.5	max. 20
Free Acidity mol/kg	24	22	24	23.3	max. 50
Fructose %	41.9	41	39.5	40.8	not fixed limit
Glucose %	32.4	31.3	32.7	32.7	not fixed limit
frcts +glcs %	74.3	73.4	71.1	72.9	min. 60
Sucrose %	0.66	0.34	0.71	0.57	max.5
glcs /frcts	1.3	1.27	1.28	1.28	not fixed limit
glcs /water	1.3	1.3	1.3	1.3	not fixed limit
[a] Protein %	0.4	0.38	0.37	0.38	min.0.1
Starch	ND	ND	ND	ND	not acceptable
Defect samples	5	12	7	Total unacceptable samples 24	

[a] Total protein of honey is between 0.1% to 0.65% (Hassan Nazarian, Razieh Taghavizad and Ahmad Majd. (2010). Origin of honey proteins and method for its quality control. Pakistan Journal of Botany, 42(5), 3221-3228.).

The moisture content of honey is highly important factor contributing to its stability against fermentation and granulation during storage (Singh and Bath, 1997). The present study also demonstrated that the average moisture content in the Northern and Western region is lower than in the South region. Southern region is hot and very humid subtropical climate, but the northern region has low humidity and very cold climate. The different moisture content of honey depends on harvest season, the degree of maturity reached in the hive and moisture content of original plant (Finola, Lasagno, Marioli, 2007).Moisture shows a mean value of 16.5 %, showing a mean value lower than that reported in Turkey - 18.9% (Guler, Z. 2005), in France - 18.1% (Devillers et al. 2004) and in Poland (Przybylowski and Wilczynska. 2001).

The diastase activity and the HMF content are widely recognized as parameters indicating the freshness of honey (Mendes et al., 1998). The total mean of diastase activity is 11.2 units. Samples from Talysh Mountains have lower values than those from Caucasus regions. Diastase activity level is very different around the world. A higher level of diastase activity were registered in France - 22.4 (Devillers et al, 2004.), Argentina – 19.7 (Cantarelli et al, 2008) also in Italy -39.1 (Esti et al.1997). No significant differences were found in the protein and acidity levels between the three regions of origin.

Samples of honey from the Greater Caucasus region showed significantly lower HMF content than the samples from the Lesser Caucasus and Talysh Mountains. The variation in the activity of diastases and HMF may be related to source of honey as well as climate of region (Singh and Bath, 1997). The mean value of HMF in this study was 7.4 mg /kg, higher than that obtained in Turkey 4.52 mg /kg (Turhan. 2007), but lower than those obtained in Argentina -14.8 mg /kg (Finola et al. 2007), as well as a report from the Italy- 7.6 mg /kg (Esti et al. 1997) showed almost the same result as our result.

In this study, the combined levels of glucose and fructose varied from 68.4% to 78.9%. Glucose + fructose means belonging Greater Caucasus (74.3%) were found less high than in other regions. The mean value of glucose and fructose was found 72.9%. Our results for glucose and fructose value showed approximately similarity with the results from Algeria - 72,6% (Ouchemoukh et al, 2007). Higher than in Turkey - 68.4% (Guler, Z. 2005) and in Argentina -68,1% (Cantarelli et al, 2008).

In this study, the mean value of sucrose was found 0.57%. This result is lower than the results from Argentina - 4,05% (Cantarelli et al, 2008), Turkey - 3,03% (Turhan. 2007), France - 0.74% (Devillers et al, 2004.) Poland - 1,23% (Przybylowski Wilczynska and 2001), Italy -1.09 (Esti et al al.1997).

Starch is important aspect in assessing the genuineness of honey, according to EU standards, the presence of starch and hydrolyzed starch is not acceptable. In this study, all samples were in the acceptable range.

CONCLUSIONS

In a study of 53 samples of Azerbaijani honey, which was produced in three different regions of Azerbaijan, some consideration may be given to the professional level of beekeepers, who sometimes do not allow high quality production and marketing of honey in the country, in fact, only 54% of the samples to achieve good quality, while about 45% of them show one or more defects (According to EU standards). An increased and more effective extension service will be necessary to improve the beekeepers' knowledge about honey quality features and adequate production and storage technologies. On the other hand, better control of the marketed honey is needed for consumer protection. The researches on Azerbaijani honey should be further developed, in order to better understand the actual extent and interpretation of some of the analytical results obtained, which may be related to bee race, environment, climate, bee forage, etc., and to learn more about the local bee flora. Moreover, the achievement of a good knowledge of the product would provide the scientific support for the introduction of a national norm for honey.

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STORAGE RETENTION OF STILBENE, ELLAGIC ACID, FLAVONOL, AND PHENOLIC CONTENT OF MUSCADINE GRAPE (VITIS ROTUNDIFOLIA MICHX.) CULTIVARS

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A B S T R A C T

The presence of ellagic acid and other nutraceutical compounds in muscadine grapes add value and enhance the marketability of this southern U.S. specialty crop. Due to its nutraceutical profile, muscadines may potentially become the next "super fruit". The objective of this study was to determine the retention of important phytochemical compounds including anthocyanins, phenolics, flavonols, stilbenes and organic acids from whole muscadine grape berries and individual fruit parts following cold storage. Stilbene, ellagic acid, flavonol, and phenolic compounds were analyzed in berries of 11 muscadine grape cultivars following 14 days of cold storage at 4˚C. The major phenolic compounds were identified by their retention times and characteristic spectra. Quantification was made by utilizing calibration curves of external standards for each of the analyzed compounds including trans- and cis-resveratrol, trans- and cis-piceid, ellagic acid, myricetin, quercetin and kaempferol. Total phenolics decreased in 6 cultivars but increased in 5 cultivars, suggesting differences in decay development and fruit deterioration. Anthocyanin content showed an overall decrease in all cultivars except 'Eudora'. Stilbenes showed an overall decrease across cultivars, but flavonol content was cultivar and compound specific. Free ellagic acid increased in all cultivars, except 'Pollyanna', and total ellagic acid increased or remained constant in all cultivars.

Keywords: nutraceutical, piceid, polyphenolic, resveratrol, *Muscadinia rotundifolia*.

INTRODUCTION

Muscadine grapes (Muscadinia rotundifolia; syn Vitis rotundifolia Michx.) are native to the southeastern U.S. and are the most widely cultivated Vitis species in the region due to their inherent resistance to numerous fungal pathogens and to Pierce's disease (Xylella fastidiosa). They thrive in soil and climate conditions not generally favorable for bunch grape production (Talcott and Lee, 2002). Vines grow vigorously reaching a length of 30 meters or more in the wild. The grapes are produced in small clusters, have a unique fruity flavor, and are considered a southern delicacy. Muscadines differ from other Vitis species since they have an extra pair of chromosomes (Patel, 1955). They have a notably thicker skin than other Vitis species that protects them from heat, UV radiation, humidity, insects and fungi. Although their consumption is primarily limited to the southern U.S. region, their unique aroma, flavor, and more importantly their high nutraceutical content make this under-utilized fruit a candidate for expanding markets of fresh fruit, wine, juice, and additives for healthier processed foods.

Stilbenes are a small class of phenylpropanoids characterized by a 1,2-diphenylethylene backbone. Stilbenes are synthesized in grape berries under natural environmental conditions (Jeandet et al., 1991), but are increased by the up-regulation of defense genes encoding pathogenesis-related proteins (Chong et al., 2009). The cis- and trans-isomers of resveratrol, a pharmacologically important stilbene, are present in the skin during all ripening stages, but are almost totally absent from the pulp (Chong et al. 2009). Specific accumulation of resveratrol in the berry skin results from the localization of stilbene synthase (STS), the pivotal enzyme for stilbene biosynthesis. As a defense mechanism, stilbenes display potent antifungal effects as well as function in dormancy and growth inhibition in

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plants (Croteau et al., 2000). From a pharmacological perspective, scientists have been reporting for decades the various ways that stilbene and resveratrol can positively affect health (Arichi et al., 1982; Brakenhiem et al., 2001; DeSanti et al., 2000a; DeSanti et al., 2000b; El-Mowafy, 2002; Jang et al., 1997; Kimura et al., 1985; Kinsella et al., 1993; Lu and Sorreno, 1999). Piceid, resveratrol 3-O-β-D-glucoside, also exhibits activity comparable to resveratrol (Romero-Perez et al., 1999). Resveratrol and piceid exist in the cis- form, an isomer of the trans- form. In early studies trans-resveratrol (TRes) was shown to inhibit platelet aggregation, inhibit the oxidation of low-density lipoproteins, reduce the level of triacylglycerol, and protect the liver from lipid peroxidation (Romero-Perez, 1999). Since glycosidase is known to be present in the digestive tract, it is possible that piceid could be converted to resveratrol and absorbed during digestion (Hackett, 1986). Therefore it is important to consider all isomers and glucosides of TRes.

Muscadine grapes possess several unique and distinguishing chemical compounds, including ellagic acid. Ellagic acid is commonly present in other fruits, such as raspberry, strawberry, and blackberry, but is absent in all other Vitis species. Ellagic acid in muscadine grapes is expressed as free ellagic acid (FEA), ellagic acid glycosides, and ellagitannins (Talcott and Lee, 2002). The presence of ellagic acid and its derivatives in plants has been widely studied because of its antiproliferative and antioxidant properties. The antiproliferative properties are due to its ability to directly inhibit DNA binding of certain carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons (PAHs) (Lesca, 1983). Ellagic acid also has a chemoprotective effect in cellular models by reducing oxidative stress (Talcott and Lee, 2002; Lesca, 1983; Patrana-Bonilla et al., 2003; Mertens-Talcott et al., 2003; Stoner and Morse, 1997; Khanduja et al., 1999).

Another unique attribute of muscadine fruit chemistry is the presence of anthocyanins such as 3,5-diglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin in non-acylated forms (Flora, 1978; Goldy *et al*., 1986; Lamikanra, 1988). Though absorption of anthocyanins appears to be low in humans (Prior, 2004), it seems likely that cells in which they function in defense of oxidative stress must concentrate the anthocyanins or one of their derivatives (Galli *et al*., 2002). Anthocyanins are known to protect blood vessels in humans. They also play a role in cancer prevention. There are more than 80 publications that discuss the ability of different anthocyanins to prevent different kinds of cancer (Hartle *et al*., 2005).

Phenylpropanoids, or phenolics are a large family of secondary metabolites involved in plant response to abiotic and biotic stresses. Phenolics are ubiquitous in the plant kingdom and are the most abundant secondary metabolites found in plants (Amakura *et al*., 2000). Many phenolics not only protect the parent plant, but also exhibit significant pharmacological benefits. Phenolic compounds play an important role in overall food properties, as they are generally involved in defense against ultraviolet radiation or as phytoalexins (Amakura *et al*., 2000; Dixon, 1986; Singleton, 1980; Zaat *et al*., 1987). Phenolics may also play a role in the regulation of plant metabolism (Laks and Pruner, 1989). Polyphenols represent the third most abundant constituent in grapes and wines after carbohydrates and fruit acids (Singleton, 1980). Phenolics are mainly distributed as 28-35% in skin, 60-70% in seed and less than 10% in the pulp of the grape (Shi *et al*., 2003). Phenolics contribute to the bitterness and astringency of fruits and are also considered to be the most important compounds affecting flavor and color differences in wines. Analysis of total phenolics is used to estimate the antioxidant capacities of fresh fruits and vegetables (Thaipong *et al*., 2006). The higher total phenolic content present in muscadine grapes compared to other *Vitis* species is attributed to high ellagic acid, gallic acid, and flavonoid glycoside concentrations (Lesca, 1983; Mertens-Talcott *et al*., 2003; Patrana-Bonilla *et al*., 2003; Talcott and Lee, 2002; Yilmaz and Toledo, 2004).

