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REVIEW

OCCURRENCE AND PRODUCTION OF FURAN IN COMMERCIAL FOODS

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ABSTRACT

Furan (C_4H_4O) is a compound classified as "possibly carcinogenic to humans" by International Agency for Research on Cancer. As precursors, ascorbic acid, unsaturated fatty acids, amino acids and carbohydrates have been suggested to induce furan formation. Human exposure occurs mainly through consume of coffee, canned foods and Baby food. Average intake of furan is 1.5 μg/day for children aged 4-6 years and 27 μg/day for adults. Currently no limits for furan in food were fixed by European legislation. Since the carcinogenicity of furan, levels in food should be kept as low as reasonably achievable.

Keywords: baby food, food safety, furan

1. INTRODUCTION

Furan (C_4H_4O) is a small cyclic ether with aromatic character. It is a lipophilic coumpond with low molecular weight, high volatility, and 31°C as boiling point (NTP 1993) (Fig. 1). Furan and its derivatives (2-methylfuran, 2-ethylfuran, 2-pentylfuran, 2,5-dimethylfuran, 2-butylfuran, 2,3-benzofuran) have known to occur in heat-treated foods and drinks, and to contribute to the sensory property of foods (MAGA, 1979; MERRIT *et al*., 1963; EFSA 2010).



Figure 1: Chemical structure of furan.

Classified as *possibly carcinogen to humans* (group 2-B) by the International Agency for Research on Cancer (IARC, 1995), furan is carcinogenic for rats and mice. In the opinion of European Food Safety Authority (EFSA) the weight of evidence indicates that furan-induced carcinogenicity is probably attributable to a genotoxic mechanism (EFSA, 2004).
Furan is formed during heating process used for the manufacture of foods, and has been detected in hot-air dried, baked, fried and roasted food items, such as cereal products and coffee, as well as canned or jarred prepared foods (US FDA, 2004a; EFSA, 2005; ZOLLER *et al*., 2007). There appear to be multiple precursors (sugars, amino acids, ascorbic acid, poly-unsaturated fatty acids), and many pathways to the furan production (Fig. 2). The principal are:

- thermal degradation of certain amino acids;
- thermal degradation/Maillard reaction of reducing sugars;
- thermal oxidation of ascorbic acid, poly-unsaturated fatty acids and carotenoids (YAYLAYAN, 2006);
- thermal degradation of the common precursors in the process of roasting.

The primary source of furan in food is the thermal degradation of carbohydrates such as glucose, lactose, and fructose (MAGA, 1979).

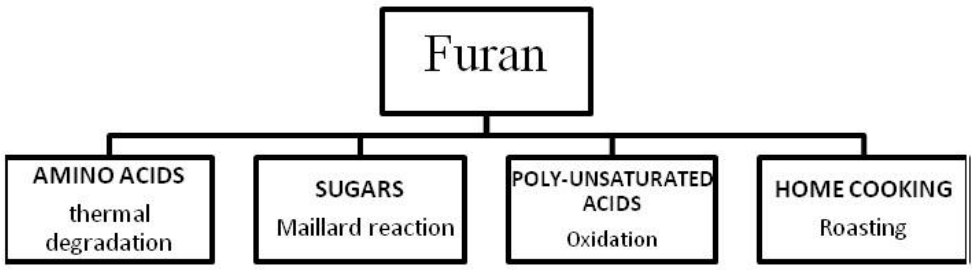


Figure 2: Precursors and sources of furan in food.

Amino acids serine and cysteine are able to metabolize acetaldehyde and glycolaldehyde, which react by aldol-condensation, then produce aldotetrose derivatives and furan. Alanine, threonine, and aspartic acid can generate only acetaldehyde; they require the presence of serine, or cysteine and reducing sugars, also, to produce glycolaldehyde and then furan (PEREZ and YAYLAYAN, 2004).

The reactions between several amino acids and carbohydrates on heating form aldotetrose derivatives, then after cyclisation can form furan (PEREZ and YAYLAYAN, 2004; LIMACHER *et al*., 2008). When ascorbic acid was mixed in model systems with single amino acids (glycine or serine), sugar (erythrose) or unsaturated fatty acids (e.g. linoleic), the mixtures produced far less furan on heating than ascorbic acid alone did (MARK *et al*., 2006).

Experimentally monounsaturated acid (oleic) did not form furan (BECALSKI and SEAMAN, 2005), and if furan is formed from unsaturated fatty acids the yield increases as the degree of unsaturation increases.

Furan production has been linked with free radicals autoxidation process (PEREZ and YAYLAYAN, 2004). So, Ferric ions increased furan formation in linoleic acid by 79%, and in trilinolein by 29% (MARK *et al*., 2006), addition of commercially available antioxidants (such as tocopherol acetate) reduced the formation of furan (PEREZ and YAYLAYAN, 2004). Moreover the effects of lipid oxidation, catalysts such as Fe(II) or antioxidants are sometime contradictory. Given the complicated and competing reaction pathways available in autoxidation processes (some of which may lead to furan, and some not) limiting autoxidation may not always lead to a corresponding reduction in furan formation (Fig. 3).

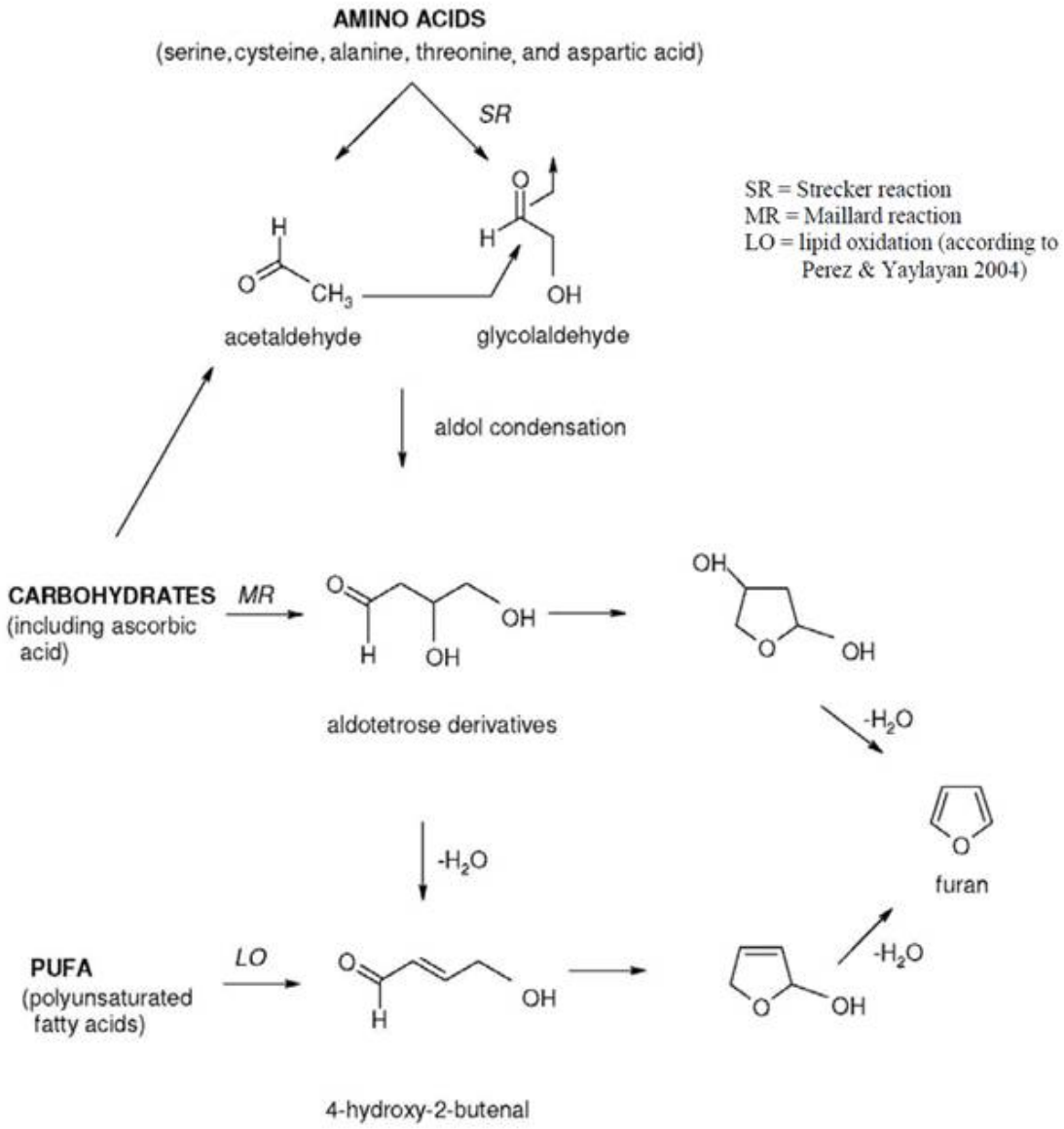


Figure 3: Proposed pathways of formation of parent furan from three main groups of sources: amino acids, carbohydrates, and polyunsaturated fatty acid (VRANOVÀ and CIESAROVÀ, 2009).

Due to its low polarity, furan can pass trough biological membranes and enter various organs, it is rapidly and extensively absorbed from the intestine, and the lung. Repeated doses accumulate in the liver (EFSA, 2004a) and kidney, and in fewer quantities in intestine, stomach, blood and lung (BURKA *et al*., 1991).
Absorbed furan is metabolized rapidly by cytochrome P-450 (CYP) enzymes via ring opening to form (Z)-2-butene-1,4-dialdehyde. The rapid hepatic metabolism seems to limit, however, its systemic delivery. The capacity to furan's bioactivity is also of relevance for the distribution, since it can result in an irreversible binding to the respective tissue. Within the first 24 hours after a single oral application of furan (8 mg/kg bw), 80% of the total radioactivity was eliminated via the lung, urine and feces (BURKA *et al*., 1991).

In the 2-year rat study, animals of each sex (n=70) were administered furan at 2, 4, or 8 mg/kg bw 5 days per week. Mean body weights of male rats that received 8 mg/kg furan were lower than controls. Increased incidences of numerous non-neoplastic liver lesions (biliary tract fibrosis, hyperplasia, chronic inflammation, and proliferation and hepatocyte cytomegaly, cytoplasmic vacuolization, degeneration, nodular hyperplasia, and necrosis) were present in treated rats. Cholangiocarcinoma of the liver occurred in all groups of dosed rats. A separate 2-year study was conducted in which 50 male rats were administered 30 mg/kg furan 5 days per week. Cholangiocarcinoma of the liver occurred with an overall incidence of 100% (40/40) and hepatocellular carcinoma occurred with an overall incidence of 15 % (6/40) (BURKA *et al*., 1991; NTP, 1993).
Both in vitro and in vivo studies show that metabolic activation by cytochrome P-450 (CYP) enzymes is involved in furan-induced toxicity (KEDDERIS *et al*., 1993). It is likely that furan or (Z)-2-butene-1,4-dial reacts with DNA in target cells and can play a role in furan induced tumors.
Furan causes loss of ATP after bioactivation to metabolites which cause an irreversible uncoupling of hepatic mitochondrial oxidative phosphorylation, this activates cytotoxic enzymes, including endonucleases, that produce DNA double-strand breaks prior to cell death (MUGFORD *et al*., 1997; KEDDERIS and PLOCH, 1999).
Furan was able to induce:
a) gene mutations, chromosome aberrations and sister chromatid exchanges (SCE) in cultured mammalian cells, and chromosomal aberrations in mice bone marrow cells;
b) hepatocellular tumours after *ras* oncogene activation, suggesting that furan, or a reactive metabolite, can directly activate protooncogenes (REYNOLDS *et al*., 1987);
c) chronic hepatic cytotoxicity in the genesis of liver tumours in infant male mice (JOHANSSON *et al*., 1997);
d) monocytic cell leukemias with minimal hyperplasia of bone marrow.
Hepatocytes from human livers donors oxidized furan at rates equal to, or greater than, those of rat hepatocytes in vitro (KEDDERIS *et al*., 1993) (Fig. 4).

Species	Blood furan (μM)	Liver perfusion[b] (μmol/min)	Furan oxidation[c] (μmol/min)
Rat	0.80	0.017	0.213
Mouse	0.80	0.005	0.122
Human	0.68	1.02	25.6–37.7

[a] Simulations of exposure to 10 ppm furan for 4 hr using the physiologically based dosimetry models for humans, mice (Table 1 and Fig. 1), and rats (Kedderis *et al.*, 1993).
[b] The rate of liver perfusion with furan equals the blood furan concentration times the liver blood flow rate.
[c] The rate of furan oxidation in the liver under these conditions equals the initial rate of metabolism (V_{max}/K_M) times the blood furan concentration.

Figure 4: Blood flow limitation of furan biotransformation (KEDDERIS *et al.*, 1993).

In alcohol consumption the furan metabolism is higher due induction of cytochrome P450 2E1 in humans by ethanol (PERROT *et al*., 1989). However there is small difference

between the levels of human exposure and doses that induce carcinogenic effects in experimental animals.

1.1. Hydroxymethyl-2-furfural heat-induced formation and occurrence in food

Furan and 5-hydroxymethylfurfural (HMF) are compounds that are formed in a variety of heat-treated commercial foods. Such a widespread occurrence of furan and HMF in many types of food is due to the fact that they are products of different reactions following multiple routes and involving different precursors and intermediate (ANESE and SUMAN, 2013). In particular, HMF can be formed as an intermediate in the Maillard reaction, which occurs when carbohydrates are heated in the presence of amino acids or proteins (MAURON, 1981), or, alternatively, by thermal dehydration of a sugar under acidic conditions (KROH, 1994). At low pH, glucose or fructose may undergo 1,2 enolization and dehydration to form 3-deoxyosone, which is the key intermediate in HMF formation. Fructose is more reactive than glucose in the formation of HMF (LEE and NAGY, 1990). According to PEREZ LOCAS and YAYLAYAN (2008), HMF can form from fructose or sucrose via the generation of a highly reactive fructofuranosyl cation. At high temperatures and in dry systems this cation can quickly be converted into HMF.
HMF formation in foods has been found to be affected by sugar type, pH, water activity and the presence of divalent cations (GOKMEN *et al*., 2008). As furan and HMF formation is concomitant to that of color and flavor of heated foods, it is very difficult to mitigate their formation without compromising the food sensory acceptability. Changes in process parameters, i.e. heating regime modification, and formulation can be regarded as strategies that can be applied to prevent furan and HMF formation (ANESE and SUMAN, 2013).
Particularly breakfast cereals, coffee, honey as well as pasteurized juices or pulps etc. are subjected to intensive HMF formation.

- Cereal products: RUFÍAN-HENARES *et al*. (2006) revealed that the HMF concentration varied between 6.59 and 240.51 mg/kg (w/w). The highest average concentration of HMF was found in maize-based breakfast cereal (42.81±7.92 mg/kg), followed by wheat (40.79±8.57 mg/kg) and rice (32.14±10.79 mg/kg) products. Authors have also compared products with and without the addition of honey and stated that HMF concentration was higher in the former group, 43.44±10.35 versus 34.24±6.17 mg/kg, respectively. HMF formation was also studied as one of the factors influencing browning of infant cereals. Increases in HMF concentration were investigated at different stages of cereals processing (toasting, hydrolysis, drying) in model systems (FERNANDEZ-ARTIGAS *et al*., 1999). The hydrolysis process was connected with increases in HMF concentration. The drying stage, however, did not contribute to overall HMF synthesis probably due to short processing times.
- Coffee: On the basis of analysis of 22 coffee samples MURKOVIC and PICHLER (2006) stated that HMF concentration in the investigated products ranged from 300 to 1900 mg/kg. They found that roasting coffee at 240°C caused a rapid increases in HMF (up to 900 mg/kg) in the first 3 min. Further roasting was connected with decreases in HMF contents probably because of the occurrence of consequent degradation reactions. ARRIBAS-LORENZO and MORALES (2010) analysed 35 commercial roasted coffee brands as well as 19 soluble coffee brands. They estimated four levels of HMF: 110, 625, 1734, and 2480 mg/kg for natural, blend (mixture of torrefacto and natural coffee), torrefacto (coffee roasted with sugar addition) and soluble coffee, respectively. The largest differentiation in HMF level was found in soluble coffee clusters (min. 691, max. 4023 mg/kg). The authors

established that the different modes of coffee brewing (espresso, filtered, Italian, soluble) influence potential content of HMF. A possible mitigation strategy is represented by the physical removal of furan and HMF by means of vacuum treatments. The vacuum technology has already been studied as a tool to remove HMF from roasted coffee, by exploiting the chemical and physical properties of these molecules (QUARTA and ANESE, 2012). In this case, thinking of a possible industrial exploitation of this technology for coffee, the product coming from the tunnel oven could be moved to a hydration step (e.g. carried out by means of a spray of pressurized water) followed by a vacuum step. The vacuum-treated food with reduced furan and/or HMF and water contents can be then subjected to eventual flavor enrichment and finally packaged (ANESE and SUMAN, 2013).

- Fruit and vegetable products: These products are usually rich sources of sugars and organic acids as well as amino acids. Processing of this group of foodstuffs thus leads to the formation of significant amounts of HMF. BURDURLU and KARADENIZ (2003) investigated the influence of extract, storage time and temperature on non-enzymatic browning of apple juice concentrates. Browning index was correlated with HMF concentration. Juice samples were stored at different temperatures (5, 20, 37°C) for four months. For juices stored at 5°C as well as 20°C, increases in HMF level were minor (increase from 0.62 up to 4.37 mg/kg). HMF formation was much more significant at 37°C reaching 190 and 963 mg/kg. On the basis of the results, the authors confirmed the usefulness of HMF both as a heat processing index and an indicator of storage conditions. Recently, SALDO *et al*. (2009) demonstrated that processing apple juice by means of ultrahigh-pressure homogenization could represent an alternative to conventional pasteurization. Ultrahigh-pressure homogenization, while causing a significant decrease in microbial counts, allowed HMF formation to be reduced. HMF concentration in pasteurized juice was indeed 100-fold higher than in ultra-high-pressure homogenized and raw counterparts.
- Honey: in case of honey, the level of HMF is strictly normalized (COUNCIL DIRECTIVE, 2001). According to normalization, HMF concentration should not exceed 40 mg/kg with exception of honeys from tropical climate (not more than 80 mg/kg). Increased amounts of HMF can result from improper processing or prolonged storage (TOSI *et al*., 2002; 2004; 2008; FALLICO *et al*., 2004). An increased of HMF level in honey was also found to be connected with initial pH (acidity) (FALLICO *et al*., 2004). TOSI *et al*. (2002; 2004; 2008) investigated the kinetics of HMF formation and changes in enzymatic activity during honey heating. It was shown that the initial HMF concentration did not influence the kinetics of its formation. Even after intensive heating (90°C for 20 min) HMF concentration did not reach 40 mg/kg. FALLICO *et al*. (2008) however, pointed out that under different storage conditions degradation HMF can occur in honey samples. Rate constants of degradation process at temperatures between 25 and 50°C (for citric as well as chestnut honey) were higher than the corresponding rate constant of formation. These findings should be taken under consideration in proper legislation process (FALLICO *et al*., 2008).
- Dairy products: Sterilization processes are the origin of HMF in dairy products and may be connected with their colour change (browning). The total HMF-value has generally been applied to distinguish heat-treated milk (pasteurized, UHT, concentrated and powdered). ALBALA-HURTADO *et al*. (1998) studied changes of HMF concentration during storage of infant milk. Powdered infant milk had more HMF than corresponding liquid milks (34.7 and 12.2 μg/kg (w/v), respectively, after 9 month, 37°C). The influence of different temperatures on HMF formation

during the storage of UHT milk was studied by CAIS-SOKOLINSKA *et al*. (2004). There were no significant differences in HMF concentration in milk stored at 4 and 8°C, but storage at room temperature caused a two fold increase in its amount when compared with freshly sterilized product (CAIS-SOKOLINSKA *et al*., 2004). ORAL *et al*. (2011) compared changes in total HMF fraction in sweet whey powder (SWP) and skim milk powder (SMP) at 3 different temperatures (25, 35 and 45°C) and moisture (2.5, 5, 7.5% for SWP and 5.5, 7.7, 10% for SMP) during eight month storage period. Small formation of HMF was observed during storage at 25°C in all studied samples but a great increase in HMF during storage at 45°C was observed, which indicates a large dependence of the formation of HMF on time and temperature of storage and moisture content. Since milk powder is used in the formulation of infant formula, instant beverage, bakery products, chocolate, its HMF content should be under consideration for end product quality and nutritional value.

Several techniques have been used for HMF detection in foods. Liquid chromatography is the most widely method used. These techniques utilize ultraviolet (UV) detection because of the strong absorption of HMF at 280-285 nm. However, many compounds naturally present or formed in foods during processing may also absorb at this wavelength. Poor chromatographic resolution of these compounds may adversely affect the quantification of HMF during UV detection (GOKMEN *et al*., 2006). For this reason several techniques involve the protein hydrolysation step process. ORAL *et al*. (2014) studied the HMF binding capacity of the most common proteins (e.g. caseine) in infant formulas and determined separability from them by acid digestion. Consequently, the HMF levels of the samples were evaluated separately either by no treatment or by the acid-heat treated method. The HMF values of the 10%, 5% and 2.5% casein solutions prepared with HMF stock solution (131.89 mg/L) were found as 116.31, 122.31 and 127.48 mg/L respectively, without acid hydrolysis. After acid hydrolysis, the HMF levels of the casein solutions were determined as 115.52, 121.10 and 124.51 mg/L in 10%, 5% and 2.5% respectively. Acid application have not got any statistical importance at all concentration for making free of bounded HMF ($p < 0.05$). Also, HMF was produced by the chemical reaction between acid and carbohydrates (certain food ingredients) during the acid hydrolysis stage. To avoid this situation, the acid hydrolysis phase of the analysis should be omitted. In the samples prepared without acid hydrolysis stage, the level of HMF did not change with varying of sample concentration. Also, during the acid hydrolysis stage the HMF levels in samples are thought to be dependent on the amount of sample. This is because, during the degradation of proteins with acid, acid is consumed more due to the solution having a high concentration. Therefore, in this study, HMF level was lower in 10% than in 2.5% concentrations due to the decrease in the amount of acid per food compound (carrageenan, sucrose, inulin and fructose). As a result, it was concluded that HMF cannot be evaluated by the traditional method which is based on the principle of separating it from proteins for measurement (ORAL *et al*., 2014).

Although HMF has been used for years as a quality indicator of thermally processed foods, recently some toxicological concerns are raised. HMF has a number of structural alerts (furan ring, α,β-unsaturated carbonyl group, and allylic hydroxyl group) that pose possible genotoxic and carcinogenic risks (ANESE and SUMAN, 2013).

However, further studies suggest that HMF does not pose a serious health risk, but the subject is still a matter of debate (GOKMEN *et al*., 2006).

HMF can initiate and promote the growth of aberrant crypt foci (ACF) in rat colons in a dose-dependent manner (ZHANG *et al*., 1993). HMF induced a significant number of chromosome aberrations and a significant lowering of mitotic activity in cultured Chinese hamster V79 cells (NISHI *et al*., 1989). In a two years study conducted by the National

Toxicology Program, HMF was found to increase the incidence of hepatocellular adenomas in female B6C3F1 mice, whereas no carcinogenic activity was observed in male or female F344/N rats as well as in male B6C3F1 mice (NTP, 2010). In addition, HMF was associated with increased lesions of the olfactory and respiratory epithelium of the nose in male and female rats and mice (NTP Technical Report, 2010). Its derivative 5-sulfidemethylfurfural (SMF), exhibited direct mutagenicity in human lymphoblasts and induced 8-azaguanine-resistant mutants in Salmonella *typhimurium* TM677 in a dose-dependent manner (SURH *et al*., 1994). Histopathological analyses revealed that SMF induced moderate damage to liver tissue and notable damage to the kidneys (nearly all proximal tubules in SMF exposed animals were destroyed). The molecular mechanism underlying this selective toxicity of SMF for proximal tubules is unknown (BAKHIYA et al., 2009). On the basis of kinetic data (MONIEN *et al*., 2009), it was estimated that between 452 and 551 mg/kg of the initial HMF dose (500 mg/kg) was converted in mice into SMF which was subsequently circulated. The sulfotransferases (SULT) are the enzymes that converted HMF to SMF. Human SULT isoforms have a widespread tissue distribution and are expressed in many tissues including liver, lung, brain, skin, platelets, breast, kidney, and gastrointestinal tract (SALMAN *et al*., 2009). Moreover, humans express SULT in ex-trahepatic tissues more extensively than rodents do and may therefore be more sensitive to HMF (TEUBNER *et al*., 2007). Some studies on mutagenicity or carcinogenicity of other HMF derivatives showed that furfuryl alcohol and furfural were not observed to be mutagenic in Salmonella *typhimurium* strains TA98, TA100, TA102, TA1535, or TA1537 (NTP, 1999).
It should be noted that studies which demonstrate the positive and protective role of HMF are also available. WANG *et al*. (2010) revealed that HMF protects LO2 hepatocytes cell from oxidative damage. YAMADA *et al*. (2011) have been suggested that HMF could be useful for the treatment or prevention of type I allergic diseases. Due to the fact that there is inconclusive evidence regarding HMF's potential toxicity to human health, it cannot be determined whether HMF should be considered unsafe or whether the benefits of its use in industry outweighs the risks it may pose. Additional studies are needed to elucidate the potential effects that long-term exposure to HMF could have on human health.

1.2. Formation of furan in food

Different factors, such as temperature, pH, water activity, storage and time conditions, presence/absence of inhibitors and activators may influence the furan levels in food.
In industrial field, at temperature around 200°C, the furan levels increase with the temperature increasing. However when it exceeds the value of 200°C the concentration of furan is independent from the temperature values. In domestic cooking furan formation depends on the type of cooking. Frying (150°-200°C) produces higher levels, if compared with the baking. Furan has been associated with the flavor of foods. Since it is a highly volatile compound, an important part of the furan formed during thermal treatment will be lost during food handling and food preparation by evaporation, depending on the food matrix.
The combination of higher temperatures and lower water content leads to a higher furan content. Furan seems likely to form in browned products, especially if the water activity is low. In particular high levels of furan were founded in bread toasted from a brown to very dark colour.
Furan levels may formed at various pH and temperatures in experimental model systems (NIE *et al*., 2013), and pH has a significant effect on thermally induced furan formation, if temperature is greater than 110°C. At pH 7.00 higher levels of furan were observed than at pH 9.40 and 4.18, suggesting that pH is an important factor influencing furan formation as

a function of thermal treatment and temperature. For instance, after heating for 30 minutes at 150°C in experimental model at pH 7.00, 9.40, and 4.18 the furan concentration was founded at 304, 238, and 40 ng/mL, respectively. Also it is reported (FAN, 2005a) that less furan is formed at pH 3.00 than at pH 7.00 for glucose solution. At pH 7.00 furan content increase rapidly from 34 to 304 ng/mL with temperature increasing from 120° to 150°C. These data suggest that temperature is also a major factor affecting furan formation in model system (NIE *et al*., 2013).
Under sterilization conditions, fructose-glycine system produces high level of furan, while less furan significantly is formed in glucose-glycine system (NIE *et al*., 2013) (Fig. 5). It seems that formation of furan by Maillard reactions is dependent, as activators, on the amino acids and sugars used. The addiction of phenylalanine to glucose results in an increase of about 50% of furan. Moreover the presence of the amino acids alanine, threonine, and serine results in higher furan amounts. In contrast, the furan amount decreases by 20% in a binary mixture of fructose and phenylalanine (LIMACHER *et al*., 2008).
Carbohydrates and amino acids are also commonly used as additives in food products, thus if carbohydrates and amino acids are required together in formulation, sucrose would be a better choice than glucose and fructose to reduce the accumulation of furan by Maillard reactions during the heating (LIMACHER *et al*., 2008).

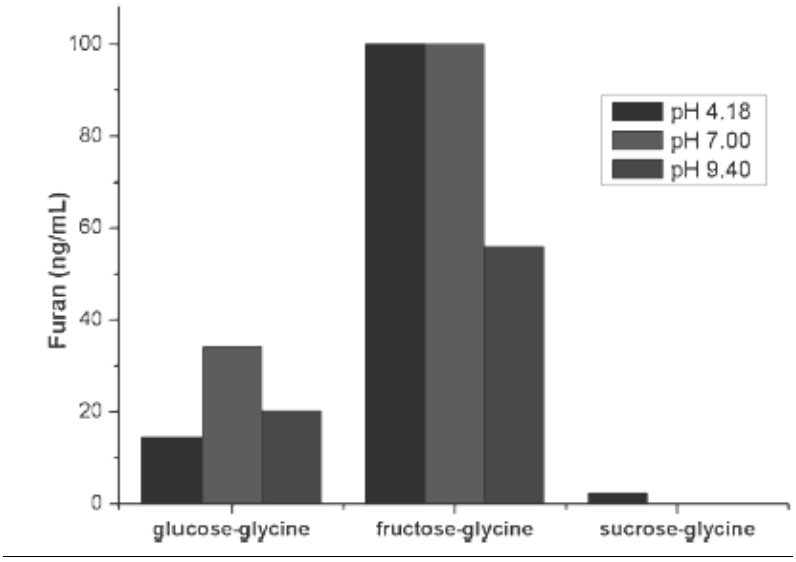


Figure 5: Furan formation in a glucose-glycine, fructose-glycine and sucrose-glycine heating model system at 120° C for 30 minutes, simulating sterilization conditions (NIE *et al*., 2013).

Furan may be produced from polyunsaturated fatty acids during thermal and UV-C treatment. (FAN, 2015). Furan is also produced from linolenic acid emulsion during storage at 25 °C. At pH 9 more furan was formed than at pHs 3 or 6 during longer storage (FAN, 2015).
Part of the furan concentrations founded in commercially available food products might originate from chemical deterioration reactions during storage (PALMERS et al., 2015). A range of individual vegetable purées was stored at two different temperatures to investigate the effects of storage on the furan concentrations of shelf-stable, vegetable-based foods. After 5 months of storage at 35°C (temperature-abuse conditions), a general

increase in furan concentrations was observed. The furan formation during storage could be reduced by storing the vegetable purées at a refrigerated temperature of 4°C, at which the furan concentrations remained approximately constant for at least 5 months. Following storage, the vegetable purées were briefly reheated to 90°C to simulate the effect of the final preparation step before consumption. Contrary to storage, furan concentrations decreased as a result of evaporative losses. Consequently, both refrigerated storage and the reheating step prior to consumption showed the potential of mitigation measures for furan formation in vegetable-based foods (e.g. canned vegetables, ready-to-eat soups, sauces or baby foods). On the contrary methylfuran concentrations rapidly decreased during storage (PALMERS et al., 2015).

Therefore, the retention or release of furan by different food constituents was systematically evaluated. The presence of oils in foods decreases the volatilization of furan and may as such increase the actual intake of furan to a large extent (BECALSKI and SEAMAN, 2005).

For oils, furan concentrations are similar in olive oil and in corn oil. Palm oil contains significantly more furan. It is believed that the high amounts of carotenoids in crude palm oil are responsible for this difference (SHAHIDI, 2005), since carotenoids were identified as important precursors of furan by BECALSKI and SEAMAN (2005). It is possible that furan have been formed by lipid oxidation and has accumulated over time during oils storage.

FROMBERG *et al*. (2009) evaluated the influence of the browning process in fried meat and fish meat balls using either vegetable oil or butter. Furan was found at low level in the heavily fried fish meat balls using butter (3.1 ng/g) as frying agent and in medium fried fish meat balls using vegetable oil (2.5 ng/g) as frying agent fried. However furan was not found above the limit of quantification of 2.4 ng/g in the crust of the medium fried meat balls using vegetable oil as frying oil. On the other hand furan was found in the crust (2.4 ng/g) of the heavily fried meat balls using butter, but not in the heavily fried meat balls when the whole meat balls were analysed. Surprisingly, FROMBERG *et al*. (2009) founded furan in the minced ingredients before frying, even if they were not able to find the explanation for the context (Fig. 6).

	Lightly fried	Medium fried	Heavily fried	Medium fried	Crust from heavily fried	Crust from medium fried	Minced ingredients before frying
Frying agent	Butter	Butter	Butter	Oil	Butter	Oil	
Meat balls	<2.4	<2.4	<2.4	<2.4	2.4	-	6.6
Fish meat balls	<2.4	<2.4	3.1	2.5	2.4	<2.4	<2.4

Figure 6: Levels of furan [ng/g] in home fried meat balls and fish balls (FROMBERG *et al*., 2009).

Some conservation treatments such as irradiation may facilitate the production of high concentrations of the contaminant. FAN (2005a) reported that ionising radiation induced the formation of furan in apple and orange juices. Furan levels increased linearly as the radiation dose increased from 0 to 5 kGy. Furthermore, in the first 3 days of storage after the irradiation treatment, the furan levels continued to increase in both apple and orange

juices. According to FAN (2005ab), the increase in furan during the earlier storage period may be due to the residual effect of irradiation.
To monitor the presence of furan in food, the Commission Recommendation 2007/196/EC requests the Member States to collect data on heat-treated commercial food products. In response to the Commission request, a total of eighteen Member States have so far submitted analytical results for furan content in food to the European Food Safety Authority (EFSA). A total of 4186 complete results were reported for foods sampled between 2004 and 2009 (Table 1). Data were sorted into 21 different food categories (5 coffee and 16 non coffee categories). The 'baby food' category was subcategorised into 6 groups according to the ingredient combination and the category 'others' was subcategorised into more homogenous subgroups in order to extract further information (EFSA, 2010).

Table 1: Number of samples collected by Member States (indicated by ISO country code) between 2004 and 2009 for the analysis of furan content in food (EFSA, 2010)

Food group	AT	BE	CY	DE	DK	ES	FI	GB	GR	HU	IE	IT	LT	NL	NO	PL	SK	SI	Total
Coffee instant[1]	0	25	0	20	7	0	0	2	0	0	1	2	1	0	0	0	0	0	57
Coffee roasted bean[1]	0	1	0	0	0	0	0	5	0	1	5	0	0	0	0	0	2	1	15
Coffee roasted ground[1]	8	12	5	0	1	4	0	15	12	0	3	17	0	0	0	0	8	3	88
Coffee not specified[1]	0	25	11	420	3	0	1	0	0	0	12	5	1	0	0	0	0	0	478
Coffee ready-to-drink	8	0	0	0	0	0	0	0	12	0	31	0	0	0	0	0	10	7	68
Baby food	12	78	15	619	4	8	20	20	14	0	214	22	0	10	24	252	0	10	1322
Infant formula	0	0	0	0	0	0	0	0	0	0	1	9	0	1	0	0	0	0	11
Vegetables	0	59	0	13	11	6	2	11	1	0	7	9	3	9	4	0	16	5	156
Fruits	2	35	0	30	2	0	3	0	1	0	5	13	2	10	0	0	5	0	108
Vegetable juices	0	17	0	25	0	0	0	0	0	0	0	0	0	0	0	0	3	0	45
Fruit juices	0	14	0	129	6	0	34	0	7	15	8	16	1	13	0	0	3	0	246
Fish	2	29	0	0	0	0	0	0	4	0	6	0	0	2	0	0	0	0	43
Cereal products	40	68	0	38	0	0	1	0	0	0	32	1	1	0	0	0	0	0	181
Meat products	26	61	0	15	1	0	0	4	2	1	11	0	0	0	0	0	0	12	133
Milk products	0	44	0	9	0	0	0	0	0	0	0	0	0	11	0	0	0	0	64
Beer	3	15	0	67	0	0	0	0	6	0	0	0	0	7	0	0	4	0	102
Soy sauces	0	46	0	2	0	0	0	3	0	0	0	0	0	0	0	0	0	0	51
Soups	4	16	10	136	10	0	0	1	0	0	32	0	0	16	0	0	12	8	245
Sauces	4	59	4	105	0	0	3	8	5	0	30	2	1	2	8	0	3	11	245
Baked beans	2	3	0	0	1	2	1	6	2	0	36	0	0	0	2	0	1	0	56
Other products	2	118	0	272	3	0	0	53	0	0	0	13	0	7	0	0	3	0	471
Total	113	725	45	1900	49	20	65	128	66	17	434	109	10	88	38	252	70	57	4186

The report data have identified as types of foods most responsible of dietary exposure (content> 100 mg / kg) (Table 2):
- coffee,
- baby foods,
- sauces and soups.

Table 2: Furan content in food per main food category (EFSA, 2010).

Product category	Furan content µg/kg							
	N	P05	P25	Median	Mean	P75	P95	Max
Coffee instant[1]	57	2.8-3	72	271	569	850	2118	2200
Coffee, roasted bean[1]	15	38	905	3998	3611	5303	6407	6407
Coffee, roasted ground[1]	88	19	296	1695	1786	2610	5749	6900
Coffee, not specified[1]	478	13	933	1819	1850	2481	4783	6500
Coffee ready-to-drink	68	0-5	0-5	11	37-40	53	154	360
Baby food	1322	0-2	4-6.6	22	28-29	42	79	224
Infant formula	11	0-2.2	0-2.5	0-2.5	0.2-3.2	0-2.5	2.2-10	2.2-10
Baked beans	56	0-4	5-10	21	23-24	32	57	80
Beer	102	0-1.6	0-2	0.14-3	3.3-5.2	4-8	13	28
Cereal product	181	0-0.3	0-4	5-10	15-18	20-20.5	60	168
Fish	43	0-0.3	0-1.2	2.4-4.3	17-18	16	86	172
Fruit juice	246	0-0.7	0-1.42	0-2	2-5	1.4-5	8-10	90
Fruits	108	0-1	0-2.4	0-5	2-5	2-6	13	36
Meat products	133	0-0.32	0-2	3-10	17-19	22	85	160
Milk products	64	0-0.18	0-0.32	0.4-0.9	5-6	7	20	80
Sauces	245	0-2.3	0-3.2	2.4-8	8.5-11	11	30	175
Soups	245	0-1.4	2.5-5	17	23-24	34	72	225
Soy sauce	51	0-3	13	19	23-24	33	55	78
Vegetable juice	45	0-1	0-3	0-5	2-6	0-9	14	20
Vegetables	156	0-0.32	0-3	0-5	7-9	6-8	39	74
Others	471	0-0.32	0-2.4	7-8	15-16	22	61	164

Among all products tested, the highest furan content was reported in roasted coffee beans with an average of 3.611 µg/kg. Furan and furan derivatives have long been known as intrinsic components of coffee flavours. Green coffee beans contain only traces of furan. The furan levels in the roasted coffee are correlated with the roast colour (GUENTHER *et al*., 2010). Furan retention studies were also conducted with coffee, since it is believed to be the major source of furan in adults diet. In coffee, furan retention was mainly caused by the lipophilic fraction. Defatted coffee brew showed a significantly lower retention of furan than coffee brew: the furan response increased significantly from 78 to 89% after defatting. On the one hand, oils are precursors of furan. Palm oil contains significantly more furan and it is responsible for the high content of furan in roasted coffee (LACHENMIER *et al*., 2009).

Automatic coffee machines produced brews with the highest levels of furan, because a higher ratio of coffee powder to water is used giving a lower dilution factor, and because the closed system favors retention of furan. Much lower levels were produced by standard home coffee-making machines and by manual brewing (GOLDMAN *et al*., 2005; ZOLLER *et al*., 2007).

Furan is found in a wide assortment of foods including potato chips, tortilla chips (FDA, 2004a), dried fruits, popcorn, corn crisp, cereal product (puffed rise) might be consumed by children and findings of furan in these products may cause food safety concern (FROMBERG *et al*., 2009).

Milk based processed food showed low mean furan content (6 µg/kg), but interestingly a maximum furan content of 80 µg/kg was found in sweetened condensed milk (EFSA, 2006). Maximum values exceeding a level of 100 µg/kg were found in fish products such as mackerels and sardines in tomato sauce, in meat products like canned duck with lentils or rabbit with prunes, in soups such as tomato soup and in gravy (FROMBERG *et al*., 2009).

Department of Nutrition, National Food Institute has developed a recipe database with potential furan containing dishes which was used as a platform for analyzing furan in

commonly eaten freshly prepared home cooked dishes. The recipes have been chosen on basis of knowledge upon potential furan containing ingredients after heating (ZOLLER *et al*., 2007) as well as knowledge on dietary habits in the European region (MÄNNISTÖ *et al*., 2003; ELMADFA and WEICHSELBAUM, 2005; DEBACKER *et al*., 2007; FAGT *et al*., 2008). As worst-case scenarios, foods were home cooked using canned ingredients which contained furan. However, this did not lead to elevated levels of furan in the prepared home cooked foods. For ready-to-eat foods with an initial level of furan, cooking reduced the level of furan in the food to about half the original content probably due to evaporation of furan during heating. Nevertheless furan is relatively stable in heated foods left for cooling where the losses of furan were insignificant (ZOLLER *et al*., 2007; FROMBERG *et al*., 2009) (Figg. 7, 8).

Recipe	Canned	Vegetables	Meat/poult	Fish	Milk/dairy	Egg	Cereals	Comments on ingredients and preparation
Oatmeal porridge[1]							●	Oats heated by cooking (oats are steamed, dried and toasted)
Oatmeal porridge in microwave oven[1]							●	Oats heated by microwave cooking (oats are steamed, dried and toasted)
Tomato soup[1]	●	●						Canned tomatoes and onion
Minestrone[1]	●	●	●					Canned tomatoes and several vegetables
Fish meatballs[1]				●		●		Minced fish
Ragu/Sauce Bolognese[1]	●	●	●					Minced meat, canned tomatoes and tomato puree, vegetables
Gullasch[1]	●	●	●		●			Beef, canned tomatoes
Meat balls[1]			●			●		Minced meat
Wok fried pork with vegetables[1]	●	●	●					Pork fried in wok with canned vegetables and soy sauce
Omelette[1]						●		Very traditional dish eaten all over Europe

1. Fogt *et al*. 2007.

Figure 7: Main ingredients and preparation procedure in selected recipes (FOGT *et al*., 2007).

Meat sauce (finished)	< 2.4
Vegetable oil	5.1
Beef bouillon	< 2.4
Tomato puree	< 2.4
Tinned tomato	6.0

Figure 8: Levels of furan in homemade meat sauce including ingredients [ng/g] (FROMBERG *et al*., 2009).

High levels of furan were found in toasted bread slices (e.g. 83 μg/kg furan for dark toasted bread) and this was correlated to the browning level (FROMBERG *et al*., 2009). The dark and black toasted bread had high to very high levels of furan and the degree of browning of the toasted bread has very high influence on the amount of furan in the food item and therefore influence on the amount of furan consumed. The furan level might be

associated with the use of ascorbic acid in the flour used for the bread combined with a baking process leaving low levels of water in the final products. The untoasted bread analysed did not contain furan above 2.4 ng/g (FROMBERG *et al*., 2009). The crust always contained more furan than the entire bread, with a 3- to 20-fold difference depending on the surface-to-volume ratio (ZOLLER *et al*., 2007).
When an initial level of furan is present in the food item heated, changes in furan levels appear when the food item is heated. Heating the food item almost reduced the furan level in the food by 50%, however the furan level in the foods do not change when left for cool for one hour, and furan therefore seems to be stable in the food when it is not heated (FROMBERG *et al*., 2009) (Fig. 9).

	Finished soup	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Tomato soup	<2.4	<2.4	<2.4	2.4	<2.4	2.8	2.4
Minestrone	<2.4	<2.4	3.8	<2.4	<2.4	<2.4	3.0

Figure 9: Development in the furan concentrations over time in homemade soups [ng/g] (FROMBERG *et al.*, 2009).

The exceptions are vigorous boiling or cooking where furan can be lost, presumably by evaporation and by entrainment in the large volumes of steam that are released. On the other hand, warming the food even in lightly lidded containers can increase furan levels, so any additional formation appears to be balanced by evaporative losses (HANSIP *et al*., 2006).

1.3. Presence of furan in baby food

Children are sensible consumers, for this reason food safety and quality are essential. In EFSA report (EFSA, 2010), the highest maximum concentrations of furan for the non-coffee categories were found in baby food with 224 μg/kg.
Children have high capacity of absorption nutrients and non-nutrients (FIMP, 2011) but they have a reduced capacity of detoxification compared to an adult organism (GINSBERG, 2004). The metabolic differences baby\adult decrease with increasing age, but still have an influence through adolescence, for which there is still a high degree of risk of exposure to toxic agents (MADHAVAN and NAIDU, 1995; GINSBERG, 2004).
With the exception of coffee products, commercial complementary foods (6-24 months) were the food group with the highest furan concentrations. The problem is restricted only to commercially sterilized baby foods, while freshly cooked home-made complementary food was found to be furan-free (LACHENMEIER *et al*., 2009). The exposure assessment for babies is therefore challenging as it is not the total consumption that has to be evaluated, but more specifically, only the consumption of commercial products.
In EFSA report (2010) were analyzed, between 2004 and 2009, 11 samples of Infant Food (infant formula) and 1322 samples of Baby Food, divided in 6 sub-categories. Jarred baby food and infant formulae are of particular interest as they may form the sole diet for many infants and furan has been reported in those products (EFSA, 2010) (Fig. 10).

Food group	AT	BE	CY	DE	DK	ES	FI	GB	GR	HU	IE	IT	LT	NL	NO	PL	SK	SI	Total
Baby food	12	78	15	619	4	8	20	20	14	0	214	22	0	10	24	252	0	10	1322
Infant formula	0	0	0	0	0	0	0	0	0	0	1	9	0	1	0	0	0	0	11

Figure 10: Number of samples collected by Member States between 2004 and 2009 for the analysis of furan content in food (EFSA, 2010).

The mean furan content in infant formulae was 3 μg/kg, the furan content of jarred commercial baby food with an overall mean content of 29 μg/kg and a maximum value of 224 μg/kg was similar to previously reported data (CREWS and CASTLE, 2007).
Mean furan content in baby food containing only fruits is 5 μg/kg and 40 μg/kg in baby food containing only vegetables. BIANCHI *et al*. (2006) assumed that this difference in furan content could be due to different heating treatment as fruit samples are generally pasteurised whereas the vegetables are generally sterilized (EFSA, 2010) (Fig. 11).

Baby food subcategory	Furan content μg/kg							
	N	P05	P25	Median	Mean	P75	P95	Max
Cereal based	132	0-2.4	0-10	19	19	38	59	96
Meat and vegetables	447	7-8.1	24	38	39-40	51	78	129
Vegetables only	201	9.3-9.5	21	31	39-40	50	82	224
Fruits and vegetables	69	0-2	0-3	4-5	10-12	16	42	66
Fruits only	239	0-1.3	0-2.1	0-4.4	2.5-5	3.6-6	8.8-10	58
Non classified	234	0-2	6.2-7.3	17	31-32	40	130	215

Figure 11: Furan content in baby food sub categories (EFSA, 2010).

Baby food containing mainly vegetables and meat show mean furan concentrations of 40 μg/kg whereas cereal based baby food show lower mean values (19 μg/kg).
Furan was analysed in 21 different baby-food samples purchased from the Finnish markets. The mean levels of furan varied between 4.7 and 90.3 μg/kg (JESTOI *et al*., 2009).
US FDA (2004a) internet database is rather extensive, and data from years 2004-2005 show furan concentrations in fruit-based baby-foods below 8 μg/kg, vegetables and mixed vegetables up to 112 μg/kg and meat containing mixed baby and toddler foods up to 90 μg/kg.
In another study the retention of furan was significantly higher in baby food ''beef and vegetables'' and even more in baby food ''spinach''. Baby food ''spinach'' and baby food ''beef and vegetables'' contained 2 and 1% corn oil, respectively. Although the total fat content of baby food ''beef and vegetables'' (3.4%) was higher than the total fat content of baby food ''spinach'' (1.9%), the retention of furan by baby food ''spinach'' was significantly higher than the retention by baby food ''beef and vegetables''. These results lead to the assumption that the addition of a low amount of oil significantly increased the retention of furan, and this to a much higher extent than the total fat content. This implies that the presence of oils influences the actual intake of furan. As, from a nutritional point of view, elimination of oils from baby food is not an option, it would be better to add the oils after heat-processing, right before consumption of the baby food (LACHENMEIER et al., 2009; JESTOI *et al*., 2009). The problem is restricted only to commercially sterilized

baby foods, while freshly cooked home-made baby food was found to be furan-free (LACHENMEIER *et al*., 2009).
Therefore, the differences in furan retention caused by various food constituents are an important problem to be systematically studied.

1.4. Dietary furan intake

The Danish National Survey of Dietary Habits and Physical Activity 2000-2004, calculated the exposure of furan. The dietary survey comprised a random sample of 4120 individuals aged 4-75 years. Dietary intake was obtained using a 7 d pre-coded food diary. The amounts of food consumed were given in household measures (cups, spoons, slices, etc.) or estimated from photos of different portion sizes showing four to six different portions. The mean food intakes were calculated for each individual using the General Intake Estimation System (GIES) version 0.995a (Danish Institute for Food and Veterinary Research, Søborg, Denmark). In this analysis were used data from children 4-6 years (n=335) and adults 15-75 years (n=4692). Calculations of the furan exposures showed a median intake of 1.1 μg/day for children (mean 1.5 μg/day) and a median intake of 33.5 μg/day for adults (mean 27 μg/day) (FROMBERG *et al*., 2009).

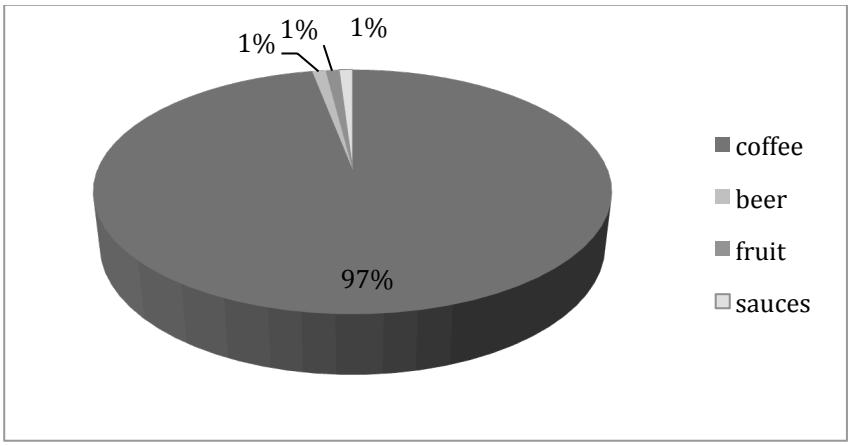


Figure 12: Foods contributing to the median intake of furan for adults of 34 μg/day (FROMBERG *et al*., 2009).

For adults the main contributor (95%) to the exposure of furan is coffee with an average daily intake of more than 0.6 L of coffee (the coffee is mostly made of 40 g medium roasted ground coffee per litre water) (JOHANSSON *et al*., 1998; FAGT *et al*., 2008) (Fig. 12).
As children do not have a high intake of coffee, the foods contributing to the intake are from other sources. The main food group contributing to furan is the breakfast cereal as high levels of furan was found in the breakfast cereals combined with children's high consumption (FROMBERG *et al*., 2009) (Fig. 13).

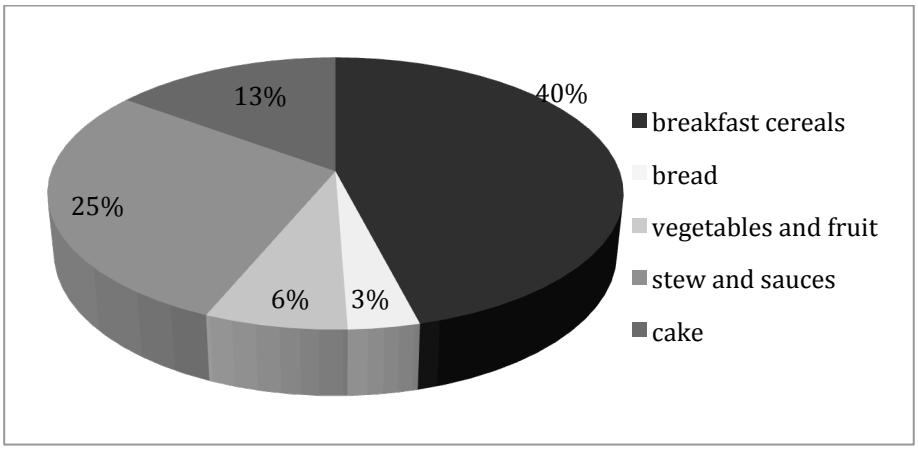


Figure 13: Foods contributing to the median intake of furan for children of total 1.1 μg/day (FROMBERG *et al.*, 2009)

The DONALD data show that the consumption of jarred baby foods in children 3 months aged is rather low. The highest exposure occurs in the children 9 months aged, and may reach a daily intake over 1 μg /kg bw. Afterwards, the exposure again decreases, due to the fact that both bodyweight and the use of non-jarred foods increase. The EFSA assumed a daily mean exposure of 1.01 μg /kg bw for children 9 months aged (BFR, 2009; KIM *et al.*, 2009; BAKHIJA and APPEL, 2010; LACHENMEIER *et al.*, 2012). The only exception is the study of Kim et al. (2009a) from Korea, which reported a considerably lower exposure (0.017-0.084 μg/kg). The difference between Korea and the high exposures in Europe might be explained by cultural differences (e.g. less consumption of commercially jarred foods) or that major sources were overlooked in the study. The data show a potential public health concern for this contaminant mostly for consumers 9 months aged (EFSA, 2005).
The exposure data were then used to characterize risk using the margin of exposure method based on a benchmark dose lower confidence limit for a 10% response (BMDL10) of 1.28 mg/ kg bw/ day for hepatocellular tumours in rats (CARTHEW et al., 2010). The margin of exposures (MOEs) was below the threshold of 10000, which is often used to define public health risks (LACHENMEIER *et al.*, 2012).
The foremost question that should be considered in the exposure estimation of furan in complementary foods is the treatment of the different subgroups of complementary foods, as beverages in particular contain lower concentrations than other groups. In the past, this differentiation was not made. Therefore, depending on the proportion of analysed beverages, the average furan content might be underestimated mostly in children 9 months aged.

2. CONCLUSIONS

We can conclude that furan is present in a variety of heat-treated commercial foods for adults (coffee, sauce and soup) and infants (jarred and canned baby food, breakfast cereal). Preliminary studies indicate that home-prepared food, with the exception of coffee, hardly contain any furan above the limit of detection (EFSA, 2010).
For the home cooked foods, foods rich in carbohydrates are most likely to form furan, probably due to a Maillard browning reaction of the food. High levels were found in

toasted bread and the content was correlated to the browning level, therefore not to toast the bread to a dark brown color might reduce the intake of furan. Even the worst case scenarios using ingredients containing furan for the home cooked foods did not lead to evaluated levels of furan during cooking. For ready-to-eat foods with initial occurrences of furan, cooking reduces the level of furan in the food to about half the initial concentration. Nevertheless, furan is stable in hot food items and the loss of furan present in the food before heating compared to the content after boiling the food is negligible. The furan level remained stable for one hour after heating and it can therefore be concluded that furan is stable in the food items. It was not until the food was reheated that the level of furan decreased (FROMBERG *et al*., 2009).

It appears to be possible to reduce the furan content in some food by volatilisation through heating and stirring of canned or jarred foods in an open saucepan. Recent studies have suggested that a simple approach to avoiding furan would be to heat infant foods in an open can while applying stirring (JESTOI et al., 2009; LIU and TSAI, 2010). This would really result in a considerable evaporation of furan, if parents would adhere to this practice. The first studies regarding this phenomenon reported losses of 29-55% in vegetable purees during different warming procedures in microwave ovens (ZOLLER *et al*., 2007), or even losses of up to 85% reported during heating opened jars over a period of 5.5 h in boiling water, and a reduction of ca.50% if the baby food jar was opened but not heated (GOLDMANN *et al*., 2005).

Reduction of furan in foods is likely to be more challenging compared to other process contaminants, for two reasons. First, there may be little room for maneuver to lower heating times and temperatures because the processes of pasteurisation and sterilisation are indispensable for the microbiological safety of foods. Second, furan has a wide range of precursors. Ascorbic acid shows the highest potential to form furan, followed by polyunsaturated fatty acids and then sugars (STADLER, 2006). Ascorbic acid and polyunsaturated fatty acids are regarded as desirable food components because of their health benefits.

The best approaches appear so far to involve intervention in the reaction mechanisms. For example, formation of furfural from ascorbic acid in model orange juice was repressed by the presence of ethanol and mannitol acting as free radical scavengers (SHINODA *et al*., 2005). Reduction of atmospheric oxygen reduces the autoxidation of unsaturated fatty acids and also reduces furan formation from several precursors, notably ascorbic acid, as the addition of sulphite does (MARK *et al*., 2006). Therefore, modification of the atmospheres within heating systems might be effective in reducing furan in foods.

Since the carcinogenicity of furan is probably attributable to a genotoxic mechanism (EFSA, 2004) levels in food should be kept ALARA as low as reasonably achievable. Currently, the limits for furan in food have not been established yet by European legislation.

FDA recommends that consumers eat a balanced diet, choosing a variety of foods that are low in trans-fat and saturated fat, and rich in high-fibre grains, fruits, and vegetables (FDA 2004c). Under the circumstances described previously, the continuation of the research is desirable for achieving safer and healthier foods.

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REVIEW

THE HEALTH EFFECTS OF OLEUROPEIN, ONE OF THE MAJOR PHENOLIC COMPOUNDS OF OLIVES, OLEA EUROPAEA *L.*

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ABSTRACT

Olives (*Olea europaea* L.) and olive oils have a significant place in the daily diet in the Mediterranean Region and contain significant amounts of polyphenolic compounds, of which oleuropein, hydroxytyrosol and tyrosol are dominant. Harvest time, geographical region and climate all affect the phenolic content. Experimental, clinical and epidemiological studies show the beneficial effects of oleuropein, such as antioxidant, antimicrobial, anticancer anti-inflammatory, antineuropathic and other properties. In particular, olive leaves, roots, virgin olive oil and olive mill waste (vegetation and wastewater) are potential sources of oleuropein. This review focuses on the pharmacological effects of oleuropein and extraction procedures for oleuropein.

Keywords: olive, phenolic compounds, oleuropein, pharmacological effects, *Olea europaea*, extract

1. INTRODUCTION

Olives are fruits and are a significant part of the daily diet, playing a vital role in the agricultural sector especially in the Mediterranean countries and having high economic value in this region. In addition to their popular usage as table olives, they have become extremely valuable because they have important nutrient properties and have shown positive benefits for human health. The majority of olives produced globally are used in oil production (approximately 85-90%), and the rest is used in the production of table olives. Although Turkey comes behind Spain, Italy and Greece in oil flesh and table olive production (FAO, 2011), It is reported that olive producing regions in this country are in order of Aegean (55.11%), Marmara (27.72%), Mediterranean (14.94%) and Black Sea region (2.22%) and that 75-80% of total oil production of olive oil is made in the Aegean region (ARSLAN and OZCAN, 2011). It is reported that as of 2010, Turkey has 6 million of olive trees and 2 million of table olive trees, and 73% of total olive areas and olive production is made of oil types and 27% comprises table olives. Olive oil production was approximately 1,040,000 tons and table olive production was 375,000 tons according to statistical data in 2011 (DOGAKA, 2011).

Olive trees (*Olea europaea L.*) are included in the Oleaceae family. The most important characteristic of olive fruits is their high oil content, chemical composition and a unique bitterness in comparison to other fruits with hard seeds (OTHMAN *et al*., 2008; MAFRA and BARROS, 2006). The typical bitterness of olive fruits results from their rich content of phenolic compounds, especially of oleuropein, in olive cultivars (Fig. 1). These phenolic compounds and oleuropein content of olive cultivars increase the interest in their antioxidant properties and their positive effects on health (ARSLAN and OZCAN, 2011; BARBARO *et al*., 2014;CARDENO *et al*., 2013). It has been reported that, in addition to their mono-unsaturated fatty acid content, lower-concentration compounds are the reason for their positive effects on nourishment. The amount of phenolic compounds ranges between 1-2% in fresh fruits, and that these are mainly secondary metabolites in olives (OTHMAN *et al*., 2008). Phenolic acids, phenolic alcohols, flavonoids and secoiridoids are also important phenolic compounds in olive cultivars. Iridoids and secoiridoids are compounds that are bound to glycosides and are formed from the secondary metabolism of terpenes as precursors of various indole alkaloids. It is reported that the secoiridoides in Oleaceae are usually derived from the oleoside type of glycosides, which are characterized by an exocyclic 8,9-olefinic functionality. Oleuropein is an ester that forms from 3-4-dihydroxyphenyl ethanol and hydroxytyrosol and has the oleosidic skeleton. It is reported that demethyloleuropein, oleuropein aglycone, elenolic acid and verbascoside are essential compounds of hydroxycinnamic acid derivative in olive cultivars (ROMANI *et al*., 1999; SERVILLI *et al*., 2004; ROMERO *et al*., 2002; ESTI *et al*., 1998; AMIOT *et al*., 1986).

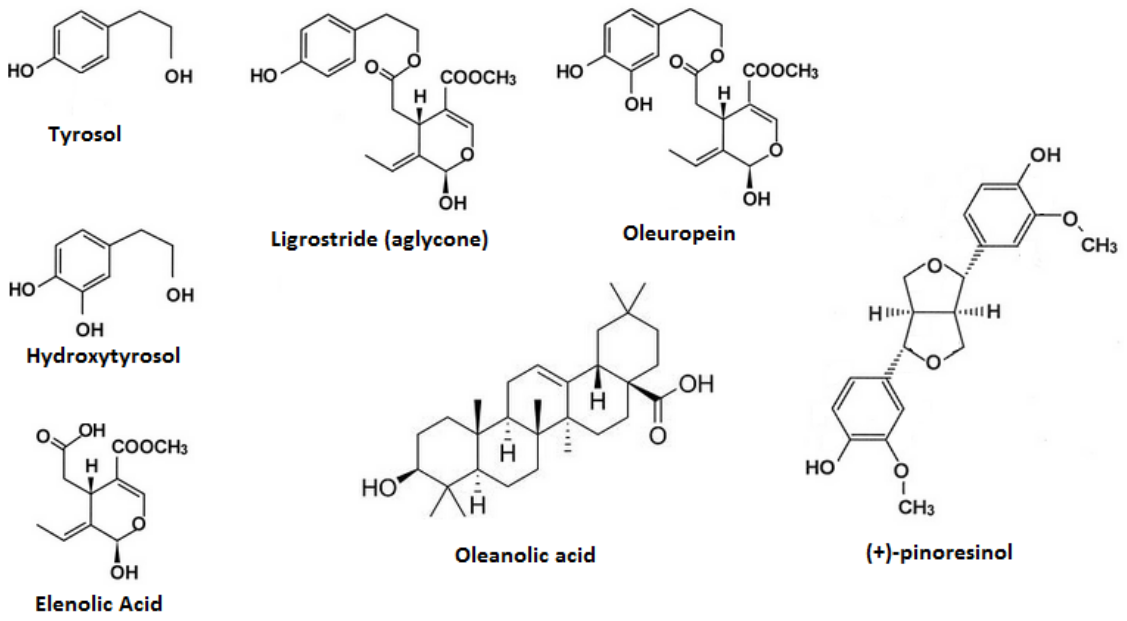


Figure 1: Chemical structures of some of the phenolic compunds that are found in the *Oleacea* family (YILDIZ and UYLASER, 2011).

