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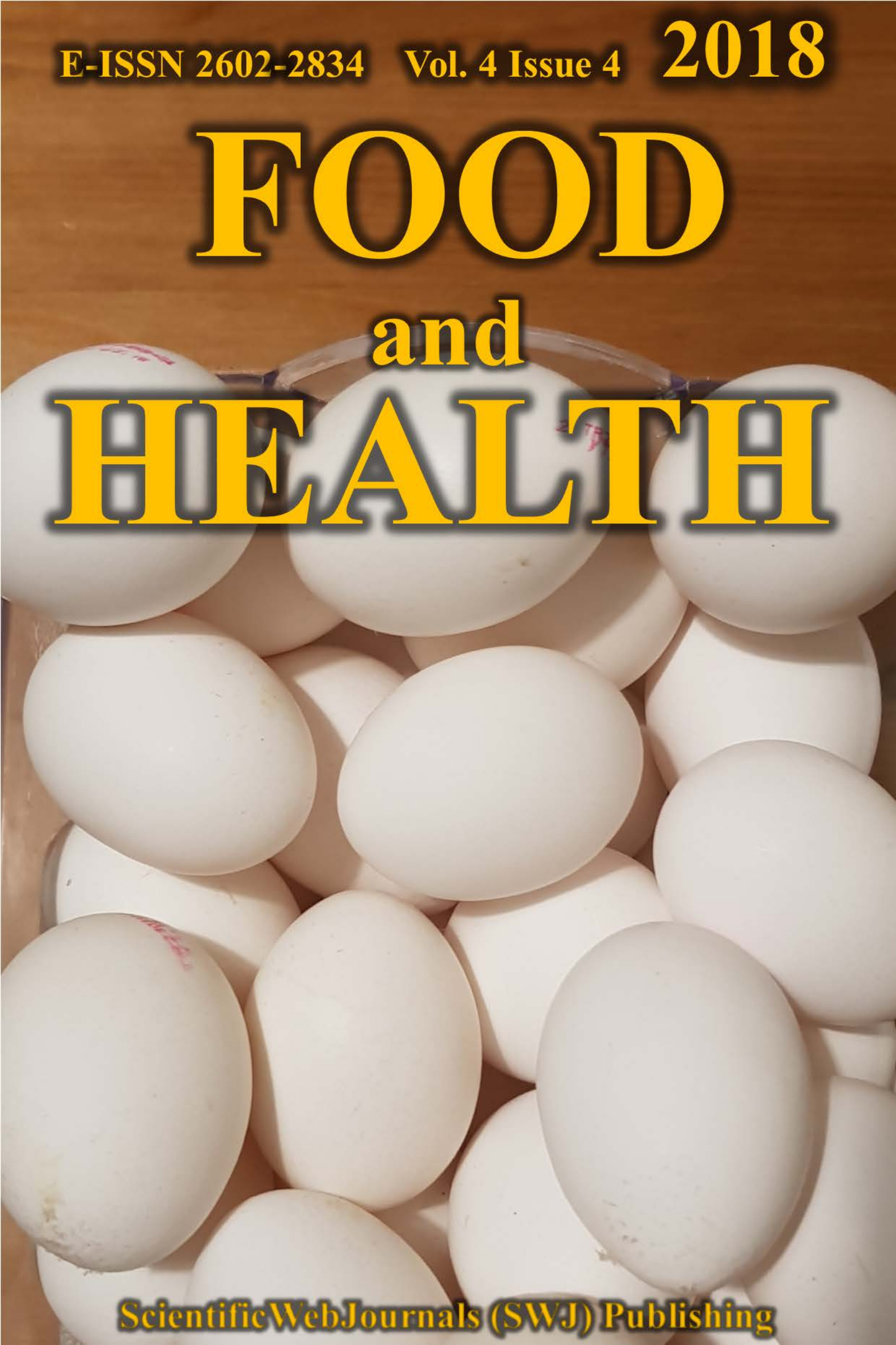

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THE EFFECT OF USING FROZEN RAW MATERIAL AND DIFFERENT SALT RATIOS ON THE QUALITY CHANGES OF DRY SALTED ATLANTIC BONITO (*LAKERDA*) AT TWO STORAGE CONDITIONS

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ABSTRACT

This study identifies the effect of freezing raw material on the storage quality of salted Atlantic bonito (*Lakerda*) at refrigerated (4 ±1°C) and ambient (17 ±3°C) conditions. It also shows the effect of different salt:fish ratios on the shelf-life and biogenic amine development during storage. The products with the lowest salt content corresponded to the lowest sensory acceptance. Previously frozen raw material (FRM) had higher salt uptake compared to freshly salted fish (FSF). Water phase salt (WPS %) level usually reached to suggested seafood safety levels (20%) within the 1st week. There were significant differences ($p<0.05$) amongst the samples treated with different salt ratios and stored at different temperatures. Higher salt content caused higher thiobarbituric acid value indicating acceleration of lipid oxidation. Lower biogenic amine values were observed with products produced from FRM. Overall results demonstrated the advantage of using FRM for dry salting of Atlantic bonito in terms of food quality.

Keywords: Atlantic bonito, Frozen fish, Dry salting, Lakerda, Quality changes, Salt concentration

Introduction

Atlantic bonito (*Sarda sarda*, Bloch 1793) is known as a commercially important fish species in the world (Turan *et al*., 2006). It is an epipelagic and highly migratory fatty fish which belongs to *Scombridae* family. This species has a wide geographical distribution and occurs throughout Atlantic Ocean, the Mediterranean and its adjoining seas (Zaboukas *et al*., 2006; Ateş *et al*., 2008). The world production of this species was 33651 tons in 2014, and Turkish production was 19031 tons in the same year (FAO, 2016a; TUİK, 2016). The catch in Turkey occurs primarily in the Black Sea and Marmara Sea (Ateş*et al*., 2008).

Atlantic bonito contains high amount of fat and therefore, it is more prone to oxidation and spoilage compared with less fatty fish (Zaboukas *et al*., 2006). Since it belongs to scombroid fishes, which are typically implicated in histamine seafood poisoning, it carries high histamine health risk if improperly handled (Lehane and Olley, 2000). The high level of free histidine in dark muscle, is susceptible to bacterial decomposition and thus to an accumulation of histamine (FDA, 2011; FAO-WHO, 2013). The past research demonstrated the presence of biogenic amines, particularly unsafe histamine levels in various commercially produced salted Atlantic bonito products (Köse *et al*., 2012; Koral *et al*., 2013). Koral and Köse (2012) reported limited shelf-life for fresh Atlantic bonito stored at refrigerated temperatures without ice as 4 days. Using ice only extended shelf-life for 3 more days. Due to short storage life of fresh Atlantic bonito at chilled storage, it is often marketed either as frozen or as salted and smoked products. *Lakerda* (a traditional salted fish product of Turkey and Greece) is originated from large Atlantic bonito with the sizes of 50-60 cm, later smaller sizes of bonito 30-40 cm are used due to reduction in its large size population (Kahraman *et al*. 2014). It is marketed either at refrigerated storage or at room temperature if processed and sold by retail processors (Koral *et al*., 2013).

Atlantic bonito is caught seasonally and therefore, is usually frozen or salted during high fishing season due to its limited shelf-life and high histamine health risk. On the other hand, this species has a better market value and consumer acceptance when marketed either as smoked or salted in comparison to frozen unprocessed products. Therefore, further processing of frozen Atlantic bonito into salted or smoked products is of interest to seafood industry. Previous studies on salted Atlantic bonito was usually carried out on *lakerda* which is mainly processed from this species (Köse *et al*., 2012). Recently, *lakerda* production was also applied to different fish species such as mackerel and salmon (Köse *et al*., 2012). It is consumed without further heating and belong to group of the ready-to-eat products (Erkan *et al*., 2009). There are at least 5 different *lakerda* production methods observed by our research team. The main processing line involves dry salting of the raw material for 1-5 days, then the processors either continue with dry salting by replacing the brine or carry on with brining. The products are matured within a month and then the products are stored in oil, brine or other seasoned solutions in plastic packs, glass jars or as vacuum packed.

Different factors can affect the quality and safety of *lakerda*. Past research on dry salted Atlantic bonito was usually concentrated on estimating the shelf life of *lakerda*. Its shell-life is usually around 3 months in cold storage although varying shelf lives were reported by different studies depending on the storage temperature, and processing and/or packaging methods (Köse *et al*. 2012). Lüleci (1991) obtained 60 days of shelf-life for this product stored in brine at 4°C. Erkan *et al.*(2009) investigated the effect of vacuum packing on the shelf-life of *lakerda* from previously frozen Atlantic bonito. They stored the products in different packing methods such as in glass jar containing oil, in glass jar with brine and vacuum pack in brine. Sensory results of their research showed that all the products spoilt after 14th week at cold storage. Therefore, they demonstrated that packaging methods used did not make significant differences in sensory values. Turan *et al.* (2006) and recently, Kocatepe *et al.* (2014) also investigated the shelf-life of dry salted Atlantic bonito (*lakerda*) at refrigerated storage. However, both studies did not determined the sensory values, and the shelf stability of the products was judged using chemical and microbiological quality parameters. Therefore, their results cannot be evaluated into storage life without sensory values.

Studies on the effect of using different salt:fish ratios on the quality and safety of dry salted Atlantic bonito during storage are scarce. Since bonito is usually frozen immediately after catch prior to further processing during high fishing season, it is important to know the effect of freezing raw material on the quality of *lakerda*. Moreover, previous investigations showed that processing and marketing *lakerda* at room temperature are commonly applied by the retail processors while refrigerated storage is more common at factory scale producers (Koral *et al*., 2013). Therefore, it is also important to identify the effect of storage temperatures in terms of food safety and quality of *lakerda*. No study exists either on the effect of freezing raw material or storage temperature on the quality and safety of *lakerda* made from Atlantic bonito.

The aim of this study was to identify the effect of freezing of raw material on the storage quality of *lakerda* produced from Atlantic bonito at refrigerated (4 ±1°C) and ambient

(17 ±3°C) conditions. Moreover, we also aimed to determine the effect of different salt:fish ratios during salting on the shelf-life and biogenic amine development during storage.

Materials and Methods

Sampling plan and sample preparations

Atlantic bonito was obtained from Trabzon (Turkey) whole market and transported to the laboratory in cold chain within 1h. The mean size of fish used was 39.95±1.63 cm, the average weight was 672.00±85.24 g. After heading and gutting, the fish were washed three times with chilled water in ice and kept in chilled water in 1h, and washed again. The raw material was firstly divided into two batches. The 1stbatch was used as control group where fresh fish was used for salting. The 2ndbatch was frozen at -40°C, then stored at -20°C for a month. Then, the fish was defrosted in a cold store room (4 ±1°C) for 16 hours before processing (Figure 1). Each batch was subdivided into 3 groups before dry salting employing three different salt:fish ratios as 1:3, 1:4, 1:6 (kg:kg). The fish were cut into pieces as 4-5 cm in width before placing into glass jars, with alternating layers of salt and fish. After salting, each group was split into two subgroups, -one was stored at ambient (17 ±3°C) temperature and the other was kept at cold storage at 4 ±1°C for 3 months (Table 1). Chemical, physical and sensory analyses were carried out to determine changes in quality and the level of biogenic amine changes.

Chemicals and Reagents

Salt (Rock salt; Billur Tuz, İzmir, Turkey) was obtained from a supermarket.All chemicals and solvents used were analytical and chromatographic grade, respectively. They are purchased from Sigma-Aldrich and Merck.

Chemical Analysis

Moisture content was determined by oven drying of 5g fish muscle at 105°C until a constant weight was obtained (AOAC 1995, Method 985.14). Results were expressed as g water/100g muscle. Dry matter value was calculated from the results of moisture contents. Mohr method was used to determine salt content (NaCl) in fish muscle as described in Rohani *et al*. (2010). Water Phase Salt (WPS) was calculated from the amount of salt in the product relative to the product moisture and salt content, using the following equation (Losikoff, 2008);

WPS% = [salt %/(salt %+ moisture%)]x100

The method of Lücke and Geidel (1935) was used to determine total volatile base-nitrogen (TVB-N) content as described by Goulas and Kontominas (2005). TBA values, expressed in mg malonaldehyde (MDA/kg), were estimated by using the method of Tarladgis *et al*. (1960) described by Smith *et al*. (1992). The method of Boland and Paige (1971) was used for trimethylamine (TMA) analysis. Biogenic amines were analysed using high performance liquid chromatography (HPLC) method according to Köse *et al*. (2011) as modified from Eerola *et al*. (1993). HPLC equipment was Shimadzu Prominence LC-20 AT series (Japan) HPLC with autosampler (SIL20AC, Shimadzu, Japan), Diode Array Detector (SPD-M20A, Shimadzu, Japan) and Intertsil column (GL Sciences, ODS-3, 5 µm, 4.6x250 mm). This method is an originated from EU suggested methods (EC Directive 2005a). Triplicated sampling was carried out and measured separately per group at each sampling point.

Sensory Analysis

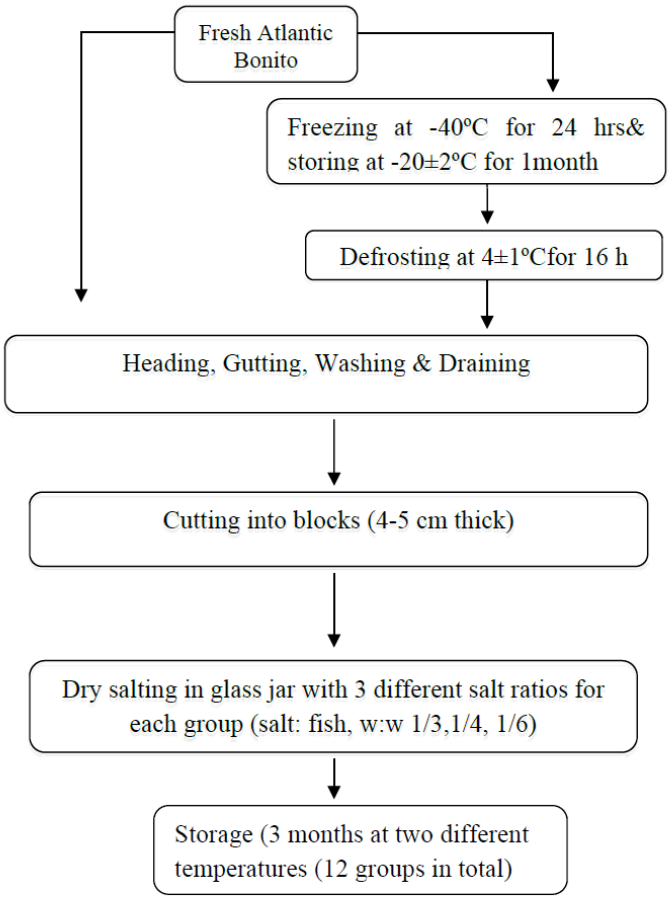
Sensory analyses were performed by using modified method derived from the methods of Amerina *et al*. (1965), Karaçam *et al*. (2002) and Archer (2010). Salted fish samples were assessed on the basis of appearance, odour and texture characteristics. Eight trained panellists judged the overall acceptability of the samples using ten point descriptive scale. According to the scale, sensory evaluation of samples is as 10–9: excellent, 8–7: good, 6–5: medium, 4: the 'limit point' for acceptable⁄unacceptable and <4: unacceptable.

Other Measurements

Water activity (a_w) was measured using an AQUALAB TE3 model water activity meter according to principals described in Minegishi *et al*. (1995). The pH measurements were taken with a digital pH meter (Jenco 6230N, CA, USA) by placing the electrode into the samples where 5g of fish flesh had been homogenized with 10 mL of distilled water. Readings were carried out for both a_w and pH in triplicate.

Statistical Analysis

The data obtained were analysed by analysis of variance (one way ANOVA) and when significant differences were found, comparisons among means were carried out by using a Tukey and Mann Whitney U test (data not provided in the normality of assumptions) under the program called JMP 5.0.1 (SAS Institute. Inc. USA) and SPSS (SPSS Inc., Chicago, IL) (SokalandRohlf, 1987).A significance level of 95% ($p<0.05$) was used throughout analysis.



Experimental Groups

Storage Temperature	Ambient Temperature (17 ±3°C)						Refrigerated Temperature (4 ±1ºC)					
Raw Material Type	Fresh Raw Material			Frozen Raw Material			Fresh Raw Material			Frozen Raw Material		
Salting Groups (salt:fish ratio w:w)	1/3	1/ 4	1/6	1/3	1/ 4	1/6	1/3	1/ 4	1/6	1/3	1/ 4	1/6

Figure 1. Processing outline and experimental groups

Results and Discussion

Tables 1-3 show the changes in the contents of pH, a_w, dry matter, salt and WPS % of *lakerda* samples originated from fresh and previously frozen raw material during storage at two different temperatures.

The percentages of dry matter in the fish flesh increased significantly($p<0.05$) in all groups during the 1st week from 36.86% up to 53.14% and from 37.12% up to 54.87% for fresh and frozen raw material groups, respectively depending on the ratio of salt:fish used (Table 1).The rise in the values continued significantly ($p<0.05$) for some salt:fish ratio groups during the 2nd week.The highest dry matter value was found with the highest salt concentration due to higher diffusion rate of salt and water in and out of fish flesh, respectively. Moreover, dry matter values were usually higher for the products produced from frozen raw material in comparison with fresh raw material with significant variations within some salt:fish ratio groups ($p<0.05$).

The percentages of salt and WPS contents showed a similar trend as dry matter contents of *lakerda* (Table 2). As expected, the highest salt content was obtained in products salted with the highest salt concentration.The values in-

creased significantly ($p<0.05$) during storage with some exceptions. Significant variations were also observed amongst all groups ($p<0.05$) indicating the significant effect of the storage temperature and the ratios of salt:fish used on the salt uptake. Apart from its preserving effect on spoilage, salting is also used to prevent seafood health hazards by both its direct effect on pathogenic microorganisms and decreasing the water activity of the food to limit microbial growth or toxin formation (Köse, 2010). Erkan *et al*.(2009) reported that *lakerda* is characterized by a salt content of 15%. Our results showed that the suggested salt content was reached for the samples treated with the ration of 1/3 (salt:fish) at the first week of storage at both temperatures in a range of 16.1-17.3% with the exception of the products prepared from frozen fish kept at refrigerated temperature that reached a salt level as 15.5% on the 8th week. The results also indicated that such amount of salt can be obtained with a ration of 1/4 salt:fish if fresh raw material used and product stored at ambient temperature. The lowest salt uptake was obtained for products prepared from frozen raw material and kept at refrigerated temperature.

Water phase salt is known as the amount of salt in the product relative to the product moisture content (Losikoff, 2008). Above 15-20% WPS in the products is usually necessary to prevent seafood health hazard (Köse, 2010). Although salt content retards bacterial spoilage, halotolerant and halophilic histamine forming bacteria have been reported to grow well in 12% NaCl broth (Lakshmanan *et al*. 2002). Among these halotolerant bacteria, *Staphylococcus sp., Vibrio* sp. and *Pseudomonas sp.* have been identified as the major halotolerant histamine forming bacteria (Lakshmanan *et al*., 2002; Hernández-Herrero *et al*., 1999). FDA (2011) reported *Staphylococcus aureus* as the highest salt tolerant bacteria which can grow at WPS as high as 20% although toxin formation is prevented above 10%. WPS level of freshly salted Atlantic bonito reached to safety levels within 1st week after salting for salt:fish ratios of 1/3 and 1/4, at both temperatures. However, WPS level reached to 20% for the salt:fish ratio 1/6 on the 4thweek at ambient temperatures and after two months at refrigerated temperatures (4±1°C). Similar trend was found for *lakerda* processed from previously frozen raw material. Koral *et al*. (2013) reported halophilic bacteria counts were usually within acceptable levels for commercial *lakerda* products sold at refrigerated temperatures, while the high values were found for the products obtained from retail processors kept at ambient temperatures.

In comparison to the present study, Ormancı and Colakoglu (2017) obtained higher salt levels and WPS% in their *lakerda* samples matured at different temperatures (4, 15 and 20°C) with the highest value corresponding to 15°C as 29.8% WPS. It is known that such products are eaten without desalting or cooking. Therefore, such high values are not usually preferred by the consumers. Variations in the levels of salt and WPS were reported for commercially salted *lakerda* products (Koral *et al*., 2013). Our results were within the range of the values reported for commercial *lakerda* products both from Greece and Turkey (Koral *et al*., 2013).

Water activity (a_w) is another growth limiting factor for microorganisms. Salting decreases a_w and has inhibition effect on pathogenic bacteria. According to FDA guideline (FDA, 2011), minimum a_w to allow the growth of *S. aureus* is 0.83 and toxin formation is 0.85 using salt. Water activity of fresh Atlantic bonito was 0.994. The a_w values significantly ($p<0.05$) dropped down to 0.783 (min) - 0.885 (max) within the 1st week of storage for all experimental groups (Table 3). The $a_w$values were usually found within the safety limit on the 1st week as suggested by FDA to prevent bacteria growth or toxin formation. The results showed that the higher the salt contents, the lower a_w values were found. In our previous studies, we determined varying a_w levels from commercial *lakerda* produced from Atlantic bonito and other fish products from Turkish and EU origin indicating the variations in the methodology used (Köse *et al*., 2012; Koral *et al*., 2013). The a_w results obtained by Ormancı and Colakoglu (2017) supported our findings.

The pH values of freshly processed raw material were found higher than the products originated from previously frozen raw material in a range of 5.75-7.24 and 5.67-6.12, respectively (Table 3). The pH of fish immediately after being caught was reported to be between 6.0 and 6.5. The fish were acceptable up to a pH of 6.8 but were considered to be spoiled above a pH of 7.0 (Huss, 1988). This pH is also used for safety regulations for such products since pH below 5 is reported to prevent most pathogenic bacteria growth or toxin formation (Köse, 2010; FDA, 2011). The levels of pH obtained for all groups were above 5 indicating this parameter cannot be used to judge the product safety of *lakerda* prepared from the salt:fish ratios applied in the current study. Although a significant decrease occurred during storage in the pH values of products originated from frozen raw material ($p<0.05$), the changes were usually found insignificant ($p>0.05$) for fresh raw material group with the exception of samples representing 1/6 group stored at ambient temperature starting from 6th week. This result indicates that spoilage is possible at low salt contents at warm temperatures.

The highest pH value was obtained as 7.4 at the end of storage period for *lakerda* produced from fresh raw material using 1/6 ratio and stored at ambient temperature. This group also corresponded to the lowest sensory values (Table 4). The pH values obtained by Ormancı and Colakoglu (2017) supported our findings.

Table 4 and the figures 2 and 3 represent sensory scores of *lakerda* processed from fresh and frozen Atlantic bonito. The results for texture, odour and appearance of the samples showed that the products with the lowest salt content had the lowest sensory acceptance (Table 4). Significant differences ($p<0.05$) occurred amongst all groups relating to all sensory parameters tested throughout the storage. The sensory scores decreased significantly during time depending on the ratios of salt:fish used (Figure 2 and 3). The products corresponding to 1/3 and 1/4 salt:fish ratio groups were within the good quality throughout the storage period indicating the suitability of these salt ratios for *lakerda* production for both raw material and storage temperature groups. However, at ambient temperature, the samples corresponded to 1/6 salt:fish ratio group were unacceptable after 1st month of storage according to sensory scores for both types of raw material used. The cold storage prolonged shelf-life of these products at refrigerated temperature particularly for the group obtained from previously frozen raw materials.It is also noted that sensory scores of *lakerda* processed from previously frozen Atlantic bonito were higher than freshly processed Atlantic bonito with the exception of salting group of 1/6 stored at ambient temperature (17±3°C). The products obtained with frozen raw materials and then kept at refrigerated temperatures were in acceptable quality throughout storage period for all types of salting groups. Therefore, the results suggest that freezing raw material prior to processing and refrigeration after processing is necessary if longer shelf life is required. Such advantage is more profound for the lowest salt:fish ration at refrigerated storage since the products were within acceptable quality at the end of 3 months' storage while being unacceptable for freshly processed group at the end of 2nd month.

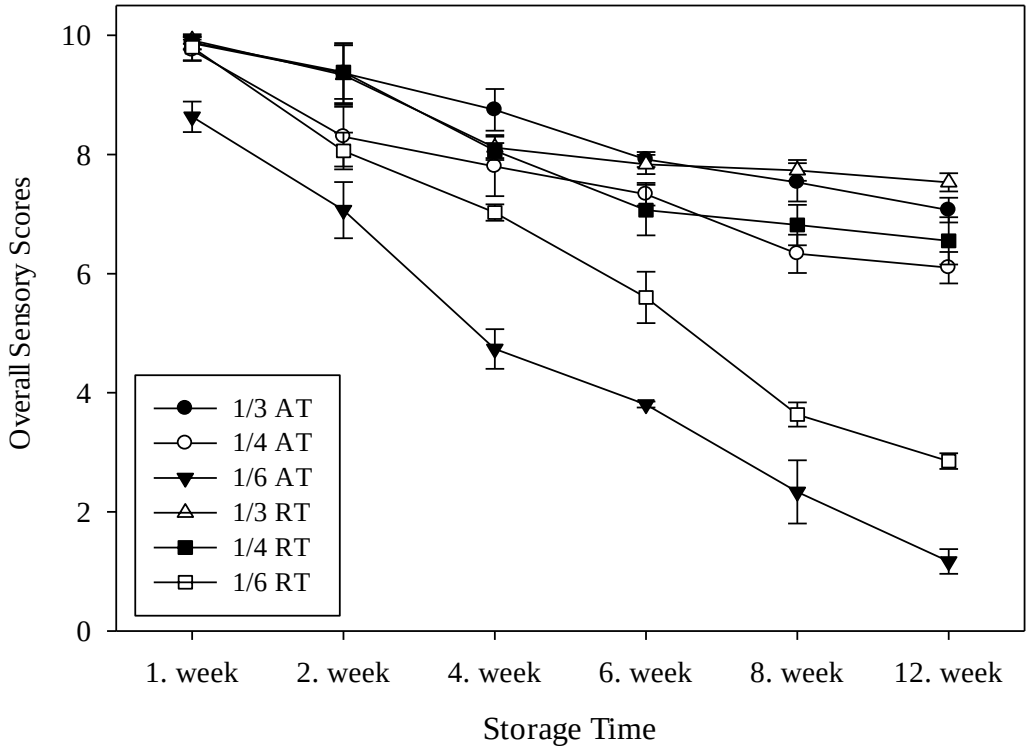
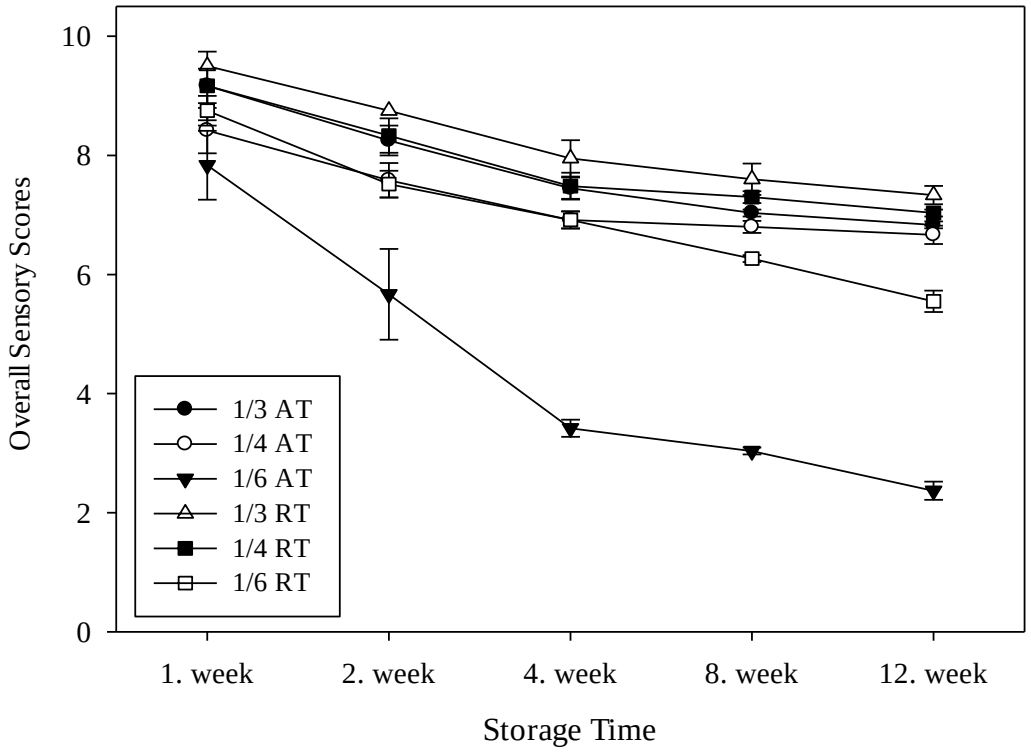


Figure 2. Overall sensory scores for *lakerda* (dry salted Atlantic bonito) processed from fresh raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures



AT: Ambient temperature, RT: Refrigerated temperature

Figure 3. Overall sensory scores for *lakerda* (dry salted Atlantic bonito) processed from previously frozen raw materials, stored at ambient (17±3°C) and refrigerated (4±1°C) temperatures

Different studies reported different shelf lives for *lakerda* depending on the storage temperature, and processing and/or packaging methods. Our results obtained for the samples stored at refrigerated conditions supported the results reported by Erkan *et al*. (2009) for brine, vacuum- and oil-packed *lakerda* samples produced from Atlantic bonito stored at the same conditions. The amount of salt used prior to brining in their study was higher than the present study. Therefore, the present study suggests that salt:fish ratio higher than 1:3 may not add beneficial effect to the sensory life of *lakerda* although the shelf-life can also be affected by the different production methodology in two different studies. Lüleci (1991) obtained 60 days of shelf-life for *lakerda* made from Atlantic bonito stored in brine at 4°C. Recently, Caglak *et al*. (2016) reported a very short shelf-life for *lakerda* at refrigerated storage packed in plastic bags as less than 8 days. They reported that vacuum and modified atmosphere packing extended the shelf-life up to 23 days. Although their method is similar to the current study in terms of dry salting at the beginning, later they used brine solution for maturation which differs from the present study. Therefore, the amount of salt used is not very clear which makes it difficult to compare with the current study. Turan *et al*. (2006) and recently, Kocatepe *et al*. (2014) also investigated shelf-life of dry salted Atlantic bonito which was processed as *lakerda* at refrigerated storage. However, both studies did not determined the sensory values, and the shelf stability of the products was judged using chemical and microbiological quality. Such results can only be evaluated in support of sensory values. In our previous study, we observed 4 days of shelf life for hot-smoked Atlantic bonito at ambient temperature (17 ±3°C), and 10 days at refrigerated temperature (Koral *et al*., 2010). Therefore, *lakerda* has an advantage over such products in terms of extending the shelf-life of this species.

The results of TVB-N, TMA and TBA are shown in Table 5. The TVB-N values increased significantly ($p<0.05$) for all groups throughout the storage, and also significant differences ($p<0.05$) were observed amongst different salting and storage groups with some exceptions. Varying levels of TVB-N have been suggested for different fish products to assess their freshness in literature (Connell, 1990; Huss, 1988). European Union set varying TVB-N limits as 25-35 mg/100 g for unprocessed fishery products shall be regarded

as unfit for human consumption where organoleptic assessment has raised doubts as to their freshness (EU Directive, 2005b and 2008). However, Atlantic bonito is not included in EU regulation. Therefore, TVB-N levels can be used only in support of sensory values. Our results showed that TVB-N did not support sensory values (Table 5). TVB-N values of the lowest salt concentration group reached to the unacceptable level set by EU regulation on the 4th week for each raw material group stored at ambient temperatures (17±3°C). The TVB-N values were within the permitted level (min: 18.21 mg/100 g –max: 22.06 mg/100 g) for other groups. Therefore, the results shows the advantage of refrigerated storage for keeping better chemical quality of dry salted Atlantic bonito. Lower TVB-N values were obtained by Erkan *et al*.(2009) and Kocatepe *et al*.(2014), while TVB-N levels reported by Turan *et al*.(2006) were close to some of our experimental groups. The TVB-N levels in fisheries products are affected by different factors including the initial condition of the fish which explains the differences in the different studies (Huss, 1988).

TBA values increased significantly throughout storage ($p<0.05$). There was also significant differences ($p<0.05$) amongst the groups with some exceptions. TBA is a quality parameter particularly relates to lipid oxidation. The lowest TBA value was found with the products originated from frozen raw material and treated with the lowest salt content (1/6 salt:fish ratio). The products treated with 1/3 salt:fish ratio and kept at ambient temperature reached to unacceptable level at the end of storage for freshly processed Atlantic bonito. Therefore, this study indicates that high salt content can accelerate lipid oxidation, while freezing and frozen storage to prior to salting can retard oxidative changes in fish products. Erkan *et al*. (2009) reported a decrease in TBA values during storage of *lakerda* from Atlantic bonito at refrigerated temperatures with some fluctuations. Therefore, their results did not support our findings. Kocatepe *et al*. (2014) observed higher TBA contents. The differences may have caused due to differences in processing stages and the initial condition of the fish prior to processing.

Trimethylamine values of *lakerda* showed similar trend with TVB-N values. The highest TMA values were found in the products treated with the lowest salt concentration and kept at ambient temperature (17±3°C). At the end of storage, TMA values were 10.72 and 11.12 mg/100 g for fresh and frozen raw material groups, respectively. Significant differences ($p<0.05$) were found during storage and amongst the groups. TMA is another chemical parameter commonly used to determine fish spoilage. It is a pungent volatile amine often associated with the typical "fishy" odour of spoiling seafood. Its presence in spoiling fish is due to the bacterial reduction of trimethylamine oxide which is naturally present in the living tissue of many marine fish species. Although TMA is believed to be generated by the action of spoilage bacteria, the correlation with bacterial numbers is often not very good (Huss, 1995). A suggested acceptable level is reported as 12 mg/100 g (Goulas and Kontominos, 2005). TMA values were still below the suggested upper limit for this parameter. The results of Turan *et al*.(2006) and Erkan *et al*.(2009) supported our values for some experimental groups.

Table 6 and 7 show the changes in the biogenic amine values of dry salted Atlantic bonito (*lakerda*) during storage. The results of the conducted study showed that dry salting prevents the formation of biogenic amines, particularly histamine. The levels of histamine were below 29 ppm throughout the storage despite of differences in the groups of salt:fish ratio and storage temperatures. This study also showed that although spermidine levels of fresh and processed Atlantic bonito were higher at the beginning of storage, it generally decreased until the end of storage for all types of samples. The highest biogenic amine values corresponded to Atlantic bonito treated with the ratio of 1/6 salt:fish content stored at ambient temperature. Therefore, the results demonstrated the beneficial effect of high salt concentration and refrigeration on preventing histamine formation in salted Atlantic bonito. Histamine values were also found very low and none of the products exceeded the permitted levels set by FDA, EU and Turkish authorities.

Ormancı and Colakoglu (2017) reported that all biogenic amines analysed decreased significantly ($p<0.05$) during ripening of *lakerda* from Atlantic bonito with the exception of spermine at both refrigerated and ambient temperatures. However, they also obtained an increase for most biogenic amines at different days depending on the ripening temperatures. Although initial histamine value in fresh fish used in their study was higher than the value obtained in present study, lower histamine contents were recorded by the authors for the samples ripened at both 17 and 20°C. Histamine formation can be affected by various factors.