Much work has been done on health benefits of individual chemicals found in muscadine grapes, but little information is available showing the concentrations of these compounds present in a large number of muscadine grape cultivars; most studies have only looked at a few of the most widely grown muscadines varieties, such as 'Noble', 'Ison', and 'Carlos'. A more comprehensive look at additional muscadine grape cultivars could reveal some "hidden treasures" in regard to their nutraceutical properties. A previous study (Marshall et al., 2012) examined the important phytochemical concentrations in the muscadine fruit tissue of 21 cultivars at harvest. From these results a subset of 11 cultivars were selected for analysis after 14 days of storage. The criteria for selection were based on

the levels of physical, textural marketability of the fruit after storage.

This study was initiated to determine the concentration and retention of total phenolics and anthocyanins in whole muscadine grapes, as well as total ellagic acid (TEA), stilbenes and the flavonols present in juice, pulp and skin of 11 cultivars of muscadine grapes grown at the Thad Cochran Southern Horticultural Laboratory (TCSHL) in Poplarville, Mississippi after 14 days of storage. Very little compound was recovered in the juice and pulp; therefore, those results are not represented. A comprehensive analysis of these compounds has not previously been reported for muscadine grapes in the gulf coast region, and would provide a valuable resource for growers in this area considering growing muscadine cultivars that maximize functional food benefits.

MATERIALS AND METHODS

Fruit Preparation and Storage: All muscadine grape cultivars were collected from the McNeill, MS vineyard of the Thad Cochran Southern Horticultural Laboratory (TCSHL) in August of 2007. Vines of numerous muscadine grape cultivars were established in a planting in 1994 where vines were planted 6.4 m apart in 3.7 m rows and trained to Geneva double curtain trellis. Training consisted of a single main stem raised to a wire 1.5 m in height with two arms extended 3.2 m each direction from the main stem to form the vine's permanent framework on the double-trellis system. Vines received applications of 227 g/vine of 8N-3.4P-8.8K in March, 114g of 13N-13-P-13K in May, and 114g of 33N annually and were drip irrigated as needed. Weed and insect pest management was conducted using recommended practices (Braswell et al, 2006) and no fungicides were applied.

Approximately 4.5 L of fruit were randomly collected from each cultivar at optimum maturity based on soluble solids content (Brix°). Approximately 100 berries were contained in each of three clam shells placed into growth chambers in a completely randomized design. Growth chambers were set at 4°C. Clam shells were removed from chambers after 14 days of storage for analysis. Two homogeneous fruit subsamples of 10 berries were haphazardly taken from each clamshell and were immediately separated into skins, seeds, juice and pulp. Berries were cut in half around the equator with a scalpel. Seeds were extracted and set aside. Berry halves were pinched gently to separate skin from juice and pulp. Juice partition was separated from pulp by hand squeezing through four layers of cheesecloth. Fruit parts were separated into three replications, lyophilized, then ground finely with a coffee grinder and stored at -20°C until further analysis.

Extraction and Analysis of Stilbene, and Free Ellagic Acid: Samples of 1 g of lyophilized juice, pulp, or skin were put into centrifuge tubes with 10 mL of 80:20 ethanol:water (LeBlanc, 2006). Solutions were homogenized for 1 min, and then incubated for 30 min at 60°C with agitation every 5 min (LeBlanc, 2006). After heating, the tubes were removed and centrifuged at 1,000 rpm for 15 min. Supernatant was filtered through a fine mesh filter to remove the large particles and brought to a volume of 10 mL with 80:20 ethanol:water. An aliquot was then filtered in vacuo through a 0.2 µM Nucleopore Track-Etch membrane (Milipore, Billerica, MA) and 1 mL was pipetted into sample vials for initial analysis by HPLC for free ellagic acid, ellagic acid glucosides, the stilbenes trans and cis resveratrol, and trans and cis piceid. HPLC analysis was carried out on a Dionex UVD 340S HPLC system (Dionex, Sunnyvale, CA) with a 4 channel diode array detector. The system was equipped with a Sunfire C18 column 5.0 µm ODS (3.0 x 250 mm) (Waters Corp, Milford, MA). The injection volume for both standards and samples was 20 µL. The flavonols and ellagic acid were quantified at 360 nm, trans-compounds at 306 nm and the cis-compounds at 285 nm. The mobile phase was solvent A, methanol/formic acid/water (10:1:89, v/v/v); solvent B, acetonitrile; and solvent C, water. A multi-step gradient suitable for phenolic separation was used as follows: at 0 min, 100% solvent A; at 35 min 30% solvent A and 70% solvent B; and at 50 min 100% solvent C with a 5 min post-run with solvent C. The flow rate was 0.3 mL/min that we determined to be necessary to achieve good peak separation, **with a total runtime of 59 minutes**. Samples were protected from light at all times to hinder degradation of the phenolic compounds and the conversion of cis-stilbenes to trans- isomers.

Acid Hydrolysis for Flavonols and Total Ellagic Acid: Two (2.0) mL of the previous sample were then added to 2 mL 2N HCL (166.66 mL 80:20 ethanol: water and 33.34 mL HCl) and placed in a water bath for 60 min at 95°C to achieve acid hydrolysis of flavonoid glycosides to aglycons (Talcott and Lee, 2002). The samples were then vortexed for 30 sec and refiltered in vacuo through a 0.2 µm Nucleopore Track-Etch membrane and 1 mL was again pipetted into sample vials for final analysis by

HPLC for flavonoids (myricetin, quercetin and kaempferol) and total ellagic acid. The HPLC analysis was carried out on the same system as above, using the same column, mobile phase and multi-step gradient program. Total runtime for separation was 27 minutes.

All compounds were identified by using UV/VIS spectral interpretation and retention time of authentic standards (Sigma-Aldrich Chemical Co, St. Louis, MO). All external standards were prepared to 10, 50, and 100 ppm, which was the expected range of compounds within our samples. Quantification was made by calculating the area under the curve based on calibration curves of external standards for each of the analyzed compounds, trans resveratrol (TRes) and cis resveratrol (CRes), cis piceid (CPic), free ellagic acid (FEA), total ellagic acid (TEA), myricetin, quercetin, and kaempferol. Detection limits are calculated by area under the curve of the standard: TRes - 1 µg/g, EA - 10 µg/g, myricetin, quercetin, kaemferol – 8 µg/g, Cpic – 6 µg/g. All results were expressed as µg/g dry weight.

Total Phenolics: Total phenolics were identified by the Folin-Ciocalteau (Singleton, and Rossi, 1965) assay with gallic acid equivalents. Fifty grams of previously frozen (-20 °C) fruit was blended to a fine puree, and filtered through cheesecloth. Filtered puree (0.5 g) was added to a 50 mL tube along with 25 mL of cold extraction solvent (4:4:2:0.001 acetone : methanol : deionized water : formic acid) and mixed thoroughly. Samples were filtered with #4 Whatman filter paper (Fisher Scientific, Waltham, MA), and two replications of 1.0 mL were each placed in a glass test tube. Folin-Ciocalteau reagent (1.0 mL) was added, mixed and allowed to stand for 3 min. Then 1.0 mL of 1 N sodium carbonate was added, mixed, and the sample was allowed to stand for 7 min. Seven (7.0) mL water was added, mixed, capped and allowed to stand for 30 min at 40°C in the dark (Waterhouse, 2002) before reading the absorbance at 726 nm in a Beckman-Coulter DU 730 UV-VIS spectrophotometer (Beckman Coulter, Inc., Brea, CA). Total phenolic content was expressed in fresh weight as gallic acid equivalents in mg/100 g sample, using a standard curve generated with 50, 100, 150, and 300 mg/L gallic acid.

Total Anthocyanins: Total anthocyanins were analyzed with a modified Giusti and Wrolstad (2001) pH shift assay. Fifty (50.0) g of previously frozen fruit was blended to a fine puree, and filtered through cheesecloth. Filtered puree (0.5 g) was added to a 50 mL tube along with 25 mL of cold extraction solvent (4:4:2:0.001 acetone : methanol : deionized water : formic acid) and mixed thoroughly. Samples were filtered with #4 Whatman filter paper, and 0.5 mL of each extracted sample was placed into 15 mL polypropylene tubes. Tubes consisted of 2 replications for pH 1.0 and 2 replications for pH 4.5. To adjust pH, 4.5 mL of appropriate pH buffer was added. Tubes were capped, vortexed and allowed to stand for 30 minutes at 40°C (LeBlanc, 2006) in the dark before reading the absorbance at 510 nm and 700 nm (to correct for haze) against a blank cell of distilled water in a Beckman-Coulter DU 730 UV-VIS spectrophotometer. The absorbance of the diluted sample (A) was as follows: $A = (A_{510} - A_{700})$ pH 1.0 - $(A_{510} - A_{700})$ pH 4.5. The fresh weight anthocyanin content was then calculated as the total of monomeric anthocyanin pigment (mg/L) = $(A \times MW \times DF \times 1{,}000) / (\varepsilon \times 1)$, where A is the absorbance of the diluted sample and DF is the dilution factor. MW and ε refer to the predominant pigment content contained in the sample. For muscadine, cyanid-3-glucoside was used therefore MW = 449.2 and ε = 26,900.

Statistical Analysis: Data represents the mean of three replicate **with two subsamples (n=6) analyses** by ANOVA to calculate F probabilities. Mean separation achieved using LSD test ($P < 0.05$) with SAS software 9.2 (SAS, 2008).

RESULTS AND DISCUSSION

Stilbene: Stilbene concentrations vary among muscadine cultivars as well as over time. After 14 days of storage, trans-resveratrol (TRes) was found in the skin of all 11 cultivars tested ranging in amount from 3.83 µg/g in 'Southland', a black cultivar, to 23.36 µg/g in 'Sweet Jenny', a bronze cultivar (Figure 1). Eight of the cultivars retained the TRes that was measured at harvest with an insignificant increase in three of the cultivars. Three cultivars ('Albemarle', 'Eudora', and 'Pollyanna') exhibited a decrease in concentration. 'Pollyanna' had the greatest concentration of TRes at harvest with 66.0 µg/g, but this amount significantly decreased during storage to 14.12 µg/g.

Interestingly, the TRes concentrations were not influenced by skin color. Bronze berries had equally as much resveratrol as did the black berries within the cultivars tested. Jeandet *et al*. (1991) found that *Vitis vinifera* grape skin cells had the ability to suddenly decrease production of resveratrol after veraison. Immature grapes were capable of synthesizing resveratrol, but this ability steadily decreased with

maturity. It is also known that anthocyanins derive from the same phenylalanine/polymalonate pathway and share common precursors with resveratrol. Holkroft and Kader (1999) found that in strawberries, which are also non-climacteric, the biosynthetic pathway for anthocyanin is still operational after harvest and the storage at low temperatures did not inhibit the process. Therefore, if anthocyanin synthesis continued after harvest, one would expect the resveratrol content to be adversely affected. Jeandet *et al*. (1995) conclude that there was a direct negative relationship between stilbene phytoalexin formation and anthocyanin content in grape skin cells. Therefore the loss of resveratrol after a 14 day storage period was expected, yet eight of the cultivars retained the TRes that was measured at harvest with an insignificant increase in three of the cultivars.