Factors such as variety, geographical location, maturing progress, harvest time and seasonal conditions all affect phenolic substance amounts in olives. It was stated that the total precipitation content during the period until harvest time affected phenolic substances (ROMERO *et al*., 2003; ESTI *et al*., 1998), and there were decreases in phenolic concentrations of olive oils from fruits of trees cultivated in irrigated areas or under high rainfall conditions when compared to the oils from non-irrigated or low rainfall regions (TOVAR *et al*., 2002; YOUSFI *et al*., 2006; BOTIA *et al*., 2001). Contrary to this situation, it was indicated that phenolic contents were higher in olives in the Alanya region, which receives a relatively high rainfall (ARSLAN and OZCAN, 2011). The phenolic contents of olives that were cultivated in higher altitude locations were greater than those from lower altitude regions (MOUSA *et al*., 1996), and that high altitude did not affect the content of phenolic substances of Sariulak olives that were grown in different regions. It was stated that harvest time affected the phenolic substances in olives; and phenolic contents changed based on olive harvests made at different times during September-December months. It was indicated that at the second harvest period around the October-November months, rutin, verbascoides, taxifolin and tyrosol contents increased, and in addition, phenolic contents decreased toward the last periods of the harvest time. It was reported that among the phenolic substances present in Sariulak olives, apigenin, cinnamic acid and p-coumaric and 4-hydroxybiphenyl carboxylic acid contents decreased, whereas ferulic and vanillic acid contents increased as the harvest time progressed (ARSLAN and OZCAN, 2011).

2. OLEUROPEIN AND ITS FORMATION

Olive fruits contain a high level of mono-unsaturated oils and there are significant amounts of phenolic substances. Phenolic compounds are good antioxidants and contribute to the prevention of illnesses due to their important biological activity characteristics (OTHMAN *et al*., 2008). The antioxidant capacities of phenolic substances

originate from their reducing characteristics, which enable them to react with mono oxygen (RICE-EVANS *et al*., 1997).
It is stated that there are approximately 40 phenolic compound when olive plants are considered as a whole as a tree, branch, root, and olive fruit. Among the phenolic compounds the concentrations of, especially, oleuropein, hydroxytyrosol, lutolein, rutin, trans cinnamic acid and tyrosol are higher compared to other phenolic substances (YORULMAZ *et al*., 2013; ARSLAN, 2012; ARSLAN and OZCAN. 2011). Oleuropein, dimethyl-oleuropein, ligstride and oleoside compounds indicate dominance of phenolic oleosides in olive fruits and, in addition to this, verbascoside is the main derivative of hydroxycinnamic acid in olives. Oleuropein is the most important phenolic compound in olive cultivars and its concentrations can reach up to 140 mg g^{-1} (db) levels in some olive species; and the content ranged between 60-90 mg g^{-1} (db) of dry matter in young olive cultivars (AMIOT *et al*., 1986; LE TUTOUR and GUEDON, 1992).
Oleuropeins, classified within the secoiridoids, exist in varying concentrations in olive plants, mostly in tree stems, branches, leaves and olive fruits. Iridoid and secoiridoids that are bound to glycosides are compounds formed in the secondary metabolism of terpenes which are the precursors of various indole alkaloids. It is reported that oleuropein (Fig. 2) is an ester of 2-ethanol (3,4-dihydroxyphenyl) and oleuropein aglycone have an oleoside structure known as secoiridoide glucoside (SOLER-RIVAS *et al*., 2000).

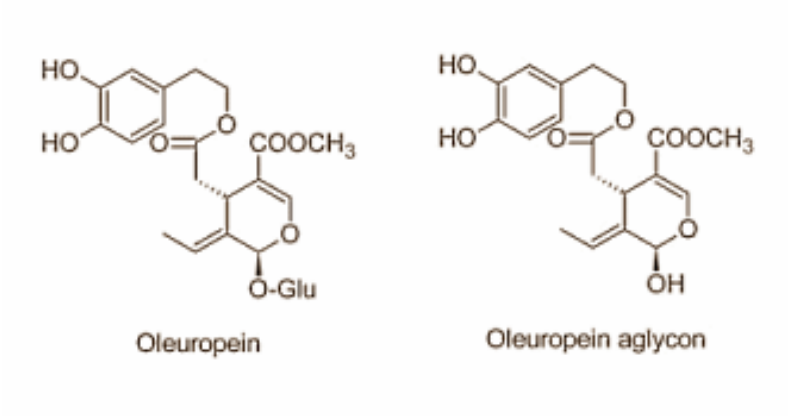


Figure 2: Chemical structures of oleuropein and oleuropein aglycone (Yıldız and Uylaser, 2011).

Oleuropein production in olives (Fig. 3) occurs by branching in the mevalonic acid cycle in the secondary metabolism of terpenes during the formation of oleosides (DAMTOFT *et al*., 1992). Secoiridoids are derived from these compounds and the carbon skeleton is derived from mevalonic acid. Geraniol, 10-hydroxygeraniol and iridoidal are regarded as the precursors of loganin. Deoxyloganic acid, 7-epiloganic acid and loganic acids are the precursors of oleuropein, which are incorporated in to the ligstrosides (DAMTOFT *et al*., 1993).

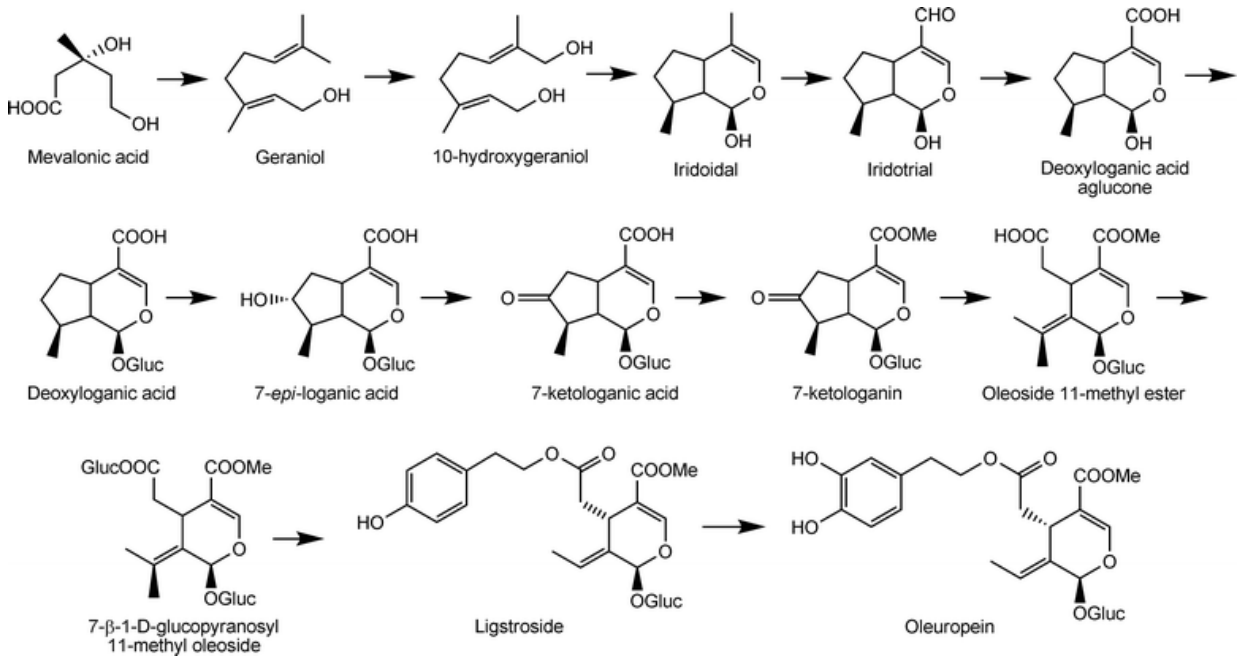


Figure 3: Formation of oleuropein in the Olea family (YILDIZ and UYLASER, 2011).

In studies conducted on the contents of oleuropein which is responsible for the bitter taste in olive cultivars, its content ranged between 2.1-134 mg g^{-1}(db) in olive leaves, 11-18.9 mg g^{-1}(db) in olive branches, 1.9-6 mg g^{-1} (db) in olive roots, 0.3-21.7 mg g^{-1} (db) in olive fruits and 0-11.2 mg g^{-1} (db) in extra virgin olive oil (ANSARI et al., 2011; SAVOURNIN *et al*., 2001; TAYOUB *et al*., 2012; MALIK and BRADFORD, 2006; ALTINYAY and ALTUN, 2006; YORULMAZ *et al*., 2013). In non-virgin oils, oleuropein contents range between 0-0.4 mg g^{-1} after oil extraction and oleuropein is also found in pomace and in the liquid portion (vegetation and waste water) except for oil obtained as a result of olive oil pressing that is found in ratios between 6.5-8.7 mg g^{-1} (GOLDSMITH *et al*., 2014; ALLOUCHE *et al*., 2004; YILDIZ and UYLASER, 2011). Oleuropein formation in olive fruits takes place at the order of growth stage, green maturation stage and black maturation stage. During the growth stage, oleuropein accumulates in olive fruits and amounts of chlorophyll and oleuropein decrease at the green maturation stage. At the black maturation stage in olives, a decrease in oleuropein contents takes place and anthocyanin pigments are formed (AMIOT *et al*., 1989). It is proposed that oleuropein content is high at the beginning of the fruit growth in young olive fruits (YORULMAZ *et al*., 2013). Although there is a significant amount of oleuropein in olives that are harvested when the olive fruits are green, the oleuropein content decreases rapidly with the black maturation phase (BIANCO *et al*., 1993). Although phenolic compounds in table olives are initially higher, in the production of table olives the oleuropein concentration decreases during maturation and disappears at full ripeness. The oleuropein concentration also decreases sharply after salt treatment and disappears after lye treatment. It is also reported that the phenolic contents of table olives showed different phenolic profiles such as hydroxytyrosol concentration of olive drupes, which decline rapidly during the black maturation phase, which are explained by the fact of climate, soil type and growing region (OTHMAN *et al*., 2008).

3. OLEUROPEIN AND ITS PHARMACOLOGICAL EFFECTS

The significant effects of phenolic compounds that are present in plants have increased interest for the use of these substances in the preparation and processing of foodstuffs. Olive fruits and oil that are produced in the Mediterranean region have a key position in the daily diet and they are good resources in terms of oleuropein, which is the most prominent phenolic substance (ARSLAN and OZCAN, 2011). Oleuropein, which is found in varying quantities in olive fruits, branches, leaves and roots of olive trees, has many positive effects on human health according to clinical, epidemiological and experimental investigations conducted on the health effects, such as antioxidant (VISIOLI *et al*., 2002; GONZALES-HIDALGO *et al*., 2012), anti-atherogenic, anti-inflammatory (VISIOLI *et al*., 1998), anti-cancer, antimicrobial and antiviral effects (TRIPOLI *et al*., 2005; FREDRICKSON and GROUP, 2000).

Effects of oleuropein on lipid oxidation are shown in the decrease in by-products (malondialdehyde and 4-hydroxynonenal) that react with 2-thiobarbituric acid (TBA) and produced by oxidation. It is stated that antioxidant potential of oleuropeins is related to the ability of radical stability development by forming a hydrogen bond within the molecule between the free hydrogen of hydroxyl groups and phenoxy radicals and the higher content of oleic acid that are present in virgin oils compared to other edible oils and the presence of high level of antioxidant compounds in olives and olive oils (hydroxytyrosol and oleuropein) have less sensitivity to oxidation in comparison to n-6 unsaturated fatty acids. (BARBARO *et al*., 2014). Oleuropein has protective effects in the decrease of plasmatic levels of cholesterol in the total, free and ester forms in rabbits for improving the resistance of low density lipoproteins to oxidation (CONI *et al*., 2000) and also oleuropeins have the ability to clear nitric oxide (NO). In addition, this causes an increase in inducible nitric oxide synthesis in cells (DE LA PUERTA *et al*., 2001). Hypochlorous acid, which is formed as a result of oxidation by neutrophil myeloperoxidases at the site of inflammation and can cause damage to proteins including enzymes (VISIOLI *et al*., 2002).

There are many studies that have considered the effects of oleuropein on health. The consumption of virgin olive oil, which is rich in nutrients, have an observed antitumor effect and decrease the proliferation of cancer cells, especially of the colon, breast and skin (CARDENO *et al*., 2013; PSALTOPOULOU *et al*., 2011). It is reported that an application of a 400 μM dose of oleuropein and hydroxytyrosol cause a significant decrease in the cell proliferation of colon cancer (HT-29) 24-hour after treatment (CARDENO *et al*., 2013). It is stated that the oleuropein aglycone compound is the most effective phenolic compound for decreasing the viability of cancer cells in breast, colon and kidney cancer types (MENENDEZ *et al*., 2007;HAMDI and CASTELLON, 2005). Oleuropein that has been extracted from olive leaves has marked antitumor effects on prostate, breast and hepatoma cancer cells, depending on the amount of dose, 24-72 hours after application (KOCKAR *et al*., 2010). The effects of oleuropeins on the various cancer types and cells are shown in Table 1.

Table 1: Oleuropein-induced anti-tumor effects in different cancer cells.

Cell line	Cancer type	References
MCF-7;MDA;T-47D	Breast adenocarcinoma and ductal carcinoma	(HAMDI and CASTELLON, 2005)
786-O	Renal cell adenocarcinoma	(HAMDI and CASTELLON, 2005)
RPMI 7951	Melanoma	(HAMDI and CASTELLON, 2005)
HT-29,Caco-2,LoVo	Colorectal adenocinoma	(CARDENO *et al*., 2013;CORONA *et al*., 2007)
PC-3,MCF-7,HEP3b	Prostate,Breast,Hepatoma	(KOCKAR *et al*., 2010)
LNCaP and DU145	Prostate cancer	(ACQUAVIVA *et al*., 2012)
A549	Lung carcinoma	(MAO *et al*., 2012)

The antitumor effect of oleuropein is related to its anti-angiogenic function. There are many different varieties of tumors that are connected with other cells and these are formed by complex interactions in a permeable microstructure. The development of a tumor mass is formed in the 'nutrition' environment, and in growing initially forms a deoxygenated environment. The formation of new blood vessels occurs by stimulating multiplication and activities of endothelial cells (BARBARRO *et al*., 2014). Skin that is exposed to solar UV radiation is damaged and also skin thickness and elasticity are reduced, which makes it more vulnerable to skin cancers. It is reported that oleuropein and olive leaf extracts from were administered orally twice for 30 weeks in hairless mice. Both the extract and oleuropein (0.3 and 1 g kg^{-1}) significantly inhibit the increases in skin thickness, reductions in skin elasticity, skin carcinogenesis and tumor growth (KIMURA and SUMIYOSHI, 2009).

Antimicrobial agents are used to control microbial growth and many synthetic antioxidant substances are used for this purpose. There is an increase in the use of natural substances instead of synthetic substances due to their side effects on human health. Oleuropein has been considered as a natural antioxidant substance to reduce the growth rate of microorganisms. It has been reported that the phenolic compound oleuropein in virgin olive oil has antimicrobial activity towards *L. plantarum*, *B. cereus* and *S. enteritidis* among both Gram positive and Gram negative bacteria (CICERALE *et al*., 2012; AZIZ *et al*., 1998). The toxic effect of oleuropein is higher for Gram (+) bacteria than for Gram (-) bacteria, especially *S. aureus* and *E. coli* (FURNERI et al., 2002). Oleuropein also has antimycoplasmal properties and consequently has positive effects against mycoplasma bacteria strains, which are resistant to antibiotic applications (FURNERI *et al*., 2002). Although the antimicrobial activity mechanism of the oleuropein component has not been explained completely, it has been suggested that the microbial activity mechanism of oleuropein originates from the presence of an ortho-diphenolic (catechol) system in the environment and that it changed the ability of a glycoside group, enabling it to enter through the cell membrane and reach the target region (SAIJA and UCCELLA, 2001). It was expressed that oleuropein that had been extracted from olive leaves also has antimicrobial characteristics towards *Campylobacter jejuni*, *Helicobacter pylori* and *Staphylococcus aureus*, which are resistant to methicillin, and extracts of olive leaves played a crucial role in the arrangement of the composition of stomach flora by reducing *H. pylori* and *C. jejuni* microorganisms (SUDJANA *et al*., 2009).

4. DIFFERENT ANALYTICAL APPROACHES FOR OLEUROPEIN EXTRACTION FROM OLIVE LEAVES

Oleuropein is the most significant component among phenolic compounds present in olive cultivars. It is present in a significant amount in the development phases of olive fruits, but its content starts to diminish along with maturation and decreases to near zero after the olive fruits turn from green to black. There is a demand for extracting and purifying of oleuropein existing in the olive cultivars, especially from olive leaves, because of their pharmacological characteristics and favorable effects on health. There are many methods for extracting such phenolic substances from olive leaves, such as extraction by using organic solvents at high temperature and pressure, liquid-liquid extraction (JAPON-LUJAN and LUQUE DE CASTRO, 2006), super-critical liquid extraction (SAHIN *et al*., 2011), polar substances extraction (MALIK and BRADFORD. 2008), solid phase extraction, acidified de-ionized water extraction (STAMATOPOULOS *et al*., 2012), steam blanching, hot water and ultrasound extraction (ANSARI *et al*., 2010).
Oleuropein is more readily extracted from olive leaves due to their higher content of oleuropein compared to the fruits. Prior to the extraction of oleuropein, olive leaves are dried at temperatures not exceeding 60°C and then the dried leaves are crushed to granules with their dimensions ranging between 0.9-2 mm. Following this, the phenolic substances and oleuropein are extracted. The stability and content of oleuropein differs, depending on the extraction method used. It is reported that a higher amount of oleuropein is extracted by using 80% methanol compared to extraction by hexane, ethanol, hot water or a mixture of methanol/hexane (SAHIN *et al*., 2011; MALIK and BRADFORD, 2008). The oleuropein is quite stable for 30 days at room conditions and also stable in aqueous extracts for 7 days when stored at room temperatures but is degraded after 17 days (MALIK and BRADFORD, 2008). Drying of olive leaves at 25°C has no effects on the oleuropein and verbascosides contents at room conditions but drying leaves at greater than 60°C causes a decrease in phenolic substances (MALIK and BRADFORD. 2008). The extraction of oleuropein from leaves by using acidified de-ionized water (pH: 3, 60°C) has a marked effect on the color of the leaf extract (green) and oleruopein content; and also this method has the advantage of the non-toxic characteristics of acidified de-ionized water (ANSARI *et al*., 2011). Among the treatments of steam blanching, hot water and UV-C irradiation, the steam extraction method provides an increase in oleuropein yield from 25 to 35 times compared to non-steam blanching samples whereas antioxidant activity increases from 4 to 13 times. The particle size of leaves (1-3 mm) used affects the extraction of phenolic contents and oleuropein and the ethanolic extraction of hot water blanched leaf fractions produces higher amounts of oleuropein compare to the untreated samples (STAMATOPOULOS *et al*., 2012). Ethanol, methanol and water are considered as co-solvents in 20% (v/v) for the extraction of oleuropein from olive leaves, whereas the maximum oleuropein yield is obtained by supercritical fluid (CO_2) extraction modified with 20% methanol (SAHIN *et al*., 2011).

5. RESULTS AND CONCLUSIONS

Olives have a crucial place in the daily diet in the Mediterranean region. When they are examined in terms of ingredients, in addition to mono-unsaturated fatty acids, there are 15-40 phenolic substances in olive fruits, leaves, branches and roots; and oleuropein is found to be the most prominent substance among the phenolic compounds. The amount of rainfall, geographical region, harvest time and altitudes affect the phenolic contents in olive cultivars and there is a close relationship between phenolic compounds and olive

maturation. It is crucial that when prompted to maximum extract of oleuropein, color changes of olive fruits should be monitored in terms of phenolic contents and oleuropein. Oleuropein has many pharmacological effects such as antioxidant, antimicrobial, anti-inflamatory, antitumor and neuroprotective effects on neural disorders emerging with aging (Table 2).

Table 2: Biological activities of oleuropein that found in olive fruits,leaf and virgin olive oils.

Activity	Effects
Antioxidant	Inhibition of oxidation of LDL,improvement of radical stability
Anti-tumor	Antiproliferative,anti-migration,angiogenesis and apoptosis effect
Antimicrobial	Bacterial cell membrane damage
Neuroprotective	Tau fibrilization inhibition,oxidative stress reduction
Anti-inflammatory	Lipoxygenase and cytokine inhibition

It has been observed that oleuropein extracted from olive cultivars can be used in the prevention of proliferation of different cancer cells such as breast, skin, lung, colon cancers and also neuronal disorders occurring due to aging. It is possible to obtain oleuropein naturally and due to its pharmacological effects it is likely to be used as an antioxidant and antimicrobial agent in the food preparation or preservation and health sectors. Among the available extraction methods of oleuropein from olive cultivars, especially from leaves, polar substances are being used according to color, antioxidant activity and stability of oleuropein at room conditions. Some pre-treatments of the extraction procedure can be applied to increase extraction yield from olive leaves such as use of supercritical fluid, steam blanching and grinding dried olive leaves to the size of 0.9-3 mm.

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REVIEW

THE HERBACEOUS CHARACTER OF WINES

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ABSTRACT

The herbaceous (vegetative) character of wine can be sensorially pleasant or not depending on different circumstances. This character can be specific to a particular grape cultivar (an endogenous varietal character) or produced during the winemaking procedure, from either an endogenous or exogenous cause. Several volatile wine components containing oxygen (aldehydes and alcohols with 6 carbon atoms), nitrogen (alkylmethoxypyrazines), and selected sulfur compounds (thiols, sulfides, disulfides) are reviewed and described. The effects of micro-oxygenation and storage in wood on the herbaceous post-fermentative character are discussed. Finally, the preventive tools and winemaking technologies used to reduce or to control this sensory character are described.

Keywords: green leaf volatiles, herbaceous, methoxypyrazines, thiols, vegetative, wine aroma

1. INTRODUCTION

The aroma complexity of wine is the result of several different factors (AMERINE and ROESSLER, 1976; RAPP, 1998). It consists of many hundred volatile compounds of various origin, which are present at concentrations ranging from fractions of ng/L up to mg/L and are able to interact with the olfactory epithelium to generate a sensory perception (ROBINSON *et al*., 2014a,b). Many of these odorous substances derive directly from the grapes (RAPP, 1998) and their formation is affected by both the grape metabolism and the viticultural ecosystem. Other impactful odorant compounds originate from the pre-fermentation biochemistry occurring during grape crushing and maceration. The 'fermentative aromas' (RAPP and MANDERY, 1986) originate from the metabolism of the microorganisms responsible for the alcoholic and malolactic fermentations. The chemical reactions occurring during the finishing and ageing of the wines also play a crucial role in the final bouquet of wine. Moreover, the effect of the addition of chemicals and adjuncts, the characteristics of the cooperage used for the processing and storage and the closure systems are also determining factors.

The fascination exercised by wine on the consumer is due in large part to the complexity of these orthonasal cues and to the retronasal perceptions evoked at the first sip. Due to the huge variability of odor notes, the volatiles are the components that often best define the parameters of quality and typicality of wine. Moreover, some aroma components are robust markers of identity, playing an important role in the protection of local products from commercial frauds (BOSELLI *et al*., 2008). A focus on 'varietal winemaking' is becoming increasingly important for countries characterized by an extremely variegated viticulture in terms of pedo-climatic conditions and cultivated genotypes which results in a large number of wines with specific geographical determination (e.g., over 400 in Italy).

Occasionally, wines may express excessive sensations of herbaceous ('vegetative') off-flavor, resulting in a reduction in fruit intensity and detrimentally impacting palate structure. Consumers rarely appreciate wines with pronounced herbaceous flavors; these products are described as possessing immature character, lacking refinement and elegance, and as such are not sought after by the modern consumer. In order to reduce or avoid these olfactory qualities in wines it is of critical importance to know the origins and causes of these characters.

This paper reviews the chemical compounds known to be responsible for the herbaceous character of wines and reports on the preventive tools and winemaking technologies used to minimize or control 'herbaceousness'. The herbaceous aroma in wines is described in reference to the genesis of the molecules responsible for the olfactory perception. These components can be ascribed to three different chemical classes:

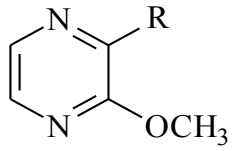
- alkylmethoxypyrazines (endogenous varietal and exogenous herbaceous character)
- aldehydes and alcohols with 6 carbon atoms (pre-fermentative herbaceous character)
- selected low molecular weight sulphur compounds (herbaceous character in fermentation)

The contribution of any of these compounds to the final aroma depends on their concentration, their perception threshold and the extent to which they are modulated by interactions with both volatile and non-volatile components of wine.

2. HERBACEOUS CHARACTER DUE TO METHOXYPYRAZINES

2.1. Endogenous (varietal) herbaceous character

The varietal herbaceous character is usually described as 'green pepper', or 'tomato leaf' and is mainly derived from a group of nitrogen-containing compounds, the alkylmethoxypyrazines, which were first discovered in bell peppers (BUTTERY *et al*., 1969) and are present in green plant tissues, including grapes (BAYONOVE *et al*., 1975). Methoxypyrazines are used by insects and plants in chemical defense or, conversely, they may act in insects as pheromones.
The alkylmethoxypyrazines (MP) are chemical compounds based on the heterocyclic aromatic structure of pyrazine, originating from the metabolism of amino acids (Fig. 1).



R= CH_2CH_3, ETMP
R= $CHCH_3CH_2CH_3$, SBMP
R= $CH(CH_3)_2$, IPMP
R= $CH_2CH(CH_3)_2$, IBMP

Figure 1: Chemical structure of alkylmethoxypyrazines. ETMP: 3-ethyl-2-methoxypyrazine; SBMP: 3-sec-butyl-2-methoxypyrazine; IPMP: 3-isopropyl-2-methoxypyrazine; IBMP: 3-isobutyl-2-methoxypyrazine.

The main MP found in grapes, musts and wines are 3-ethyl-2-methoxypyrazine (ETMP); 3-sec-butyl-2-methoxypyrazine (SBMP); 3-isopropyl-2-methoxypyrazine (IPMP); and 3-isobutyl-2-methoxypyrazine (IBMP). The nature of the alkyl radical largely determines the olfactory perceptions of these compounds (SALA *et al*., 2004) as summarized in Table 1.

Table 1: Odor descriptors and olfactory threshold in water (ng/L) of the main alkylmethoxypyrazines (MP) according to SALA *et al*. (2004).

Alkylmethoxypyrazines	Odor description	Threshold
ETMP	Raw potato, earthy, green, bell pepper	400-425
SBMP	Green, ivy leaves, bell pepper	1-2
IPMP	Earthy, cooked asparagus, green pepper	2
IBMP	Earthy, green, bell pepper, musty	0.5-2

Their aroma is generally described as 'bell pepper', 'leafy', 'ivy leaves', 'vegetable', 'peas', 'asparagus', 'ginseng', 'roasted', 'musty' and 'raw potatoes'.
MP represents a narrow, delineated group of extremely powerful odorants characterized by extremely low sensory perception thresholds (e.g., 1-2 ng/L in distilled water; KOTSERIDIS *et al*., 1999). IBMP is typically the most predominant MP in grape and wine and, as such, is a major contributor to the vegetative character (SALA *et al*., 2004; ALLEN

et al., 1991; LACEY *et al*., 1991). This vegetative character is most commonly, although not exclusively, associated with Sauvignon blanc, Cabernet Sauvignon and other Bordeaux varietals (ALLEN *et al*., 1991).

IPMP may also be present in certain grapes and thus found in the derived wine as a varietal character. However, it is the major exogenous contributor to the pool of methoxypyrazines because it is the most abundant and potent of the MP released by ladybugs, insects of the family Coccinellidae that can be accidentally incorporated with grapes during harvest operations (BOTEZATU *et al*., 2012), as reported in the next section.

Different pathways leading to the MP responsible for the herbaceous character of wines have been proposed. One utilizes the amino acid leucine (for IBMP) or valine (for IPMP) and an unknown 1,2-dicarbonyl compound leading to the formation of 3-alkyl-2-hydroxypyrazine (HP). The final steps of these pathways involve the methylation of HP to form the associated MP, catalyzed by an O-methyltransferase enzyme (DUNLEVY *et al*., 2010; DUNLEVY *et al*., 2013). A very wide range of MP levels can be found in grapes due to changes in their concentration throughout the ripening process. Although the content of both IPMP and IBMP increases during early berry development, IPMP was found to decrease before veraison, whereas IBMP decreased rapidly after veraison in Cabernet Sauvignon grapes (HASHIZUME and SAMUTA, 1999). Similarly, MP levels were found to decrease progressively and rapidly with grape maturity both in Cabernet Sauvignon and Merlot (ROUJOU DE BOUBEE *et al*., 2000). In Sauvignon blanc grapes, LACEY *et al*. (1991) found the same trend, although ALLEN *et al*. (1991) found an increase in IBMP during ripening.

A positive linear relationship was found between the content of IBMP in grapes at different ripening stages and the derived wines from the Bordeaux region (produced from Merlot, Cabernet Franc and Cabernet Sauvignon grapes) (KOTSERIDIS *et al*., 1999). RYONA *et al*. (2010) hypothesized that the IBMP formation during ripening was due to the methylation of 3-isobutyl-2-hydroxypyrazine (IBHP) to IBMP. Therefore, IBHP is a key intermediate in both the formation and degradation of IBMP (HASHIZUME *et al*., 2001; HARRIS *et al*., 2012) although previous studies attributed this decrease to the photolability of IBMP (HASHIZUME and SAMUTA, 1999; RYONA *et al*., 2008). Further research is necessary in order to better understand the formation of these compounds and control their concentrations in wines.

The excessive green bell pepper aroma found in red wines containing IBMP is generally considered unfavorable to wine quality. However, the presence of this compound at low levels is often noted to augment the quality of certain wines obtained from red varieties (e.g., Cabernet Franc, Cabernet Sauvignon, Carmenere, Merlot) or from white varieties (Sauvignon blanc, Semillon) by adding to the intrinsic flavor complexity of these varietals (ALLEN *et al*., 1995; BELANCIC and AGOSIN, 2007). The presence of IBMP can be a positive quality factor when it is not dominant but is in balance and complemented by other herbaceous and fruity aromas (SALA *et al*., 2004).

The content of MP in the wine depends primarily on grape composition (ROUJOU DE BOUBEE *et al*., 2002). Viticultural factors may also influence the MP content in grapes and wines. Understanding the mechanisms that influence the formation of wine volatile compounds through grape growing, winemaking and storage is important in developing strategies for the production of wines with specific sensory attributes that appeal to target markets (ROBINSON *et al*., 2014b).

IBMP concentrations were found to be significantly negatively correlated with the number of buds per vine (CHAPMAN *et al*., 2004). The levels of MP can also be controlled in the vineyard by either extending the maturation or by defoliation (SALA *et al*., 2004). High correlations between the decrease of malic acid and the decrease of IBMP as the grapes ripened, irrespective of other cultural and climatic variables, has been reported (ROUJOU

DE BOUBEE *et al*., 2000), indicating that IBMP levels could serve as markers indicative of poor ripening.
In general, light-exposed clusters are reported to have reduced IBMP levels at harvest, although there is some ambiguity in the literature (RYONA *et al*., 2008). Light exposure seems to have two opposing effects on the concentration of MP in grapes: (a) by promoting the biological formation of MP in immature grapes, and (b) by reducing the MP in ripening grapes (whether by photodegradation or initiating demethylation) (HASHIZUME and SAMUTA, 1999; HARRIS *et al*., 2012; HEYMANN *et al*., 1986). The actual content of MP in grapes would result from the total sum of these two processes.
Light exposure has previously been shown to affect other wine constituents, such as superior alcohols, phenols and esters (D'AURIA *et al*., 2003), dimethylsulfide (MARAIS and POOL, 1980), terpenes and norisoprenoids (CALO' *et al*., 1996). Cluster exposure to sunlight will inhibit IBMP accumulation pre-veraison (RYONA *et al*., 2008) and several studies have reported that pre-veraison cluster light exposure will result in decreased IBMP levels at harvest (DUNLEVY *et al*., 2010; RYONA *et al*., 2008). MARAIS and POOL (1980) reported a decrease of the IBMP levels in Sauvignon blanc by up to 50% in light-exposed berries across several sites in South Africa studied over two years, and they showed a reduction in IBMP at both veraison and harvest. Thus, the incident light conditions can affect the IBMP levels in the final wine.
The effect of the light exposure has been linked to the number of leaf layers (SALA *et al*., 2004). SCHEINER *et al*. (2010) evaluated the effects of basal leaf removal timing and severity on IBMP concentration in grape berries. The complete leaf removal significantly reduced the IBMP concentrations and consequently the herbaceous character. Early leaf removal reduced the IBMP concentrations more than later removal.
Another factor that can affect IBMP concentrations is the vine water status. SALA *et al*. (2005) reported that irrigation increased the IBMP content significantly; similar results have been reported by other authors (MENDEZ-COSTABEL *et al*., 2014a). Rainfall during the growing season may result in higher IBMP concentrations in the grapes at harvest, due to increased and prolonged vegetative growth (ROUJOU DE BOUBEE *et al*., 2000). Lack of rainfall has been shown to decrease perceptual green characters in wine (MENDEZ-COSTABEL *et al*., 2014b). In summary, all of these studies demonstrate the complex relationship between viticultural practices and varietal aroma. It is difficult to predict the final wine aroma because of the multiple compounds and pathways involved.

2.2. Exogenous herbaceous character

Lady beetles (*Coleoptera: Coccinellidae*) have been identified as a second source of high MP content in wine. This off-flavor has been termed 'ladybug taint' (LBT) (PICKERING *et al*., 2005). LBT is a wine defect caused by the inadvertent incorporation of ladybeetles into the wine during the winemaking process. *Harmonia axyridis*, also known as the multicolored Asian ladybeetle (MALB), and *Coccinella septempunctata* (seven spots) are the species considered responsible for causing the taint in many wine regions in Europe and in the United States (BOTEZATU *et al*., 2012; KOGEL *et al*., 2015; GALVAN *et al*., 2008).
Coccinellidae can emit a potent mix of odor-active compounds that may serve various behavioral functions, including defense, aggregation and mate-attraction (AL ABASSI *et al*., 1998; CUDJOE *et al*., 2005). The MP composition varies among species, although IPMP is the major exogenous contributor to LBT in wines. BOTEZATU *et al*. (2012) reported that *Coccinellidae* could release dimethyl-, isopropyl-, sec-butyl- and isobutyl-2-methoxypyrazine into wine at concentrations that negatively impact product quality when the ladybeetles are incorporated with grapes during the harvest operations. PICKERING *et al*. (2005) showed that the inclusion of *Harmonia axyridis* during fermentation of white and

red musts significantly modified the wine aroma and affected the sensory properties. White wines acquired a peanut, bell pepper and asparagus flavor, while in red wines peanut, asparagus, bell pepper, earthy and herbaceous flavors predominated. The same authors observed a correlation between the decrease of the positive fruit, floral aroma and the increase of the number of beetles added both in white and red wines. Further research is needed to investigate treatments aimed at reducing the impact of these beetles and manipulating their concentration in wines for specific commercial targets.

2.3. Techniques mitigating the herbaceous character due to methoxypyrazines

The increase of the MP content happens primarily on the first day after destemming and crushing of the grapes, before the start of the alcoholic fermentation. It is possible that the duration of maceration may influence the levels of MP in the final wines (SALA *et al*., 2005). Various strategies have been investigated to reduce the levels of MP in the finished product.

The Spinning Cone Column is a modern, fast and efficient technology for extracting volatile components, such as ethanol to obtain low-alcohol wines, at high speed and low temperature (PICKERING, 2000). It was first applied by dairies in New Zealand to remove grassy pyrazine flavors from whey and it has been suggested that this method could be adapted to remove MP from wine (SCHMITT, 2014). Other strategies have focused on methods to reduce MP in finished white and red wines without altering the concentrations of other volatiles. The levels of IPMP (as well as other volatiles) were lowered by activated charcoal addition to white wine. Treatment with oak chips reduced the intensity of ladybug taint in both white and red wines, while other applications generally had no effect on white wine and limited effect on red wines (PICKERING *et al*., 2006). The treatment of grape juice and must with silicone (a non-polar sorbent) prior to fermentation was found to reduce MP without altering the concentrations of the main fermentation-derived compounds. Therefore, it was used as a strategy for reducing all the MP without affecting the other positive volatile compounds (RYONA *et al*., 2012).

The exposure of wine to oxygen during and after bottling is a crucial interval affecting the wine aroma compounds. The closure and the packaging can effect post-bottling modifications with resultant changes in MP concentrations during aging. All types of closure reduced significantly MP concentrations in wine (SILVA *et al*., 2011). The greatest decrease in the MP content was evident in multilayer aseptic packages, due to the higher sorption capacity and gas permeability of this material (BLAKE *et al*., 2009). Light and storage temperature did not influence significantly the MP decomposition in wines during a 12-month ageing period (BLAKE *et al*., 2010).

Thermovinification (or hot maceration) is a process used to increase productivity, modify the aroma profile and reduce the risk of microbial contamination. It has the potential to reduce IPMP in MALB infested grapes (PICKERING *et al*., 2008). Heating of *Coccinellidae*-affected grape must (Riesling and Pinot noir) prior to fermentation as a possible remedial intervention resulted in a moderate decrease of all MP (KOGEL *et al*., 2015).

An alternative approach is the flash-détente technique, also reported in the literature as flash vacuum expansion. In this technique, plant materials are heated quickly (60-90°C) and then exposed to high vacuum (20-50 hPa) (BRAT *et al*., 2000). Flash-détente has been also applied to crushed and destemmed grapes which were heated at 80-85°C, submitted at 20-50 hPa and then cooled down to 30°C (AGERON *et al*., 1995; MOUTOUNET and ESCUDIER, 2000). The heating of the must followed by sudden expansion and instantaneous evaporation of water explosively ruptures the cellular tissue structure resulting in the extraction of pigments, phenols, aroma compounds and other dry matter present in the cells. Initially, this technique was used to promote the release of

anthocyanins and tannin (HE *et al*., 2012; ESCUDIER *et al*., 1998); however, its ability to remove deleterious volatiles was soon recognized. According to VINSONNEAU *et al*. (2006) the flash-détente technique can improve the quality of red wines aged for 9 months; the most significant decrease of the herbaceous character was observed for Cabernet Sauvignon and Cabernet Franc. It must be noted that the sequential application of a heating treatment and of high vacuum is not a selective approach and contributes to the evaporation of both positive and undesired components, including MP (JONES and GNEKOW, 2011). For this reason, it is also possible to remove aldehydes and alcohols with 6 carbon atoms, which are pre-fermentative herbaceous aroma compounds formed during destemming (ESCUDIER, 2001; RAZUNGLES, 2010; FAVAREL, 2013) as discussed in the next section.

3. PRE-FERMENTATIVE HERBACEOUS CHARACTER

Aldehydes and alcohols with 6 carbon atoms (C6) are volatile, odorous molecules which can contribute to the herbaceous aroma in wine. They are mainly generated through the enzymatic breakdown of C18 polyunsaturated fatty acids contained in plant membranes. Their concentrations in must can be in the order of several hundreds of $\mu g/L$ (MORENO and PEINADO, 2012) or even more than 13000 $\mu g/L$ (FERREIRA *et al*., 2000), with very variable odour thresholds (400-8000 $\mu g/L$) (GUTH, 1997). Their levels depend on several factors, including the grape variety and ripeness, treatments prior to fermentation, and temperature/duration of contact with the skins. Therefore, they can be considered pre-fermentative volatile compounds. Their cut-grass-like aroma is the characteristic odor of freshly damaged green leaves; therefore, these compounds are often referred as Green Leaf Volatiles (GLV) (HARSCH *et al*., 2013). The C6 alcohols frequently found in grapes include hexanol, (Z)-3-hexenol and (E)-2-hexenol. As well as (E)-2-hexenol, (E)-3-hexenol may also be found in wine at levels of $\mu g/L$ (GIGOT *et al*., 2010).
The C6 aldehydes commonly identified in grapes are hexanal and (E)-2-hexenal; also C7 compounds such as heptanal, and (E)-2-heptenal (KALUA and BOSS, 2008) have been found, but at lower concentration with respect to C6 aldehydes. At low concentrations (generally less than 0.5 mg/L threshold), these C6 volatiles compounds contribute positively to the overall aroma of the wine. However, at higher levels they lead to unwanted organoleptic notes such as herbaceous, green fruit, and crushed leaf and may also impart a bitter flavor (D'ONOFRIO, 2011; FERRANDINO *et al*., 2012).
These C6 compounds may be present in free volatile form or in bound form, as glycosides (CABRITA *et al*., 2006; DI STEFANO *et al*., 1998; LANATI *et al*., 2000; SEFTON, 1998). The C6 alcohols were identified only as free volatile compounds in Casorzo Malvasia and Schierano Malvasia, while in the aromatic Candia Malvasia they were present both as free and glycosylated forms, although the former predominated (BORSA *et al*., 2008).
Several studies were conducted in order to distinguish grape varieties on the basis of their C6 compounds composition. RAPP *et al.* (1993) have shown that the content of (E)-3-hexenol and its isomer (Z)-3-hexenol are the most important analytical parameters to discriminate monovarietal wines of Riesling, Müller-Thurgau, Kerner, Scheurebe, Ehrenfelser and Bacchus. Also, MORET *et al.* (1984), references these two compounds among the significant parameters discriminating Venetian white wines. More recently, OLIVEIRA *et al.* (2006) showed that (E)-3-hexenol/(Z)-3-hexenol ratio clearly discriminates Loureiro wines from those of Alvarinho, Avesso and Trajadura from Portugal. Moreover, 1-hexanol/(E)-3-hexenol and 1-hexanol/(Z)-3-hexenol ratios may also be able to discriminate Vinhos Verdes monovarietal wines (north of Portugal).

Aldehydes are the major C6 compounds in ripe grapes of Riesling, while in Cabernet Sauvignon C6 alcohols prevail (KALUA and BOSS, 2010). Analyzing the evolution of C6 volatile compounds during berry development of Cabernet Sauvignon, it was found that aldehydes increased significantly during the middle development stage but then decreased throughout the late development stage. The alcohols were present throughout the growth of berry, but reached significant levels only towards the end of ripening (KALUA and BOSS, 2010). The greater increase of the alcohols over the aldehydes across the course of ripening is a positive aspect because alcohols have a higher perception threshold than the aldehydes, so the resulting herbaceous character is less pronounced (D'ONOFRIO, 2011).
The C6 aldehydes and alcohols derive from the oxidation of grape polyunsaturated fatty acids such as oleic acid (C18:1ω9), linoleic acid (C18:2ω6) and linolenic acid (C18:3ω3) initiated by the lipoxygenase pathway when the berries are crushed. Four enzymatic activities are sequentially involved in this pathway. First, an acyl-hydrolase frees the fatty acids with 18 carbon atoms from membrane lipids. Next, a lipoxygenase catalyzes the fixation of oxygen. The peroxides obtained are then split into C6 aldehydes (DRAWERT *et al*., 1966). Some of these may be reduced to their corresponding alcohols by an alcohol dehydrogenase in the grape (CROUZET, 1986).
The C6 compounds increase from veraison to harvest, with the greatest concentration expressed at harvest (KALUA and BOSS, 2010; YANG *et al*., 2009). For the neutral cultivar Nebbiolo grown in Piedmont (Italy), the pre-fermentative volatile compounds present in highest concentration were hexanal and E-2-hexenal (FERRANDINO *et al*., 2012). The greater concentration of E-2-hexenal compared to Z-3-hexenal could be explained by an increased activity of 3Z,2E-enal-isomerase, which catalyzes the reaction from Z-3-hexenal to E-2-hexenal (KALUA and BOSS, 2010). In addition, the low concentration of these two aldehydes in the pre-veraison berries could be explained by an increased activity of the alcohol dehydrogenase that converts these aldehydes to the corresponding alcohols. During ripening, the concentration of aldehydes increases. Therefore, some C6 compounds that contribute to the grape aroma may be produced pre-veraison, rather than post-veraison.
The evolution of (E)-2-hexen-1-ol and (E)-2-hexenal to form thiols with tropical scents, such as 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), has been recently confirmed during fermentation (HARSCH *et al*., 2013). This pathway will further reduce the perceptual herbaceousness, both by reducing the C6 pool as well as by formation of the 'fruity' thiols which will serve to mask herbaceousness.

3.1. Avoiding the pre-fermentative herbaceous character: from the harvest to the winemaking process

The overall perceptual herbaceous character is related to the ripening stage of the grapes, but can arise in the must due to forceful mechanical action on the clusters. Therefore, it is important to consider the level of ripeness, the type of harvest, the transport, the crushing, the time and temperature of maceration, the type and the degree of pressing, the type of pumps, the degree of oxygenation and the use of enological adjuvants with regards to herbaceous expression. High temperature pretreatment of must immediately following crushing ("hot break") was shown to result in five- to six-fold higher concentrations of (E)-2-hexenal in Concord grape juice as compared to conventional hot press, especially with immature fruit, resulting in persistence of green aromas in the juice. In contrast, de-pectinization, pressing, and pasteurization decreased the content of (E)-2-hexenal of the juice of Concord grapes due to the reduction to (E)-2-hexenol (IYER *et al*., 2010).

3.2.1. The harvest

Harvest timing is an important factor in minimizing/preventing the formation of herbaceous compounds. Additionally, accumulation of the C6 alcohols and aldehydes derived from the enzymatic oxidation of linoleic and linolenic acid can be reduced by limiting mechanical operations starting with the harvest. Mechanical harvesting increases herbaceous character due to uncontrolled enzymatic oxidation (NARDIN *et al*., 2006); consequently, those grapes must be pressed quickly. In contrast, manual harvesting reduces the mechanical stress on the clusters with concomitant lower losses plus lower contamination with leaves, earth, water and other foreign substances. While mechanical harvesting certainly speeds up the overall process of winemaking, it comes at the expense of the mechanical stresses on the cluster and the acceleration of the oxidation reactions.

3.2.2. Destemming and crushing

Destemming (separating and removing the stalks from the grape berries) and crushing (breaking the grape in order to release the juice and some pulp material) are the next mechanical processes involved in winemaking. The influence of crushing on the herbaceous aroma in must and in the corresponding wine is well established (RIBEREAU-GAYON *et al*., 2005; HASHIZUME and SAMUTA, 1997). Usually, destemming and crushing are performed by a single machine, a crusher-destemmer or destemmer-crusher, so-named in relation to the succession of the two processing stages. The destemmer-crusher first removes the stalks and then breaks open the grapes, thus avoiding the contact of the expressed juice with the stems. In contrast, in the crusher-destemmer undesired flavors can potentially be extracted from the stalks, leading to an increase of herbaceous aromas and bitter tastes. Furthermore, the presence of stalks reduces the intensity of the red wine color due to the absorption of anthocyanins (NARDIN *et al*., 2006).
It is important to use equipment that reduces mechanical stresses, such as excessive pressure, abrasions and lacerations, in order to reduce the enzymatic oxidation activity on fatty acids. In this way, the polyphenol and potassium extraction from the stalks is also minimized. The use of inert gases or vacuum systems in concert with the crushing and pressing of the clusters is a very popular approach to remove oxygen and slow down the oxidation of the must and increase the formation of volatile 'fruity' esters and glutathione (BOSELLI *et al*., 2010; DI LECCE *et al*., 2013).
In the case of the wines produced by carbonic maceration, crushing is completely skipped and the tank is filled with whole clusters by means of a conveyor belt that minimizes the damage to the plant tissues. The herbaceous and bitter flavors may arise in some varieties as the stalks are not separated and removed in this process. Although wines produced by using carbonic maceration can be obtained by grapes harvested prematurely, the aromatic profiles of such wines do not exhibit herbaceous characters but instead show pleasant fruity notes (ETAIO *et al*., 2011). This phenomenon is explained by the limited production of C6 aldehydes and alcohols due to both the lack of oxygen in the tank and the lack of crushing of the grapes.

3.2.3 Pre-fermentative maceration

The enzyme that catalyzes the peroxides scission to aldehydes is associated with the membranes, consequently the aldehyde levels are proportional to the intensity of maceration of these solid parts. To limit their concentration in red wines, it is important to control the maceration temperature, not to employ long pre-fermentative maceration and to continually monitor the maceration stage.

4. HERBACEOUS CHARACTER IN FERMENTATION

Sulfur is present in many organic compounds within the grape such as amino acids, peptides, proteins, vitamins and co-enzymes and may also result from sulfur spraying (KWASNIEWSKI *et al*., 2014). Volatile sulfur compounds (VSC) may then originate from yeast metabolism during the alcoholic fermentation. These molecules are characterized by a strong aromatic impact at low concentrations due to very low odor thresholds (MESTRES *et al*., 2000).

VSC can be distinguished based on their functional groups (thiols, sulfides, disulfides etc.). However, there is another classification based upon the boiling point (b.p.) of 3-methylthiopropanol (b.p. 90°C): light sulfur compounds show a b.p. lower than 90°C, whereas heavy sulfur compounds have a b.p. higher than 90°C (MESTRES *et al*., 2002). The light sulfur compounds such as hydrogen sulfide, ethanethiol and methanethiol arise from the degradation of the sulfur-carbon bond of methionine or cysteine derivatives (LANDAUD *et al*., 2008). They usually have an unpleasant effect on the quality of the wine, are characterized by off-flavors of 'rotten egg', 'sewer', 'cooked vegetable' or 'cooked cabbage', 'onion', 'garlic' or 'rubber' and show high volatility and low perception thresholds ($\mu g/L$) (MESTRES *et al*., 2000; GONIAK and NOBLE, 1987). Dimethyl sulfide at low concentration is considered less undesirable since it shows aromas of 'cooked corn' and 'asparagus' (BELL and HENSCHKE, 2005).

Hydrogen sulfide, ethanethiol and methanethiol are present in reduced wines and, due to their low boiling points, can be volatilized by racking and aeration (MOREIRA *et al*., 2002). In Table 2, the sensory properties of light sulfur compounds are listed according to different authors and their boiling point is reported (RIBEREAU-GAYON *et al*., 2005; LANDAUD *et al*., 2008; BENKWITZ *et al*., 2012). For a review of the lower molecular weight sulfur compounds see MESTRES *et al.* (2000).

Table 2: Sensory properties and boiling point of light sulfur compounds (C_1-C_2) according to RIBEREAU-GAYON *et al*., 2005; LANDAUD *et al*., 2008; BENKWITZ *et al*., 2012.

Light sulfur compounds	Chemical Formula	Flavor note	Perception threshold (μg/kg)	Boiling point (°C)	Probable precursor
Hydrogen sulfide	H_2S	rotten egg	10-80	-61	Cysteine, sulfate, sulfite
Methanethiol	CH_3SH	cooked cabbage, onion	0.3	6	Methionine
Ethanethiol	CH_3CH_2SH	onion, rubber	1.1	35	Unknown
Dimethyl sulfide	CH_3SCH_3	cooked corn, asparagus	25	35	Cysteine

Other volatile sulfur compounds include the higher molecular weight grape-derived thiols, such as 3MH, 3MHA, 4-mercapto-4-methyl-pentan-2-one (4MMP), and 4-mercapto-4-methyl-pentan-2-ol (4MMPOH), all of which contribute positively to the varietal aroma of wine and are thus referred to as varietal sulfur compounds (BELL and HENSCHKE, 2005).

The interaction of methoxypyrazines with varietal thiols is still unclear (WYNGAARD *et al*., 2014). CAMPO *et al.* (2005) showed that the fruity character is related to the presence of

varietal thiols (3MHA) and is negatively affected by the presence of methoxypyrazine. The vegetative aroma due to IBMP, such as 'bell pepper' has been shown to be slightly masked by the fruity aroma, as berry flavorings were added to a neutral red wine containing added bell pepper (HEIN *et al*., 2009).
As mentioned earlier, the volatile thiols also have an important sensory role due to their biosynthesisis metabolizing C6 compounds (C6-thiol precursors); ROLAND *et al.* (2010) demonstrated that (E)-2-hexen-1-ol played a key role in the formation of the glutathionylated conjugate 3-*S*-glutathionylhexan-1-ol (G3MH). This conversion diminishes the perceptual herbaceousness by reducing the C6 pool while concomitantly increasing the masking pool. HARSCH *et al.* (2013) found that the addition of sodium hydrosulfide (NaSH) to grape juice greatly increased the formation of 3MH and 3MHA from (E)-2-hexen-1-ol and (E)-2-hexenal precursors during the fermentation of Sauvignon blanc musts under commercial winemaking conditions. While these two precursors exhibit a thiol-forming potential during fermentation, (E)-2-hexenal and (E)-2-hexen-1-ol are quickly catabolized in the very early stages following yeast addition, whereas the synthesis of H_2S (the thiol source) by yeasts begins after the conversion of sugars. This timing problem may be responsible for the low content of 3MH and 3MHA in finished Sauvignon blanc wines in spite of the high potential for their formation.

4.1. Oxidation of volatile thiols

Oxygen plays an important role in controlling the sensory defects associated with sulfur compounds. Micro-oxygenation can reduce undesired sulfur compounds, such as methanethiol and ethanethiol, to levels below their odor threshold, with no significant increase in dimethyl disulfide (GOMEZ-PLAZA *et al*., 2011). This may be explained by the role of quinones as oxygen oxidizes *trans*-caftaric acid and other phenolic compounds and leads to the formation of the corresponding *o*-quinones (VIDAL and AAGAARD, 2008; WATERHOUSE and LAURIE, 2006). Furthermore, glutathione plays an important role during the oxidation of white must, forming the 2-S-glutathionyl-caftaric acid or Grape Reaction Product (GRP) which is very common in wines (BOSELLI *et al*., 2006). This derivative is not oxidized by polyphenol oxidase and does not modify the color of the must (DU TOIT *et al*., 2007; ROLAND *et al*., 2010).
Pre-fermentative operations could provide a positive contribution to the aroma profile of white wine, such as Sauvignon blanc, by increasing the G3MH precursor. At this stage of winemaking, the cysteinylated and glutathionylated precursors of varietal thiols are not oxidizable because of the chemical stability of the thioether bond under oxidative conditions (ROLAND *et al*., 2011).
Varietal thiols, however, are highly reactive compounds and their concentrations can decrease during storage (HERBST-JOHNSTONE *et al*., 2011). Oxygen and iron can oxidize these compounds into their disulfide forms. In addition, they can participate in chemical reactions with the products of phenolic oxidation (*o*-quinones) catalyzed by transition metals (e.g., iron and copper) (HERBST-JOHNSTONE *et al*., 2011; NIKOLANTONAKI *et al*., 2010; NIKOLANTONAKI and WATERHOUSE, 2012).

5. CONTROL OF THE HERBACEOUS CHARACTER AFTER THE ALCOHOLIC FERMENTATION

The aging stage of a wine is a delicate technological phase linked to a variety of chemical-physical modifications that help define the final organoleptic characteristics. Micro-oxygenation is a technique that may be used during this stage, employing a controlled

flow of oxygen in tanks to simulate the complex reactions that occur during wine maturation in barrel (CELOTTI and ZUCCHETTO, 2003). This treatment results in many changes to the organoleptic qualities of the wine; these changes have been empirically separated into two main phases (MOUTOUNET *et al*., 2001):
Structuring: this takes place from the end of the alcoholic fermentation to the beginning of malolactic fermentation (MLF). In this phase, oxygen is consumed by polymerization reactions, which increase the aggressiveness and intensity of tannins on the palate. At the same time a decline in the aromatic intensity and complexity of the wine is observed. The changes in the chemical composition of wine caused by MLF are complex and often involve an enhancement of the fruity aroma and 'dairy' note and a decrease of the herbaceous character (LIU, 2002).
Harmonization: in this phase the intensity and the aromatic complexity increases, whereas the herbaceous character decreases. Furthermore, there is an increase in the color intensity, especially the purple hue. It is important to end the micro-oxygenation at the second stage in order to prevent excessive oxidation of the wine components leading to off-flavors. It is critical to prevent violent oxygenation during either stage to avoid generalized, uncontrolled oxidation.
The sensory changes occurring in red wines exposed to different micro-oxygenation regimens and with different woods have been described (CELOTTI and ZUCCHETTO, 2003; MOUTOUNET *et al*., 2001). It was emphasized how the controlled application of oxygen, as well as the wood contact in barrels, may decrease the astringency and the herbaceous characters of wine. Barrel-ageing of wines is costly and laborious and is thus not suitable for all wines and wine styles. Micro-oxygenation is an affordable alternative for accelerating the stabilization of color and phenolic compounds and for improving the sensory quality of red wines (LLAUDY *et al.*, 2006; PARISH *et al.*, 2000). Oxygen addition led to an increase of the fruity and floral notes, whereas the herbaceous character significantly decreased also in Cencibel red wines (CEJUDO-BASTANTE *et al*., 2011). However, HERNANDEZ-ORTE *et al*. (2009) reported a strong dependence of the effects of microxygenation on the grape variety (Cabernet Sauvignon or Tempranillo), the use of barrels or steels vats and the period of maturation of the wines.
Sensory analysis was used to follow important perceptual sensations, such as astringency and herbaceousness, which are modified by controlled oxygenation. The herbaceous character showed a fairly rapid decrease during initial micro-oxygenation; however, for longer treatment times there was a general increase in the herbaceous note. The best results were obtained with up to 2 months of treatment of micro-oxygenation without the addition of tannin. The same studies (CELOTTI and ZUCCHETTO, 2003; MOUTOUNET *et al*., 2001) highlighted the difficulty in accurately determining the oxygen dosage and the appropriate treatment times. However, it was demonstrated that the application of sensory analysis in concert with careful analytical control of the process allowed the optimal time for terminating the treatment to be decided.

6. CONCLUSIONS

The herbaceous character of wines is of important concern to the wine scientist, to the winemaker and finally to the wine consumer. Different classes of compounds with very different chemical structures can contribute to this particular sensory feature of wines. Alkylmethoxypyrazines, for instance, are key contributors to vegetativeness in Sauvignon blanc, as are some selected volatile thiols. The herbaceous character can be specific to a particular grape cultivar (an endogenous varietal character) or produced during the winemaking procedure, from either an endogenous or an exogenous cause. The positive or

negative (off-flavor) attributes associated with the herbaceous character depend not only on the chemical composition (qualitatively and quantitatively) of the headspace of wine and/or the wine itself, but also on the odor activity values of the different volatile components as determined by sensory techniques.
For future perspectives, a deeper understanding of the origin of these herbaceous characters and, perhaps more importantly, their prediction in the finished wine (based upon chemical quality markers) remains a challenging subject, which deserves continued investigation.
This would be of benefit, not only to the wine scientist, but also to the global winemaking industry.

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PAPER

EFFICACY OF NEUTRALISED ELECTROLYSED WATER AND MILD HEAT AGAINST FOODBORNE PATHOGENS ISOLATED FROM *FICUS CARICA*

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ABSTRACT

Problems with microorganism toxins in dried fig exports are becoming very important. Chlorine-based sanitizers are effective way of controlling microorganisms, but on the other hand their reaction with natural organic and inorganic matter may potentially form carcinogenic products. Therefore, different sanitizers for the disinfection of food and food contact surfaces are required as an alternative to chlorine-based sanitizers. Some earlier studies revealed that neutralised electrolysed water (NEW) may be a potential substitute for cleaning and sanitizing agents in packaged products. In order to make a contribution to solve toxins problems, the antibacterial and antifungal effect of neutralised electrolysed water (NEW) on the foodborne pathogens were evaluated in this study. Spores of *Aspergillus flavus* and *Penicillium expansum* were isolated from the surface of fig fruits. *Escherichia coli and Bacillus cereus* known to occur on the surface of figs were also evaluated. Vegetative cells and spores of bacterium and fungi were exposed to five different concentrations of NEW (100, 75, 25, 5 and 1%) at three different temperatures (22, 50 and 70°C) for 1, 3 and 5 min. According to the results, at 22°C, 1% neutralised electrolysed water exposure for 1 min effectively decreased the number of vegetative cells of *E. coli* and *B. cereus* by approximately 8.5 log cfu/ml and 6.3 log cfu/ml, respectively. At 50°C, 5% neutralised electrolysed water exposure for 1 min significantly reduced the *A. flavus* and *P. expansum* spore numbers by 5.54 log cfu/ml and 7 log cfu/ml, respectively. With the effect of mild temperature (22-50°C), low chlorine neutralised electrolysed water (9.22 mg/l - 33.85 mg/l available chlorine concentrations) showed a strong antibacterial and antifungal activity against foodborne pathogens. As a conclusion, neutralised electrolysed water can be used widely as a sanitizer in fig enterprises, instead of high cost chlorine based disinfectants.

Keywords: *Aspergillus flavus, Bacillus cereus, Penicillium expansum,* sanitizer

1. INTRODUCTION

Figs (*Ficus carica L.*) are an economically important and highly valued fruit with high contents of fibre, minerals and polyphenols. They are mainly grown in the Aegean region of Turkey. With the yearly production of 275,002 tons, Turkey ranks the first in the World in fig production and exportation (TUIK, 2012). Turkey owns the 26% of the world's fig production and 36% of the world's fig exportation (ÇALIŞKAN and Polat, 2012). Thirty percent of figs produced in Turkey are marketed as fresh figs, while the remaining fruit is consumed as dried figs. Due to the high content of carbohydrate and water activities of semi-dried figs on the tree and fallen figs collected from the soil, figs can be considered a good substrate for mycotoxin formation. Water activities for semi-dried on the tree, fallen figs collected from the soil, figs taken from the drying stage and those from warehouses have been reported as 0.88- 0.94; 0.76-0.87; 0.70-0.80 and 0.69-0.73a_w, respectively (KARBANCIOGLU-GULER and HEPERKAN, 2009). The most common toxigenic fungi reported in Turkish dried figs are *Aspergillus* section *Nigri*, *Fusarium spp.*, and *Aspergillus* section *Flavi Penicillium spp.*; their toxin types are aflatoxin, citrinin, fumonisin, patulin, and ochratoxin A (HEPERKAN, 2006). Figs are usually contaminated with *Escherichia coli, Bacillus cereus* and *Bacillus cereus* spores. The numbers of these microorganisms on dried figs were reported to vary from a few hundreds to thousands per gram of fruit (FRAZIER and WESTHOFF, 1988; AKBAS and OZDEMIR, 2008). Poor storage conditions such as damp environments and high storage temperatures may cause bacterial growth to reach to a level of about 10^7-10^8 cfu/g for dried figs. The main purpose of all methods applied for food preserving is to prevent or limit microbial and enzymatic changes in foods. It was estimated that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalisations, and 5,000 deaths in the United States each year (MEAD *et al*., 1999).

According to the Environmental Protection Agency (EPA) (2001), chlorine-based sanitizers are effective way of controlling microorganisms, but on the other hand their reaction with natural organic and inorganic matter may potentially form carcinogenic products (FAWELL, 2000; ALLENDE *et al*., 2009). Many of these products may cause cancer and generates reproductive and developmental problems in laboratory animals. Therefore, different sanitizers for the disinfection of food and food contact surfaces are required as an alternative to chlorine-based sanitizers.

Electrolysed water generation technology was initially developed in Russia (MAMADZHANOV *et al*., 1974), and then modified in the 1980s by Japanese food industry researchers (HATI *et al*., 2012). In this technology, by using tap water and salt, system generates two stream simultaneously, one is alkaline and the other is acidic, with the characteristics of cleaning and sanitising solutions, respectively. Researchers evaluated the effectiveness of these solutions as a sanitizing and cleaning agent. Studies revealed that they can be a potential effective substitute for cleaning and sanitizing agents in packaged products (IWASAWA *et al*., 1993; HAYASHIBARA*Et al*., 1994).