Table 1. The changes in the contents of dry matter (%) of *lakerda* (dry salted Atlantic bonito) processed from fresh and previously frozen raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

RM	ST	SFR	Fresh bonito	1st week	2nd week	4th week	6th week	8th week	12th week
Fresh Raw Material	AT	1/3	36.86±0.28	*53.14±0.17$^{c}_{A}$	53.33±0.55$^{c}_{A}$	*54.58±0.44$^{d}_{A}$	53.67±0.48$^{d}_{A}$	53.91±0.03$^{c}_{A}$	*51.69±0.49$^{c}_{B}$
		1/4		50.58±0.53$^{b}_{A}$	52.13±0.08$^{c}_{A}$	*51.99±0.67$^{c}_{A}$	52.09±0.68$^{c}_{A}$	*50.98±1.62$^{b}_{A}$	*49.55±0.62$^{b}_{A}$
		1/6		*44.86±0.72$^{a}_{A}$	*47.30±0.23$^{a}_{B}$	*48.98±0.28$^{b}_{B}$	48.98±0.22$^{b}_{B}$	*48.64±0.52$^{b}_{B}$	*46.03±0.48$^{a}_{C}$
	RT	1/3		52.13±0.35$^{c}_{A}$	53.14±0.21$^{c}_{B}$	*53.09±0.55$^{d}_{B}$	53.22±0.12$^{d}_{B}$	52.88±0.34$^{c}_{B}$	54.98±1.32$^{d}_{B}$
		1/4		*50.99±0.51$^{b}_{A}$	50.66±0.50$^{b}_{A}$	*50.94±0.18$^{c}_{A}$	51.10±0.86$^{c}_{A}$	52.88±0.34$^{c}_{B}$	52.69±0.26$^{c}_{A}$
		1/6		45.42±0.65$^{a}_{A}$	*46.37±0.21$^{a}_{A}$	*46.49±0.18$^{a}_{A}$	47.01±0.35$^{a}_{A}$	*45.91±0.22$^{a}_{A}$	*48.22±0.16$^{b}_{B}$
Frozen Raw Material	AT	1/3	37.22±0.26	*54.87±0.25$^{d}_{A}$	55.35±0.82$^{d}_{A}$	*57.09±1.38$^{d}_{A}$	NA	54.88±1.22$^{b}_{A}$	*55.52±0.38$^{d}_{A}$
		1/4		51.14±0.40$^{c}_{A}$	51.83±0.71$^{bc}_{A}$	*54.54±0.25$^{c}_{A}$	NA	*53.46±0.86$^{b}_{A}$	*53.65±0.15$^{c}_{A}$
		1/6		*47.87±0.21$^{b}_{A}$	*50.33±0.38$^{b}_{B}$	*51.89±0.84$^{b}_{B}$	NA	*52.95±1.09$^{b}_{B}$	*52.80±0.44$^{b}_{B}$
	RT	1/3		51.86±0.80$^{c}_{A}$	52.76±0.52$^{c}_{A}$	*55.14±0.22$^{c}_{B}$	NA	53.80±0.28$^{b}_{A}$	53.58±0.12$^{c}_{A}$
		1/4		*46.26±0.37$^{b}_{A}$	51.21±0.11$^{b}_{B}$	*52.21±0.02$^{b}_{B}$	NA	49.44±0.25$^{a}_{B}$	52.01±0.32$^{b}_{B}$
		1/6		43.91±0.82$^{a}_{A}$	*48.35±0.64$^{a}_{B}$	*47.25±0.20$^{a}_{B}$	NA	*48.60±0.15$^{a}_{B}$	*49.55±0.28$^{a}_{B}$

A: Analysis, RW: Raw Material Type, ST: Storage Temperature, SFR: The ratio of salt:fish(w:w), AT: Ambient Temperature, RT: Refrigerated Temperature, NA: Not Analysed, ± SD: n: 3, The different superscript lowercase letters (a,b,c..) represent statistical differences amongst different salting subgroups under each raw material group at the same storage time ($p<0.05$). The different subscript uppercase letters (A,B,C…) represents statistical differences during storage period of the same group ($p<0.05$). '' on each data represents that there is statistical difference between the data obtained for fresh and frozen raw material groups at the same storage time and the ration of salt:fish.*

Table 2. The changes in the contents of salt (%) and WPS % of *lakerda* (dry salted Atlantic bonito) processed from fresh and previously frozen raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

A	RM	ST	SFR	Fresh	1st week	2nd week	4th week	6th week	8th week	12th week
Salt (%)	Fresh	AT	1/3	0.61±0.02	16.37±0.13$^{e}_{A}$	16.42±0.07$^{e}_{A}$	16.23±0.11$^{e}_{A}$	17.22±0.10$^{f}_{B}$	17.32±0.06$^{e}_{B}$	16.14±0.12$^{d}_{A}$
			1/4		*15.51±0.06$^{d}_{A}$	*15.92±0.04$^{d}_{B}$	*15.47±0.05$^{d}_{A}$	15.44±0.06$^{d}_{A}$	*15.36±0.15$^{c}_{A}$	*15.21±0.05$^{c}_{A}$
			1/6		*11.33±0.03$^{b}_{A}$	12.31±0.14$^{b}_{B}$	13.08±0.08$^{b}_{C}$	13.24±0.03$^{b}_{C}$	13.60±0.14$^{a}_{D}$	13.16±0.10$^{a}_{C}$
		RT	1/3		*16.16±0.05$^{e}_{A}$	16.19±0.14$^{e}_{A}$	*16.50±0.04$^{f}_{B}$	16.59±0.08$^{e}_{B}$	*16.32±0.06$^{d}_{A}$	*16.05±0.06$^{d}_{A}$
			1/4		*13.17±0.05$^{c}_{A}$	*13.77±0.22$^{c}_{B}$	*13.90±0.03$^{c}_{B}$	14.58±0.04$^{c}_{C}$	*14.44±0.12$^{b}_{C}$	14.04±0.22$^{b}_{B}$
			1/6		*9.61±0.07$^{a}_{A}$	11.78±0.10$^{a}_{B}$	*11.32±0.09$^{a}_{C}$	12.82±0.04$^{a}_{D}$	*13.49±0.26$^{a}_{E}$	*13.66±0.16$^{b}_{E}$
	Frozen	AT	1/3	0.66±0.08	16.36±0.38$^{e}_{A}$	16.38±0.37$^{d}_{A}$	16.36±0.21$^{d}_{A}$	NA	16.98±0.10$^{d}_{A}$	16.33±0.12$^{e}_{A}$
			1/4		*13.89±0.10$^{c}_{A}$	*14.49±0.26$^{c}_{B}$	*14.68±0.12$^{c}_{B}$	NA	*14.04±0.18$^{b}_{A}$	*14.33±0.42$^{c}_{B}$
			1/6		*12.68±0.30$^{b}_{A}$	12.24±0.11$^{b}_{A}$	13.11±0.05$^{b}_{B}$	NA	13.16±0.25$^{ab}_{B}$	13.63±0.15$^{b}_{C}$
		RT	1/3		*14.14±0.10$^{d}_{A}$	*14.70±0.08$^{c}_{B}$	*14.91±0.06$^{c}_{B}$	NA	*15.51±0.26$^{c}_{C}$	*15.28±0.36$^{d}_{BC}$
			1/4		*12.19±0.19$^{b}_{A}$	*12.45±0.22$^{b}_{A}$	*13.03±0.14$^{b}_{B}$	NA	*13.68±0.34$^{b}_{C}$	13.92±0.24$^{b}_{C}$
			1/6		*10.55±0.31$^{a}_{A}$	11.41±0.17$^{a}_{B}$	*11.99±0.07$^{a}_{B}$	NA	*12.69±0.24$^{a}_{C}$	*12.57±0.27$^{a}_{C}$
Water Phase Salt (WPS%)	Fresh	AT	1/3	0.96±0.04	25.89±0.22$^{e}_{A}$	26.03±0.31$^{e}_{A}$	*26.33±0.32$^{e}_{A}$	27.10±0.32$^{f}_{B}$	27.31±0.05$^{e}_{B}$	*25.04±0.15$^{c}_{A}$
			1/4		*23.88±0.13$^{d}_{A}$	24.96±0.02$^{d}_{B}$	24.37±0.31$^{d}_{A}$	24.37±0.19$^{d}_{A}$	23.87±0.42$^{d}_{A}$	*23.16±0.12$^{b}_{A}$
			1/6		*17.04±0.14$^{b}_{A}$	*18.94±0.11$^{b}_{B}$	20.41±0.01$^{b}_{C}$	20.61±0.03$^{b}_{C}$	*20.94±0.34$^{b}_{C}$	*19.60±0.24$^{a}_{C}$
		RT	1/3		*25.24±0.19$^{e}_{A}$	25.68±0.24$^{e}_{A}$	*26.02±0.18$^{e}_{A}$	26.18±0.10$^{e}_{A}$	25.73±0.07$^{d}_{A}$	*26.28±0.17$^{d}_{A}$
			1/4		*21.18±0.11$^{c}_{A}$	21.82±0.20$^{c}_{A}$	*22.08±0.06$^{c}_{A}$	22.97±0.36$^{c}_{B}$	*22.69±0.17$^{c}_{B}$	22.88±0.10$^{b}_{B}$
			1/6		*14.97±0.25$^{a}_{A}$	18.01±0.12$^{a}_{B}$	*17.46±0.16$^{a}_{C}$	19.48±0.06$^{a}_{D}$	19.95±0.24$^{a}_{D}$	*20.87±0.16$^{a}_{E}$
	Frozen	AT	1/3	1.04±0.04	26.60±0.34$^{d}_{A}$	26.84±0.09$^{d}_{A}$	*27.61±0.39$^{d}_{B}$	NA	27.34±0.24$^{e}_{B}$	*26.85±0.32$^{e}_{A}$
			1/4		*22.13±0.26$^{c}_{A}$	23.12±0.58$^{c}_{A}$	24.41±0.05$^{c}_{B}$	NA	23.17±0.15$^{c}_{A}$	*23.61±0.25$^{c}_{A}$
			1/6		*19.57±0.44$^{b}_{A}$	*19.77±0.02$^{b}_{A}$	21.41±0.36$^{b}_{B}$	NA	*21.85±0.16$^{b}_{B}$	*22.40±0.16$^{b}_{C}$
		RT	1/3		*22.70±0.41$^{c}_{A}$	*23.74±0.10$^{c}_{B}$	*24.95±0.02$^{c}_{C}$	NA	25.13±0.12$^{d}_{C}$	*24.76±0.12$^{d}_{C}$
			1/4		*18.48±0.14$^{b}_{A}$	20.33±0.25$^{b}_{B}$	*21.42±0.17$^{b}_{C}$	NA	*21.29±0.16$^{b}_{C}$	22.48±0.14$^{b}_{D}$
			1/6		*15.83±0.20$^{a}_{A}$	18.09±0.04$^{a}_{B}$	*18.52±0.03$^{a}_{B}$	NA	19.80±0.32$^{a}_{C}$	*19.94±0.08$^{a}_{C}$

A: Analysis, RW: Raw Material Type, ST: Storage Temperature, SFR: The ratio of salt:fish(w:w), AT: Ambient Temperature, RT: Refrigerated Temperature, NA: Not Analysed, ± SD: n: 3, The different superscript lowercase letters (a,b,c..) represent statistical differences amongst different salting subgroups under each raw material group at the same storage time ($p<0.05$). The different subscript uppercase letters (A,B,C…) represents statistical differences during storage period of the same group ($p<0.05$). '' on each data represents that there is statistical difference between the data obtained for fresh and frozen raw material groups at the same storage time and the ration of salt:fish.*

Table 3. The changes in the contents of pH and water activity (a_w) of *lakerda* (dry salted Atlantic bonito) processed from fresh and previously frozen raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

A	RM	ST	SFR	Fresh Bonito	1st week	2nd week	4th week	6th week	8th week	12th week
Water Activity (a_w)	Fresh	AT	1/3	0.994 ±0.001	*0.753±0.001$^{a}_{A}$	*0.760±0.001$^{a}_{B}$	0.753±0.001$^{a}_{A}$	0.756±0.001$^{a}_{C}$	*0.747±0.001$^{a}_{D}$	0.749±0.001$^{b}_{D}$
			1/4		*0.867±0.001$^{f}_{A}$	*0.789±0.001$^{b}_{B}$	*0.766±0.001$^{b}_{C}$	0.769±0.001$^{b}_{C}$	*0.756±0.001$^{b}_{D}$	0.750±0.001$^{b}_{E}$
			1/6		*0.809±0.001$^{c}_{A}$	*0.861±0.001$^{e}_{B}$	*0.843±0.001$^{f}_{C}$	0.831±0.001$^{e}_{D}$	*0.863±0.001$^{f}_{B}$	*0.860±0.001$^{c}_{B}$
		RT	1/3		*0.783±0.000$^{b}_{A}$	*0.788±0.000$^{b}_{B}$	*0.774±0.001$^{c}_{C}$	0.770±0.001$^{b}_{C}$	*0.766±0.001$^{c}_{D}$	0.746±0.001$^{a}_{E}$
			1/4		*0.815±0.000$^{d}_{A}$	*0.807±0.001$^{c}_{B}$	*0.785±0.001$^{d}_{C}$	0.772±0.002$^{b}_{D}$	*0.777±0.000$^{d}_{E}$	0.751±0.002$^{b}_{F}$
			1/6		*0.861±0.000$^{e}_{A}$	*0.842±0.000$^{d}_{B}$	*0.819±0.001$^{e}_{C}$	0.812±0.001$^{d}_{D}$	*0.840±0.001$^{e}_{B}$	*0.817±0.001$^{d}_{C}$
	Frozen	AT	1/3	0.992 ±0.001	*0.765±0.001$^{a}_{A}$	*0.755±0.001$^{a}_{B}$	0.751±0.001$^{a}_{C}$	NA	*0.754±0.001$^{b}_{B}$	0.749±0.001$^{a}_{C}$
			1/4		*0.790±0.001$^{b}_{A}$	*0.778±0.001$^{b}_{B}$	*0.753±0.001$^{a}_{C}$	NA	*0.760±0.001$^{c}_{D}$	0.750±0.001$^{a}_{C}$
			1/6		*0.834±0.002$^{d}_{A}$	*0.819±0.001$^{d}_{B}$	*0.787±0.002$^{d}_{C}$	NA	*0.781±0.002$^{e}_{D}$	*0.780±0.002$^{d}_{D}$
		RT	1/3		*0.819±0.001$^{c}_{A}$	*0.777±0.001$^{b}_{B}$	*0.761±0.001$^{b}_{C}$	NA	*0.751±0.001$^{a}_{D}$	0.749±0.001$^{a}_{D}$
			1/4		*0.855±0.001$^{e}_{A}$	*0.850±0.002$^{e}_{B}$	*0.766±0.001$^{c}_{C}$	NA	*0.758±0.001$^{c}_{D}$	0.754±0.001$^{b}_{E}$
			1/6		*0.885±0.005$^{f}_{A}$	*0.813±0.001$^{c}_{B}$	*0.791±0.001$^{d}_{C}$	NA	*0.770±0.001$^{d}_{D}$	*0.765±0.001$^{c}_{E}$
pH	Fresh	AT	1/3	6.16 ±0.02	*5.83±0.01$^{a}_{A}$	*5.95±0.02$^{a}_{B}$	*5.93±0.01$^{a}_{B}$	5.67±0.01$^{a}_{C}$	5.75±0.01$^{a}_{D}$	5.83±0.02$^{a}_{A}$
			1/4		*5.90±0.01$^{b}_{A}$	6.07±0.01$^{b}_{B}$	*5.94±0.01$^{a}_{C}$	5.76±0.02$^{b}_{D}$	*5.88±0.01$^{b}_{A}$	5.85±0.01$^{a}_{E}$
			1/6		6.07±0.01$^{d}_{A}$	*6.05±0.03$^{b}_{A}$	*6.03±0.02$^{b}_{AB}$	5.97±0.02$^{d}_{B}$	*6.30±0.01$^{d}_{C}$	*7.24±0.03$^{c}_{D}$
		RT	1/3		5.94±0.01$^{c}_{A}$	*5.98±0.02$^{a}_{A}$	*5.96±0.01$^{a}_{A}$	5.88±0.01$^{c}_{B}$	*5.89±0.01$^{b}_{B}$	5.83±0.01$^{a}_{C}$
			1/4		*5.86±0.01$^{a}_{A}$	*6.04±0.02$^{b}_{B}$	*6.01±0.02$^{b}_{B}$	5.92±0.02$^{d}_{C}$	*5.90±0.03$^{b}_{C}$	*5.96±0.03$^{b}_{C}$
			1/6		5.98±0.01$^{c}_{A}$	*6.10±0.02$^{b}_{B}$	*6.06±0.02$^{b}_{B}$	5.95±0.03$^{d}_{A}$	*5.96±0.01$^{c}_{A}$	*5.95±0.01^{b}
	Frozen	AT	1/3	6.12 ±0.04	*5.92±0.01$^{a}_{A}$	*5.80±0.00$^{b}_{B}$	*5.78±0.02$^{a}_{B}$	NA	5.71±0.03$^{b}_{C}$	5.80±0.01$^{a}_{B}$
			1/4		*6.03±0.02$^{c}_{A}$	5.77±0.01$^{a}_{B}$	*5.81±0.01$^{a}_{B}$	NA	*5.67±0.01$^{a}_{C}$	5.81±0.03$^{a}_{B}$
			1/6		6.05±0.04$^{c}_{A}$	*5.89±0.03$^{d}_{B}$	*5.85±0.02$^{b}_{B}$	NA	*5.81±0.02$^{c}_{B}$	*5.82±0.02$^{a}_{B}$
		RT	1/3		5.97±0.01$^{b}_{A}$	*5.77±0.00$^{a}_{B}$	*5.88±0.01$^{b}_{C}$	NA	*5.79±0.01$^{c}_{B}$	5.82±0.01$^{a}_{B}$
			1/4		*5.96±0.01$^{b}_{A}$	*5.82±0.01$^{b}_{B}$	*5.88±0.03$^{bc}_{C}$	NA	*5.80±0.03$^{c}_{B}$	*5.84±0.02$^{a}_{B}$
			1/6		6.00±0.01^{bc}	*5.85±0.00$^{c}_{B}$	*5.91±0.03^{c}	NA	*5.86±0.03^{c}	*5.88±0.03^{a}

A: Analysis, RW: Raw Material Type, ST: Storage Temperature, SFR: The ratio of salt:fish(w:w), AT: Ambient Temperature, RT: Refrigerated Temperature, NA: Not Analysed, ± SD: n: 3, The different superscript lowercase letters (a,b,c..) represent statistical differences amongst different salting subgroups under each raw material group at the same storage time (p<0.05). The different subscript uppercase letters (A,B,C…) represents statistical differences during storage period of the same group (p<0.05). '' on each data represents that there is statistical difference between the data obtained for fresh and frozen raw material groups at the same storage time and the ration of salt:fish.*

Table 4. Sensory scores of *lakerda* (dry salted Atlantic bonito) processed from fresh and previously frozen raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

A	RM	ST	SFR	1st week	2nd week	4th week	6th week	8th week	12th week
Texture	Fresh	AT	1/3	$9.75\pm0.10^{b}_{A}$	$*9.30\pm0.15^{c}_{B}$	$*8.35\pm0.18^{c}_{C}$	$8.05\pm0.06^{d}_{C}$	$*7.40\pm0.05^{e}_{D}$	$7.00\pm0.08^{d}_{E}$
			1/4	$*9.55\pm0.30^{b}_{A}$	$*8.30\pm0.16^{b}_{B}$	$7.30\pm0.16^{b}_{C}$	$7.25\pm0.23^{c}_{C}$	$*6.10\pm0.06^{c}_{D}$	$*6.00\pm0.07^{c}_{D}$
			1/6	$*8.35\pm0.\ 10^{a}_{A}$	$*7.60\pm0.21^{a}_{B}$	$*4.35\pm0.36^{a}_{C}$	$3.80\pm0.16^{a}_{D}$	$*2.25\pm0.22^{a}_{E}$	$*1.10\pm0.12^{a}_{F}$
		RT	1/3	$9.85\pm0.25^{b}_{A}$	$*9.15\pm0.00^{c}_{B}$	$8.05\pm0.08^{c}_{C}$	$7.65\pm0.00^{c}_{D}$	$7.55\pm0.34^{e}_{D}$	$7.40\pm0.14^{e}_{D}$
			1/4	$*9.75\pm0.20^{b}_{A}$	$*9.35\pm0.41^{c}_{A}$	$*8.05\pm0.05^{c}_{B}$	$7.55\pm0.41^{c}_{C}$	$7.20\pm0.04^{d}_{C}$	$7.00\pm0.14^{d}_{C}$
			1/6	$*9.55\pm0.05^{b}_{A}$	$*8.40\pm0.20^{b}_{B}$	$7.15\pm0.16^{b}_{C}$	$5.10\pm0.00^{b}_{D}$	$*3.40\pm0.26^{b}_{E}$	$*2.80\pm0.06^{b}_{F}$
	Frozen	AT	1/3	$9.50\pm0.50^{b}_{A}$	$*8.50\pm0.50^{c}_{B}$	$*7.25\pm0.25^{b}_{C}$	NA	$*7.00\pm0.15^{c}_{C}$	$6.80\pm0.15^{d}_{C}$
			1/4	$*8.50\pm0.50^{b}_{A}$	$*7.25\pm0.25^{a}_{B}$	$7.00\pm0.50^{b}_{BC}$	NA	$*6.80\pm0.32^{bc}_{C}$	$*6.50\pm0.24^{c}_{C}$
			1/6	$*7.50\pm0.50^{a}_{A}$	$*5.00\pm0.00^{a}_{B}$	$*3.50\pm0.50^{a}_{C}$	NA	$*3.00\pm0.24^{a}_{C}$	$*2.50\pm0.16^{a}_{D}$
		RT	1/3	$9.50\pm0.50^{b}_{A}$	$*8.75\pm0.25^{c}_{A}$	$7.75\pm0.50^{c}_{B}$	NA	$7.50\pm0.30^{d}_{B}$	$7.30\pm0.40^{e}_{B}$
			1/4	$*9.00\pm0.00^{b}_{A}$	$*8.50\pm0.50^{c}_{A}$	$*7.70\pm0.25^{c}_{B}$	NA	$7.30\pm0.20^{cd}_{C}$	$7.00\pm0.25^{e}_{C}$
			1/6	$*8.50\pm0.50^{b}_{A}$	$*7.75\pm0.25^{b}_{A}$	$7.00\pm0.25^{b}_{B}$	NA	$*6.30\pm0.50^{b}_{C}$	$*5.50\pm0.45^{b}_{D}$
Odour	Fresh	AT	1/3	$*9.95\pm0.05^{b}_{A}$	$*8.90\pm0.10^{c}_{B}$	$*8.90\pm0.01^{b}_{B}$	$7.80\pm0.13^{d}_{C}$	$*7.90\pm0.06^{d}_{C}$	$7.30\pm0.12^{d}_{C}$
			1/4	$*9.90\pm0.10^{b}_{A}$	$7.80\pm0.13^{b}_{B}$	$*7.80\pm0.11^{c}_{B}$	$7.20\pm0.19^{d}_{C}$	$6.70\pm0.29^{c}_{D}$	$*6.40\pm0.21^{c}_{D}$
			1/6	$*8.85\pm0.15^{a}_{A}$	$*6.90\pm0.20^{a}_{B}$	$*4.95\pm0.04^{a}_{C}$	$3.75\pm0.21^{a}_{D}$	$*1.85\pm0.16^{a}_{E}$	$*1.00\pm0.10^{a}_{F}$
		RT	1/3	$9.95\pm0.05^{b}_{A}$	$8.95\pm0.08^{c}_{B}$	$7.95\pm0.03^{c}_{C}$	$7.90\pm0.10^{d}_{C}$	$7.75\pm0.25^{d}_{C}$	$7.50\pm0.15^{d}_{C}$
			1/4	$*9.95\pm0.05^{b}_{A}$	$8.95\pm0.07^{c}_{B}$	$7.95\pm0.06^{c}_{C}$	$6.75\pm0.23^{c}_{D}$	$*6.70\pm0.09^{c}_{D}$	$*6.40\pm0.15^{cE}$
			1/6	$*9.95\pm0.05^{b}_{A}$	$7.98\pm0.02^{b}_{B}$	$6.88\pm0.13^{b}_{C}$	$5.85\pm0.14^{b}_{D}$	$*3.75\pm0.27^{b}_{E}$	$*2.75\pm0.07^{b}_{F}$
	Frozen	AT	1/3	$*8.50\pm0.50^{b}_{A}$	$*8.00\pm0.00^{c}_{AB}$	$*7.50\pm0.50^{c}_{B}$	NA	$*7.10\pm0.10^{cd}_{B}$	$6.90\pm0.40^{c}_{B}$
			1/4	$*8.00\pm1.00^{ab}_{A}$	$7.75\pm0.25^{bc}_{A}$	$*7.00\pm0.00^{b}_{B}$	NA	$6.90\pm0.08^{c}_{C}$	$*6.80\pm0.08^{c}_{C}$
			1/6	$*7.50\pm0.50^{a}_{A}$	$*5.50\pm0.50^{a}_{B}$	$*3.50\pm0.50^{a}_{C}$	NA	$*3.10\pm0.20^{a}_{C}$	$*2.40\pm0.42^{a}_{D}$
		RT	1/3	$9.50\pm0.50^{cd}_{A}$	$8.75\pm0.25^{d}_{B}$	$7.80\pm0.13^{c}_{C}$	NA	$7.40\pm0.10^{e}_{D}$	$7.20\pm0.24^{c}_{D}$
			1/4	$*9.50\pm0.50^{cd}_{A}$	$8.50\pm0.50^{d}_{B}$	$7.50\pm0.50^{c}_{CD}$	NA	$*7.40\pm0.16^{d}_{D}$	$*7.10\pm0.30^{c}_{D}$
			1/6	$*9.00\pm0.20^{c}_{A}$	$7.50\pm0.50^{b}_{B}$	$6.75\pm0.25^{b}_{C}$	NA	$*6.20\pm0.20^{b}_{D}$	$*5.75\pm0.15^{b}_{E}$
Appearance	Fresh	AT	1/3	$9.90\pm0.10^{b}_{A}$	$*9.90\pm0.10^{d}_{A}$	$*9.00\pm0.00^{d}_{B}$	$7.90\pm0.03^{d}_{C}$	$7.30\pm0.30^{d}_{D}$	$6.90\pm0.20^{e}_{D}$
			1/4	$*9.80\pm0.20^{b}_{A}$	$*8.80\pm0.13^{c}_{B}$	$*8.30\pm0.34^{c}_{C}$	$7.55\pm0.04^{d}_{D}$	$*6.20\pm0.20^{c}_{E}$	$*5.90\pm0.30^{c}_{E}$
			1/6	$8.70\pm0.30^{a}_{A}$	$6.70\pm0.23^{a}_{B}$	$*4.90\pm0.01^{a}_{C}$	$3.85\pm0.15^{a}_{D}$	$*2.90\pm0.08^{a}_{E}$	$*1.40\pm0.32^{a}_{F}$
		RT	1/3	$9.95\pm0.05^{b}_{A}$	$*9.90\pm0.10^{d}_{A}$	$8.35\pm0.35^{c}_{B}$	$7.95\pm0.04^{d}_{C}$	$7.90\pm0.08^{e}_{C}$	$7.70\pm0.18^{f}_{C}$
			1/4	$*9.95\pm0.05^{b}_{A}$	$*9.85\pm0.15^{d}_{A}$	$8.20\pm0.20^{c}_{B}$	$6.90\pm0.01^{c}_{C}$	$*6.55\pm0.11^{c}_{D}$	$*6.25\pm0.16^{d}_{E}$
			1/6	$*9.90\pm0.10^{b}_{A}$	$*7.80\pm0.20^{b}_{B}$	$7.05\pm0.05^{b}_{C}$	$5.85\pm0.11^{b}_{D}$	$*3.75\pm0.28^{b}_{E}$	$*3.00\pm0.18^{b}_{E}$
	Frozen	AT	1/3	$9.50\pm0.50^{b}_{A}$	$*8.25\pm0.25^{cd}_{B}$	$*7.60\pm0.25^{d}_{C}$	NA	$7.00\pm0.30^{d}_{D}$	$6.80\pm0.20^{c}_{D}$
			1/4	$*8.75\pm0.25^{a}_{A}$	$*7.75\pm0.25^{c}_{B}$	$*6.75\pm0.50^{b}_{C}$	NA	$*6.70\pm0.30^{c}_{C}$	$*6.70\pm0.10^{c}_{C}$
			1/6	$8.50\pm0.50^{a}_{A}$	$6.50\pm0.50^{a}_{B}$	$*3.25\pm0.50^{a}_{C}$	NA	$*3.00\pm0.10^{a}_{C}$	$*2.20\pm0.32^{a}_{D}$
		RT	1/3	$9.50\pm0.50^{b}_{A}$	$*8.75\pm0.25^{d}_{B}$	$8.30\pm0.25^{c}_{C}$	NA	$7.90\pm0.15^{e}_{D}$	$7.50\pm0.15^{d}_{E}$
			1/4	$*9.00\pm0.50^{ab}_{A}$	$*8.00\pm0.00^{c}_{B}$	$7.25\pm0.50^{c}_{C}$	NA	$*7.20\pm0.12^{d}_{C}$	$*7.00\pm0.10^{c}_{C}$
			1/6	$*8.75\pm0.50^{a}_{A}$	$*7.30\pm0.38^{b}_{B}$	$7.00\pm0.25^{bc}_{B}$	NA	$*6.30\pm0.16^{b}_{C}$	$*5.40\pm0.20^{b}_{D}$

A: Analysis, RW: Raw Material Type, ST: Storage Temperature, SFR: The ratio of salt:fish(w:w), AT: Ambient Temperature, RT: Refrigerated Temperature, NA: Not Analysed, ± SD: n: 8, The values below 4.0 is unacceptable. The different superscript lowercase letters (a,b,c..) represent statistical differences amongst different salting subgroups under each raw material group at the same storage time ($p<0.05$). The different subscript uppercase letters (A,B,C…) represents statistical differences during storage period of the same group ($p<0.05$). '' on each data represents that there is statistical difference between the data obtained for fresh and frozen raw material groups at the same storage time and the ration of salt:fish.*

Table 5. The changes in the values of TVB-N, TBA and TMA of *lakerda* (dry salted Atlantic bonito) processed from fresh and previously frozen raw materials, stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

A	RM	ST	SFR	Fresh	1st week	2nd week	4th week	6th week	8th week	12th week
Total Volatile Bases Nitrogen (TVB-N) (mg/100g)	Fresh	AT	1/3	12.61 ±0.18	*12.96±0.35$^{a}_{A}$	*17.16±0.35$^{a}_{B}$	*18.91±0.70$^{a}_{C}$	20.66±0.35$^{a}_{D}$	25.06±1.05$^{c}_{E}$	27.36±0.05$^{b}_{F}$
			1/4		17.16±0.35$^{c}_{A}$	*18.56±0.35$^{a}_{B}$	*20.31±0.70$^{b}_{C}$	22.41±0.70$^{b}_{D}$	*27.46±0.35$^{c}_{E}$	*31.23±0.30$^{c}_{F}$
			1/6		*20.31±0.10$^{e}_{A}$	*28.72±0.70$^{c}_{B}$	*42.37±1.05$^{c}_{C}$	49.03±1.40$^{c}_{D}$	*71.79±1.05$^{d}_{E}$	*101.10±2.05$_{F}$
		RT	1/3		*14.36±0.35$^{b}_{A}$	17.86±0.35$^{a}_{B}$	*18.21±1.40$^{a}_{B}$	18.91±0.70$^{a}_{B}$	22.06±0.35$^{a}_{C}$	*23.46±0.28$^{a}_{D}$
			1/4		16.11±0.70$^{c}_{A}$	18.21±0.70$^{a}_{B}$	*18.91±0.70$^{a}_{BC}$	20.31±0.70$^{a}_{C}$	23.46±0.35$^{b}_{D}$	*28.66±0.15$^{b}_{E}$
			1/6		18.56±0.35$^{d}_{A}$	*20.21±0.00$^{b}_{B}$	21.66±0.35$^{b}_{C}$	23.71±0.70$^{b}_{D}$	26.51±0.70$^{c}_{E}$	*31.12±0.20$^{c}_{F}$
	Frozen	AT	1/3	13.31 ±0.14	*15.06±0.35$^{a}_{A}$	*18.21±0.00$^{bc}_{B}$	*21.01±0.70$^{b}_{C}$	NA	23.66±0.25$^{b}_{D}$	26.16±1.05$^{a}_{E}$
			1/4		17.16±0.35$^{b}_{A}$	*20.66±0.35$^{d}_{B}$	*22.06±0.35$^{c}_{C}$	NA	*25.26±0.12$^{c}_{D}$	*29.20±0.20$^{b}_{E}$
			1/6		*17.51±0.70$^{b}_{A}$	*23.46±1.05$^{e}_{B}$	*37.82±1.40$^{d}_{C}$	NA	*48.70±0.50$^{d}_{D}$	*78.80±1.05$^{c}_{E}$
		RT	1/3		*15.76±0.35$^{a}_{A}$	17.16±0.35$^{a}_{B}$	*19.26±0.35$^{a}_{C}$	NA	22.36±0.15$^{a}_{D}$	*25.16±0.28$^{a}_{E}$
			1/4		17.16±0.35$^{b}_{A}$	18.56±0.35$^{c}_{B}$	*20.66±0.35$^{b}_{C}$	NA	23.56±0.25$^{b}_{D}$	*26.46±0.10$^{a}_{E}$
			1/6		18.56±0.35$^{b}_{A}$	*19.26±0.35$^{c}_{A}$	21.36±0.35$^{b}_{B}$	NA	26.50±0.40$^{c}_{C}$	*29.86±0.10$^{b}_{D}$
Thiobarbuturucacid (TBA) (mg MDA/kg)	Fresh	AT	1/3	0.49 ±0.06	*0.94±0.02$^{e}_{A}$	2.18±0.02$^{d}_{B}$	*3.97±0.04$^{e}_{C}$	5.87±0.01$^{f}_{D}$	*7.76±0.01$^{e}_{E}$	*8.16±0.06$^{f}_{F}$
			1/4		*0.76±0.01$^{d}_{A}$	*2.11±0.03$^{d}_{B}$	*3.75±0.01$^{d}_{C}$	4.16±0.01$^{c}_{D}$	*6.89±0.03$^{e}_{E}$	*7.36±0.12$^{e}_{F}$
			1/6		*0.71±0.01$^{c}_{A}$	1.96±0.01$^{c}_{B}$	*2.98±0.01$^{c}_{C}$	3.78±0.01$^{b}_{D}$	*6.29±0.04$^{d}_{E}$	*6.88±0.14$^{d}_{F}$
		RT	1/3		*0.78±0.01$^{d}_{A}$	*1.96±0.02$^{c}_{B}$	*3.74±0.01$^{d}_{C}$	5.01±0.03$^{e}_{D}$	*5.87±0.02$^{c}_{E}$	*6.36±0.08$^{c}_{F}$
			1/4		*0.67±0.01$^{b}_{A}$	*1.70±0.01$^{b}_{B}$	*2.80±0.02$^{b}_{C}$	4.69±0.03$^{d}_{D}$	*5.33±0.02$^{b}_{E}$	*5.86±0.12$^{b}_{F}$
			1/6		*0.57±0.01$^{a}_{A}$	*1.56±0.01$^{a}_{B}$	*2.56±0.02$^{a}_{C}$	2.98±0.03$^{a}_{D}$	*3.36±0.02$^{a}_{E}$	*4.72±0.06$^{a}_{F}$
	Frozen	AT	1/3	0.71 ±0.06	*1.12±0.01$^{c}_{A}$	2.12±0.03$^{c}_{B}$	*3.03±0.01$^{e}_{C}$	NA	*5.26±0.12$^{c}_{D}$	*6.16±0.16$^{d}_{E}$
			1/4		*0.94±0.02$^{b}_{A}$	*1.95±0.02$^{b}_{B}$	*2.73±0.02$^{d}_{C}$	NA	*4.89±0.08$^{b}_{D}$	*5.36±0.16$^{a}_{E}$
			1/6		*0.82±0.02$^{a}_{A}$	1.86±0.02$^{a}_{B}$	*2.56±0.02$^{b}_{C}$	NA	*4.79±0.08$^{b}_{D}$	*6.78±0.18$^{e}_{E}$
		RT	1/3		*1.05±0.03$^{c}_{A}$	*2.11±0.02$^{c}_{B}$	*2.98±0.01$^{e}_{C}$	NA	*4.82±0.12$^{b}_{D}$	*5.78±0.18$^{c}_{E}$
			1/4		*0.82±0.02$^{a}_{A}$	*1.96±0.02$^{b}_{B}$	*2.63±0.02$^{c}_{C}$	NA	*4.58±0.22$^{a}_{D}$	*5.56±0.08$^{b}_{E}$
			1/6		*1.05±0.03$^{c}_{A}$	*1.89±0.01$^{a}_{B}$	*2.38±0.02$^{a}_{C}$	NA	*4.46±0.12$^{a}_{D}$	*5.28±0.16$^{a}_{E}$
Trimethylamine (TMA) (mg/100g)	Fresh	AT	1/3	1.93 ±0.12	*2.28±0.03$^{c}_{A}$	*2.59±0.05$^{b}_{B}$	*2.73±0.06$^{a}_{C}$	3.28±0.08$^{b}_{D}$	*3.37±0.04$^{a}_{E}$	*3.86±0.08$^{b}_{F}$
			1/4		*2.56±0.01$^{d}_{A}$	*2.82±0.05$^{c}_{B}$	*3.00±0.06$^{c}_{C}$	3.40±0.03$^{c}_{D}$	*3.82±0.04$^{c}_{E}$	*4.32±0.14$^{c}_{F}$
			1/6		*2.91±0.04$^{e}_{A}$	*3.12±0.08$^{d}_{B}$	*5.74±0.08$^{d}_{C}$	7.18±0.12$^{d}_{D}$	*9.01±0.07$^{d}_{E}$	*10.72±0.06$^{d}_{F}$
		RT	1/3		*1.97±0.07$^{a}_{A}$	*2.25±0.05$^{a}_{B}$	*2.76±0.03$^{a}_{C}$	3.11±0.07$^{a}_{D}$	*3.30±0.04$^{a}_{E}$	*3.56±0.07$^{a}_{F}$
			1/4		*2.15±0.03$^{b}_{A}$	*2.59±0.04$^{b}_{B}$	*2.84±0.03$^{b}_{C}$	3.23±0.03$^{ab}_{D}$	*3.56±0.03$^{b}_{E}$	*3.96±0.05$^{b}_{F}$
			1/6		*2.42±0.04$^{d}_{A}$	*2.82±0.03$^{c}_{B}$	*3.00±0.04$^{c}_{C}$	3.41±0.03$^{c}_{D}$	*3.82±0.04$^{c}_{E}$	*4.52±0.04$^{c}_{F}$
	Frozen	AT	1/3	2.58 ±0.12	3.22±0.01$^{c}_{A}$	*3.48±0.03$^{b}_{B}$	*3.62±0.04$^{bc}_{C}$	NA	*4.36±0.16$^{c}_{D}$	*4.86±0.08$^{b}_{E}$
			1/4		*3.29±0.03$^{c}_{A}$	*3.56±0.03$^{bc}_{B}$	*3.65±0.03$^{c}_{C}$	NA	*4.82±0.14$^{d}_{D}$	*5.32±0.10$^{c}_{E}$
			1/6		*4.23±0.04$^{d}_{A}$	*4.38±0.04$^{d}_{B}$	*6.52±0.03$^{b}_{C}$	NA	*8.21±0.07$^{f}_{D}$	*11.12±0.16$^{e}_{E}$
		RT	1/3		*2.89±0.03$^{a}_{A}$	*3.11±0.07$^{a}_{B}$	*3.38±0.02$^{a}_{C}$	NA	*3.48±0.06$^{a}_{C}$	*4.46±0.07$^{a}_{D}$
			1/4		*2.92±0.04$^{a}_{A}$	*3.18±0.04$^{a}_{A}$	*3.56±0.03$^{b}_{B}$	NA	*3.82±0.13$^{b}_{C}$	*4.78±0.15$^{b}_{D}$
			1/6		*3.05±0.01$^{b}_{A}$	*3.63±0.03$^{c}_{B}$	*3.76±0.09$^{d}_{B}$	NA	*5.18±0.08$^{e}_{C}$	*6.68±0.24$^{d}_{D}$

A: Analysis, RW: Raw Material Type, ST: Storage Temperature, SFR: The ratio of salt:fish(w:w), AT: Ambient Temperature, RT: Refrigerated Temperature, NA: Not Analysed, ± SD: n: 3, The different superscript lowercase letters (a,b,c..) represent statistical differences amongst different salting subgroups under each raw material group at the same storage time ($p<0.05$). The different subscript uppercase letters (A,B,C…) represents statistical differences during storage period of the same group ($p<0.05$). '' on each data represents that there is statistical difference between the data obtained for fresh and frozen raw material groups at the same storage time and the ration of salt:fish.*

Table 6. The changes in the biogenic amine contents of *lakerda* (dry salted Atlantic bonito) processed from fresh raw material stored at ambient (17 ±3°C) and refrigerated (4 ±1°C) temperatures.