Cis-piceid was found in six of the cultivars tested at 14 days with the greatest amount found in 'Darlene' (25.36 µg/g), 'Janet' (19.26 µg/g) and 'Albermarle' (16.45 µg/g). 'Albemarle' showed a significant increase in concentration of cis-piceid after 14 days in storage from 0.00 to 16.45 µg/g.

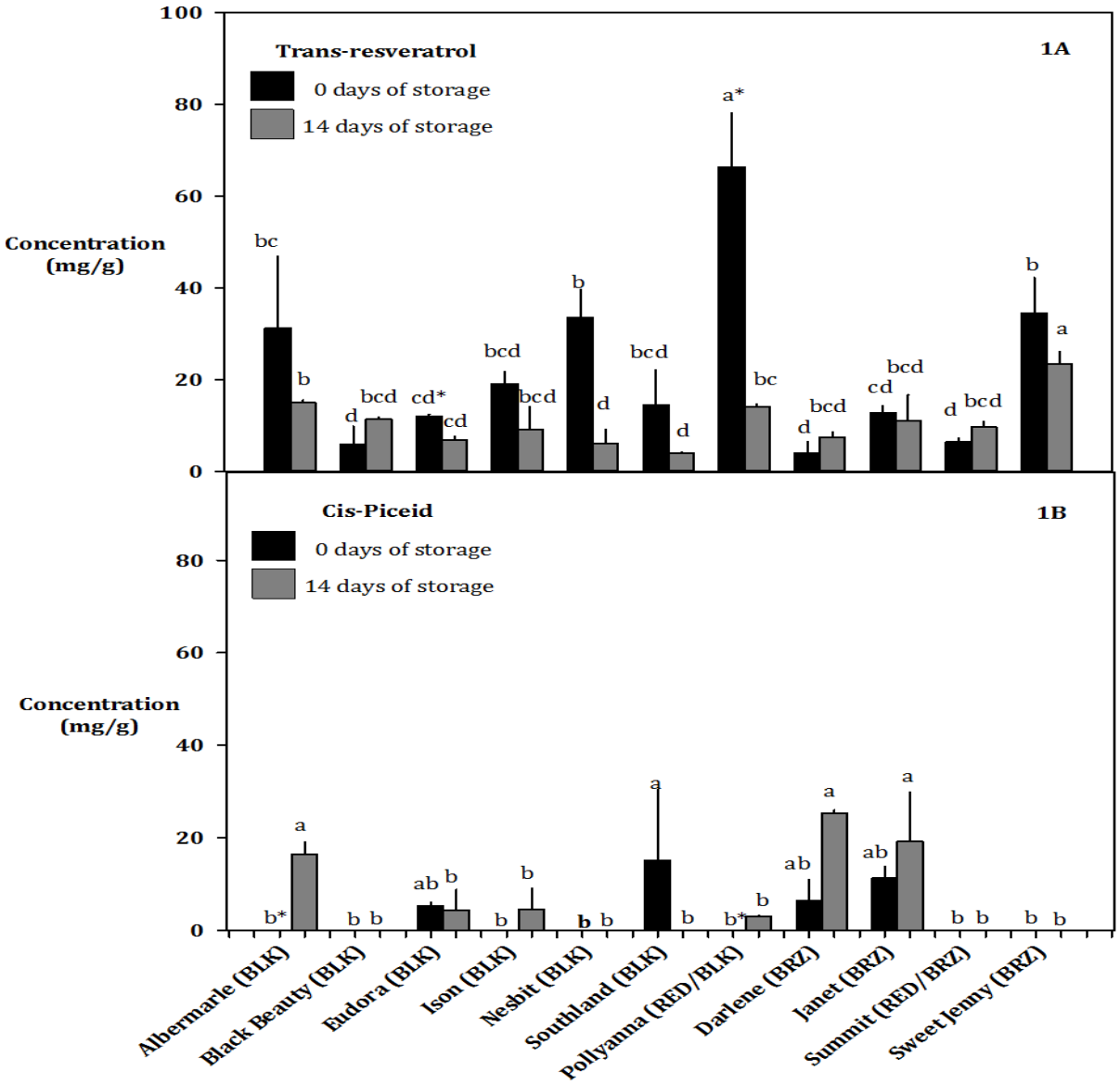


Figure 1. Stilbene (trans-resveratrol and cis-piceid) content in the skin of 11 muscadine grape cultivars at 0 and 14 days of storage. Letters that are different indicate mean cultivar differences across each storage period (0 days and 14 days) according to LSD test with a $P \leq 0.05$. Asterisks represent differences in stilbene content for berries stored at 0 and 14 d for each cultivar. Black fruited cultivars are indicated by BLK, Bronze cultivars = BRZ, a red/bronze cultivar = RED/BRZ and a red/black cultivar = RED/BLK.

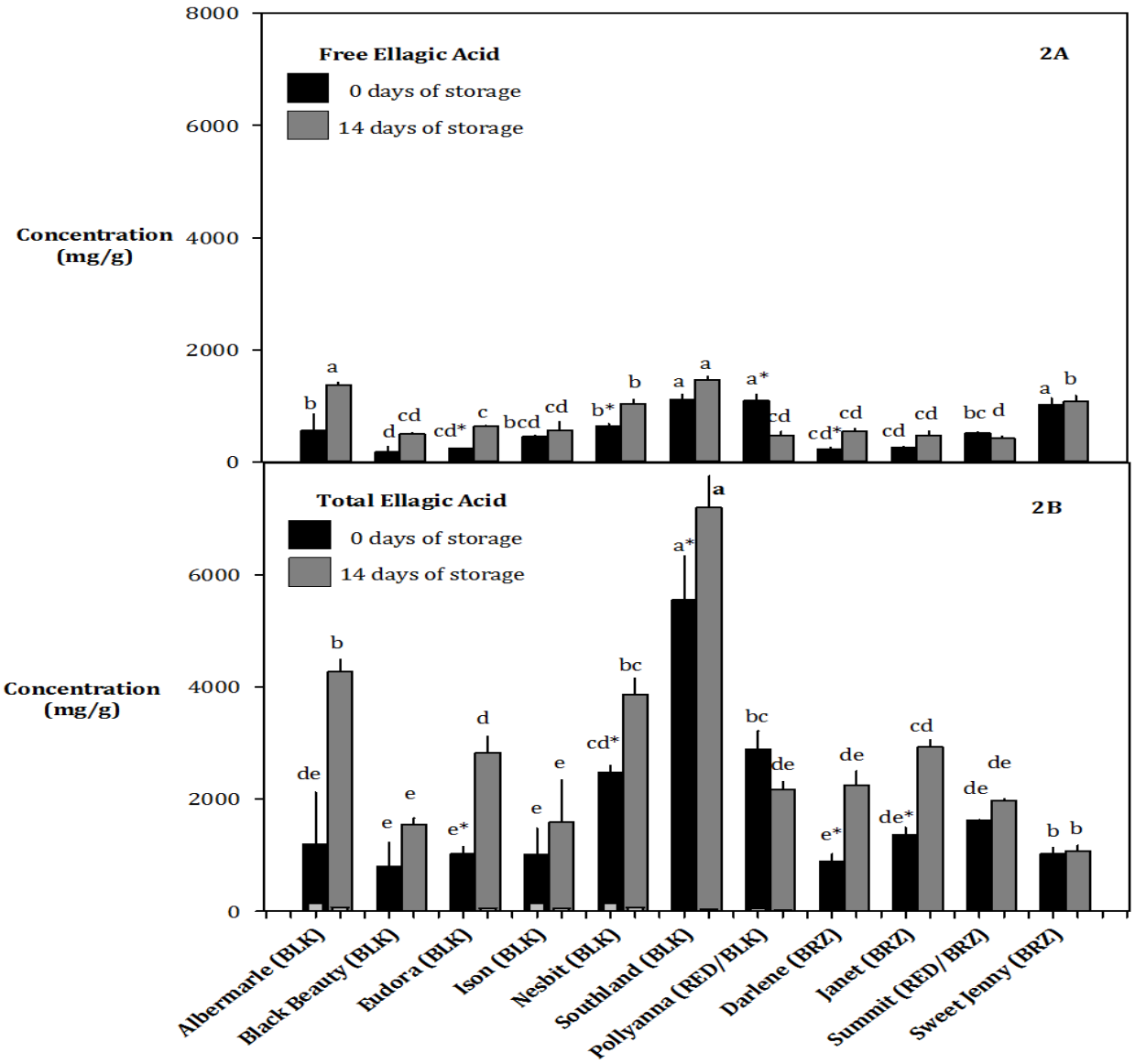


Figure 2. Free ellagic acid (FEA) and total ellagic acid (TEA) content in the skin of 11 muscadine grape cultivars after 0 days and 14 days of storage. Letters that are different indicate mean cultivar differences across each storage period (0 days and 14 days) according to LSD test with a $P \leq 0.05$. Asterisks represent differences in ellagic acid content for berries stored at 0 and 14 d for each cultivar. Black fruited cultivars are indicated by BLK, Bronze cultivars = BRZ, a red/bronze cultivar = RED/BRZ and a red/black cultivar = RED/BLK.

Ellagic Acid: The free (aglycone) form of ellagic acid (FEA), and the total ellagic acid (after acid hydrolysis) increased in the skin of 'Nesbit', 'Albemarle' and 'Eudora' during the 14-day storage period (Figure 2). FEA decreased in only 'Pollyanna', and remained consistent in the other 7 varieties. In the pulp, FEA was found in 'Southland' (52.05 µg/g), 'Summit' (25.31 µg/g), and 'Sweet Jenny' (23.95 µg/g), and in the juice of 'Ison' (8.61 µg/g), 'Southland' (10.11 µg/g), and 'Sweet Jenny' (37.51 µg/g), but not in any other cultivar.

After 14 days of storage, total ellagic acid (TEA) significantly increased in the skin of 'Janet' from 1364.1 µg/g to 2930.1 µg/g, in 'Nesbit' from 2480.3 µg/g to 3873.0 µg/g, and in 'Southland' from 5554.7 µg/g to 7205.4 µg/g (Figure 2). 'Southland' contained the highest concentration of TEA of the cultivars tested. This is consistent with the results from day 0 where 'Southland' had the greatest amount of any variety tested with 5554.7 µg/g total ellagic acid. TEA was even found in the pulp of 'Eudora' (40.71 µg/g), 'Southland' (83.63 µg/g), 'Summit' (37.32 µg/g) and 'Sweet Jenny'

(76.47 µg/g). TEA was found in the juice of all varieties except 'Ison' with the highest concentration in 'Southland' with 110.64 µg/g.