The electrolysed water acidic solution is reported to be highly effective in deactivating the *E. coli*, *S. enteritides, S. aureus, C. albicans* and *L. monocytogenes* strains (VENKITANARAYANAN, 1999; ILERI *et al*., 2006). The disinfection mechanism of the acidic electrolysed water can be attributed to several factors, including the destruction of bacterial protective barriers, the increase in membrane permeability, the leakage of cellular inclusions, and the decrease in activities of some key enzymes (ZENG *et al*., 2010). However, the potential application of Strong Acid Electrolyzed Water (SAEW) is limited because of its low pH ($\leq$ 2.7). At this low pH, dissolved Cl_2 gas can be rapidly lost due to volatilization, decreasing the bactericidal activity of the solution by the time (LEN *et al*., 2000) and may affect human health and the environment adversely. Moreover, the high

acidity of strong acid electrolyzed water may cause the corrosion of equipment and consequently limit its practical application.
In Japan, strong acid electrolyzed water (pH 2.5±0.2; 20-60 mg/l available chlorine concentrations (ACC)) and slightly acidic electrolysed water (pH 5.0-6.5; 10-30 mg/l ACC) usage in food products is authorised by the Japanese Ministry of Health and Welfare (ZACHARIA *et al*., 2010).
Recently, slightly acidic or neutralised electrolysed water has been of interest, as it minimises human health and safety risks from Cl_2 degassing, reduces corrosion of surfaces, and limits phototoxic side effects (GUENTZEL *et al*., 2008). In spite of these advantages, neutralised electrolysed water *in vitro* inactivation of different food pathogens has not been intensively studied.
Our previous study is among the very few preliminary one aimed to unveil the bactericidal effectiveness of acid electrolysed water against different food spoilage and pathogenic microorganisms (ILERI *et al*., 2006).
In Turkish enterprises, routine fig processing as follows; initially dried figs are washed with salty (3-6% NaCl) high-temperature water (60-80ºC) and then processed further and finally packaged. The presence of salt increases the heat resistance of spores due to reduced water activity leading to spore dehydration (JUNEJA and SOFOS, 2002). While this treatment may not be enough to decrease the microbial population of figs.
Thus it was suggested that neutralised electrolysed water was used instead of salty and high temperature water in fig enterprises to the optimum temperature and the optimum concentration of NEW under laboratory conditions.
Therefore, the effectiveness of NEW on spores of *Aspergillus flavus* and *Penicillium expansum* isolated on the surface of fig fruits, vegetative cells of *Escherichia coli* ATCC 35218 and *Bacillus cereus* ATCC 11778 known to occur on the surface of figs and *Bacillus cereus* ATCC 11778 spores was evaluated under controlled conditions.

2. MATERIALS AND METHODS

2.1. Bacterial cultures and the preparation of inocula

E. coli ATCC 35218 and *B. cereus* ATCC 11778 were plated on Nutrient Agar (NA) and incubated at 37°C for 24 h. after incubation, *E. coli* ATCC 35218 and *B. cereus* ATCC 11778 suspensions were prepared by transferring several colonies to a 10 ml of NaCl solution (0.9%, w/v) with the sterile inoculation loop and vortexed using a thermal mixer. The 1 mL aliquots of undiluted and diluted cultures were transferred to a cuvette, and optical densities of the solutions at 600 nm were measured. Aliquots of the samples were also used to perform serial dilutions. Dilutions were plated on NA plates and incubated at 37°C overnight. Colonies were counted to determine viable bacterial cell counts (cfu/mL) at each dilution ratio, and then a standard curve was prepared by using absorbance and viable cell count.

2.2. Preparation of spore solution of *B. cereus*

B. cereus ATCC 11778 was grown on nutrient agar (Difco, Becton Dickinson, Detroit, MI, USA) containing 500 ppm Bacto Dextrose and 3 ppm manganese sulphate for 7 days at 30°C. After this incubation, sporulation was determined by the Schaeffer-Fulton method (SCHAEFFER and FULTON, 1933). Each agar plate with spores was rinsed twice with approximately 10 ml of sterile-distilled water while each plate was revolved on a

turntable, and the agar surface was gently agitated with a glass spreader to facilitate spore removal or the harvesting of the spores.
The accumulated spore suspensions were washed four times by centrifugation at 4000 rpm for 20 min and re-suspended in sterile NaCl solution (0.9%, w/v). Tween 80 (0.05%, v/v) (Sigma, St. Louis, MO, USA) was added to spore suspensions in order to minimise the clumping of spores and improve the accuracy of spore counts. All transfers, inoculations and harvesting for the preparation of spore suspensions were performed under laminar flow conditions.
The spore counts were enumerated by direct microscopic counting on a Thoma slide. For verification of the number of spores in the suspension, the spore suspension was first heat-shocked at 80°C for 10 min to kill vegetative cells. Next, the number of viable spores was estimated by spread-plating 0.1 ml of spore suspension on nutrient agar at 30°C for 24 h. The spore suspension was stored at 4°C until use (AKBAS and OZDEMİR, 2008).

2.3. Preparation of spore solution of *Aspergillus flavus* and *Penicillium expansum*

Aspergillus flavus was isolated from fig fruits in the laboratory of Adnan Menderes University. *A. flavus* was cultured on potato dextrose agar plates (Merck) at 25°C for one week. After a portion (about 15 ml) of 0.1 M dibasic sodium phosphate adjusted with 0.05 M citric acid monohydrate of pH 7.0 (PC) with 0.005% (v/v) Tween 80 was poured onto the plate, spores were suspended in PC buffer by rubbing gently with a glass rod. Clumped spores in PC buffer were scattered by sonication for 2 min. The suspension was filtered through eight layers of sterile cheese cloth to remove hyphae. The spore concentration of the suspension was estimated with a haemocytometer. The suspension was diluted with PC buffer to obtain a concentration of 107 cells per ml (FUJİKAWA and ITOH, 1996).
Penicillium expansum was isolated and identified from fig fruits at the microbiology laboratory of Adnan Menderes University. Spore suspensions were prepared on Petri dishes for 1 week with PDA and 50 mg/l of streptomycin. After one week of incubation at 25°C, spores were collected and suspended in sterile Ringer's solution. After filtering through eight layers of sterile cheese-cloth, spores were counted and brought to a final concentration of 107 cell per ml (SPADARO *et al*., 2002).

2.4. Preparation of treatment solutions

NEW was obtained by electrolysis of a mixture of NaCl (20 g/l) and tap water using STEL-10H-120-01 generator (STEL - 10H- 120-01, Russia) at 40.0 V, 9.0 A and produced at a rate of 250 ml/22 sec. A cylindrical electrochemical cell for processing solutions comprised an inner, hollow, tubular anode, an outer, cylindrical cathode, and a permeable, tubular, ceramic diaphragm that was arranged between the anode and cathode and divided the inter-electrode space into anode and cathode chambers so that a working section of the cell was formed. There was a hole connecting the internal chamber with the channel for the liquid withdrawal. The internal chamber was connected by the annular channel to the horizontal channel used for feeding liquid subjected to the treatment.
The NEW dilutions were prepared by using sterile tap water at rates of 100% (undiluted), 75%, 25%, 5% and 1%, while deionised water (DW) alone was used as control. Analytical indices (ORR, pH and ACC) of the treated solutions were measured immediately after NEW preparation and before each bactericidal and fungicidal experiment. The pH was measured with a pH meter (HI 2211-02, HANNA, USA), and ORP was measured with an ORP meter (HI98120, HANNA, USA). The pH meter was calibrated using commercial

standard buffers at pH 4.0 and 7.0 (Merck Ltd., Germany). The ACC was measured on the basis of the iodometric method reported by DYCHDALA (1983).

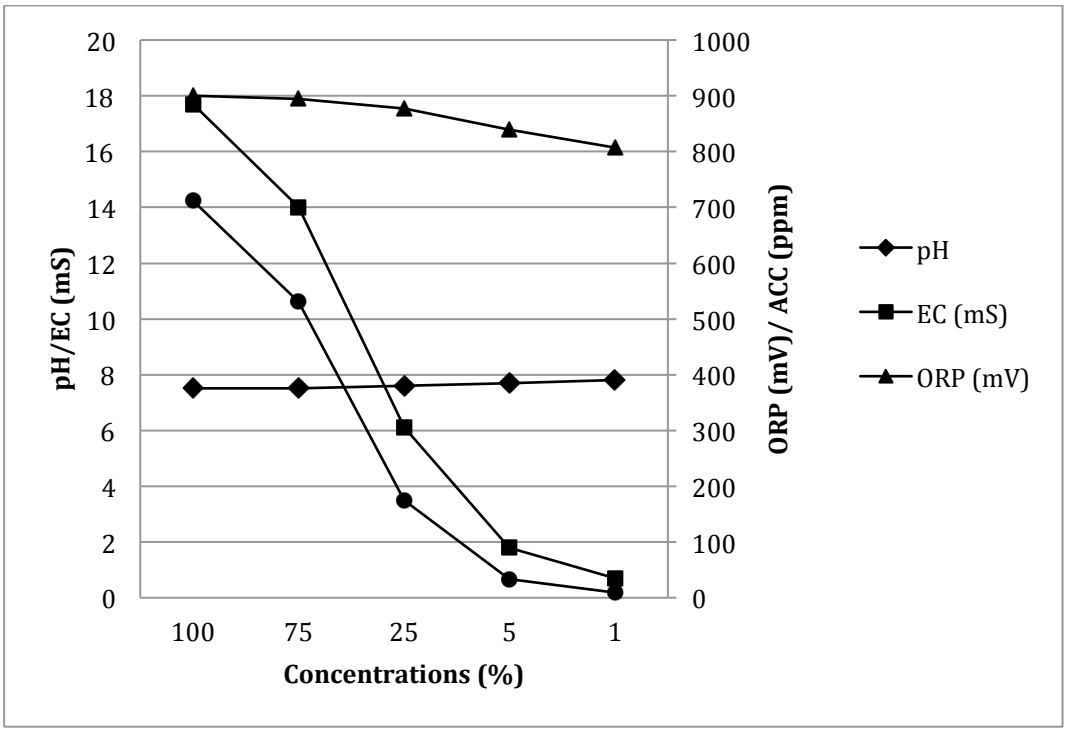
2.5. *In vitro* inactivation of microorganisms by NEW

In vitro inactivation experiments for their disinfection potential against vegetative cells of *E. coli* ATCC 35218, *B. cereus* ATCC 11778 and spores of *A. flavus*, *P. expensum*, and *B. cereus* were carried out at 22, 50 and 70°C for 1, 3, and 5 min. The bacteria and spore suspensions were prepared as described above. Different concentrations of the NEW solutions were prepared with sterile tap water. Before use, each NEW was diluted and placed in a water bath at 22, 50 and 70°C to reach the desired temperature before inoculation. One millilitre of each vegetative cell and spore suspension was separately added to 9 mL of different concentrations of NEW (1, 5, 25, 75, and 100%, v/v) and sterile DW. After inoculation, 1 mL aliquots were periodically collected at the end of incubation time (1, 3, and 5 min) at 22, 50 and 70°C, and transferred into the membrane filter funnel containing sterile DW (50 ml). The samples were drawn completely through the filter. The funnel was rinsed with sterile DW (150 ml). The membrane filter was removed from the funnel and placed into the prepared Petri dish. Agar plates were incubated at the proper temperature (bacterium and fungi, 35±2 and 30°C, respectively) and for the appropriate time period (bacterium and fungi, 24 and 72 h, respectively).

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical properties of tested solutions

One of the first applications of electrolysed water was the sterilisation of medical instruments in hospitals; it has since been used in the medical, dental, agriculture, dairy and food industries. Based on the literature review carried out as part of this study, it was found that applications in the food industry have been the most frequently researched.
Applications of electrolysed sanitising solutions for microbial inactivation in fresh lettuce have been reported by PARK *et al*. (2001), KOSEKI *et al*. (2003), DELAQUIS *et al*. (2004) and ONGENG *et al*. (2006). It was also used for the disinfection of tomatoes (Bari *et al*., 2003), spinach (RAHMAN *et al*., 2010), cucumbers (KOSEKI *et al*., 2004) and strawberries (KOSEKÎ *et al*., 2003; UDOMPIJITKUL *et al*., 2007).
To provide ease of use in fig enterprises, tap water was used for the preparation of different concentrations of NEW. The ORP, pH and ACC for the treated solutions are shown in Figure 1. The pH, ORP and ACC for five different concentrations of NEW solution (100, 75, 25, 5 and 1%) used in this study were pH 7.45-7.84, 900-807 mV and 712.6-9.2 mg/l, respectively.
Differences between the pH and ORP of different concentrations of EAS were very small, but the difference between ACC was significant. The DW was used as a control solution in this study; it had a pH of 7.58, an ORP of 353 mV, and no available chlorine was detected.



Concentrations (%)	100	75	25	5	1
pH	7,5	7,5	7,6	7,7	7,8
EC (mS)	17,7	14	6,12	1,8	0,7
ORP (mV)	900	894	877	840	807
ACC (ppm)	712,6	530,2	174,7	33,9	9,2

Figure 1: Physicochemical properties of tested solutions.

3.2. Antimicrobial effects of different dilutions of NEW on pure cultures of *E. coli, B. cereus and spores of B. cereus, A. flavus and P. expansum*

For vegetative cells, very rapid killing was observed. At all of the concentrations tested, NEW reduced bacterial numbers to the detection limit shown below within 1 in at 22°C (Figs. 2, 3), corresponding to a $\log_{10}$ reduction factor of 8.5 and 6.3 (within 1 min) for *E. coli* and *B. cereus*, respectively.

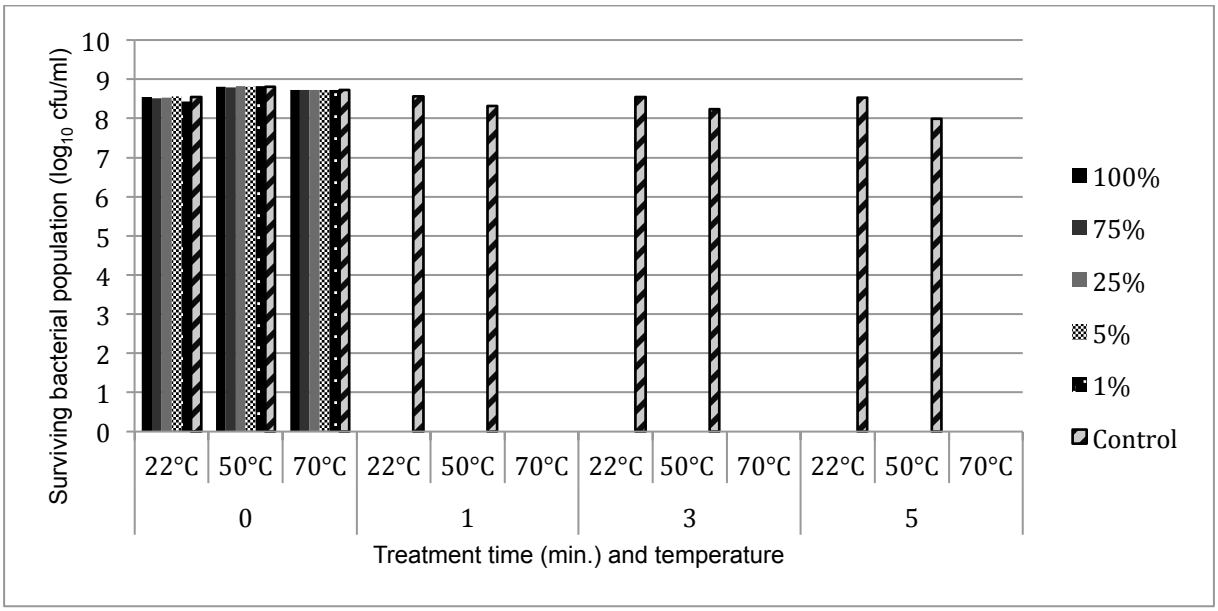


Figure 2: The effectiveness of different concentrations of NEW against *E.coli* ATCC 35218 *vs* treatment time and temperature.

At 50°C, the 1 min treatment of control group of *B. cereus* achieved the 0.6 $\log_{10}$ cfu/ml reduction of their population. However, under the same conditions, a pure culture of *B. cereus* vegetative cells using 1% NEW (available chlorine of 9.22 mg/l) was reduced by 6.6 $\log_{10}$ cfu/ml (8.06 $\log_{10}$ cfu/ml to 1.5 $\log_{10}$ cfu/ml). The decrease in the control group depends on temperature and was found to be 1.6 $\log_{10}$ cfu/ml at the same temperature for 5 min (Fig. 3).

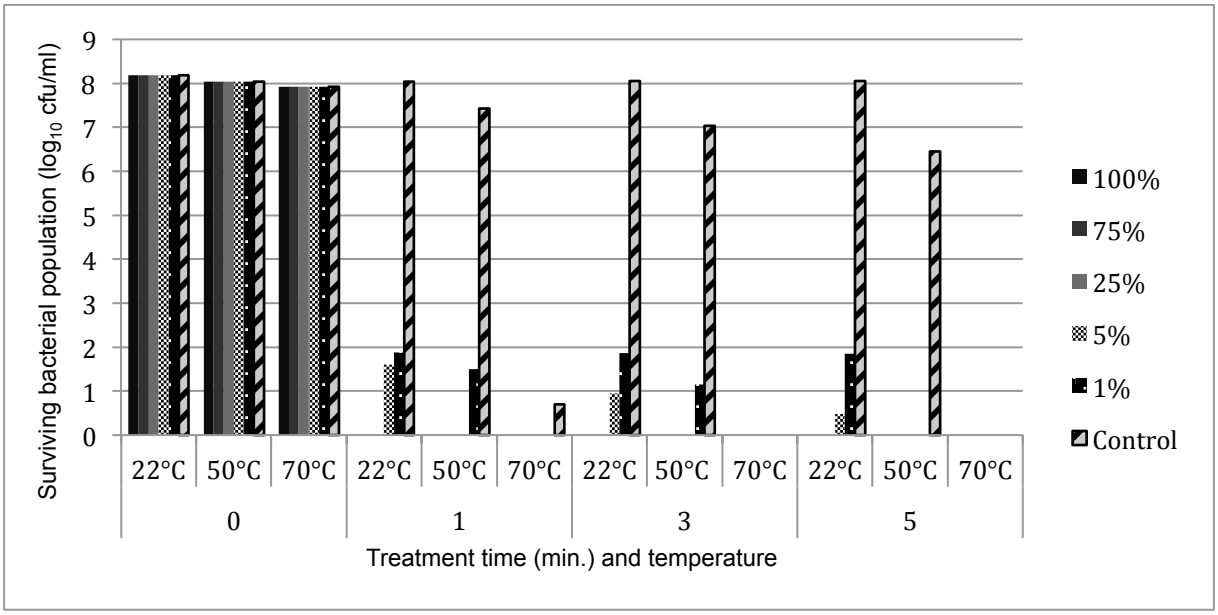


Figure 3: The effectiveness of different concentrations of NEW against *B. cereus* ATCC 11778 *vs* treatment time and temperature.

At 70°C, a reduction of 7.2 and 7.92 $\log_{10}$ cfu/ml in the control group was observed for 1 and 5 min, respectively (Fig. 3). ISSA-ZACHARIA *et al*. (2010) reported that SAEW (pH 5.6, 23 mg/l ACC; and 940 mV ORP) effectively reduced the populations of *E. coli, S.aureus* and *Salmonella spp*. by 5.1, 4.8, and 5.2 $\log_{10}$ cfu/ml, respectively. KIM *et al*. (2000) observed that *B. cereus* was more resistant to treatment than *E. coli* O157:H7 and only 3 log reductions were achieved after 10 s of ROX EO water treatment. Kiura *et al*. (2002) showed that reduction of the population of *B. subtilis* by 3.5 $\log_{10}$ cfu/ml was achieved by 5 min of acid electrolysed water (AEW) treatment (pH 2.32, 4.95 mg/l ACC).
The effect of ozonation was investigated to reduce *E. coli, B. cereus* and *B. cereus* spores in dried figs. *B. cereus* spores, *E. coli* and *B. cereus* counts were decreased by 3.5 log numbers at 1.0 ppm ozone concentration for 360 min ozone treatment (AKBAS and OZDEMIR, 2008).
The effectiveness of different concentrations of NEW was evaluated against spores of *A. flavus* and *P. expansum* using varying treatment times and temperatures *in vitro* (Figs. 4 and 5). It was observed that spores were more highly resistant to the activity of NEW than vegetative cells. At 22°C, the treatment of spores of *A. flavus* for 1 min using NEW (25%, 174.7 mg/l) resulted in a reduction of 5.83 $\log_{10}$ cfu/ml. Under the same conditions, the reduction of spores of *P. expansum* was 6.04 $\log_{10}$ cfu/ ml. Antimicrobial effectiveness of NEW associated with contact temperature increased up to 50°C, but the control group was not affected by this increase in temperature. At 50°C, the numbers of spores of *A. flavus* and *P. expansum* were reduced by more than 5.54-7 $\log_{10}$ cfu/ml within 1 min of exposure to NEW (33.9 mg/l ACC) (Figs. 4 and 5).

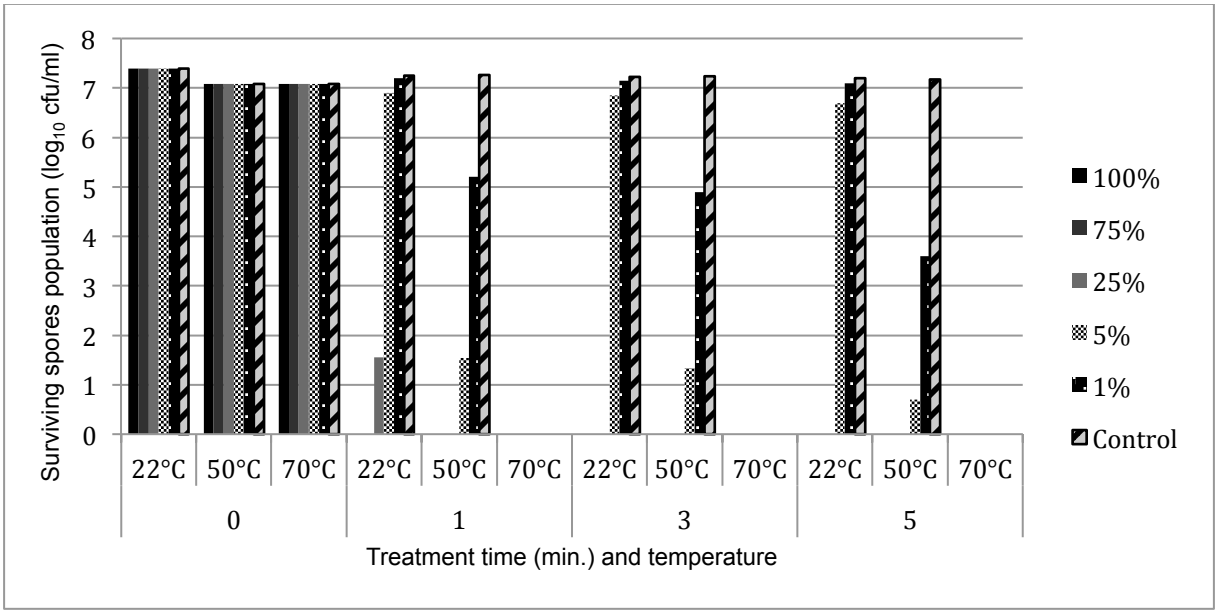


Figure 4: The effectiveness of different concentrations of NEW against spores of *A. flavus vs* treatment time and temperature.

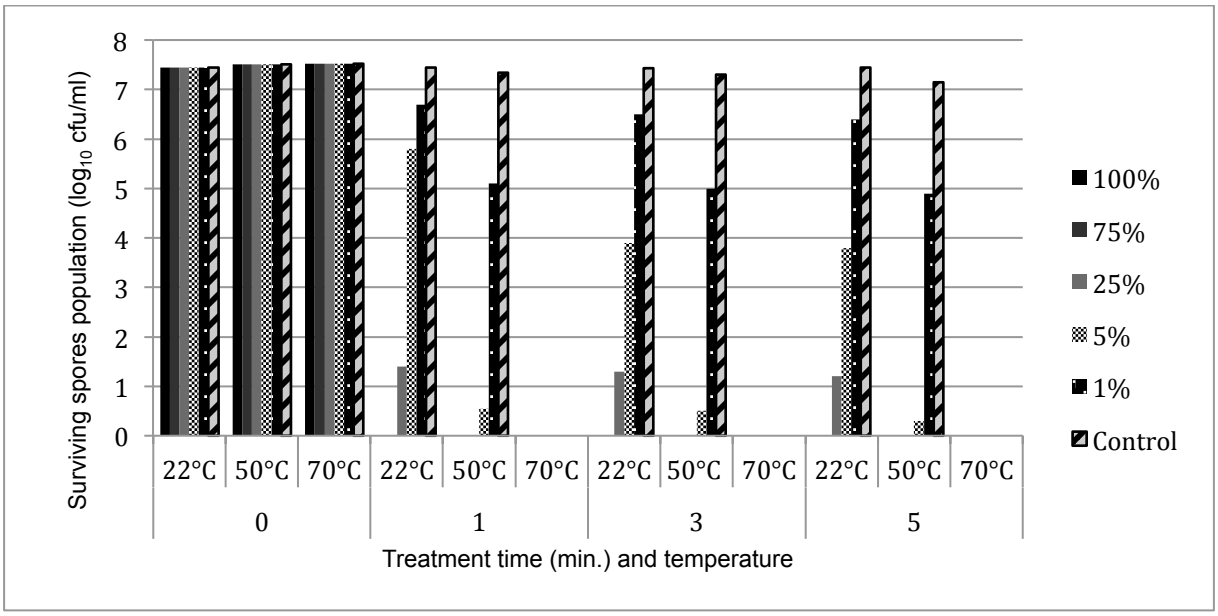


Figure 5: The effectiveness of different concentrations of NEW against spores of *P. expansum vs* treatment time and temperature.

At 70°C, populations of the control groups decreased to undetectable levels. Therefore, the effectiveness of NEW was not determined at this temperature (Figs. 4 and 5). Chlorine at levels of 100 to 200 ppm is currently recommended for the control of postharvest pathogen spores in dump tanks and other recirculating water systems (WILLET *et al*., 1989). However, the use of chlorine has disadvantages, such as the corrosion of metal equipment, reliance on manual monitoring of chlorine concentrations, sensitivity to organic load, effectiveness within a narrow pH range, and the formation of harmful chlorinated by-products (ROBERTS and REYMOND, 1994).

EAW is an alternative to chlorine and chlorine compounds; it has the lowest amount of free chlorine and a very wide pH range. Therefore, it has been reported that EAW can be effectively used for the control of postharvest pathogen spores (OKULL and LABORDE, 2004; AL-HAQ and SUGİYAMA, 2004; KOSEKI *et al*., 2004). In their studies on a laboratory scale, Audenaert *et al*. (2012) demonstrated that electrolysed oxidising water (EOW) has the potential to control *Fusarium* spp. in wheat grains.

It was observed that *B. cereus* endospores were substantially more resistant to the activity of NEW and temperature than fungi spores. Control groups of *B. cereus* spores were not affected by high temperature (70°C), but reduction of *B. cereus* spores exposed to NEW was increased at the same temperature. At 22°C, decreases in viable spores were initially rapid with a more than 5 $\log_{10}$ cfu/ml reduction observed after 1 min of exposure to undiluted NEW (100%). Under the same conditions, log reduction of 4.77 $\log_{10}$ cfu/mL was obtained when the concentration of NEW was decreased from 100% to 75%.

A longer exposure time was more effective in reducing the spore germination. After 3 min of exposure to 100, 75 and 25%, log reductions of 6.26 log10 cfu/ml, 4.84 $\log_{10}$ cfu/ml and 4.3 $\log_{10}$ cfu/ml, respectively, were observed.

At 22°C and 100% NEW, the number of viable spores dropped below the experimental detection limit within 5 min, which corresponds to the log reduction factor of 7.04. After 5 min of exposure to 75% NEW, 1.86 $\log_{10}$ cfu/ml viable spores were still observed (Fig. 6). It was determined that NEW, which has a low concentration and low content of ACC, was

more effective against spores of *B. cereus* at high temperatures. At 70°C, 5 $\log_{10}$ cfu/ml reductions were achieved after 1 min of 5% NEW treatment (Fig. 6).
Robinson *et al*. (2010) reported that, at the concentration of 99%, analytes produced a log reduction factor of greater than five in viable *B. atrophaeus* endospores within 90 s, and at concentrations of 10% or lower, the sporicidal effect was not statistically significant.

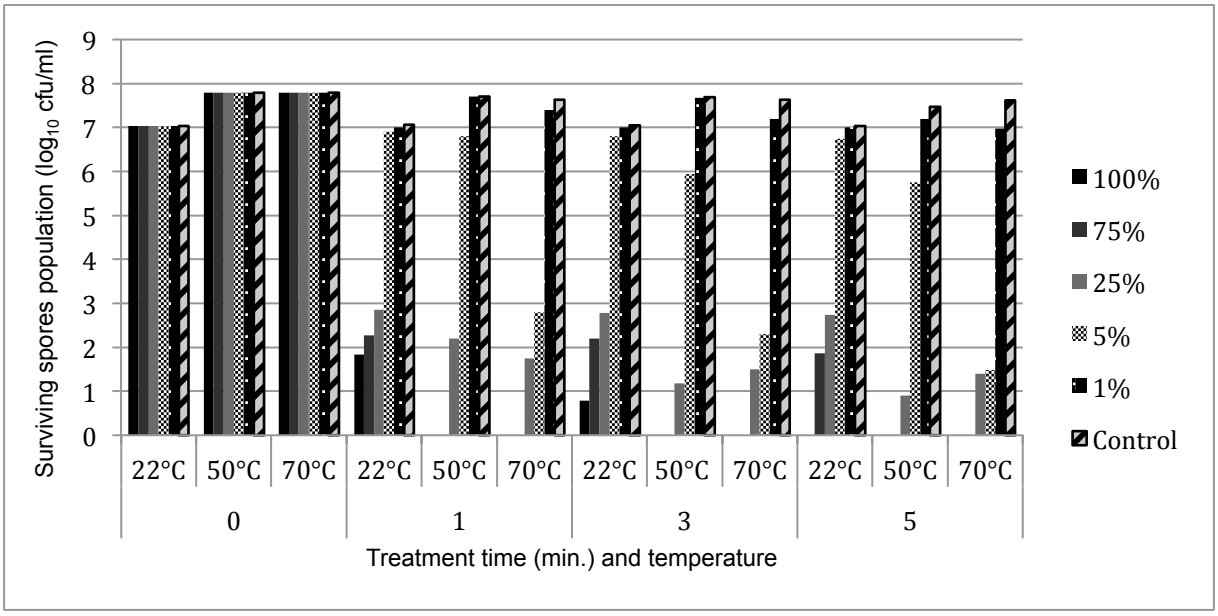


Figure 6: The effectiveness of different concentrations of NEW against spores of *B. cereus* ATCC 11778 *vs* treatment time and temperature.

Physicochemical properties and bactericidal activity of NEW and AEW under four different storage conditions were investigated by Cui *et al*. (2009). They reported that NEW with a near-neutral pH (pH 6.5) and low ORP value (550 mV) had a bactericidal activity (pH <3.0, ORP > 1000 mV) similar to AEW. Different researchers have reported similar results to those from the study of CUI *et al*. (2009) (MARAIS and BROZEL, 1999; KIURA *et al*., 2002; ROBINSON *et al*., 2010). They observed that available chlorine is one of the main contributing factors for the antimicrobial activity. Our results also indicated that the concentration of free chlorine in EOW was more substantial for the microbial growth reduction than pH.

4. CONCLUSIONS

The NEW solution was synthesised as the result of the electrochemical activation. The solution of high reaction ability is in the meta-stable state and contains strong oxidants such as ClO_2, HClO, ClO^-, Cl^-, HO_2, O_3, H_2O_2, H_3O^+, Cl_2O etc. It should be noted that somatic cells of animals and humans have a system of enzymatic protection against the action of the peroxidation factors mentioned. Microbial cells, especially anaerobic ones, are deprived of this protection. This fact determines the high selectivity of the NEW action on the micro flora. The findings of this study demonstrated that the bactericidal efficacy was retained against vegetative cells at dilutions as low as 1% and against bacteria and fungi spores at dilutions as low as 5% NEW. Also, 5% NEW with a pH of 7.7, ACC of 33.9 mg/l

and ORP value of 840 mV can effectively inactivate pure cultures of spores of *B. cereus* and fungi *in vitro*. In addition, as a result of being non-corrosive, more stable for storage, inexpensive, and posing less potential health hazards to workers due to a lack of Cl_2 degassing, 5% NEW has more potential for application in fig enterprises than AEW (acidic and includes high available chlorine) and chlorine-based disinfectants, which have high associated costs.

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PAPER

TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT PROPERTIES OF SOLINA (*TRITICUM AESTIVUM* L.) AND DERIVATIVES THEREOF

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ABSTRACT

This study aims to characterize grains and derivatives of Solina, an Italian traditional winter soft wheat (*Triticum aestivum* L.). Total polyphenol content and antioxidant properties were investigated in grains, whole flours and bread, both in aqueous-organic extracts and residues.
Results showed the important contribution of hydrolysable polyphenols, isolated in the residue of the aqueous-organic extract, to antioxidant properties (over 90% in grains and derivatives), and the key role played by milling and baking in antioxidant properties. Moreover, the analysis of potentially bioactive antioxidants remaining in the residues showed to be required for a comprehensive determination of antioxidant capacity in cereals. The study highlights that Solina represents a valuable Italian traditional wheat cultivar with proved antioxidant properties.

Keywords: antioxidants, Total Polyphenol Content (TPC), traditional foods, soft wheat Solina, whole wheat flours, bread

1. INTRODUCTION

Nowadays the traditional character of foodstuffs is a value-adding characteristic which contributes to defining quality thereof and is also often associated with positive health benefits. Traditional foods, in fact, not only play an important role in the cultural and nutritional food patterns of every country because their cultivation or preparation have been passed down from generation to generation, and/or because they have been consumed locally for years and become part of daily life in communities; they are often considered healthy and wholesome and are also often associated with positive health benefits because of their chemical profile.
Solina is a traditional Italian winter soft wheat (*Triticum aestivum* L.), typical of the province of L'Aquila, in the Abruzzo region, within the National Park of Gran Sasso. Its cultivation in Abruzzo dates back to 1500 a.C. and there is evidence of a strong connection between this variety and the life of people from Abruzzo who keep appreciating it for the sensory properties it provides to bakery products. It is grown in mountainous areas and shows a high adaptability to marginal areas, as well as resistance to cold. That ancient cultivar was, in fact, defined by last century agronomists "*Wheat hybernum*". Solina also shows a high stability in yields, and according to agronomists it is also compatible with organic farming methods because it does not require a large amount of nitrogen; moreover it is able to tolerate and compete strongly with weeds for its size and tillering capacity (PORFIRI *et al*., 2001).
In this study, grains of the Solina cultivar, whole flours and bread samples thereof were examined in order to evaluate the phenolic components and the antioxidant properties of this traditional crop.
Some technological and chemical parameters of grains were also considered, so as to identify a comprehensive portrait of this Italian wheat cultivar.
According to literature, grains, in particular whole grains, provide a wide range of nutrients and phytochemicals that work in synergy to maintain human health (LIU, 2007; OKARTER and LIU, 2010). Whole grain consumption has been associated with reduced risk of chronic diseases, such as cardiovascular diseases and cancer (SCHATZKIN *et al*., 2007; MELLEN *et al*., 2008; AUNE *et al*., 2013). These beneficial properties have been rediscovered by consumers and producers (ARVOLA *et al*., 2007).
As the content of bioactive molecules is affected by genetic and growing conditions in grains as well as by processing conditions in foods (SLAVIN *et al*., 2000), samples of Solina grains, flours and bread formulated with this soft wheat cultivar were studied.

2. MATERIALS AND METHODS

2.1. Sampling

Analysis were performed on grain, whole wheat flour and bread samples. Grains were supplied by three farms located in three different spots of the same area: Castelvecchio Subequo, Capestrano and Introdacqua. According to sample location they were labeled as Solina 1, Solina 2 and Solina 3, collected respectively at Castelvecchio Subequo, Capestrano and Introdacqua.
The grains of each collected sample were divided in two aliquots. An aliquot was milled by a water cooled mill (Janke & Kunkel IKA Labortechnik, Staufen, Germany) and stored at 4°C until chemical analyses were performed. An aliquot was tempered for 24 h to 15.5% moisture content, then milled in an experimental mill MLU-202 Bühler (Switzerland)

equipped with three breaks and three reduction rolls and six screens to obtain whole mill flours.
Solina bread was also studied. Five loaves of bread (four long loaves and one round loaf), produced according to a traditional bread making process, were purchased in bakeries located in the same area of grain collection. They were coded as reported in Table 1.

Table 1. Code, description and some chemical parameters of bread samples*.

Code	Description	Composition		
		Moisture (%)	Protein (%)	Ash (%)
A	Bread long loaf (900 g)	9.9	11.5	1.90
B	Bread long loaf (1660 g)	7.8	11.2	2.74
C	Bread round loaf (900 g)	10.8	12.5	2.96
D	Bread long loaf (810 g)	11.0	11.6	2.65
E	Bread long loaf (1700 g)	10.4	11.6	1.81

*Data are expressed as mean of replicate measurements (n=3); differences within measurements were in the range reported in the method.

2.2. Chemicals and standards

Common reagents and standards were purchased from Sigma–Aldrich Srl (Milan, Italy), Extrasynthèse (Genay, France), Carlo Erba (Milan, Italy) and BDH Laboratory Supplies (Poole, UK) and their purity degree was chosen according to the analysis to be performed. Double-distilled water (Millipore, Milan, Italy) was used throughout the study.

2.3 General chemical analysis

Grain quality was determined after removing impurities, i.e., broken grains, heat-damaged grains, shriveled grains, vegetable impurities, vitreous grains, on 100 g seeds by visual evaluation. Test weight (TW), thousand kernel weight (1,000 KW) and kernel hardness (KH) were assessed. TW was determined by a Shopper chondrometer equipped with 250 mL cylinder. 1,000 KW was determined by counting and weighing 1,000 kernels, while kernel hardness (KH), diameter and moisture of grains were determined by the SKCS 4100 apparatus (Perten Instruments, Sweden) according to the standard method AACC 55-31 (2003).
Total proteins were determined by Kjeldahl method according to ICC standard method No 105/2 (2003) using 5.70 as specific conversion factor. Ash content was determined on the inorganic residue remaining after the incineration of the sample in a muffle furnace at the temperature of 900°C according to the ICC method No 104/1 (2003), while moisture was determined according to ICC standard method No 110/1 (2003). Moisture is reported as g/100 g fresh weight (f. w.), while both protein and ash content are reported as g/100 g dry matter (d. m.).

2.4. Sample extraction for evaluation of Total Polyphenol Content (TPC) and antioxidant properties

Total polyphenols were extracted as described by DURAZZO *et al*. (2013), with some modifications. Extractable polyphenols were determined on aqueous-organic extracts, while non extractable polyphenols were determined on solid residues of aqueous-organic extraction. In details, some non-extractable polyphenols, i.e. hydrolysable polyphenols, were isolated and determined following a specific and suitable acid hydrolysis as reported below.

Extractable polyphenols

About 3.0 g, 3.5 g, 4.5 g of grains, flours and bread, respectively, were placed in a test tube and added with 20 mL of acid methanol/water (50:50 v/v, pH= 2). Tubes were swirled at room temperature for 3 min, then mildly shaken for 1 h in a water bath at room temperature as well. Tubes were centrifuged at 2500 x g for 10 min, and the supernatant was recovered. Then, 20 mL acetone/water (70:30, v/v) were added to residue and the extraction repeated as reported above. Methanolic and acetonic extracts were combined and centrifuged at 2800 x g for 15 min. The resulting mixture was then used for the determination of total polyphenol content and antioxidant properties.

Hydrolysable polyphenols

The residue left after the above described extraction was dried in a ventilated oven at 25°C. 250 mg, 300 mg, 400 mg of grain, flour and bread residue, respectively, were mixed with 20 mL of methanol and 2 mL of sulfuric acid (18 M). Samples were gently stirred for 1 min and shaked at 85°C for 20 h in a water bath.
Samples were then centrifuged (2500 x g for 10 min), and the supernatant was recovered. After two washings with minimum volume of distilled water and re-centrifuging as necessary, the final volume was taken up to 50 mL. The tube was centrifuged at 2800 x g for 20 min and the resulting supernatant was used for the determination of total polyphenol content and antioxidant properties.

2.5. Determination of Total Polyphenol Content (TPC)

The TPC was determined using the Folin-Ciocalteau procedure (SINGLETON et al., 1999). Briefly, appropriate dilutions of extracts were oxidised with Folin-Ciocalteau reagent, and the reaction was neutralised with sodium carbonate. The absorbance of the resulting blue colour solution was measured at 760 nm against an appropriate blank after 2 h of reaction at room temperature at dark. Gallic acid was used as standard.

2.6. Antioxidant properties evaluation

The determination of antioxidant properties was carried out by FRAP (Ferric Reducing Antioxidant Power) assay according to methods of BENZIE and STRAIN (1996) and PULIDO *et al*. (2000) and by using a Tecan Sunrise® plate reader spectrophotometer. The method is based on the reduction of Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) complex to ferrous at low pH.

2.7. Statistical Analysis

All analyses were performed in triplicate. Data are presented as mean±standard deviation (s.d.).
Data obtained by Official Methods are presented as mean. Statistica for Windows (Statistical package; release 4.5; StatSoft Inc., Vigonza PD, Italy) was used to perform One-way Analysis of Variance (ANOVA). Significant differences between grain and flours of the same origin were evaluated by the Student's t test.

3. RESULTS AND DISCUSSIONS

The inspection of grain impurities in the samples is an important control procedure necessary to verifying wheat quality and it is a preliminary step prior to the milling phase. The percentage of ripe grains were higher than 95% in all samples. This confirms the good quality of analysed grains.
The values obtained for the main physical parameters (kernel hardness, test weight, thousand kernel weight, diameter) and the chemical composition (moisture, protein, ash) of grain samples are reported in Table 2. As concerns physical parameters, no significant differences were found among grain samples. The values obtained for TW (74.1 - 80.2 Kg/hL), 1,000 KW (>43 g) and grain diameter (2.11-2.47 mm) show that kernels are wholesome, sound, not sprout damaged.

Table 2. Physical and chemical parameters of grains*.

Sample	KH	TW (kg/hL)	1,000 KW (g)	Diameter (mm)	Moisture (g/100 g)	Protein (g/100 g d.m.)	Ash (g/100 g d.m.)
SOLINA 1	19.70	74.1	44.6	2.47	12.4	13.1	2.04
SOLINA 2	16.69	80.2	54.4	2.46	12.6	11.9	1.75
SOLINA 3	33.70	79.0	43.4	2.11	12.4	13.7	2.00

*Data are expressed as mean of replicate measurements (n=3); differences within measurements were in the range reported in the method.

As regards kernel hardness, the data reported in Table 2 confirm our samples belong to the "soft" species and their variation may depend on the different growing locations. According to POMERANZ and WILLIAMS (1990), in fact, the main factors that affect the grain hardness are growing location (soil type, elevation, planting type, irrigation, fertilizers and cultivation practice), growing season (precipitation and temperature during maturation and post ripening), storage conditions, protein content, moisture and kernel size. Regarding the main chemical parameters, significant differences were observed: Solina 2 showed the lowest protein and ash content. Those data show, therefore, that environmental and growing factors not only influence hardness, but also chemical parameters such as protein and ash content.
In Table 3, total polyphenol content (TPC) and antioxidant properties (FRAP) are reported for grains and whole wheat flours. Values refer to aqueous-organic extracts and residues. In grains, TPC values ranged between 165.57 and 183.75 mg/100g d.m. in aqueous-organic

extracts, and between 1084.42 and 1325.32 mg/100g d.m. in residues, whereas the range for FRAP values was 6.34-6.60 μmol/g d.m. in aqueous-organic extracts and 85.06-109.12 μmol/g d.m. in residues. TPC and FRAP values in aqueous-organic extracts showed no differences between the grain samples supplied by the three farmers. On the other hand, as to the residue, the TPC resulted significantly different in the grain samples and the highest value was obtained for Solina 1; the values for antioxidant properties in Solina 1 were higher than those obtained for Solina 2 and Solina 3 that were, at their turn, comparable. As reported by several authors (ADOM *et al*., 2003; CARCEA *et al*., 2009) differences in the bioactive molecule distribution and antioxidant properties in wheat, as well as in foodstuffs in general, could be related to genetic factors, agronomic practices and environmental conditions. It is interesting to notice that in grains the FRAP values in residues are about 15 fold higher than in aqueous-organic extracts. This trend was also confirmed by the data obtained for TPC. The hydrolysable polyphenols, isolated in residue, belong to several classes of phenolic compounds, i.e. hydrolysable tannins, phenolic acids and hydroxycinnamic acids, and consist on ferulic acid, caffeic acid, sinapic acid, etc. These phenols are linked to carbohydrates and protein by covalent bonds, hydrogen bonds and/or hydrophobic interactions and they are released from food matrix after a strong acid hydrolysis (PÉREZ JIMÉNEZ and TORRES, 2011; PÉREZ-JIMÉNEZ *et al*., 2013). These compounds represent a significant fraction in some groups of foods, among which cereals and have appreciable antioxidant properties and possibly specific health properties. However, they have been generally underestimated and studies are still required to tackle this issue (SAURA-CALIXTO, 2012). In addition, recent studies have shown that non-extractable polyphenols significantly contribute to the beneficial properties associated to dietary fibre (VITAGLIONE *et al*., 2008; SAURA-CALIXTO, 2011).

Table 3: TPC and FRAP values in aqueous-organic extracts and their residues in grains and flours*.

Products	Aqueous-organic extract (extractable polyphenols)					
	TPC (mg/100g d.m.)			FRAP (μmol/g d.m.)		
	Grain	Flour	P value$^{\Omega}$	Grain	Flour	P value$^{\Omega}$
SOLINA 1	170.43±28.61	129.28±13.64^{a}	0.01	6.60±0.47	2.75±0.17^{b}	0.0001
SOLINA 2	165.57±26.11	130.87±15.85^{a}	0.001	6.34±0.27	2.35±0.14^{a}	0.0001
SOLINA 3	183.75±14.07	155.24±12.74^{b}	0.01	6.47±0.60	2.31±0.07^{a}	0.0001
Mean	**173.25±24.05**	**138.46±18.16**	**0.0001**	**6.47±0.46**	**2.48±0.24**	**0.0001**

Products	Residue (hydrolysable polyphenols)					
	TPC (mg/100g d.m.)			FRAP (μmol/g d.m.)		
	Grain	Flour	P value$^{\Omega}$	Grain	Flour	P value$^{\Omega}$
SOLINA 1	1325.32±57.99^{c}	981.01±50.27^{b}	0.0001	109.12±10.54^{b}	114.25±3.24^{b}	n.s.
SOLINA 2	1084.42±66.99^{a}	811.65±38.57^{a}	0.0001	85.06±9.90^{a}	99.20±11.14^{a}	0.01
SOLINA 3	1198.73±37.49^{b}	791.50±80.14^{a}	0.0001	90.40±4.30^{a}	118.29±4.26^{b}	0.0001
Mean	**1202.82±114.39**	**866.82±104.77**	**0.0001**	**92.07±12.22**	**111.61±10.13**	**0.0001**

*mean ± s.d.; Anova, Tukey HSD Test: by column, means followed by different letters are significantly different ($p < 0.05$).

▫ Student's t test ; n.s.=not significant

In our study, the determined hydrolysable polyphenols (isolated in the residue of the aqueous-organic extract of grains) showed to account for 87-89% of total polyphenols and contribute to the total antioxidant properties for about 93%. These data match those obtained in the other studies on cereals (PEREZ-JIMENEZ and SAURA-CALIXTO, 2005; CHANRASEKARA and SHAHIDI, 2010).
Regarding the effect of milling process on TPC content, whole flours exhibited a more significant reduction than in grains, both in the aqueous-organic extract and in the residue. Results showed that antioxidant properties also appear to be affected by the milling process: a decrease in FRAP values was, in fact, observed in aqueous-organic extract (mean value: 6.47±0.46 *vs* 2.48±0.24 μmol/g d.m.; P<0.0001), whereas an increase in FRAP values was reported in residues (mean value: 92.07±12.22 *vs* 111.61±10.13 μmol/g d.m.; P<0.0001). The increase of FRAP values in residue after milling process could be due to a better efficiency of extraction of bound compounds with antioxidant properties, related to improvement of solvent penetration for the enlargement of the particle surface. These data confirm the importance of milling process in retention of components contributing to antioxidant properties (DUODU, 2011).
Nowadays, several investigations have been carried out to study the effects of cereal processing technologies, such as milling, baking, extrusion, etc., on antioxidant properties (LIYANA-PATHIRANA and SHAHIDI, 2007; RAGAEE *et al*., 2014). As a consequence, TPC (mg/100g d.m.) and FRAP (μmol/g d.m.) in aqueous-organic extracts and their residues were also studied in Solina bread samples, as reported in Table 4. It is worth mentioning that whole wheat bread represents a rich source of bioactive compounds as reported in literature (JENSEN *et al*., 2011; ABDEL-AAL and RABALSKI, 2013).

Table 4. TPC and FRAP values in aqueous-organic extracts and their residues in bread*.

Loaves	TPC (mg/100g d.m.)		FRAP (μmol/g d.m.)	
	Aqueous-organic extract	Residue	Aqueous-organic extract	Residue
Code A	63.13±6.03^{cd}	832.91±46.21^{a}	8.57±0.34^{d}	106.21±16.96^{a}
Code B	49.44±6.98^{b}	894.07±62.64^{a}	3.93±0.17^{a}	120.27±30.77^{ab}
Code C	52.87±6.93^{bc}	1326.90±48.00^{b}	6.14±0.34^{b}	141.90±8.33^{b}
Code D	36.96±5.59^{a}	1237.41±69.35^{b}	4.14±0.37^{a}	114.19±3.52^{a}
Code E	71.23±6.04^{d}	877.73±88.77^{a}	7.80±0.26^{c}	126.60±7.80^{ab}

*mean ± s.d.; Anova, Tukey HSD Test: by column, means followed by different letters are significantly different ($p < 0.05$).

In bread samples, as it was observed in raw materials, the major contribution to antioxidant properties was given by hydrolysable polyphenols (93-97%). Moreover, the comparison of the TPC and FRAP values of the residue in bread samples and the corresponding values in grains showed that a decrease of TPC, from grains to bread, corresponded to an increase of FRAP values. Several authors have observed that some antioxidants can be generated during non-enzymatic browning reactions, such as the Maillard reaction (MANZOCCO *et al*., 2001).

4. CONCLUSIONS

Our results showed that in grains and derivatives thereof, hydrolysable polyphenols (isolated in the residue of the aqueous-organic extract) represent an important fraction, with appreciable antioxidant properties and possibly health properties. In particular, this research highlighted that the analysis of potentially bioactive antioxidants, remaining in the residues, is required for an adequate and comprehensive determination of antioxidant capacity in cereals. Results also pointed out the key role played by the milling and baking process in antioxidant properties.
So, in conclusion, Solina wheat represents a valuable Italian traditional wheat cultivar with proved antioxidant properties. It also contributes to highlighting the benefits that traditional foodstuffs may have Europe-wide: they represent foods with an increased nutritional value and bring a strong contribution to land protection and conservation of biodiversity.

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PAPER

QUALITY CHANGES OF SPOTLESS SHAD DURING STORAGE AT DIFFERENT CONDITIONS

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ABSTRACT

This study investigates the effect of using ice in combination with refrigeration on the sensory, physico-chemical and microbiological attributes of spotless shad during storage. Spotless shad kept in ice under refrigerated conditions had better sensory, physico-chemical and microbiological quality as compared with control groups. The shelf life of samples kept at ambient temperature without ice was 2 days. Using ice and refrigeration only extended the shelf-life for 3 days and 4 days, respectively, while ice application with refrigeration further increased the shelf-life by 10 days. Physico-chemical and microbiological results usually supported sensory values. Histamine values were below EU (European Union) and FDA permitted levels for the shelf-life of fish.

Keywords: spotless shad, biogenic amines, quality changes, sensory values, shelf life

1. INTRODUCTION

Spotless shad, *Alosa immaculata,* Bennett, 1835 is the largest representative species of the *Clupeidae* family, it has a pelagic life and is classified under diadromous fish species (FAO, 2014). This shad species is the most abundant in Eastern Europe both in inland and marine waters (NAVODARU *et al*., 2003). It is a commercially important species in Turkey (TUİK, 2013) and some other European countries (FAO, 2014). The fishing period of shad was recorded to take place between March and October for the Black Sea. Various catching methods and materials are used for shad (ERGÜDEN *et al*., 2007) and the total catch value was 1,541 tons for the year 2013 in Turkey (TUİK, 2013). Some other European countries also reported a total catch of about 1,029 tons (FAO, 2014). However, this species is commonly caught by local fishermen and therefore, most of their catch is likely not to be recorded.

Fish spoilage relates to both seafood quality and safety that further affects the market value of the product as well as its nutritional value. Spoilage is mainly caused by bacterial activity, other than sensory quality loss (such as its appearance, flavour, odour and texture), some compounds, such as Biogenic Amines (BAs), can be formed by bacterial decarboxylation of the precursor amino acids, thus, leading to food safety issues. Most reports have focused on histamine, which is reported to cause scombrotoxin poisoning, closely linked to the consumption of fish kept in abused time/temperature conditions and species belonging to different families, including *Clupeidae*, due to high content of free histidine in their muscle (LEHANE and OLLEY, 2000; EU DIRECTIVE, 2005). Putrescine and cadaverine are also known to enhance the toxicity effect of histamine. Other than food safety issues, several BAs are known to be associated with fish decomposition and the formation of cancerous nitrosamines (LEHANE and OLLEY, 2000; PONS-SÁNCHEZ-CASCADO *et al*., 2006).

Although, previous studies demonstrated the advantage of using chilled storage conditions for various fish species and the different shelf lives were observed (VARLIK and HEPERKAN, 1990; VECIANA-NOGUÉS *et al*., 1990; CARACHE *et al*., 2002; KÖSE and ERDEM, 2004; CHOTIMARKORN, 2014; KORAL and KÖSE, 2012), and limited studies are available for the quality changes of spotless shad stored at chilled temperatures. So far, DUYAR *et al*. (2012) investigated the shelf life of this species at refrigerated temperature and obtained 6 days of shelf life. In our previous study, we studied physico-chemical and sensory quality of shad, obtained in a market in the months of July and August and kept in refrigerated storage in open air for 3 days. We gathered that the quality of shad varies depending on the initial quality of fresh fish (KÖSE *et al*., 2000). However, storage quality and development of biogenic amines under various chilled temperature conditions of this species are still unknown. Previous literature, including our past studies, showed that keeping fish in ice during refrigerated storage improves the storage quality, while extending the shelf life. Similarly, this type of application retarded BAs development (KORAL and KÖSE, 2012).

Shad is known to contain high fat, up to 26%, which affects its storage quality more than less fatty fish (BORAN and KARAÇAM, 2011). Moreover, this species is highly susceptible to the formation of histamine and other biogenic amines, which result in seafood health risk for the consumers. Therefore, we aimed to investigate the effect of different ice application under refrigerated temperature of this species on its shelf life and biogenic amine development.

2. MATERIALS AND METHODS

2.1. Materials and experimental design

Fresh, spotless shad samples used in this study were caught in the South-Eastern Black Sea in the month of January 2013. The samples that were previously kept in ice and placed in EPS (expanded polystyrene) boxes for 3hours after the catch, were collected directly from a boat at the port of Trabzon, Turkey. The fishes were then kept in EPS boxes containing ice and were transferred to the laboratory within 1hour. The fishes which weighed 30kg (125 fishes) in total were immediately washed with tap water and then, the fishes were randomly divided into 4 groups. Each group was kept in plastic containers. Ice application on fish was carried out layer by layer. The containers were covered with plastic wrap (cling film). The sample groups were;

(i) Control: Fish stored at ambient temperature without ice (C): The container temperature: 17.0°C ± 3.0°C, Fish average temperature: 16.0°C ± 4.0°C. The average length and weight of the fishes weighing a total of 5.40 kg (24 fishes) are 29.59 cm ± 1.98 cm, 225.59 g ±37.38 g, respectively.

(ii) Fish stored in ice, ratio is at 1:1 (w: w), and at ambient temperature (IAT) 17.0°C ± 3.0°C: Fish temperature fluctuated between 4.0°C and 14.0°C with daily ice replacement (Fish temperature dropped to 4°C in 2 hours after ice addition). The average length and weight of the fishes weighing a total of 7.2 kg (30 fishes) are 30.19 cm ± 2.30 cm, 238.98 ± 53.17 g, respectively.

(iii) Refrigerated (in a refrigerator: Arçelik, 8810NF, Turkey) fishes without ice (RT): Storage in cold air at 4.8°C ± 0.3°C. Fish temperature 3.8°C ± 0.4°C. The average length and weight of the fishes weighing a total of 7.2 kg (30 fishes) are 29.88 ± 2.15 cm, 241.2 ± 59.94 g, respectively.

(iv) Iced storage in refrigerator (IRT) at 4°C ±1°C: Fish and ice ratio, (1:1 w:w). Fish temperature, 0.5°C ± 0.3°C. The average length and weight of the fishes weighing 10kg (40 fishes) are 30.58 cm ± 1.76 cm, 246.25g ± 40.8 g, respectively. Ice was replaced daily.

Sampling was carried out daily by randomly choosing spotless shad, until the products were spoilt based on sensory results. The ice was the flake iced type obtained freshly from an ice maker (Hoshizaki, FM-80EE, Amsterdam, Holland). The internal temperatures of the fishes in each group were measured on a daily basis using a digital thermometer (Taylor, 9842N Waterproof Digital Thermometer, USA) by injecting the upper dorsal part of the fish.

3. METHODS

3.1 Sensorial evaluation

Sensory analysis was carried out using eight trained panellists who judged the freshness of the samples using a 10-point scale (ARCHER, 2010). The panellists were comprised of 6 males and 2 females (5 academicians and 3 administrative staff). The subjects were qualified after passing the screening tests stated by BOTTA (1995). The panellists are 20 in number and they were chosen from previously trained people who are regular shad consumers based on the criteria given by BOTTA (1995).

Table 1 shows the sensory score sheets used to evaluate spotless shad samples. This structured category scale is based on the traditional freshness, quality grading system for

the whole iced and refrigerated spotless shad. According to scale, 4 is the limit for acceptable/unacceptable, <4: unacceptable. The results were presented as the means of data obtained from 8 panellists.

Table 1: Sensory evaluation criteria of shad samples.

Score	Appearance					Odour	Texture
	Eyes	Gills	Skin	Flesh	Appearance of Belly Walls		
10	Slightly convex, clear, black pupil.	Dark red, purple, clear slime	Bright colours, iridescent, few loose scales.	Glossy, rosy hue, fresh bright blood on fillet.	No belly burst	Oily, marine, fresh blood, sulphide, weak odour.	Firm, stiff, smooth
9	Flat, slightly convex, clear black pupil.	Dark red, pink, slightly faded.	Bright, slight iridescence, few loose scales.	Slight translucency, rosy hue, bright blood on fillet.	No belly burst.	Oily, marine, fresh fruit, sulphide.	Loss of stiffness, still firm, smooth.
8	Flat, slightly convex, slightly cloudy	Dark red, pink, slight brown, slight reddening on gill covers	Loss of brightness, some loose scales.	Slight translucency, slight discoloration of belly flaps.	No belly burst or very slight belly burst	Oily, musty, leathery, slight sulphide.	Loss of stiffness, slight softening, smooth.
7		Red, pink, slight bleaching, slight reddening on gill covers.	Dull, slight bronzing, some loose scales.	Slightly opaque, slightly brown, slight discoloration of belly flaps.	Slight belly burst.	Oily, musty, sulphide, slightly sour.	Limp, slightly soft, slightly gritty.
6	Flat, slightly cloudy	Red, pink, brown, slight bleaching, reddening on gill covers.	Dull, slight bronzing, some dirty scales.	Opaque, dull, brown, reddening on belly flaps.		Musty, stale fruit, stale grass, malty, sour.	
5	Flat, slightly sunken, slightly cloudy, grey pupil.	Pink, brown slime, bleached, reddening on gill covers.	Dull, bronzing, dirty scales.	Opaque, brown, discoloured belly flaps and tail.	Definite belly burst.	Stale fruit, stale grass, sour, malty, slightly rancid.	
4	Flat, slightly sunken, cloudy, bloodshot, discoloured.					Sweaty, sour sink, stale meat,H_2S	Limp, soft, gritty.
3	Flat, slightly sunken, wrinkled, discoloured, blood shot.	Pink, bleached, brown slime, bronzing on gill covers.	Dull, bleached, brown slime.	Opaque, general discoloration.		Sweaty, cheesy, sour sink, byre-like, rotten fruit.	

Triplicate samples (3 randomly selected fish) were taken per day at other intervals from each group to evaluate their quality criteria at the sensory laboratory section. Fresh spotless shad was also included in the evaluation, starting from the 2nd day, to support the judgement of the assessors as a reference sample (although, the panellists were blind to its code). Different samples were simultaneously presented in plates coded. They were expected to give the highest score for the blinded reference sample (freshly caught spotless shad). After the sensory analysis, the fish were further used for physico-chemical analysis.

3.2. Physico-chemical analysis

Total Volatile Basic Nitrogen (TVB-N) was determined using the method of Lucke and Geidel as described by INAL (1992). Thiobarbituric acid value (TBA) was estimated according to SMITH *et al*. (1992). The method of BOLAND and PAIGE (1971) was used for analyzing Trimethylamine Nitrogen (TMA-N). The pH measurements were taken with a digital pH meter (Jenco 6230N, CA, USA) by placing the electrode into the homogenized samples (KÖSE *et al*., 2012). Biogenic amines were analyzed using a High Performance Liquid Chromatography (HPLC) method according to KÖSE *et al*. (2012) and KORAL and KÖSE (2012). HPLC equipment was Shimadzu Prominence LC-20 AT series (Japan) with auto sampler (sıl20ac, Shimadzu, Japan), a Diode Array Detector (SPD-M20A, Shimadzu, Japan) and Intertsil column (GL Sciences, ODS-3, 5μm, 4.6×250mm). All physico-chemical analysis was carried out in triplicate sampling, and results were represented as means ±SD.

3.3. Bacterial analysis

Total aerobic Viable Bacteria (TVB) were counted using plate count agar according to KÖSE and ERDEM (2004). Mesophilic and psychrophilic microorganisms were counted after incubation at 4°C for 8 days for psychrophilic microorganisms and at 37°C for 48 hours for mesophilic microorganisms. Histamine-forming Bacteria (HFB) were determined in accordance with the method used by KÖSE (1993). Total mesophilic and psychrophilic HFB were determined using the same condition applied for TVB with the exception of using Niven's medium, instead of plate count agar. Triplicate samples were used for each type of analysis, while each analysis was performed in duplicate. Counts of bacteria were expressed as log cfu/g. The results were presented as means of all counts ±SD.

3.4. Statistical analysis

All statistical analysis was performed using JMP software (Version 8.02, 2009, SAS Institute. Inc., Cary, NC, USA) (SOKAL and ROHLF, 1987). Analysis of variance (ANOVA) was used to compare the results within the groups as well as during storage period. When significant differences were found, comparisons among data were carried out using Turkey test. A significant level of 95% ($p<0.05$) was used throughout the analysis.