Storage Time	Storage Temp.	SFR Groups	Tryptamine (ppm)	Phenethylamine (ppm)	Putrescine (ppm)	Cadaverine (ppm)	Histamine (ppm)	Tyramine (ppm)	Spermidine (ppm)	Spermine (ppm)
		Fresh	5.62±0.05^{c}	14.67±0.29^{a}	3.89±0.03^{g}	1.51±0.17^{d}	<0.86*	22.40±0.16^{c}	162.59±0.40^{c}	1.44±0.03^{a}
1st week	**Ambient**	**1/3**	5.75±0.14$^{c}_{B}$	*30.42±0.60$^{c}_{D}$	*3.21±0.05$^{e}_{D}$	*1.11±0.06$^{b}_{C}$	*3.23±0.05$^{b}_{B}$	12.81±0.17$^{b}_{D}$	*114.46±0.66$^{b}_{C}$	*8.58±0.10$^{d}_{D}$
		1/4	*6.50±0.08$^{d}_{B}$	*22.91±0.08$^{b}_{C}$	*3.42±0.12$^{f}_{D}$	*2.74±0.05$^{f}_{C}$	*6.17±0.16$^{c}_{A}$	11.37±0.01$^{a}_{D}$	*109.45±0.68$^{b}_{D}$	4.48±0.08$^{c}_{C}$
		1/6	*6.43±0.06$^{d}_{B}$	*29.99±0.14$^{c}_{D}$	*3.00±0.12^{d}	*1.50±0.06$^{d}_{A}$	*9.34±0.22$^{d}_{A}$	12.92±0.13$^{b}_{D}$	*114.62±0.63$^{b}_{D}$	*3.49±0.07$^{b}_{B}$
	Refriger-ator	**1/3**	*4.62±0.16$^{a}_{A}$	*36.39±0.18$^{f}_{D}$	*2.09±0.03$^{b}_{D}$	*0.39±0.02$^{a}_{A}$	*0.95±0.03$^{a}_{A}$	11.12±0.20$^{a}_{B}$	*110.02±0.54$^{b}_{D}$	*4.40±0.25$^{c}_{D}$
		1/4	5.15±0.11$^{b}_{A}$	*32.13±0.52$^{d}_{D}$	*1.93±0.06$^{a}_{C}$	*1.89±0.06$^{e}_{C}$	*0.94±0.01$^{a}_{A}$	*11.40±0.17$^{a}_{C}$	103.82±0.42$^{a}_{D}$	4.51±0.05$^{c}_{D}$
		1/6	*4.73±0.06$^{a}_{B}$	*34.93±0.20$^{e}_{D}$	*2.72±0.06$^{c}_{D}$	*1.25±0.04$^{c}_{B}$	<0.86*	*11.61±0.09$^{a}_{C}$	*110.13±0.44$^{b}_{D}$	*4.75±0.14$^{c}_{D}$
4th week	**Ambient**	**1/3**	*7.41±0.07$^{b}_{D}$	*21.58±0.09$^{c}_{B}$	*2.38±0.08$^{c}_{B}$	*0.82±0.07$^{b}_{B}$	*6.44±0.13$^{d}_{C}$	*10.57±0.32$^{c}_{B}$	*100.21±1.16$^{d}_{B}$	*3.19±0.06$^{a}_{A}$
		1/4	*7.34±0.23$^{b}_{C}$	*24.03±0.02$^{d}_{D}$	*2.41±0.05$^{d}_{C}$	0.40±0.02$^{a}_{A}$	*7.35±0.23$^{e}_{B}$	*10.80±0.06$^{c}_{C}$	*97.75±0.07$^{c}_{C}$	*3.52±0.04$^{b}_{A}$
		1/6	*9.53±0.43$^{c}_{D}$	*24.36±0.32$^{d}_{C}$	*2.85±0.13^{e}	*2.54±0.11$^{d}_{C}$	*12.01±0.12$^{f}_{B}$	*8.90±0.07$^{a}_{C}$	*80.25±0.33$^{a}_{C}$	*3.50±0.06$^{b}_{B}$
	Refrigerator	**1/3**	5.53±0.12$^{a}_{B}$	*15.87±0.09$^{b}_{C}$	1.70±0.05$^{b}_{B}$	1.59±0.04$^{c}_{C}$	*1.21±0.04$^{a}_{B}$	*9.58±0.08$^{b}_{A}$	88.10±0.15$^{b}_{B}$	*3.07±0.06$^{a}_{A}$
		1/4	5.53±0.13$^{a}_{B}$	*15.71±0.07$^{b}_{B}$	*1.59±0.04$^{a}_{B}$	*0.40±0.02$^{a}_{A}$	1.50±0.05$^{b}_{B}$	*8.74±0.16$^{a}_{B}$	*79.70±0.14$^{a}_{B}$	*3.48±0.09$^{b}_{A}$
		1/6	5.41±0.10$^{a}_{C}$	*14.07±0.03$^{a}_{C}$	*2.28±0.08$^{c}_{C}$	0.36±0.01$^{a}_{A}$	*1.72±0.07$^{c}_{A}$	*12.22±0.21$^{d}_{D}$	*105.62±0.51$^{e}_{C}$	*3.58±0.07$^{b}_{B}$
8th week	**Ambient**	**1/3**	*6.75±0.11$^{c}_{C}$	*28.02±0.10$^{f}_{C}$	*2.78±0.07$^{d}_{C}$	*0.42±0.03$^{a}_{A}$	*6.98±0.07$^{d}_{D}$	*11.65±0.24$^{c}_{C}$	*102.15±0.20$^{d}_{B}$	3.66±0.13$^{a}_{B}$
		1/4	*7.15±0.11$^{d}_{C}$	*17.23±0.05$^{d}_{B}$	*1.84±0.06$^{b}_{B}$	*0.41±0.02$^{a}_{A}$	*8.20±0.05$^{e}_{D}$	*9.93±0.19$^{b}_{B}$	*86.07±0.29$^{b}_{B}$	*3.43±0.16$^{a}_{A}$
		1/6	*7.74±0.15$^{e}_{C}$	*19.75±0.48$^{e}_{B}$	*2.82±0.07^{d}	*2.31±0.06$^{c}_{B}$	*17.18±0.05$^{f}_{D}$	*6.43±0.24$^{a}_{B}$	*61.77±0.26$^{a}_{B}$	*3.53±0.03$^{a}_{B}$
	Refrigerator	**1/3**	*5.62±0.06$^{a}_{B}$	*4.97±0.05$^{a}_{A}$	*1.97±0.02$^{c}_{C}$	*0.45±0.05$^{a}_{A}$	*1.44±0.01$^{a}_{C}$	11.46±0.19$^{c}_{B}$	*95.97±0.26$^{c}_{C}$	3.38±0.07$^{a}_{B}$
		1/4	*5.90±0.09$^{b}_{C}$	*12.48±0.08$^{c}_{A}$	*1.88±0.05$^{b}_{C}$	*1.92±0.03$^{b}_{C}$	*2.25±0.10$^{b}_{C}$	*11.35±0.21$^{c}_{C}$	*94.95±0.31$^{c}_{C}$	*3.64±0.05$^{a}_{B}$
		1/6	*5.50±0.06$^{a}_{C}$	*5.93±0.06$^{b}_{A}$	*1.65±0.01$^{a}_{B}$	*0.37±0.01$^{a}_{A}$	*2.62±0.06$^{c}_{B}$	*10.84±0.05$^{c}_{B}$	*87.90±0.08$^{b}_{B}$	*3.25±0.04$^{a}_{A}$
12th week	**Ambient**	**1/3**	4.75±0.03$^{b}_{A}$	*0.85±0.04$^{b}_{A}$	0.53±0.02$^{a}_{A}$	0.91±0.02$^{b}_{B}$	3.03±0.02$^{c}_{A}$	5.18±0.08$^{c}_{A}$	*66.56±0.23$^{e}_{A}$	*4.91±0.02$^{d}_{C}$
		1/4	*4.82±0.08$^{b}_{A}$	*0.72±0.01$^{a}_{A}$	*0.52±0.01$^{a}_{A}$	*0.73±0.02$^{a}_{B}$	*7.88±0.03$^{e}_{C}$	*4.18±0.06$^{b}_{A}$	*56.38±0.25$^{b}_{A}$	*4.28±0.02$^{c}_{B}$
		1/6	*4.75±0.06$^{b}_{A}$	*2.90±0.09$^{c}_{A}$	*1.22±0.01^{c}	4.65±0.05$^{e}_{D}$	*16.95±0.02$^{f}_{C}$	*2.90±0.09$^{a}_{A}$	*14.42±0.13$^{a}_{A}$	*2.23±0.02$^{a}_{A}$
	Refrigerator	**1/3**	4.56±0.02$^{a}_{A}$	*13.16±0.02$^{e}_{B}$	0.72±0.01$^{b}_{A}$	*1.08±0.01$^{c}_{B}$	1.67±0.03$^{a}_{C}$	*11.68±0.10$^{d}_{B}$	*59.93±0.20$^{c}_{A}$	*3.93±0.03$^{b}_{C}$
		1/4	4.63±0.05$^{a}_{A}$	*16.16±0.08$^{f}_{C}$	*0.59±0.01$^{a}_{A}$	*0.71±0.01$^{a}_{B}$	2.88±0.08$^{b}_{D}$	*5.25±0.04$^{c}_{A}$	*63.75±0.08$^{d}_{A}$	*4.28±0.01$^{c}_{C}$
		1/6	*4.58±0.02$^{a}_{A}$	*10.15±0.09$^{d}_{B}$	*0.48±0.01$^{a}_{A}$	*1.26±0.01$^{d}_{B}$	*4.38±0.09$^{d}_{C}$	*5.18±0.06$^{c}_{A}$	*71.30±0.08$^{f}_{A}$	*4.20±0.05$^{c}_{C}$

*±SD, n=3, *: SFR: The ratio of salt:fish (w:w), The levels were under detection limit. The different lowercase letters (a,b,c..) represent statistical differences amongst groups at the same storage time (p<0.05). The different uppercase letters (A,B,C…..) represents statistical differences during storage period of the same group (p<0.05). '*' on each data represents that there is statistical difference between the data obtained for frozen raw material group relating to the same storage time and the same salt concentration ratio.*

Table 7. The changes in the biogenic amine contents of *lakerda* (dry salted Atlantic bonito) processed from frozen raw material stored at ambient (17 ±3°C) and refrigerated (4±1°C) temperatures.

Storage Time	Storage Temp	SFR Groups	Tryptamine (ppm)	Phenethylamine (ppm)	Putrescine (ppm)	Cadaverine (ppm)	Histamine (ppm)	Tyramine (ppm)	Spermidine (ppm)	Spermine (ppm)
		Fresh	4.52 ± 0.04^{a}	30.77 ± 0.35^{f}	2.07 ± 0.06^{c}	1.76 ± 0.02^{b}	<0.86*	5.92 ± 0.06^{a}	96.67 ± 0.21^{c}	4.69 ± 0.09^{b}
1st week	**Ambient**	**1/3**	$5.98\pm0.14^{e}{}_{B}$	$*13.54\pm0.15^{c}{}_{C}$	$*2.11\pm0.10^{c}{}_{D}$	$*0.40\pm0.02^{a}{}_{A}$	$*1.30\pm0.09^{b}{}_{A}$	$13.75\pm0.13^{d}{}_{D}$	$*98.59\pm0.44^{c}{}_{D}$	$*5.45\pm0.23^{d}{}_{D}$
		1/4	$*5.37\pm0.08^{c}{}_{C}$	$*13.94\pm0.05^{c}{}_{C}$	$*1.98\pm0.07^{c}{}_{C}$	$*0.76\pm0.04^{b}{}_{B}$	$*1.52\pm0.02^{c}{}_{A}$	$11.86\pm0.19^{b}{}_{C}$	$*79.68\pm0.11^{a}{}_{C}$	$4.45\pm0.20^{a}{}_{B}$
		1/6	$*5.19\pm0.06^{b}{}_{B}$	$*9.79\pm0.09^{b}{}_{B}$	$*2.50\pm0.09^{d}{}_{D}$	$*2.26\pm0.06^{c}{}_{C}$	$*7.09\pm0.04^{d}{}_{A}$	$12.93\pm0.12^{c}{}_{D}$	$*99.40\pm0.28^{c}{}_{D}$	$*4.42\pm0.15^{a}{}_{A}$
	Refrigerator	**1/3**	$*5.52\pm0.04^{d}{}_{C}$	$*2.28\pm0.04^{a}{}_{A}$	$*1.59\pm0.04^{a}{}_{B}$	$*1.53\pm0.06^{b}{}_{A}$	<0.86*	$11.70\pm0.19^{b}{}_{D}$	$*91.93\pm0.13^{b}{}_{C}$	$*4.69\pm0.09^{b}{}_{D}$
		1/4	$5.30\pm0.17^{c}{}_{C}$	$*25.93\pm0.06^{e}{}_{D}$	$1.83\pm0.06^{b}{}_{C}$	$*2.69\pm0.09^{d}{}_{C}$	$*1.17\pm0.08^{b}{}_{A}$	$*13.50\pm0.40^{d}{}_{D}$	$104.76\pm0.13^{d}{}_{D}$	$4.51\pm0.05^{a}{}_{B}$
		1/6	$*5.28\pm0.08^{bc}{}_{C}$	$*17.73\pm0.17^{d}{}_{D}$	$*1.75\pm0.03^{b}{}_{C}$	$*1.47\pm0.02^{b}{}_{C}$	$*1.07\pm0.06^{a}{}_{A}$	$*13.30\pm0.26^{cd}{}_{D}$	$*97.24\pm0.20^{c}{}_{D}$	$*4.92\pm0.06^{c}{}_{C}$
4th week	**Ambient**	**1/3**	$*5.64\pm0.15^{a}{}_{B}$	$*11.49\pm0.38^{b}{}_{B}$	$*1.79\pm0.06^{b}{}_{C}$	$*0.39\pm0.02^{a}{}_{A}$	$*1.72\pm0.07^{b}{}_{B}$	$*12.19\pm0.23^{c}{}_{C}$	$*84.79\pm0.34^{a}{}_{C}$	$*4.50\pm0.15^{d}{}_{C}$
		1/4	$*5.49\pm0.18^{a}{}_{C}$	$*6.41\pm0.18^{a}{}_{A}$	$*2.13\pm0.12^{e}{}_{C}$	$0.39\pm0.01^{a}{}_{A}$	$*2.01\pm0.04^{c}{}_{B}$	$*13.32\pm0.24^{d}{}_{D}$	$*89.79\pm0.20^{b}{}_{D}$	$*4.00\pm0.05^{a}{}_{A}$
		1/6	$*5.46\pm0.34^{a}{}_{B}$	$*16.77\pm0.28^{d}{}_{D}$	$*1.92\pm0.04^{d}{}_{C}$	$*0.64\pm0.04^{b}{}_{A}$	$*19.95\pm0.61^{e}{}_{B}$	$*11.75\pm0.13^{c}{}_{C}$	$*89.23\pm0.33^{b}{}_{C}$	$*4.34\pm0.22^{c}{}_{A}$
	Refrigerator	**1/3**	$5.77\pm0.12^{a}{}_{D}$	$*17.41\pm0.25^{e}{}_{D}$	$1.63\pm0.06^{a}{}_{B}$	$1.57\pm0.05^{c}{}_{A}$	$*1.08\pm0.04^{a}{}_{A}$	$*5.72\pm0.15^{b}{}_{A}$	$87.61\pm0.36^{b}{}_{B}$	$*4.23\pm0.05^{b}{}_{C}$
		1/4	$5.61\pm0.07^{a}{}_{D}$	$*17.60\pm0.29^{e}{}_{C}$	$*1.81\pm0.04^{c}{}_{C}$	$*1.81\pm0.02^{d}{}_{B}$	$1.70\pm0.08^{b}{}_{B}$	$*5.59\pm0.10^{b}{}_{B}$	$*97.93\pm0.06^{d}{}_{C}$	$*4.35\pm0.04^{c}{}_{B}$
		1/6	$5.62\pm0.06^{a}{}_{D}$	$*13.84\pm0.05^{c}{}_{C}$	$*1.88\pm0.08^{cd}{}_{C}$	$0.36\pm0.01^{a}{}_{A}$	$*2.50\pm0.05^{d}{}_{B}$	$*5.34\pm0.05^{a}{}_{C}$	$*93.62\pm0.18^{c}{}_{C}$	$*4.07\pm0.05^{a}{}_{B}$
8th week	**Ambient**	**1/3**	$*4.55\pm0.13^{a}{}_{A}$	$*14.56\pm0.03^{d}{}_{D}$	$*0.36\pm0.01^{a}{}_{A}$	$*0.66\pm0.01^{a}{}_{B}$	$*2.41\pm0.06^{b}{}_{C}$	$*3.93\pm0.02^{a}{}_{A}$	$*39.21\pm0.43^{a}{}_{B}$	$3.60\pm0.05^{b}{}_{A}$
		1/4	$*4.60\pm0.02^{a}{}_{A}$	$*16.47\pm0.19^{e}{}_{D}$	$*0.42\pm0.01^{b}{}_{A}$	$*1.07\pm0.06^{d}{}_{C}$	$*3.05\pm0.08^{e}{}_{C}$	$*5.44\pm0.05^{c}{}_{A}$	$*61.21\pm0.07^{d}{}_{B}$	$*4.49\pm0.10^{e}{}_{B}$
		1/6	$*4.50\pm0.05^{a}{}_{A}$	$*14.08\pm0.04^{d}{}_{C}$	$*0.55\pm0.01^{c}{}_{A}$	$*0.83\pm0.03^{c}{}_{B}$	$*24.36\pm0.06^{f}{}_{C}$	$*4.83\pm0.02^{b}{}_{B}$	$*50.63\pm0.35^{b}{}_{B}$	$*4.32\pm0.06^{e}{}_{A}$
	Refrigerator	**1/3**	$*4.92\pm0.03^{c}{}_{B}$	$*2.86\pm0.03^{b}{}_{B}$	$*0.63\pm0.01^{d}{}_{A}$	$*2.27\pm0.01^{e}{}_{B}$	$1.21\pm0.06^{a}{}_{B}$	$11.52\pm0.16^{d}{}_{C}$	$*56.55\pm0.43^{c}{}_{A}$	$3.44\pm0.02^{a}{}_{A}$
		1/4	$*4.94\pm0.04^{c}{}_{B}$	$*4.82\pm0.03^{c}{}_{A}$	$*0.35\pm0.04^{a}{}_{A}$	$*0.64\pm0.01^{a}{}_{A}$	$*2.72\pm0.02^{c}{}_{C}$	$*4.60\pm0.02^{b}{}_{A}$	$*63.32\pm0.45^{d}{}_{B}$	$*4.11\pm0.06^{d}{}_{A}$
		1/6	$*4.79\pm0.02^{b}{}_{A}$	$*1.24\pm0.02^{a}{}_{A}$	$*0.39\pm0.01^{a}{}_{A}$	$*0.75\pm0.01^{b}{}_{B}$	$*2.98\pm0.21^{d}{}_{C}$	$*4.75\pm0.07^{b}{}_{B}$	$*61.73\pm0.39^{d}{}_{B}$	$*3.84\pm0.05^{c}{}_{A}$
12th week	**Ambient**	**1/3**	$4.78\pm0.10^{b}{}_{A}$	$*7.12\pm0.09^{d}{}_{A}$	$0.56\pm0.06^{a}{}_{B}$	$0.98\pm0.01^{a}{}_{C}$	$3.12\pm0.04^{b}{}_{D}$	$5.46\pm0.08^{c}{}_{B}$	$*36.50\pm0.40^{a}{}_{A}$	$*3.90\pm0.06^{b}{}_{B}$
		1/4	$*5.12\pm0.06^{d}{}_{B}$	$*7.68\pm0.10^{e}{}_{B}$	$*0.68\pm0.08^{b}{}_{B}$	$*2.16\pm0.06^{b}{}_{D}$	$*5.15\pm0.08^{d}{}_{D}$	$*5.98\pm0.05^{d}{}_{B}$	$*56.18\pm0.24^{e}{}_{A}$	$*4.86\pm0.04^{e}{}_{C}$
		1/6	$*5.68\pm0.08^{e}{}_{B}$	$*6.08\pm0.04^{b}{}_{A}$	$*1.65\pm0.10^{d}{}_{B}$	$4.13\pm0.03^{d}{}_{D}$	$*29.16\pm0.16^{e}{}_{D}$	$*3.32\pm0.06^{a}{}_{A}$	$*38.54\pm0.15^{b}{}_{A}$	$*4.78\pm0.04^{d}{}_{B}$
	Refrigerator	**1/3**	$4.45\pm0.03^{a}{}_{A}$	$*5.86\pm0.02^{a}{}_{C}$	$0.68\pm0.02^{b}{}_{A}$	$*3.78\pm0.04^{c}{}_{C}$	$1.48\pm0.03^{a}{}_{C}$	$*10.12\pm0.12^{f}{}_{B}$	$*58.50\pm0.32^{f}{}_{A}$	$*3.66\pm0.08^{a}{}_{B}$
		1/4	$4.65\pm0.10^{b}{}_{A}$	$*6.56\pm0.08^{c}{}_{B}$	$*0.76\pm0.04^{b}{}_{B}$	$*5.12\pm0.08^{e}{}_{D}$	$2.95\pm0.06^{b}{}_{D}$	$*6.40\pm0.08^{e}{}_{C}$	$*53.12\pm0.12^{d}{}_{A}$	$*4.42\pm0.06^{c}{}_{B}$
		1/6	$*4.98\pm0.06^{c}{}_{B}$	$*9.19\pm0.04^{f}{}_{B}$	$*0.88\pm0.08^{c}{}_{B}$	$*5.88\pm0.03^{f}{}_{D}$	$*3.78\pm0.10^{c}{}_{D}$	$*4.15\pm0.06^{b}{}_{A}$	$*42.48\pm0.30^{c}{}_{A}$	$*4.96\pm0.15^{e}{}_{C}$

*±SD, n=3, *: SFR: The ratio of salt:fish (w:w), The levels were under detection limit. The different lowercase letters (a,b,c..) represent statistical differences amongst groups at the same storage time (p<0.05). The different uppercase letters (A,B,C.....) represents statistical differences during storage period of the same group (p<0.05). '*' on each data represents that there is statistical difference between the data obtained for fresh raw material group relating to the same storage time and the same salt concentration ratio.*

The results of previous studies with commercially processed products showed that high biogenic amine levels can also be obtained with the products containing high salt concentration and kept at cold storage (Köse *et al*., 2012; Koral *et al*., 2013). So, the levels of biogenic amine content can also depend on handling and storage of raw materials prior to salting or other processing ways. It was also demonstrated that fresh Atlantic bonito kept at different chilled conditions (Koral and Köse, 2012) had reached to unacceptable histamine values at certain time of storage period. Therefore, handling and storing raw material at suitable time and temperature conditions are important to avoid health risk associated with histamine.

Conclusion

This study showed that previously frozen raw material had higher salt uptake in comparison with freshly salted Atlantic bonito with the increasing level in parallel to increasing time and salt concentration. Higher WPS% levels for both raw material groups were found at ambient temperature in comparison to refrigerated conditions.WPS level reached to suggested seafood safety level (20%) within the same week of salting for all experimental group with the exception of 1/6 group at refrigerated temperature. The $a_w$values significantly dropped within the 1stweek after salting reaching to seafood safety levels (below 0.83) for all groups at the end of storage. The results showed that although there were significant differences amongst samples treated with different salt ratios and different storage applications, such differences did not affected products' chemical acceptability for salt:fish ratios of 1/3 and 1/4. However, higher the salt used, higher TBA content was observed at both temperatures of both groups. This might have caused due to salt accelerating the lipid oxidation.

Our results demonstrated that dry salting prevents the formation of biogenic amines, particularly histamine in salted Atlantic bonito. In general, lower biogenic amine values were observed with products originated from previously frozen Atlantic bonito compared to freshly processed fish. Histamine values were also found very low and none of the products exceeded the permitted levels.

The results showed that the products with the lowest salt contenthad the lowest sensory acceptance in both raw material groups. Although some significant differences occurred between salt ratios for 1/3 and 1/4 in terms of product quality at both temperatures, such differences did not make a great effect on the sensory quality since each group was within acceptable quality throughout the storage period. Therefore, both salt:fish ratio groups were found suitable for *lakerda* production from Atlantic bonito. The salt:fish ratio of 1/6 was only found suitable for previously frozen raw materials which were also kept in refrigerated conditions after salting. Therefore, this study demonstrates that using previously frozen raw material may extend the shelf life of dry salted fish products.

Finally, the overall results of this study indicate that dry salting using previously frozen Atlantic bonito have an advantage in relation to both food quality and food safety, particularly if low salt contents intended to be used. Moreover, freezing and frozen storage will help to kill parasites in this type of products contributing to additional benefit in terms of seafood safety.

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İMMÜNOTERAPİ VE PROPOLİSİN KANSER İMMÜNOTERAPİSİNDE KULLANIM POTANSİYELİ

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ÖZ

Son yıllarda kanser hastalığı oldukça yaygın olarak görülmektedir. Halk sağlığı için büyük tehlike oluşturan kanser hastalığının tedavi yöntemleri üzerinde yenilikçi araştırmalar yapılmaktadır. İmmünoterapi immün yanıtları artıran veya azaltan terapötik ajanlar ile ortaya çıkan hastalığın tedavisidir. İmmünoterapinin kanser tedavisinde etkili olduğu bilinmektedir. Bu amaç doğrultusunda kullanılabilirliği araştırılan, bir arı ürünü olan propolis antitümör etkiler göstermesi ile dikkat çekmektedir. Bunun yanı sıra antibakteriyel, antimikrobiyal, antifungal, antiviral ve sitotoksik etkileri olduğu bilinmekte olan propolisin son yıllarda immünomodülatör etkileri incelenmektedir. Araştırmacılara göre propolis kanser immünoterapisinde umut vadeden bir bileşendir. Bu derlemede immünoterapi ve propolisin kanser immünoterapisinde kullanımı hakkında bilgi verilmesi amaçlanmaktadır.

Anahtar Kelimeler: İmmünoterapi, Propolis, Kanser, İmmünomodulatör

ABSTRACT

IMMUNOTHERAPY AND POTENTIAL USE OF PROPOLIS IN CANCER IMMUNOTHERAPY

In recent years cancer incidence has increased. Innovative studies have been carried out on the treatment methods of cancer, a major threat to public health. Immunotherapy is the treatment of the disease with therapeutic agents that increase or decrease immune responses. Immunotherapy is known to be effective in the treatment of cancer. A bee product, propolis that has been investigated for its availability in this respect draws attention because of its antitumor effects. In addition, immunomodulatory effects of propolis, which is known to have antibacterial, antimicrobial, antifungal, antiviral and cytotoxic effects, have been investigated in recent years. According to researchers, propolis is a promising compound in cancer immunotherapy. In this review, it is aimed to give information about immunotherapy and the use of propolis in cancer immunotherapy.

Keywords: Immunotherapy, Propolis, Cancer, Immunomodulator

Giriş

İmmünite, vücuda giren veya verilen yabancı bileşenlere (mikroorganizma, toksin, toksoid, protein, polisakkarit, vb.) karşı vücudun bütün savunma mekanizmaları ile karşı koyması, direnç göstermesi, kendini koruması ve zararlı maddeyi elimine etmesi olarak tanımlanabilmektedir (Herbert ve Cohen, 1993; Arda vd., 1998; Schenk, 2002; Göç, 2015).

İmmün sistem ise bu direncin ortaya çıkmasını sağlayan hücreler, dokular ve moleküllerin tümünü kapsamaktadır. İmmün sistemin en önemli fizyolojik işlevleri; enfeksiyonları engellemek, yerleşen enfeksiyonları yok etmek, konağı ölü hücrelerinden arındırmak ve doku onarımını başlatmaktır. Son yıllarda araştırmacılar kişiye özel kanser tedavisi yaklaşımında immün sistemi mercek altına almıştır. Böylelikle immün sistem araştırmaları sayesinde araştırmacılar bazı tümörlerin büyümesini önlemek ve tümör hücrelerine karşı immün yanıtı uyararak kanseri tedavi etmek üzere yöntemler geliştirmektedirler. Bu yöntemler temelde konağın immün yanıtını tümör hücrelerini yok edecek şekilde etkileyen ajanlar ile geliştirilmektedir. Tüm yararlı fonksiyonlarının aksine normalin dışında gelişen immün yanıt ciddi morbidite ve mortalite ile sonuçlanabilen birçok enflamatuvar hastalığın nedeni olabilmektedir (Arda vd., 1998; Göç, 2015; Jantan vd., 2015).

Gelişmiş ülkelerde, ölüm nedenlerine göre bir sıralama yapıldığında kanser ikinci sırada yer almaktadır. Özellikle gelişmiş ülkelerde, popülasyon yaşlarının artışı, fiziksel aktivite oranının azalması, sigara gibi zararlı alışkanlar ve beslenme tarzlarının da değişmesi gibi birçok nedenle yüksek olan kanser oranı giderek daha da artmaktadır (Jemal vd., 2011). Türkiye Halk Sağlığı Kurumu Kanser Daire Başkanlığının 2014 yılında yayınladığı Dünya Kanser Raporu'nda, toplanan verilerden yola çıkarak, 20 yıl sonrasında tespit edilecek kanser vakaların sayısının 22 milyon olacağı ve bu süreçte kanser kaynaklı ölümlerin sayısının ise 13 milyona ulaşacağı öngörülmektedir. Kemoterapi ve radyo terapinin, kanser tedavisinde oldukça etkili silahlar olduğu kanıtlanmıştır. Fakat günümüzde bu yöntemlerin kullanımı akut toksisiteler ve tümörlerin ilaç dirençli fenotipler oluşturma kabiliyeti gibi nedenlerle sınırlanmaktadır. Bunlara ek olarak, kanserle mücadelede en büyük sorunlardan biri olan geç teşhis konulması durumunda da tedavide başarı oranları oldukça düşüktür (Wang vd., 2014). Bu nedenle de her geçen gün, tedavi başarı oranlarını artıracak, daha yeni tedavi yöntemlerinin önemi ortaya çıkmaktadır (DeSantes vd., 2009). İmmünoterapinin, kemoterapi ve radyoterapi gibi tedavi yöntemlerinin aksine, doğrudan tümör hücrelerini hedef alıyor olması, diğer sağlıklı vücut hücrelerine zarar vermemesi bu alanda oldukça güçlü bir alternatif oluşturmasını sağlamıştır (Barbaros ve Dikmen, 2015).

İmmünoterapi immün yanıtları artıran veya azaltan terapötik ajanlarla ortaya çıkan hastalığın tedavisidir. Çoğu geleneksel tedavi yöntemlerinden daha başarılı sonuçlar verdiği, özellikle de uygulandığı her bireyde başarılı sonuçlar elde edilemeyen tedavi yöntemleri için, umut vadeden alternatif bir yöntem oluşturmuştur (Yamaguchi, 2016). Kanser immünoterapisine yönelik çalışılmakta olan pek çok yeni strateji, konağın kendi immün yanıtının tümör hücrelerine karşı güçlendirilmesi esasına dayanmaktadır. İmmünoterapide genellikle organizmada immün sistemin fonksiyonlarını artırıcı veya azaltıcı etki gösteren immünomodülatör bileşenler kullanılmaktadır. Böylece yetersiz immünitenin görüldüğü hastalıklarda veya otoimmün hastalıklarda tedavi amacıyla immünite kontrol altına alınarak tedavi edilebilmektedir. Örneğin aşılara ilave edilen adjuvan maddeler immüniteyi artıran etkiye sahipken, doku transplantasyonları sırasında uygulanan bazı ilaçlar immün sistemin tüm antijenlerine karşı bir süre tepki göstermemesini ve böylece immüniteyi azaltıcı etkiler sağlamaktadır (Arda vd., 1998; Schenk, 2002; Jantan vd., 2015). Benzer şekilde kanser immünoterapisi; tümör antijenlerine karşı aktif immün yanıtların artırılması veya antitümör antikorların veya T hücrelerinin pasif immünite oluşturmak amacıyla uygulanmasını içermektedir. İmmün yanıtların son derece spesifik olması nedeniyle, tümöre özel bağışıklığın, hastaya zarar vermeden seçici olarak tümörü yok etmede kullanılabileceği uzun zamandır ümit edilmektedir. İmmünoterapi hala tümör immünologlarının ana hedefidir ve tedaviye yönelik pek çok yaklaşım, deney hayvanlarında ve insanlar üzerinde denenmektedir (Schenk, 2002; Göç, 2015). Yüksek miktarlarda biyolojik ajanın üretimini sağlamayı mümkün kılan rekombinant DNA teknolojisi gibi biyoteknoloji üzerine yapılan keşifler ise, immünoterapinin tam anlamıyla bir kanser tedavisi olarak kullanımını daha ileri boyuta taşımıştır (Muehlbauer vd., 2006). Geçtiğimiz yıllarda, birçok biyolojik ajan Amerikan Gıda ve İlaç Dairesi (FDA) tarafından onaylanmıştır. Günümüzde çeşitli bileşenler ve/veya mikroorganizmalar kullanılarak kanser tedavisinde immünolojik yaklaşımlar üzerinde dikkat çekici çalışmalar yapılmaktadır.

İmmünoterapötik olarak kullanılan ve henüz immünomodülatör etkileri araştırılmakta olan bileşenler; immüniteyi azaltıcı ve immüniteyi artırıcı olarak ikiye ayrılmaktadır. Günümüzde tüketicilerin daha çok doğal gıda ve gıda takviyelerine yönelmesi bilimsel araştırmalara farklı yönlerde ivme kazandırmış ve yöresel, doğal ürünlerin immünoterapide kullanım potansiyelleri araştırılmaya başlanmıştır. Bu bağlamda, antik çağlardan itibaren farklı amaçlarla kullanılan bir arı ürünü olan propolis birçok araştırmanın odak noktası olmuştur. Propolis arılar tarafından bitki tomurcuklarından toplanan ve arıların enzimleri ile değişime uğrayan reçineli

bir materyaldir. Rengi yeşilden kırmızıya hatta koyu kahverengiye kadar değişim gösterebilmektedir. Propolisin kendine özgü bir kokusu vardır ve yapışkan bir dokuya sahiptir. İklim ve bölgelere göre büyük farklılıklar gösterebilen propolisin yapısı genellikle %30 mum, %50 reçine ve bitki balsamı, %10 esansiyel ve aromatik yağlar, %5 polen ve diğer bileşenlerden oluşmaktadır (Burdock, 1998). Arıların propolisi peteklerindeki delikleri mühürlemek ve işgalci böceklerin ölülerinin kovan içinde çürümesini engellemek için kullandıkları bilinmektedir. Ayrıca propolis antiseptik ve antimikrobiyal etkileri sayesinde koloniyi çeşitli hastalıklardan korumaktadır (Burdock, 1998; Salatino vd., 2005).