Flavonols: Myrcetin concentration in the skin (Figure 3) remained relatively constant in eight of the cultivars tested. 'Janet' and 'Sweet Jenny', bronze cultivars, had the highest concentrations of myricetin at harvest. Yet, myricetin increased in 'Janet' from 998.8 µg/g to 1690.0 µg/g after 14 days, and decreased in 'Sweet Jenny' from 974.5 µg/g to 257.6 µg/g. Myricetin also increased from 369.1 µg/g to 452.1 µg/g in 'Nesbit', a black muscadine. Both bronze and red colored muscadines possess myricetin, a flavonol present in red but not white V. vinifera grapes (Flora, 1978).

Quercetin, also a flavonol, has been shown to protect against DNA mutations, colon cancer, and heart disease (Hollman and Katan, 1999). (Patel, 1955; Talcott and Lee, 2002). The greatest concentrations of quercetin in muscadine skin were found in 'Nesbit' (826.1 µg/g) and 'Sweet Jenny' (866.1 µg/g). After 14 days in storage, quercetin increased in 'Nesbit' to 1126.7 µg/g, but decreased in 'Southland' (black), 'Summit' (red/bronze), and 'Sweet Jenny' (bronze).

Kaempferol was found in much lesser concentrations in the skin of all cultivars overall, and not detected at all in 'Black Beauty'. The greatest concentrations were found in 'Nesbit' at harvest (221.9 µg/g) and after storage (265.7 µg/g), which was a significant increase. Kaempferol concentrations also increased in response to storage in 'Southland', from 20.6 µg/g to 54.7 µg/g, and 'Summit', from 92.6 µg/g to 139.1 µg/g.

Overall, total flavonols were highest at harvest in 'Sweet Jenny', but then decreased significantly during storage. After 14 days, the highest concentrations were found in 'Janet' and 'Nesbit'.

Total Phenolics: Total phenolics content (TPH) of whole fruit did not change significantly in six of the eleven cultivars (Figure 4). 'Ison' (black), 'Pollyanna' (red-black) and 'Nesbit' (black) had significant reductions in total phenolic content. Two cultivars, 'Southland' (black) and 'Sweet Jenny' (bronze), showed an increase in total phenolic content after storage.

Among the cultivars, total phenolics content were more consistent after storage than at harvest. Fruit ranged from 721.90 mg/100g in 'Southland' to 318.54 mg/100g in 'Summit'. 'Southland' had one of the lowest concentrations of total phenolics at harvest with 447.54 mg/100g, but continued to produce phenolics during storage.

In addition to differences among cultivars between initial and stored concentrations, some cultivars exhibited dramatic changes in total phenolic content in response to storage. 'Nesbit' (black) and 'Pollyanna' (red-black) had the highest phenolic contents at day 0 with 1061.65 mg/100g and 1057.89 mg/100g, respectively, but this amount dropped dramatically by day 14 and was 466.79 mg/100g and 360.07 mg/100g, respectively. The other cultivars that had smaller initial values experienced less change in total phenolic content in response to storage.

Black muscadines that were tested in this study contained higher levels of total phenolics than bronze berries, suggesting they would contain a higher antioxidant capacity. Takeda *et al*. (1983) also found an increase in phenol content of 'Fry' muscadine grapes stored at 4.5°C, and correlated these increases to decay development and fruit deterioration because of translocation of sugars in to the fruit ceases and sugars and organic acids are converted to carbon dioxide, heat and intermediate organic compounds.

Anthocyanins: *Six of the cultivars remained consistent in anthocyanin content (Table 4). The same three cultivars, 'Ison', 'Pollyanna', and 'Nesbit', that decreased in total phenolics also decreased in anthocyanin content. Yet, 'Eudora' (black) and Janet (bronze) increased in anthocyanin content over the 14 days of storage.*

The results also showed that the high phenolic value did not necessarily correspond to high amounts of anthocyanins. 'Pollyanna', a red-black variety, had one of the highest initial phenolic values but only intermediate anthocyanin levels. This is not surprising, as anthocyanins are primarily found in the skin of grapes, and are responsible for pigmentation. The primary anthocyanins in muscadine grapes are nonacylated 3,5-diglucosides of six anthocyaninidin bases.

Fruit anthocyanin content did differentiate nicely by color. The black cultivars contained the highest levels of anthocyanins as expected. 'Albemarle' (66.66 mg/100g) and 'Nesbit' (63.32 mg/100g) had the highest concentrations, followed by 'Eudora' (50.51 mg/100g), 'Ison' (48.43 mg/100g), and 'Southland' (32.84 mg/100g). None of the bronze cultivars had appreciable anthocyanin concentrations.

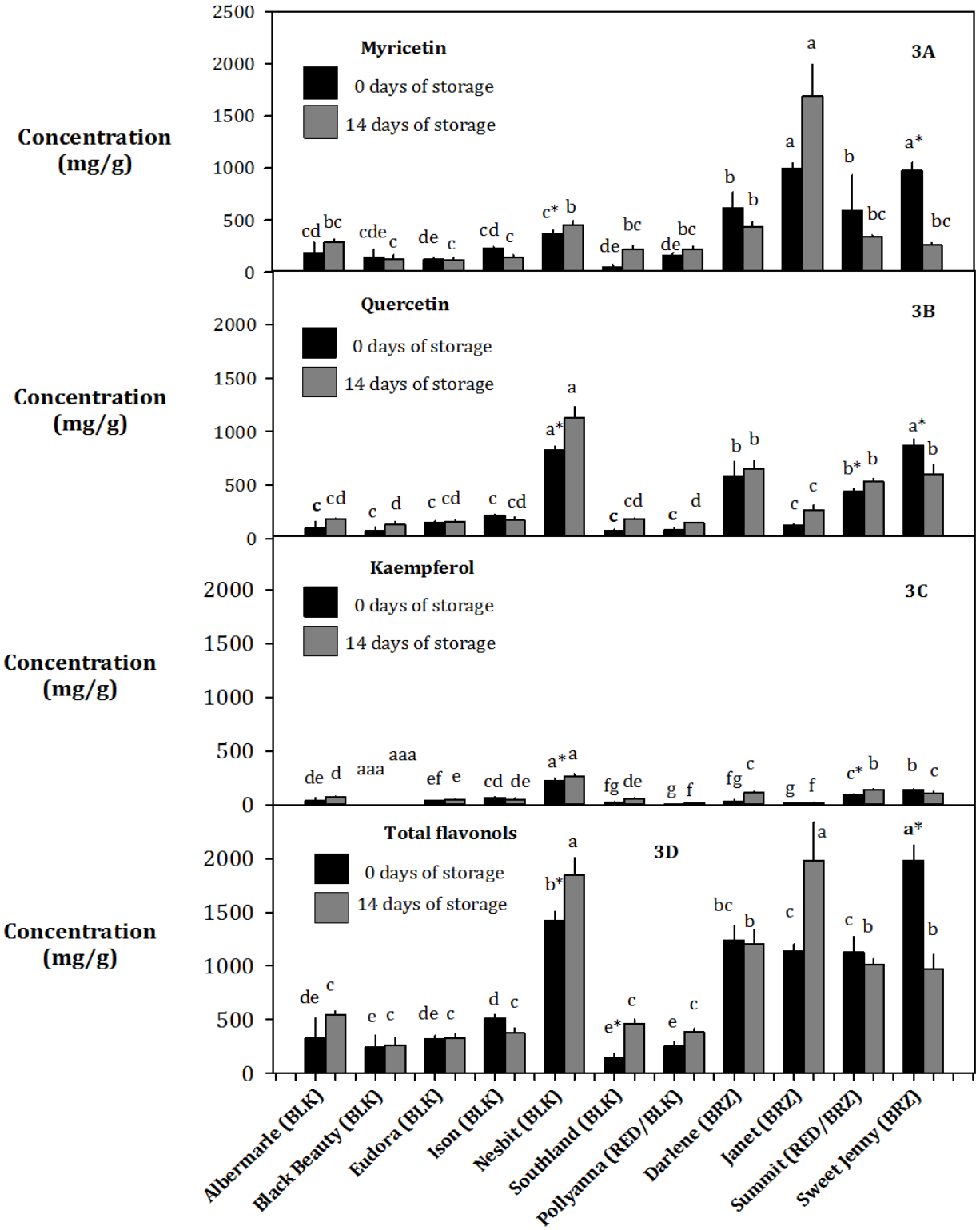
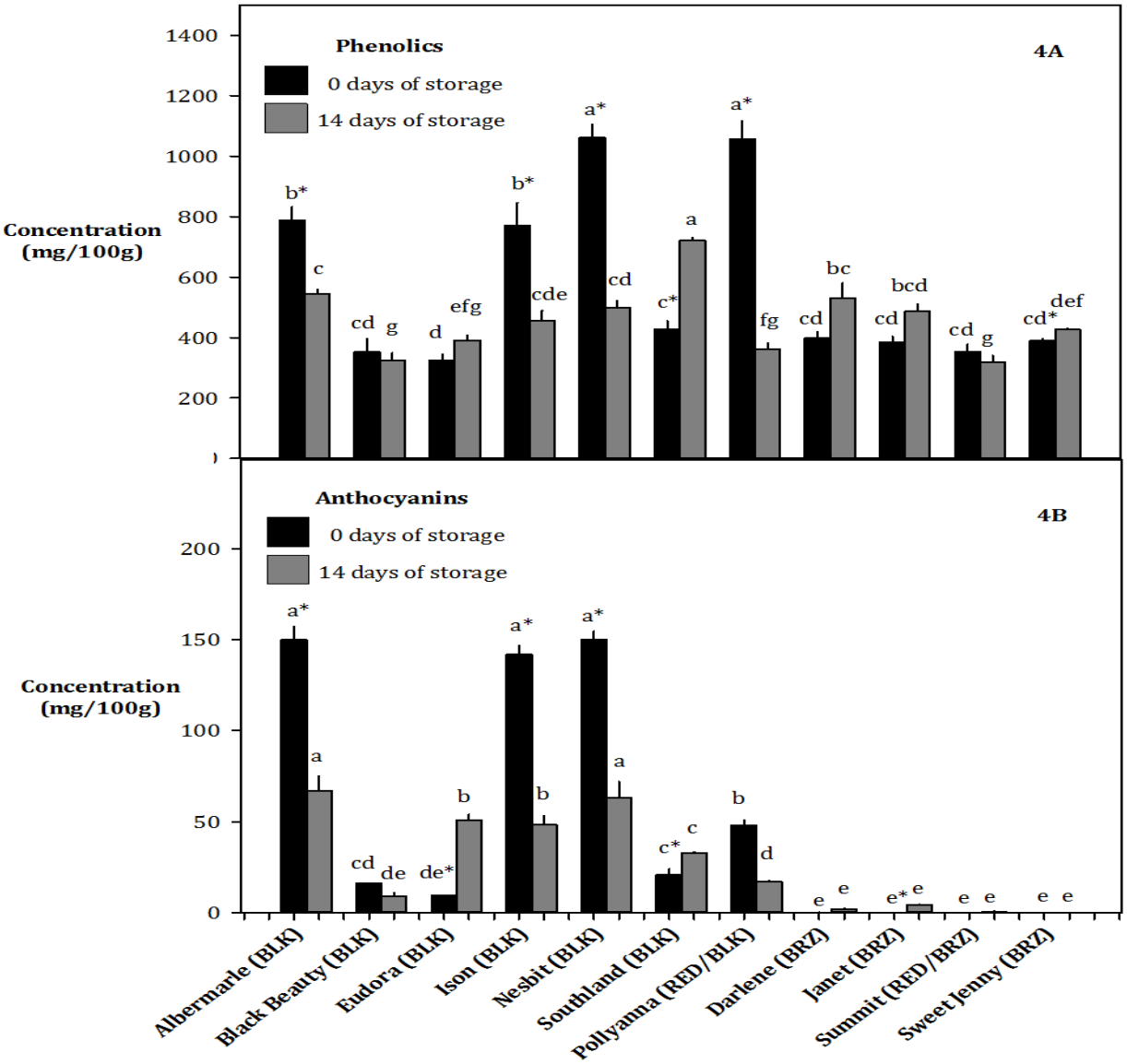


Figure 3. Flavonol content of skin for 11 muscadine grape cultivars after 0 and 14 days of storage. Letters that are different indicate mean cultivar differences across each storage period (0 days and 14 days) according to LSD test with a $P \leq 0.05$. Asterisks represent differences in flavonol content for berries stored at 0 and 14 d for each cultivar. Black fruited cultivars are indicated by BLK, Bronze cultivars = BRZ, a red/bronze cultivar = RED/BRZ and a red/black cultivar = RED/BLK.