4. RESULTS AND DISCUSSION

Sensory analysis is important in any food quality evaluation program because the ultimate criterion for judgment is the human response (XU *et al*., 2014). In this study, sensory evaluation was based on appearance, texture and odour and the results are presented in Table 2. Sensory scores decreased significantly in both control and experimented samples

with increasing storage ($p<0.05$). Significant changes occurred between the control group and others, starting from the 1st day of storage ($p<0.05$) for the relating sensory attributes.

Table 2: Sensory values of shad groups stored under various conditions.

Day of storage	Sample type	Appearance	Odour	Texture	Over all mean
0	Fresh	9.66±0.21	9.80±0.10	9.62±0.36	9.69±0.09
1	C	$8.28\pm0.19^{a}_{A}$	$7.28\pm0.15^{a}_{A}$	$8.30\pm0.16^{a}_{A}$	$7.95\pm0.58^{a}_{A}$
	IAT	$9.84\pm0.09^{b}_{A}$	$9.28\pm0.29^{b}_{A}$	$9.30\pm0.23^{b}_{A}$	$9.47\pm0.32^{b}_{A}$
	RT	$9.68\pm0.23^{b}_{A}$	$9.40\pm0.37^{b}_{A}$	$9.76\pm0.11^{b}_{A}$	$9.61\pm0.19^{b}_{A}$
	IRT	$9.78\pm0.13^{b}_{A}$	$9.80\pm0.10^{b}_{A}$	$9.94\pm0.09^{b}_{A}$	$9.84\pm0.13^{b}_{A}$
2	C	$6.04\pm0.11^{a}_{B}$	$6.12\pm0.16^{a}_{B}$	$7.22\pm0.22^{a}_{B}$	$6.46\pm0.66^{a}_{B}$
	IAT	$8.18\pm0.13^{b}_{B}$	$8.16\pm0.11^{b}_{B}$	$9.10\pm0.22^{b}_{A}$	$8.48\pm0.54^{b}_{B}$
	RT	$9.12\pm0.13^{c}_{B}$	$9.12\pm0.23^{c}_{A}$	$9.50\pm0.10^{c}_{A}$	$9.24\pm0.45^{c}_{B}$
	IRT	$9.48\pm0.22^{c}_{A}$	$9.64\pm0.45^{c}_{A}$	$9.74\pm0.09^{c}_{A}$	$9.65\pm0.10^{c}_{A}$
3	C	$4.08\pm0.08^{a}_{C}$	$3.22\pm0.13^{a}_{C}$	$4.14\pm0.11^{a}_{C}$	$3.81\pm0.51^{a}_{C}$
	IAT	$7.18\pm0.08^{b}_{C}$	$7.16\pm0.17^{b}_{C}$	$8.16\pm0.18^{b}_{B}$	$7.50\pm0.57^{b}_{C}$
	RT	$9.06\pm0.15^{c}_{C}$	$8.70\pm0.16^{c}_{C}$	$8.88\pm0.25^{c}_{B}$	$8.71\pm0.45^{c}_{C}$
	IRT	$8.74\pm0.15^{c}_{B}$	$8.62\pm0.09^{c}_{B}$	$9.10\pm0.11^{c}_{B}$	$8.77\pm0.16^{c}_{B}$
4	C	$3.04\pm0.05^{a}_{D}$	$2.02\pm0.11^{a}_{D}$	$3.04\pm0.11^{a}_{D}$	$2.70\pm0.59^{a}_{D}$
	IAT	$6.12\pm0.13^{b}_{D}$	$6.10\pm0.10^{b}_{D}$	$7.22\pm0.13^{b}_{C}$	$6.48\pm0.64^{b}_{D}$
	RT	$8.18\pm0.11^{c}_{D}$	$8.24\pm0.19^{c}_{D}$	$8.56\pm0.19^{c}_{B}$	$8.32\pm0.49^{c}_{D}$
	IRT	$8.44\pm0.29^{c}_{B}$	$8.40\pm0.14^{c}_{B}$	$8.70\pm0.07^{c}_{C}$	$8.50\pm0.08^{c}_{B}$
5	C	$1.06\pm0.09^{a}_{E}$	$0.54\pm0.11^{a}_{E}$	$0.88\pm0.15^{a}_{E}$	$0.83\pm0.26^{a}_{E}$
	IAT	$5.04\pm0.15^{b}_{E}$	$5.26\pm0.11^{b}_{E}$	$6.30\pm0.21^{b}_{D}$	$5.53\pm0.67^{b}_{E}$
	RT	$7.14\pm0.11^{c}_{E}$	$7.28\pm0.13^{c}_{E}$	$7.26\pm0.11^{c}_{C}$	$7.23\pm0.08^{c}_{E}$
	IRT	$8.26\pm0.15^{d}_{B}$	$8.12\pm0.08^{c}_{C}$	$8.38\pm0.19^{c}_{D}$	$8.12\pm0.17^{d}_{C}$
6	IAT	$3.34\pm0.42^{a}_{F}$	$4.36\pm0.21^{a}_{F}$	$4.12\pm0.13^{a}_{E}$	$3.94\pm0.53^{a}_{F}$
	RT	$6.08\pm0.15^{b}_{F}$	$6.40\pm0.19^{b}_{F}$	$6.16\pm0.15^{b}_{D}$	$6.21\pm0.17^{b}_{F}$
	IRT	$8.04\pm0.32^{c}_{B}$	$7.92\pm0.29^{c}_{C}$	$7.80\pm0.08^{c}_{E}$	$7.82\pm0.12^{c}_{D}$
7	IAT	$2.98\pm0.08^{a}_{G}$	$3.22\pm0.16^{a}_{G}$	$3.16\pm0.09^{a}_{F}$	$3.12\pm0.12^{a}_{G}$
	RT	$5.14\pm0.22^{b}_{G}$	$5.00\pm0.19^{b}_{G}$	$4.90\pm0.07^{b}_{E}$	$5.02\pm0.11^{b}_{G}$
	IRT	$7.16\pm0.17^{c}_{D}$	$7.14\pm0.19^{c}_{D}$	$7.04\pm0.09^{c}_{F}$	$7.11\pm0.06^{c}_{E}$
8	IAT	$1.12\pm0.15^{a}_{H}$	$1.00\pm0.07^{a}_{H}$	$1.02\pm0.11^{a}_{G}$	$1.05\pm0.06^{a}_{H}$
	RT	$3.02\pm0.23^{b}_{H}$	$3.02\pm0.25^{b}_{H}$	$3.20\pm0.33^{b}_{F}$	$3.45\pm0.13^{b}_{H}$
	IRT	$6.78\pm0.33^{c}_{E}$	$7.04\pm0.15^{c}_{D}$	$6.84\pm0.23^{c}_{G}$	$6.89\pm0.14^{c}_{F}$
9	RT	$0.76\pm0.18^{a}_{I}$	$1.06\pm0.23^{a}_{I}$	$0.72\pm0.40^{a}_{H}$	$0.85\pm0.19^{a}_{I}$
	IRT	$6.34\pm0.15^{b}_{F}$	$6.50\pm0.19^{b}_{E}$	$6.58\pm0.16^{b}_{H}$	$6.47\pm0.13^{b}_{G}$
10	IRT	$5.98\pm0.31_{F}$	$6.20\pm0.31_{EF}$	$6.18\pm0.16_{I}$	$6.12\pm0.16_{H}$
11	IRT	$5.44\pm0.29_{FG}$	$5.92\pm0.29_{F}$	$5.56\pm0.10_{I}$	$5.81\pm0.10_{I}$
12	IRT	$4.90\pm0.23_{G}$	$4.96\pm0.27_{G}$	$5.04\pm0.11_{J}$	$4.97\pm0.07_{I}$
13	IRT	$3.94\pm0.21_{H}$	$3.92\pm0.08_{H}$	$3.78\pm0.27_{K}$	$3.88\pm0.09_{J}$

'4' is the limit for acceptability/unacceptability of the samples.

Different superscript small letters (a,b,c) represents statistical differences among groups ($p<0.05$). Different superscript capital letters (A,B,C,D,..) represents statistical differences amongst different days within the same group during storage ($p<0.05$).C: Control, IAT: Fish stored in ice at ambient temperature; RT: Refrigerated fish without ice; IRT: Fish stored in ice at refrigerator temperature.

The changes were also found to be significant between samples stored in ice at ambient temperature and the samples stored at refrigerated temperatures with and without ice, starting from the 2nd day of storage ($p<0.05$). The sensory scores of both groups kept at refrigerated temperatures were similar up to the 4th day, thereafter, the variations occurred. According to the overall sensory data, the control group sample got spoilt on the 3rd day, whereas using ice extended its shelf life for 3 more days. Refrigerated temperature without ice also supported sensory values having a shelf life of 7 days, although, a dryness on the surface of the fish occurred. The samples kept in ice at refrigerated temperature received the highest sensory scores compared to other samples with a shelf life of 12 days. This may be attributed to the stable as well as cold temperature of the fish under this condition.

In our previous study with fresh Atlantic bonito, we demonstrated that filleting can improve the sensory quality of fish for about 6 days if kept in ice and under refrigerated temperature (KORAL and KÖSE, 2012). We also demonstrated that sensory quality of shad can vary at refrigerated temperature depending on the initial quality of fish (KÖSE *et al*., 2000). DUYAR *et al*. (2012) investigated the quality changes of shad (*Alosa tanaica*) at refrigerated storage packed in stretch film without ice. According to sensory values, they obtained one day higher shelf life than present study for the shad samples kept at refrigerated temperature without ice. The difference can be explained in the protecting effect of using stretch film, since we obtained dryness on the surface of fish at open air in the refrigerator. XU *et al*. (2014) determined the effect of electrolyzed oxidizing water and chitosan on the microbiological, physico-chemical, and sensory attributes of American shad (*Alosa sapidissima*) during refrigerated storage (4±1°C) kept in air-proof polyethylene pouches. They showed that the shelf life of fish can further be improved by 9-10 days during refrigerated storage with this treatment.

The pH can be used as a spoilage indicator of fish and fish products (XU *et al*., 2014). The pH of fresh fish after death is usually reported as close to neutral and varies between 6.0 and 7.1 during post-mortem storage depending on the fish species, diet, season, type of muscle, and other factors (HUSS, 1988; XU *et al*., 2014). The pH of fresh shad was recorded as 6.8 and it has increased significantly throughout storage period for all groups ($p<0.05$) (Table 3). The pH values of control group were significantly higher than the experimental groups and reached 7.18 at the time of spoilage. Similar result was obtained on the 5th day for the group stored in ice at ambient temperature, while higher values were recorded for fish samples stored at refrigerated temperatures on the day when the fish was organoleptically unfit for consumption. Although, increase in pH values correlates well with the spoilage during storage, the values do not support sensory values. Although, different authors obtained lower pH values for fresh shad species, they likewise obtained similar trend for pH values during spoilage (KÖSE *et al*., 2000; DUYAR *et al*., 2012; XU *et al*., 2014).

TVB-N has been used as a quality indicator of fish products (HUSS 1988; CONNELL 1990). It includes ammonia; primary, secondary, and tertiary amines; and products of protein breakdown. An increase in TVB-N is attributed to an increase in the activity of endogenous enzymes and bacterial growth (XU *et al*., 2014). The European Union sets varying TVB-N limits as 25-35 mg/100 g for unprocessed fishery products that is regarded as unfit for human consumption, where organoleptic assessment has raised doubts as to their freshness (EU DIRECTIVE 2005 and 2008). The changes in the TVB-N values during storage are shown in Table 4. The values increased significantly with the increasing storage time for all groups ($p<0.05$). This increase may be attributed to the production of ammonia. Significant differences were also observed between control group and the experimental groups beginning from the 1st day of storage ($p<0.05$). TVB-N value of the control group was close to the suggested limit on the 3rd day of storage, when the fish

became organoleptically unfit for consumption. The levels reached 82.6 mg/100g on the 4th day. However, TVB-N values did not support sensory results obtained for the experimental groups and the levels reached unacceptable limit on the 6th and 8th day of storage for samples stored in refrigerator and ice at ambient temperature, respectively. TVB-N values of the samples stored in ice at refrigerated temperature were found within the acceptable quality throughout the storage. This result confirms our earlier findings with fresh bonito on the beneficiary effect of using ice during refrigerated storage (KORAL and KÖSE, 2012). Previous studies also demonstrated that filleting and addition of electrolyzed, oxidized water and chitosan can retard the development of TVB-N in fish (KÖSE and KORAL, 2012; XU *et al*., 2014). However, higher TVB-N values were found by KÖSE *et al*. (2000) with shad species kept at refrigerated temperature and packed in stretch film without ice. The higher values can be explained by the sampling season, being carried out in summer in previous study, while sampling was done in winter in the current study, since the water temperature can affect the initial microbial load and variations in bacterial species, leading to differences in TVB-N formation. DUYAR *et al*. (2012) also obtained higher TVB-N values for *A. Tanaica,* which was kept in refrigerated temperature without ice application. However, they did not state the sampling time and the initial conditions of fish before storage. Therefore, we assumed that the initial conditions of fish may be the reason for differences in the results.

Trimethylamine 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, its 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). Initial TMA-N values was 0.28 mg/100g and it significantly increased throughout the storage period for all experimental groups (Table 4). Significant variations were observed between control and other groups, beginning from the 1st day of storage, and also within the groups ($p<0.05$). The lowest TMA-N values were found in the group stored in ice at refrigerated temperature, while the highest levels were received by the control group. The values were within the acceptable levels for this parameter throughout the storage period. Therefore, TMA values did not support the sensory results for this species. The results were in agreement with our previous findings for fresh bonito stored under refrigerated conditions (KORAL and KÖSE, 2012).

TBA is a by-product of lipid oxidation. Therefore, it is a common method for assessing lipid oxidation in fish products (XU *et al*., 2014). The TBA value for a good quality chilled fish is reported to be between 5 and 8 mg malonaldehyde/kg, while the levels of 8 mg malonaldehyde/kg fish flesh are generally regarded as the limit of acceptability for most fish species (SCHORMÜLLER, 1969). The values of TBA increased significantly depending on time, and significant variations were also observed between the control group and the other groups ($p<0.05$), beginning from the 1st day of storage (Table 4). Significant differences were observed within the experimental groups beginning from the 2nd day of storage. The control group reached an unacceptable limit on the 4th day, while fish stored at refrigerated temperature without ice got spoilt on the 5th day. The sample kept in ice and stored at ambient temperature had a slightly favourable condition and TBA value reached an unacceptable level on the 7th day. Fish kept in ice and in refrigerated conditions had the lowest values that were within the acceptable quality throughout the storage period. These results suggest that storage of fish in ice should also be applied in refrigerated/cold storage conditions to avoid lipid oxidation. Cold storage without ice application is not suitable to retard oxidation. TBA values were only in support of control group in terms of its overall acceptability of sensory values. Lower values were recorded from previous

studies for bonito and Indian shad during refrigerated storage (KÖSE *et al*., 2000; KÖSE and KORAL, 2012; MASSA *et al*., 2012; XU *et al*., 2014). The differences in TBA values might be attributed to the fat content in different fish species. The results of DUYAR *et al*. (2012) supported our findings for TBA results.

The results of the total mesophilic and psychrophilic viable bacteria and HFB are shown in Table 5. Initial counts of mesophilic and psychrophilic total viable bacteria, and the total mesophilic and psychrophilic HFB of fresh fish were observed as 1.95, 2.18, 2.15 and 2.45 log cfu/g, respectively. Significant differences were found amongst the groups along with the increasing levels throughout the storage with some exceptions ($p<0.05$). Storage in ice and under refrigerated conditions significantly decreased the total mesophilic viable bacteria and HFB load of the samples in the first day of storage, then significant increase occurred in the counts during further storage ($p<0.05$). The lowest mesophilic counts were obtained for samples containing ice at refrigerated temperatures within a variation of 1.30-3.18 log cfu/g.

Table 3: The changes in pH values of spotless shad groups stored under various conditions.

Day of storage	C	IAT	RT	IRT
Fresh	6.80±0.08	6.80±0.08	6.80±0.08	6.80±0.08
1	$7.02\pm0.02^{a}_{A}$	$6.88\pm0.03^{b}_{A}$	$6.87\pm0.04^{b}_{A}$	$6.86\pm0.05^{b}_{A}$
2	$7.14\pm0.03^{a}_{B}$	$6.90\pm0.06^{b}_{A}$	$6.97\pm0.02^{b}_{B}$	$6.88\pm0.03^{b}_{A}$
3	$7.18\pm0.09^{a}_{C}$	$6.94\pm0.04^{b}_{A}$	$7.07\pm0.04^{c}_{C}$	$6.90\pm0.03^{b}_{A}$
4	$7.37\pm0.02^{a}_{D}$	$7.02\pm0.03^{b}_{B}$	$7.17\pm0.02^{c}_{D}$	$6.94\pm0.04^{d}_{A}$
5	$7.64\pm0.02^{a}_{E}$	$7.08\pm0.06^{b}_{B}$	$7.21\pm0.03^{c}_{E}$	$6.98\pm0.04^{d}_{A}$
6	*	$7.12\pm0.03^{a}_{BC}$	$7.26\pm0.03^{b}_{E}$	$6.99\pm0.04^{c}_{A}$
7	*	$7.18\pm0.04^{a}_{C}$	$7.38\pm0.02^{b}_{F}$	$7.00\pm0.02^{c}_{A}$
8	*	$7.24\pm0.08^{a}_{C}$	$7.50\pm0.09^{b}_{G}$	$7.05\pm0.01^{c}_{B}$
9	*	*	$7.40\pm0.07^{a}_{D}$	$7.09\pm0.02^{b}_{C}$
10	*	*	*	$7.18\pm0.03_{D}$
11	*	*	*	$7.23\pm0.04_{D}$
12	*	*	*	$7.26\pm0.05_{D}$
13	*	*	*	$7.34\pm0.06_{E}$

*: Not Analyzed, Different superscript small letters (a,b,c) represents statistical differences among groups ($p<0.05$). Different superscript capital letters (A,B,C,D,…) represents statistical differences amongst different days within the same group during storage ($p<0.05$).C: Control, IAT: Fish stored in ice at ambient temperature; RT: Refrigerated fish without ice; IRT: Fish stored in ice at refrigerator temperature.

Table 4: The changes in the levels of chemical quality parameters of spotless shad groups stored at various conditions.

Day of storage	Sample type	Chemical Criteria		
		TVB-N (mg/100 g)	TMA (mg/100 g)	TBA (mg MDA/ kg)
0	Fresh	9.11±0.30	0.28±0.13	0.46±0.28
1	C	$14.71\pm0.28^{a}_{A}$	$1.36\pm0.06^{a}_{A}$	$0.83\pm0.10^{a}_{A}$
	IAT	$11.21\pm0.28^{b}_{A}$	$1.08\pm0.18^{b}_{A}$	$0.57\pm0.05^{b}_{A}$
	RT	$12.61\pm0.15^{b}_{A}$	$0.53\pm0.04^{c}_{A}$	$0.63\pm0.08^{b}_{A}$
	IRT	$10.51\pm0.16^{c}_{A}$	$0.39\pm0.08^{d}_{A}$	$0.47\pm0.09^{b}_{A}$
2	C	$21.01\pm0.16^{a}_{B}$	$2.58\pm0.10^{a}_{B}$	$1.68\pm0.06^{a}_{B}$
	IAT	$14.85\pm0.34^{b}_{B}$	$1.78\pm0.05^{b}_{B}$	$1.09\pm0.08^{b}_{B}$
	RT	$14.48\pm0.26^{b}_{B}$	$1.01\pm0.08^{c}_{B}$	$1.47\pm0.09^{c}_{B}$
	IRT	$11.91\pm0.15^{c}_{A}$	$0.65\pm0.13^{d}_{B}$	$0.63\pm0.02^{d}_{B}$
3	C	$28.72\pm0.18^{a}_{C}$	$2.94\pm0.08^{a}_{C}$	$4.62\pm0.23^{a}_{C}$
	IAT	$14.71\pm0.22^{b}_{B}$	$2.08\pm0.16^{b}_{C}$	$2.06\pm0.17^{b}_{C}$
	RT	$17.02\pm0.20^{c}_{C}$	$1.33\pm0.19^{c}_{C}$	$3.55\pm0.05^{c}_{C}$
	IRT	$13.31\pm0.12^{b}_{B}$	$0.77\pm0.08^{d}_{B}$	$0.90\pm0.14^{d}_{C}$
4	C	$82.65\pm0.21^{a}_{D}$	$3.86\pm0.18^{a}_{D}$	$9.98\pm0.16^{a}_{D}$
	IAT	$17.51\pm0.10^{b}_{C}$	$2.98\pm0.06^{b}_{D}$	$4.86\pm0.12^{b}_{D}$
	RT	$21.18\pm0.20^{c}_{D}$	$2.42\pm0.03^{c}_{D}$	$7.13\pm0.07^{c}_{D}$
	IRT	$14.01\pm0.12^{d}_{B}$	$0.86\pm0.14^{d}_{C}$	$1.18\pm0.10^{d}_{D}$
5	C	$119.07\pm0.16^{a}_{E}$	$5.89\pm0.21^{a}_{E}$	$14.31\pm0.08^{a}_{E}$
	IAT	$18.21\pm0.23^{b}_{D}$	$3.86\pm0.07^{b}_{E}$	$6.14\pm0.18^{b}_{E}$
	RT	$24.86\pm0.15^{c}_{E}$	$3.25\pm0.18^{c}_{E}$	$11.15\pm0.07^{c}_{E}$
	IRT	$16.11\pm0.10^{d}_{C}$	$1.01\pm0.09^{d}_{D}$	$1.38\pm0.16^{d}_{E}$
6	IAT	$23.81\pm0.14^{a}_{E}$	$5.14\pm0.22^{a}_{F}$	$7.67\pm0.03^{a}_{F}$
	RT	$29.40\pm0.20^{b}_{F}$	$4.67\pm0.12^{b}_{F}$	$13.24\pm0.05^{b}_{F}$
	IRT	$17.51\pm0.26^{c}_{D}$	$1.25\pm0.14^{c}_{E}$	$2.02\pm0.16^{c}_{F}$
7	IAT	$26.62\pm0.28^{a}_{F}$	$7.68\pm0.18^{a}_{G}$	$10.09\pm0.18^{a}_{G}$
	RT	$33.26\pm0.20^{b}_{G}$	$5.49\pm0.09^{b}_{G}$	$15.35\pm0.19^{b}_{G}$
	IRT	$20.31\pm0.17^{c}_{E}$	$1.40\pm0.03^{c}_{F}$	$2.46\pm0.16^{c}_{G}$
8	IAT	$32.92\pm0.15^{a}_{G}$	$8.12\pm0.07^{a}_{H}$	$11.72\pm0.17^{a}_{H}$
	RT	$42.82\pm0.20^{b}_{H}$	$6.99\pm0.08^{b}_{H}$	$18.36\pm0.25^{b}_{H}$
	IRT	$21.71\pm0.22^{c}_{E}$	$1.57\pm0.07^{c}_{G}$	$3.06\pm0.14^{c}_{H}$
9	RT	$37.82\pm0.22^{a}_{H}$	$9.28\pm0.16^{a}_{I}$	$13.19\pm0.28^{a}_{I}$
	IRT	$22.41\pm0.12^{b}_{F}$	$1.71\pm0.10^{b}_{H}$	$3.86\pm0.18^{b}_{I}$
10	IRT	$24.51\pm0.30_{G}$	$1.86\pm0.08_{H}$	$4.98\pm0.20_{I}$
11	IRT	$25.21\pm0.28_{G}$	$1.81\pm0.14_{H}$	$5.55\pm0.55_{i}$
12	IRT	$25.91\pm0.16_{G}$	$2.03\pm0.12_{I}$	$6.14\pm0.14_{J}$
13	IRT	$26.62\pm0.34_{G}$	$2.42\pm0.10_{I}$	$6.82\pm0.18_{K}$

Different superscript small letters (a,b,c) represents statistical differences among groups ($p<0.05$). Different superscript capital letters (A,B,C,D,…) represents statistical differences amongst different days within the same group during storage ($p<0.05$).C: Control, IAT: Fish stored in ice at ambient temperature; RT: Refrigerated fish without ice; IRT: Fish stored in ice at refrigerator temperature. TVB-N: Total volatile basic nitrogen, TBA: Thiobarbituric acid value, TMA-N: Trimethylamine nitrogen.

The recommended limit for Total Viable bacteria Counts (TVC) for fresh fish consumption is reported to be between 6-7 log cfu/g (ICMSF 1992; CHOTIMARKORN, 2014). The results of this study suggest that although, mesophiles reached the unacceptable level on the 3rd day of storage for the control group, psychrophilic bacteria counts were still within an acceptable level for further storage, possibly due to unfavourable conditions. Better quality was obtained from the samples kept in ice at ambient conditions in comparison with samples stored without ice at refrigerated conditions, in terms of bacteria growth, indicating the washing effect of ice during melting at ambient conditions. TVC exceeded the limit for psychrophilic bacteria of the samples stored under refrigerated conditions without ice on the 7th day, while samples with ice at ambient conditions were still acceptable up to the 9th day. However, the best quality was found for shad which was kept in ice and under refrigerated conditions, the TVC was still within the acceptable level throughout the storage period. Sensory results supported TVC for control group and the bacteria load are still under the suggested limit for other groups within their acceptable sensory quality. With the exception of TVB-N, the results of mesophilic and psychrophilic viable counts did not support chemical quality parameters. UDDIN *et al*. (2001) observed a higher bacteria load from the muscle of Indian shad (*Tenualosa ilisha*) stored in ice, and placed in insulated polystyrene and wooden boxes. They also reported higher counts for fish stored in wooden boxes than fish stored in the initial. The total bacterial count in the muscle of the fish stored in insulated boxes was 3.5 x 10^6 cfu/g and it increased to 1.8 x 10^9cfu/g after 18 days, when the fish became organoleptically unfit for consumption. They also reported that the bacterial flora of the fishes stored in wooden boxes showed comparatively higher initial bacteria counts and increased rapidly during storage. No study exists on the microbial changes of fresh, spotless shad, either at ambient or chilled conditions. Therefore, this study presents new data on the possible microbial activities during storage of this species under different conditions.

Initial levels of HFB in fish is important since previously formed histamine decarboxylases, are likely to continue to decarboxylase histidine to histamine, even when histamine decarboxylase positive bacteria are no longer viable (KÖSE, 2010). In this study, initial HFB counts were 2.15 log cfu/g for mesophiles and 2.45 log cfu/g for psychrophilic bacteria. The counts also increased significantly throughout the storage period with the exception of mesophiles, which significantly dropped on the 1st day for samples stored in ice at ambient temperatures and without ice at refrigerated temperatures ($p<0.05$). Significant variations were observed between control group and other groups as well as within the experimental groups ($p<0.05$). The highest HFB counts was recorded for control group, while the lowest were observed in samples stored in ice at refrigerated temperatures.

The results of BAs contents are shown in Table 6. Biogenic amines contents varied significantly depending on storage life and the groups, with some exceptions ($p<0.05$). Histamine is the only BAs that is regulated for fish and fisheries products. The European Regulation (EU) permits up to 100 mg/kg for fresh and processed fish, and 200 mg/kg for fishery products which have undergone enzyme maturation treatment in brine (EU DIRECTIVE, 2005), while FDA allows for less than 50 mg/kg (FDA, 2011). Shad is listed among the regulated fish species due to its high histidine content (FDA, 2011). Histamine contents in samples representing control and in all experimental groups, were under the detection limit at the beginning of the storage trial and the 1st day of storage, and significantly increased throughout storage ($p<0.05$). Detectable levels started on the 2nd day for control group as 7.41 mg/kg and the values reached an unacceptable level set by the FDA on the 5th day. Detectable values of histamine were obtained on the 3rd day for the samples stored in ice at ambient temperature and without ice at refrigerated temperatures as 4.93 mg/kg and 1.50 mg/kg, respectively.

Table 5: Microbial changes of the shad groups stored under various conditions.

Day of storage	Sample type	Total Viable Bacteria (log cfu/g)		Total Histamine Forming Bacteria (log cfu/g)	
		Mesophiles	Psychrophilic	Mesophiles	Psychrophilic
0	Fresh	1.95±0.07	2.18±0.10	2.15±0.23	2.45±0.13
1	C	$3.12\pm0.14^{a}{}_{A}$	$2.20\pm0.09^{a}{}_{A}$	$3.36\pm0.12^{a}{}_{A}$	$2.74\pm0.04^{a}{}_{A}$
	IAT	$2.95\pm0.10^{a}{}_{A}$	$2.76\pm0.11^{b}{}_{A}$	$1.95\pm0.15^{b}{}_{A}$	$2.57\pm0.07^{b}{}_{A}$
	RT	$1.60\pm0.08^{b}{}_{A}$	$2.43\pm0.12^{a}{}_{A}$	<1.47	$2.72\pm0.16^{a}{}_{A}$
	IRT	<1.47	$2.72\pm0.07^{b}{}_{A}$	$2.36\pm0.17^{d}{}_{A}$	$2.30\pm0.15^{b}{}_{A}$
2	C	$5.22\pm0.13^{a}{}_{B}$	$2.24\pm0.10^{a}{}_{A}$	$4.41\pm0.23^{a}{}_{B}$	$2.98\pm0.10^{a}{}_{B}$
	IAT	$3.27\pm0.15^{b}{}_{B}$	$3.04\pm0.12^{b}{}_{B}$	$2.81\pm0.09^{b}{}_{A}$	$2.85\pm0.13^{a}{}_{B}$
	RT	$1.90\pm0.12^{c}{}_{B}$	$3.44\pm0.07^{c}{}_{B}$	$1.60\pm0.12^{c}{}_{B}$	$4.31\pm0.15^{b}{}_{B}$
	IRT	$1.60\pm0.05^{d}{}_{B}$	$2.61\pm0.20^{d}{}_{B}$	$1.78\pm0.08^{c}{}_{B}$	$2.18\pm0.02^{c}{}_{A}$
3	C	$6.34\pm0.25^{a}{}_{C}$	$2.26\pm0.04^{a}{}_{A}$	$5.18\pm0.13^{a}{}_{C}$	$3.10\pm0.09^{a}{}_{B}$
	IAT	$3.45\pm0.07^{b}{}_{C}$	$3.00\pm0.28^{b}{}_{B}$	$3.73\pm0.11^{b}{}_{B}$	$3.30\pm0.21^{a}{}_{C}$
	RT	$2.30\pm0.10^{c}{}_{C}$	$3.85\pm0.17^{c}{}_{C}$	$2.23\pm0.14^{c}{}_{C}$	$4.28\pm0.01^{b}{}_{B}$
	IRT	$1.85\pm0.03^{d}{}_{C}$	$2.68\pm0.12^{d}{}_{B}$	$1.95\pm0.10^{d}{}_{B}$	$2.89\pm0.12^{c}{}_{B}$
4	C	$7.26\pm0.29^{a}{}_{D}$	$2.43\pm0.12^{a}{}_{B}$	$5.58\pm0.27^{a}{}_{D}$	$3.32\pm0.14^{a}{}_{C}$
	IAT	$3.95\pm0.10^{b}{}_{D}$	$3.30\pm0.21^{b}{}_{C}$	$3.40\pm0.07^{b}{}_{C}$	$3.45\pm0.13^{a}{}_{C}$
	RT	$4.19\pm0.13^{c}{}_{D}$	$5.21\pm0.12^{c}{}_{D}$	$3.57\pm0.09^{b}{}_{D}$	$5.36\pm0.09^{b}{}_{C}$
	IRT	$2.20\pm0.09^{d}{}_{D}$	$2.80\pm0.06^{d}{}_{A}$	$2.48\pm0.23^{c}{}_{A}$	$2.93\pm0.12^{c}{}_{B}$
5	C	$9.43\pm0.21^{a}{}_{E}$	$2.38\pm0.04^{a}{}_{B}$	$6.23\pm0.17^{a}{}_{E}$	$3.40\pm0.18^{a}{}_{C}$
	IAT	$4.12\pm0.17^{b}{}_{D}$	$3.54\pm0.13^{b}{}_{D}$	$3.60\pm0.05^{b}{}_{B}$	$3.69\pm0.12^{a}{}_{D}$
	RT	$4.24\pm0.22^{b}{}_{D}$	$5.48\pm0.19^{c}{}_{E}$	$3.83\pm0.10^{b}{}_{E}$	$5.60\pm0.13^{b}{}_{D}$
	IRT	$3.02\pm0.18^{c}{}_{E}$	$2.93\pm0.04^{d}{}_{C}$	$2.57\pm0.20^{c}{}_{A}$	$3.19\pm0.06^{c}{}_{B}$
6	IAT	$4.42\pm0.05^{a}{}_{E}$	$3.70\pm0.09^{a}{}_{E}$	$3.85\pm0.14^{a}{}_{B}$	$4.26\pm0.30^{a}{}_{E}$
	RT	$5.04\pm0.17^{b}{}_{E}$	$6.30\pm0.32^{b}{}_{F}$	$3.60\pm0.13^{a}{}_{D}$	$5.90\pm0.15^{b}{}_{E}$
	IRT	$2.91\pm0.02^{c}{}_{E}$	$3.04\pm0.09^{c}{}_{C}$	$2.86\pm0.17^{b}{}_{C}$	$3.30\pm0.05^{b}{}_{C}$
7	IAT	$4.82\pm0.13^{a}{}_{F}$	$4.19\pm0.15^{a}{}_{F}$	$3.93\pm0.15^{a}{}_{B}$	$4.39\pm0.17^{a}{}_{E}$
	RT	$5.28\pm0.07^{b}{}_{F}$	$7.39\pm0.06^{b}{}_{G}$	$3.78\pm0.12^{a}{}_{E}$	$6.69\pm0.01^{b}{}_{F}$
	IRT	$2.48\pm0.11^{c}{}_{D}$	$2.77\pm0.07^{c}{}_{A}$	$2.48\pm0.16^{b}{}_{A}$	$3.37\pm0.15^{c}{}_{C}$
8	IAT	$5.60\pm0.06^{a}{}_{G}$	$4.24\pm0.11^{a}{}_{F}$	$4.20\pm0.29^{a}{}_{D}$	$5.48\pm0.04^{a}{}_{F}$
	RT	$5.85\pm0.09^{b}{}_{G}$	$7.85\pm0.13^{b}{}_{H}$	$4.40\pm0.08^{a}{}_{F}$	$6.48\pm0.09^{b}{}_{G}$
	IRT	$2.70\pm0.12^{c}{}_{D}$	$2.82\pm0.08^{c}{}_{A}$	$2.00\pm0.17^{b}{}_{B}$	$3.54\pm0.08^{c}{}_{D}$
9	IAT	$6.94\pm0.30^{a}{}_{H}$	$4.46\pm0.10^{a}{}_{G}$	$4.18\pm0.05^{a}{}_{D}$	$6.18\pm0.12^{a}{}_{G}$
	IRT	$2.95\pm0.11^{b}{}_{E}$	$3.11\pm0.10^{b}{}_{C}$	$2.30\pm0.12^{b}{}_{A}$	$3.27\pm0.06^{b}{}_{C}$
10	IRT	$2.90\pm0.08_{E}$	$2.98\pm0.11_{C}$	$3.11\pm0.09_{D}$	$3.59\pm0.08_{D}$
11	IRT	$3.18\pm0.07_{F}$	$2.89\pm0.06_{A}$	$3.30\pm0.07_{E}$	$3.79\pm0.10_{E}$
12	IRT	$3.10\pm0.10_{F}$	$3.04\pm0.03_{C}$	$3.18\pm0.05_{D}$	$3.28\pm0.09_{C}$
13	IRT	$3.00\pm0.11_{E}$	$3.29\pm0.14_{C}$	$3.23\pm0.16_{E}$	$3.48\pm0.07_{D}$

Different superscript small letters (a,b,c) represents statistical differences among groups ($p<0.05$). Different superscript capital letters (A,B,C,D,…) represents statistical differences amongst different days within the same group during storage ($p<0.05$).C: Control, IAT: Fish stored in ice at ambient temperature; RT: Refrigerated fish without ice; IRT: Fish stored in ice at refrigerator temperature.

According to FDA, the values were unacceptable on the 8th and 9th day, for samples stored at refrigerated temperature without ice and ambient temperature in ice, respectively. The lowest histamine values were recorded for the samples stored in ice at refrigerated temperature. The detectable levels were obtained for this group on the 7th day, and the values were under 10 mg/kg throughout the storage period. Therefore, this condition is found to be the most suitable method used to store fresh shad in order to avoid histamine health risk. Histamine values were well correlated with sensory and chemical quality parameters in terms of food safety. AL-BULUSHI *at al*. (2009) reported that mesophilic bacterial count of log 6–7 cfu/g has been associated with 50 mg/kg histamine. The suggested level reached by the control group on the 3rd day. On the other hand, the results of mesophilic HFB count on the 5th day were correlated with the amount of histamine obtained on the relating day for control group. The results of mesophilic bacteria counts obtained for samples stored in ice at ambient temperature supported the statement of AL-BULISHI *at al.* (2009), while the counts of psychrophilic total viable bacteria and HFB were in agreement with this statement, for the group which stored in refrigerator without ice, indicating a favourable temperature for such bacteria. Since both bacteria counts and histamine values were well below the permitted levels for the group stored in ice and at refrigerated temperature, the results gotten from this group also supports the above statement.

Although, significant variations were obtained for tryptamine levels of some groups on certain days, the values were found insignificant amongst the groups. Tryptamine values were observed to be below 7 mg/kg throughout the storage. The values of phenylethylamine decreased at the beginning of storage, thereafter, fluctuations in the levels occurred. Putrescine value of fresh fish was 0.73 mg/kg at the beginning of trial, then, the levels increased sharply up to 72.5 mg/kg for control group. Although, the putrescine levels of other groups also increased significantly, the rate was significantly slower for these groups, with the lowest values being at 2.49 mg/kg, representing the samples kept in ice at refrigerated temperature at the end of the storage period. Higher values were observed for cadaverine with the highest levels corresponding to control group. The values reached up to 166.69 mg/kg for control group on the 5th day, while the levels were 56 mg/kg and 120.02 mg/kg on the 8th and 9th day, for groups stored in ice at ambient temperature and without ice at refrigerated temperature, respectively. Fig 1. shows the samples stored in ice at refrigerated temperature which also contained the lowest cadaverine value and the result was 25.18 mg/kg at the end of storage.

Tyramine is a naturally occurring monoamine compound derived from the amino acid, tyrosine. Fresh fish contains little or no tyramine, but a large amount can be found in spoiled or fermented fish (FAO, 2013). Tyramine values also increased significantly during storage ($p<0.05$) with the highest values representing the control group at 54.63 mg/kg on the 4th day. The values of other groups were significantly lower in comparison to control group ($p<0.05$). The lowest values were attributed to the samples stored at refrigerated temperatures. Spermidine values of fresh fish was 12.32 mg/kg, and the levels decreased significantly in all groups throughout storage ($p<0.05$). Significant variations were also observed amongst the groups ($p<0.05$). The changes in the spermine values are usually insignificant up to the 6th day of storage with some exceptions, thereafter, fluctuations in the values were observed. The highest spermine level was determined to be 4.70mg/kg on the 7th day for the sample stored in ice at ambient temperature.

Biogenic amines in foods are reported to be indicators of freshness or spoilage in fish. Moreover, some migraines are known to BAs, particularly, tyramine and phenylethylamine. Although, tyramine might also act as a histamine potentiator (TAYLOR and LIEBER, 1979), the contribution of this biogenic amine to histamine poisoning is not clear. However, around 100-800 mg/kg of tyramine and 30 mg/kg of

phenylethylamine have been reported to be toxic doses in foods (KORAL and KÖSE 2012). Our study showed that the levels of tyramine and phenylethylamine did not reach toxic levels within the storage period of this research

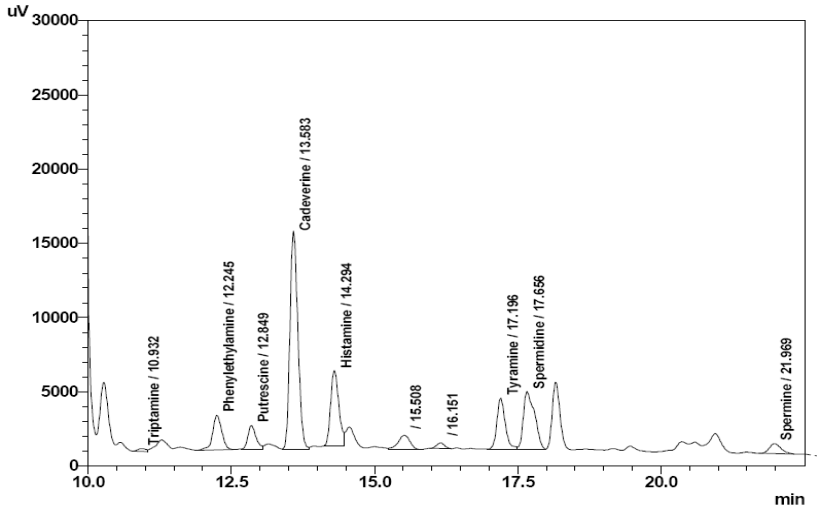


Fig. 1: Chromatogram belongs to shad samples (IRT group, 13th days).

Table 6: The values of biogenic amines for the shad samples stored under various conditions.

Day of Storage	Sample Groups	Results of Biogenic amines (mg/kg)							
		Tryptamine	Phenylethylamine	Putrescine	Cadeverine	Histamine	Tyramine	Spermidine	Spermine
0	Fresh	5.60±0.21	18.21±0.26	0.73±0.13	2.80±0.08	<0.86*	2.12±0.19	12.32±0.11	2.49±0.23
1	C	$5.04±0.62^{a}_{A}$	$17.11±0.44^{a}_{A}$	$1.32±0.16^{a}_{A}$	$4.28±0.37^{a}_{A}$	<0.86*	$1.83±0.21^{a}_{A}$	$9.34±0.20^{a}_{A}$	$2.37±0.18^{a}_{A}$
	IAT	$6.45±0.50^{b}_{A}$	$15.82±0.54^{b}_{A}$	$1.37±0.14^{a}_{A}$	$4.82±0.23^{b}_{A}$	<0.86*	$2.10±0.15^{a}_{A}$	$11.70±0.42^{b}_{A}$	$2.67±0.10^{a}_{A}$
	RT	$5.98±0.31^{b}_{A}$	$16.07±0.35^{b}_{A}$	$0.95±0.12^{b}_{A}$	$5.35±0.29^{c}_{A}$	<0.86*	$1.95±0.21^{a}_{A}$	$10.52±0.34^{a}_{A}$	$3.34±0.19^{b}_{A}$
	IRT	$6.02±0.03^{b}_{A}$	$17.62±0.05^{a}_{A}$	$0.78±0.04^{c}_{A}$	$3.40±0.14^{d}_{A}$	<0.86*	$2.01±0.01^{a}_{A}$	$12.28±0.10^{c}_{A}$	$3.65±0.02^{b}_{A}$
2	C	$5.13±0.23^{a}_{A}$	$8,74±0,21^{a}_{B}$	$6,83±0,22^{a}_{B}$	$31,44±0,80^{a}_{B}$	$7.41±0.15_{A}$	$6,97±0,13^{a}_{B}$	$5,29±0,10^{a}_{B}$	$2,73±0,18^{a}_{A}$
	IAT	$5.43±0.18^{a}_{B}$	$16,83±0,39^{b}_{B}$	$2,94±0,24^{b}_{B}$	$15,60±0,71^{b}_{B}$	<0.86*	$4,74±0,22^{b}_{B}$	$9,75±0,36^{b}_{B}$	$2,67±0,31^{a}_{A}$
	RT	$5.85±0.37^{a}_{A}$	$17,75±0,49^{b}_{B}$	$1,66±0,11^{c}_{B}$	$9,25±0,37^{c}_{B}$	<0.86*	$2,82±0,20^{c}_{B}$	$9,74±0,35^{b}_{A}$	$3,08±0,11^{b}_{A}$
	IRT	$5.35±0.49^{a}_{B}$	$18,86±0,42^{c}_{B}$	$2,38±0,11^{d}_{B}$	$6,57±0,30^{d}_{B}$	<0.86*	$1,96±0,11^{d}_{A}$	$11,06±0,52^{c}_{A}$	$3,32±0,40^{b}_{A}$
3	C	$5.15±0.21^{a}_{A}$	$8,87±0,27^{a}_{B}$	$12,76±0,65^{a}_{C}$	$42,25±0,93^{a}_{C}$	$15.88±0.60^{a}_{B}$	$14,31±0,83^{a}_{C}$	$4,11±0,28^{a}_{C}$	$2,36±0,11^{a}_{A}$
	IAT	$4.93±0.32^{a}_{C}$	$12,59±1,00^{b}_{C}$	$8,89±0,44^{b}_{C}$	$35,70±0,71^{b}_{C}$	$4.93±0.13^{b}_{A}$	$6,67±0,47^{b}_{C}$	$6,18±0,28^{b}_{C}$	$1,81±0,11^{b}_{B}$
	RT	$5.73±0.38^{b}_{A}$	$18,06±0,77^{c}_{B}$	$1,70±0,11^{c}_{B}$	$7,59±0,52^{c}_{C}$	$1.55±0.11^{c}_{A}$	$3,27±0,16^{c}_{C}$	$9,30±0,44^{c}_{A}$	$2,85±0,21^{c}_{A}$
	IRT	$5.71±0.42^{b}_{B}$	$17,25±0,64^{c}_{A}$	$0,92±0,10^{d}_{A}$	$4,82±0,28^{d}_{C}$	<0.86*	$2,02±0,15^{d}_{A}$	$8,13±0,25^{d}_{B}$	$3,34±0,30^{d}_{A}$
4	C	$5.48±0.25^{a}_{A}$	$8,10±0,28^{a}_{B}$	$55.91±1,32^{a}_{D}$	$126.32±1,38^{a}_{D}$	$35.86±0.64^{a}_{C}$	$54,63±0,89^{a}_{D}$	$4,12±0,30^{a}_{C}$	$2,83±0,25^{a}_{A}$
	IAT	$4.91±0.13^{b}_{C}$	$12,20±0,40^{b}_{C}$	$3,94±0,11^{b}_{D}$	$25,83±0,86^{b}_{D}$	$8.26±0.60^{b}_{B}$	$6,57±0,33^{b}_{C}$	$6,35±0,37^{b}_{C}$	$3,10±0,14^{a}_{C}$
	RT	$5.17±0.23^{a}_{A}$	$16,90±0,28^{c}_{A}$	$2,70±0,12^{c}_{C}$	$10,41±0,61^{c}_{B}$	$1.88±0.14^{c}_{A}$	$3,16±0,28^{c}_{C}$	$7,53±0,32^{c}_{B}$	$2,92±0,16^{a}_{A}$
	IRT	$5.18±0.29^{a}_{B}$	$17,54±0,61^{c}_{A}$	$1,69±0,18^{d}_{C}$	$8,13±0,24^{d}_{D}$	<0.86*	$2,76±0,14^{c}_{B}$	$8,25±0,42^{d}_{B}$	$2,37±0,12^{b}_{B}$
5	C	$5.38±0.42^{a}_{A}$	$9,85±0,35^{a}_{C}$	$72,57±0,98^{a}_{E}$	$166,69±1,81^{a}_{E}$	$48.82±1.68^{a}_{D}$	$46,31±0,98^{a}_{E}$	$3,39±0,25^{a}_{D}$	$2,27±0,15^{a}_{A}$
	IAT	$4.92±0.47^{a}_{C}$	$16,04±0,80^{b}_{B}$	$4,36±0,29^{b}_{E}$	$27,80±0,71^{b}_{E}$	$16.88±0.25^{b}_{C}$	$6,37±0,36^{b}_{C}$	$7,65±0,40^{b}_{D}$	$2,44±0,30^{a}_{A}$
	RT	$5.25±0.34^{a}_{A}$	$16,76±0,39^{b}_{A}$	$3,36±0,21^{c}_{D}$	$12,70±0,57^{c}_{D}$	$2.59±0.18^{c}_{B}$	$3,16±0,25^{c}_{C}$	$8,50±0,72^{b}_{B}$	$2,64±0,31^{a}_{A}$
	IRT	$5.10±0.20^{a}_{B}$	$15,31±0,63^{b}_{C}$	$3,52±0,47^{c}_{D}$	$19,35±0,94^{d}_{E}$	<0.86*	$3,67±0,43^{c}_{C}$	$6,87±0,25^{b}_{C}$	$2,62±0,33^{a}_{B}$

Different superscript small letters (a,b,c) represents statistical differences among groups ($p<0.05$). Different superscript capital letters (A,B,C,D,..) represents statistical differences amongst different days within the same group during storage ($p<0.05$).C: Control, IAT: Fish stored in ice at ambient temperature; RT: Refrigerated fish without ice; IRT: Fish stored in ice at refrigerator temperature*: LOD(Limit of detection for histamine). (LOD values for histamine:0.86; for putrescine:0.56; for tryptamine: 1.80; Phenylethylamine: 1.50; Cadeverine: 0.66; Tyramine: 0.87; Spermidine: 0.65; Spermine: 0.71 mg/kg.).

Limited studies exist for the biogenic amine contents of spotless shad. In our previous studies on salted shad products, obtained from Turkey and Greece, we determined histamine values to be below 20 mg/kg, although, higher amounts of tyramine and cadaverine which is about 85mg/kg and 100 mg/kg, respectively, were observed for products stored at ambient temperatures, while low values correspond to the samples kept at chilled conditions (KÖSE *et al*., 2011; KORAL *et al*., 2013).

5. CONCLUSIONS

Good sensory quality was observed in fresh shad with the addition of ice during refrigerated storage. Ice application and refrigerated storage increased the shelf-life for 10 days. The worst sensory results, represented control group which was without ice and ambient temperature storage shelf life less than three days. Storing fresh shad in ice also helped to improve its physico-chemical quality and decrease biogenic amine development. Therefore, keeping fresh shad in ice under refrigerated storage is advised, in order to delay histamine formation and retard quality loss prior to processing or fresh market. The results suggest that using ice can improve the shelf-life of shad, stored at ambient and refrigerated temperatures as relating to food quality and safety.

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PAPER

ANTIMICROBIAL ACTIVITY STUDIES AND CHARACTERIZATION OF CELLULOSE ACETATE FILMS CONTAINING ESSENTIAL OILS

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ABSTRACT

This work aimed to evaluate the antimicrobial effectiveness of a biodegradable composite, cellulose acetate film with the incorporation of various combinations of cinnamon, oregano and sweet fennel essential oils (EOs) against *Escherichia coli*, *Staphylococcus aureus* and *Penicillium* spp. Moreover, thickness, the mechanical and water vapor barrier properties of these films were evaluated. The films with the incorporation of pure oregano EO and the combination oregano + cinnamon EOs presented the best results against all microorganisms tested. The water vapor transmission rate of the film was reduced with the addition of the EOs and the thickness of the film increased. The EO increased the flexibility of the films, and the film with pure oregano EO was the most resistant, showed 30,9 N of puncture strength. The results suggest that the EOs incorporated as natural antimicrobial agents can potentially be used in food packaging, reducing the risk of microbial contamination of food.

Keywords: active packaging, cinnamon, food packaging, food safety, oregano, sweet fennel

1. INTRODUCTION

Microorganisms may be responsible for a reduction in food quality, reducing shelf-life and causing economic losses. In addition, contaminated food can cause toxic infections. Microbial counts on fresh or processed solid or semi-solid foods are usually higher on the surface of these foods; therefore these surfaces require effective protection (MELO, 2003).
Thus, the development of materials that can serve as a protective barrier for food associated with other techniques, such as selection and control of raw materials, use of good manufacturing practices, and the use of additives that have been appropriately tested are of fundamental importance to ensure food safety, being able to provide consumers with guarantee of quality and safe food for consumption (DEVLIEGHERE *et al*., 2004; MASTROMATTEO *et al*., 2010).
The materials used for the production of active packaging include different biopolymer matrices, such as proteins, lipids and polysaccharides (MCHUGH *et al*., 1994; CHINNAN and PARK, 1995; ROJAS-GRAU *et al*., 2007; ATARÉS *et al*., 2010a). Such packaging must be environmentally acceptable, suitable for a wide range of food products and allow the incorporation of additives, including antimicrobial agents (ROSSI-MÁRQUEZ *et al*., 2009; SHOJAEE-ALIABADI *et al*., 2013).
The use of active antimicrobial materials for food packaging suggest that the product may have lower concentrations of preservatives or even be free of it, since the packaging with its additives will carry out this function. Thus, the consumer will have access to more natural products especially if the preservative used is also a natural product, such an essential oil (NAIDU, 2000).
Essential oils (EOs) can be used in the development of materials for antimicrobial active packaging because they have an excellent potential to act as a natural microbiological barrier (LAMBERT *et al*., 2001; ABDOLLAHZADEH *et al*., 2014). Several EO have been tested, such as: oregano, cinnamon, thyme, clove, satureja hortensis, lemongrass, ginger, anise, turmeric, guava leaf, nutmeg and lime (ROJAS-GRAU *et al*., 2007; ROSSI-MÁRQUEZ *et al*., 2009; ATARÉS *et al*., 2010a; MATAN, 2012; MANSO *et al*., 2011).
EOs are liquid, volatile, and rich in phenolic compounds. They can be synthesized by aromatic plants as their secondary metabolites (KAUL *et al*., 2003; RODRÍGUEZ *et al*., 2007). EOs have been widely used for their antibacterial, fungicidal, antiparasitic, insecticidal and medicinal applications (BAKKALI *et al*., 2008). In addition, EOs are approved for direct contact with food because they are considered to be GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA) (BURT, 2004).
EOs (each containing several components) when mixed in various combinations, may produce a synergic effect. This synergistic effect can increase the antimicrobial activity (ROMANO *et al*., 2009; GIBRIEL *et al*., 2013).
Antimicrobial active packaging must meet certain requirements such as: be effective against a large spectrum of microorganisms, show efficiency at low concentrations, not cause undesirable changes in the sensory characteristics of the product, and must be in accordance with the current legislation (SOARES and GONÇALVES, 2008).
The analysis of antimicrobial properties is important for predicting the behavior of antimicrobial films in food systems. Thus, this study aimed to evaluate the *in vitro* antimicrobial activity of cellulose acetate film incorporated with essential oils of cinnamon, sweet fennel and oregano, that are natural antimicrobial agents, against bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungus (*Penicillium* spp). The thickness, the mechanical and water vapor barrier proprieties in this cellulose acetate film were also analyzed for characterization them.

2. MATERIALS AND METHODS

2.1. Essential oils

Three EOs of plants were used: oregano, sweet fennel and cinnamom. The oils were acquired commercially from ®Ferquima (Ferquima Indústria e Comércio Ltda, Vargem Grande paulista, São Paulo-Br). In Table 1 the common name, plant variety, major components, and part of the plant material used for the extraction of the chosen EOs are presented. All selected EOs were produced by distillation and kept at room temperature in an amber glass bottle.

Table 1: Description of common name, plant variety, major components and part plant used to obtaining essential oils according to the supplier.

Common name	Plant variety	Major components	Part plant
Cinnamon	*Cinnamomum zeylanicum* Blume	cinnamaldehyde	Leaves
Sweet fennel	*Foeniculum vulgare* var. dulce (Mill.) Batt. & Trab.	Anetolhole	Seeds
Oregano	*Origanum vulgare* (L.)	Carvacrol	Leaves

2.2. Microorganisms

The microorganisms evaluated were the bacteria: *Escherichia coli* (ATCC 1122) (Gram-negative), *Staphylococcus aureus* (ATCC 6538) (Gram- positive) and the fungus *Penicillium* spp.
The microbial cultures were obtained from the American Type Culture Collection (ATCC) in the Universidade Federal de Viçosa, Minas Gerais, Brazil.
Bacterial cultures and fungus were pre-activated in brain heart infusion broth (BHI). Bacteria were grown on plate count agar (PCA) at 35°C/48 h and fungus was grown on Potato Dextrose Agar (PDA) at 25°C/5 days.
Microbial cultures were maintained at refrigeration temperatures as stock culture, in order to obtain freshly cultured microbial suspension. This microbial suspension, following, was used to microbial activity assay by disc diffusion method.

2.3. Preparation of active films

Cellulose acetate matrix films were obtained by casting according to MELO (2003) with same modifications.
Film-forming solutions were prepared by dissolving cellulose acetate (10% w/v) in acetone. Essential oils that were either pure or in combination (1:1 or 1:1:1) were added to the filmogenic solutions at a concentration of 50% w/v. Six different films were developed: 1) film incorporated with oregano + sweet fennel + cinnamon EOs; 2) film incorporated with oregano + sweet fennel EOs, 3) film incorporated with oregano + cinnamon EOs; 4) film incorporated with sweet fennel+ cinnamon EOs; 5) film incorporated with only oregano EO; and 6) negative control, film without any EOs.
Preliminary analysis by disk diffusion method using individually the oregano, sweet fennel and cinnamon EO, presented effective microbial activity for these oils, highlighting the oregano EO (data no shown).

The filmogenic solutions were cast on clean, flat glass plates with a glass rod to a pre-determined height. The films were dried at temperature room and then removed from the glass plates.

2.4. *In vitro* antimicrobial activity of the active films

The agar disk diffusion method was used to determine the antimicrobial activity (CLSI, 2003).
Microbial suspensions were prepared and adjusted in brain heart infusion broth (BHI). Inoculums of the bacteria were 100 μL of a suspension containing approximately 10^4 colony-forming units (CFU) per mL for bacteria, or 10^4 spores/mL for the fungus. The inoculums were spread on the surface of Mueller-Hinton agar plates. Then a sterile film disk (10 mm diameter) incorporated with the EO or EOs under test was placed in the center of the agar plate. A film disk without any EOs was used as negative control (ESPITIA, 2009). The 6 films were previously exposed to UV light (Prodicil, 110v, 254nm) for 15 minutes for sterilization.
The plates with bacteria were then incubated at 35±2°C for 48h, while plates with fungus were incubated at 25±2°C for 5 days. Following incubation, the total diameter of the inhibition zones (colony-free perimeter) was measured with a caliper. The data were expressed as inhibition zones (cm), including the disk area. Each experimented was done in triplicate.

2.5. Film characterization

2.5.1. Thickness

The films thickness was measured with a digital micrometer (Mitutoyo -Japan). The mean thickness was calculated from five measurements.

2.6. Water vapor transmission rate (WVTR)

The WVTR of films was measured gravimetrically, using the ASTM E96-95 standard test method. Diffusion cells containing anhydrous calcium chloride desiccant (0% RH) were sealed by the test film. The cells were stored in desiccators, each at a constant RH (75%) with a saturated salt solution and temperature of 25°C. The cells were weighed at 24 h intervals (over a 5 day period). Transported water vapor was determined from the weight gain of the diffusion cell at a steady state of transfer. The slope of the weight versus time plot (day) was divided by the effective film area (m^2) to obtain the WVTR using the equation below. At least 3 replicates were produced for each film type.

$$WVTR = \frac{\Delta W}{A}$$

Where WVTR is the water vapor transmission rate ($g/d\ m^2$) and A is the area of the exposed film surface (m^2).

2.7. Mechanical analysis

Puncture tests were performed to evaluate the mechanical strength of the film using a Texture Analyzer (TA.XT plus, Stable Micro Systems - Haslemere, England). For the puncture test, film discs (6.4 cm diameter) were fixed to a support with a circular opening

of 10 mm in diameter and 15 mm in depth. A cylindrical probe of 5 mm diameter was moved perpendicularly to the film surface at a constant speed of 1 mm/s until it passed through the film. The force-deformation curves were obtained and force (N) and deformation (mm) values at the puncture point were recorded to represent the puncture strength (N) and deformation (mm) of the film. Ten replications were performed for the mechanical tests.

2.8. Statistical Analyses

The experiment was carried out in a completely randomized design. All analyses were conducted in three replicates in triplicate. Differences between the results of the inhibition zones of the essential oils were evaluated by analysis of variance (ANOVA) using the *F*-test. The Tukey test was used for the antimicrobial analyze and the Duncan test was applied to films characterization. Differences were considered significant when $p < 0.05$, using the SAS® software 9.0 (SAS Institute Inc., NC, USA).

3. RESULTS AND DISCUSSION

3.1. Evaluation antimicrobial of active films incorporated with essential oils

Antimicrobial efficiency was observed in all formulations of the active film incorporated with EO. It was different of the control film (without EO), that not showed antimicrobial activity. The inhibition zones microbial of several films incorporated with OEs against the tested microorganisms are shown in Table 2.

Table 2: Inhibition zones (cm) formed by actives films incorporate with essential oils against bacterial strains *E. coli* and *S. aureus* and fungus *Penicillium* spp., analyzed at an ideal temperature for the microorganisms to develop: bacteria, 35 ± 2ºC/48 h and fungus 25 ± 2ºC/5 days.

Active films	Mean diameter of inhibition zones (cm)[+]		
	***Penicillium* spp**	***E. coli***	***S. aureus***
Oregano Pure	2.67[a]	0.99[ab]	3.75[a]
Oregano + Cinnamon	2.74[a]	1.14[a]	2.7[b]
Oregano + Sweet fennel	1.36[b]	0.50[c]	2.08[b]
Oregano + Cinnamon+ Sweet fennel	1.50[b]	0.73[bc]	1.11[c]
Cinnamon + Sweet fennel	1.15[b]	0.03[d]	0.4[c]

*In each column, different superscript letters are significantly different (p< 0.05), by Tukey test.
[+]No includes disc diameter (10 mm).

E. coli bacteria was more resistant than *S. aureus* and *Penicillium* spp. to the treatments with active films in this work. The lower antimicrobial activity against gram - negative bacteria, *i.e. E.coli*, can be attributed to the fact that they are in general more resistant due to the external lipopolysaccharide wall surrounding the peptidoglycan cell wall (ZINOVIADOU *et al*., 2009).
In relation to inhibition of the *S. aureus* microorganism, the active film with oregano EO was the most efficient (p <0.05), followed by 2 different films: film incorporated with

oregano + cinnamon EOs and the film incorporated with oregano + sweet fennel EOs. There were no differences (p>0.05) found for these two latter films (Table 2).
The film with combination oregano + cinnamon + sweet fennel EOs and the film with combination sweet fennel + cinnamon EOs were both statistically similar (p> 0.05). They showed lower activity than the other films against the microorganism *S. aureus* (Table 2).
The films incorporated with oregano + cinnamon EOs and the film incorporated with oregano EO alone proved to be the most efficient against the microorganism *E. coli*. The film with the combination of cinnamon + sweet fennel EOs was the least effective (Table 2) against this bacteria.
Fungus *Penicillium* spp. was most sensitive to the film incorporated with oregano EO alone and to the film with combination of oregano + cinnamon EO (Table 2).
The films into oregano and the film with combination cinnamon + oregano presented the highest antimicrobial activity against bacteria and fungus tested . In the work of Ye *et al* (2013) was showed that the active components, cinnamaldehyde and carvacrol expressed high antibacterial activities both to *E. coli* and *S. aures*, besides of have synergistic antimicrobial activities for the bacterias.
The efficiency of the oregano EO against some microorganisms has already been reported by PEREIRA *et al*. (2008), PESAVENTO *et al*. (2015) and MARTUCCI *et al*. (2015). Positive results were also observed by EMIROGLU *et al*. (2010), when a combination of thyme EO with oregano EO was incorporated into Soy-based films. These authors showed an *in vitro* antimicrobial effect against *S. aureus* and *E. coli* 0157: H7.
According to preliminary studies (data no shown), the inhibitory effect of oregano EO and combinations of EOs when incorporated into the cellulose acetate films was lower than that found in pure oregano EO and combinations of EOs. The possible causes that would explain this result could be a partial loss of volatile compounds during film preparation. It may also be due to dilution of the EOs in the filmogenic solutions.
Various tests with EOs incorporated in films have obtained results that indicate a real potential to use such films in the food industry for packaging. EMIROGLU *et al*. (2010) evaluated the effect of a soy edible film incorporated with natural antimicrobial, thyme and oregano EOs on fresh ground beef patties; while OUSSALAH *et al*. (2007) used the Alginate based film incorporated with winter savory, oregano and cinnamon EO to wrap ham and bologna.