Mısırlıların, Yunanların, Romalıların propolisi cilt yaralarını tedavi etmek amacıyla kullandıkları bilinmektedir. Propolis antienflamatuvar özelliği sayesinde yara ve ülser tedavi edici etkisi ile dikkat çekmiştir (Ghisalberti, 1979). Yüzyıllardır çeşitli tedavi edici etkileri olduğu bilinen bir madde olmasına rağmen, bilim insanları günümüzde hala propolisin yeni özellikleri ve aktif bileşenlerinin çeşitli etkilerinin mekanizmaları üzerinde araştırmalar yapmaktadır (Sforcin, 2007). Yapılan araştırmalarda propolisin çeşitli tümör hücreleri üzerinde sitotoksik etkiler gösterdiği tespit edilmiştir (Grunberger vd., 1988; Awale vd., 2008; Carvalho vd., 2011; Popovic vd., 2012 Chen vd., 2014). Sitotoksik etkilerinin yanı sıra farklı tümör hücrelerini apoptoza (programlı hücre ölümü) sürüklediği (Choudhari vd., 2013; Novak vd., 2014) ve tümör hücresi enjekte edilen farelere propolis verilmesi sonucu tümör büyüme hızlarının ve tümör hacimlerinin azaldığı bildirilmiştir (Orsolic vd., 2005; Inoue vd., 2008).

Bu çalışmada immünoterapi hakkında genel bilgiler verilmiş ve propolisin kanser immünoterapisindeki kullanım potansiyeli ile ilgili yapılan çalışmalar derlenmiştir.

İmmünite, İmmün Sistem ve Etki Mekanizmaları

İmmünite doğal ve adaptif olarak iki ana başlıkta incelenmektedir. Yapısal ve genetik özelliklerine göre canlılarda doğal olarak çeşitli savunma mekanizmaları bulunmaktadır. Doğal immünitede deri ve mukozaların anatomik yapısı, mukozaların mukus salgısı, solunum sistemindeki silli epitel hücrelerinin dışarıya yönelik hareketi, bağırsağın peristaltik hareketi gibi birçok savunma mekanizması mevcuttur. Bu mekanik savunma mekanizmalarının yanı sıra doğal immünitenin hücresel boyutu da mevcuttur. Doğal immünitenin immün hücreleri nötrofiller, dendritik hücreler, makrofajlar ve doğal öldürücü hücrelerdir (NK). Mikroorganizma ve ürünlerinin konakla direkt ilişki kurmasıyla veya yapay yollarla patojenlere ait antijenlerin konağa verilmesi ile kazanılan immünite ise adaptif immünitedir. Adaptif immünitenin immün hücreleri ise T ve B lenfositlerdir (Arda vd., 1998; Özbal, 2000; Göç, 2015). T lenfositler, hücresel tip immün cevapta etkili olan timüs kontrolünde farklılaşmaktadır. B lenfositlerin olgunlaşmasını sağlayan lenfoid organlar ise memelilerde doğumdan önce dalak ve karaciğer, doğumdan sonra ise mide-bağırsak sistemi mukoza altı lenfoid dokular (tonsiller, appendiks, peyer plakları) ile kemik iliğidir (Arda vd., 1998; Özbal, 2000). İmmün sistem ise lenfoid organların ve immün cevabın oluşturulmasında görev alan hücrelerin tümünü ifade etmektedir. İmmün sistem yanıtı mekanizmasına göre hümoral (sıvısal) ve hücresel olarak ikiye ayrılmaktadır. Antijeni spesifik olarak tanıyan ve ortadan kaldırılmasını sağlayan, B lenfositlerin plazma hücrelerine farklılaşarak salgıladıkları antikorlar sayesinde ortaya çıkan immün yanıt hümoral immünite; antijeni spesifik olarak tanıyan T lenfositler sayesinde oluşan immün yanıt ise hücresel immünitedir (Muehlbauer vd., 2006; Mayer ve Nyland, 2016).

Doğal immünitenin hücresel komponentlerinden olan nötrofiller akut inflamatuvar etkilerin çoğunda inflamasyon bölgesine ilk ulaşan ve vücuda giren patojen mikroorganizmalarla ilk karşılaşan hücrelerdir. Yüzeyindeki çeşitli reseptörlerle yabancı mikroorganizmalara bağlanıp fagosite ederler. Fagositoz mekanizmasında büyük molekül yapılı maddeler, ölü hücreler ve yabancı mikroorganizmalar bir miktar hücre zarı ile birlikte hücre içine alınmaktadır. Doğal immünitenin diğer bir hücresel bileşeni olan dendritik hücreler ise immün cevabın düzenlenmesinde önemli rol oynayan ve beyin, testis ve göz haricinde tüm dokularda bulunan antijen eksprese eden hücrelerdir. İmmatür karakterdeyken antijeni yakalama ve işleme tabi tutma özelliği olan dendritik hücrelerin çevresel uyaranlar sayesinde olgunlaşma süreci ilerledikçe, T hücre uyarımı yapabilmektedir. Dendritik hücreler bu görevlerine ek olarak B hücrelerinin fonksiyonlarının oluşumunda etkili oldukları için hümoral immünitenin gelişiminde önemli rol oynamaktadırlar. Bir diğer doğal immünite hücresel komponenti ise makrofajlardır. Makrofajlar bağ dokuda, karaciğerde, akciğerde, sinir sisteminde, seröz boşluklarda, lenfoid organlarda, kemik ve eklemlerde bulunmaktadır. Makrofajlar 1 μm'den büyük molekülleri fagositoz yoluyla hücre içine alarak sindirebilmektedir ve bu özellikleri sayesinde makrofajlar çöpçü hücreler olarak da adlandırılmıştır. NK hücreleri ve K hücreleri de doğal immünetinin bir parçası olarak görev yapmaktadırlar ve uyarıldıklarında hücrelerin birbirleriyle iletişimini sağlayan protein ve peptidlerin bir grubu olan sitokinleri salgılayarak veya yabancı mikroorganizmalara karşı sitotoksik etki göstererek hızlı bir immün yanıt oluşturmaktadırlar. NK hücreleri enfeksiyonlara karşı doğal immün yanıtı oluşturmaya ek olarak, dentritik hücrelerle birlikte patojenlere karşı oluşan

adaptif immün yanıtı oluşturabilmektedirler (Özbal, 2000; Muehlbauer vd., 2006; Göç, 2015).

Doğal immünitenin hücresel komponentleri olduğu gibi adaptif immünitede de B ve T lenfositleri mevcuttur. B lenfositleri yabancı antijenleri tanıyarak çeşitli antikorları salgılayıp patojen mikroorganizmaları etkisiz hale getirebilmekte veya fagositik hücreleri aktive edebilmektedir. Kandaki toplam B lenfosit sayısı $3x10^8$/L'dir. B lenfositlerinin diğer lenfositlerden en önemli farkı membranında immünoglobulin reseptörleri taşımalarıdır ve bu reseptörlerin çok sayıda olması nedeniyle yüzey görünümleri T lenfositlerin aksine pütürlüdür (Arda vd., 1998; Özbal, 2000). Diğer bir lenfosit grubu olan T lenfositler ise fonksiyonlarına göre indükleyici, aktivatör, baskılayıcı ve sitotoksik olarak dörde ayrılmaktadır. Kan dolaşımındaki lenfositlerin %70-80'i T lenfositlerdir. T lenfositlerinin yüzeylerindeki reseptörler sayesinde gruplandırılmaları ve adlandırılmaları mümkün olmaktadır. Bu adlandırma çeşitli CD antikorları taşımaları ile yapılmaktadır. CD_4 T hücreleri (yardımcı) ve CD_8 T hücreleri (sitotoksik) T lenfositlerin ana alt gruplarını oluşturmaktadırlar (Şengül, 2008). CD_4 reseptörlü yardımcı T hücrelerinin kandaki sayısının $0.5\text{-}1.6x10^9$/L, CD_8 reseptörlü sitotoksik T hücrelerinin sayısının $0.3\text{-}0.9x10^9$/L olduğu bilinmektedir (Arda vd., 1998; Özbal, 2000).

Enflamasyon ve immünolojik olaylar sırasında bazı immün sistem hücreleri tarafından sitokin adı verilen hormon benzeri polipeptid moleküller sentezlenmektedir. Sitokinler immün sistem hücrelerinin aktivitelerine yön veren ve hücreler arasında iletişimi sağlayan küçük protein yapıdaki birimlerdir. İnterferon gama (IFN- γ), interlökin 2 (IL-2), tümör nekroz faktörü alfa (TNF- α) ve tümör gelişme faktörü beta (TGF- β) gibi sitokinler konağın antijenlere karşı reaksiyonlarını, lökosit ve bazı hücrelerin gelişmesini, hareketini, farklılaşmasını sağlayan immünomodülatör moleküllerdir. Tablo 1'de bazı sitokinlerin kaynağı ve aktivitesi gösterilmiştir.

Tablo 1. Bazı sitokinleri üreten hücreler ve bu sitokinlerin etkileri

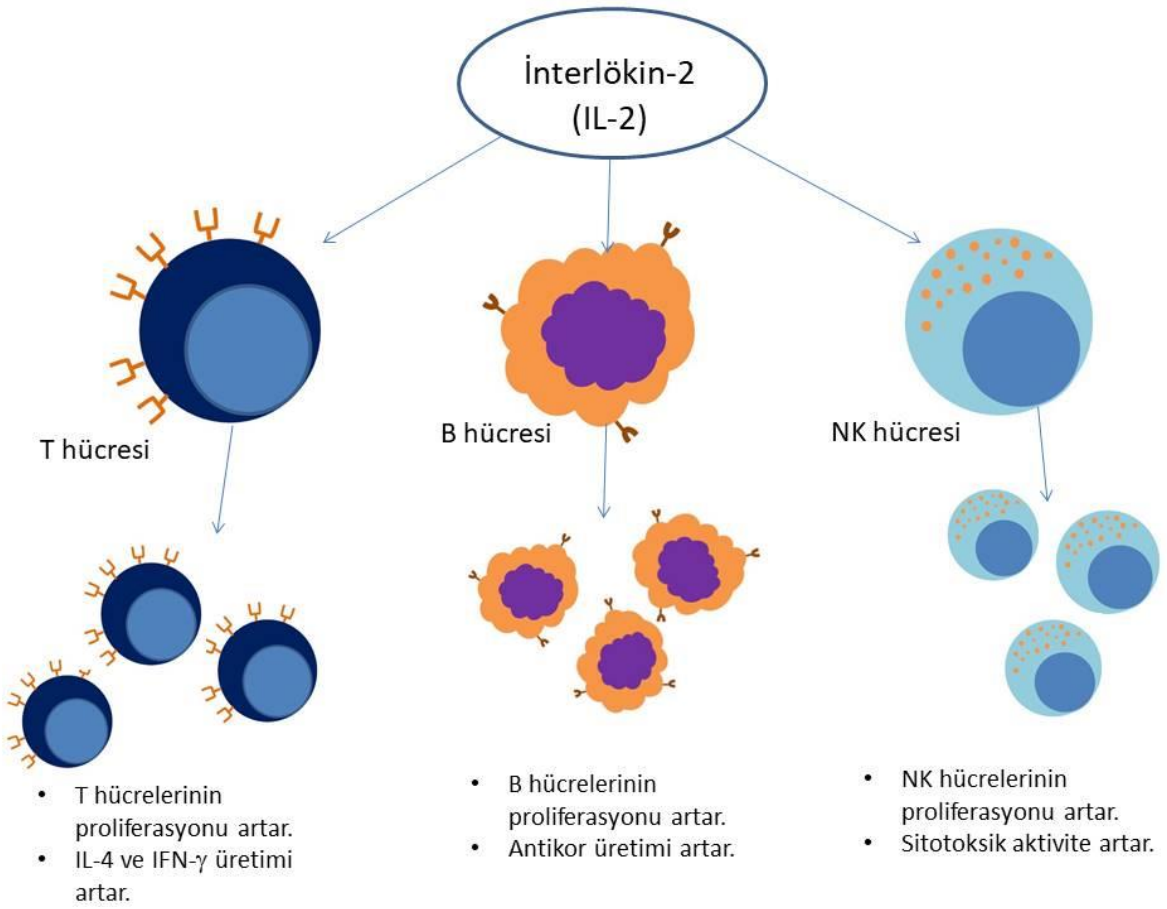
Table 1. The cells producing some cytokines and their effects

Sitokin	Kaynak hücre/hücreler	Etki	Kaynak
IL-1 α-β	Makrofaj, T ve B lenfositleri	İmmünitede artış, T lenfositlerinde farklılaşma	Özbal, 2000
IL-2	T lenfositleri	T, B ve NK hücrelerinde farklılaşma	
IL-4	T lenfositleri	T ve B lenfosit farklılaşma	Göç, 2015
IL-9	T lenfositleri	T lenfositlerinde proliferasyon	
IFN- γ	T lenfositleri, NK hücreleri	İmmünomodülatör etki	Özbal, 2000
TNF-α	T lenfositleri	Enflamasyon, tümörisidal etki	
TNF- β	T lenfositleri	Tümörisidal etki	
TGF- β	T lenfositleri, makrofajlar	İmmünosüpresyon	

İmmün sistem hücrelerinin aktivitesini artıran sitokinlerden biri IL-2'dir. IL-2'nin T, B ve NK hücrelerini uyararak bu hücrelerin proliferasyonlarında artış sağlamaktadır. Şekil 1'de IL-2'nin T, B ve NK hücreleri üzerindeki etkisi şematik olarak gösterilmiştir.

Kanser İmmünoterapisi

Kanser hastalığı, kontrolden çıkan hücrelerin sürekli olarak çoğalması sonucu oluşur. Kanser oluşumunda X ışınları, ultraviyole ışınları gibi fiziksel faktörler ile 3-metilkolantren, benz-alfa-piren gibi kimyasal faktörlerin dışında onkojenik virüslerin de rolü vardır. Bir sağlıklı hücrenin herhangi bir dış etken sonucu kontrolden çıkarak hızla büyümesiyle oluşan kanserli hücrede, normal hücrede bulunmayan doku antijenleri eksprese edilmektedir (Özbal, 2000; Göç, 2015). Örnek bir kanser hücresi olan 4T1 fare meme kanser hücre hattı ile laboratuvarımızda yürüttüğümüz çalışmalardan elde ettiğimiz görüntüler Şekil 2'de gösterilmiştir. Şekilde görüldüğü üzere hücreler apoptoza uğramadan çoğalmaya devam etmiştir.



Şekil 1. IL-2'nin T, B ve NK hücreleri üzerindeki etkisi

Figure 1. The effect of IL-2 on T,B and NK cells

Şekil 2. 4T1 fare meme kanser hücrelerinin apoptoza uğramadan kontrolsüz çoğalması

Figure 2. Uncontrolled proliferation of 4T1 mouse breast cancer cells without apoptosis

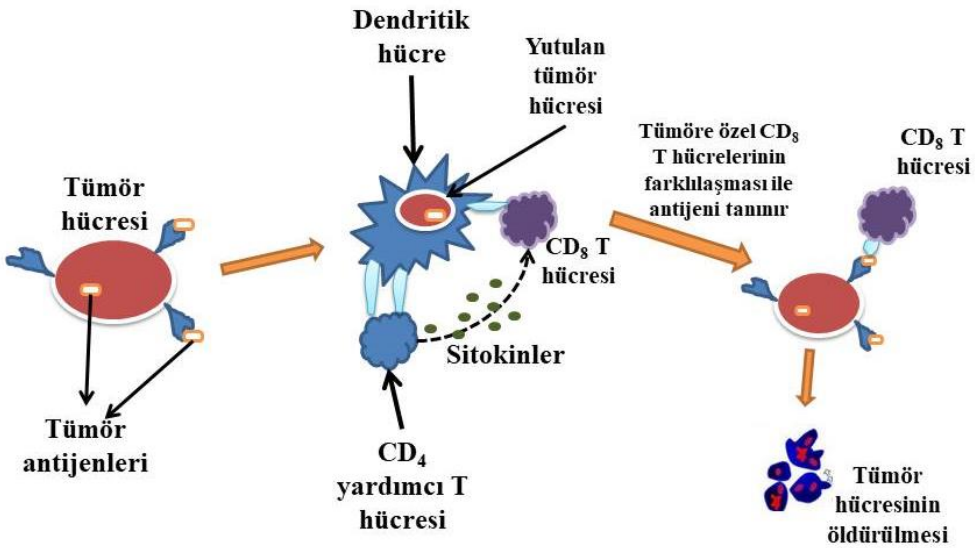
Konağın immün sistemi yabancı doku antijenlerini kolayca tanımasına rağmen tümör dokusunu organizmadan kolayca atamamaktadır. İnsanda saniyede milyarlarca hücre çoğalmakta ve bu hücrelerin yüzlercesi mutasyonla farklı hücre oluşturmaktadır. Bu farklı hücrelerin temizlenmesinde hücresel immün yanıt mekanizması rol almaktadır. Tümör hücrelerinin gelişmesinin baskılanmasında yardımcı ve sitotoksik T hücreleri, NK ve K hücreleri sorumludur. Tümör antijenleri tarafından uyarılan T lenfositler çeşitli sitokinler salgılayarak sitotoksik T hücrelerini, makrofajları, B lenfositlerini, K ve NK hücrelerini aktive etmektedir. Uyarılan bu hücrelerin sentezlediği TNF- α tümör hücreleri için sitotoksik etki göstermektedir. Ayrıca T lenfositlerinin salgıladıkları IFN-γ ile uyarılan K ve NK hücreleri tümör hücrelerini öldürmektedir (Özbal, 2000). Tümör hücrelerine karşı immün yanıt şematik olarak Şekil 3'te gösterilmektedir.

Kanser immünoterapisine yönelik çalışılmakta olan pek çok yeni stratejinin prensibi konağın kendi immün yanıtının tümör hücrelerine karşı güçlendirilmesine dayanmaktadır. Bu kapsamda farklı bitkisel, kimyasal ve biyokimyasal bileşenler üzerine çalışmalar yapılmaktadır (Schenk, 2002; Göç, 2015).

Kanser ve Çeşitli Hastalıkların İmmünoterapisinde Kullanılan Bazı Doğal İmmünomodülatör Bileşenler

Çeşitli hastalıkların tedavi edilmesi amacıyla kullanılan kimyasal immünomodülatör bileşenlere alternatifler geliştirmek amacıyla araştırmacılar bitkisel, mikrobiyal ve hayvansal bileşenler üzerinde yenilikçi çalışmalar yürütmektedir. Bu kapsamda birçok bitkisel kaynaklı bileşiğin in vivo ve in vitro olarak immünomodülatör etkileri araştırılmıştır. Kanser immünoterapisi araştırmalarında ise daha spesifik çalışmalar mevcuttur (Jantan vd., 2015).

İmmünomodülatör etkileri olduğu bilinen birçok bitkisel kaynaklı bileşenin kanser immünoterapisinde kullanımı araştırılmaktadır. Bu bileşenlerden biri olan kurkumin, zerdeçal bitkisinden elde edilen, antioksidan ve antienflamatuvar özellikleri olduğu bilinen ve son yıllarda kanser önleyici etkileri araştırılan bir pigmenttir. Kanser hücreleri üzerinde antiproliferatif ve proapoptotik etkileri olduğu bilinmektedir (Mukhopadhyay vd., 2001). Bir diğer doğal immünomodülatör bileşen ise resveratrol olarak bilinen doğal bir fitoaleksindir. Bu bileşen genellikle kırmızı üzüm, yer fıstığı ve ananasta bulunmaktadır. Apoptoza bağlı olarak kanser hücrelerinin gelişimini engellediği bilinmektedir (Whyte vd., 2007; Kundu vd., 2008). Bitkisel kaynaklı doğal immünomodulatör maddelerden olan soya kaynaklı izoflavonlar genistein, daidzein ve glisitein içermektedir. Bu bileşenlerden genisteinin kanser hücrelerinin proliferasyonunu, invazyonunu ve metastazını engellediği bilinmektedir (Barnes, 1997; Li ve Sarkar, 2002). Bu bileşik gruplarına ek olarak alkoloidler, esansiyel yağlar, flavonoidler, flavonlar, flavonoller, izoflavonlar örnek olarak gösterilebilir (Jantan vd., 2015).



Şekil 3. CD_8 T hücrelerinin tümör hücresini öldürmesi

Figure 3. Killing of tumor cell by CD_8 T-cells

Uzun yıllardır bitki bileşeni olarak bilinen ancak doğada en çok bakteri ve fungusların hücre duvarlarında bulunduğu ortaya çıkan bir başka immünomodulatör madde ise β-glukandır (Chan vd., 2004). Yirmi yılı aşkın süredir yapılan araştırmalar sayesinde, β-glukanın oldukça güçlü immün stimülant olduğu ve tümörlere karşı antagonistik etkisi olduğu görülmüştür. Bunların yanı sıra, kolesterol ve trigliserit seviyesini düşürme, kan şekerini normal düzeylerine getirme, cilt yaralarını iyileştirme gibi birçok yararı da gözlenmektedir (Akramiené vd., 2007; Pohorska vd., 2016). Besin alımını ve iştahı düzenlediği ve kilo kaybına yardımcı olduğunu gösteren çalışmalar nedeniyle obezite tedavisinde de etkili olabilme potansiyeli mevcuttur (Pohorska vd., 2016). Günümüzde, β-glukanların antitümör etkileri üzerine yoğunlaşılmıştır ve son araştırmalar konakta tümör oluşumuna karşı doğal ve adaptif immün yanıtları artırdığını göstermektedir. Giderek artan araştırma verilerine göre, β-glukanlar tümör ilişkili dendritik hücre fonksiyonlarını, antitümör aktiviteleri artıracak şekilde düzenleyebilmektedir (Ning vd., 2016). Mikrobiyal kaynaklı immünomodülatörlerden bir diğeri ise özellikle meme kanseri tedavisinde kullanılan ilaçların yapımında kullanılan ve *Streptomyces peucetius* ATCC 27952 tarafından sentezlenen doksorubisin, antrasiklin tipte bir antibiyotiktir (Niraula vd., 2010). Doksorubusin meme ve akciğer kanseri gibi solid tümörlerin tedavilerinde kullanılmaktadır ve meme kanseri tedavisinde de en aktif ilaçlardan biri olarak kabul edilmektedir (Jones vd., 2003).

Bitkisel ve mikrobiyal kaynaklı immünomodülatör bileşenlerin yanı sıra hayvansal kaynaklı olan immüomodülatörler de mevcuttur. MS hastalığının tedavisinde kullanılan timik hormonlar bu bileşenlerden bazılarıdır. Bir ön hormon olan D vitamininin hayvansal kaynaklı kolekalsiferol (Vitamin D_3) ve bitkisel kaynaklı ergokalsiferol (vitamin D_2) olmak üzere iki öncülü vardır (Holick ve Garabedian, 2006). 1.25-dihidroksi vitamin D güçlü antiproliferatif, prodiferansiyatif, proapoptotik ve immünomodülatör etki gösterdiği bilinmektedir (Van Etten ve Mathieu, 2005).

Hayvansal kaynaklı immünomodulatör maddelerden önemli örneklerden bazıları da çeşitli arı ürünleridir. Arı ürünleri immünomodulatör etkileri başta olmak üzere antitümör ve antiinvaziv etkileri sayesinde kanser immünoterapisinde kullanım potansiyeline sahiptir. Bir arı ürünü olan polenin temel bileşenleri fenolik asit türevleridir. Arı poleninin immün sistem stimüle edici aktivitesi ve antitümör etkisi olduğu belirtilmiştir (Dudov vd., 1994). İmmünoterapide kullanılan arı zehrinin ise IL-4 sitokininin salgılanmasını azalttığı; IL-5 ve IFN-γ sentezlenmesini art1rarak immünomodulatör etki gösterdiği bildirilmiştir (Jutel vd., 1995). Önemli bir arı ürünü olan propolis ise gallik asit, kateşin, kafeik asit, kuersetin, sinnamik asit, naringenin, apigenin, galangin, kafeik asit fenil ester (CAPE) gibi önemli fenolik bileşikler içermektedir. Birçok çalışma ile propolisin antitümör, antikanser, antienflamatuar, antiproliferatif etkileri kanıtlanmıştır ve yeni çalışmalar sürdürülmektedir (Matsuno vd., 1997; Kimoto vd., 1998; Banskota vd, 2001).

Propolisin Kimyasal Yapısı ve Biyoaktif Özellikleri

Günümüzde propolis yenilikçi bir koruyucu ve biyoaktif gıda takviyesi olarak kullanılmaktadır. Propolis, Türk Standartları Enstitüsü tarafından ‘’İşçi arıların, kovan içerisindeki besinleri, yavru arıları ve kendilerini çeşitli patojen mikroorganizmalardan (virüs, bakteri, fungus) korumak amacıyla bitkilerin yaprak, gövde, tomurcuk vb. kısımlarından topladığı reçinemsi maddeleri ve bitki nektarlarını, başlarında yer alan salgı bezlerinden salgılanan enzimler ile biyokimyasal değişikliğe uğratarak oluşturdukları, ‘arı tutkalı’ olarak da adlandırılan ürün.” olarak tanımlanmıştır (TSE 12910, 2003). Bal arıları topladıkları propolisi ağızlarında nemlendirip yumuşatarak ve aynı zamanda bazı enzimler ekleyerek propolisi pelet haline getirir ve peleti ön bacaklarını kullanarak arka bacaklarındaki polen sepetine aktarırlar (Doğan ve Hayoğlu, 2012). Polen sepeti propolis ile doldurulduğunda kovana taşınmaktadır. Propolis genç işçi arılar tarafından 25-30 dakikada boşaltılmaktadır (Pehlivan vd., 2012). Ortalama propolis üretimi her yıl, her koloni için 10 gramdan 300 grama kadar değişebilmektedir. Fakat yine de bu miktar arılara, iklime, bitki çeşitliliğine ve tuzaklama mekanizmalarına bağlı olarak farklılıklar gösterebilmektedir (Doğan ve Hayoğlu, 2012).

Esas olarak kovanda etkili bir antiseptik madde olarak işlev gören reçine, mum ve uçucu yağlardan oluşmaktadır (Viuda-Martos vd., 2008). Yaygın olarak şekerlerin, biyofarmasötiklerin ve kozmetik ürünlerin bir bileşeni olarak kullanılmakta olup, gıda ve içeceklerde doğal koruyucu ve biyoaktif bileşiklerin kaynağı olarak raf ömrünü ve tüketicinin sağlığını iyileştirici doğal ürünler kategorisinde de popülerlik kazanmaktadır. Bu bağlamda propolis farklı formlarda satışa sunulmaktadır. Kapsül, tablet, sulu/etanol ekstraktı veya ham propolis olarak satın almak mümkündür. Ayrıca birçok ürün içerisine eklenerek farklı formülasyonlarda da karşımıza çıkmaktadır (Marcucci vd., 1995; Osesa vd., 2016).

Propolis sahip olduğu çeşitli biyolojik özelliklerinden dolayı anti-inflamatuar, antimikrobiyal, antioksidan, antitümör, anti-ülser ve anti-HIV gibi soğuk algınlığı, yaralar ve ülserler, romatizma, diyabet ve diş çürüğü, burkulmalar, kalp gibi hastalıkları önlemek ve tedavi etmek için yaygın

olarak kullanılmaktadır (Huang vd., 2014; Vagish-Kumar, 2014).

Farklı ekstraksiyon metotları propolisin çeşitli biyolojik etkilerinin aktivitesinde farklılıklara neden olmaktadır. Her solvent farklı bileşenlerin çözünmesini ve ekstraksiyonunu sağlamaktadır (Sforcin, 2007). Bugüne kadar yapılan çalışmalar ile propolisin 300'den fazla bileşeni tanımlanmıştır ancak kimyasal kompozisyonu oldukça karmaşıktır. Üstelik bu kompozisyon propolisin toplandığı bitki kaynağına, mevsime, yerel floraya göre farklılıklar göstermektedir. Değişken kimyasal yapısı propolisin medikal kullanım için standardizasyonunda sorun teşkil etmektedir (Ghisalberti, 1979; DeCastro, 2001). Propolisler farklı ülkelerde farklı statülere sahiptirler. Almanya, İsviçre gibi bazı Avrupa ülkelerinde ilaç olarak kabul edilirken diğer birçok ülkede gıda takviyesi olarak adlandırılır (Atayoğlu, 2012).

Propolisin en çok kullanılan formu olan etanolik ekstraktının (EEP) kuru ağırlığının %50'sinden fazlasını fenolik bileşikler oluşturmaktadır. Bu %50'lik paya en az 4 çeşit kafeik asit esteri [kafeik asit benzil ester, salisilik asit benzil ester, sinnamik asit benzil ester ve kafeik asit fenetil ester (CAPE)] dahildir (Hepşen vd., 1996). Sinnamik alkol, sinnamik asit, vanillin, benzil alkol, benzoik, kafeik ve ferulik asit propoliste bulunan fenolik maddelerdendir (Yılmaz vd., 2004). Propolis içerisinde bulunan bazı temel bileşenler Tablo 2'de belirtilmiştir (Kumova vd., 2002).

Gaz kromatografisi (GC), gaz kromatografisi-kütle spektrometresi (GC-MS) ve ince tabaka kromatografisi (TLC) analizleri ile Brezilya'dan toplanan propolis örneklerinin içerikleri belirlendiğinde, ana bileşenlerinin fenolik bileşikler (flavonoidler, aromatik asitler ve benzo[*a*]pirenler), diterpenler ve triterpenler, esansiyel yağlar olduğu belirlenmiştir. Flavonoidlerin (kaemferol, 5,6,7-trihidroksi-3,4'-dimetoksi flavon, aromadendrin-4'-metil eter) bu propolis örneğinde az miktarda belirtilmiştir (Boudourova-Krasteva vd., 1997). Çeşitli kaynaklara sahip propolislerin farklı kimyasal yapıda olması, bu propolis örneklerinin biyolojik özelliklerinin benzersiz olabileceği öngörüsünü yaratmaktadır (Bankova, 2005). Avrupa'ya özgü propolislerde antibakteriyel ve antifungal aktivitenin flanononlar, flavonlar, fenolik asitler ve onların esterlerinden ileri gelirken, Brezilya'ya özgü propolislerde ise bu aktiviteler prenillenmiş p-kumarik asitler ve diterpenler sayesinde görülmektedir (Sforcin, 2007). Çeşitli solventler ile elde edilen propolis ekstraktlarının grip virüsünün ve vaksiniya virüsünün üremesini azalttığı ve antiviral etkileri olduğu bilinmektedir (Maksimova-Todorova vd., 1985). Propolisin in vitro olarak çeşitli hücre hatları üzerindeki sitotoksik etkileri yapılan çalışmalar tarafından belirlenmiştir. Özellikle kanser hücre hatları ile ilgili yapılan çalışmalar ışığında in vivo çalışmalar yürütülmüştür (Grunberger vd., 1988; Awale vd., 2008; Carvalho vd., 2011; Popovic vd., 2012; Chen vd., 2014). Sitotoksik etkilerinin yanı sıra farklı tümör hücrelerini apoptoza sürüklediği bilinmektedir (Choudhari vd., 2013; Novak vd., 2014). Deney hayvanları ile yapılan çalışmalar ise propolisin tümör hücresi enjekte edilen farelere propolis verilmesi ile sonucu tümör büyüme hızlarının azaltılabildiği hatta tümör hacimlerinin azaldığı bildirilmiştir (Orsolic vd., 2005; İnoue vd., 2008). Propolis ile beslenen farelerin immün sistem hücrelerindeki proliferasyon artışı ise propolisin etkili bir immünomodulatör olduğunu göstermektedir (Park vd., 2004).

Tablo 2. Propolisin tanımlanan bazı temel bileşenleri (Kumova vd., 2002)

Table 2. Some defined components of propolis

Bileşenler			
1	Flavonoidler	10	Alkoller, ketonlar ve fenoller
2	Hidroksiflavonlar	11	Heteroaromatik bileşikler
3	Hidroksiflavononlar	12	Terpen ve sekuterpenler ve türevleri
4	Kalkonlar	13	Alifatik hidrokarbonlar
5	Benzoik asit ve türevleri	14	Sekuterpen ve triterpen hidrokarbonlar
6	Asitler	15	Steroller ve steroid hidrokarbonlar
7	Esterler	16	Mineraller
8	Benzaldehit türevleri	17	Şeker
9	Sinnamil ve sinnamik asit ile türevleri	18	Amino asitler

Tüm olumlu özellikleri yanısıra propolisin bazı bireylerde alerjik tepki oluşturabildiği bilinmektedir. Hausen'in araştırmasında propolis ile temas sonucu oluşan alerjinin sebebinin 4 çeşit kafeat olduğunu bildirmiştir. Bunlar fenil etil kafeat, benzil kafeat, 3-metil-2-bütenil kafeat ve geranil kafeattır (Hausen, 2005). Propolisin alerjik etkilerinin azaltılması amacıyla 2012 yılında İtalya'da yapılan bir çalışmada ham propoliste ve etanolik propoliste bulunan, bazı kişilerde alerjik etkilere sebep olan kafeat esterlerinin laktik asit bakterilerinin kullanıldığı biyotransformasyon yöntemi ile giderilmesi amaçlanmıştır. Bu yöntemin prensibi ise propolisin Lactobacillus helveticus'un 'sinnamol esteraz' aktivitesinden yararlanılarak alerjenik moleküllerin biyotransformasyon yolu ile azaltılmasına dayanmaktadır. Propolise uygu-

lanan bu işlemin propolisin flavonoid içeriğini ve antimikrobiyal aktivitesini etkilemediği bildirilmiştir (Gardana vd., 2012).

Propolisin Antitümör Etkisi

Antik çağlardan beri bal ve propolis, bilinen fonsiyonel etkileri nedeniyle, terapötik ajanlar olarak kabul edilmiştir. Araştırmacılar propolisin in vivo ve in vitro olarak antitümör etki gösterdiğini bildirmişlerdir (Sforcin, 2007). Antitümör etkileri incelemek amacıyla çeşitli tümör hücreleri üzerinde yapılan in vitro çalışmalar, genellikle sitotoksisite analizlerini ve apoptotik etkilerin analizlerini kapsamaktadır. Bu bağlamda 2014 yılında yapılan bir çalışmada Brezilya'ya özgü propolis, etanol ile ekstrakte edilip fare melanom hücreleri (B16F10) üzerindeki sitotoksik etkisi incelenmiş ve propolis ekstraktının B16F10 hücrelerinin %50'sini inhibe eden konsantrasyonun (IC_{50} değeri) 32,6 µg/mL olduğu belirlenmiştir. Aynı çalışmada propolis ekstraktının B16F10 hücreleri üzerindeki apoptotik etkisi incelenmiş; 10 µg/mL konsantrasyondaki propolis esktraktının B16F10 hücrelerinin %18,4'ünü apoptoza sürüklediği ve 50 µg/mL konsantrasyondaki propolis esktraktının B16F10 hücrelerinin %34,5'ini apoptoza sürüklediği bildirilmiştir (Novak vd., 2014). Benzer bir çalışmada ise propolisin MeOH ekstraktının insan pankreas kanser hücreleri (PANC-1) üzerindeki etkisi incelenmiştir ve 10 µg/mL konsantrasyonundaki propolis ekstraktının PANC-1 hücrelerinde %100 oranında nekroz tipteki morfolojik değişimler sonucu ölüm gözlendiği belirtilmiştir (Awale vd., 2008). Propolisin kanola yağı ile ekstrakte edilerek 3 farklı kanser hücresi üzerindeki sitotoksik etkilerinin incelendiği çalışmada IC_{50} değerleri; insan promiyelositik lösemi hücrelerinde (HL-60) 28,87 µg/mL, insan kolon kanser hücrelerinde (HTC-8) 40 µg/mL ve insan meme kanseri hücrelerinde (MDA/MB-435) 22,19 µg/mL olarak tespit edilmiştir (Carvalho vd., 2011). Benzer bir çalışmada Tayvan'a özgü 8 tip propolisten elde edilen etanol ekstraktlarının 2-20 µg/mL konsantrasyonları arasında insan melanom hücrelerinin %50'sini inhibe ettiği belirtilmiştir (Chen vd., 2004). Propolisin farklı ekstraktlarının in vitro olarak antitümör etkiler göstermesinin yanı sıra propolisten izole edilen bazı bileşenlerin de benzer etkileri gösterdiği bilinmektedir. Örneğin propolisin aktif bir bileşeni olan CAPE'nin insan meme kanseri hücreleri (MCF-7) için IC_{50} değerinin 5 µg/mL olduğu ve 10 µg/mL konsantrasyondaki CAPE'nin insan cilt melanom hücrelerinde (SK-MEL-28) %100 oranında inhibisyon sağladığı belirtilmiştir (Grunberger vd., 1988). Tablo 3'te propolis ve propolisin etken maddelerinin in vitro olarak incelenen antitümör etkileri gösterilmiştir.