Phenolics and Anthocyanins: Figure 4. Total Phenolics and anthocyanin content of whole muscadine grapes after 0 and 14 days of storage. Letters that are different indicate mean cultivar differences across each storage period (0 days and 14 days) according to LSD test with a $P \leq 0.05$. Asterisks represent differences in phenolic and anthocyanin content for berries stored at 0 and 14 d for each cultivar. Black fruited cultivars are indicated by BLK, Bronze cultivars = BRZ, a red/bronze cultivar = RED/BRZ and a red/black cultivar = RED/BLK.

CONCLUSION

Levels of ellagic acid, phenolics, and stilbenes vary greatly in muscadine grape skins. Some compounds are retained after 14 days of storage while others increase or even decrease in concentration. These changes not only depend on the compounds themselves, but also cultivar. There are no explanations as to why some cultivars increase in concentrations while others decrease. Other fruit have been found to react differently over several seasons due to environmental conditions. This is also true of muscadines. Stilbene biosynthesis, for example, is induced in response to a wide range of biotic and abiotic stress factors (Chong, *et al.*, 2009). Smith (2013) found that berries from the least efficacious fungicide treatment had nearly ten times more resveratrol than the fruit from the most efficacious fungicide treatment. All of the muscadines in this study were treated exactly the same and harvested within the same season at the same time.

Being the only Vitis species to possess ellagic acid, muscadine grapes are unique. Ellagic acid was found in

the skins of all tested cultivars and in the pulp and juice of a few others. Ellagic acid and its derivatives are being investigated as potential chemopreventatives because extracts of fruits containing ellagic acid derivatives are more powerful than individual substances for inhibiting cancer cell proliferation. This is because multiple phenolic compounds in fruit act synergistically with ellagic acid, and affect biological processes that inhibit cancer initiation and cancer cell growth (Mertens-Talcott et al., 2003; Mertens-Talcott and Percival, 2005; Mertens-Talcott et al., 2005). One such flavonol synergist might be quercetin, which relaxes the blood vessel wall (Rendig et al., 2001) and increases the production of enzymes that dissolve blood clots (Abou-Agag et al., 2001). The combination of these two compounds changes the activity of regulatory proteins and enzymes called MAP kinases that regulate cell division and viability (Mertens-Talcott and Percival, 2005). The potential health benefits of ellagic acid and flavanol synergists in the muscadine grape add value to the crop and enhance its marketability.

This information can be used by muscadine geneticists to develop a healthier berry. Also, the presence of resveratrol in the pulp of 'Eudora' and 'Janet' provides possibilities for breeding cultivars with enhanced nutraceutical benefits. Typically muscadine geneticists select for sweetness, firmness, skin thickness, disease resistance, and other attributes that are visual or palatable. These and other performance characteristics for many of the cultivars in this study are detailed in a previous study (Stringer *et al*., 2008). With an ever-increasing drive for healthier foods, this current study shows that muscadines have high concentrations of health-benefitting phytochemicals, but the concentrations can be quite different between cultivars at harvest and after storage. 'Southland' stood out as an unexpected champion increasing in total phenolics, total ellagic acid, quercetin and kaempherol while in storage. 'Southland' also contained the highest ellagic acid content of all the cultivars measured.

Of note, muscadine berries were carefully cut and separated; therefore, juice partition was not obtained by crushing with seeds and skins as commonly done in commercial juice or wine production. If juice were obtained from crushing with skins and/or seeds, the juice would probably contain higher levels of compounds similar to those found in other studies (Amakura *et al*., 2000). Seed partitions are not reported. Previous research (Patrana-Bonilla *et al*., 2003; Romero-Perez *et al*., 1999) show a majority of the phenolic compounds in muscadines are found in the skins and seeds. Yet the compounds found in seeds are different than those found in skin. Seeds contain even more nutraceutical compounds not addressed in this (Patrana-Bonilla *et al*., 2003).

Muscadine grapes are seeded grapes. Muscadines contain 3-6 large seeds in the center of the pulp. Yet, the nutraceutical benefits that consumers will obtain from the consumption of muscadine far exceed the inconvenience of removing seeds. Many fruit must be first peeled, cut, or somehow prepared for eating. With the combination of ellagic acid, resveratrol, and flavonols such as quercetin and myrcetin, muscadine grapes are a healthy choice for a whole food snack.

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BOVINE AND FISH GELATIN COATINGS INCORPORATING TANNINS: EFFECT ON PHYSICAL PROPERTIES AND OXIDATIVE STABILITY OF SALMON FILLETS

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A B S T R A C T

Fish gelatin provides an alternative source of gelatin for Halal and Kosher applications and is prion and zoonotic agent free. However, applications of fish gelatin have been limited due to inferior mechanical and barrier properties. The physical properties of fish gelatins can be improved by crosslinking using natural polyphenolic compounds such as tannic acid. The objectives of this study were to develop fish gelatin films incorporated with tannic acid and evaluate their antioxidant, thermal, tensile, water vapor permeability and water solubility properties. Also, the effect of tannin-incorporated gelatins on the oxidative stability of salmon fillets was examined at 4 and 10 °C. Comparative data with bovine gelatins were generated. Fish gelatin (6.75% wt/wt) films were prepared at a gelatin:tannic acid ratio (wt/wt) of 1:0.05, 1:0.10 and 1:0.15. Tensile strength of bovine gelatin control was approximately 49 MPa and that of fish gelatin was 21 MPa. Tensile strength of bovine gelatins increased with tannic acid incorporation ($P > 0.05$) and did not vary significantly for fish gelatin films. Percent elongation of films increased and elastic modulus decreased with tannic acid incorporation. Water solubility of bovine gelatin films was reduced significantly ($P < 0.05$) and there was no significant effect of tannin on the solubility of fish gelatin films. The water vapor permeability was not significantly different for both the gelatins ($P > 0.05$) and the values ranged between 1.62 and 2.01 g mm/kPa h m^2. Bovine and fish gelatin films with highest level of tannic acid showed an increase in glass transition temperature of approximately 12 and 6 °C, respectively. Films with tannic acid possessed antioxidant activity and were able to reduce oxidation (TBARS values) in gelatin coated refrigerated salmon held for 12 days.

Keywords: fish gelatin, bovine gelatin, salmon, tannin, tannic acid, antioxidant, lipid oxidation.

INTRODUCTION

Shelf life of meat and seafood can be extended if microbial growth and oxidative reactions are limited. As consumer rejection of synthetic additives is becoming more common, natural preservative systems are sought. Due to high water activity, neutral pH, presence of autolytic enzymes and relatively high concentration of free fatty acids, fish such as salmon can be spoiled easily (Duna *et al*., 2010). Storage under controlled atmosphere can be effective for fish. For instance, in muscle foods stored under modified atmosphere, the microbial counts were well within the limit for 3 weeks, but deterioration in muscle color was observed after 12 days (Antionewski *et al*., 2007). Lipid oxidation can lead to off odors due to rancid volatiles, loss of muscle color due to oxidation of oxymyoglobin and drip accumulation (liquid oozing out of stored muscle) leading to deterioration in appearance (Antionewski *et al*., 2007). Fish muscle contains polyunsaturated fatty acids (He *et al*., 1997) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Several health benefits are associated with these fatty acids; however, these foods are easily susceptible to oxidation and strategies to control oxidation would be beneficial for extending the shelf life for these perishable foods.

Use of antioxidant systems such as plant phenolics and plant extracts on fish fillets to reduce oxidation was investigated. Direct treatment of fish fillets with antioxidants were reported in several studies. Vacuum packed sardines (rosemary extract) (Ozogul *et al*., 2010), bonito fillets (grape seed and green tea extracts)

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(Yerlikaya and Gokoglu 2009), blue sprat (tea polyphenol) (Seto *et al*., 2005) and salmon fillets (isoeugenol solution) (Tuckey *et al*., 2009) were treated with antioxidants to retard oxidation. Instant green tea powder was added to mackerel fillets (Alghazeer *et al*., 2008), and green tea extracts were added to minced mackerel muscle (He *et al*., 1997; Tang *et al*., 2001) and minced carp (Dembele *et al*., 2010) to extend fish shelf life. Solutions of chitosan with vitamin E were able to reduce oxidation in lingcod fillets (Duna *et al*., 2010).

Use of edible coatings and films incorporated with active components (such as antioxidants or antimicrobials) will result in prolonged activity of the active components on foods than direct application by dipping or spraying (Min *et al*., 2005). Edible films can act as barriers to oxygen, water and limit lipid oxidation. Muscle foods can be wrapped in a preformed gelatin film, dipped or sprayed with gelatin film forming solution. On industrial scale, dipping muscle foods in gelatin solutions seems to be more practical. Significant improvements in oxidative stability of fish fillets dipped in chitosan-megrim skin gelatin solution (Caballero *et al*., 2005) and gelatin-benzoic acid solution (Ou *et al*., 2002) have been observed. Treated plastic films are also effective. For example, low density polyethylene films with barley husk extracts were able to reduce the TBARS values of salmon fillets stored for 12 month at -20 °C (Abreu *et al*., 2010). Chitosan microcapsules with horseradish extract coated onto ethylene vinyl acetate film (Jung *et al*., 2009) resulted in an extended shelf life of Spanish mackerel stored at 5 °C for 9 days due to reduced oxidation.

Bovine and porcine gelatins are well studied for use as coatings to extend quality of meat and seafood products. Recently, fish gelatin gained importance as an alternative source to mammalian gelatin due to socio-cultural and safety/sanitary reasons (Gomes Estaca *et al*., 2009). Fish processing waste can account to about 75% of total catch and 30% of this consists of fish skin and bones with gelatin (Gomez-Guillen *et al*., 2002). However, use of fish gelatins is limited as they have inferior rheological properties due to low number of proline and hydroxyproline residues and fewer inter and intra chain crosslinks compared to mammalian gelatins (Gomez Estaca *et al*., 2009). These films are naturally hydrophilic and absorb large quantities of water, resulting in more plasticization and inferior properties, which affect the applications of these films for extending shelf life of muscle foods.