3.2. Film characterization

3.2.1. Thickness and water vapor transmission rate (WVTR)

Adding EOs causes an increase in the thickness of the films. There was a difference (p>0.05) between the thicknesses of the control film (without EO) and the active films incorporated with EO. However, the thickness of the different films after incorporating the EOs was similar, ranging from 29 to 44mm, as shown in Table 3.
The water resistance of the films improved with the incorporation of the EOs, *i.e.*, there was a reduction in the WVTR. The WVTR of the films with EOs presented a significant difference (p< 0.05) when compared to the control films (without EO). However, the WVTR films with EOs (Table 3) was same. Films incorporated with EOs showed a better barrier against water vapor. They had a lower permeability, and therefore are indicated for moisture control for moisture sensitive foods as they would give better protection.
The hydrophobic nature of EOs could potentially increase the hydrophobicity of cellulose acetate film. Studies on the incorporation of lipids as EOs into films have shown improvements in their water vapor barrier properties (ZINOVIADOU *et al*., 2009; BAHRAM *et al*., 2012).

Table 3: Water vapor transmission rate (WVTR) and thickness of differents active films incorporate with essential oils.

Active Films	Thickness (μm)	WVTR ($g.m^{-2}d^{-1}$)
Negative Control (without EO)	$29.0^{a} \pm 1.6$	$259.61^{a} \pm 69.46$
Oregano Pure	$42.8^{b} \pm 3.2$	$162.92^{b} \pm 27.23$
Oregano + Cinnamon	$38.5^{b} \pm 1.0$	$175.61^{b} \pm 14.54$
Oregano + Sweet fennel	$44.3^{b} \pm 4.7$	$192.10^{b} \pm 1.95$
Oregano + Cinnamon + Sweet fennel	$42.8^{b} \pm 3.2$	$197.86^{b} \pm 7.71$
Cinnamon + Sweet fennel	$40.0^{b} \pm 0.4$	$152.80^{b} \pm 37.35$

*In each column, different superscript letters are significantly different ($p < 0.05$), by Duncan test.

3.3 Mechanical analysis

The kind of EOs and its concentration affect the mechanical properties of films differently. The magnitude of such differences is dependent on the added compound (SHOJAEE-ALIABADI *et al*., 2014).
Puncture tests were performed to evaluate the mechanical strength of the film. Table 4 shows the influence of EO incorporations on the mechanical properties of cellulose acetate films. The presence of EO droplets in the film matrix affected both puncture strength and deformation at the break point of the films.

Table 4: Mechanical Analysis of different active films incorporate with essential oils Puncture tests: puncture strength (PS) and deformation at breaking point (DB).

Active films	DB (mm)	PS (N)
Negative Control (without EO)	$2.71^{a} \pm 0.76$	$23.42^{a} \pm 1.24$
Oregano Pure	$3.09^{b} \pm 0.38$	$30.94^{b} \pm 6.28$
Oregano + Cinnamon	$4.18^{c} \pm 0.71$	$26.53^{a} \pm 1.87$
Oregano + Sweet fennel	$3.28^{bd} \pm 0.19$	$24.37^{a} \pm 0.29$
Oregano + Cinnamon + Sweet fennel	$3.98^{c} \pm 0.51$	$24.65^{a} \pm 0.01$
Cinnamon + Sweet fennel	$3.58^{d} \pm 0.11$	$18.07^{c} \pm 6.59$

*In each column, different superscript letters are significantly different ($p < 0.05$), by Duncan test.

The puncture strength (PS) ranged from 18.1 to 30.9 N, for films for combinations of cinnamon + sweet fennel EOs and pure oregano EO, respectively. The film with pure oregano EO was the most resistant, thus, it had a greater force at breaking point.
Films incorporated with a combination of oregano + cinnamon + sweet fennel EOs and the oregano + cinnamon EOs were the most elastic, *i.e.* they had a higher deformation at the breaking point, while the control film presented the smallest deformation at breaking point (Table 4). Therefore, the presence of EOs in film matrices, makes them more elastic, resulting in more stretchable films, depending on the EO concentrations (SHOJAEE-

ALIABADI *et al*., 2013). It occurs because, the EO acted as a plasticizer and increased the flexibility of the polymer chains (ATEF *et al*., 2015).
The kind and amount of phenolic compounds present in oregano, cinnamon and sweet feenel EOs cause different binding and interactions between cellulose acetate molecules and essential oil (LEE *et al*., 2015). As resulted, the films containing these EO had different mechanical properties.
The work reported by Atarés *et al*. (2010a) who conducted tests using Scanning Electron Microscopy and surface morphology in addition to other tests, observed that films with ginger EO was less resistant and less elastic than those with cinnamon EO.
Atarés *et al*. (2010b) evaluated the use of cinnamon and ginger EOs in low proportions (less than 1:0.1 ratio of lipid to protein) in Sodium Caseinate based films. The authors observed that this quantity did not cause any impact on the mechanical properties and only caused a small impact on the water vapor permeability of the film.
When agents are incorporated into packaging materials, polymeric matrix physical and mechanical properties are changed, there are modifications specific to each combination of antimicrobial - polymer. Consequently when a new active packaging material is developed, it is born with new specific characteristics (SOARES and GONÇALVES, 2008).

4. CONCLUSIONS

Essential oils can be recognized as an appropriate natural food preservative, and may be used to replace the synthetic preservatives. Films incorporated with pure oregano EO and the combination of oregano + cinnamon EOs appear as an alternative to extend the commercial validity of foods. Therefore, there is the possibility to replace the synthetic chemical preservatives direct used in foods for antimicrobial active packaging incorporated with EOs for food preservation.
Active packaging based on cellulose acetate incorporated with EOs besides boosting food safety, maintaining their quality and safety, contributes to reducing the environmental impact of traditional packaging because it is based on renewable sources of raw materials.
The technology of active materials is an emerging and promising area of research and it can confer multiple benefits for a wide range of products. However, more research needs to be conducted for different applications in order to evaluate the technological, economic and safety potentials.

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PAPER

VOLATILES AND ACCEPTABILITY OF LIQUEURS FROM KUMQUAT AND GRAPEFRUIT

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ABSTRACT

The aim of this work was to produce liqueurs from "minor" citrus fruits, such as kumquat and grapefruit, characterize their volatile fraction and evaluate their acceptability by a consumer test. A *limoncello* sample (LP) was produced under the same conditions and used for comparison. All the new liqueurs were found to be richer in limonene and poorer in oxygenated compounds than the LP. The volatile fraction was mostly represented (85%) by limonene in grapefruit liqueur. Liqueur from kumquat peel (KP) was the richest in volatile compounds, whereas the one from kumquat whole fruit (KWF) was the poorest. This latter also had the particular feature to be the richest in sesquiterpene alcohols. Octanal and decanal, and two acetals deriving from these aldehydes (1,1-diethoxyoctane and 1,1-diethoxydecane) were most prevalent in KP and LP. The consumer test showed that all liqueurs were judged to be acceptable. Nevertheless, *limoncello* remained the most preferred, while the KWF liqueur obtained the best flavour score in the group of minor citrus fruits.

Keywords: consumer acceptance, grapefruit, kumquat, liqueur, volatile compounds

1. INTRODUCTION

Citrus fruits are either consumed as fresh or processed to obtain juices, jams and, in small amounts, to produce liqueurs such as the so-called *rosolio. Rosolios* are liqueurs obtained by alcoholic maceration of flowers or fruits with the final addition of water and sugar. The most famous Italian *rosolio* made from citrus fruits, is *limoncello.* It is highly requested on the international market and it is manufactured by the alcoholic maceration of lemon (*Citrus limon* L.) peel. The composition of the volatile fraction plays a fundamental role in the development of citrus liqueurs aroma. In particular, the ratios carbonyls-to-oxygenated compounds, alcohols-to-oxygenated compounds, and esters-to-oxygenated compounds are indices of flavouring quality (POIANA *et al.*, 2006). Several investigations have been carried out on the volatile compounds of *limoncello* in order to find the molecular markers to establish quality and genuineness. VERSARI *et al.* (2003) reported that the addition of essential oils to *limoncello* causes an increase of oxygenated compounds and a loss of hydrocarbons. They also reported that compounds such as ethyl acetate, acetaldehyde, and 2-methyl-1-propanol should be related to the occurrence of microbiological activity in the sugar syrup. CRUPI *et al.* (2007) found differences in terms of type, amount, and variation range of volatile compounds in 12 commercial *limoncello* samples and in 2 types of *limoncello* produced in a laboratory. POIANA *et al.* (2006) reported variations in the volatile profiles of the alcoholic extract of lemon fruit to be a function of the geographic area and season. Besides *limoncello*, there are only a few liqueurs made from citrus fruits, such as r*osolio* from tangerine and orange, mainly produced for local markets. To our knowledge, no effort has been made for producing liqueurs from "minor" citrus fruits, such as kumquat (*Fortunella margarita* L.) and grapefruit (*Citrus paradisi* L.).

Kumquat is a vigorous and prolific small bushy tree that produces oval or round fruits with a smooth, bright orange rind (BARRECA *et al.*, 2011). Unlike other citrus fruits, kumquat fruit is eaten without discarding the peel, and this has nutritional relevance since this part is particularly rich in flavonoids (GATTUSO *et al.*, 2007; TRIPOLI *et al.*, 2007). Many citrus flavonoids exhibit antioxidant activity, inhibit angiogenesis, and slow down cancer cell migration and proliferation (BARRECA *et al.*, 2009; BENAVENTE-GARCÍA and CASTILLO, 2008). There are only a few studies on the volatile constituents of kumquat. BERNHARD and SCRUBIS (1961) found limonene to be the most abundant compound in kumquat oil extracted by steam distillation. Aldehydes, ketones, free alcohols, terpene esters, α-pinene, and myrcene were also reported in this study. KOYASAKO and BERNHARD (1983) identified 71 volatiles in oil obtained by simultaneous distillation and extraction; UMANO *et al.* (1994) reported 84 volatiles in steam-distillation extracts; CHOI (1995) identified 82 volatiles in oil extracted by cold pressing. More recently, PENG *et al.* (2013) identified a total of 43 compounds in the volatile fractions of kumquat essential oils extracted by different methods. The principal constituents of the oils were similar, and differences were only found for minor compounds such as linalool, terpinen-4-ol and α-terpineol.

Grapefruit is a citrus fruit that contributes to human health mainly thanks to its high contents of ascorbic acid and fiber (PEIRÓ *et al.*, 2006). Unfortunately, the presence of some flavonoids, such as naringin, is responsible for the bitter taste that limits acceptance by consumers. Nevertheless, fresh or processed grapefruit may be conveniently mixed with other foods to formulate desirable and wholesome products.

The present study aimed to assess the possibility of using kumquat and grapefruit for the production of innovative types of *rosolio*. The experimental liqueurs were produced on a laboratory-scale and were subjected to volatile profile characterization by headspace-solid

phase microextraction (HS-SPME) and evaluation of consumer acceptance in comparison with *limoncello*.

2. MATERIALS AND METHODS

2.1. Liqueur making

Fresh fruit peels were utilized for the preparation of kumquat (*Fortunella margarita*) (KP), grapefruit (*Marsh seedless*) (GP), and lemon (*Femminello comune*) (LP) liqueurs. For this purpose, about 500 g of each fruit was accurately peeled, and the peels, consisting of the flavedo part, were put into a jar containing 500 mL of ethanol (95% v/v) and left to steep for 2 weeks. After this period, the peels were taken out of the alcohol and syrup made with 500 mL of water plus 400 g of sugar was added to the ethanol extract. The liqueurs obtained were let to rest for 2 months in the dark at room temperature for maturation. During preparation, it was observed that peeling the kumquat was very difficult, due to the small size of the fruit (about 15 g). In order to assess the possibility of avoiding such step, a liqueur from the maceration of the whole fruit (KWF) was also prepared. In this latter preparation, 500 g of the fruit was directly put into 500 mL of ethanol (95% v/v) and left to steep for 2 weeks; then the preparation followed the same steps as described for the other liqueurs.

2.2. Volatile fraction extraction and GC/MS analysis

Headspace-solid phase microextraction (HS-SPME) was chosen as the extraction technique for the present study, since it had been successfully applied to determine the volatile composition of kumquat essential oils (PENG *et al.*, 2013) and lemon liquor (CRUPI *et al.*, 2007). Volatile compounds were extracted using a preconditioned 2-cm-long 50/30 mm divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, Pa., U.S.A.). Two mL of each liqueur were put in a 12-mL crimped vial, with 0.4 g NaCl added, and conditioned for 10 min at 37°C and stirred with a magnetic bar. Then the fiber was exposed in the headspace of the vial for 20 min. Desorption of analytes from the SPME fiber took place in a split/splitless injector set at 250°C with a split ratio of 1:25 using a 3 min desorption time. Separation of volatile compounds was performed using an Agilent 6890 gas chromatograph (GC) coupled with an Agilent 5975 mass spectrometer (MS) (Agilent, Wilmington, Del., U.S.A.) using a HP5-MS column (30 m × 0.25 mm × 0.25 mm). The chromatographic conditions were: (i) oven, 40°C (2 min) to 190°C at 5°C min^{-1}, to 230°C at 15°C min^{-1}, held 2 min; (ii) detector, source temperature 240°C; transfer line temperature 240°C; (iii) carrier gas, helium at constant flow of 1.0 mL min^{-1}. The impact energy was 70 eV. Data were acquired using full-scan mode in the range of 20-250 m/z at an acquisition rate of 5 Hz. Volatile compounds were tentatively identified by comparing the experimental spectra with those reported in the NIST Library and with those obtained by pure external standard injection when available. Each sample was analyzed in triplicate and results were reported as a mean of area counts x 10^{6}. The repeatability of the SPME-GC/MS method was lower than 10% in terms of relative standard deviation (RSD).

2.3. Acceptance and preference testing

The consumer test was carried out in a conference room where temporary partitions were erected to create isolated booths able to separate testers during analysis, in compliance with the Standard no. 8589 of the International Organization for Standardization (ISO

1988). Testing was performed at room temperature (20 °C) with appropriate and adequate artificial lighting, simulating daylight. A total of 75 consumers (age 19–47, mean 23.1; 45 males and 30 females) were recruited to participate in the consumer test. A 3-digit random code was assigned to the liqueurs, which were served at room temperature in 80-mL white polyethylene glasses. For each sample, about 10 mL was served. Mineral water was at each participant's disposal to cleanse mouth during testing. The evaluation form used had 3 sections: the first required information about the sex and age of the panelists; the second section required the evaluation of color, odor, and flavor using a 6-point hedonic scale (1 = extremely dislike; 6 = like extremely); the last section asked the consumers to rank the samples according to overall appreciation. The use of flavor, instead of taste, as a sensorial descriptor was chosen because our purpose was to assess the blend of taste and smell sensations evoked in the mouth.

2.4. Statistical analysis

The results of color, odor, and flavor assessment were subjected to a one-way analysis of variance (ANOVA). Moreover, differences in the preference rank sums between all possible pairs of products were considered. Should any of these (absolute) differences exceed a critical value, the preferences for that pair of products would differ from one another at the stated statistical significance level (n = 75, $P \leq 0.05$, critical value = 40.6) (BASKER, 1988).

3. RESULTS AND DISCUSSIONS

3.1. Volatile fraction

Figures 1 and 2 show the total ion current profile of volatile compounds of liqueurs obtained from kumquat peel (Fig. 1A), kumquat whole fruit (Figure 1B), grapefruit peel (Fig. 2A), and lemon peel (Fig. 2B). Clearly, the 4 liqueurs were different under a qualitative point of view.

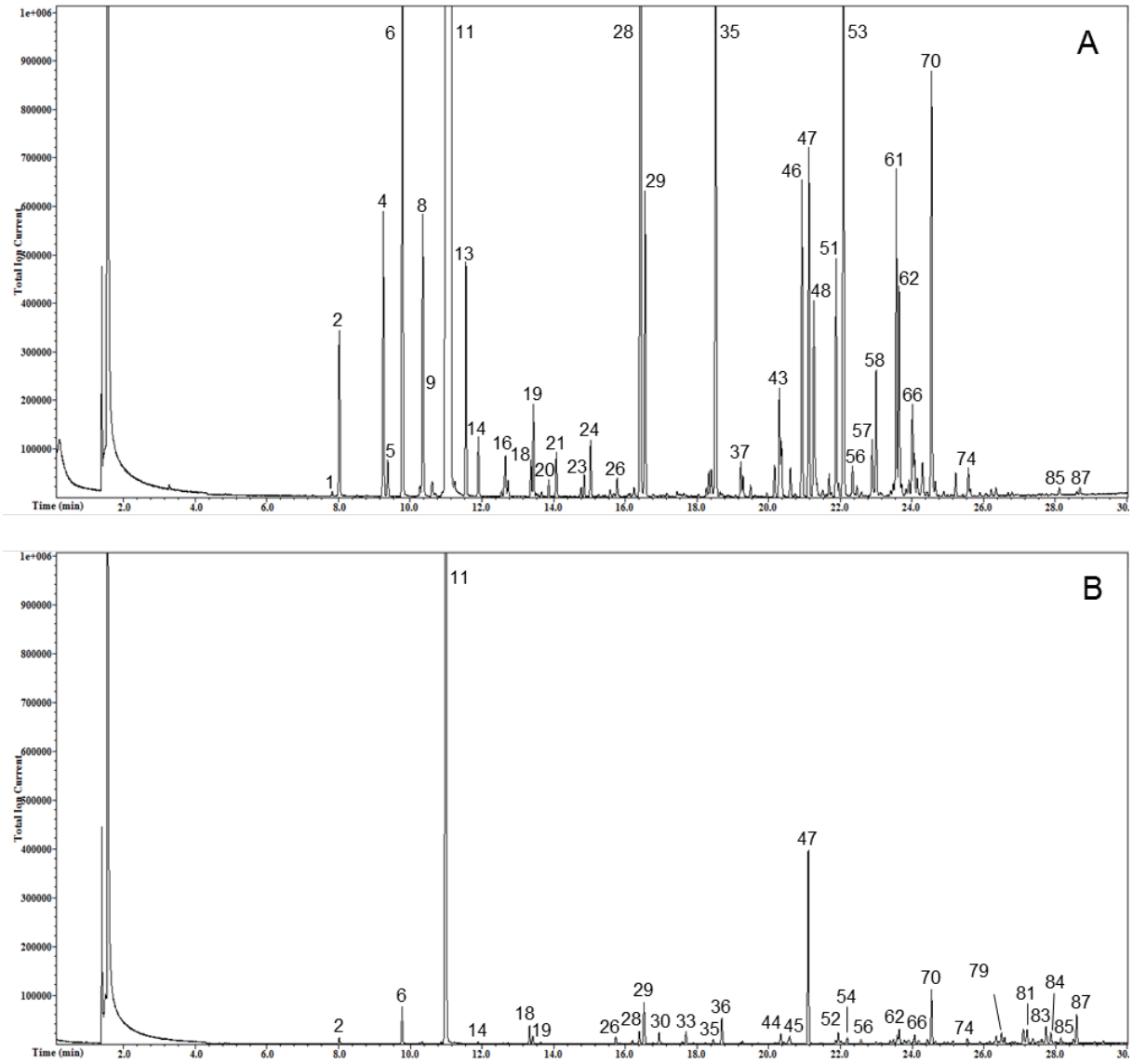


Figure 1: SPME-GC/MS profiles of kumquat peel liqueur (A) and kumquat whole fruit liqueur (B). The peak numbers refer to the compounds in Table 1.

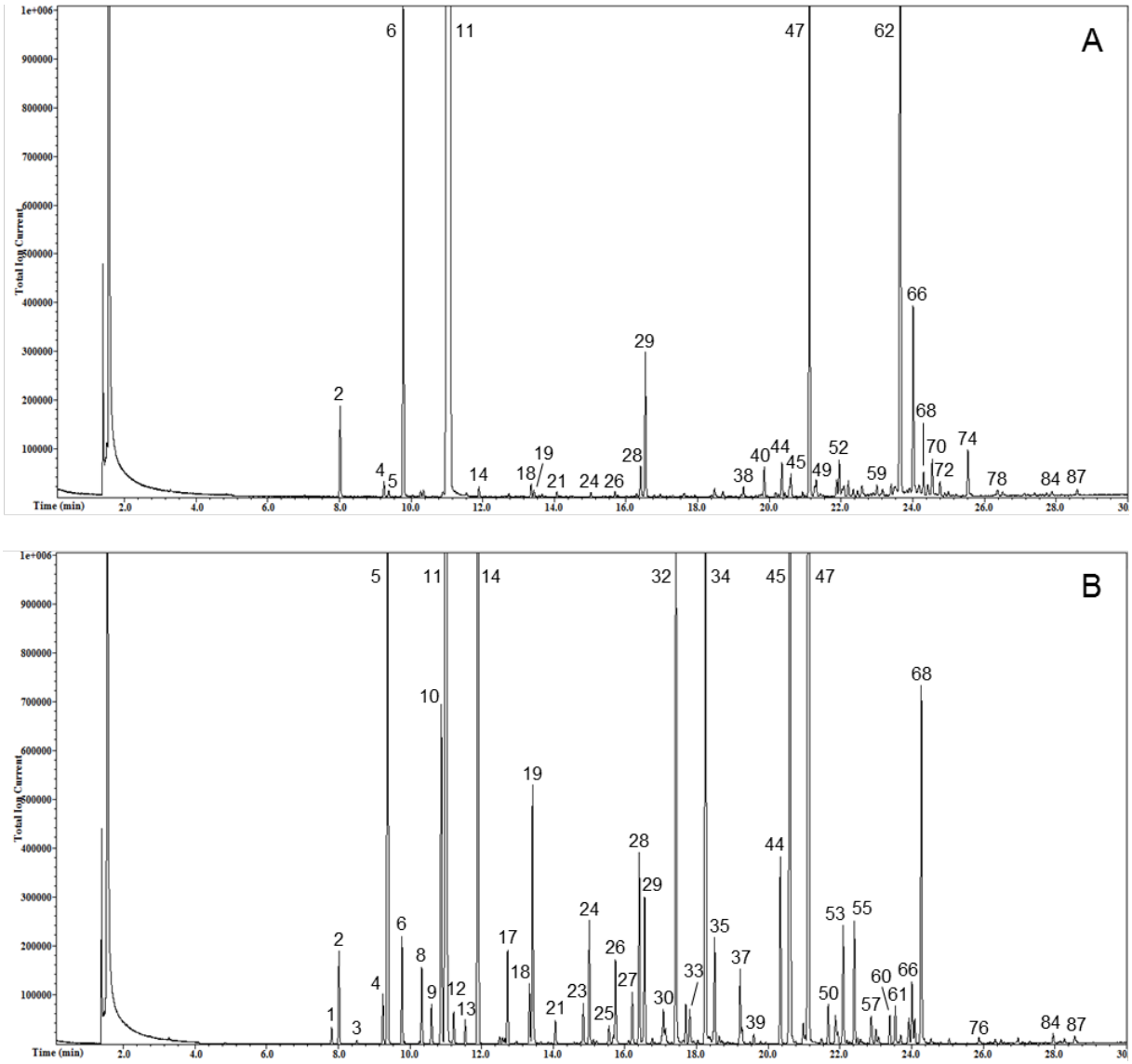


Figure 2: SPME-GC/MS profiles of grapefruit peel liqueur (A) and lemon peel liqueur (B). The peak numbers refer to the compounds in Table 1.

Table 1 summarizes the mean values for the volatile compounds expressed as both absolute and relative percentage area. The total area and the sums of the areas of monoterpenes (MTs), sesquiterpenes (STs), and oxygenated compounds (OCs) are also reported in Table 1.

Table 1: Volatile composition of liqueurs.

No.	Compound	KP		KWF		GP		LP		[b]ID
		[a]Area	%	Area	%	Area	%	Area	%	
1	α-Thujene	0.20	0.01	-	-	-	-	0.50	0.07	MS
2	α-Pinene	7.63	0.47	0.25	0.18	3.88	0.40	3.95	0.54	MS, ES
3	Camphene	-	-	-	-	-	-	0.15	0.02	MS, ES
4	β-Sabinene	12.67	0.78	-	-	0.66	0.07	2.16	0.29	MS, ES
5	β-Pinene	1.73	0.11	-	-	0.29	0.03	39.85	5.43	MS
6	β-Myrcene	36.77	2.26	1.43	1.05	20.92	2.14	4.33	0.59	MS, ES
7	α-Phellandrene	0.50	0.03	-	-	0.28	0.03	0.10	0.01	MS
8	Octanal	14.34	0.88	0.08	0.06	0.33	0.03	3.30	0.45	MS, ES
9	α-Terpinene	0.89	0.05	-	-	0.07	0.01	1.72	0.23	MS, ES
10	p-Cymene	-	-	0.08	0.06	0.22	0.02	16.43	2.24	MS, ES
11	Limonene	1239.37	76.07	104.96	76.96	833.53	85.29	231.73	31.56	MS, ES
12	β-*cis*-Ocimene	0.88	0.05	-	-	-	-	1.46	0.20	MS
13	β-*trans*-Ocimene	9.66	0.59	-	-	-	-	1.02	0.14	MS
14	γ-Terpinene	2.62	0.16	0.13	0.10	0.42	0.04	64.83	8.83	MS
15	*trans*-Sabinene hydrate	0.25	0.02	-	-	-	-	0.30	0.04	MS
16	1-Octanol	2.40	0.15	0.05	0.04	-	-	0.30	0.04	MS, ES
17	α-Terpinolene	0.76	0.05	0.06	0.04	0.15	0.02	4.07	0.55	MS
18	Linalool	1.55	0.10	0.79	0.58	0.53	0.05	2.56	0.35	MS, ES
19	Nonanal	4.02	0.25	0.37	0.27	0.30	0.03	11.73	1.60	MS, ES
20	Nonanol	0.21	0.01	0.07	0.05	0.13	0.01	-	-	MS, ES
21	γ-Terpinolene	2.06	0.13	-	-	0.22	0.02	1.00	0.14	MS
22	Camphor	0.39	0.02	-	-	-	-	0.08	0.01	MS
23	β-Citronellal	1.00	0.06	-	-	-	-	1.85	0.25	MS, ES
24	*cis*-Sabinene hydrate	2.60	0.16	-	-	-	-	6.00	0.82	MS
25	1-Decanol, 2-hexyl-	0.39	0.02	-	-	-	-	0.08	0.01	MS
26	(-)-4-Terpineol	0.93	0.06	0.36	0.26	-	-	4.14	0.56	MS
27	α-Terpineol	0.55	0.03	0.15	0.11	0.24	0.02	2.43	0.33	MS, ES
28	Decanal	53.75	3.30	0.59	0.43	1.44	0.15	8.25	1.12	MS, ES
29	Acetic acid, octyl ester	13.76	0.84	2.04	1.50	6.16	0.63	7.50	1.02	MS
30	Nerol	-	-	0.50	0.37	0.10	0.01	1.99	0.27	MS, ES
31	β-Citronellol	0.24	0.01	-	-	0.07	0.01	0.89	0.12	MS, ES

32	Z-Citral (neral)	0.24	0.01	-	-	-	-	27.04	3.68	MS
33	*trans*-Geraniol	-	-	0.60	0.44	-	-	1.78	0.24	MS, ES
34	E-Citral (geranial)	0.43	0.03	0.06	0.04	-	-	39.13	5.33	MS
35	1,1-Diethoxyoctane	39.04	2.40	0.23	0.17	0.14	0.01	5.19	0.71	MS
36	Anethol	0.21	0.01	1.26	0.92	0.31	0.03	0.36	0.05	MS, ES
37	Undecanal	1.57	0.10	0.05	0.04	0.14	0.01	3.27	0.45	MS
38	Nonyl acetate	0.96	0.06	0.09	0.07	0.47	0.05	0.79	0.11	MS
39	Methyl geranoate	0.12	0.01	-	-	-	-	0.45	0.06	MS
40	δ-Elemene	-	-	-	-	1.37	0.14	-	-	MS
41	*trans*-Carvyl acetate	0.18	0.01	-	-	-	-	-	-	MS
42	Copaene	1.48	0.09	-	-	0.24	0.02	-	-	MS
43	α-Terpinenyl acetate	5.03	0.31	-	-	0.10	0.01	-	-	MS
44	Citronellyl acetate	2.76	0.17	0.53	0.39	1.76	0.18	8.45	1.15	MS
45	Neryl acetate	1.47	0.09	0.57	0.42	1.61	0.16	87.54	11.92	MS, ES
46	Isoterpinolene	14.77	0.91	-	-	0.21	0.02	0.89	0.12	MS
47	Geranyl acetate	16.74	1.03	8.40	6.16	24.00	2.46	89.29	12.16	MS, ES
48	β-Cubebene	10.60	0.65	-	-	0.48	0.05	-	-	MS
49	β-Elemene	0.63	0.04	-	-	0.84	0.09	-	-	MS
50	Citronellal	1.00	0.06	-	-	-	-	1.86	0.25	MS, ES
51	Acetic acid, decyl ester	10.53	0.65	0.17	0.12	0.83	0.08	1.70	0.23	MS
52	Limonen-10-yl acetate	0.61	0.04	0.52	0.38	1.63	0.17	-	-	MS
53	*trans*-Caryophyllene	37.14	2.28	0.18	0.13	0.75	0.08	5.82	0.79	MS
54	α-Santalol	-	-	0.32	0.23	0.77	0.08	-	-	MS
55	α-Bergamotene	-	-	-	-	-	-	5.45	0.74	MS
56	*cis*-Caryophyllene	0.56	0.03	0.20	0.15	0.31	0.03	0.29	0.04	MS
57	Neryl propionate	2.98	0.18	-	-	0.21	0.02	1.64	0.22	MS
58	α-Humulene	6.12	0.38	0.08	0.06	0.27	0.03	0.76	0.10	MS
59	β-Santalene	-	-	-	-	0.70	0.07	0.37	0.05	MS
60	Geranyl propionate	0.30	0.02	0.19	0.14	0.74	0.08	1.27	0.17	MS, ES
61	1,1-Diethoxydecane	14.40	0.88	-	-	-	-	1.79	0.24	MS
62	Germacrene D	10.20	0.63	0.73	0.54	47.56	4.87	0.10	0.01	MS
63	*trans*-β-Farnesene	0.61	0.04	0.20	0.15	0.26	0.03	0.45	0.06	MS
64	β-Selinene	0.43	0.03	0.30	0.22	0.37	0.04	-	-	MS

65	Valencene	0.91	0.06	-	-	0.71	0.07	1.35	0.18	MS, ES
66	Bicyclogermacrene	4.93	0.30	0.53	0.39	10.03	1.03	3.06	0.42	MS
67	*cis*-α-Bisabolene	2.00	0.12	0.24	0.18	1.08	0.11	1.18	0.16	MS
68	β-Bisabolene	2.10	0.13	-	-	1.40	0.14	16.20	2.21	MS
69	γ-Cadinene	0.24	0.01	0.23	0.17	0.77	0.08	-	-	MS
70	δ-Cadinene	21.33	1.31	2.45	1.80	1.90	0.19	0.24	0.03	MS
71	Longifolene	0.81	0.05	0.16	0.12	-	-	-	-	MS
72	α-Chamigrene	-	-	-	-	0.84	0.09	-	-	MS
73	γ-Bisabolene	-	-	0.07	0.05	0.27	0.03	0.23	0.03	MS
74	Germacrene B	1.45	0.09	0.19	0.14	2.47	0.25	-	-	MS
75	Nerolidol	0.34	0.02	-	-	0.14	0.01	-	-	MS
76	Palustrol	0.24	0.01	0.10	0.07	-	-	0.25	0.03	MS
77	Caryophyllene oxide	0.36	0.02	0.15	0.11	-	-	-	-	MS
78	Dodecanoic acid, ethyl ester	0.49	0.03	0.47	0.34	0.50	0.05	0.24	0.03	MS
79	Veridiflorol	-	-	0.60	0.44	-	-	0.19	0.03	MS
80	Globulol	-	-	0.29	0.21	0.21	0.02	-	-	MS
81	Fonenol	-	-	0.81	0.59	0.15	0.02	-	-	MS
82	t-Cadinol	0.09	0.01	0.22	0.16	-	-	-	-	MS
83	Aromadendrene	0.11	0.01	0.87	0.64	0.17	0.02	-	-	MS
84	Cedrenol	0.04	0.01	0.52	0.38	0.27	0.03	0.44	0.06	MS
85	Hinesol	0.46	0.03	0.26	0.19	-	-	-	-	MS
86	Torreyol	-	-	0.21	0.15	-	-	-	-	MS
87	α-Bisabolol	0.20	0.01	1.43	1.05	0.34	0.03	0.49	0.07	MS
	Total	1629.33	-	136.39	-	977.26	-	734.25	-	
	Monoterpenes	1333.36	81.83	106.91	78.39	860.85	88.09	380.49	51.82	
	Sesquiterpenes	101.65	6.24	6.43	4.71	72.79	7.45	35.50	4.83	
	Oxygenated compounds	194.32	11.93	23.05	16.90	43.62	4.46	318.26	43.34	

KP: kumquat peel; KWF: kumquat whole fruit; GP: grapefruit peel; LP: lemon peel. ID: identification. [a]Compounds quantified as total area counts x 10^6 (mean of 3 repetitions). [b]MS: identification based on the NIST MS library; ES: identification based on authentic external standards analysed by mass spectrometry.

The KP liqueur was the richest in volatile compounds (73 molecules identified), followed by the LP (65), the GP (61), and the KWF (54). The KP also had the highest total integrated peaks area, followed by the GP, the LP, and the KWF. Even though the study was not quantitative, under our experimental conditions the area of the KP appears to be about 12-fold larger than that of the KWF, and this could be a consequence of the peeling operation due to: i) the breakage of the cells containing the essential oils that favored the better

extraction of the volatile compounds during maceration; ii) the higher contact area between the peel and alcohol since only peels had been used in the maceration. In comparison to the LP, the KP total area was about 2 times higher, the KWF was about one fifth lower, and the GP was about 30% higher. Among the volatile compounds, MTs group was the most abundant in all the samples, constituting 78-80% of the total, with the exception of the LP, where these compounds were found to be about one-half of the total. In fact, the LP was characterized by a remarkable presence of OCs, which represent about the second half of the total volatile compounds. As concerns single volatiles, limonene was the predominant compound identified, even though the peak area strongly varied among liqueurs (from about 105×10^6 in KWF to about 1239×10^6 in KP). In terms of relative abundance, this monoterpene represented about 85% of the total area in the GP and about 76% in kumquat samples (both in the KP and the KWF), which are much higher than in the LP (about 32%). The abundance of limonene was expected, since it is the principal component of the volatile fraction of various citrus fruits (VERSARI *et al.*, 2003; CRUPI *et al.*, 2007; DUGO *et al.*, 2010; ASIKIN *et al.*, 2012), including kumquat, in which it represents more than 90% of volatile compounds of the peel essential oil (UMANO *et al.*, 1994; CHOI, 2005). The low concentration of limonene detected in the LP liqueur, compared to the KP and the GP, could be explained by the different content in the corresponding essential oils. As reported by CACCIONI *et al.* (1998), the lemon essential oils were characterized by a limonene concentration of 60–71% of the total volatile compounds, while the limonene concentrations in the essential oil of other citrus, such as grapefruit, orange, and bitter orange, were always higher than 90%. This monoterpene is associated with odor descriptors such as lemon-like, lemon, and orange, but presents a high odor threshold (CHOI, 2005; POHJANHEIMO and SANDELL, 2009). As regards the other MTs, the most representative ones, with peak area > 15×10^6, were ß-myrcene, isoterpinolene, ß-sabinene, and ß-trans-ocimene in KP, and α-terpinene, ß-pinene, geranial, neral, and *p*-cymene in the LP. It is well known that neral and geranial (terpenoid isomers known as citral) are responsible for the strong lemon aroma; they were not detected or detected at only very low level in the KP, the KWF, and the GP samples. Apart from limonene, ß-myrcene was the monoterpene found in appreciable amounts in the GP and the KWF samples (20.92×10^6 and 1.43×10^6, respectively). As far as STs are concerned, the KP contained more compounds with peak area > 10×10^6, such as trans-caryophyllene, δ-cadinene, β-cubebene, and germacrene D, whereas germacrene D and β-bisabolene were found in the GP and in the LP, respectively. This suggests that the KP liqueur is characterized by greater aroma complexity compared with the other samples investigated. The KWF had the particular feature to be the richest in STs alcohols (α-bisabolol, fonenol, veridiflorol, cedrenol, and globulol). These compounds are the primary constituents of the essential oil, conferring a weak sweet floral aroma, and are used commercially in various fragrances. Among the OCs, geranyl acetate and neryl acetate were detected at the highest level in the LP (peak area value about 90×10^6). They were also found in the other liqueurs under examination, but at lower levels. These esters were associated with fresh and citrusy notes (THI MINH TU *et al.*, 2002), and are used in flavor and perfumery products to impart floral and fruity aromas. The KP liqueur also contained high levels of octanal and decanal (peak area values about 14.34×10^6 and 54×10^6, respectively). This finding does not agree with the results of previous studies carried out on the volatile fraction of kumquat essential oil, in which octanal and decanal were not detected or detected only at trace level (UMANO *et al.*, 1994; PENG *et al.*, 2013). These two aldehydes are commonly detected both in *Citrus sphaerocarpa* peel oil (THI MINH TU *et al.*, 2002) and in orange essential oil (HÖGNADÓTTIR and ROUSSEFF, 2003). At GC/Olfactometry analysis, octanal was associated to sweet, citrusy, lemon and green descriptors, whereas decanal was perceived as sour, metallic, lemon and fatty (THI MINH TU *et al.*, 2002; HÖGNADÓTTIR and

ROUSSEFF, 2003). In our study, the presence of 2 acetals corresponding to the 2 aldehydes, 1,1-diethoxyoctane, and 1,1-diethoxydecane, was ascertained. 1,1-Diethoxyoctane has an odor of fatty, oily, green citrus with woody, spicy and fruity nuances (MOSCIANO, 1994a), whereas 1,1-diethoxydecane presents an odor defined as waxy, green, aldehydic and orange with cognac and coconut nuances (MOSCIANO, 1994b). The 2 acetals were most prevalent in the KP and the LP liqueurs, and their presence was due to the high level of the aldehydes. In fact, acetals originate from the reaction between alcohols and aldehydes, giving rise to an unstable semiacetal, which evolves to a stable derivative after reacting with a second alcohol molecule (HEYDANEK and MIN, 1976). PLUTOWSKA *et al.* (2010) found acetals as minor compounds in alcoholic beverages and spirits, with a possible role in enhancing the bouquet of the product.

3.2. Acceptance and preference testing

Figure 3 reports the results of the sensory analysis. No significant difference among samples was perceived regarding color, which ranged between 3.4 and 3.9 (for the GP and the LP, respectively). The LP was the most appreciated sample as to flavor. This result could be due to the higher concentration of volatile compounds with a high olfactory impact (in particular OCs) and the greater familiarity of consumers with this traditional Italian liqueur. Regarding the liqueurs obtained from kumquat, the odor scores were 2.89 for the KP and 3.20 for the KWF, while the flavor scores were 3.01 and 3.32 highlighting a certain appreciation by consumers. This could be linked to the high amounts of sesquiterpene alcohols. The grapefruit liqueur was more significantly preferred for its odor in comparison to the two kumquat liqueurs.

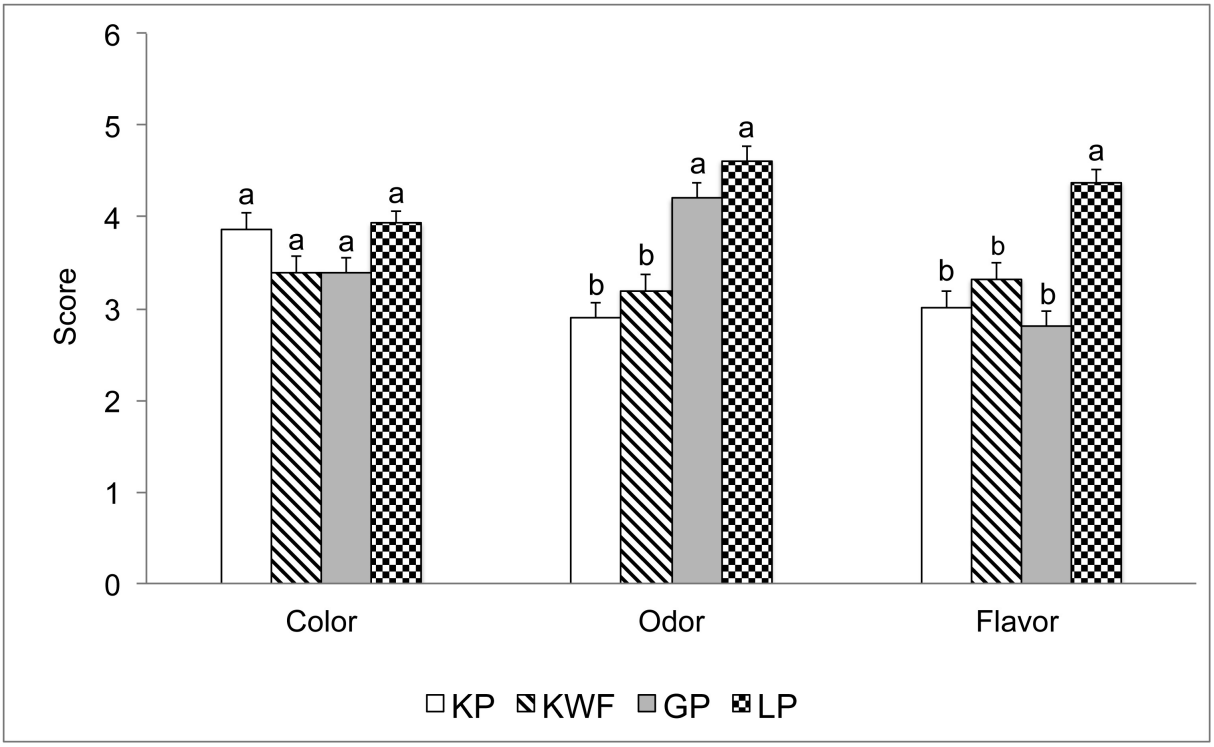


Figure 3: Mean score values and statistic analyses of the consumer acceptance of the liqueurs. KP, kumquat peel; KWF, kumquat whole fruit; GP, grapefruit peel; LP, lemon peel. Values having different subscript letters are significantly different (P<0.05).

Table 2 shows the ranking of preferences expressed by the panelists (third section of the evaluation form). In the same table the result of the statistical analysis obtained comparing the differences among the rank sums of the single liqueur with the critical value, as proposed by BASKER (1988), is reported. The rank sums were obtained by adding the preference ranking scores (from 1 to 4 starting from the most appreciated liqueur) expressed by each panelist. As expected, the LP proved to be the most appreciated liqueur, whereas the KWF and the KP were more appreciated than the GP. On the whole, the taste panel data highlighted the possibility of using kumquat for the production of *rosolio*, even though adjustments in the preparation process (fruit to ethyl alcohol ratio; duration of the maceration step) should be carried out to improve the odor and flavor perception. The use of the whole fruit instead of the peel determined an increase in the preference by the taste panel members; therefore, the peeling step could be avoided.

Table 2: Rank sums of preferences and results of the statistical analysis (BASKER, 1988) of the liqueurs.

			KP	**KWF**	**GP**	**LP**
		Rank sums	*206*	*183*	*230*	*122*
KP	BC	*206*	0	23	24	**84**
KWF	B	*183*	23	0	**47**	**61**
GP	C	*230*	24	**47**	0	**108**
LP	A	*122*	**84**	**61**	**108**	0

ABC: Different letter indicates significant differences (n = 75, $P \leq 0.05$, critical value = 40.6).
Bold font indicates values higher than critical value.

4. CONCLUSIONS

The main result of this study was the assessment of the feasibility of using minor citrus fruits for producing innovative liqueurs. With respect to *limoncello*, the kumquat and grapefruit liqueurs had greater concentrations of limonene and lower concentration of oxygenated compounds, the latter having high relative flavor activity. These differences justify the preference given to the liqueur obtained from lemon peel, even though all the innovative liqueurs were judged to be acceptable. Among them, the kumquat liqueur obtained from whole fruit seems to be the most promising, probably because it had the highest content of sesquiterpene alcohols.

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PAPER

EFFECT OF TEMPERATURE, GUM CONCENTRATION AND GUM RATIO ON CREEP-RECOVERY BEHAVIOUR OF CARBOXYMETHYL CELLULOSE-GUAR GUM MIXTURES: MODELING WITH RSM AND ANN

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ABSTRACT

In the present study, the effects of temperature (15, 25, 35°C), gum concentration (1.5, 2.0 and 2.5 %) and gum ratio (25:75, 50:50, 75:25) on the creep-recovery rheological properties of carboxymethyl cellulose **(**CMC)-guar gum (GG) mixtures were investigated. Within the studied range of experimental design, recovery index (ΔJ) value of CMC-GG gum solution was analyzed based on the design factors. Experimental recovery index responses were modelled using RSM (R^2= 0.9711) and ANN (R^2= 0.9829).

Keywords: artificial intelligence, creep and recovery test, response surface methodologyyrheological properties

1. INTRODUCTION

Gums are widely used in food industry to stabilize emulsion, prevent ice recrystallization (KAYACIER and DOGAN, 2006). The use of two or more gums is a very widespread practice in the food industry due to synergistic interaction between two or more different gums (KAYACIER and DOGAN, 2006). Guar gum (GG) is used as an economical thickening and stabilizing agents in the food industry and is often combined with xanthan, locust bean gum (LBG), or carboxymethyl cellulose (CMC) to increase synergistic changes in viscosity or gelling behaviour (PRADO *et al*., 2005). In generally, gum or gum combination is used in coating, drug delivery and food additives such as dressings, sauces, cereal products, fruit filling, confectionary products and frozen and baked products (PAI *et al*., 2002).

Synergistic interactions of mixed gums have an attractive in the food industry due to reducing production costs and improving product quality and mouth feel (MAO and RWEI, 2006; NORZIAH *et al*., 2006). The determination of viscoelastic properties of product is very important for prediction of product stability in many processing, transportation and storage (STEFFE, 1996). To determine viscoelastic properties of the samples, creep-recovery test is a very crucial method for providing knowledge about the internal structure of material (DOLZ *et al*., 2008). Several studies have been conducted on creep of food materials for evaluating in emulsions (DOLZ et al., 2008; YıLMAZ *et al*., 2012), salad dressing (ZHANG *et al*., 2008), milk (BAYARRI *et al*., 2009), tomato juice (AUGUSTO *et al*., 2013), instant pudding (DOGAN *et al*., 2014). DOGAN *et al*. (2014) also evaluated the effect of four different gums (carrageenan, alginate, guar and xanthan gum) and their different combinations on creep-recovery properties of the pudding samples.

Foods are very complex systems due to possible interactive effects of factors such as temperature, gum type, gum concentration etc. To reduce the number of extensive experimentation and learn about complex interactive effects, modeling and optimization is an inevitable stage for a process (BAS and BOYACı, 2007; MINGZHI *et al*., 2009). Therefore, optimization of the mixed gum formulation is important for both economical and rheological characterization of product (DOGAN *et al*., 2014). For this purpose, response surface methodology (RSM) is the mostly used statistical methods in optimization of rheological properties parameters. However, RSM does not adequately explain nonlinear models (Ross, 1999). To overcome this problem, nonlinear models based artificial intelligence models such as artificial neural networks (ANN) can be considered. ANN has been successfully applied in modeling of creep-recovery behavior for used in formulation of instant pudding (DOGAN *et al*., 2014), deformation of the grape molasses (TOKER and DOGAN, 2013) and construction of predictive models in meat emulsion (YıLMAZ, 2012).

There are many studies have been conducted on the synergistic behavior of gum mixtures like carrageenan-locust bean gum (TAKO and NAKAMURA, 1986), Xanthan-locust bean gum (COPETTI *et al*., 1997), Xanthan-modified guar gum (PAI and KHAN, 2002), CMC-arabic gum (GARCIA-ABUIN *et al*., 2010). However, there is a limited number of studies in the literature using creep and recovery test in order to evaluate and optimize gum combinations. To the best of our knowledge, no published literature was found on the effect of gum concentration, temperature and gum ratio on mixed gum (CMC-GG) optimization by response surface methodology approach based on creep-recovery parameters of the gum combination.

The aim of the this study was to observe the effects of gum concentration, temperature and gum ratio and their interaction terms on the creep-recovery characteristics of CMC-GG mixture samples and then to perform desirability function to determine optimum percentage deformation of mixture by considering several factors simultaneously.

2. MATERIALS AND METHODS

2.1. Materials

Polymer powders of guar gum (GG) and sodium carboxylmethyl cellulose (CMC) (Sigma-Aldrich Corp, St Louis, MO), with nominal molecular weight of 700,000 g/mol were kindly provided by Dr. Kerim YAPICI, Nanotechnology Engineering, Sivas, Turkey.

2.2. Preparation of gum solutions

The CMC and GG powders were dissolved in distilled water seperately at 25°C for 6 h using a magnetic stirrer with gentle shaking in order to prepare 1.5, 2 and 2.5% (w/v) stock solutions of CMC and GG. The CMC stock solution was thoroughly mixed with GG solution at different volume ratios, CMC:GG, such as 75:25; 50:50 and 25:75 respectively.

2.3. Rheological measurements

Creep and recovery tests of the samples measurements were carried out by a stress controlled rheometer (Malvern Kinexus Pro, UK) fitted by a cone-and-plate system. Cone and plate geometries are well suited for studying viscoelastic (stress growth, creep and dynamic) properties of foods. Diameter and angle of the cone were chosen 50 mm and 1°, respectively. The gap between the cone-and-plate was fixed at 0.05 mm for all measurements. A peltier plate assembly was used for temperature controlling purpose during the measurements with ±0.1°C precision.
Firstly amplitude sweep over a stress was performed under at a constant frequency of 1 Hz frequency at 25°C to find linear viscoelastic region (LVR). Then, stress sweep tests were conducted between 0.1 and 10 Hz to determine LVR. Creep – recovery tests were carried out under the constant shear stress of 0.05 Pa (in LVR) at three different temperatures (15, 25, and 35°C) to measure the deformation of mixtures. As known, creep-recovery tests include two parts, one of which is creep phase in which 0.05 Pa. Constant stress was applied for 120 s and the compliance values were recorded as a function of time. In the second part, which is recovery part, the applied stress was removed and then compliance values were also obtained as a function of time for 120 s. In creep-recovery test, the stress response of samples under constant stress in a total time of 240 s was measured. Each measurement was replicated two times. The effect of gum concentration, temperature and gum ratio on the creep-recovery characteristics data of CMC-GG mixed gel was examined. Recovery index or recovery percentage (ΔJ) is used to quantify the viscoelastic response of a materials as given in Eq. (1). The following equation as proposed by DOLZ *et al*. (2008) was used to calculate the percentage deformation of each gum mixtures.

$$\Delta J = 100\frac{J(120) - J(240)}{J(120)} \qquad (1)$$

In this equation J(120) and J(240) refer to the values of the compliance at the final creep phase and at the final recovery phase, respectively.

2.4. Experimental design and response surface methodology (RSM)

One of the models to describe chemical processes in analytical methods is quadratic model

in comparison with linear and cubic equation. The design consists of three replicated center points. The individual and combined effects of temperature, gum concentration and gum ratio on recovery percentage were studied using Box–Behnken response surface method (BOX and BEHNKEN, 1960) (BBD) using MINITAB 16.0 (Minitab Inc. State College, PA, USA) (Table 1).

Table 1. Factors and their coded levels of BBD.

Independent variable	Representation	Low (-1)	Center point (0)	High (+1)
Temperature (°C)	X1	15	25	35
Gum concentration (%)	X2	1.5	2.0	2.5
Mixed gum ratio	X3	25:75	50:50	75:25

All creep and recovery tests were conducted in duplicate.
The collected data were fit to a quadratic mathematical model expressed as:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + E$$

where Y is the response (ΔJ, %), b's are regression coefficients. The independent variables; temperature, gum concentration and gum ratio were coded as X_1, X_2, and X_3, respectively. The analysis of variance (ANOVA) and regression analysis were performed to define the coefficients of the predictive model and significant terms using MINITAB 16.0 (Minitab Inc. State College, PA, USA). The optimum conditions for maximizing the apparent viscosity was determined using Response Optimizer tool in MINITAB 16.0 (Minitab Inc. State College, PA, USA).

2.5. Model validation

The constructed model was verified by additional trials at the optimal rheological properties of CMC-GG gum mixtures carried out in triplicate at the design conditions.

2.6. Artificial neural network (ANN) modeling

The input variables used in the ANN models were temperature (X_1), gum concentration (X_2), and gum ratio (X_3). The ANN model was generated as output variable recovery index (ΔJ, Y_1). Several ANNs with different numbers of hidden layer neurons was developed for describing creep behavior of gum mixtures. Input data were randomized into three sets: learning, validation and testing. Usually 30% of data are used for testing and remaining 70% for training and validation (MEHDIZADEH and MOVAGHERNEJAD, 2011). The experimental data including 30 data points was divided so that 70% was used to train the model, 15% was used to validate the model and 15% was used to test the generalization ability of the model. Activation function of hidden layer was "logsig" and the one in output layer was "purelin" as shown in Equations (2-3).

$$\text{logsig}(x) = \frac{1}{1 + e^{-x}} \qquad (2)$$

$$\text{logsig}(x) = \frac{1}{1 + e^{-x}} \quad (3)$$

Training of the network was performed with the function of "trainlm", that updates weight and bias values according to Levenberg-Marquardt optimization. In learning of network "learngd" as adaption learning function was used. The maximum training epochs were 1000, and mean square error was 0.0001. The other parameters of neural network were taken as defaults of neural network toolbox, MATLAB R2011a. The performance of the ANN was statistically measured by the mean squared error (MSE), average relative deviation (ARD) and the coefficient of determination (R^2). These statistics were used to assess the performance of model predictions compared to the actual data. The R^2 represents how well the approximated function predicts the response versus just using the response mean. Values closest to 1 are the best. The MSE is a representation of the difference between the predicted and actual values and gives a sense of how close the predicted values are to the observed values in the units of those values. Lower values of MSE are best and it was calculated using the formula in Equation 4. The ARD represents the percent error. Lower values are best and it was calculated using the formula in Equation 5.

$$\text{MSE} = \frac{\sum_{1=1}^{n} \left(X_{\text{pre,i}} - X_{\text{exp,i}}\right)^2}{n} \quad (4)$$

$$ARD = \frac{100}{n} \sum_{i=1} \frac{\left(X_{pre,i} - X_{\text{exp},i}\right)}{X_{\text{exp},i}} \quad (5)$$

where $X_{pre,i}$ is the predicted output from observation i, $X_{exp,i}$ is the experimental (target) output from observation i, $\bar{X}$ is the average value of experimental output, and n is the total number of data.

2.7. Selection of the best model

To compare the constructed models and decide on the best one among them, performances of the models was measured using root mean square error (RMSE), mean absolute error (MAE), and coefficient of determination (R^2), which are common comparison tools in modeling. A statistical difference measure test was also carried out to evaluate the performance of the model by calculating root mean square error (RMSE) and mean absolute error (MAE) values as follows:

$$RMSE = \left(\frac{1}{N} \sum_{i=1}^{N} \left(X_{pred,i} - X_{\text{exp},i}\right)^2 \right)^{0.5} \quad (6)$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} \left| X_{pred,i} - X_{\text{exp},i} \right| \quad (7)$$

where X_{exp} is the experimental value and X_{pred} is the predicted value of recovery index (ΔJ, %) value, N is the number of data.

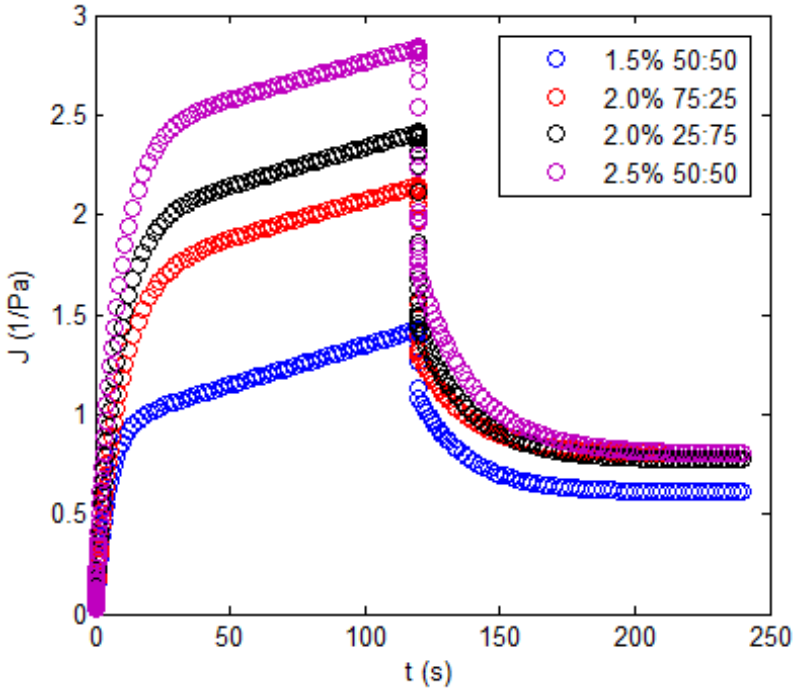
2.8. Statistical analysis

Statistical analyses were accomplished using MINITAB 16.0 to test the significance of different rheological properties of mixed gums. The pairwise comparisons were made by Tukey's test with a significance level of 0.05.

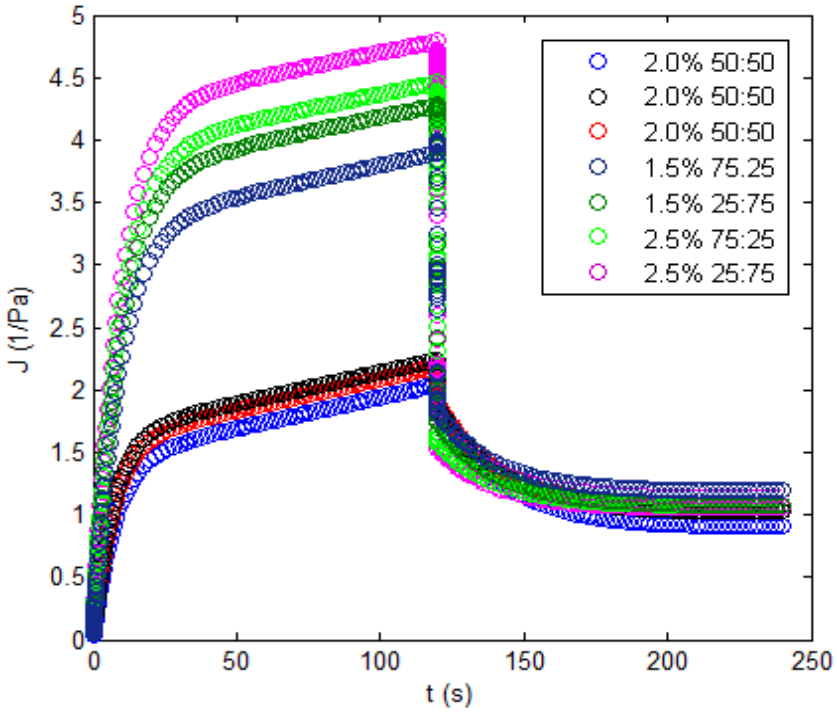
3. RESULTS AND DISCUSSION

3.1. Effect of concentration, ratio and temperature on the creep behaviour of gum mixtures

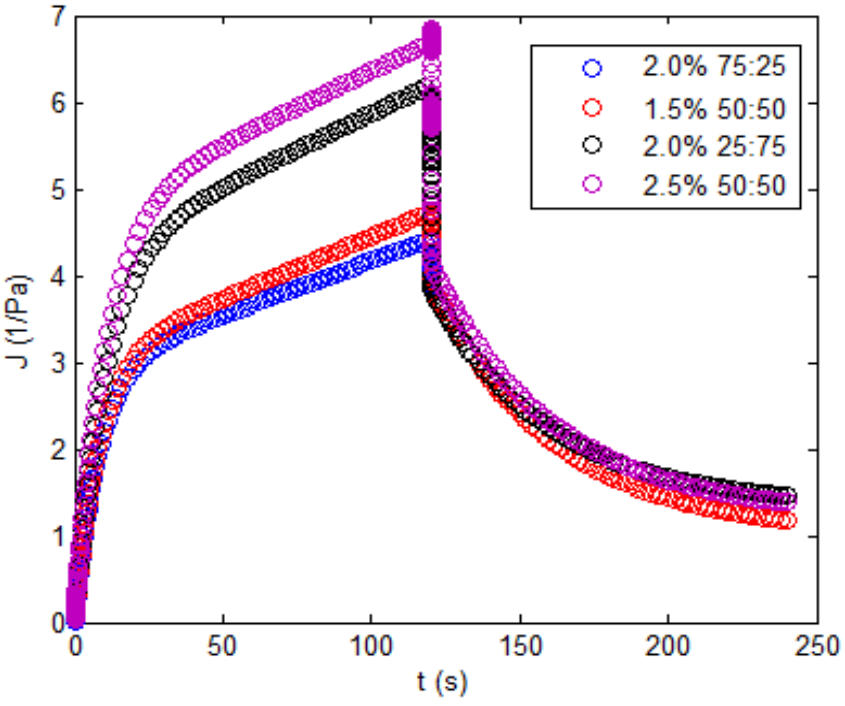
Fig. 1a-c shows graphical representation of creep and recovery experimental results for corresponding to all the mixed gum studied at 15, 25, 35°C respectively, in a time interval between 0 and 240 s. Following the application of stress, 0.05 Pa, deformation occured and deformation was recorded as function of time, J=f(t), during the rheological creep and recovery measurements. At time, t=120, J(120) corresponds maximum deformation value. In Fig. 1c, J(120) values are nearly 7 Pa^{-1} for 2.5% 50:50 at 35°C. On the other hand, it has reached to 2.8 Pa^{-1} at 15°C for the same level as seen in Fig. 1a. This implies that J(120) values gets higher value due to weaking internal structure of gum mixtures and thus higher deformability and more softening structure at 35°C. SOZER (2009) also reported that high compliance value shows weak structure of material.