Propolisin in vivo olarak antitümör etkilerinin belirlenmesi amacıyla genellikle Balb/c ırkı fareler kullanılmaktadır ve propolis etken maddeleri gavaj yoluyla, kas veya tümör dokusu içerisine enjeksiyon ile farelere verilmektedir. Antitümör etkilerin belirlenmesinde genel olarak tümör boyutlarının ölçümü ile tümör gelişme hızları belirlenmekte veya farelerin toplam vücut ağırlıklarındaki artış veya azalma ölçülmekte ve böylece dolaylı olarak tümör gelişimi belirlenmektedir. Aynı amaç kapsamında sitokin salınımı, T lenfositlerin proliferasyonu, dalak ve timüs bezlerinin ağırlık değişimleri gibi immün sistem uyarılarının ölçülmesi yoluyla da antitümör etkiler incelenebilmektedir.

Yapılan bir çalışmada fare kanser hücreleri ($4x10^6$ hücre/mL) enjekte edilen deneklere enjeksiyondan 24 saat sonra gavaj yolu ile 320 mg/kg ve 960 mg/kg dozlarında suda çözünebilen propolis ektraktı 10 gün boyunca günde 5 defa olacak şekilde uygulanmıştır. Deneme sonunda tümör gelişiminin propolis uygulaması ile yavaşlatıldığı ve tümör hacimlerinin propolis uygulanan gruplarda kontrol grubuna göre daha az olduğu belirtilmiştir (İnoue vd., 2008). Diğer bir çalışmada B16F10 hücreleri ($1x10^6$ hücre/mL) farelere enjekte edildikten sonraki 40 gün boyunca günde iki defa propolisin etanol ekstraktı (10 mg/kg) enjeksiyon ile uygulanmıştır. Bu çalışma sonunda uygulamanın tümör gelişimini yavaşlattığı belirtilmiştir (Novak vd., 2014). Aynı yıl yapılan bir diğer çalışmada antikanser bir ilaç olan Irinotecan (IRI) ve propolis ekstraktının sinerjistik etkisi incelenmiştir. Denemede Swiss ırkı erkek albino fareler kullanılmış ve Ehrlich tümör hücreleri $1x10^6$ hücre/fare olacak şekilde kas içine enjekte edilmiştir. Ardışık 3 gün boyunca propolis ekstraktı (100 mg/kg) ve IRI (50 mg/kg) farelere enjekte edilmiştir. Araştırmacılar bu deneme sonunda propolis uygulamasının IRI'nin etkinliğini artırdığını ve tümör gelişimini yavaşlattığını bildirmişlerdir (Lisicic vd., 2014). Filho vd. (2014) tarafından fareler üzerinde yapılan çalışmada, 9,10-dimetil-1,2-benzantrasin (DMBA) ile indüklenen cilt kanseri oluşturulmuştur. On altı hafta boyunca propolisin hidroalkolik ekstraktının (50 ve 100 mg/kg) gavaj olarak verilmesi ile tümör gelişiminin kısmen inhibe edilebildiği ve propolis ekstraktının kemopreventif (Gelişmekte olan tümörün gelişiminin kontrol altına alınmasını sağlayan etkidir ve/veya kalıtsal olarak kansere yakalanma riski taşıyan kişilerin kullandığı ilaçların bir özelliğidir.) etki gösterdiği belirlenmiştir. Benzer bir çalışmada güçlü bir kolon karsinojeni olan 1,2-dimetilhidrazin ile indüklenen kolon kanserine karşı, farelere gavaj yoluyla 10, 30 ve 90 mg/kg dozlarında verilen propolisin etanolik ekstraktının koruyucu etkisi incelenmiştir. Çalışma sonunda uygulanan üç farklı dozdan yalnızca 30 mg/kg doz ile propolis ekstraktının kolon karsi-

nojenlerine karşı vücudu koruyucu etki gösterdiği ve preneoplastik lezyon gelişiminde baskılanma görüldüğü bildirilmiştir (Bazo vd., 2002). In vivo olarak yapılan çalışmalarda çeşitli propolis ekstraktlarının dışında propolisin içeriğindeki bazı bileşenlerin (CAPE, kuersetin, kafeik asit) de antitümör etkileri test edilmiştir. Osrolic vd. (2015), fare meme kanseri hücresi [MCa (1x 10^5 hücre/mL)] enjekte edilen farelere 50 ve 150 mg/kg dozlarda enjeksiyon ile CAPE, kafeik asit ve suda çözünebilen propolis ekstraktı uygulamıştır. Deneme sonunda tümör oluşumu ve gelişiminde yavaşlama, farelerin yaşam sürelerinde artış gözlendiği belirtilmiştir. Ancak suda çözünebilen propolis ekstraktının kefeik asit ve CAPE'nin gösterdiği etkilerden daha zayıf bir etki oluşturduğu bildirilmiştir. Aynı araştırmacının benzer bir çalışmasında deneklere fare meme kanseri hücreleri [MCa (2x10^5 hücre/mL)] enjekte edilmiş ve ardından denemenin 5.,10. ve 15. günlerinde farelere gavaj yoluyla 50 ve 150 mg/kg dozlarında suda çözünebilen propolis ekstraktı, kafeik asit ve CAPE uygulanmıştır. Deneme sürecindeki 14 gün boyunca ise farelere gavaj yoluyla 1200 mg/kg dozunda kuersetin uygulanmıştır. Çalışma sonucunda uygulanan propolis ve propolis bileşenlerinin tümör gelişimini ve metastazını baskıladığı belirtilmiştir (Orsolic vd., 2004). Balb/c farelere herhangi bir tümör hücresi enjekte edilmeden yapılan bir çalışmada 5, 10, 20 mg/kg dozlarda gavaj yoluyla uygulanan CAPE'nin immünomodulatör etkileri incelendiğinde; 20 mg/kg dozdaki uygulamanın IFN-γ, IL-2, IL-4 salınımında ve CD_8 T hücrelerinde artış görülmüştür. Bu etkiler ile CAPE'nin immün sistemi module ederek antitümör ve antikanser etkiler gösterdiği belirtilmiştir (Park vd., 2004). Tablo 4'te propolis ve propolisin etken maddelerinin in vivo olarak incelenen antitümör etkileri gösterilmiştir.

Tablo 3. Propolis ve propolisin etken maddelerinin in vitro olarak incelenen antitümör etkileri

Table 3. In vitro investigation of antitumor effects of propolis and its active components

Etken madde	Konsantrasyon (µg/mL)	Etki	Kaynak
Propolis ekstraktı	32.6	Fare melanom hücrelerinde (B16F10) %50 oranında inhibisyon	Novak vd., 2014
Propolis ekstraktı	10	İnsan pankreas kanser hücrelerinde (PANC-1) %100 oranında ölüm	Awale vd., 2008
Propolis ekstraktı	28.87	İnsan promiyelositik lösemi hücrelerinde (HL-60) %50 oranında inhibisyon	Carvalho vd., 2011
	40	İnsan kolon kanser hücrelerinde (HTC-8) %50 oranında inhibisyon	
	22.19	İnsan meme kanseri hücrelerinde (MDA/MB-435) %50 oranında inhibisyon	
CAPE	5	İnsan meme kanseri hücrelerinde (MCF-7) %50 oranında inhibisyon	Grunberger vd., 1988
	10	İnsan cilt melanom hücrelerinde (SK-MEL-28) %100 oranında inhibisyon	
8 farklı propolis ekstraktı	2-20	İnsan melanom hücrelerinde %50 oranında inhibisyon	Chen vd., 2004
Propolis ekstraktı	10	Fare melanom hücrelerinin (B16F10) %18,4'ünde apoptoz	Novak vd., 2014
	50	Fare melanom hücrelerinin (B16F10) %34,5'inde apoptoz	

Tablo 4. Propolis ve propolisin etken maddelerinin in vivo olarak incelenen antitümör etkileri

Table 4. In vivo investigation of antitumor effects of propolis and its active components

Etken madde	Uygulanma dozu (mg/kg)	Uygulanma şekli	Etki	Kaynak
CAPE	20	Oral olarak	T lenfosit proliferasyonunda artış, farelerin timüs bezi ve dalak ağırlıklarında artış, IFN-γ, IL-2, IL-4 salgılanmasında artış	Park vd., 2004
CAPE, kafeik asit, suda çözünebilen propolis ekstraktı	50 150	Enjeksiyon	Tümör oluşumu ve gelişiminde yavaşlama, farelerin yaşam sürelerinde artış	Orsolic vd., 2005
Propolis ekstraktı	10	Enjeksiyon	Tümör gelişiminde yavaşlama	Novak vd., 2014
Propolis ekstraktı	100	Enjeksiyon	Tümör gelişiminde yavaşlama	Lisicic vd., 2014
Propolis ekstraktı	320 960	Oral olarak	Tümör gelişiminde yavaşlama	İnoue vd., 2008
Propolis ekstraktı	100 50	Oral olarak	Tümör gelişiminde kısmi inhibisyon	Filho vd., 2014
Kafeik asit,CAPE, kuersetin	50 150 1200	Oral olarak	Tümöre karşı antimetastatik etki	Orsolic vd., 2004
Propolis ekstraktı	30	Oral olarak	Kolon karsinojenlerine karşı vücudu koruyucu etki, preneoplastik lezyon gelişiminde baskılanma	Bazo vd., 2002

Propolisin akut ve kronik toksisitesiyle ilgili yeterli çalışma bulunmamaktadır. İki yüz ve 5000 mg/kg vücut ağırlığı/gün dozlarındaki propolis uygulaması deney hayvanlarında toksik ölümlere sebep olmamıştır ve gerekli hesaplamalar yapıldıktan sonra insanlar için toksik etki yaratmayan güvenli dozun 1.4 mg/kg vücut ağırlığı olduğu bildirilmiştir. Araştırmacı propolisin farelerdeki LD_{50} değerinin 2-7.3 g/kg arasında değiştiğini belirtmiştir. Araştırmacı, 90 fare ile yapılan denemeler ile propolisin NOAEL değerini (Gözlenebilen hiçbir yan etki göstermeyen doz) 1400 mg/kg vücut ağırlığı/gün olduğunu bildirmiştir (Burdock, 1998). Propolisin insanlar üzerindeki bazı etkilerini incelemek amacıyla yapılan bir araştırmada 30 gün boyunca sağlıklı gönüllülere toz propolis ekstraktı suda çözündürülerek içirilmiş ve vücudun serbest radikallere karşı savunma olarak ürettiği süperoksidaz dismutaz enzimi aktivitesi ile toplam kolestrol, yüksek ve düşük yoğunluklu lipoprotein kolesterol, trigliseritler, glukoz, ürik asit, ferritin ve transferrin, plazmadaki malondialdehit konsantrasyonu gibi bazı kan parametreleri değerlendirilmiştir. Günlük propolis alınımının etkilerinin cinsiyete göre farkılık gösterdiği görülmüştür. İlk 15 günde erkeklerin %23.2'sinde malondialdehit konsantrasyonunun düştüğü ve 30. günde kadınların %20.9'unda süperoksidaz dismutaz enzimi aktivitesinin arttığı görülmüştür. Ancak propolis uygulamasının deneme kapsamında ölçülen kan değerlerinde anlamlı bir değişikliğe sebep olmadığı belirtilmiştir (Jasprica vd., 2007).

Ayrıca yapılan çalışmalar, kemoterapötik ajanlarla kombine edilen ham, suda çözünür propolisin potansiyel olarak postkemoterapötik reaksiyonları en aza indirirken güçlendirilmiş bağışıklığı maksimize ettiği ve tedavi etkinliğini ilaç etkileşimi oluşturmadan arttırdığını ortaya koymuştur (Orsolić ve Basić, 2005; Patel, 2016; Suzuki vd., 2002; Vagish-Kumar, 2014).

Sonuç

Gelişen teknoloji ve yapılan çalışmalar sayesinde immünoterapi kanser tedavi yöntemlerine alternatif oluşturma potansiyeline sahiptir. Kanser tedavisinde her hastaya özel bir tedavi yaklaşımının daha etkin sonuçlar vermesi göz önüne alındığında, konağın kendi immün sistemini module ederek hastalıkların tedavi edilmesini sağlayan immünoterapinin gelecek yıllarda sıklıkla kullanılacağı düşünülmektedir. Bu kapsamda immünoterapötik etkileri olduğu kanıtlanan propolisin de medikal kullanımının yaygınlaşabileceği düşünülmektedir. Antimikrobiyal, antifungal, antiviral, antitü-

mör etkileri gibi yararları olduğu kanıtlanan arı ürünü propolis, farklı ülkelerde gıda takviyesi veya ilaç olarak adlandırılmaktadır. Türkiye'de bal, arı poleni, arı sütü gibi çeşitli arı ürünleri kullanılmaktadır buna rağmen propolis bu ürünlerden daha az bilinmekte veya tüketiciler tarafından diğer arı ürünleri ile karıştırılmaktadır. Propolisin birçok olumlu sağlık etkileri mevcuttur ve çeşitli hastalıkların önlenmesi ve tedavisinde etkili olduğu bilinmektedir. Propolisin bu etkilerini sağlayan bileşenleri mevsimsel etkilerin ve bitki örtüsündeki değişimlerin sonucunda çeşitlilik göstermektedir. Ülkemizde zengin bir bitki örtüsü vardır ve arıcılık oldukça yaygındır. Araştırmalarımız sonucunda propolisin bilinçli üretiminin ve tüketiminin artırılması gerektiği düşünülmüştür. Bu kapsamda oldukça yararlı bir arı ürünü olan propolisin daha çok tanıtılması gerekmektedir. Ancak propolisin tüm olumlu yönlerine karşın, yetkili üreticilerin veya uzman doktorların önerdiği miktarlarda kullanılması gerekmektedir.

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A COMMON GENETIC ETIOLOGY FOR IMPULSIVITY AND OVEREATING

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ABSTRACT

In its most basic form impulsivity, a heritable trait, is defined as a tendency to act without control and has been implicated in the onset, symptomatic expression, and maintenance of overeating. Specifically, high impulsivity and its related constructs such as poor inhibitory control and high sensitivity for reward and environmental cues have been shown to perpetuate binging and overeating. Thus, several studies have been conducted to investigate the possible common genetic etiologies for high impulsivity and overeating. The purpose of this review is to summarize the genetic findings indicating an association between impulsivity and overeating.

Keywords: Overeating, Impulsivity, Polymorphism, Genetic, Impulse control, Binge eating

Introduction

Impulsivity, a personality trait broadly defined as acting without thinking, is routinely associated with high- risk decision-making and behaviors, and is suggested to be a mediator modulating eating behavior towards overeating and lack of control in eating behavior. Specifically, research showed prevalence of impulsivity in patients with binge-eating disorder (BED) (Schag, Schonleber, Teufel, Zipfel, & Giel, 2013) which is a psychiatric disorder characterized by frequent episodes of binge eating and loss of control over food intake. In line with this, studies have shown impulsivity to be positively correlated with body mass index (BMI) (Meule & Blechert, 2017), difficulty in weight maintenance (Weygandt et al., 2015), and tasty but unhealthy food choices (Kakoschke, Kemps, & Tiggemann, 2015). Furthermore, in children, higher impulsivity was also shown to associate with a greater risk of becoming obese in adulthood (Fields, Sabet, & Reynolds, 2013), and with difficulty in weight loss (Nederkoorn, Jansen, Mulkens, & Jansen, 2007).

Impulsivity is a multidimensional construct with several facets, characterized primarily by disinhibited and rapid spontaneous response without consideration of possible consequences (i.e. urgency and lack of perseverance) and heightened drive towards pleasurable stimuli (i.e. reward sensitivity). Altered reward sensitivity (measured by longer cue gazing duration and difficulty in inhibiting saccades toward cues) has been shown in persons with BED (Schag et al., 2013) and a sub-group of those with bulimia nervosa (BN; another psychiatric disorder characterized by cycle of binging with compensatory self-induced vomiting) that is frequently categorized as ‘‘multi-impulsive” (Wonderlich et al., 2005). Similarly, enhanced appetite towards reward-signaling cues (i.e. food cues), termed as ‘cue-reactivity’ (Jansen et al., 2008), as well as attention and memory biases towards food cues were all observed in obese persons (Meule, de Zwaan, & Muller, 2017), and persons with binging type of eating disorders (Schmitz, Naumann, Biehl, & Svaldi, 2015). Moreover, adolescents with binge eating habits and lack of control over eating were shown to exhibit greater reward sensitivity, engage in impulsive behaviors, and possess a tendency to engage in rash behavior when distressed (Fields et al., 2013). Overall, these findings indicate a strong association between overeating and impulsivity trait that may partially implicate causality.

The search for a causal link between impulsivity and overeating has led researchers to attempt to determine a common genetic etiology. This review briefly summarizes the recent findings on the known genetic tendencies that suggest impulsivity as a heritable trait that is intermediary and shares genetic components with overeating, highlighting the need to address the impulsivity trait for more effective obesity interventions.

Dopamine-Related Genes

Dopamine (DA) is known to play a critical role in reward-related processes and the key modulator within the meso-limbo-cortical system of which the activity has been implicated in the actual reward processing and immediate reward value. It has been shown that both acute exposure to and anticipation of food intake result in DA release (Volkow & Baler, 2015). In addition, greater activity in the mesolimbic dopaminergic regions of the meso-limbo-cortical system has been reported in response to food cues in obese persons (vs. lean persons) (Stoeckel et al., 2008) and in obese persons with BED (vs. non-BED) (Geliebter et al., 2006). Moreover, impaired mesolimbic dopaminergic signaling in overeating and lower *dopamine receptor* (*D2DR*) availability in obese persons have also been shown (Wang et al., 2001).

It is known that the activity in the dopaminergic mesocortical pathway of the meso-limbo-cortical system is involved in impulsive action, behavioral inhibition, reward prediction error (cognitive flexibility and decision-making as well as mediating neural responses for sensory specific processes for taste (Volkow & Baler, 2015). Increased activity in this pathway has been shown to be negatively correlated with impulsive action (Uher & Treasure, 2005) and reduced activity in this pathway has been associated with palatable food intake (Stice, et al., 2008a) and higher body weight (Batterink, et al., 2010). Thus, the functional relevance of DA to both impulsivity trait and overeating encouraged the researchers to conduct studies to investigate the polymorphisms on the expression of the encoding DA- related genes in relation to overeating and the impulsivity trait.

It is known that the *ANKK1* (*ankyrin repeat and protein kinase domain-containing protein*) gene is involved in DA synthesis and promotes the gene for DRD2 (Neville, Johnstone, & Walton, 2004). The *TaqIA* polymorphism on *ANKK1* gene, specifically on the *A1* allele (*TaqIA* $A1^+$) causes low DA synthesis and is associated with diminished (30-40%) *DRD2* density, overall leading to reduced DA function (Jonsson et al., 1999). The *TaqIA* $A1^+$ polymorphism was shown in patients with BED (Davis et al., 2012), and has been associated with overeating, prospective weight gain (Stice, Spoor, Bohon, & Small, 2008b), and higher BMI as well as stronger response to food reinforcement in

obese persons (Epstein et al., 2007). It was shown to predict the weight loss outcomes in children (Chan et al., 2014) and the neural activity in response to ingestion of palatable foods, independent of BMI (Felsted, et al., 2010). *TaqIA A1*$^{+}$ polymorphism was also shown to associate with impulsivity and reward seeking (Chen et al., 2007*).*

A functional polymorphism which produces a valine (val)/methionine (met) substitution at *codon 158 (val158met variant)* on the gene encoding the catechol-*O*-methyl transferase (*COMT*) -an enzyme responsible for the DA catabolism in the meso-limbo-cortical system, causes greater enzymatic activity, which results in higher DA degradation and catabolism and thus low DA levels (Bilder, Volavka, Lachman & Grace, 2004). The *Val*^{158}*Met* polymorphisms have been associated with total adiposity (i.e. abdominal), fat intake, unhealthy food choices and overall desirability to food as well as susceptibility for BED (Leehr et al., 2016; Wallace et al., 2015). It is also associated with impulsive action decreased function in the prefrontal cortex, a region of the brain for executive function and decision making, and greater responsivity to reward in a reward seeking/risk taking task (Bilder et al., 2004; Lancaster, et al.,2012).

An interaction effect has been reported between *DAT1* gene mutation, causing lower DA transmission and *COMT* polymorphism on cognitive flexibility and reward-related neural activity (Yacubian et al., 2007) as well as maladaptive eating patterns including binging (Hersrud & Stoltenberg, 2009). Another study showing effect of the *DAT1* genotype independent of *COMT* genotype (Aarts et al., 2010), however, in this study, the group sizes did not allow the authors to do analyze separately, instead they have used *COMT* genotype as a covariate in their analysis. A similar interaction effect has been reported between *COMT (Val158Met)* and dopamine *D4 receptor (DRD4)* on low cognitive inhibition and binging (Heinzel et al., 2012). The exon-3 seven-tandem repeats (*7R*) allele of the *DRD4* gene is known to cause low DA activity, possibly via decreased receptor expression and maturation, which then leads to significantly higher amounts of DA being required to induce the same response produced by the other alleles (Asghari et al., 1995). The *DRD4 7R allele* has been associated with insufficient prefrontal cortex function for response control leading vulnerabilities for impulsivity and impulsivity-related psychiatric conditions as well as binge eating and concomitant weight gain (Steiger et al., 2016).

Collectively, these studies indicate that genetic disruptions in the mesolimbic DA signaling cause impairments in reward processing, thus cause sensory deprivation of the brain's reward or pleasure mechanisms, and purport the individual's biochemical ability to derive reward from a threshold of what people normally achieve. This eventually may lead to behavioral compensation of reward-seeking and thus promote overeating. Moreover, impaired behavioral control, perhaps arising from genetic disruptions in mesocortical DA activity, may be promoting overeating by inhibiting the individual's ability to suppress food intake through diminished behavioral impulse control.

Serotonin-Related Genes

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) is well known to affect mood, personality, and eating behavior. Mutations of the genes encoding for 5-HTP transporter and 5-HTP *receptor (HTR2A and HTR2B)* have been implicated in the impulsivity trait and overeating (Bevilacqua & Goldman, 2013; Kuikka et al., 2001). Decreased 5-HT activity has been associated with binging behavior in eating disorders. Specifically, downregulation of 5-HT transporters was found in obese women with BED (Kuikka et al., 2001), and upon recovery the 5-HT transporter was reported to be upregulated (Tammela et al., 2003). This suggests that the effect of 5-HT is transient and may be a consequence of psychopathology. Consistent with these, obesity was shown to associate with polymorphisms on the genes encoding for 5-HT transporter (Zhao, et al., 2013) and 5-HT2A receptors (Erritzoe et al., 2009), causing decreased 5-HT levels. Decreased 5-HT activity has also been associated with the impulsivity trait (Stoltenberg, Christ, & Highland, 2012). Together these may suggest that polymorphisms leading to low 5-HT activity may be specific to lack of control over eating episodes in persons with high impulsivity. Although an association between the dysregulation in the 5-HT system and overeating has been reported, the direction of the effect and a possible mediator effect of the impulsivity trait as an endophenotype for overeating has yet to be determined by further studies.

***FTO* Gene**

Variants in the *FTO (*fat mass and obesity associated) gene were the first single nucleotide polymorphisms robustly associated with high BMI. It is known to be the best candidate to predict genetic obesity (Chuang et al., 2015) and to regulate dopaminergic activity (Hess et al., 2013). Recent studies have shown that, similar to dopamine, the *FTO* gene displays a differential role in food intake perhaps through altered reward processing as well as diminished impulse control. The carriers of the certain variants in the *FTO* gene were also shown to predict larger volumes of nucleus accumbens, a reward-related brain area, (Rapuano et al.,

2017), reduced prefrontal cortex function during aging (Chuang et al., 2015), reduced frontal lobe volume (Ho et al. 2010). It is possible that the *FTO* gene to be involved in the modulation of the prefrontal cortex responses leading to greater impulsivity and reward seeking. This may partially contribute to the mechanism underlying the possible causal effect *FTO* genotype on obesity.

Opioid Receptor Gene

The *G allele (G+)* of the *A118G* polymorphism of the μ-opioid 1 receptor encoding gene (*OPRM1*) has been found to be prevalent in obese persons with BED (vs. obese non-BED persons) (Davis et al., 2009).

Studies have suggested abnormal opioid transmission in prefrontal cortex and nucleus accumbens may lead to deficits in impulse control (Selleck et al., 2015) and altered acitivity in these areas have also been shown to be related to problems in impulse control in overeating and binge eating (Dong et al., 2016). Thus, individuals may be prone to elevated food-related hedonic responses through dopaminergic and opioidergic influences on reward-related processes.

Neuregulin 3 Gene

Recent preclinical data has shown that expression for *neuregulin 3 (Nrg3)* genes in the amygdala -a key region for fear and emotion processing, and in the prefrontal cortex may be involved in the development of the impulsivity trait (Pietrzykowski & Spijker, 2014). The amygdala and its efferent projections to mesolimbic pathway have been implicated in incentive learning and reward value processing (Blaiss & Janak, 2009). Recent findings show that the amygdala is also playing a role in addiction as well as impulsive choice and actions (Depue et al., 2014). The basolateral nucleus of amygdala encodes emotional events with reference to their particular sensory-specific features and motivational or affective significance, and it has been known to receive afferents from visceral brainstem and hypothalamus and to send projections to dopaminergic meso-cortico-limbic structures. Although it is premature to assume that an effect of the *Nrg3* gene expression causes a link between impulsivity and overeating in humans, it is a novel candidate gene requiring further attention.

Conclusion

In light of these findings, although the underlying mechanisms remain unknown, a causal link between impulsivity trait and overeating seems possible. The interplay between the genetic and neurobiological impulsivity markers, and the neuropeptides and gut hormones could be addressed in future studies with the goal of tracking common genetic factors and their contributions to the neurobiological bases. Elucidation of possible mediation of the eating behavior by the impulsivity trait may allow us better understand the resistance to lifestyle interventions.

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BIOACTIVE AND PHYSICOCHEMICAL PROPERTIES OF WILD FRUIT POWDER ADDED SPONGE CAKE

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ABSTRACT

This study had investigated the effects of the addition of wild fruit (elaeagnus, hawthorn, medlar, myrtle) on the physicochemical and functional properties of sponge cakes. For this purpose, fruits powders at the level of 5 and 10% were used in cakes which were determined by sensory acceptance test. Myrtle had the highest TPC and DPPH activity while elaeagnus had the lowest values. Analyses of the cake samples were carried out at 1$^{st\ h}$, 7th and 14th d. Texture profile analysis (TPA) revealed that the addition of fruit powder resulted in decrease in the hardness and chewiness values of cake samples compared to the control group. Among the samples, the control group had the highest L^* and b^* values and the samples containing medlar powder had a higher redness value. As the storage time increased, L^* and a^* values were also increased, whereas b^* values decreased. The examination of TPC and DPPH activity of the cake samples at 1st, 1st h and 14th d showed that the addition of fruit powders caused an increase in both parameters. The results of the present study suggested that the use of specific proportions of wild fruit powders in cakes positively affects the physicochemical and bioactive properties of sponge cakes.

Keywords: Elaeagnus, Functional cake, Hawthorn, Medlar, Myrtle

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Introduction

Functional foods supply the body's basic nutrients and provide additional benefits to human physiology and metabolic functions. Thereby, these foods contribute to preventing diseases and achieving a healthier life (İşleroğlu & Yıldırım, 2005). Research studies and consumer demands show that natural products can be used to improve the textural and functional properties of cakes. Gupta, Bawa, and Semwal (2009) reported that the use of barley flour in cakes affects nutritional and functional properties. In other studies, soapwort extract was replaced with egg white Celik, Yılmaz, Işık, and Üstün (2007) and banana powder was replaced with flour (Park, Lee, & Chun, 2010). In low calorie sponge cakes, usage of erythritol and turmeric powder was reported to decrease the stiffness value in sponge cakes (Seo, Park, & Jang, 2010). By the replacement of wheat flour with gamma aminobutyric acid, the bioactive characteristics of cakes were increased and this was found to be beneficial to human health. Lee and Lin (2008) found that replacement of sugar with 75% isomaltooligosaccharide syrup decreased the stiffness and total bacteria count resulting from long storage.

Hawthorn (*Crataegus* spp.), belonging to the Rosaceae family has been used in the pharmaceutical and food industries in China and European countries. Hawthorn berries contain high amounts of caffeic, malic, tartaric, citric acid and organic acid making up 3-6% of the total dry fruit (Chang, Zuo, Chow, & Ho, 2006). Hawthorn flowers and fruits contain epicatechin, hyperoside and chlorogenic acids, which are responsible for free radical scavenging activity (Tadić et al., 2008)

Medlar (*Mespilus germanica* L) is a fruit belonging to the family of Rosaceae. Ayaz, Demir, Torun, Kolcuoglu, and Colak (2008) proved its phenolic content to decrease with ripening. Elaeagnus (*Elaeagnus angustifolia* L.) has 4-hydroxybenzoic and caffeic acids as its principal phenolic compounds. In Iranian folk medicine, it is used for its anti-inflammatory and analgaesic properties. Decoction and infusion of its fruit is considered to be a good remedy for fever, jaundice, asthma, tetanus and rheumatoid arthritis (Ahmadiani et al., 2000).

Myrtle *(Myrtus communis*) fruits and leaves contain phenolic acids, such as ellagic, gallic, caffeic and flavonoids including cathechin, myricetin, hesperidin, esculin and patuletin in methanol extracts. Myrtle can be used as a natural antioxidant as it shows strong antioxidant properties and has a high level of phenolic content. Amensour, Sendra, Abrini, Perez-Alvarez, and Fernandez-Lopez (2010) reported phenolic compounds to be the major contributors of the antioxidant activities of *Myrtus communis.* Moreover, this fruit could be used as an easily accessible source of natural antioxidants and as a food supplement.

The cake, which can be produced with several methods, is very important in bakery product industry since the production and the consumption of it increase continuously as a result of the increase in population, urbanization, and easement of access and application of new technologies. Cake products can be produced in wide variety of formulations all over the world. The differences in the formulation of the cakes make them attractive not only for their pleasing flavors but also for their appearance. Sponge cake has a special and important place in the variety of cakes (Dizlek, 2003; Dizlek & Altan, 2015).

The aim of this study was to utilize hawthorn, medlar, elaeagnus and myrtle as wild fruit powders in the production of sponge cakes by partially replacing them with wheat flour. To the best of the author's knowledge there is no report on the use of wild fruits in the formulation of sponge cake. Therefore, the effects of wild fruit replacement on the chemical and textural properties, total phenolic content, antioxidant activity and staling of sponge cakes were investigated.

Materials and Methods

Materials

Hawthorn (*Crataegus* spp.), medlar (*Mespilus germanica* L) and elaeagnus (*Elaeagnus angustifolia* L.) samples were obtained from Kayseri, Turkey and myrtle *(Myrtus communis* L.) samples were obtained from Mersin, Turkey. Sugar, eggs, vanillin, salt, flour, baking powder and surfactant (monoglyceride and diglyceride ester) were purchased from local markets.

Methods

Chemical Analyses

The fruits were dried and grounded at room temperature for a month, and then the samples were sieved through a 0.5 mesh. Moisture of the cake, fruit and the fruit ash contents were determined following AACC methods (AACC, 2000).

Cake Preparation

The creaming process was used for the preparation of the samples as described Özer, Dizlek, Kola, and Altan (2004). Initially, 100 g eggs were mixed in a mixer at a speed of 1 for 2 minutes (Kitchen Aid Classic, USA). Then 19.3 g surfactant and 60 g water were added and mixed at the same speed and time. After this, 144 g sugar was added and mixed for 2 min at the same speed. Two hundred grammes of wheat

flour (or wheat flour and fruit powder), 6.9 g of baking powder, 0.8 g of salt and 1.5 g of vanillin were added and mixed in the same way. Finally, the batter was mixed for 30 s at speed 4. A standard amount of batter (40 g) was placed in 8-cup non-stick muffin pans and baked for 30 min at 210 °C in a laboratory oven with air circulation (Kenwood, Model NW796, China). After baking, the cake samples were carefully taken out of the muffin pans and cooled at room temperature for an hour (Dizlek, 2015). The cakes were packed in polypropylene bags and were stored at room temperature in a dry place. Fruit powders were used at 5% and 10% levels in the sponge cake form and these cakes were compared to control cakes which were fruit-free in the composition. These are abridgments:

H5: 5% Hawthorn, H10: 10% Hawthorn, M5: 5% Medlar, M10: 10% Medlar, E5: 5% Elaeagnus, E10: 10% Elaeagnus, My5: 5% Myrtle, My10: 10% Myrtle.

Texture and Colour Properties of Cakes

Texture profile analysis was performed using at texture analyzer (Stable Micro System, TA-XT2Plus, England). The upper parts of the cakes were removed and cake crumb texture profile analyses were performed. A 50 mm diameter probe was used and the device was calibrated to 5 g weight. The initial force was 10 g and force was applied to the samples twice. Between the first and second, landings were set to 5 seconds delay, the probe was reduced to 10 mm/sec until the center of sample's deformation was 40%. The pre-test speed of 1mm/sec, test speed of 1mm/sec and post-test speed of 10mm/sec were set up and hardness, springiness, chewiness and adhesiveness were obtained 1 h, 1 d, 7 d and 14 d after baking.

Colour analyses of the crust and crumb of the cake samples were determined with a colour measurement device (Konica-Minolta, CR400, Japan). The device was calibrated with the standard calibration scale, then readings were taken through samples and values were recorded in the form of L^* (0=black, 100=white), a^* (+value=red, -value=green) and b^* (+value=yellow, -value= blue).

Total Phenolic Content of Cake Samples

Cakes were cut into slices and dried in the oven at 40 °C for 24 h. Then they were sieved through a 35 mesh screen. One gram of cake and 10 mL of 80% methanol were added and shaken at 200 rpm at 37 °C for 2 h. The mixture was centrifuged (Nüve, NF 800R, Turkey) at 3100 *g* for 10 min. The filtrate was used for analyses.

The Folin Ciocalteu procedure of Sudha, Baskaran, and Leelavathi (2007) was followed. One hundred microlitres of sample and 900 µL water were added and then 1 mL of 10% diluted Folin-Ciocalteu reagent and 2 mL of 10% Na_2CO_3 solution were added. At room temperature, the mixture was incubated in dark place for an hour. For a control sample, 0.5 mL of distilled water was used. The absorbance was read at 765 nm by using a spectrometer (Shimadzu UV-1700, Japan). The data were expressed as gallic acid equivalents (GAE) in mg per g of dry-material.

Free Radical-Scavenging Activity of Cake Samples

The procedure of Wronkowska, Zielińska, Szawara-Nowak, Troszyńska, and Soral-Śmietana (2010) was used for estimation. DPPH (2,2-diphenylpicrylhydrazyl) solution was prepared by dissolving 10 mg of DPPH in 25 mL of 80% methanol. Two hundred and fifty microliters of DPPH solution and 2.11 mL of 80% methanol were added and 100 µL of methanolic extract was mixed. The mixture was incubated at room temperature in the dark. The absorbance was measured at 517 nm by using a spectrometer (Shimadzu UV-1700, Japan). The ability to scavenge the DPPH radical was calculated by the following formula:

Free-radical scavenging activity (%): $[1- (A_s/A_0)] \times 100$

Where A_0 is the absorbance of the control and A_s is the test sample.

Sensory analysis

Sensory analysis of the cake samples was conducted to identify fruit powder rate by ten panelists in the Department of Food Engineering at Erciyes University, Kayseri, Turkey. For this purpose, fruits' powders at the level of 5 and 10% were used in cakes, which were preliminarily determined by sensory acceptance test. Cake samples were evaluated for overall acceptance on a nine-point hedonic scale ranging from 1 (extremely dislike) to 9 (extremely like). In addition, samples were evaluated for appearance, odour, flavour, texture and overall acceptability.

Statistical Analysis

Statistical differences between values were evaluated by the Tukey multiple comparison test at the level of $p<0.05$ using the SPSS (17.0.1 (SPSS Inc., Chicago, Illinois, US) software package.

Results and Discussion

Chemical Analyses

The moisture and ash content of the wild fruit samples and wheat flour are presented in Table 1. Özcan, Hacıseferoğulları, Marakoğlu, and Arslan (2005) found that hawthorn had an ash content of 2.28%. Hacıseferoğulları,

Özcan, Sonmete, and Özbek (2005) have reported that medlar had 2% ash content. Aydın and Özcan (2007) found the ash content of myrtle as 0.72%. Differences among the chemical compositions of fruits may be due to variability of growing conditions and variety. We investigated the effects of bioactive and physicochemical properties of wild fruits on cake samples. Lu, Lee, Mau, and Lin (2010) indicated that cake with green tea extract and the control group exhibited no differences in terms of moisture content. In this study, at the end of the 14 d storage; moisture content of cake samples containing medlar, 5% elaeagnus and myrtle were close to control group; whereas cakes with hawthorn powder had higher moisture content. As expected, the moisture content of cake samples decreased statistically at the end of the 14th d. ($p<0.05$).

In the preliminary experiments, the sensory analyses showed that sponge cakes containing high level of fruit powder rated lower scores. Therefore, 5 and 10% fruit powder were decided to be replaced with wheat flour.

As shown in Figure 1, cake samples containing 10% hawthorn powder had the highest moisture content (30%) at the first analyses. The sample of cake containing 5% hawthorn powder is the closest sample to the control group.