Gelatins can be crosslinked with a variety of cross-linking agents. Most of these studies are intended to reduce solubility of gelatins and enhance strength properties. Crosslinking will result in the formation of new covalent bonds between reactive groups leading to enhanced physical properties. Often, plant phenolics are a good choice as crosslinkers as they are natural compounds and also possess antioxidant activity. Tannic acid is a plant phenolic with multiple phenolic groups and can react with proteins resulting in improved film forming ability. Crosslinking bovine gelatin films with tannic acid (Zhang *et al*., 2010b) resulted in reduction of film solubility by about 80%. Equilibrium moisture uptake of crosslinked films was lower than that of the control films. However, crosslinking did not limit the water uptake of films. Other crosslinkers including enzymes like transglutaminase (Piotrowska *et al*., 2008), white grape juice and coffee (Strauss *et al*., 2004), phenolic extract from Acacia bark (Haroun and Toumy, 2010), genipin (Bigi *et al*., 2002) and caffeic acid (Zhang *et al*., 2010a) were used to crosslink food grade gelatins. These systems reduced the water solubility and altered properties of films to different extents. From the scope of the literature, use of fish gelatin coatings and films to extend shelf life of fish products has been limited. The objectives of this work is to develop gelatin coatings incorporated with tannins and investigate the effect on fish gelatin properties and suitability of these as antioxidative coatings on salmon fillets.

MATERIALS AND METHODS

Film preparation with tannic acid: Commercial cold water fish gelatin (Norland Fish Products, Cranbury, NJ) and bovine skin gelatin (Sigma, St. Louis, MO, USA) were used in film preparation. Film forming solution (FFS) was formulated to contain 6.75% gelatin, and tannic acid (Sigma, St. Louis, MO, USA) at concentrations of 0, 5, 10 and 15% (wt/wt of gelatin). Initially, gelatin and tannic acid solutions were prepared separately by dissolving in warm deionized water. Tannic acid solution was added, in 8-10 increments, to the gelatin solution while being stirred at approximately 70 °C for 30 min. The FFS was denatured in 90 °C water bath for 30 min and cooled to room temperature. Glycerol (Sigma, St. Louis, MO, USA) was added to the FFS at a concentration of 25% (wt/wt of gelatin) followed by degassing. Amount of dry solids in each film preparation was kept constant (3 g) to maintain uniform film thickness. The FFS was poured

into Teflon plates and left to dry for 24 h at 25 °C, 15% RH. Dried films were stored at 50% RH until use.

Tensile properties of films: Tensile properties including tensile strength (TS), elastic modulus (EM) and percent elongation at break (%E) were measured using standard method D 882-01 (ASTM, 2001). To facilitate mounting on the clamps of a texture analyzer (TA-XT2, Stable Micro System Ltd., Surrey, UK), the prepared films were cut into 50 mm (long) x 8 mm (wide) strips and conditioned in a 50% RH chamber for 2 days at 22±2 °C before testing. A 5-kg load cell and crosshead speed of 50 mm/min was used for determining the tensile properties.

Water vapor permeability of films: Modified Gravimetric Cup method based on ASTM standard E 96-92 (McHugh *et al*., 1993) was used to determine the water vapor transmission rate (WVTR) of films. Desiccation chambers fitted with fans to attain air velocity of 152 m/min were maintained at 0% RH and 22±2 °C. Polymethylmethacrylate circular test cups with lid and screws were filled with deionized water (6 ml). Circular discs were cut from the films and placed in between the circular test cup lids. They were screwed tight to form a seal. Reduction in weight due to loss of water was recorded every 2 hours for 8 hours and the last reading was at 24th hour. Calculated water loss with time was divided by cup area (m^2) to give water vapor transfer rate (g/h-m^2). Permeance (g/kPa-h-m^2) was obtained by dividing water vapor transfer rate with partial pressure at the inner surface of the film. Permeance was multiplied by average film thickness to yield water vapor permeability (WVP) (g-mm/kPa-h-m^2).

Water solubility of films: Film soluble matter was determined by the modified method of Zhang *et al*. (2010b). Film (1 g) was taken in a centrifuge tube and 50 ml water at 90 °C was added to it. The centrifuge tubes were immersed in water bath at 90 °C for 30 min. The tubes were then cooled and centrifuged (Sorvall Legend Mach 1.6, Thermo Scientific, Waltham, MA) for 15 min at 2000 *g*. The supernatant was discarded and the pellet was dried for 6 h at 106 °C. The % soluble matter was the difference in the weight of the film taken and the dried pellet times one hundred.

Glass transition temperature (T_g) of films: Glass transition temperatures of films were measured using a Modulated Differential Scanning Calorimeter (MDSC) (DSC Q200, TA Instruments, New Castle, DE). Sample (10±1 mg) was taken in standard DSC pan, placed in the furnace with nitrogen flow rate of 20 ml/min. Sample was subjected to heating rate of 10 °C/min between temperature range of -50 to 150 °C. Heat flow data was collected and analyzed using instrument software (Universal Analysis 2000, v. 4.3, TA Instruments, New Castle, DE) to calculate the T_g.

Antioxidant activity of films: Film antioxidant activity was determined following DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Bao *et al*., 2009) using 0.072 mM/L DPPH reagent in ethanol. Films were stored in a 50% RH chamber at 23±1 °C and sampled on day 0, 10 and 20. Percent DPPH quenched was calculated by [1-(AS-AO)/AC)]*100); where AS, AC, AO are the absorbance values of sample, control, and solution of 5.5 ml ethanol and 500 µl sample, respectively.

Effect of tannin incorporated gelatins on the oxidative stability of salmon fillets: Atlantic salmon fillets (*Salmo salar*) were obtained from a local retailer (Pullman, WA). Fillets were sliced using a sterile knife into pieces of approximately 15±0.5 g and stored at 3°C for one day until use. Pieces of same shape and size were sliced so that all pieces would have similar surface area (approx. 25 cm^2). Fillet pieces were dipped in fish and bovine gelatin solution with tannic acid. Samples were dried under a fan for 15 min. After drying, samples were placed in foam meat trays (Genpack, Gens Falls, NY) and covered with a Saran® wrap to mimic packaging in retail display. Samples were stored at 4 and 10 °C for 12 days and analyzed for TBARS every third day. Treatments included uncoated fillets (positive control), fillets dipped in control FFS (negative control) and fillets dipped in FFS solutions with 5, 10 and 15% tannic acid.

Modified protocol for TBARS assay was adopted from Kim *et al*. (2012). Briefly, 15±0.3 g fish fillet was blended (Waring Blender, Model HGBTAC30, Warring Commercial, Torrington, CT) with 40 ml of 1.5% trichloroacetic acid (Sigma, St. Louis, MO) for 15 seconds. Blended mixture (20 ml) was taken and centrifuged (Sorvall Legend Mach 1.6, Thermo Scientific, Waltham, MA) for 15 min at 3300 *g*. Supernatant (2ml) was added to 2 ml of 20 mM thiobarbituric acid (Sigma, St. Louis, MO) solution. The mixture was heated in a 95 °C water bath for 30 min and cooled to room temperature. Solution was filtered through 0.45 µm Whatman (Maidstone, Kent, UK) GD/X syringe filters. The absorbance of samples was taken at

532 nm and TBARS values (mg MDA/Kg fish) were calculated using the extinction coefficient.

Statistical analysis: Pooled ANOVA assuming randomized complete block design was used for salmon oxidation studies. Data analysis was done using statistical analysis software version 9.2 (SAS Institute, Cary, NC). For determining TBARS, each treatment consisted of triplicate samples at every time point. Pair wise comparisons equivalent to Fishers LSD were used for physical properties of the films. Each mean ± standard error was the average of 2, 5, 6, 8 and 10 replicates for glass transition temperature, DPPH radical scavenging activity, water vapor permeability, tensile properties and water solubility of films, respectively.

RESULTS AND DISCUSSION

Tensile properties: Incorporation of tannic acid to gelatin films altered tensile properties. Tensile strength increased ($P > 0.05$) and % elongation decreased ($P > 0.05$) with increasing concentration of tannic acid in bovine gelatin films (Fig 1, 2). In case of fish gelatin films, there was a slight decrease ($P < 0.05$) in tensile strength compared to control. Percent elongation of fish gelatin samples with intermediate concentrations of tannic acid (5% and 10%) increased ($P > 0.05$) compared to control and decreased ($P < 0.05$) for films with 15% tannic acid. Increase in the TS and decrease in the % elongation of crosslinker-incorporated gelatin films has been reported by other authors (Cao *et al*., 2006; Kim *et al*., 2005; Rivero *et al*., 2010). Increase in tensile strength can be due to the crosslinkers stabilizing the film matrix, which in turn decreases film elongation. Incorporation of tannic acid increased ($P > 0.05$) film stiffness (Fig 3), which is evident by an increase in the elastic modulus of bovine and fish gelatin samples.

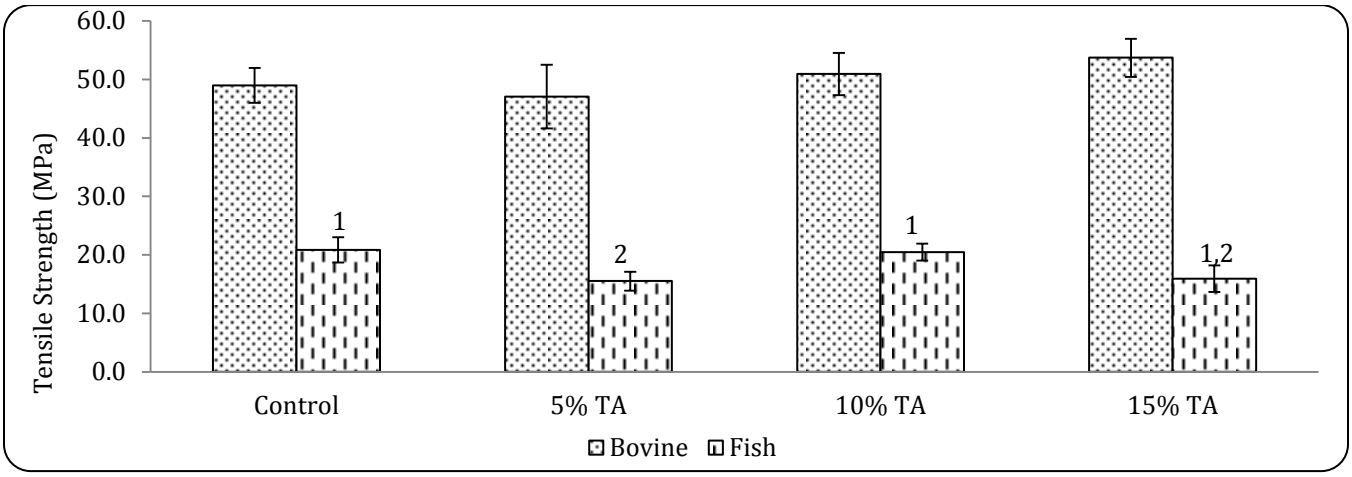

Figure 1. Tensile strength (MPa) of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Means with same numbers and without numbers are not significantly different ($P > 0.05$).