A) T=15°C



B) T=25°C

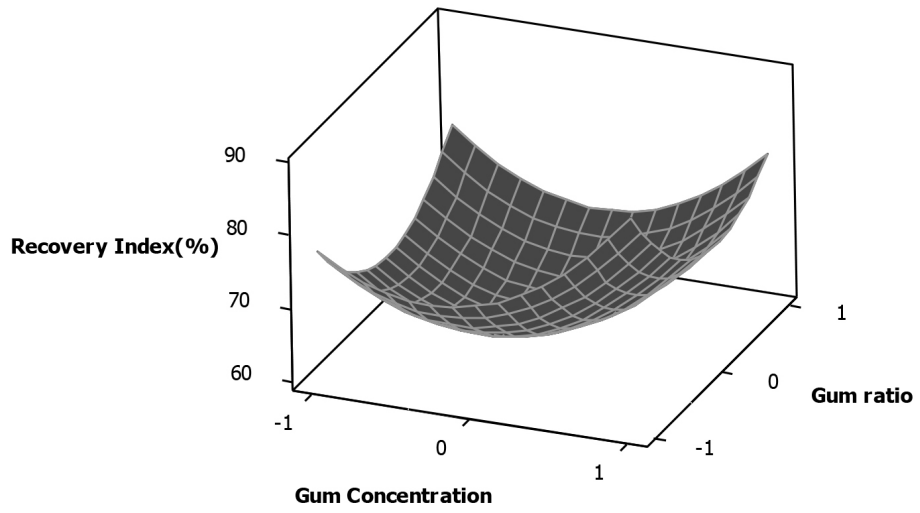


C)T=35°C

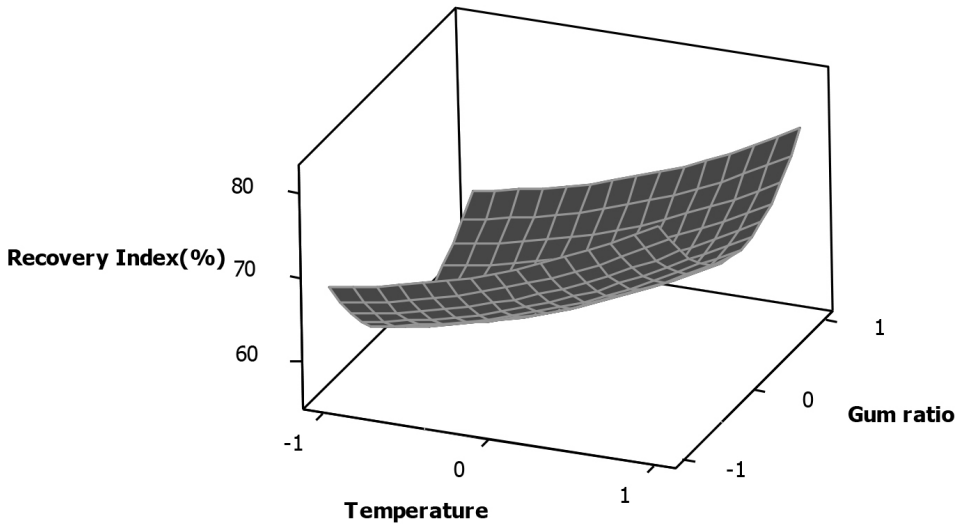
Fig. 1. Creep and recovery plot of gum mixtures for different temperature levels a) T=15°C b) T=25°C c)T=35°C.

CMC and GG is used as thickener and stabilizer in food processing. During food processing, temperature takes critical role due to affected rheological properties of food systems. Upon increasing temperature, it causes a change deformation structure of the gum mixtures. Gum mixture structure is weakened with increase of temperature. Creep compliance value, J(t), increases with temperature due to the related with the softness of the material structure (HAYTA and SCHOFIELD, 2005). These data also show that the recovery index in all gum mixtures is positively correlated with temperature at the fixed gum concentration and ratio (Fig. 2b-c). The drop in viscoelasticity at low temperature level comes from strong or stiffer structure of gum mixtures. On the other hand, there is no significant change for the effect of gum ratios on the recovery indexes for the fixed gum concentration at 15°C (p>0.05).

a)



b)



c)

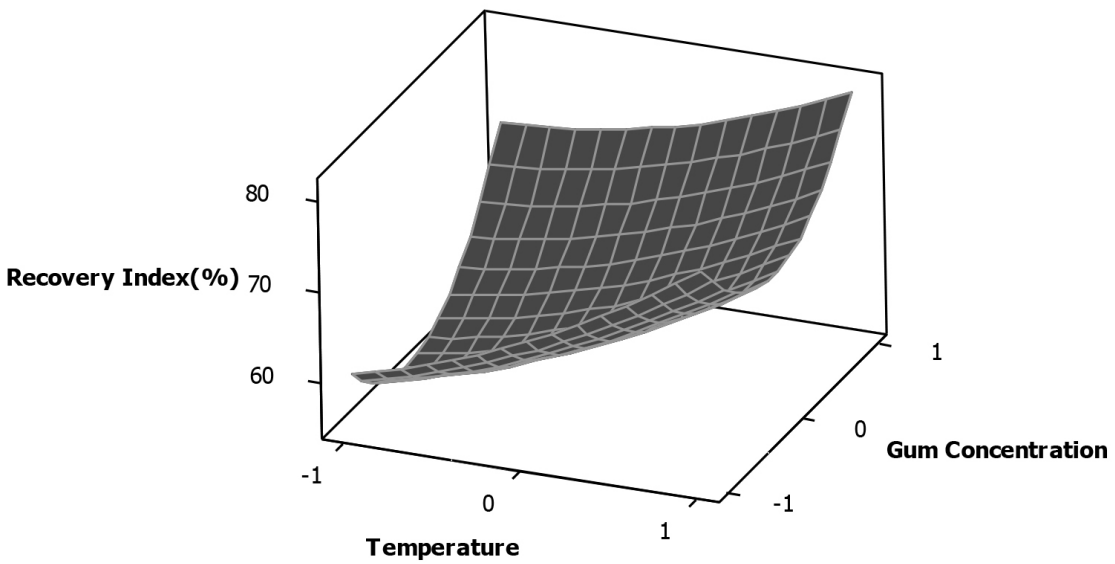


Fig. 2. Response surface showing the effect of a) gum concentration vs gum ratio, b) temperature vs gum ratio, c) temperature vs gum concentration on rheological properties of gum mixtures.

As CMC-GG mixtures concentration increased, recovery index values get higher and viscoelastic response of the mixtures is pronounced (Fig.2a). It is resulted from intermolecular interaction between CMC-GG mixture depended strongly concentration of the mixture. For 2.5% (w/v) gum mixture showed higher compliance, J(t), values indicating higher deformation than concentration of 1.5 and 2% (w/v) for all temperatures as depicted in Figs. 1a-c.

Recovery index, calculated as in Eqn. 1, has changed with gum ratio for 1.5% (w/v) and 2% (w/v) gum mixtures. When the mixed gum has 2% 50:50, designated as center model points, lower deformation is significantly achieved in Fig. 1a and b. Conversely, the strongest synergistic effect on recovery indexes has been observed when changed with mixed ratio (25:75 and 75:25) tabulated as in Fig. 1b. It is associated with increased elastic nature of gum mixture especially in 25:75 gum ratio in higher presence of GG at all temperature levels. Guar gum which highly extend in gum mixture entangles with network structure and binds with CMC. Furthermore, guar gum more dominates the structural arrangement in higher gum mixture indicating that is highly elastic nature and recovery indexes attains to 87.50% at 25°C shown in Table 2.

The results also suggested that hydrophilic intrinsinc interaction between CMC-GG (TIPVARAKARNKOON and SENGE, 2008) which leads to the increasing softening gum structure. Therefore, viscoelastic properties of the mixtures in the aqueous medium are enhanced. Even in lower concentration of gum mixture, significant synergetic effect was also observed for 1.5% 25:75 and 75:25 mixtures in Fig. 1b. It could be related that lower degree entanglement of gum mixture at lower concentration permits to the more elastic effect contribution on mixture nature at 25°C.

Table 2. Experimental measured values according to Box-Behnken response surface method (RSM) versus predicted values of RSM and ANN models for recovery index (%).

Temperature (°C) (X_1)	Gum conc. (%) (X_2)	Mixed gum ratio (X_3)	Recovery Index (%) (Y_1)		
			Experimental	RSM pred.	ANN pred.
35	1.5	50:50	77.24±3.04 ^{bc}	77.90	76.45
25	2.0	50:50	61.52±1.89 ^{f}	60.84	61.36
15	1.5	50:50	58.80±2.45 ^{f}	60.25	58.75
15	2.0	25:75	68.44±0.73 ^{de}	68.24	68.00
35	2.0	25:75	81.68±1.48 ^{ab}	81.86	80.71
25	2.5	75:25	77.91±0.30 ^{bc}	78.96	77.09
15	2.5	50:50	72.08±0.06 ^{cd}	71.42	71.49
25	1.5	25:75	78.11±0.60 ^{bc}	77.05	77.29
35	2.5	50:50	82.48±1.97 ^{ab}	81.01	81.48
35	2.0	75:25	76.00±0.86 ^{bc}	76.63	75.26
25	2.0	50:50	59.96±2.93 ^{f}	60.84	59.86
25	2.0	50:50	61.06±1.27 ^{f}	60.84	60.91
15	2.0	75:25	63.64±3.52 ^{ef}	63.01	63.39
25	2.5	25:75	87.50±0.16 ^{a}	88.56	86.30
25	1.5	75:25	77.27±3.05 ^{bc}	76.19	76.48

Data within the same column with different superscript letter are significantly different (p <0.05).

When higher presence of CMC (75:25) at the 25°C, recovery index value (77.91%) gets smaller value as compared value at 25:75 ratio revealing that more CMC addition led a smaller viscoleastic deformation of mixture and viscous behaviour of mixture increase at the 25°C. Hence, viscous flow deformation gains to importance for the higher CMC content of solution and viscous properties of mixing also increase at 35°C As seen in Fig. 1c, maximum compliance value of 1.5% 50:50 of solution is nearly same than 2.0% 75:25 of solution. It suggests also that strong viscoelastic effect of GG plays major role in determining the direction of synergetic effect of mixed gums at 35°C.
For comprehensive understanding of interactions between concentration, mixing ratio, temperature study on optimization of creep properties of CMC-GG mixtures was achieved to get most resistant and stable product during the efficient food product design.

3.2. Identification of optimized rheological characteristics of carboxymethyl cellulose-guar gum mixtures conditions through response surface modeling

RSM is a frequently used technique for modeling and determining optimal process conditions. Estimation of response surface analysis of experimental results for prediction of recovery index value (ΔJ) in gum mixtures based on temperature (°C) (X_1), gum concentration (%, w/v) (X_2) and gum ratio (v/v) (X_3) per BBD (Table 2), were further elucidated to identify the best process conditions in the ranges tested. The highest recovery index of about 87.50% was obtained at 25°C, 2.5% gum concentration, and 25:75 of gum ratio, whereas the poorest conditions were 15°C, 1.5% gum concentration, and 50:50 gum ratio, giving about 58.80% of recovery index (Table 2).
Second-order polynomial equations (quadratic model) were established to identify the relationship between recovery index and three dependent variables: temperature (°C) (X_1),

gum concentration (%, w/v) (X_2) and gum ratio (v/v) (X_3). Table 3 shows the effect of process variables and interactions on recovery index.

Table 3. ANOVA table for BBD model.

Term	Coefficient	T Value	P Value
Intercept	60.84	76.69	**0.000**
Block	-0.576	-1.623	0.121
Temperature (°C) (X_1)	6.805	14.01	**0.000**
Gum conc. (%) (X_2)	3.569	7.347	**0.000**
Mixed gum ratio (X_3)	-2.612	-5.376	**0.000**
X_1* X_1	2.024	2.830	**0.011**
X_2* X_2	9.780	13.68	**0.000**
X_3* X_3	9.570	13.38	**0.000**
X_1* X_2	-2.013	-2.929	**0.009**
X_1 *X_3	-0.220	-0.320	0.752
X_2* X_3	-2.186	-3.182	**0.005**

R-Sq = 0.9711%; R-Sq(adj) = 0.9581 %; R-Sq(pred) = 0.9299 %.

Another run with excluded insignificant terms according to Table 4 expressed by Eqn. 8:

$$Y=60.84+6.81X_1+3.57X_2-2.61X_3-2.01\ X_1X_2-2.19\ X_2X_3+2.02X_1^2+9.78\ X_2^2+9.57\ X_3^2 \quad (8)$$

where Y is predicted recovery index (ΔJ, %). Equation 8 was found fairly adequate to represent the data with R^2 of 0.97. The insignificant lack of fit for recovery percentage was (P = 0.46 >0.05), which also proved that the model fit the experimental data well.

Table 4. Significance of term coefficients for BBD; A: Temperature (°C); B: Gum concentration (%); C: Mixed gum ratio.

Term	Coefficient	Standard Error Coefficient	T Value	P Value
Constant	60.84	0.775	78.47	0.000
A	6.805	0.475	14.33	0.000
B	3.569	0.475	7.518	0.000
C	-2.612	0.475	-5.501	0.000
A*B	-2.013	0.672	-2.997	0.007
B*C	-2.186	0.672	-3.256	0.004
A^2	2.024	0.699	2.896	0.009
B^2	9.780	0.699	13.994	0.000
C^2	9.570	0.699	13.693	0.000

All factors showed significant effects (p<0.05) on recovery percentage and the interactions between temperature-temperature, concentration–concentration, ratio-ratio interactions, temperature–concentration and concentration-ratio showed significant effects (Table 4). The most important factors determining the recovery index were temperature with highest

coefficient (6.81), which indicates that it is the most dominant factor influencing the overall recovery index value followed by gum concentration (3.57) and gum ratio (2.61) (Table 4).
Estimation of recovery index at varying process variables (temperature, gum concentration, and gum ratio) are represented in response surfaces plots shown in Fig. 2a-c. Fig. 2a shows the effect of gum concentration and gum ratio on the recovery index at mid-point temperature of 25°C. The recovery index of the gum -mixtures was strongly influenced by both variables. A decrease in gum ratio from 75:25 to 25:75 gave the highest recovery indexes of 87.50 % at about 25°C and 2.5 % gum concentration was observed (Fig. 2a). Gum ratio had the most negative effect on the recovery index, meaning that the increase in gum ratio caused decrease in deformation when stress was applied to gum mixture. On the other hand, the effect of temperature and gum ratio on recovery indexes is shown in Fig. 2b, where gum concentration was set at 2.0 % (w/v) as the center point. A linear increase in recovery indexes was observed as temperature increased (Fig. 2b). Similarly, the positive effect of temperature was observed in Fig. 2c, showing the effect of temperature and gum concentration on recovery indexes.
Therefore, to determine the optimal treatment conditions, the response optimizer tool in MINITAB® 16.1 (Minitab Inc.) was used. Optimization was performed regarding maximum recovery index value since low recovery index shows that mixed gums had higher resistance or lower deformation when stress is applied. Higher resistance of the sample against stress is important for product quality during handling and storage (DOGAN *et al*., 2014). Conditions for obtaining optimum recovery percentage of 95.38%, as predicted by the model, were found to be 35°C and 2.5 (%, w/v) and 50:50 gum ratio. These conditions were experimentally tested in triplicate to validate the models predictive ability. A recovery percentage of 90.34±2.79% was obtained and there was no significant difference between the experimental and model predicted values (p>0.05).

3.3. PREDICTIVE MODELING OF CREEP BEHAVIOUR USING ANN

3.3.1. Effect of architecture and topology on neural network

Selection of network topology in ANN modeling is the key issue. Several parameters such as the number of hidden layers, the number of neurons, the transfer function, the epochs and learning rate affected the network topology. The number of hidden neurons is one of the most important parameters of ANN modeling. A high number of neurons performs satisfactorily for training data but may fail for testing data (over-fitting), while a few hidden neurons cause unsatisfactory convergence (under-fitting) (TOKATLI *et al*., 2009). In this study, the number of neurons in the hidden layer was chosen by a trial and error method, varying the neurons from 3 to 12.
Several ANN models with different network topologies were trained, tested, and their performance was evaluated to select the best network topology. The R^2, RMSE, and ARD statistics from training and testing data for different ANN topologies is summarized in Table 5.
Table 5 shows that the 3-9-1 topology was chosen as the best topology (Fig. 3) with the minimum MSE and ARD, and maximum R^2 values. In case of training data set, the coefficient of determination (R^2) and ARD values were 0.9889 and 0.003%, respectively, whereas in testing data set, R^2 was 0.9866 and ARD was 0.009% (Table 5). ANN with 3-9-1 topology was also performed (R^2 values of 0.9889, 0.9784, 0.9866 and MSE values of 1.8478, 6.3565, 4.5024 in training, testing and checking (validation) periods, respectively) (Table 6).

Table 5. Performance of different Artificial Neural Network (ANN) model in estimation of Recovery Index (%).

	R^2		MSE		ARD (%)	
Network	**Training**	**Testing**	**Training**	**Testing**	**Training**	**Testing**
3:3:1	0.9820	0.6355	2.49	145.79	0.005	0.080
3:4:1	0.9942	0.9364	0.88	11.50	0.0003	0.004
3:5:1	0.9859	0.9664	2.17	4.24	0.002	0.039
3:6:1	0.9321	0.9091	12.85	26.97	0.006	0.025
3:7:1	0.9889	0.8964	1.85	4.46	0.002	0.002
3:8:1	0.9806	0.9445	3.38	10.67	0.0005	0.016
3:9:1	**0.9889**	**0.9866**	**1.85**	**4.50**	**0.003**	**0.009**
3:10:1	0.9926	0.9692	1.27	6.21	0.005	0.043
3:11:1	0.9910	0.9888	1.30	11.69	0.002	0.037
3:12:1	0.9970	0.9372	4.47	12.07	0.004	0.031

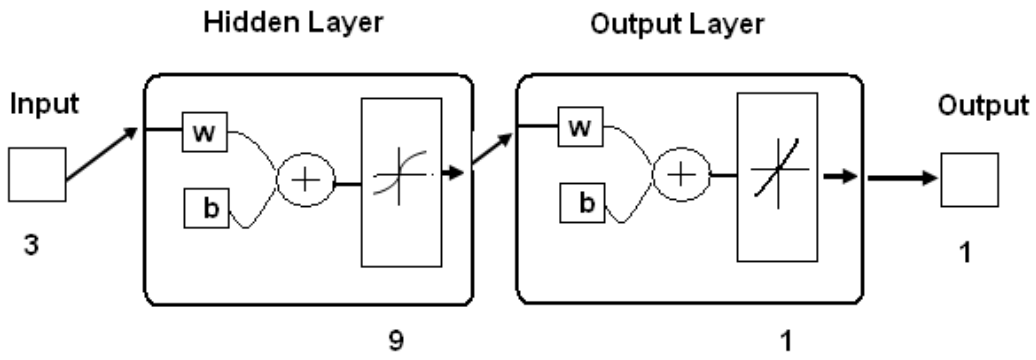


Fig. 3. Schematic representation of ANN to simulate the rheological properties of gum mixtures.

Table 6. Performance of Neural Network Model For Prediction of Recovery Index (ΔJ) Values of Gum Mixture

	Statistical Parameter	Training	Testing	Validating	Overall
Recovery Index (%)	R^2	0.9889	0.9784	0.9866	0.9829
	MSE	1.8478	6.3565	4.5024	3.0417

3.4 Comparison of RSM and ANN models

Fig. 4 shows the plot of predicted recovery index by RSM and ANN against the experimental values. The ANN model exhibited a better relationship between experimental and predicted recovery index values than the RSM model. The results showed that the ANN predictions were closer to experimental values than those of RSM.

Though both the models based on RSM and ANN performed well and offered stable response in prediction; the ANN based approach was better in predicting and fitting than the RSM model for testing the data. Thus, the accuracy of ANN model was higher and has better fitted data than the RSM model.

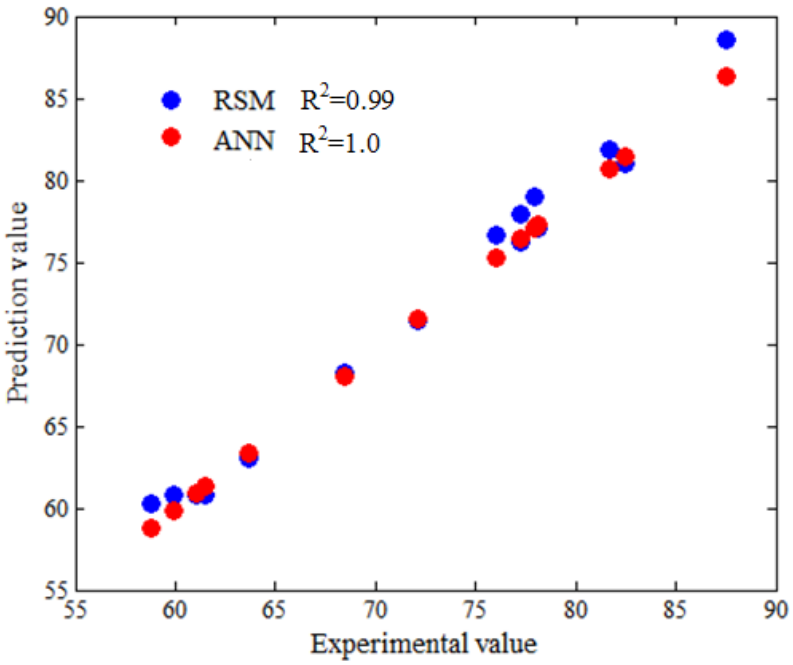


Fig. 4. Verification predicted values versus actual experimental values of RSM and ANN models for rheological properties of gum mixtures.

4. CONCLUSIONS

Detailed creep and recovery viscoelastic measurements at contant 0.05 Pa revealed that recovery index (ΔJ) remarkably changed according to temperature, gum concentration and gum ratio. Elastic or viscous effects contribution on the gum mixture structure affected the creep properties of CMC-GG mixtures. Within the studied range of experimental design, higher content of GG of mixed gum solution increased ΔJ value at all levels. In addition to mixed gum ratio, synergisim effect of gum mixtures was observed especially at 25°C. On the other hand, deformation properties of gum mixtures significantly changed with temperature.

Furthermore, optimization and modeling of gum mixtures are taken into consideration to obtain both best quality of product and economical point of the view. Experimental recovery index responses were optimized using prediction models such as response surface methodology (RSM) and artificial neural network (ANN) models. Several ANN topologies were evaluated in order to predict creep test for gum mixtures. These two methods were also compared for their predictive abilities. The best fit model was identified in ANN model with the highest coefficient of determination (R^2) and lowest MSE values. Therefore, ANN can be considered as a prediction tool for rheological properties of gums mixtures.

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GALACTOMYCES GEOTRICHUM ISOLATED FROM WATER KEFIR: INTERACTION WITH *LACTOBACILLUS KEFIR*

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ABSTRACT

In this study, a yeast-like fungus strain wk1 isolated from water kefir grains was identified as *Galactomyces geotrichum*, which had great adhesion ability with *Lactobacillus kefir*. Exposure to lithium chloride aroused the marked loss of attached bacteria, while acetic acid treatment led to a thicker adhesion. Heat and sodium hydroxide treatment had non-significant effect on the interaction. Furthermore, *G. geotrichum* wk1 showed its hydrophobic and basic phenotype with strong electron donor property, contributing to the extremely strong auto-aggregation ability. These properties were greatly weakened after all pretreatments, except LiCl treatment, coincided with the low auto/co-aggregation ability. The results in this study may be helpful to understand the interactions of participating bacteria and fungi in the kefir grains and its role for the grain formation.

Keywords: aggregation, interaction, *Galactomyces geotrichum*, hydrophobicity, kefir, *Lactobacillus kefir*

1. INTRODUCTION

Water kefir, or sugary kefir, is a fizzy and cloudy lacto-fermented homemade beverage, low acid, somewhat sweet and slightly alcoholic, which is prepared by inoculation of sucrose solution or fruit juice with kefir grains containing different species of health-promoting bacteria and fungi (GULITZ *et al*., 2011; ALTAY *et al*., 2013). Nowadays, water kefir with great probiotic characteristics has been taken great attention from scientists and consumers due to the trends towards natural, nutritional, health-promoting and flavor rich beverages, and also the advantages of sucrose breakdown compared to common soft drinks, making it a perfect alternative for those who wish to avoid dairy or soft drinks, or just want another tasty addition to their probiotic menu (MOREIRA *et al*., 2008; SILVA *et al*., 2009).

However, unlike milk kefir, there are only few data available about the analysis of the water kefir consortia and the interactions of participating microbes, especially fungi. Several reports have explored the microbes present in water kefir. GULITZ *et al*. (2011) obtained a collection of 453 bacterial isolates from water kefir with the viable cell numbers (cells/g) ranging from 1.2×10^{6} to 5.6×10^{8}, and the viable yeast cells from 5.8×10^{6} to 2.7×10^{7}. Among yeasts, *Saccharomyces cerevisiae*, *Lachancea fermentati*, *Hanseniaospora valbyensis* and *Zygotorulaspora florentina* were identified, and *Z. florentina* is the yeast species which is the predominant yeast species. Other researchers (WITTHUHN *et al*., 2005; MAGALHAES *et al*., 2011) also reported similar results. Lactic acid bacteria and yeasts were considered to be important for microbiota and grain formation.

In an unpublished previous study, we collected water kefirs of different long term traditional household preparations. Microorganisms in water kefir grains were isolated using standard microbiological media, and one strain isolate of *Galactomyces geotrichum* was obtained. To the best of our knowledge, this is the first time that *Galactomyces geotrichum* was isolated from water kefir. In addition, interestingly, during the isolation, we found that the strain always appeared with the adhesion of lactic acid bacteria around its surface, which indicated it may play an important role to the formation of stable microbiota in kefir grains due to the interaction with lactic acid bacteria.

In general, the current studies on the microbial population and symbiotic interactions are carried out and laid special emphasis on the role of lactic acid bacteria on the basis of the biofilm production (WANG *et al*., 2012), lectin-like adhesion (GOLOWCZYC *et al*., 2009) and surface layer collagen binding protein (YADAV *et al*., 2013), and so on. Up to date, there's no detailed report on the interaction between *G. geotrichum* and lactic acid bacteria. On the other hand, previous researches on the adhesion or biosorption ability of fungi were mainly focused on the removal of dye, pesticide, heavy metals, and so on (BHATTI and AMIN, 2013; ALMEIDA and CORSO, 2014; HAROUNE *et al*., 2014; TANG *et al*., 2014). Few studies were carried out to evaluate the interaction occurred between fungi and bacteria. Herein, the objective of the present study was to describe preliminary investigation on the interaction of the isolate *G. geotrichum* and *Lactobacillus kefir* 12-2, which was also isolated from water kefir, to evaluate its possible contribution to the formation of kefir grains.

2. MATERIALS AND METHODS

2.1. Strains, media and cultivation

Water kefir grains were collected from ordinary families in Wuhan, Hubei province, China. Water kefir was prepared in sucrose solution (100 g/L), and the fermentation

carried out at 25°C for three days. The supernatant was discarded and the grains were used for further study. 10 g water kefir grains were diluted with 90 mL 0.1% (w/v) sterile peptone water, and then mechanically homogenized by a stomacher for 60 s. Serial dilutions were prepared by mixing 1 mL grain suspension with 9 mL of peptone water. The different serial dilutions were plated on MRS (Difco, USA) and YPD agar plates to isolate lactic acid bacteria and fungi in grains respectively. *Lactobacillus kefir* 12-2 (identification data not shown) was obtained and cultured in MRS broth at 37°C without shaking. The fungus (strain number wk1) was cultured in YPD broth at 28°C with shaking (150 rpm).

2.2. DNA extraction and sequence analysis of PCR amplicon

For the DNA extraction from overnight cultures of single colony, the Yeast DNAiso Kit (Takara Bio, China) was used according to the instruction. The DNA was amplified with primers NS1 (GTA GTC ATA TGC TTG TCT C) and NS8 (TCC GCA GGT TCA CCT ACG GA) spanning 18S rDNA gene (White *et al*., 1990). A total of 50 μL PCR reaction system contained 2 μL of the extracted DNA solution, 5 μL of 10×PCR buffer (50 mM KCL, 15 mM $MgCL_2$, 100 mM Tris-HCL, pH9.0), 2.5 U Taq polymerase, 0.2 mM each deoxyribonucleoside triphosphate, 25 pmol of each primer.
The amplification program was carried out under following conditions: 94 °C for 5 min for initial denaturation, then 34 cycles of 40 s at 94°C, 40 s at 50°C, and 40 s at 72°C, followed by a final extension step for 5 min at 72°C. The PCR products were electrophoretically analyzed by 1% (w/v) agarose gel. Direct sequencing of the PCR product was performed on a DNA sequencer. Sequence was submitted and deposited in GenBank database, and was compared as a query sequence with those deposited in GenBank database by means of blast algorithm.

2.3. Adhesive interaction assay

The adhesion ability of the fungus was evaluated using *Lactobacillus kefir* 12-2 as a model microorganism. The microorganism cultures were centrifuged at 3000×*g* for 5 min to remove the supernatant. The cells were washed twice and resuspended in sterile saline solution (0.75% w/v NaCl). The OD_{600} was adjusted to 0.8-1.0. Equal volumes of the fungus and *L. kefir* 12-2 cell suspension were mixed and incubated for 3 h at 30°C. After being spread on slide glass and stained with 1% crystal violet solution for 30s, the cell mixture was examined under a light microscope (Nikon Eclipse E100) with 10× ocular lens and 100× oil immersion lens.
In order to evaluate the adhesion activity of the nonviable or partially damaged cells, different pretreatments were used. Heat treatment at 121°C for 20 min was used to inactivate the fungus cells. Nonviable cells were then tested according to the method as above. For the assay to obtain partially damaged cells through the extraction of cell wall polysaccharide like glucans with hot-water (CORRADI DA SILVA *et al*., 2008), 1 M sodium hydroxide (NaOH) or 0.5 M acetic acid (AA), instead of saline solution, were used to resuspend the fungus cells, followed by incubation at 90°C for 3h. Besides, 5 M lithium chloride (LiCl) was also used but incubated at 37°C for 1 h. After pretreated in the three solutions, the fungus cells were centrifuged and re-suspended twice in 0.75% (w/v) NaCl solution, and then exposed to bacteria to evaluate the effect of cell damage on the adhesion ability of the fungus. Native cells without any treatment were defined as viable control.

2.4. Auto-aggregation and co-aggregation assay

Auto-aggregation and co-aggregation abilities were studied spectrophotometrically according to the method described before (COLLADO *et al*., 2008) with some modifications.
Microorganism suspensions were prepared as described before. The cell suspension was incubated at 30°C for 5 h. The supernatant was separated and the absorbance at 600 nm was measured using a spectrophotometer (Mapada UV-1800, Shanghai, China). Auto-aggregation (%) was expressed as $[1 - (A_T / A_0) \times 100]$, where A_T represents the absorbance of the supernatant at different times and A_0 is the initial absorbance at time T=0.
For the co-aggregation assay, 2 ml of cells of the fungus and *L. kefir* 12-2 were mixed and incubated at 30°C for 5 h. Absorbance values at 600 nm were measured at different times. Co-aggregation (%) was calculated as $[1 - A_{mix} /(A_1 + A_2)/2] \times 100$, where A_1 and A_2 represent the absorbance in control tubes containing only wk1 or the lactobacilli strain respectively, and A_{mix} represents the absorbance of the mix suspension at certain monitored times.

2.5. Cell surface hydrophobicity

Cell suspensions were prepared as described above. 3 mL of cell suspensions were mixed by vortexing with 1 mL of xylene, chloroform and ethyl acetate, respectively, and then incubated for 30 min at room temperature. The absorbance of the aqueous phase was measured at 600 nm. The hydrophobicity (%) was calculated as $(1\text{-}A_T/A_0) \times 100$, where A_T and A_0 represent the absorbance at time T = 30 min and 0, respectively.

2.6. Statistical analysis

All the experiments were carried out three times independently. The data are presented as means ± standard deviation. Analysis of variance (ANOVA) was carried out with SPSS Statistics software v20.

3. RESULTS AND DISCUSSION

3.1. Morphological and molecular characteristics of the yeast

The strain wk1 was cultured in YPD agar at 28°C for 5 days and the morphological characteristics of the fresh microculture were observed under microscopic observation. The strain presented a whitish and powdery circular colony without pigments (Fig. 1A). Mycelia are hyaline, septate, branched, 4.5-15 μm in width. Conidia, single or in chain, are long cylindrical and tip dulled, with the size of 4.7-10 μm × 8.1-22 μm (Fig. 1B).
Wk1 was further identified by amplication and sequencing of 18S rDNA with primers NS1 and NS8. An amplicon of 1694 bp was obtained and submitted to NCBI GenBank database. The sequence was deposited with the accession number KM357929. Sequence comparison showed the strain sharing 99% similarity with *Galactomyces geotrichum*. Molecular identification evidenced the microscopic characteristics very well. *G. geotrichum* is commonly the dominant species in milk products like yoghurt, cheese and so on (CHAVES-LÓPEZ *et al*., 2012; AKABANDA *et al*., 2013). DE HOOG and SMITH (2004) considered that *G. geotrichum* is a closely related, but a different teleomorph of the well-known yeast-like fungus, *Geotrichum candidum*. Since 2004, a recent taxonomic revision concluded that the old *Galactomyces geotrichum* complex contained four separate species

(POTTIER *et al*., 2008). *Galactomyces geotrichum* is wildly considered as teleomorph of *Galactomyces candidum*. ALPER *et al*. (2011) obtained 18 *G. candidum* strains from various environmental niches. The phylogenetic lineage analysis presented that all these 18 strains and *Galactomyces geotrichum* were located within a well-supported clade with 99% bootstrap value. The microbiota in milk/water kefir grains was related to its origin, to a great extent. The local endemic environment is very important to the biodiversity of the kefir grains. To the best of our knowledge, this is the first time to report that *G. geotrichum* was found in water kefir.

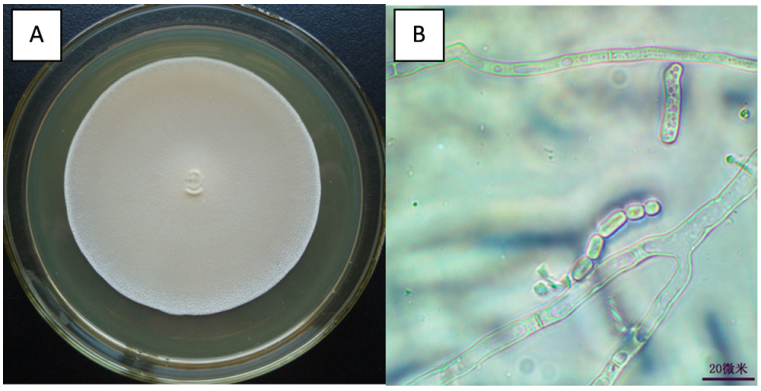


Figure 1: Colony on YPD agar plate (A) and micrograph (B) of *G. geotrichum* wk1. Scale bar 20 μm.

3.2. Adhesive interaction with lactic acid bacteria

During the isolation of microbes from kefir grains, the binding between *G. geotrichum* wk1 and lactic acid bacteria was observed. To investigate the attachment of LABs on the surface of *G. geotrichum* wk1, the two kinds of microbes isolated from water kefir grains were mixed and co-incubated. *G. geotrichum* wk1 exhibited the surprising interaction ability to all the tested LABs (data not shown). The adhesion with *L. kefir* 12-2 in this study was shown in Fig 2. *L. kefir* 12-2 arranged in lines and layers around the surface of *G. geotrichum* wk1, seems like forming a bacterial film outside.

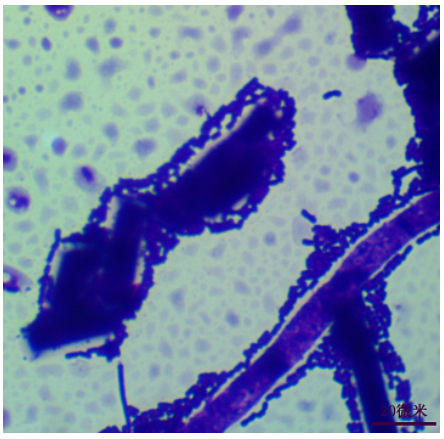


Figure 2: Interaction between *G. geotrichum* wk1 and *L. kefir* 12-2. Scale bar 20 μm.

3.3. Effect of pretreatment on the adhesion capacity

In order to investigate the interaction between inactivated or partially damaged *G. geotrichum* wk1 and *L. kefir*, different pretreatments including heating and treated with NaOH, AA and LiCl were performed. Results showed that adhesive patterns were significantly different between native and damaged fungal cells.

Pretreatment of heating at 121°C for 20 min inactivated the cells, and changed the cell morphology mildly, causing the blunting of the ends of conidia and the decrease of the binded bacteria, to some extent (Fig. 3A). Treatment with NaOH and AA, offering basic and acid conditions to *G. geotrichum* respectively, led to totally different effects on the interaction of *G. geotrichum* and *L. kefir*.

Based on the fact that fungal cell walls are mainly composed of polysaccharides such as α- and β-glucans, mannans and chitin (AHRAZEM *et al*., 2002), and alkali or acid were often used to extract the polysaccharides from the fungal cell wall at high temperature (CORRADI DA SILVA *et al*., 2013; CHEN *et al*., 2013), we deduced that exposure of *G. geotrichum* to NaOH led to the degradation of the components in cell wall, mostly alkali-soluble polysaccharides. CORRADI DA SILVA *et al*. (2008) obtained three D-glucans from the mycelium of the fungus *Botryosphaeria rhodina* MAMB-05 by sequential extraction with hot-water and hot aqueous KOH (2% w/v). In our experiment, we set the high temperature condition at 90°C. After exposure to NaOH at 90°C, *G. geotrichum* was observed to have a weaker binding with crystal violet, which was reflected by the obviously weak appearance of the cells in the microscopic figure. But the damage of cell in NaOH did not weaken the adhesive capacity of wk1 greatly (Fig. 3B). In contrast, AA treatment led to a thicker binding of *L. kefir* around *G. geotrichum* (Fig. 3C). This finding may explain the stable microbiota consortia in the acid environment of water kefir.

Unlike NaOH and AA treatment, LiCl treatment aroused a marked loss of attached bacteria (Fig. 3 D). In many previous studies, LiCl was used to remove the surface layer proteins which are located in the paracrystalline layer outside the microorganism cell wall and involved in the adhesive properties (REN *et al*., 2012). The usage of LiCl did have a significant effect on the removal of the surface layer proteins on the fungal cell wall and a consequent loss of attached bacteria, but a few lactobacilli cells still existed outside the surface of *G. geotrichum* wk1, indicating complex interactions between the fungus and lactobacilli. Components on the cell surface, such as polysaccharides and surface layer proteins, are part of these interactions. Other surface molecules involved in the adhesion of *G. geotrichum* wk1 possibly exist (WAŚKO *et al*., 2014). In addition, the treatment of lactobacilli cells with LiCl will be further performed to evaluate the contribution of fungal or bacterial surface layer proteins on the interaction. The adhesion activity of *G. geotrichum* with *L. kefir* observed in this study may be attributed to the complex adhesin molecules present on the cell surface of *G. geotrichum*, which are tolerant to heating and basic/acid condition.

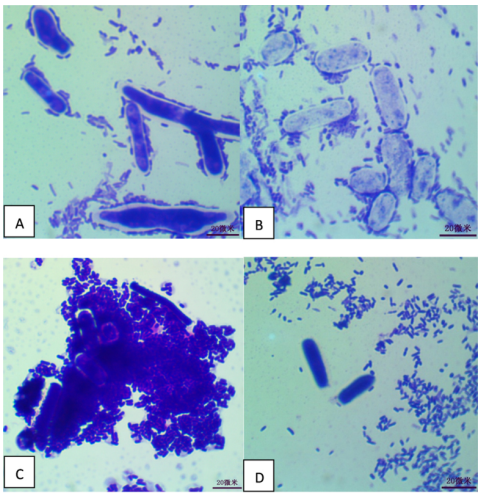


Figure 3: Effect of different treatment on the interaction between *G. geotrichum* and *L. kefir*. (A) Heating at 121°C for 20 min; (B) 1 mol/L NaOH; (C) 0.5 mol/L AA; (D) 5 mol/L LiCl . Scale bar 20 μm.

3.4. Hydrophobicity and aggregation activity

In general, cell wall associated properties, including hydrophobicity, auto-aggregation and coaggregation, were strongly related with interaction between microorganisms (BOONAERT and ROUXHET, 2000; CHEN *et al*., 2010). These properties are attributed to carboxylic groups and Lewis acid-base interactions (REN *et al*., 2012). Solvents with different polarity, xylene, chloroform and ethyl acetate, were used to evaluate the hydrophobic or hydrophilic properties of the adhesin molecules on the cell surface. Strong affinity for apolar xylene indicates good hydrophobicity. High affinity for monopolar and acidic chloroform represents basic and electron donor properties, by contrary, high affinity for monopolar and basic ethyl acetate represents acidic and electron acceptor properties (REN *et al*., 2012; SUN *et al*., 2012). In this study, a strong affinity to xylene (83.49%) and chloroform (87.5%) as well as a low affinity to ethyl acetate (42.22%) indicated the hydrophobic and basic phenotype of the fungus *G. geotrichum* wk1, with strong electron donor property. Different treatments changed the surface properties greatly (Table 1). *G. geotrichum* wk1 even appeared hydrophilic after the treatment of NaOH and AA, with the negative hydrophobicity values.

Table 1: Hydrophobicity, electron donor and acceptor properties of *G. geotrichum* wk1.

	Affinity (%)		
	Xylene	**Chloroform**	**Ethyl acetate**
untreated	83.49±3.18^{a}	87.50±4.67^{a}	42.22±2.76^{b}
Heated	30.09±4.27^{b}	55.77±5.14^{c}	-25.57±2.12^{d}
NaOH-treated	-37.87±3.63^{c}	28.10±5.46^{e}	-50.60±3.81^{e}
AA-treated	-100.28±5.18^{d}	36.65±3.16^{d}	-18.47±4.71^{c}
LiCl-treated	80.81±2.76^{a}	76.57±2.98^{b}	69.19±2.17^{a}

Means (n=3) with different letters in the same column are significantly different (P<0.05).

G. geotrichum wk1 exhibited the extremely strong auto-aggregation ability, reaching 97.48% after 3 h (Fig. 4). All the pretreatments in this study weakened the auto-aggregation abilities of *G. geotrichum* during the first 2 hours, but cells with heating treatment presented an equivalent auto-aggregation capacity with the initial native cells since 3 hours later. Similar tendency existed in the co-aggregation study (Fig. 5), except that cells treated with LiCl had higher co-aggregation ability than cells with NaOH and AA treatment.

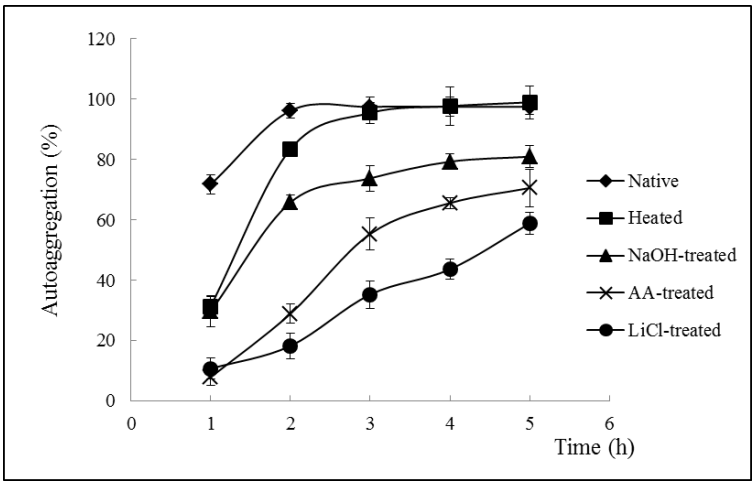


Figure 4: Auto-aggregation abilities of *G. geotrichum* during 5 h incubation.

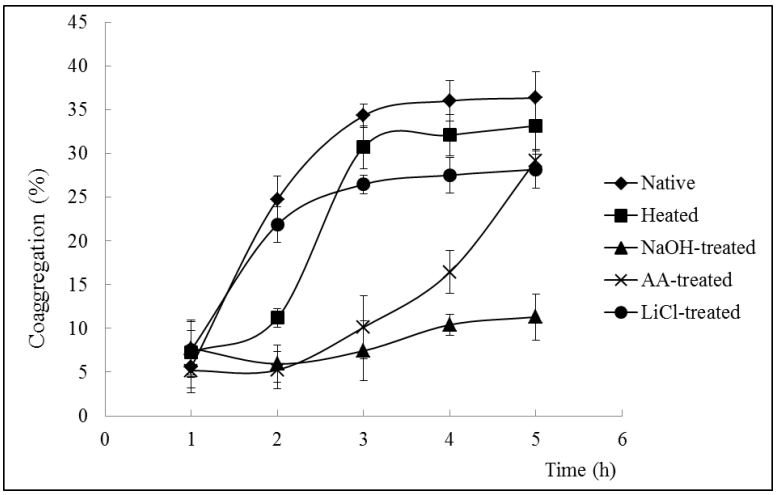


Figure 5: Co-aggregation abilities of *G. geotrichum* with *L. kefir* during 5 h incubation.

The data of cell surface properties obtained here by spectrophotometric method largely correlated with the results of microscopic imaging, except the aggregation characteristics after AA treatment. Spectrophotometric data were affected by complex cell suspension system, reflecting not only the auto-aggregation and co-aggregation properties, but also cell characteristics itself. So in this study, microscopic imaging is a more intuitive method to evaluate of the adhesion capacity.

4. CONCLUSIONS

The interaction between *G. geotrichum* and *L. kefir* on the basis of adhesion pattern was studied in this paper. Previous studies were mostly focused on the contribution of lactic acid bacteria to the kefir grain formation, for example, due to the production of polysaccharides. Researches on the role of yeast or fungi were rarely carried out. The present study showed that *G. geotrichum* wk1 could strongly bind with lactic acid bacteria, suggesting a potential role in the maintaining the symbiotic microbiota of kefir grains. The adhesive capacity decreased slightly after higher temperature and NaOH treatment, and weakened greatly by LiCl treatment, while enhanced by AA treatment. The adhesive activity seemed to be dependent on the cell surface-associated properties, such as hydrophobicity, auto-aggregation and co-aggregation. The findings in this study may be helpful to understand the interactions of participating bacteria and fungi in the kefir grains and the possible mechanism of the grain formation. These results prompted the search for the adhesin molecules on the surfaces of *G. geotrichum* wk1, as well as the quantitation of the adherent LABs, which are currently in progress in our laboratory. In addition, better techniques including electron microscopy will be used to evaluate the morphological changes and the existence of adhesion molecules more clearly.

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PAPER

IMPROVING BREAD QUALITY USING CO-CULTURES OF *SACCHAROMYCES CEREVISIAE*, *TORULASPORA DELBRUECKII* JK08, AND *PICHIA ANOMALA* JK04

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ABSTRACT

Co-cultured *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* JK08, and *Pichia anomala* JK04 were used as a leavening agent. The leavening ability, crumb structure, texture profile, crumb aroma, and sensory properties of bread were evaluated. The leavening ability of the co-cultures tested was lower than for *S. cerevisiae* alone. Leavening containing a co-cultures produced bread crust and crumb that were slightly yellow in colour and bright. Generally, co-cultured bread produced a larger diversity and higher abundance of volatile organic compounds. Superior colour properties, favorable aroma, and decent textural and structural features resulted in higher sensorial ratings for co-cultured bread. We suggest the use of co-culture as leavening agents for improved bread quality.

Keywords: bread quality, co-culture, *Pichia anomala*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*

1. INTRODUCTION

Bread is a food that not only has a long history, but also a long future. Over 12,000 years ago, the first bread was probably developed by deliberate experimentation using wheat flour and water. Product development and process innovation in bread making is still an active field. Consumer interest in bringing unique and alternative breads to the table is driving the production of a wide variety of breads (CAUVAIN, 2012; MONDAL and DATTA, 2008). A multitude of studies conducted over the 20th century focused on improving bread quality and have explored the immense terrain of recipes (GARDNER *et al*., 2002; ROSELL *et al*., 2009; MOVAHED *et al*., 2012; NILUFER-ERDIL *et al*., 2012), process innovations (KARAOĞLU *et al*., 2006; BOSMANS *et al*., 2013) and the use of novel microorganisms as leavening agents (CABALLERO *et al*., 1995; PLESSAS *et al*., 2005; CHOI and CHOI, 2009; MO and SUNG, 2014). Although the experience of bread quality is highly personal, it may be described as the sum of the sensory pleasures associated with flavour, taste, texture, and appearance (CAUVAIN, 2012).

The technological role of yeast in bread making has been well established. The main role of yeast is to produce gas by degrading the sugars available in the flour or that are added to the recipe. The gas produces air bubbles that are contained by the stretchy gluten proteins in the flour, which causes the dough to rise and produces an aerated structure in the resulting bakery product. During fermentation, the yeast produces abundant alcohols and other volatile compounds that impart unique tastes and flavours to the bread (STEAR, 1990; GÉLINAS, 2009; ALI *et al*., 2012; PACHECO *et al*., 2012).

Over the last two decades, there has been great interest in using new strains of baker's yeast that produce particular aromas, anti-molding properties, or desirable nutritional characteristics to fulfill the needs of the baking industry (GÉLINAS, 2009). Furthermore, the use of co-cultures as leavening agents has been reported to confer favorable effects in baking products. Examples include the use of *Lactobacillus plantarum* or *Pediococcus cerevisiae*, combined with *Saccharomyces cerevisiae*, as a co-culture for improving bread quality and delaying staling (ELHARIRY *et al*., 2011); employing lactic acid bacteria and *S. cerevisiae* as a starter to enhance the nutritional content and shelf life of cassava-wheat bread (OGUNBANWO *et al*., 2008); the use of *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus* mixed with the lactose-fermenting yeast *Kluyveromyces marxianus* to improve bread quality and increase shelf-life (PLESSAS *et al*., 2008); and the use of starter culture combinations of *Lactobacillus fermentum*, *S. cerevisiae*, and *Candida krusei* to enhance aroma in Ghanaian maize dough fermentation (ANNAN *et al*., 2003).

Torulaspora delbrueckii and *Pichia anomala* have unique traits that improve the quality of bakery products. As reported by HERNANDEZ-LOPEZ *et al*. (2003), *T. delbrueckii* strains IGC5321 and IGC5323 exhibited higher leavening ability and CO_2 production than *S. cerevisiae* after exposure to hyperosmotic and freeze-thaw stress. MO and SUNG (2014) reported that *P. anomala* SKM-T enhanced flavour properties and extended shelf life. The use of *T. delbrueckii, P. anomala*, and *S. cerevisiae* as co-cultured leavening agents has not yet been reported. Here, we investigated the effects of co-cultured *S. cerevisiae*, *T. delbrueckii* JK08, and *P. anomala* JK04 as a leavening agent on bread quality. Leavening ability, structural crumb features, texture profile, crumb aroma, and sensory properties of bread were evaluated.

2. MATERIALS AND METHODS

2.1. Microorganism strains and bread ingredients

T. delbrueckii JK08 (TD) and *P. anomala* JK04 (PA) were isolated from Korean traditional starter (*Nuruk*), and then identified and collected at the Institute of Fermentation Biotechnology, Kyungpook National University, Daegu, South Korea. The strains were sub-cultured in yeast extract-peptone-dextrose (YPD, Difco, Le Pont de Claix, France) agar (Oxoid, Hampshire, United Kingdom) and stored at 2-4°C. *S. cerevisiae* (SC) was isolated from compressed instant baker's yeast (saf-instant S.I., Lesaffre, Marcq, France). Commercial wheat flour (Beksul, CheilJedang, Seoul, South Korea) contained 13.93% protein and 0.47% ash, with a pH of 4.35. Sugar and salt were purchased from a supermarket in Sangju, South Korea.

2.2. Preparation of cultures

Preparation of cultures was described previously (WAHYONO *et al*., 2015). The strains were sub-cultured in 5% YPD broth and cultivated in a rotary shaker (JSSI-300C, JS Research, Gongju, South Korea) at 30°C for 48 h, with shaking at 180 rpm. The yeast cells were collected by centrifugation (Hanil Supra 22K, Hanil, Incheon, South Korea) at 4000 × g for 10 min at 4°C. The supernatant was discarded, and the pellet was re-suspended in distilled water, vortexed thoroughly, and stored at 4 °C. Cell density was measured using a hemocytometer (Neubauer chamber, Celeromics, Cambridge, United Kingdom). To measure cell density, 10^{-1} to 10^{-4} dilutions of culture were prepared; 10 µl of diluted culture was loaded into the hemocytometer chamber. The chamber was observed at 100× magnification using a binocular microscope (CX31RTSF, Olympus, Tokyo, Japan). Cells in four different regions of the chamber were counted. Cell density was calculated using the following formula:

$$c = \frac{N}{n \cdot d} \times 10\,000 \qquad (1)$$

where c is the concentration (expressed as number of cells per millilitre), N is the number of cells, n is number of squares counted, and d is the dilution factor. Number 10 000 represents a constant converter to millilitre.

2.3. Leavening ability

The leavening ability of co-cultures was measured as described previously (WAHYONO *et al*., 2015). Bread dough containing 20 g flour, 20 ml water, and 4 × 108 yeast cells per ml of water was mixed thoroughly in a 100-ml graduated cylinder and then incubated at 30°C for 210 min. This was done in triplicate and each sample was observed every 30 min. The maximum leavening rate (ml/h) was calculated from the highest volume reached in 210 min divided by the time the highest volume was first recorded.

2.4. Bread-making procedure

Baking was carried out in a bread maker (National SD-BT102, Panasonic, Osaka, Japan) using the 4-h standard bread-making setting. The basic recipe consisted of wheat flour, salt, sugar, and drinkable water. A single culture SC and co-cultures of *S. cerevisiae* + *T.*

delbrueckii JK08 (SCTD), *S. cerevisiae* + *P. anomala* JK04 (SCPA), and *S. cerevisiae* + *T. delbrueckii* JK08 + *P. anomala* JK04 (SCTDPA) were used as leavening agents. The working ratio for co-cultures of *S. cerevisiae* + *T. delbrueckii* JK08 (SCTD) and *S. cerevisiae* + *P. anomala* JK04 (SCPA) were 50:50, respectively. The co-culture ratio for *S. cerevisiae* + *T. delbrueckii* + *P. anomala* JK04 (SCTDPA) was 30:35:35. In the bread dough, the SC cell density was adjusted to approximately 5×10^8 cells per ml of water added to the dough. For TD and PA, the cell densities were approximately 10^9 cells per ml of water added. Dough compositions are given in Table 1. The bread maker protocol included a first mixing for 20 min, resting for 25 min, and a second mixing for 10 min, followed by a first fermentation at 35°C for 50 min. A third mixing was performed for 3 min, followed by a second fermentation at 35°C for 40 min, and then a fourth mixing for 2 min. Proofing was done at 40°C for 50 min and finally, loaves were baked at 210°C for 40 min. Baking was performed in triplicate. After baking, the bread loaves were tempered at ambient temperature (28-30°C) before analysis.

2.5. Moisture content, specific volume, and bread yield efficiency

The moisture content of the bread crumb was determined by the oven-drying method (CZUCHAJOWSKA *et al*., 1989). Specific volume was determined by the seed displacement method (AACC 10-05, 2000). Bread yield efficiency was calculated as described by MOVAHED *et al*. (2012) using the following formula:

$$P_2 = \left(\frac{W_3}{W_2}\right) \times 100 \qquad (2)$$

where P2 is bread yield efficiency, W3 is bread weight and W2 is flour weight.

2.6. Chromaticity of bread crust and crumb

The crust and crumb colour were measured using the CR-400 Chroma Meter (Konica-Minolta, Tokyo, Japan); *L* (lightness), *a* (redness), and *b* (yellowness) values (Hunter colour) were measured for six regions of bread crust and crumb. The whiteness index (*WI*) was calculated according to HSU (2003) and LIN *et al*. (2009).

2.7. Texture profile analysis

Texture profile analysis (TPA) was carried out in triplicate for two slices of bread. TPA was performed using a texture analyzer (CT3 4500, Brookfield, Middleboro, USA). Bread samples were sliced to approximately 25-mm thickness. Hardness, cohesiveness, springiness, and chewiness of the center of the bread slices were measured (BLANDINO *et al*., 2013). The settings and conditions were carried out as described previously by ULZIIJARGAL *et al*. (2013), with some modifications: the acrylic cylindrical probe had a 38.1 mm diameter (TA4/1000), the pretest speed was 2 mm/s, the test speed was 2 mm/s, the post-test speed was 2 mm/s, the distance was 10 mm (40% compression), and the trigger load was 50 g.

2.8. Bread crumb image analyses

The structural features of the bread crumb were analyzed using ImageJ software (1.47v, National Institutes of Health, Bethesda, MD, USA). Structural features included bread cell

density, mean cell area, and the fraction of cell area to total area. Bread crumb images were captured using a scanner (Epson Perfection V370 Photo, Epson, Japan) at a resolution of 800 × 800 dpi. Images were calibrated to reflect actual size using a known scale, were cropped to 60 × 60 mm, filtered using a bandpass filter, and converted into binary images using the *convert to mask* feature for differentiating between the cell and non-cell area. Before analyzing particles (cells), the particle size was set from 0.01 mm^2 to infinity and the circularity was set from 0 to 1. This particle size corresponds to a particle diameter of 0.1 mm, which can be resolved by the human eye (PONGJARUVAT *et al*., 2014).

2.9. Analysis of volatile compounds in bread crumbs

Volatile compounds were analyzed as described previously by PLESSAS *et al*. (2008), using gas chromatography and mass spectrometry (7890A GC-MS; Agilent, Santa Clara, CA, USA) with a flame ionization detector (FID). The separation was performed with a DB-WAX column (60 m × 250 μm × ϕ 0.25 mm) (Waters, Milford, MA, US). The detector was an Agilent 5975C Inert XL MSD with Triple-Axis Detector. Helium was used as a carrier gas with a constant flow of 1 ml/min. Using solid-phase microextraction technique (SPME), 1 g of each bread sample was put into a 20-ml vial accessible to the SPME needle through the vial septum. Then, the vial was submerged in a water bath at 60°C and the SPME fiber (50/30 μm DVB/CAR/PDMS, Supelco, Bellefonte, PA, USA) was exposed to the headspace of the vial for 60 min. When the extraction process was finished, the SPME fiber was inserted into the injector port (set at 280°C) of the gas chromatograph (GC) for thermal desorption of volatile compounds for 5 min in splitless mode. The GC temperature program was set as follows: 35°C for 5 min, increased by 5°C/min to 50°C (held for 5 min), increased by 5.5°C/min to 230°C (held for 5 min). Volatile compound identification was based on comparison of GC retention times and peak areas with spectral data from the Wiley9Nist 0.8 library (Wiley9Nist 0.8 library, mass spectral search program, version 5.0, USA).

2.10. Sensory evaluation

Bread samples were prepared from a freshly baked loaf (6-8 h after baking). The bread samples were cut about 50 × 20 × 25 mm and served on a small paper plate. Sensory evaluations were conducted by 15 semi-trained consumers who were students and professors at Kyungpook National University, South Korea. The sensory attributes tested included appearance, colour, flavour, mouthfeel, and overall acceptability. The sensory attribute scale used for assessing the bread was as follows: 1, extremely dislike; 4, neither like nor dislike; and 7, extremely like (ULZIIJARGAL *et al*., 2013).

2.11. Statistical analysis

To examine statistical significance, the data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at the $p < 0.05$ level of significance. The correlation among structural features and textural profiles of bread crumb was analyzed using a 2-tailed Pearson correlation at $p < 0.05$. The analysis was carried out using SPSS for windows (ver. 19, IBM, New York, New York, USA). The graphs were constructed using Microsoft Excel (2007v, Microsoft, Redmond, Washington, USA).

3. RESULTS AND DISCUSSION

3.1. Leavening ability

The leavening ability of co-cultures was compared to that of single cultures (SC) in lean dough containing wheat flour and water (Fig. 1). The leavening rates were significantly different among the cultures tested ($p < 0.05$). For SC, the dough was greatly leavened after only 30 min of incubation, but for co-cultures, the dough was greatly leavened after 60 min of incubation. The leavening rates for SC, SCTD, SCPA, and SCTDPA were 55.4, 41.6, 36.5, and 31.6 ml/h, respectively. In a previous study, we found that the leavening rate for single cultures of *T. delbrueckii* JK08 and *P. anomala* JK04 were 8.67 and 2.29 ml/h, respectively. The lower performance of *T. delbrueckii* JK08 and *P. anomala* JK04 may be due to slower growth relative to *S. cerevisiae* (WAHYONO *et al*., 2015). BELY *et al*. (2008) also reported lower performance for *T. delbrueckii* 27828 and *T. delbrueckii* 31703 in terms of fermentation rate in comparison to *S. cerevisiae*. On the contrary, HERNANDEZ-LOPEZ *et al*. (2003) reported that *T. delbrueckii* strains IGC5321 and IGC5323 exhibited higher leavening ability and CO_2 production than *S. cerevisiae* after exposure to hyperosmotic and freeze-thaw stress. We demonstrated that incorporating TD and PA with SC produced a co-culture with greatly improved leavening ability for a longer leavening period (> 120 min). ELHARIRY *et al*. (2011) revealed that incorporating *S. cerevisiae* and *L. plantarum* in a sourdough system delivered favorable effects such as improving the leavening ability, as well as the sensory and physical properties of the bread.

Table 1: List of ingredients used in bread making.

Ingredients	Quantity	Yeast addition (total cells)[a]		
		S. cerevisiae	***T. delbrueckii*JK08**	***P. anomala*JK04**
Wheat flour (g)	280			
Sugar (g)	16.8			
Salt (g)	5.6			
Water (ml)	200			
Leavening agent;				
SC		1×10^{11}	-	-
SCTD		5×10^{10}	1×10^{11}	-
SCPA		5×10^{10}	-	1×10^{11}
SCTDPA		3×10^{10}	7×10^{10}	7×10^{10}

[a]Calculated according to the yeast concentration and ratio determined in the bread-making procedures in the materials and methods.

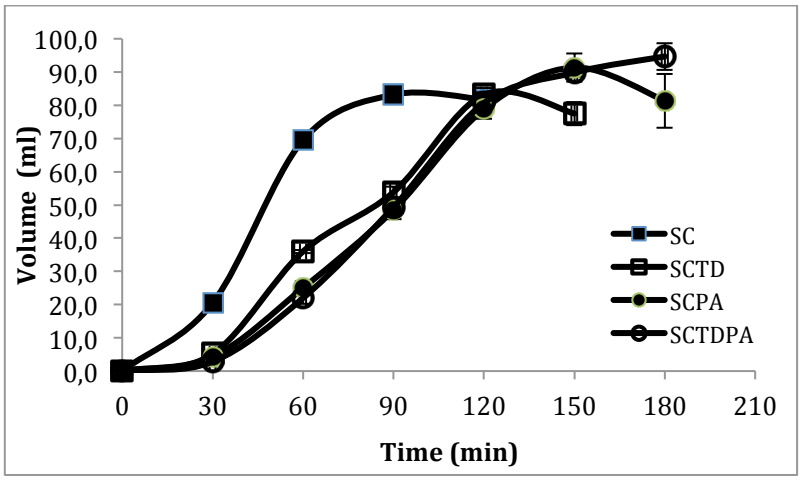


Figure 1: Leavening rates for co-cultures or a single culture in lean dough. Results are means±SD of triplicate.

3.2. Physical properties

The co-cultures did not affect the moisture content or bread yield, but significantly affected the bread-specific volume ($p < 0.05$, Table 2). The specific volumes of breads produced with the cultures tested were in the range of 4.01-4.37 cm^3/g. In comparison to breads leavened with co-cultures, the bread leavened with SC produced the greatest specific volume (4.37 cm^3/g), which is consistent with the observation that SC exhibited the highest leavening activity (55.4 ml/h). We previously reported that lower leavening abilities for *T. delbrueckii* JK08 and *P. anomala* JK04 produced significantly lower specific volumes in comparison to *S. cerevisiae* (WAHYONO *et al*., 2015). These results strongly suggest a connection between the yeast's leavening ability and the specific volume of the resulting bread.

No significant differences were observed in bread-specific volume for the co-cultures tested. Even though there were significant differences in leavening rates for the co-cultures tested, the time period for fermentation in the bread-making process was not long enough for optimum leavening activity for SCTD, SCPA, SCTDPA (120, 150, and 180 min, respectively). However, the specific volume of breads produced by co-cultures is well within the range of standard bread, with specific volumes that ranged from 3.5 to 6 cm^3/g (ULZIIJARGAL *et al*., 2013).