Table 1. Chemical analysis of wild fruit and wheat flour

Sample	Moisture (%)	Ash (%)*
Wheat Flour	$13.95^{a}\pm 0.2$	$0.60^{d}\pm 0.1$
Hawthorn	$11.4^{c} \pm 0.4$	$4.9^{a} \pm 0.1$
Medlar	$7.8^{e}\pm 0.4$	$2.8^{b} \pm 0.1$
Elaeagnus	$13.3^{b} \pm 0.3$	$1.9^{c} \pm 0.1$
Myrtle	$8.1^{d} \pm 0.2$	$2.4^{b} \pm 0.1$

a–e, means within a column with different letters are significantly different ($P<0.05$).
Results are given as the mean values ± standard deviation.

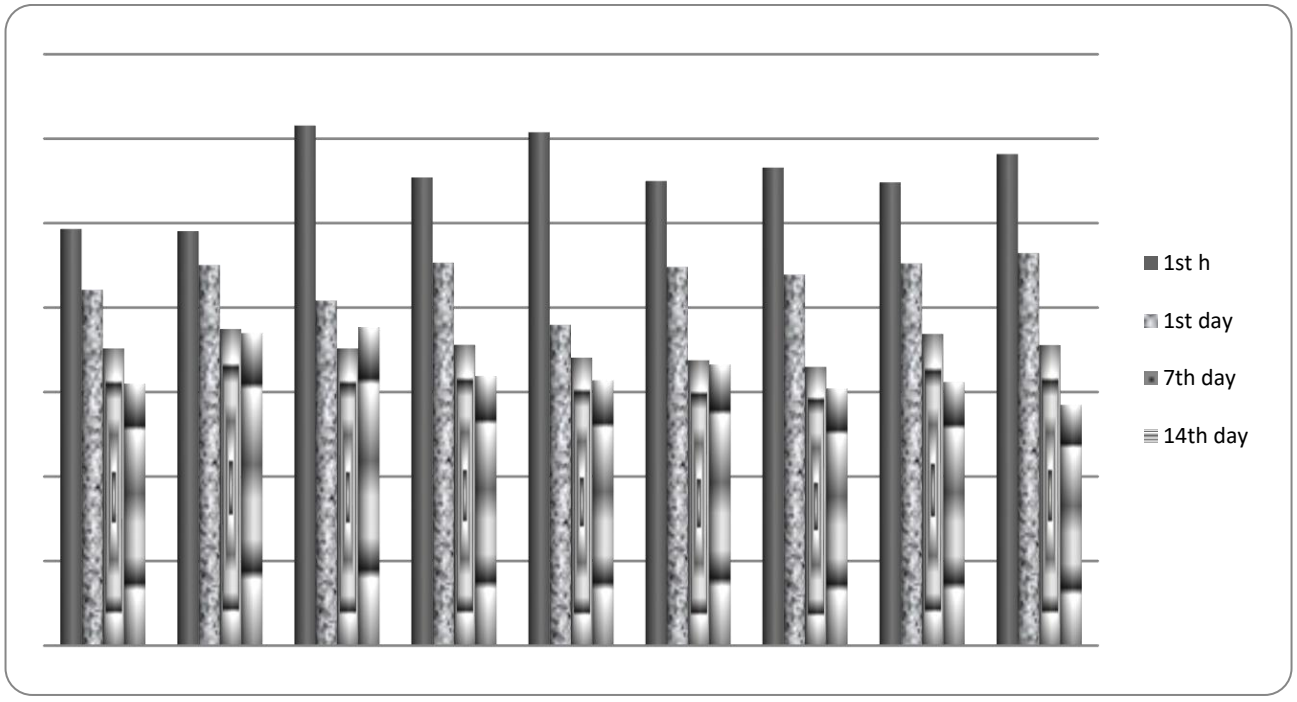
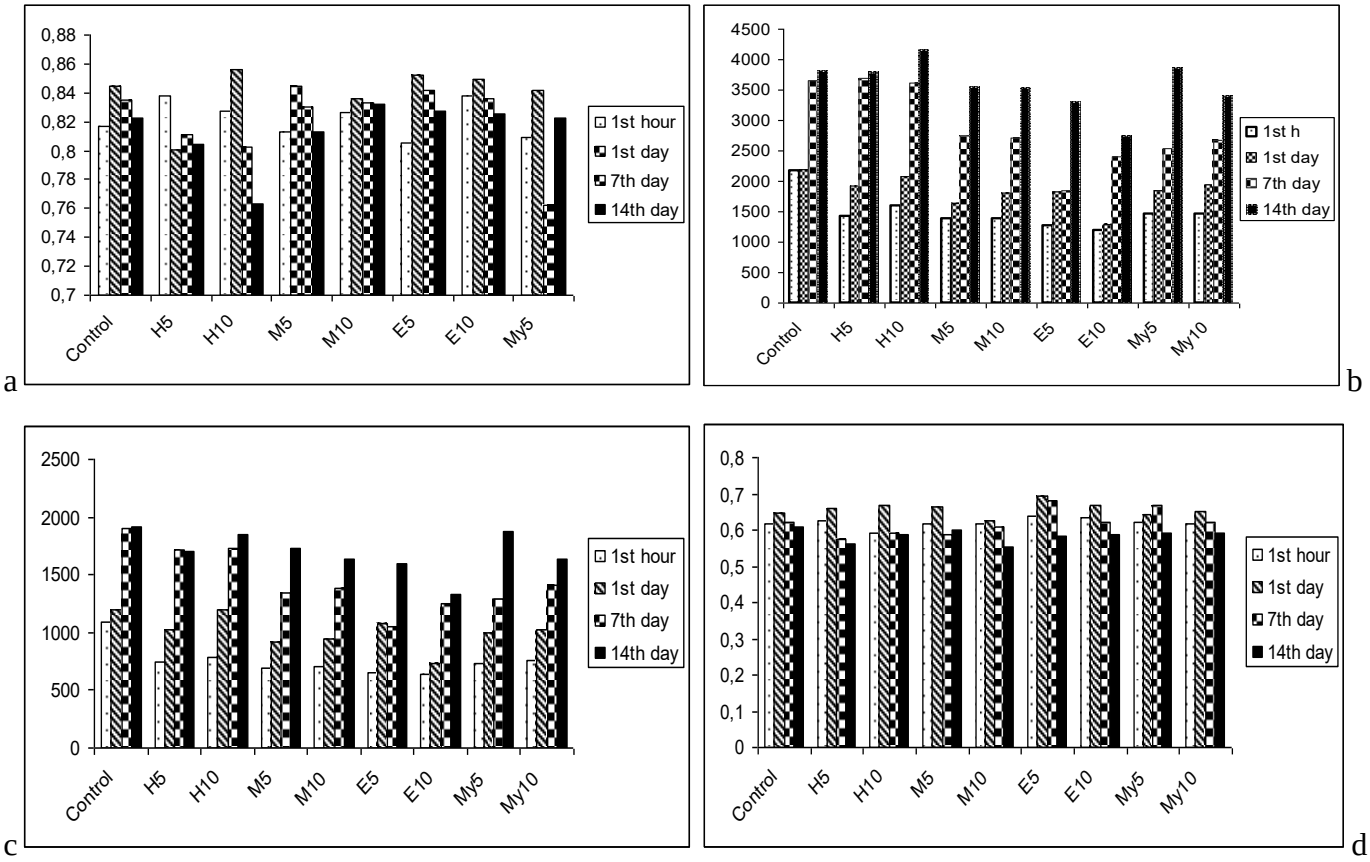


Fig. 1. Moisture content of sponge cake samples

Textural Characteristics of Cake Samples

Figure 2 displays the TPA results of cakes examined in the 1st h after baking. Fruit powder containing cakes had a lower stiffness value than the control group and the values were identical after the 1st and 7th d. At the end of 2 weeks periods, H10 cake samples had higher values than the control group. In this study, cake samples containing fruit powders had higher moisture values during all storage periods. The springiness and cohesiveness values were statistically insignificant ($p>0.05$) among the cake samples. As in hardness values, the chewiness values of cakes containing fruits were lower than the control group. These values were determined for all storage periods. Use of fruit powder affected the hardness value and resulted in an increase in the shelf life. In particular, the use of elaeagnus powder positively affected hardness value at the end of 14th d storage.



H5: 5% hawthorn powder, H10: 10% hawthorn powder, M5: 5% medlar powder, M10: 10% medlar powder, E5: 5% elaeagnus powder, E10: 10% elaeagnus powder, My5: 5% myrtle powder, My10: 10% myrtle powder

Figure 2. TPA profile of the sponge cakes after 1st h, 1st, 7th and 14th d: (**a**) crumb springiness; (**b**) crumb hardness; (**c**) crumb chewiness; (d) crumb cohesiveness

Ertaş and Çoklar (2008) used different types of molasses instead of sugar and found that after 21 d storage, cakes containing molasses had lower values than the control group. In a study, in which barley flour was replaced with wheat flour, at the 96th and 120th h, the hardness values of cake samples containing 30% barley flour were lower than the control. Ronda, Gómez, Blanco, and Caballero (2005) used some sugar alcohols and oligosaccharides instead of sugar in sponge cake. Especially when isomaltose was used, the stiffness value was lower than the control; while oligofructose, polydextrose and mannitol had higher values than the control. In one study, the use of 10% banana powder resulted in hardness values get close to the control, however when the powder level increased, the hardness value also increased. Jia, Kim, Huang, and Huang (2008) found that when 10, 40 and 70% levels of almond flour were replaced with wheat flour; stiffness was significantly reduced with the increase in almond flour.

Colour Properties of Cake Samples

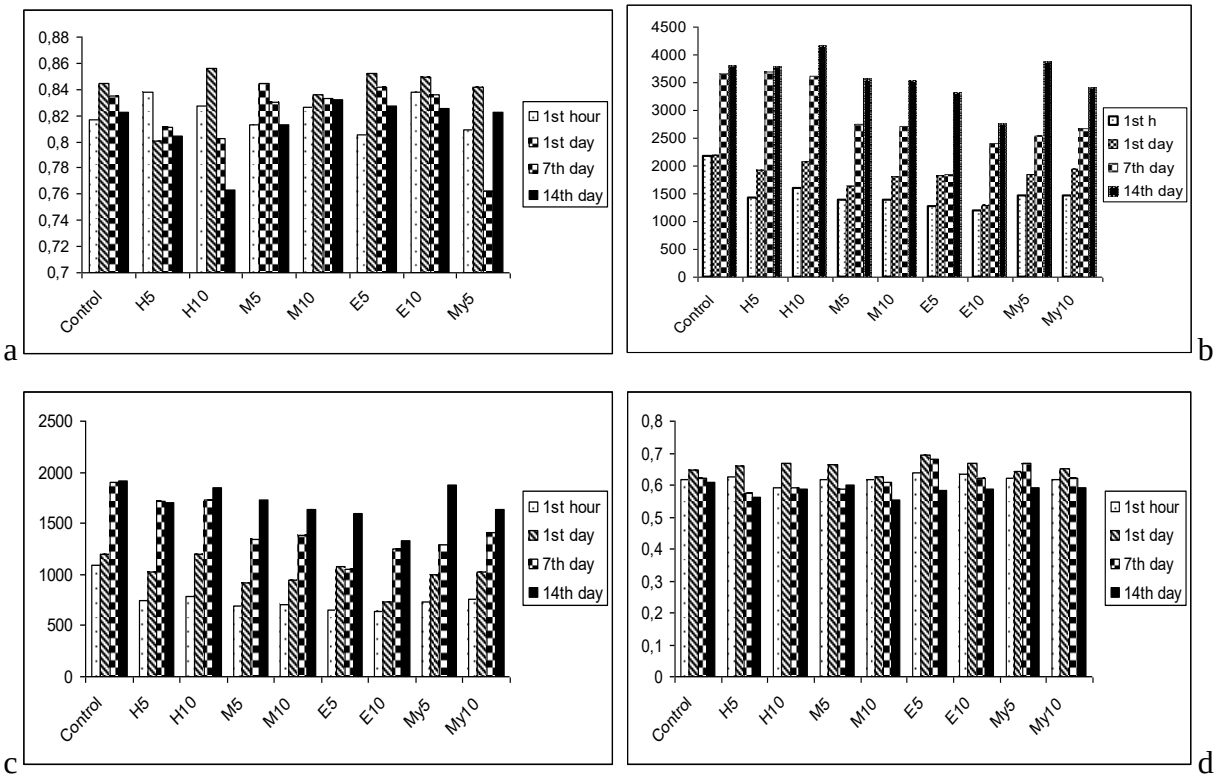
Colour parameters are important for formulations or processing. In cake analysis (Table 3), cake colour was measured as crust and crumb values and the lightness of the crust was found to be lower than that of the crumb due to exposure to high temperature. The control group had the highest *L** values in crumb at 1st h after baking and H5 sample had the nearest value to control; while M10 had the lowest *L** value. The highest difference in crumb redness value was obtained from the cake sample containing 10% medlar powder, which was an expected result because of the colour contribution of medlar fruit. Fruit powder containing cake samples had a higher *a** value than the control group. It was determined that *L** and *a** values increased with the addition of fruit powder.

As shown in Table 4, the highest *L** value was measured in the control group in crust. The *L** value of the control and H5 samples decreased at the end of the storage period of 14 d. In this study, E5 had the highest *a** value after 1 h baking. Among the sponge cake samples, statistically significant ($p<0.05$) differences were found during the storage period. H5 had the highest value 1 h after baking; while E10 had the highest value at the end of the 14th d.

Capriles et al. (2008) reported that control group had the highest *L** value and with the increase of amaranth flour the value decreased. Lu et al. (2010) also reported that the *L** value decreased with the addition of green tea extract. Capriles et al. (2008) pointed out that the use of amaranth flour in cakes decreased the *L** value. Lu et al. (2010) reported that the addition of green tea extract powder to cake samples lowered the *L** value of the crumb compared to the control sample.

Total Phenolic Content of Cake Samples

As expected, the TPC content significantly ($p<0.05$) increased with the addition of fruit powder. The TPC of myrtle was the highest; but in cake samples M10 had the highest TPC value (Figure 3). The control had a value of 266 mg GAE/100 g dry sample and M10 had 1678.9 mg GAE/100g dry sample 1 h after baking. In one study; TPC increased from 2.07 mg/g to 3.15 mg/g with the addition of 25% apple pomace (Sudha et al., 2007). In this study, at the end of 14 day storage period, the TPC of the samples decreased.



H5: 5% hawthorn powder, H10: 10% hawthorn powder, M5: 5% medlar powder, M10: 10% medlar powder, E5: 5% elaeagnus powder, E10: 10% elaeagnus powder, My5: 5% myrtle powder, My10: 10% myrtle powder

Figure 2. TPA profile of the sponge cakes after 1st h, 1st, 7th and 14th d: (**a**) crumb springiness; (**b**) crumb hardness; (**c**) crumb chewiness; (d) crumb cohesiveness

Table 2. Crumb colour values of sponge cake samples

Samples	*L**				*a**				*b**			
	1st hour	1st day	7th day	14th day	1st hour	1st day	7th day	14th day	1st hour	1st day	7th day	14th day
Control	79.67^{Ba} ± 0.44	79.95^{Ba} ± 0.57	79.91^{Ba} ± 0.32	80.74^{Aa} ± 0.51	4.43^{Ad} ± 0.24	4.46^{Ad} ± 0.33	4.21^{Ad} ± 0.23	4.06^{Ade} ± 0.36	24.81^{Ca} ±0.56	26.18^{Ba} ±0.68	27.80^{Aa} ± 0.66	28.34^{Aa} ± 0.33
5% Hawthorn	70.50^{Ab} ± 0.52	70.65^{Ab} ± 0.51	70.43^{Ab} ± 0.39	70.68^{Ab} ± 0.35	2.94^{ABe} ± 0.24	2.92^{ABe} ± 0.21	2.63^{Be} ± 0.13	3.32^{Ae} ± 0.48	23.68^{Bb} ± 0.17	22.54^{Cc} ± 0.43	25.30^{Ab} ± 0.61	22.86^{ABc} ± 0.98
10% Hawthorn	69.21^{Ac} ± 0.57	68.95^{Ac} ± 0.72	67.09^{Bc} ± 0.80	64.66^{Cd} ± 0.44	4.73^{Ad} ± 0.25	3.95^{Bd} ± 0.34	4.69^{Ad} ± 0.54	4.82^{Ad} ± 0.59	22.38^{Cc} ± 0.47	24.28^{Bb} ± 1.14	25.64^{Ab} ± 0.59	24.52^{BCb} ± 0.39
5% Medlar	62.26^{CBe} ± 0.92	63.27^{Bd} ± 0.46	61.22^{Ce} ± 0.62	64.76^{Ad} ± 1.15	6.30^{Bc} ± 0.46	7.06^{Ab} ± 0.47	7.04^{ABb} ± 0.39	7.08^{Ab} ± 0.53	18.66^{Abe} ± 0.44	14.73^{Cf} ±0.50	18.40^{Ae} ± 0.49	16.41^{Bg} ±0.38
10% Medlar	57.79^{Bf} ± 0.57	56.34^{Cf} ± 0.38	57.70^{Bf} ± 0.95	61.10^{Ae} ± 0.31	8.95^{Aa} ± 0.26	8.96^{Aa} ± 0.42	9.30^{Aa} ± 0.39	9.03^{Aa} ± 0.71	17.83^{Aef} ± 0.54	17.85^{Ae} ± 0.48	18.04^{Ae} ± 0.62	17.59^{Af} ± 0.36
5% Elaeagnus	69.54^{Ac} ± 0.31	68.19^{Bc} ± 0.75	66.38^{Cc} ± 0.89	66.36^{Cc} ± 0.47	2.98^{ABe} ± 0.42	2.72^{Ce} ± 0.29	4.61^{Ad} ± 0.19	3.35^{Be} ± 0.22	19.11^{Bde} ±0.55	19.13^{Bd} ± 0.51	20.96^{Ad} ± 0.89	21.68^{Ad} ± 0.28
10% Elaeagnus	64.69^{Bd} ± 0.38	63.79^{Cd} ± 0.40	66.45^{Ac} ± 0.86	67.19^{Ac}± 0.26	4.86^{Ad} ± 0.27	4.08^{ABd} ± 0.37	4.43^{BCd} ± 0.46	3.66^{Ce} ± 0.36	17.33^{Cg} ± 0.42	19.15^{Bd} ± 0.46	21.23^{Acd} ± 0.51	19.56^{Be} ± 0.32
5% Myrtle	63.17^{Be} ± 0.72	63.24^{Bd} ± 0.57	64.97^{Ad} ± 0.60	64.81^{Ad} ± 0.60	6.72^{Ac} ± 0.24	6.33^{Bc} ± 0.16	5.39^{Cc} ± 0.25	5.67^{Cc} ± 0.19	20.10^{Bd} ± 0.74	18.53^{Cde} ± 0.46	22.31^{Ac} ± 0.61	19.56^{Be} ± 0.53
10% Myrtle	59.93^{Bf} ± 0.50	59.21^{Be} ± 0.52	60.76^{Af} ± 0.86	60.75^{Ae} ± 0.34	7.96^{Ab} ± 0.18	7.10^{Bb} ± 0.61	7.49^{ABb} ± 0.32	7.18^{Bb} ± 0.32	18.76^{Ce} ±0.72	22.23^{Ac} ± 0.59	22.10^{Acd} ± 0.52	21.13^{Bd} ± 0.39

a–f: means within a column with different letters are significantly different (P<0.05). A-C: means within a row with different letters are significantly different (P<0.05). Results are given as the mean values ± standard deviation.

Table 3. Crust colour values of sponge cake samples

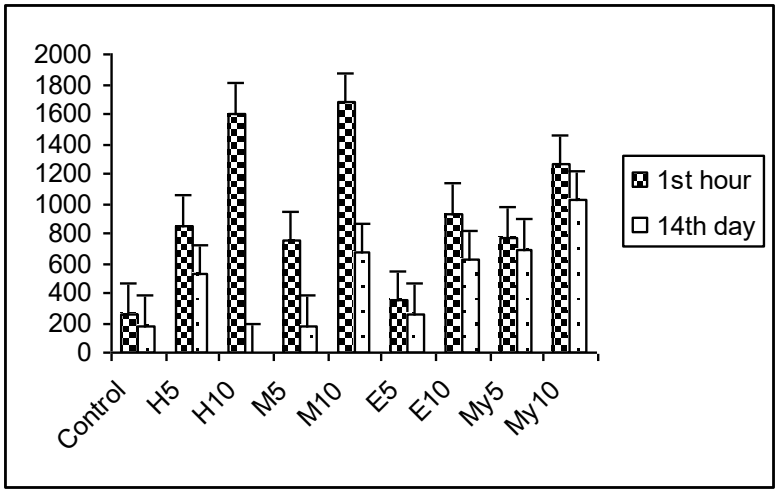
Samples	*L**				*a**				*b**			
	1st hour	1st day	7th day	14th day	1st hour	1st day	7th day	14th day	1st hour	1st day	7th day	14th day
Control	79.26^{Aa} ± 0.41	73.25^{Ca} ± 0.42	74.68^{Ba} ± 0.62	74.78^{Ba} ± 0.61	11.40^{Ae} ± 0.25	8.04^{Bb} ± 0.52	6.34^{Ce} ± 0.28	4.68^{De} ± 0.35	32.33^{Ab} ± 0.36	32.02^{Aa} ± 0.97	29.59^{Bc} ± 0.66	30.22^{Bbc} ± 0.32
5% Hawthorn	66.32^{Ab} ± 0.66	63.94^{Bb} ± 0.65	64.19^{Bb} ± 0.36	64.58^{Bb} ± 0.58	11.97^{Ade} ± 0.35	12.15^{Aa} ± 0.39	10.66^{Bde} ± 0.31	11.16^{Bc} ± 0.47	33.42^{Aa} ± 0.31	28.06^{Dc} ± 0.39	30.61^{Bbc} ± 0.53	29.39^{Cc} ± 0.66
10% Hawthorn	63.71^{Ac} ± 0.59	61.48^{Cc} ± 0.50	62.79^{Bc} ± 0.46	62.65^{Bc} ± 0.34	12.18^{Bd} ± 0.38	13.11^{Aa} ± 0.26	10.37^{Cd} ± 0.53	9.85^{Cd} ± 0.36	26.96^{Ce} ± 0.60	33.14^{Aa} ± 0.69	30.34^{Bbc} ± 0.31	29.96^{Bbc} ± 0.29
5% Medlar	60.51^{Bd} ± 0.42	60.29^{Bd} ± 0.43	60.74^{ABd} ± 0.85	61.70^{Acd} ± 0.63	13.05^{Ac} ± 0.52	11.74^{Ba} ± 0.47	10.53^{Cde} ± 0.34	11.31^{Bc} ± 0.13	31.76^{Ab} ± 0.63	24.13^{Ce} ± 0.61	27.45^{Bd} ± 0.28	27.21^{Bcd} ± 0.80
10% Medlar	56.81^{Bg} ± 0.53	54.84^{Ch} ± 0.46	59.36^{Ae} ± 0.80	59.15^{Af} ± 0.36	13.37^{Ac} ± 0.41	13.49^{Aa} ± 0.29	11.32^{Bc} ± 0.42	10.38^{Cd} ± 0.31	30.27^{Ac} ± 0.50	29.57^{Ab} ± 0.83	27.94^{Bd} ± 0.58	27.73^{Bd} ± 0.61
5% Elaeagnus	59.71^{Bde} ± 0.36	57.20^{Cg} ± 0.28	60.52^{Ad} ± 0.77	60.72^{Ade} ± 0.35	14.92^{Ab} ± 0.17	14.05^{Aa} ± 0.55	11.96^{Cab} ± 0.69	12.79^{Ba} ± 0.31	26.88^{Ce} ± 0.71	25.93^{Cd} ± 0.50	31.08^{Ab} ± 1.17	29.44^{Bc} ± 0.37
10% Elaeagnus	55.02^{Dh} ± 0.29	57.20^{Cf} ± 0.28	59.18^{Bc} ± 0.57	60.13^{Aef} ± 0.57	15.93^{Aa} ± 0.46	15.53^{Aa} ± 0.48	12.64^{Ba} ± 0.56	12.32^{Bb} ± 0.31	25.00^{Bf} ± 0.56	32.83^{Aa} ± 0.65	32.59^{Aa} ± 0.51	32.81^{Aa} ± 0.34
5% Myrtle	58.78^{Cf} ± 0.60	60.30^{Bd} ± 0.75	64.43^{Ab} ± 0.41	60.86^{Bde} ± 0.70	12.91^{Ac} ± 0.28	12.17^{Ba} ± 0.62	10.321^{Dd} ± 0.36	11.3^{Cc} 7± 0.22	28.38^{Ad} ± 0.63	24.36^{Ce} ± 0.62	28.22^{Ad} ± 0.21	26.47^{Be} ± 0.12
10% Myrtle	59.03^{Be} ± 0.26	58.48^{Be} ± 0.67	60.77^{Ad} ± 0.40	60.88^{Ade} ± 0.45	12.93^{Ac} ± 0.19	12.15^{Ba} ± 0.10	11.86^{Cab} ± 0.34	11.24^{Dc} ± 0.27	27.21^{Ae} ± 0.45	29.65^{Ab} ± 0.43	30.46^{Abc} ± 0.45	30.62^{Ab} ± 0.31

a–f: means within a column with different letters are significantly different (P<0.05). A-D: means within a row with different letters are significantly different (P<0.05). Results are given as the mean

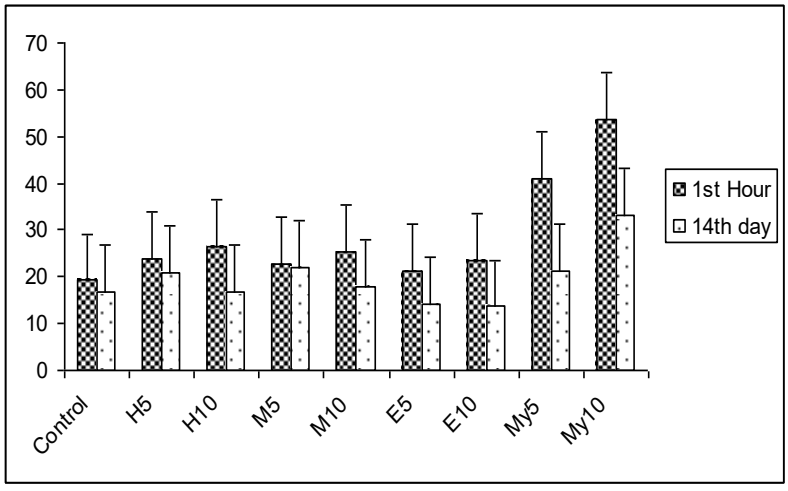
DPPH Activity of Cake Samples

As in TPC, the DPPH activity of the control group was lower than fruit powder containing sponge cake samples (Figure 3). Myrtle had the highest DPPH activity and the myrtle containing My10 sample had the highest value among the cake samples. Fruits have high antioxidant activity and this property decreased, however continued in sponge cakes.

Chang et al. (2006) investigated the effect of storage temperature on phenolics stability in hawthorn fruits and found that phenolic compounds were stable at 4°C, but they were unstable at temperature above 40°C. In particular, at room temperature (23°C) after 6 months storage, 50% degradation was observed in epicatechin and procyanidin-B_2. In addition, phenolic stability was reported to decrease at 4°C, 23°C and 40°C after 6 months storage in hawthorn drink. Catechins lost 70% of their initial components at room temperature after 6 months storage. For hawthorn fruit, it is more effective to store at low temperatures. And also, these results are similar with DPPH activity results. Lu et al. (2010) showed that green tea extract increased the antioxidant activity of cakes. It was identified that after 14 d storage the antioxidant activity of all cake samples decreased, but cakes containing elaeagnus had lower antioxidant activity than control group. In one study regarding the antioxidant activity of polyphenols in extracts of myrtle used for the preparation of myrtle liqueur, the initial value of myricetin-3-*O*-rhamnoside was determined as 1.7 mg/mL; however the value was reported to decrease to 0.5 mg/mL after 12 months storage.



a



b

H5: 5% hawthorn powder, H10: 10% hawthorn powder, M5: 5% medlar powder, M10: 10% medlar powder, E5: 5% elaeagnus powder, E10: 10% elaeagnus powder, My5: 5% myrtle powder, My10: 10% myrtle powder

Figure 3. (a) TPC and (b) DPPH activity of the sponge cakes

Conclusions

In this study, the effect of wild fruit powders was evaluated in terms of sponge cake properties. The result of the present investigation revealed that wild fruits such as hawthorn, medlar, elaeagnus and myrtle can be used in bakery products to improve functional properties after conducting preliminary sensory analysis to assess product acceptability. Although sponge cakes are exposed to high temperatures for a long period of time during baking, samples are able to maintain their TPC and DPPH activity. In particular, elaeagnus may be used as asolution for the stiffness, which negatively affects the shelf life of bakery products. The findings of this research implied that wild fruits in powdered form or their extract can be considered as functional ingredients to provide functional improvements in bakery products.

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DETERMINATION OF ORGANOCHLORINATED PESTICIDE AND POLYCHLORINATED BIPHENYL CONGENERS RESIDUES IN CHICKEN EGGS BY GAS CHROMATOGRAPHY

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ABSTRACT

The aim of this study was to determine the concentrations of organochlorinated pesticides (OCPs) and polychlorinated biphenyls (PCBs) congeners in chicken eggs obtained from various locations in Turkey by gas chromatography and valitadion of the results using different detector systems (MS, MS/MS and ECD). In total, eighteen OCP and PCB compounds were analysed in hundred egg samples. Only β-HCH, 4,4-DDE and PCB138 were found in nine egg samples at concentrations of 5.1-7.2 µg/kg, 8.4-30 µg/kg and 4.2 µg/kg respectively. The detected concentrations of these compounds were found to be lower than the maximum residue levels (MRLs) set by EU. The recoveries, relative standard deviations (RSD), limit of detection (LOD) and limit of quantification (LOQ) were found in the range of 83-111%, 0.9-14.1%, 1.2-3.5 µg/kg and 0.3-10.0 µg/kg respectively.

Keywords: Organochlorinated pesticides, Polychlorinated biphenyls, Chicken egg, Gas chromatography

Introduction

Environmental contamination of persistent organic compounds (POPs) has been widely reported and documented (Virgínia C Fernandes et al., 2011; Ritter et al., 1995). Polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs) are semi volatile, ubiqutous compounds and resistant to biochemical and physical degredation mechanisms (Mansilha et al., 2010; Ritter et al., 1995). They can be accumulated in adipose tissues and fat layers of organisms due to their lipophylic structure (Ritter et al., 1995; Vallack et al., 1998). Exposure to these compounds may lead to cancer formation, neurotoxic disorders, reproductive and behavioral adverse effects (Mansilha et al., 2010). Since these compounds can be accumulated in fat, they can be delivered through foods having certain amount of fat to the human beings (Bernhoft et al., 1997; Polder et al., 2016). They can still be found in the environment and in food materials at levels that may cause harmful effects on human health (such as disruption of hormonal activity) due to their persistent and lipophylic nature despite the production and use of these toxic substances have been banned or restricted in most countries since early 1970s (Virgínia C Fernandes et al., 2011). It has been reported that residual distribution of these pollutants such as dichlorodiphenyltrichloroethane (DDT), PCBs, dieldrin, chlordane, hexachlorobenzene (HCB) and hexachlorohexanes (HCH) are widely found in foods containing fat (Jeong et al., 2014; Liu et al., 2007). Some studies have revealed their occurence in dairy products such as cheese, milk, butter, yoghurt (Keikotlhaile et al., 2010; Salem et al., 2009) and in human milk (Çok et al., 2011; Nasir et al., 1998) as well. Substances reported to be found in these foods include HCHs, DDTs and endosulfan.

Exposure to OCPs and PCBs and accumulation in the adipose tissues occurs through the food chain contamination and environmental pollution (Ahmad et al., 2010). Pesticide contamination from pesticide containing feeds to chicken meat and egg has been reported (Aulakh et al., 2006; Kilic et al., 2011; Olanca et al., 2014; Tao et al., 2009). OCP residues in feed material ingested by chickens and therefore results in the occurence in meat tissues and eggs which are then consumed by consumers. Due to their lipophylic structure, they tend to be accumulated in body tissues. According to some researchers, the proportions of intake of DDTs and HCHs into the body through inhalation and dermal contact are 5.1% and 13.5% of the total intakes respectively, Ingestion through diet was reported as around 94.9% of the total (Kilic et al., 2011).

Chicken egg, and chicken meat tend to be the most popular food items in many countries. However, these food materials are reported as main sources of OCPs by researchers worldwide (Aulakh et al., 2006; Darko & Acquaah, 2007; Fontcuberta et al., 2008) due to their significant amount of fat components. Therefore, regular screening of these foodstuffs is necessary to inform both the consumers and traders to inctease the level of awareness. Although the contamination and toxicity of OCPs and PCBs have been extensively investigated in many developed countries, very few studies are available in the literature on OCP and PCB levels in foods in Turkey (Kilic et al., 2011). Thus, this work was carried out to investigate the degree of contamination with HCH-isomers, heptachlor, aldrin, dieldrin, hekzachlorobenzene, total DDT and polychlorinated biphenyls (PCBs) congener's residues in chicken eggs.

The maximum residue levels (MRLs) for pesticide residues in various foodstuff permitted in the EU are given in respective legislations. The MRLs in Turkish legislation are the same as in EU legislation. The MRLs for OCP and PCB compounds investigated in this study were set as varied from 10 to 50 µg/kg for different compounds (Table 1) (EC 2005; 2008; 2011; Turkish Legislation 2011).

Table 1. MRL values set by EU

Compound Name	MRL (µg/kg)	EC regulation No
Aldrin	20	Reg. (EC) No 839/2008
α-HCH	20	Reg. (EC) No 149/2008
β-HCH	10	Reg. (EC) No 149/2008
γ-HCH (lindane)	10	Reg. (EC) No 149/2008
Dieldrin	20	Reg. (EC) No 839/2008
Heptachlor	20	Reg. (EC) No 149/2008
HCB	20	Reg. (EC) No 149/2008
Sum of DDT and DDE	50	Reg. (EC) No 149/2008
Biphenyl	10	Reg. (EU) No 978/2011

The occurence and level of POPs in various food materials has been studied using different analysis methods (Barriada-Pereira et al., 2005; Bolanos et al., 2007; Cortes-Aguado et al., 2008; Wong et al., 2010). However, little information about comparison of detection capacities and limits of GC system coupled with different detectors for these substances has been found in the literature (Fernandes et al. 2012; Olanca et al. 2014). In this work GC method using ECD, MS and MS/MS detector systems have been used for determination of PCB and OCP residues in hundred egg samples obtained from different regions of Turkey. The results obtained from different detector systems have been elucidated and discussed.

Materials and Methods

Chemicals, Reagents and Standards

OCPs and PCBs standards [Aldrin, dieldrin, hexachlorobenzene (HCB), α-hexachlorocyclohexane (α-HCH), β-hekzaklorosiklohekzan (β-HCH), γ-hekzaklorosiklohekzan (γ-HCH), heptachlor, 4,4-dichlorodiphenyldichloroethylene (4,4DDE), 2,4-dichlorodiphenyltrichloroethane (2,4DDT), 4,4-dichlorodiphenyldichloroethane (4,4DDD), 4,4 dichlorodiphenyltrichloroethane (4,4 DDT), 2,4,4'-Trichlorobiphenyl (PCB28), 2,4,6-Trichlorobiphenyl (PCB30), 2,2',5,5'-Tetrachlorobiphenyl (PCB52), 2,2',4,5,5'-Pentachlorobiphenyl (PCB101), 2,2',3,4,4',5'-Hexachlorobiphenyl (PCB138), 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB153), 2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB180), 2,2',3,3',4,5,5',6-Octachlorobiphenyl (PCB198)] were obtined from Dr. Ehrenstrofer GmbH (Ausburg, Germany). Isooctane secondary and working calibration standard solutions of OCPs and PCBs were prepared to spike egg samples to the required concentrations. Working solutions were prepared in isooctane at 1000 mg/L. PCB 198 (1000 mg/L in isooctane) purchased as internal standard (IS).

The solvents used (isooctane, petrol ether, and acetone) were pesticide residue analysis grade, obtained from Merck&Co., Inc. (Kenilworth, N.J., U.S.A). The absorptive materials used in our study were silica gel (60-70 mesh) and alimuna purchased from Merck&Co., Inc. (Kenilworth, N.J., U.S.A). Silica and alumina were activated after drying at 200°C for 15 h prior to use.

Silanized glass wool (research grade), provided by Serva (Heidelberg, Germany) was used to plug the matrix solid-phase dispersion (MSPD) column. Anhydrous sodium sulphate (pro-analysis) were obtained from Merck&Co., Inc. (Kenilworth, N.J., U.S.A).

Eggs

100 egg samples were collected from local shops and supermarkets in different locations of Turkey (Table 2) representing various production areas from various regions.