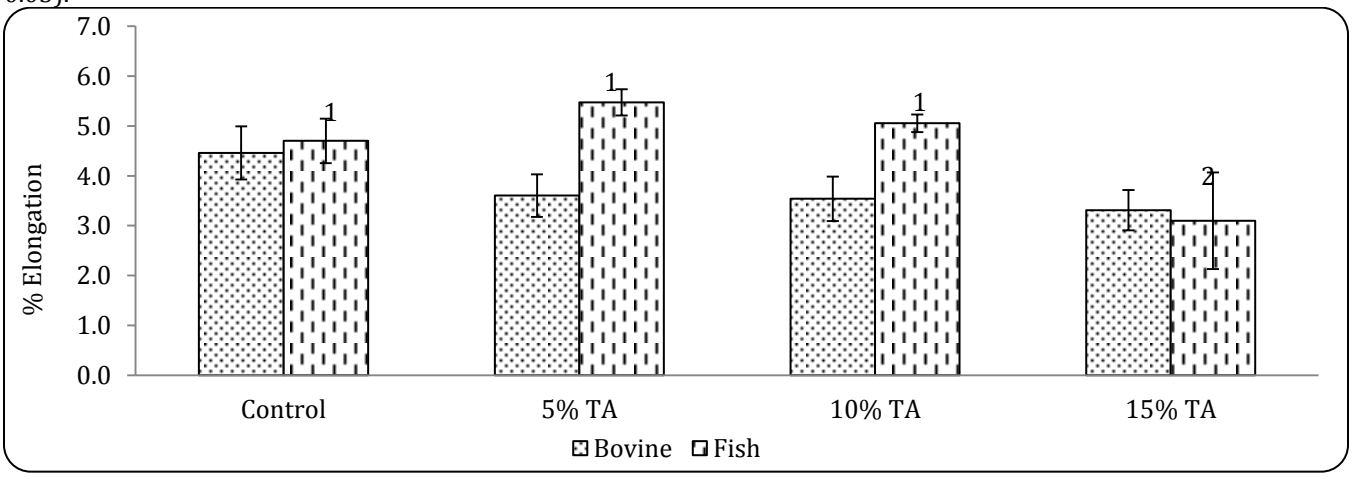

Figure 2. Percent elongation of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Means with same numbers and without numbers are not significantly different ($P > 0.05$).

Tensile strength increased from 84 to 88 MPa as the concentration of tannic acid increased from 0 to 40 mg/g gelatin in bovine gelatin films (Cao *et al*., 2006). The same study showed a reduction of approximately 2 MPa in the elastic modulus of the samples with increase in tannin concentration. Kim *et al*. (2005) reported an increase of tensile strength from 44 to 68 MPa and no significant change in the elastic modulus of the samples with the incorporation of condensed tannins in gelatin chitosan matrix. Rivero *et al*. (2010) reported an increase in tensile strength of chitosan films incorporated with tannic acid. Crosslinkers change physical properties to varying degrees. However, direct comparison of data among different gelatin samples is not reliable due to different formulations, test conditions and gelatin composition.

Tensile strength and elastic modulus of bovine gelatin samples are significantly higher compared to fish gelatin samples (Fig 1, Fig 3). This can be explained by the difference in the amino acid composition. Proline and hydroxylproline content is higher in bovine gelatin compared to fish gelatin (Avena-Bustillos *et al*., 2006, 2011; Gomez-Gullien *et al*., 2007; Gomez-Estaca *et al*., 2009). These amino acids stabilize the triple helix structure resulting in increased tensile strength and elastic modulus. Hydroxyproline has major role in stabilizing the triple helix structure due to H-bonding to its hydroxyl group. High content of proline and hydroxyproline is believed to be the reason for highly viscous properties for mammalian gelatins (Aveena-Bustillos *et al*., 2006).

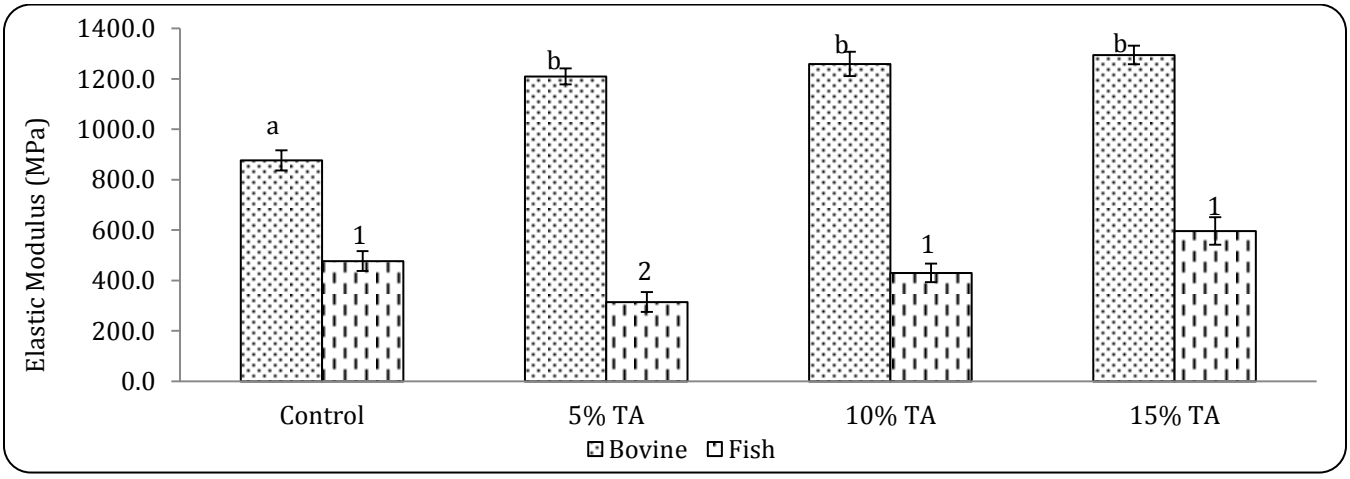

Figure 3. Elastic modulus (MPa) of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=8). Letters and numbers represent significant difference in the means of fish and bovine gelatin samples, respectively. Means with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Water vapor permeability: Incorporation of tannic acid did not make a significant ($P > 0.05$) difference in the WVP of the bovine and fish gelatin films. However, samples with 15% tannic acid have lower WVP compared to controls. Difference was significant in case of bovine gelatin films. Tannic acid has numerous hydroxyl groups which can interact with water (Cao *et al*., 2006). As a result, the WVP of samples did not change significantly. No significant change in the WVP of tannic acid crosslinked bovine gelatins (Cao *et al*., 2006) and transglutaminase crosslinked fish gelatins (Piotrowska *et al*., 2008) was reported.

Water solubility: Solubility of bovine gelatin films reduced significantly ($P < 0.05$) with incorporation of tannic acid. The percent soluble matter was reduced from 55.4±3.9 to 12.9±1.7 with incorporation of 15% tannic acid in bovine gelatin films. In case of fish gelatin, solubility did not change significantly ($P > 0.05$) with incorporation of tannic acid. Film solubility increased for intermediate concentrations of tannic acids and reached value close to the control for fish gelatin films with 15% tannic acid. Increased degree of crosslinking can result in decreased combination of gelatin with water (Cao *et al*., 2006).

Approximately 8% decrease in solubility of tannin crosslinked chitosan was reported by Rivera *et al*. (2010). Solubility of bovine gelatin films crosslinked with tannic acid (3 wt %) decreased significantly (Zhang *et al*., 2010b). Formation of covalent crosslinks in the film matrix was suggested as possible reason for reduction in film solubility. Reduction in solubility from 99 to 27% was reported

for cod fish gelatin films crosslinked with 0.3 mg/ml transglutaminase (Piotrowska *et al*., 2008). Kolodziejska *et al*. (2006) reported reduction in solubility of chitosan-fish gelatin films crosslinked with transglutaminase. It is likely that the film solubility depends on the extent and type of crosslinking and the method used to determine solubility (boiling time and temperature).

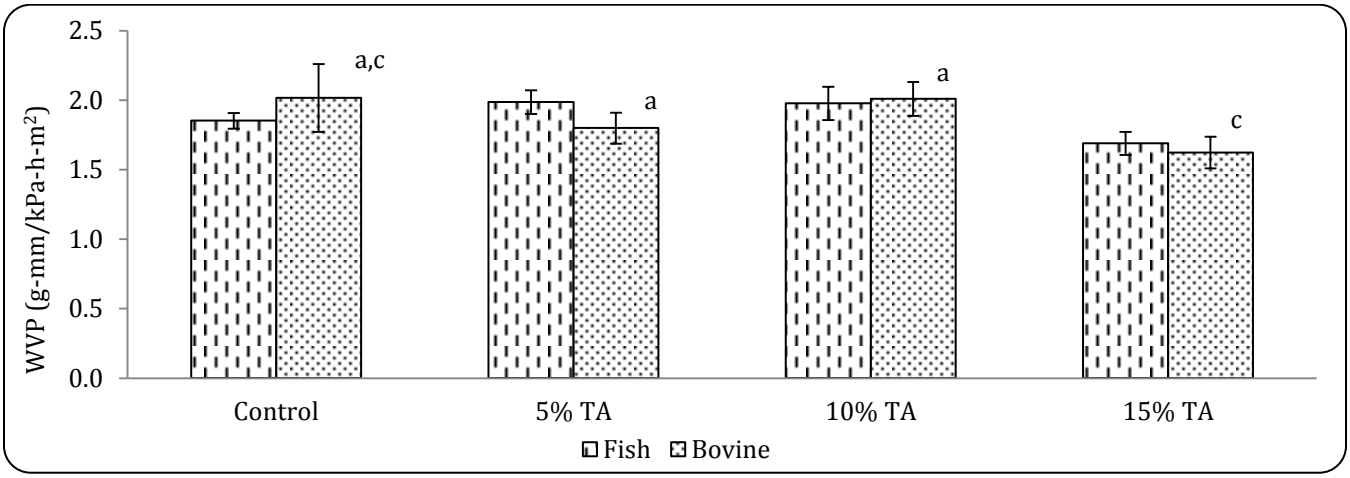

Figure 4. Water vapor permeability of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=6). Means with same letters and without letters are not significantly different ($P > 0.05$).