The co-cultures significantly affected the chromaticity of the bread crust (Table 3). SCTD-leavened bread showed the greatest *L*, *b*, and *WI* values. The *L* value for SCTD was significantly greater than that for SC or SCPA. The *b* value for SCTD was only significantly greater than that for SC. The *WI* value for SCTD was significantly higher than those for the other cultures tested. SCTDPA produced the highest *a* value, which was significantly greater than that of SC or SCTD. In contrast, SC-leavened bread produced the lowest *L*, *a*, *b*, and *WI* values. These results were consistent with previous work that demonstrated greater *L* and *b* values for bread crust when *T. delbrueckii* JK08 was used as a leavening agent (WAHYONO *et al*., 2015). For crumb colour, the co-cultures significantly affected the *L* and *b* values, but not the *a* and *WI* values (Table 3). SCPA produced the greatest *L* value, which was significantly greater than that of SC. It also produced the greatest *b* value, which was significantly higher than that of SC or SCTD. The greatest *L* and *b* values of bread crumb arose when *P. anomala* JK04 was used as a leavening agent, while lower *L* and

b values (darker colour) were produced when *S. cerevisiae* was used (WAHYONO *et al*., 2015). In short, TD produced greater lightness and unsaturated yellowish colour in the bread crust, and PA produced similarly a coloured bread crumb. On the other hand, SC produced a darker and saturated yellowish colour in bread crust and crumb. HERNANDEZ-LOPEZ *et al*. (2003) reported that commercial baker's yeast (*S. cerevisiae*) exhibited greater maltase and invertase activity than *T. delbrueckii* IGC5321. Consequently, SC produced more reactive saccharides, which may contribute to darker colour formation. The saccharides and nitrogen-containing substances involved in the browning reaction create the dark-coloured pigment melanoidin, which confers a darker colour to the bread crust and crumb (STEAR, 1990).

Table 2: Physical properties of bread leavened with co-cultures or a single culture[a].

Yeast	Crumb moisture content (%)	Specific volume (cm^3/g)	Bread yield efficiency (%)
SC	46.33 ± 0.50^{A}	4.37 ± 0.15^{A}	147.97 ± 0.75^{A}
SCTD	46.58 ± 0.59^{A}	4.08 ± 0.05^{B}	148.86 ± 1.00^{A}
SCPA	46.83 ± 0.35^{A}	4.15 ± 0.01^{B}	147.85 ± 0.21^{A}
SCTDPA	46.61 ± 0.16^{A}	4.01 ± 0.09^{B}	148.13 ± 0.75^{A}

[a]Means with the same superscript letter in a column are not significantly different at the level $p < 0.05$.

Table 3: Chromaticity of bread crust and crumb leavened with co-cultures or a single culture[a].

Yeast	Crust colour				Crumb colour			
	L	*a*	*b*	Whiteness index (*WI*)	*L*	*a*	*b*	Whiteness index (*WI*)
SC	38.22 ± 1.22^{C}	6.26 ± 0.72^{C}	15.85 ± 0.97^{B}	35.90 ± 0.88^{B}	56.85 ± 2.88^{B}	-2.52 ± 0.11^{A}	8.53 ± 0.78^{C}	55.93 ± 2.76^{A}
SCTD	41.63 ± 0.93^{A}	6.92 ± 0.67^{BC}	17.77 ± 0.24^{A}	38.59 ± 1.00^{A}	59.08 ± 0.11^{AB}	-2.56 ± 0.05^{A}	9.25 ± 0.38^{BC}	57.97 ± 0.03^{A}
SCPA	39.39 ± 0.34^{BC}	7.79 ± 0.26^{AB}	17.00 ± 0.28^{A}	36.57 ± 0.35^{B}	60.22 ± 0.75^{A}	-2.42 ± 0.02^{A}	10.34 ± 0.13^{A}	58.82 ± 0.71^{A}
SCTDPA	40.12 ± 0.83^{AB}	8.23 ± 0.22^{A}	17.66 ± 0.46^{A}	37.02 ± 0.64^{B}	58.37 ± 1.55^{AB}	-2.43 ± 0.10^{A}	9.58 ± 0.47^{AB}	57.21 ± 1.40^{A}

[a]Means with the same superscript letter in a column are not significantly different at the level $p < 0.05$.

3.3. Structural features of bread crumb

The structural parameters of bread crumb are expected to influence its mechanical behavior. By using image analysis, the structural features of bread crumb can be quantified (ZGHAL *et al*., 2002). Hence, we carried out an image analysis of bread crumb and the results are presented in Table 4. The digital binary images of bread crumb from which the structural features could be extracted are shown in Fig. 2. The use of co-cultures significantly affected the cell density and the mean cell area of bread crumb, but not the fraction of cell area to total area. The cell density of bread crumb leavened with SCTD (58.89 1/cm) was the greatest among cultures tested, and was significantly greater than that leavened with SC (50.04 1/cm). Inversely, the mean cell area of bread crumb leavened with SC (0.90 mm^2) was the largest, significantly larger than that leavened with SCTD (0.73 mm^2). These results suggest that as mean cell area increases, cell density decreases. The

large mean cell area for SC-leavened bread is consistent with its high specific volume. In other words, bread leavened with SC was more porous than bread produced using co-cultures, probably because of superior leavening ability and higher CO_2 production (WAHYONO *et al*., 2015). Alternatively, PONGJARUVAT *et al*. (2014) reported that high specific volume is tightly correlated with cell density and cell area fraction.

We performed correlation analysis for structural features and mechanical parameters (TPA) of bread crumb. We found that the cell density, mean cell area, and fraction of cell area to total area were correlated with cohesiveness, but not hardness, springiness, or chewiness (Table 5). Structural features were not strongly correlated with overall bread quality, which is more strongly affected by attributes such as odor and appearance (LAMPIGNANO *et al*., 2013).

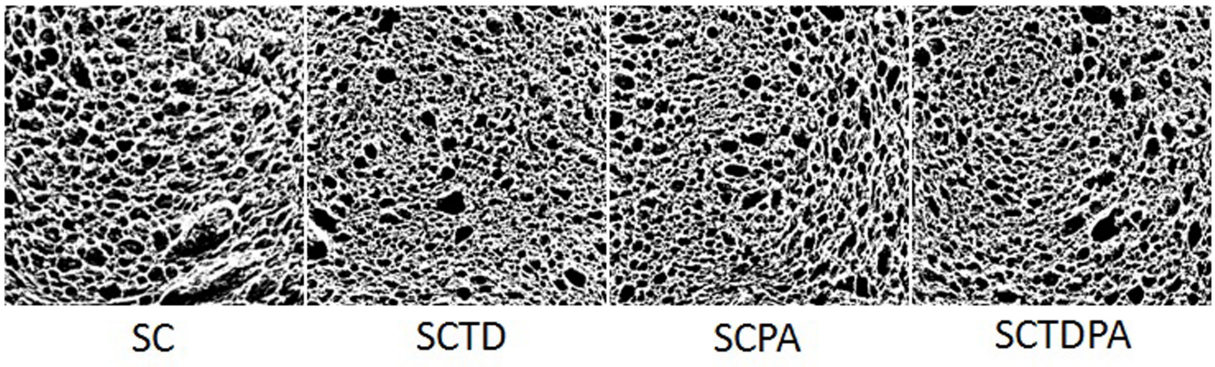


Figure 2: The digital binary images of breads crumb leavened with co-cultures or a single culture.

Table 4: Structural features of bread crumb leavened with co-cultures or a single culture quantified by image processing[a].

Yeast	Cell density (1/cm^2)	Mean cell area (mm^2)	Fraction of cell area to total area (%)
SC	50.04±5.59^{B}	0.90±0.10^{A}	44.87±0.40^{A}
SCTD	58.89±2.92^{A}	0.73±0.03^{B}	42.83±2.06^{A}
SCPA	52.55±3.37^{AB}	0.85±0.09^{AB}	44.34±1.87^{A}
SCTDPA	53.38±3.59^{AB}	0.84±0.04^{AB}	44.57±1.03^{A}

[a]Means with the same superscript letter in a column are not significantly different at the level $p < 0.05$.

Table 5: Pearson correlation of structural features and textural profiles of bread crumb.

	Hardness	Springiness	Cohesiveness	Chewiness	Cell density	Mean cell area
Springiness	0.360					
Cohesiveness	-0.485	-0.391				
Chewiness	0.981**	0.353	-0.319			
Cell density	0.450	0.365	-0.633*	0.380		
Mean cell area	-0.435	-0.432	0.732**	-0.340	-0.950**	
Cell fraction	-0.175	-0.326	0.658*	-0.043	-0.273	0.541

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

3.4. Texture profile analyses

TPA was used to evaluate the textural properties of bread crumb (Fig. 3). The co-cultures altered hardness, chewiness and cohesiveness, but these changes were insignificant, except for cohesiveness. SCTD-leavened bread was of lower cohesiveness than bread leavened with other cultures (Fig. 3), consistent with our previous study, demonstrating that *T. delbrueckii* JK08 produces bread crumb with lower cohesiveness (WAHYONO *et al*., 2015). As stated earlier, the cohesiveness of bread crumb was the only parameter that correlated with its structural features. According to SCANLON and ZGHAL (2001), the crumb textural properties were largely determined by the bread crumb structural features. Fine and uniformly-sized cells produce a softer texture. Here we have demonstrated that a greater mean cell size conferred greater cohesiveness and vice versa. However, this result should be further evaluated in light of previous work that established that crumb cohesiveness is controlled by moisture content and the strength of networks surrounding the cell pore (CAUVAIN, 2004). We have shown that the use of co-cultures produced bread of quality and textural properties comparable to bread leavened using a single culture.

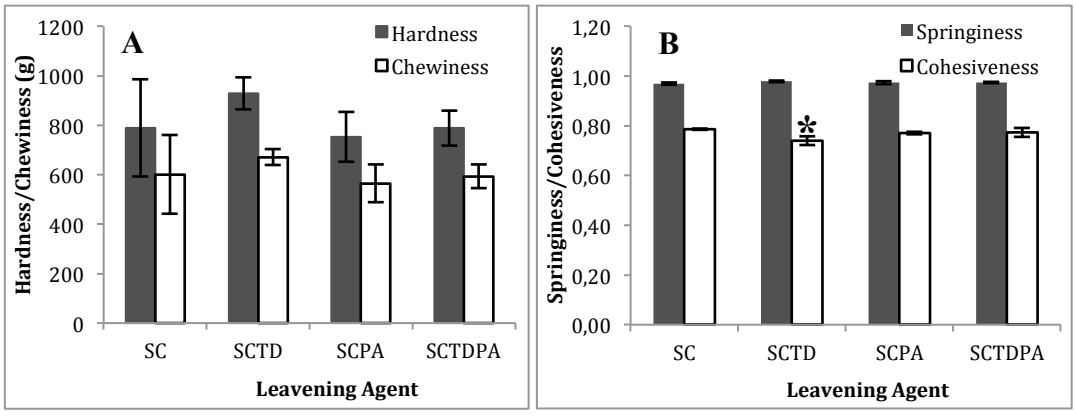


Figure 3: Texture profiles of bread leavened with co-cultures or a single culture.
(A) Hardness and chewiness of bread crumb. (B) Springiness and cohesiveness of bread crumb. Results are means ± SD of triplicate.

(*) Significantly different at the level $p < 0.05$.

3.5. Volatile compounds of bread crumb

A total of 54 volatile compounds were identified in the bread crumb leavened with SCTDPA, whereas 50, 47, and 49 volatile compounds were identified in the bread crumb leavened with SC, SCTD, and SCPA, respectively (Table 6). SCTDPA-leavened bread not only produced more unique volatile compounds, but in greater abundance, as indicated by the greater peak area. In most cases, the bread leavened by co-cultures produced more volatile compounds than that of a single culture.

The volatile compounds were predominately alcohols, aldehydes and esters. Isobutyl alcohol (i-BuOH), isoamyl alcohol (i-AmOH), and phenethyl alcohol (PEA) were the predominant alcohol groups. i-BuOH levels were highest in SCTDPA bread (20,169) and the lowest in SC bread (1,986). Inversely, i-AmOH levels were the highest in SCPA bread (11,168) and lowest in SCTDPA bread (6,265). As reported by KIM *et al*. (2013), *P. anomala* Y197-13 produced high i-AmOH levels and conferred a banana flavour that significantly affected the flavour and taste of turbid rice wine. WATANABE *et al*. (1990) reported that bread containing high levels of i-AmOH was less favorable than bread containing high levels of i-BuOH. The bread leavened with *P. anomala* SKM-T exhibited a higher PEA content and was preferred over *S. cerevisiae* (MO and SUNG, 2014) due to the favorable honey and flower odor of PEA (JENSEN *et al*., 2011).

The predominant aldehydes in bread crumb were n-hexanal, furfural, and benzaldehyde. The bread leavened with the co-cultures containing PA (SCPA and SCTDPA) produced the highest amounts of these compounds. n-hexanal was the most abundant aldehyde and conferred a green flavour. The second most abundant was benzaldehyde which produces an almond odor (BIRCH *et al*., 2013a). Fulfural is characterized by a burnt and sweet, caramel-like, odor (PROST *et al*., 2012). The other volatile compounds that contributed to either favorable (n-octanal, n-decanal) or unfavorable (n-heptenal) odors were comparable among all cultures tested.

Esters typically have pleasant, fruity, or sweet odors (BIRCH *et al*., 2013b). In most cases, SCTDPA leavened bread contained a greater abundance and higher diversity of ester compounds. Compounds including isoamyl acetate (fruity), ethyl caproate (fruity, wine, apple, banana, brandy), ethyl octanoate (fatty, fruity), ethyl decanoate, and ethyldodecanoate were enhanced in the bread leavened with co-cultures containing PA (SCPA and SCTDPA). SC and TD enhanced particular compounds such as ethyl acetate and methyl salicylate, respectively. In alcoholic beverages, ethyl acetate can produce unfavorable sensory qualities. Mixed cultures of *S. cerevisiae* and *P. anomala* (mutant type) produced higher ethyl acetate-hydrolyzing esterase activities. This enzyme is crucial in the formation of acetate ester, which delivers superior flavour (KURITA, 2008).

The bread leavened by co-cultures was obviously superior to that leavened by a single culture with regard to the volatile compound content. These compounds were produced mainly from the metabolism of yeasts during dough fermentation and flour lipid oxidation (BIRCH *et al*., 2013b). These processes are influenced by the availability of free, reactive amino acids, sugars, alcohols, enzyme activity, and the degree of polymerization and hydration of substrates due to mixing to baking (STEAR, 1990). SADOUDI *et al*. (2012) reported that the use of co-cultures altered the production of volatile compounds in wine, because co-culture interactions influenced the entire metabolic pathway.

Table 6: Volatile compounds contained in the bread crumb leavened with co-cultures or a single culture.

No	Group	RT	Compound	Flavour Description	Peak Area*			
					SC	SCTD	SCPA	SCTDPA
1	Acids	30.40	Acetic acid	Acid, pungent [a]	1,973	1,838	2,483	4,043
2		33.28	Isobutyric acid		339	275	287	408
3		35.61	2-Methylbutanoic acid	Sweaty [e]	680	603	514	939
4		40.00	2-Methylpropanoic acid	Sweat, butter[a]	81	59	83	176
5	Alcohols	18.63	Isobutyl alcohol		1,986	7,494	13,946	20,169
6		23.30	Isoamyl alcohol	Banana [f]	8,011	8,174	11,168	6,265
7		26.30	2-Ethyl-1-decanol		ND	ND	ND	301
8		27.69	2-Methyl-3-pentanol		146	180	67	88
9		27.90	1-Hexanol	Flower [a]	465	610	527	674
10		28.14	2-Nonanol		153	159	ND	87
11		28.63	3-Ethoxy-1-propanol	Fruity [a]	87	236	ND	116
12		32.76	1-Dodecanol		228	163	280	684
13		35.42	2-Furanmethanol		309	552	615	1,857
14		40.86	Phenethyl alcohol	Honey, Flower [a]	9,506	12,292	8,391	12,643
15	Aldehydes	18.01	n-Hexanal	Green [d]	5,369	19,874	37,144	71,305
16		22.38	n-Heptanal	Fatty, rancid [d]	233	262	528	302
17		25.99	n-Octanal	Citrus [d]	207	263	333	495
18		27.11	2-Heptenal		600	1,086	1,594	2,335
19		30.88	Furfural		1,646	2,068	2,764	7,529
20		31.79	n-Decanal	Citrus [d]	151	230	237	379
21		32.56	Benzaldehyde	Almond [d]	3,507	5,475	6,389	8,374
22		33.64	5-Methyl-2-furfural		132	47	131	529
23		38.80	2,4-Decadienal	Fatty, waxy [b]	38	94	103	224
24	Alkenes	8.96	2,4-Dimethyl-1-heptene		62	ND	ND	396
25		29.86	3-Ethyl-2-methyl-1,3-hexadiene		36	58	83	120
26	Benzenes	15.92	Methyl benzene		269	330	551	413
27		24.95	Ethenylbenzene		762	134	8,026	6,265
28		29.97	1,3-bis(1,1-dimethylethyl)benzene		381	563	85	1,503
29	Esters	9.10	Ethyl acetate	Pineapple [a]	1,688	120	635	5,590
30		14.66	Isobutyl acetate	Ethereal, fermented	ND	ND	ND	134

				odor [b]				
31		19.87	Isoamyl acetate	Fruity [e]	305	666	5,290	7,923
32		24.16	Ethyl caproate	Fruity, wine, apple, banana, brandy [c]	305	51	4,641	3,903
33		25.43	n-Hexyl acetate		37	34	124	256
34		30.10	Ethyl octanoate	Fatty, Fruity [a]	625	796	4,646	12,307
35		30.75	Amyl caproate		7	ND	84	351
36		34.96	Ethyl decanoate		204	171	4,066	9,553
37		36.12	Ethyl-9-decenoate		ND	ND	104	1,658
38		38.27	Methyl salicylate		220	1,686	316	1,876
39		38.91	2-Phenethyl acetate	Roasty[e]	235	101	209	868
40		39.22	Ethyldodecanoate		336	281	1,069	1,407
41	Furans	24.06	2-Pentylfuran	Floral, fruity [d]	309	246	887	566
42		31.99	2-Acetylfuran		282	339	450	1,243
43	Ketones	12.73	2,3-Butanedione	Buttery, caramel [d]	353	592	460	542
44		22.29	2-Heptanone		ND	ND	58	67
45		25.89	3-Hydroxy-2-butanone	Butterscotch [d]	867	744	922	1,149
46		26.37	1-Octen-3-one	Mushroom [g]	48	263	419	765
47	Phenols	40.61	Butylatedhydroxytoluene		1,585	2,788	4,507	2,683
48	Pyrazines	25.33	Methylpyrazine		185	179	228	546
49		27.33	2,6-Dimethylpyrazine	Hazelnut [e]	68	73	219	314
50		27.49	Ethylpyrazine		58	95	95	223
51	Terpenes	37.04	(-)-.beta.-Bisabolene		220	ND	341	222
52	Others	28.90	Dimethyl trisulfite		51	120	217	263
53		37.64	Naphthalene		79	143	446	288
54		41.42	1,4-Methanobenzocyclodecene		75	139	265	275

*the values of volatile compounds calculated from the peak area divided by 1000.
ND, not detected
[a]. JENSEN *et al*. (2011); [b]. MO and SUNG (2014); [c]. DAIGLE *et al*. (1999); [d]. BIRCH *et al*. (2013a); [e]. PROST *et al*. (2012); [f]. KIM *et al*. (2013); [g]. BIRCH *et al*. (2013b)

3.6. Sensory properties

The average results of the sensory evaluation of appearance, colour, flavour, mouthfeel, and overall acceptability are shown in Fig. 4. In most cases, the co-cultures slightly enhanced all the sensory attributes, except for appearance. All attributes produced satisfactory scores in the range of 4.73–5.57 out of a total 7 points. On average, the co-

cultures produced marked improvement over the single culture, which scored in the range of 4.07-5.71. SCTDPA leavened bread was superior in overall acceptability (5.57), which is attributable to higher ratings in flavour (5.27) and mouthfeel (5.30). High flavour ratings are probably due to the high abundance of favorable volatile compounds (Table 6). SCTD leavened bread had a superior colour rating (5.53). These results demonstrate that incorporating SC, TD, and PA as leavening agents conferred beneficial characteristics to bread. SC contributed to improving bread appearance through greater leavening ability, and TD and PA contributed to enhanced flavour, colour, and mouthfeel.

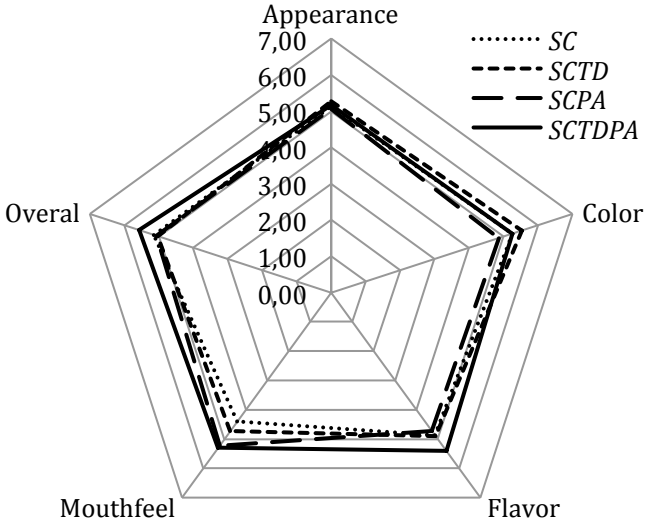


Figure 4. Radar plot of the sensory properties of bread leavened with co-cultures or a single culture. Result reflects the means of scores from 15 semi-trained panelists.

4. CONCLUSIONS

We have shown that the use of mixed cultures of *S. cerevisiae*, *T. delbrueckii* JK08, and *P. anomala* JK04 enhanced bread quality. The bread leavened by the co-cultures produced textural and structural properties comparable to single cultures of S. *cerevisiae*. The co-cultured bread had a superior aroma and enhanced sensorial qualities. Thus, the use of co-cultures as leavening agents has great promise in fulfilling the consumer need for unique and high-quality bread.

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PAPER

CHARACTERIZATION OF BIOACTIVE COMPOUNDS IN ROSEHIP SPECIES FROM EAST ANATOLIA REGION OF TURKEY

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ABSTRACT

The objective of this work was to determine some bioactive compounds for four different rosehip species (*Rosa* L.), growing in the East Anatolia region of Turkey. It was determined that the average fruit weights of the species varied between 9.8 g (*R. dumalis*) and 34.5 g (*R. canina*). The total soluble solids showed statistically significant variations among the rosehip species (14-22 ˚Brix). The acidity was inversely proportional to total soluble solids and ranged between 1.00% (*R. canina*) and 2.67% (*R. villosa*). The highest total phenolic, L-ascorbic acid contents and the highest total antioxidant capacity were found in *R. canina*. The total phenolic, total anthocyanin, total dry matter, and L-ascorbic acid contents and the total antioxidant capacity of the rosehip species ranged as follows 1081-6298 mg gallic acid equivalent/100 g, 2.43-3.72 mg/100 g, 40.1–56.7%, 24.93-754.48 mg/100 g, and 10.04-97.95 mmol trolox equivalent/g, respectively. Glucose was the most common sugar in *Rosa* species (5.99-12.48 g/100 g), the major organic acid in the rosehip species was citric acid (0.48-1.05 g/100 g). A dendogram based on some pomological and biochemical characteristics of the rosehip species were grouped into 2 main clusters. Findings on the biochemical characteristics of the species will provide insights to plant breeders /growers and for further research.

Keywords: antioxidant, organic acids, phenolic, rosehip, sugars

1. INTRODUCTION

Rosehip plants are not selective in terms of climate and soil requirements and grow in several areas, including Europe, Africa, Middle and West Asia and Russia (NILSON, 1997; ILISULU, 1992). Rosehips grow in almost all regions of Turkey and are well-known and consumed fruits in Anatolia. They are perennial plants belonging to the genus *Rosa* in the Rosaceae family. The genus *Rosa* includes numerous species and varieties, and each country has its own endemic rosehip species. *Rosa pisiformis* and *Rosa dumalis* subsp. *antalyensis* are endemic species for Turkey (ERCISLI, 2005). Out of about 100 rosehip species occurring all around the world, 27 species grow in Turkey (TURKBEN, 2003; ERCISLI and GULERYUZ, 2005).

Red fruits are rich in phytochemicals such as phenolic substances, flavonoids, anthocyanin and carotenoids (QIAN *et al*., 2004; TRAPPEY *et al*., 2005; CIESLIK *et al*., 2004). Rosehips contain more and a greater variety of phytochemicals compared to other fruit species (HALVORSEN *et al*., 2002; OLSSON *et al*., 2004). Also, they contain minerals, high-capacity antioxidants, carotenoids, phenolic compounds, tocopherol, bioflavonoids, tannins, pectins, organic acids, amino acids, ascorbic acid, and fatty acids (GAO *et al*., 2000; DEMIR and OZCAN, 2001; LARSEN *et al*., 2003; CHRUBASIK *et al*., 2008; JABLONSKA *et al*., 2009; BARROS *et al*., 2010). The fact that *Rosaceae* fruits have important physiological functions may be due to abundant phenolic substances, because it is known that the spectra of biochemical activity of phenolic substances, including their antioxidant activity, antimutagenic and anti-carcinogenic effects, are wide (TAPIERO *et al*., 2002; NAKAMURA *et al*., 2003). These compounds also contribute to the quality and nutritional value of the plant (ERCISLI, 2007). Moreover, it has been reported that rosehip fruits are used to cure illnesses such as influenza, other infections, inflammatory diseases, chronic pain and ulcer and that they have a protective effect on health (GUIMARAES *et al*., 2010).

Despite species variation, rosehips contain about 20- to 30-fold more vitamin C compared to oranges. Besides, rosehips, which are a valuable source of minerals, are quite rich in phosphorus and potassium (NOJAVAN *et al*., 2008; SZENTMIHALYI *et al*., 2002; KOVACS *et al*., 2004). Therefore, rosehip fruits are widely used in food and pharmaceutical industries. In Turkey, numerous foods such as marmalade, jam, churchkhela, nectar and tea are made from rosehip fruits (ERCISLI and GULERYUZ, 2005; YILDIZ and ALPASLAN, 2012). Besides, rosehip fruits are added to probiotic beverages, fruit yogurts and soup (DEMIR *et al*., 2014).

Recently, naturality and bioavailability have been considered among the most important characteristics of food products (ERCISLI, 2007). In Turkey, rosehip fruits grow naturally, without requirement of chemical compounds and fertilizers. In this study, we aimed to determine and compare some important bioactive compounds and biochemical features of four different rosehip species growing naturally in high altitudes of Ardahan city located in Eastern Anatolia in Turkey. So far, there is little information about sugar and acidity in rosehips, and no previous scientific studies have been carried out on rosehip species in the region.

2. MATERIALS AND METHODS

2.1. Plant material

Mature fruits of *R. pimpinellifolia*, *R. villosa*, *R. canina*, and *R. dumalis* were collected at the same ripening stage in two locations in Ardahan Province in September 2014 (Table 1). The fruits were immediately transferred to the laboratory in polyethylene bags and stored

at –20°C until analysis. Rosehip species have been identified based on fruit, flower and leaf of the collected genotypes as described by DAVIS (1972). All analyses except sugar analyses were carried out in triplicate. In total, 75 fruits were used for each species, and each replicate consisted of 25 fruits.

Table 1: The collection areas of species.

Species	Collection Areas
R. pimpinellifolia L.	Ardahan, Çıldır, Gölebakan village, Fields, 2010m, September 2014
R. canina L.	Ardahan, Posof Baykent village, Alaybeyi located, 1950m, September 2014
R. villosa L.	Ardahan, Posof Baykent village, Alaybeyi located, 1950m, September 2014
R. dumalis L.	Ardahan, Posof Gönülçalan village, Gönülçalan forest, 2000m, September, 2014

2.2. Fruit weight, total soluble solids, total dry matter, pH and titratable acidity

Ten hips of every species were weighted on a digital scale with a sensitivity of 0.01 g (TX-4202L, Shimadzu, Japan). The seeds of hips of every species were counted (n=10). Total soluble solids in ten hips of every species were determined using a digital refractometer (Mettler Toledo 30P, USA) and expressed in ˚Brix at 22°C. The total dry matter in ten hips of every species was measured according to the AOAC (1984) reference method. Acidity in ten hips of every species was determined titrimetrically according to CEMEROGLU (1992) and expressed as a percentage of citric acid.

2.3. Total anthocyanin, total phenolic content and total antioxidant capacity

Determination of the total anthocyanin content was done according to GIUSTI and WROLSTAD (2001) with slight modifications. Fresh fruits (5 g) were homogenized in 10 mL of methanol containing 1% HCl for 2 min, then kept overnight, and filtered through Whatman No. 2 filter paper. Two extracts were prepared, one with potassium chloride buffer, pH 1.0 (1.86 g of KCl in 1 L of distilled water), and the other with sodium acetate buffer, pH 4.5 (54.43 g of $CH_3CO_2Na.3H_2O$ in 1 L of distilled water). Absorbance of the extracts was measured at 510 and 700 nm (SQ2800, Unico UV visible Spectrophotometer, USA) after 15 min of incubation at room temperature. The content of total anthocyanin was calculated from the molar absorption of cyanide 3-glucoside.
The total phenolic content was determined by the Folin-Ciocalteu method (SPANOS and WROLSTAD, 1992). A fruit sample (5 g) was homogenized (T18, IKA Homogeniser, Germany) in 25 mL of ethanol and centrifuged (NF 400, Nüve, Turkey) at 3.500 *g* for 3 min. The supernatant was collected, purified by filtration through filter paper, and 2 mL of 10% Folin-Ciocalteu reagent was added to 0.4 mL of the extract, followed by incubation for 2-3 min. Then, 1.6 mL of 7.5% Na_2CO_3 solution was added to the mix and incubated for 1 hour in the dark. Absorbance was measured at 765 nm on a spectrophotometer (SQ2800, Unico UV visible Spectrophotometer, USA) against the blank solution (0.4 mL of water, 2 mL of Folin-Ciocalteu reagent, and 1.6 mL of Na_2CO_3). The total amount of phenolic

compounds was calculated as a mg gallic acid equivalent (GAE)/100 g by using the gallic acid standard.
The ferric reducing antioxidant power (FRAP) assay was performed according to BENZIE and STRAIN (1996). Samples (1 g) were homogenized in 50 mL of 80% methanol solution in a flask wrapped in aluminum foil. The flasks were incubated in an incubator shaker (IKA, Germany) at 30°C and 150 *g* for 24 hours. The samples were centrifuged at 3.200 *g* for 20 min. The supernatant was collected, and 200 µL of supernatant was mixed by vortexing (IKA, Germany) with 3 mL of the FRAP reagent (300 µM acetate buffer, pH 3.6, 10 µM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 µM HCl, and 20 µM $FeCl_3$, 10:1:1 (v/v/v)). The samples were incubated in a water bath (ST30, Nüve, Turkey) at 37°C for 30 min, and the absorbance was determined at 593 nm. Standard curve was prepared using different concentrations of trolox and expressed in mmol trolox equivalent (TE)/g frozen sample.

2.4. Sugar and organic acid contents

Determination of sugar contents in rosehips was done according to MIRON and SCHAFFER (1991) by HPLC (HP Agilent 1100 series, USA) using a Shim-Pack HRC NH2 column (300 × 7.8 mm, 5 µm) with a refractive index detector (RID). Frozen samples (1 g) were powdered in liquid nitrogen with a mortar and pestle, transferred to an Eppendorf tube, and 20 µL of aqueous ethanol (80%, v/v) was added. The mixture was placed in an ultrasonic bath (Sonorex Digital 10P, Switzerland) sonicated for 15 min at 80°C, then filtered, and the procedure was repeated three times. All filtered extracts were combined and evaporated to dryness in a boiling water bath. The residue was dissolved with 2 mL of distilled water and filtered before HPLC analysis. The sugar contents in the samples were calculated using calibration curves plotted by using external standards.
Identification of organic acids and determination of their contents were done by HPLC using an HPX 87H (300 × 7.8 mm, 5 µm) column and a UV detector. For carboxylic acid and L-ascorbic acid detection, 1 g of a frozen sample was powdered in liquid nitrogen with a mortar and pestle and mixed with 20 mL of aqueous meta-phosphoric acid (3%) at room temperature for 30 min on a shaker. The acidic extract was filtered, made up to 25 mL with the same solvent, and then used for HPLC analysis. External standards were used to identify and calculate organic acid contents from the retention times and calibration curves (BOZAN *et al*., 1997).

2.5. Statistical analysis

All results were analyzed using the SPSS (version 15) statistical analysis package and the mean ± standard error values obtained from triplicate measurements. Data were subjected to analysis of variance (ANOVA) and significant differences between the groups were determined by the multiple comparison procedure according to DUNCAN (1955). Differences at $p<0.05$ were considered significant. The Cluster analysis applied to evaluate relationships among species was performed by Ward's method using Euclidean distances.

3. RESULTS AND DISCUSSIONS

3.1. Pomological and biochemical characterization

The fruit weights of the samples, total soluble solids, total dry matter, pH and acidity values are given in Table 2. Statistically significant differences ($p<0.05$) in these parameters between the species were determined (Table 2). It was also determined that the average

fruit weights of the species varied between 9.8 g (*R. dumalis*) and 34.5 g (*R. canina*), and that the average seed numbers of 10 hips varied between 10 (*R. villosa*) and 23 (*R. canina*). The total soluble solids showed statistically significant variations among the rosehip species (Table 2). The lowest total soluble solids were found in *R. villosa* (14 ˚Brix), while the highest value was found in *R. canina* (22 ˚Brix), followed by *R. pimpinellifolia* and *R.dumalis* (20 ˚Brix). The total dry matter content of the fruits was between 40.1% (*R. villosa*) and 56.7% (*R. canina*) (Table 2). Demir and Ozcan (2001) found that total dry matter amounts in *R. canina* were in the range between 20.5 and 23.5%. Ercisli (2007) reported that the total soluble solids of different rosehip species growing in the Erzurum region ranged between 29.4 and 37.3 ˚Brix, and that the highest total soluble solids were determined in *R. dumalis* (37.3 ˚Brix), while the lowest content was found in *R. villosa*. The author also found that the highest total dry matter content was shown by *R. dumalis* (40.4%) and the lowest total dry matter content was shown by *R. villosa* (29.4%). The total soluble solids of rosehip species were reported to range between 14 and 40 ˚Brix in several studies carried out in different regions of Turkey (Sen and GuneS, 1996; Misirli *et al*., 1999; Demir and Ozcan, 2001). In our study, the acidity was inversely proportional to total soluble solids and ranged between 1.00% (*R. canina*) and 2.67% (*R. villosa*). The lowest pH value was observed in *R. villosa* (2.86), while the highest pH value was observed in *R. canina* (3.50). Demir and Ozcan (2001) demonstrated that the acidity of *R. canina* hips collected from two different regions was 1.17% in Hadim and 1.44% in Kastamonu, while the pH values were 5.12 in Hadim and 4.34 in Kastamonu. Different rosehip species, cultivars, climate and geographical conditions are known to affect total soluble solids, acidity and pH values (ERCISLI, 2007). Also, high altitude causes acidity levels to increase in fruits.

Table 2: Some pomological and biochemical properties of rosehip species.

Species	Localities	Fruit Shape	Flesh Colour	Peel Colour	Fruit weight (g)
R. pimpinellifolia	Çıldır	Round	Purple	Black	17.3±0.6^{b}
R. villosa	Posof	Round	Orange	Red	10±0.1^{c}
R. canina	Posof	Elliptic	Orange	Red	34.5±0.9^{a}
R. dumalis	Posof	Elliptic	Orange	Red	9.8±0.2^{c}
Species	**Average seeds/1 hip**	**Total soluble solids (˚Brix)**	**Total Dry matter (%)**	**Acidity (%)**	**pH**
R. pimpinellifolia	11±0.9^{ab}	20±0.9^{ab}	55.3±2.5^{a}	1.30±0.10^{b}	3.00±0.05^{b}
R. villosa	10±1.1^{ab}	14±0.8^{b}	40.1±6.7^{b}	2.67±0.09^{a}	2.86±0.04^{c}
R. canina	23±0.7^{a}	22±1.4^{a}	56.7±5.5^{a}	1.00±0.02^{b}	3.50±0.05^{a}
R. dumalis	18±0.8^{a}	20±1.6^{ab}	55.9±8.2^{a}	1.45±0.02^{ab}	3.06±0.01^{b}

Different letters (a-d) for same line are statistically significantly differences among sampling dates by Duncan's multiple range test at $p<0.05$.

3.2. Determination of total anthocyanin, total phenolic content and total antioxidant capacity

The total anthocyanin, total phenolic content and total antioxidant capacity of the rosehip species are given in Table 3. *R. pimpinellifolia,* known as a 'black rosehip' in the region, had

the highest anthocyanin content (3.72 mg/100 g), whereas *R. dumalis* and *R.villosa* had the lowest values (2.43 and 2.45 mg/100 g, respectively). It was previously reported that the major anthocyanin in *R. canina* fruits was cyanidin-3-O-glucoside (Guimaraes *et al*., 2013). Guerrero *et al.* (2010) found that the total anthocyanin content in rosehip fruits was 0.38 mg/100 g, and the total phenolic content was 145.7 mg/100 g. Anthocyanins give color to fruits and they have therapeutic and antioxidant activity. Cyanidin-3-O-glucoside was reported to have the highest oxygen radical scavenging effect (WANG *et al*., 1997).

In our study, the lowest total phenolic content was found in *R. pimpinellifolia* (1081 mg GAE/100 g), and the highest content was found in *R. canina* (6298 mg GAE/100 g). Various researchers determined that the amounts of total phenolic compounds were between 176–9600 mg GAE/100 g in ripe rosehips (ERCISLI, 2007; Su *et al*., 2007; EGEA *et al*., 2010; FATTAHI *et al*., 2012; ROMAN *et al*., 2013). Similar to our data, YOO *et al.* (2008) found the total phenolic content in rosehips to be 815.5 mg GAE/100 g, and FATTAHI *et al*. (2012) reported it to be 176.48–225.65 mg GAE/100 g. DEMIR *et al*. (2014) detected the highest total phenolic content among rosehip samples collected in Gumushane, Turkey in *R. dumalis* subsp. *boissieri* (5200 mg GAE/100 g) and the lowest total phenolic content in *R. canina* (3100 mg GAE/100 g). The total phenolic content results obtained in our study were found to be higher than those reported in the literature. The differences may be due to different extraction methods, the ripening stage of the hips, environmental conditions, the harvest season, altitude or plant genotype.

The FRAP (Ferric reducing antioxidant power) method was developed by BenziE and Strain (1996) and is based on the reduction by antioxidants of Fe^{3+} complexed by TPTZ (tripyridyl triazine) to Fe^{2+} in a low-pH environment. The results showed that there were statistically significant differences ($p<0.05$) in the total antioxidant capacities between the rosehip species. *R. pimpinellifolia* was found to have the lowest antioxidant capacity (10.04 mmol TE/g), and *R. canina* was found to have the highest antioxidant capacity (97.95 mmol TE/g). The values found in our study were lower than those found by DEMIR *et al*. (2014). The authors reported that the total antioxidant capacity of *R. dumalis* subsp. *boissieri* was 194.36 mmol TE/g and that of *R. canina* was 103.56 mmol TE/g. These differences may be due to factors such as the geographical area, the degree of ripening, climate conditions and experimental conditions. CUNJA *et al*. (2015) reported that the highest antioxidant capacity was observed in *R. canina* fruits harvested in September and that frost damage occurring in the following months decreased antioxidant capacity. In addition, it was shown that antioxidant capacities of *R. canina* fruits ranged from 63.35 to 127.8 μM TE/100 g as determined by the DPPH method.

Table 3: Total anthocyanin, total phenolic content, total antioxidant capacity (FRAP), organic acid and sugar contents of rosehip species.

		R. pimpinellifolia	*R. villosa*	*R. canina*	*R. dumalis*
	Total anthocyanin (mg/100g)	3.72±0.06[a]	2.45±0.03[c]	2.75±0.07[b]	2.43±0.09[c]
	Total phenolic (mg GAE/100g)	1081±12.8[d]	2944±70.8[c]	6298±116.7[a]	4411±16.9[b]
	FRAP (mmol TE/g)	10.04±0.47[d]	37.84±1.55[b]	97.95±2.12[a]	26.45±6.98[c]
	L ascorbic acid (mg/100g)	24.93±4.0[d]	119.83±3.3[c]	754.48±100.2[a]	254.81±12.5[b]
Sugars	*Sucrose (g/100g)*	0.38[c]	0.42[b]	0.55[a]	0.41[b]
	Glucose (g/100g)	5.99[d]	12.48[a]	8.05[b]	6.79[c]
	Fructose (g/100g)	4.38[c]	4.90[b]	5.03[a]	4.15[d]
	Sorbitol (g/100g)	4.17[c]	6.25[a]	5.15[b]	3.94[d]
	Total sugar(g/100g)	14.92[d]	24.05[a]	18.78[b]	15.29[c]
	Oxalic acid (g/100g)	0.14±0.01[c]	0.25±0.02[b]	0.38±0.04[a]	0.29±0.01[b]
Organic acids	*Tartaric acid (g/100g)*	0.21±0.08[c]	0.26±0.03[c]	0.65±0.13[a]	0.30±0.01[b]
	Malic acid (g/100g)	0.65±0.04[b]	0.45±0[c]	0.48±0.06[c]	0.73±0.16[a]
	Citric acid (g/100g)	0.48±0.05[c]	1.05±0.05[a]	0.94±0.14[b]	0.95±0.3[b]
	Succinic acid (g/100g)	0.092±0.01[a]	0.007±0[c]	0.010±0[b]	0.006±0[c]
	Fumaric acid (g/100g)	0.015±0[b]	0.011±0[c]	0.033±0.01[a]	0.014±0[b]

Different letters (a-d) for same line are statistically significantly differences among sampling dates by Duncan's multiple range test at $p<0.05$.

3.3. L-Ascorbic acid, sugar and organic acid contents

Sugar and organic acid contents are the most important factors determining fruit quality and taste. Organic acids increase bioavailability of ascorbic acid by inhibiting ascorbic acid oxidation (PADAYATTY and LEVINE, 2001; KOBUS *et al*., 2005). In this study, there were significant differences in L-ascorbic acid, sugar and organic acid contents between the rosehip species, as presented in Table 3. The L-ascorbic acid contents of the species were found to range between 24.93 mg/100 g (*R. pimpinellifolia*) and 754.48 mg/100 g (*R. canina*). The L-ascorbic acid values obtained in our study were higher than those reported in the literature. ROMAN *et al*. (2013) revealed that the ascorbic acid contents in ripe rosehips ranged between 112.2 and 360.2 mg/100 g. Barros *et al*. (2010) found the ascorbic acid content in *R. canina* to be 68.04 mg/100 g. NOJAVAN *et al.* (2008) determined that the ascorbic acid content increased upon ripening to 417.5 mg/100 g in rosehip species and that the value was 6-fold of that found in oranges. DEMIR *et al*. (2014) determined that the ascorbic acid content was lowest in *R. dumalis* (65.75 mg/100 g) and highest in *R. gallica* (160.30 mg/100 g). In addition, CELIK *et al*. (2009) found the ascorbic acid contents in rosehip species in Van, Turkey to be 604-1.032 mg/100 g. It was also shown that there were significant differences between rosehip species in ascorbic acid content, which could be affected by ecologic factors, the degree of ripening and soil conditions (MABELLINI *et al*., 2011; ADAMCZAK *et al*., 2012). Rosehip species growing in high altitude regions are rich in ascorbic acid due to higher light exposure and lower oxygen amounts. Light exposure increases the amount of carotene and thus protects ascorbic acid in the fruit, while the lack of oxygen reduces oxidative stress and lessens ascorbic acid breakdown (YAMANKARADENIZ, 1983). Ascorbic acid contents of rosehips vary depending on climate conditions, fruit types and years (DEMIR and OZCAN, 2001).

Glucose was found to be the most common sugar in *Rosa* species, and the lowest glucose content was found in *R. pimpinellifolia* (5.99 g/100 g) while the highest content was found in *R. villosa* (12.48 g/100 g). The amounts of sucrose ranged in the species between 0.38–0.55 g/100 g (*R. pimpinellifolia* and *R. canina*, respectively), the fructose contents ranged between 4.15-5.03 g/100 g (*R. dumalis* and *R. canina*, respectively), and the sorbitol contents ranged between 3.94-6.25 g/100 g (*R. dumalis* and *R. villosa*, respectively). Also, the lowest total sugar amount was found in *R. pimpinellifolia* (14.92 g/100 g) while the highest amount was found in *R. villosa* (24.05 g/100 g). Other studies reported glucose contents in rosehip fruits to range between 7.45-12.94 g/100 g and fructose contents to range between 7.96-18.44 g/100 g. Similar to our results, sucrose contents were reported to range between 0.88-5.61 g/100 g and total sugar contents were reported to range between 12.05-20.46 g/100 g (YORUK *et al*., 2008; BARROS *et al*., 2011; ROSU *et al*., 2011; OZRENK *et al*., 2012). Likewise, DEMIR *et al*. (2014) revealed glucose amounts in *Rosa* species to range between 9.54 g/100g (*R. dumalis*) and 17.25 g/100g (*R. gallica*) and fructose amounts to range between 10.78 g/100g (*R. dumalis*) and 18.84 g/100g (*R. canina*). The fructose and sucrose values obtained in our study were found to be lower than those reported in the literature, whereas the total sugar amounts were found to be higher. The differences in organic acid and sugar values between our and other studies might be due to different soil and climate conditions of the region and the differences in experimental analysis. Also, differences in the harvest season are thought to affect the results.

The major organic acid in the *Rosa* species was citric acid (0.48 to 1.05 g/100 g). In this study, we found that oxalic acid was most abundant in *R. canina* (0.38 g/100 g) and least abundant in *R. pimpinellifolia* (0.14 g/100 g). Fumaric acid was also most abundant in *R. canina* (0.033 g/100 g) and least abundant in *R. villosa* (0.011 g/100g). The tartaric acid values among the species were 0.21-0.65 g/100 g, the malic acid values were between 0.45 and 0.73 g/100 g, and the succinic acid values were between 0.006-0.092 g/100 g. In a

previous study, the citric and malic acid amounts in *Rosa* species were 4.76-9.12 g/100 g and 0.45-1.10 g/100 g, respectively (DEMIR *et al*., 2014). ADAMCZAK *et al*. (2012) found that the citric acid content in *R. tomentosa* was 4.34 g/100 g. Thus, the organic acid values obtained in our study were lower compared to those found in previous studies.
A dendogram based on the pomological and biochemical characteristics studied of the rosehip species can be seen in Fig. 1.

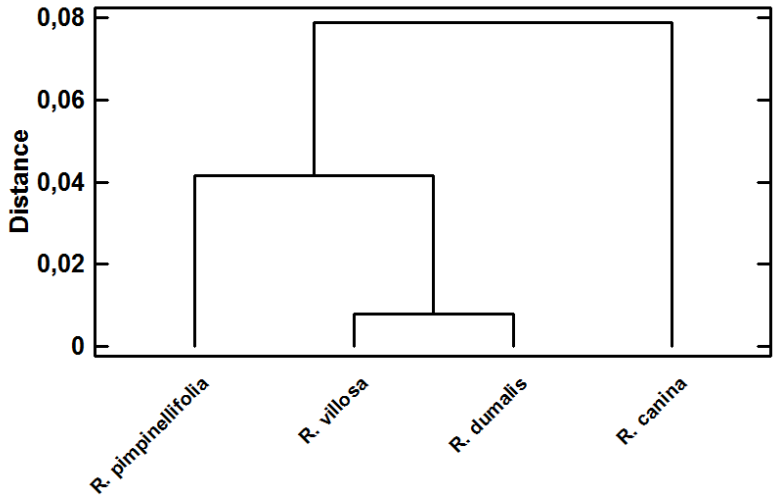


Figure 1: Cluster analyse of rosehip species according to their pomological and biochemical properties.

The species were grouped into 2 main clusters. In the first cluster, *R. villosa* and *R. dumalis* were found to be the closest species based on the characteristics analyzed. Fruit weights, total anthocyanin contents and succinic acid contents of both species were low. *R. pimpinellifolia* was found in the same cluster, while *R. canina* fell in a separate cluster, for its characteristics were different from those of the other species.
This study aimed to determine and compare some important bioactive compounds and biochemical features of 4 different rosehip species growing naturally in Ardahan (Eastern Anatolia, Turkey) and mostly consumed by the locals. The differences in acidity and sugar contents of the species, compared with previous studies, are thought to be due to different altitudes. Moreover, it was found that the L-ascorbic acid, total anthocyanin and total phenolic content values, known to increase with altitude, were high in this study. The total antioxidant capacities of these species were also high. This study is important as a foundation for further research. Besides, knowing biochemical characteristics of the species will facilitate the work of plant breeders and growers. It is known that bioactive components of fruits positively affect health. It is suggested that rosehip fruits are good sources of bioactive compounds and phytonutrients. Their consumption may prevent some illnesses and protect health.

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COMPARISON OF E-NOSES: THE CASE STUDY OF HONEY

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ABSTRACT

Authentication is a major research theme in food analysis. Electronic noses (e-Noses) represent an effective tool for food authentication and - among others - for the determination of food origin. In this work we compare the performance of two e-Noses (metal-oxide-sensor based *vs* mass-spectrometry based) in the determination of geographic origin of honey. We analyzed 14 honey samples from South Tyrol, an Italian alpine region, which were compared with other 12 commercial samples from diverse European origins. Both e-Noses afforded 85% of correct identifications. Mass spectrometry provided a deep analytical insight, thanks to the possibility to determine mass peaks with good accuracy.

Keywords: South Tyrol Italian honeys, geographic origin, PTR-ToF-MS, e-Nose

1. INTRODUCTION

Gas-chromatographic methods represent the benchmark for food volatile analysis. In spite of its robustness and analytical power, gas-chromatographic analysis, being a separation-based technique, is time-consuming and has a low analytical throughput. An alternative analytical approach is based on the employment of electronic Noses (e-Noses). In the e-Nose, an array of electrochemical sensors (GARDNER, 1999) or a mass spectrometer (PÉREZ PAVÓN *et al*., 2006) provide a fingerprint of the headspace of a given sample. Typically an e-Nose, trained using samples of known origin, can be employed to recognize and predict sample identity on the basis of a specific fingerprint. Unlike GC-MS, the e-Nose provides little information as to the actual composition of the sample headspace; on the other hand e-Noses are generally easy to use, they provide a high analytical throughput and they are relatively inexpensive.

Proton Transfer Reaction-Mass Spectrometry (PTR-MS), similarly to an MS-based e-Nose, performs a rapid and direct analysis of the headspace of the sample. Unlike in MS-based e-Noses the use of a *soft* ionization approach allows to minimize fragmentation, thus increasing the informational content of the mass spectral fingerprint (Hansel *et al*., 1995). The coupling of PTR-MS to Time-of-Flight (ToF) mass analyzers has further enhanced the performance of the technique, allowing for high mass and time resolution. PTR-ToF-MS has already been employed in the determination of food origin, with applications, among others, on cheese (GALLE *et al*., 2011), ham (DEL PULGAR *et al*., 2011) and coffee (YENER *et al*., 2015).

Honey is traditionally consumed and appreciated worldwide, mainly because of its organoleptic properties and nutritional value. Even though the main constituents of honey are sugar and water, a great variety of aroma compounds can also be encountered.

Gas Chromatography-Mass Spectrometry (GC-MS) was often employed to describe the composition of honey headspace, and up to 400 distinct VOCs were reported is a single honey type (GUYOT *et al*., 1998). The two main factors affecting the quality of honey are its botanical and geographical origin. The botanical origin of the nectar and plant secretions is a major source of aroma compounds and aroma precursors but geographical origin, through the influence of soil and climate, also plays an important role. Several GC-MS studies were carried out with the aim to study and predict the botanical and/or geographical origin of honeys (CUEVAS-GLORY *et al*., 2007; KAŠKONIENĖ and VENSKUTONIS, 2010). This approach mostly applies in the case of honeys deriving from a single plant species (*unifloral* honeys) and researchers have claimed the discovery of plant specific-markers (CASTRO-VÁZQUEZ *et al*., 2006; JERKOVIĆ *et al*., 2006; VERZERA *et al*., 2014; JERKOVIĆ and KUŚ, 2014); these most often include terpenes, norisoprenoids, nitriles or phenolic compounds and their derivatives (MANYI-LOH *et al*., 2011a). Less frequently, putative markers of geographic origins have also been detected (RADOVIC *et al*., 2001).

e-Noses have been effectively employed in discriminating honeys from different botanical and/or geographical origins (BENEDETTI *et al*., 2004; DYMERSKI *et al*., 2014; ZAKARIA *et al*., 2011). Sometimes the e-Nose was coupled to another technique, such as Fourier Transform-Infra Red (FT-IR) spectroscopy (SUBARI *et al*., 2012) or Electronic Tongue (BURATTI *et al*., 2004). The coupling of analytical techniques based on different physical-chemical principles and the "merged" dataset thus generated often allowed for an enhancement in discrimination capability.

Recently, PTR-MS coupled to a quadrupole mass analyzer was employed in the classification of honeys having different botanical origins (KUŚ and VAN RUTH, 2015): the technique was not always able to perform a correct classification, affording an average prediction accuracy of 77%. In another recent paper (SCHUHFRIED *et al*., 2016), a PTR-MS

instrument using a Time-of-Flight (ToF) mass analyzer was employed in the headspace analysis of 70 mono-floral honeys of diverse origins. The higher mass resolution of the ToF detector, along with the use of multivariate classification techniques, provided 90-100% correct predictions based on botanical origin.
The assessment of food typicality represents an issue of major relevance to the food industry and a challenging task from an analytical point of view. The objective of the present work is thus twofold: (i) find new analytical tools for the valorization of local food production and (ii) compare the performance of two e-Noses based on different physical-chemical principles. Quite interestingly, the two instruments provide similar performance, with advantages and drawbacks on both sides: the MOS-based e-Nose is more portable and low-cost but it does not provide information as to sample headspace composition, whereas the MS-based instrument gives a fairly detailed analytical insight.

2. MATERIALS AND METHODS

2.1 Honey samples

Honey commercial samples were provided by the Servizio Veterinario dell'Azienda Sanitaria dell'Alto Adige (Bolzano, Italy). The sample set consisted of 26 honeys, out of which 14 originating from South Tyrol and 12 from other European countries (namely Italy, Romania, Spain, Germany and Czech Republic). From the point of view of botanical origins, the sample set was rather heterogeneous, including multi-flower and forest samples as well as monofloral honeys (namely from acacia, chestnut, dandelion, lime and eucalyptus). The year of production of the honeys was 2013. All samplings were performed from the same jar, typically containing 250-500 g of sample. For a more detailed description of the sample set please refer to Table 1, supplementary material.

2.2 PTR-ToF-MS

Headspace measurements were performed using a commercial PTR-ToF 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria). The instrumental conditions in the drift tube were the following: drift voltage 550 V, drift temperature 110°C, drift pressure 2.33 mbar affording an E/N value of 140 Townsend (1 Td = 10^{-17} V*cm^2). Sampling was performed with a flow rate of 40 sccm using a heated (110°C) PEEK transfer line. Measurements were performed in an automated fashion by means of a multipurpose GC automatic sampler (Gerstel GmbH, Mulheim am Ruhr, Germany). The analytical method was mutuated from a previously validated method for coffee powder headspace analysis (YENER *et al*., 2014), with some minor adaptations (not shown). Honey aliquots (1.0 g) were transferred into 40-ml glass screw-capped vials, suitable for volatile analysis. All measurements were performed in triplicate. The measurement order was randomized to avoid possible systematic memory effects. All vials were incubated at 40°C for 30 min before PTR-MS analysis. Each sample was measured for 30 s, at an acquisition rate of one mass spectrum per second. Data processing of ToF spectra included dead time correction, internal calibration and peak extraction steps performed according to a procedure described elsewhere (CAPPELLIN *et al*., 2010). In this case this allowed to reach a mass accuracy better than 0.001 Th, which is sufficient for sum formula determination. The baseline of the mass spectra was removed after averaging the whole measurement and peak detection and peak area extraction was performed by using modified Gaussian to fit the data (CAPPELLIN *et al*., 2011). To determine the concentrations of volatile compounds in ppbv = nL of VOC L^{-1} headspace the formulas described by LINDINGER and JORDAN

(1998) were used by assuming a constant reaction rate coefficient (k_R=2×10^{-9} cm^3/s) for H_3O^+ as a primary ion.
The PTR-ToF-MS dataset was submitted to an initial step of filtration based upon concentration. The selection of mass peaks exceeding an arbitrary threshold has often proven to be an effective empirical approach to improve the discrimination ability of PTR-MS data (APREA *et al*., 2015). A concentration threshold arbitrarily set at 1 ppbV, was applied. When peaks having an estimated concentration higher than 1 ppbV were selected, a subset of 55 peaks was generated. For the purpose of multivariate analysis, the mass spectral fingerprint obtained for every sample was normalized by the corresponding total emission. Normalization has already been proven useful (YENER *et al*., 2015), generally allowing to compensate variations in total emission, at the same time preserving the mass spectral fingerprint typical of each sample or sample class.

2.3 Electronic Nose

Analyses were performed with a PEN3 e-Nose (Airsense Analytics, Schwerin, Germany). The instrument has ten Metal Oxide Semiconductor (MOS) sensors displaying different specificity profiles (Table 2). The e-Nose was equipped with an automated sampling device (headspace sampler HSS32 from Airsense Analytics). The analytical procedure for e-Nose honey analysis was validated in a previous work (ZULUAGA *et al*., 2011). Honey samples were measured directly, with no prior dilution. One-gram aliquots were transferred into 10-ml vials, suited for volatile compound analysis. Samples were equilibrated for 20 minutes at 40°C and then analyzed. The e-Nose program was based upon measurement cycles of 150 seconds, separated by 450 seconds of sensor flushing with clean air. Inlet flow was set at 400 ml/min. E-Nose sensor specificities, as stated by the producer, are reported in Table 2.

Table 1: Main characteristics of the honey samples used in the study.

Sample designation	Geographic origin	Botanical origin
H01	South Tyrol	acacia
H02	South Tyrol	forest
H03	South Tyrol	forest
H04	South Tyrol	multiflower
H05	South Tyrol	forest
H06	South Tyrol	mixed (forest/flower)
H07	South Tyrol	multiflower
H08	South Tyrol	multiflower
H09	South Tyrol	multiflower
H10	South Tyrol	chestnut
H11	South Tyrol	multiflower
H12	South Tyrol	multiflower
H13	South Tyrol	multiflower

H14	South Tyrol	multiflower
H15	South Tyrol	multiflower
H16	South Tyrol	dandelion
H17	South Tyrol	multiflower
H18	South Tyrol	multiflower
H19	Italy	chestnut
H20	Italy	chestnut
H21	Italy	lime
H22	Italy, Romania	acacia
H23	Italy, Spain, Romania	forest
H24	Italy	eucalyptus
H25	EU	multiflower
H26	Germany	multiflower
H27	Czech Republic	multiflower
H28	Czech Republic	multiflower
H29	Czech Republic	forest

Table 2: E-nose sensor specificities, as declared by the manufacturer.

Sensor	**Specificity**
S1	Aromatic compounds
S2	Broad range, very sensitive, reacts with nitrogen oxides
S3	Ammonia, aromatic compounds
S4	Mainly hydrogen, selectively (breath gases)
S5	Alkenes and less polar aromatic compounds
S6	Methane, broad range
S7	Sulfur compounds, terpenes, limonene and pyridine
S8	Alcohols, broad range
S9	Sulfur organic compounds
S10	Reacts on high concentrations (>100ppm), sometimes very selective (methane)

2.4. Software

All data analysis was performed using MATLAB (Statsoft, Natick, MA) and R software (the R Foundation for Statistical Computing, Vienna, Austria).

2.5. Statistical analysis

All results are to be intended as means of triplicate measurements. For Principal Component Analysis (PCA), all variables were normalized using the respective standard deviations. Linear Discriminant Analysis (LDA) was carried out with the aid of the R package MASS (Venables, Ripley, and Venables 2002). LDA models were cross-validated by "leave-one-out" method.

3. RESULTS AND DISCUSSIONS

3.1. Classification of honey samples based on Metal Oxide Sensors

In this study, the headspace of 26 honey samples was analyzed with a portable electronic nose. Such device employed an array of ten electronic gas sensors (metal oxide sensors) able to detect and distinguish headspace volatiles via a pattern-recognition algorithm. Typical signals generated by the e-nose with honey samples are shown in Fig. 1.

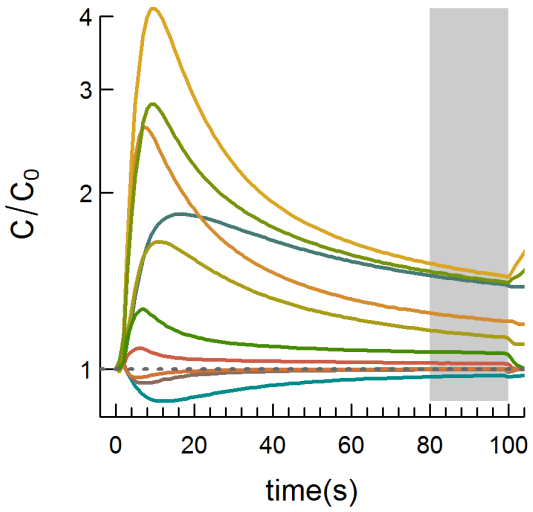


Figure 1: Typical e-Nose profile obtained on a honey sample. Signals were averaged between 80 and 100 seconds, as indicated by the shaded area.

The responses from each sensor during 120 s allowed to extract two data: the maximum signal and a plateau value (recorded arbitrarily between 80 and 100 s of measurement). Based on a preliminary data analysis (results not shown), the two data sets displayed a high degree of covariance; the plateau values, which displayed better repeatability, where thus used in subsequent analysis, whereas maximum values were discarded. Fig. 2 shows the score plot of the principal component analysis (PCA). PCA is an unsupervised pattern recognition tool very useful to plot in a reduced dimensional space (*i.e.* generally, the first two principal components are sufficient to explain most of the variance contained in the original dataset), observe any potential similarities between the samples and, in case, identify the most important variables responsible for such similarities. In detail, the score values of the first two principal components (accounting for the 91% of the total

variability) allow to establish a relatively good separation between samples from South Tyrol from those of other origins, mostly obtained through the employment of component 2.
Looking at the loading values (Fig. 2), the observed discrimination capacity is mainly explained by just 5 metal oxide sensors, and namely S2, S6, S7, S8 and S9. The higher response obtained with sensor S8 in honeys of various European origins is in agreement with mass spectrometric data, showing a higher ethanol content (paragraph 3.2). Sensors S7 and S9, which are specific for sulfur compounds, show more intense signals in South Tyrol honeys. Instead, sulfur compounds do not seem to be important for honey discrimination by PTR-MS, thus suggesting that the analytical responses provided by the two e-Noses are somewhat complementary.