Instruments

GC-ECD chromatographic analysis of OCPs and PCBs was performed using Shimazdu 14A gas chromatography (Kyoto, Japan) equipped with a 63Ni electron capture dedector (ECD). Analytes were seperated with Zebron ZB-35 column (30x0.50µmx0.25mm) containing 5% phenylmethylpolysiloxane with phase thickness of 0.25µm (Phenomenex, U.S.A). The temperature program used for the anaylsis was: from 50 °C (3 min) to 170 °C (0 min), and to 290°C (3 min) at 4 °C/min. The injector was set to 270 °C in the split mode. Helium was carrier at 2 mL/min and nitrogen was used at the make-up gas pressure 75 kPA. Idenfitication of peaks was based on comparison of the retention times of compounds in the standard solutions. Quantification of the analyzed compounds was performed using the internal standard and the GC/MS system.

Table 2. The regions where the egg samples collected

Region	City	Number of samples collected
Black Sea Region	Samsun	5
Central Anatolia	Karaman	10
Central Anatolia	Ankara	5
Central Anatolia	Çorum	5
Central Anatolia	Kayseri	10
Central Anatolia	Yozgat	3
Egean Region	Afyon	9
Egean Region	Denizli	7
Egean Region	İzmir	8
Egean Region	Manisa	5
Marmara Region	Balıkesir	10
Marmara Region	Bursa	7
Marmara Region	İstanbul	10
Marmara Region	Kırklareli	3
Marmara Region	Sakarya	3

GC/MS chromatographic analysis of OCPs and PCBs was done using Thermo DSQ GC/MS instrument (Austin, Texas, U.S.A), equipped with ZB-35 column. Helium was used as the carier at 1.5 mL/min. The ion source and transfer were kept 280 °C respectively. Electron impact ionization mode with 70 eV electron energy was selected. The screening analysis was performed in the selected-ion monitoring (SIM) mode monitoring at least two characteristic ion for each compound. In some experiments and for confirmation purpose, scan aquisition mode (m/z 50-450) was used. The oven programme was the same as applied for GC-ECD analysis.

GC-MS/MS analysis was done using Thermo Finnigan Polaris Q Ion Trap instrument (San Jose, CA, USA). The oven programme was the same as applied for GC-ECD analysis. Ion source temperature was set to 250 ºC, and transfer line temperature to 280 ºC. Emission current was 250 µA at SIM mode, and multiplier voltage was 1500 V.

Solid Phase Preperation for Clean-up

100 parts by weight of alumina to 8.8 parts of water was added and shaked until the clumpings disappeared. It was kept for 24 hours in the dark at room temperature to equilibrate the final water content at 9 % (Hogendoom and Goewie, 1998). 5.1 grams of silica gel (70-230 mesh) (Merck, Kenilworth, N.J., U.S.A) was weighed and held at 200 °C for about 15 hours (overnight) in an oven. Then it was placed in the desiccator until cooling to the room temperature.

Sample Preparation

Sample preparation was done according to the method given elsewhere (Valsamaki et al., 2006). The samples were taken to the laboratory and homogenized using a blender. 20g of homogenized sample was taken into a centrifuge tube before adding 30 mL diethylether. Then the mixture was vortexed for 30 seconds. The tube containing mixture was centrifuged at 4000 rpm for 10 minutes. Diethylether phase was seperated and dried under nitrogen gas flow at 40°C. The remaining oil part was then passed through alumina and silica gel columns for cleaning-up.

Extraction and Clean-up

10 µg/kg of standart solution which was prepared using selected OCPs and PCBs was spiked into 1 g of oil extracted from egg sample. 2 mL of petroleum ether was added and vortexed for 30 seconds before transferring into the columns containing varied amounts of alumina/silica and alumina/florisil mixes (4 g alumina/5 g silica, 4 g alumina/5 g florisil, 8 g alumina/10 g silica, 8 g alumina/10 g florisil). The elutions were mixed with 1 mL of hexane and analysed using GC-ECD and GC-MS.

Validation

The analytical method developed for determination of PCBs and OCPs in chicken egg samples was validated according to the EU Decision 2002/657/EC by using GC-ECD and GC-MS. For this purpose, selectivity, specifity, linearity, precision (intra-day and inter-day reproducibility) accuracy were determined. Also in GC-MS/MS recovery was studied for confirmation of three instruments RSDs.

Results and Discussion

The tests were conducted using GC-ECD and GC-MS in three replicates and % recoveries were calculated as given in Table 3. According to the results obtained from GC-ECD analysis, it can be stated that using 8 g alumina/10 g silica and 8 g alumina/10 g florisil columns resulted in lower % recoveries compared to 4 g alumina/5 g silika and 4 g alumina/5 g florisil columns especially for 2,4-DDT and PCBs 138, 153 and 180. The best % recoveries were obtained from both GC-ECD and GC-MS when combination of 4 g alumina/5 g silica was used in extraction and clean-up step for 24DDT, PCB153 and PCB180.

Table 3. GC-ECD and GC-MS % recoveries after clean-up process

Pesticides	GC-ECD				GC-MS	
	Recovery (%)				Recovery (%)	
	A	B	C	D	A	B
HCB	109.75	102.60	107.19	114.90	97.23	97.73
Dieldrin	106.15	89.07	83.90	102.70	92.53	114.70
24DDT	84.05	88.43	64.28	73.00	110.73	73.23
PCB28	113.35	101.50	169.80	144.63	107.70	112.40
PCB52	106.10	106.17	112.13	119.33	99.33	115.40
PCB101	98.75	95.70	91.17	94.80	101.10	113.83
PCB118	113.03	105.63	89.53	93.30	101.77	126.33
PCB138	81.53	84.30	70.00	72.03	96.87	106.63
PCB153	91.85	85.90	55.75	74.80	96.67	81.90
PCB180	118.10	106.02	73.83	100.00	91.13	59.35

A: 4 g alumina/5 g silica. B: 4 g alumina/5 g florisil. C: 8 g alumina/10 g silica. D: 8 g alumina/10 g florisil

The selectivity of the methods used was assessed by the analysis of six blank samples. No peaks of interfering compounds were observed within the intervals of the retention time of the analytes in any of these samples. Additionally, spiked samples with mix of standarts prepared at concentration of 5 µg/kg in isooctane were analysed using GC-MS. A typical chromatogram of spiked egg oil sample obtained from GC-MS is given in Figure 1.

Linearity was obtained from the triplicate injections matrix-matched calibration standard solutions at 5 levels (0.5, 10, 15 and 20 µg/kg) by using internal standard method. The correlation coefficients (r^2) were calculated in the range of 0.9564-0.9999 for GC-ECD and in the range of =0.9701-0.9994 for GC-MS.

The accuracy was evaluated by recovery tests; analyzing fortified blank samples at the same concentration levels used in the precision tests (5, 10 and 15 µg/kg in oil) for egg samples for GC-ECD and GC-MS. The accuracy and precision of the results of the method (Table 4) confirm to the values given in Decision 2002/657/EC. Thus, the mean accuracy values obtained in the recovery tests were between 86 and 116% and for intra-day (n=6) study, RSDs were obtained in the range of 1.10-15.31% and for inter-day study RSDs were obtained in the range of 2.73- 17.51% from validation results obtained using GC-ECD as given in Table 4.

The mean accuracy values obtained in the recovery tests were between 81 and 116 % and for intra-day (n=6), RSDs were obtained in the range of 0.30-7.20 and for inter-day study RSDs were obtained in the range of 1.20-10.10% from validation results obtained using GC-MS (Table 4). The precision of the method was determined in two stages: repeatability (intra-day) and intermediate precision (inter-day). Repeatability was expressed by the RSD of the results from six replicates analysed on the same day by the same analyst using the same instrument. The intermediate precision was expressed by the RSD of the results of eighteen analyses performed on three different days (n=3), six analyses/day, by the same analyst using the same instrument.

Recovery tests at 10 µg/kg concentration (n=10) was done in GC-ECD, GC-MS and GC-MS/MS systems. The mean recoveries and RSDs were given at Table 5. Recovery was obtained in the range of 70-120% and RSDs were obtained below at 20%.

GC-ECD, GC-MS and GC-MS/MS methods have been applied to hundred egg samples and the analyses of OCP and PCB congener's residues were determined. As a result of an efficient clean-up step, the interfering substances and background noise have been eliminated. Thus, the determination of each compound has been succeeded in high accuracy and precision. Nine egg samples showed the presence of β-HCH, 4,4-DDE and PCB138. Quantification of the substances was carried out through the matrix-matched calibration curves by GC-ECD, obtained in terms of µg/kg of sample according to the recovery values given in Table 5. The highest concentration found was 30 µg/kg of 4,4-DDE in a sample obtained from Karaman. Other regions that OCP and PCB residues found in samples were Kayseri, Balıkesir, and Yozgat. Other than nine egg samples, the other results were always lower than the LOD values given in Table 6. The detected amounts of β-HCH, 4,4-DDE and PCB 138 in nine egg samples were in the range of 5.1-7.2 µg/kg, 8.4-30 µg/kg and 4.2 µg/kg respectively.

The main analytical problem in chromatographic analysis of foods has been reported as the complexity of the matrix (Fugel et al., 2005) together with interfering co-extractive substances. These substances may deteriorate the chromatographic column (Garrido Frenich et al., 2006). Therefore, the analysis of OCPs and PCBs in egg samples involved a sample preparation step including a clean-up steps prior to extraction process.

The multi-residue methodology for the determination of 11 OCP and 7 PCB substances in egg samples by GC-ECD, GC-MS and GC-MS/MS using a clean-up process has been applied. Using matrix matched calibration procedure avoided matrix interference effects. Recoveries were found to be between 83% and 111%. The LOQs of substances analysed were lower than the MRL established for eggs in the European Union. In agreement with the findings of Olanca et al. (2014), the detected amounts of OCP and BCB substances found in nine egg samples were found to be lower than MRLs set by EC (EC 2005, 2008, 2011).

Chan et al. (1996) analysed 51 PBC and 17 OCP in *Thaleichthys pacificus* oil using GC-MS and they found β-HCH, 4-4 DDE and PCB138 at the range of 5-10, 30-70, and 2-6 ng/g lipid respectively depending on the location where samples were collected. The amount of residues they have detected in fish oil samples seems lover when compared with the results of this study (from 4.2 to 30 µg/kg egg sample). The reason might be the differences in the concentration of these residues in feeding material of chicken and fish.

Table 4. Method performance of GC-ECD and GC-MS (spike levels: 5,10 and 15 µg/kg)

Analyte	5 µg/kg						10 µg/kg						15 µg/kg					
	Average recovery (%)		Intra-Day precision (RSD %, n=6)		Inter-Day precision (RSD %, n=18)		Average recovery (%)		Intra-Day precision (RSD %, n=6)		Inter-Day precision (RSD %, n=18)		Average recovery (%)		Intra-Day precision (RSD%, n=6)		Inter-Day precision (RSD %, n=18)	
	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD	GC-MS	GC-ECD
Aldrin	86	94	4,10	3,40	4,20	8,79	98	93	3,10	1,40	4,93	4,05	109	93	7,20	2,20	9,60	4,33
α-HCH	100	104	2,60	7,90	4,13	10,00	99	90	4,00	9,30	5,20	11,00	116	98	6,00	2,70	10,00	2,73
β-HCH	108	98	4,80	15,31	7,90	15,67	97	90	6,10	4,90	7,90	6,95	104	96	2,40	4,00	9,50	8,87
γ-HCH (lindane)	116	102	3,10	7,90	3,80	14,26	99	107	2,10	3,90	3,57	4,75	105	100	5,73	4,00	8,20	4,50
Dieldrin	102	104	4,63	1,10	8,30	7,93	85	107	2,60	8,90	4,00	10,05	94	102	6,60	3,50	9,67	4,17
Heptachlor	100	116	2,73	14,57	3,00	17,51	97	90	3,10	6,70	4,07	12,80	105	96	7,10	2,60	8,00	4,13
Heptachlorepoxide	96	98	1,70	8,45	2,17	10,81	93	97	1,10	1,80	3,13	3,05	111	100	1,30	4,50	3,47	4,97
HCB	106	96	1,50	4,40	3,50	5,76	100	103	2,10	5,70	4,37	7,00	108	98	6,00	3,70	7,40	4,13
2,4-DDT	100	94	2,70	1,80	3,00	6,05	79	110	2,30	4,10	2,63	4,20	86	100	5,00	4,50	7,57	6,20
44-DDT	100	96	3,77	3,80	4,30	7,50	75	105	5,00	4,05	5,07	8,20	95	100	6,20	4,50	7,10	8,45
44-DDD	110	86	1,90	8,45	1,90	14,28	90	103	1,40	1,10	1,97	5,70	95	102	6,50	2,40	8,47	3,73
44-DDE	102	102	0,80	6,18	1,30	8,44	94	103	2,00	4,70	2,37	7,80	94	96	5,70	2,75	7,53	3,27
PCB 28	102	95	2,23	7,66	2,30	11,51	96	109	1,90	1,60	2,23	3,70	97	100	5,77	3,60	6,80	6,60
PCB 30	106	92	2,00	4,02	3,37	9,82	101	104	2,20	1,50	3,47	2,95	116	101	6,80	3,20	10,10	4,53
PCB 52	104	94	0,60	5,00	1,93	7,67	96	100	2,20	1,90	2,40	4,05	97	101	5,20	2,10	5,97	5,47
PCB 101	100	99	1,50	6,32	1,60	8,60	93	105	1,70	0,90	2,07	7,20	92	100	4,20	3,80	5,83	5,93
PCB 118	106	96	2,30	5,34	4,40	10,57	93	105	0,30	1,30	1,43	2,55	91	99	3,30	1,30	5,60	3,40
PCB 138	108	94	1,30	3,20	1,73	6,62	96	105	1,40	0,20	2,67	3,50	95	94	6,10	3,50	7,27	3,60
PCB 153	104	93	1,13	4,15	1,30	5,45	81	100	2,30	0,40	3,03	4,85	91	101	5,20	1,70	6,07	5,87
PCB 180	106	94	0,83	5,20	1,20	6,76	84	97	1,77	0,50	2,20	2,50	88	94	0,70	2,10	6,77	3,10

RSD: Relative standard deviation

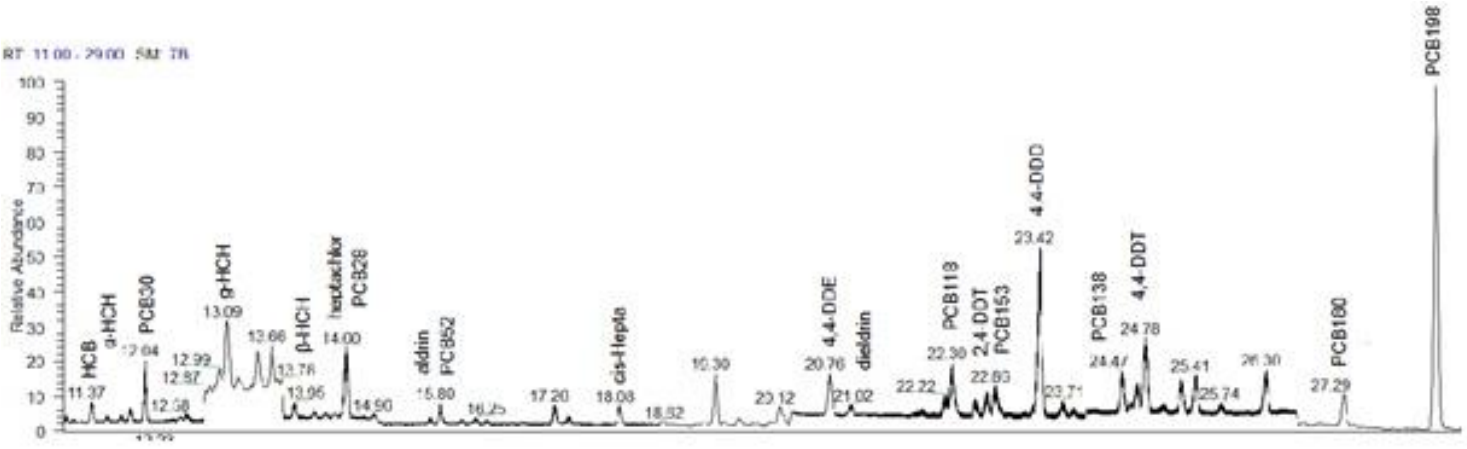

Figure 1. GC–MS chromatogram of mixture of OCPs and PCBs (5 µg/kg)

Table 5. Recovery efficiencies of methods (spike level: 10 µg/kg)

Compound Name	Recovery (%)			RSD (%)		
	ECD	MS	MS/MS	ECD	MS	MS/MS
Aldrin	94	98	95	7.2	3.8	7.1
α-HCH	90	97	104	10.4	4.9	14.1
β-HCH	89	98	102	8.5	5.6	7.7
γ-HCH (lindane)	104	99	111	4.1	2.8	9
Dieldrin	108	86	101	7.4	2.5	4.9
Heptachlor	91	99	96	7.1	2.9	7.3
HCB	102	97	95	7.0	5.0	7.9
2.4-DDT	110	84	99	5.7	2.4	8.2
4.4-DDT	103	83	94	7.5	5.4	11.3
4.4-DDD	101	92	102	6.5	0.9	7.6
4.4-DDE	103	94	96	5.4	1.8	7.2
PCB 28	109	97	104	5.5	2.0	8.4
PCB 30	103	99	111	4.1	2.9	6.6
PCB 52	100	96	101	5.4	1.7	5.7
PCB 101	105	94	101	8.9	1.6	9.7
PCB 138	103	95	89	8.6	1.3	8.8
PCB 153	99	85	92	8.7	2.4	8.5
PCB 180	100	84	96	9.0	1.6	8.1

Table 6. Limit of detections (LOD), limit of quantitations (LOQ) and correlation cofficients (R^2) of GC-MS and GC-ECD

	Linearity (R^2)		LOD (µg/kg)		LOQ (µg/kg)	
Compound Name	**GC-ECD**	**GC-MS**	**GC-ECD**	**GC-MS**	**GC-ECD**	**GC-MS**
Aldrin	0.9862	0.9930	1.5	2.4	5.2	8.1
α-HCH	0.9933	0.9973	2.5	1.7	5.2	5.8
β-HCH	0.9564	0.9854	2.5	1.5	7.5	4.9
γ-HCH (lindane)	0.9848	0.9982	2.5	1.2	8.4	4.0
Dieldrin	0.9873	0.9837	1.5	1.0	5.2	3.3
Heptachlor	0.9934	0.9897	2.2	1.9	7.5	6.3
HCB	0.9882	0.9935	1.5	0.9	5.2	3.0
2.4 DDT	0.9827	0.9753	3.1	1.0	10.0	3.5
4.4 DDT	0.9932	0.9701	3.5	1.6	7.2	5.2
4.4 DDD	0.9991	0.9829	2.5	0.9	5.1	3.0
4.4 DDE	0.9873	0.9900	1.5	1.2	5.2	4.0
PCB 28	0.9939	0.9943	2.2	1.1	7.5	3.6
PCB 30	0.9981	0.9994	2.3	0.9	7.2	3.1
PCB 52	0.9956	0.9904	2.4	1.0	8.2	3.3
PCB 101	0.9922	0.9909	1.9	1.2	6.3	3.9
PCB 138	0.9949	0.9864	1.5	0.7	5.1	2.3
PCB 153	0.9956	0.9881	1.2	0.6	4.1	2.1
PCB 180	1.0000	0.9877	3.0	0.3	9.8	1.1

Ahmad et al. (2010) analysed Organochlorine pesticide (OCP) residues in eggs and meat samples from Jordan using GC-ECD. They found that 28% (38/134) of the examined eggs were contaminated with OCP residues and according to their study, mainly HCHs and DDTs were the most prominently noticed compounds. Percentage recovery in eggs after fortification at 100 µg/kg were in the range of 80-99% and LOD values were reported as 4-5 µg/kg which are slightly higher than LOD values obtained in this work. They detected HCH substances in 15 egg samples out of 134 at concentrations ranging from 6 µg/kg to 1.3 mg/kg egg. They have also reported DDE and DDT residues at 5 µg/kg-0.6 mg/kg concentrations. Some egg samples they analysed had higher residue concentrations than the samples analysed in this study which might be explained as the effect of geographic location where the samples collected.

Valsamaki et al. (2006) analysed 20 OCP and 8 PCB in chicken eggs using GC-ECD and GC-MS. The average recoveries they reported are ranging from 82 to 110 % which are in a good agreement with the recoveries obtained in this study. They have reported the LOD and LOQ values as in the range of 0.3-0.7 µg/kg and 1.0-2.3 µg/kg respectively. In this study the LOD and LOQ values were found to be almost three fold of these values. The reason might be the difference in techniques used in sample preparation and clean-up procedures.

Conclusions

According to the data obtained by using GC-ECD, GC-MS and GC-MS/MS methods, it can be stated that the best repeatibility and recovery were provided by GC-MS technique. The clean-up procedure using 4 g alumina and 5 g silica columns gave best recoveries. Consequently, the best performance was obtained from clean-up process using alumina and silica combined columns prior to GC-MS method.

In this study, β-HCH, 4,4-DDE and PCB138 were detected in nine egg samples out of hundred samples ranging from 4.2 to 30 µg/kg. Although the detected amounts of residues were below the maximum residue levels (MRLs) permitted in foods, they have been banned for several years in countries of the European Union. Some of them are still present in the environment because of their persistent nature. The health risk of POP exposure through egg consumption was discussed elsewhere (Polder et al., 2016). Therefore, continuous monitoring of OCPs and PCBs residues in food materials is necessary and the monitoring procedures has been well established in many developed countries.

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CHEMOMETRIC EVALUATION OF THE GEOGRAPHICAL ORIGIN OF TURKISH PINE HONEY

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ABSTRACT

The aim of the study was to characterize Turkish pine honey samples and classify them according to their geographical origin. *Marchalina hellenica*, which lives on *Pinus brutia*, is the main source of pine honey in Turkey. The honeybee (*Apis mellifera*) collects honeydew for making pine honey.In this study, 26 pine honey samples from five different districts of Muğla were classified as high quality pine honey via melissopalynological analysis and subjected to chemical analysis to evaluate physicochemical parameters. To classify honey samples according to their geographical origin, multivariate analysis of variance (MANOVA) and linear discriminant analysis (LDA) were performed on the experimental data. By using 13 variables (three microscopic quantities, seven physicochemical parameters, and three selected volatile compounds) as predictors for LDA, all honey samples were correctly classified according to their geographical origin. To the best of our knowledge, there is no information in the literature on the classification of Turkish pine honey according to geographical origin; thus, the outcomes of this study are important for the characterization, classification, and authenticity of Turkish pine honey. In addition, these results can used the comparison and standardization of honeydew honey varieties in the world.

Keywords: Chemical parameters, *Marchalina hellenica*, Melissopalynology, Multivariate analysis, Pine honey

Introduction

Honey is a natural food, and its quality and composition are important for healthy human consumption. Honey can be broadly categorized as floral or honeydew honey. Floral honey is derived from honeybees collecting nectar from plants, whereas honeydew honey is derived from honeybees collecting sweet substances mainly from the excretions of plant-sucking insects (*Hemiptera*) on the living parts of plants or secretions of the living parts of plants (Sanz et al., 2005; Karabagias et al., 2014). Clover, thyme, acacia, and citrus are some examples of floral honey varieties, while pine and fir are examples of honeydew honey varieties.

The majority of the world's pine honey (about 90%) is produced in the Muğla region of Turkey because of the suitable climatic conditions and relative humidity for *Marchalina hellenica* (syn. *Monophlebus hellenicus*) (Coccoidea: Homoptera) and its natural host, *Pinus brutia*. *M. hellenica* is a type of insect that lives on *P. brutia* and is the main source of honeydew. This insect is found only in Turkey and Greece (Santas, 1979). In Turkey, about 30% of all honey is produced in the region of Muğla as the region has nearly 60,000 ha of *Pinus brutia* forest (Şahin, 2000). Turkey produces an estimated 90,000 tons of honey annually (FAOSTAT, 2014) and 25,000 to 30,000 tons of this is pine honey. Most of the pine honey is exported all over the world (Maybir, 2015). Therefore, the quality and authenticity of pine honey is as important as floral honey. Microscopic analysis and chemical analysis show the honey quality and authenticity. Honeydew honey is generally characterized by honeydew elements composed of microscopic algae, fungus spores. If a honey with the ratio "number of honeydew elements (HDE)"/ "number of total pollens (P)" is greater than 3, is considered as honeydew honey (Louveaux et al., 1978; Soria et al., 2004). If the HDE/P is 4.5, is called high density superior quality honeydew honey (Sorkun, 2008).

Moisture, 5-hydroxymethylfurfuraldehyde (HMF), free acidity (FA), lactonic acidity (LA), total acidity (TA), and pH analyses are some of the important criteria for evaluating honey. Among them, HMF is an indication of the quality of honey. HMF is produced from sugars by the decomposition of monosaccharides (Leshkov et al., 2006; Simeonov et al., 2016) when honey is heated or stored for a long time the concentration of HMF significantly increases (Silva et al., 2016).

Moisture is another important factor that determines honey quality as it is the second largest constituent of honey. Moisture affects the physical properties of honey, such as viscosity and crystallization, as well as other parameters such as color, flavor, taste, specific gravity, solubility, and conservation (Escuredo et al., 2013; Silva et al., 2016).

FA, LA, TA, and pH are the other parameters that determine the authenticity of honey. According to White (1975), the pH of honey should be between 3.2 and 4.5. Honey contains between 0.17–1.17% organic acids and between 0.05–0.15% amino acids (D'Arcy, 2007). They are responsible for the characteristic taste and acidity of honey (Krell, 1996). The natural acidity of honey inhibits the growth of microorganisms, as the optimum pH for most organisms is between 7.2–7.4 (Karabagias et al., 2014; Silva et al., 2016).

FA is related to the deterioration of honey. It is characterized by the presence of organic acids in equilibrium with lactone, internal esters, and some inorganic ions such as phosphates, sulfates, and chlorides (Moreira et al., 2010). Electrical conductivity (EC) depends upon the mineral content, organic acids, proteins, and other substances in honey (D'Arcy, 2007). Conductivity is a useful criterion to determine the botanic origin of honey and thus is frequently used in routine analyses (Bogdanov, 2002). The EC value of floral honey is lower than that of honeydew honey (Bogdanov, 1999). Honey contains at least four broad groups of components that have antioxidant activity, polyphenols or phenolic compounds (flavonoids and phenolic acids), enzymes (e.g. glucose oxidase and catalase), ascorbic acid, and peptides (Nicholls & Miraglio, 2003). Volatile compounds are also important for honey quality, and they vary according to botanical origin (Karabagias et al., 2014).

In this study, we experimentally determined microscopic quantities such as the number of honeydew elements (HDE), the number of total pollen (P), and HDE/P. In addition, the HMF, moisture, FA, LA, TA, pH, and volatile contents of pine honey samples were analyzed. Besides the analytical results from the present study, EC (Özkök & Çıngı, 2010) and volatile compounds (Özkök et al., 2016) values from our previous studies were also used for statistical analyses. To the best of our knowledge, there is no information in the literature on the classification of Turkish pine honey according to geographical origin; thus, the outcomes of this study are important for the characterization, classification, and authenticity of Turkish pine honey.

Materials and Methods

Collection of Honey Samples

Honey samples were collected from five areas (Milas, Ortaca, Marmaris, Fethiye, and Datça) around Muğla city

where pine honey beekeeping is extensively practiced. Suitable apiaries were chosen according to vegetation diversity and distance between the villages. Samples were stored in glass containers, shipped to the laboratory, and maintained at 4°C until analysis.

Melissopalynological Analysis (Microscopic Analysis)

Analytes for the identification of P and HDE in 10 g of honey were obtained according to procedure of Louveaux et al., 1978 and Sorkun, 2008.

10 g honey was mixed with 20 mL of distilled water in a tube together with a tablet containing 12542 *Lycopodium* spores. To dissolve the tablet, tubes were incubated for 10–15 min in a water bath at 45°C. After the tablet was fully dissolved, few drops of basic fuchsine were added to stain the pollens and spores, and the mixture was centrifuged at 3500 rpm for 45 min. Water from the centrifuged tubes was removed, and the tubes were dried upside down on a drying mat to fully drain the fluid. Then, 1 mL of 50% glycerine was added to the precipitate of each tube and mixed homogenously. Subsequently, 0.01 mL was withdrawn from this mixture and plated on a lamella. The material was covered by a lamella (18 × 18 mm^2), and two separate analytes were obtained for microscopic analysis.

Examination of the Number of Total Pollen (P)

Pollen and spore analytes were examined and counted under a Nicon Eclipse E400 light microscope at 20× and 40× magnification. During the counting process, analytes were examined starting from the top left corner to eventually cover the whole area (18 × 18 mm^2); the numbers of pollens and *Lycopodium* spores in this area were counted separately. Counts of two separate analytes were obtained, and their averages were applied to the formula below:

$$\text{Number of total pollen}\,\frac{\text{P}}{10}\,\text{g} = \frac{\textit{Pollens counted} \times 12542\,*}{\textit{Lycopodium}\ \text{spores counted}}$$

*Number of spores found in one *Lycopodium* tablet

Examination of the Number of Honeydew Elements (HDE)

In the same analytes in which P was counted, HDE was also counted. During this process, starting from the top left corner to eventually cover the whole area (18×18 mm^2), the numbers of spores, hyphea, and any algae present were counted. The HDE content in 10 g of honey was determined by the following formula:

$$\text{Number of honeydew elements (HDE)}/10\ \text{g} = \frac{\text{Number (spore + hyphea + algae) counted} \times 12542}{\textit{Lycopodium}\ \text{spores counted}}$$

HDE/P Ratio

Based on the results of microscopic identification, all honey samples were identified as high density-superior quality pine honey and thus appropriate for chemical analysis.

HMF Analysis

Bogdanov (2002)'s HMF method was followed for the HMF analyses. Initially, 5 g of honey was dissolved in 25 mL water and transferred to a 50 mL volumetric flask. Then, 0.5 mL of Carrez solution I (15 g of potassium hexacyanoferrate dissolved in water and made up to 100 mL) was added, and the solution was mixed. Subsequently, 0.5 mL of Carrez solution II (30 g of zinc acetate made up to 100 mL with water) was added, mixed, and made up to the mark with water. The mixture was filtered through paper, rejecting the first 10 mL filtrate. Then, 5.0 mL of the resulting filtrate was pipetted into each of two test tubes; 5.0 mL of 0.2% sodium bisulfite solution was added to the second test tube and mixed well. The absorbance of the sample solution was determined against the reference solution at 284 and 336 nm in 10 mm quartz cells within 1 h. HMF values were calculated according to the following formula:

$$\text{HMF mg/kg} = \frac{(\text{Absorbance284} - \text{Absorbance336}) \times 149.7 \times 5 \times \text{Dilution factor (D)}}{\text{Weight (W)}}$$

Moisture Analysis

Moisture analysis was performed according to a refractometric method. The homogenate of 1 g pine honey sample was measured by a refractometer. Each sample was measured twice, and the average value was determined.

FA, LA, TA, and pH Analysis

FA, LA, TA, and pH analyses were performed according to a procedure described by Bogdanov (2002). Initially, 5 g of pine honey was dissolved in a few milliliters of water. The solution was then transferred quantitatively to a 50 mL volumetric flask and filled to the mark with water. After mixing well, 25 mL of the solution was pipetted into a 250 mL beaker. A bar magnet was added, and the initial pH (pHi) was noted. The solution was stirred gently and titrated first with sodium hydroxide solution (up to 10 mL), then (into the same beaker) with sulfuric acid solution (up to the second equivalence point). The results were calculated according to formula.

FA is expressed in milliequivalents of sodium hydroxide required to neutralize 1 kg of honey.

$$FA = V \times T \times (50/25) \times (1000/M)$$

LA is expressed in the same units:

$$LA = [(10 - V) \times T - 0.05 \times V'] \times (50/25) \times (1000/M)$$

TA is expressed in the same units:

$$TA = FA + LA$$

Chemometric Methods

Multivariate statistical analysis of the experimental data was conducted using SPSS statistical software version 23.0 (SPSS Inc., Chicago, IL, USA). Discriminant analysis was performed using multivariate analysis of variance (MANOVA) followed by linear discriminant analysis (LDA). All data were scaled with Fischer's method, and all models were cross-validated using the leave-one-out method. A 26 × 13 data matrix, corresponding to 26 pine honey samples and 13 experimental variables (HDE, P, HDE/P, moisture, pH, FA, LA, LA/FA, EC, HMF, eicosane, 2-furanmethanol, and lidocaine contents) were used to predict the geographical origin of honey samples.

Results and Discussion

Microscopic, physicochemical parameters, volatile compounds analysis results of 26 honey samples showed Figure 1, Table 1, 2 and 3.

For the geographical classification of 26 pine honey samples from five different districts (five samples from Datça, five samples from Fethiye, six samples from Marmaris, seven samples from Milas, and three samples from Ortaca), 13 experimentally determined quantities (HDE, P, HDE/P, moisture, pH, FA, LA, LA/FA, EC, HMF, eicosane, 2-furanmethanol, and lidocaine) were used as predictors for multivariate statistical analysis. All of the 13 predictors were subjected to MANOVA to elucidate the effect of geographical origin on the microscopic and chemical properties of pine honey samples.

According to Codex Alimentarius Committee on Sugars (2001) a maximum value of HMF for mixed or processed honey 40 mg/kg and a maximum value of HMF if the honey and blends of honey originate from regions with a tropical climate 80 mg/kg. In this study, the HMF analysis results of 26 samples revealed a minimum of 0.14 mg/kg, a maximum of 44.54 mg/kg, and an average of 4.93 mg/kg. Unsuitable samples could indicate overheating or inadequate storage conditions.

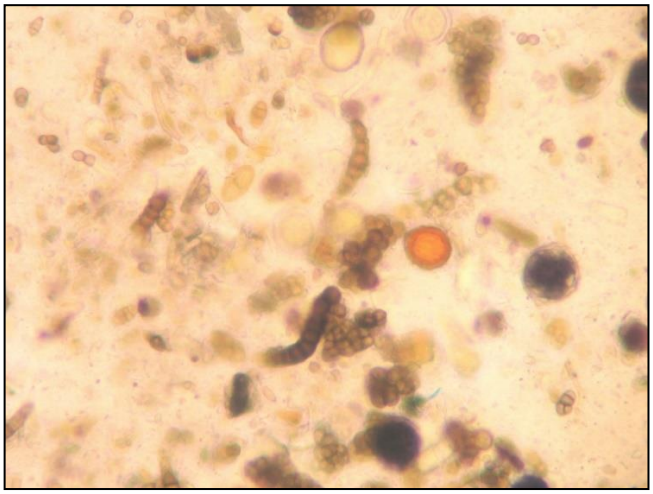

Figure 1. Turkish Pine honey

Table 1. Outcomes of HDE/P analysis of honey samples

Sample No	District	HDE	P	HDE/P	Identification
1	Datça	59408	9995	5.94	High density superior quality pine honey
2	Datça	55770	10940	5.09	High density superior quality pine honey
3	Datça	66041	10778	6.12	High density superior quality pine honey
4	Datça	77949	5124	15.2	High density superior quality pine honey
5	Datça	92446	9507	9.7	High density superior quality pine honey
6	Fethiye	498544	4703	106	High density superior quality pine honey
7	Fethiye	146542	8449	17.3	High density superior quality pine honey
8	Fethiye	89884	10451	8.6	High density superior quality pine honey
9	Fethiye	116040	5889	19	High density superior quality pine honey
10	Fethiye	192481	23292	8.26	High density superior quality pine honey
11	Marmaris	166366	9222	18.04	High density superior quality pine honey
12	Marmaris	189892	11758	16.15	High density superior quality pine honey
13	Marmaris	66284	12736	5.2	High density superior quality pine honey
14	Marmaris	118880	26391	4.5	High density superior quality pine honey
15	Marmaris	168200	28623	5.87	High density superior quality pine honey
16	Marmaris	128168	12370	10.36	High density superior quality pine honey
17	Milas	75542	16802	4.5	High density superior quality pine honey
18	Milas	213810	2388	89.53	High density superior quality pine honey
19	Milas	67215	731	92	High density superior quality pine honey
20	Milas	110731	7231	15.3	High density superior quality pine honey
21	Milas	135453	5495	24.6	High density superior quality pine honey
22	Milas	214864	9241	23.2	High density superior quality pine honey
23	Milas	167851	5860	28.6	High density superior quality pine honey
24	Ortaca	55944	1832	30.5	High density superior quality pine honey
25	Ortaca	123341	12888	9.5	High density superior quality pine honey
26	Ortaca	121619	5320	22.8	High density superior quality pine honey

Table 2. Physicochemical parameters for pine honey samples

Sample No	Moisture (g/100g)	pH	FA (meq/kg)	LA (meq/kg)	TA (meq/kg)	LA/FA	HMF (mg/kg)	EC* mS/cm
1	15.2	4.81	7.36	15.60	22.96	2.12	2.00	1.49
2	15.5	4.67	11.04	15.60	26.64	1.41	1.47	1.52
3	17.2	5.11	9.20	15.60	24.80	1.70	0.42	1.24
4	15.5	5.19	9.20	15.60	24.80	1.70	0.95	1.42
5	17.5	5.24	9.20	10.40	19.60	1.13	2.53	1.51
6	14.0	4.22	14.72	15.60	30.32	1.06	15.26	0.89
7	14.2	4.74	14.72	20.80	35.52	1.41	1.35	1.31
8	14.6	5.17	9.20	18.20	27.40	1.98	0.57	1.19
9	17.0	6.32	18.40	13.00	31.40	0.71	0.50	1.21
10	21.5	4.40	18.40	18.20	36.60	0.99	1.70	1.06
11	17.0	4.17	20.24	26.00	46.24	1.28	12.61	1.83
12	18.4	3.98	20.24	20.80	41.04	1.03	8.26	1.71
13	16.5	4.18	12.88	18.20	31.08	1.41	44.54	1.15
14	18.4	4.26	20.24	20.80	41.04	1.03	5.56	1.66
15	14.0	4.33	20.24	20.80	41.04	1.03	3.67	1.51
16	15.2	4.56	20.24	20.80	41.04	1.03	7.17	1.42
17	15.5	4.83	12.88	18.20	31.08	1.41	2.80	0.94
18	15.0	4.98	12.88	18.20	31.08	1.41	2.40	1.46
19	15.0	4.92	9.20	18.20	27.40	1.98	1.57	1.38
20	15.8	5.08	11.04	18.20	29.24	1.65	2.57	1.27
21	15.0	5.26	7.36	15.60	22.96	2.12	0.14	1.22
22	16.2	4.61	9.20	18.20	27.40	1.98	2.14	1.09
23	16.2	4.59	11.04	18.20	29.24	1.65	1.25	1.17
24	12.0	4.52	16.56	20.80	37.36	1.26	1.70	1.61
25	15.2	5.31	14.72	20.80	35.52	1.41	2.07	2.19
26	17.0	5.22	9.20	15.60	24.80	1.70	3.00	2.26
Average	**16.0**	**4.80**	**13.45**	**18.00**	**31.45**	**1.45**	**4.93**	**1.41**
Min.	**12.0**	**3.98**	**7.36**	**10.40**	**19.60**	**0.71**	**0.14**	**0.89**
Max.	**21.5**	**6.32**	**20.24**	**26.00**	**46.24**	**2.12**	**44.54**	**2.26**

* Data is taken from Özkök & Çıngı, (2010).