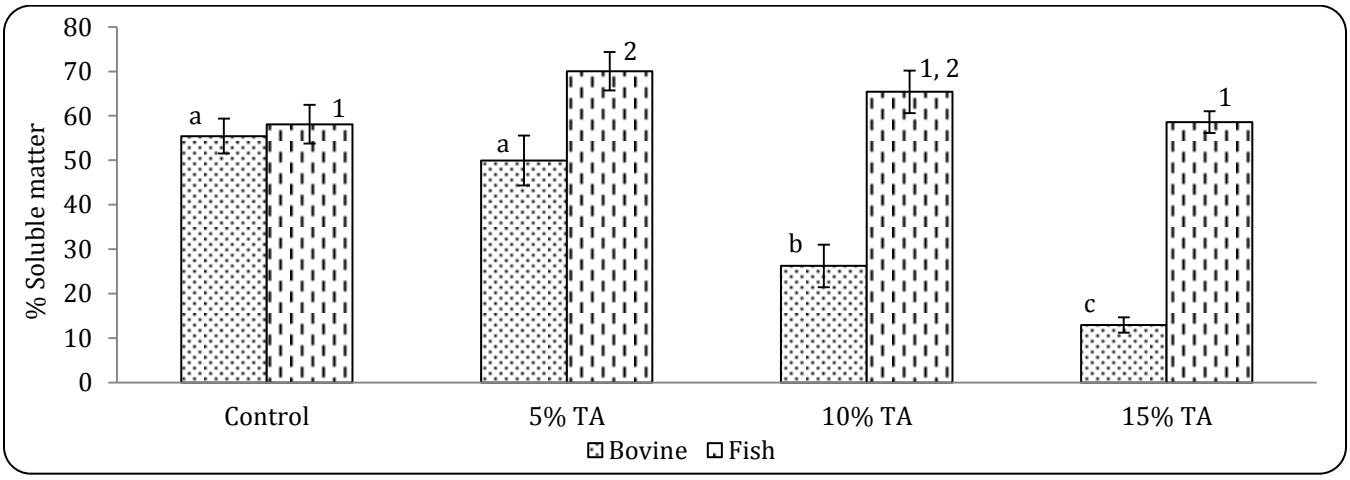

Figure 5. Water solubility of tannic acid incorporated bovine and fish gelatin samples. Values represent means ± standard error of means (n=10). Letters and numbers represent significant difference in the means of bovine and fish gelatin samples, respectively. Means with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Glass transition temperature: Material transition from a glassy or brittle state into a rubbery state over a temperature range is described by glass transition. Incorporation of tannic acid increased the glass transition temperature by 12 and 6 °C for bovine and fish gelatin films, respectively. When tannins are incorporated into the films, the film matrix becomes more rigid, which in turn increases the energy needed for transition into a rubbery state.

Antioxidant activity of films: Bovine and fish gelatin controls showed 10% DPPH radical scavenging activity.

Table 1. Glass transition temperature (T_g) of bovine and fish gelatin films with tannic acid.

Treatments	Bovine gelatin films	Fish gelatin films
	Glass Transition temperature (°C)	
Control	26.5±0.39	31.6±1.4
5% TA	33.68±1.05	28.7±1.0
10% TA	33.42±1.09	34.4±2.7
15% TA	38.75±0.77	37.8±0.7

Values represent means ± standard error of means (n=2).

Bovine gelatin controls retained this level of activity throughout the 20 day storage while the fish gelatin

controls did not. Samples with tannic acid possess 70 to 90% radical scavenging activity. Level of tannic acid in the films had no significant effect ($P > 0.05$) on the radical scavenging activity of the films. Cao *et al*. (2006) reported an improvement in the properties of tannic acid crosslinked bovine gelatin films during 90-day storage. It was proposed that tannic acid interacts with gelatin in a step-by-step manner, resulting in improved properties (Cao *et al*., 2006; Frazier *et al*., 2003). Similar phenomenon might be responsible for changes observed in this study to retain radical scavenging activity during storage. However, it should be noted that effect of storage on other film properties was not investigated in this study.

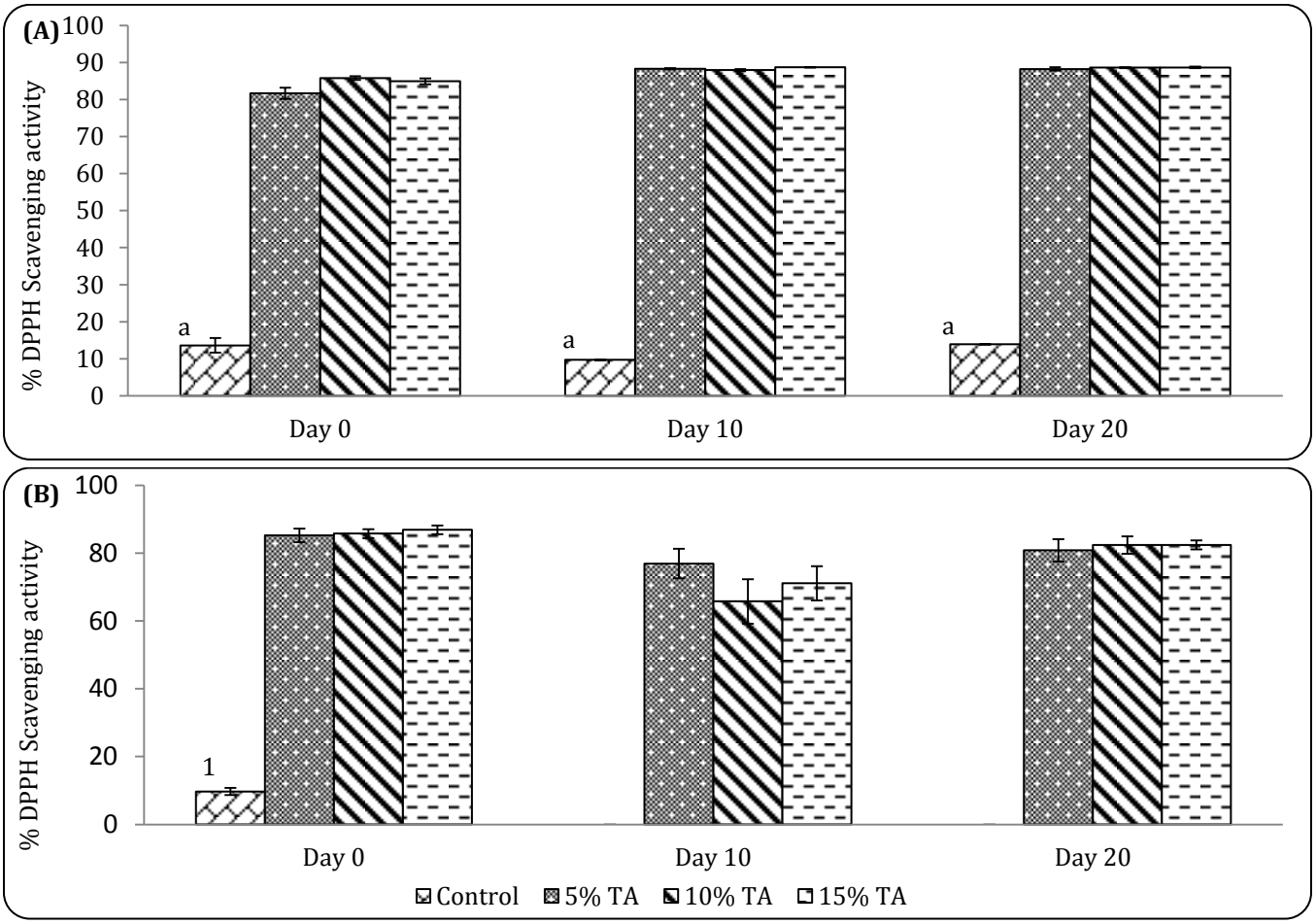

Figure 6. DPPH radical scavenging activity of tannic acid (0, 5, 10, and 15%) incorporated bovine (A) and fish (B) gelatin films. Letters and numbers represent significant difference in the means of bovine and fish gelatin samples, respectively. Means (n=5) with same letters or numbers and without letters or numbers are not significantly different ($P > 0.05$).

Effect of tannin incorporated gelatins on the oxidative stability of salmon fillets: There was a tendency for salmon fillets treated with bovine and fish gelatin solutions to show lower TBARS values compared to untreated controls (Table 2), but the trend was not strong and the results were similar after day 9. At 4 °C storage, bovine and fish gelatin treatments resulted in lower TBARS values than untreated samples and controls at day 3, 6 and 9. However, the differences were not significant ($P > 0.05$). Similar results were reported for samples stored at 10 °C.

It is likely that the treatment effect is being masked by the variability in the assay. Variability in TBARS can be due to several reasons. Degradation of fatty acids results in the formation of malondialdehyde (MDA). It is a minor component of fatty acids with double bonds. The MDA is used as indicator of oxidative rancidity in biological samples. Monoenolic form of MDA reacts with methylene groups of TBA and forms a complex (Mendens *et al*., 2009), which has absorption maxima at 532 nm. However, TBA also reacts with other compounds like alkenals,

alkadienes, pyridines, pyrimidines, sucrose and urea (Shahidi and Zhong, 2005). The generic term TBARS is used to describe these substances. Also, TBA reacts with other food compounds including carbohydrates, amino acids and pigments (Mendens *et al*., 2009). The TBARS assay can lack accuracy in certain food systems due to these factors. Underestimation of oxidation products in the sample can occur when MDA reacts with amino acids, proteins and glycogen in the food (Jo and Ahn, 1998). However, this method is widely used to assess oxidative spoilage in foods due to its simplicity.

Table 2. TBARS (mg MDA/Kg fish) values of salmon fillets stored at 4 and 10 °C treated with bovine and fish gelatin solutions incorporated with 5, 10 and 15% (wt/wt of gelatin) tannic acid.

		4 °C		10 °C	
Storage time	Treatment	Bovine gelatin	Fish gelatin	Bovine gelatin	Fish gelatin
		mg MDA/Kg fish			
Day 0		1.16±0.15		1.29±0.09	
	Untreated	1.33±0.55	1.24±0.29	0.77±0.34	0.95±0.43
	Control	1.07±0.18	1.38±0.18	0.75±0.22	1.03±0.49
Day 3	5% TA	0.41±0.16	0.39±0.19	0.42±0.23	0.30±0.12
	10% TA	0.51±0.20	0.27±0.12	0.34±0.07	0.22±0.10
	15% TA	0.2±0.06	0.24±0.13	0.36±0.13	0.18±0.05
	Untreated	0.55±0.09	0.66±0.36	0.39±0.12	0.73±0.27
	Control	1.02±0.24	0.61±0.11	0.59±0.01	0.23±0.06
Day 6	5% TA	0.30±0.03	0.31±0.15	0.05±0.00	0.18±0.11
	10% TA	0.13±0.04	0.09±0.03	0.15±0.05	0.04±0.00
	15% TA	0.08±0.03	0.06±0.01	0.12±0.05	0.04±0.01
	Untreated	1.01±0.66	0.82±0.31	0.64±0.29	0.19±0.12
	Control	0.86±0.20	0.80±0.28	0.56±0.30	0.20±0.14
Day 9	5% TA	0.26±0.03	0.19±0.02	0.38±0.13	0.30±0.13
	10% TA	0.46±0.16	0.40±0.31	0.04±0.00	0.07±0.03
	15% TA	0.78±0.69	0.53±0.45	0.61±0.13	0.12±0.06
	Untreated	0.21±0.02	0.56±0.16	0.19±0.06	0.10±0.03
	Control	0.86±0.19	1.17±0.53	0.32±0.13	0.16±0.09
Day 12	5% TA	0.29±0.15	0.84±0.64	0.17±0.09	0.09±0.02
	10% TA	0.58±0.26	0.21±0.07	0.22±0.16	0.12±0.05
	15% TA	0.57±0.25	0.14±0.04	0.07±0.05	0.15±0.02

Values represent means ± standard error of means (n=3).

CONCLUSION

Incorporation of tannic acid resulted in reduction in solubility of bovine gelatin films and other minor changes to fish and bovine gelatin film properties. Treatments reduced the TBARS values of salmon fillets at best during the first 9 days of storage. However, general trend in data was not evident due to variability in the assay. Perhaps a more sophisticated assay such as HPLC would be more suitable to study treatment effects of the added antioxidant.

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