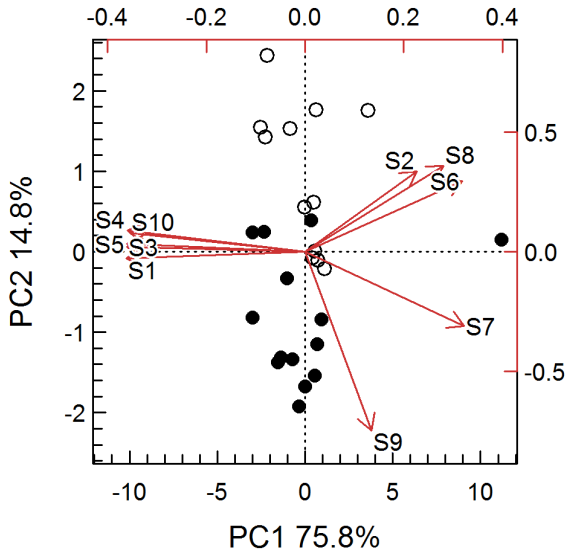


Figure 2: Principal Component Analysis of the autoscaled data obtained by e-Nose (● = South Tyrol, ○ = Other). Alphanumeric codes correspond to e-Nose sensors.

3.2. Classification of honey samples based on PTR-ToF-MS

We next investigated the potential enhancement offered by a more advanced electronic nose based on mass spectrometry. Here we used an on-line proton-transfer-reaction mass spectrometry based on hydronium ions as ion source reagents directly connected to an analyzing time-of-flight mass spectrometer system (PTR-ToF-MS). Fig. 3 shows a typical mass spectrum obtained for a honey sample, in the range 15-215 Th of mass-to-charge ratio (*m*/*z*). The high mass resolution provided by the Time-of-Flight mass analyzer enabled the detection of more than 204 mass peaks**.** Upon filtering based on average concentration a subset of 55 mass peaks, having concentrations higher than 1ppbV, was selected and employed in further analyses.

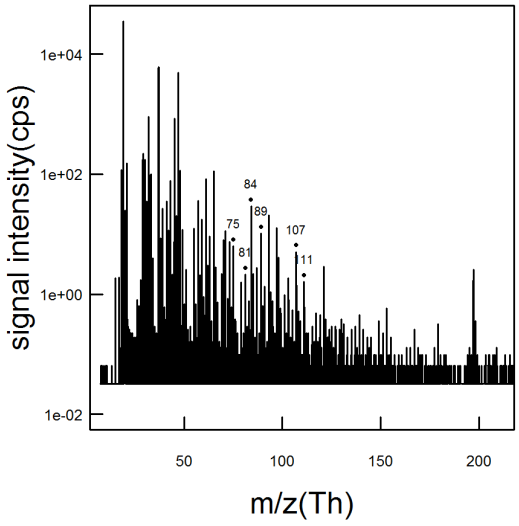


Figure 3: Typical mass spectrum obtained on a honey sample. The position of some selected mass peaks is highlighted by the corresponding nominal masses.

PCA was next used to reduce the dataset dimensionality, observe sample similarities and highlight the most important mass fragments (Fig. 4). The score values of the first two principal components (accounting for the 53% of the total variability) allow visualize a partial separation between the samples from South Tyrol respect those from other origin. Separation was achieved thanks to both Principal Components 1 and 2. The visual inspection of Principal Component higher than two did not provide an improvement in discrimination ability. To understand which mass fragment is responsible for the observed clustering of the samples, the loading values were then analyzed; a subset of 10 mass peaks was defined (Fig. 4), referring to the variables whose loadings showed the highest absolute values for either Principal Component.

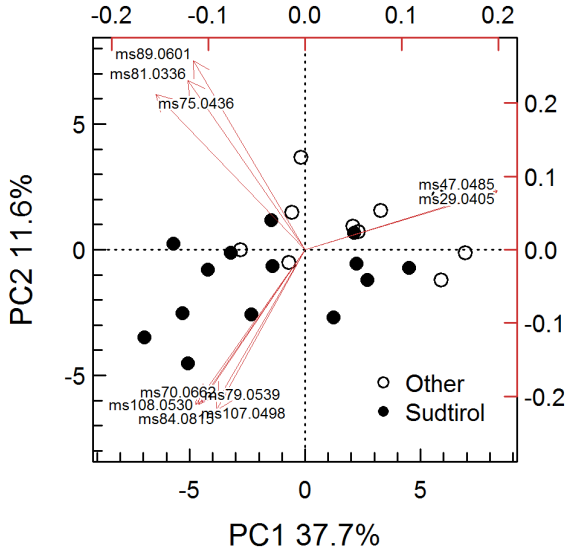


Figure 4: Principal Component Analysis of the autoscaled data obtained by PTR-ToF-MS (● = South Tyrol, ○ = Other). Alphanumeric codes correspond to mass-to-charge ratios as measured by PTR-ToF-MS.

The mass spectrometric data obtained by means of the PTR-TOF has a resolution of 4000 Dm/m or higher. This, after calibration, typically allows for the determination of masses up to the third decimal digit, eventually ensuring the assignment of a sum formula to most mass peaks. The cross-matching of mass spectral data with published databases of honey volatiles (KAŠKONIENĖ and VENSKUTONIS 2010; MANYI-LOH, Ndip, and Clarke 2011b; WOLSKI *et al*. 2006) and fragmentation patterns of pure compounds (APREA *et al*. 2007; BUHR, van Ruth, and Delahunty 2002; DEMARCKE *et al*. 2009) allowed to tentatively assign some of the detected mass peaks to known constituents of the headspace of honey. All these compounds are well representative of factors having a key impact in affecting honey quality and characteristics such as floral origin, oxidation, fermentation *etc.* Mass peaks *m*/*z* 84.082 (with a fragment at *m*/*z* 70.066), *m*/*z* 81.034 and *m*/*z* 111.044 were tentatively attributed to an N-heterocycles and carbonyls which, even though not necessarily reported in honey, are known as Maillard reaction intermediates and might participate in non-enzymatic browning reactions that take part in the oxidative alteration of many food products, including honey (NURSTEN 2005). Mass peak *m*/*z* 107.049 (along with fragment at *m*/*z* 79.054 and 13C isotopologue at *m*/*z* 108.053) was tentatively assigned to benzaldehyde, a compound previously reported in some unifloral honeys (MOREIRA and DE MARIA 2005) and associated to almond and burnt sugar sensory notes (ACREE and ARN 2004). Other relevant mass peaks could be assigned to well-known fermentation products already detected in honey (WOLSKI *et al*. 2006). They namely were ethanol (*m*/*z* 47.048 and fragment at *m*/*z* 29.040), methyl-acetate and methyl-formate (*m*/*z* 75.044), and acetoin, butyric acid, butyrolactone and ethyl-acetate (*m*/*z* 89.060 and fragment *m*/*z* 71.049).

3.3. Comparison of the e-Noses

Two classification models based on linear discriminant analysis (LDA) were next build up on the basis of the most important variables selected by PCA for e-Nose based on MOS sensors and PTR-TOF-MS, respectively. LDA is a supervised pattern recognition tool especially developed for qualitative classification problems. When the signal from the selected MOS sensors were used, the resulting model afforded correct identifications for 82% and 87% of South Tyrol honeys and samples of other origin, respectively, with an overall 85% of correct identifications. Instead, when the model was built with the selected fragments from PTR-TOS-MS, then, the resulting classification model was able to correctly identify 92% and 78% of honeys samples from South Tyrol and other origin, respectively, with an overall 85% of correct identifications. Further detail about the discrimination is provided in the supplementary material (Table 3). The results show that a portable e-Nose, once validated, may have classification performance similar or even better than that achievable with instruments based on high resolution mass spectrometry. Contrarily to what expected, the higher amount of fragments detected by the high resolution mass spectrometers do not result with a higher capacity to discriminate samples. Apparently, the capacity of discriminating the samples is hindered by an increased noise or uncertainty around the signal of individual fragments.

Table 3: LDA confusion matrices, as obtained using e-Nose and PTR-ToF-MS (left and right, respectively).

		Model based upon MOS-based e-Nose		**Model based upon PTR-ToF-MS**	
		Predicted		**Predicted**	
Original		**Other**	**South-Tyrol**	**Other**	**South-Tyrol**
	Other	10	2	11	1
	South-Tyrol	2	12	3	11

4. CONCLUSIONS

This research work presents an unprecedented analytical approach based on two different types of electronic nose. This approach was employed to address the question of the typicality of honeys from South Tyrol (Italy). PTR-ToF-MS, with its high mass resolution, allowed for a rapid, yet thorough characterization of the honey headspace, permitting to pinpoint several candidate aromatic markers. The MOS-based electronic nose provided a cost effective solution to the same problem, being more portable and less expensive than the latter instrument. The sample set was of limited size (26 honeys) and the work is thus intended to be a preliminary study. Botanical diversity, which is known to play a major role on headspace composition, was probably underestimated, and the corroboration of these first results by means of a more extended survey is indeed advisable.
The work also addresses the strategic theme of the typicality of food products issued from a small alpine region (South Tyrol). The work demonstrates how the development of novel analytical approaches can enable researchers and institutions to validate the typicality of regional products, representing an undoubted source of added value to all local productions.

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PAPER

FUNCTIONAL FOODS: TRENDS AND DEVELOPMENT OF THE GLOBAL MARKET

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ABSTRACT

In the functional foods market, the products targeting health and mental well-being have prompted the food industry to increase research and development of these new foods.
Despite the uncertainties of existing regulations, outlining the context of a rapidly expanding market in main countries.
This paper provides an overview of the current situation of the global market of functional foods. The objective of this analysis is to determine whether, and in what way, the field of functional foods can actually be an opportunity both for food companies, in terms of economic benefit, and for consumers, as an opportunity healthy products.

Keywords: consumers, consumption trends, functional foods, health claims, market

1. INTRODUCTION

Since the early 21st century, our society has been witnessing a continuous increase in life expectancy and, at the same time, greater attention to quality. Consumers are increasingly concerned about their health and pay more attention to their lifestyle and the healthiness of their diet (SZAKÁLY *et al*., 2012).
The increase in demand for such foods can be explained by the increasing cost of healthcare, the steady increase in life expectancy, and the desire to improve their quality (SIRÓ *et al*., 2008).
There is no official definition of Functional Foods (FFs) common to all States, but the EU-project "Functional Food Science in Europe" (FUFOSE) gives an appropriate working definition: "A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern" (HAWKES, 2004).
Functional foods in addition to the nutritional characteristics have properties that affect positively on one or more physiological functions. This feature is related to the bioactive compounds and also depends on the various technological treatments applied to food (ARVANITOYANNIS and VAN HOUWELINGEN-KOUKALIAROGLOU, 2005). The most important challenge is to ensure that the functional ingredients will survive and remain "active" and "bio-available" after the processing and storage (DAY *et al*., 2009).
In some cases heat treatments can generate an enhancement of antioxidant activity which is associated with the formation of brown coloured melanoidins (MANZOCCO *et al*., 2000; DI MATTIA *et al*., 2007).
The beneficial effects on the body can be attributed to both the functional molecules naturally present in foods that the compounds added to the industrial food matrices. An extensive line of research is focusing on the genetic modification that will expand the options for the enrichment of foods with novel ingredients or existing ones modified (KATAN and DE ROOS, 2004).
The main substances that give the characteristic of "functionality" to the food are vitamins, flavonoids, fiber, omega-3, minerals and bacterial cultures (KESERVANI *et al*., 2010).
Functional foods are the first to bear health claims. According to the Codex Alimentarius, a claim means "any representation which states, suggests or implies that a food has particular characteristics relating to its origin, nutritional properties, nature, production, processing, composition or any other quality". The two main types of claims regard: (a) what the food contains, i.e. nutrient content claims and comparative claims and (b) what the product does in terms of health, well-being and performance, i.e. health claims (RICHARDSON *et al*., 2003). These health claims have become a means to communicate to consumers the health benefits of foods that contain specific formulations, conveying relevant information that would otherwise remain unknown.
Communication has a great impact on consumers' knowledge and attitudes (VERBEKE, 2008). Health claims are key factors for the development of the functional food market. They play a central role in driving purchase decisions, and help consumers make more informed food choices, especially if the product is made of new ingredients or performs actions beneficial to health that are poorly understood (ANNUNZIATA and VECCHIO, 2012). Consumers' purchasing decisions are influenced by many factors. Some studies have shown, for example, life satisfaction and age influence the choice of functional foods (CARRILLO *et al*., 2013). These new trends have dramatically changed the industry. Food companies are investing in this sector, with new marketing and communication strategies

and changing their food innovation process. The market for functional foods is dynamic and growing. Japan, the homeland of the FFs, is a leader in the field, followed by the United States and Europe (BLEIEL, 2010).
This article presents an overview of global markets for functional foods, in order to have a concrete vision of the evolution of this market over time.
The aim is to identify which areas are more prone to the development of this market, paying more attention to those countries that contribute significantly to the growth of this sector.

2. CONSUMER ACCEPTANCE AND MAIN SEGMENTS OF FUNCTIONAL FOODS

The growing demand for healthy lifestyles is becoming an important factor for consumers. Some studies have found a number of variables affecting the consumption and purchasing habits of consumers. These are the degree of healthiness of their diet the existence of special needs related to problems of health and nutrition information on the label (ANNUNZIATA and PASCALE, 2009).
The agro-food companies have responded to this new trend and have developed a growing variety of new products with instructions and pictures related to health: functional foods.
It is not easy to understand the level of knowledge gained by the consumer in respect of these foods and the reasons behind the decision to buy/not to buy.
Some studies have identified the main factors that influence the consumer's decision to purchase.
Authors divide these factors into three groups: consumer characteristics, purchasing situation and product characteristics (BRÖRING, 2010). Other studies distinguish among sensory attributes of food (e.g. aroma or texture), physiological (e.g. hunger or appetite) and psychological factors (e.g. mood, beliefs or attitudes) (STEIN and RODRÍGUEZ-CEREZO, 2008).
Another study has suggested the main trends that drive the success of functional foods, such as: age, sex, education and demographic changes (URALA and LAHTEENMAKI, 2007), the containment of health care costs (MILNER, 2000), media, access to more information, nutrition labeling (KOTILAINEN et al., 2006), increasing emphasis on healthy diet and global prevention of chronic diseases, innovations in food technology and brand differentiation and greater emphasis on value for money.
The high sale price of functional foods (compared to the average of the corresponding conventional food) is due to the fact that the manufacturer has to cover the high costs of production associated with the particular technologies involved in the development of the product. In addition, the manufacturer of functional foods must also invest many resources in marketing the product, as the sales of functional foods lead to good profits only if the manufacturer is able to identify the right target consumers for each specific product (BONANNO, 2012).
So even the high costs incurred in the marketing of functional foods must be covered by the selling price of such foods.
The consumer is aware of this, but some of them think that this increase is a marketing ploy of companies that leverage on the term "functional" to justify the high prices.
A considerable number of studies argues that, in general, consumers are more willing to incur expenditure for food products with beneficial effects on health (LARUE *et al*., 2004).
Four different categories of consumers that perceive and associate a different value to functional products have been identified (DI PASQUALE *et al*., 2011).

The first group represents the category of "uninformed consumers". These people mostly belong to the age group 50-64 (51%), characterized by a medium-high level of education and a prevalence of low average income. Consumers say they do not know these "new" foods and have never purchased them. They are uninformed and do not give importance to the relationship between food and health.
The second group is identified as "consumers concerned about their health". This group is characterized by people with highest average age (49 years old), with a high level of education and income. These are consumers who seem to be familiar with functional foods; they have bought them, and continue to do so. They believe that a proper diet is not enough to provide adequate health benefits and that it is necessary to supplement their diet with concentrated nutrients or, in this context, agree with the statement "functional foods bring real health benefits".
The third group is called "conscious consumers". Their average age is 40 years with a higher level of education (63% are college graduates), and their income is medium-high. These consumers are firmly convinced of the real benefits of functional foods.
The last group includes "non health conscious consumers". They are aware of functional foods due to advertising. This group has never purchased or shown interest in these foods. Therefore, we can infer how information plays a fundamental role in the creation of preferences (in particular as regards the influence of promotional campaigns on purchase behavior).
Information search and evaluation of alternatives are the two phases that precede the purchase of a FF.
With traditional media, the consumer is increasingly aware and informed. He is able to choose what and where to buy without requesting any outside opinion.
E-commerce is a new medium of information for consumers. Before buying, he has a greater selection and availability of products (FORMAN *et al*., 2009). There is greater convenience by eliminating travel costs and enabling purchases irrespective of geographic location (CAIRNCROSS, 1997). For this reason, many companies are looking to e-commerce as a tool to spread awareness and innovation by focusing on clarity, fairness, honesty and transparency.
Functional foods are driven especially by a main segment: dairy (HILLIAM, 1998; MENRAD, 2003).
The reasons of this choice by consumers are mainly due to the strong dairy tradition of many countries, but also to the attention paid to these products. The results of the development of these skills are increasingly present also on supermarket shelves that currently provide a wide variety of functional foods. Commercially the probiotic has been adopted by almost all the major brands. Also considering the continued growth of the market in recent years despite the global crisis, it is not hard to imagine a great future for this sector in the times to come.
In addition to dairy were identified other segments: soft drinks, bakery products and baby food. These segments are followed by micro-segments such as food intolerance, diet, gut, defense, energy reintegration, anti-cholesterol, calcium and bakery healthy reintegration (NIELSEN, 2009). According to a research of the Mintel International (MINTEL, 2013), although the segment of dairy products has approached quickly to the maturity of the market, 44% of consumers are still looking for yogurt with additional benefits.

3. NUTRITION AND HEALTH CLAIMS

The history of functional foods is not very old. In 1980 a Japanese company was always more interested in the prevention of diseases related to a healthy lifestyle, out of its

awareness of the rapid aging process. This gave a strong impetus to food science and to the politicians in the food sector (ARAI, 2001).
Later, in 1991, these foods were defined by the acronym FOSHU, Foods for Specified Health Use, namely foods having beneficial effects on human health due to the presence of particular elements or the lack without of allergenic constituents.
The first FOSHU product was approved in 1993. Over 500 have been approved since then (JONES and JEW, 2007).
Most food industries have paid attention to the concept of functional food introduced by the research projects of the MESC (Ministry of Education, Science and Culture), started in 1984. In the US, special attention was given to claims that accompany the products with a functional role. Three important changes occurred in 1990, 1994 and 1997. They affected the dissemination of information to consumers about the relationship between diet and health in food regulations. The first of these is the Nutrition Labeling and Education Act of 1990 (NLEA). The NLEA allows statements on food labels that characterize the relationship of any food or food component to a disease or health-related condition. Such "health claims" must be pre-approved by the FDA before their use.
The second and probably most important, and controversial, change in food regulations was the passage of the Dietary Supplement Health and Education Act of 1994 (DSHEA). This act regulates dietary supplements as foods, not food additives, defining them as "vitamins, minerals, herbs or other botanicals, amino acids, or other dietary substances for use by man to supplement the diet by increasing the total dietary intake, including concentrates, metabolites, constituents, extracts, or any combination of the above".
To expedite the health claims approval process and thus hasten the availability of health messages to consumers, Congress enacted the FDA Modernization Act (FDAMA) in 1997. This legislation streamlines the FDA pre-approval process by enabling the use of so-called "authoritative statements" on food labels as health claims. Historically, companies that have attempted to launch a functional food in Europe have faced a variety of legislation regulating the approval of products, the kinds of nutrition information required on labels, and the types of functional and health claims that were allowed in connection with a product (BECH-LARSEN and SCHOLDERER, 2007). After a first attempt at harmonization, which has prohibited all product-related communications from attributing properties for prevention, treatment or cure of human diseases to food (EUROPEAN PARLIAMENT AND COUNCIL OF EUROPE, 2001), the situation changed. On July 2003, the European Commission proposed a harmonized regulation COM/2003/0424 on nutrition and health claims made on foods, including dietary supplements (FAO, 2007). In December 2006 the Council and Parliament adopted the Regulation 1924/2006 on nutrition and health claims made on foods. For the first time, this Regulation lays down harmonized rules across the European Union for the use of nutrition claims such as "low fat", "high fiber" or health claims such as "reducing blood cholesterol" (EUROPEAN PARLIAMENT AND COUNCIL OF EUROPE, 2006). This regulation ensures clear and accurate information based on evidence accepted by the whole scientific community. In order to bear claims, foods will need to have appropriate nutrient profiles, which will be set. This Regulation introduces a new category of claims, i.e., "Reduction of disease risk claims". This means any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease. Europe instead displays requirements for a high level of scientific substantiation. The European Food Safety Authority (EFSA) is involved in implementing the new regulation. This Authority has published the specific guidances in 2012, prepared by the Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA), on the scientific requirements for the substantiation of particular health claims: post-prandial blood glucose responses/blood glucose control, weight management,

energy intake and satiety, protection against oxidative damage, cardiovascular health, bone, joints, and oral health, neurological and psychological functions and physical performance. These guidances help companies who want to submit health claims for authorization. The assessment of a health claim by EFSA is the first step in the authorization process. Only those claims, which are scientifically substantiated, will finally be authorized for use. The final approval of a health claim is the responsibility of the European Commission and Member States, based on the scientific assessment expressed in the opinion of EFSA's Panel.
Recently EFSA refused food claims from label resulting in a cut of the potential market of functional foods. In fact continues the discussion between the companies and EFSA on the process of evaluation of health claims according to Reg. (EC) 1924/06. EFSA has published the scientific opinions related to the validation of two health claims (art. 13 (5) of Reg. 1924/06) and one novel food (EFSA, 2015).
The scientific opinions will now be discussed in EU committees of competence for the publication of the Regulations of the two health claims and of the novel food.

4. WORLD MARKET OF FUNCTIONAL FOODS

Describe the functional foods market is very complex because there are many factors that affect the industry. In general, Fig. 1 shows a graph since divided into four quadrants that identifies the main areas of high/low risk and a high/low growth in terms of potential investments. Brazil, China, India, East and Southeast Asia are the most attractive regions from the growth and risk viewpoints. They are countries with competitive economies. Other developing areas that is the Middle East and North Africa are less attractive due to higher risk.
The market of functional foods is growing rapidly and is highly dynamic. In many ways it may even be characterized as an experimental environment (BECH-LARSEN et al., 2007).
The increase in life expectancy, resulting in an increase in the number of the elderly and the desire for an improved quality of life, as well as the increasing costs of health care, has stimulated governments, researchers, health professionals and the food industry to study how such changes can be managed more effectively.

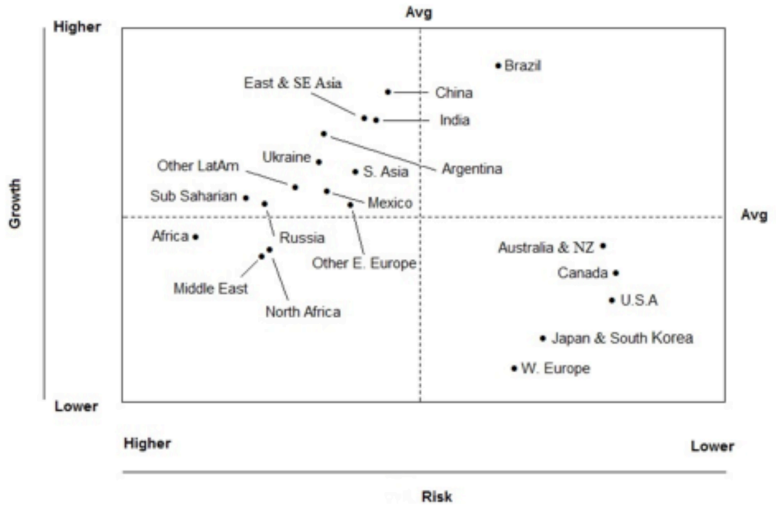


Figure 1: Large emerging markets - Brazil, China, India and South-East Asia regions are most attractive (McKinsey-IFAMA Agribusiness & Food Survey, 2012).

It is difficult to have a precise estimate of the data on the total turnover and the volume of functional foods sold.
Some experts have observed the global market for functional foods and were able to give estimates of revenues over the years.
A study, for example, has estimated that the global market of FFs varies from US$ 33 billion (HILLIAM, 2000) to US$ 32 billion (URALA and LAHTEENMAKI, 2004). In 2002, Sloan estimated a growth to US$ 47.6 billion (SLOAN, 2002). Other estimates indicate a total global market value of functional foods in the range of US$ 34 billion in 2004 to US$ 73 billion in 2003 (KOTILAINEN et al., 2006), and of US$ 81 billion in 2005 (JUST-FOOD, 2007). In 2010, Euromonitor estimated the value of the global market for functional foods in US$ 168 billion, that is 2.5 times the size of vitamins and dietary supplements market (EUROMONITOR, 2010a). Moreover, the growing desire to use food to help prevent chronic diseases (KHAN *et al*., 2013) has led to a rapid growth of the food industry, with a compound annual growth rate of 8.6% in the 10 years to 2012 (EUROMONITOR, 2010b; SZAKÁLY et al., 2012).
There are three regions where sales of functional foods are concentrated: Japan, United States and Europe (MENRAD, 2003; DATAMONITOR, 2004). Williams *et al*. (2006), which indicates that the demand for functional foods within developing countries is growing, presenting a lucrative opportunity to develop domestic markets.
The demand for functional foods appears to be heterogeneous throughout different countries. The industry growth is affected by the selling price and, consequently, the average per capita expenditure globally. In 2013 Euromonitor estimated that the average per capita expenditure is around US$ 36 from country to country, reaching a maximum value of approximately US$ 272 with a worldwide turnover in the sector of approximate US$ 252 billion (EUROMONITOR, 2013). Fig. 1 shows the global distribution of functional foods market shares.
As can be inferred from Fig. 2, the main market for functional foods is Asia Pacific. Revenues for the field of FFs in Asia and the Pacific islands constitute as much as 34% of total revenue worldwide. This is not surprising, given that Japan alone is one of the main markets for FFs. The aggregate of Asia Pacific covers a vast territory, which includes the various countries where the market for functional foods is growing rapidly, as, for example, China, South Korea and Malaysia. For this reason, the revenues for the field of FFs in the Asian territory are so crucial in the world. The second largest market in the world is the North American one, substantially consisting of the U.S. and Canada. These two countries alone reach 25% of total revenue worldwide. Particularly in the U.S., the field of functional foods is a real business. Their marketing is favored by a legislative approach as well as a very permissive advertising. In addition to that the growth of the sector is boosted by the size of the territory and the population. These factors contribute to the high revenue.

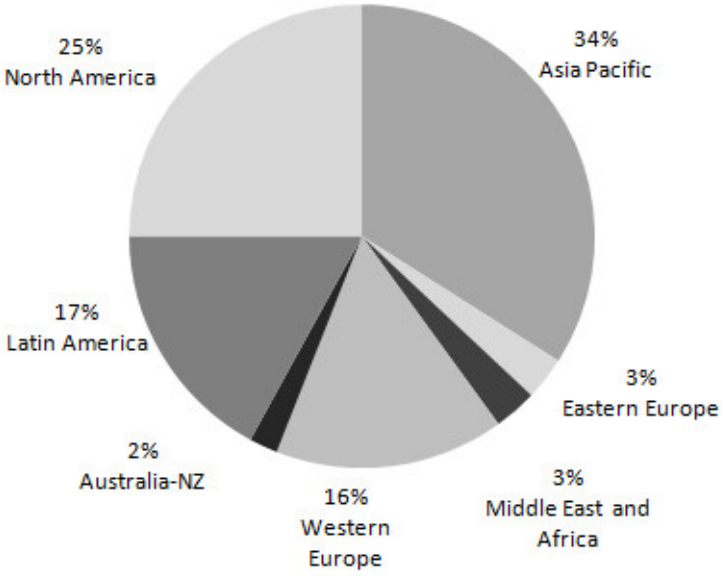


Figure 2: Percentage breakdown of total revenue worldwide (Euromonitor, 2013).

The demand for functional foods in Europe varies considerably from country to country, being affected by culture and culinary tradition (CASTELLINI *et al*., 2002).
Western Europe has a wide range of functional foods. The variety of FFs is able to meet the specific needs of particular groups of consumers. This has led to the development of a profitable market, which has been relatively stable over time, with a 16% of total revenue worldwide. The main source of revenue for the area of functional foods is the UK with 20% of total revenues which corresponds to about US$ 7.4 billion, followed by Germany with 14% and France whit 13% of total revenues, which corresponds both to about US$ 4.9 billion. Spain and Italy account for 12 and 11% of total revenues respectively, which correspond to about US$ 3.7 billion.
In Latin America, the functional foods and food production are relatively "new", but very promising. In fact, as can be seen from Fig. 2, the revenues for this sector are really high. Whereas the marketing of functional foods in South America started very recently and revenues for the field of functional foods currently make up 17% of total revenue worldwide, it can be said that Latin America is a market on which investment in functional foods should continue. From the point of view of the percentage breakdown of functional foods revenues worldwide, the markets of Australia, New Zealand (NZ), Africa, the Middle East and Eastern Europe can be considered negligible, since all together they make up just 8% of total revenue worldwide. Regarding Australia, the low revenue for functional foods is certainly due to the small size of the population and, therefore, the small number of potential consumers. As far as Africa and the Middle East are concerned, the market for functional foods is hampered by extreme poverty and socio-cultural backwardness of most of the territory, as well as by the uprisings and conflicts which unfortunately involve many countries of this vast geographic area. Let's not forget that for most of the African population there is no need to eat healthy, rather to just eat. Therefore, apart from a few rare exceptions, the market for functional foods in Africa and the Middle East is not favorable. In Eastern Europe the functional food market accounts for approximately 3% of total revenues overall and it includes some interesting countries from the viewpoint the development of the functional food market.
Russia alone, for example, constitutes 51% of total revenues for functional foods, followed by Poland, which has 17% of total revenues and the Czech Republic with approximately

9% (EUROMONITOR, 2013). On the other hand, for most of the other countries that make up the vast east European territory, the marketing of functional foods has just-timidly begun or is even non-existent. So, the market of functional foods in the East European region is very fragmented and in need of a better organization and an adequate promotion in order to foster development.

5. GLOBAL TRENDS OF THE FUNCTIONAL FOODS MARKET

The global market of functional foods in recent years is growing as is evident in Fig. 3. Steady real term growth of 7.2% is expected to continue to 2017 (EUROMONITOR, 2013).

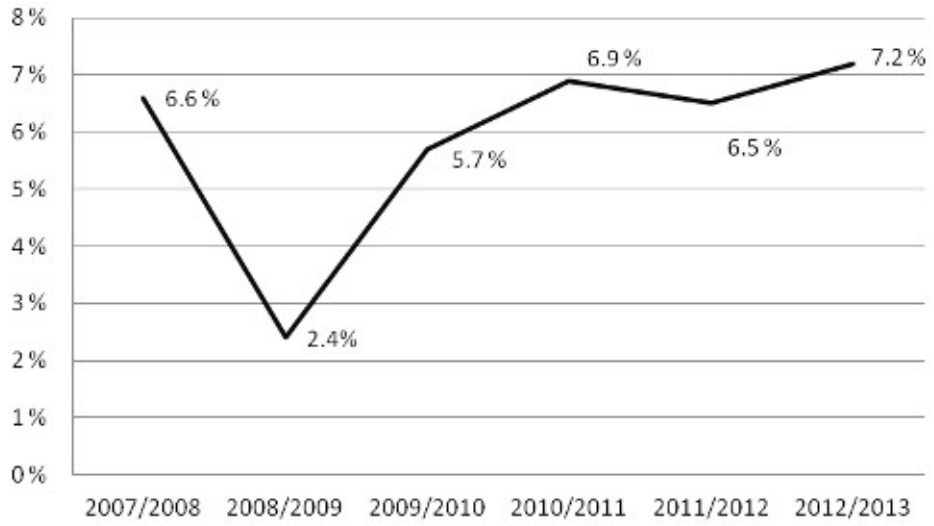


Figure 3: Growth rates of the global market of functional foods (Euromonitor, 2013).

The situation of the functional foods market varies from country to country. In order to have a more complete view of the world market, Table 1 shows the performance of the sector in terms of annual growth rate.
This boom not only underlines the dominance and maturity of the Asian market, which in 2013 recorded as many as 11.2 percentage points. As shown in Table 1, the market has been consistently strong, only in 2010-11 did it register a small decline but this has not destabilized its growth. Asia is a vast territory, led by China and Japan. Let's not forget the Pacific Islands, which contribute to the growth of the market, despite the small percentages.
The economic and social context influences this development. It was found that the increase in income guides the growth of the country. In addition, the population in Japan is aging faster than in any other part of the world. Senior consumers are more interested in maintaining a healthy lifestyle, willing to spend money on products with warranty sure to be beneficial (AGRICULTURE AND AGRI-FOOD CANADA, 2011). Several studies claim that the market for functional foods in China is expected to expand further in the near future, becoming increasingly important globally. However, the consumer markets in Asia are not fully liberalized and remain very fragmented.
Despite being the second largest market, North America sees a decidedly unbalanced trend. From 2007-08 to 2008-09 the retail value of functional foods decreased by less than 4 percentage points. The reason may depend on the economic recession that hit the United States, which has crippled the global economy. Despite this, America has recorded

positive values, reaching about 6% in 2011, but then suffered a further decline. Subsequently there was a new growth up to 4.6% in 2013.
Latin America is considered a promising region. In 2008-09 there was a slight decrease of 4 percentage points probably due to the economic crisis. From 2009 to 2012-13 the trend was positive and this does nothing but emphasize how this turns out to be potentially attractive in terms of growth and investment.
Finally in the Middle East and Africa the economic and social context make the growth of these markets difficult and unstable. However the situation is not entirely negative. There are also areas with high growth potential such as Saudi Arabia, South Africa and the State of Israel. These aggregate with Australia and New Zealand have a minimum percentage impact on the industry.
The growth trend in Eastern and Western Europe is varied over the years. Definitely better for Western Europe with a market that has established itself over time even if the growth value is lower compared to Eastern Europe (emerging market). Indeed, the latter recorded a sales boom in 2007-2008 before suffering a collapse in the ensuing year, which coincided with the global economic crisis, and a sharp recovery as early as 2010. Although the annual growth rate in Eastern Europe is higher than that of Western Europe, it must be emphasized instead as the average annual consumptions per capita are higher in this area (EUROMONITOR, 2013).

Table 1. Annual percentage growth rate in the functional foods sector worldwide (Euromonitor, 2013).

	2007-08	2008-09	2009-10	2010-11	2011-12	2012-2013
Asia Pacific	6.2	6.3	8.9	8.1	10	11.2
North America	4.8	- 4.2	1.4	5.5	1.4	4.6
Latin America	11.1	8.5	12.8	12.9	12.4	13.1
Middle East and Africa	14.9	6.6	9.1	9.8	9.3	9.7
Australia-NZ	6.7	6.6	7.9	8.1	5.2	6.1
Western Europe	5.4	2.4	1.5	2.3	2.5	2.8
Eastern Europe	20.6	1.6	7.7	11	9.2	10.2

6. THE EMERGING MARKETS

For emerging markets there is plenty of opportunity for investment in functional foods. It provides an absolute increase of US$ 23 billion by 2017, 40% more than the growth forecast for developed markets. In the study of the functional food market, greater attention has been paid to two major countries: Brazil and Mexico. These favorable socio-economic conditions have attracted companies looking to invest in functional foods. Brazil is one of the leading countries in the production and consumption of food with a market for functional foods growing by 10% per year, three times more than the market for conventional foods. In Brazil a recent face-to-face survey to 450 consumers (DE BARCELLOS and LIONELLO, 2011) has indicated that the functional food market is incipient, but is developing rapidly with a growing interest in sophisticated and health-oriented products (EUROMONITOR, 2007). The demographic trend is one of the factors which particularly affects the growth of the sector. Brazil has a young and growing

population of 196 million people that could reach the 200 million mark by 2015. This means Brazil will retain its position as the world's fifth most populous country. The boom in the Brazilian economy is coupled with a constant improvement in the population's wealth. According to the Brazilian Institute of Geography and Statistics (IBGE), consumer income from all sources has grown, propelling many Brazilians into the middle class. The middle class has grown from 38% to 51% since 2003, and purchasing power has also risen accordingly (JERGER, 2012).
This has resulted in an increased demand for high-quality food-stuffs. Functional foods have recorded impressive growth rates.
Despite the good economic conditions of the country, the birth rate has decreased in the 2006-2011 period. The total population of babies and infants is expected to drop to 7.5 million by 2020, or by 13.4% compared to 2011. This would lead to a decrease in the sales volume of functional foods.
Similarly, the population of children between three and eight years old experienced a 5.7% decrease, from 2006 to 2011, and is expected to drop another 19.2% by 2020, the largest decline of any population segment (AGRICULTURE and AGRI-FOOD CANADA, 2013).
Despite these shrinking numbers, kids will continue to influence demand for functional foods. To counteract the increase of obesity in this target of population, healthy products such as functional products, organic food, cereals, yoghurts, dairy drinks and fruit-flavored water, will see improvements in their sales volume, as will products with less salt, low/no fat and low/no sugar.
In 2011, the target of consumers aged 18-29 years was 40.6 million, decreasing to an expected 39.3 million by 2020. Despite the decline, by the end of 2020 this segment will represent the second-largest age group in Brazil, or 19.3% of the total population in Brazil. Brazilians in this age group are seeking more ways of keeping fit. For example, through sports or performing arts such as dancing. This lifestyle is another opportunity for functional products that can supplement the needs of adults pre or post-workout. For example, consumption of fortified/functional sports drinks experienced strong growth of nearly 40% in value terms from 2009 to 2011, and sales value is expected to grow by another 55.6% from 2011 to 2015.
Finally, the target consumers aged 30-44 reached a total population of 42.9 million in 2011 and are forecast to reach 48.7 million by 2020, to represent 23.9%, or the largest age segment of the total population. This is a more mature population group with the large majority starting to have children or already having a family. Due to families and career responsibilities, Brazilians at this age have busy lifestyles and do not have much time to prepare meals at home from scratch. This lack of time in day-to-day living is expected to drive up demand within the food industry for healthy and fortified/functional ready-meals. Mexico along with Brazil influences the growth of the sector. To influence the sector is the demographic trend of the Mexican population. Middle-aged adults (40-64 years) are the most financially stable consumers in Mexico. As most have already settled down with homes and families, they have significantly more disposable income than younger generations. In addition, middle-aged consumers are the most populous demographic in Mexico. Their market dominance is expected to continue, with the number of middle-aged adults reaching nearly 30 million in 2015 and roughly 35 million in 2020. As middle-aged consumers are very concerned about healthy lifestyles, they will continue to demand fortified meals, food and drinks adapted to their age, such as skim milk, calcium-enriched foods and drinks, vitamins and nutritional supplements, as well as high-fibre, sugarless, and low fat/cholesterol/sodium products. Particular attention is given to women's health, especially during the period of pregnancy (EUROMONITOR, 2012).
A campaign carried out by the Mexican government in 2011 stressed the importance of an adequate intake of folic acid in women. This has led major companies to produce

functional foods suitable for expectant mothers (AGRICULTURE AND AGRI-FOOD CANADA, 2012). We must say that in Mexico, between 2005-2011, there has been a declining birth rate equal to 5% but this is a fact that does not affect the functional food market. The economic downturn has families save money, but not on their child's health. Although consumers continue to have fewer children per family, parents will have more resources to spend on each child leading to increased purchases of functional foods thus allowing to maintain a steady growth of the industry. In terms of sales, in 2011 Mexico totaled a record US$ 12.7 billion providing for an increase of US$ 16.3 billion in 2015.

7. CONCLUSIONS

The agribusiness sector has undergone a profound change in the last two decades. The deepening global crisis that has hit the economy has considerably changed consumers' habits and as a result, companies have been forced to revise their action plan.
Increased attention to consumer's health, has given the scientific research and business, a stimulus to develop products with therapeutic features that go beyond the role played by traditional foods. Research in this field has given a strong contribution and made sure that the food market can evolve.
These products have had an immediate success in most of the industrialized countries and in the developing ones, therefore we can say that they can actually be an opportunity both for the industry in economic terms, and for the consumer in terms of health.
Looking at the situation of the world market, the leading exponent in the field of FFs is Asia Pacific, followed by North America, Latin America and finally Western Europe. The field of "functional" food has a potential that still has to expand, but it calls for the solution of the main issues related to regulations and information aspects. Therefore, investments in the field of functional foods will prove beneficial only if specific legislation, an international collaborative approach and a direct and effective strategy for communication between producer and consumer will be developed.

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SHORT COMMUNICATION

THE CASE OF THE 2014 CROP SEASON IN TUSCANY: A SURVEY OF THE EFFECT OF THE OLIVE FRUIT FLY ATTACK

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ABSTRACT

In this work we compare the chemical composition of olive fruits (*cv Moraiolo*) and the sensory and chemical quality of olive oils extracted in two crop seasons: the 2013 crop season, characterized by a very minor attack by the *Bactrocera oleae*, and the 2014 crop season, characterized by one of the strongest attacks of *Bactrocera oleae* in the last decades. Results show that during the 2014 crop season the pulp/stone ratio, moisture, phenolic content, oil content, and sugar content were lower than in the 2013 crop season. Moreover, the olive oils from the 2014 crop season were characterized both by higher free acidity values, peroxide value and K_{232}, and by lower values of antioxidants (phenolic compounds and tocopherols). The olive oils from the 2013 crop season did not present any defects, while among those from the 2014 crop season, 41% were defective and did not fall into the extra virgin olive oil category.

Keywords: extra virgin olive oil, *Moraiolo* cultivar, olive degradation, olive fly, olive oil quality

1. INTRODUCTION

In recent decades, extra virgin olive oil (EVOO) has been attracting the interest of the scientific community, also due to its well-recognized nutraceutical and sensory properties (CECCHI *et al*., 2015). These properties go to define the quality of EVOO and are affected by various factors, one of which is the olive fruits' health status (MIGLIORINI *et al*., 2012).
One of the main risk factors for the health of olive fruits is related to the attack of pathogens and phytophagy, the most dangerous aggressor being the olive fly, *Bactrocera oleae* (PEROVIC *et al*., 2007; WANG *et al*., 2009; WANG *et al*., 2013).
In most crop seasons in the inland areas of Tuscany, attacks by the olive fly are scarce, so the effect on EVOO quality is low. Instead, in some crop seasons, olive fly attacks can affect up to 100% of the olive fruits in some areas, with very heavy consequences on the sensory and nutritional properties of EVOO.
Various parameters can be used to make a quantitative assessment of olive fly attacks. Among these parameters, active infestation only accounts for the stages of development of the larvae up to the second age and is used to establish the threshold beyond which a chemical treatment is justified (this threshold is 10% of active infestation). The total infestation takes into account all the types of infestation caused by olive fly and, therefore, can be better correlated to the variations in the chemical parameters of the olive fruits (CALECA *et al*., 2007). In fact, the main risk factors for the fruits' state of health are those that cause the rupture of the skin. The main consequences of such a rupture are: (i) exposure to oxygen of the olive fruits' chemical constituents; evaporation of water from the fruit no longer protected by the outer waxy layer; (ii) alteration of the metabolic processes of the fruit; (iii) attacks from exogenous and endogenous enzymes. Each of these phenomena affects the olive fruits' chemical composition, which can compromise the quality parameters of the EVOOs (SERVILI *et al*., 2007), as also stated in studies conducted on oils obtained by milling different mixtures of healthy and damaged olives on a laboratory scale (KOPRIVNJAK *et al*., 2010; PEREIRA *et al*., 2004; MRAICHA *et al*., 2010) or on oils from partially damaged olives in different industrial mills (GOMEZ-CARAVACA *et al*., 2008). The decrease in the oil quality mainly depends on: i) the kind of infestation, ii) the percentage of damaged fruits, iii) the cultivar, and iv) the fruits' stage of development (GUCCI *et al*., 2012).
The 2014 crop season was characterized by a very intense attack by the olive fly and, in Tuscany, almost all the olive plantations were totally damaged. One of the main reasons for this attack was the many climatic anomalies during the Fall 2013 - Fall 2014 period (LaMMA, 2013; LaMMA, 2014a; LaMMA, 2014b; LaMMA, 2015).
The main goal of this study was to represent the chemical composition of the olive fruits and the extracted EVOOs in light of the olive fruit fly attack in the 2014 crop season in Tuscany.

2. MATERIALS AND METHODS

2.1. Data relating to olive fly infestation

Data relating to active infestation and total infestation were collected from the Azienda Agricola Buonamici organic farm (Fiesole, Florence, Italy) by AgroAmbiente (2014), a Tuscany regional government website which collects data on olive infestation.

2.2 Olive fruit samples

During the 2013 and 2014 crop seasons 10 olive trees of both the *Frantoio* and *Moraiolo* cvs were selected from the Azienda Agricola Buonamici organic farm (Fiesole, Florence, Italy) and Fattoria Altomena (Pelago, Florence, Italy). Olive fruit samples, of approximately 500 g, were collected from these plants on a weekly basis: from September 8 to November 24 in 2013, and from September 1 to October 27 in 2014. All the samples were analyzed as soon as they were delivered to the laboratory.

2.3 Oil samples

The oil samples consisted of olive oils produced in Tuscany, mainly in inland areas, which were analyzed by the Laboratorio Chimico Merceologico (Promofirenze, Special Agency of the Florence Chamber of Commerce, Florence, Italy) in the 2013 and 2014 seasons. The oils were from the same farms in the two crop seasons, with the exception of the farms which did not harvest the olives in 2014 because they had been completely ruined by the olive fly.

2.4 Chemical analyses on the olive fruits

The total phenolic content, water content and oil content in the olive fruit samples were evaluated as previously reported (CECCHI *et al.*, 2013).
One hundred olive fruits were weighed and pitted to calculate the pulp/stone ratio. The stones were weighed, and the pulp weight was calculated as the difference between the weight of the whole olive fruit and the weight of the stones. The pulp/stone ratio was calculated by dividing the pulp weight by the stone weight.
To measure the sugar content of the olive fruits, eight grams of olive paste were cold extracted (6 ± 2°C) with distilled water in a 200 ml flask for 2 hours. The content of the flask was filtered through paper and 10 ml of the solution obtained were diluted with water in a 20 ml flask. The sugar content was analyzed with an enzymatic method using the ChemWell automatic analyzer (Awareness Technology, ChemWell 9210, Palm City, FL). Three enzymatic kits were used to measure respectively (i) the sum of two monosaccharide contents, namely glucose and fructose; (ii) the disaccharide sucrose content; and (iii) the polyol mannitol content. All the kits were purchased from R-Biopharm (Darmstadt, Germany). Measurements were performed by means of external calibration standards: fructose and glucose (purity > 99%, Sigma Aldrich SrL, Milan, Italy), and mannitol (purity > 98%, Sigma Aldrich Srl, Milan, Italy). The results provided by the instrument were expressed in g/l; they were also converted into sugar content on a dry matter basis (g/kg dm) as the average of two readings carried out for each sample. The sucrose contents were determined by multiplying the difference between the sum of glucose, fructose, and sucrose contents, and the sum of glucose and fructose contents, by 0.95.

2.5 Chemical and sensory analyses on the olive oils

The free acidity, peroxide number, and UV spectrophotometric indices (K_{232}, K_{270}, ΔK) of the oil samples were determined according to the analytical methods (EEC Reg. 2568/1991).
The tocopherols of the olive oil samples were determined according to the ISO 9936:2006/Corr. 1:2008 analytical method (ISO 9936/2008), while the total phenolic compounds were quantified according to the COI/T.20/Doc. no. 29 analytical method (COI/T.20/Doc.29, 2009).

Sensory evaluation of the olive oil samples was performed according to EEC 2568/91 regulations and the following amendments (EEC Reg. 2568/1991). The form used for the sensory evaluation was developed according to the EEC 2568/91 regulation (EEC Reg. 2568/1991) and the COI/T.20/Doc. No 15/Rev. 8 February 2015 document (COI/T.20/Doc. 15/ Rev.7., 2015).

2.6 Statistical analyses

The F-Test was used for each parameter to test if the variances in the data from the two crop seasons were unequal. Statistical significance was determined using Microsoft Excel statistical software (*t*-test: two-sample assuming unequal variances and using two-tailed distribution). Results of $P < 0.05$ were deemed as differences.

3. RESULTS AND DISCUSSIONS

Table 1 compares levels of active and total olive fly infestation in the 2013 and 2014 crop seasons in Tuscany. As shown, in the 2013 crop season, the active infestation never exceeded three attacks per 100 olives, remaining below the threshold for chemical treatment (10 attacks per 100 olive fruits). Instead, in the 2014 crop season, at the end of July, the active infestation had already passed this threshold. Regarding the total infestation, it remained below six attacks per 100 olives throughout the 2013 crop season, while during the 2014 crop season it exceeded 100 attacks per 100 olive fruits.
To understand the effect of the attack on the olive fruits' state of health and on the quality of the extracted EVOO, it is enough to observe that at the peak of attack in the 2014 crop season, there was an active infestation of approximately 40% while the total infestation even exceeded 100%; this suggests that at least 60% of the fruits had an infestation due to the presence of exit holes or larvae older than the second age, with an obvious detrimental effect on the quality of the fruit itself.

Table 1. Levels of active and total olive fly infestation in the 2013 and 2014 crop seasons. Data are shown as number of attacks per 100 olive fruits.

	2013		2014	
	Active infestation	Total infestation	Active infestation	Total infestation
Jul 17	-	-	4	4
Jul 23	0	0	6	6
Jul 31	0	0	12	12
Aug 07	0	0	15	16
Aug 14	0	0	5	18
Aug 20	0	0	9	22
Aug 27	1	2	13	30
Sep 04	1	2	20	47
Sep 11	1	2	26	72
Sep 18	3	5	32	79
Sep 25	3	3	36	88
Oct 02	3	6	40	95
Oct 09	2	3	42	111

Many factors seem to have contributed to the intense olive fly attack in Tuscany in the 2014 crop season. First, the 2013 crop season was a year of vegetative charge, as a consequence, in some cases the harvest was not completed; while the 2014 crop season was a year of vegetative discharge. Second, Tuscany was affected by major climatic anomalies during the period of Fall 2013 - Fall 2014. The mild winter temperatures meant that the flies could overwinter. Therefore, they were able to lay their eggs on the olive fruits remaining on the ground, especially in the olive groves where the harvest in 2013 had not been completed; in addition, the June "heat wave" limited the olives' productivity, due to the concomitant start of the fruit set. Finally, the mild temperatures combined with the high summer rainfall created the perfect precondition for a wide and rapid spread of the fly from generation to generation (LaMMA, 2014b).

Figures 1 and 2 show the chemical content trends during ripening of the cv *Moraiolo* olive fruits from Azienda Agricola Buonamici in the two crop seasons. The data regarding the cv *Frantoio* olives and the olives from Fattoria Altomena gave the same conclusions, so they are not shown in this short communication.

In the 2014 crop season the pulp/stone ratio did not increase during the ripening period, in contrast to what was observed in 2013 (Fig. 1). This may be due to several effects of the olive fly attack, such as the modification or impairment of the growth processes of the fruit and decrease in pulp owing to the trophic effect caused by the parasite. The natural consequence of the lower contribution of the pulp to the total weight of the olive fruit was a lower moisture content in the olives (Fig. 1). The moisture contents were comparable until September 22, when the pulp/stone ratio in the two olive oil seasons was not very different. After that date, the moisture in the 2014 crop season plummeted dramatically to values of up to 30% less than in 2013.

The sugar content of the olives was also affected by the olive fly attack (Fig. 2). In the 2013 crop season we can observe a sugar content of 40-45 g/kg of dry matter at the optimal ripening time (MIGLIORINI *et al.*, 2011; CECCHI *et al.*, 2013). Instead, in the 2014 crop season (October 22) the sugar content was already less than 10-15 g/kg of the dry matter. This drastic reduction may be due to both to the parasite compromising the biochemical maturation process and the evident loss of pulp.

A similar effect was seen for the oil content of the olive fruits (Fig. 2) and, consequently, the theoretical yields in the mill. In October we observed a break in the biochemical phenomenon of oil accumulation, together with a removal of pulp due to the fly's trophic activity.

Finally, the phenolic content of the 2014 crop season was always lower than that of the 2013 crop season, reaching a difference of 35% at the end of October (Fig. 2). This was probably due to the combined effect of the fly attack and the high water availability (PANNELLI *et al.*, 1994) in the 2014 crop season.

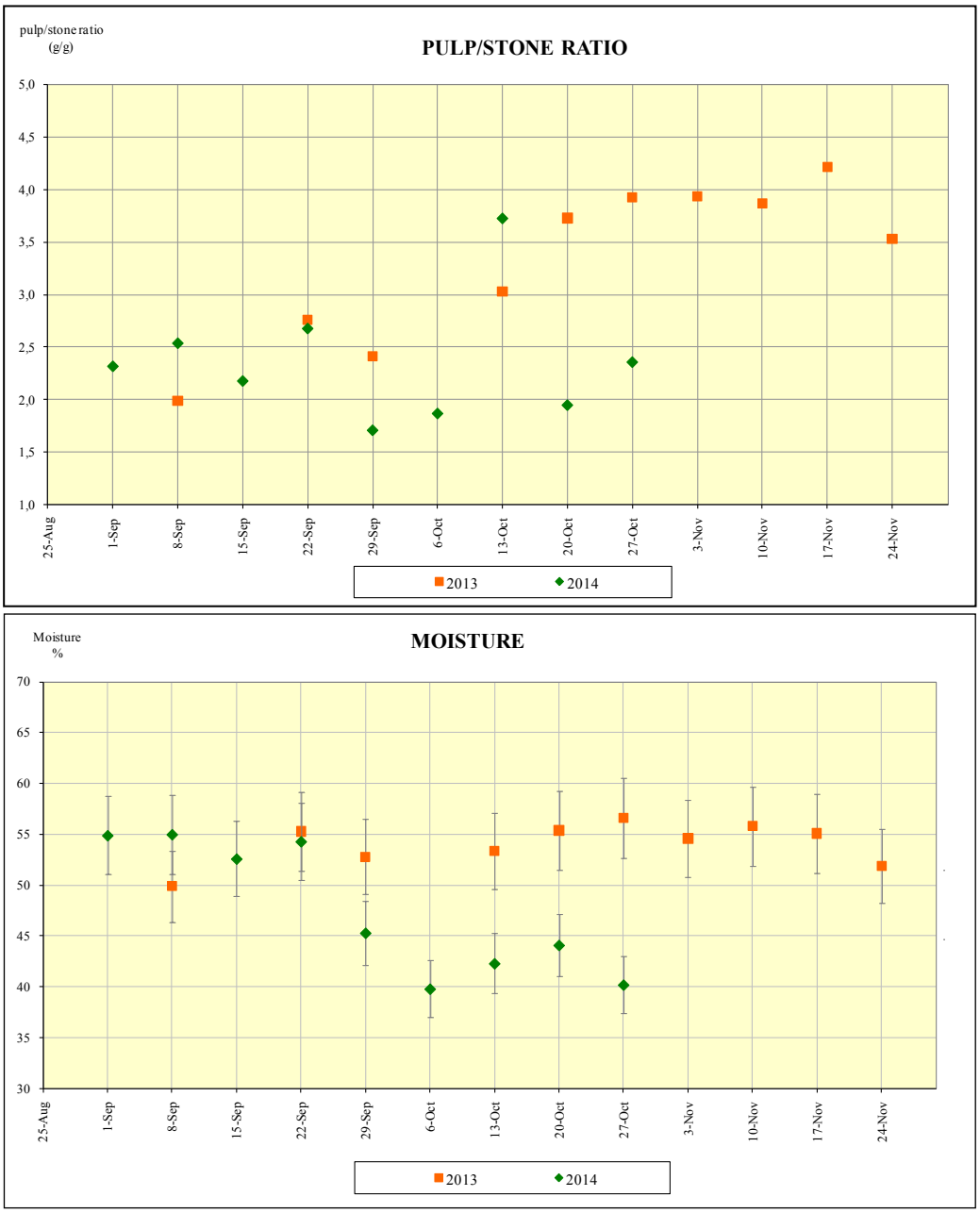
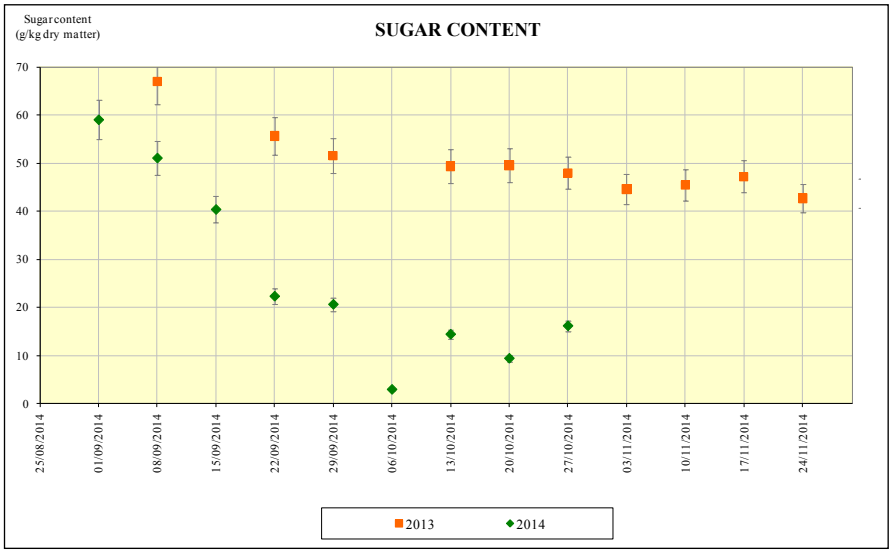
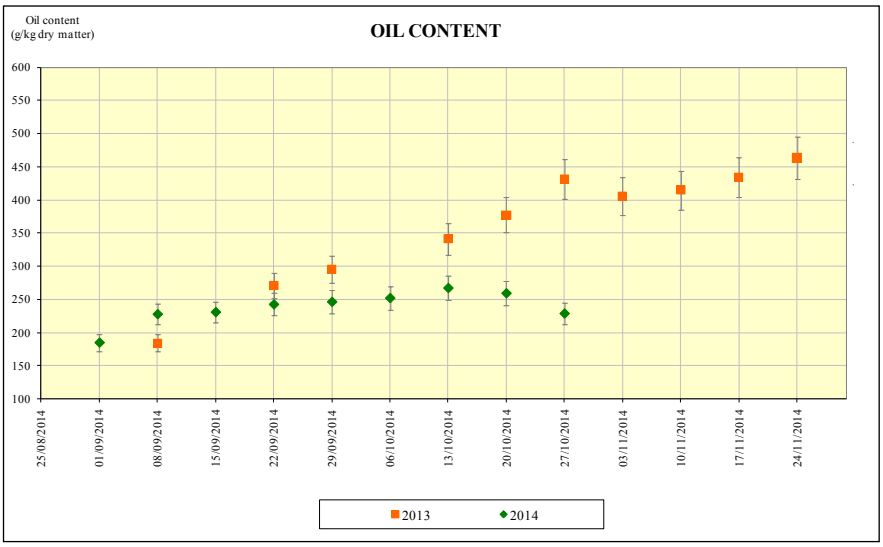


Figure 1: Pulp/stone ratio and moisture content trends in cv *Moraiolo* olive fruits during ripening in the 2013 and 2014 crop seasons.





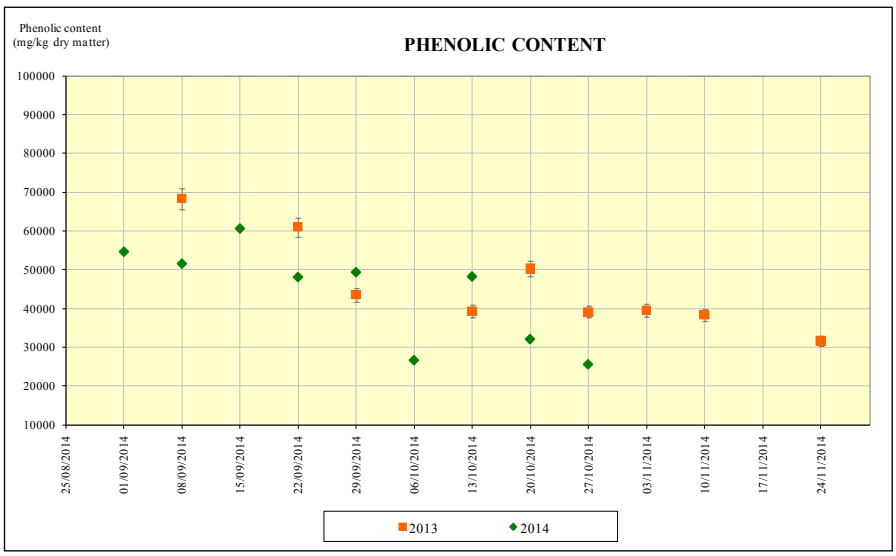


Figure 2. The chemical content trends in cv *Moraiolo* olive fruits during ripening in the 2013 and 2014 crop seasons.

The olive fly attack had a strong negative impact on the oil quality. Table 2 shows the results of the parameters used to describe oil quality. In the 2014 crop season, the olive fly attack, causing the oil to come into contact with the water in the olive fruits and then with the exogenous and endogenous lipases, may have induced the hydrolytic degradation of the triglycerides, with a clear increase in free acidity. Consequently, in the 2014 crop season, as much as 16% of the analyzed oils was found to be outside the legal limits which oil must respect to be classified as extra virgin (EEC Reg. 2568/1991), while in the 2013 crop season only one oil sample was found to be outside the legal limit of 0.80% free acidity.
The attack of the olive fly may have caused the fatty acids' exposure to strong oxidative conditions; hence, a sharp increase in both the peroxide number and the value of K_{232} occurred.

Table 2. Results of the parameters used to describe oil quality in the 2013 and 2014 crop seasons. For the mean value of each parameter, columns with letters indicate significant differences at $P < 0.05$.

	Free Acidity (%)	Peroxide number ($meqO_2/kg$)	Tocopherol content (mg/kg)	Phenolic content (mg_{tyr}/kg)	K232	K270	ΔK
2013 Crop Season							
Number of samples	356	354	202	212	235	235	235
Mean value	0.21 a	5.2 a	277 a	372 a	1.74 a	0.13 a	0.000 a
Maximum value	0.91	19.8	616	817	2.30	0.20	0.007
Minimum value	0.11	1.4	173	122	1.24	0.08	0.001
Standard deviation	0.08	2.08	67	128	0.15	0.02	0.001
2014 Crop Season							
Number of samples	221	212	35	52	76	76	76
Mean value	0.51 b	7.8 b	202 b	330 a	1.90 b	0.16 b	0.004 b
Maximum value	4.21	29.2	352	637	2.62	0.53	0.053
Minimum value	0.11	2.6	120	53	1.56	0.08	0.000
Standard deviation	0.39	3.5	49	140	0.22	0.07	0.008

Regarding the antioxidant components of the oil, the tocopherols were also compromised by the infestation, while the decrease in phenolic content was not statistically significant, confirming the unclear correlation between the % of fly attack and the phenolic content reported previously (GOMEZ-CARAVACA *et al*. 2008).
Regarding the oil sensory analysis, during the 2013 crop season all of the oil samples were free from defects, so they fell into the extra virgin olive oil category. In the 2014 crop season, instead, due to the major olive fly attack, 41% of the analyzed oils were defective, so they did not fall into the extra virgin olive oil category. Among these, 29% could be categorized as virgin olive oils, while the remaining 12% fell into the "*lampante*" olive oil category.
It also has to be taken into account that several farms did not harvest the completely ruined olives in 2014. This probably caused an underestimation of the negative effects of the olive fly attack on the oils from this crop season in Tuscany.
In conclusion, this research confirmed that the olive fruit fly is a strong risk factor for the quality of olive oil. A strong infestation by the olive fly causes a significant degradation in the chemical properties of the olive fruits and, as a result, poor oil quality.

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