Table 3. Volatile compounds found in pine honey samples (% content)*

	Aldehydes		Alcohols	Ketones	Hydrocarbons				Acids		Esters	Others
Sample no	Furfural	2-Furan carboxaldehyde	2-Furanmethanol	3,5-dihydroxy-6-methyl-2H-pyran-4(3H)-one	Eicosane	Heptacosane	Benzene	Octadecane	Benzoic acid	1,2-Benzene dicarboxylic acid	Octadecenoic acid methyl ester	Lidocaine
1	nd	nd	nd	nd	1.02	nd	28.94	nd	nd	nd	1.78	5.64
2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.74
3	nd	nd	1.46	2.41	1.44	nd	nd	nd	nd	nd	nd	2.43
4	nd	nd	1.64	2.57	nd	1.19	nd	nd	1.26	nd	nd	4.67
5	nd	nd	1.35	2.26	nd	nd	nd	nd	nd	nd	0.52	3.46
6	nd	nd	1.92	nd	nd	nd	nd	nd	nd	nd	nd	0.95
7	nd	nd	nd	nd	0.46	nd	nd	nd	nd	1.81	nd	2.01
8	nd	nd	nd	nd	nd	nd	0.75	nd	nd	nd	nd	0.98
9	nd	nd	1.33	2.77	0.38	nd	4.86	nd	nd	nd	0.44	2.50
10	1.33	nd	0.57	1.98	nd	nd	nd	nd	nd	nd	nd	2.09
11	nd	nd	0.96	1.38	0.33	nd	nd	0.44	nd	nd	nd	2.41
12	nd	nd	nd	nd	nd	nd	27.67	nd	nd	nd	nd	1.02
13	nd	nd	1.91	2.33	0.11	0.14	nd	nd	nd	nd	nd	3.21
14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.59
15	nd	nd	1.14	1.50	nd	nd	nd	nd	nd	nd	nd	5.19
16	0.35	nd	1.30	2.43	0.29	0.36	nd	nd	2.28	nd	nd	3.90
17	nd	nd	0.93	nd	3.3	nd	nd	nd	nd	nd	nd	2.82
18	0.49	nd	1.10	2.14	nd	nd	nd	nd	nd	nd	nd	2.27
19	nd	nd	1.20	2.01	nd	nd	nd	nd	0.64	nd	nd	3.10
20	nd	13.05	1.29	3.31	1.43	nd	nd	nd	nd	nd	0.36	1.55
21	nd	nd	1.57	nd	0.70	0.94	nd	nd	nd	nd	nd	2.04
22	nd	nd	1.02	nd	1.36	nd	nd	nd	nd	nd	nd	2.19
23	nd	nd	1.15	nd	0.37	1.30	13.53	nd	nd	nd	nd	1.78
24	nd	nd	nd	nd	1.79	1.89	nd	nd	1.86	nd	nd	7.53
25	nd	nd	1.40	2.47	1.07	nd	nd	nd	1.47	nd	nd	8.86
26	0.51	5.80	1.78	2.01	nd	nd	nd	nd	nd	nd	nd	2.91

[nd] not determined [*] Data is taken from Özkök, Sorkun, & Salih, (2016).

The average pH of honey is 3.9, but it is higher generally for honeydew honey (White & Doner, 1980). The pH analysis results of our honeydew honey samples revealed an average 4.80, a minimum of 3.98, and a maximum of 6.32. Escuredo, Fernandez-Gonzalez & Carmen (2012) reported pH values of between 3.5 and 5.0 for honey samples from Northwest Spain. Similarly, Karabagias et al., (2014) found pH values of between 4.42 and 5.20 for Greek pine honey samples.

In the present study, FA ranged from 7.36 meq/kg to 20.24 meq/kg. FA values should be lower than 50 meq/kg according to the Council Directive 2001/110/EC. All samples (100% of the samples) in our study meet these standards. Higher values could indicate the fermentation of sugars into organic acids. On the other hand according to Silva et al., (2016) the presence of different organic acids, geographical origin, and harvest season can affect honey acidity. LA results revealed an average of 18.00 meq/kg, a minimum of 10.4 meq/kg, and a maximum of 26 meq/kg. TA results revealed an average of 30.81 meq/kg, a minimum of 14.84 meq/kg, and a maximum of 46.24 meq/kg. Karabagias et al., (2014) found that FA ranged between 18.08 meq/kg and 41.54 meq/kg, LA ranged between 1.59 meq/kg and 5.59, and TA ranged between 23.75 meq/kg and 44.94 meq/kg. White & Doner (1980) reported FA values of between 30.29 and 66.02 meq/kg for honeydew honey samples. Bacandritsos (2004) reported a TA value of 36.1 meq/kg for pine honey. Our results were found to be consistent with these results.

Based on Pillai's trace (V= 2.982, F=2.704, p= 0.000 < 0.05) and Wilk's Lambda (Λ=0.000, F=5.714, p=0.000<0.05) statistics, MANOVA revealed that there was a significant multivariate effect of geographical origin on the combination of 13 predictors. However, according to separate univariate ANOVAs, only six of them (FA, LA, LA/FA, pH, EC, and lidocaine) were significant (p<0.05) for the classification of honey samples. Therefore, two different discriminant analyses were performed. The first discriminant analysis was conducted using all 13 predictors (Figure 2a), and the second using only the six significant predictors from ANOVA (Figure 2b).

By using all 13 predictors, LDA revealed two statistically significant discriminant functions:

First function: Wilk's Lambda=0.000, $\chi 2$=136.412, df=52, p=0.000<0.05

Second function: Wilk's Lambda=0.009, $\chi 2$=75.988, df=36, p=0.000<0.05

The first discriminant function accounted for 61.0% of the total variance while the second accounted for 35.8%. As shown in Fig. 1a, all honey samples were correctly classified according to their geographical origin. Overall, 100% of original and 76.9% of cross-validated grouped cases were correctly classified.

By using the six significant predictors from ANOVA, LDA revealed two statistically significant discriminant functions: Wilk's Lambda=0.019, $\chi 2$= 77.727, df=24, p=0.000<0.05

Second function: Wilk's Lambda=0.141, $\chi 2$=38.146, df=15, p=0.001<0.05

The first discriminant function accounted for 62.9% of the total variance while the second accounted for 31.9%. Based on FA, LA, LA/FA, pH, EC, and lidocaine, honey samples from Milas and Ortaca were clearly distinguished from the other groups in which the correct classification rates for Marmaris, Datça, and Fethiye were 83.3%, 80%, and 60.0%, respectively. Overall, 84.6% of original and 69.2% of cross-validated grouped cases were correctly classified.

The results of two discriminant analyses demonstrated that the use of non-significant predictors greatly increased the discrimination rate, and significant multivariate predictors could be as important as significant univariate predictors for sample discrimination.

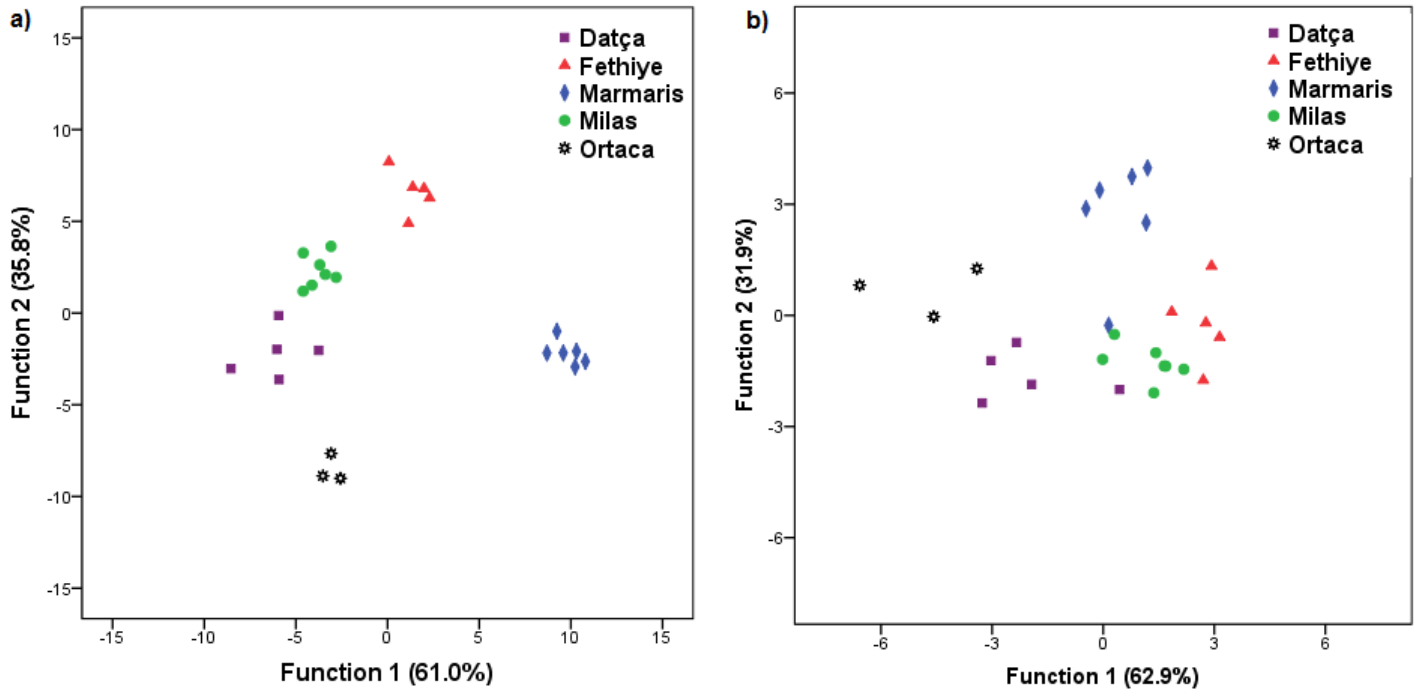

Figure 2. Discriminant functions scatter plot based on a) 13 parameters b) 6 ANOVA significant parameters

Conclusion

This study showed for the first time a comprehensive analysis of Turkish pine honey. All honey samples were correctly classified according to their geographical origin based on microscopic properties, physicochemical properties, and volatile contents. The findings of this study are important for the characterization and authenticity of Turkish pine honey. In addition, these results can support the comparison and standardization of honeydew honey varieties in the world.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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WHAT DO THEY KNOW ABOUT FOOD SAFETY? A QUESTIONNAIRE SURVEY ON FOOD SAFETY KNOWLEDGE OF KITCHEN EMPLOYEES IN ISTANBUL

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ABSTRACT

Lack of hygiene knowledge and perception of food handlers, play a big role in outbreaks. The purpose of this study was to evaluate knowledge and awareness of food handlers with regard to food safety in Istanbul. The survey was conducted involving 400 kitchen employees working in 22 kitchens in Istanbul. The findings have been analysed with respect to gender, educational level and work experience variables in the SPSS program. According to results; 90.8% of participants know that, food hygiene means to remove the illness-causing factors in food. It was determined that the level of knowledge of food handlers did not differ according to gender. According to the hypothesis that we obtained that there is a difference according to education levels. Also, knowledge of the food handlers was significantly different according to the job position and to the duration of the food handler in a food establishment.

Keywords: Food safety, Knowledge, Awareness, Food hygiene

Introduction

Food borne diseases still continue to be a major public health concern all over the world even in developed countries (Cates et al., 2009). Each year, it is reported that millions of people suffer from food-borne diseases because of consumption contaminated food (Sanlier, 2009; Cates et al., 2009; Senior, 2009). According to European Food Safety Authority (EFSA, 2010) report, 48.7% of foods borne illnesses are associated with food services. Consumers, become more concerned with food safety and quality of food ingredients because of the outbreaks caused by food borne disease agents. European Food Safety Authority and the European Centrefor Disease Prevention and Control reported that, only in the year of 2013, 5196 food-borne and waterborne outbreaks, 5946 hospitalizations and 11 deaths in the European Union (EU). Among these, 22.2% of out breaks were occurred in food establishments such as restaurants, cafes, pubs, bars and hotels (EFSA & ECDC, 2015).

News about the disease outbreaks, lectures on food safety in schools, reports and announcements from authority cause consumers to have awareness and knowledge on food borne diseases. In such a case, consumer awareness, hazard possibility coming from foods and quality searching, make food handlers to obey hygiene rules and to take care what they do. A study in USA, suggested that improper food handler practices contributed to approximately 97% of food borne illnesses (Howeset al., 1996). As Sharif & Al-Malki (2010) reported; three factors are playing important role in food poisoning outbreaks concerning food handlers: knowledge, attitude and the other one is practice. Several authors have identified that good levels of knowledge on food safety among food handlers and the effective application of such knowledge in food handling practices are essential in ensuring the production of safe food (Mortlock, Peters, & Griffith, 1999). As Todd et al. (2007) mentioned, the most reported cases related to food-borne disease are because of inadequate temperature control, infected food handlers and bare hand food manipulation, contaminated raw ingredients, cross-contamination and inadequate heat processing. In order to prevent these errors, food handlers' knowledge and awareness is surely very important. Because of that we examined food handlers' knowledge on such subjects. In the study performed by Smigic et al. (2016), food handlers' knowledge and gaps related to these critical food safety issues were investigated. On the other hand, they also investigated and compared the level of food safety knowledge among food handlers in three different countries, Serbia, Greece and Portugal. In the study, the knowledge score (KS) was calculated by dividing the sum of correct answers by the total number of questions. As the conclusion of this research; the average KS for all participants was 70.5%. The best KS was obtained for Portuguese food handlers (72.6%), then Serbian food handlers (71.3%) and Greek food handlers get lower scores (69.1%). Pichler et al. (2014) was planned a study to detect the most important gaps in knowledge on food safety among food handlers in Vienna, Austria. According the results of this study; the average knowledge score for all food handlers was 76%. Knowledge gaps that determined in this research were concerning correct temperatures for cooking, holding and storing foods. There are many studies about the knowledge and practices of food safety which was done in different types of food processing plants and variety of food handlers (Bolton et al., 2008; Dewaal, 2003; Howells et al., 2008; McCarthy et al., 2007; Gomes-Neves et al., 2007; Marais, Conradie & Labadarios, 2007; Sanlier, 2009; Tokuç et al., 2009; Walker, Pritchard, & Stephen, 2003; Giritlioglu, Batman &Tetik et al., 2011).

Food handlers' training is seen as an important strategy to increase the knowledge and awareness. As Clayton et al. (2002) reported; if food handlers develop a correct perception of hygiene, it will be possible to accomplish the risk of food borne illnesses. On the other hand, a number of studies indicate that although training may increase the knowledge of food safety, it does not always result in a positive change in food handling behaviours (Howes et al. 1996).

As Bas et al. (2006) mentioned, there are many facts imposing risk on food safety in Turkish food and beverage industry, due to industrialization and mass production, fast food consumption, street vendors and growing international trade. Turkish Food Hygiene Regulation (Anonymous, 2011) notified many hygiene rules that food industry should obey in order to obtain healthy and safety food. On the other hand, according to this regulation, Turkish food business must provide food hygiene trainings related with work activities of their staff.

Many researchers concluded that safe food is the most important subject of the day and strictly related with good hygiene practices and the knowledge of the employees that carry the food production process. With the idea we got from this truth, we aimed to evaluate knowledge and awareness of all employees on food safety and hygiene rules and personal hygiene that hugs all food safety practices, in all types of kitchens, with a large scale in district of Istanbul. Also we focused on what they don't know about safe food. We tried to contact with food handlers working at all stages

in caterings, restaurants, hotels, kebab houses, school kitchens. The results of this study will provide information for the national food safety training strategy.

Materials and Methods

Questionnaire Design

In order to determine the perception of hygiene and food safety knowledge of kitchen employees, a self-administrable, Likert type questionnaire has been used. The questionnaire was prepared based on the previous study conducted by Çakıroğlu and Uçar (2008) and the questions were developed with the help of literature review on food safety and food quality. The questionnaire consisted of 38 statements in three groups. The groups are about; socio-demographic characteristics (7 questions), food safety and hygiene rules (17 statements) and personal hygiene (12 statements). The questionnaire includes a set of negative sentences in addition to the positive ones. Responses to the positive sentences have been graded as follows: 'I certainly agree', 5 points; 'I agree', 4 points; 'undecided', 3 points; I don't agree', 2 points and 'I certainly don't agree', 1 point. In the negative sentences, the grades have been assigned in a reverse order.

Participating the Business and Delivery of the Questionnaires

This survey was conducted from March to December involving 400 kitchen employees working in 22 kitchens in Istanbul, a city in Turkey. Medium and large scaled enterprises' kitchens which includes at least 10 food handlers, were selected for the survey.Assessments were comprised of catering establishments, school food services, hotels, kebab houses, and restaurants. The employees in the selected kitchens were asked to complete self-administrable questionnaire in order to collect research data.

Evaluating the Questionnaires

The findings have been analyzed with respect to gender, educational level and work experience variables in the *Statistical Package for Social Sciences* (SPSS) program. In evaluating the hygiene perception grades, "Independent-samples *T* test" for the gender variable, "One-way Anova" analysis and "scheffe test" for the other variables have been applied. Frequencies, averages and standard deviations have been calculated.

Results and Discussion

The demographic characteristics of 400 persons who participated in the survey are given in Table 1. When Table 1 is examined, it was seen that 76% of the participants in food businesses were male workers, 75.8% were in the age range of 19-40, 42.2% were high school graduates, 26.5% were journeyman and 34.8% of the participants were working in a food service for more than 10 years. 64.8% of them were educated on food safety and 77% of them had periodic controls in their institution.

Table1. The demographic characteristics of participants

	f	%
Gender		
Female	96	24
Male	304	76
Age		
<18	19	4.8
19-40	303	75.8
41-60	74	18.4
>60	4	1
Education		
Primary school	163	40.8
High school	169	42.2
University	66	16.5
Master	2	0.5
Position of work		
Executive chef	45	11.2
Sous chef	57	14.3
Chef de party	81	20.2
Journeyman	106	26.5
Busboy	72	18
Steward	39	9.8
Years in food service		
< 1year	55	13.8
1-5 years	124	31
6-10 years	82	20.4
>10 years	139	34.8
Education on food safety		
Yes	259	64.8
No	141	35.2
Periodic controls		
Yes	308	77.0
No	81	20.3
No answer	11	2.7

The answers to the statements that measure the knowledge and awareness of food safety and hygiene rules that must be observed in food enterprises are as shown in Table 2. The reliability analysis of the statements on food safety and hygiene rules was examined with the Cronbach alpha test and the value found to be 0,85. According to this value, it can be said that the answers given to the questions are consistent and the questions are reliable. When the results of this table are evaluated, some important points can be expressed as follows: 90.8% of participants know that, food hygiene means to remove the illness-causing factors in food. 5% of participants didn't agree this statement. This result shows that they mostly know food hygiene is needed for healthy

food but on the other hand there are still food handlers that do not know neither what food hygiene means. Giritlioglu at al. (2011) performed a questionnaire survey to assess the knowledge and practice of food safety and hygiene of 82 students in university cookery programs in Turkey. The results showed that although the students regarded the issues of food safety and personal hygiene as important, they had inadequate knowledge in these areas. Totally 17% of participants commented as I certainly don't agree, I don't agree and undecided for "*Bacteria can be transmitted to food via poorly cleaned equipment*" statement and as seen with this result there is a lack of information about this subject. Also this result is similar with the answers for another statement; "*Hot served foods should be kept at 60 °C or above, cold served foods should be kept at 4 °C and below*". The proportion of those who answered "*There is no harm in keeping animal originated food such as meat, milk, eggs at room temperature*" is quite high with 16.5%. Participants answered as I certainly don't agree, I don't agree and undecided for the statement "*Water used for every business in the kitchen should be drinkable*" at the ratio of 16.2%. most of the participants (83.8%) had no information that drinkable water is needed in kitchen for any purpose although this situation is underlined in Turkish Food Hygiene Regulation (Anonymous, 2011). 36% of participants answered as I certainly don't agree, I don't agree and undecided for the statement "*Cooked foods can be kept at room temperature for more than 2 hours before serving*". In addition, 18 people (4.5%) left this question blank. 23.3% of the kitchen workers responded by saying "*Frozen foods can be frozen again after thawed*", undecided, agree and strongly agree. The phrase "*Frozen foods can be thawed at room temperature*" was answered as undecided, I agree and I strongly agree at the ratio of 54.2 % and was left blank at the ratio of 5.8 %. About more than half of food handlers have false knowledge on safety of frozen foods. The answers that were given to statements on frozen foods also indicated that there is a lack of knowledge on frozen foods. Foods should never be defrosted in this way why the reason bacteria can multiply rapidly between 4-60 °C. In the study of Al-Shabib, Mosilhey & Husain (2016), 85% of workers were aware about the fact that defrosted foods cannot be refrozen again. According to Sani &Siow (2014), about 75% of the respondents had knowledge about refreezing defrosted food. "I agree, I strongly agree and undecided" answers were given to "*There is no harm in terms of human health for some of the molds growing on the food*" is at the ratio of 30.2%. This is a big ratio for not to have knowledge about health harms caused by mycotoxins of moulds.

The reliability analysis of the statements on knowledge and awareness of personal hygiene was examined with the Cronbach alpha test and the value found to be 0.79. According to this value, it can be said that the answers given to the questions are consistent and the questions are reliable. The answers to the statements that measure the knowledge and awareness of personal hygiene are as shown in Table 3.When the results of this table are evaluated, some important points can be expressed as follows:

The percentage of those who are certainly agree, agree and undecided with the phrase "*Kitchen worker who is directly related to the food can touch the food with his bare hand*" is quite high with 38.7%. Totally 29 % of participants commented as I certainly don't agree, I don't agree and undecided for "*In our nose there are bacteria which can cause food poisoning*". Food intoxication by *Staphylococcus aureus* is considered as the third most important cause of food borne diseases in the world (Normanno et al., 2005) and this bacterium can be presence in nose microflora. The reason for asking respondents' idea about this was to understand if they know about this pathogen. In a similar study performed by N.A. Al-Shabib, Mosilhey & Husain (2016), 52.9 % of respondents knew *S. aureus* as a food pathogen. 27.4 % of participants certainly didn't agree, didn't agree and had no decision if it is also needed to wash their hands except of washing just before starting work. 21 % of the food handlers that participated to our survey don't know that open wounds and abscess can be sources for bacteria causing food poisoning. Similar to this result, 22.8 % of participants certainly didn't agree, didn't agree and had no decision if they have to start to work after they have closed their wounds with waterproof tape. The rate of kitchen workers, who think that food handlers suffering from flu, diarrhea, influenza or other illnesses, may work in the kitchen, is 15.5 %. 84.5 % of respondents knew that this is not appropriate. This result is concordant with Codex Alimentarius Commission (2003) report. According to that it is stated that sick food handlers are not allowed to work or deal with foods. In the study of Al-Shabib, Mosilhey & Husain (2016), 29-31 % of workers handle foodstuffs when sick or having wounds and cuts. This percentage evaluated by the researchers as not very high. Food handlers also don't know that each kitchen worker is a tool for the transportation of bacteria to food (17.5 %), they have to remove their rings, watches, bracelets before starting work (15.8 %) and must not smoke (14.3 %). As Abdul-Mutalib et al. (2012) reported, more than 40 % of their respondents wear jewellery while working. High percentage (86.2%) of food handlers was also aware of wearing watches, earrings and rings in the study of Al-Shabib,

Mosilhey& Husain (2016). This result shows similarity with our result.

Table 2. Answers to food safety and hygiene rules knowledge and awareness statements

	I certainly don't agree		I don't agree		Undecided		I agree		I certainly agree		No answer	
Statements	f	%	f	%	f	%	f	%	f	%	f	%
Food hygiene means to remove the illness-causing factors in food	7	1.8	13	3.2	17	4.2	116	29	247	61.8	-	
There is no inconvenience that the waste materials are kept in the kitchen together with the foodstuffs	221	55.3	85	21.3	25	6.2	28	7	30	7.4	11	2.8
Cooked and uncooked foods should be prepared with separate equipment and should be stored separately	9	2.2	19	4.8	32	8	102	25.5	230	57.5	8	2
Bacteria can also be transmitted to food via poorly cleaned equipment	18	4.5	27	6.8	23	5.7	108	27	224	56	-	
The case of food poisoning caused by a meal prepared in your company causes damage to the company's reputation	15	3.8	12	3	27	6.7	71	17.7	275	68.8	-	
Hot served foods should be kept at 60 °C or above, cold served foods should be kept at 4 °C and below	8	2	15	3.7	51	12.8	107	26.8	212	53	7	1.7
There is no harm in keeping animal originated food such as meat, milk, eggs at room temperature	183	45.8	124	31	24	6	37	9.2	25	6.3	7	1.7
Be sure that the meats that are accepted to the establishment are brought to operation under the cold chain	8	2	14	3.5	33	8.2	103	25.8	232	58	10	2.5
Sufficient number of showers and washbasins must be available in the food facility according to the density of the staff	11	2.8	8	2	19	4.7	107	26.8	245	61.2	10	2.5
The water used for every work in the kitchen must be drinkable	13	3.2	13	3.2	39	9.8	94	23.5	233	58.3	8	2
Bacteria multiply very quickly in the foods that are kept at room temperature and reach the level that can cause food poisoning	15	3.7	12	3	17	4.2	103	25.8	242	60.5	11	2.8
Cooked foods can be kept at room temperature for more than 2 hours before serving	120	30	118	29.5	43	10.8	50	12.5	51	12.7	18	4.5
Frozen foods can be frozen again after thawed	208	52	81	20.2	28	7	32	8	33	8.3	18	4.5
Typhoid fever is an important digestive system disease and may infect people by consuming chicken, milk, eggs	25	6.3	28	7	78	19.5	122	30.5	114	28.5	33	8.2
Frozen foods can be thawed at room temperature	102	25.5	58	14.5	33	8.2	92	23	92	23	23	5.8
The most important signs in food poisoning are; diarrhoea, nausea, vomiting, fever, abdominal pain, fatigue and loss of appetite	11	2.8	2	0.5	18	4.5	112	28	138	59.5	19	4.7
There is no harm in terms of human health for some of the moulds growing on the food	195	48.8	61	15.2	37	9.2	50	12.5	34	8.5	23	5.8

Table 3. Answers to personal hygiene knowledge and awareness statements

	I certainly don't agree		I don't agree		Undecided		I agree		I certainly agree		No answer	
Statements	f	%	f	%	f	%	f	%	f	%	f	%
Each kitchen worker is a tool for the transportation of bacteria to food	9	2.2	13	3.3	48	12	152	38	178	44.5	-	
The kitchen worker, who is directly related to the food, can touch the food with bare hands	138	34.5	92	23	41	10.2	69	17.3	45	11.2	15	3.8
In our nose there are bacteria which can cause food poisoning	18	4.4	27	6.8	71	17.8	116	29	157	33.2	11	2.8
Open wounds and abscess can be sources for bacteria causing food poisoning	10	2.5	34	8.5	40	10	132	33	184	46	-	
Kitchen workers must go through health check every 6 months	10	2.5	17	4.3	31	7.8	87	21.7	255	63.7	-	
It is enough for the kitchen staff to wash their hands in the kitchen just before starting work	176	44	73	18.3	27	6.8	55	13.7	55	13.7	14	3.5
If the kitchen worker is suffering from flu, diarrhoea, influenza or other illnesses, there is no problem working in the kitchen	196	49	114	28.5	20	5	24	6	38	9.5	8	2
Appropriate hand washing is made with hot water and by brushing the nails with soap and disinfectant by rubbing the hands.	8	2	7	1.7	19	4.8	106	26.5	251	62.8	9	2.2
Personnel should show maximum care on hygiene when entering or leaving food processing areas	14	3.5	8	2	22	5.5	100	25	238	59.5	18	4.5
There is no objection to smoking in the food processing area	246	61.5	68	17	21	5.2	22	5.5	35	8.8	8	2
Staff working in food production should start to work after they have closed their wounds with waterproof tape	29	7.2	35	8.8	27	6.8	137	34.2	155	38.8	17	4.2
Employees do not need to remove their rings, watches, bracelets before starting work	195	48.8	84	21	29	7.2	32	8	31	7.8	29	7.2

The most often reported food handlers' mistakes were handling of food by an infected person or by a person carries food-borne pathogens, touching the food with bare-hand, improper hand washing and insufficient cleaning of equipment that are in contact with foods (Nørrung & Buncic, 2008).

The following hypotheses were established to determine whether the knowledge of food handlers on "Food Safety and Hygiene Rules" and "Personal Hygiene" differs according to the socio-demographic characteristics and the results were given in Table 4. The independent samples t test and the ANOVA test were conducted to determine differences in significance level of 0.05.

When the hypotheses shown in the Table 4 are evaluated; it was determined that the level of knowledge of employees did not differ according to gender (Food Safety and Hygiene Rules knowledge; female=3.6195 ±0.9011, male=3.8185 ±0.8891 and Personal Hygiene knowledge; female=3.9444 ±0.6324, male=3.9715 ±0.6987). It was seen that the regular audits and inspections of the food establishments and the trainings related to their fields have great importance in increasing the knowledge level of the kitchen workers. As the result of another research, it was observed that food safety training increased knowledge on food safety issues (Lynch, Elledge, Griffith, & Boatright, 2003). A meta-analysis has shown that food safety training increases knowledge and improves attitudes about hand hygiene practices (Soon, Baines, & Seaman, 2012). On the other hand, it is important not to forget that more knowledge does not always lead to positive changes in food handling procedures (Bas¸ Ersun, &Kıvanç, 2006; Ansari-Lari, Soodbakhsh, &Lakzadeh, 2010; Park, Kwak, & Chang, 2010). Along with training, there are many other factors that may affect the knowledge of food handlers, such as age, education or work experience (Pichler, Ziegler, Aldrian, &Allerberger, 2014). The hypothesis that there is a difference according to education levels in terms of Food Safety and Hygiene Rules knowledge has been tested with One-way Anova and found to be dif-

ferent. According to the Tukey HSD test; while the education level of high school and primary education was in the same group with the lower average (3.7000 ±0.9535, 3.7132 ±0.8941, respectively); university graduates (4.0848 ±0.6607) were included in a separate group. Personal Hygiene knowledge also differs according to education level. As a result of the Tukey HSD test; while the educational status of primary and high school graduates (3.8681 ±0.6817, 3.9359 ±0.7143, respectively) were in the same group with lower mean; university graduates (4.2696 ±0.5035) were in a separate group. According to the Anova test results; knowledge on Food Safety and Hygiene Rules was different according to the job position. Among the post hoc tests, the Tukey HSD test was conducted and according to the results of stewards and sous chefs (3.3575 ±1.1178 and 3.6749 ±0.8207, respectively) were in a group, while those who work as busboy, journeyman, chef de party and executive chef (3.7061 ±0.8951, 3.8113 ±0.8957, 3.8954 ±0.7987, 4.0341 ±0.8279, respectively) were found to be in the other group with a higher average. In this case, knowledge of stewards and sous chefs was lower than others. Personal hygiene knowledge of food handlers was also different according to the job position. Tukey HSD test was performed among Post Hoc tests and those who were working in the steward position were separated from the others with the lowest mean (3.7607 ± 0.7322) alone. Those who worked as sous chef, busboy, journeyman, chef de party and executive chef were in a group (3.9094 ±0.6678, 3.9347 ±0.7068, 3.9387 ±0.7119, 4.0422 ±0.6277, 4.1870 ±0.5986 respectively). Food Safety and Hygiene Rules knowledge differs according to the duration of the food handler in a food establishment. According to the results of the Tukey HSD test; the ones who worked for 6-10 years (3.8903 ±0.8008) and more than 10 years (4.0479 ±0.8008) were in same group, while those who worked in one food operation less than 1 year (3.3412 ±1.1097) and those who worked for 1-5 years (3.5716 ±0.9209) 0.7116) were in the same group. As the number of working period in food operation increases, the level of knowledge of Food Safety and Hygiene Rules seems to increase. Similar results were obtained for Personal Hygiene knowledge. According to the results of the study performed by R. Garayoa et al. (2011), hygiene knowledge levels were slightly better among people who are graduated from middle- or high-school and for those who had worked 10 or more years in the catering sector.

Table 4. Comparison the knowledge of food handlers according to the socio-demographic characteristics

Hypotheses	Test	Result	Decision
H_1: The knowledge on the Food Safety and Hygiene Rules of kitchen workers differs according to gender	Independent t Test	t=-1.905 df=398 p=0.057	Rejected
H_2: Personal Hygiene knowledge of kitchen workers differs according to gender	Independent t Test	t=-0.338 df=398 p=0.736	Rejected
H_3: The knowledge of kitchen workers on Food Safety and Hygiene Rules differs according to the their training situation	Independent t Test	t=7,428 df=398 p=0.000	Accepted
H_4: The knowledge of kitchen workers on Personal Hygiene differs according to their training situation.	Independent t Test	t=6.880 df=398 p=0.000	Accepted
H_5: The knowledge on the Food Safety and Hygiene Rules of the kitchen staff varies according to whether or not periodic checks are carried out at the institution where they work	Independent t Test	t=6.979 df=387 p=0.000	Accepted
H_6: The knowledge onPersonal Hygieneof the kitchen staff varies according to whether or not periodic checks are carried out at the institution where they work	Independent t Test	t=5.650 df=387 p=0.000	Accepted
H_7: The knowledge of kitchen workers' on Food Safety and Hygiene Rules differs according to the educational situation	One way Anova	F=5.157 df=2 p=0.006	Accepted
H_8: The knowledge of kitchen workers' on Personal Hygienediffers according to the educational situation	One way Anova	F=8.902 df=2 p=0.000	Accepted
H_9: The knowledge of kitchen workers' on Food Safety and Hygiene Rules differs according to the job position of food handler	One way Anova	F=3.085 df=5 p=0.010	Accepted
H_{10}: The knowledge of kitchen workers' on Personal Hygiene differs according to the job position of food handler	Oneway Anova	F=2.022 df=5 p=0.075	Rejected
H_{11}: The knowledge of kitchen workers' on Food Safety and Hygiene Rules differs according to their professional experience (the working time of the food business)	One way Anova	F=12.139 df=3 p=0.000	Accepted
H_{12}: The knowledge of kitchen workers' on Personal Hygiene differs according to their professional experience (the working time of the food business)	One way Anova	F=7.906 df=3 p=0.000	Accepted

Conclusion

Findings in this study provide very important information on the level of food safety knowledge and major knowledge gaps. 90.8% of participants know that, food hygiene means to remove the illness-causing factors in food however critical knowledge gaps were determined such as handling of frozen foods, hand washing, statements in national legislation, some important and critical food borne diseases and their agents, proper food storage temperatures and some of the important, critical hygiene rules. It is clear that in order to overcome the deficiencies in knowledge of kitchen workers' food safety and hygiene rules and personal hygiene, in-service training must be supported and maintained regularly in accordance with legal regulations. With the results of this study; it was seen that the regular audits and inspections of the food establishments and the trainings related to their fields have great importance in increasing the knowledge level of the kitchen workers. It was determined that the level of knowledge of employees did not differ according to gender. On the other hand; according to the hypothesis that we obtained that there is a difference according to education levels in terms of "Food Safety and Hygiene Rules knowledge". Also, knowledge of the employees was significantly different according to the job position and to the duration of the food handler in a food establishment.

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